INVESTIGATION INTO THE ROLE OF c-MYC IN DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS

This thesis is submitted in part fulfilment of the requirements for the degree of Master of Science in the Department of Molecular Biosciences, Division of Biochemistry, University of Adelaide, Australia.

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THESIS SUMMARY

The protein products of the *myc* family of proto-oncogenes regulate a wide variety of cellular processes, such as proliferation, apoptosis and cell growth. The importance of this family of genes is highlighted by the observation that their absence in mouse development results in significant defects and death during early embryogenesis.

Investigation into the events of early embryogenesis can be modelled in the laboratory via the use of mouse embryonic stem (mES) cells, which are derived from the inner cell mass (ICM) of the mouse embryo and are equivalent to 4.5 days post coitum (dpc). An important characteristic of ES cells is that they are pluripotent, meaning that they have the potential to form every embryonic cell type. Maintenance of a pluripotent state, in culture is achieved by the exposure to the cytokine, Leukemia Inhibitory Factor (LIF). Differentiation of ES cells can achieved in cell culture in various ways, one of which leads to the formation of structures called embryoid bodies. These can be used as an *in vitro* model for embryonic differentiation of cells of the pluripotent ICM to the three primary germ layers, Ectoderm, Endoderm and Mesoderm. In the embryo and embryoid bodies it has be demonstrated that the differentiation of pluripotent cells into the germ layers there is a deceleration in the cell cycle times and adoption of a more tightly regulated cell cycle.

It was the focus of this research to investigate the role of c-Myc protein in the differentiation of pluripotent embryonic stem cells into the three primary germ layers. To analyse the association of c-Myc protein with changes in cell cycle kinetics Western Blot Analysis was utilised. In this experiment, two distinct protein species were detected in early time points but were down-regulated as differentiation proceeded. Based on the

estimated size of the proteins detected it was proposed that one of the bands was full-length c-Myc and the other was the shorter, differentially translated c-Myc subtype, c-MycS. Specific antibodies generated in the course of this research demonstrated that the shorter band detected in the Western Analysis was c-MycS, which illustrated that translation of the c-Myc protein is differentially regulated during differentiation of ES cells.

This thesis also describes the impact of c-Myc over-expression on differentiation and the maintenance of pluripotency. This effect was analysed during embryoid body differentiation experiments utilising Northern blot detection of RNA markers, which showed delayed differentiation kinetics. LIF titration assays demonstrated that c-Myc over-expression decreased dependence on LIF for ES cells to maintain pluripotency.

DECLARATION

NAME: Duane Rivett PROGRAM: Masters of Scienc

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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SIGNATURE: ..... DATE: 24/02/04

CHAPTER 1

INTRODUCTION

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INTRODUCTION

1.1 Mammalian Embryonic Development

Embryogenesis begins with fertilisation of the egg. This single cell is the progenitor of most extra-embryonic membranes and all the cell lineages that comprise the embryo and the adult. The initial stages of mammalian development entail an increase in cell number without alteration in cellular differentiation potential (reviewed in Hogan *et al.*, 1994). The first differentiation event occurs at approximately 2.5 days post coitum (dpc), at the 16 cell morula stage, where the outer cells give rise to the extra-embryonic trophectoderm lineage while the inner cells form the first pluripotent cell population of the embryo, the inner cell mass (ICM) (Gardner, 1983). Pluripotence is defined as retaining the potential to contribute progeny to all embryonic lineages. Between 2.5 and 3.5 dpc, fluid accumulates within the embryo that extrudes the ICM to one side of the embryo, forming the blastocelic cavity (Watson and Kidder, 1988). By 4.5 dpc the outer ICM cells, adjacent to the cavity, have differentiated into an additional extra-embryonic lineage, termed primitive endoderm, while the remainder of the ICM forms the pluripotent epiblast. At 4.5 dpc the embryo implants into the uterus (reviewed in Hogan *et al.*, 1994). Following implantation, the pluripotent cells of the epiblast undergo a period of rapid proliferation between 5.5 and 6.5 dpc, where cell cycle times drop as low as 5-7 hours (Snow, 1977; Poelmann, 1980; Lawson *et al.*, 1991). Furthermore, during this stage of development, the pluripotent epiblast cells differentiate into an additional pluripotent lineage, termed primitive ectoderm. Primitive ectoderm is a pseudo-stratified epithelial monolayer of cells surrounding a central pro-amniotic cavity. The pro-amniotic cavity forms around 5.0 dpc by the induction of programmed cell death in the central pluripotent cells through the

action of a diffusible 'death' signal, eminating from a lineage of the primitive endoderm, the visceral endoderm, that lies adjacent to the pluripotent cell mass. The outer pluripotent cells survive this apoptotic signal through interaction with an extra-cellular matrix associated survival signal in the basement membrane separating visceral endoderm and primitive ectoderm (Coucouvanis and Martin, 1995).

The primitive ectoderm is the pluripotent cell substrate for the process of gastrulation at approximately 6.5 dpc. It is at this stage of development when cells first lose pluripotency and form the three primary germ layers, ectoderm, endoderm and mesoderm (Ginsberg *et al.*, 1990). The onset of gastrulation is defined by the appearance of the primitive streak on the posterior most end of the embryo, where pluripotent cells differentiate into definitive mesoderm. The emerging embryonic mesoderm migrates between the primitive ectoderm and the visceral endoderm from the most posterior embryonic region to the distal tip of the embryo. In the period of 4.5-6 dpc cell cycle times are thought to be as low as 10 hours. Following mesoderm formation the cell cycle transition times have slowed to predicted rates of 22.2 hours at day 7 (Snow, 1977). This indicates that as the pluripotent cells of the embryo differentiate into non-pluripotent cells, there is major remodelling of the cell cycle.

1.2 The Myc Family

Mammalian cells respond to extracellular stimuli via the activation of immediate early (IE) genes, which produce proteins that elicit the appropriate cellular responses. One of such group of IE genes are the *myc* family (Winkles, 1998). The protein products of the *myc* family of proto-oncogenes are nuclear localised and involved in a wide variety of cellular functions such as cell proliferation, growth and apoptosis (Ryan and Birnie, 1996). They have been extensively investigated as a target for chromosomal translocations,

rearrangements, mutations and amplifications, which enhance its activity in wide variety of cancers (Lutz *et al.*, 2002). In fact, *myc* was discovered through its homology to the transforming gene, *v-myc*, of the avian myelocytomatosis virus, MC29 (Vennstrom *et al*, 1982).

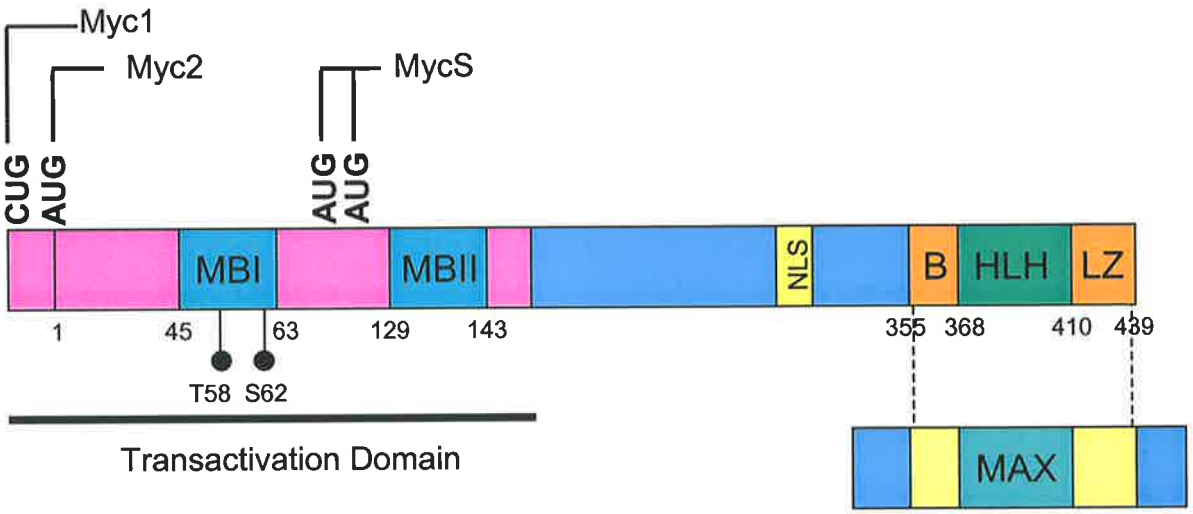
There are several members of the *myc* family, the most characterised of which are c-, L- and N-*myc*. The transactivation domain, located at the N-terminus (amino acids 1-143), contains two 'myc boxes', MBI and MBII, which are areas of strong homology between the family members (Kato *et al.*, 1990; Atchley and Fitch, 1995). The C-terminus of the protein contains a basic helix-loop-helix, leucine zipper (b-HLH-LZ) domain, which enables dimerisation to partner proteins and binding to promoters of genes containing a consensus CACGTG sequence, termed the E-box (reviewed in Ryan and Birnie, 1996; Grandori *et al.*, 2000). Myc proteins also contain a centrally positioned nuclear localisation signal (NLS) (Ryan and Birnie, 1996). A schematic diagram of the structure of Myc is shown in Figure 1.1. Myc has been shown to have a very short half-life, approximately 30 minutes, and is ubiquitinated and degraded by the 26s proteosome (Herschko and Ciechanover, 1998; Gross-Mesilaty *et al.*, 1998). This short half-life enables rapid down-regulation of the protein and tight regulation of its activity (Gregory and Hann, 2000). Myc function is dependent on binding to the b-HLH-LZ domain of the Max protein (Amati *et al.*, 1993 and 1992) which allows binding to the promoters of target genes and modulation of transcription. Another family of b-HLH-LZ proteins, the Mads, can also associate with Max to elicit transcriptional repression of Myc target genes via binding to the E-box. The onset of differentiation, and associated cell cycle withdrawal, is typically characterised by the up-regulation of Mad family members, including Mad1, Mxi1 (Mad2), Mad3 and Mad4, and the down regulation of Myc family members (reviewed in Zhou and Hurlin, 2001).

