GADD153 IS AN INDICATOR OF STRESS IN RECOMBINANT CHINESE HAMSTER OVARY CELLS

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ABSTRACT

Maintaining optimal viable cell numbers during culture of genetically engineered cells represents an important avenue for maximised yield of recombinant proteins. Within the culture environment there are many stresses which may decrease product yield, either by influencing the efficiency of individual cells or by producing decreased cell numbers by activation of apoptosis (programmed cell death). Understanding how mammalian cells perceive stress and the sequence of molecular events which link from perception to execution is important in order to identify means to generate cell cultures which are more resistant to stress. Early events in the sequence of stress perception may offer the possibility of indicators for the onset of responses. Subsequently, the indication of stress may permit an intervention to reverse the insult and to maintain a better quality of cell culture environment.

I examined a candidate gene that may serve as an indicator of stress. This was Growth Arrest and DNA Damage Inducible gene 153 [Gadd153]. Recombinant CHO DG44 19.6 cells that were placed in conditions of nutrient stress, including medium depleted of amino acids, glucose or glutamine, displayed a rapid induction of expression of Gadd153. Readdition of nutrients to the depleted medium reversed the elevated expression of Gadd153 in these cells. A second type of stress (blockade of glycosylation of proteins in the endoplasmic reticulum with tunicamycin) also produced a rapid activation of Gadd153 expression. During batch culture, as cells enter the decline phase there is an elevated expression of Gadd153.

The promoter of Gadd153 (regions –779 to +21) was linked to a reporter construct and assessed as an indicator of cell stress. This region of the promoter was used as it had previously been reported to be stress-responsive in other systems (Luethy et.al., 1990). Initially, I carried out transient transfection studies using a Gadd153-Luciferase reporter construct and this proved to be stress-responsive. To determine if the promoter had potential as a non-invasive indicator of cell stress a CHO DG44 Gadd153-d2EGFP stable cell line was produced. Preliminary investigations have shown promise as the Gadd153-d2EGFP transgene displayed a response to ER stress.

In this thesis I have presented data showing that the expression of Gadd153 is an indicator of several forms of stress which may be imposed on recombinant cells in batch culture. Amino acid and nutrient depletion and the presence of incorrectly-processed secretory proteins all enhance Gadd153 expression in a response which is rapid and reversible. I have demonstrated that the Gadd153 promoter is inducible after stress imposition and have also shown that it has potential as an online, non-invasive indicator of cell stress. This type of approach may not only indicate the factors which may be important in loss of cell viability in response to environmental stresses but will also open avenues for intervention with stress situations.

DECLARATION

No portion of work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other University or other Institute of learning.

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THE AUTHOR

Tracy Murphy BSc. is a graduate of the University of Manchester (UK) where she read Biochemistry which included a sandwich year at Smithkine Beecham Pharmaceuticals. After graduation, she begun her research for the degree of Ph.D at the University of Manchester, sponsored by the British Biological Research Council (BBSRC) and a CASE award with British Biotech Limited.

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I wish to dedicate this thesis to my Mum and Dad and family. I am forever grateful for their love, encouragement and support and especially for lifting my spirits whilst writing this thesis. Finally, I would like to thank Peter for his huge amount of patience, love and emotional support throughout this time.

CONVENTION AND ABBREVIATION

The standard abbreviation of the Biochemical Journal (as described in "Notes to Authors, Biochemical Journal (1993) 289, 1") have been used throughout this thesis. All other abbreviations are defined in the text as appropriate; abbreviation encountered in more than one section of text are listed below:

Symbols

α	alpha
β	beta
γ	gamma

Abbreviation	Item
ATF2	Activating transcription factor 2
AP-1	Activator protein-1
ASK1	Apoptosis signal-regulating kinase1
bp	Base pairs
BSA	Bovine serum albumin
CAT	Chloramphenicol acetyl transferase
cDNA	Complementary DNA
C/EBP	CCAAT Enhancer Binding Protein
CHO	Chinese Hamster Ovary
CHOP	C/EBP Homologous Protein
CREB	cAMP-response element binding protein
DAPI	4'6-Diamidino-2-phenylindole dihydrochloride
DEPC	Diethulpyrocarbonate
DHFR	Dihydrofolate reductase
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulphoxide
DOC	Downstream of CHOP
DTT	Dithiothretol
ECL	Enhanced chemiluminescence

EDTA	Ethylenediaminotetraacetic acid
eIF	Elongation initiation factor
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinase
FCS	Foetal calf serum
g	Gravity
Gadd	Growth arrest and DNA damage
GFP	Green fluorescent protein
GS	Glutamine synthetase
HAC1	Homology to ATF/CREB
HEK	Human embryonic kidney
hrs	Hours
IGF	Insulin like growth factor
IKB	Interferon-kappa-β
IKK	Interferon-kappa-β kinase
IL	Interleukin
IPTG	Isopropyl β-D-thiogalactoside
IRE1	Inositol requiring and ER to nucleus signalling
JNK	c-Jun N-amino terminal kinase
kb	Kilobase
kDa	Kilodalton
LB	L-broth
LDH	Lactate dehydrogenase
MOPS	4-morpholine proposulphonic acid
mRNA	Messenger RNA
MMS	Methyl Methanesulphonate
MSX	Methionine sulphoximine
MTX	Methotrexate
NFκβ	Nuclear factor kappa-β
NIK	Nuclear factor kappa-β inducing kinase
OD	Optical density
PBS	Phosphate-buffered saline

PDGF	Platelet-derived growth factors
PCA	Perchloric Acid
PERK	Endoplasmic reticulum-resident kinase
PKC	Protein kinase C
s	Seconds
SAPK	Stress activated protein kinase
SDS	Sodium dodecyl sulphate
SSC	Standard saline citrate
ssDNA	Single-stranded DNA
SV40	Simarian virus 40
tRNA	Transfer RNA
U	Units
UPR	Unfolded Protein Response
18S rRNA	18S ribosomal RNA
28S rRNA	28S ribosomal RNA
w/v	Weight per volume
v/v	Volume per volume

CHAPTER 1 INTRODUCTION

The exploitation of animal cell culture by manipulation of recombinant DNA to express biologically active molecules has led to a whole new field of drug development for the treatment of disease. In order to reach the clinic, recombinant proteins require a complex but cost effective route of production and so great emphasis is placed on process development.

Historically microbial cells have been used to express recombinant molecules, due to the simplicity of the culturing techniques required, for example, interferon production in the bacterium *Escherichia coli* (Harris,1984). Due to the complexity of large multidomain proteins being produced today, higher eukaryotes are preferred as the host to perform accurate post-translational modifications. These include disulphide bond formation, intramolecular folding and glycosylation and are required for protein efficacy *in vivo*. An example of this is in the production of vaccines where immunogenicity depends on the precise structure of the protein (Geisse et.al.,1996). Higher eukaryotes, such as Chinese hamster ovary (CHO) cells have become a cell of choice for these reasons even though there are a few disadvantages such as fragility, slow growth rate, lower densities, complex nutrient requirement and low productivity (Lavery, 1990). However, it is evident that a large proportion of the profits generated by the biotech industry are derived from animal cell culture (Mastrangelo and Betenbaugh, 1998) therefore there is much impetus for research into improving cell growth and productivity. A major focus of research has centered on modifications to the media and the bioreactor conditions such as agitation, aeration and pH. Successful outcomes have been achieved but recently attention has turned to research of cell death and the processes that control it (Cotter and Al-Rubeai, 1995). This has arisen from the discovery of the process called apoptosis or programmed cell death (Kerr et al, 1972). Research focused on the initiation and control of apoptosis may identify approaches to delay onset of cell death within culture and thus increase the longevity and productivity of recombinant cells. In this thesis I have examined the expression of Growth Arrest and DNA Damage gene (Gadd153) as a genetic marker of mammalian cell stress in batch culture. Furthermore, I tested the feasibility of using the promoter of this gene as an online monitor of cell stress. The aim of this was to provide a rapid indicator of the entry of cells into apoptosis and thus enable its intervention.

1.1 ANIMAL CELL BIOTECHNOLOGY

1.1.1 Expression of recombinant proteins in mammalian cells

Processes for the production of therapeutic molecules are based upon large scale culture, or fermentation, of cells transfected with an expression vector containing a foreign gene insert. The control of expression of the gene is at a scale which expedites recovery from the culture, and leads to purification and to homogeneity of the potential product.

The first recombinant protein to be licensed for use as a therapeutic agent was insulin in 1982 (Goeddel, 1979; Johnson, 1983) although the first recombinant product from mammalian cells to become available was tissue type plasminogen activator (tPA) (Pennica et. al.,1983; Paborsky et. al., 1990). There are now several pharmaceutical products which are produced from mammalian cell cultures and some of the current products and their therapeutic applications are shown below in Table 1.1.

The initial stage of protein production is the manipulation of DNA to build an expression vector suitable for the controlled transcription of the gene. The expression vector is inserted into a vector that is replicated in *E. coli.* A high concentration of vector can then be purified from *E.coli* culture. The expression vector containing recombinant DNA also has essential features for expressing the exogenous gene in mammalian cells. Strong promoter sequences and polyadenylation/termination sequences are required for high level expression (Ondek et.al., 1987; Schirm et.al., 1987; Manley, 1988). Bacterial plasmid replication and drug resistance sequences are included to allow vectors to be propagated in bacteria. Lastly, a selectable marker is also included to allow selection of mammalian cells that have integrated the vector. Once cloning in *E. coli* is completed the vector can be introduced into the mammalian cell using electroporation or exposure of cells to a calcium phosphate-DNA co-precipitate (Sambrook et. al.,1989). Such chimeric sequences of DNA once introduced into

Table 1.1 Some recombinant products of mammalian cells

Product	*Disease / Condition*
Lymphokines	Viral infections
Erythropoietin	Anemia, haemodialysis
Recombinant Insulin	Diabetics, insulin dependent
Urokinase	Blood clots
Granulocyte stimulating factor	Wounds, severe
Tissue plasminogen activator	Heart attacks, for survival to hospital
Transfer factor	Multiple sclerosis
Protein C	Hip surgery, protein C deficiency
Epidermal growth factor	Burns
Factor VIII	Haemophilia
Human Growth hormone	Pituitary deficiency
Alpha-interferon	Hairy -cell leukemia
Dacliximab	Organ rejection
Palivizumab	Lower respiratory tract disease
Glucagen	Diabetes- hypoglycemia
Endostatin	Prevention of blood vessel formation (tumours)
Antagon	Gonadotrophin releasing hormone antagonist
Panorex	Cytotoxic antibody
Zeffix	Hepatitis B / C, cancer therapy

host cells are subject to instability due to replication within the host cell, and this is dependent on the type of expression system (Sambrook et al, 1989).

Transient expression systems can be produced using a variety of mammalian cells. The cells are transfected with a vector containing regulatory sequences such as those derived from Simian virus 40 (SV40) plus the DNA or cDNA of interest (Fiers et al., 1978). Transient expression vectors are either constructed without a gene for selection or the cells are transfected without selection pressure.

Mammalian cells give low to moderate levels of expression under these conditions. Transfected vector is taken up into the cell nuclei but not the genome itself. The vector is therefore not replicated so gene copy number decreases after cell division. Protein expression levels decrease limiting the harvest of protein to one or two days after transfection (Sambrook et. al.,1989; Old and Primrose, 1988).

COS cells (simian CV-1 transformed cells) constitutively express the SV40 T-antigen which is required to activate the SV40 origin of replication. This facilitates the replication of vectors containing the SV40 origin of replication, as episomes within the COS cell nucleus. This results in replication of the vector, giving an overall high copy number of the transfected DNA. Replication of the vector continues until the cells die due to intolerance of high levels of the episomal-replicating DNA. This system is generally used to examine regulation of gene expression using a reporter gene, or when only small amounts of proteins are required, for example, immunofluorescent labelling for locating a non-secretory protein (Hentschel et.al.,1991). In this thesis I used HEK cells for transient transfection as they readily take up plasmid DNA in transient transfections (Roberts,G., British Biotech Ltd, unpublished observation). However, any commercially viable process for the production of proteins must be based upon a stable expression system where the vector DNA is passed from mother to daughter cells thus maximising the expression of the gene and as a safety regulation.

Stable expression uses either viral replication sequences on episomal vectors e.g. Bovine papilloma virus (BPV) in permissive cells or relies on integration of the vector into the host genome. Fragments of the BPV circular genome have been linked to an *E.Coli* plasmid vector and transfected into mouse cells. This vector can be replicated to produce a high gene copy number yet it is not integrated into the host cell genome. The vector is maintained as a stable episome producing a stable cell line (Hentschel et.al.,1991). The majority of stable expression vectors have selectable markers, these allow selection for cells which have intact vector integrated into the host cell genome. Although many copies of the vector enter the cell, the number that eventually reside in the genome following integration is

variable. It is generally thought, in most expression systems, that the greater the copy number of recombinant gene the larger the yield of the protein. This has obvious implications when trying to optimize a process.

1.1.2 Selectable and amplifiable markers

When exogenous DNA is introduced into mammalian cells, stable integration is a relatively inefficient process (Kucherlapati and Skoultchi, 1984). It is therefore necessary to have an efficient means of selecting cells that have taken up, and express, the foreign DNA. Several biochemically selectable marker genes have been cloned and are available for use in mammalian cells.

A widely used dominant selectable marker that can be used with wild type mammalian cells, is the neomycin-resistance gene. Mammalian cells are killed by the antibiotic G418, an aminoglycoside similar to neomycin and kanamycin. It blocks protein synthesis in eukaryotic cells (Colbere-Garapin et.el., 1981). Aminoglycoside antibiotics (G418, neomycin, kanamycin) can be inactivated by a phosphotransferase gene encoded by the bacterial transposon Tn5 (neo^R). Southern and Berg (1982) showed that when the neomycin resistance gene was linked to the SV40 virus early gene expression sequences, they were able to produce mouse and monkey cells resistant to the antibiotic G418. Another example of a dominant selectable marker is the use of the glutamine synthetase gene (GS) (Bebbington et.al., 1992). GS catalyses the formation of glutamine from glutamate and ammonia and provides the only pathway for the synthesis of glutamine (Meister, 1980). Thus, in the absence of exogenous glutamine, GS is an essential enzyme. GS can be used as a dominant selectable marker in cells that already contain GS activity and thus the system is not restricted to particular mutant cell lines.

Non-dominant markers include thymidine kinase (Colbere-Garapin et.al.,1979), adenine phosphoribosyltransferase, hypoxanthine phosphoribosyltransferase and dihydrofolate reductase (DHFR) (Bebbington and Hentschel, 1987). These non-dominant markers can only be used in mutant cells that lack endogenous enzyme

activity of the marker. DHFR is an enzyme that converts dihydrofolate to tetrahydrofolate, which is required for nucleotide biosynthesis in the absence of hypoxanthine and thymidine. CHO dhfr$^-$ cells lack endogenous DHFR enzymes (Bebbington and Hentschel, 1987) and require hypoxanthine and thymidine for growth. Thus, cells transfected with a vector containing DHFR can survive in media without hypoxanthine and thymidine.

Gene amplification is also a strategy used for promoting high level gene expression. In general, the amplifiable marker encodes an enzyme essential for cell survival and the enzyme inhibitor will be used to select for cells that are resistant to the inhibitor. By gradually increasing the concentration of the inhibitor, variants that are resistant to higher levels of the inhibitor can be selected. The most commonly used system uses methotrexate (MTX) to select for amplification of DHFR genes in CHO cell lines that lack endogenous DHFR enzymes (Bebbington and Hentschel, 1987). DHFR catalyses the reduction of dihydrofolate to tetrahydrofolate which is an important step in the synthesis of purines and thymidylate (Daubner et. al., 1985). The drug methotrexate (MTX), is a folate analogue that kills cells by binding to the catalytic site of DHFR. At high concentrations MTX blocks this conversion but at lower concentrations it will select for cells producing large amounts of DHFR (Yin and Schimke, 1996). Also, as the cells divide and replicate their DNA there is selection pressure, from the MTX, for those that replicate the drug resistant gene (DHFR). When the replication occurs a large section of DNA is repeated, leading to amplification of the gene for the recombinant protein which is adjacent to the DHFR gene. Overall, the aim is to increase specific recombinant protein production in a stable cell line and this takes a great deal of time since several rounds of selection are generally required to achieve maximal expression levels (Page and Synenham, 1991).

The GS system is also used for gene amplification and has proven to be more productive than the DHFR system due to higher levels of expression in the initial transfectants (Cockett et. al., 1990). GS is the essential enzyme required for the catalysis of glutamate and ammonia to form glutamine and is inhibited by methionine sulphoximine (MSX). As with the DHFR system, cells transfected with the GS vector amplify the inserted gene alongside the GS gene as they become

resistant to increasing concentrations of MSX. This system has an advantage over the DHFR expression system in that it can be used even when the cells are still expressing the cellular GS gene, however under this background the approach has poor selection (Sanders and Wilson, 1984).

1.1.3 Derivation of CHO cell lines for recombinant protein production

This project involved the use of a number of CHO cell lines and their derivation history is summarized in Figure 1.1. CHO cells were originally isolated by Puck et.al. (1958) from fibroblasts and are proline auxotrophs and hence require proline for survival. CHOK1 was the original subline isolated (Kao and Puck, 1967). CHOK1 cells were generated after mutagenesis using ethyl methanesulfonate, 6-thioguanine and gamma radiation. Mutants lacking a functional DHFR activity, CHO Duk cells were selected (Urlaub and Chasin, 1980).

CHO Pro^- is another subline derived from the original CHO cells and is auxotrophic for proline (Kao and Puck, 1967). It was from this cell line that CHO $Pro3^-$ subline was derived (Dewey, 1975). Flintoff (1976) selected MTX-resistant mutants from CHO $Pro3^-$ in two independent steps. The first step yielded a mutant MtxRI that produced wild-type levels of an altered DHFR enzyme. The mutant enzyme bound MTX with a lower affinity and its catalytic activity was 10-fold resistant to inhibition of the drug (Flintoff and Essami, 1980). The growth of this mutant was found to be resistant to a low level of MTX (Flintoff, 1976). A further mutant was isolated from the MtxRI cell line that was resistant to even higher levels of MTX. This mutant MtxRIII, contained 8-10-fold higher levels of DHFR activity (Flintoff, 1976) and had corresponding amplified levels of dhfr gene sequences (Wigler et.al., 1980; Flintoff et.al., 1982). Most, if not all, of the elevated DHFR activity in this amplified mutant was found to be the type that was resistant to MTX inhibition. In addition, Urlaub and Chasin (1980) had presented evidence that the dhfr locus is diploid in CHO cells therefore the MtxRIII genotype was interpreted as consisting of a wild-type gene as one allele and about 10 copies of a gene with a missense mutation in the other allele. Flintoff (1976) attempted to reverse this process by selecting a relatively MTX-sensitive mutant starting with these MtxRIII

cells. Treatment with a low dose of MTX in conjunction with tritiated deoxyuridine as a suicide agent had previously been shown to efficiently select for MTX-sensitive cells among a MTX-resistant population of CHO cells (Urlaub and Chasin, 1980). The DHFR activity is not blocked completely in MTX-resistant cells and therefore tritiated deoxyuridine is incorporated into DNA causing cell death, whereas MTX-sensitive cells are not able to incorporate this into their DNA which results in their survival. This method had previously proven to be efficient for selecting cells with one copy of the dhfr gene instead of two (Urlaub and Chasin, 1980).

MtxRIII cells were exposed to ionising radiation as this was known to cause changes in the dhfr locus in CHO cells (Graf and Chasin, 1982). Two independent mutagenesis treatments were performed and surviving cells were subjected to several rounds of MTX plus tritiated deoxyuridine selection. Clones were isolated and screened for DHFR activity. Two clones were isolated that were hemizygous for dhfr gene (UA2 and UA4). UA2 and UA4 cell lines were then used to perform double deletions by mutagenisis with γ-irradiation. Several independent populations were then subjected to selection for complete DHFR deficiency using tritiated deoxyuridine (Urlaub and Chasin, 1980). Two mutants were isolated which were derived from UA4 (DG41 and DG42). Southern blot analysis revealed that there was a complete lack of dhfr DNA in them. This supported the idea that only one copy of this gene is present in the progenitor strain, that is UA4 was hemizygous.

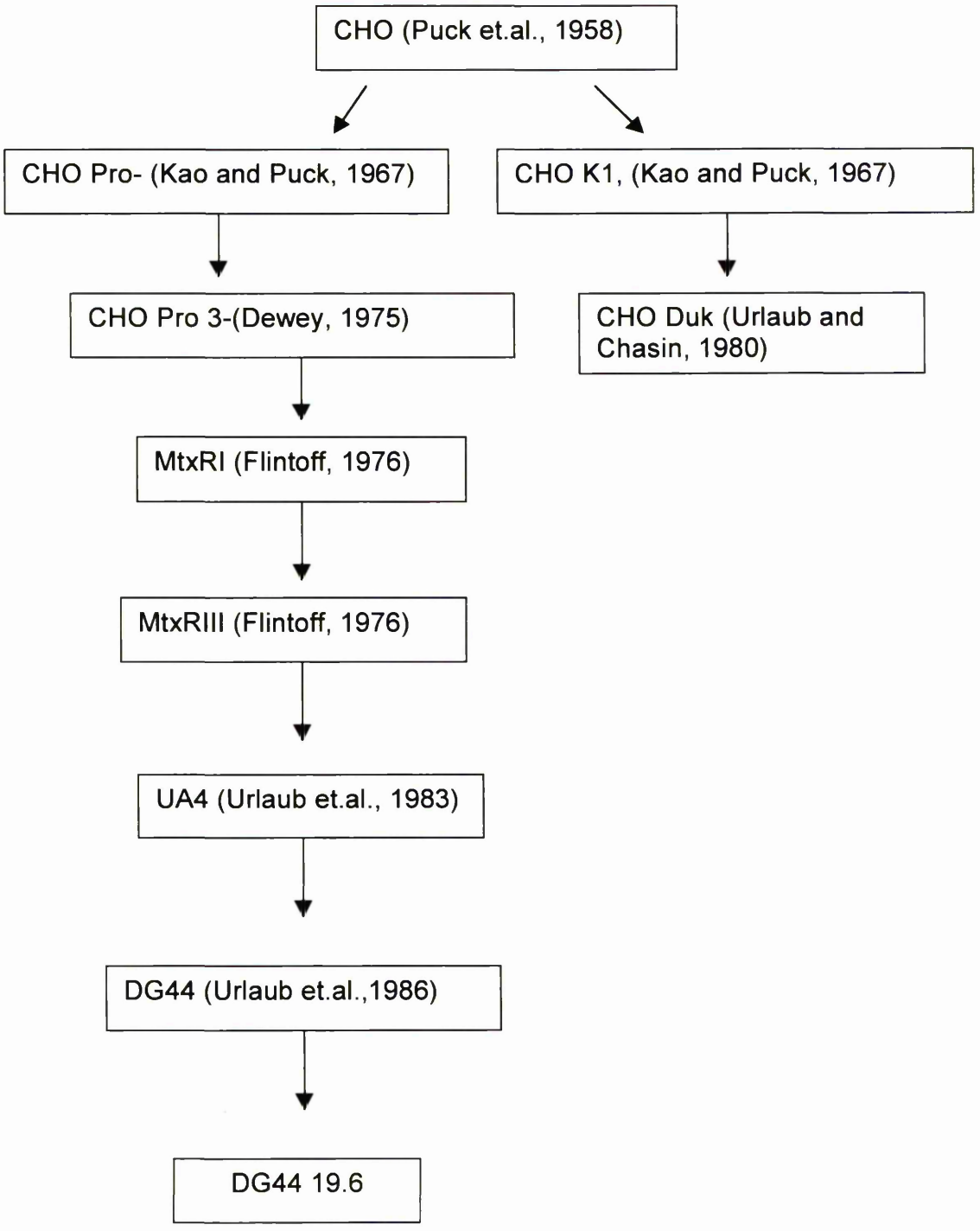
The mutagenesis had resulted in the loss of all 20 copies of the amplified allele, and cytogenetic analysis showed that a large deletion had occurred in the region of the dhfr gene on chromosome 2 (Funanage et.al., 1986). Only one dhfr allele remains in this cell line, localised to the arm of chromosome Z-2. Gamma-ray mutagenesis of UA4 gave rise to a DHFR-deficient cell line called DG44 which has suffered extensive deletions and inversions at this locus (Urlaub et.al., 1986).

The target gene DHFR protein dihydrofolate reductase is an enzyme whose action in the cell can be either necessary, dispensable or lethal depending on the composition of the culture medium. All the CHO cell lines used carry lesions at

their dhfr locus such that functional DHFR is no longer produced. Hence, in order to grow, these cell lines require exogenous thymidine and hypoxanthine (to be utilized in the salvage pathway in the synthesis of guanine and adenine). The isolation of these cell lines has allowed the use of DHFR-containing vectors to act as selection markers, thus allowing them to grow without added thymidine and hypoxanthine. The DHFR vector can also be amplified by using MTX, hence greatly increasing the amounts of heterologous protein produced (Section 1.1.2). DG44 cells were utilized by British Biotech Limited (BBL) in the production of a recombinant cell line containing a recombinant protein expression vector linked to the DHFR cDNA. The recombinant cDNA coded for thrombin activatable plasminogen (TAPgen) a proprietary molecule to BBL (Comer et.al., 1995). Cells were transfected by electroporation and clones were isolated under MTX selection. DG44 19.6 is a once cloned, suspension adapted cell line capable of expressing TAPgen to high titres in stirred tank reactors using serum-free media. This cell line was investigated in this thesis.

Initially, insect cell culture was used for the expression of plasminogen, this is due to the lack of plasminogen activators, which are present in mammalian cell cultures (Whitefleetsmith et.al.,1989; Davidson et.al.,1990). Subsequently, plasminogen has also been expressed in baby hamster kidney (BHK) cells and CHO cells. It can be co-expressed with α-2 antiplasmin to inhibit any plasmin activity and allow secretion of full-length recombinant plasminogen (Busby et.al.1991).

Figure 1.1 Derivation of CHO Duk and CHO DG44 Cells



1.1.4 The Influence of medium on mammalian cell growth and the production of recombinant proteins in batch culture

Animal cell culture has become an important approach for the production of biologically functional proteins for human therapy. The quantity and quality of protein production is influenced by the culture environment which changes over the course of cell cultivation (Mercille and Massie, 1994; Singh et.al., 1994; Perreault and Lemieux, 1994; Simpson et.al., 1998). The culture environment must be optimised and controlled within an optimal region to maximise productivity. The factors that affect the culture environment and cell growth are nutrient concentrations, by-product accumulation, pH and osmolarity. These factors must be taken into consideration when designing culture media for fed-batch animal cell cultures.

Since Eagle (1959) developed the minimum essential medium (MEM) for animal cell growth, empirical trial and error methods have been used in the subsequent development of culture media. MEM consists of glucose, amino acids, vitamins in a balanced salt solution and growth of cells was greatly enhanced by addition of serum, especially foetal calf serum because of its growth-promoting properties. Depending on tissue origin, the nutritional requirements of cells are different (Freshney, 1994). Eagle (1959) found that 13 amino acids were required for proliferation of cells, arginine, cysteine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine and valine. Glutamine had the highest rate of utilisation (Butler et. al., 1983). It has been shown that glutamine is used as an energy source as most of its carbon skeleton is oxidised in the tricarboxylic acid cycle (Reitzer et. al., 1979). It also accounts for 40 % of the energy requirements of CHO cells (Donnerly and Scheffler, 1976). The amide nitrogen of glutamine serves as a source for the biosynthesis of purines, pyrimidines, amino acids and amino acid sugars (Tate and Meister, 1977). Other carbohydrates besides glucose are known to have growth-promoting properties such as fructose, galactose and mannose (Eagle, 1958).

Serum is often added to the culture medium. It contains a large number of constituents (Table 1.2), many of which are at very low concentrations or may yet

to be determined making serum composition difficult to define. It has a high protein concentration, many of these proteins are carriers for minerals, fatty acids and hormones, and many of its constituents appear to be essential for the culture of cells.

Table 1.2 Typical constituents of growth medium derived from serum

Constituent type	***Example***
Attachment and matrix factors	Fibronectin
Hormones	Insulin, hydrocortisone
Growth factors	Platelet derived growth factor, insulin like growth factor
Serum proteins	Serum albumin, fetuin, thrombin, transferin, globulins
Lipids and lipid precursors	Cholesterol, linoleic acid, choline
Protease inhibitors	α-2 macroglobin
Metabolites and nutrients	amino acids, gluose, ketoacids
Trace elements	iron, copper, zinc, selenium

The disadvantages of using serum besides its high cost, especially in large scale culture, are the possible presence and action of many minor components that are undefined. Serum also interferes in recombinant protein purification due to many different protein species, and it contains growth inhibitors which may be detrimental to the overall aim of its use (Lambert et.al.,1989; Zavorski et.al.,1993). Such disadvantages have led to the development of serum-free media. These can contain serum extracts such as fetuin, which may also not be fully defined. Serum-free media has been utilised with CHO cells for production of human soluble thrombodulin (Ogata et.al.,1993), human interferon γ (Hayter et.al.,1992) and for long term cultivation (Gasser et.al., 1985). Serum-free media are often produced by supplementation of a basal medium such as Dulbecco's Modified Eagle's Medium (DMEM), RPMI 1640, or Ham's F12. The basal medium provides soluble low molecular weight nutrients e.g. carbohydrate, vitamins, amino acids, nucleosides and inorganic salts. Supplements most commonly added to CHO cell

culture to replace serum include hormones, such as insulin (a small polypeptide which has multiple effects on cellular metabolism, for example in the uptake of glucose, Komolov, 1978), and peptide growth factors, such as insulin-like growth factor 1 and II (ILGF-1 and II) (Dulak and Temin, 1973), platelet-derived growth factor (PDGF) (Heldin et.al., 1979), fibroblast growth factor (FGF) (Gospodarowicz, 1974) and epidermal growth factor (EGF) (Gospodarowicz, 1978). In addition to their roles as mitogens, PDGF and IGF's may function as survival cytokines, preventing c-myc-induced cell death under low serum conditions (Harrington et. al., 1994). Other proteins and polyamides are also required for survival and include transferrin (an iron carrier protein, Guilbert and Isocove, 1976), albumin (which acts as a nutrient carrier and will also bind toxic components, Freshney, 1994), and fibronectin (a multimeric protein found on the plasma membrane of cells associated with cell adhesion, cell aggregation and changes in cell morphology, Yamada et.al., 1978). Protease inhibitors such as aprotonin, soybean trypsin inhibitor and leupeptin, are also added to media to prevent degradation of the recombinant proteins being produced. In addition, selenium, zinc, iron and copper also appear to be required for cell growth in serum-free media (Freshney, 1987; Zavokski, 1993).

1.2 APOPTOSIS

In order to optimise animal cell culture processes it is important to address how these cells die in batch culture as once this is established it may allow for the intervention of cell death. Apoptosis and necrosis constitute two distinct mechanisms of cell death which differ in morphology, mechanism and incidence. Figure 1.2 outlines the two mechanisms.

1.2.1 The process of apoptosis

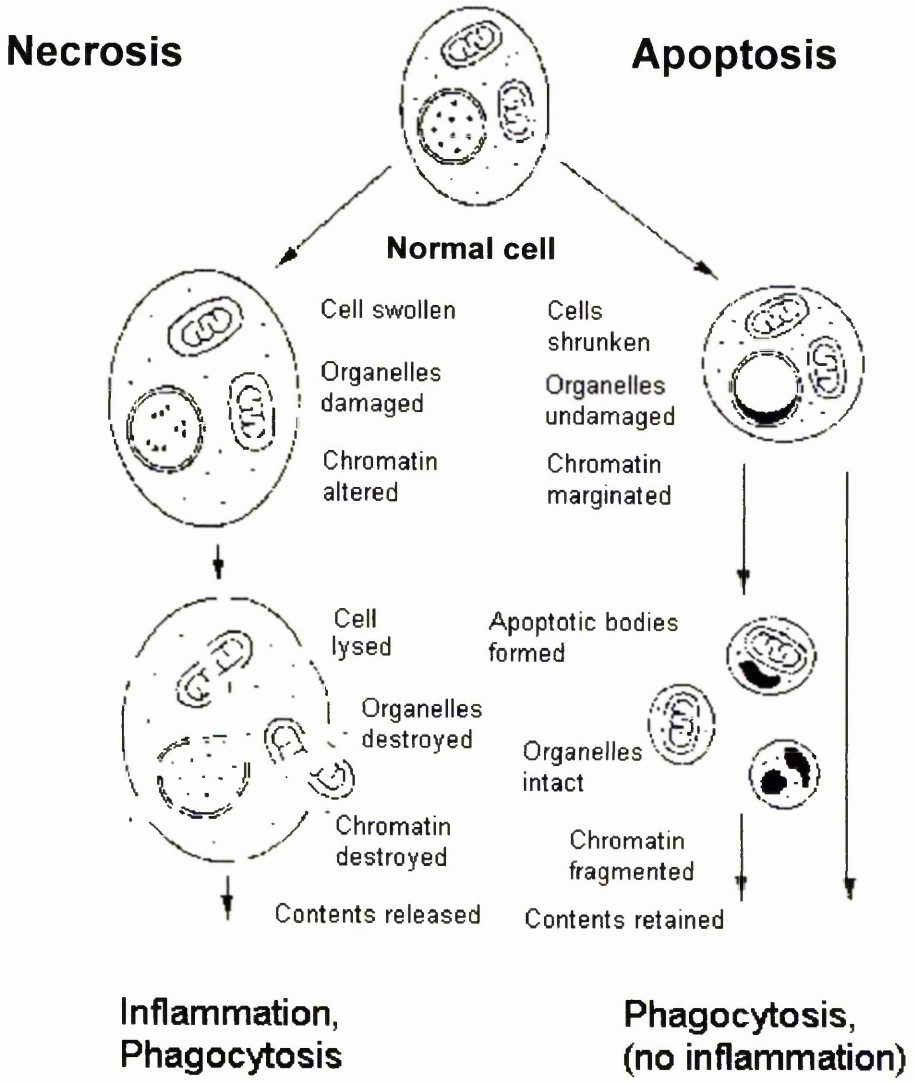
Apoptosis is an active energy-dependent process triggered by mild stresses such as media limitation or serum deprivation (Kerr et.al.,1972). This mode of cell death is characterised by cell shrinkage (Cohen, 1993). Simultaneously the cytoplasm condenses and this contraction is associated with loss of intracellular

fluid and ions (Lockstrin and Beaulation, 1981). This is due to the endoplasmic reticulum dilating, forming vesicles and fusing with the plasma membrane to release their contents into the extracellular environment (Morris et.al.,1984). Apoptotic bodies form within the cell membrane which enclose cytoplasm and organelles which pinch off the cell surface. This process is known as "membrane blebbing" (Barr and Tomei, 1994). The most characteristic features of apoptosis are the changes within the nucleus. Chromatin condenses into dense granular caps under the nuclear membrane. These are then cleaved into several membrane-bound apoptotic bodies (Arends et.al.,1990). Apoptotic bodies are phagocytosed by neighbouring cells *in vivo* (Cotter and Al-Rubeai, 1995). Finally nuclear DNA is cleaved into discrete fragments of 180 bp by endogenous nucleases. This is where the genome is cleaved in the linker region between adjacent nucleosomes. Caspase activation (Section 1.2.6) is thought to be responsible for the final execution of apoptosis by cleaving specific death substrates such as poly (ADP)-ribose polymerase (PARP), the inhibitor of caspases-activated Dnase (ICAD) also known as DFF45, gelsolin and lamins (Li and Yuan, 1999). Such proteolysis is directly responsible for the characteristic morphological changes associated with apoptosis.

In contrast to apoptosis, necrosis is a passive process in response to an overwhelming stress or damage to the cell (Singh et.al., 1994). In response to stress the cell suffers physiological damage that disrupts osmotic balance, due to the influx of ions. This causes water uptake, swelling of the cell until the membrane ruptures and ultimately the cell bursts (Wyllie et.al., 1980). *In vivo* there is an inflammatory response in surrounding cells due to the release of cellular debris (Schwartz et. al., 1993).

The involvement of apoptosis in recombinant animal cell batch culture and its implications will be discussed later in Section 1.3.

Figure 1.2 Diagram showing the morphological differences between cells undergoing apoptosis and necrosis



(adapted from Williams et al, 1992)

Degeneration by necrosis is usually the result of cell damage. Irreversible damage to cell organelle membranes results in the release of cellular debris. Apoptosis is an active option taken by the cell. Apoptotic bodies are efficiently phagocytosed in vivo without the release of cellular contents.

1.2.1.1 Detection of apoptosis

Model systems have been developed which allow for the study of apoptosis at a biochemical level. An example of such a system is the treatment of immature thymocytes with glucocorticoids, potent activators of apoptosis (Wyllie and Morris, 1982). A morphological change in the nucleus was observed as a result of degradation of the genome. This could be catagorised into two phases; domain cleavage were the genome is cleaved into 200-300 and 30-50 kb pieces, by domain nucleases (Walker et.al.,1991, Dusenbury et.al. 1991) followed by the formation of an oligonucleosomal ladder were further cleavage occurs in the linker regions between adjacent nucleosomes into 180-200 bp by fragmentation nucleases. Detection of a 'DNA ladder' by electrophoresis serves as a classic biochemical marker of the early stages of apoptosis (Afanasev et.al., 1986).

Recently, new protocols for measuring apoptosis have emerged involving flow cytometry, western blotting, light microscopy and fluorescent microscopy. Flow cytometry is used to analyse the extent of apoptosis by using Hoechst 33342 and propidium iodide. This assay distinguishes apoptotic, membrane permeable and viable cells. Apoptotic cells shrink and condense, thus forward light scatter decreases and 90 ° light scatter may increase. Apoptotic cells exhibit increased blue Hoechst fluorescence and low red fluorescence since they initially exclude propidium, viable cells however, exhibit low blue and red fluorescence (Ormerod, 1994). Another method is used which is known as the *TUNEL* assay (terminal deoxynucleotidyl transferase-mediated dUTP nick and end labelling). This involves the labelling of fragmented DNA in cell lines, cultures, cell smears, cell dots and frozen or paraffin-embedded tissue sections. In the plasma membrane phospholipids change with initiation of a protein that binds phosphatidyl serine. Phosphatidyl serine is located on the inner leaflet of the plasma membrane. Entry into apoptosis leads to a translocation to the extracellular side. Thus appearance of a phosphatidyl serine on the extracellular side can be exploited as a marker for apoptotic cells (Fadok et. al., 1992). Annexin V has a high affinity for phosphatidyl serine and therefore can be used to detect apoptotic cells (Homburg, 1995). Annexin can be conjugated to fluoroscein to produce Annexin-V-FLUOS to

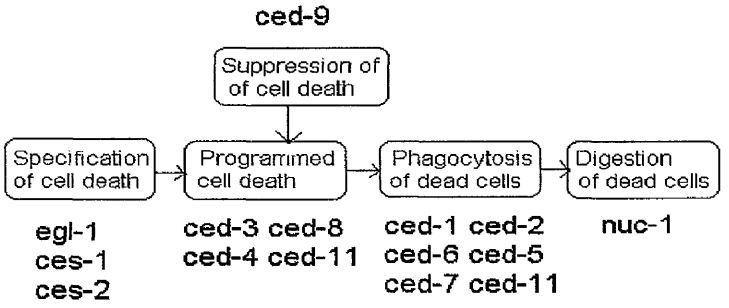
discriminate apoptotic cells. Finally, a method to detect apoptosis-induced proteolysis is with an antibody against PARP Poly (ADP-ribose) polymerase. This is a nuclear enzyme involved in DNA repair (Nicholson et.al., 1995). In many cell types, an early biochemical event in apoptosis is the proteolytic cleavage of PARP by a caspase acting at a highly conserved cleavage site (Lazebnik et.al.,1994). Anti-PARP, a rabbit polyclonal antibody was prepared to detect proteolysis of PARP in cell extracts by western immunoblotting. The appearance of 31 kDa and 85 kDa cleavage fragments is indicative of protease activity (Lazebnik et.al.,1994).

1.2.2 Genes involved in the regulation of apoptosis

Initially, gene products which regulate apoptosis were identified in *Caenorhabditis elegans* (*C. elegans*) the nematode worm. The simplicity of this organism made it a very attractive and powerful model system to determine the mechanisms involved. The discovery of the cell death genes (ceds) in *C.elegans* led to the identification of homologous genes in several species that play major roles in the control of apoptosis.

131 cells out of 1090 somatic cells formed in the adult hermaphrodite, undergo programmed cell death (Ellis et.al.,1991). Mutant nematodes have been identified with defects in different parts of the cell death process and this has allowed a genetic pathway of cell death to be produced.

Figure 1.3 Genes involved in the apoptotic pathway in *C. elegans*



Several groups of genes have been elucidated. This analysis has provided a useful framework for investigation of the molecular mechanisms of apoptosis in mammalian cells. Several nematode cell death genes have been cloned and sequenced (Yuan, 1995). It was found that Ced-9, a suppressor of apoptosis was homologous to the mammalian Bcl-2 family of proteins (Section 1.2.4) and Ced-3 is homologous to the interleukin-1β-converting enzyme (ICE) family of cysteine proteases (Section 1.2.6). ICE's are a family of cysteine proteases with Cys 285 at the active site of the enzyme which activate pro-interleukin-1β by cleavage at 2 aspartate residues at positions 27 and 116 (Thornberry et. al., 1992). It was found that CrmA, a 38 kDa protein encoded by the cow pox virus inhibited ICE in rat fibroblasts which consequently inhibited apoptosis (Ray et.al.,1992). Introduction of Bcl-2 into ICE-expressing cells also provided cell survival (Ray et. al.,1992).

A unified nomenclature for this evolutionarily conserved family that catalyze the cleavage of target proteins at sites downstream of specific aspartic acid residues has been adopted using the term 'caspase' (for cysteine–containing aspartic acid-ases) as the root for serial names. The Ced-4 gene has also been cloned and sequenced (Yuan and Horvitz, 1992) and a mammalian homologue has been isolated (Apaf1) which is known to activate caspase-9, a Ced-3 homologue (Lui et.al., 1996; Yang et.al., 1997; Zou et.al., 1997). *C.elegans* has been useful in predicting the basic elements involved in apoptosis in mammals but there appears to be more complex pathways involved in higher mammals as, for example, Reaper found in Drosophila (White at.al., 1994) is unidentified in *C.elegans*.

1.2.3 The regulation of apoptosis

In response to extracellular stimuli eukaryotic cells recruit signal transduction pathways. It is by this mechanism that cells respond to adverse environments to activate apoptosis. This can be a result of a number of stresses including the appearance or disappearance of hormones or cytokines (Crompton and Cidlowski, 1986; Williams et.al., 1990), changes in direct intercellular interactions (Howe et.al., 1998), DNA damage, UV-irradiation, ionising radiation, heat shock oxidative stress (Clutton, 1997) or nutrient stress and endoplasmic reticulum stress

(Kaufman, 1999). A choice is made within the cell between self-destruction or survival. Some of the signals associated with induction of apoptosis are often involved in promotion of proliferation or differentiation in other cell contexts (Wyllie, 1997). A number of external signals cause execution of apoptosis however, there is much evidence to suggest that signalling pathways converge to one, or very few, common final pathways (Thornberry, 1996). Figure 1.4 outlines some of the pathways involved in apoptosis.

1.2.4 The Bcl-2 family of proteins

Human genes encoding Ced-9-related proteins are known as the Bcl-2 family. The currently known members include two distinct types; pro-survival and pro-apoptotic. At least a dozen members of the Bcl-2 family have been identified in mammalian cells (Figure 1.5). Several others have been identified in viruses: Epstein-Barr virus BHFR1, African swine fever virus (ASFV) and herpes virus saimiri ORF 16 gene (Hale et.al., 1996).

Bcl-2 is the prototype for the subfamily that promotes cell survival in response to a number of stresses including growth factor withdrawal, irradiation and cytotoxicity. This anti-apoptotic function is opposed by Bax and its subfamily members, which at high concentrations, can trigger apoptosis (Brown, 1996). The formation of heterodimers or homodimers between Bcl-2 family members regulates apoptosis in a positive or negative manner (Yang and Korsmeyer, 1996).

All members possess at least one of four motifs known as Bcl-2 homology domains (BH1 to BH4). These regions are required for function as mutations abolished the ability to prevent or delay apoptosis (Sato et.al., 1994; Yin et.al., 1994). Three members of the pro-apoptotic family, Bax, Bak and Bok, resemble Bcl-2 closely but lack the BH4 domain (Hsu et.al., 1997). The other 'killers' possess only the short BH3 domain including Bad (Strasser et.al., 1997; Zha et.al., 1997) and Bim (O'Connor et.al., 1998). This domain is essential for death-enhancing functions of the pro-apoptotic subfamily.

Figure 1.4 Pathways involved in apoptotic cell death

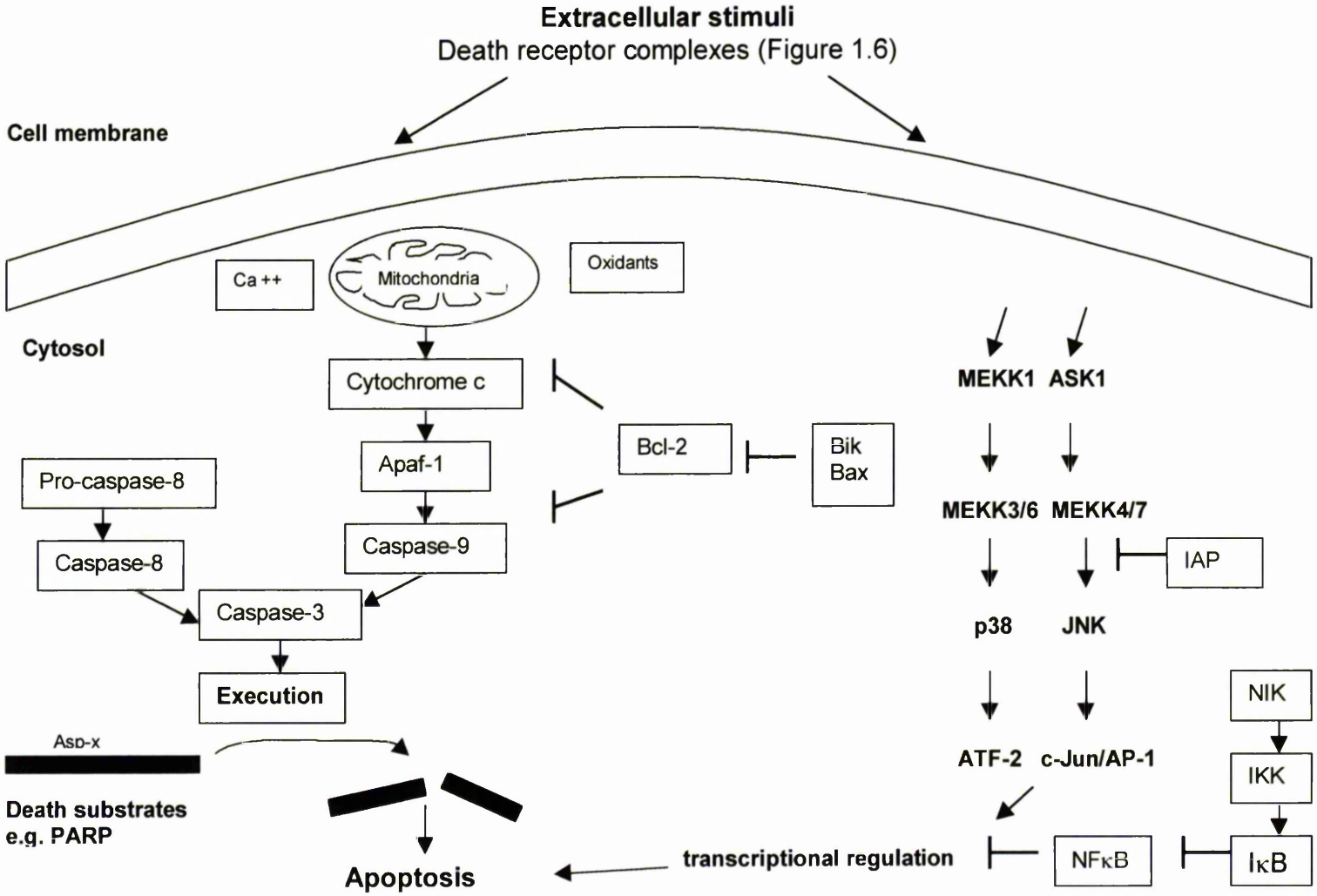
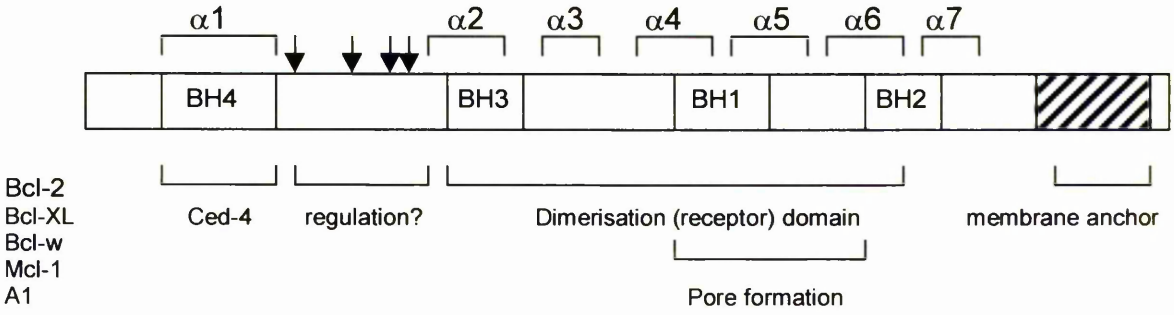
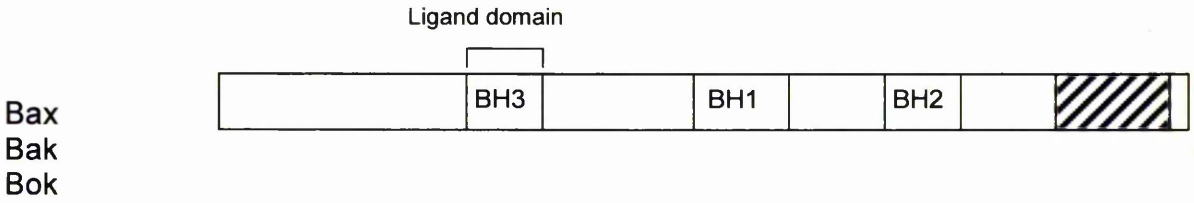


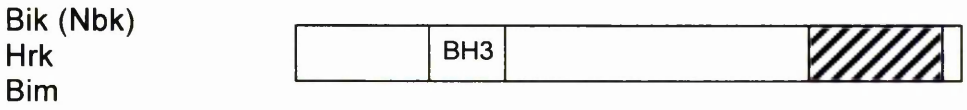
Figure 1.5 Members of the Bcl-2 family

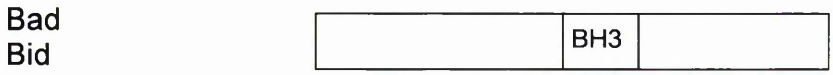
Pro-survival



Pro-apoptotic







The Bcl-2 family. α 1 to α 6 depict regions corresponding to α-helices in the tertiary structure. α5 and α6 are hydrophobic helices implicated in pore formation. BH1 to BH4 (Bcl-2 Homologous domains) are domains are conserved domains between family members, however BH4 is absent in some. Arrows indicate sites of Ser and Thr residues in Bcl-2 phosphorylated by Jun kinase. Three types of protein antagonise the action of the pro-survival family. An example of the first type is Bax, which lacks a BH4 domain, then second and third types (such as Bik and Bad) bear no homology to Bcl-2 except for a short BH3 domain, and can be distinguished by whether or not they bear a hydrophobic COOH terminus (hatched).

Structural studies of Bcl-$_{XL}$ have shown that there is a physical basis for heterodimerisation. BH1, BH2 and BH3 regions create an elongated hydrophobic cleft on the surface of the molecule (Muchmore et.al., 1996) which allows a BH3 amphipathic helix to bind (Sattler et.al., 1997). Pro-apoptotic members bind to this BH3 death promoting region and neutralise the survival function of this subfamily of proteins. When the BH3 region of Bcl-$_{XL}$ was mutated this prevented the formation of a heterodimer with Bad which resulted in enhanced cell survival (Kelekar et.al., 1997). It is becoming clear that there is specificity of interaction between pro- and anti-apoptotic subfamily members. Whereas Bad specifically interacts with Bcl-$_{XL}$ rather than Bcl-2 (Yang et.al, 1995) and Bok inhibits MCl-1 and viral homologue BHRF1 but not Bcl-2 or BcL-$_{XL}$ (Hsu Yu et.al., 1997).

Recently in C. elegans a new death gene has been discovered, egl-1, which encodes a BH3-domain-containing protein that interacts with Ced-9 to cause cell death (Conradt and Horvitz, 1998). This evidence suggests that the mode of cell death regulation in C. elegans is similar to that described above for the Bcl-2 family.

1.2.4.1 Regulation of apoptosis by the bcl-2 family of proteins

In *C.elegans*, Ced-9 and its mammalian homologue Bcl-$_{XL}$ can interact with Ced-4 (the mammalian homologue of which is Apaf-1) (Chinnaiyan et.al., 1997; Spector et.al., 1997; Wu et.al., 1997; James et.al., 1997). Ced-4 can also bind Ced-3 and the Ced-4/Ced-3 interaction stimulates Ced-3 activation (Chinnaiyan et.al., 1997; Seshagiri et.al., 1997; Wu et.al., 1997) (Figure 1.4). It is thought that a complex of Ced-9/Bcl-2 with a Ced-4-like caspase activator and procaspase might represent a regulatory death molecule or 'apoptosome' (Hengartner, 1997) where Bcl-2 blocks apoptosis by inhibiting the activity of the Ced-4-like molecule. Evidence of this in mammalian cells is that Apaf-1 requires cytochrome c and dATP to associate with procaspase-9 (Li et.al., 1997) and to alter Apaf-1 conformation to expose its caspase recruitment domain (CARD) and cause its activation. This sequence of events is dependent on Bcl-2 and its function (Hofmann et.al., 1997). Bcl-2 can

prevent the release of cytochrome c from the mitochondria (Gross et.al., 1999). However, there is no evidence that Bcl-2 binds to Apaf-1.

Bad is phosphorylated via Akt (del Peso et.al., 1997) in cells stimulated with IL-3 and the product is sequestered in the cytosol by binding to 14-3-3 protein (Zha et.al., 1996). Hence phosphorylated Bad cannot bind Bcl-$_{XL}$ and inhibit its survival function. There is evidence that Bcl-2 may also be regulated by phosphorylation (Haldar, 1996; Blagosklonny et.al., 1997; Ito et.al., 1997). Activation of jun kinase (JNK), which accompanied many forms of apoptosis was shown to promote phosphorylation in four sites between BH4 and BH3, a region implicated in negative regulation of its activity (Chang et.al., 1997).

Apoptosis is regulated by changes in mitochondrial flux and loss of voltage potential across the inner membrane (the permeability transition) reflecting opening of the mitochondrial pores (Kroemer et.al., 1997). Bcl-2 can modulate these processes but the mechanism is not understood. It has been suggested that Bcl-2 may modulate mitochondrial pore components (Zoratti and Szabo, 1995). Furthermore, cytochrome c release has been reported to be involved with caspase activation but it remains controversial whether release precedes permeability transition and whether the release is due to channel formation or rupture of the outer membrane (Gross et.al., 1999).

1.2.5 Tumour supressor genes and oncogenes

Induction and inhibition of apoptosis involves a complex network of regulatory signals. Mutations in the specific genes involved can cause illegitimate cell survival, the main cause of tumours and cancer (Wyllie, 1997). To prevent such an occurrence, genes regulating apoptosis are themselves oncogenes and tumour suppresser genes, the expression of which must be efficiently controlled.

1.2.5.1 p53

The phosphoprotein p53 is a tumour supressor, implicated in induction of both growth arrest and apoptosis following DNA damage. p53 null mice develop normally but are prone to a variety of spontaneous tumours by 6 months of age (Donehower et.al., 1992). p53 is a transcription factor (Hariskoshi et.al., 1995). It also functions as a cell-cycle regulator, inducing cell cycle arrest following DNA damage (Kasten et.al., 1991). A radiation-induced increase in p53 levels lead to up regulation of a number of proteins including Gadd45, (growth arrest and DNA damage protein 45) (Canman and Kastan, 1995). The gene for Gadd45 is induced following ionisation radiation and is believed to be important in suppression of cell-cycle progression and is dependent on normal p53 function (Zhan et.al., 1994). Apoptosis is a frequent consequence of DNA damage often via p53 dependant pathway (Cohen et.al., 1992).

p53 causes cell cycle arrest in G1, in cells which have damaged DNA (Ryan et.al.,1993). This allows time for repair before mitosis to avoid mutageneic lesions. However, if the DNA damage is beyond repair then apoptosis will be triggered.