Figure 1.1

A diagramatic representation c-Myc protein which shows:

- the domains of the protein, including the Myc box 1 (MBI) and Myc Box 2 (MBII), the nuclear localisation sequence (NLS) and the basic helix loop helix leucine (bHLHLZ) domain.
- that the bHLHLZ domain is where Myc associates with Max
- the translational start sites of the c-Myc1, c-Myc2 and c-MycS subtypes,
- the Threonine 58 and Serine 62 residues, which are phosphorylated as a method of Myc protein stability control.

Figure adapted from Xiao *et al.*, 1998, Claassen and Hann, 1999 and Grandori *et al.*, 2000


Myc1
Myc2
MycS
CUG
AUG
AUG
AUG
MBI
MBII
NLS
B
HLH
LZ
1
45
63
129
143
355
368
410
439
T58
S62
Transactivation Domain
MAX

1.2.1 Myc/Max/Mad Associated Transcriptional Control

Various studies have shown Myc activates and represses various genes involved with cell cycle, cell growth, adhesion and apoptosis (Coller *et al.*, 2000; Grandori and Eisenman, 1997; Jansen-Durr *et al.*, 1993; O'Connell *et al.*, 2003). Activation of Myc-mediated transcription is dependent on Max association and also the N-terminal transactivation domain. Full length c-Myc has been shown to activate *in vivo* reporter constructs linked to full-length promoters from putative target genes and synthetic, E-box containing promoters (reviewed in Grandori *et al.*, 2000). It has previously been demonstrated that the transactivation domain (amino acid 1-143) contains two 'Myc boxes', of which only MBI (aa 44-63) is absolutely required for transcriptional activation, whilst MBII (aa 129-143) has no transcriptional activity (Kato *et al.*, 1990; Atchley and Fitch, 1995). The function of MBII in gene transcription has been shown to be involved with its association with TRAPP (Transformation/transcription domain associated protein) (Brough *et al.*, 1995; McMahon *et al.*, 1998), which has roles in recruiting the histone acetyl transferase enzyme, hGCN5 and Tip60. Acetylation of histones at target promoters results in an open chromatin conformation and promotes transcription by allowing progression of transcriptional machinery along the DNA (McMahon *et al*, 2000; Frank *et al.*, 2003). High levels of Myc-associated deacetylation has been directly shown at the *TERT* (Xu *et al.*, 2001) and *Cyclin D2* promoters (Bouchard *et al.*, 2001). Conversely, only a modest up-regulation of histone acetylation is observed at Myc-associated *cad* and *odc* promoters when there is a strong up-regulation of transcription associated with re-entry to the cell cycle (Eberhardy *et al.*, 2000). At these promoters the Myc/Max complex recruits a P-TEFb, a kinase that phosphorylates RNA polymerase II as a method of promoting transcription (Eberhardy and Farnham, 2001).

The role of Myc as a transcriptional activator is opposed by the action of the Mad proteins (Ayer *et al.*, 1993). The N-terminus of the Mad proteins contains a region essential for the repressive role of Mad, termed the Sin3 Interaction Region (SIR) which binds Sin3 (Ayer *et al.* 1995, Kasten *et al.*, 1996; reviewed in Zhou and Hurlin, 2001). Sin3 associates with histone deacetylases (HDAC), which enhance gene silencing via deacetylation of histone tails and closed chromatin conformation (reviewed in Ahringer, 2000).

Recent characterisation of Myc transcriptional regulation has revealed that whilst Myc has roles as a transcriptional activator it also has many roles as a transcriptional repressor. Miz-1 is a transcription factor that activates transcription of the cyclin dependent kinase inhibitors, $p15^{INK4B}$ and $p21^{cip1}$. The association of Myc/Max dimers to Miz-1 at the initiator (Inr) regions of the *p15*^{INK4B} and $p21^{cip1}$ genes prevents transcription by blocking interaction of Miz-1 to its co-activator, p300 (Staller *et al.*, 2001; Wu *et al.*, 2003). This method of transcriptional repression is dependant on MBII and is shared by other Inr binding transcription factors (Li *et al.*, 1994), such as TFII-I (Roy *et al.*, 1993). This evidence, coupled with the results from Kato *et* al., 1990, suggests that MBI is involved with transcriptional activation while MBII is involved with gene repression. Consequently, it has been shown that Myc is involved in the repression of a wide variety of genes, predominantly involved with activation of differentiation and cell cycle arrest (Amundson *et al.*, 1998; Coller *et al.*, 2000; Lee *et al.*, 1997; Claassen and Hann, 2000; Philipp *et al.*, 1994).

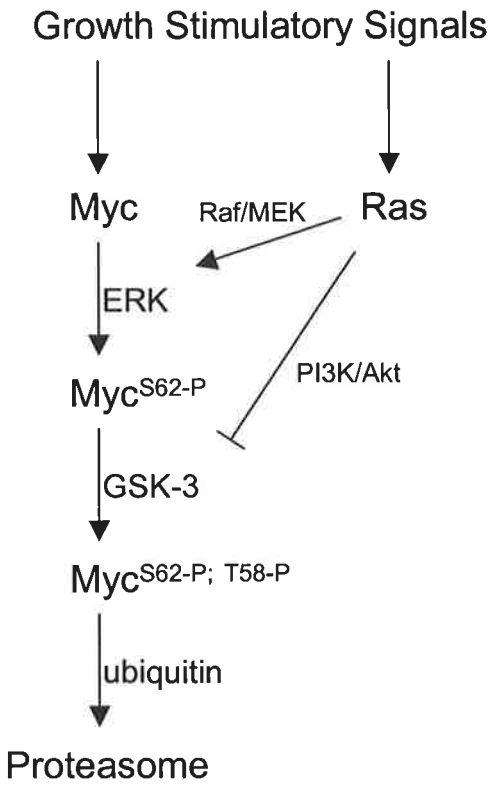
1.2.2 Activation of Myc

Myc is widely considered a general downstream effector of proliferation signals as mitogen stimulation causes increases in its transcription, translation and protein stability

(Spencer and Groudine, 1991). Platelet Derived Growth Factor (PDGF), Colony Simulating Factor (CSF), Epidermal Growth Factor, Interleukin (IL) -7, IL-2 and other mitogens, have all been shown to up-regulate *myc* transcription and translation (See Spencer and Groudine, 1991 for a review). Myc protein stability is also increased in response to mitogenic stimulation. This is mediated via the activation of Ras, which is a small GTP-binding protein critical to cell growth control and response to mitogenic stimuli. Downstream of Ras activation is a signalling cascade involving MAPK (ERK) and Raf, which conveys mitogen-activated cell cycle progression signals from the cell membrane to the nucleus (Sears *et al.*, 1999). It has been shown in primary mouse embryo fibroblasts that over-expression of both Ras and Myc is sufficient to cause transformation (Land *et al.*, 1983). Ras is also involved in the inhibition of ubiquitin-mediated Myc degradation via the regulation of N-terminal phosphorylations in the MBI region of Myc at Threonine (Thr) 58 and Serine (Ser) 62. Phosphorylation of Ser 62 stabilises the protein, enhancing its half-life, while phosphorylation of Thr 58, which is dependent on Ser 62 phosphorylation, targets the protein for degradation via the ubiquitin/proteosome pathway. Ras up-regulates Raf/MEK that results in ERK-mediated Ser 62 phosphorylation. Thr 58 phosphorylation is thought to be mediated via the constitutively expressed kinase, GSK-3, which is inactivated by the Ras up-regulated PI-3K/AKT pathway (Sears *et al.*, 2000). This process is summarised in figure 1.2. It has been shown that the half-life of Myc can be increased from 30 minutes to over 1 hour via the mutation of Thr 58 (Sears *et al.*, 2000), which has also been shown to be the most common point mutation in Myc found in tumours (Huang *et al.*, 1995). Mutation of Ser 62, however, results in a decrease in the proteins half life to approximately 10 minutes (Sears *et al.*, 2000).

Figure 1.2

The role of Ras in the stabilisation of the c-Myc protein in response to growth stimulatory signals. Adapted from Sears *et al.*, 2000.


Growth Stimulatory Signals
Myc
Raf/MEK
Ras
ERK
PI3K/Akt
Myc S62-P
GSK-3
Myc S62-P; T58-P
ubiquitin
Proteasome

1.2.3 The Role of Myc in Cell Cycle Regulation

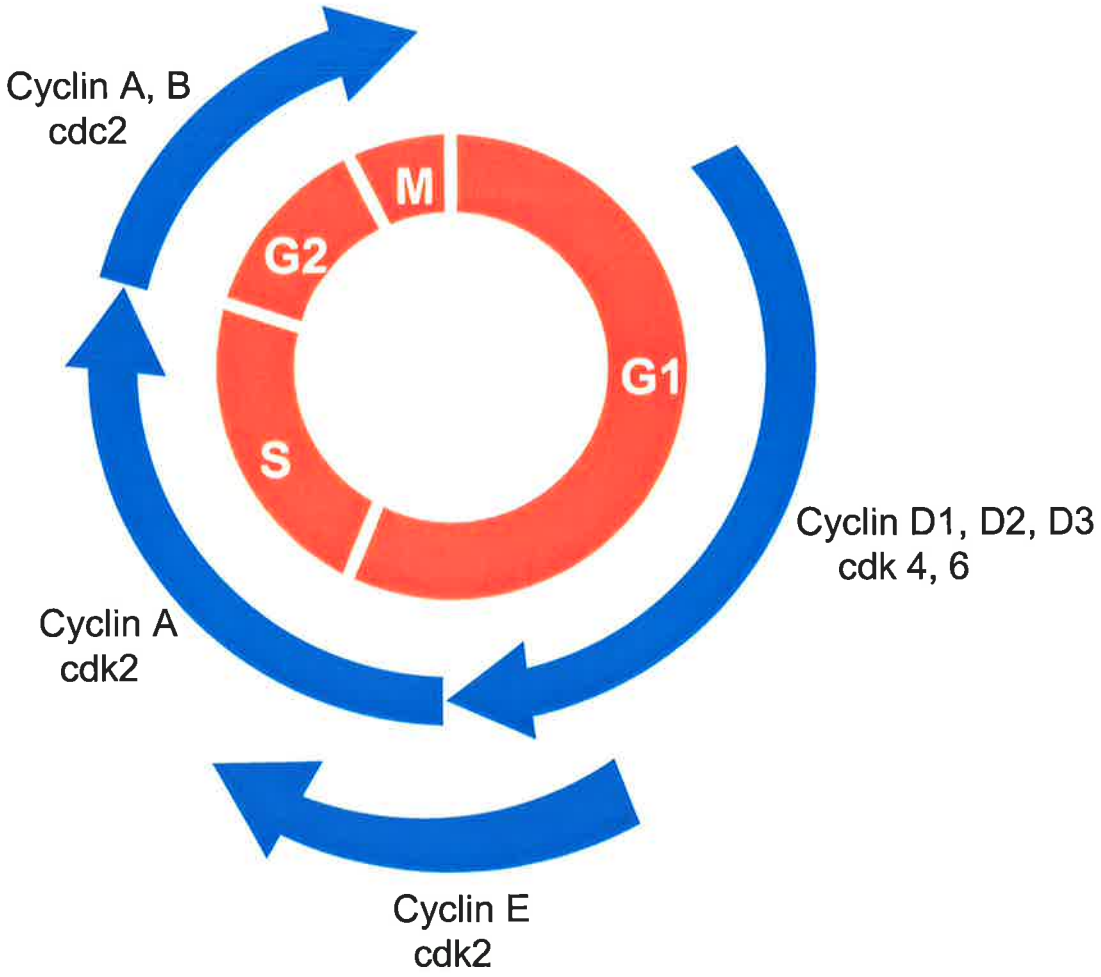
1.2.3.1 The Mammalian Cell Cycle

The mammalian cell cycle is a tightly regulated process, which allows the ordered duplication of chromosomes and cell division. The cell cycle is divided into 4 discreet phases, termed G1, S, G2 and M-phase. Chromosome replication occurs in S-phase and cell division occurs in M-phase. These phases are separated by G1 and G2-phase, in which the cell will sense its environment to assess whether it should enter the next phase (reviewed in Donjerkovic and Scott, 2000). The ordered progression and rate of the cell cycle is controlled via the ordered activation of a family of kinases, termed the cyclin dependent kinases (cdk). Activation of the catalytic cdk subunit is dependant on association with the regulatory Cyclin subunit, allowing phosphorylation of targets involved in cell cycle progression. While the level of the cdk typically stays constant throughout the cell cycle, Cyclin levels are restricted to discreet phases (reviewed in Morgan, 1997). The cell cycle is summarised in figure 1.3.

Following mitosis, when the two daughter cells are separated, all cdk activity is destroyed. It is not until mid-G1 that Cyclin D proteins are up-regulated, resulting in the association with cdk4 and cdk6 and the phosphorylation of G1-targets. One of the targets of the G1-phase cyclin/cdk complexes is the Retinoblastoma protein (Rb), which is a repressor of the transcription factor, E2F (Helin *et al.*, 1993). This cdk-dependent phosphorylation releases the Rb protein from its complex with E2F allowing transcription of genes that control G1-S phase progression. One such gene transcribed by E2F is *CyclinE*. Active CyclinE/cdk2 complexes are able to phosphorylate targets involved with entry into S-phase, including Rb (reviewed in Ekholm and Reed, 2000).

Figure 1.3

Control of progression through the phases of the Mammalian Cell Cycle. These events are reviewed in Morgan, 1997.


Cyclin A, B
cdc2
M
G2
G1
S
Cyclin D1, D2, D3
cdk 4, 6
Cyclin A
cdk2
Cyclin E
cdk2

Cdk activation is also regulated by two other methods, other than Cyclin association. Activation is opposed by the action of cdk inhibitors such as $p21^{cip1}$ and $p27^{kip1}$ that associate with cyclin/cdk complexes (reviewed in Sherr and Roberts, 1999). Phosphorylation on specific Cdks can result in either activation or repression of Cyclin/Cdk complexes (Poon *et al.*, 1994; Solomon *et al.*, 1993; Mitra and Schultz, 1996).

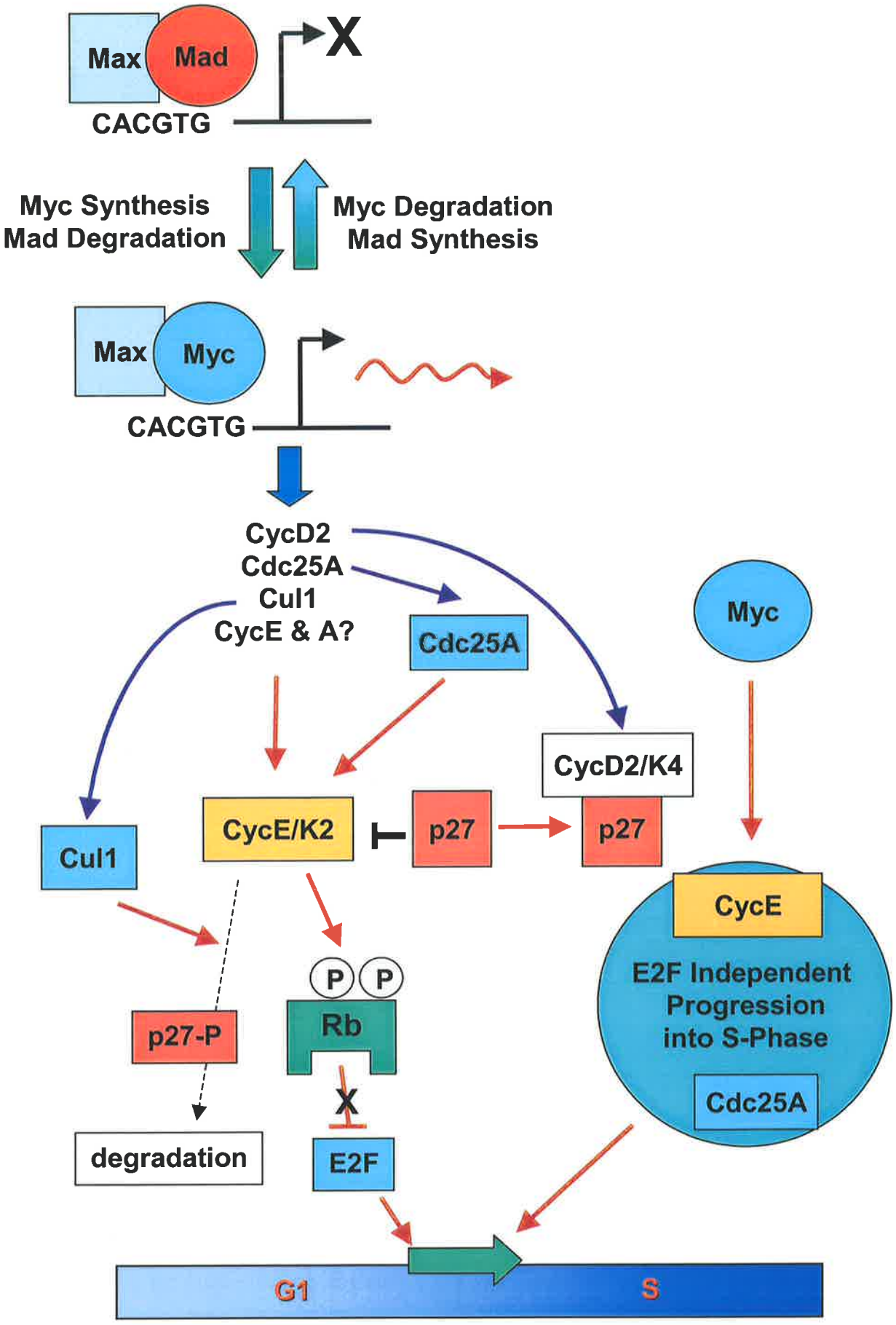
1.2.3.2 Myc-mediated Cell Cycle Progression