1.2.5.2 c-myc

c-Myc is a transcription factor important for cell proliferation, yet it is also involved in cell death (Green et.al.,1994). Under growth-promoting conditions, such as in the presence of IL-2 or other cytokines, c-myc induces proliferation. However the

continued presence of c-myc under conditions of growth arrest, such as in murine myeloid cells deprived of growth factor, will trigger apoptosis which is p53-dependent (Askew et.al., 1991; Evan et.al.,1992). This is because c-Myc is a transcriptional activator of p53 (Reisman et.al., 1993). It has been shown that c-myc is not an ultimate death gene but it can influence other signals to determine the cell decision to die or survive.

1.2.6 Caspase cascade and downstream targets

The execution phase of apoptosis requires the participation of caspases (Figure 1.6). Caspases are synthesized as inactive precursors (zymogens) containing either long or short N-terminal prodomains in addition to the two mature subunits. Sequential cleavage of the proenzymes often by upstream caspases causes the formation of the active enzyme consisting of a small and large subunit that forms a heterodimer (Cryns and Yuan, 1998). This heterodimer forms a tetramer by binding to an identical heterodimer. The catalytic cysteine residue lies within a conserved QACXG pentapeptide of the large subunit, but both subunits contribute crucial residues to the catalytic site (Nicholson and Thornberry, 1997; Cohen, 1997; Salvesen and Dixit, 1997).

There have been at least 10 caspases found in mammals and it is believed that this may be diversified by the formation of mixed tetramers. Each caspase is thought to have a limited number of targets. Thornberry et.al. (1997) defined the substrate specificity of each human caspase. Three subsets were distinguished by their preference for particular amino acids before the cleavage site. Those in group I (caspase 1, 4 and 5) prefer bulky hydrophobic residues at this position (consensus WEHD), while those in group II, (caspase 2, 3 and 7) require aspartate (consensus DExD), and those in group III, (caspase 6, 8, 9 and 10), favoured a branched chained aliphatic residue (consensus (IVL)ExD).

Activation of Caspase-8 initiates apoptosis by activating downstream effector caspases such as Caspase-3 (Figure 1.6). The effector caspases cleave many cellular substrates including structural proteins (nuclear lamins, actin and gelsolin),

signalling proteins and regulators of DNA replication or transcription (PARP, DNA-dependent protein kinase) (Thornberry and Lazebnik, 1998). Cleavage of these substrates underlies many of the biochemical and morphological events of apoptosis. One such caspase substrate is the inhibitor of caspase-activated DNase (ICAD), also called DFF (Enari et.al., 1998; Sakahira et.al., 1998; Lui et.al., 1997). ICAD binds to CAD (caspase-activated DNase) and keeps it in the cytosol. Cleavage of ICAD by caspases allows CAD to migrate to the nucleus where it executes internucleosomal digestion of DNA (Enari et.al., 1998; Sakahira et.al., 1998). Other cleavage events by caspases cause protein activation rather than destroying protein function. Caspases have been shown to activate sterol regulatory element binding proteins and a number of protein kinases including protein kinase C δ, MEKK1 (an apical kinase of the JNK pathway), and PAK-1 (Cory and Adams, 1998).

1.2.7 Signalling via death domain complexes

A number of receptors have been identified which transduce signals to activate the caspases including Fas (APO1, CD95) (Itoh et.al., 1991), tumour necrosis factor receptor 1 (TNF-R1) (Tartaglia et. al.,1991) and the recently identified death receptors, DR3 (Chinnaiyan et. al., 1996; Kitson et. al.,1996), DR4 (Pan et.al., 1997), DR5 and DR6 (Screaton et.al., 1997) (Figure 1.6).

A consensus sequence found on the intracellular domains was a cysteine-rich repeat, a sequence also found in Reaper in Drosophila (White et. al., 1994). This has been named the death domain (DED) (Golstein et. al., 1995). It is thought that when ligands bind externally such as FasL, Apo2L or TNF, or when there is an extracellular stress (UV), this leads to the recruitment of intracellular death domain proteins to form a death-inducing signalling complex. This then goes on to activate intracellular caspases. Despite the diversity of the extracellular stimuli the execution of apoptosis is usually mediated through the activation of a common sequence of caspases (Ashkenazi and Dixit, 1999).

The complex network of signal transduction pathways in some cell types triggers either apoptosis via caspase activation, or activation of NFκB for survival (Figure 1.4). This mechanism may be controlled by decoy receptors, which function as inhibitors of cell death signalling, known as DcR1 and DcR2 both of which are cell surface molecules (Pan et.al., 1997; Marsters et.al., 1997). Furthermore, co-transfection and overexpression approaches have been utilised to deduce proteins involved with domain protein complex function, including the complex associated with TNF-R1. TRADD (TNF-R1 associated death domain protein), found to be part of the complex, may act as an adapter protein, and may mediate the balance between two antagonistic pathways leading either to death via apoptosis or life via activation of NFκB (Hsu et. al., 1996). The role of TRADD may be to recruit RIP (receptor interacting protein) to the TNF-R1 complex (Stanger et. al., 1995; Ting et. al., 1996). RIP may also play an essential role in mediating the activation of NFκB by TNF. Recruitment of TRAF-2 (TNF-R associated factor) by TRADD, however, is implicated in the activation of JNK/AP-1 pathway in conjunction with ASK1 (Lee et.al., 1997; Nishitoh et.al., 1998). Other proteins, such as Caspase-8 may act as a linkage between the signalling complex and the caspases.

1.2.8 Protein kinase signalling and the activation of downstream targets

Activation of the protease cascade via ligation of Fas cell-surface receptor involves recruitment of ICE-like protease to the receptor complex (Nagata, 1996). However, mechanisms regulating initiation of the apoptotic pathway by the multitude of other pro-apoptotic stimuli are less clear. Many forms of cellular stress can result in apoptosis and these stresses can cause activation of recently identified cellular signalling cascades of protein kinases, the stress activated protein kinases (SAPKs) also called c-Jun N-terminal Kinases (JNKs) and the p38/RK kinases (Kyriakis and Avruch, 1996; Zanke et.al.,1996) (Figure 1.4). The components of these pathways are distinct from the components of the extracellular signal regulated kinase (ERK) pathway. However, they all belong to the same family of mitogen activated protein kinases (MAPK) which contain Tyr/Thr phosphorylation sites required for activity. These pathways amplify the original signal by the activation of sequential phosphorylation reactions. The

emerging picture is that eukaryotes as diverse as yeast and mammals have independently-regulated, parallel signal transduction pathways and activate different members of the MAPK family.

ERK family members are activated by proliferative signals. However, many forms of cellular stress, such as DNA damage, UV radiation, ionising radiation, heat shock and oxidative stress as well as extracellular stimuli acting through receptors (Kyriakis and Avruch, 1996) lead to activation of the SAPK and p38 pathways

1.2.8.1 SAPKs

The SAPKs are activated in response to various stresses in mammalian cells. They were first discovered as the dominant MAP2 kinase activated in rat liver in response to injection of cycloheximide *in vivo* (Kyriakis and Avruch, 1990). Several SAPK have been cloned from mouse sources, including SAPKαI, SAPKαII, SAPKAβ, SAPKAγ and also JNK-1 and JNK-2 from human (Kyriakis and Avruch, 1990). Three SAPK genes have been identified (α, β, γ). These genes are diversified by alternative splicing into at least eight isoforms that are translated into either 54 kDa or 46 kDa polypeptides. JNK-1 is identical to SAPK p46γ, JNK-2, SAPKp54αII (Kyriakis et.al.,1994). All isoforms are proline-directed Ser/Thr kinases and are related to ERK 1/2 of MAPK but are only 40 % homologous. They participate in different pathways and have distinct substrate specificities (Kyriakis and Avruch, 1990). SAPK's are components of novel signal transduction pathways that are activated by oncoproteins, TNF-α (Kyriakis and Avruch, 1990), interleukin-1, UV-irradiation, cellular stresses and osmotic shock (Moriguchi et. al., 1995). The transcription factor c-jun, is a target for SAPK's and becomes phosphorylated on Ser 63 and Ser 73 (Pulverer et. al., 1991) and this event enhances transactivational function (Smeal et. al., 1991). Another important candidate for SAPK substrate is ATF2, a member of the CREB cAMP response element binding protein. ATF2 unlike CREB is activated by stresses that activate SAPK, such as UV radiation (Gupta et.al.,1995).

SAPK's are doubly phosphorylated on a TPY site by a dual-specificity kinase, SEK-1 (SAPK-1 or ERK kinase 1) which shares 45 % homology to ERK activator MEK (Derijard et. al., 1995; Lin et. al., 1995). MEKK-1 lies upstream of SEK-1 and thus SAPKs, without any change in ERK 1/2 activity (Yan et. al., 1994). MEKK-1 in turn, is thought to be regulated by a homologue of Ras. For the SAPK pathways the Ras homologues are Rac-1 and Cdc42Hs, two small G-proteins. Expression of constitutively active mutants of Rac-1 or Cdc42Hs in COS-7 cells increased SAPK activity from 5-10 fold without an increase in ERK activity (Coso et. al., 1995; Minden et. al., 1995).

1.2.8.2 p38

Another MAPK activated by stress (UV or increased cellular stress) is p38 or RK (reactivating kinase) or MPK2, a homologue of the yeast HOG1 gene (Han et.al.,1994; Rouse et.al., 1994; Freshney et.al., 1994). It is activated by phosphorylation on both Thr and Tyr on a TGY site. Two activators of p38 have been characterised, activator of JNK-1 and MKK-3, proteins apparently specific for p38 (Denjard et. al., 1995; Lin et. al., 1995). The known downstream targets of p38 are a Ser/Thr kinase MAP-activated protein kinase (MAPKAP-2) and the transcription factor ATF2 (Rouse et. al., 1994; Freshney et. al., 1994). MAPKAP-2 phosphorylates small heat shock protein Hsp25/Hsp27 *in vitro* (Stokoe et. al.,1992). Hsp25/Hsp27 activation is a prominent part of the response to inflammatory cytokines, physiological stress and growth factors. Another p38 isoform, mxi-2, phosphorylates max (c-myc binding protein) in vitro (Zervous et.al.,1995). p38 has also been shown to phosphorylate Gadd153 in response to stress (Wang, 1996).

1.2.8.2.1 AP-1

AP-1 is a sequence-specific transcription activator composed of members of the Jun and Fos protein families (Angel and Karin, 1991). These proteins which belong to the bZIP group of DNA binding proteins associate to form a variety of homo- and heterodimers that bind to a common site. AP-1 was first identified by

its role in human metallothionein II_A gene regulation (Lee et.al., 1987). It was shown to be a transcription factor that mediates gene induction by the phorbol ester tumour promoter 12-O-tetradecanoylphorbol-13 acetate (TPA) and hence the name TRE (TPA response element) for its DNA recognition site (Angel et.al., 1987). AP-1 activity can also be induced by many other stimuli, including growth factors, cytokines, T cell activators, neurotransmitters and UV irradiation (Angel and Karin, 1991). Several mechanisms are involved in the induction of AP-1 activity and may be classified into those that increase the abundance of AP-1 components and those that stimulate the transcriptional activity of the components. MAPK are involved with the regulation of AP-1 activity. Following mitogenic stimulation, Elk-1, a transcription factor, is rapidly phosphorylated by ERK1/2 (Gillie et.al., 1992; Marais et.al., 1993). This results in the formation of a ternary complex composed of Elk-1, serum response factor (SRF) and the serum response element of the c-Fos promoter (Gillie et.al.,1992). This results in an induction of c-Fos which upon translocation to the nucleus combines with pre-existing Jun proteins to form AP-1 dimers. These are more stable than c-Jun homodimers and increased stability results in higher levels of AP-1 binding activity as it shifts the equilibrium towards dimer formation essential for DNA binding.

The c-Jun promoter contains a TRE sequence with one base change and therefore it is recognized by c-Jun-ATF2 heterodimers rather than AP-1 (van Dam et.al., 1993). Unlike c-Jun, ATF2 is a constitutively expressed protein. However, most cell types contain some c-Jun protein prior to stimulation. Following exposure to stimuli that activate members of the JNK family both c-Jun and ATF2 are rapidly phosphorylated (Derijard et.al., 1994; Devary et.al., 1992; Gupta et.al., 1995). This leads to c-Jun transcriptional induction. Thus, part of the increase in AP-1 activity in response to JNK-activating stimuli (such as TNFα or UV) is due to increased c-Jun synthesis and possibly c-Fos synthesis. Another part of the increase in AP-1 activity is due to c-Jun phosphorylation. In general the activities of both pre-existing and newly synthesized AP-1 components are modulated through their phosphorylation. Different types of MAPK, including ERK and JNK contribute to induction of AP-1 activity in response to a diverse array of extracellular stimuli and each MAPK has a specific substrate. These apparently contradictory observations can be consolidated by assuming that the function of

the AP-1 complex is to activate the transcription of a variety of target genes in response to stimulation of cell surface receptors that are connected to several different transduction pathways. Thus, rather than being a specialized executor of a unique response to exogenous stimuli, the AP-1 complex plays a general role in the transduction of signals from the membrane to the nucleus.

Overall, a major question remains to be answered in relation to the links between SAPK pathways, caspases and the Bcl-2 family of proteins. At present it is known that there are different pathways involved in triggering apoptosis.

1.3 THE PREVENTION OF APOPTOSIS AND CONSEQUENCES FOR RECOMBINANT PROTEIN EXPRESSION

Eukaryotic cells are more fragile than bacteria and the environment typically associated with large scale culture often leads to significant levels of cell death. It is now known that a significant proportion of cells used in the animal cell biotechnology industry in culture systems die via apoptosis , including hybridoma, CHO and myeloma cells. The apoptotic death occurs in response to nutritional and environmental stresses during growth in bioreactors (Al-Rubeai and Singh, 1998; Mastrangelo and Betenbaugh, 1998; Hu and Aunins, 1997).

Intervention with the onset of apoptotic death offers avenues for extended survival of recombinant animal cells at the latter stages of batch culture, a time when cell numbers are maximal and productivity is high. This offers the potential for significant enhancement of product yield and several approaches (from medium manipulation to introduction of anti-apoptotic genes) have been used to inhibit apoptosis (Franek and Dolnikova, 1991; Duval et.al., 1991; Singh et.al., 1996; Levine et.al., 1993; Nunez et.al., 1990; Oslen et.al., 1996; Reed, 1994; Shimizu et.al., 1996; Goswami et.al., 1999).

Many cell types, including NS0 myeloma, CHO and hybridomas, will undergo apoptotic cell death following a limitation of serum, depletion of glucose or exhaustion of particular amino acids (Mercille and Massie, 1994; Mastrangelo and Betenbaugh, 1998; Singh et.al., 1996). Studies have been undertaken to identify

nutritional strategies that can enhance the viability and productivity of cell lines used in biotechnological processes.

Fortification of the culture medium with amino acids or other key nutrients has been shown to suppress apoptosis and significantly extend culture lifetimes (Franek and Dolnikova, 1991; Duval et.al., 1991). As protein production is primarily a function of the viable cell population this approach can improve the yield of recombinant proteins. Recently, it was demonstrated that hybridoma cells on the verge of starvation-induced apoptosis can be rescued by the addition of single amino acids (Franek and Sramkova, 1996). In some instances medium supplementation does not lead to enhanced cellular productivity. Franek and Dolnikova (1991) observed specific productivity in hybridomas was greatest in nutrient-poor medium. However, total production was reduced compared to cells in fortified medium, owing to increased levels of apoptosis due to nutrient-starvation. The production of heterologous proteins, in some cases, may be greatest in growth-arrested or slow growing cells because the cells resources are utilised for protein production rather than for cell proliferation (Franek and Dolnikova, 1991; Suzuki and Ollis, 1990; Al-Rubeai et.al., 1992). Controlled feeding and cell cycle arrest strategies are limited by the abilities of these conditions to stimulate apoptosis.

Genes involved in the regulation of apoptosis continue to be discovered (Section 1.2.2). The overexpression of the anti-apoptotic proto-oncogene Bcl-2 (Section 1.2.4) in a number of cell lines has been used to suppress apoptosis in response to typical stresses in the bioreactor environment, such as nutrient limitation, accumulation of toxins, viral infections, oxygen deprivation and hydrodynamic stress (Singh et.al., 1996; Levine et.al., 1993; Nunez et.al., 1990; Oslen et.al., 1996; Reed, 1994; Shimizu et.al., 1996; Goswami et.al., 1999). Recently, Goswami et. al. (1999) reported that overexpression of Bcl-2 was able to significantly extend the viability of serum-free suspension adapted recombinant CHO cells in batch culture and when insulin or transferrin were withdrawn. In addition, studies of Bcl-2 expression in a Burkitts Lymphoma cell line and two studies of murine hybridomas indicated that Bcl-2 substantially extended the duration of batch cultures by reducing the rate of cell death during decline phase

(Singh et.al., 1996; Simpson et.al., 1997; Itoh et.al., 1995), and following individual exclusion of each amino acid (Simpson et.al., 1998). Importantly, in terms of biopharmaceutical productivity, the extension of batch cultures resulted in a substantial improvement in antibody productivity. In contrast, using the same cell line as Simpson et. al., Fassnacht et. al., (1998) reported that there was no significant difference between antibody productivity between control and Bcl-2 transfected cell lines. However, further studies indicated that Bcl-2 overexpression substantially increased antibody productivity in the same hybridoma in high cell density perfusion culture systems (Fassnacht et.al., 1999). Recent studies of NSO and CHO cell lines, both expressing the same chimeric antibody product showed that cells exhibited significantly improved robustness following Bcl-2 transfection, but no improvement in antibody productivity in batch cultures (Tey et.al., 1999a,b). In addition, Bcl-2 transfected NS0 cells exhibited a substantial increase in maximum cell number and antibody titre compared to control cells (Tey et.al., 1999b). Bcl-2 overexpression also failed to delay apoptosis in NSO myeloma cells in decline phase batch culture or under serum limitation (Murray et.al., 1996), and in CHO Duk cells in batch culture or exposed to staurosporine or hydrogen peroxide (Ang, 1996).

Recently, Simpson et. al (1999) reported that the overexpression of Bcl-2 decreased growth rate and prolonged G1 phase in continuous chemostat cultures of hybridoma cells. They suggested that the lack of increased antibody production after suppression of apoptosis during death phase of batch cultures in some cell culture systems is due to exhaustion of key nutrients. They suggested that overexpressing Bcl-2 to enhance productivity will only work when the suppression of the apoptotic pathway is combined with the perfusion of cultures with fresh medium, or feeding with amino acids, in order to supply the surviving cells with the precursors necessary for gene expression.

It is known that Bcl-2 is able to block two factors involved in the initiation of apoptosis, cytochrome c (Kluck et.al., 1997; Lui et.al., 1996; Yang et.al., 1997) and Apoptosis Inducing Factor (AIF) (Kroemer et.al., 1997; Susin et.al., 1996). It has been suggested that both these factors reside between the inner and outer membranes of the mitochondria and are released in response to apoptotic stimuli,

whereupon they initiate subsequent steps in the apoptotic pathway (Susin et.al., 1996; Lui et.al., 1996). Bcl-2, is able to block apoptosis by blocking the release of these agents from the mitchondria. Cytochrome c has been shown to activate caspases (Zou et.al., 1997). However, the pathway by which AIF causes apoptosis appears to be caspase independent (Susin et.al., 1999). Goswami et.al. (1999) performed experiments to determine if apoptotic pathways existed in CHO cells that are dependent on Bcl-2 and caspases. They found that whereas blocking caspase activity with caspase inhibitors did not inhibit apoptosis occurring via the AIF pathway overexpression of Bcl-2 did. Furthermore, use of a combination of caspase inhibitors and overexpression of Bcl-2 provided a significantly greater protection to the cells. Even when both inhibitor regimes were used cells still eventually died by apoptosis. Their data suggests pathways exist in CHO cells that are either dependent upon, or independent of both caspases and Bcl-2 in CHO cells. Overall this group proposed that pathways involved in triggering apoptosis may include a Bcl-2 dependent pathway involving the activation of caspases, a caspase pathway that is activated independently of Bcl-2, and also a caspase independent pathway. The specific pathway triggered may be dependent upon exposure to different types of stimuli (Goswami et.al., 1999).

Other Bcl-2 family members, such as Bcl-$_{XL}$ have also shown promise for inhibition of apoptosis. Overexpression of this protein delayed the onset of apoptosis in response to nutrient deprivation, irradiation, and glucocorticoid exposure (Boise et.al., 1993; Chao et.al., 1995). Analogues of Bcl-2, E1B-19K from adenovirus and BHRF1 from Epstein Barr virus offer additional alternatives (Rao et.al., 1992; Tarodi et.al., 1994). Other avenues of apoptosis prevention have been explored, such as inhibiting caspases by overexpression of known inhibitors CrmA from cow pox virus and p35 from baculovirus. Under these conditions apoptosis in response to virus infection, nutrient limitation and growth factor withdrawal was delayed (Brooks et.al., 1995; Clem et.al., 1996; Gagliardini et.al., 1994). Other methods for inhibiting apoptosis include the use of chemical inhibitors that block specific pathways involved in the progression towards cell death. Chemicals used to inhibit specific caspases are among a number of cell death inhibitors discovered to date. Z-VAD.fmk, YVAD.cmk, BD.fmk and DEVD-CHO all contain cleavage sites

that match that of a caspase family substrate and each has been used to limit apoptosis (Sarin et.al., 1996; Deshmukh et.al., 1996; Vanags et.al., 1996).

Although much remains to be understood of the factors that regulate the entry of cells into apoptosis there is an emerging picture of initiating mechanisms which switch on the final execution pathway (Dickson, 1998; Anderson, 1997). There are cell specific differences in the responses of cells to intervention regimes and it is difficult to predict an approach that will prevent apoptosis whilst increasing product harvest. However, it would clearly be advantageous to minimise apoptosis or at least have an indicator of the likelihood of entry of cells into apoptosis. At the early stages, after stress perception but prior to commitment to apoptosis, cells could be rescued from apoptosis allowing continuation of healthy and productive culture.

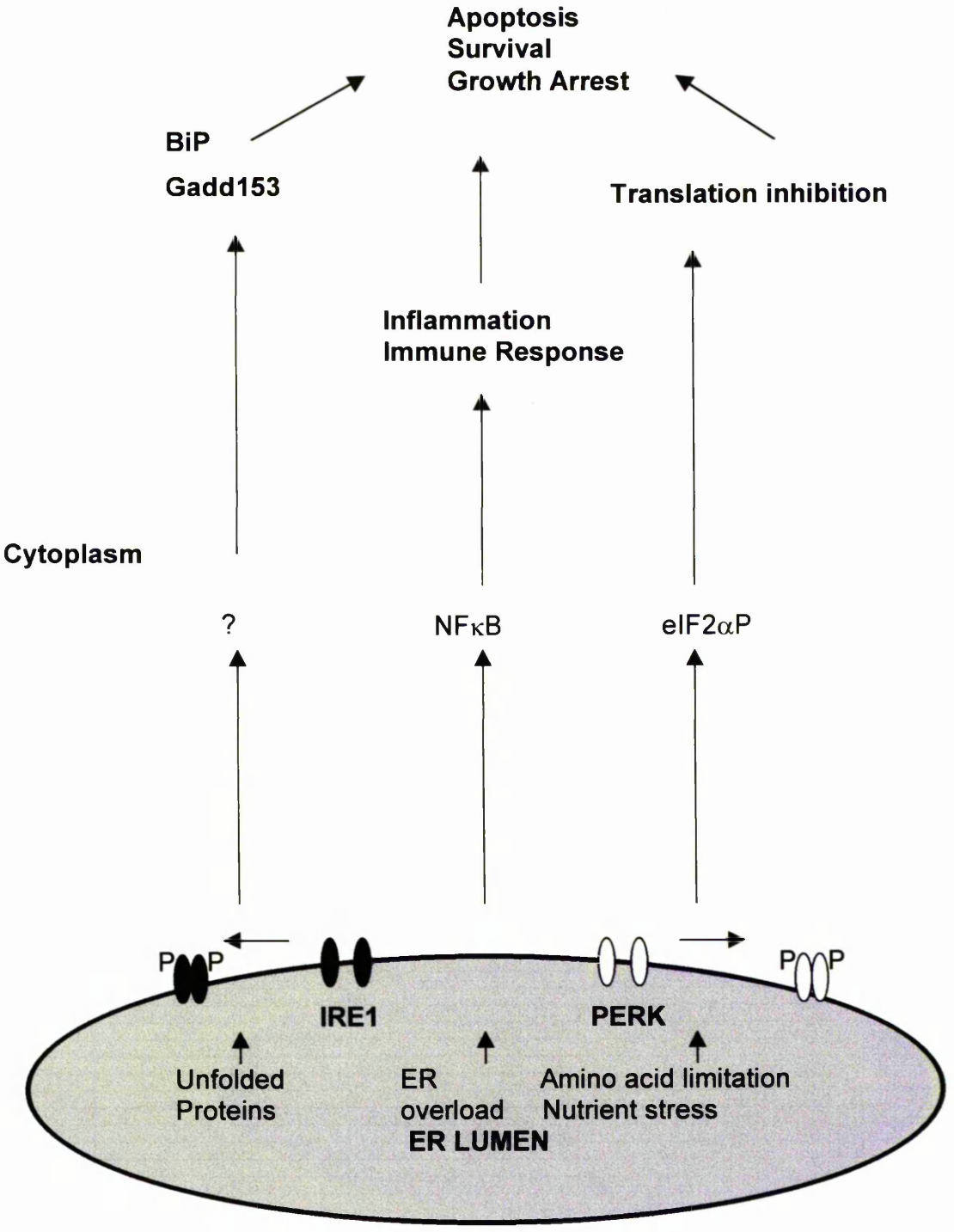
1.4 CELLULAR RESPONSE TO ER STRESS

The endoplasmic reticulum can be thought of as an organelle of eukaryotic cells which has a number of distinct functions. It has an oxidizing environment which is ideal to promote the folding of membrane and secretory proteins destined for the cell surface, lysosomes or the golgi apparatus and it is also involved with sterol lipid biosynthesis. In addition to this, the ER functions as a major signal-transducing organelle responding to alterations in homeostasis due to a variety of stimuli. Signals are transduced from the ER to the cytoplasm and to the nucleus resulting in adaptation for survival or induction of apoptosis. Death-inducing signals emanate from the ER in response to various stresses. Different responses have been found to occur dependent on the stress and are termed the unfolded protein response (UPR) (Section 1.4.2) and the ER overload response (EOR) (Section 1.4.3). Figure 1.7 shows a summary of the pathways involved in the ER stress response.

1.4.1 Protein-folding in the ER

Chaperones facilitate and promote the productive folding of proteins and protein complexes within the ER. They provide two major functions. Firstly, they catalyze

Figure 1.7 Coordination of translation and transcriptional controls in response to ER stress



(adapted from Kaufman, 1999)

protein folding reactions, increasing the rate without changing the pathway to final confirmation, an example of which is Protein Disulphide Isomerase (PDI), and secondly, they maintain the protein in a protein folding-competent state. This means that they prevent protein-folding intermediates from aggregating and stabilise energetically unfavorable conformations of polypeptides to minimise irreversible dead-end protein misfolding, an example of which is Immunoglobulin Binding Protein (BiP) (Dill and Chan, 1997). BiP was first identified as an ER resident protein that bound to heavy chain immunoglobulins and inhibited their secretion in the absence of light chains in pre-B lymphocytes (Haas and Wabl, 1983). In this manner, BiP prevented the secretion of incompletely assembled immunoglobulins. BiP was also found to be expressed at a high level in virally-transformed cells under conditions of glucose deprivation (Lee, 1987). Due to this finding, BiP and other similar proteins were termed the glucose-regulated protein family (GRP's) and BiP (KAR2 in yeast, *S.cerevisiae*) is known as GRP78, as it has a molecular weight of 78 kDa (Tokunaga et.al., 1992). BiP displays a weak ATPase activity that is stimulated by peptides that contain hydrophobic amino acids such as Leu and Phe (Flynn et.al., 1991; Blond-Elguindi et.al., 1993). BiP reacts transiently with these hydrophobic patches preventing aggregation and maintaining a protein folding-competent state. Many other chaperones, including UDP-glucose/glycoprotein glucosyltransferase (UGT) calreticulin and GRP94/endoplasmin, are involved in assuring that only properly folded proteins exit the ER.

GRPs are expressed constitutively in all cells and they are upregulated in response to stresses such as glycosylation inhibition, the presence of reducing agents, heavy metals, amino acid analogues, glucose starvation and pertubation of intracellular Ca^{2+} homeostasis (Kaufman, 1999). ER stress can also take the form of overexpression of mutant proteins or subunits and of the overexpression of some wild-type proteins (Kozutsumi et.al., 1988; Dorner et.al., 1989). In response to this ER stress and induction of the UPR, chaperones such as GRPs and PDI are upregulated and they are therefore considered an ER stress-specific inducible family of proteins.

The UPR is also activated in response to lipid deprivation in eukaryotic cells causing an upregulation in synthesis of lipids and new membrane structures to restore homeostasis. The ER membrane-bound transcription factor sterol regulatory element binding protein (SREBP) is released by sterol-regulated proteolysis and activates genes downstream containing SRE elements which encode proteins involved in fatty acid and cholesterol biosyntheisis.

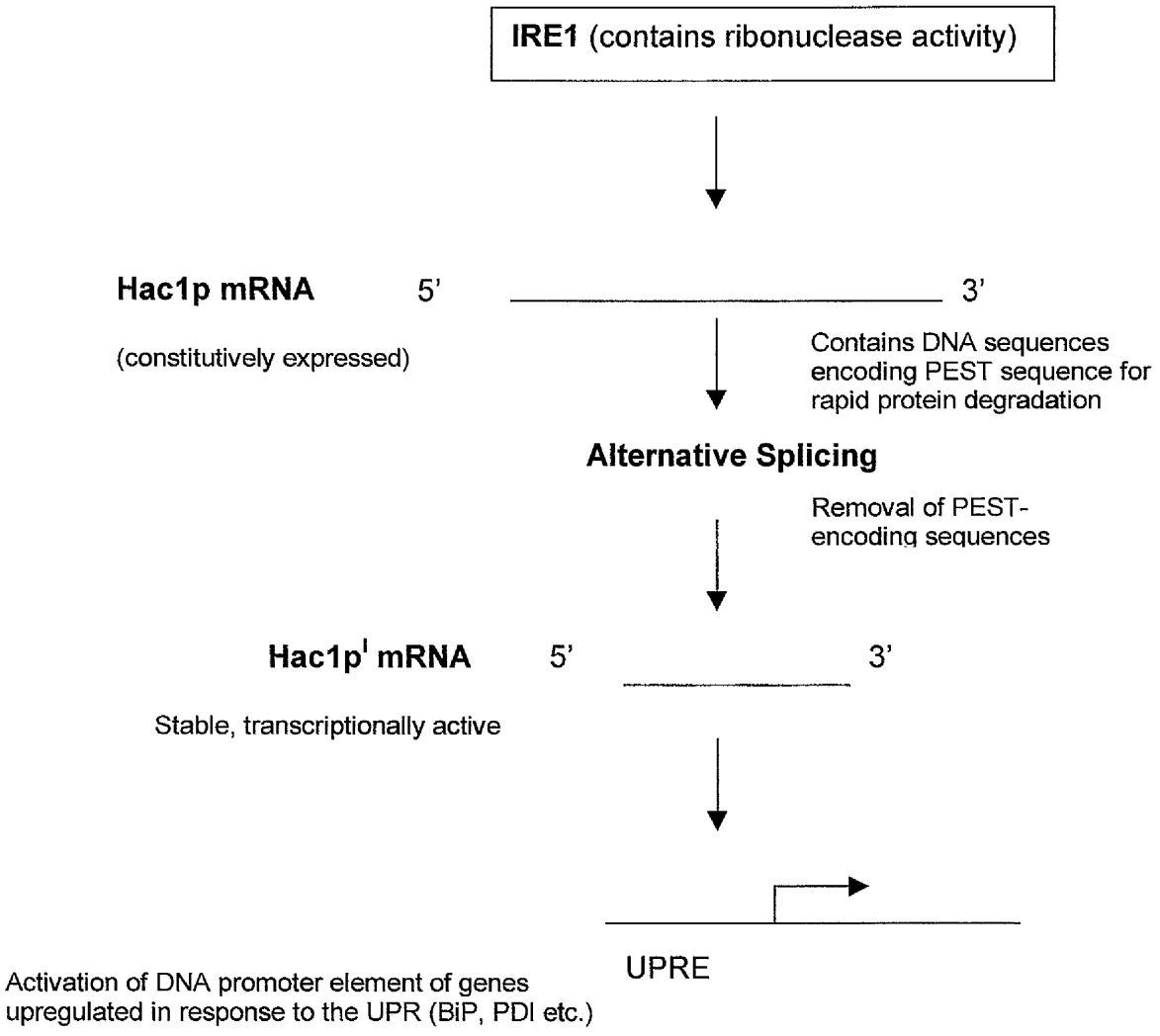
1.4.2 The unfolded protein response (UPR)

The UPR has been shown to exist in mammalian and yeast cells. Although the mechanisms responsible in yeast have been relatively well characterised, much less is understood about the mammalian system. Mediators of the response in yeast include the protein kinases, Gcn2p and Ire1p. Gcn2p is a dedicated eIF2α kinase activated after amino acid deprivation to induce transcription of genes encoding enzymes required for amino acid biosynthesis (Hinnebusch, 1996). Ire1p plays a different role, being the proximal sensor of the UPR in yeast which contains ribonuclease activity (Cox et.al., 1993; Mori et.al., 1993).

The UPR was initially identified in yeast and much of the work associated with understanding the UPR has been carried out in this organism. The UPR in yeast causes activation of the Ser/Thr kinase, Ire1p, which dimerises and trans-phosphorylates itself to elicit an endoribonuclease activity. Its structure is similar to that of a mammalian transmembrane receptor. A recent model of Ire1p has been proposed whereby the kinase resides in the membrane of the ER with its N-terminal facing the lumen. This domain acts as a sensor of stress. Mammalian receptor kinases are activated by dimerisation, and this is the case for Ire1p. Under non-stressed conditions Ire1p is present as an inactive monomer, but after stress it oligomerises, activating the kinase that transphosphorylates and induces the UPR signalling cascade (Shamu and Walters, 1996; Mori et.al., 1993).

Ire1p acts upstream of Hac1p (HAC: homologous to ATF and CREB) (Figure 1.8), since Hac1p mRNA is not spliced in yeast strains lacking the kinase (Cox and Walter, 1996). Hac1p was first identified through binding to an unfolded protein

Figure 1.8 IRE1 downstream targets



response DNA binding element (UPRE) in yeast during ER stress and has been found to be required for the activation of the UPR as Δ hac1p yeast strains cannot induce UPR (Cox and Walter, 1996). Hac1p is regulated by alternative splicing (Figure 1.8). $Hac1p^{U}$ is synthesized constitutively, however, after UPR induction the Hac1p mRNA is spliced to give $Hac1p^{i}$ (Cox and Walter, 1996). This alternative splicing requires tRNA ligase (RLG1) activity but not the spiceosome normally responsible for mRNA processing (Sidrauski et.al., 1996). $Hac1p^{U}$ contains a PEST sequence, to target it for degradation and appears to be more rapidly degraded than $Hac1p^{i}$ accounting for its lack of transcriptional activity in wild type cells. Cox and Walter (1996) proposed a biphasic model were activation of preformed $Hac1p^{U}$ by modifying it to $Hac1p^{i}$ which is more stable and transcriptionally active.

In the last couple of years two mammalian Ire1p homologs have been identified, IREα and IREβ (Wang et.al., 1998). The cDNAs encoding human hIRE1αp and murine mIREβp were characterised and shown to be similar to the yeast (Wang et.al., 1998). They have been proposed to sense stress in the ER and subsequently to activate transcription of the GRPs. The murine and human genetic loci have been termed ern1/ern2 and ERN1 and ERN2 respectively (Tirasophon et.al., 1998; Wang et.al., 1998).

At present a mammalian homolog of HAC1p has not been detected. The activity of the human IRE1αp on a HAC1p substrate was tested *in vitro*. Surprisingly, the human IRE1αp was able to cleave the 5' splice site of HAC1p mRNA at the identical site as the yeast IRE1p (Tiraphon et.al., 1998). The kinase activity was required for cleavage and this finding supports the idea that oligomerisation and trans-phosphorylation of IRE1p precedes and is required for endoribonuclease activation. However, the 3' splice site of the HAC1p substrate was not cleaved by the human IRE1αp (Tirasophon et.al., 1998). It has been suggested that murine IRE1βp may cleave the 3' splice site of yeast HAC1p mRNA and proposed that IRE1βp may form a heterodimer with IRE1αp capable of cleaving both 5' and 3' sites (Tirasophon et.al., 1998). The conservation of kinase and endoribonuclease activities of human IRE1αp compared to yeast Ire1p suggest that they are critical

for transmitting the UPR signal form ER to nucleus. To test the functional requirement for endoribonuclease activity human IRE1αp defective in this activity but retaining kinase activity was overexpressed in mammalian cells (Welihinda, et.al., 1999). Treatment with a glycosylation inhibitor, tunicamycin known to cause the UPR in many cell types (Larrson et.al., 1993; Perez-Sala and Mollinedo, 1995; Dricu et.al., 1996) failed to activate the BiP promoter and the UPR, thus proving that endoribonuclease activity is required for signalling. Another ER stress signalling kinase has been identified, pancreatic eIF2α kinase or PKR-ER related kinase PERK and will be described in Section 1.4.4.

Compared to the yeast UPRE, the mammalian UPRE appears to be more complex. The mammalian UPRE is termed the ER-stress response element (ERSE). This is a common 19 nucleotide sequence motif [CCAAT(N)$_9$CCACG] with a GC rich 9 bp spacer (Yoshida et.al., 1998; Roy and Lee, 1999). It is thought to be responsible for transcriptional induction of BiP, GRP94 and calreticulin in response to ER stress (Yoshida et.al., 1998). A protein complex ER-stress response factor (ESRF) was recently shown to binds to an ERSE in a stress-inducible manner (Roy and Lee, 1999). Identification of the components of ERSF should provide insights into the components that regulate expression from the mammalian ERSE.

1.4.3 The ER-overload response (EOR)

The EOR occurs when there is an overexpression of transmembrane ER-localised proteins (Meyer et.al., 1992; Pahl and Baeuerla, 1995, 1996, Pahl et.al., 1996). This frequently occurs after viral infection producing massive amounts of glycoprotein. In these circumstances Ca^{2+} is released from the organelle. Several mechanisms have been proposed to elicit calcium release and generation of reactive oxygen intermediates (ROI) in response to the EOR (Pahl and Baeuerla, 1996). Overexpression or alteration in membrane proteins may also disrupt the ER membrane and permit Ca^{2+} or glutathione (a weak oxidant) to leak out. Alternatively, membrane alterations may effect the activity of the Ca^{2+}-ATPase to block calcium uptake by the ER. Calcium release leads to the production of ROIs

by the peroxidase moeity of lipoxygenases and cyclooxygenases (Tam et.al., 1995). This generation of ROIs has been shown to cause a downstream transcriptional induction of NF-κB, an important mediator of the human immune and inflammatory response (Figure 1.4 and Figure 1.7) (Baeuerle and Henkel, 1994). The mechanism of action is unknown but increasing the cellular concentration of ROIs causes the phosphorylation and subsequent degradation of I-κB the inhibitory subunit of NF-κB, releasing an active form of NF-κB (Beg et.al., 1993; Sun et.al., 1994). NF-κB heterodimerises, then translocates into the nucleus and increases transcription of target genes involved in the pro-inflammatory and immune response, such as cytokines, haemopoietic growth factors and cell adhesion molecules (Baeuerle and Henkel, 1994). In addition, experiments involving peroxidase inhibition by treatment with tepoxalin prevented NF-κB activation by thapsigargin, thus supporting the involvement of ROI in the calcium-dependent activation of NF-κB (Tam et.al., 1995). As NF-κB activation causes the induction of genes involved in cell survival it has been implicated as having an anti-apoptotic role in response to the EOR.

1.4.4 Apoptosis inducing signals from the ER

1.4.4.1 Translation inhibition

It has recently emerged that different death inducing signals can be generated in response to ER stress (Figure 1.7). Signals include translational inhibition mediated through phosphorylation of the translational initiation factor eIF2α and also IRE1-mediated transcription of CHOP/Gadd153.

An immediate response to ER stress is to inhibit protein translation initiation, protecting cells from further accumulation of proteins to conserve nutrients and energy. Generally, the rate of translation initiation in a cell depends on mRNA abundance, the number of ribosomes, the availability of initiator $tRNA^{met}$ and the amount and activity of eukaryotic initiation factors (eIFs) (Kaufman, 1999). The major mechanism for translational control is the reversible phosphorylation of eIFs,

especially eIF2 (Pain, 1996). The function of eIF2 is to bring the initiator $mRNA^{met}$ to the 40S ribosome where polypeptide chain synthesis is initiated by the formation of a ternary complex with 40S ribosome (eIF2-GTP-$mRNA^{met}$) and the hydrolysis of GTP to GDP (Hershey, 1991). To promote another round of initiation GDP must exchange with GTP by catalysis with guanine nucleotide exchange factor, eIF2B. However, phosphorylation of the α subunit of eIF2 on serine 51 stabilises the eIF2-GDP-eIF2B complex preventing the exchange reaction (Pain, 1996). The phosphorylation of the eIF2α subunit immediately decreases the level of functional eIF2 preventing initiation events on all mRNAs in the cell (Donze et.al., 1995). It has also been demonstrated that apoptosis is triggered in response to phosphorylation of eIF2α under severe conditions of stress (Srivastava et.al., 1998), however the mechanism involved in the downstream activation of caspases has not been examined.

Conditions that disrupt ER function also inhibit protein synthesis and this correlates with increased eIF2α phosphorylation (Prostko et. al., 1992). It is known that the kinases upstream of eIF2α, double-stranded-RNA-dependent protein kinase (PKR) and PKR-ER related kinase (PERK), are activated under conditions of protein synthesis inhibition. PERK, identified independently by Shi et.al. (1998) and Harding et.al. (1999), is an eIF2α kinase localized to the ER. Harding et.al. (1999) demonstrated that PERK phosphorylates eIF2α on serine 51 *in vitro* and *in vivo*. Furthermore, treatment of cells with tunicamycin increased autophosphorylation of overexpressed PERK (Harding et.al., 1999). This supports PERK as a selective eIF2α kinase activated in response to ER stress. It has been noted that PERK has similar properties to Gcn2 and Ire1p in yeast, as it contains a kinase and a luminal ER sensing domain (Harding et.al., 1999).

In addition to translation inhibition, the disruption of ER function leading to apoptosis is triggered by other forms of stress. The transcription factor CHOP (C/EBP homologous protein) or Gadd153 (growth arrest and DNA damage Inducible gene) has been implicated in mediating signals in response to ER stress (Bartlett et.al., 1992; Chen et.al., 1992; Price and Calderwood, 1992; Sunnerhagen et.al., 1995; Carlberg et.al., 1996; Brewer et.al., 1997; Dricu et.al.,

1997). The regulation of Gadd153 and its involvement in the ER stress response will be discussed in Section 1.5.2.

1.5 GROWTH ARREST AND DNA DAMAGE GENE (Gadd153)

1.5.1 Discovery and properties of Gadd153

Genes involved in regulation of cell differentiation and growth arrest in response to various stimuli are potential candidates for apoptosis regulation. Fornace et. al (1989) cloned a unique class of genes from CHO cells which showed induced expression in response to both growth arrest and DNA damage (Gadd). They identified five family members, Gadd45, Gadd153, Gadd34, Gadd7 and Gadd33. It was proposed that Gadd genes could play roles in mammals similar to that of several genes involved in growth arrest in bacteria and yeast (Luethy et.al., 1992). Examples of homologous bacterial genes are sulA, an SOS gene which codes for a protein associated with growth arrest (Lavin and Schroeder, 1988), and RAD9 responsible in yeast, at least in part, for the delay of cell cycle progression following DNA damage (Weinert and Hartwell, 1988).

Gadd153 encodes a 19 kDa protein, often called CHOP, related to the CCAAT/enhancer-binding protein (C/EBP) family of transcription factors (Ron and Habener, 1992). The C/EBPs are bZIP proteins characterised by a conserved leucine zipper domain, through which C/EBP monomers dimerise, adjacent to a basic domain involved in DNA sequence recognition and binding. The C/EBP family consists of five members (C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ and Gadd153). Although Gadd153 forms stable heterodimers with other C/EBPs, its basic region deviates significantly from the consensus defined by other members of the C/EBP family in that it contains proline and glycine substitutions in conserved residues believed to be essential for the interaction of these proteins with DNA-binding sites (O'Neil et.al., 1990; Shuman et.al, 1990). Indeed Gadd153-C/EBP heterodimers fail to bind to several known C/EBP sites *in vitro* suggesting that Gadd153 may function as a negative regulator of C/EBP transcription factors (Ron and Habener, 1992). There is no evidence that Gadd153 forms homodimers. However, Gadd153-C/EBP heterodimers are

capable of binding unique DNA sequences that are distinct from classical C/EBP sites (Ubeda et.al., 1996). Furthermore, it was recently demonstrated that Gadd153 forms stable dimers with other non-C/EBP transcription factors, such as activating transcription factor (ATF3) (Chen et.al., 1996). The latter is encoded by a gene that is itself stress inducible, raising the possibility of stress signalling by Gadd153-ATF3 heterodimers (Chen et.al., 1996).

The Gadd153 gene was found to span five kilobases and contains 4 exons in CHO cells (Park et.al.,1992). The 5' flanking region of the gene, within 420 bp of the transcription initiation site, contains a number of cis-elements associated with transcriptional regulation. These include a Hogness box (ATAAAA) (Lersch et.al., 1989), an inverted GCCAAT box which is a putative binding site for one or more C/EBPs (Mitchell and Tijian, 1989; Chodish et.al., 1988), seven SP1 sites for the binding of RNA polymerase II transcription factor SP1 (Mitchell and Tijian, 1989) and an AP-1 site (Stein et.al., 1989). The Gadd153 800 bp promoter region is rich in G and C content (> 70 %) and contains an unusually long stretch of alternating CpG residues. This promoter region was ligated to the bacterial chloroamphenicol acetyltransferase (CAT) gene. A number of cell lines were transfected with this construct and treated with stress. Inducible expression of CAT was detected but only when the promoter was in its endogenous orientation (Luethy et. al., 1990; Bruhat et.al., 1997).

1.5.2 The regulation of Gadd153

Gadd153 transcription can be regulated by C/EBPs. Evidence to support this was displayed when the overexpression of C/EBPβ transactivated the Gadd153 promoter in HepG2 heptoma cells (Sylvester et.al., 1994). In addition, Fawcett et. al. (1996) reported that multiple protein complexes interact with the Gadd153 C/EBP DNA binding site following cellular stress, including C/EBPβ. This group also demonstrated that C/EBPβ transactivated the Gadd153 promoter in PC12 cells but that physical interaction between Gadd153 and C/EBPβ attenuated this activation, providing evidence for an auto-regulatory mechanism whereby Gadd153 gene transcription is controlled by its own protein product (Fawcett et.al.,

1996). Other protein complexes were found to interact with the Gadd153 C/EBP DNA binding site including ATF4 complexes (also known as CREB2), and also ATF3 complexes. These two complexes bind sequentially to this site in PC12 cells during the first six hours of exposure to stress (Fawcett et.al., 1999). ATF4 bound after 2 hours as Gadd153 mRNA levels increased, and enhanced binding of ATF3 complexes by 6 hours as Gadd153 expression declined. The AP-1 element has also been shown to contribute to transcriptional activation in the Gadd153 promoter in HeLa cells (Guyton et.al., 1996).

Besides regulation at the transcriptional level, Gadd153 it is also controlled by other mechanisms including mRNA stability following gene induction (Jackman et.al., 1994) and post-translationally by phosphorylation (Wang and Ron,1996). Recently, Wang and Ron (1996) purified Gadd153 from COS-1 cells and found that it undergoes inducible phosphorylation on two adjacent serine residues, *in vitro*. Gadd153 was found to be phosphorylated by p38, but not by JNK (Section 1.2.8). A specific inhibitor of p38 MAP kinase, SB203580, abolished the stress-inducible phosphorylation of Gadd153 (Wang and Ron, 1996). Phosphorylation of Gadd153 on these residues enhances the ability of the protein to function as a transcription inhibitor and is also shown to be required for full inhibitory effect of adipose cell differentiation (Wang and Ron, 1996).

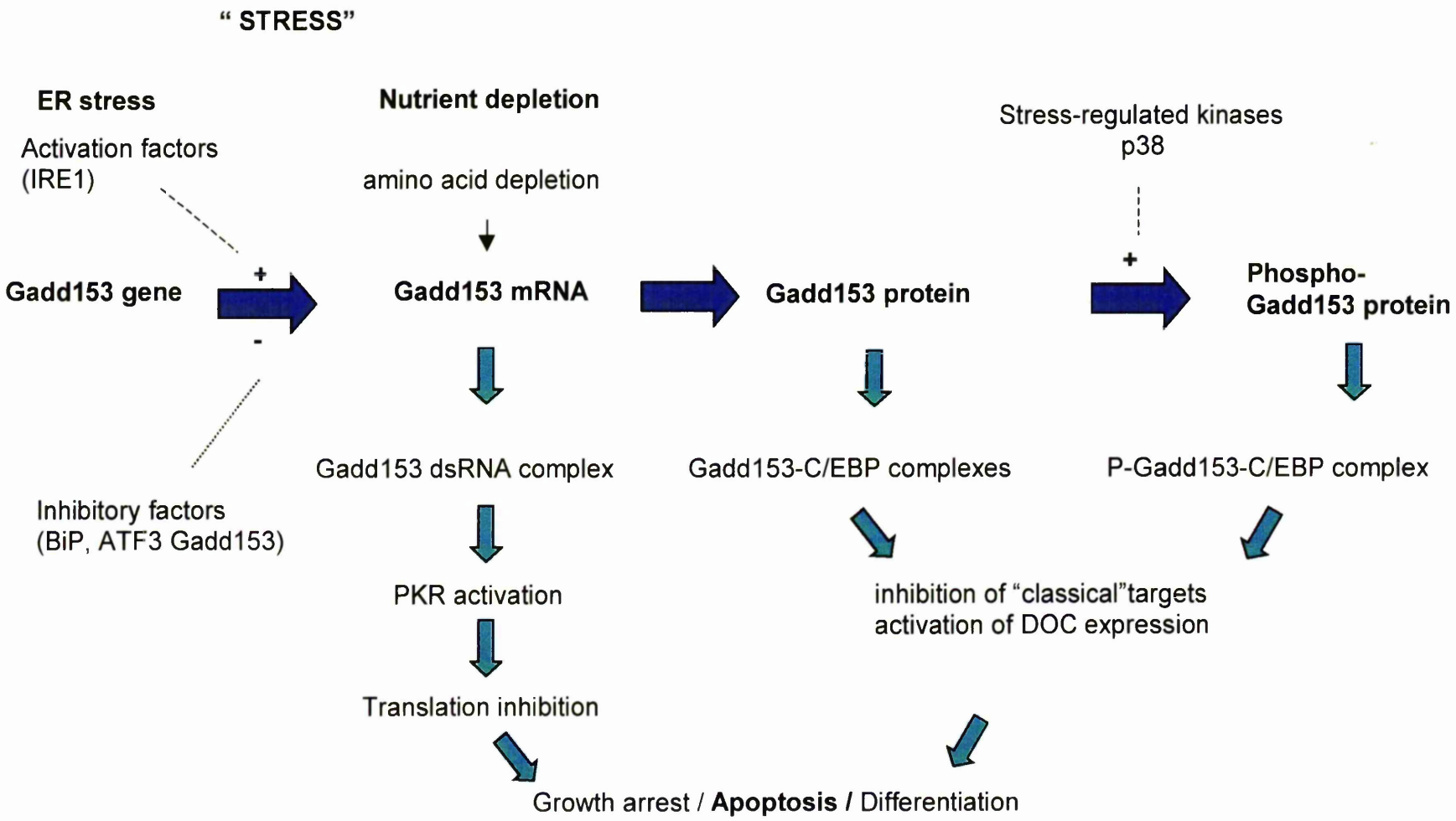
It appears that Gadd153 is regulated by multiple levels of positive and negative signals mediated at the transcriptional, post-transcriptional and post-translational levels during the mammalian stress response.

1.5.3 The cellular functions of Gadd153

1.5.3.1 Gadd153 and apoptosis

A number of investigations have been carried out to determine the role of Gadd153 in the response to cell stress and onset of growth arrest and/or cell death. Figure 1.9 shows a summary of its overall function and roles in the cell. Most evidence supports a role for Gadd153 in negative regulation of cell growth

Figure 1.9 Regulation and downstream targets of Gadd153



and induction of apoptosis. Murine myeloblastic leukemia cells expressed high levels of Gadd153 and died via apoptosis following expose to genotoxic stresses such as methylmethanesulphonate (MMS) or UV radiation (Zhan et. al., 1994). G_1/S arrest of the cell cycle was induced when Gadd153 was microinjected into NIH3T3 cells (Barone et. al., 1994; Zinszner et. al., 1994). Gadd153 was induced via a p53-independent pathway in M1 cells undergoing apoptosis. Furthermore ectopic expression of Gadd153 in these cells caused the execution of apoptosis (Matsumoto et. al., 1996). It has been shown in HeLa cells that activation of Gadd153 expression by MMS is independent of protein kinase C and tyrosine kinases using specific inhibitors (Luethy and Holbrook, 1994). Disruption of the Gadd153 gene is associated with the human cancer, myxoid liposarcoma, a tumour of adipose tissue. A t(12;16) chromosomal translocation results in the expression of an oncogenic fusion protein between Gadd153 and the amino terminus of an RNA binding protein TLS or FUS (Crozat et. al., 1993; Rabbitts et. al., 1993). This suggests that Gadd153 may play a role in an inducible growth arrest pathway triggered by DNA damage or metabolic stress.

1.5.3.2 Gadd153 and ER stress

Investigations have shown that Gadd153 transcription is induced in response to ER stress and there is much evidence to support that the effect is to cause growth arrest and induction of apoptosis (Section 1.4.4).

The deletion of Gadd153 from the mouse genome led to a modest reduction in cell death on activation of the UPR (Zinszner et.al., 1998). In addition, overexpression of BiP prevented Gadd153 induction (Wang et.al., 1996) however, this may be due to BiP interfering with UPR signalling (Morris et.al., 1997). Recently, the regulation of Gadd153 transcription in response to ER stress was investigated by performing transfection studies with hIreα and mIreβ (Wang et.al., 1998). This group demonstrated that both signalling molecules activated Gadd153 transcription, although the physiological significance of this is not known. Furthermore, Ubeda et.al. (1999), investigated mechanisms by which Gadd153 mediates its response to ER stress. It appeared that Gadd153 and methionyl-tRNA synthetase (MetRS)

genes overlapped in a conserved region that controls mRNA stability and therefore they speculated that under stressed conditions, such as amino acid deprivation, Gadd153 and MetRS form a double stranded mRNA hybrid between the two transcripts. In addition this group have demonstrated that an *in vivo* a double stranded RNA-duplex of more than 30 bp activated a double-stranded RNA-dependent signal pathway that controlled the initiation of protein synthesis. This is comparable with previous studies by Haro et.al. (1996) who demonstrated that PKR was activated when bound to segments of double-stranded RNA that resulted in the phosphorylation of eIF2α to inhibit translation initiation.

Overall, these experiments have provided much evidence to support a role for Gadd153 in response to ER stress.

1.5.3.3 Gadd153 and metabolic stress

Gadd153 expression is also increased in response to a number of growth arrest conditions including serum starvation, amino acid depletion, glucose deprivation, oxidative stress and the acute phase response.

Carlson et.al. (1993) demonstrated that glucose deprivation caused an induction of Gadd153 mRNA in HeLa cells and in 3T3 cells. In 3T3 cells, this induction of the Gadd153 protein was found to inhibit adipogenesis in a low glucose environment. A significant induction of Gadd153 transcription was also observed in H4-II-E heptoma cells in response to depletion of single amino acids (Marten et.al., 1994) and there was a similar stimulation of Gadd153 mRNA in HeLa, HepG2 and Caco-2 cells exposed to leucine-depleted medium (Bruhatt et.al., 1997). Recently, Huang et.al. (1999) demonstrated that LLC-PK1 cells incubated in nutrient- and serum-deprived medium had elevated Gadd153 mRNA expression which could be suppressed by readdition of glutamine alone.