One way Myc is thought to promote cell cycle progression is through induction of cyclin gene transcription. In mouse fibroblasts under serum starved conditions, over-expression of Myc has been shown to induce the transcription of the *Cyclin E* and *A* genes and progression into S-phase (Jansen-Durr *et al.*, 1993). Myc is also a direct transcriptional activator of the *Cyclin D2* (Bouchard et al., 1999) and *Cul1* gene (O'Hagan *et al.* 2000). Up-regulation of Cyclin D2/cdk4 complexes results in the sequesteration of the cdk inhibitor, p27, from Cyclin E/cdk2 complexes (Bouchard et al., 1999) and Cul1 induces p27 ubiquitination and degradation (O'Hagan *et al.* 2000) resulting in increase in net CyclinE/cdk2 activity. Myc also directly activates the *Cdc25A* gene, the protein product of which, is involved in removing inhibitory phosphates from cdk2, inducing activation of the Cyclin E/cdk2 complex (Sexl *et al.*, 1999). Myc-mediated Cdc25A and Cyclin E up-regulation is also thought to drive G1- to S-phase progression of the cell cycle in the absence of E2F function (Santoni-Rugiu *et al.*, 2000) though a mechanism for this process is yet to be defined. These events are summarised in figure 1.4.

Myc has also been shown to repress genes involved with inhibition of cell cycle progression. As discussed previously, Myc represses the Miz1-mediated transcription of cdk inhibitors $p15^{INK4B}$ (Staller *et al.*, 2001) and $p21^{cip1}$ (Wu *et al.*, 2003). Myc also

Figure 1.4

c-Myc has various roles in promoting cell proliferation via G1-S phase progression. These are involved with direct and indirect transcriptional activation and possibly other non-transciptional roles. Figure constructed using information from Jansen-Durr *et al.*, 1993, Bouchard *et al.*, 1999, O'Hagan *et al.*, 2000, Santoni-Raguni *et al.*, 2000 and Sexl *et al.*, 1999.


Max
Mad
X
CACGTG
Myc Synthesis
Mad Degradation
Myc Degradation
Mad Synthesis
Max
Myc
CACGTG
CycD2
Cdc25A
Cul1
CycE & A?
Cdc25A
Myc
CycD2/K4
Cul1
CycE/K2
p27
p27
CycE
E2F Independent
Progression
into S-Phase
Cdc25A
P
P
Rb
p27-P
X
degradation
E2F
G1
S

represses the transcription of Growth Arrest and DNA Damage genes, *gadd34*, *gadd45* and *gadd153* (Amundson *et al.*, 1998; Marhin *et al.*, 1997), Growth Arrest gene, *gas1* (Lee *et al.*, 1997) and cdk inhibitor, *p21* (Claassen and Hann, 2000). MBII has also been shown to directly associate and sequester p21 protein as a method of reciprocal regulation which results in lower available levels of, both, p21 and Myc (Kituara *et al.*, 2000).

1.2.4 The Role of Myc in Differentiation

It has long been known that cell cycle modification is a consequence/prerequisite for differentiation (reveiwed in Gao and Zelenka, 1997). As Myc is involved with activating the proliferation (see 1.2.3.2) Myc down-regulation is also a feature of differentiation, especially in terminally differentiating cells, which exit the cell cycle completely (reviewed in Zhou and Hurlin, 2001 and Henriksson and Luscher, 1996). It has been shown that in various cell types that the onset of differentiation results in a switch from Myc/Max dimers to Mad/Max dimers (Xu *et al.*, 2001; Lin *et al.*, 2000; Queva *et al.*, 1998). This switch results in the down-regulation of cell cycle activators and loss of inactivation of cell cycle repressors (Xu *et al.*, 2001; Lin *et al.*, 2000; Queva *et al.*, 1998; Wu *et al.*, 2003), and in many cases is essential to differentiation (Lin *et al.*, 2000; MacLean-Hunter *et al.*, 1994). The down-regulation of Myc in differentiation is also thought to be involved with the silencing of various genes, via the loss of Myc-associated histone deacetylation at specific sites (Lee *et al.*, 2003), and the up-regulation of Mad-associated HDAC activation (Queva *et al.*, 1998; Xu *et al.*, 2001). Interestingly, it has been shown that reintroduction of Myc into quiecent differentiated cells can cause re-entry into the cell cycle (Jansen-Durr *et al.*, 1993).

Another role Myc has in differentiation is its effects on cell adhesion and cell growth. Recent studies have demonstrated that Myc regulates genes and proteins involved with translation, such as ribosomal subunits, adhesion molecules and metabolic enzymes (Shiio *et al.*, 2003; Frye *et al.*, 2003; Coller *et al.*, 2000; O'Connell *et al.*, 2003; Amundson *et al.*, 1998; Iritani and Eisenman, 1999). These results indicate that action of Myc is involved with structural changes to the shape of the cell, its attachment to it's immediate microenvironment and metabolic functional changes within the cell during differentiation.

1.2.5 Myc Alternative Translation Variants

Understanding the regulation of Myc has recently become more complicated as several translational variants of the protein have been characterised. These variants arise through initiation of translation from alternative start sites. Variants include c-Myc1, which arises from an upstream CUG, c-Myc2, which is the most common form of the protein, arising from translation from the ATG, and the downstream ATG-initiated, c-MycS subtypes (Spotts *et al.*, 1997; Xiao *et al.*, 1998 and see Figure 1.2). The c-MycS protein, which lacks the N-terminal 100 amino acids, lacks the highly conserved MBI and has been detected in human, murine and avian cells. Like its full-length counterparts, c-MycS proteins, are nuclear-localised short-lived phosphoproteins, which have the ability to dimerise with Max. These proteins, as expected, are incapable of inactivating Myc target genes, and have been associated with inhibition of full-length Myc-mediated transcription (Spotts *et al.*, 1997).

While c-MycS proteins appear unable to activate transcription, it has become increasingly clear that the cellular outcome of their expression is not unlike that of full-length Myc. These proteins retain the ability to repress *gas1* and *gadd45* (Xiao *et al.*, 1998) and

increase cdk2 kinase activity (Hirst and Grandori, 2000). c-MycS is still able to initiate both proliferation and apoptosis in immortalised rodent cells (Xiao *et al.*, 1998) and has been shown to be up-regulated, to levels comparable to full-length, in rapidly dividing cells (Spotts *et al.*, 1997). c-MycS is also more rapidly degraded than full length c-Myc (Spotts *et al.*, 1997) possibly because it does not contain the site of stabilising phosphorylation at Ser 62 (Sears *et al.*, 2000).

1.2.6 Myc and Embryonic Development

During embryonic development *myc* RNA is widely expressed. c-*myc* RNA expression in the gastrulating embryo is readily detected throughout the entire conceptus (embryonic and extra-embryonic regions). At 7.5 dpc c-*myc* expression was detected at high levels in the extra-embryonic lineages whilst in the primitive ectoderm proliferative zone, showed only modest expression. N-*myc* was detected at moderate levels in the 6.5 dpc embryo and becomes highly expressed in the primitive streak and the primitive ectoderm of the 7.5 dpc embryo. N-*myc* expression decreased during mesoderm formation and differentiation into the epithelioid cells of the head process, somites and the nascent heart (Downs *et al.*, 1989; Queva *et al.*, 1998).

Mice carrying functional mutation of the c-*myc* allele have impaired development and die by 10.5 dpc. Generally, the embryos are small and form most cell types but display heart defects, fluid-filled pericardia and neural abnormalities caused by improper closure of the neural tube. The death of the embryos is thought to occur due to malnutrition caused by poor vascularisation of the yolk sac (Davis *et al.*, 1993). N-Myc knockout mice develop normally until approximately 10.5 dpc but die at approximately 11.5 dpc, possibly caused by failure of the major organs to develop (Sawai *et al.*, 1993). L-Myc is expressed in

embryogenesis in areas of proliferation and differentiation however L-Myc knockout mice are viable, with few developmental defects (Hatton *et al.*, 1996). This suggests that other Myc family members may play redundant roles in development. This compensation hypothesis is supported by the work of Malynn *et al.*, 2000, when they replaced the c-*myc* gene with an N-*myc* gene and demonstrated that knock-in mice developed normally. This suggests that all Myc proteins may play similar roles in development but are expressed in different spatial patterns. In contrast to the *myc* knockout embryos, *Max* knockout mice cannot develop past the blastocyst stage of development. These mice die in early post-implantation and at this stage are 50 – 70% smaller than wild-type and *Max* +/- heterozygous embryos. It was suggested that the embryos in early development utilised a maternal store of Max protein as was demonstrated by the high levels of Max protein in the unfertilised egg and the 0.5 dpc embryo (Shen-Li *et al.*, 2000).