As discussed earlier (Section 1.5.3.2) Gadd153 can be induced by conditions which adversely effect the ER. Investigations were carried out by Jousse et.al. (1999) to determine if amino acid limitation regulates Gadd153 expression in a

pathway that was dependent on the UPR. It was found that amino acid depletion did not upregulate BiP expression (a typical UPR marker) but did induce Gadd153 mRNA expression. However, treatment with tunicamycin did upregulate BiP and Gadd153 in the same cells. Furthermore, deletion studies of the Gadd153 promoter revealed a regulatory element between –649 and –190 bps that was involved in mediating gene activation in response to amino acid depletion but did not play a role in induction of the gene by UPR. This data supports the hypothesis that Gadd153 is upregulated in response to amino acid deprivation through a pathway that is distinct from ER stress in the signalling cascade. In yeast it is known that amino acid depletion causes an accumulation of uncharged tRNA species that cause the activation of a kinase Gcn2 (Section 1.4.4.1). It has been suggested that a similar casacade may exist in mammalian cells that can respond to accumulation of uncharged tRNA species and that they participate in the upregulation of numerous genes which may include Gadd153 (Hinnesbusch, 1994).

1.5.4 Downstream targets of Gadd153

Recently, work has focused on identifying Gadd153 downstream targets. Investigations have been carried out to determine if Gadd153 is involved in the activation of gene transcription. In addition, the role of Gadd153 mRNA as a potential signalling molecule has also been investigated. The presently known targets are Downstream of CHOP (DOCs) shown in Figure 1.9.

Initially, Ubeda et. al. (1996) isolated DNA sequences from a random oligonucleotide library to which Gadd153-C/EBP heterodimers were capable of binding. It appeared that in most physiological settings Gadd153 did not accumulate to levels that were sufficient to completely inhibit dimerisation of other C/EBPs present in the cell. Thus, it seems likely that after binding to C/EBPs, Gadd153 mediates its action by activating target promoters to alter gene expression. A number of observations support this hypothesis. High expression of Gadd153 leads to cell cycle arrest (Zhan et.al., 1994) and this property is diminished when the integrity of the putative DNA binding basic region of Gadd153

is disrupted (Barone et.al., 1994). Also the stress-inducible phosphorylation of Gadd153 by p38 MAPK (Section 1.2.8.2) enhanced transcriptional activation of the protein (Wang and Ron, 1996). Based on this knowledge Wang et.al. (1998) identified a number of genes whose induction by ER stress was Gadd153-dependent. They used representational difference analysis to compare the complement of genes expressed in stressed wild-type mouse embryonic fibroblasts with those expressed in cells nullizygous for Gadd153. Gadd153 expression was shown to be absolutely required for the activation by stress of a novel set of target genes referred to as downstream of CHOP (DOCs) (Wang et.al., 1998). DOC6 encodes a putative protein of 819 amino acids, and has close sequence homology to gelsolin (48 % identity). Gelsolin has been found to undergo cleavage by Caspase-3 during programmed cell death, and the N-terminal fragment generated by this cleavage causes the collapse of the actin cytoskeleton, a feature of apoptosis (Kothakota et.al., 1997). DOC6 shares a potential DXXD caspase cleavage site with gelsolin. It has been proposed that an N-terminal cleavage fragment of DOC6 may have similar effects in the actin cytoskeleton in cells exposed to ER stress (Wang et.al., 1998).

DOC4 appears to be a mammalian sequence homologue of a Drosophila protein Temn/Odz. Data suggests that Tenm/Odz has signalling functions involved in development of the early fly embryo (Baumgartner, 1994). A secreted fate for DOC4 is suggested by the fact that the N-terminus of the protein is identical to that of a novel secreted form of the paracrine hormone, heregulin, known as γ heregulin (Schaefer et.al., 1997). Wang et. al. (1998) found that DOC4 protein remained in close association with the cells that produced it and proposed a model whereby expression of DOC4 by stressed cells serves to propagate a signal to adjacent cells. They further speculate that this signal may be important to the phenotypic alterations in cell fate that accompany the regeneration processes that occur when tissues are subjected ER stress. Therefore, DOC6 may cause effects that contribute to apoptosis whereas DOC4 may have signalling properties that effect the process of regeneration.

DOC1 and DOC3 were found to be fragments of a mouse homolog of human and sheep carbonic anhydrase VI (CA-VI). The latter encodes a secreted form of an

enzyme that catalyses the reversible hydration of CO_2 and is known to be expressed at high levels in the salivary gland (Fernley et.al., 1989). Recently, studies by Sok et.al. (1999) identified that the mouse homolog codes for a stress-inducible form of CA-VI downstream of Gadd153 and encodes a protein which is retained intracellularly. The activation of this protein is dependent on Gadd153 and C/EBPβ as activation is absent in cells deficient in either gene. The residues known to form the active site of secreted CA-VI have been conserved in this novel intracellular CA-VI. Therefore it is assumed to contain carbonic anhydrase and esterase activities (Sly and Hu, 1995). It is known that carbonic anhydrase catalyses the reversible hydration of CO_2 to H_2CO_3 (Tashian, 1989). It was hypothesized that inside the cell, where a net production of CO_2 takes place, the cells CO_2 hydration may have the effect of acidifying the intracellular environment i.e. H_2CO_3 into H^+ and HCO_3^-. It is known that cellular pH effects many cellular processes such as the membrane pore-forming activity of pro-apoptotic regulator Bax. This is pH dependent, increasing with decreasing pH (Antonsson et.al., 1997). It is thought that during stress Gadd153 and CA-VI-dependent intracellular acidification may contribute to apoptosis by increasing the pore-forming activity of pro-apoptotic mediators such as Bax.

As mentioned earlier (Section 1.5.3.2), it has been hypothesised by Ubeda et.al. (1999) that Gadd153 may also mediate a response to stress via the formation of a double stranded RNA complex containing Gadd153 mRNA. This complex may be an activator of PKR in response to amino acid limitation to inhibit protein translation.

1.6 BIOLUMINESCENT AND FLUORESCENT REPORTERS OF GENE EXPRESSION IN MAMMALIAN CELLS

Bioluminescence is a natural phenomenon that has been manipulated for use in research. Bioluminescent markers are used as a molecular biological tool in the construction of genetic reporters. This technology could be applied to monitor genes involved in apoptotic cell death. This would have major implications for the biotechnology industry.

1.6.1 Luciferases

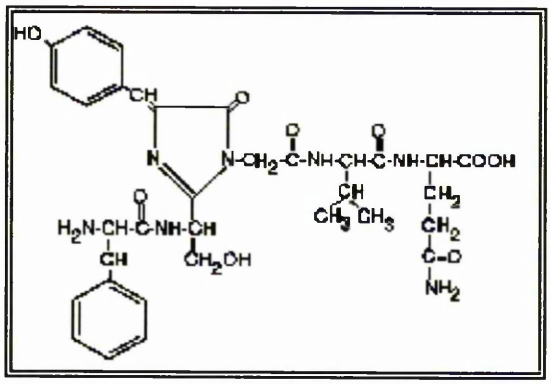
Firefly-luciferase is a monomeric enzyme of 61 kDa and catalyzes a two step oxidation reaction to yield light usually in the green to yellow region (550-570 nm). It offers features that make it a useful reporter. It shows little toxicity or deleterious effects on normal cellular metabolism, is conveniently extracted from plant and animal tissue, and is easily assayed using a luminometer. The sensitivity, wide linear range (eight orders of magnitude), extremely low background, rapid turnover of activity and the relative ease of the luciferase assay has made it attractive to many researchers in studying temporal changes in gene expression (Alam and Cook, 1990; Bronstein et.al., 1994; Brandes et.al., 1996).

Another type of luciferase is *Renilla-luciferase*, a monomeric enzyme that catalyzes the oxidation of coelenterazine to yield coelenteramide and a blue light at 480 nm. Even though as a reporter the *Renilla-luciferase* provides many of the same benefits as firefly-luciferase it offers no particular advantages and its assay chemistry is somewhat more limited. Although it is not generally preferred over firefly-luciferase, it has recently become popular as a companion reporter for experiments where different reporters are required. This so called Dual-Luciferase™ Reporter Assay developed by Sherf et. al. (1996) allows rapid sequential quantification of both *firefly* and *Renilla* luciferases from the same sample whereby *Renilla-luciferase* is most commonly used as an internal control to compensate for experimental variables such as transfection efficiency.

1.6.2 Green Fluorescent Protein (GFP)

GFP, from the jellyfish *Aequorea Victoria*, is also used as a reporter of gene expression. When expressed in either eukaryotic or prokaryotic cells and illuminated with UV or blue light, GFP yields a bright green fluorescence at 509 nm (Ward et.al., 1980). The major advantage of using GFP is its ease of detection and no substrates or co-factors are required (Heim et.al., 1994). Unlike luciferase it is possible to use GFP in many species for live cell detection purposes. The fluorescence of GFPs is dependent on the key sequences Ser-Tyr-Gly (amino acids 65-67). This sequence undergoes spontaneous oxidation to form a cyclised chromophore (Inouge and Tsuji, 1994) (Figure1.10). Enhanced GFP (EGFP) is easier to detect as it has a single, strong, red-shifted excitation peak at 488 nm. It contains mutations of Ser to Thr at amino acid 65 and Phe to Leu at position 64 and is encoded by a gene with human-optimized codons (Cormack et.al., 1996; Haas et.al., 1996; Yang et.al., 1996). Crystallographic structures of wild-type GFP and the mutant EGFP (S65T) reveal that GFP tertiary structure resembles a barrel (Ormo et.al., 1996; Yang et.al., 1996). GFP is a single chain polypeptide of 238 amino acids (Prascher et.al., 1992). Most of these amino acids form β sheets that compacted through an antiparallel structure to form a barrel. An α-helix containing the chromophore is located inside the barrel which shields it from the external environment. The compact structure makes GFP very stable under a variety of conditions, such as high temperature, alkaline pH and chemical reagents (Bokman and Ward, 1981; Ward, 1981; Robart and Ward, 1990). In addition, the signal from GFP does not have any enzymatic ampification; hence sensitivity of GFP is lower than firefly luciferase. For these reasons GFP has limited applications in transcriptional studies.

Figure 1.10 GFP chromophore (Cody et.al., 1993)



1.6.2.1 Destabilised EGFP an its applications

As the stability of GFP was found to limit its application in gene reporter studies studies were carried out to decrease this stability without losing this fluorescence. It is known that most proteins require ubiquitin modifications for degradation, catalyzed by a number of ubiquitation enzymes (Goldberg and Rock, 1992; Gottesman and Maurizi, 1992; Hershko and Ciechanover, 1992; Rechsteiner et.al., 1993). Some proteins, however, do not require ubiquitin modifications such as mouse ornithine decarboxylase (MODC) (Bercovich et.al., 1989; Rosenburg-Hasson et.al., 1989; Murakami et.al., 1992). MODC is the key enzyme in the biosynthesis of polyamines. This is one of the most short-lived proteins having a half-life of 30 minutes. The C-terminus of MODC contains a PEST sequence and its deletion prevents its rapid degradation (Ghoda et.al., 1989). Recently, Li et.al., (1998) fused the degradation domain of MODC to the C-terminus of EGFP. The degradation domain of MODC was found to dramatically decrease the half-life of EGFP in mammalian cells to 2 hours. They then mutated key amino acids in the PEST sequence of the fusion protein and identified several mutants with different half-lives. Furthermore, linkage of the fusion protein to the binding sequences of the transcription factor NFκB allowed detection of the TNFα-mediated NFκB induction in HEK293 cells. The use of destabilised EGFP (dEGFP) as a transcription reporter makes gene induction study possible in real time with living cells (Li et.al., 1998).

1.7 AIMS OF PROJECT

This study has involved an investigation of the feasibility of using a genetic reporter as a monitor of stress in recombinant CHO cell stress. The objective of using this monitor would be to decipher the metabolic and cell signalling events that characterise the early onset of decline phase of batch culture.

Initial work involved investigation of the nature of the cell death pathway in CHO cells at the end of the culture period when cells enter their decline phase. Apoptosis represents an active death pathway where genes have been identified

to regulate the process. Cell death via apoptosis has opened up the possibility of genetically engineering cells to contain an inducible marker of cellular stress.

A further objective was therefore to identify a target gene expressed in a temporal manner upstream of the initiation of apoptosis. A candidate gene (Gadd153) was identified and its endogenous expression was characterised at transcriptional and translational levels in relation to the kinetics of induction and reversibility in response to stresses that cells could encounter in the bioreactor environment. The promoter of this gene was then used in conjunction with a fluorescent marker to test the feasibility of such an approach as an on-line, non-invasive monitor of cellular stress, which may allow for the intervention of apoptosis.

CHAPTER 2 MATERIALS & METHODS

2.1 SOURCE OF MATERIALS

2.1.1 General Chemicals

High grade chemical reagents were used. The source of these chemicals and other products used during this study can be found in Appendix A.

2.1.2 Mammalian cell lines

The serum-dependent, adherent DG44 CHO cell line, its derivative DG44 19.6 (a suspension-adapted, serum-free, recombinant cell line) and HEK cells were provided by Dr. Nigel Woods, British Biotech, Oxford, UK.

CHO Duk cells were provided by Dr. N. Jenkins, Biological Laboratory, University of Kent, Canterbury, UK. The progenitor CHO cells had been irradiated and selected to be phenotypically mutant in the dihydrofolate reductase protein and hence CHO Duk cells were routinely cultured in the presence of hypoxanthine and thymidine (Urlaub and Chasin, 1986).

2.1.3 Bacterial strains

XL1-Blue MRF' strain of *E. coli* is a recombinant-deficient suppressing strain used for plating and growing plasmids, which permits α- complementation (F' episome) with the amino terminus of β-galactosidase encoded in PUC-derived vectors.

JM109 is a rec A- strain which lacks the *E. coli* K restriction system. Thus undesirable restriction of cloned DNA and recombination with host chromosomal DNA were prevented. The endonuclease A- mutation in this strain facilitates improved yield and quality of isolated plasmid DNA. JM109 is deficient in β-galactosidase activity due to deletions in both genomic and episomal copies of the lacZ gene which permits α- complementation (F' episome) with the amino terminus of β-galactosidase encoded in PUC-derived vectors.

The TOP10 bacterial strain was used for general cloning and blue/white screening without IPTG in conjunction with the pCR-TOPO cloning kit (Appendix A).

2.1.4 Solutions

General

All solutions were made using ion-exchange purified water obtained from an Elix 10 system. In the case of molecular biology reactions, solutions were prepared in Milli Q water. All buffers used in the isolation and processing of RNA were made up in Milli Q water which had been treated with the ribonuclease inhibitor, diethylpyrocarbonate (DEPC) at a concentration of 0.05% (v/v) overnight. The solutions were then autoclaved. All solutions were brought to the correct pH by using either hydrochloric acid or sodium hydroxide. The pH was measured at room temperature using an Orion Model digital pH meter with a glass electrode. TE buffer used was made up of 10 mM Tris and 1mM disodium EDTA and the pH was adjusted to 8. All solutions were sterilised by autoclaving unless otherwise stated.

2.1.5 Cell culture media

All solutions were sterilised through a 0.2 μ filter.

2.1.5.1 Growth media for DG44 19.6 CHO cells

For standard culture, DG44 19.6 cells were maintained in growth medium (CHO-S-SFM II), a complete, serum-free, low protein (less than 100 μg/ml) media. The formulation of CHO-S-SFM II is proprietary information of GibcoBRL. I supplemented the basic medium by addition of 25nM Methotrexate and 30 KIU/ml aprotonin.

Other formulations of CHO-S-SFM II were produced by GibcoBRL to assess the consequences of media that lacked either (i) all amino acids or (ii) glucose and glutamine. These media were then made iso-osmotic.

2.1.5.2 Growth media for DG44 CHO Cells

Parental cells were maintained in growth medium (HAMS-F12 + FCS) consisting of HAMS-F12 media supplemented with foetal calf serum (10 % v/v), MEM non-essential amino acids and glutamine. The constituents of HAMS-F12 are shown in Appendix B. Selective medium was prepared by adding G418 sulphate at a concentration of 1 mg/ml to the non-selective media, HAMS-F12+FCS.

2.1.5.3 Growth media for CHO Duk and HEK cells

For standard culture, cells were maintained in growth medium (DMEM + FCS) consisting of Dulbecco's Modified Eagle Medium supplemented with foetal calf serum (10 % v/v), MEM non-essential amino acids and glutamine. CHO Duk cells were also supplemented with hypoxanthine and thymidine (HT supplement). The constituents of DMEM are shown in Appendix B.

2.2 GENERAL CELL CULTURE

2.2.1 Maintenance of cells

All adherent cells were grown at 37°C with a 5% CO_2/ 95% air overlay. Cells were observed under light microscopy. All handling of cells was carried out under strict aseptic conditions in a laminar flow tissue culture cabinet.

Cells were harvested by removing the growth medium, and by washing cell sheets once in phosphate-buffered saline (PBS). Cells were detached from the culture flask by incubating the PBS-washed cell sheet for three minutes with 2 ml of trypsin-EDTA and subsequently tapping the flask to fully detach the cells. An aliquot of the detached cell suspension was counted (Section 2.2.4). The cells were then centrifuged at 130 g for three minutes and resuspended in the appropriate fresh growth medium in flasks to give the required cell concentration.

For suspension-adapted cells an aliquot of cells was taken to determine the cell concentration by counting (Section 2.2.4). The cells were then resuspended in

fresh growth medium in shake flasks (Appendix A) to give the required cell concentration.

2.2.2 Cryopreservation

2.2.2.1 Cryopreservation for adherent cells

Cells were frozen from time to time to maintain stocks. Exponentially growing cells were trypsinised as described (Section 2.2.1). The cell pellet was resuspended in cell medium appropriate to the cell line. The medium was supplemented with dimethylsulphoxide 10 % (v/v). The cell suspension was aliquoted in 1 ml sterile cryotubes and left on ice for 10 minutes. The tubes were placed in a polystyrene box and left at -80°C overnight. The tubes of cell stocks were then frozen in liquid nitrogen the following day for long-term storage.

2.2.2.2 Cryopreservation of suspension cells

CHO DG44 19.6 cells were frozen from time to time to maintain stocks. A volume containing 1 x 10^6 cells was centrifuged at 130 g for 3 minutes. The cell pellet was resuspended in appropriate growth medium supplemented with 25 nM MTX and 30 KIU/ml aprotonin. DMSO was added to give a 10 % (v/v) solution. The process of freezing was as described for adherent cells (Section 2.2.2.1).

2.2.3 Reviving cells from liquid nitrogen

2.2.3.1 Reviving adherent cells

To revive frozen cell stocks, aliquots of cells were removed from the liquid nitrogen and thawed by incubating at 37°C. Pre-warmed media (37°C) was carefully added to the tubes and this was mixed gently. The cell suspension was centrifuged at 130 g for 3 minutes, then resuspended in fresh medium, transferred to a T-flask and placed in the cell culture incubator.

2.2.3.2 Reviving DG44 19.6 CHO cells

To revive frozen cell stocks, aliquots of cells were removed from the liquid nitrogen and thawed by incubating at 37°C. Pre-warmed media (37°C) was carefully added to the tubes and this was mixed gently. The cell suspension was centrifuged at 130 g for 3 minutes, then resuspended in fresh media, transferred to a shake flask, gassed for 1 second with 100 % CO_2 and then placed in a 37 °C incubator shaking at 100 rpm.

2.2.4 Cell concentration and viability determination

Cell samples were diluted 1:1 with 1 % (w/v) trypan blue in 1 x PBS and counted by light microscopy using a haemocytometer (Improved Neubauer). Cells which excluded the blue dye were assumed to be viable, thus total and viable cell concentrations were determined.

2.2.5 Analytical procedures

2.2.5.1 ATP Assay (Stanley & Williams, 1969)

To harvest cell extracts for determination of ATP content suspension cells were centrifuged at 130 g for 3 minutes, washed in PBS and placed in a microfuge tube. 20 % PCA (w/v) was added to the tubes to give a final 2 % PCA solution. Acidified extracts were stored on ice for 15 minutes before centrifugation (12,000 g, 2 minutes, at room temperature) to remove precipitated material which contained DNA (Section 2.2.6). Portions (250 µl) of the supernatants were removed and neutralised with 0.5 M Triethanolamine (TEA) containing 2 M KOH (TEA/KOH). One drop of Universal Indicator was usually added to samples prior to addition of TEA/KOH. Neutralised extracts were either subject to immediate analysis or stored at –20 °C for subsequent analysis.

ATP was analysed by Luciferase. Luciferin/Luciferase was prepared by homogenisation of 50 mg of dried firefly lantern extracts (Type FLE-50) in 10 ml of

water by 8-10 passes of a glass-teflon homogeniser. Extraction continued with a subsequent incubation of extracts in the dark for 30 min at 4 °C and after this time the mixture was centrifuged (17,000 g, 10 min, 4 °C). Portions (1 ml) of the supernatant containing Luciferin/Luciferase were stored at –20 °C until use. The ATP assay buffer constituents were prepared as 3 stock solutions and these were mixed on the day of use (in a ratio of 1:1:1). Assay buffer consisted of (final concentration) 33.3 mM $NaHAsO_4$ (pH 7.4), 26.6 mM $MgSO_4$, 3.3 mM KH_2PO_4 (pH 7.4).

Neutralised PCA-treated cell extracts were thawed and centrifuged (12,000 g, 2 min, room temperature) to remove precipitated $KClO_4$. Portions (10 μl and 20 μl) of supernatant were added to 500 μl of assay buffer and the tubes were kept in the dark for 30 min to remove background luminescence. Luciferin/Luciferase (20 μl) was added to each tube, and after mixing the luminescence was measured immediately for 6 seconds in a LKB Wallac Luminometer (Appendix A). Standards (0-100 pmol ATP) were processed in parallel with the samples.

2.2.5.2 DNA Assay (Burton, 1956)

Acid-precipitable material containing DNA from ATP extraction (Section 2.2.5). was resuspended in 1 ml of 0.5 M-PCA. DNA was extracted by acid hydrolysis at 80 °C for 30 minutes. Cellular DNA content was measured in PCA-precipitated pellets by hydrolysis as above and subsequent treatment as follows. Assay reagent (2 ml) was added to each sample and, after mixing, reactions were incubated overnight at room temperature. Residual particulate matter was removed by centrifugation (1,700 g, 10 min, 25°C) and the absorbance at 595 nm was recorded.

Assay reagent constituents were 100 ml glacial acetic acid to which was added 1.5 g Diphenylamine, 1.5 ml H_2SO_4 and 0.5 ml of 2 % (v/v) acetaldehyde. DNA was quantified by reference to standards (0-50 μg DNA). Stock DNA (1 mg/ml) was prepared by dissolving Calf Thymus DNA in 5 mM NaOH. This stock was stored at -20°C. DNA (0-50 μl) was added to 1 ml 0.5 M PCA and processed in parallel with samples.

2.3 DNA MANIPULATION

All methods described in the following sections were adapted from Sambrook et al. (1989), unless otherwise stated.

2.3.1 General handling of nucleic acids

2.3.1.1 Determination of nucleic acid and bacterial concentration

A sample of the solution to be analysed was taken and, in the case of nucleic acids, it was diluted with a further addition of the same buffer. The true sample reading was calculated by subtracting a buffer blank reading at the stated wavelength. For a solution of nucleic acids, a clean preparation was considered to be one which had a ratio of absorbance at 260 nm to 280 nm of 1.8 or greater. For estimation of the amount of DNA, an absorbance of 1 at 260 nm was equivalent to 50 μg/ml. For estimation of the amount of RNA, an absorbance of 1 at 260 nm was equivalent to 40 μg/ml. For a suspension of bacteria, readings were taken at 550 nm to check that they were in the log phase of growth, i.e. a reading of 0.3-0.5.

Table 2.1 Bacterial strains and plasmids

Plasmid	***Sources***	***Antibiotic selection***	***Bacterial strain***	***Used for;*** *(see Section 2.5)*
Gadd153-CAT	Dr. N.J. Holbrook (Luethy et.al.,1990)	Ampicillin	XL-blue	PCR of Gadd53 800 bp promoter
pGadd153	Sian Leech (made in this laboratory)	Ampicillin	XL-blue	Gadd153 cDNA probe
pGL3	Promega (Appendix C)	Ampicillin	JM109	To generate Gadd153-Luciferase
pd2EGFP	Clontech (Appendix C)	Kanamycin	JM109	To generate Gadd153-d2EGFP

CMV-pRL	Promega (Appendix C)	Ampicillin	JM109	Control transfectant in dual luciferase assays
pCR-TOPO	Invitrogen (Appendix C)	Ampicillin	TOP10	Cloning and sequencing Gadd153 promoter
Gadd153-TOPO1/2	Constructed in this project	Ampicillin	TOP10	For obtaining the Gadd153 promoter by restriction digest
Gadd153-Luciferase	Constructed in this project	Ampicillin	JM109	Transient transfection to measure Gadd153 promoter induction.
Gadd153-d2EGFP	Constructed in this project	Kanamycin	JM109	Stable and transient transfections to measure Gadd153 promoter induction

2.3.2 Extraction of genomic DNA from CHO cells

Medium was removed from the cells and the cell sheets were washed in 5 ml PBS. Cells were trypsinised (Section 2.2.1) and the cell suspension was harvested by centrifugation at 130 g for 4 minutes. The resultant cell pellet was extracted immediately or stored at -20°C for later extraction. For extraction of DNA, 3 ml of extraction buffer (0.1 M EDTA, pH 8.0 containing 0.5% (w/v) Sarkosyl) was added to the pellets which were gently resuspended. The mixtures were incubated at 55°C for 2 hours with DNase-free proteinase K (added to a final concentration of 0.2 mg/ml), with periodic swirling. The DNA was then purified (Section 2.3.3).

2.3.3 Purification of genomic DNA

Genomic DNA was purified using the phenol-chloroform method. A volume of 3 ml of phenol:chloroform:isoamyl alcohol solution, made in a ratio of 25:24:1 (v/v)

respectively, was added to the DNA solution. The tubes were turned end-over-end for 10 minutes and, when an emulsion had formed, the tubes were centrifuged for 15 minutes at 3000 g at room temperature. The aqueous phase was transferred to a clean tube and the extraction was repeated twice with aqueous phases being saved each time. DNA was precipitated at room temperature from the deproteinised aqueous phase by the addition of 0.1 volume of 3M sodium acetate (pH 5.5) and 2 volumes of ethanol. DNA immediately formed a precipitate and could be removed from the tube by centrifugation in a bench-top centrifuge at 3000 g at room temperature. Washed pellets were air-dried and they were resuspended in 250 μl of TE buffer (Section 2.1.4). The absorbance at 260 and 280 nm was measured (Section 2.3.1.1).

2.3.3.1 Phenol:chloroform extraction

This method was routinely used to purify nucleic acids from protein contaminants. Phenol (equilibrated to pH 8 with 0.5 M Tris pH 8) was added to chloroform and isoamylalcohol in a ratio of 25:24:1 respectively. This solution was added in a 1:1 ratio to samples and the tubes were centrifuged at 17,000 g for 3 minutes (unless otherwise stated) to separate the organic and aqueous phases. The aqueous phase containing the nucleic acid was removed, taking care not to disturb denatured proteins at the interphase.

2.3.3.2 Ethanol precipitation

Two volumes of ice-cold absolute ethanol and 0.1 volumes of sodium acetate (pH 5.2) were added to the sample and incubated at -20°C for 30 minutes. Following this, the mixture was centrifuged at 12,000 g at 4 °C for 30 minutes. The pellet was washed with 70 % (v/v) ethanol, left to air dry for a few minutes then resuspended in 20 μl of sterile deionised water.

2.3.3.3 Restriction digestion of DNA

All restriction digests were performed at 37°C for 2 hours unless otherwise stated. The amount of enzyme required to digest 10 μg of DNA was 1 unit (U). Restriction digests of DNA were performed using restriction enzymes with the appropriate 10 × reaction buffer (final concentration being 1 ×).

2.3.3.4 DNA Gel Electrophoresis

Solutions

TBE (10 ×) pH 8	890 mM Tris
	0.5 mM EDTA
	890 mM Boric acid
Loading Buffer (10 x)	20 % (w/v) Ficoll 400
	4 % (w/v) bromophenol blue
	1 mM EDTA
	50 % (v/v) glycerol
Ethidium bromide	5 mg/ml ethidium bromide

Appropriate amounts of agarose were dissolved in 1 × TBE to give an appropriate final concentration of agarose (usually 1 % w/v) by microwave. The solution was then cooled to 60°C. Ethidium bromide was added to give a final concentration of 0.5 mg/ml. After the gel had set, the DNA samples were mixed with loading buffer (diluted 10-fold) before loading onto the gel. The gel was loaded then electrophoresed at 70 V for about 1 hour. DNA was visualised under UV transillumination.

2.4 BACTERIAL CULTURE

2.4.1 Bacterial culture media

The bacterial culture media used was L-Broth (LB) which consists of 10 mg/ml tryptone, 5 mg/ml yeast extract, 5 mg/ml NaCl and 1 mM NaOH. Bacteria were also grown on LB-agar plates which were made from LB supplemented with 1.5% (w/v) agar. Both LB and LB agar culture media were autoclaved before use. For blue/white selection of transformants, 100 μl of 100 mM IPTG and 20 μl of 50 mg/ml X-Gal were spread over the surface of the antibiotic-containing agar plates and allowed to absorb for 30 minutes at 37°C prior to use. Appropriate antibiotics (ampicillin, 50 μg/ml or kanamycin, 50 μg/ml; final concentrations) were added to the LB or LB-agar for bacterial selection.

2.4.2 Generation of competent cells

A single bacterial colony was grown up overnight in 10 ml of LB (Section 2.4.1) at 37°C with shaking. Some of the overnight culture, 2 ml, was added to 100 ml of LB and incubated at 37°C with agitation until the optical density (OD) at 550 nm was about 0.3 (indicating that the cells were in the log phase of growth). Cells were transferred to pre-cooled tubes and these were kept on ice for 10 minutes. Cells were centrifuged at 7000 g for 2 minutes in a JA-20 rotor (all centrifugation steps were at 4°C). The bacterial pellet was resuspended in 2 ml of ice-cold 0.1 M $CaCl_2$ for each 50 ml of original culture. To increase competency cells were left on ice for 4 hours. Frozen stocks were prepared with 15 % (v/v) glycerol. Quickly, portions of the suspension were dispersed into chilled sterile tubes and snap-frozen at -70°C.

2.4.3 Transformation and selection

Competent cells (Section 2.4.2) were transformed with different amounts of plasmids i.e. 10 ng to 100 ng. This involves the addition of 100 μl portions of

competent cells to sterile universal tubes, on ice, containing the above amount of plasmid. The mixture was left on ice for 10 minutes and then the bacteria were heat-shocked at 40°C for 2 minutes, a procedure that allows exogenous DNA to enter the bacterial cell. LB (1 ml) was then added to each universal tube and these were placed in a shaker at 250 rpm for 1 hour at 37°C. Transformants were selected by inoculating LB or IPTG/X-Gal agar plates (Section 2.4.1) containing appropriate antibiotics, with portions taken from each transformation tube (1, 10, 25 μl made up to 50 μl with LB). Plates were incubated overnight at 37°C. The following day colonies were selected. They were used to inoculate 10 ml of LB, with appropriate antibiotic, and then incubated overnight at 37°C with agitation. The bacterial clones were either used for mini-preparation of plasmid DNA (Section 2.4.4.1) or used to inoculate 40 ml LB, containing the appropriate antibiotics for midi preparation of plasmid DNA (Section 2.4.4.2).

2.4.4 Preparation of plasmid DNA

Two types of plasmid isolations were used, (i) midi-preparation for the isolation of large quantities of RNA-free, high quality plasmid DNA by using a QIAGEN Kit (Appendix A) and (ii) mini-preparation for the isolation of a smaller quantity of plasmid DNA when absolute quality of the DNA was not required. For both methods, the main stock solutions were as follows;

Solution I	Solution II
25 mM Tris	0.2 M NaOH
50mM glucose	1 % (w/v) SDS
10 mM EDTA	
(pH 8)	

Solution III

5 M potassium acetate, pH 4.8.

2.4.4.1 Mini-preparation of plasmid DNA

1 ml of the overnight cultures (Section 2.4.3) was removed and centrifuged at 17,000 g for 1 minute. The pellet was resuspended in 200 μl of solution I and left on ice for 10 minutes. 200 μl of solution II was added and the suspension was mixed very gently and left on ice for a further 10 minutes. 150 μl of solution III was added and mixed gently. The mixture was centrifuged at 17,000 g for 10 minutes and the supernatant was removed to a fresh tube. The nucleic acids were recovered by sequential phenol-chlorophorm extraction and ethanol precipitation (Sections 2.3.3.1 and 2.3.3.2). After washing the pellet with 70 % (v/v) ethanol, pellets were air-dried and resuspended in 30 μl of sterile deionised water.

2.4.4.2 Midi-preparation of plasmid DNA

For large scale preparation of plasmid DNA, the QIAGEN midi kit (Appendix A) procedure was used. Bacteria were harvested by centrifugation at 5500 g in a JA-14 rotor for 10 minutes. Bacterial pellets were resuspended in 4 ml of P1 buffer for 5 minutes at room temperature. 4 ml of P2 buffer was added and mixed gently to lyse the cells. 4 ml of P3 buffer was added to precipitate unwanted material and the mixture was left on ice for 15 minutes. The preparation was centrifuged at 20,000 g for 30 minutes at 4 °C in a JA-20 rotor. The supernatant containing plasmid DNA was removed and re-centrifuged. A QIAGEN-tip was equilibrated with 4 ml of QBT buffer and allowed to empty by gravity flow. Supernatant was then applied to the QIAGEN-tip and this was followed by two washes with QC buffer. Finally the plasmid DNA was eluted with QF buffer. DNA was precipitated by addition of 0.7 volumes of isopropanol, mixing and centrifugation at 15,000 g for 30 minutes at 4 °C. Supernatant was removed from the pellet and it was washed with 70 % (v/v) ethanol, air-dried and dissolved in a suitable volume (100-500 μl) of TE buffer, pH 8. Verification of plasmid harvest was achieved by running samples prior to, or after digestion, with appropriate enzymes on a 1 % agarose gel (Sections 2.3.3.3 and 2.3.3.4).

2.5 CONSTRUCTION OF VECTORS

2.5.1 Preparation of Gadd153 promoter insert

The general strategy of vector construction is shown in Figure 2.1. Prior to use as a starting material for subsequent cloning Gadd153-CAT was digested with Cla I and Hind III to confirm the presence of the 800 bp Gadd153 promoter insert (Figure 2.2).

Forward and reverse primers with Eco RI overhangs and Hind III overhangs were designed to amplify the Gadd153 promoter region from the Gadd153-CAT plasmid by PCR (Appendix C). PCR products were analysed by separating on an ethidium bromide stained 1 % agarose gel. The fragment of the correct size (800 bp) was purified using Geneclean II kit (Figure 2.3A and 2.4A) (Section 2.5.2).

2.5.2 Extraction of DNA using Geneclean II kit

The band of the correct size was excised from the agarose gel and placed in 4.5 volumes of sodium iodide stock solution (6 M) and 0.5 volume of TBE modifier. The mixture was incubated in a 50 °C water bath for about 5 minutes until all the agarose had dissolved. 15 μl of well-mixed glass milk was then added. The solution was mixed and placed on ice for 5 minutes to allow binding of DNA to the silica matrix of the glass milk, with mixing every 1 to 2 minutes to ensure that the Glass milk stayed in suspension. The mixture was centrifuged at 17,000 g for 2 minutes and the sodium iodide supernatant was then discarded. The pellet was washed 3 times with ice-cold NEW WASH. During each wash, the pellet was resuspended by pipetting back and forth whilst digging into the pellet with a pipette tip. After it was resuspended, the mixture was centrifuged for 5 minutes at 17,000 g and the supernatant was discarded. The pellet was resuspended in 15 μl of water. The solution was then incubated at 50 °C for 3 minutes and centrifuged at 17,000 g for 30 seconds, eluting the DNA. The supernatant containing the DNA was carefully removed. 2 μl of DNA solution was separated on a 1 % agarose gel containing ethidium bromide (Section 2.3.3.4) to determine the presence and abundance of DNA using the UV transilluminator.

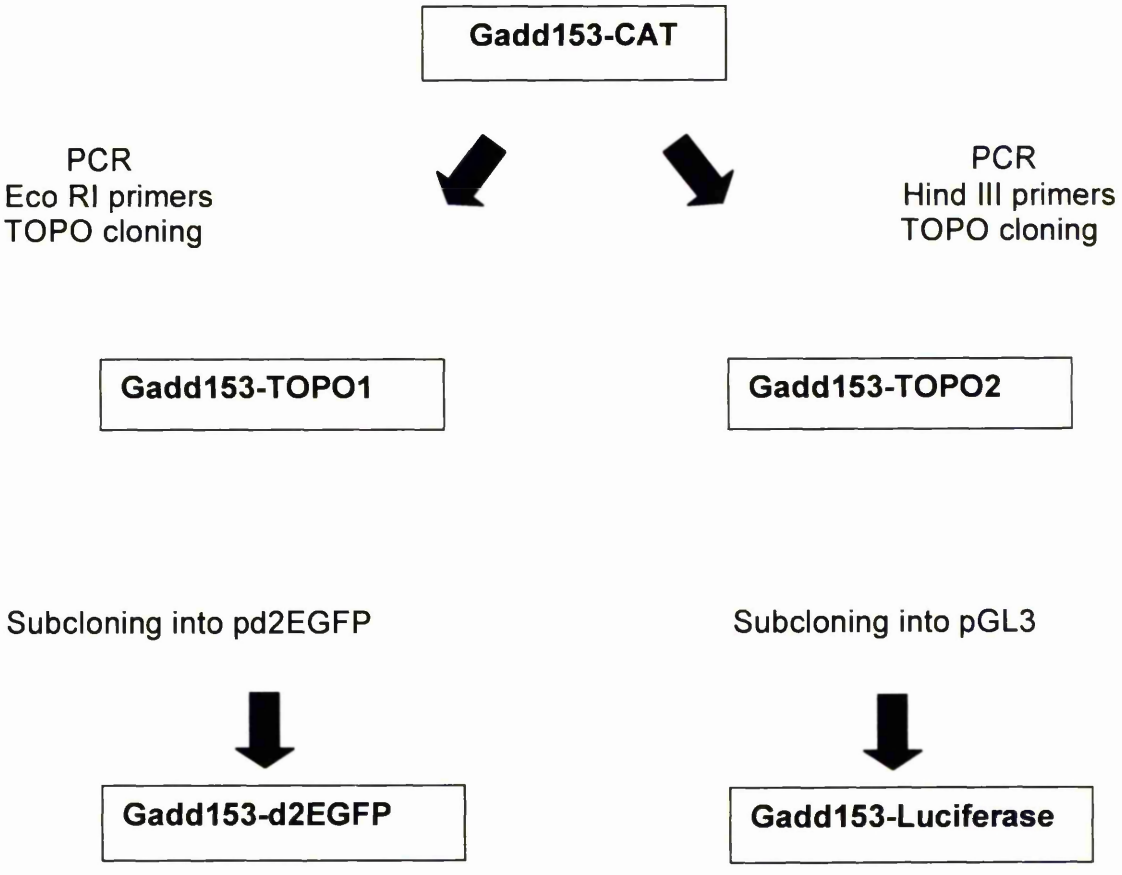
2.5.3 Ligation of Gadd153 promoter fragment into a TOPO cloning vector

The TOPO-Cloning Kit (Appendix A) was used to clone the purified 800 bp Gadd153 promoter PCR product so that the DNA sequence could be verified.

The ligation reaction contained; 2 μl of the purified PCR product (~10 ng/μl), 1 μl of pCR-TOPO vector (Figure 2.3A and 2.4A) and enzyme made up to 5 μl with sterile water. A mock reaction was performed in parallel containing no PCR product. The reactions were mixed gently and incubated for 5 minutes at room temperature. Before proceeding to the next stage the tubes were briefly centrifuged (12,000 g, 10 seconds, room temperature) and placed on ice.

To initiate the One Shot Transformation Reaction, 2 μl of 0.5 M β-mercaptoethanol was added to each vial of TOP10 competent cells and mixed gently with a pipette. A volume of TOPO Cloning reaction (2 μl) of was added to the cells and they were incubated on ice for 30 minutes. The cells were then heat-shocked for 30 seconds at 40 °C without shaking and returned to ice for a further 2 minutes. The mixture was brought to room temperature and 250 μl of LB medium was added and mixed. The tubes were capped tightly and shaken at 37 °C for 30 minutes. The cells were then returned to ice before being spread (25-100 μl) on prewarmed, ampicillin-containing, LB agar plates (Section 2.4.3). The plates were incubated overnight at 37 °C. This procedure was very efficient producing hundreds of colonies. Ten colonies were picked for analysis. Selected clones were cultured overnight in LB medium containing 50 μg/ml ampicillin. Plasmid DNA was isolated using mini-preparations (Section 2.4.4.1) and selection for positive clones involved restriction analysis using Eco RI with Gadd153-TOPO1 (Figure 2.3B) and Hind III with Gadd153-TOPO2 (Figure 2.4B) for appearance of an 800 bp insert. A QIAGEN midi prep was used to produce purified plasmid DNA without RNA or DNA contaminants. The purified plasmid was then used for sequence analysis of the insert (Section 2.7.5). For sequence data see Appendix C.

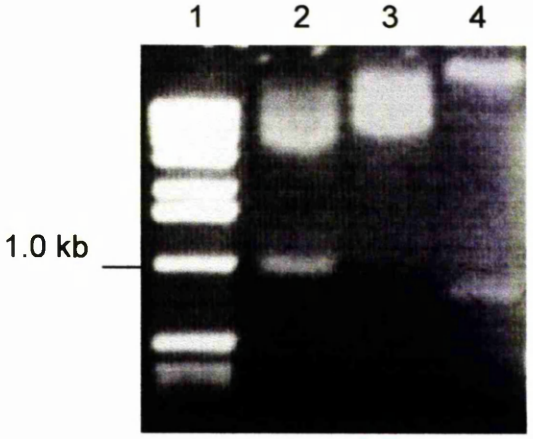
Figure 2.1 General strategy for the construction of expression vectors



The 800 bp Gadd153 promoter was amplified by PCR by Eco RI primers and Hind III primers and cloned in TOPO cloning vectors (Gadd153-TOPO1 and Gadd153-TOPO2). The inserts were then subcloned into pd2EGFP and pGL3 backbones to produce Gadd153-d2EGFP and Gadd153-Luciferase. The sequences of Gadd153-CAT, Gadd153-d2EGFP and Gadd153-Luciferase were aligned and shown to be identical (Appendix C).

Figure 2.2 DNA agarose gel showing digest of Gadd153-CAT plasmid

(A)

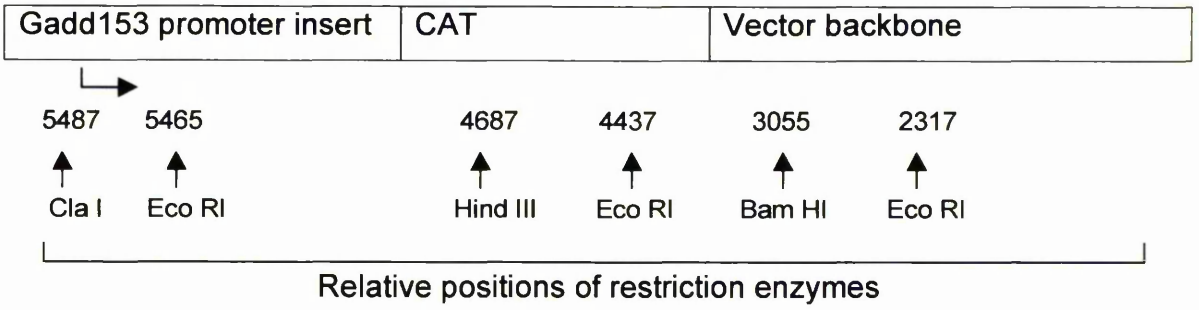


Lane

1. DNA molecular weight markers
2. Eco RI digest
3. Bam HI + Cla I digest
4. Cla I + Hind III digest

(B)

Total length 5487 bp



Relative positions of restriction enzymes

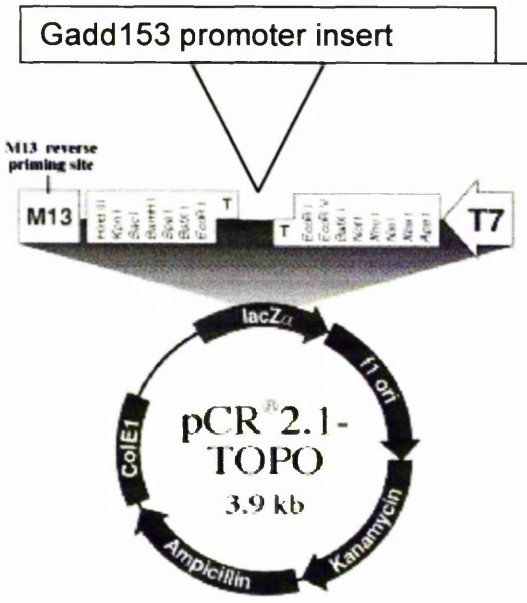
(C) Fragments obtained with restriction enzyme digests

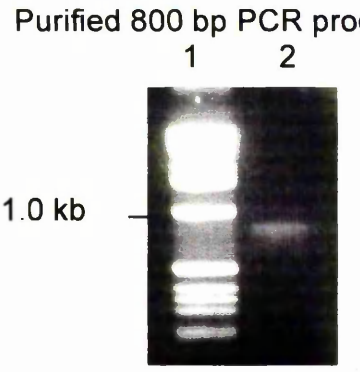
Restriction enzyme	**DNA fragment sizes (bp)**
Eco RI	1050, 2120, 2317
Bam HI + Cla I	3055, 2431
Hind III + Cla I	800, 4687

20 μg of Gadd153-CAT was digested with Eco RI and double digests were performed with Bam HI & Cla I and Hind III & Cla I (Section 2.3.3.3). The digested DNA was subjected to separation on DNA agarose gel electrophoresis, as shown in (A) (Section 2.3.3.4). The schematic (B) shows the relative positions of restriction sites along the plasmid. DNA fragment sizes obtained from restriction digests with various enzymes are shown in (C).

Figure 2.3 Insertion of the Gadd153 promoter (800 bp) fragment into a pCR-TOPO vector

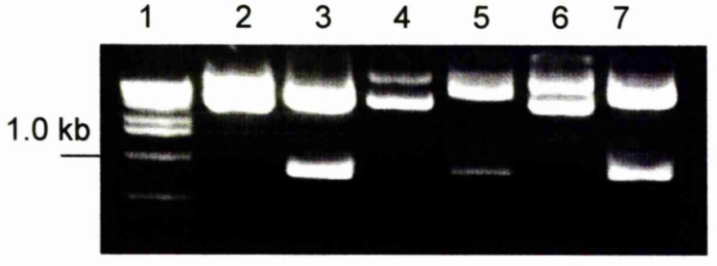
(A)





Lane
1. Molecular weight markers
2. Gadd153 promoter 800 bp insert

(B) Eco RI digests of 3 selected clones



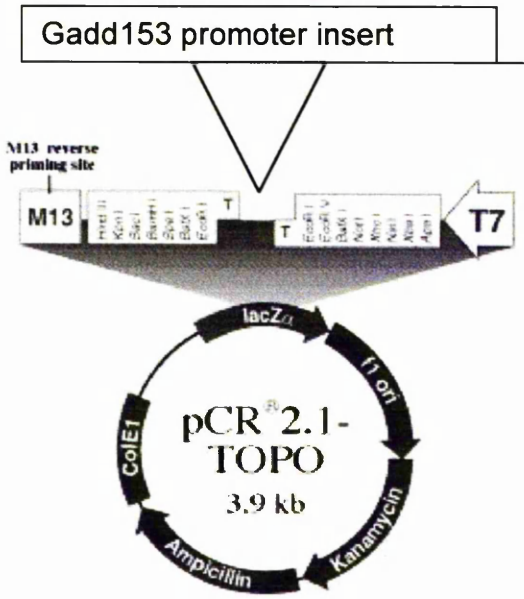
Lane
1. Molecular weight markers
2. Clone 1 undigested
3. Clone 1 Eco RI digest
4. Clone 2 undigested
5. Clone 2 Eco RI digest
6. Clone 3 undigested
7. Clone 3 Eco RI digest

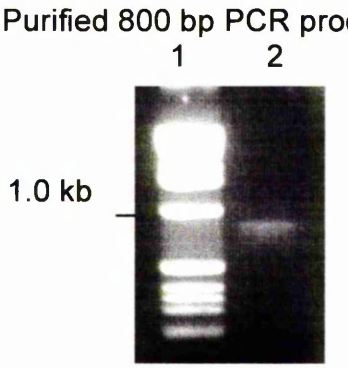
(A) Primers were designed (Appendix C) and the 800 bp Gadd153 promoter insert was amplified by PCR (Section 2.7.4) from the Gadd153-CAT vector. The fragment was purified (Section 2.5.2) and ligated into the pCR 2.1-TOPO cloning vector (Section 2.5.3). TOP10 bacteria were transformed and positive clones were selected (Section 2.4.3).

(B) DNA was extracted by mini-preparation (Section 2.4.4.1) and the presence of an insert was determined by restriction analysis with Eco RI (Section 2.3.3.3) and subjection to 1 % agarose gel electrophoresis (Section 2.3.3.4). The vector inserts were sequenced from clones 1 and 3 (Section 2.7.5). Sequences obtained were compared to that of Gadd153-CAT and showed 100 % homology (Appendix C). A maxi preparation of Clone 1 was performed and renamed Gadd153-TOPO1 (Section 2.5.3).

Figure 2.4 Insertion of the Gadd153 promoter (800 bp) fragment into a pCR-TOPO vector

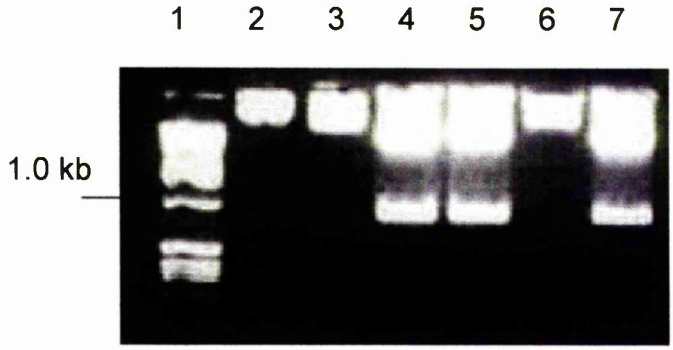
(A)





Lane
1. Molecular weight markers
2. Gadd153 promoter 800 bp insert

(B) Hind III digests of 3 selected clones



Lane
1. Molecular weight markers
2. Clone 1 undigested
3. Clone 2 undigested
4. Clone 1 Hind III digest
5. Clone 2 Hind III digest
6. Clone 3 undigested
7. Clone 3 Hind III digest

Primers were designed (Appendix C) and the 800 bp Gadd153 promoter insert was amplified up by PCR (Section 2.7.4) from the Gadd153-CAT vector. The fragment was purified (Section2.5.2) and ligated into the pCR 2.1-TOPO cloning vector (Section 2.5.3) as shown in (A). TOP10 bacteria were transformed and positive clones were selected (Section 2.4.3). DNA was extracted by mini-preparation (Section 2.4.4.1) and the presence of an insert was determined by restriction analysis with Hind III (Section 2.3.3.3) and subjection to separation by 1 % agarose gel electrophoresis (Section 2.3.3.4) as shown in (B). The vector inserts were sequenced from clones 1 and 3 (Section 2.7.5). A maxi preparation of Clone 1 was performed and renamed Gadd153-TOPO2 (Section 2.5.3).

2.5.4 *Linearisation and dephosphorylation of plasmids*

pGL3 (promoterless Luciferase plasmid) was linearised with Hind III (Figure 2.5) and p2EGFP-1 (promoterless Destabilised Enhanced Green Fluorescent Protein plasmid) was linearised with Eco RI (Figure 2.6). The Gadd153 promoter insert was cut from Gadd153-TOPO1 and 2 by digesting with Eco RI and Hind III respectively (Figure 2.5 and 2.6). DNA fragments required for vector construction were excised from the gels and purified using Geneclean II (Section 2.5.2). The terminal phosphate groups on pGL3 and pd2EGFP fragments were eliminated to decrease the probability of self-ligation. This was achieved by incubation of the linearised plasmids with 1 U/μl alkaline phosphatase and the appropriate incubation buffer. The mixture was incubated at 37 °C for 30 minutes and the DNA was recovered using Geneclean II (Section 2.5.2).

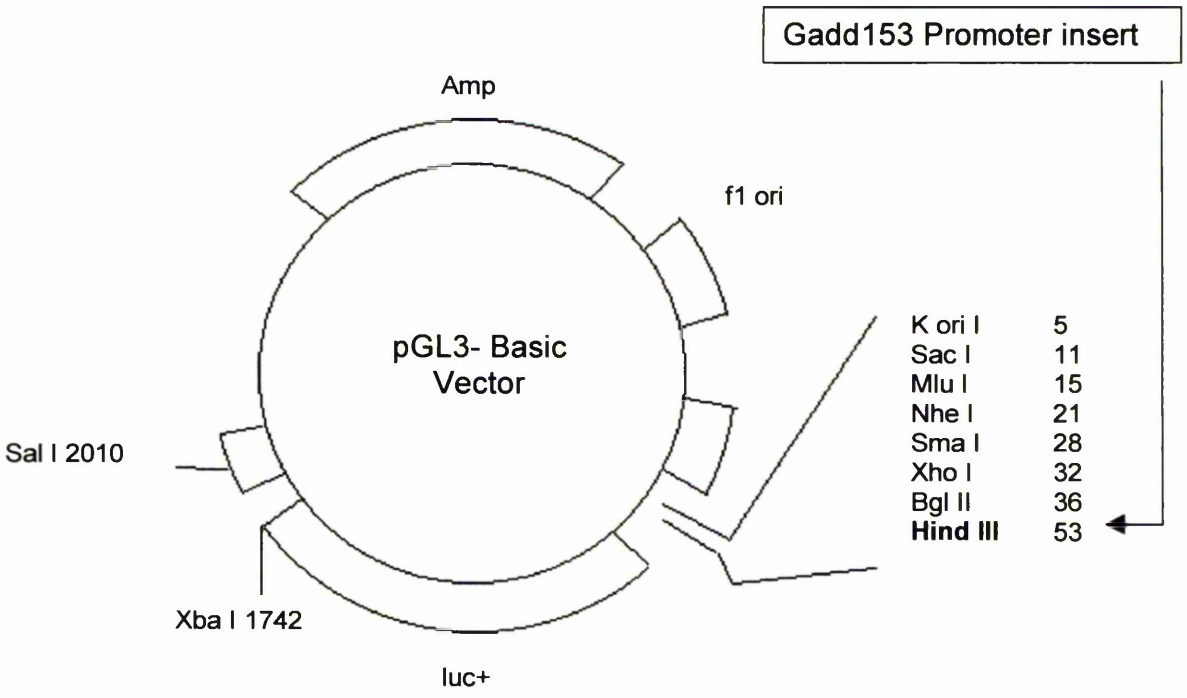
2.5.5 *Ligation and isolation of clones*

Ligation was performed by incubation of dephosphorylated vectors with the 800 bp Gadd153 promoter inserts with 20 U T4 DNA ligase and T4 DNA ligase incubation buffer at 15 °C overnight. Different molar ratio of vectors and insert were used i.e. 3:1, 1:3, 1:1 maintaining the amount of vector at 1 μg. To stop the reaction it was heated to 65 °C for 10 minutes and then placed on ice. This mixture was then used to transform competent bacterial cells (Section 2.4.3). The strain of competent cells used for the transformation of both Gadd153-Luciferase and Gadd153-d2EGFP vectors was JM109. The transformants for Gadd153-Luciferase were selected on ampicillin-containing LB plates and colonies were picked and grown in 10 ml LB supplemented with ampicillin at 37 °C with shaking overnight (Section 2.4.3). Gadd153-d2EGFP transformants were selected on kanamycin-containing LB plates and colonies were picked and grown in 10 ml of LB supplements with kanamycin at 37 °C with shaking overnight (Section 2.4.3). Plasmid DNA from the selected colonies was purified using the mini-preparation method (Section 2.4.4.1). Plasmid DNA from Gadd153-Luciferase colonies were digested with Hind III (Figure 2.7A). A single Gadd153-d2EGFP colony was recovered and plasmid DNA was digested with various enzmes (Figure 2.8A)

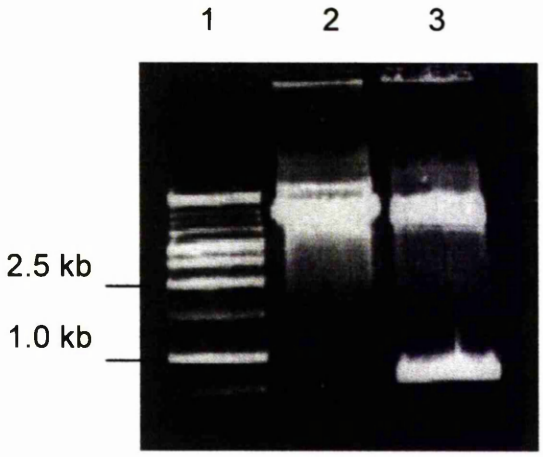
(Section 2.3.3.3). Digested DNA was electrophoresed on ethidium bromide stained gel (Section 2.3.3.4) to check for insert. Digestions were performed to show insert orientation for Gadd153-d2EGFP, shown in Figure 2.8A and for Gadd153-Luciferase, shown in Figure 2.9.