1.3 Embryonic Stem Cells

Embryonic Stem (ES) cells are derived from the ICM of the mouse pre-implantation blastocyst. ES cells can be maintained indefinitely in culture in the presence of the cytokine, leukemia inhibitory factor (LIF) (Nichols *et al.*, 1990). ES cells retain pluripotency, as demonstrated by their ability to contribute to all the embryonic cell lineages, including the germ line, when re-injected into blastocysts (Robertson *et al.*, 1986). ES cells also express the pluripotent cell marker, *Oct4* (Rosner *et al.*, 1990), and the ICM cell marker, *Rex1* (Rogers *et al.*, 1991). Cell division times in ES cells are very rapid (~12 hours) in comparison to somatic cells, which demonstrate cell cycle times in excess of 24 hours (Stead *et al.*, 2002). Observations in our laboratory demonstrate that these rapid cell cycle times are associated with constitutively active cyclin E/cdk2 and cyclin A/cdk2, which are thought to drive the rapid cell cycles associated with this period of development (Stead, personal communication). The cell cycle kinetics in ES cells is

compared to a somatic cell cycle in figure 1.5. There are also no detectable levels of cyclin dependent kinase inhibitors, p21 and p27 (Stead *et al.*, 2002)

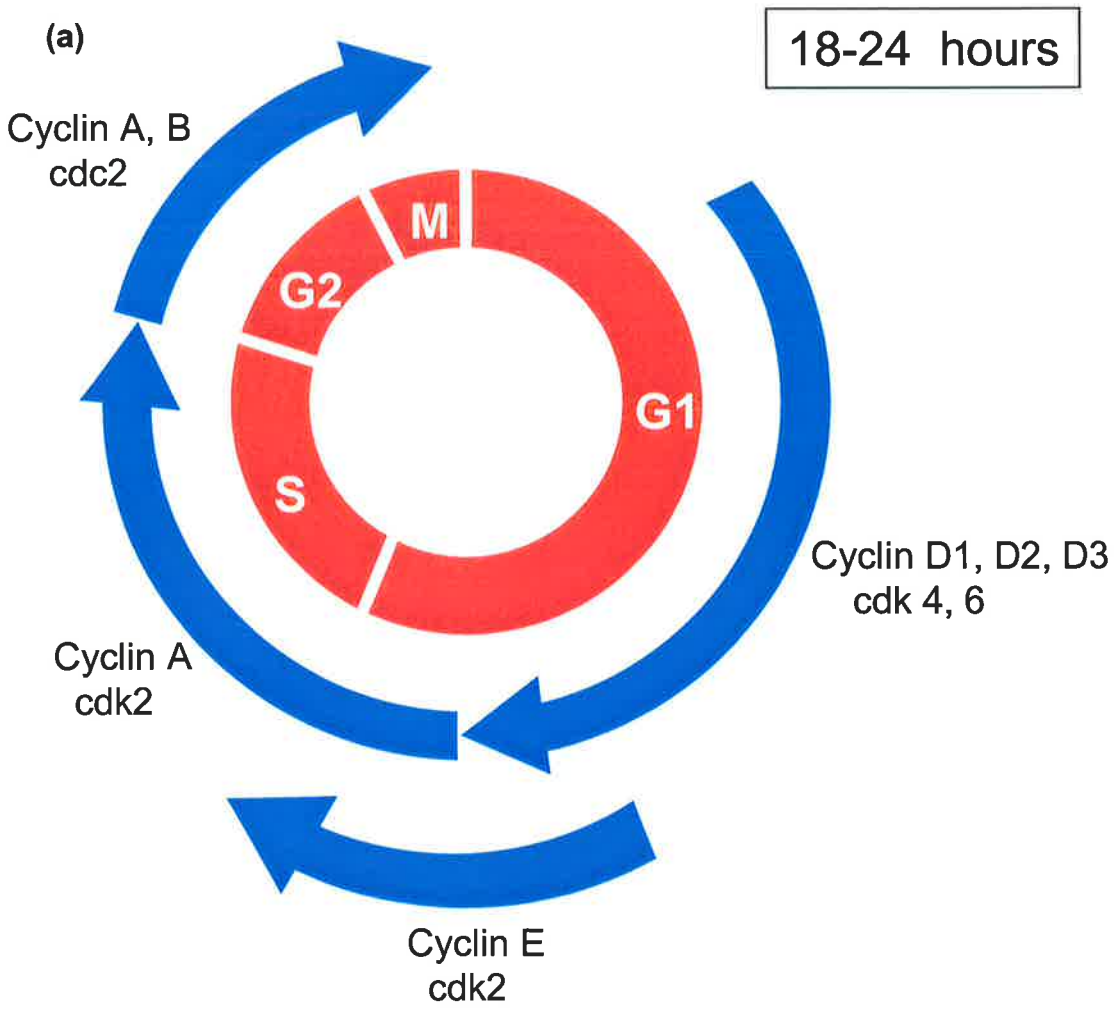
Culture of ES cells in the conditioned medium, MedII, leads to the formation of Early Primitive Ectoderm-like (EPL) cells (Rathjen *et al.*, 1999). This differentiation event *in vitro* mimics the formation of primitive ectoderm *in vivo*. Like primitive ectoderm, EPL cells retain pluripotency, as measured by *Oct4* expression and express the primitive ectoderm marker, *Fgf5*. EPL cells also retain the potential to form the primary germ layers of the embryo, *in vitro* (Rathjen *et al.*, 1999, Lake *et al.*, 2000). Culture of ES or EPL cells as aggregates in suspension, termed embryoid bodies (EB), induces ordered differentiation mimicking the *in vivo* events. ES cell EBs form lineages of all three primary germ layers (Doetschman *et al.*, 1985; Rathjen *et al.*, 1999; Lake *et al.*, 2000). EPL cell EBs form >95% mesoderm as determined by their expression of the nascent mesoderm marker *brachyury* (Lake *et al.*, 2000). It has been shown previously in our laboratory that as ES cells undergo differentiation into mesoderm, both cyclin E/cdk2 and cyclin A/cdk2 activity decreases (Stead, personal communication).

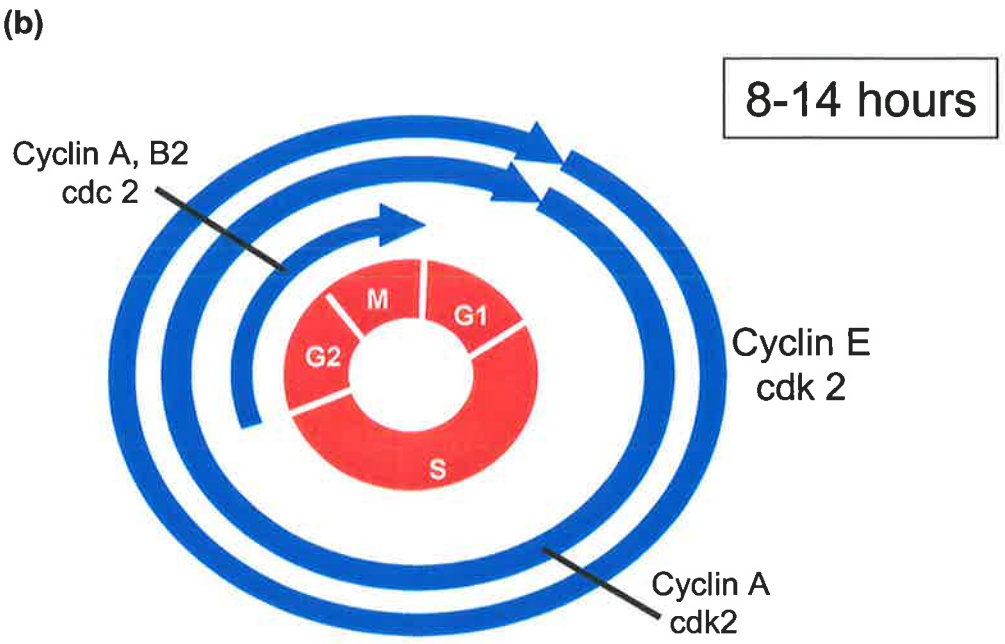
1.3.1 ES Cell Differentiation and Myc

The role of Myc in ES cell differentiation has been previously studied using a fusion protein termed RLF/L-Myc, found in human small cell lung carcinoma (SCLC). This protein is produced by a chromosomal translocation and fusion of the cellular gene *rlf* to the L-*myc* gene creating a protein, which contains the full length L-Myc with a 79 amino acid extension from the RLF protein. Interestingly, expression of the RLF/L-Myc in ES cells delays the formation of beating muscle and nerves in ES cells grown to form EBs. In

Figure 1.5

c-Myc has various roles in promoting cell proliferation via G1-S phase progression. These are involved with direct and indirect transcriptional activation and possibly other non-transciptional roles. Figure constructed using information from Jansen-Durr *et al.*, 1993, Bouchard *et al.*, 1999, O'Hagan *et al.*, 2000, Santoni-Raguni *et al.*, 2000 and Sexl *et al.*, 1999.


(a)
18-24 hours
Cyclin A, B
cdc2
M
G2
G1
S
Cyclin D1, D2, D3
cdk 4, 6
Cyclin A
cdk2
Cyclin E
cdk2


(b)
8-14 hours
Cyclin A, B2
cdc 2
M
G1
G2
S
Cyclin E
cdk 2
Cyclin A
cdk2

cells expressing high levels of the fusion protein cell death occurs at approximately 6 days (MacLean-Hunter *et al.*, 1994).

1.4 Aims

c-Myc is a potent cell cycle activator with various roles in proliferation and differentiation. Given that c-Myc is also essential to embryonic development it may be deduced that c-Myc may have a role in the activation of the rapid cell cycle times seen in the pluripotent cell layers in the embryo and ES cells. Thus it was of primary interest to analyse the endogenous expression of Myc protein in ES cells and during their differentiation into embryoid bodies. Investigation of c-Myc expression levels during embryoid body differentiation would enable elucidation of what functions of c-Myc may be required in the differentiation of the pluripotent cell types of the embryo. Generation of antibodies that would allow discrimination between different translational variants of c-Myc would increase the understanding of how c-Myc may control the dynamic processes seen during embryogenesis.

The switch from Myc/Max to Mad/Myc dimers during terminal differentiation has been shown in various cell lines, such as HL60s (Xu *et al.*, 2001) and B Lymphocytes (Lin *et al.*, 2000). As over-expression of Myc has also been shown to be sufficient for promoting quiescent cells to re-enter the cell cycle (Jansen-Durr *et al.*, 1993) it was of interest to see when c-Myc was required to be down-regulated in order for the cells to differentiate.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2
MATERIALS AND METHODS

2.1 Abreviations

Ab	Antibody
ATP	Adenosine tri-phosphate
APS	Ammonium persulphate
bp	Base pairs
BSA	Bovine serum albumin
β-ME	β-mercaptoethanol
cDNA	complementary DNA
cdk	Cyclin dependent kinase
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetra Acetic Acid
EPL	Early Primitive Ectoderm-like
ES	Embryonic Stem
EtBr	Ethidium Bromide

EtOH	Ethanol
FBS	Foetal Bovine Serum
Fgf	Fibroblast Growth Factor
dGTP	dexyguanosine triphosphate
GST	Glutathione S-Transferase
HRP	Horse radish peroxidase
ICM	Inner Cell Mass
IP	Immunoprecipitation
ITSS	Insulin-transferrin-sodium-selenite
kb	Kilobase pair
kDa	Kilo Dalton
LIF	Leukemia Inhibitory Factor
M	Moles per litre
mM	Millimoles per litre
µM	Micromoles per litre
µg	Microgram
µl	Microlitre
mA	Milliamperes
MBI	Myc Box I
MBII	Myc Box II
MDP	N-Acetyl-Muramyl-L-Ala-D-Iso-Gln-OH
mLIF	Mouse Leukaemia Inhibitory Factor
MOPS	Morpholinopropanesulfonic acid
MQ	Milli-Q
Mr	Molecular weight

mRNA	Messenger RNA
OD	Optical Density
4-OHT	4-Hydroxytamoxifen
PAS	Protein A Sepharose
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline Triton X-100
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
Rb	Retinoblastoma
RNA	Ribonucleic Acid
rpm	revolutions per minute
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
TBE	Tris Borate EDTA
TEMED	N,N,N',N'-Tetramethyl-Ethenediamine
TPCK	Tosyl-L-phenylalanine chlormethyl ketone
TTP	Tyrosine Triphosphate
Tween-20	Ployoxyethylene-sorbitan-Monolaurate
UV	Ultra violet
V	Volts

2.2. Tissue Culture

2.2.1 Materials

4-Hydroxytamoxifen	Sigma Chemical Co.
β-ME	Sigma Chemical Co.
ES DMEM	Gibco BRL
DMEM	Gibco BRL
DMSO	Sigma Chemical Co.
ESGRO/LIF	Chemicon
FBS	Commonwealth Serum Laboratories
Gelatin	Sigma Chemical Co.
Puromycin	Sigma Chemical Co
Trypsin	Gibco BRL

2.2.2 Tissue Culture plastic ware

100mm plates	Falcon
150cm^2 flasks	Falcon
75cm^2 flasks	Falcon
Pipettes (25ml/10ml)	Falcon
6 well trays	Falcon
24 well tray	Falcon

2.2.3 Buffers

PBS	pH 7.5, NaCl (8 g), KCl (0.2g), KH_2PO_4 (0.2g), Na_2HPO_4 (1.15 g) in 100ml H_20

2.2.4 Solutions

Alkaline Phosphase Fixative	4.5mM citric acid; 2.25mM sodium citrate; 3mM sodium chloride; 65% methanol; 4% para-formaldehyde
L-glutamine	100mM L-glutamine in MQ H_20, filter sterilised and stored as 10ml aliquots at –20°C
Trypsin-EDTA	Trypsin 1:250 (Difco) (1g), 10ml versene solution (10x concentrate) to 100ml with MQ H_2O
PBS/β-ME	100mM β-ME in sterile PBS. Fresh solution prepared fortnightly
PBS/Gelatin	0.2% (w/v) gelatin in PBS. Sterilised by autoclaving (20 psi for 25 minutes at 140°C)
Trypan blue	0.4 g Trypan blue, 0.81 g NaCl, 0.06 g K_2HPO_4 in 100ml MQ H_2O, pH 7.4 filter sterilised

2.2.5 Cell Culture medium

ES DMEM	DMEM (Gibco, BRL), pH 7.4, containing high glucose, supplemented with 10% FBS
ES Complete Medium	ES DMEM (Gibco, BRL), containing high glucose, supplemented with 10% FBS, 0.1mM β-mercaptoethanol (β-ME), 10% KNOCKOUT^{TM}SR (Gibco, BRL), 1mM glutamine and 1000 U/ml of mLIF
ES Incomplete medium	ES DMEM (Gibco, BRL), containing high glucose, supplemented with 10% FBS, 0.1mM B-ME.
LIF Titration Medium	ES DMEM (Gibco, BRL), containing high glucose, supplemented with 10% FBS, 0.1mM β-mercaptoethanol (β-

	ME), 1mM glutamine, 10% KNOCKOUT^{TM}SR (Gibco, BRL) and the desired U/ml of mLIF
MedII Medium	Conditioned medium produced by growing HepG2 cells to subconfluency. Medium was harvested and filter sterilised (0.22 μm) and stored at 4°C for up to 14 days (Rathjen et al., 1999).

2.2.6 Cell Lines

All cells were maintained in tissue culture incubators at 37°C in 10% CO_2.

D3 ES: Derived from the ICM of the pre-implantation 129 strain mouse embryo blastocyst (Doetschman et al, 1985). Kindly donated by Dr Lindsay Williams, Ludwig Institute, Melbourne.

CMES: D3 ES cells as described above over-expressing a full-length human c-Myc cDNA under the control of the EF-1α promoter. Construct transfected hc-Myc::pEF-IRES-puro6. A complete map of pEF-IRES-puro 6 is contained in Appendix 1.

c-MycER ES: D3 ES cells as described above over-expressing a full-length human c-MycER cDNA (as described by Littlewood *et al.*, 1995) under the control of the synthetic CAG promoter as described in. Construct transfected pCAGIpuro::MycER. A complete map of pCAGIpuro is contained in appendix 2.

MycT58ER ES: D3 ES cells as described above over-expressing a full-length human c-MycT58ER cDNA under the control of the synthetic CAG promoter. MycER ES cell line with Threonine 58 of c-Myc, Quikchange mutated to Alanine. Construct transfected pCAGIpuro::MycT58ER. As described in Cartwright *et al.*

HL60: Promyelocytic leukemia from the periferal blood of a 36 year old Caucasian female Received from Angel Lopez, Institute for Medical and Vetinary Science, Adelaide.

2.2.7. Miscellaneous

Freezing vials	Nunclon
Sterile bottles	Schott
0.2 μm sterile bottle top filters	Corning
Bactriological petri dishes	Techno-plas
Microscopy	Nikon ELW D0.3 phase contrast
Laminar Flow Hood	Gelman Sciences

2.3. Tissue Culture Methods

2.3.1 Gelatinised Tissue Culture plates

All tissue culture plates used for ES and EPL cells were gelatinised with 0.2% (w/v) gelatin in PBS. Plates were covered with gelatin solution and left for at least 30 minutes at room temperature. The gelatin solution was removed and the plate washed in PBS immediately before use.

2.3.2 Determination of cell number

Cells were harvested to a single cell suspension as described in 2.3.5 and 2.3.6. A 20μl aliquot of the single cell suspension was diluted in 20μl of trypan blue. Cell numbers were counted under 200x phase contrast magnification using a haemocytometer. Trypan blue stained dead cells were omitted from the cell count.

2.3.3 Stable ES Cell Line Establishment Via Electroporation

2.3.3.1 Electroporation and Selection

Cells were harvested to a single cell suspension as described in 2.3.5 and 2.3.6 and a viable cell count was performed as described in 2.3.2. The cells were then resuspended to 3-5x10^7 cells in 900µl of cold (4°C) 1x PBS. 10µg of linearised DNA was then added to the solution and the solution was transferred to a electroporation cuvette (Biorad). The cells were subjected to 500µF/0.2kV in a Biorad Gene Pulser with a Biorad Capacitance Extender. Then they were transferred to 10ml of complete ES medium, plated into 4 gelatinised 100mm dishes grown overnight as described in 2.3.5. The following day 1µg/ml puromycin was added to the medium to allow selection of stably expressing colonies. The medium was aspirated and fresh medium was added everyday for approximately one week when resistant colonies started to form.