Gadd153-Luciferase was digested with Apa I and Bam HI giving bands with the molecular weight 2061 and 3557 bp respectively. The wrong orientation would have shown bands at 2776 and 2842, probably not distinguishable from each other. Gadd153-d2EGFP was digested with Apa I showing a band of 4943 bp. The band of 122 bp is not visible on this image but could be seen under the transilluminator. The wrong orientation would have shown bands at 4572 and 808 bp and this is obviously not the case. The insert was definitely present in this clone as it was sequenced, therefore I conclude that it was correct.

Figure 2.5 Digestion of pGL3 and Gadd153-TOPO2 to obtain DNA fragments required for the construction of Gadd153-Luciferase



Hind III digests

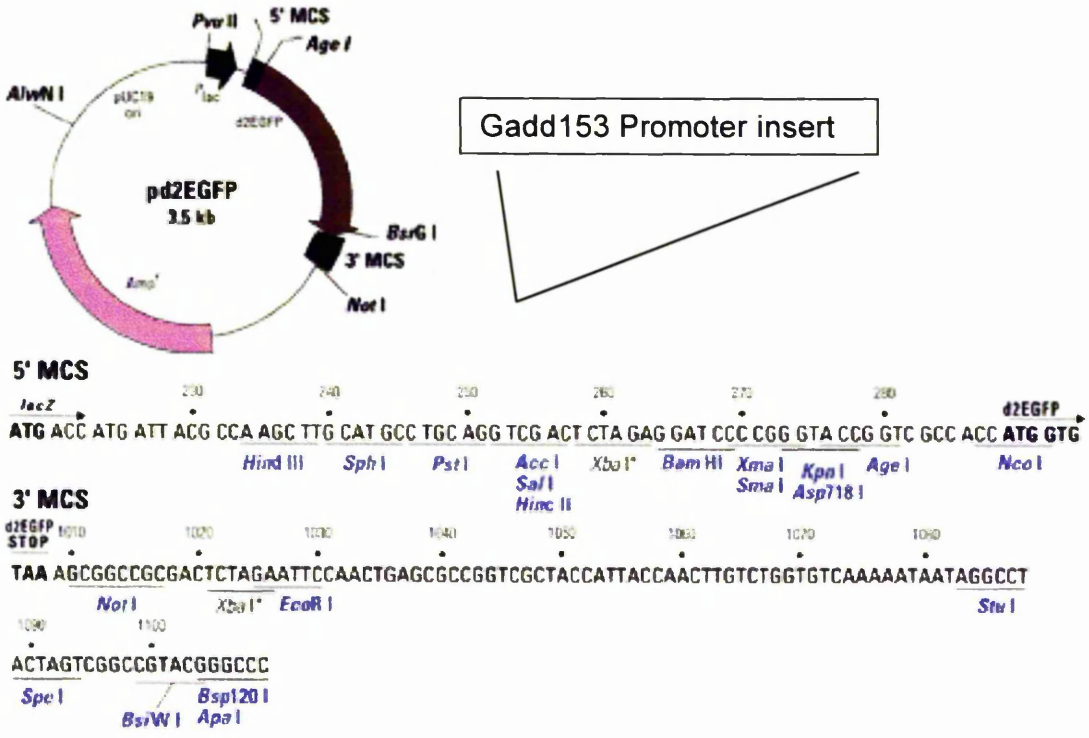


Lane

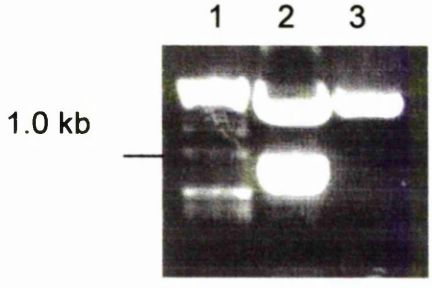
1. Molecular weight markers
2. pGL3 Hind III digest (4818 bp band obtained)
3. Gadd153-TOPO2 Hind III digest (3900 bp and 800 bp bands obtained)

The sequenced Gadd153 promoter 800 bp fragment was excised from 20μg of Gadd153-TOPO2 using Hind III and 20 μg of pGL3 was linearised with Hind III (Section 2.3.3.3). The digests were subjected to 1 % agarose gel electrophoresis (Section 2.3.3.4). The two respective bands obtained (4818 bp and 800 bp) were excised from the gel and purified (Section 2.5.2). Ligation between the Gadd153 promoter insert and extracted, linearisedpGL3 was performed overnight. The ligation reaction was transformed into JM109 bacteria (Section 2.5.5).

Figure 2.6 Digestion of pd2EGFP and Gadd153-TOPO1 for DNA fragments required for construction of Gadd153-d2EGFP



Eco RI digests of Gadd153-TOPO1 and pd2EGFP



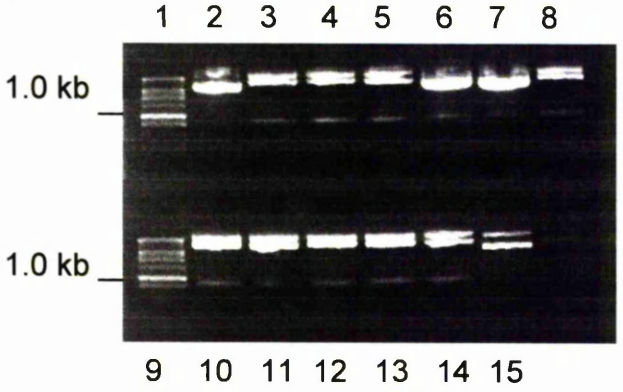
Lane

1. Molecular weight markers
2. Gadd153-TOPO1 Eco RI digest
   (3500 bp and 800 bp bands obtained)
3. pd2EGFP Eco RI digest
   (3500 bp band obtained)

The sequenced Gadd153 promoter fragment was isolated from 20μg of Gadd153-TOPO1 using Eco RI and 20 μg of pd2EGFP was linearised with Eco RI (Section 2.3.3.3). The digests were subjected to 1 % agarose gel electrophoresis. The two respective bands obtained (3500 bp (pd2EGFP) and 800 bp) were excised from the gel and purified (Section 2.5.2). Ligation reaction between the Gadd153 promoter insert and extracted, linearised pd2EGFP was performed overnight (Section 2.5.5). The ligation reaction was transformed into JM109 bacteria (Section 2.4.3)

Figure 2.7 Identification of clones containing Gadd153-Luciferase with (800 bp) promoter insert

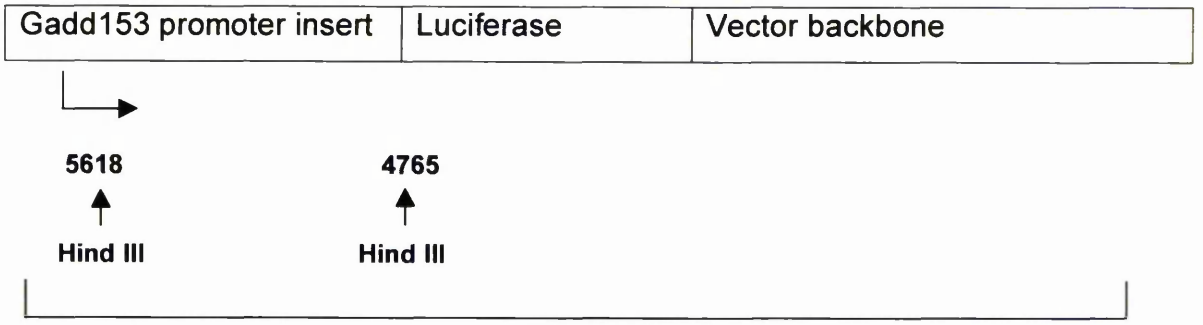
(A)



Lane
1. Molecular weight markers
2. Undigested plasmid
3. Clone 1
4. Clone 2
5. Clone 3
6. Clone 4
7. Clone 5
8. Clone 6
9. Molecular weight markers
10. Clone 7
11. Clone 8
12. Clone 9
13. Clone 10
14. Clone 11
15. Clone 12

(B)

Total length 5618 bp



Relative positions of restriction enzymes

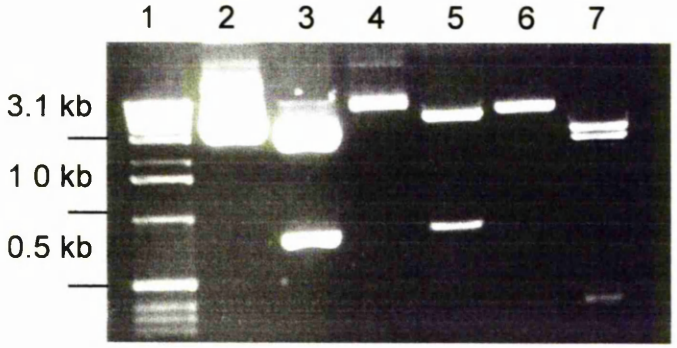
(C) Fragments obtained with restriction enzyme digests

Restriction enzyme	DNA fragment sizes (bp)
Hind III	800, 4818

Competent JM109 bacteria were transformed with overnight ligation reaction. Twelve colonies were selected and plasmid DNA was isolated from mini- preparations (Section 2.4.4.1). DNA from each colony was subjected to restriction analysis (Section 2.3.3.3) with Hind III for presence of the 800 bp insert as shown in (A). All clones selected contained an 800 bp insert apart from Clone 12. A schematic in (B) shows the relative positions of restriction sites along the plasmid. Fragment sizes obtained in insert containing plasmids are shown in (C).

Figure 2.8 Determination of Gadd153-d2EGFP plasmid and the presence and orientation of (800 bp) insert

(A)

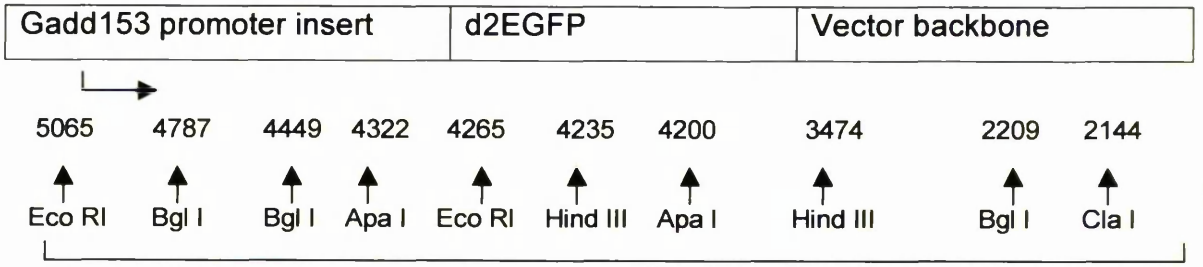


Lane

1. Molecular weight markers
2. Undigested plasmid
3. Gadd153-d2EGFP Hind III digest
4. Gadd153-d2EGFP Cla I digest
5. Gadd153-d2EGFP Eco RI digest
6. Gadd153-d2EGFP Apa I digest
7. Gadd153-d2EGFP Bgl I digest

(B)

Total length 5065 bp



Relative positions of restriction enzymes

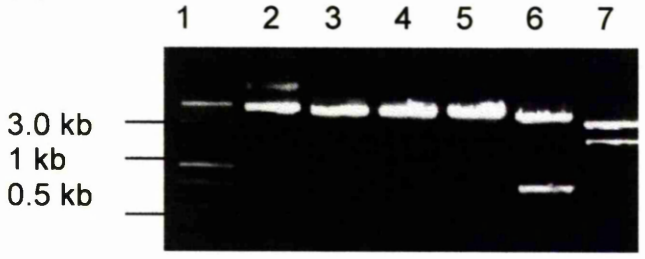
(C) Fragments obtained with restriction enzyme digests

Restriction enzyme	**DNA fragment sizes (bp)**
Hind III	761, 4304
Cla I	5065
Eco RI	800, 4265
Apa I (right orientation)	122, 4943
Apa I (wrong orientation)	808, 4257
Bgl I	338, 2240, 2487

Competent JM109 bacteria were transformed with the overnight ligation reaction (Section 2.5.5). The colony which grew was cloned and mini- preparation was performed (Section 2.4.4.1). 20 μg of plasmid DNA was subjected to restriction analysis with Hind III, Cla I, Eco RI, Apa I and Bgl I to determine presence and orientation of insert shown in (A). The schematic in (B) shows relative positions of restriction sites on the plasmid. Fragment sizes obtained are shown in (C). The insert was sequenced to check it was identical to database Gadd153 promoter sequence (Section 2.7.5).

Figure 2.9 Determination of Gadd153-Luciferase plasmid and promoter insert orientation

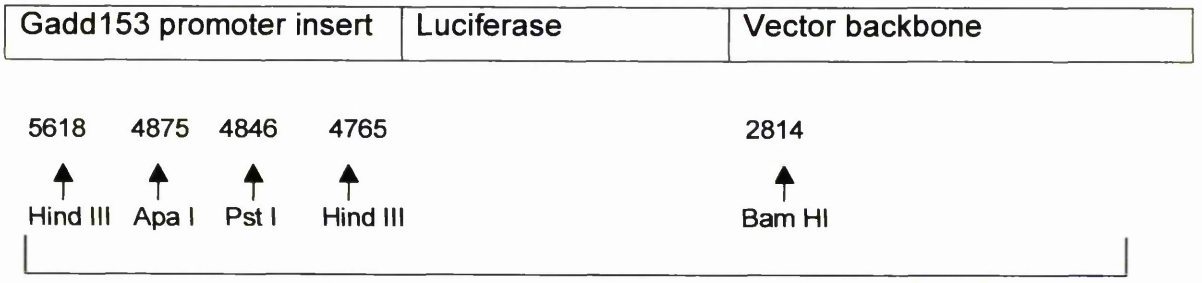
(A)



Lane
1. Molecular weight markers
2. Undigetsed plasmid
3. Clone 3 Apa I digest
4. Clone 3 Bam HI digest
5. Clone 3 Pst I digest
6. Clone 3 Hind III digest
7. Clone 3 Apa I + Bam HI digest

(B)

Total length 5618 bp



Relative positions of restriction enzymes

Restrction enzyme	**DNA fragment sizes (bp)**
Hind III	800, 4818
Apa I	5618
Bam HI	5618
Pst I	5618
Apa I + Bam HI (right orientation)	2061, 3557
Apa I + Bam HI (wrong orientation)	2776, 2842

Clone 3 (Gadd153-Luciferase) was selected and plasmid DNA isolated by midi-preparation was subjected to extensive restriction analysis, shown in (A) (Section 2.3.3.3). The plasmid (5 μg) was cut with ApaI, Bam HI, Pst I and Hind III. To determine the insert orientation the plasmid was cut with Apa I and Bam HI confirming the correct orientation of the 800 bp insert. The schematic in (B) shows the relative positions of restriction sites along the plasmid. DNA fragment sizes from digests are shown in (C). The insert was sequenced to check it was 100 % identical to database Gadd153 promoter sequence (Section 2.7.5).

2.6 TRANSFECTION OF DNA INTO MAMMALIAN CELLS

2.6.1 Electroporation

This procedure was used to transfect CHO DG44 cells with the Gadd153-d2EGFP plasmid.

2.6.2 Preparation of DNA and cells

DG44 CHO cells were maintained in exponential growth in HAMS-F12 +FCS (Section 2.1.5.2) for at least 9 days after removal from frozen stocks prior to transfection. The Gadd153-d2EGFP plasmid was purified using a QIAGEN midi Prep kit (Section 2.4.4.2) and checked to ensure it was free of chromosomal RNA and DNA. The plasmid was linearised with Bgl II. The DNA was then ethanol-precipitated (Section 2.3.3.2) and resuspended in sterile distilled water at a concentration of 0.5 mg/ml. All handling of the DNA after the precipitation stage was done in a laminar flow cabinet.

2.6.3 Stable transfection of cells

Cells were counted on the day of transfection such that 1 x 10^7 cells were used for each transfection. Two transfections were performed (i) no DNA (mock) (ii) Gadd153-d2EGFP plasmid. The cells were centrifuged at 130 g for 5 minutes and then they were washed once in cold PBS (50 ml). Cells were maintained on ice after this stage. Cells were resuspended such that cell concentration was 10^7 cells/ml. 1 ml portions were mixed with plasmid DNA (40 μg) or mock solutions (an appropriate volume of sterile water) in a electroporation cuvette. The cell-DNA mixture was then left on ice for 5 minutes. Two consecutive pulses at 1500 V, 3 μF were delivered to cuvettes. Cuvettes were returned to ice for a further 5 minutes and the contents were added to 30 ml of HAMS-F12 + FCS, prewarmed to 37 °C. 10ml of this mixture was distributed into a 96-well tissue culture tray with 200 μl in each well. 10 ml of this cell suspension was further diluted with 10 ml of

HAMS-F12 + FCS and distributed to two further 96-well plates. The remaining 10 ml of cell suspension was further diluted with 30 ml of HAMS-F12 + FCS and distributed over four 96-well plates. All the plates were placed in the incubator. After 24 hours, 0.1 ml of HAMS-F12 + FCS supplemented with G418 to a final concentration of 1 mg/ml was added to each well and the plates were replaced in the incubator until substantial cell death had occurred (about 14 days) and discrete surviving colonies appeared.

2.6.4 Cloning

The plates from the transfections by were examined carefully under an inverted microscope and the wells with a single colony of cells were identified and harvested to 24-well plates and ultimately expanded into T-flasks. 30 clones were recovered and frozen in liquid N_2.

2.6.5 Transient transfections with adherent cells

HEK 293 cells were maintained in exponential growth in DMEM +FCS (Section 2.1.5.3) for at least 9 days after removal from frozen stocks prior to transfection. Plasmid DNA was ethanol-precipitated (Section 2.3.3.2) and resuspended in sterile distilled water at a known concentration. All handling of the DNA after the precipitation stage was performed in a laminar flow cabinet.

On the day prior to transfection, cells were seeded at 4 x 10^5 in 2 ml of DMEM+FCS in 6 well plates. On the day of transfection the cells had reached a density of 70–80 % confluence. Gadd153-Luciferase (100 ng/well) and CMV-pRL (10 ng/well) or Gadd153-d2EGFP (1 μg/well) was diluted with DMEM containing no serum, proteins or antibiotics to a total volume of 2 ml. QIAGEN SuperFect Transfection Reagent was added to the DNA solution at a volume appropriate to the particular concentration of DNA. This was vortexed for 10 seconds and left to incubate for 5-10 minutes at room temperature to allow complex formation (specific experimental details in Chapter 3, Section 3.3). During this incubation period growth medium was gently aspirated from cells and cell sheets were washed once with 1x PBS. The transfection medium was then diluted to the

appropriate volume with DMEM+FCS to treat all cells at a volume of 1 ml/well. Cells were incubated for 3 hours at to facilitate DNA uptake and after this transfection incubation period medium was removed by gentle aspiration and cells were washed twice in 1x PBS. Cells were then subjected to incubation regimes as detailed in the specific experimental sections (Chapter 3, Section 3.3) for up to 15 hours.

2.7 RNA ISOLATION AND NORTHERN HYBRIDISATION

2.7.1 RNA isolation using Trizol Reagent

This method was routinely used to purify RNA. Cells were washed in PBS once and then lysed directly in a culture dish by adding 1 ml per 5-10 x 10^6 of cells of Trizol reagent by repetitive pipetting. Suspension cells were treated similarly but were initially centrifuged at 130 g for 4 minutes, washed in PBS and then resuspended in Trizol.

2.7.1.1 Phase separation

Samples were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform was added per 1 ml of Trizol Reagent and vigorous shaking by hand was continued for 15 seconds followed by an incubation of 2 to 3 minutes at room temperature. The samples were then centrifuged at 12,000 g for 15 minutes at 2 to 8 °C. Following centrifugation, the upper, colourless, aqueous phase containing RNA was transferred to a fresh tube. RNA was precipitated by the addition of isopropyl alcohol (0.5 ml per ml of Trizol reagent initially added). Samples were incubated at room temperature for 10 minutes and centrifuged at 12,000 g for 10 minutes at 2 to 8 °C. The RNA precipitate, sometimes invisible, formed a gel like pellet on the side of the bottom of the tube. The pellet was then washed once in 75 % (v/v) ethanol, vortexed and centrifuged for 5 minutes at 7,500 g at 2 to 8 °C. RNA was air-dried and then re-dissolved in a minimal amount of DEPC water (RNase-free)

(Section 2.1.4). Quantification of RNA was assessed as described in Section 2.3.1.1.

2.1.1 Northern hybridisation

RNA was extracted as described in Section 2.7.1. A volume of RNA (10 μg) was added to denaturing solution to give a final volume of 20 μl. The mixture was heated at 65°C for 15 minutes. It was mixed with 2 μl of loading buffer and loaded onto a 1 % (w/v) agarose gel. The agarose was melted in DEPC-treated water, 10× gel buffer and 37 % (v/v) stock formaldehyde in a ratio of 15:2:3. Electrophoresis was performed in 1 × gel buffer and at a constant voltage of 70 V for 2 hours.

Solutions

(10×) Gel running buffer:	0.4 M MOPS, pH 7 100 mM sodium acetate 10 mM EDTA
Denaturing buffer:	35 μl formaldehyde 100 μl formamide 20 μl 10x gel running buffer
Loading buffer:	1 mM EDTA in 2 % ficoll in 50 % glycerol (w/v) % (w/v) bromophenol blue

Separation of RNA was visualised by staining the gel in a solution containing ethidium bromide (0.1 ml of a 10 mg/ml stock added to 500 ml of DEPC-treated water). The gel was stained for 15 minutes and then destained for 30 minutes in DEPC-treated water. The gel was examined on a UV transilluminator. Two rRNA bands (18S and 28S) were clearly visible and confirmed the intactness of RNA.

2.1.2 Preparation of probes

DNA probes used for Northern hybridisation were prepared as described in Section 2.8.3 by PCR of Gadd153 fragment from pGadd153 (Appendix C). Labelling of probes used a random primed DNA labelling kit (Appendix A) for linear cDNA (Gadd153) and a nick translation kit (Appendix A) for plasmid probes (18s ribosomal RNA).

2.1.2.1 Random primed DNA labelling (Vogelstein, 1983)

The standard random primed assay involved denaturing 25 ng Gadd153 cDNA in a microfuge tube by heating for 10 minutes at 100 °C. This was followed by cooling on ice. The reaction mixture consisted of 1 μl each of 0.5 mM dTTP,0.5 mM dCTP and 0.5mM dGTP, 2 μl of reaction mixture (Appendix A), 5 μl of 50 μCi [α^{32}P] dATP (3000 Ci/mol) and it was made up to 20 μl with sterile distilled water. This was added to the DNA along with 1 μl of Klenow enzyme and incubated for 30 minutes at 37 °C. To stop the reaction, the mixture was heated to 65 °C for 10 minutes. Removal of non-incorporated deoxyribonucleoside trisphosphates was performed by ethanol precipitation (Section 2.3.3.2). Before addition to H-mix the probe was heated to 100 °C for 5 minutes and momentarily placed on ice.

2.1.2.2 Nick translation labelling (Rigby, 1977)

The standard nick translation assay involved using 0.1 μg plasmid DNA in a microfuge tube on ice. The reaction mixture consisted of 1 μl each of 0.4 mM dTTP,0.4 mM dCTP and 0.4 mM dGTP, 2 μl of 10x buffer (Appendix A), 2 μl of 20 μCi [α^{32}P] dATP (3000 Ci/mol) and it was made up to 20 μl with sterile distilled water. To start the reaction 2 μl of enzyme mixture was added and incubated for 35 minutes at 15 °C. To stop the reaction it was heated to 65 °C for 10 minutes. Removal of non-incorporated deoxyribonucleoside trisphosphates was performed by ethanol precipitation (Section 2.3.3.2). Before addition to H-mix the probe was heated to 100 °C for 5 minutes and momentarily placed on ice.

2.1.3 Northern hybrisation

Primarily the gel was soaked in 10 × SSC (1.5 M sodium citrate, 0.15 M sodium citrate, pH 7) for 30 minutes and the transfer of RNA to nylon filter was achieved using capillary action overnight. Transfer was confirmed by examination of the filter for 18S and 28S rRNA species. The filter was then baked in a vacuum oven for 2 hours at 28mmHg at 80°C. For hybridisation of probe to filter two methods were routinely used as described by Church and Gilbert, (1984) and Denhardt, (1992).

2.1.3.1 Hybridisation of probe to filter (I) (Church and Gilbert, 1984)

DNA probes were labelled as described in Sections 2.7.3.1 and 2.7.3.2. The DNA probe was boiled for 5 minutes and it was then placed immediately on ice. The filter was prehybridised in hybridisation solution, which consisted of 0.5 M $NaHPO_4$, pH 7.2 containing 1 mM EDTA, 1 % (w/v) BSA and 7 % (w/v) SDS, for 30 to 60 minutes at 65 °C in the hybridisation oven/tubes. This solution was removed and the probe, mixed with a fresh aliquot of hybridisation solution, was added to the filter. Hybridisation was allowed to proceed at 65 °C overnight in a Techne HB1 hybridisation oven. After the hybridisation period, the solution containing the probe was removed and the membrane was washed in initial wash buffer (40 mM $NaHPO_4$, pH 7.2 containing 1 mM Na_2EDTA, 5 % SDS and 0.5 % BSA) for 5 to 15 minutes. Subsequent washes were performed with wash buffer (40 mM $NaHPO_4$ pH 7.2 containing 1 mM Na_2EDTA and 1 % SDS). All steps were performed at 65 °C with buffers being prewarmed to that temperature before use. After checking background radioactivity on filters, autoradiography is performed as described in Section 2.7.5. Filters were stripped by addition of boiling 0.1 % SDS to filters and leaving this to cool to room temperature with agitation. Filters were checked for complete removal of radioactivity by leaving overnight on a phosphorimage plate.

2.1.3.2 Hybridisation of probe to filter (II) (Denhardt, 1992)

Baked filters were soaked briefly in 250 ml 5 x SSC and prehybridisation was carried out overnight in 8 ml of hybridisation mix (H-mix) in a Techne hybridisation oven at 42°C. H-mix consisted of 50% (v/v) formamide, 4 x SSC, 100μg/ml ssDNA (from salmon sperm), 1 x Denhardt's solution (from a 50 x stock which consisted of ficoll, polyvinyl-pyrrolidone and BSA (fraction V), at concentrations of 1% (w/v) and 0.1% (w/v) SDS, respectively).

After labelling, the DNA probe (Sections 2.7.4.1 and 2.7.4.2) was added to 5 ml of H-mix. This mix was previously boiled for 10 min, then it was cooled on ice to a temperature of about 50°C and added to the filter in the hybridisation tube (drained of prehybridisation mix). Incubation was performed overnight at 42°C.

After the hybridisation period, the radiolabelled hybridisation solution was removed and the filters were washed in the following sequential regime; one wash in 2 x SSC, at room temperature for 20 min, two washes in 1 x SSC, one at 42 °C and then 65 °C, each for 15 min, one wash in 0.5 x SSC, at 55 °C for 20 min, two washes in 0.25 x SSC at 42 °C for a further 15 min. All washes were performed in the hybridisation oven. All wash buffers (no matter what SSC concentration) contained 0.1% (w/v) SDS and the volume used for each wash was 9 ml. Radioactivity associated with the filter was measured after each wash with a radioactive monitor, and washing was stopped when background counts (measured at the corner of the filter) were less than 5 cps as determined by the monitor. The filter was then rinsed in 2 x SSC at room temperature to remove SDS. Excess liquid was blotted off, then the filter was sealed in a thin plastic bag and analysed by phosphorimage analysis for 30 min for quantification. The filter was then exposed to an X-ray film overnight or longer at -70°C, in a cassette using intensifying screens (Section 2.7.5).

2.1.4 Development of X-ray Films, phosphorimage analysis and Densitometry

X-ray films were processed in a dark room at room temperature using a developer. Phosphorimage analysis was performed over a period of 30 minutes during which

time the filter, sealed in a plastic bag, was exposed to a phosphorimage-plate. mRNA and protein levels were quantified by a scanner and densitometry software package (Appendix A)

2.2 REVERSE TRANSCRIPTION AND cDNA ANALYSIS

2.2.1 Synthesis of first strand cDNA

Reverse transcription of mRNA from cells was performed using the T-Primed First-Stand Synthesis Kit. Each reaction tube supplied contained dATP, dCTP, dGTP dTTP, Murine Reverse Transcriptase, Rnase/Dnase-free BSA and Not 1-$d(T)_{18}$ primer. Total RNA was prepared from cells using the method described in Section 2.7.1. RNA (1 μg) was denatured at 65°C for 5 minutes in a volume of 12 μl with total volume made up in DEPC-treated water. The RNA solution and reaction tubes were incubated at 37°C for 5 minutes. The RNA solution was then transferred to the reaction tube and incubated at 37°C for a further 5 minutes. The contents were mixed by gentle vortexing and the reaction was allowed to proceed at 37°C for 60 minutes.

2.2.2 Primer design

A BLAST search (which scans the nucleotide databases) was used to find the DNA sequence of genes or cDNA of interest. Sequences obtained included the Gadd153 promoter sequence and a Gadd153 cDNA probe sequence (Appendix C). Primers were designed against two highly conserved regions of the cDNA. When using a plasmid template, primers were designed against either side of the insert of interest. Primer sequences were checked for suitability by performing a sequence alignment using the same BLAST programme.

2.2.3 Polymerase Chain Reaction (PCR)

Templates used were plasmid DNA or first stand cDNA from RT reactions (Section 2.8.1). To denature the RNA-cDNA duplex and to inactivate reverse transcriptase the reaction was heated to 90 °C for 5 minutes and then chilled on ice. The following components were mixed in a sterile 0.5 ml microfuge tube: 30 μl of sterile dH_2O, 10 μl of 10 x amplification buffer (500mM KCl, 100 mM Tris. HCl, pH 8.3, 15 mM $MgCl_2$ and 0.1 % (w/v) gelatin), a mixture of dNTPs, each at a concentration of 1.25 mM, 100 pmoles of upstream primer, 100 pmoles of downstream primer and up to 2 μg DNA template. Finally this was made up to 100 μl with sterile dH_2O and 40 μl of light mineral oil was added to prevent evaporation.

Touch-down PCR was used to amplify the desired targets and the protocol is shown below. These are the standard conditions used that worked well for a wide range of templates and oligonucleotide primers but annealing temperatures were optimised for particular primers used (Appendix C).

The samples were placed on the thermal cycler and heated to 94 °C to denature the DNA completely. While the mixture was at 94 °C, 0.5 μl of Taq DNA polymerase (5 units/μl) was added (Appendix A). Amplification was then carried out using typical conditions for denaturation, annealing and polymerisation:

Cycle	*Denaturation*	*Annealing*	*Polymerisation*
First cycle	5 minutes at 94 °C	2 minutes at 57 °C	3 minutes at 72 °C
Subsequent cycles (x30)	1 minute at 94 °C	2 minutes at 57°C	3 minutes at 72 °C
Last cycle			10 minutes at 72 °C

For the last cycle, extension of the poly-A tail was at 72 °C for 10 minutes. The samples were transferred to -20 °C for storage. Gel electrophoresis was routinely used to analyse a sample of amplified DNA from the reaction mixture (Section 2.3.3.4).

2.2.4 DNA Sequencing

DNA sequence was determined using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Appendix A). For each reaction the following reagents were mixed in a microfuge tube: 8 μl of terminator ready reaction mix, template, PCR product (10-30 ng/μl) 3.2 pmole each of forward and reverse primers were then added to the mixture and made up to 20 μl with dH_2O. This mixture was overlaid with 40 μl of light mineral oil. The tubes were placed on the thermal cycler and the following cycles were performed: 96 °C for 30 seconds, 59 °C for 15 seconds and 60 °C for 4 minutes. This cycle was repeated a further 25 times until finally cooled to 4 °C. Following this procedure the products were purified. 2 μl of 3 M sodium acetate, pH 4.6 and 50 μl 95 % ethanol were added and then the entire contents were transferred into new microfuge tubes, vortexed and placed on ice for 10 minutes. The tubes were centrifuged at 12,000 g for 15-30 minutes. The ethanol was then carefully aspirated with a pipette and the pellet was washed in 250 μl of 70 % (v/v) ethanol. The alcohol was aspirated to leave a pellet at the bottom of the tube.

The sequence was obtained by using an automated sequencer, a facility within The School of Biological Sciences. A volume of 6 μl of sample buffer (deionised formamide, 25 mM EDTA (pH 8) containing 50 mg/ml blue dextran in a ratio of 5:1 formamide/blue dextran) was added per sample. This was vortexed and then pulse-centrifuged. The sample was heated to 90 °C for 2 minutes to denature it and placed on ice until ready to use (Perkin Elmer, protocol number 402078, 1995), this method was for *ABI 373* and *ABI 377 Prism*). *ABIView* a computer package, was used to analyse the sequence obtained.

2.2.5 DNA extraction from agarose gels

The QIAEX II Agarose Gel Extraction Protocol method was used to extract DNA from agarose in the production of cDNA probes only, all other DNA extraction was performed using the Geneclean II kit (Section 2.5.2). Firstly, DNA was electrophoresed on agarose gels and the band required was excised using a clean sharp scalpel. As much excess agarose was removed as possible and then the

DNA fragment was placed in a pre-weighed microfuge tube. The gel slice weight was determined by the difference. To this 3 volumes of buffer QX1 was added to 1 volume of DNA i.e. 300 μl to 100 mg of gel slice. 10 μl of resuspended QIAEX II was added to ≤ 2 μg DNA. The mixture was then incubated at 50 °C for 10 minutes to solubilise the agarose and bind the DNA. The solution was vortexed every 2 minutes to keep QIAEX II in suspension. The pH optimum for DNA binding, pH 7.5, is indicated by a yellow colour. If the colour of the mixture turned orange or purple, 10 μl of sodium acetate, pH 5.0, was added and mixed to return the pH to 7.5. After the incubation period the sample was centrifuged for 30 seconds and the supernatant was removed with a pipette. The remaining pellet was washed in 500 μl of buffer QX1, resuspended by vortexing and centrifuged at 12,000 g for 30 seconds and the supernatant was removed. The pellet was then washed twice with 500 μl of buffer PE using the same procedure as above. The pellet was then air-dried for 10-15 minutes until it turned white. DNA was eluted by addition of 20 μl of 10 mM Tris-HCL, pH 8.5 or H_2O with vortexing. The solution was incubated at room temperature for 5 minutes to enhance recovery.

2.3 IMMUNOLOGICAL DETECTION OF PROTEIN

2.3.1 Isolation of Total Cellular Protein

Cells were washed three times in 1x PBS. The cells were scraped and lysed into 100-200 μl of RIPA buffer, which consisted of 1% (v/v) NP40, 0.5% (w/v) sodium deoxycholate and 0.1% (w/v) SDS made up in 1x PBS. To this buffer was supplemented with a protease inhibitor and phosphatase inhibitor cocktail immediately before use. The inhibitor cocktail consisted of 0.5 μM PMSF, 10 μM sodium orthovanadate and 10 μM aprotonin.

2.3.2 Protein Assay

The Bio-Rad assay kit (Appendix A) was routinely used for measurement of protein in cell lysates and nuclear fractions. A microassay procedure was used

which measured between 0–3.5 μg protein. Using a 0.1 mg/ml stock of bovine serum albumin several dilutions were made to create a standard curve from 1–3.5 μg in a volume of 60 μl. A standard curve was prepared each time the assay was performed. Protein samples to be assayed were diluted until they were on the standard curve scale. Samples were assayed such that 1 μl of diluted sample is added to 59 μl of water. Duplicate standard samples and unknown samples were assayed for protein concentrations in a microtitre plate by addition of 60 μl of a diluted Bio-Rad reagent (1:3 in water). After incubation at room temperature for 5 minutes the samples were measured at 570 nm on a plate reader.

2.3.3 Preparation of samples for Sodium Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sample buffer was prepared at twice the required final strength (0.13 M Tris, 4% (w/v) SDS, 0.14 mM bromophenol blue, 20% (v/v) glycerol, pH 6.8) and 50 μl 2-mercaptoethanol was added per ml of sample buffer. Equal volumes of 2 x sample buffer was added to each cell extract. The mixtures were boiled for 5 min and then either separated immediately on SDS-PAGE (Section 2.9.3.1).

2.3.3.1 SDS-PAGE

Solutions

Acrylamide Stock: 30% (w/v) acrylamide solution
0.8 % (w/v) bisacrylamide

Separating buffer: 1.5 M Tris, pH 8.8
0.4 % SDS

Stacking buffer: 0.5 M Tris, pH 6.8
0.4 % SDS

Ammonium Persulphate	10% (w/v)
TEMED	100 % (v/v)
Electrophoresis buffer	0.05 M Tris 0.2% SDS 0.4 M Glycine

A 10% acrylamide separating gel was prepared by mixing 3.3 ml acrylamide stock, 2.5 ml separating buffer and 4.2 ml water for use with the Bio Rad mini gel system. Polymerisation was initiated by addition of 100 µl 10 % (w/v) ammonium persulphate and 10 µl TEMED. The gel mixture was quickly poured into gel slabs, leaving space at the top for the stacking gel. A 4 % acrylamide stacking gel was prepared by mixing 1.32 ml acrylamide stock, 2.5 ml stacking buffer and 6.18 ml of water. Polymerisation was initiated by addition of 100 µl 10 % (w/v) ammonium persulphate and 10 µl TEMED. The gel mixture was poured immediately into gel slabs on top of separating gels. A teflon comb was placed into each stacking gel and the gel was then allowed to set. When the gel has set, the comb was removed and the gel sandwich could be placed in the gel tank. Electrophoresis was performed in electrophoresis buffer. Gels were electrophoresed at 60 V until the bromophenol blue dye reached the interphase between stacking and separating gels, then the voltage was increased to 200 V until the dye was near the end of the separating gels. In addition to samples, protein markers were also included on all gels (see Appendix A).

2.3.4 Electroblot Transfer of Proteins to Nitrocellulose

Solutions

Blotting buffer	0.02 M Tris, pH 7.4 0.2 M Glycine 20 % (v/v) methanol
TBS/Tween	0.01 M Tris, pH 7.4

	0.14 M NaCl
	0.1 % Tween 20 (v/v)
Blocking Buffer	5% milk marvel in TBS/Tween, pH7.4
1x Ponceau stain	0.5 g Ponceau S,1 ml glacial acetic acid made up to 100 ml with deionised water

At the end of electrophoresis the gel apparatus was disassembled and the gel was soaked in blotting buffer. The transfer was achieved using a Bio-Rad Semi-dry electroblotting system. Two Biorad filters and one nitrocellulose filter were trimmed to obtained an identical area to the separating gel and they were soaked in blotting buffer. One pre-soaked Bio-Rad filter paper was placed in a layer on the anode of the electroblot. Care was taken not to trap any air bubbles. A filter paper followed by the gel then the nitrocellulose and subsequently another filter paper were stacked and the apparatus was assembled. The proteins were transferred vertically using a current of 5 mA/cm^2 of the gel area for 30 minutes. Subsequent to transfer the nitrocellulose membranes were stained with 1x Ponceau stain for 1 minute then rinsed with deionised water. Protein transfer to the filter was assessed and then the filter was placed in blocking buffer. It was left at room temperature for 30 minutes with constant agitation. Following the blocking step, primary antibody at an appropriate dilution in blocking buffer was added (Appendix A). The reaction was allowed to proceed for overnight at 4 °C with constant agitation. The filter was then washed with blocking buffer 4 times, each time for 5 minutes. This washing step allowed complete removal of unbound antibody. The secondary antibody (Appendix A) was then added in a dilution of 1 in 1000 in blocking buffer and the reaction was allowed to proceed at room temperature for one hour. After this incubation, the filter was washed in TBS-Tween 4 times each for 5 minutes. 2 ml of a 1:1 mixture (made up immediately before use) of the Amersham peroxidase ECL reagent mixes was pipetted onto the protein surface of the filter and this was allowed to remain for 1 minute. Excess substrate was removed, the filter was wrapped in saran-wrap and it was exposed to X-ray film for intervals of between 1 minute and 30 minutes. For development and quantification procedure see Section 2.7.5.

2.9.5 Stripping of filter

Filters were stripped by incubation with stripping buffer (62.5 mM Tris, pH 6.7 containing 100 mM 2-mercaptoethanol and 2% (w/v) SDS) at 50 °C for 30 minutes with agitation. The filter was washed twice in large volumes of TBS/Tween and was incubated with blocking buffer overnight at 4 °C. The filter was ready for reprobing with other antibodies.

2.10 ELECTROMOBILITY SHIFT ASSAY (EMSA)

2.10.1 Preparation of nuclear protein extracts (Rana and Farmer, 1994)

Cells were washed in 2 x ice-cold PBS and lysed in NP-40 lysis buffer which consisted of 10 mM Tris –HCl, pH 7.4 supplemented with 10 mM NaCl, 3 mM $MgCl_2$ and 0.5 % Nonidet P-40. Cell lysates were incubated on ice for 15 minutes and then nuclei were pelleted by centrifugation at 1000 g for 5 minutes at 4 °C. Pellets were washed in 5 ml of lysis buffer and the procedure was repeated. The cell nuclei were resuspended in 1 ml nuclear suspension buffer which consisted of 50 mM Tris-HCl, pH 7.5 containing 10 mM Mg Ac, 40 % (v/v) glycerol and 1 mM DTT. Suspensions were stored at –80 °C until use. To extract nuclear proteins nuclei were thawed on ice and then centrifuged at 1000 g for 10 minutes at 4 °C. Pellets were resuspended in 50 µl ice-cold protein extraction buffer which consisted of 10 mM HEPES, pH 7.5 supplemented with of 0.4 M NaCl, 5 mM EDTA, 0.2 mM PMSF and 1 mM DTT. The mixture was incubated on ice for 15 minutes with occasional vortexing and then centrifuged at 1000 g for 15 minutes at 4 °C. The supernatant was stored in 10 µl portions at –80 °C after addition of glycerol to 15 % (v/v). The protein concentration was determined by using the Bio-Rad protein assay kit (Section 2.9.2).

2.4.2 Radio-labelling of double stranded oligonucleotide

100 pmoles of double stranded oligonucleotide (Appendix C) was incubated in 1 μl of 20 x annealing buffer which consists of 200 mM Tris-HCl, pH 7.9 supplemented with 40 mM $MgCl_2$, 1 M NaCl and 20 mM EDTA. To this mixture was added 1 μl each of 0.5 mM dTTP,0.5 mM dCTP and 0.5mM dGTP, 5 μl of 50 μCi [α^{32}P] dATP (3000 Ci/mol) and it was made up to 20 μl with sterile distilled water. To begin the labelling reaction 1 μl of Klenow enzyme was added and incubated for 30 minutes at 37 °C. To stop the reaction it was heated to 90 °C for 5 minutes and cooled to 30 °C. This step was performed prior to use of the oligonucleotide in an electromobility shift assay.

2.4.3 EMSA assay

Nuclear extracts (10 μg protein) were incubated at 4 °C for 15 minutes with 3 μg of poly (dI.dC), 2 μl of carrier mix consisting of 50 mM $MgCl_2$ and 340 mM KCl and delta buffer consisting of, 25mM HEPES, pH 7.6 supplemented with 0.1 mM EDTA, 40 mM KCl, 8 % (w/v) ficoll and 1 mM DTT, to give a final volume of 18 μl. The NaCl concentration was adjusted to 200 mM. A volume of 1 μl of labelled oligonucleotide (Section 2.10.2) was added to the mixture and incubated for 30 minutes at 4 °C. Samples were then separated for 3 hours at 150 V on a 6 % polyacrylamide gel containing a 0.5 x Tris-borate-EDTA buffer. The gel was placed on a gel drier to dry and then covered in plastic film before being exposed to a phosphorimage plate for an appropriate amount of time (Section 2.7.5).

2.5 FLUORESCENCE MICROSCOPY

2.5.1 Preparation of mowiol mountant

Mowiol mountant was prepared by the addition of 6 g of water-free glycerol, 2.4 g mowiol 4-88 and 6 ml water. This mixture was mixed for 2 hours at room temperature. 12 ml of 0.2 M Tris.HCl, pH 8.5 was added and the temperature was

increased to 50 °C. The solution was centrifuged at 5000 g for 20 minutes and portions of the supernatant were stored at -20 °C.

2.5.2 DAPI staining

2.5.2.1 Adherent cells

Cell nuclei were examined by staining with DAPI (4', 6-diamidino-2-phenylindole). At appropriate intervals the growth medium was removed and coverslips were rinsed in PBS. The cells on the coverslips were fixed with 3.7 % (w/v) paraformaldehyde for 30 mins at 37 °C. The cells were washed with 70 % (v/v) ethanol, then rinsed in water. A drop of DAPI (1 mg/ml) was added to the coverslip. After 15 seconds, the coverslip was rinsed in water and mounted on a slide using a mixture of 9 parts mowiol mountant and 1 part p-phenylenediamine (10 mg/ml). The cell fluorescence was examined using Leica Fluorescence microscope (Appendix A). Images were captured using a digital microscope camera connected to an Apple Macintosh computer using IP Spectrum and Adobe Photoshop software.

2.5.2.2 Suspension cells

Cells (1×10^{6}) were centrifuged at 130 g for 5 minutes and the supernatant was discarded. The cell pellet was rinsed in PBS and then resuspended in 200 μl of 3.7 % paraformaldehyde and incubated for 30 minutes. 200 μl of cell suspension was put on polylysine-coated coverslips and left for 1 hour for cells to attach to the coverslips. Polylysine-coated coverslips were prepared by spreading polylysine (100 mg/ml) on the coverslips and this was left in a dust free environment. Attached cells were treated as with adherent cells described in Section 2.11.2.1.

2.5.3 Preparation of Gelvatol (GFP mountant)

Solution I	Solution II
0.02 M Na_2HPO_4	0.02 M KH_2PO_4,
0.14 M NaCl	20 g of vinyl alcohol (mw 25,000)
0.04 % (w/v) NaN_3	

Gelvatol mountant consisted of 72 % (v/v) Solution I and 28 % (v/v) Solution II with a final pH of 7.7.

2.5.4 Detection of EGFP fluorescence

Cells were grown on sterile coverslips. At appropriate intervals the growth medium was removed and cells were rinsed in PBS. 2ml of 4% (w/v) paraformaldehyde was then added directly to the cell sheets. After 30 minutes incubation at room temperature fixed cells were washed twice with PBS and then mounted onto a glass slide with 10 μl of Gelvatol (Section 2.11.3). The coverslips were sealed with molten agarose and left to dry for 30 minutes The cell fluorescence was examined using Leica Fluorescence microscope (FITC filter) excitation of EGFP is at 488nm (Appendix A). Slides were examined immediately or were stored for up to a week at 4 °C before examination.

2.6 DETECTION OF REPORTER CONSTRUCT EXPRESSION

2.6.1 FACS analysis

Cells were transfected as described in Section 2.6.5. Following transfection and treatment cell growth medium was aspirated and cells were washed once with PBS. They were then trypsinised (Section 2.2.1), resuspended with 1 ml of DMEM +FCS and centrifuged at 130 g for 3 minutes. Media was aspirated and cells were resuspended in 500 μl of prewarmed 1x PBS to 37°C. Harvested cells (10,000 per sample) were analysed for fluorescent intensity using a Becton Dickson

FACSVantage cytometer (Appendix A). EGFP was excited at 488 nm, and emission was detected using a 510/20 bandpass filter. Cell debris was excluded by electronic gating and the percentage of Green Fluorescent Protein positive cells in the population was calculated using Lysis II software (Appendix A).

2.6.2 Dual-luciferase assay

The Dual-luciferase Assay (Appendix A) is based on the cotransfection of Gadd153-luciferase and CMV-pRL plasmids. CMV-pRL is cotransfected as an internal control to determine transfection efficiency between samples.
Initially, Luciferase Assay Reagent II (LARII) a lyophylized luciferase assay substrate was resuspended in 10 ml of Luciferase Assay Buffer II and stored at -70°C in 1 ml aliquots. The second assay substrate Stop & Glo was resuspended in 200 μl of Stop & Glo Substrate Solvent to make a 50x stock solution stored at -70°C. This reagent was diluted to 1x with the same solvent for use in the assay.

2.6.2.1 Preparation of cell lysates for luciferase assay

Passive Lysis Buffer (5x) (PLB) contained in the assay kit was diluted to 1x working concentration with distilled water on the day of experiment and kept at 4°C. Growth medium was gently aspirated from cells which were ready for harvest after transfection and treatment. They were washed once with PBS and then 500 μl of 1x PLB was added to cover the monolayer of cells. Homogeneous lysates were rapidly prepared by manual scraping of cells from the culture dish with plastic cell lifters. Cells were harvested by pipetting up and down to produce a homogeneous suspension and were placed into a microfuge tube. The cells were then subjected to 2 freeze-thaw cycles to accomplish complete cell lysis. The suspension was centrifuged at 12,000 g for 30 seconds at 4 °C and cleared lysates were placed in fresh tubes and stored at -70°C until assayed.

2.6.2.2 Assay procedure

The assays were performed in microtitre plates using a luminometer plate reader (Appendix A). LAR II was predispensed into wells of a 96-well plate 25 μl/well to complete the desired number of dual luciferase reactions. The luminometer was programmed to perform a 2 second pre-measurement delay followed by a 10 second measurement period for each reporter assay. A volume of 5 μl of cell lysate was added to the wells containing LAR II and placed in the luminometer to initiate a reading. The luminometer plate reader was attached online to a printer to record firefly luciferase activity measurement. Once reading was established 25 μl of the second reagent was dispensed (Stop & Glo) and then the plate was replaced in the luminometer and a second reading initiated. The *Renilla* luciferase activity measurement was recorded. Final readings were calculated by determining the ratio of luciferase activity to *Renilla* luciferase activity.

2.7 STATISTICAL METHODS

The standard deviation (SD) from the mean was calculated from the formula:

$$SD = \sqrt{\sum (x-m)^2 / n-1}$$

The standard errors of the mean (SEM) was calculated from the formula:

$$SEM. = \frac{SD}{\sqrt{n}}$$

where m = arithmetic mean of n observations

x = an observed value

n = number of observed observations

n-1 = number of degrees of freedom

Students t-TEST

A Student's t-Test was used to determine the probability of whether one population of samples were significantly different to another population of samples.

Results were expressed as significantly different if the probability was <0.05 represented by * in the figures presented.

CHAPTER 3 RESULTS

3.1 GROWTH AND CHARACTERISTICS OF CHO DUK AND CHO DG44 19.6 CELLS

In this Section, the initial characterisation of growth and death of CHO DUK cells and CHO DG44 19.6 cells in batch culture will be presented. Significantly, the data shows that both cell lines die in normal batch culture through an apoptotic mechanism.

3.1.1 Growth curves

A typical growth curve is shown for CHO Duk cells in Figure 3.1. After seeding at an original density of $1x10^5$ cells/ml, the cells experienced an initial lag phase and subsequently entered into an exponential growth phase. After reaching a maximum density of approximately 1.8×10^6 cells/ml, the cells maintained in stationary phase for about 48 hours and cells then began to die and viability decreased in a time-dependent manner. This profile is typical of cells in batch growth. As viability decreased the number of non-viable cells found in the medium increased at a similar rate. The doubling time of these cells was 17 hours which is consistent with that reported by Anwar (1994) and Ang (1996).

A typical growth curve for serum-free batch culture is presented for DG44 19.6 cells in Figure 3.2. Similar to CHO DUK cell growth an initial lag phase was observed after seeding the cells at an original density of 2×10^5 cells/ml but they subsequently entered into exponential growth phase. After reaching a maximum density of approximately 1.8×10^6 cells/ml, the cells maintained stationary phase for about 24 hours and then started to die and viability decreased in a time dependent manner. The doubling time of these cells was 15 hours. This data is consistent with other suspension-adapted CHO cells grown in serum-free conditions (Goswani et.al., 1999).

Figure 3.1 Growth curve of CHO Duk cells in batch culture

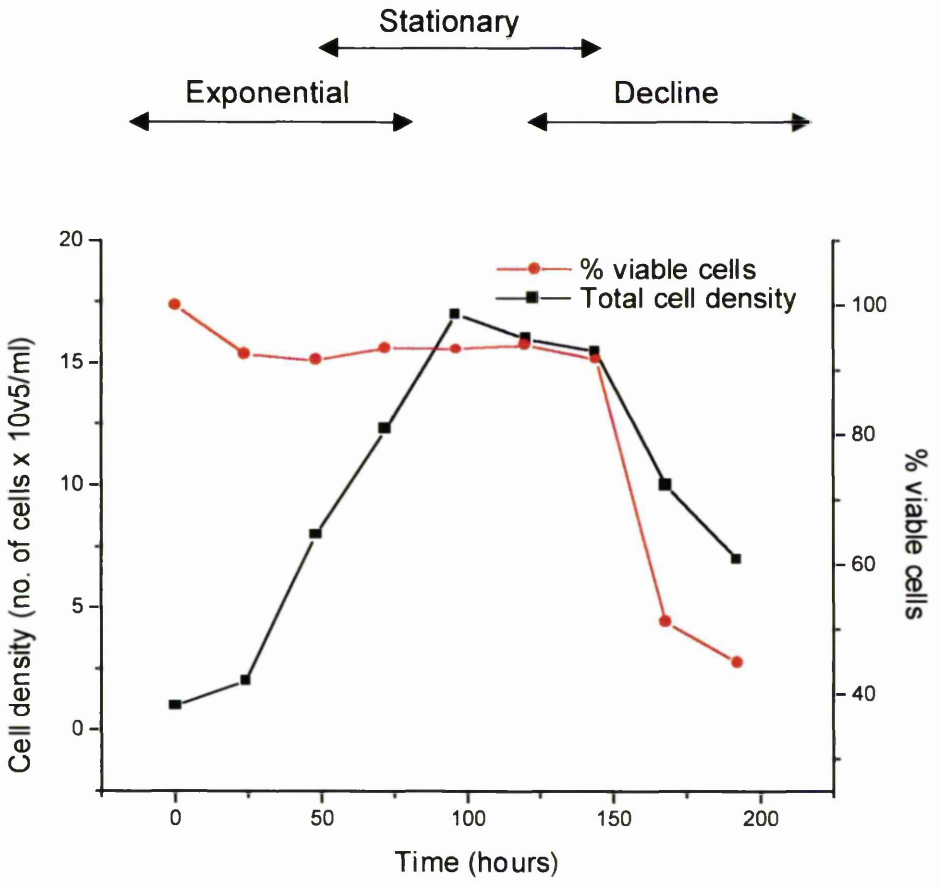


Figure 3.1 shows the total cell density and the % viability plotted against time. To set up growth curves, exponentially growing cells were harvested (Section 2.2.1) and seeded at a density of $1x10^5$ cells /ml in T-flasks. Cell counts were performed using trypan blue exclusion (Section 2.2.1) at the appropriate time points. Data presented is the average value for 3 counts for 3 independent experiments as the error was always less than 5 %.