2.3.3.2 Picking Colonies

When the resistant ES colonies were large enough to pick the medium was aspirated and the plate was washed with PBS. Under an inverted microscope the colonies were dislodged from the plate and pulled up with a P200 pipette set to 30µl. The colony was then placed in 50µl of trypsin, left for 2 minutes and the cells were dispersed by the P200 pipette. The cells were then transferred to a gelatinised 24 well tray and grown overnight in ES complete medium. The following day the cells were returned to selection medium containing 1µg/ml puromycin. The cells were then passaged as described in 2.3.5.

2.3.4 Thawing stored cell lines

2.3.4.1 ES cells

A vial of cells was removed from liquid nitrogen and placed in a 37°C water bath. As soon as the cells were thawed they were diluted in 10ml of fresh culture medium and spun at 1200 rpm for 4 minutes. Approximately 5 x 10^5-1x 10^6 cells were then resuspended in the appropriate medium and placed in a gelatinised 100mm dish, at 37°C, 10% CO_2 in a humidified incubator. The following day, medium was replaced with fresh medium.

2.3.4.2 Additional cells lines

A vial of cells was removed from liquid nitrogen and placed in a 37°C water bath. As soon as the cells were thawed they were diluted in 10ml of fresh culture medium and plated in a 75cm^2 flask. Thawed cells were cultured at 37°C, 5% CO_2 in a humidified incubator. Medium was replaced with fresh medium the following day.

2.3.5 Maintenance of ES and EPL cell lines

Embryonic stem (ES) cells were cultured on tissue culture grade plastic (Falcon) pre treated with 0.2% gelatin/PBS. Semi confluent ES cell plates were washed with PBS before the addition of trypsin (1ml) prewarmed to 37°C for 2 minutes. A single cell suspension was obtained by manual pipetting and transferred into 4ml of ES complete medium. Cells were spun at 1200 rpm for 2 minutes, gently resuspended in 10ml of fresh ES complete medium and reseeded at a density ranging from 5 x 10^5 to 1 x 10^6 cells per 100mm dish and maintained in a humidified incubator at 37°C, 10% CO_2 as described in Smith, (1991). ES cells were passaged every 2-3 days and maintained for a maximum of 30 passages.

EPL cells were induced and maintained by culturing 1 x 10^6 ES cells in a 100mm dish pre-treated with 0.2% gelatin/PBS (w/v) containing 50% ES Incomplete medium, 50% MEDII medium. EPL cells were usually derived after 2 days of culture. EPL cells were passaged every 2 days for a maximum of 3 passages at 1 x 10^6 cells/100mm dish using the same method described for ES cells. All experiments performed with EPL cells in this thesis used EPL cells formed after 2 days. This was judged morphologically (flattened cells, single cell layer) and confirmed by northern analysis as described in 2.9.

2.3.6 Growth of Cells for LIF Titration Analysis

2.3.6.1 Cell Culture

ES cells to be analysed by were plated into gelatinised 24 well trays. 500 cells were plated per well and were allowed to grow for 24 hours in complete medium in a humidified incubator at 37°C, 10% CO_2. The following day the medium was aspirated and the wells were washed twice with 1x PBS to remove all traces of complete medium. LIF Titration Medium was then added to each well at the desired concentration of mLIF and the cells were allowed to grow for 6 days. In the case of the experiments with the MycER ES cell lines, 4-hydroxytamoxifen was added to the LIF Titration Medium at 100nM and the medium was changed every second day. The medium was also changed every second day in the minus 4-hydroxytamoxifen control.

2.3.6.2 Alkaline Phosphatase Detection

Alkaline Phosphatase was detected utilising an 86-R Alkaline Phosphatase Detection kit (Sigma). This was done by aspirating the medium and fixing the cells in Alkaline Phosphatase Fixative for 5 minutes. The cells were then washed several times in H_2O preceding the addition of the developing solution. The developing solution is prepared via

mixing 200µl of FRV-Alkaline with 200µl of Sodium Nitrate. After 2 minutes 9ml of H_2O and Napthol were added. When desired level of staining occurred the cells were washed several times in H_2O and allowed to air dry.

2.3.7 Maintenance of HL-60 cells

HL-60 cells were grown as described by Steinman *et al.*, 1998. HL-60 cells were cultured in $75cm^2$ flasks in RPMI media supplemented with 10% FBS. Cells were not grown past $8x10^6$ cells/ml and split every 4-5 days. When splitting, 5ml of cells was transferred to a new $75cm^2$ flask and 45ml of RPMI+10% FBS was added. The cells were grown in a humidified incubator at 37°C with a 10% $C0_2$ concentration.

2.3.8 Preparation of Embryoid Bodies (EB's)

ES cells cultured under standard conditions were removed from tissue culture plastic as described previously (2.3.4). After forming a single cell suspension, ES cells were plated into bacteriological dishes at a density of 1 x 10^6 cells per $10cm^2$ dish and cultured in ES incomplete medium. ES cells do not contact the bacteriological dish and form suspended, free floating aggregates known as embryoid bodies. EB's were cultured for 7 days during which, fresh and media was replaced every 2 days.

2.3.9 Harvesting Cells and Embryoid Bodies

All cells were removed from tissue culture plastic by treating cells with 1-2ml trypsin/PBS for 4 minutes. Following dissociation from the plastic, trypsin was inactivated with 5-8ml of incomplete medium. Cells were centrifuged at 1200 rpm for 2 minutes and washed 3 times in PBS. After removal of the PBS cell pellets were stored at –80°C.

Suspended embryoid bodies were transferred to 30ml tubes and allowed to settle. The embryoid bodies were washed three times in PBS and allowed to settle before removing the PBS and storing the pellets at –80°C.

2.4 Molecular Biology

2.4.1 Radiochemicals

α-P^{32}dATP of a specific activity 3000 Ci/mmol and concentration of 10 mCi/ml (Geneworks)

2.4.2 Chemicals

Acetic Acid: BDH

Ammonium Acetate: BDH

APS: Sigma Chemical Co.

Bis-Acrylamide: Geneworks

B-ME: Sigma Chemical Co.

Bradford Reagant: Biorad Laboratories

Bromophenol Blue: Biorad Laboratories

BSA: Sigma Chemical Co.

dATP: Boehringer Mannheim

dCTP: Boehringer Mannheim

dGTP: Boehringer Mannheim

dTTP: Boehringer Mannheim

DTT: Sigma Chemical Co.

EtBr: Sigma Chemical Co.

EDTA: Sigma Chemical Co.

Ethanol: BDH

Formaldehyde (37%): BDH

Formamide: BDH (deionised and stored at –20°C)

Glycerol: BDH

Glycine: BDH

Glutathione Agarose: Scientifix, Zymatrix

HCl: BDH

Isopropanol: BDH

Leupeptin: Sigma Chemical Co

Magnesium Chloride: Sigma Chemical Co.

Methanol: BDH

MOPS: Sigma Chemical Co.

NP-40: Sigma Chemical Co.

Potassium Chloride: Sigma Chemical Co.

Potassium Acetate: BDH

PMSF: Sigma Chemical Co.

Tween 20: Sigma Chemical Co.

Ponceau S: Sigma Chemical Co.

Potassium Acetate: BDH

Sodium Acetate: BDH

Sodium Azide: Sigma Chemical Co.

Sodium Hydroxide: BDH

Sodium Orthovanadate: Sigma Chemical Co.

SDS: Sigma Chemical Co.

TEMED: Sigma Chemical Co.

Tris: Sigma Chemical Co.

Tris-HCl: BDH

Triton-X-100: Sigma Chemical Co.

2.4.3 Enzymes

Restriction endonucleases supplied by New England Biolabs, Pharmacia and Geneworks

Klenow: Amersham

2.4.4 Buffers

Lysis buffer	50mM Hepes, pH 7.9, 250mM KCl, 0.1mM EDTA, 0.1mM EGTA, 0.4mM NaF, 0.4mM $NaVO_4$, 10% glycerol, 0.1% NP40, 0.5 nM PMSF, 1µg/ml leupeptin, 1mM DTT
Western Transfer Buffer	12.5mM Tris-HCl, 100mM Glycine, 0.05% SDS, 20% Methanol
1x SDS PAGE running buffer	250mM Glycine, 25mM Tris, 0.1% SDS
10x MOPS	200mM MOPS pH 7.0, 50mM NH_4Ac, 10mM EDTA
2 X SDS load buffer	100mM Tris pH 6.8, 20% glycerol, 4% SDS, 200mM DTT
10 x DNA/RNA loading dye	15% Ficoll 400, 0.25% BPB, 0.25% Xylene cyanol
6 x SDS Loading Buffer	360mM Tris pH 6.8, 600mM DTT, 12% SDS, 0.006% bromophenol blue (BPB), 60% glycerol
PBS	130mM NaCl, 10mM Na_2HPO_4, 30mM NaH_2PO_4, pH 7.0