Figure 3.2 Growth curve of CHO DG44 19.6 cells in batch culture

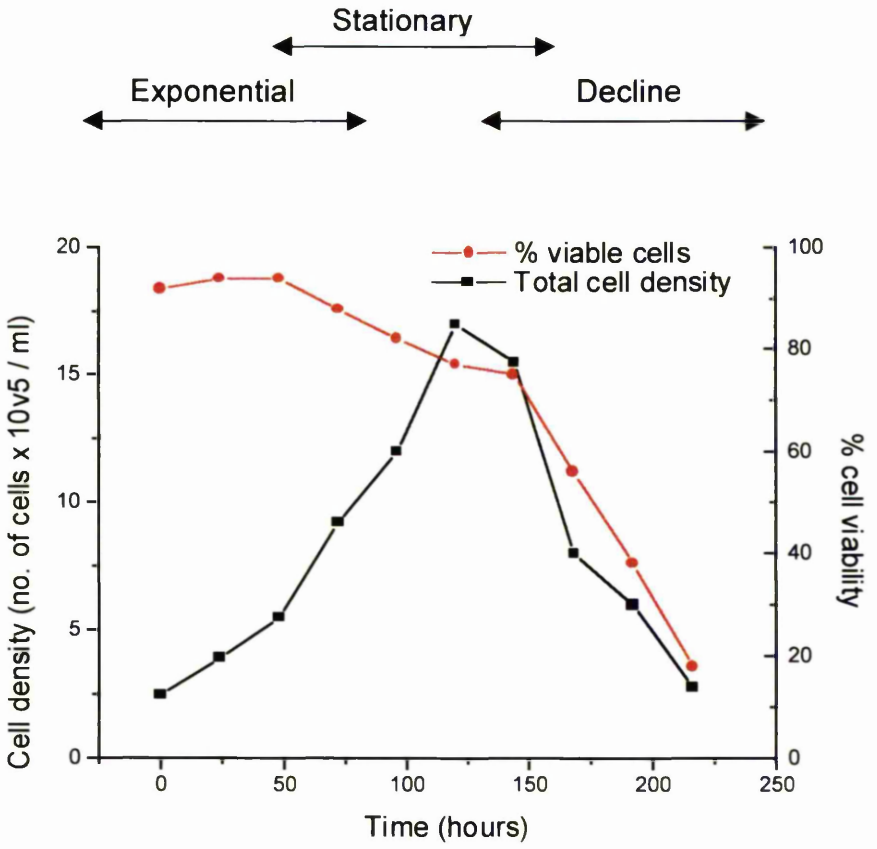


Figure 3.2 shows total cells and % viability plotted against time. To set up growth curves, exponentially growing cells were harvested (Section 2.2.1) and seeded at a density of $2x10^5$ cells / ml in shake flasks. Cell counts were performed using trypan blue exclusion (Section 2.2.1) at the appropriate time points. Data presented is the average value for 3 counts from 3 independent experiments as the error was always less than 5 %.

3.1.2 Assessment of cell death as apoptotic

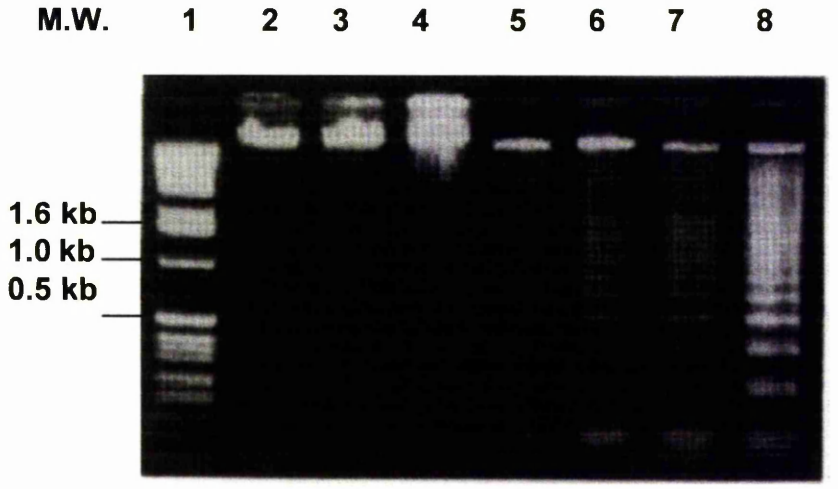
3.1.2.1 Analysis of genomic DNA

DNA was extracted from CHO Duk and CHO DG44 19.6 cells and the integrity of DNA was determined by electrophoretic size separation in ethidium bromide-stained agarose (Figures 3.3 and 3.4). From 0 to 48 hours of batch culture, there was a high molecular weight band visible indicating the presence of intact genomic DNA. However, smaller molecular weight species were visible in cell samples isolated from 92-120 hours of culture, coinciding with the time when cell viability started to drop (Figures 3.1 and 3.2). This distinct pattern became more apparent as the cells proceeded further into decline phase. DNA fragmentation into discrete sizes is typical of cells dying by apoptosis where nucleosome fragmentation occurs (Afanasev et. al., 1986).

3.1.2.2 Morphological analysis by fluorescence microscopy

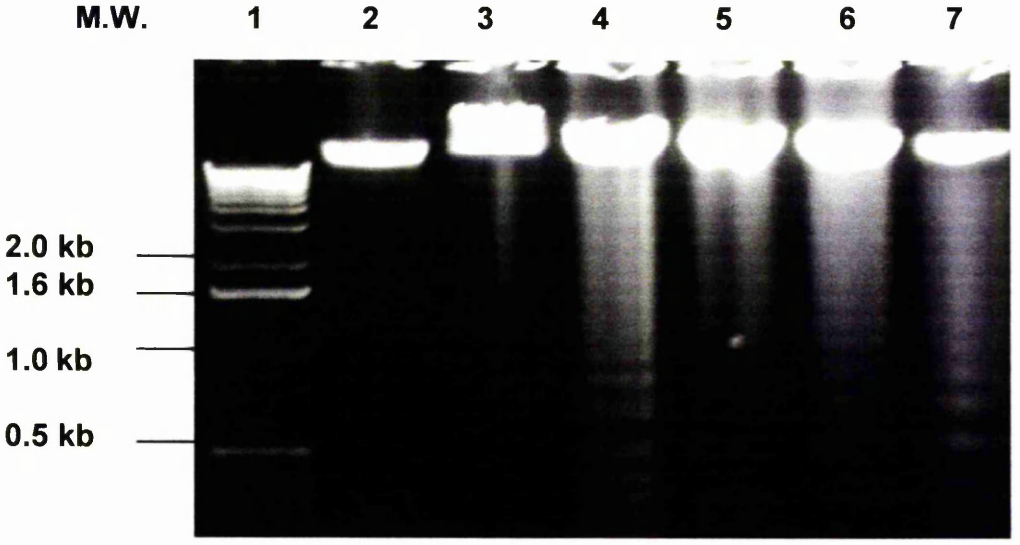
Cells were harvested at three stages of growth: early exponential phase (24 hours of growth), mid-exponential phase (96 hours of growth, CHO Duk only) and decline phase (192 hours of growth). Cells were fixed and stained with DAPI (Section 2.11.2.1). Figures 3.5A and 3.6A, show that in early-exponential phase, CHO Duk and DG44 19.6 cells are in interphase as indicated by their decondensed chromatin which is diffusely stained. Figure 3.5B shows CHO Duk cells in mid-exponential phase undergoing interphase, indicated again by decondensed chromatin which was diffusely stained. These cells are considerably denser after rounds of cell division. Figures 3.5C and 3.6B show both cell lines in decline phase and the nuclei of most of these cells are brightly stained, showing condensed chromatin. CHO Duk cells floating in the media at 96 hours of culture showed condensed chromatin staining. Such cells were not observed in any of the fields of view for cells isolated in exponential growth phase (a total of at least 500 cells).

Figure 3.3 DNA agarose gel electrophoresis of DNA extracted from cells during batch culture of CHO Duk cells



Genomic DNA was extracted as described in Section 2.3.2 at the time points indicated; lane 1; DNA molecular weight markers, lane 2; 44 hours, lane 3; 54 hours, lane 4; 69 hours, lane 5; 92 hours, lane 6; 117 hours, lane 7; 162 hours and lane 8; 216 hours. 10 μg of DNA from each time point was loaded onto a 1 % agarose gel and DNA gel electrophoresis was performed (Section 2.3.3.4).

Figure 3.4 DNA agarose gel electrophoresis of DNA extracted from cells during batch culture of CHO DG44 19.6 cells



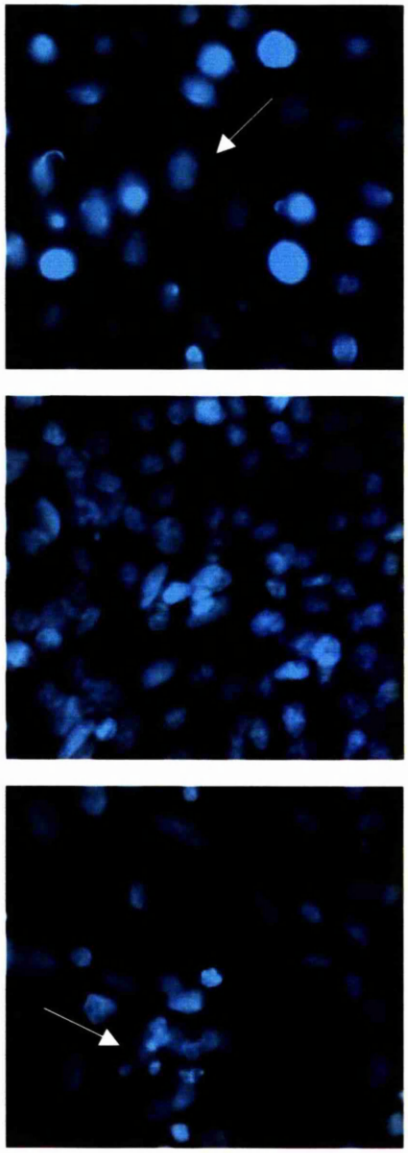
Genomic DNA was extracted as described in Section 2.3.3 at the time points indicated; lane 1; DNA molecular weight markers, lane 2; 48 hours, lane 3; 120 hours, lane 4; 144 hours, lane 5; 168 hours, lane 6; 216 hours and lane 7; 240 hours. 10 μg of DNA from each time point was loaded onto a 1 % agarose gel and DNA gel electrophoresis was performed (Section 2.3.3.4).

Figure 3.5 Fluorescent images of DAPI stained CHO Duk cell nuclei collected throughout phases of batch culture

(A)

(B)

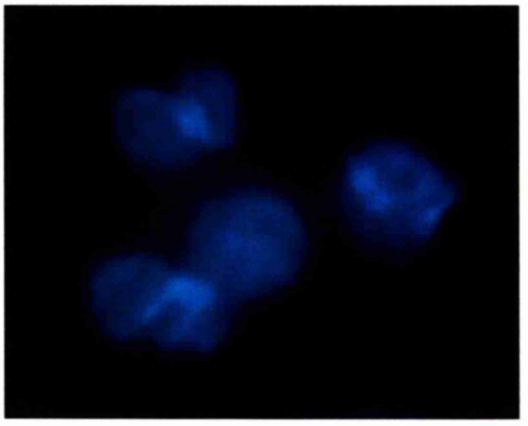
(C)



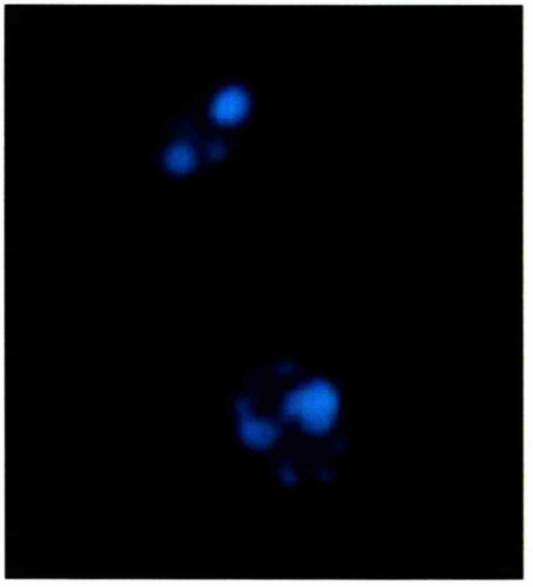
Exponentially growing CHO Duk cells were harvested and seeded at a density of $1x10^5$ cells/ml on sterile coverslips (Section 2.2.1). At intervals cells were fixed and stained with DAPI (Section 2.11.2.1). (A) represents nuclei from cells collected after 48 hrs of culture and (B) represents nuclei from cells collected after 72 hrs of culture and (C) represents nuclei from cells collected after 192 hrs of culture. All images represent typical fields observed with more than 500 cells. The highlighted cells in (A) represents a dividing cell which is typical of cells extracted from the exponentially growing phase. The arrow highlighted cells in (C) show obvious chromatin condensation and fragmentation characteristic of apoptotic cells.

Figure 3.6 Fluorescent images of DAPI stained CHO DG44 19.6 cell nuclei collected throughout phases of batch culture

(A)



(B)



Exponentially growing CHO DG44 19.6 cells were harvested and seeded at a density of $2x10^5$ cells/ml (Section 2.2.1). At appropriate intervals the suspension cells were DAPI stained after attachment polylysine coated coverslips (Section 2.11.2.2). (A) represents nuclei from cells collected after 48 hrs of culture and (B) represents nuclei from cells collected after 192 hrs of culture. The images represent typical fields observed with more than 500 cells. The cells in (A) represent healthy cells typical of cells extracted from the exponentially growing phase. The cells in (B) show obvious chromatin condensation and fragmentation which are characteristic of apoptotic cells.

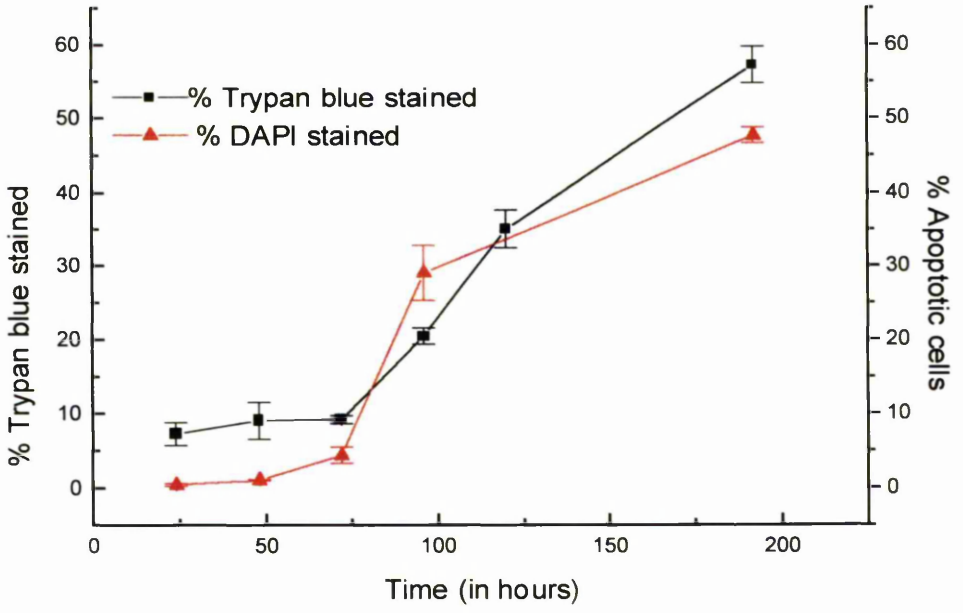
3.1.3 Quantification of apoptosis through batch culture

To obtain a quantitative analysis of apoptosis, cells from different stages of growth were harvested and cell nuclei were stained with DAPI to define the extent of apoptosis in the cell population. Cell viability was assessed simultaneously by trypan blue exclusion. Cells stained with DAPI were examined by fluorescence microscopy and cells with condensed and fragmented nuclei (i.e. apoptotic) were scored as a percentage of cells in the field of view.

The viability of CHO Duk cells, by trypan blue staining, started to drop after 100 hours of culture (consistent with Figure 3.1) and the percentage of apoptotic cells in the population began to increase at the same time. The percentage of non-viable cells measured by trypan blue staining after 192 hours was 57.2 ± 2.5 % and the cells were 47.6 ± 1.1 % apoptotic (Figure 3.7). The slightly lower percentage of dead cells measured by fluorescence microscopy compared to trypan blue staining might be due to secondary necrosis occurring in late stages of apoptosis.

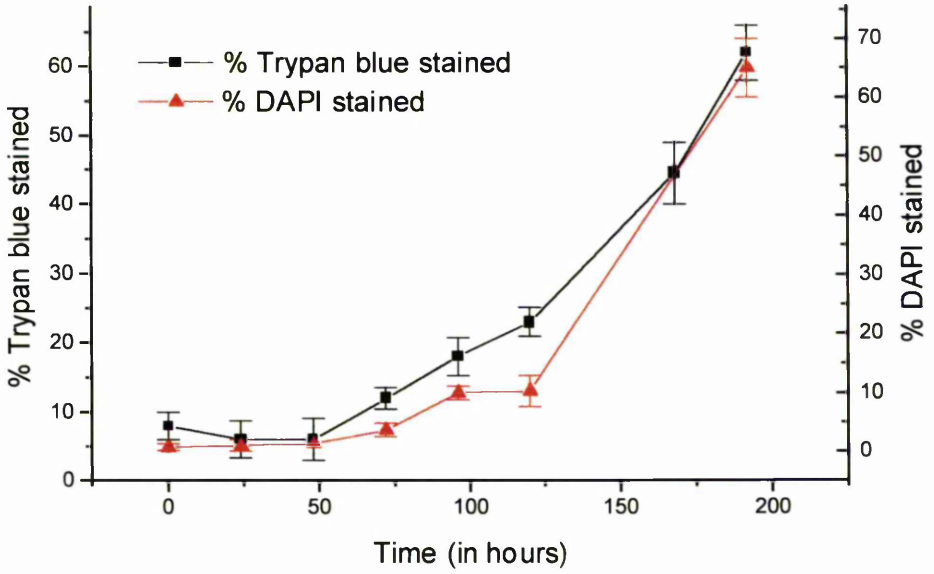
A similar pattern was observed in CHO DG44 19.6 cells were viability began to drop after 100 hours of culture (consistent with Figure 3.2) and the percentage of apoptotic cells in the population began to increase at the same time. The percentage of non-viable cells measured by trypan blue staining after 192 hours was 61.9 ± 13.2 % and cells were 65 ± 5 % apoptotic showing that cell death is occurring entirely by apoptosis (Figure 3.8).

Figure 3.7 Relationship between non-viable (trypan blue stained) and apoptotic (DAPI stained) CHO Duk cells with time in culture



Cell viability was assessed by trypan blue exclusion as described in Section 2.2.1. Cell counts were performed in triplicate. Cells with condensed chromatin observed by fluorescence microscopy were scored, by counting, as apoptotic after staining the nuclei with DAPI (Section 2.11.2.1). A minimum of 500 cells were examined for each determination. Data is presented as mean ± S.E.M. of 3 independent experiments.

Figure 3.8 Relationship between non-viable (trypan blue stained) and apoptotic (DAPI stained) CHO DG44 19.6 cells with time in culture



Cell viability was assessed by trypan blue exclusion as described in Section 2.2.1. Cell counts were performed in triplicate. Cells with condensed chromatin were observed by fluorescence microscopy and scored as apoptotic by counting after staining the nuclei with DAPI (Section 2.11.2.2). A minimum of 500 cells were examined for each determination. Data is presented as mean ± S.E.M. of 3 independent experiments.

3.1.4 Summary – apoptosis in batch culture

My observations have shown that onset of cell death in CHO Duk and CHO DG44 19.6 cells during batch culture occurs through an apoptotic mechanism. However, there have been conflicting reports about the significance of apoptosis in the death of CHO cells in batch culture. One group has presented evidence that CHO cells die in batch culture through apoptosis (Moore et. al.,1995; Moore et.al., 1997) whereas a second group failed to find evidence of apoptosis during batch culture of specific CHO cell lines (Singh et.al., 1994) but they have since reported apoptosis as the mode of cell death for other CHO cell lines (Perani et.al., 1997). This aspect is enlivened by the fact that apoptosis is a process which does occur in CHO cells in response to a number of stresses (Section 4.1). Thus it is clear that the cellular machinery to engage apoptosis is present in CHO cells (Section 1.3).

In this study, a similar pattern of apoptotic cell death occurred irrespective of cell status. CHO Duk cells are non-recombinant, serum-dependent, adherent cells whereas CHO DG44 19.6 cells are recombinant, suspension-adapted and grown in serum-free medium. This data indicates that cells die via apoptosis even in the absence of transfection or selection procedures.

The aim of the rest of this thesis was to examine factors that inform on the mechanisms that activate apoptosis or to provide indicators of the likelihood of entry of cells into apoptosis. It is envisaged that, at the early stages, after stress perception but prior to commitment to apoptosis, cells could be rescued from apoptosis allowing continuation of healthy and productive culture. The following Result sections describe the manipulation and utilisation of a candidate gene, Gadd153, as an indicator of the early onset of stress perception by CHO cells.

3.2 ASSESSMENT OF GADD153 IN RESPONSE TO STRESS IN CHO DG44 19.6 CELLS

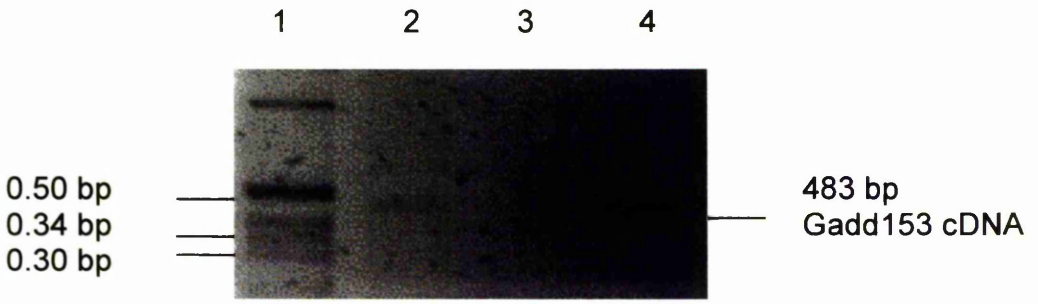
3.2.1 Induction of Gadd153 expression in batch culture

To determine the response of Gadd153 to different phases of batch culture RNA was isolated throughout batch cultures. Initially RT-PCR was performed on RNA to determine if the Gadd153 transcript was present. Figure 3.9 shows that a 483 bp Gadd153 cDNA was visible by UV transillumination after 144 hours of batch culture. This preliminary result was indicative that Gadd153 was induced as these cells entered decline phase. Northern hybridisation was performed as further confirmation (Figure 3.10). This shows a single band of about 0.9 kb which was undetectable or at very low levels in early batch culture but significantly induced by 144 hours (Figure 3.10A). This corresponds to a number of reports were Gadd153 mRNA was observed to migrate with a size of 0.9 kb from HeLa cells (Carlson et.al.,1993), H4-II-E heptoma cells (Marten et.al., 1994), HepG2 and Cac0-2 cells (Bruhatt et.al., 1997) and LLC-PK1 cells (Huang et.al., 1999). When reprobed with 18S rRNA cDNA, the expected 1.9 kb band corresponding to 18S rRNA was observed in all lanes, indicative of equivalent loading of RNA in each lane. The relative percentage of Gadd153 mRNA expression with time is shown in Figure 3.10B. This was calculated from the ratio of Gadd153 mRNA to 18S ribosomal RNA. Hence, endogenous Gadd153 was detectable and had increased 1.7-fold at the Northern level by 144 hours.

3.2.2 Induction of apoptosis

Nutrient deprivation may be one of the primary causes of cell death during the decline phase of batch culture. Many cell types will undergo apoptotic cell death following depletion of glucose or exhaustion of particular amino acids (Mercille and Massie, 1994; Singh et.al. 1996). Other stresses involved in cell death involve ER stress and the onset of UPR (Section 1.4.2). As the recombinant cells used in this study were selected for high recombinant protein expression and secretion they could be experiencing ER-associated problems such as UPR.

Figure 3.9 RT-PCR of Gadd153 from RNA in CHO DG44 19.6 cells throughout batch culture

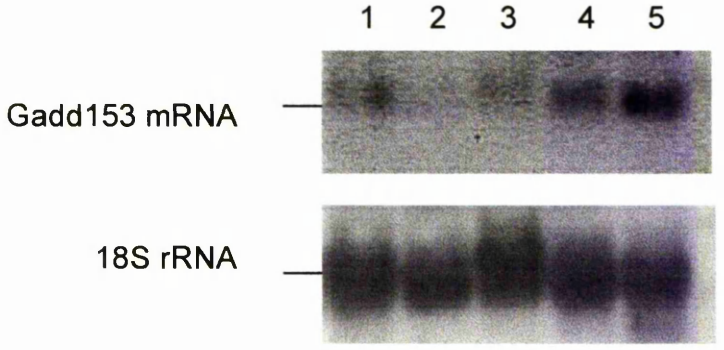


Exponentially growing cells were seeded at 2 x 10 ^{5} cells/ml and RNA was isolated throughout batch culture (Section 2.7.1). RT-PCR of 1 μg of RNA was performed using Gadd153 primers (Section 2.8). PCR products were electrophoresed a 1 % agarose gel and visulaised by UV transillumination (Section 2.3.3.4). The species above correspond to: lane 1; 1 kb DNA molecular weight markers, lane 2; 96 hours batch culture, lane 3; 120 hours batch culture and lane 4; 144 hours batch culture.

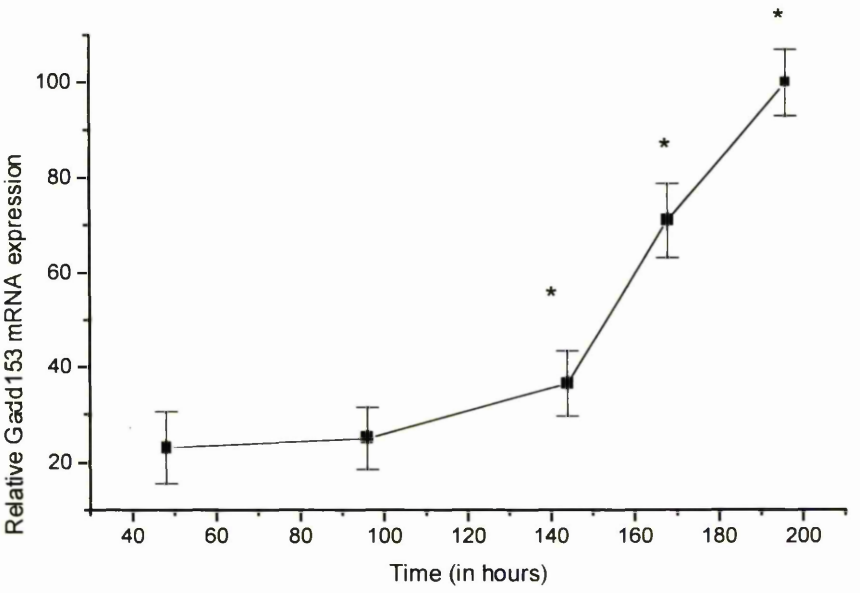
Exponentially growing CHO DG44 19.6 cells were seeded at $2x10^5$ cells/ml in shake flasks (Section 2.2.1). Total RNA was isolated from cells as described in Section 2.7.1 at time points throughout batch culture. 20 μg of each RNA sample was separated on an agarose gel and transferred to nylon (Section 2.7.4.1). After marking the positions of 18S and 28S rRNA species, filters were probed with an α-^{32}P-labelled Gadd153 cDNA (Section 2.7.3.1) and subjected to autoradiography (Section 2.7.5). Filters were stripped and reprobed with an α-^{32}P-labelled 18S rRNA probe and again subjected to autoradiography. A representative blot is shown in (A) and the time points of the RNA species in each lane are as follows: lane 1; 48 hours, lane 2; 72 hours, lane 3; 96 hours, lane 4; 144 hours and lane 5; 216 hours. The graph shown in (B) shows Gadd153 mRNA expression throughout batch culture. Gadd153 mRNA and 18S rRNA expression levels were calculated as fractions out of 1 were the highest value represented 1. From these values a ratio of Gadd153 mRNA to 18S rRNA was determined for each sample. Each value is expressed as a percentage and is termed the relative Gadd153 mRNA expression. All values are presented as mean $\pm$ S.E.M. of 3 independent experiments. Significance of difference between 48 hours sample and samples taken after this time were tested using a Student's t-test ($p< 0.05$).*

Figure 3.10 Relationship between induction of Gadd153 mRNA expression with time in batch culture

(A) Representative blot of Gadd153 mRNA expression in batch culture



(B) Graph showing Gadd153 mRNA induction in relation to time in batch culture



In the following sections the effects of nutrient depletion and ER stress were examined in relation to cell viability, apoptosis and Gadd153 expression of CHO DG44 19.6 cells. To investigate nutrient stress cells were incubated in media which was either glucose-free, glutamine-free, or depleted of both glucose and glutamine and also media which was free of all amino acids (all media was iso-osmotic). To investigate ER stress tunicamycin was added to the cell culture. Tunicamycin inhibits protein N-linked glycosylation within the ER. This is known to be a potent inducer of the UPR and can lead to growth arrest and programmed cell death in many cell types (Larsson et.al. 1993; Perez-Sala and Mollinedo, 1995; Dricu et.al., 1996).

3.2.2.1 Measurement of cell viability, energy status and extent of apoptosis

When cells were subjected to stress the following factors were investigated (i) cell viability (ii) extent of apoptosis (iii) cellular energy status and (iv) Gadd153 expression (at mRNA and protein level).

Control untreated cells displayed no significant change in cell viability over 24 hours, maintaining values of between 92 and 95 % viability (Figure 3.11). Cells placed in glutamine-depleted medium maintained similar high viability over the same time period. This is an expected observation as CHO cells carry the gene coding for glutamine synthetase (GS) which allows for the synthesis of glutamine when in demand by the cell (Bebbington, 1992). Culture of cells in medium depleted of glucose caused a 24 % decrease in viability by 24 hours indicating that in this medium glucose is more essential than glutamine in maintenance of cell viability. This observation is consistent with that of Zanghi et.al. (1999) who found that glucose deprivation in CHO cells is a more potent inducer of cell death than depletion of any single amino acid. In medium depleted of glucose and glutamine there was a steady decrease in viability for 18 hours followed by a further rapid decline. Cell viability had decreased by 76 % by 24 hours. Culture of cells in medium depleted of all amino acids produced a potent killing action on the cells with a 88% decrease in viability by 24 hours. Tunicamycin addition to

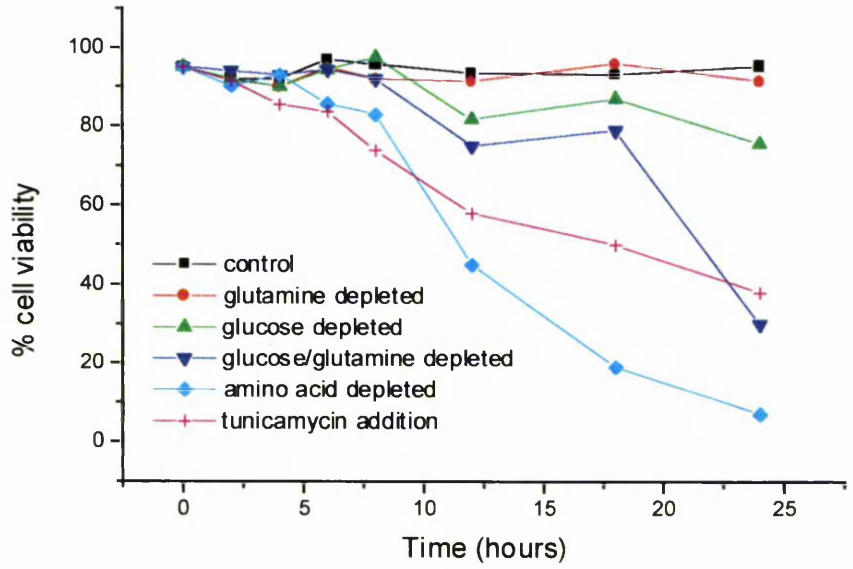
control medium caused a rapid and steady decline in viability so that cells were only 40 % viable by 24 hours.

A similar order of effectiveness was observed in relation to the induction of apoptosis (Figure 3.11B) and loss of cellular ATP in response to stresses (Figure 3.11C). After 6 hours the percentage of apoptotic cells was negligible for all treatments. However, by 12 hours there was an increase in apoptotic cell death in all cases with an order of apoptosis: amino acid depletion > glucose and glutamine depletion > tunicamycin treated > glucose depletion > glutamine depletion. By 24 hours cells in medium depleted of glutamine and glucose or all amino acids were all apoptotic. At the same time treatment with tunicamycin had resulted in 80 % of the cells becoming apoptotic. Depletion of a single nutrient produces smaller changes in apoptosis. Glucose-depleted cells were 20 % more apoptotic than glutamine depleted cells which were only 10 % apoptotic.

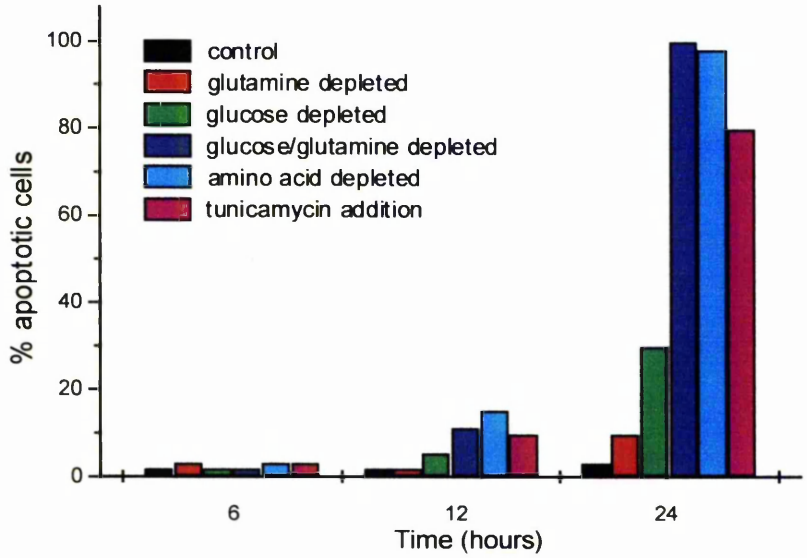
Cellular ATP was determined in response to each treatment (Figure 3.11C). Control cells maintained high levels of ATP throughout 24 hours of culture. These cells maintained an ATP content of 158 pmol ATP / μg DNA. This concentration of ATP was similar to reports of healthy HeLa cells by Carlson et.al. (1993). After 6 hours of treatment in glucose-depleted medium and in medium depleted of glucose and glutamine ATP contents were 40 % and 20 % of control, respectively. After 12 hours of culture ATP contents were unchanged in control and in cells in glutamine-depleted medium but ATP contents of cells in medium depleted of glucose or glucose and glutamine continued to decrease. By this time the ATP content of cells in amino acid-depleted medium had decreased dramatically (to only 5 % of control). Treatment with tunicamycin decreased ATP to 53 % of control after 24 hours of culture. However, cells from all treatments (except for control and glutamine-depletion) had ATP levels of 5 % of control or less by this time.

Figure 3.11 Cell viability and onset of apoptosis in response to various stresses

(A)



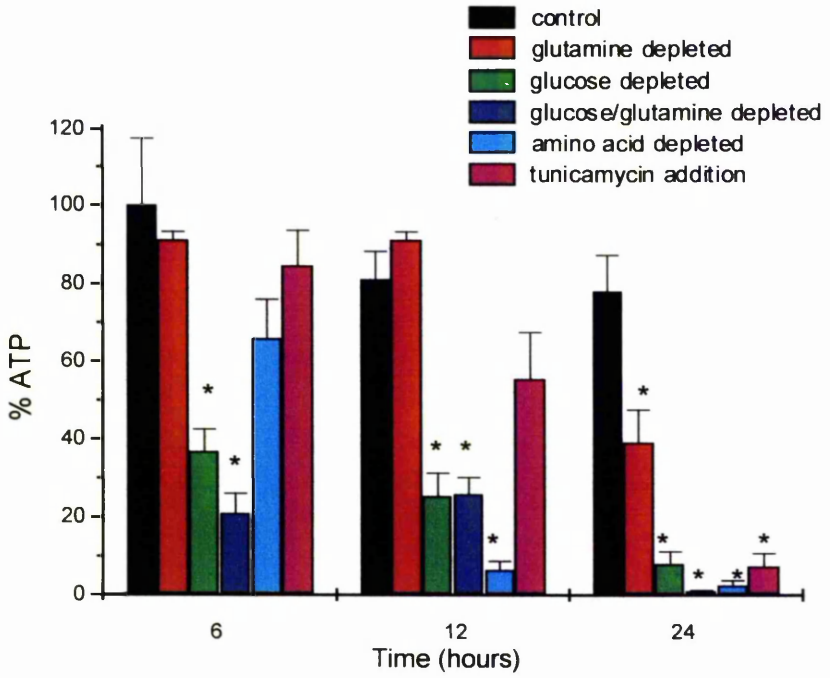
(B)



Exponentially growing CHO DG44 19.6 cells were seeded at $2x10^5$ cells/ml in shake flasks (Section 2.2.1). At mid-log phase cells were centrifuged, washed in PBS and resuspended in 50 ml of treatment media as follows; control, glutamine-depleted, glucose-depleted, glucose/glutamine-depleted, amino acid-depleted and tunicamycin addition. When present, tunicamycin was added at a concentration of 4 μg/ml. Cell viability was assessed by trypan blue exclusion at 0, 6, 12 and 24 hours (Section 2.2.1), shown in (A). Data is presented as the mean of 3 independent experiments were the error was always less than 5 %. Cells with condensed chromatin observed by fluorescence microscopy were scored as apoptotic by counting after staining the nuclei with DAPI at the same times indicated above (Section 2.11.2.2), shown in (B). A minimum of 500 cells were examined for each determination. Data is presented as mean of 3 counts from one experiment.

Figure 3.11 (cont.) Cell ATP levels in response to various stresses

(C)



Exponentially growing CHO DG44 19.6 cells were seeded at $2x10^5$ cells/ml in shake flasks (Section 2.2.1). At mid-log phase cells were centrifuged, washed in PBS and treated as described in the legend to Figure 3.11. Cell ATP concentration was assessed by a ATP firefly-luciferase assay as described in Section 2.2.5.1. Cell ATP concentration was standardised by measuring the total DNA in each sample using a DNA assay (Section 2.2.5.2). Data presented is the average ± S.E.M. from 3 independent experiments. Significance of difference between control and treated samples was tested using a Student's t-test ($p < 0.05$).*

3.2.2.2 Measurement of Gadd153 mRNA expression

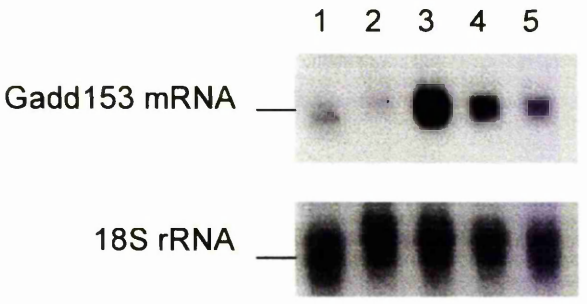
As a starting point to determine if nutrient stress could induce Gadd153 expression, and for subsequent determination of the time course of induction, initial experiments were performed by examination of the response of cells to culture in amino acid-depleted medium. This treatment was used as it had previously displayed the most potent effect on the entry of cells into apoptotic death (Section 3.2.2.1).

Figure 3.12A shows a representative Northern blot of Gadd153 mRNA expression from RNA isolated from cells placed in amino acid-depleted medium. An expected 0.9 kb RNA species showed good hybridisation. When reprobed with 18S rRNA cDNA, the expected 1.9 kb band was observed showing the respective loading of RNA in each lane. At the Northern level, expression of Gadd153 mRNA at 2 hours was negligible but by 4 hours had increased 9-fold. This was a somewhat transient response as by 10 hours expression levels had fallen by 30 % (Figure 3.12B).

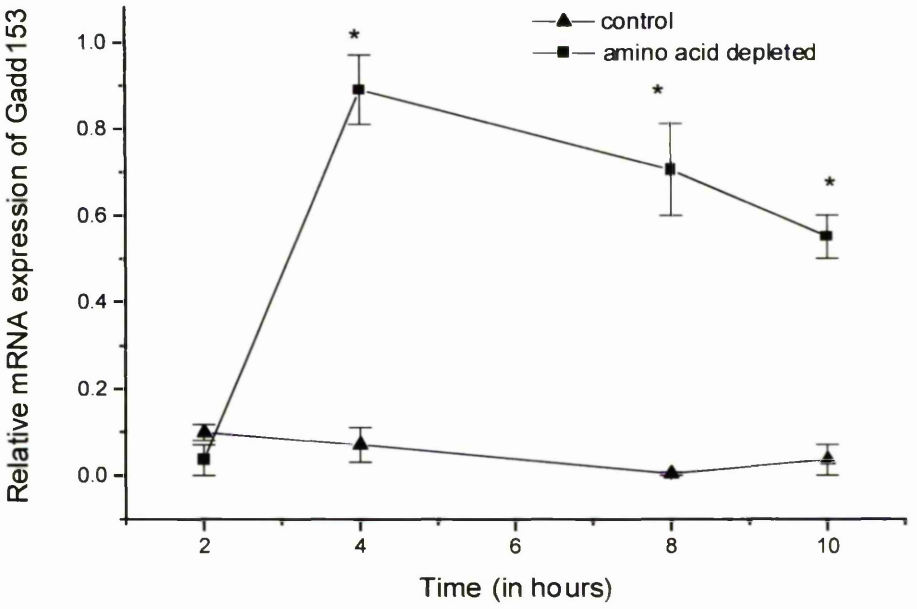
As Gadd153 response to amino acid depletion had been shown to be rapid, assessment of the effects of other treatments was made after 4 and 8 hours of treatment. A representative Northern blot of Gadd153 mRNA induction with all treatments is shown in Figure 3.13A. Data from replicate experiments is presented in Figure 3.13B. Control cells displayed a low and constant basal level of Gadd153 expression. A 5-fold induction of Gadd153 mRNA was observed for cells in glutamine-depleted medium within 4 hours of challenge and the mRNA remained elevated for a further 4 hours of culture. The pattern of response was similar in cells placed in amino acid-depleted medium. However cells responded more slowly to this stress than previously demonstrated (Figure 3.12) reaching only a 6 to 8 fold increase in Gadd153 expression over 8 hours. Cells in glucose-depleted medium displayed an 8-fold induction of Gadd153 mRNA expression at 4 hours although the extent of stimulation had fallen slightly by 8 hours. This is consistent with the report of Carlson et.al. (1993) who observed Gadd153 mRNA induction in HeLa cells within 4 hours of glucose deprivation. The most marked response in my CHO cell system was in the case of cells placed in medium depleted of both glucose and glutamine or in medium supplemented with

Figure 3.12 Induction of Gadd153 mRNA expression in CHO DG44 19.6 cells after amino acid depletion

(A) Representative blot of Gadd153 mRNA



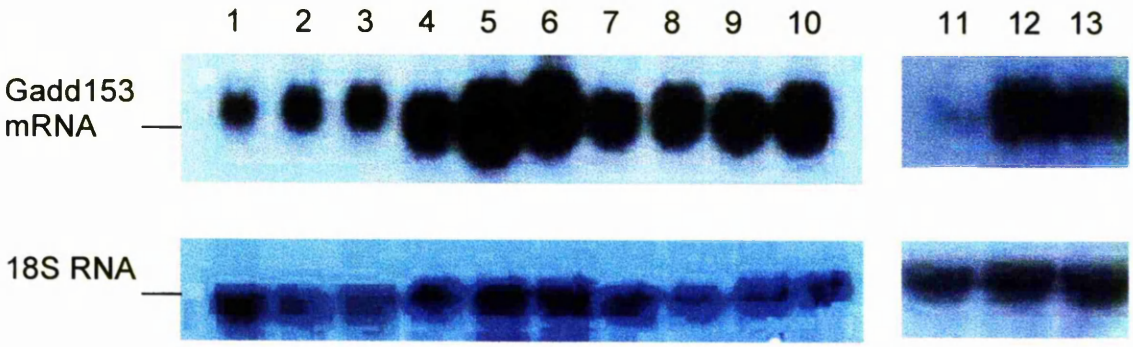
(B) Graph showing effect of amino acid depletion on Gadd153 mRNA expression



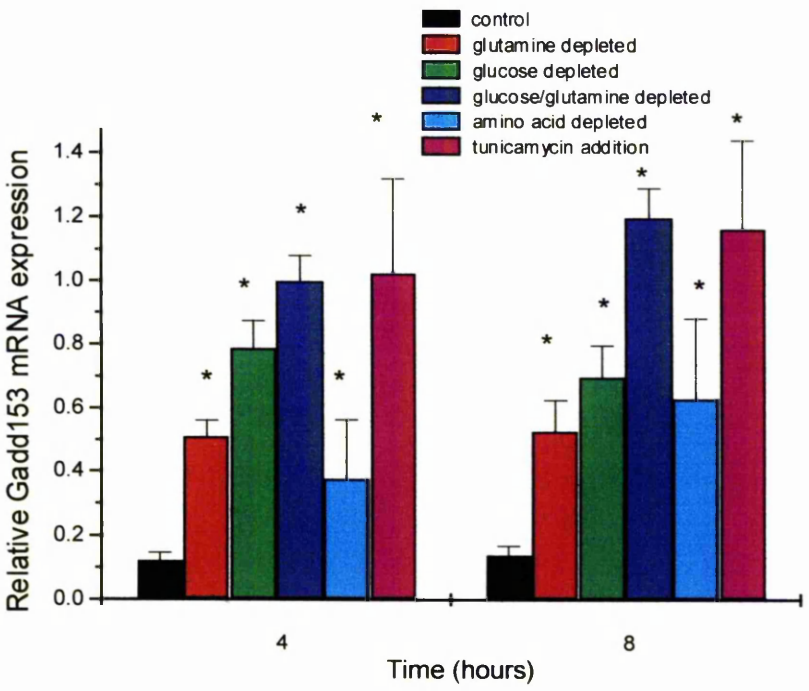
Exponentially growing cells were seeded at a density of $2x10^5$ cells/ml in shake flasks. At mid-log phase cells were washed in 1x PBS and resuspended in treated as described in the legend of Figure 3.11. When present tunicamycin was added at a final concentration of 4 μg/ml. Cells were harvested at 4 and 8 hours and total RNA was isolated (Section 2.7.1). 20 μg of RNA from each sample was separated on an agarose gel and transferred to nylon (Section 2.7.2). After marking the positions of 18S and 28S rRNA species, filters were probed with an α-^{32}P-labelled Gadd153 cDNA (Section 2.7.4.1) and subjected to autoradiography (Section 2.7.5). Filters were stripped and reprobed with an α-^{32}P-labelled 18S rRNA probe again subjected to autoradiography. The RNA species in each lane are as follows lane 1; control 4 hours, lane 2; control 8 hours, lane 3; amino acid-depleted 4 hours, lane 4; amino acid-depleted 8 hours, lane 5; tunicamycin addition 4 hours, lane 6; tunicamycin addition 8 hours, lane 7; glutamine-depleted 4 hours, lane 8; glutamine-depleted 8 hours, lane 9; glucose-depleted 4 hours, lane 10; glucose-depleted 8 hours, lane 11; control 8 hours, 12; glucose and glutamine-depleted 4 hours, and glucose and glutamine-depleted 8 hours). The relative Gadd153 mRNA expression was the ratio of Gadd153 mRNA to 18S rRNA values are presented as mean ± S.E.M. of 3 independent experiments. Significance of difference between control and treated samples was tested using a Student's t-test ($p < 0.05$).*

Figure 3.13 Induction of Gadd153 mRNA in response to various stresses in CHO DG44 19.6 cells

(A)



(B)



tunicamycin (Figure 3.13). In this case Gadd153 mRNA expression increased 10-fold by 4 hours and 12-fold by 8 hours. This potent, rapid effect of tunicamycin agrees with other researchers who have shown that tunicamycin can cause a significant increase in Gadd153 mRNA expression in NIH-3T3 cells and HeLa cells (Price and Calderwood 1992; Jousse et.al. 1999). Jousse et.al. (1999) also noted that stimulation of Gadd153 mRNA expression in response to tunicamycin treatment or glucose depletion of HeLa cells was more profound than the response to depletion of any single amino acid.

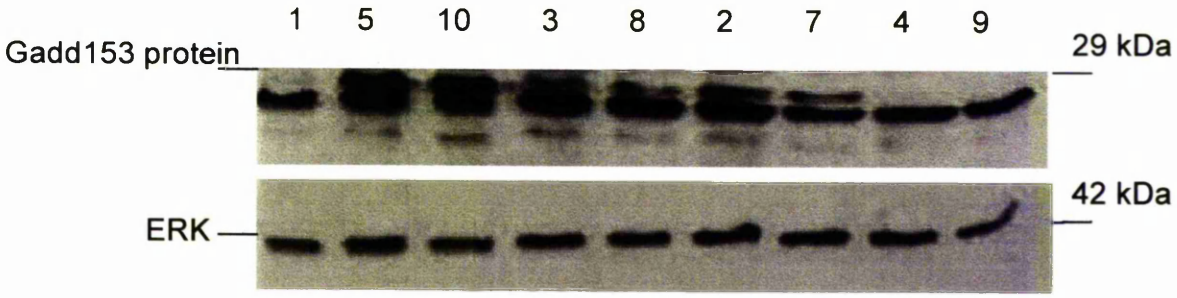
3.2.2.3 Measurement of Gadd153 expression at protein level

Experiments were performed to determine how Gadd153 protein expression responded to nutrient and ER stress. A representative Western blot analysis is shown in Figure 3.14A. The band obtained is consistent with reports by Ron and Habener (1992) and Carlson et.al. (1993) who observed that Gadd153 protein migrates on SDS-PAGE separation with an apparent molecular mass of 29 kDa. For detection of the protein in these experiments they used a rabbit anti-Gadd153 polyclonal antibody that was made in-house against a synthetic peptide corresponding to amino acids 2 to 12 of the Gadd153 protein. I was unable to obtain this antibody so for this study a commercially available antibody was used. This was a Santa Cruz affinity-purified rabbit polyclonal antibody raised against a synthetic peptide corresponding to amino acids 149-168 mapping at the C-terminus of Gadd153 of human origin. The lower molecular weight band detected may be an abundant member of the C/EBP family showing non-specific binding. Replicate experiments were performed to generate a statistical analysis (Figure 3.14B). Cells in glucose-depleted or glutamine-depleted medium exhibited a 3-fold increase in Gadd153 protein expression and this remained constant for a further 6 hours. Tunicamycin caused a most dramatic response (a 19-fold increase by 6 hours and a 32-fold increase by 12 hours). Amino acid depletion, however, had no effect on Gadd153 protein expression. Depletion of all amino acids is likely to inhibit the translation of new proteins in this system. The extent of Gadd153 protein expression correlates with Gadd153 mRNA for each treatment

Exponentially growing CHO DG44 19.6 cells were seeded at $2x10^5$ cells/ml in shake flasks. At mid-log phase cells were washed in 1x PBS and then resuspended in treated as described in the legend of Figure 3.11. When present, tunicamycin was added to give a final concentration of 4 μg/ml. After 6 and 12 hour incubations cells were harvested and protein samples were isolated (Section 2.9.1). 100 μg of protein samples were separated on SDS-PAGE (Section 2.9.3.1) and Western blotting was performed using anti-rabbit Gadd153 polyclonal antibody (Section 2.9.4). Filters were stripped (Section 2.9.5) and reprobed with anti-mouse pan-ERK antibody. Samples analysed were as follows:1; control 6 hours, 2; glutamine-depleted 6 hours, 3; glucose-depleted 6 hours, 4; amino acid-depleted 6 hours, 5; tunicamycin addition 6 hours, 6; control 12 hours, 7; glutamine-depleted 12 hours, 8; glucose-depleted 12 hours, 9; amino acid-depleted 12 hours and 10; tunicamycin addition 12 hours. A representative blot is shown in (A) with results of 3 independent experiments shown in (B). Relative Gadd153 protein expression was calculated as the ratio of Gadd153 protein to ERK protein. Data is presented as mean ± S.E.M. of 3 independent experiments. Significance of difference between control and treated samples was tested using a Student's t-test ($p < 0.05$).*

Figure 3.14 Induction of Gadd153 protein expression under various stresses in CHO DG44 19.6 cells

(A) A representative Western blot of Gadd153 protein expression



(B) Graph showing induction of Gadd153 protein expression

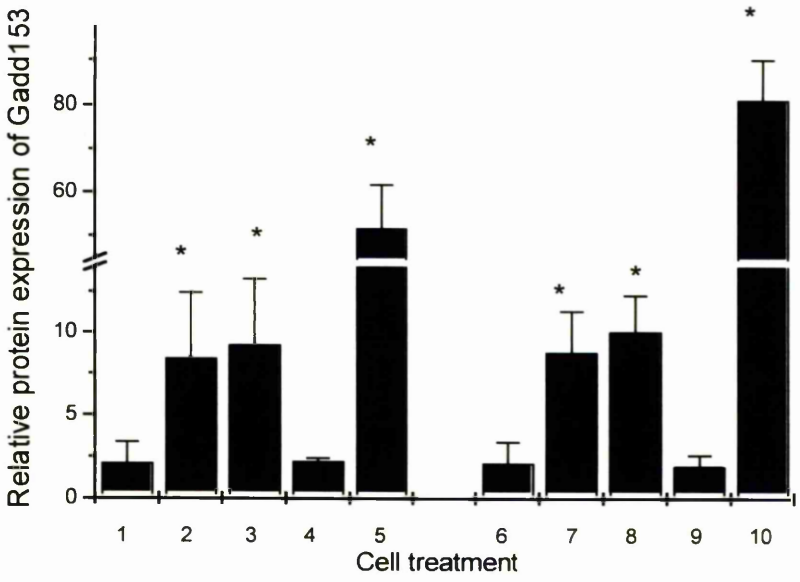
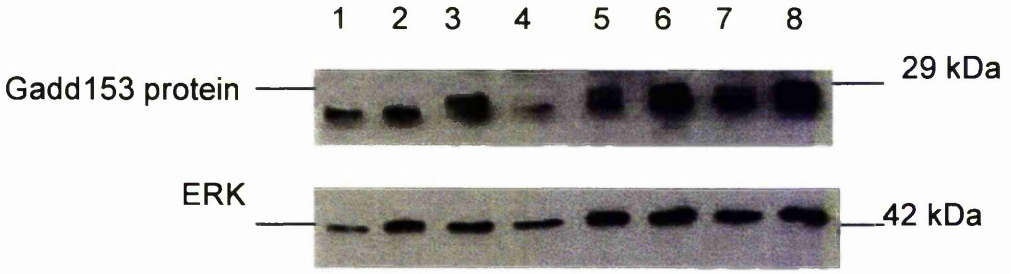


Figure 3.15 Gadd153 protein expression increased with time after depletion of glucose and glutamine in CHO DG44 19.6 cells



Exponentially growing CHO DG44 19.6 cells were seeded at $2x10^5$ cells/ml in shake flasks. At mid-log phase cells were washed in 1x PBS and then treated as described in the legend of Figure 3.11. After 18 and 24 hour incubations cells were harvested and protein samples were isolated (Section 2.9.1). 100 μg of protein samples were separated on SDS-PAGE (Section 2.9.3.1) and Western blotting was performed using anti-rabbit Gadd153 polyclonal antibody (Section 2.9.4). The filter was stripped (Section 2.9.5) and reprobed with anti-mouse pan-ERK antibody. Samples were analysed as follows: 1; control 18 hours, 2; control 24 hours, 3; glucose-depleted 18 hours, 4; glucose-depleted 24 hours, 5 glutamine-depleted 18 hours, 6; glutamine-depleted 24 hours, 7; glucose/glutamine-depleted 18 hours, 8; glucose/glutamine-depleted 24 hours.

except amino acid depletion. The lag in Gadd153 mRNA expression and appearance of protein is due to the time required for the protein to be translated. It has previously been reported that amino acid limitation inhibits protein translation initiation (Dever et.al., 1995), for this reason you would not expect a correlation between mRNA and protein expression under this treatment.

As the effects of glutamine-depletion and glucose-depletion relatively low compared to tunicamycin treatment at 12 hours, an extended time course was performed. Figure 3.15 shows a representative Western blot of an experiment in which the treatment incubation time was extended to 24 hours. At later times of treatment the intensity of Gadd153 protein expression increased in all conditions of nutrient depletion including glucose or glutamine.

3.2.3 Temporal relationship of events occurring in CHO DG44 19.6 cells in response to nutrient and ER stress imposition

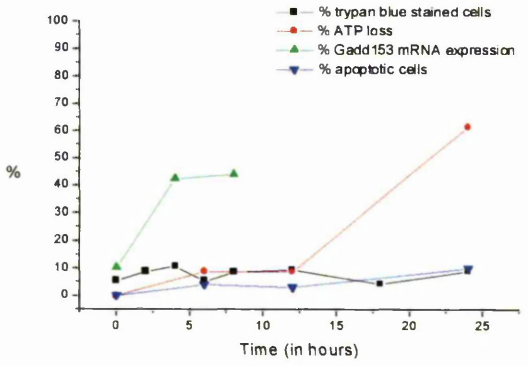
Gadd153 mRNA induction was an early event in cellular response to stress imposition (Figure 3.16). The extent of induction did not necessarily correlate with the degree of apoptosis that occurred in the hours that followed. There were two distinct patterns of response. Amino acid depletion, depletion of glucose plus glutamine and treatment with tunicamycin caused a rapid induction of Gadd153 mRNA followed by a loss of ATP and slightly later by a concomitant decrease in cell viability and enhanced apoptosis. A different pattern was observed in cells placed in glutamine-depleted and glucose-depleted medium. In these cases the slightly lower extent of induction of Gadd153 mRNA resulted in a relatively prolonged lag period before occurrence of ATP loss, cell viability loss and eventual onset of apoptosis.

These results suggest that Gadd153 mRNA induction indicates on the likelihood that apoptosis will occur but that the extent of induction is not directly related to the rapidity of the onset of apoptosis. The rate of change of each parameter in the different treatment conditions gave an ordered sequence of events: initial induction of Gadd153 mRNA, followed by a lowering of cell ATP content and finally, an almost simultaneous loss of cell viability and onset of apoptosis. The

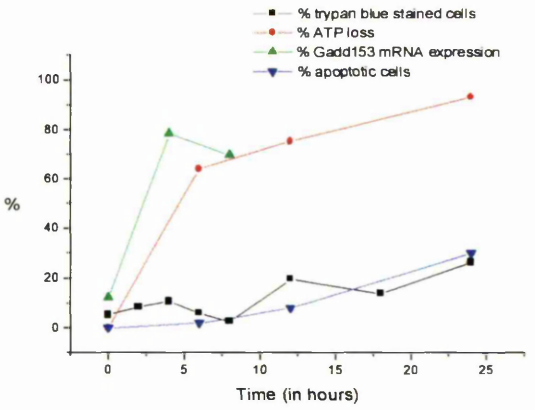
The summary of data from Figures 3.11 to 3.12 are represented a graph for each cell treatment. Information on procedures, detection and analysis are given in Figures 3.11 to 3.13. The relative percentage of Gadd153 mRNA expression was calculated by taking the highest value as 100 % and determining the other values respectively. The relative percentage of all parameters are indicated by a colour code shown in the right hand corner of each graph.

Figure 3.16 Time course of induction of Gadd153 mRNA, apoptosis, loss of cell viability and ATP with various stresses

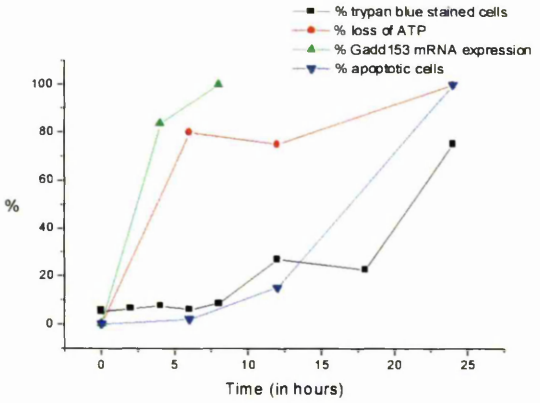
Glutamine depletion



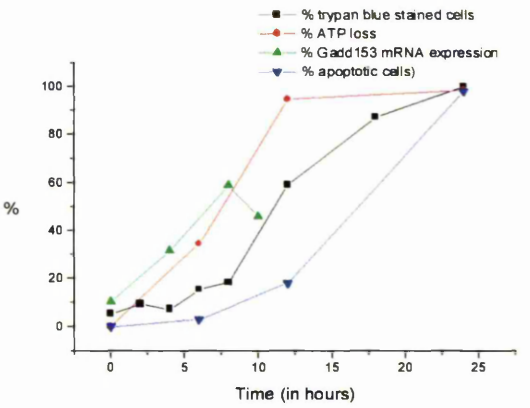
Glucose depletion



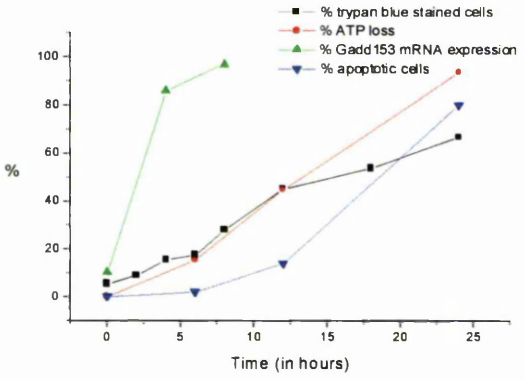
Glucose & Glutamine depletion



Amino acid depletion



Tunicamycin addition



overall order of potency appeared to be that amino acid depletion > glutamine plus glucose depletion > tunicamycin treatment > glucose depletion > glutamine depletion.

3.2.4 Relationship between Gadd153 mRNA induction, loss of ATP and onset of apoptosis with a decrease in cell viability

To determine if there were correlations between Gadd153 mRNA induction, ATP loss, onset of apoptosis and cell viability, data obtained from previous experiments (Figure 3.11 to 3.13) were pooled together and correlative graphs were plotted, as shown in Figure 3.17.

There appears to be a relationship between the extent of Gadd153 induction and cell viability. However, cells that were in medium depleted of amino acids showed low Gadd153 mRNA expression despite extensive loss of cell viability and so do not fit with the pattern described for the other treatments.

Cellular ATP content was compared to cell viability. There appears to be a correlation in that cellular ATP and cell viability decrease simultaneously. However, points for tunicamycin treatment lie off scale as cell viability decreased yet cellular ATP content remained relatively high.

The extent of apoptosis was compared to cell viability and there is a clear correlation between apoptotic cells and cell viability indicating that cell death under all stresses used occurs via apoptosis.

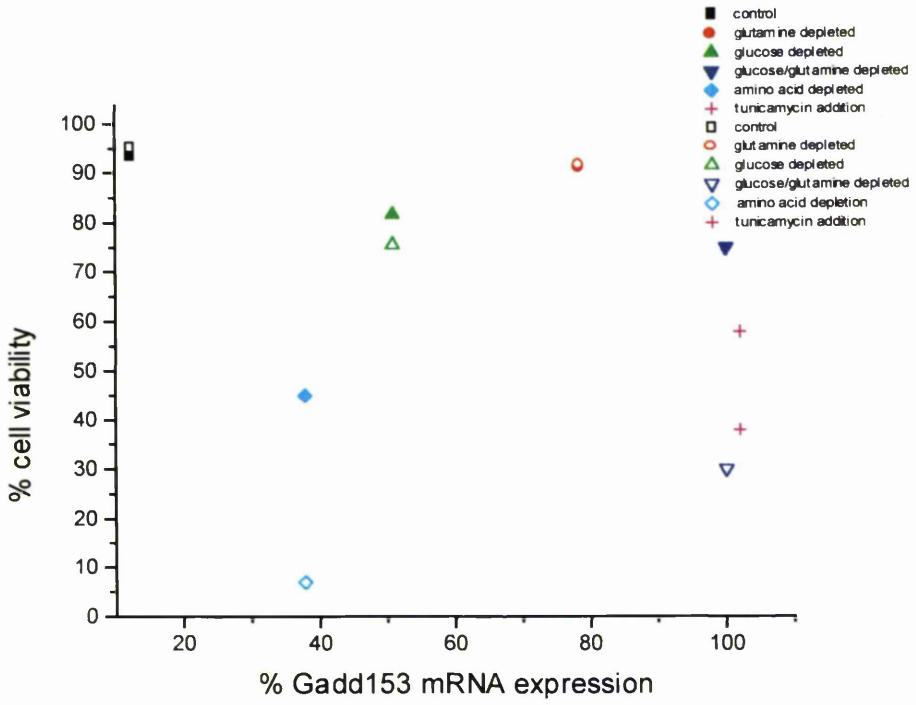
3.2.5 Refeeding experiments: reversal of nutrient stress and consequence for cell survival

Experiments were performed to determine if refeeding nutrient deprived cells had any effect on cell fate in terms of viability, ATP content and apoptosis. The effects on Gadd153 mRNA and protein expression were also examined.

Data was pooled together from Figures 3.11 and 3.13 to determine the association between cell viability after various treatments with respect to the following parameters: (A) Gadd153 mRNA induction, where solid symbols represent % viability at 12 hours versus Gadd153 mRNA expression at 4 hours and open symbols represent % viability at 24 hours versus Gadd153 mRNA expression at 8 hours, (B) ATP content, where solid symbols represent % viability at 12 hours versus % ATP content at 12 hours and open symbols represent % viability at 24 hours versus % ATP content at 24 hours and (C) onset of apoptosis, where solid symbols represent % viability at 12 hours versus % apoptosis at 24 hours and open symbols represent % viability at 24 hours versus % apoptosis at 24 hours.

Figure 3.17 Relationship between induction of Gadd153 mRNA, loss of ATP and onset of apoptosis with cell viability after treatment with stress

(A) % Gadd153 mRNA expression versus % cell viability



(B) % ATP content versus % cell viability

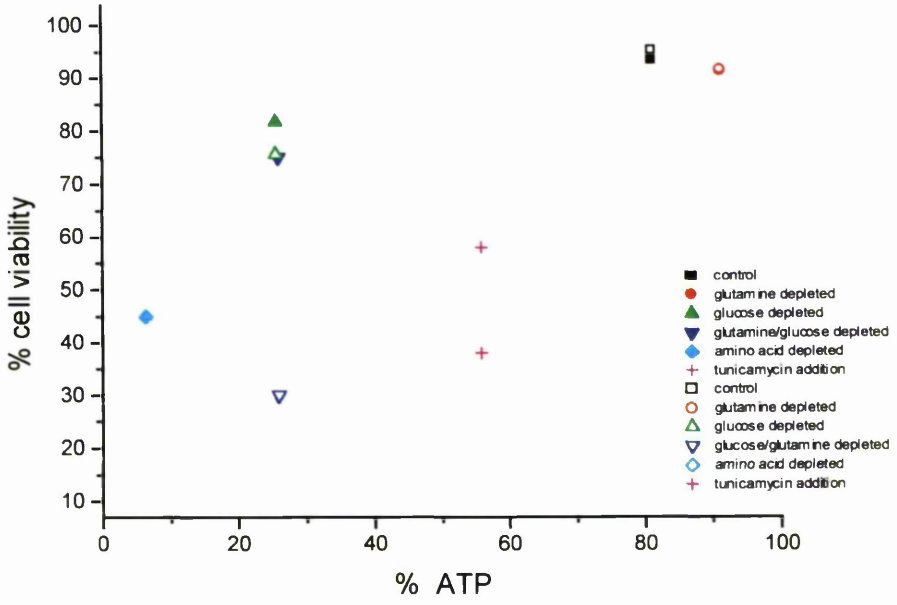
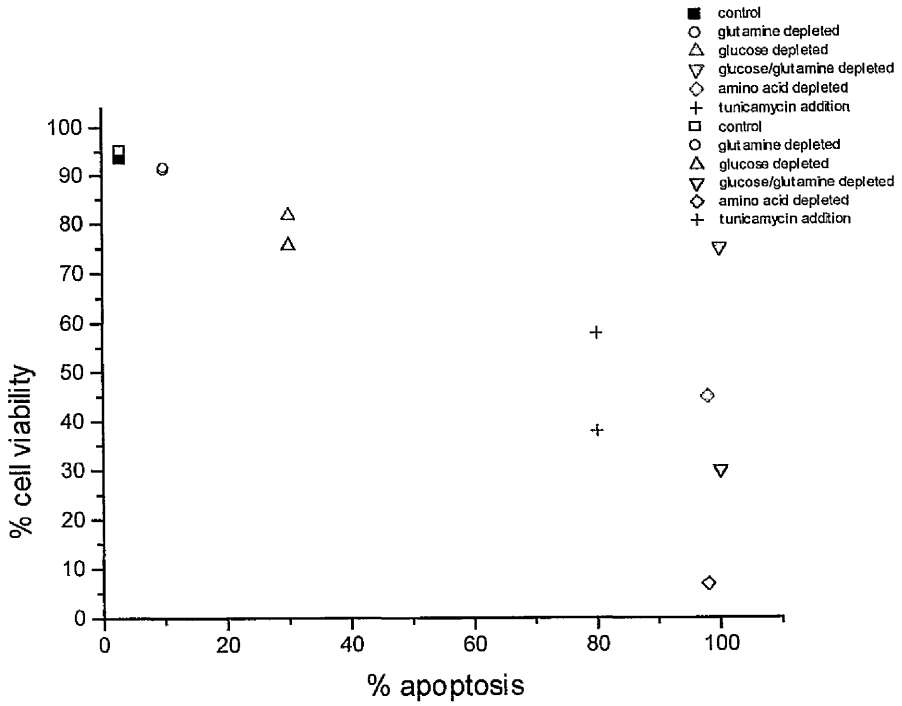


Figure 3.17 (continued)

(C) % Apoptosis versus % cell viability



3.2.5.1 Measurement of cell viability, energy status and extent of apoptotic cell death

Cellular ATP levels, viability and extent of apoptosis were measured in cells in complete medium and medium which had been depleted of glucose, glutamine, both glucose and glutamine or amino acids. In addition the same parameters were measured for cells in complete medium supplemented with tunicamycin. To determine if changes in cellular ATP, viability and apoptosis in response to stress regimes could be reversed or stabilised, nutrients were added back to the cell cultures after 12 hours of treatment and cells were allowed a further 12 hours before harvest for analysis. Data from these experiments is shown in Table 3.1.

Control cells, incubated in complete medium, maintained high viability (93-98%). Cells cultured in glutamine-depleted medium also maintained high viability throughout the time course but a decrease was observed in response to glucose depletion (71 %) and in response to depletion of both glucose plus glutamine (19 %). Readdition of nutrients maintained cell viability to control levels. Amino acid depletion had the most potent effect, decreasing viability to 7 % after 24 hours. However, refeeding partially prevented this dramatic change and cells maintained a viability of 65 % by 24 hours. Cells treated with tunicamycin displayed a decrease in cell viability after 24 hours (38 %).

Cellular ATP content exhibited a more sensitive or rapid decrease in response to stress. Control cells maintained normal ATP content throughout the 24 hour period but cells in glutamine-depleted medium displayed a loss of ATP (19 % by 24 hours) and refeeding only marginally prevented this decrease (25 %). Cells in glucose-depleted medium had a low ATP content (7% by 24 hours). Complete loss of cellular ATP content was observed by 24 hours in cells that were cultured in medium depleted of glucose and glutamine. Readdition of nutrients maintained ATP content to 31 % (i.e. equivalent to the 6 hour value). Amino acid depletion decreased ATP content to 2 % of control by 24 hours and refeeding only partially stabilised this decline (27 %). Cells treated with tunicamycin contained 7 % of control ATP content.

The extent of apoptosis was assessed for all treatments and displayed a less dramatic response to stress relative to the other parameters measured. Under

Table 3.1 Prevention of cell death and maintenance of cell viability by readdition of nutrients to depleted CHO DG44 19.6 cells

Cell treatment	Culture incubation time (hr)	Readdition time (hr)	% Cell viability	% Apoptosis	% ATP
Control	0 6 24	- - -	93 97 98	2 2 3	100 ± 1.0 99 ± 1.4 82 ± 1.6 *
Glutamine depleted	6 24 24	- - 12-24	95 97 97	2 10 2	80 ± 0.7 * 19 ± 1.0 * 25 ± 1.4 *
Glucose depleted	6 24 24	- - 12-24	94 71 97	2 30 2	36 ± 0.6 * 8 ± 0.1 * 24 ± 0.9 *
Glucose / glutamine depleted	6 24 24	- - 12-24	94.3 19 96	2 100 2	32 ± 0.7 * 0 * 31 ± 0.8 *
Amino acid depleted	6 24 24	- - 12-24	92 7 65.6	3 98 60	65 ± 0.7 * 2 ± 1.8 * 27 ± 0.38 *
Tunicamycin addition	6 24	- -	97 38	2 80	84 ± 0.3 * 7 ± 0.9 *

Exponentially growing cells were seeded at a density of $2x10^5$ cells/ml. At mid-log phase cells were washed in 1x PBS and treated as described in the legend of Figure 3.11. When present, tunicamycin was added to a final concentration of 4 μg/ml. Cells were harvested at 0, 6 and 24 hours for analysis of cell viability by trypan blue exclusion (Section 2.2.1) and apoptosis by counting DAPI stained condensed nuclei (Section 2.11.2.2). Cell ATP content was also analysed by performing an ATP firefly luciferase assay (Section 2.2.5.1). This was standardised by determining cell DNA content (Section 2.2.5.2). At 12 hours incubation half the nutrient depleted samples were refed and allowed to recover for a period of 12 hours before harvesting the cells for analysis. Data presented is shown as the mean % cell viability, % apoptosis of 3 counts of one experiment and the mean % ATP content (where 100 % was equal to 158 pmol/ATP/μg DNA) ± S.E.M of 3 independent experiments. Significance of difference between control and treated samples at each time point was tested using a Student's t-test ($p < 0.05$).*

control conditions apoptotic cells were negligible throughout 24 hours of culture. Incubation of cells in glutamine-depleted medium increased apoptotic cells (10 % by 24 hours) but after refeeding, entry into apoptosis was blocked and values were similar to that found in controls. Cells in glucose-depleted medium were 30 % apoptotic by 24 hours whereas cells in glucose plus glutamine-depleted medium were all apoptotic by this time. In both cases negligible amounts of apoptotic cells were detected after refeeding at 12 hours. Similarly, all cells were apoptotic after amino acid depletion however, after refeeding 60 % of cells were still apoptotic. Cells treated with tunicamycin displayed a steady increase in apoptosis to 80 % by 24 hours.

3.2.5.2 Reversibility of Gadd153 mRNA expression

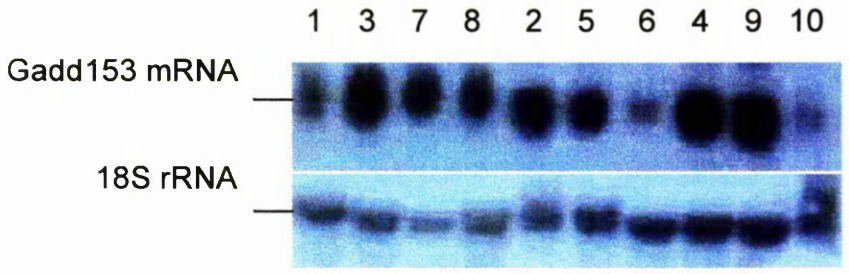
Experiments were devised to determine if the induction of Gadd153 mRNA was reversible and the relationship of this to cell survival. After an incubation period of 6 hours of stress, half the treated cells were placed in complete culture medium (refed) and incubated for a further 6 hours prior to harvest. The remaining cells were subjected to continued incubation in the appropriate 'stress' medium. Samples were taken at 6 hours (control and treated) and 12 hours (control, treated and treated-refed). A representative Northern blot of Gadd153 mRNA expression is shown in Figure 3.18A. The results of 3 independent experiments are shown in Figure 3.18B. A low basal level of Gadd153 mRNA expression was observed in control cells. The measurements of Gadd153 mRNA expression in treated cells were similar to that described in Section 3.2.2.2. Cells placed in glutamine-depleted medium had an increased expression of Gadd153 mRNA at 6 and 12 hours relative to control reaching a value of 3–fold of control. However, after readdition of glutamine at 6 hours and a 6 hour recovery period, expression had returned to basal levels. Depletion of glucose and readdition gave a similar reversible pattern of expression. Depletion of both glucose and glutamine gave the most marked increase in Gadd153 induction, almost 50 % greater than with glutamine depletion alone or glucose depletion alone. However, after placing cells in complete medium Gadd153 expression was restored to control values.

The reversibility of Gadd153 mRNA expression in response to nutrients agrees with the report of Carlson et.al (1993) for HeLa cells deprived of glucose. Huang et.al. (1999) also found that Gadd153 mRNA expression is decreased by refeeding glutamine to nutrient-deprived LLC-PK1 cells.

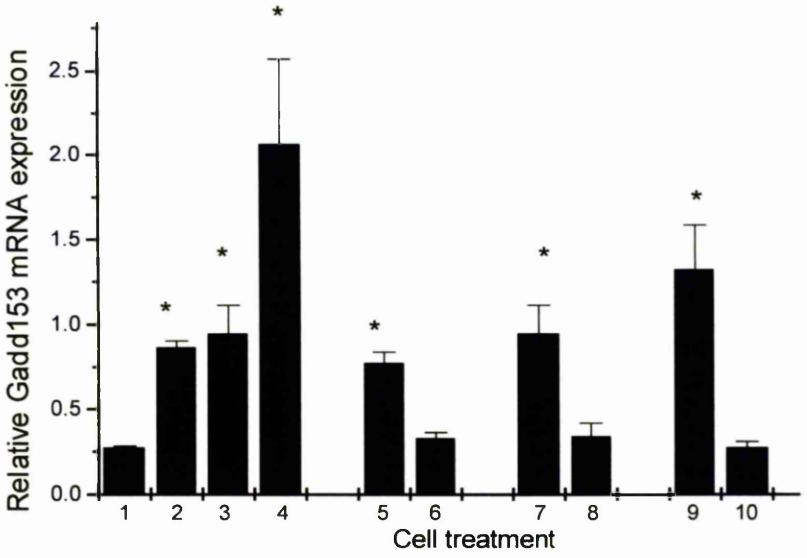
Exponentially growing cells were seeded at a density of $2x10^5$ cells/ml in shake flasks. At mid-log phase cells were washed in 1x PBS and resuspended and treated as in legend of Figure 3.11. Cells were harvested at 6 and 12 hours. After 6 hours incubation half the nutrient depleted samples were refed and given a further 6 hours to recover before harvesting the cells. Total RNA was isolated from cell samples as described in Section 2.7.1. 20 μg of RNA of each sample was separated on an agarose gel and transferred to nylon (Section 2.7.2). After marking the positions of 18S and 28S rRNA species, filters were probed with an α-^{32}P-labelled Gadd153 cDNA (Section 2.7.4.1) and subjected to autoradiography (Section 2.7.5). Filters were stripped and reprobed with an α-^{32}P-labelled 18S rRNA probe again subjected to autoradiography. Samples were analysed as follows: 1; control 12 hours, 2; glutamine-depleted 6 hours, 3; glucose-depleted 6 hours, 4; glutamine/glucose-depleted 6 hours, 5; glutamine-depleted 12 hours, 6; glutamine-depleted 6 hours + 6 hours refed, 7; glucose-depleted 12 hours, 8; glucose-depleted 6 hours + 6 hours refed, 9; glutamine/glucose-depleted 12 hours, 10; glutamine/glucose-depleted 6 hours + 6 hours refed. The relative Gadd153 mRNA expression values were calculated as the ratio of Gadd153 mRNA expression to 18S rRNA expression. Values are presented as mean $\pm$ S.E.M. of 3 independent experiments. Significance of difference between control and treated (depleted plus depleted and refed) samples at each time point was tested using a Student's t-test ($p < 0.05$).*

Figure 3.18 Readdition of nutrients to stressed CHO DG44 19.6 cells causes Gadd153 mRNA expression to be downregulated

(A)



(B)



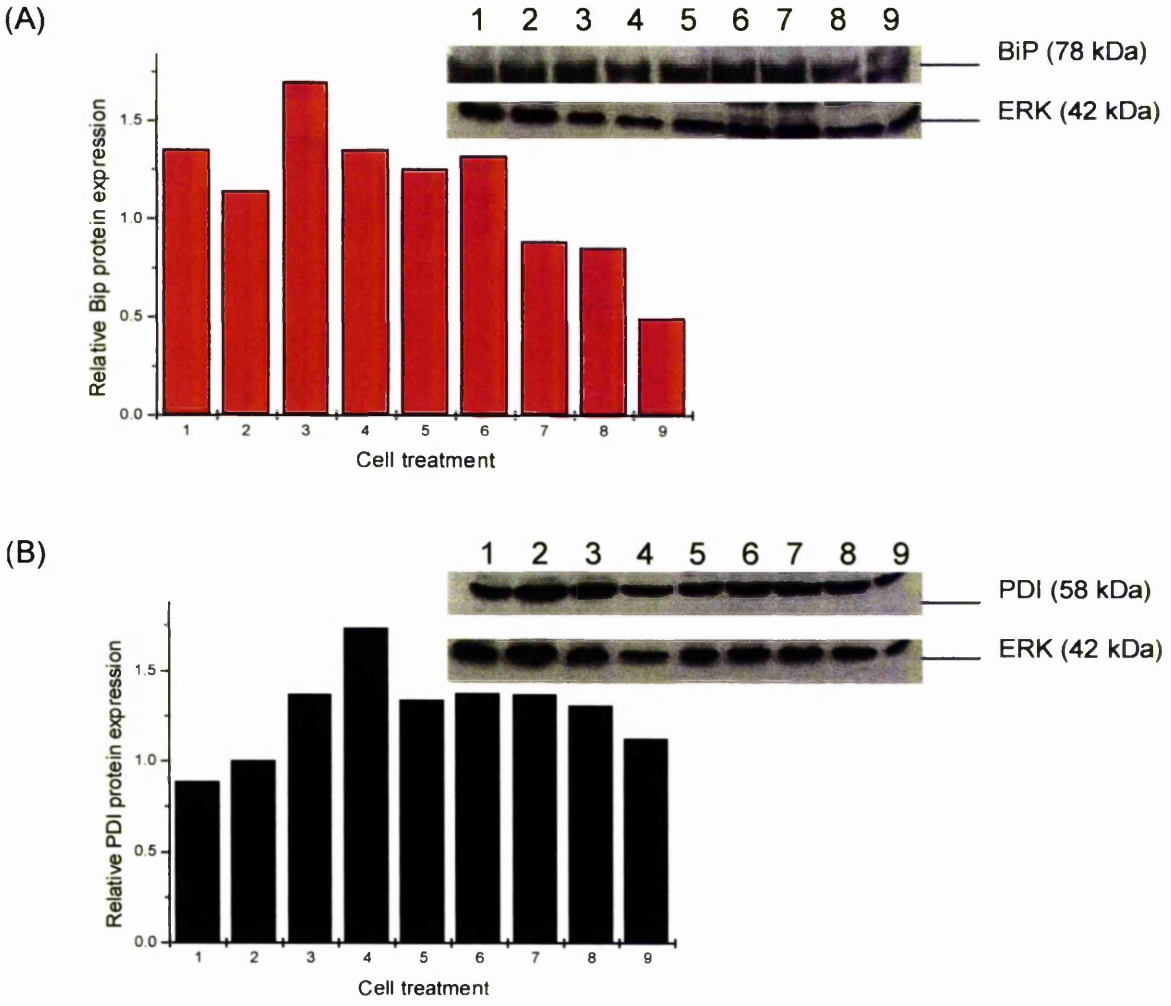
3.3 ARE RECOMBINANT DG44 19.6 CELLS EXPERIENCING STRESS DUE TO UPR OR NUTRIENT LIMITATION?

3.3.1 BiP and PDI protein expression after treatment with various stresses

The presence, and extent, of BiP and PDI expression at protein level was assessed in CHO DG44 19.6 cells in response to stresses shown to influence Gadd153 expression (Figure 3.20). Figure 3.20A is a Western blot of BiP protein expression and the relative loading is represented by a standard ERK blot. The graph shows the relative BiP protein expression with respect to ERK protein expression. Tunicamycin treatment showed a slight elevation in expression after 12 hours. This corresponds with reports by Morris et.al. (1997) who treated CHO cells with tunicamycin and obtained a similar elevated expression of BiP. The ease of detection of BiP in controls conflicts with the report by Morris et.al. (1997) who failed to detect BiP in exponentially growing CHO cells. In the present study glucose depletion and glutamine depletion caused no elevation in BiP expression compared to controls in DG44 19.6 cells. Amino acid depletion, however, caused a decrease in BiP protein expression which correlates with a decrease in Gadd153 protein expression under the same conditions (Figure 3.14). This treatment seems to allow transcription but prevents translation due to the lack of amino acids. Figure 3.20B shows a Western blot of PDI protein expression and the relative loading is represented by the standard ERK blot. The graph shows the relative PDI protein expression with respect to ERK protein expression. PDI was detected in controls. Tunicamycin treatment showed the most marked elevation in expression after 12 hours compared to controls. The other treatments showed slight elevation in PDI expression yet there was a slight decrease in cells placed in amino acid-depleted medium.

Reports by Dorner et.al. (1989) show that when protein misfolding occurs, and unfolded proteins accumulate and aggregate in the ER in recombinant CHO cells, there is a signal that selectively activates transcription of all the genes encoding GRPs, such as BiP, as well as other ER-resident proteins, such as PDI. As BiP and PDI were detected with ease in untreated control cells in the present study it appears they may be experiencing ER stress.

Figure 3.20 Graphs showing BiP and PDI protein expression in CHO DG44 19.6 cells after various stresses

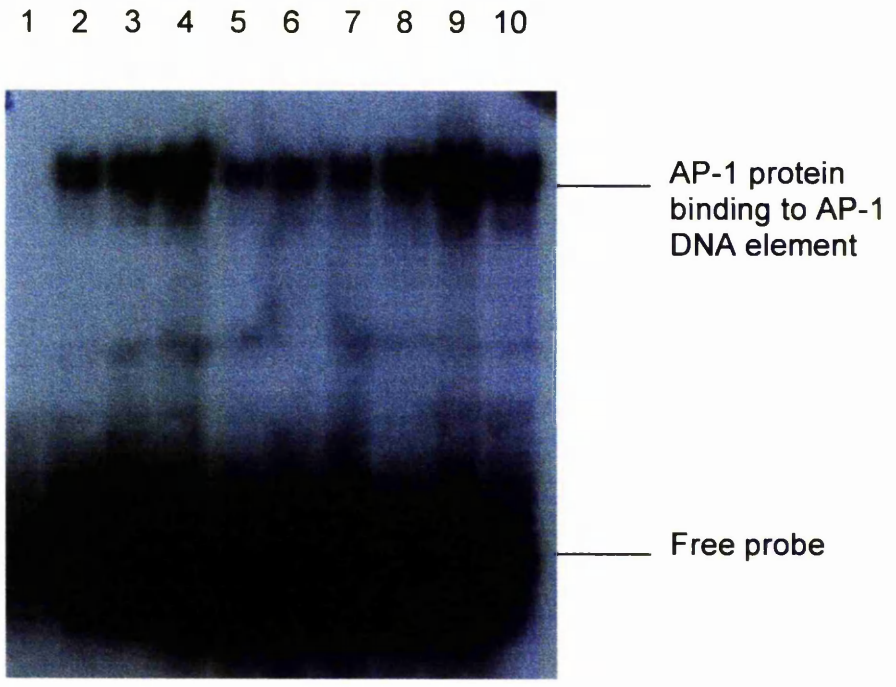


Exponentially growing cells were seeded at $2x10^5$ cells/ml. At mid-log phase cells were washed in 1x PBS treated as described in the legend of Figure 3.11. When present, tunicamycin was added to a final concentration of 4 μg/ml. After 6 and 12 hour incubations cells were harvested and protein samples were isolated (Section 2.9.1). 100 μg of protein samples were separated on SDS-PAGE (Section 2.9.3.1) and Western blotting was performed using anti-rabbit polyclonal antibodies Bip and PDI (Section 2.9.4). Filters were stripped (Section 2.9.5) and reprobed with anti-mouse pan-ERK antibody. Samples were analysed as follows: 1; control 12 hours, 2; tunicamycin addition 6 hours, 3; tunicamycin addition 12 hours, 4; glutamine-depleted 6 hours, 5; glutamine-depleted 12 hours, 6; glucose-depleted 6 hours, 7; glucose-depleted 12 hours, 8; amino acid-depleted 6 hours, and 9; amino acid-depleted 12 hours. Graph (A) represents BiP protein expression with BiP and ERK Western blots and (B) is graph PDI with PDI and ERK Western blots.

3.3.2 AP-1 constantly binds to an AP-1 DNA binding element

Analysis of AP-1 binding under control conditions and in response to tunicamycin-induced ER stress was investigated in CHO DG44 19.6 cells (Figure 3.21). No binding activity was observed in the absence of added protein although DNA complexes were observed at equivalent extents with samples from control and tunicamycin-treated cells. Any slight changes in the AP-1 complex band intensity could be explained by small differences in loading of protein between samples. Reports by Guyton et.al. (1996) demonstrate that Gadd153 transcription is upregulated in response to oxidative stress in HeLa cells. This induction was dependent on binding of AP-1 to the AP-1 DNA binding element within the Gadd153 promoter as its deletion abolished Gadd153 induction. Although binding activity does not mirror transcriptional activity of this complex factor completely, we would expect from this previous report, an increase in AP-1 binding in response to stress. A high level of binding in untreated controls suggests that the cells may be experiencing stress, and again this could be due to the fact that they are producing and secreting a heterologous protein. However, cells require mitogens to survive therefore these cells may have adapted by expressing high levels of AP-1 which is also required to transduce growth and proliferative signals as they are cultured in a serum-free medium (Section 1.4.5.2.1).

Figure 3.21 EMSA showing a constant binding of nuclear protein to an AP-1 DNA binding site in stressed and non-stressed cells



Exponentially growing cells were seeded at $2x10^5$ cells/ml. At mid-log phase cells were washed in 1x PBS and resuspended in treatment media described below. Tunicamycin was added to a final concentration of 4 μg/ml. Cells were harvested for nuclear protein isolation after 3 and 5 hours (Section 2.10.1). 10 μg of nuclear protein was mixed with a [α ^{32}P] dATP labelled, double stranded oligonucleotide (AP-1 DNA binding site) and incubated for 30 minutes to allow binding (Section 2.10.2). Samples were separated on a 6 % polyacrylamide gel. After 3 hours the gel was dried, covered in plastic film and exposed to a phosphorimage plate for an appropriate amount of time before being subjected to autoradiography. Contents of each lane of the autoradiograph are as follows: lane 1, free probe, , lane 2; control 0 hours, lane 3; control 1 hour, lane 4; control 2 hours, lane 5; control 3 hours, lane 6; control 4 hours, lane 7; tunicamycin addition 1 hour, lane 8; tunicamycin addition 2 hours, lane 9; tunicamycin addition 3 hours and lane 10; tunicamycin addition 4 hours.

3.3.3 Gadd153-C/EBP heterodimers bind to novel DNA binding sites

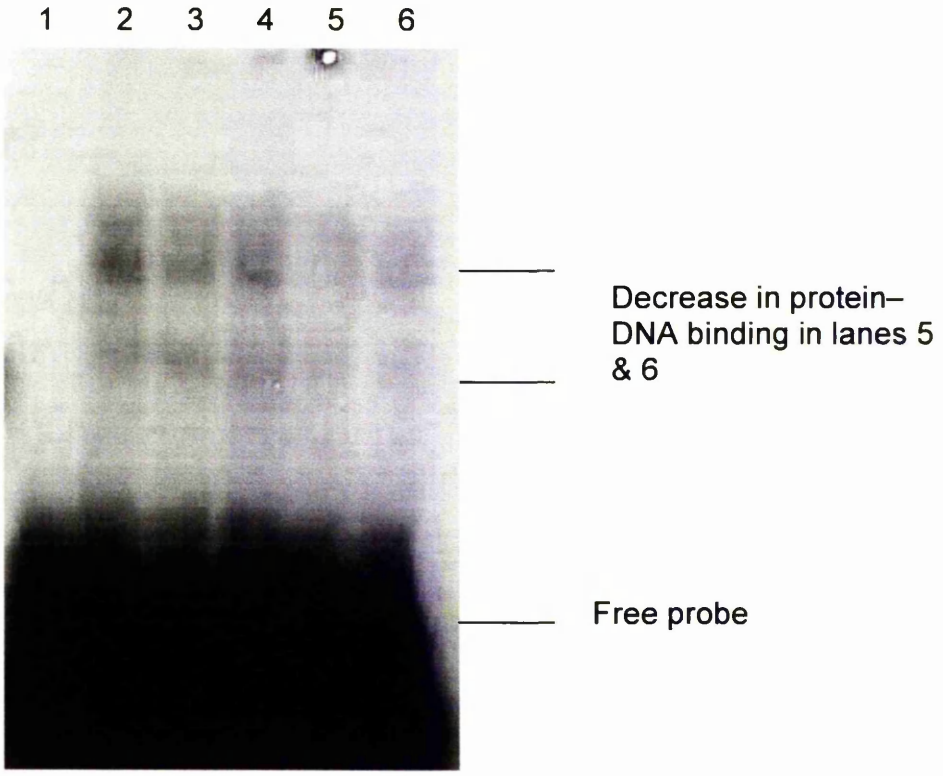
Experiments were performed to determine if induction of Gadd153 protein in response to nutrient or ER stress could generate changes in the formation of binding to classical and alternate C/EBP DNA binding elements in CHO DG44 19.6 cells.

An EMSA was performed to determine the relative binding of protein to a classical C/EBP binding element (Figure 3.22). In control samples two distinctive bands were observed and were present in all lanes at a constant amount. Amino acid depleted samples, and controls, displayed no change in DNA-protein complex binding for cells incubated up to 5 hours under each treatment. This is consistent with the Western blotting data showing no detection, therefore limited translation, of Gadd153 protein under these conditions (Figure 3.14). However samples from cells treated with tunicamycin displayed a gradual decrease in the density of the bands between 3 and 5 hours indicating that C/EBP binding to classical DNA binding elements had been attenuated but not completely inhibited.

An EMSA was performed to determine relative binding of protein to a non-classical C/EBP (alternative Gadd153) binding element (Figure 3.23). In control samples two distinctive bands were observed and these were present in all lanes at a constant amount. Samples from cells in amino acid-depleted medium, exhibited a pattern similar to controls which was constant for 5 hours. Treatment of cells with tunicamycin gave rise to a novel DNA-protein complex. Previous Western blotting analysis had indicated that Gadd153 induction by tunicamycin treatment was observed by 6 hours (Figure 3.14).

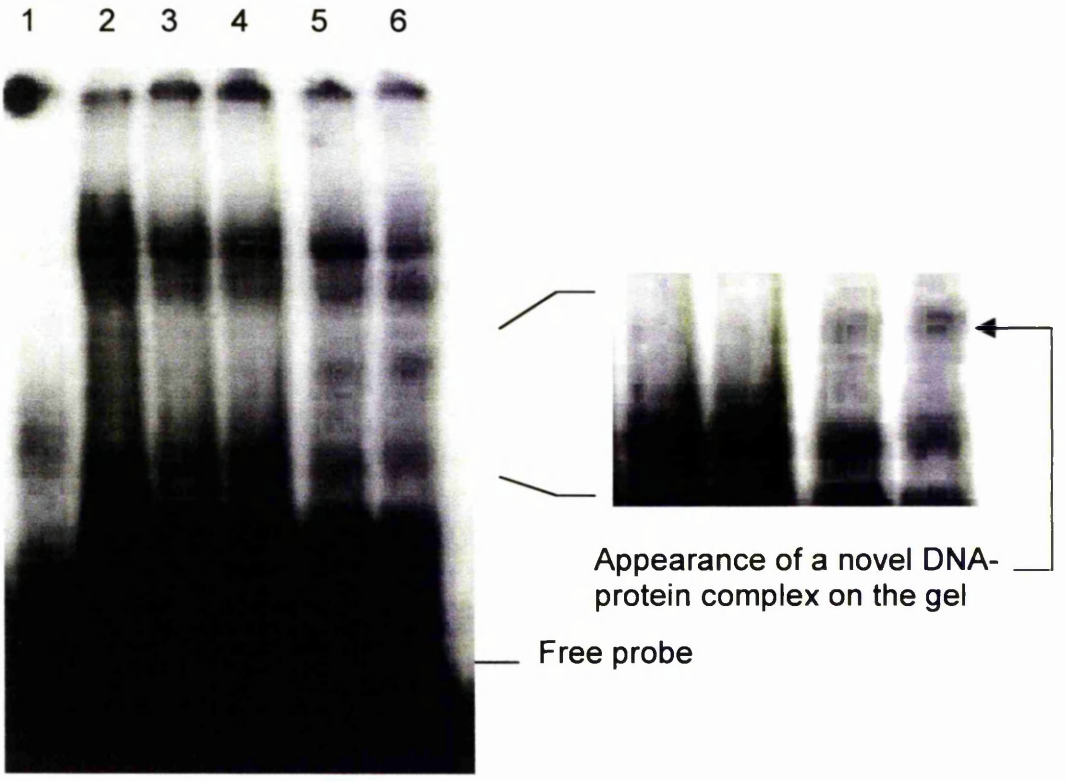
The EMSA data presented in Figures 3.22 and 3.23 support the likelihood that Gadd153-C/EBP heterodimers generated in response to stress in tunicamycin-treated cells are binding to novel target DNA binding elements in CHO DG44 19.6 cells. The implications of this for regulation of apoptosis will be discussed later (Section 4.4.1).

Figure 3.22 EMSA showing loss of binding of nuclear protein to a classical C/EBP DNA binding site after treatment with tunicamycin



Exponentially growing cells were seeded at $2x10^5$ cells/ml. At mid-log phase cells were washed in 1x PBS and treated as described below. When present, tunicamycin was added at a final concentration of 4 μg/ml. Cells were harvested for nuclear protein isolation after 3 and 5 hours (Section 2.10.1). 10 μg of nuclear protein was mixed with a [α ^{32}P] dATP labelled, double stranded oligonucleotide (C/EBP DNA binding site) and incubated for 30 minutes to allow binding (Section 2.10.2). Samples were then run on a 6 % polyacrylamide gel. After 3 hours the gel was dried, covered in plastic film and exposed to a phosphorimage plate for an appropriate amount of time before being subjected to autoradiography. Contents of each lane of the autoradiograph is as follows: 1; free probe, 2; control 0 hours, 3; amino acid-depleted 3 hours, 4; amino acid-depleted 5 hours, 5; tunicamycin addition 3 hours and 6; tunicamycin addition 5 hours.

Figure 3.23 EMSA showing a change in binding of nuclear protein to a Gadd153 alternative DNA binding site after treatment with tunicamycin



Exponentially growing cells were seeded at $2x10^5$ cells/ml. At mid-log phase cells were washed in 1x PBS and treated as described below. When present, tunicamycin was added at a final concentration of 4 μg/ml. Cells were harvested for nuclear protein isolation after 3 and 5 hours (Section 2.10.1). 10 μg of nuclear protein was mixed with a [α ^{32}P] dATP labelled, double stranded oligonucleotide (Gadd153 alternative binding site) and incubated for 30 minutes to allow binding (Section 2.10.2). Samples were then run on a 6 % polyacrylamide gel. After 3 hours the gel was dried, covered in plastic film and exposed to a phosphorimage plate for an appropriate amount of time before being subjected to autoradiography. Contents of each lane of the autoradiograph is as follows: 1; free probe, 2; control 0 hours, 3; amino acid-depleted 3 hours, 4; amino acid-depleted 5 hours, 5; tunicamycin addition 3 hours and 6; tunicamycin addition 5 hours.

3.4 GADD153 PROMOTER-GENE CONSTRUCTS AS REPORTERS OF STRESS DURING CULTURE

3.4.1 Regulation of Luciferase activity under the control of the Gadd153 promoter after nutrient and ER stress

As tunicamycin treatment has been shown to be a potent activator of Gadd153 expression (at both RNA and, especially, protein levels Figures 3.13 and 3.14) the addition of this component was used to determine the regulatory properties of the Gadd153 promoter within the Gadd153-Luciferase construct. For transient transfection studies Human Embryonic Kidney (HEK) cells were used as they were more efficient in their uptake of plasmid DNA than CHO cells. Data is presented in Figure 3.24 showing that luciferase activity expressed under the control of the Gadd153 promoter was relatively constant in transfectants maintained in normal growth medium whereas by 8 hours of tunicamycin treatment luciferase activity exhibited a 3-fold increase. This result illustrates that regulation of Gadd153 transcription by tunicamycin treatment is mediated, at least in part, by the promoter sequences situated between nucleotide position –779 and +21. This data agrees with reports by Luethy et.al. (1990) who found this region of the Gadd153 promoter in a Chloramphenicol Acetyltransferase (CAT) reporter construct to be inducible in transiently transfected HeLa, CHO and Jurkat cells after treatment with various stresses.

In further experiments, following the transfection procedure, cells were treated with two basal medium compositions; DMEM-FCS or a serum-free medium, CHO-S-SFM II (complete, depleted of glucose, depleted of glutamine, depleted of all amino acids). Cells in DMEM-FCS were treated with and without tunicamycin. The cells were then incubated for 8 hours before harvesting for analysis of luciferase activity (Figure 3.25). Tunicamycin treatment gave a 6-fold increase in luciferase activity compared to controls. Incubation of cells in medium depleted of glutamine or glucose gave a 1.5-fold increase in luciferase activity compared to relevant controls (CHO-S-SFM II), whereas there was no induction of luciferase activity in cells in medium depleted of amino acids. The extent of induction of the reporter construct was relatively similar to the induction of the endogenous

Gadd153 protein in CHO DG44 19.6 cells under these conditions (Figure 3.14) whereby tunicamycin-treatment caused the largest response followed by a smaller, less significant response after glucose or glutamine depletion. Furthermore, amino acid depletion did not induce Gadd153 protein expression which correlates with this Gadd153-reporter data.

3.4.2 Regulation of Green Fluorescent Protein (GFP) expression under the control of the Gadd153 promoter after nutrient and ER stress

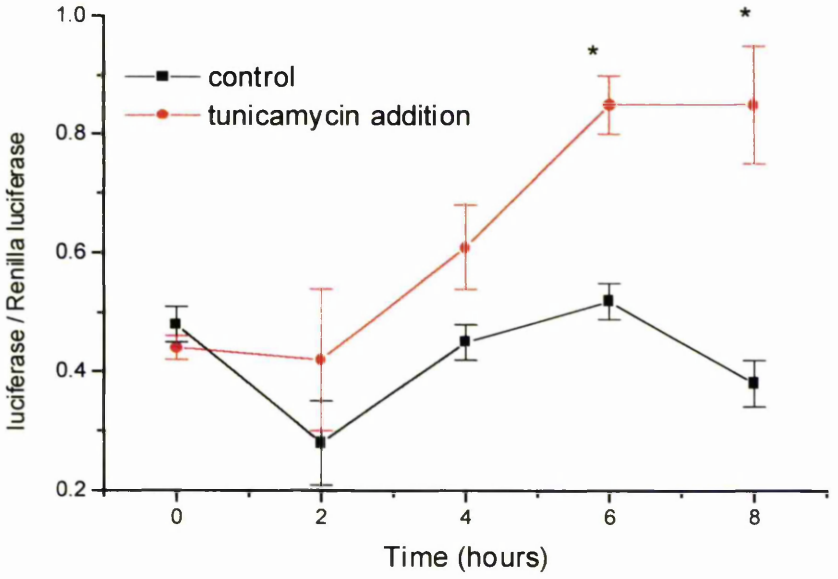
Once it was determined that the Gadd153 promoter was inducible under stress a Gadd153-d2EGFP construct was made for transient analyses. This consisted of the Gadd153 promoter ligated into a pd2EGFP vector (Figure 2.8, Section 2.5.5).

Cells were transfected with Gadd153-d2EGFP and after 8 hours they were visualised by fluorescence microscopy or subjected to FACS analysis (Figure 3.26). Untransfected cells were screened for GFP but they gave no signal on fluorescence microscopy or by FACS analysis (data not shown). Cells transfected with GFP did fluoresce and typical images are shown in Figure 3.26. Image (A) represents control transfected cells incubated in DMEM-FCS. A basal level of GFP expression was observed in these cells. As GFP diffuses throughout the cytosol cell morphology could be visualised and compared to observations by light microscopy. Control cell morphology was intact and cells exhibited the spread that is characteristic of healthy cells. Image (B) represents cells treated with tunicamycin. When observed under the microscope cells were slightly ruffled, an appearance not typical of healthy cells. GFP positive cells fluoresced intensely green. Image (C) represents cells depleted of glutamine. When observed under the microscope cells were intact and spread out and, although most had an overall healthy appearance, there were some intensely green cells that were irregular in shape. Cells placed in medium depleted of glucose and glutamine appeared more unhealthy and green than with glutamine depletion alone, represented by image (D). To determine the percentage of GFP-positive cells within each population of treated cells, FACS analysis was performed (Figure

3.27). Tunicamycin treatment gave a 2.7-fold increase in GFP-positive cells over controls. Glutamine depletion alone gave a 1.3-fold increase whereas depletion of glutamine and glucose gave a 1.5-fold increase in GFP-positive cells.

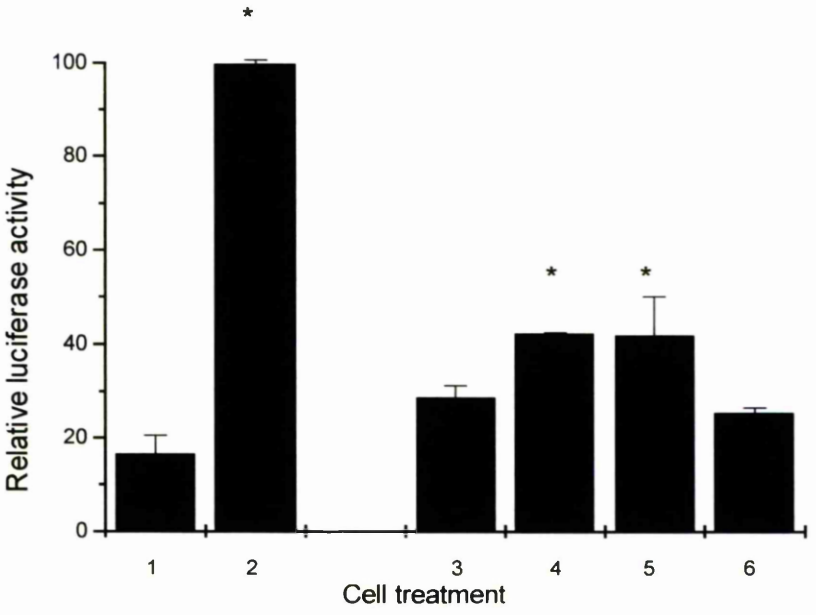
Increased numbers of GFP positive cells were observed when they had been tunicamycin-treated or placed in medium depleted in glucose and glutamine. The onset of increased GFP positive cells correlates with changes in the parameters measured for DG44 19.6 cells which were exposed to the same conditions over a similar amount of time. DG44 19.6 cell viability decreased, the number of apoptotic cells increased and the amount of Gadd153 protein expression had significantly increased (Figure 3.11 and Figure 3.14). As for glutamine depletion HEK GFP-positive cells were less abundant and similarly DG44 19.6 cells were still viable with a lower incidence of apoptosis and Gadd153 expression at this time (Figure 3.11 and Figure 3.14).

Figure 3.24 Time course of tunicamycin addition versus control on the induction of Gadd153-Luciferase in transiently transfected HEK cells



Exponentially growing HEK cells were seeded at 4x10^5 cells/ml in six well plates 24 hours prior to transient transfection. On the day of transfection cells were 60-80 % confluent. Cells were transfected using QIAGEN Superfect Transfection Reagent as described in Section 2.6.5. 100 ng of Gadd153-Luciferase and 10 ng of CMV-pRL were added per well and incubated for 3 hours. CMV-pRL was used as an internal control to assess the transfection efficiency of each sample. Cells were then washed in 1x PBS and then treated with media ± tunicamycin at 4 μg/ml. Cell lysates were isolated at appropriate time points for analysis of luciferase activity and Renilla luciferase activity using a dual reporter assay (Section 2.12.2). Data is presented as the mean ratio of luciferase activity to Renilla luciferase activity ± S.E.M. of 3 independent experiments. Significance of difference between control and tunicamycin-treated samples was tested using a Student's t-test ($p < 0.05$).*

Figure 3.25 Induction of Gadd153-Luciferase reporter construct by various stresses in transiently transfected HEK cells

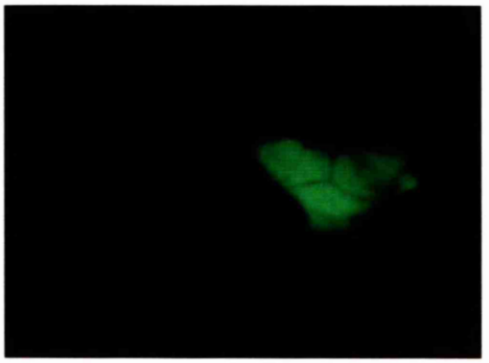


Exponentially growing HEK cells were seeded at $4x10^5$ cells/ml in six well plates 24 hours prior to transient transfection. On the day of transfection cells were 60-80 % confluent. Cells were transfected using 3 μl / well of QIAGEN Superfect Transfection Reagent as described in Section 2.6.5. 100 ng of Gadd153-Luciferase and 10 ng of CMV-pRL were added per well and incubated for 3 hours. Cells were then washed in 1x PBS and then treated with media depleted in nutrients and also with ± tunicamycin at 4 μg/ml. Cell lysates were isolated at appropriate time points for analysis of luciferase activity and Renilla luciferase activity using a dual reporter assay (Section 2.12.2). Data is presented as the mean ratio of luciferase activity to Renilla luciferase activity were tunicamycin treatment was taken to be 100 %. Values are shown as mean ± S.E.M. of 3 independent experiments. Samples were as follows 1; control (DMEM-FCS) 8 hours, 2; tunicamycin addtion (-FCS) 8 hours, 3; control (CHO-S-SFM II medium) 8 hours, 4; glutamine-depleted 8 hours, 5; glucose-depleted 8 hours; and 6; amino acid-depleted 8 hours. Significance of difference between appropriate control and treated samples was tested using a Student's t-test ($p < 0.05$).*

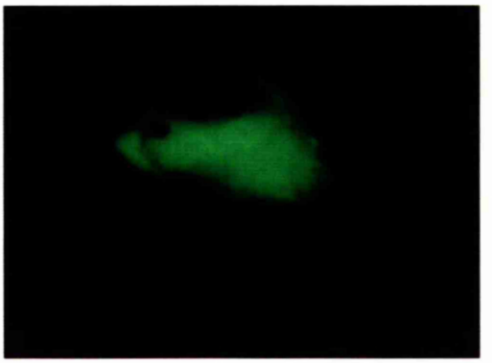
Exponentially growing HEK cells were seeded at a density of $4x10^5$ cells/ml on sterile coverslips, in six well plates, 24 hours prior to transient transfection. On the day of transfection cells were 60-80 % confluent. Cells were transfected using QIAGEN Superfect Transfection Reagent as described in Section 2.6.5. 1 μg of Gadd153-d2EGFP was added per well and incubated for 3 hours. Cells were then washed in 1x PBS and then exposed to treatment media as detailed in the text. When present tunicamycin was added to a final concentration 4 μg/ml. After 8 hours, medium was removed and cell sheets were washed in PBS and fixed in 3.7 % (w/v) paraformaldehyde (Section 2.11.4). Cells were mounted in Gelvatol for image analysis of Green fluorescent protein positive cells by fluorescence microscopy using a FITC filter, with excitation at 488 nm (Sections 2.11.3 and 2.11.4). Images show typical GFP positive cells found within a population of cells where (A) represents a control cell (DMEM-FCS), (B) represents a cell in tunicamycin-treated medium (DMEM-FCS), (C) represents a cell in glutamine-depleted (CHO-S-SFM II) medium and (C) represents a cell in glucose plus glutamine depleted (CHO-S-SFM II) medium. The percentage of the population of cells that were GFP positive was obtained for each treatment by FACS analysis and the data is shown in Figure 3.27.

Figure 3.26 Images of HEK 293 cells after transfection with Gadd153-d2EGFP and treatment with various stresses

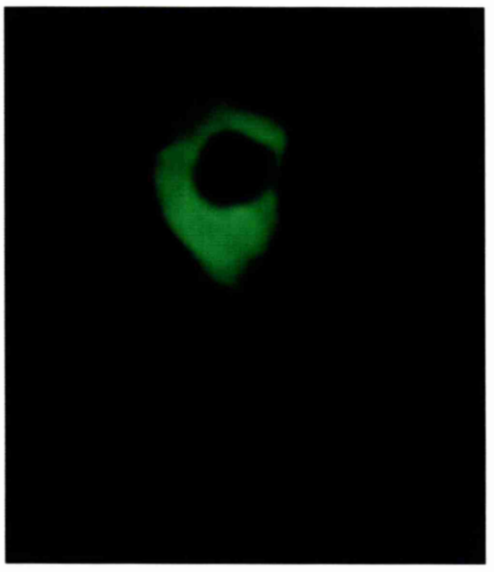
(A)



(B)



(C)



(D)

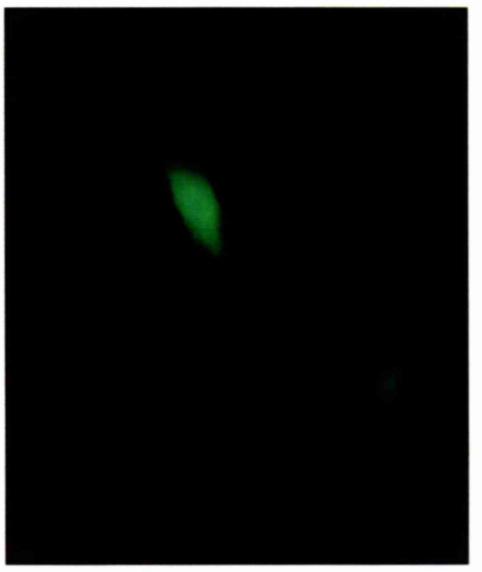
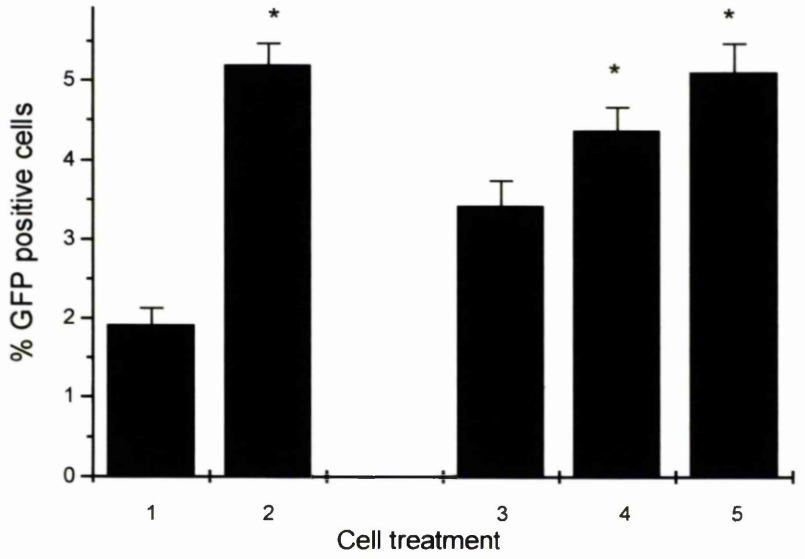


Figure 3.27 Quantification by FACS analysis of Gadd153-d2EGFP reporter construct induction by various stresses in transiently transfected HEK cells



Exponentially growing HEK cells were seeded at $4x10^5$ cells/ml in six well plates 24 hours prior to transient transfection. On the day of transfection cells were 60-80 % confluent. Cells were transfected using QIAGEN Superfect Transfection Reagent as described in Section 2.6.5. 1 μg of Gadd153-d2EGFP was added per well and incubated for 3 hours. Cells were then washed in 1x PBS and exposed to treatment media. When present tunicamycin was added to a final concentration of 4 μg/ml. After 8 hours, medium was removed and cells were harvested and resuspended in 1x PBS for analysis of Green fluorescent protein (GFP) positive cells using a FACS analysis machine and software (Section 2.12.2). Data is presented as the mean % of GFP positive cells ± S.E.M. of 3 independent experiments. Samples are as follows: 1; control (DMEM – FCS media), 2; tunicamycin addition –FCS, 3; control (CHO-S-SFM II media), 4; glutamine-depleted and 5; glucose/glutamine-depleted. Significance of difference between appropriate control and treated samples at each time point was tested using a Student's t-test ($p < 0.05$).*

3.5 PRODUCTION OF A STABLY TRANSFECTED CHO DG44-GADD153-d2EGFP CELL LINE

3.5.1 G418 transfection and cloning

The stress–responsiveness of the Gadd153 promoter (-789/+21 bp) in HEK cells (Section 3.4.2) indicated that this element, within a suitable reporter gene, could offer possibilities as an on-line indicator of cell stress.

Transfections of CHO DG44 19.6 cells were performed with Gadd153-d2EGFP and water (as a mock transfectant). After a period of 14 days in selection media no foci were visible and after a further 14 days no foci had yet appeared and all cells were dead, indicating that the transfection had been unsuccessful. The recombinant CHO cells appear very sensitive to the transfection procedure and it was decided to test the feasibility of the approach in the non-recombinant (parental) cell line. This line, CHO DG44, was transfected and after a period of 14 days foci were clearly visible. In the mock transfectants, no foci were visible. All wells of the 96-well plates were examined under light microscopy to identify wells where single cells were growing. This was to ensure a clonal population. After another 5 days, cells in chosen wells were transferred to 24-well plates then into 6-well plates and subsequently into T-flasks before being stored in liquid nitrogen. Thirty-five clones were eventually selected and grown through to this stage.

3.5.2 Growth characteristics of CHO DG44 and CHO DG44-Gadd153-d2EGFP cells

A comparison of the characteristics of growth and death of CHO DG44 cells and of CHO DG44-Gadd153-d2EGFP (clone 1) in batch culture was made.

3.5.2.1 Growth curves of CHO DG44 cells and CHO DG44-Gadd153-d2EGFP cells

Cells were maintained in HAMS F12+FCS (with G418 supplemented to medium for CHO DG44-Gadd153-d2EGFP) and cell counts performed every 24 hours and the growth profiles of the two cell lines are presented in Figure 3.28.

After seeding both cell lines at an original density of $1x10^5$ cells/ml, CHO DG44 cells experienced an initial lag phase and subsequently entered into an exponential growth phase. This was observed also for CHO DG44-Gadd153-d2EGFP cells, however the initial lag phase lasted 96 hours. CHO DG44 cells reached maximum density ($4.85x10^5$ cells/ml) by 120 hours whereas CHO DG44-Gadd153-d2EGFP cells reached this maximum density 48 hours later (Figure 3.28A). Both cell lines maintained in stationary phase for less than 24 hours and began to die with viability decreasing in a time-dependent manner (Figure 3.28B). This profile is not typical of cells in batch growth as they usually maintain stationary phase for at least 24 hours. As viability decreased the number of non-viable cells found in the media increased at a parallel rate. The doubling times were 31 hours and 44 hours for CHO DG44 and CHO DG44-Gadd153-d2EGFP cells, respectively. This is much slower than values reported by Anwar (1994) who found doubling time of CHO Duk cells to be 17 hours and earlier reports in this Thesis CHO DG44 19.6 cells had a doubling time of 15 hours (Section 3.1.1). It seems that both cell lines are under stress. This may be due to the medium in which they are growing (in the case of CHO DG44 cells) and the inclusion of G418 in medium (CHO DG44-Gadd153-d2EGFP).

3.5.3 Relationship between GFP and Gadd153 protein expression under conditions of ER stress in CHO DG44 and CHO DG44-Gadd153-d2EGFP cell lines

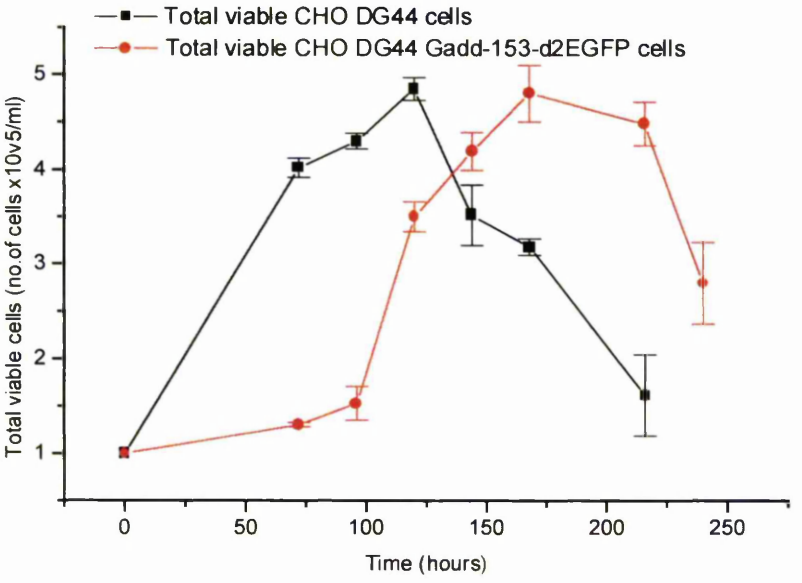
3.5.3.1 Induction of Gadd153 and GFP protein expression

Protein was isolated from CHO DG44 cells for analysis of expression of Gadd153 and GFP at protein level (Figure 3.29). DG44 CHO cells show low but detectable

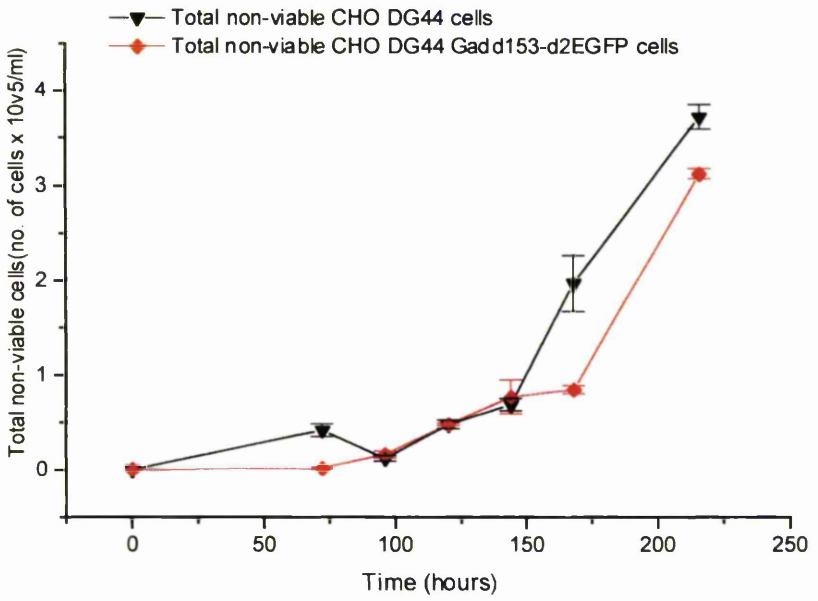
(A) shows total viable cells for CHO DG44 cells (transfected and untransfected) plotted against time and (B) shows total non-viable cells for CHO DG44 cells (transfected and untransfected) plotted against time. To set up growth curves, exponentially growing cells were harvetsed (Section 2.2.1) and seeded at a density of $1x10^5$/ml in T-75 flasks. Cell counts were performed using trypan blue exclusion (Section 2.2.1) at the appropriate time points. Data is presented as the average value for 3 counts ± S.E.M.

Figure 3.28 Comparison of cell growth of CHO DG44 cells and CHO DG44 Gadd153-d2EGFP cells

(A)



(B)

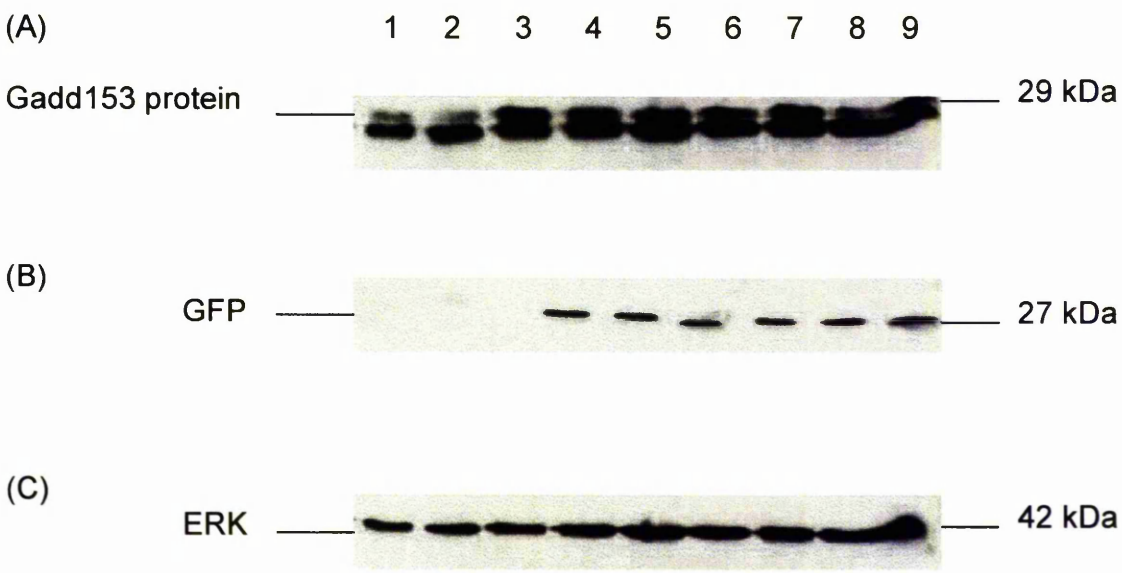


levels of Gadd153 in control conditions and in response to tunicamycin-treatment. However, by 7 hours of treatment with tunicamycin Gadd153 protein expression had increased substantially. Gadd153-d2EGFP transfected cells displayed a high level of Gadd153 protein in control conditions and this was relatively insensitive to tunicamycin treatment. Filters were stripped and reprobed for GFP using an anti-GFP antibody (Figure 3.29B). GFP was absent from parental CHO DG44 cells and was detected in equal amounts in all lanes that contained samples taken from CHO DG44-Gadd153-d2EGFP cells. Thus there was no induction of GFP expression by tunicamycin.

3.5.3.2 Involvement of G418 on induction Gadd153 and GFP protein expression

G418 is known to inhibit protein synthesis (Dawson et.al., 1986). CHO DG44-Gadd153-d2EGFP cells may be experiencing stress due to the selection procedure and this could account for the slow growth rate of the cells in batch culture. The contribution of G418 to the high basal level of Gadd153 protein expression in this cell line was examined. CHO DG44-Gadd153-EGFP cells were placed in medium containing several G418 concentrations. Cells were grown in HAMS F12+FCS with specific G418 concentrations for a period of 2 weeks. When the cells reached 80 % confluence for the third time, they were washed in PBS and cells were then resuspended in fresh media and the response to tunicamycin was examined. As a control CHO DG44 cells were treated in parallel (Figure 3.30). CHO DG44 cells displayed relatively low expression of Gadd153 in control conditions but there were increased levels in tunicamycin-treated cells. The level of Gadd153 expression in CHO DG44-Gadd153-d2EGFP cells was high in control and treated cells and this high expression in controls was dependent on the G418 selection concentration. The filter was stripped and reprobed with anti-GFP antibody (Figure 3.30B). GFP was absent from CHO DG44 cells and present in all the samples isolated from CHO DG44-Gadd153-d2EGFP cells. The amount of GFP detected was dependent on G418 addition as increased GFP was detected in cells grown in medium supplemented with 100 μg/ml G418 compared to cells grown in medium without G418.

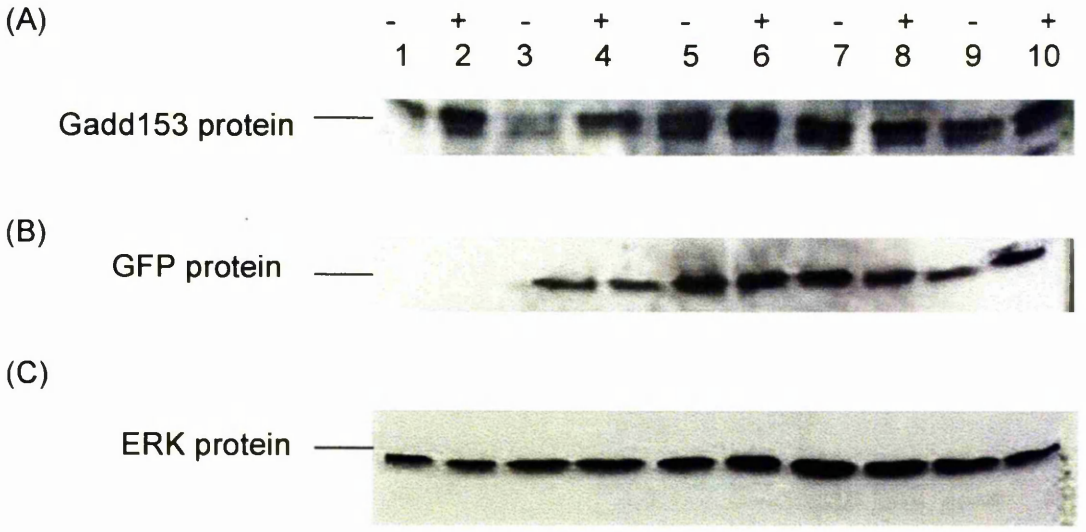
Figure 3.29 Relationship between the induction of Gadd153 protein expression and GFP expression in CHO DG44-Gadd153d2EGFP cells



Exponentially growing CHO DG44 and CHO DG44-Gadd153-d2EGFP cells were seeded at a density of 1x10^5 cells/ml in T-75 flasks. At confluence cells were washed in 1xPBS and resuspended in fresh media or media containing tunicamycin to a final concentration of 4 μg/ml. Cells were harvested after 3, 5 and 7 hours for isolation of total cellular protein (Section 2.9.1). 100 μg of protein samples were separated using SDS-PAGE (Section 2.9.3.1) and Western blotting was performed using anti-rabbit Gadd153 polyclonal antibody (Section 2.9.4) the blot is shown in (A). The filter was stripped (Section 2.9.5) and reprobed with anti-rabbit GFP antibody and the blot is shown in (B). Again, the filter was stripped and reprobed using anti-mouse pan-ERK antibody as a standard. Protein species in each lane are as follows: lane 1; CHO DG44 control 3 hours, lane 2; CHO DG44 tunicamycin addition 3 hours, lane 3; CHO DG44 tunicamycin addition 7 hours, lane 4; CHO DG44-Gadd153-d2EGFP control 3 hours, lane 5; CHO DG44-Gadd153-d2EGFP tunicamycin addition 3 hours, lane 6; CHO DG44-Gadd153-d2EGFP control 5 hours, lane 7; CHO DG44-Gadd153-d2EGFP tunicamycin addition 5 hours, lane 8; CHO DG44-Gadd153-d2EGFP control 7 hours, lane 9; CHO DG44-Gadd153-d2EGFP tunicamycin addition 7 hours.

Figure 3.30 High basal levels of Gadd153 protein and GFP expression is not associated with G418 concentration

Cell line:	CHO DG44	CHO DG44-Gadd153-d2EGFP		
[G418]:		0	0.1	0.5	1



Exponentially growing CHO DG44-Gadd153-d2EGFP cells were seeded at a density of $1x10^5$ cells/ml in T-75 flasks. At confluence cells were washed in 1xPBS, trypsinised and then resuspended in HAMS-F12 media with differing concentrations of G418 (0, 0.1, 0.5 and 1 mg/ml) in T-75 flasks at $1x10^5$ cells/ml. This procedure was repeated 3 times allowing cell growth in media containing the new concentration of G418 for 2 weeks. When cells reached confluence for the third time, they were washed in 1x PBS and treated with fresh media or media containing tunicamycin to a final concentration of 4 μg/ml. CHO DG44 cells were treated similarly from this stage onwards. Cells were harvested 8 hours for isolation of total cellular protein. 100 μg of protein samples were separated using SDS-PAGE (Section 2.9.1) and Western blotting was performed using anti-rabbit Gadd153 polyclonal antibody (Section 2.9.3.1) the blot is shown in (A). The filter was stripped (Section 2.9.5) and reprobed with anti-rabbit GFP antibody and the blot is shown in (B). Again, the filter was stripped and reprobed using anti-mouse pan-ERK antibody as a standard, shown in (C). Protein species in each lane are as follows: lane 1; CHO DG44 control, lane 2; CHO DG44 tunicamycin addition, lane 3; CHO DG44-Gadd153-d2EGFP control (0 mg/ml G418), lane 4; CHO DG44-Gadd153-d2EGFP tunicamycin (0 mg/ml G418), lane 5; CHO DG44-Gadd153-d2EGFP control (0.1 mg/ml G418), lane 6; CHO DG44-Gadd153-d2EGFP tunicamycin (0.1 mg/ml G418), lane 7; CHO DG44-Gadd153-d2EGFP control (0.5 mg/ml G418), lane 8; CHO DG44-Gadd153-d2EGFP tunicamycin (0.5 mg/ml G418), lane 9; CHO DG44-Gadd153-d2EGFP control (1 mg/ml G418) and lane 10; CHO DG44-Gadd153-d2EGFP tunicamycin (1 mg/ml G418).

3.5.4 Images of control and tunicamycin-treated CHO DG44-Gadd153-d2EGFP cells by fluorescence microscopy

GFP fluorescence intensity of CHO DG44 cells and CHO DG44-Gadd153-d2EGFP cells was analysed in response to tunicamycin-treatment. CHO DG44 cells showed a low-level background autofluorescence (Figure 3.31A). The cell morphology was healthy and fibroblastic in appearance when observed by light microscopy. CHO DG44-Gadd153-d2EGFP cells displayed a distinctly higher intensity of fluorescence in the absence of tunicamycin. This may be a result of the basal level of GFP expression. Cells maintained a morphology that was healthy similar to CHO DG44 cells (Figure 3.31B). After incubation with tunicamycin for 7 hours, the GFP fluorescence intensity of the cells had increased substantially. The most highly fluorescent cells displayed a distinct morphology observed from the diffusion of GFP throughout the cytosol of the cell. GFP-intense cells were more rounded, smaller and ruffled around the perimeter by comparison to controls (Figure 3.31C). A higher magnification of a typical cell from medium containing tunicamycin is shown in Figure 3.32. These images are consistent with the data reported by Wang et.al. (1998) who used a Gadd153-GFP reporter in a CHO cell line and examined induction of fluorescence by treatment with tunicamycin. They found that on induction of GFP the cell size measured by FACS analysis was smaller than cells that were untreated.

Exponentially growing CHO DG44 and CHO DG44-Gadd153-d2EGFP cells were seeded at a density of $4x10^5$ cells/ml on coverslips in 6 well plates. At confluence cells were washed in 1xPBS and resuspended in fresh media or media containing tunicamycin to a final concentration of 4 μg/ml. After 8 hours, medium was removed and cell sheets were washed in PBS and fixed in 3.7 % (w/v) paraformaldehyde (Section 2.11.4). Cells were mounted in Gelvatol for image analysis of Green fluorescent protein positive cells by fluorescence microscopy using a FITC filter, with excitation at 488 nm (Sections 2.11.3 and 2.11.4). Images show typical GFP positive cells found within a population of cells where (A) represents control untransfected CHO DG44 cells, (B) represents control transfected CHO DG44 cells, (C) represents 4 different fields of view of transfected CHO DG44 cells in tunicamycin-treated medium.

Figure 3.31 Images of CHO DG44-Gadd153-d2EGFP cells in control and stress conditions

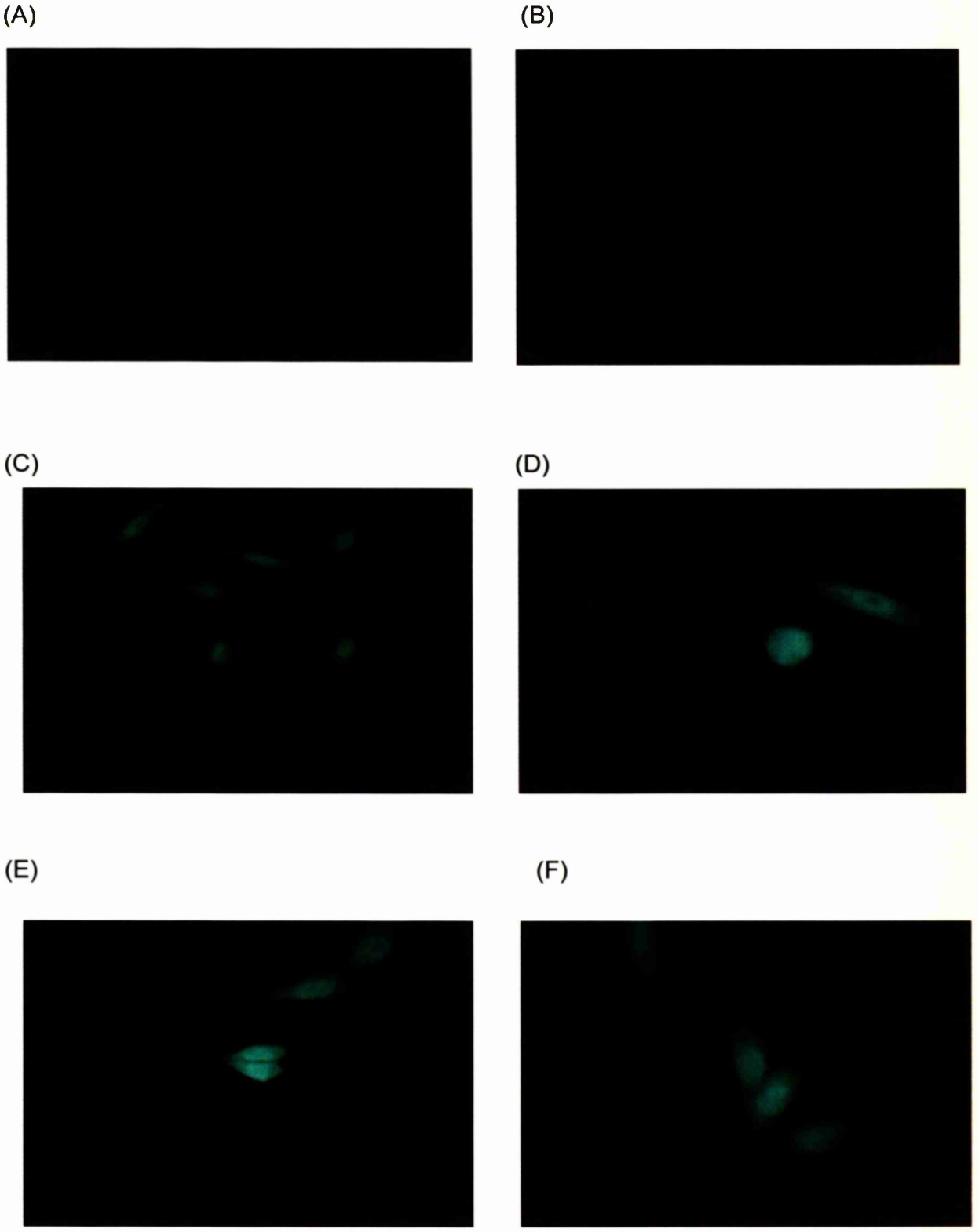
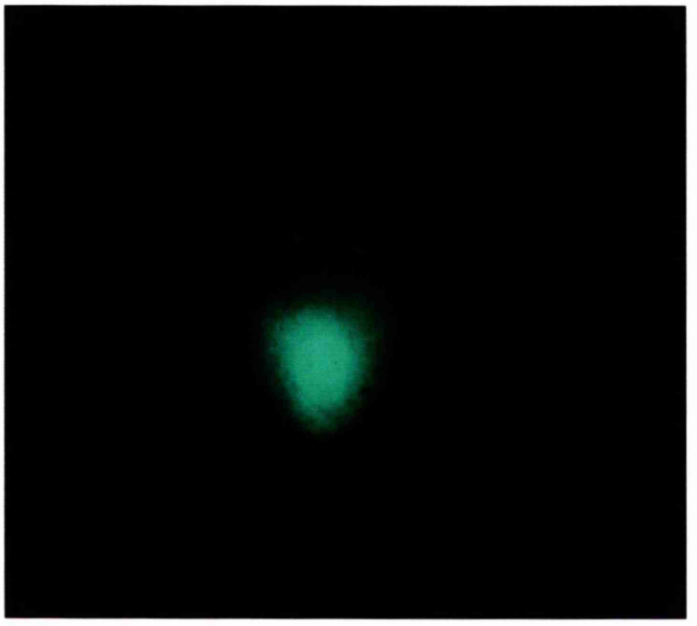


Figure 3.32 High magnification image of a GFP expressing cell under tunicamycin treatment



Exponentially growing CHO DG44 and CHO DG44-Gadd153-d2EGFP cells were seeded at a density of $4x10^5$ cells/ml on coverslips in 6 well plates. At confluence cells were washed in 1xPBS and resuspended in fresh media or media containing tunicamycin to a final concentration of 4 μg/ml. After 8 hours, medium was removed and cell sheets were washed in PBS and fixed in 3.7 % (w/v) paraformaldehyde (Section 2.11.4). Cells were mounted in Gelvatol for image analysis of Green fluorescent protein positive cells by fluorescence microscopy using a FITC filter, with excitation at 488 nm (Sections 2.11.3 and 2.11.4). This image is a high manification of a typical GFP positive cells found within a population of transfected CHO DG44 cells in tunicamycin-treated medium.

CHAPTER 4 DISCUSSION

CHO cells are a mainstay host cell for use in the biotechnology industry for manufacture of complex proteins for both therapeutic and diagnostic purposes. The quality and quantity of recombinant proteins produced is influenced by the culture environment and this is subject to progressive change with time of batch culture. The desired product is produced by viable cells and therefore maintaining a high density of viable cells is an important aspect for maximising product yield. Loss of cell viability is a major limiting factor in the production of recombinant proteins. Prevention of cell death may have major implications for the biotechnology industry and thus recent attention has been focused on the regulation of cell death with a long-term view of increased cell viability and recombinant protein productivity.

4.1 CHARACTERISATION OF VIABILITY LOSS IN CHO CELL BATCH CULTURE

It is clear from the data presented in Section 3.1 that CHO Duk and CHO DG44 cells both die via apoptosis as they enter decline phase of batch culture. DNA ladders (internucleosomal DNA cleavage) observed by DNA agarose electrophoresis coincided with the onset of decline phase (Section 3.1.2.1). However, it has been observed that the phenomenon of DNA fragmentation does not always correlate with apoptotic morphology. For example, Cohen et. al. (1992) provided evidence that dexamethasone induced apoptosis in T-cells even after the addition of zinc (a known inhibitor of calcium/magnesium dependent endonuclease activity). Apoptotic cell death was determined according to a number of criteria such as small cell size and condensed nuclear morphology observed after staining DNA with Hoechst 33342. However, the cells were shown to be devoid of DNA laddering which suggests that endonuclease activity is occurring late in the apoptotic pathway. Furthermore, Collins et. al. (1992) demonstrated that P185 murine mastocytoma cells undergo massive necrosis when treated with DEAE-dextran by examining cell morphology. However, the cells were found to exhibit a typical pattern of DNA cleavage on agarose gel electrophoresis. This evidence suggests that DNA laddering may not be a universal event in apoptosis. Other criteria, including the morphology of apoptosis, need to be used to confirm that the

cells are dying through an apoptotic mechanism. Nuclear condensation determined by fluorescence microscopy, was obvious in CHO Duk and CHO DG44 cells isolated from the decline phase of batch culture (Section 3.1.2.2). The extent of nuclear condensation correlated quite closely with the appearance of trypan blue stained cells (Section 3.1.3), suggesting that an apoptotic mechanism accounts for all cell death observed.

There have been two conflicting views on the mode of cell death of CHO cells in batch culture. In agreement with my findings, Moore et. al. (1995) reported that apoptotic cells could be identified during the course of an 11 day batch culture of CHO Duk cells in 2 litre fermenters (Moore et.al, 1995) and they later found that apoptosis could be delayed by a shift in temperature from 37°C to 30°C (Moore et.al., 1997). CHO Duk cells have also been shown to die via apoptosis by other researchers (Anwar, 1994; Ang, 1996). In addition to CHO Duk cells, other CHO cell lines have been found to die via apoptosis (Goswami et.al., 1999; Sanfeliu and Stephanopoulous, 1999; Zanghi et.al., 1999). In contrast, when Singh et. al. (1994) compared the incidence of apoptotic and necrotic cell death in CHO K1 cells, SF9 insect cells, murine plasmacytoma and hybridoma in batch culture, apoptosis was observed in murine plasmacytoma and hybridoma but not in SF9 cells or CHO K1 cells. In both reports, acridine orange was used as an apoptotic indicator as it binds to double-stranded DNA to identify chromatin condensation. The discrepancy between the results of Moore et. al. (1995) and myself on one hand and those of Singh et. al. (1994) on the other could be due to the properties of the specific CHO cell line used by Singh et.al. (1994) as they have since reported that apoptosis occurs in other CHO cell lines (Perani et.al., 1997). This group examined CHO K1 cells initially, whereas I and the other authors mentioned examined mutant cells that lacked functional dihydrofolate reductase. I used CHO Duk cells in this study, Moore et. al. (1995,1997) and Perani et.al. (1997) used recombinant cell lines derived from CHO Duk cells (Urlaub and Chasin, 1980). In addition I examined a recombinant line produced from CHO DG44 cells (Figure 1.1). In the history of CHO cell line generation, CHO K1 cells were mutagenised to generate cells which independently have yielded CHO Duk and CHO DG44 (Flintoff et.al., 1976). Thus, as a result of chromosomal rearrangements or losses, there may be phenotypic differences between CHO K1 cells and other CHO cell

lines that have been used for assessment of apoptosis and its control. Such differences in culture may have selected for cells with differing capabilities for, and sensitivities to apoptosis. It appears that the sensitivity of specific CHO cell clones to apoptotic signals generated during batch culture may vary in a manner that depends on the origin of the clone used. Furthermore, in my study of CHO Duk cells, at any one time the percentage of apoptotic cells was slightly lower than trypan blue stained cells. This could arise from the fact that late in the apoptotic pathway cells lose the integrity of their plasma membrane and become leaky allowing entry of trypan blue, a phenomenon known as secondary necrosis (Wyllie, 1992). Thus at late stages of decline phase batch culture, the percentage of apoptotic cells is almost always less than the percentage of cells which are non-viable based on trypan blue analysis. The speed with which the transition between late apoptosis to secondary necrosis occurs may be different for particular cell lines and may, in part, explain the differences between the conclusions made in this study and those presented by Singh et.al. (1994). In addition, this loss of cellular plasma membrane integrity late in the apoptotic pathway leads to cell disintegration and may account for the decrease in total cell numbers observed during CHO Duk and CHO DG44 cell culture (Figure 3.1 and 3.2).

Other evidence supports the occurrence of apoptosis as the mode of cell death in CHO cell lines (including CHO K1 cells) as apoptosis occurs in response to a number of defined stresses some of which may be associated with decline phase. Thus indicators of apoptosis have been seen in response to serum withdrawal (Zhang et.al., 1995), overexpression of c-myc (Bissonnette et.al., 1992), overexpression of Bad (Hsu et.al., 1997), overexpression of truncated hPAK65 (Lee et.al., 1997), anti-cancer drugs (Demarcq et.al., 1994; James et.al.,1994), hydrogen peroxide generation (Wang et.al., 1996), protein kinase inhibitors (Reynolds et.al., 1996), UV-irradiation (Orren et.al.,1997), vaccinia virus infection (Ink et.al., 1995) and MHC-directed cytotoxicity (Gao et.al., 1993). It is clear that the cellular machinery required to perform apoptosis is present in CHO cells. However, there remains a discrepancy on whether conditions that are generated by the decline phase of batch culture activate apoptosis or necrosis. In my study I demonstrated that two cell lines representing dhfr$^-$ mutant CHO cells, including

CHO Duk cells routinely used as a host for recombinant protein expression and also CHO DG44 19.6 cells which express a specific recombinant protein, both die via apoptosis in the decline stage of batch culture. With respect to CHO Duk cells, this confirms and extends the work of Moore et. al. (1995), illustrating that even in the absence of transfection or selection procedures CHO Duk cells die by apoptosis. In addition, the data shows that entry into apoptosis occurs in serum-containing medium, identifying that apoptosis in batch culture is not a mere reflection of the use of serum-free conditions as used by Moore et. al. (1995). It is noteworthy that a close link was seen in my studies between detachment of CHO Duk cells from matrix interactions and apoptotic cell death (Section 3.1.1). The apoptotic mechanism involved may be that first described by Frisch and Francis, (1994), described as "anoikis" which is Greek for homelessness. It is known that many cell types are dependent on adhesion to the extracellular matrix or to neighboring cells for their continued survival. Upon detachment from their contacts, endothelial (Meredith et.al, 1993) and epithelial cells (Frisch and Francis, 1994) enter into detachment-induced apoptosis (anoikis). *In vivo* this mechanism ensures that these types of cells do not normally survive in the absence of the correct interaction with extracellular matrix proteins or neighboring cells and are therefore unable to proliferate at inappropriate sites or survive in the absence of attachment, for example in the bloodstream. This mechanism has been found to involve a Ras-mediated signalling pathway via integrin engagement to activate phophatidyl inositol kinase (P I-3 kinase) which in turn activates Protein Kinase B (PKB/Akt), another kinase involved in signalling to prevent anoikis (Downward, 1998). This signalling mechanism may be significant towards a molecular explanation for the control of apoptosis in adherent CHO cell lines. However, when constitutively activated via oncogenic Ras, PKB aids cell survival and blocks anoikis in epithelial cells through a mechanism involving the downregulation of the pro-apoptotic factor Bak (Rosen et.al., 1998; Krestow et.al., 1999). This aspect of cell survival signalling may be relevant to mechanisms by which adherent cells adapt to growth and survive in suspension. The fact that a serum-free suspension culture of recombinant CHO DG44 19.6 cells also died by an apoptotic mechanism argues for the importance of apoptosis as a generic mode of cell death in decline phase of CHO cell batch culture.

Understanding more fully the molecular facets of the different lines and clones of CHO cells will be important towards identifying means of generating CHO cell lines which are more resistant to stresses that accompany growth in batch culture.

4.2 INDICATORS OF THE ONSET OF APOPTOSIS IN DECLINE PHASE BATCH CULTURE

Identification of the factors that trigger cell death in the bioreactor are of great importance to provide optimised cell culture processes. A wide range of physiological and non-physiological factors induce apoptosis and several genes have been identified which may regulate this process (Sections 1.2.2, 1.4.4 and 1.5). Despite the expansion of knowledge in this field there is much that remains to be understood about the regulation of entry into apoptosis.

Several types of intervention regimes have been tested and some of these have been shown to prevent or delay the onset of apoptosis, yet there have been cell-specific differences in responses (Section 1.3). Thus, it is difficult to predict an approach that will prevent apoptosis whilst increasing product harvest. For instance, Bcl-2 overexpression was shown to suppress apoptosis onset in cell lines such as hybridoma (Fassnacht et.al., 1998; Itoh et.al., 1995; Simpson et.al., 1997, 1998; Singh et.al., 1996) however, the specific rates of uptake of nutrients and recombinant protein production were found to be lower than in control cells in decline phase (Simpson et.al., 1998). Furthermore, it has been observed by Zhanghi et.al. (1999) that the overexpression of Bcl-2 in serum-supplemented CHO cell cultures increased the viable cell density in some cases, but it had no effect on apoptosis or death-phase kinetics under a variety of conditions, including glucose and amino acid deprivation in protein-free adapted cultures. In addition Murray et.al. (1996) demonstrated that Bcl-2 overexpression had no effect on cell viability in myeloma cells. In contrast Goswami et. al., (1999) recently reported that Bcl-2 overexpression significantly extended cell viability in serum-free CHO cell batch culture and in response to insulin and transferrin withdrawal. From these reports it is unclear whether overexpression of Bcl-2 will result in improved productivity in batch cultures of production cell lines.

Means to minimise apoptosis or to provide an indicator of the likelihood of cell entry into apoptosis would be advantageous. Such an approach might involve monitoring an indicator of early cellular stress perception, prior to commitment to apoptosis. A detectable 'indicator' could permit early 'rescue' of cells from pre-apoptotic stress and allow prolongment of continued healthy and productive culture. For a recombinant cell within a bioreactor, stress may include nutrient depletion, waste product accumulation, growth factor depletion, hypoxia, hyperoxia, osmotic stress and shear stress (Mastrangelo and Betenbaugh, 1998). Other intracellular forms of stress may impart problems for recombinant cells selected for high level protein expression and these are related to the ability of the cellular protein translational/secretory machinery to cope with the demands imposed upon it. Several stresses in animal cells appear to be perceived by a pathway involving the endoplasmic reticulum (ER), homologous to the UPR of yeast (Section 1.4.2). Very recently studies have identified a pathway by which mammalian cells respond to the 'stress' of damaged or misfolded proteins or ER overload (Hu and Aunins, 1997; Zinszner et.al., 1998; Jousse et.al., 1999). A number of environmental stresses, including nutrient depletion and oxidative damage appear to interact with this pathway at various levels to produce an overall similar response (Wang et.al., 1998). A key feature of this signalling pathway is the induction of Gadd153 (Section 1.5).

My study focused on Gadd153 as a candidate for a possible monitor of cellular stress. Gadd153 is a small (19 kDa) protein that is a member of the C/EBP family of transcription factors and is poorly expressed in non-stressed cells (Luethy et.al., 1990; Carlson et.al., 1993; Marten et.al., 1994). It has been reported that Gadd153 is upregulated in response to serum-deprivation, nutrient limitation and other stresses such as the UPR (Section 1.5.3). Investigation of endogenous Gadd153 mRNA expression in recombinant CHO DG44 19.6 cells in batch culture displayed a 1.7-fold induction of expression after 144 hours of culture and by 192 hours this induction had reached 5-8-fold (Section 3.2.1). Gadd153 induction coincided with the onset of apoptosis and loss of cell viability that signifies the entry into decline phase of batch culture (Section 3.13). A similar pattern of expression was shown, in this laboratory, to occur during NSO myeloma cell culture (Barnes, L., unpublished observation). This data provides evidence that a

relationship exists between the induction of Gadd153 mRNA and the onset of apoptosis in late stationary, or early decline phase of batch culture and indicates that a change in environmental factors may be causing stress within the cell culture. The cells perceive this stress and respond by initiating a programme of events.

Further experiments were performed to investigate relationships between the induction of Gadd153 expression, cellular energy loss, cell viability loss and onset of apoptosis in response to various stresses. The results were used to elucidate a time course of events that may provide clues into the mechanism of action of Gadd153 and its participation in initiation of apoptosis.

4.3 SIGNALLING INVOLVED IN THE ACTIVATION OF GADD153 EXPRESSION

I attempted to dissect the complexity of the batch culture system to understand the mechanistic relationship between Gadd153 and apoptosis. In order to do this various model systems were designed that would mimic some of the typical stresses that cells may be subjected to in the decline phase of batch culture. CHO DG44 19.6 cells were placed in medium deprived of different nutrients including glucose, glutamine, glucose plus glutamine and amino acids. ER stress was also induced in the cells by treatment with medium supplemented with tunicamycin, an inhibitor of N-linked glycosylation known to cause the UPR and to induce apoptosis in a number of cell types (Larrson et.al., 1993; Perez-Sala and Mollinedo, 1995; Dricu et.al., 1996). The potency of each treatment was determined at the levels of effects on cell viability, cellular energy status, extent of apoptosis and Gadd153 expression. Each parameter was measured under every stress condition. By comparative analysis of the data obtained there appeared to be an overall similarity in the order of responses to the different stresses (Section 3.2). Furthermore, the potency of the stress appeared to increase the rate of parameter change.

In response to all stresses Gadd153 mRNA was rapidly induced, followed by a decrease in cellular ATP content and finally a simultaneous cell viability decrease

and the appearance of apoptotic cells. The rate and scale of change of each parameter measured was dependent on the relative potency of the stress. From the data obtained in Section 3.2 stress conditions could be placed in an order of potency, amino acid depletion > glutamine and glucose depletion > tunicamycin treatment > glucose depletion > glutamine depletion. However, corresponding Gadd153 protein expression levels under these conditions were different in that no Gadd153 protein was expressed under conditions of depletion of all amino acids. Depletion of amino acids is known to induce Gadd153 mRNA expression in other cell lines (Bruhat et.al., 1997; Marten et.al., 1994). However, the consequence of stress for Gadd153 protein induction is only observed when sufficient amino acids are present to support protein synthesis. Bruhat et. al. (1997) showed that leucine starvation induced Gadd153 expression through transcriptional and translational mechanisms in HeLa, HepG2 and Coca-2 cells. Their findings also demonstrated that the regulation of Gadd153 expression by depletion of a single amino acid was not a consequence of protein synthesis inhibition as total protein synthesis was not significantly inhibited under these conditions. It has been suggested that cells do not respond to amino acid deprivation directly but that they sense perturbations caused by limiting amino acid levels, such as the synthesis of abnormal proteins (Wang et. al.,1996). Gadd153 is regulated by a mechanism sensitive to the level of many different amino acids. In mammalian cells the signalling pathways involved in the response to amino acid limitation have not been extensively studied. However, in yeast amino acid starvation gives rise to an accumulation of uncharged tRNA which stimulates the activity of the protein kinase GCN2 which in turn leads to the expression of numerous genes (Kilberg et.al., 1994). In mammalian cells, it has been suggested that tRNA charging is involved in the sensing of amino acid levels. A correlation was demonstrated between asparagine starvation, amino acylation of $tRNA^{asn}$ and asparagine synthetase activity (Andrulis et. al., 1979) and, recently, inhibition of leucyl-tRNA synthetase was found to coincide with an induction of Gadd153 expression in CHO cells (Jousse et.al., 1999). It appears that there may be a signalling pathway that is induced by the presence of uncharged tRNAs that mediates Gadd153 induction. A mammalian homologue of GCN2 namely PKR or PERK (Section 1.4.4), has recently been identified which may play a role in the regulation of protein synthesis inhibition in response to amino acid deprivation. The mechanisms involved in this

response may provide clues of cell signalling mechanisms that perceive and initiate cell death in cells subjected to amino acid depletion. Recently, methionyl-tRNA synthetase (MetRS) expression was found to be induced under the conditions of amino acid depletion (Lazard, 1987). Furthermore, Ubeda et. al. (1999) demonstrated that Gadd153 and MetRS mRNAs share a 3' UTR complementary sequence allowing interaction between the two mRNAs *in vivo*. In addition, the two genes are arranged in an overlapping manner on chromosome 12q13 in humans, and an AU-rich regulatory element (ARE), known to control mRNA stability, resides in this overlapping sequence. These observations suggest that a functional interaction between the two mRNAs is highly probable. It was hypothesized that under these conditions Gadd153 and MetRS form a double stranded RNA that may activate PKR (Ubeda et.al., 1999). It has previously been shown that PKR (double-stranded RNA-dependent protein kinase), binds to segments of double stranded RNA and phosphorylates the translational initiation factor eIF2α at Ser 51 to inhibit translation initiation. Phosphorylation of eIF2α by PKR is the major mechanism by which both animal and plant cells regulate protein synthesis when exposed to cellular stress (Haro et.al., 1996).

Gadd153 mRNA and protein induction was also detected in CHO DG44 19.6 cells in response to glucose depletion (Section 3.2.2.2). This data agrees with reports of HeLa and 3T3 L1 cells that were also subjected to glucose depletion (Carlson et.al., 1993). This group demonstrated that induction of Gadd153 under these conditions required newly synthesized protein as the induction was down-regulated by treatment with the protein synthesis inhibitor cycloheximide. Furthermore, they showed that Gadd153 was induced by low cellular ATP. When pyruvate was used as a replacement for glucose in the medium of HeLa cells this partially prevented the induction of Gadd153 but did not abolish it.

Glucose has been shown to be the major energy source of CHO cells (Zanghi et.al., 1999), thus deprivation of glucose is a more potent inducer of apoptosis than the depletion of any single amino acid. Glucose appears to act essentially as an energy source in CHO DG44 19.6 cells but may also participate in the glycosylation of proteins in the ER, including the recombinant, secretory protein that is being produced. Many treatments known to induce Gadd153 also induce

the Glucose Regulated Proteins (GRPs), including tunicamycin, thiapsigargin, dithiothreitol and the calcium ionophore A23187 (Bartlett et.al., 1992; Chen et.al., 1992; Price and Calderwood, 1992) as well as glucose deprivation. In addition, Pouyssegur et.al. (1977) reported that the glucose analogues, 2-deoxyglucose and glucosamine, caused the induction of GRP78 (BiP) and GRP90. This was believed to result from inhibition of protein glycosylation and not from interference with hexose transport i.e. not from energy imbalances (Lee, 1957; Watowich, 1988). Induction of these proteins is indicative of the UPR in mammalian cells. Recently, IRE1 a component of the mammalian ER stress-response pathway was cloned. It codes for a kinase which when overexpressed in 293T, COS1 and CHO cells was found to cause the induction of Gadd153 expression (Wang et.al., 1998). In agreement with their work I detected an upregulation of Gadd153 mRNA, Gadd153 protein and an indication of an upregulation of BiP protein in response to tunicamycin treatment in CHO DG44 19.6 cells.

Gadd153 mRNA and protein induction was also detected in CHO DG44 19.6 cells in response to glutamine depletion. However, this condition caused the least potent effect on all parameters measured. This, in part, may be due to the fact that CHO cells contain relatively high levels of endogenous glutamine synthetase (GS) and can therefore synthesize glutamine from glutamate in the medium when it is in demand (Bebbington, 1992). Feng et. al. 1990 have demonstrated that the specific activity of GS in mammalian cells is inversely proportional to the level of glutamine present because glutamine regulates GS at the post-transcriptional level. In addition, recent reports show that CHO cells grow more slowly in glutamine-depleted medium and enter G(0) and eventually enter apoptosis when glutamate is exhausted from the medium (Sanfeliu and Stephanopoulous, 1999). Glutamine, as well as being an alternate energy source for CHO cells, via the citric acid cycle in the formation of α-oxoglutarate, is also a precursor involved in the synthesis of purine and pyrimidine nucleotides via amidophosphoribosyl-amine transferase and carbamoyl phosphatase synthetase II, respectively (Wice et.al., 1981;1982). Glutamine is also important for protein synthesis, ammonia formation, the biosynthesis of amino acids, amino sugars and certain co-factors and also for the degradation of amino acids, as well as certain special processes such as phenylacetyl-glutamine formation (Meister, 1974; 1980). Recently, it was

reported that glutamine depletion induced Gadd153 mRNA expression but did not appear to effect cellular ATP content in LLK-PK1 cells (Huang et.al.,1999). This group proposed that Gadd153 mRNA induction by glutamine depletion is linked more closely to imbalances in the nucleotide pool than to energy stores in LLC-PK1 cells. Furthermore, glutamine depletion caused a concomitant selective decrease in intracellular CTP concentrations. In agreement with this I found that CHO DG44 19.6 cells subjected to glutamine depletion maintained cellular ATP content for a longer period of time than cells subjected to glucose depletion. However, the extent of Gadd153 mRNA expression was similar under conditions of glutamine or glucose depletion indicating that other factors, besides energy depletion, were contributing to apoptosis after glutamine depletion. It is known that extracellular nucleosides are efficiently transported into mammalian cells, were they are phosphorylated, therefore the rate of nucleotide synthesis is highly dependent on the concentration of the precursors (Loret, et.al., 1986; 1987). Glutamine is a precursor providing essential nitrogen and carbon atoms for the *de novo* ribonucleotide biosynthetic pathway (Olivares et.al., 1992). It was demonstrated that growth arrest and Gadd153 mRNA was induced by glutamine depletion that could be suppressed by the addition of cytidine, uridine and glutamine which restore CTP pools (Huang et.al., 1999).

Depletion of both glucose and glutamine from the medium of CHO DG44 19.6 cells generated an induction of Gadd153 mRNA and protein twice that obtained by depletion of either glucose or glutamine alone and to an extent that was greater than additive. The extent of apoptosis was also doubled. This result was anticipated as depletion of glutamine and depletion of glucose have previously been shown mediate a stress response by completely independent mechanisms, as described above.

4.4 RELATIONSHIPS BETWEEN GADD153 INDUCTION AND ONSET OF APOPTOSIS

It is not clear from the batch culture data in Section 3.2.1 whether apoptosis is a consequence of Gadd153 expression in the cellular response to stress. There are other possibilities that must be ruled out. For example, Gadd153 expression may

be induced in response to apoptosis-inducing events but may be involved in a parallel pathway and so not directly leading to apoptosis. However, even under this condition expression of Gadd153 may still serve as a valuable indicator of an apoptotic phenotype.

The investigation of several types of stress imposition on CHO DG44 19.6 cells demonstrated that Gadd153 mRNA induction was a rapid indicator of cellular stress. However, the extent of Gadd153 induction did not produce a precise reflection of the degree of apoptosis that occurred in the hours that followed. Furthermore, I compared the relative time taken for the parameters to change under the different stresses (Section 3.2.3). Two distinct patterns emerged. Cells depleted of either glucose or glutamine experienced a delay in the onset of cell death despite a similar induction of Gadd153 mRNA expression induced by other stress impositions. For example, amino acid depletion and glucose or glutamine depletion induced Gadd153 expression to a similar level yet apoptosis occurred twice as rapidly in amino acid-depleted cells. This data implies that different mechanisms are involved to trigger Gadd153 induction or that induction of expression is a mere indicator of apoptosis, and not directly involved in its activation.

In order to establish if there was a relationship between Gadd153 activation and onset of apoptosis in CHO DG44 19.6 cells, experiments were performed to investigate the reversibility of Gadd153 expression in relation to cell survival. In parallel the cells were analysed for cellular ATP content, extent of apoptosis and cell viability to determine if the effect of stress could be reversed or stabilised. Gadd153 mRNA induction was reversible after a 6 hour exposure to nutrient stress, followed by refeeding and a further 6 hours recovery period. It appeared that cellular ATP levels were most sensitive to the changes in cellular environment being exhausted after 24 hours by amino acid depletion and by depletion of both glucose and glutamine. Depletion of glucose alone lowered ATP content (8 % of control) more than glutamine alone (19 % of control) thus, it appears that glucose is utilised in the production of energy at a greater extent than glutamine in CHO DG44 19.6 cells. Similarly, serum-free CHO K1 cells use glucose as their major energy source (Zhanghi et.al., 1999) and this is in direct contrast to hybridoma

cells which preferentially metabolise glutamine and other amino acids over glucose as their major energy source (Singh et.al., 1994; Simpson et.al., 1998). In addition, glutamine depletion in hybridoma cells has been shown to be a more potent inducer of apoptosis than glucose depletion (Singh et.al., 1994; Simpson et.al., 1998). After refeeding CHO DG44 19.6 cells with glucose or glutamine I found that ATP levels were stabilised but did not return to control levels within 24 hours. It could be that the cells require a longer period of time to sustain the normal levels of ATP. Changes in cell viability and in the extent of apoptosis paralleled each other under all treatments and took place over a more prolonged time period than the other parameters. Under conditions where ATP levels could be maintained, cell viability and the extent of apoptosis was also maintained. The data suggests that when cellular ATP content has passed a threshold level required for survival, cells were destined to die. This was most evident after amino acid depletion where, after refeeding for 12 hours, the extent of apoptosis was relatively high reaching 60 %. Recently, Feldenburg et.al. (1999) demonstrated that partial ATP depletion induced Fas- and caspase-mediated apoptosis in MDCK cells. The reversibility of activation of such cell signalling pathways after refeeding, however, was not investigated in their study. It is possible that this pathway is being activated in CHO DG44 19.6 cells as the effect of each stress type resulted in the decrease of cellular ATP content.

My investigations attempted to define a time period between the first indication of Gadd153 induction and cellular commitment to apoptosis. It appears that starvation of nutrients for 12 hours, refeeding and recovery for 12 hours was adequate to reverse or stabilise the stress insult in terms of cellular ATP content, cell viability and the degree of apoptosis. This was evident in all cases of nutrient stress except after amino acid depletion. However, the exception of amino acid depletion was not surprising as this condition was found to have the most potent effect on cell viability and apoptosis. The effects of amino acid depletion may be causing overall protein synthesis to decrease due to protein synthesis inhibition. Goswami et. al. (1999) demonstrated that de novo protein synthesis is not necessary for CHO cells to undergo apoptosis. This implies that death-inducing and death-suppressing proteins are always present within the CHO cell, and that possibly death proteins (e.g. Bad, caspases etc.) degrade less rapidly than

survival proteins (Mercille and Massie, 1994; Mosser and Massie, 1994). Hence, in order to enter apoptosis, it is likely that cells reach a critical state that will result in the inefficient translation of putative short-lived apoptosis inhibitory proteins allowing death proteins to exert their effect. This critical state may also vary within a population of cells. It appears that once this level is reached, the apoptotic machinery may be activated irreversibly and cells rapidly undergo the subsequent morphological changes associated with apoptosis.

In agreement with my report it has been demonstrated that the use of feeding strategies aimed at constantly supplying the cells with sufficient nutrients may considerably delay the induction of apoptotic cell death (Franek and Dolnikova, 1991; Duval et.al., 1991; Robinson et.al., 1994; Franek, 1995; Franek and Chladkova-Sramkova, 1995; DiStephano et. al., 1996). However, such strategies may improve productivity but they involve the retention of the protein of interest within the culture environment for extended periods of time. Furthermore, in such pulse-batch or fed-batch cultures, it has also been reported that cells still die through an apoptotic mechanism (DiStefano et.al., 1996).

4.4.1 Activation of Gadd153 and its downstream effects

It is known that when Gadd153 protein is expressed it dimerises with other C/EBP family members (predominantly C/EBP β), interaction with classical C/EBP target genes is blocked and a different group of genes are activated (alternative C/EBP targets) (Ubeda et.al. 1996). Recently, a number of downstream effectors of Gadd153 have been isolated, known as Downstream of CHOP (DOC's). They appear to be strong candidates for genes that may precipitate the entry or progression of cells into, and through, apoptosis (Wang et.al., 1998; Sok et.al., 1999). A more detailed description of DOCs can be found in Section 1.7.5. To determine if this mechanism was occurring under stress conditions in CHO DG44 19.6 cells, EMSA analysis was performed using a radiolabelled alternative Gadd153 DNA binding element. The sequence of this oligonucleotide was identical to a calmodulin-responsive element (CaMRE) to which CHOP-C/EBP heterodimers avidly bind (Ubeda et.al., 1996). The banding pattern obtained in my

study, from cells treated with tunicamycin, correlates with reports for tunicamycin-treated NIH-3T3 cells were a band was obtained with a similar mobility, relative to the two other constant bands that were detected (Ubeda et.al.,1996). This group confirmed that the novel band consisted of a Gadd153-containing complex by introducing an anti-Gadd153 antibody to the system causing a supershift of the band up the gel. The similarity in band mobility indicates that Gadd153 protein is being expressed in response to tunicamycin stress in my system. Subsequent experiments should indicate that DOC genes are also being induced and display implications for their involvement apoptosis. Furthermore, the same nuclear extracts were incubated with labelled double stranded oligonucleotide corresponding to a classical C/EBP DNA element (angiotensinogen gene acute phase response element, APRE) which specifically binds C/EBP homodimers, such as C/EBP α/α or C/EBP α/β, but not CHOP-C/EBP heterodimers (Brasier et.al., 1990). Binding of protein to the classical C/EBP DNA binding element was diminished in tunicamycin-treated samples providing evidence that elevated Gadd153 protein could inhibit induction of any C/EBP target genes involved in cell proliferation and differentiation. It is noteworthy that in my study, under conditions of amino acid depletion (Figure 3.23), Gadd153 protein was not involved in binding and may not be expressed, therefore apoptosis could be triggered via a different pathway under these conditions. Such a pathway may involve the production of a ds RNA as a signalling molecule to activate PKR as described earlier in Section 4.3.

4.5 ARE RECOMBINANT CHO CELLS EXPERIENCING STRESS?

4.5.1 BiP and PDI protein expression – indicators of the UPR?

The endoplasmic reticulum (ER) is an environment in which protein chaperones promote and facilitate the productive folding of proteins and protein complexes. This process prevents interference with folding despite the presence of high concentrations of protein. One important function of chaperones as exemplified by protein disulphide isomerase (PDI), is catalysis of protein folding reactions, increasing the rate of folding without changing the pathway to which the protein

attains its final conformation (Gething and Sambrook, 1992). Other types of chaperone include those that maintain a protein folding-competent state, preventing aggregation of protein intermediates, an example of which is immunoglobulin binding protein (BiP). It was reported that BiP and PDI were upregulated in response to disruption of ER function induced by stresses including deprivation of glucose and inhibition of N-linked glycosylation by tunicamycin addition (Lee, 1987; Kozutsumi et.al., 1988; Dorner et.al., 1989). When protein misfolding occurs and unfolded proteins accumulate and aggregate in the ER, a signal is generated that selectively enhances the expression of BiP and PDI-like proteins and causes an unfolded protein response (UPR) (Kozutsumi et.al., 1988; Dorner et.al., 1989). It was demonstrated that overexpression of BiP prevented the induction of Gadd153 by tunicamycin (Wang et.al., 1996). The signalling involved in the prevention of Gadd153 expression has not been investigated however, it is known that overexpression of BiP prevents the induction of transcription of the GRP family and protects cells from ER stress (Morris et. al., 1997).

The presence, and extent, of BiP and PDI expression at protein level was assessed in the recombinant CHO DG44 19.6 cells in response to stresses shown to influence Gadd153 expression (Section 3.3.1). Tunicamycin treatment showed a slight elevation in BiP expression after 12 hours. This corresponds with reports by Morris et.al. (1997) who treated wild type CHO cells with tunicamycin and obtained a 5-fold elevated expression of BiP compared to controls. However, the ease of detection and basal level of BiP in controls conflicts with the report by Morris et.al. (1997) who failed to detect BiP in exponentially growing (non-recombinant) CHO cells. In the present study glucose depletion and glutamine depletion caused no elevation in BiP expression over that detected in control CHO DG44 19.6 cells. Amino acid depletion, however, caused slight decreases in both BiP and Gadd153 protein expression. As discussed earlier (Section 4.3) the prevention of translation due to the lack of amino acids limits conclusions of changes at protein level. PDI protein expression was also detected in cells growing in basal conditions. The data was similar to that obtained for BiP expression. Tunicamycin treatment showed the most marked elevation in expression after 12 hours compared to controls. The other treatments showed

slight elevation in PDI expression and again, there was a slight decrease in cells placed in amino acid-depleted medium. This data was not surprising as increases in PDI expression have previously been demonstrated in response to ER stress in a similar way to the other GRPs such as BiP (Kozutsumi et.al., 1988; Dorner, 1989).

Dorner et.al. (1989) demonstrated that when protein misfolding occurs, and unfolded proteins accumulate and aggregate in the ER in recombinant CHO cells, there is a signal that selectively activates transcription of all the genes encoding GRPs, such as BiP, as well as other ER-resident proteins, such as PDI. As untreated control cells displayed high basal levels of BiP and PDI in the present study, they may be experiencing the effects of a mild ER stress during exponential growth in batch culture in normal growth medium.

Detection at mRNA level may give further indications of the control BiP and PDI expression in basal conditions and in response to applied stresses. Jousse et.al. (1999) recently found that amino acid depletion did not activate the UPR or BiP expression. They found that downstream of the UPR and after amino acid depletion Gadd153 protein expression was induced, however they isolated two distinct cis-elements thought to be involved in the regulation of the Gadd153 promoter in response to these two different types of stress. My data indicates that recombinant CHO DG44 19.6 cells are experiencing stress caused by UPR. Control cells appear to have a high basal level of expression of UPR induced proteins (BiP and PDI). However, Gadd153 expression under basal conditions is low. It is possible that production of secretory recombinant protein is contributing to a basal stress to which cells have adapted. A more potent stress may be required to activate the transcription of Gadd153 resulting in cell growth arrest or onset of apoptosis.

4.5.2 AP-1 complex formation – indicator of stress?

The promoter of Gadd153 contains an AP-1 element that has been shown to be critical for oxidative regulation of the gene. Guyton et.al. (1996) investigated

binding of AP-1 (fos and jun) to the AP-1 element in HeLa cells after subjecting the cells to conditions of oxidative stress including UV light and hydrogen peroxide addition. Gel shift assays displayed an increase in binding of AP-1 (fos and jun) to the AP-1 element which induced transcription of Gadd153.

I performed an electromobility shift assay (EMSA) to determine whether ER stress stimulated binding of cellular factors to the Gadd153 AP-1 sequence. The AP-1 complex detected under control and tunicamycin conditions displayed binding at a similar mobility to that described by Guyton et.al. (1996). However, they did not detect binding in untreated control samples. In order to determine if fos and jun are actually bound to the AP-1 element in the present study, antibodies against fos and jun could be used to observe a supershift of the AP-1 complex on the gel. It may be that fos and jun are always bound to the AP-1 element but phosphorylation is required to activate transcription. CHO DG44 19.6 cells have been adapted to a serum-free environment and therefore may be experiencing high basal stress levels. AP-1 may be activated at basal levels and this could be what is observed. If this is the case it would suggest that AP-1 induction is not regulated by the ER stress response as there is no change in tunicamycin-treated cells.

4.6 ANALYSIS OF GADD153 AS AN ONLINE MONITOR OF CELLULAR STRESS

The induction of Gadd153 mRNA was rapid and reversible in CHO DG44 19.6 cells under a number of stress conditions (Section 3.2.5). To determine if it had potential as a non-invasive indicator of cell stress, a region of the Gadd153 promoter was used to make a reporter construct. It was previously reported that stress-responsiveness of the Gadd153 promoter resided in the –780/+20 bp region (Luethy et.al., 1990). A reporter was constructed using this region of the Gadd153 promoter ligated into a luciferase vector. The regulation of this reporter gene was examined (by transient analysis) in non-recombinant HEK cells in response to stress. HEK cells were used to assess the responsiveness of the reporter vector as they had previously been shown to be more efficient than CHO cells in the uptake of plasmid DNA (Roberts, G., British Biotech Ltd., unpublished

observation). In previous experiments tunicamycin treatment had proven to be a potent activator of Gadd153 expression at both RNA and protein levels in CHO DG44 19.6 cells (Sections 3.2.2.2 and 3.2.2.3) and therefore this treatment was used to determine the regulatory properties of the Gadd153 promoter within the luciferase reporter. Tunicamycin treatment of transfectants caused a significant induction of luciferase activity under the control of the Gadd153 promoter, whereas under normal growth conditions the reporter maintained a constant basal level of activity. This data illustrates that the regulation of Gadd153 transcription by tunicamycin treatment was mediated, at least in part, by promoter sequences situated between nucleotide positions –779 and + 21. The reporter construct was used in further experiments to determine its response to nutrient stresses. Not surprisingly, the luciferase activity was induced under the control of the Gadd153 promoter in response to glucose depletion, glutamine depletion and amino acid depletion and gave a pattern of activity that paralleled that obtained for regulated expression of the endogenous Gadd153 protein under similar conditions. Glutamine depletion and glucose depletion produced a small increase in luciferase activity but amino acid depletion produced no response. It was recently reported that glutamine depletion upregulates Gadd153 mRNA but that it is primarily controlled by mRNA stabilisation (Abcouwer et.al, 1999). This may account for the relatively low amount of luciferase activity detected in the present study. Furthermore, it was reported by Jousse et. al. (1999) that amino acid deprivation and UPR inducers, such as tunicamycin, regulate the Gadd153 promoter activity using different cis-acting elements. One cis-element is linked to Ire1 activation, whereas the other, an amino acid response element, is independent of Ire1 activation. Deprivation of single amino acids have been considered to indirectly affect protein folding in the ER and activate Gadd153 through a pathway that is common to inducers of the UPR (Wang et.al., 1996). However, the work described above by Jousse et.al. (1999) suggests that Gadd153 induction can be a result of two different pathways, one that is ER-dependent and the other ER-independent.

Jousse et. al. (1999) utilised leucine depletion to illustrate the consequence of amino acid deprivation and found that the Gadd153 promoter, when linked to a reporter gene, was sufficient to mediate the regulation of Gadd153 expression

under this condition. In contrast, I used medium depleted of all amino acids to determine the effects of amino acid deprivation on the Gadd153 promoter and did not detect any activation of the reporter. This may not reflect the inducibility of the Gadd153 promoter as protein synthesis may be inhibited by this treatment. Similarly, I found that in CHO DG44 19.6 cells Gadd153 mRNA induction was detected but this induction was not reflected at the protein level. It appears that depletion of all amino acids inhibits protein synthesis whereas leucine depletion alone may not (Jousse et.al., 1999). To confirm that protein synthesis was inhibited pulse-labelling experiments could have determined [^{35}S]-methionine incorporation into the cells. Lack of incorporation would have confirmed the extent of protein synthesis inhibition by amino acid depletion.

The same stress conditions applied to CHO DG44 19.6 cells produced different degrees of induction of endogenous Gadd153 expression and luciferase reporter expression. The lower induction of the Gadd153 promoter in the reporter assays is most likely the result of interference from the use of transient transfection that generates a relatively high basal expression due to the non-chromosomal environment of the vector DNA. In addition, there may be other DNA control elements that lie outside the promoter region incorporated into the construct or additional factors that may be involved in regulating expression. The stability of Gadd153 mRNA has been reported to be variable under different stress conditions. Recently, Abcouwer (1999) demonstrated that Gadd153 mRNA stability was increased in human breast cell lines depleted of glutamine. In addition, Jackman et. al. (1994) reported that genotoxic stress conditions increased stability of the Gadd153 transcript in proliferating CHO K1 cells and they believe this was due to a regulatory sequence known as an AU-rich element (ARE) in the Gadd153 mRNA that allowed for this greater induction. In addition, post-transcriptional mechanisms were not addressed in the luciferase assays. The extent of Gadd153 protein expression in CHO DG44 19.6 cells was greater than the stimulation of luciferase activity under the same stress conditions. Gadd153 transcriptional activity was shown to be enhanced by stress-inducible phosphorylation on serine 78 and 81 by MAP kinase p38 (Wang and Ron, 1996) and this may be contributing to post-translational stability of the protein, however there is no evidence to support this at present.

4.7 ASSESSING THE FEASIBILITY OF A GADD153-d2EGFP REPORTER GENE AS AN ONLINE MONITOR OF CELL STRESS

The promoter fragment used in the luciferase construct described above (Section 4.5) was ligated into a pd2EGFP vector. This vector was designed and developed by Li, et.al. (1998) and contains the cDNA of a Destabilised Enhanced Green Fluorescent Protein (d2EGFP). GFP is widely used reporter in studies of protein localisation by tracking fusion proteins in cells (Chalfie et.al., 1994; Marshall et.al., 1995; Cubitt et.al., 1995). However, as GFP is stable this may limit its application in gene reporter studies that require rapid reporter turnover (Li et.al., 1998). pd2EGFP was designed so that the fluorescence half-life of the protein is only 2 hours. This was achieved by fusion of amino acids 422-461 of the degradation domain of mouse ornithine decarboxylase (MODC) to the C-terminal end of EGFP (Section 1.8.2).

HEK cells transiently transfected with Gadd153-d2EGFP were analysed by fluorescence microscopy and FACS analysis for EGFP fluorescence after treatment with tunicamycin, glucose and glutamine depletion or glutamine depletion. The results obtained displayed a similar trend to that observed for the Gadd153-Luciferase reporter in that tunicamycin treatment gave the most marked stimulation. However, the level of induction of the promoter determined by EGFP fluorescence appeared to be lower than that of the Gadd153-Luciferase reporter and this may, in part, be a consequence of a higher basal level of EGFP expression compared to the basal level of luciferase expression. The folding of the GFP chromophore can be relatively slow. I used EGFP in my experiments and this has been reported to acquire fluorescence faster than wild type GFP but this may be of significance for full expression of fluorescence (Heim et.al., 1994). To maximise chromophore formation a longer incubation time than 8 hours may have been preferable before the cells were harvested for analysis. However, incubation times were extended past this time proved to be ineffective. After this time a high proportion of cells had rounded up, becoming smaller and such cells floated off into the media, contributing to cell debris and complicating FACS analysis data. It appears that these 'stressed' cells were dying of apoptosis through loss of attachment from matrix interactions similar to that observed with CHO Duk cells

CHO Duk cells (as discussed in Section 4.1). In addition, when the floating cells were isolated and observed by fluorescence microscopy they were all small, rounded up and very green providing evidence that the Gadd153 promoter construct is activated, and may be associated with the onset of apoptosis. It appears that analysis of the Gadd153-d2EGFP reporter may be restricted under certain conditions. When measuring expression of gene promoter activity that is activated by events that will cause cell death, the cells may begin to die before the chromophore is completely formed i.e. before fluorescence can be detected. Wang et.al. (1998) made a stable CHO cell line carrying a reporter that consisted of an 2.8 kb fragment of the Gadd153 promoter with GFP. They overexpressed mIre1 (a known activator of endogenous Gadd153) in the cells and then treated them with tunicamycin. A significant number of GFP-positive cells were detected and it was noted that the GFP-positive cells were smaller than untreated cells. This data agrees with my observations whereby the greenest cells were those that had rounded up, becoming smaller. It was suggested that induction of Gadd153 in these cells may be initiating programmed cell death which has been reported to occur in other cell lines (Zinzsner et.al., 1998; Matsumoto et.al., 1996). Use of the vector described in my studies with suspension-adapted cells may provide a solution to maximise the accuracy of data avoiding the problems associated with loss of attachment of cells. Conversely, the increasing concentration of EGFP expressed may have reached levels that were toxic to the cells. When using luciferase reporter technology toxicity was not an issue as only small levels of expression are required for detection (Welsh and Kay, 1997). Also, the wide linear range (eight orders of magnitude) of the luciferase assay gives low background activity allowing for accurate quantification between control and treated samples (Brandes et.al., 1996). However, this technology is invasive and requires a substrate. The major advantage of using EGFP as a reporter is that no substrate is required and therefore non-invasive detection and quantification may be possible in intact cells.

4.7.1 Stably integrated Gadd153-d2EGFP as online monitor of cell stress

The data acquired from the transient analysis of the Gadd153-reporter systems displayed an induction of the Gadd153 promoter under stress conditions. Transient transfection systems inevitably cause high background levels of reporter expression and therefore I assessed a stably integrated Gadd153-d2EGFP transgene as a potential on-line monitor of cell stress. Initially, the recombinant CHO DG44 cell line was transfected by electroporation and plated out in 96 well plates for cloning and selection with G418, however, this procedure proved to be too severe and caused all the cells to die. These cells were already subjected to a basal stress as they produce a complex recombinant protein TAPgen (Section 1.1.3) and are suspension-adapted to serum-free medium. As the importance of this work was to assess the generic value of the reporter system, the parental, serum-dependent, non-recombinant CHO DG44 cell line was used for transfection. Growth curves were compared between one of the clones selected (CHO DG44-Gadd153-d2EGFP, clone 1) and the parental CHO DG44 cell line (Section 3.5.2).

Both cell lines were grown in HAMS F12+FCS. This medium has been used previously to grow anchorage-dependent dhfr$^-$ CHO cells (Funanage and Myoda, 1986). It was selected for this series of experiments as it contains lower concentrations of most nutrients than other media therefore it was ideal for optimising cell nutrient stress conditions. In this medium maximal growth of both cell lines were less than for CHO cell lines in DMEM or CHO-S-SFM II (Section 3.1.1). The transfected CHO DG44-Gadd153-d2EGFP cells grew more slowly than CHO DG44 cells but eventually both cell lines entered into decline phase in batch culture. DAPI staining of cells in decline phase confirmed that cell death occurred via apoptosis. It is possible that supplementation of medium with G418 causes stress to the cells and may contribute to the lower growth rate of the transfected cell lines. G418, also known as neomycin, is an aminoglycoside antibiotic known to inhibit initiation, elongation and termination of protein synthesis and it can also induce misreading (Dawson et.al., 1986). A known mechanism of G418 toxicity in mammalian cells is through binding to the 80S ribosomes (Morris et.al., 1995). In addition, G418 and other related aminoglycosides have been reported to interact with other cellular components, including negatively charged

membrane phospholipids (Leclerq et.al., 1992), inositolphospholipids (Gupta et.al., 1988; Schacht, 1978; Downes and Michell, 1981) and inositolphospholipid-cleaving phospholipase C (Carney et.al., 1985; Eberhard et.al., 1990) and G418 has also been shown to result in the release of GPI-anchored proteins (Kung et.al., 1997). Despite G418 resistance of the transfected DG44 cells they may still experience some toxicity as described above.

Western analysis was used to determine if there was a relationship between the expression of Gadd153 and GFP in the transfected cell line. CHO DG44 cells were analysed for expression in parallel. A relatively high basal level of Gadd153 protein expression was detected in untransfected CHO DG44 cells in the absence of any intentional stress treatment. This may be a reflection of the relatively slow growth of the cell line. Tunicamycin addition caused a significant induction of Gadd153 expression demonstrating that this is a potent inducer in untransfected CHO DG44 cells. However, transfected CHO DG44 cells displayed this induced level of Gadd153 protein in cells whether the cells were challenged with tunicamycin or not, suggesting that some other factor was contributing to stress. In addition, GFP was detected in all transfectants at a constant level whether challenged with tunicamycin or not, but was undetectable in untransfected CHO DG44 cells. Transfected CHO DG44 cells were cultured in the presence of different concentrations of G418 to determine how this factor contributed to high GFP and Gadd153 protein expression levels. Over a range of G418 concentrations and even in the absence of G418, the cells sustained high GFP and Gadd153 protein expression. Although, it is possible that G418 concentration may contribute to the apparent 'stressed' condition of the cells, it is likely that other factors are more significant.

The growth of untransfected CHO DG44 cells was slow and may be causing the cells to enter into premature growth arrest which would explain the high basal level of Gadd153 expression detected. Incorporation of the Gadd153-d2EGFP transgene into the genome may be exerting an added 'stress' on the cells in addition to those described above. It appears that in transfected cells the reporter may be activated at all times at a low but constant level. EGFP may build up in the cells to a point were the destabilisation of EGFP is not efficient enough to

prevent toxic levels mounting. The effect of this toxicity may further activate the Gadd153 promoter-reporter until saturating levels of endogenous Gadd153 protein and EGFP are reached in the controls. If background levels had reached saturation, induction of the promoter by tunicamycin would not be detected.

To combat the stability problem of GFP destabilised EGFP (d2EGFP) is used as it has a half-life of only 2 hours. Li et. al. (1998) recently used this vector in conjunction with NFκB responsive elements as a reporter for analysis of stress perception. However, when they quantified EGFP by Western blot analysis the antibody detected both premature and mature EGFP, giving rise to high background levels. Maturation of the EGFP chromophore is post-translational and proceeds with a half time of about 25 minutes (Cormack, 1996). In my system the Gadd153 reporter displayed a high basal level of expression and therefore Western blot analysis may not be the most accurate method to distinguish between the quantity of mature EGFP present in cells challenged with and without tunicamycin. In order to minimise high background levels due to the presence of premature EGFP and to obtain more accurate reporter data other methods of detection should be utilised, such as fluorescence microscopy or those described at the end of this section.

Fluorescence microscopy was used to determine mature EGFP expression in transfected CHO DG44 cells challenged with and without tunicamycin. A low level of background autofluorescence was detected in untransfected CHO DG44 cells. In order to obtain a signal a doubling of autofluorescence is required (the threshold for detection of EGFP is ~ 100 nM [Piston, 1998]). Transfected CHO DG44 cells exceeded this amount as they displayed a distinctly higher intensity of fluorescence in the absence of tunicamycin due to a basal level of EGFP expression. The morphology of these cells remained spread out, characteristic of healthy cells. Transfected cells treated with tunicamycin appeared to be ruffled around the perimeter, rounding up and detaching and such cells fluoresced intensely green. In order to determine quantitative statistical data further studies involving other methods must be performed. The signal from EGFP does not have any enzymatic amplification, hence, the sensitivity of EGFP will probably be lower than that for enzymatic reporters, such as firefly luciferase (Piston, 1998).

However, EGFP signals can be quantified by flow cytometry, confocal scanning laser microscopy and fluorometric assays. In addition, purified EGFP can be quantified in a fluorometer-based assay in the low-nanogram range.

4.8 APPLICATIONS OF AN ONLINE MONITOR OF CELL STRESS

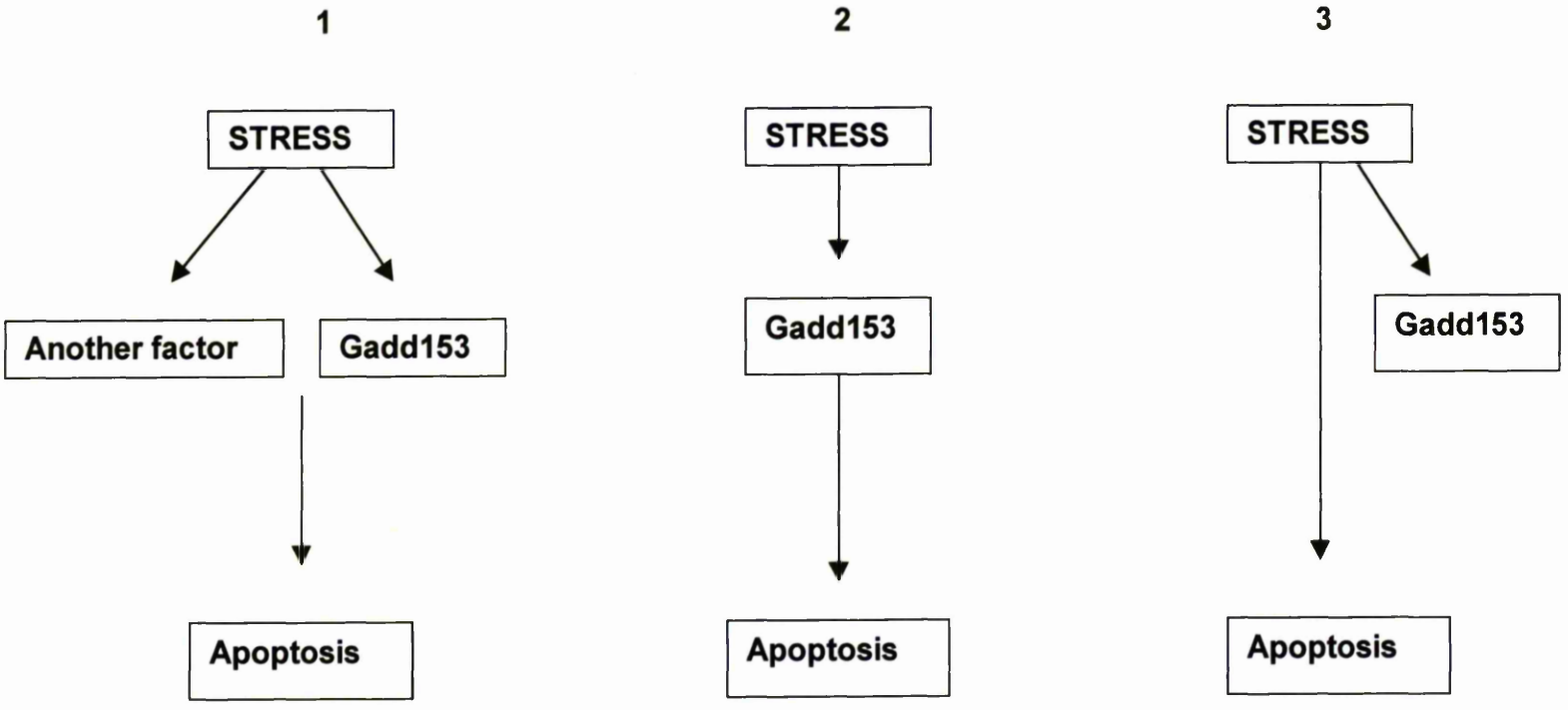
The use of the Gadd153-d2EGFP reporter as a fluorescence indicator of nutrient and intracellular stress in mammalian cells provides a technology with a number of applications for the biotech industry. Preliminary studies, shown in this thesis, are encouraging in that an increase of fluorescence was detected in cells after exposure to stresses similar to those found in decline phase of batch culture. When optimised, this technology could be applied in the improvement and selection of cell culture media constituents for different cell lines. The methodology may involve using a 96 well plate system. Cells grown in the wells, supplemented with differing concentrations of media constituents, could then be screened for their fluorescence intensity using a fluorometer plate reader. This may provide a more rapid and cost efficient process compared to current approaches. This technology could also be applied to recombinant cells in the bioreactor, where after detection of the initiation of cell stress, nutrients could be added back using a fed-batch approach at this critical time. Restoration of nutrient homeostasis may then reverse the stress insult delaying the onset of apoptosis. Measurement of fluorescence may involve the insertion of a fluoro-probe into the bioreactor, which would determine levels of fluorescence within the cell culture.

In this study I have shown that Gadd153 induction is rapid in response to stress and this can be reversed within a specific time frame. Loss of Gadd153 expression was also paralleled by a decrease in the rate of cell death. Figure 4.1 shows three hypothetical pathways in which Gadd153 may be involved in the response to a stress imposition. One hypothesis is a pathway that requires the expression of Gadd153 along with another factor in order to trigger apoptosis. A second is a pathway were apoptosis is triggered in response to Gadd153 expression and thirdly a pathway were apoptosis is triggered in response to stress independent of Gadd153 expression, but Gadd153 is expressed in parallel.

Further investigations are required to determine if Gadd153 expression actually causes apoptosis (pathway 2, Figure 4.1). This is neccessary to assess if Gadd153 is a direct indicator, or a parallel marker, of the early stages of apoptosis. To prove that Gadd153 causes apoptosis a Gadd153 gene switch construct could be made. Transfection of cells with this construct followed by a specific switch on of the exogenous Gadd153 cDNA would determine if Gadd153 expression alone is sufficient to cause apoptosis.

With the expanding knowledge of the upstream events involved in the regulation of Gadd153 gene expression and many other genes, the biochemical pathway leading to apoptosis is emerging. The major aim of this research is to determine the mechanisms of early stress perception by cells that lead to the onset of apoptosis. In this study I have demonstrated that Gadd153 expression is a rapid indicator of the onset of apoptosis and this process can be reversed within a time frame of 6-12 hours after stress imposition. Events upstream of Gadd153 may prove to be more appropriate as indicators of cell stress by increasing this time frame. Ultimately, knowledge of these events may allow for the intervention of apoptosis and therefore maintain a better quality of cell culture environment.

Figure 4.1 Is Gadd153 involved in triggering apoptosis in response to stress?



The possible pathways of apoptosis focusing on the involvement of Gadd153 are shown above. (1) represents a pathway whereby stress activation of Gadd153 expression and the expression of another factor interact to trigger apoptosis, (2) represents a pathway in which apoptosis is triggered in response to Gadd153 expression, (3) represents a pathway were apoptosis is triggered in response to stress by a pathway which is independent of Gadd153 expression, however Gadd153 is expressed in parallel via an unrelated pathway.

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APPENDICES

APPENDIX A

A1 General and specialist chemicals

Inorganic chemicals and organic solvents were purchased from BDH Chemicals Limited, Speke, Liverpool, UK or Fiscons Scientific Apparatus Limited, Loughborough, Leicestershire, UK. Other chemicals were supplied as follows:

Anachem, Beds, UK

Polyacrylamide

BDH Chemicals Limited, Speke, Liverpool, UK.

Acetaldehyde
Ammonium acetate
Boric Acid
Calcium chloride
Diphenylamine
EDTA (sodium salt
Disodium hydrogen orthophosphate
Glacial acetic acid
Glucose
Glycerol
Magnesium acetate
Perchloric acid
Phenol
Polyvinyl alcohol
Sodium acetate
Sodium chloride
Sodium hydroxide
Sodium nitrite
Sucrose
Tri-sodium citrate

Boehringer Mannheim,

ATP

Bio-Rad, Hertfordshire, UK

Bio-Rad Protein Assay Reagent

Difco Labs, Detroit, Michigan, USA

Bacto-agar
Bacto-tryptone
Bacto-yeast extract

Fulka-Chemica, Glossop, Derbyshire, UK

Formamide

Gibco BRL, Paisley, Renfrewshire, UK

Agarose (standard melting temperature)
Ammonium persulphate
Caesium chloride
CHO-S-SFM II
Dulbecco's MEM
Foetal Calf Serum (Cat # 013-06290M)
Glutamine
HAMS F12 nutrient mixture
Hypoxanthine/thymidine
Non-essential amino acids
Amino acids without glutamine
Phosphate buffered saline
Trypsin

PAA,

G418 sulphate

Pharmacia Limited, Hounslow, Middlesex, UK

Ficoll 400
Poly dI dC

Sigma Chemical Company, Poole, Dorset, UK

Ampicillin
Aprotonin
Brilliant blue
Bromophenol Blue
BSA fraction (V)
Calf Thymus DNA
4,6-Diamidino-2-phenylindole (DAPI)
Diethylpyrocarbonate (DEPC)
Dimethylsulphoxide (DMSO)
Dithiothreitol
DNA (from salmon sperm)
Dried Firefly-Lantern Extracts (FLE-50)
Ethidium Bromide
Formaldehyde (37 % (w/v))
G50-300 Sephadex
HEPES (free acid)
Kanamycin
MOPS
Mowiol
Nonidet p-40
Paraformaldehyde
p-phenylenediamine
PMSF

Polylysine
Polyvinylpyrrolidine
Sarkosyl (sodium salt)
SDS (sodium salt)
β-2-mercaptoethanol
TEMED
Tris
Trypan Blue
Tunicamycin
Tween 20

A2 Apparatus

General and disposable equipment and materials were purchased from standard suppliers. Specialist equipment was supplied as follows:

Beckman Instruments Incorporated, Fullerton, California, USA

J2-L1 centrifuge with JA-20, JA-21 and JA-18.1 rotors.
J-6B centrifuge with JS-4.2 rotor.
Heat-sealable centrifuge tubes.

Becton Dickson, San Jose, CA. USA.

FACSVantage cytometer
Lysis II software

Bio-Rad Labs, Hemel Hempsted, Hertfordshire, UK

Mini-gel electrophoresis system.
Semi-dry blot transfer system.
Mini-gel 3MM filter paper.
Gene pulser ™

Densitometer (Model GS-700 Imaging densitometer with Molecular Analyst software)

Corning Costar Corp., MA, USA

Erlenmeyer polycarbonate tissue culture flasks (250 ml) (Cat. # 430183)

Corning Limited, Shone, Staffordshire, UK

pH 120 digital pH meter.

EG + G Berthold, Postfach, Germany

Luminometer plate reader (Model Microlumat LB96P)

Fuji Photo Film Company Limited, Japan

X-ray film.

Gibco BRL, Paisley, Renfrewshire, UK

DNA mini-gel electrophoresis tanks.

Kodak, Chalon-sur-Saone, France

Intensifying Screens
X-ray film developer.
X-ray film fixer.

LKB Wallac, UK

LKB Wallac Luminometer (Model 1250)
Spectrophotometer (Model Ultraspec II)

Schleicher and Schuell, Dassel, Germany.

Nitrocellulose filters.

Techne, U.K.

Hybridisation Oven
Thermal Cycler (Model PHC-3)

Whatman Biosystems Limited, Maidstone, Kent, UK

3MM filter paper

A3 Antibodies

Anti-mouse ERK This monoclonal antibody was purchased from Affiniti Labs

Anti-rabbit phospho-ERK This polyclonal antibody was purchased from New England Biolabs.

Anti-rabbit-Gadd153 (R20)- This antibody was purchased from Santa Cruz Biotechnology, Inc (Cat.# sc-793). This is an affinity-purified rabbit polyclonal antibody raised against a peptide mapping at the carboxy terminus of the Gadd153 of human origin (differs from mouse sequence by 2 amino acids). The antibody reacts with Gadd153 of mouse, rat and human origin. It is non-cross reactive with Gadd45 or Gadd34. The dilution used for Western Blotting was 1:1000.

Anti-rabbit GFP. Living Colors Peptide Antibody (Affinity purified). This antibody was purchased from Clontech (Cat. # 8367-1). Anti-GFP is a mixture of several monospecific rabbit anti-GFP antibodies (raised against synthetic peptides selected from the native *Aqueorea victoria* green fluorescent protein) purified using GFP peptide columns. It reacts with EGFP, EBFP, EYFP, ECFP and fusion

proteins containing any of these variants. The dilution used for Western Blotting was 1:100.

Anti-rabbit Bip. This antibody was a gift from Dr. N. Bullied and was made in house. It reacts with bovine Bip and is known to cross react with canine, human and mouse Bip. The dilution for Western Blotting was 1:500-1000 dilution.

Anti-rabbit PDI. This antibody was a gift from Dr. N. Bullied and was made in house. It reacts with bovine PDI and is known to cross react with canine, human and mouse PDI. The dilution for Western Blotting was 1:500-1000 dilution.

Anti-mouse IgG. This antibody was purchased from Sigma Immunochemicals (Cat. # A-4416). It was raised in goats against the whole mouse IgG molecule, purified, and then conjugated to Sigma Horseradish Peroxidase, Type VI. The dilution used for Western Blotting was 1:1000.

Anti-rabbit IgG. This antibody was purchased from Sigma Immunochemicals (Cat. # A-6154). It was raised in goats against the whole rabbit IgG molecule, purified, and then conjugated to Sigma Horseradish Peroxidase, Type VI. The dilution used for Western Blotting was 1:1000.

A4 Molecular markers

Protein Marker (Dalton Marker VLL-L, Sigma SDS-7)
The following mixtures of proteins of known molecular weight were used as standards in SDS-PAGE gel and were purchased from Sigma Chemical Company Ltd.

Albumin, bovine	66,000 Da
Albumin,egg	45,000 Da
Glyceraldehyde-3-P-Dehydrogenase	36,000 Da
Carbonic Anhydrase, bovine	29,000 Da
Trypsinogen, bovine pancreas	24,000 Da
Trypsin Inhibitor soyabean	20,000 Da

α-lactalbumin, bovine milk 14,200 Da

DNA Marker (1 kb DNA ladder)

The following mixture of DNA fragments were used in agarose gels and was purchased from Gibco BRL (Cat.# 15615). The fragment sizes are as follows (given in bp): 12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, 506.5, 396, 344, 298, 220, 201, 154, 134, 75.

Radiochemicals

α-^{32}P-dATP: purchased from ICN Biomedical Inc. (Cat. # 33002X). Specific activity = 3000 Ci/mmol, concentration = 10mCi/ml).

APPENDIX B

Cell culture media

The constituents of Dulbecco's Modified Eagle Medium (Cat. # 41966)

Component	**mg/L**
Inorganic salts	
$CaCl_2.2H_2O$	264.00
$Fe(NO_3).9H_2O$	0.10
KCl	400.00
$MgSO_4.7H_2O$	200.00
NaCl	6400.00
$NaHCO_3$	3700.00
$NaH_2PO_4.H_2O$	141.00
Other components	
D-Glucose	4500.00
Phenol Red	15.00
Sodium Pyruvate	110.00
Amino Acids	
L-Arginine.HCl	84.00
L-Cystine	48.00
L-Glutamine	580.00
Glycine	30.00
L-Histidine.HCl.H_2O	42.00
L-Isoleucine	105.00
L-Leucine	105.00
L-Lysine.HCl	146.00
L-Methionine	30.00
L-Phenylalanine	66.00
L-Serine	42.00
L-Threonine	95.00
L-Tryptophan	16.00
L-Tyrosine	72.00
L-Valine	94.00
Vitamins	
D-Ca pantothenate	4.00
Choline Chloride	4.00
Folic Acid	4.00
i-Inositol	7.20
Nicotinamide	4.00
Pyridoxal HCl	4.00
Riboflavin	0.40
Thiamine HCl	4.00

The constituents of HAMS F12 (Cat. # 21765)

Component	**mg/L**
Inorganic salts	
$CaCl_2.2H_2O$	44.00
$CuSO_45H_2O$	0.0024
$FeSO_46H_2O$	0.83
KCl	223.60
$MgSO_4.7H_2O$	122.00
NaCl	7599.00
$NaHCO_3$	1176.00
$Na_2HPO_4.$(anhyd.)	142.00
$ZnSO_4$	0.86
Other components	
D-Glucose	1802.00
Hypoxanthine	4.00
Linoleic Acid	0.084
DL-68 Thioctic Acid	0.20
Phenol Red	120
Putrescine 2HCl	0.161
Sodium Pyruvate	110.00
Thymidine	0.70
Amino Acids	
L-Alanine	8.90
L-Arginine.HCl	211.00
L-Asparagine (free base)	13.00
L-Aspartic Acid	13.30
L-Cysteine HCl	36.00
L-Glutamic Acid	14.70
L-Glutamine	146.00
Glycine	7.50
L-Histidine.HCl.H_2O	21.00
L-Isoleucine	4.00
L-Leucine	13.00
L-Lysine.HCl	36.50
L-Methionine	4.50
L-Phenylalanine	5.00
L-Proline	34.50
L-Serine	10.50
L-Threonine	12.00
L-Tryptophan	2.00
L-Tyrosine	5.40
L-Valine	11.70
Vitamins	
Biotin	0.0073
D-Ca pantothenate	0.50
Choline Chloride	14.00
Folic Acid	1.30
i-Inositol	18.00

Nicotinamide	0.036
Pyridoxal HCl	0.06
Riboflavin	0.037
Thiamine HCl	0.30
Vitamin B_{12}	

CHO-S-SFM II is a proprietary media to Gibco BRL

APPENDIX C

C1 Bacterial strains

TOP10 F⁻ *mcr*A Δ (*mmr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 ΔLACX74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G.

JM109 F⁻ *rec*A1 *end*A1

XL-blue *rec*A1 *end*A1 *gyr*A96 *thi*-1 *hsd*R17 *sup*E44 *rel*A1 *lac [F roABlacrZDM15Tn10(Tet^r)]*

C2 Plasmid probes

18s rRNA probe: p100-D9 clone, which contains a 220 bp mouse 18s rRNA cDNA fragment, inserted at Pst I site of pBR322. The plasmid was transformed into the HB101 strain of E.Coli, and the transformants were selected for tetracycline resistance. This was a gift of Dr. Dylan R. Edwards of the department of Pharmacology, University of Calgary, Canada.

Gadd153 probe produced by Sian Leech in 1996 in this lab, using primers designed against Gadd153 cDNA from NSO myeloma cells (see Gadd153 primers). The cDNA was ligated into a TA-cloning vector.

Double stranded oligonucleotides for use in EMSA assay

Alternative Gadd153 binding site purchased from MWG Biotech

5' tgg atc cgg ctg caa tcc ccc ctc gag 3'

3' cc tag gcc gac gtt agg ggg gag ctc t 5'

AP-1 binding site purchased from MWG Biotech

5' tga ctc a 3'

3' act gag t 5'

C/EBP binding site purchased from MWG Biotech

5' t gga tcc ttg cgc aat ctg ccc tcg ag 3'

3' cct agg aac gcg tta gac ggg agc tc 5'

C3 List of Plasmids

Gadd153-CAT plasmid*:* given as a kind gift from Dr. N. J. Holbrook (Luethy, J.D. et.al. Isolation and characterisation of the hamster Gadd153 gene. J. Biol. Chem. 265: 16521-16526, 1990.) This plasmid contains ampicillin resistance and was propagated in XL-blue bacteria.

pGadd153 was made by Sian Leech in the lab.

pGL3-basic vector was purchased from Promega (Cat. # E1751). This plasmid contained ampicillin resistance and was propagated in JM109 bacteria.

pRL-CMV vector was purchased from Promega (Cat. # E2261). This plasmid contained ampicillin resistance and was propagated in JM109 bacteria.

pd2EGFP vector was purchased from Clontech (Cat. # 6008-1). This plasmid contained kanamycin resistance and was propagated in JM109 bacteria.

pCR 2.1-TOPO vector was purchased from Invitrogen (Cat. # K4500-01) as part of a kit. This plasmid contained ampicillin resistance. It was used for cloning PCR fragments and was propagated in TOP10 bacteria.

C4 Primer Sequences

All primers were synthesized at MWG Biotech

Primers used for PCR of GADD153 cDNA probe

Forward Gadd153 primer 1

5' atc ttc ata cac cac cac ac 3′ 20mer

Reverse Gadd153 primer 2

5′ cgc tcg ttc tct tca gct ag 3′ 20mer

Annealing temperature used was 57 °C.

Primers with Eco RI overhangs used for PCR of Gadd153 Promoter insert

Template used for PCR was Gadd153-Chloramphenicol

Forward Gadd153 promoter primer 1

5' gaa ttc tgg cgt gca gtg ggc gac tc 3' 26mer

Tm = 69.5 °C

Reverse Gadd153 promoter primer 2

5' gaa ttc gaa gtg tga gac tca ggc ta 3' 26mer

Tm = 63.2 °C

Annealing temperature used was 54 °C

Primers with Hind III overhangs used for PCR of Gadd153 Promoter insert

Template for PCR was Gadd153-TOPO1

Forward Gadd153 promoter primer1

5' aag ctt tgg cgt gca gtg ggc gac tc 3' 26mer

Tm 69.5 °C

Reverse Gadd153 promoter primer 2

5' agg ctt gaa gtg tga gac tca ggc ta 3' 26mer

Tm 63.2 °C

Annealing temperature used 65 °C

C5 Molecular biology kits

QIAGEN Midi preparation kit purchased from QIAGEN (Cat. # 12143)

QIAGEN Gel Extraction kit purchased from QIAGEN (Cat. # 20021)

QIAGEN Superfection Kit purchased from QIAGEN (Cat. # 301305)

TOPO Cloning kit purchased from Invitrogen (Cat. # k4500-01)

ECL-Western kit: purchased from Amersham International (Cat.# RPN 2109).

Restriction enzymes and DNA modifying enzymes: purchased from Boehringer Mannheim.

Geneclean kit II purchased from Bio 101 Inc.

TRIzol Reagent purchased from Gibco BRL (Cat.# 15596-026).

First Strand cDNA Synthesis kit purchased from Pharmacia (Cat.# 27-9261-01).

ABI PRISM Dye Terminator Cycle Sequence Ready Reaction kit purchased from Perkin Elmer Corporation (Cat.# 402078).

Nick Translation DNA labelling kit purchased from Boehringer Mannheim, (Cat.# 976 776).

Random Primed DNA labelling kit purchased from Boehringer Mannheim (Cat.# 1004760).

Dual-Luciferase Assay kit purchased from Promega (Cat. # E1910)

C6 Enzymes

Alkaline phosphatase purchased from Boehringer Mannheim (Cat.# 1758250)

DNA polymerase purchased from Boehringer Mannheim (Cat.# 642711)

Proteinase K purchased from Boehringer Mannheim (Cat.# 161519)

RNase A (Ribonuclease A): from bovine pancreas, purchased from Sigma Chemicals (Cat.# R9005)

Rnasin Ribonuclease Inhibitor: purchased from promega (Cat.# N2111)

Taq polymerase purchased from Boehringer Mannheim (Cat.# 1146165)

T4 DNA Ligase purchased from Boehringer Mannheim (Cat.# 481-220)

C7 Sequences of plasmid insert

Gadd153-Luciferase, Gadd153-d2EGFP and Gadd153-CAT aligned.

(see over page)

Gadd153 sequencing of promoter insert (database sequence compared to Gadd153-CAT/Luciferase/d2EGFP)

1. Gadd153 database sequence
2. Gadd153–CAT sequence
3. Gadd153-Luciferase sequence
4. Gadd153-d2EGFP sequence

#1
1. ACA GGAATTC TGGCGTGCAG TGGGCGACTC AGAAACGCCC AAAGGTGCTC CCCCGAGACA

.....

#61

2. GGA CCTCAGCCTC TGGGAAGCGT TAGGAGGTTA
3. TCAGCCTC TGGGAAGCGT TAGGAGGTTA
4. ACCTCAGCCTC TGGGAAGCGT TAGGAGGTTA

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#121
1. AAAGAGATGA GATCCCTTCT AAAGGGCTGG AGAAGATGTC AGTCCAGGTA GGACTAATGG

2. AAAGAGATGA GATCCCTTCT AAAGGGCTGG AGAAGATGTC AGTCCAGGTA GGACTAATGG
3. AAAGAGATGA GATCCCTTCT AAAGGGCTGG AGAAGATGTC AGTCCAGGTA GGACTAATGG
4. AAAGAGATGA GATCCCTTCT AAAGGGCTGG AGAAGATGTC AGTCCAGGTA GGACTAATGG

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#181
1. AAACTTTATC GCGGTTCCAG GGGCCTCGGN GCGCATGAGC TGGGAGGGGC CGGGAAGCTG

2. AAACTTTATC GCGGTTCCAG GG : CCTCGGC GCGCATGAGC TGGGAGGGGC CGGGAAGCTG
3. AAACTTTATC GCGGTTCCAG GG : CCTCGGC GCGCATGAGC TGGGAGGGGC CGGGAAGCTG
4. AAACTTTATC GCGGTTCCAG GG : CCTCGGC GCGCATGAGC TGGGAGGGGC CGGGAAGCTG

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#241

1. GGAGTCTGGA TGGAGGACGA AGTTGGAGGT GATGGGAGGT GGGTGGGCAG AGCCGCAGCT

2. GGAGTCTGGA TGGAGGAGCA AGTTGGAGGT GATGGGAGGT GGGTGGGCAG AGCCGCAGCT
3. GGAGTCTGGA TGGAGGAGCA AGTTGGAGGT GATGGGAGGT GGGTGGGCAG AGCCGCAGCT
4. GGAGTCTGGA TGGAGGAGCA AGTTGGAGGT GATGGGAGGT GGGTGGGCAG AGCCGCAGCT

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#301

1. CCTGGGCAGA CAAGTTCAGG AAGGACAGCC GTTGGGGCCG TTGGATACTG GGAGCTGGCG

2. CCTGGGCAGA CAAGTTCAGG AAGGACAGCC GTTGGGGCCG TTGGATACTG GGAGCTGGCG
3. CCTGGGCAGA CAAGTTCAGG AAGGACAGCC GTTGGGGCCG TTGGATACTG GGAGCTGGCG
4. CCTGGGCAGA CAAGTTCAGG AAGGACAGCC GTTGGGGCCG TTGGATACTG GGAGCTGGCG

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#361

1. CTCCGCCCTC TTCCCTCTCA TCCCCCCACC CGCGCCTCCC ACCACCGTCG GCGGCCCCTG

2. CTCCGCCCTC TTCCCTCTCA TCCCCCCACC CGCGCCTCCC ACCACCGTCG GCGGCCCCTG
3. CTCCGCCCTC TTCCCTCTCA TCCCCCCACC CGCGCCTCCC ACCACCGTCG GCGGCCCCTG
4. CTCCGCCCTC TTCCCTCTCA TCCCCCCACC CGCGCCTCCC ACCACCGTCG GCGGCCCCTG

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#421

1. CGCGTGCGCG CGCGCAGACA CCGGTTGCCA AACATTGCAT CATCCCCGCC CCCCGTCATC

2. CGCGTGCGCG CGCGCAGACA CCGGTTGCCA AACATTGCAT CATCCCCGCC CCCCGTCATC

3. CGCGTGCGCG CGCGCAGACA CCGGTTGCCA AACATTGCAT CATCCCCGCC CCCCGTCATC
4. CGCGTGCGCG CGCGCAGACA CCGGTTGCCA AACATTGCAT CATCCCCGCC CCCCGTCATC

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#481
1. CCTCCCTCGC CGCACTCTCC TTCGCCCGCC CGCGCGCGCG CGCGCGCGCG CGCGCGCATG

2. CCTCCCTCGC CGCACTCTCC TTCGCCCGCC CGCGCGCGCG CGCGCGCGCG CGCGCGCATG
3. CCTCCCTCGC CGCACTCTCC TTCGCCCGCC CGCGCGCGCG CGCGCGCGCG CGCGCGCATG
4. CCTCCCTCGC CGCACTCTCC TTCGCCCGCC CGCGCGCGCG CGCGCGCGCG CGCGCGCATG

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#541
1. ACTCACTCAC CTCCTCCGCG GAGCCTCGTG ACCCAAAGCC ACTTCCGGGT CCAAGACAAC

2. ACTCACTCAC CTCCTCCGCG GAGCCTCGTG ACCCAAAGCC ACTTCCGGGT CCAAGACAAC
3. ACTCACTCAC CTCCTCCGCG GAGCCTCGTG ACCCAAAGCC ACTTCCGGGT CCAAGACAAC
4. ACTCACTCAC CTCCTCCGCG GAGCCTCGTG ACCCAAAGCC ACTTCCGGGT CCAAGACAAC

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#601
1. GTAGCTCTCC AGCCAGAGGG CGGGGCGGAG GCGGGGGCCG AGGGGGCTCC TGAGTGGCGG

2. GTAGCTCTCC AGCCAGAGGG CGGGGCGGAG GCGGGG : CCG AGGGGGCTCC TGAGTGGCGG
3. GTAGCTCTCC AGCCAGAGGG CGGGGCGGAG GCGGGG : CCG AGGGGGCTCC TGAGTGGCGG
4. GTAGCTCTCC AGCCAGAGGG CGGGGCGGAG GCGGGG : CCG AGGGGGCTCC TGAGTGGCGG

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#661
1. ATGT : AGGGG TGGGGCGGAG TCAGTGCCAG CGTGCCGCTT TCTGATTGGC AGGCTCCTGG

2. ATGTGAGGGG TGGGGCGGAG TCAGTGCCAG CGTGCCGCTT TCTGATTGGC AGGCTCCTGG
3. ATGTGAGGGG TGGGGCGGAG TCAGTGCCAG CGTGCCGCTT TCTGATTGGC AGGCTCCTGG
4. ATGTGAGGGG TGGGGCGGAG TCAGTGCCAG CGTGCCGCTT TCTGATTGGC AGGCTCCTGG

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#721

1. GTCCCGCCCC CCAAAAGAGG GGACGGGCCC GCATAAAATA TCTTCTCTCTCG GCGCTGCAGA

2. GTCCCGCCCC CCAAAAGAGG GGACGGGCCC GCATAAAATA TCTTCTCTCTCG GCGCTGCAGA
3. GTCCCGCCCC CCAAAAGAGG GGACGGGCCC GCATAAAATA TCTTCTCTCTCG GCGCTGCAGA
4. GTCCCGCCCC CCAAAAGAGG GGACGGGCCC GCATAAAATA TCTTCTCTCTCG GCGCTGCAGA

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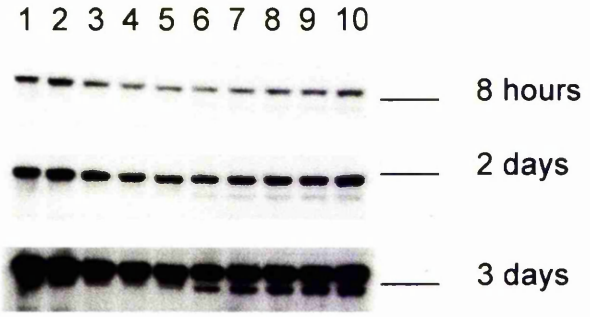
#781

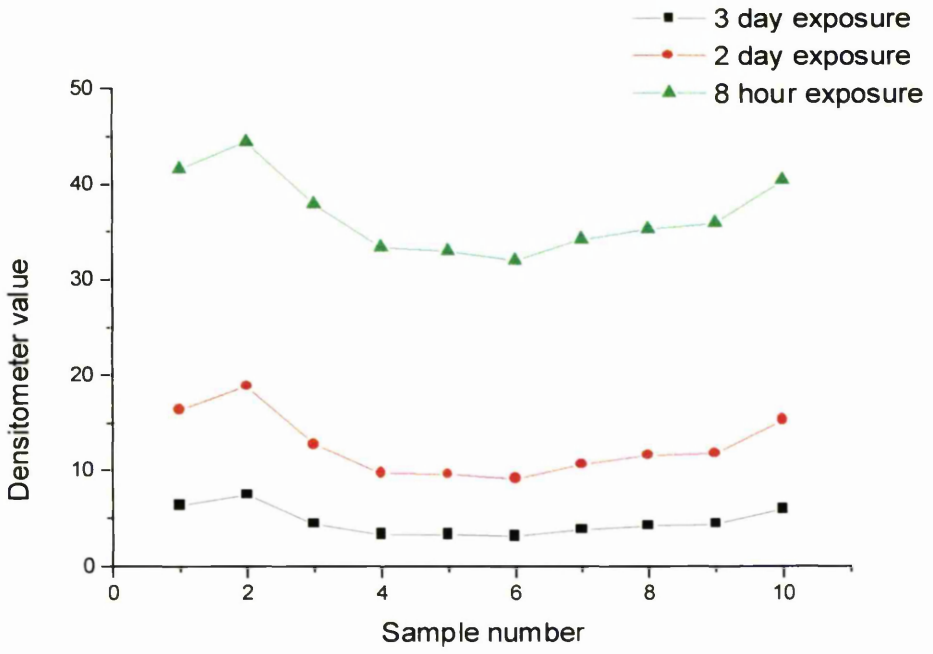
1. GGTCAGTAGC CTGAGTCTCA CACTTCGGTC

2. GGTCAGTAGC CTGAGTCTCA CACTTCGGTC
3. GGTCAGTAGC CTGAGTCTCA CACTTCGGTC
4. GGTCAGTAGC CTGAGTCTCA CACTTCGGTC

C8 Linearity study

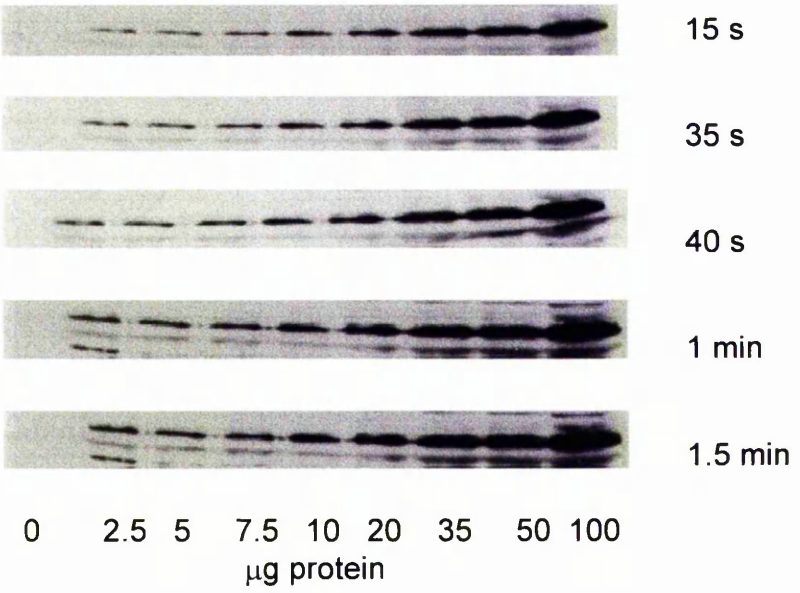
Northern Analysis





To assess the linearity of the densitometer autoradiographs of abundant 18S rRNA northern blots were developed after different exposure times shown above. The optimal exposure time (i.e. one that was non-saturating for obtaining densitometer readings) was 2 days. This exposure time was routinely used for the experiments described in this thesis and as shown was well below the saturation limit of the densitometer.

Western Analysis



A similar linearity study was carried out in the laboratory to assess the linearity of the densitometer and the ECL detection kit with concentration of protein and the time of autoradiograph exposure. The representative western blots above were probed with ERK antibody. The linear range used routinely in this thesis for densitometer readings was taken to be 35 – 45 s of exposure for up to 100 μg of protein.