PBST	130mM NaCl, 10mM $Na2HPO_4$, 30mM NaH_2PO_4, 0.1% Triton-X-100, pH 7.2
Ponceau stain	0.5% Ponceau S, 1% acetic acid in MQ H_20
Blocking Solution	5% non-fat skim milk powder in PBST
Coomassie Stain	50% MeOH (vol/vol); 0.05 brilliant (coomassie) blue R250; 10% acetic acid; 40% H_20
Coomassie Destain	30% MeOH; 10% Acetic acid; 60% H_20
STE	50mM Tris, pH 8; 150mM NaCl; 1mM EDTA

2.4.5 Solutions

Ultrahyb	Amersham
Complete freunds adjuvant	Sigma
Incomplete freunds adjuvant	Sigma
MDP	Auspep

2.4.6 Molecular Biology Kits

Megaprime Kit: Amersham

Ultraclean: MoBio

Miniprep kit: Qiagen

Midiprep kit: Qiagen

Maxiprep kit: Qiagen

ECL Chemiluminescence kit: Pierce

Western stripping kit: Alpha Diagnostics

Alkaline Phosphatase – 86-R: Sigma Diagnostics

Quickspin columns for Radiolabelled DNA Purifications: Roche Diagnostics

2.4.7 DNA Molecular weight markers

1Kb plus ladder: Life Technologies

2.4.8 Protein Molecular Weight Markers

Rainbow Markers: Amersham

Low Molecular Weight (LMW) Standard Markers: Amersham

2.4.9 cDNA Fragments Used For Probe Synthesis

Fgf5 was kindly provided by Dr. G Martin. This plasmid consisted of a fragment encoding the full length mouse Fgf5 cDNA, cloned into the *SmaI* site of pBluescript KS+ (Hebert et al., 1991).

Rex1 was kindly donated by Dr Neil Clarke. This plasmid contained 848bp of Rex1 cDNA in the *EcoRI* site.

Oct 4 cDNA in pBluescript was provided by Dr Hans Scholer. This clone contained a 462bp StuI cDNA fragment spanning positions 491 to 953 of the Oct4 cDNA sequence.

Brachyury was kindly provided by Dr Bernhard G. Herrmann. The plasmid contained a 1764 bp of Brachyury cDNA cloned into the EcoR1 site of pBluescript KS+ (Herrmann, 1991)

GAPDH (pmGap) contains 300 bp of the GAPDH cDNA sequence in pBluescript KS+ (Rathjen *et al.*, 1990).

Cyclin E was kindly provided by Nick Dyson. The plasmid contains 1.8 kb EcoR1 fragment of cyclin E cDNA.

2.4.10 Primary Antibodies

c-Myc (rabbit polyclonal N262) - sc-764	Santa Cruz Antibodies
human Cdk2 (rabbit polyclonal M2) – sc-163	Santa Cruz Antibodies

2.4.11 Secondary Antibodies

Donkey anti-rabbit immunoglobulin-HRP (NA934V)	Amersham

2.4.12 Oligoneucleotides

AbMycF	5'-dGGAATTCTGCCCCTCAACGTTAG-3'
CAG1	5'-dTCGGCTTCTGGCGTGTGACC-3'
cMyc100NAbR	5'-dCACACACTCGAGCTCCGACTGGTCGGCCGT-3'
ERseq	5'-dTGTCAAGACAAGGCAGG-3'
MycSER-f	5'-dCTGGGATCCGAATTCGCCATGGTGACC GAGCTGCTGGGAGAC-3'
MycSER-r	5'-dCACACAGGATCCCGTAGCTGTTCAAG-3'
pGEXseq3'	5'-dAAGCCACGTTTGGTGGTG-3'
pGEXseq5'	5'-dCTGCATGTGTCAGAGGTT-3'

2.4.13 Plasmids

pEF-IRES-puro 6	Kindly donated by Dr Dan Peet. For details see Appendix 1
pCAGPuro	Kindly donated by Dr Stephen Wood. For details see Appendix 2

pSRαMSVTKNEO::c-Myc	Kindly donated by Grant McArthur, Peter Macallum Cancer Institute, Melbourne. Full-length human c-Myc cDNA inserted in an EcoRI site.

2.5 Molecular Methods

2.5.1 Preparation of Whole Cell Extracts

Cells were harvested from the dish by routine methods (2.3.4) and following 2 washes in PBS were pelleted by centrifugation at 1200 rpm for 4 minutes. Residual PBS was removed and pellets stored at –80°C (2.3.8). Approximately 100µl of lysis buffer per 10^6 cells was added to cell pellets. Cell pellets were disrupted by manual pipetting, then incubated on ice for 1 hour during which they were vortexed for 5 seconds every 10 minutes. Soluble whole cell extracts were separated from insoluble cell debris by centrifugation at 14,000 rpm for 10 minutes and the supernatant collected. Protein concentration was determined colorimetrically using Bradford reagant (2.7).

2.6 Protein Detection Methods

2.6.1 SDS PAGE Analysis

SDS Page was performed as described by Laemelli, 1970. The separating gel (8-12%) was prepared using:

40% stock Bis-acrylamide solution (29:1)

375mM Tris pH8.8

0.1% SDS

0.2% APS stock solution

0.1% TEMED

Acrylamide gel percentage was altered by modifying the volume of 40% Bis-acrylamide in the gel solution. Either 0.75 or 1.5mm minigels (10 x 7cm) or 1.5mm large gels (16 x 11cm) were poured using Biorad or Oxford apparatus respectively and allowed to polymerise for approximately 30 minutes beneath an overlay of butanol. After polymerisation, butanol was removed and the stacking gel applied.

A 4% stacking gel was prepared using the same constituents described for the running gel with the appropriate balance of 40% acrylamide stock solution and 20% APS stock solution. Tris pH 6.8 was also used in replacement of Tris pH 8.8. 10, 15 or 20 well combs were inserted and the gel was left to polymerise for another 30 minutes. Gels were run in 1X SDS PAGE running buffer. Minigels were typically run at a constant voltage of 150 V while large gels were typically run at a constant voltage of 60V until the bromophenol blue dye reached the bottom of the gel.

2.6.2 Coomassie Staining

Protein gels were placed in Coomassie Stain on a rocker for at least 2 hours at room temperature. The Stain solution was poured off and Coomassie destain solution was added to the container and it was left on the rocker. Screwed up facial tissues were placed in the container also to absorb the brilliant blue. The facial tissues were replaced intermittently. When the gel was satisfactorily destained, the gel was scanned into a digital file on a HP Scanner.

2.6.3 Western Blotting

Proteins were transferred from 1.5mm unstained acrylamide gels to a 0.45µm nitrocellulose membranes in a Biorad semidry transfer machine at a constant amperage of

200mA and voltage limited at 25V in western transfer buffer. Membranes were blocked in PBS with 0.1% Tween (PBST) + 5% dry milk from 2 hours (at RT) to overnight (at 4°C). Membranes were incubated with the primary antibody (diltuted according to manufacturers instructions in PBST + 5% dry milk) overnight at 4°C. Membranes were then washed 4 times for 15 minutes in PBST by rocking, and incubated for 1 hour at RT with HRP-conjugated secondary antibody diluted according to manufacturers instructions in 5% skim milk in PBST. HRP activity was detected with an ECL detection kit (Pierce) and exposed to x-ray film (AGFA). Film was developed using an X-ray developer machine (AGFA, Curix 60). All incubations with antibodies were performed while rotating using the nutator (Clay Adams). Westerns were stripped for reprobing using the Western Stripping Kit (Alpha Diagnostics) according to manufacturers instructions.

2.7 Protein Concentration Determination (Bradford Assay)

Protein concentration was determined by a method adapted from Bradford (1976). Known standards of BSA protein (0.1, 0.5, 1, 2.5, 5, 10, 25, 50, 100µg) were pipetted into cuvettes and adjusted to 20µl with lysis buffer. The protein samples to be tested were also pipetted into cuvettes and made up to 20µl. 1ml of Bradford reagant (diluted 1:4 in MQ water) was added and mixed. A zero reference was prepared with 20µl of sample buffer mixed with 1ml of Bradford reagant. The absorbance was measured using a UV spectrophotometer at 595nm (Pharmacia LKB Ultrospec III). The absorbance was standardised by measuring the absorbance of the no protein reference and setting this at zero, then the absorbance was measured for the known BSA standards and a standard curve of absorbance at 595nm versus the protein concentration was plotted. Finally, the absorbance of the protein samples was measured and the total protein determined by finding the absorbance on the standard

curve and determining the correlating protein amount. The protein concentration was calculated by dividing the total protein by the number of microlitres assayed.

2.8 Antibody Preparation

2.8.1 Creation of GST-tagged Protein Expressing Bacterial Lines

pGEX-6P-1::N100 and pGEX-6P-1::N262 DNAs were transformed into BL21 *E. coli* cells. Ampicillin resistant colonies were grown up in Luria Broth/Ampicillin(100µg/ml)/1% glucose and glycerol stocks were prepared via adding 150µl of glycerol to 750µl of bacterial culture and stored at –80°C.

2.8.2 Test Peptide Inductions

2ml of Luria Broth/Ampicillin(100µg/ml)/1% glucose was inoculated from the glycerol stocks of the pGEX-6P-1::N100 and pGEX-6P-1::N262 BL21 strains and grown overnight at 37°C. 1ml of the overnight culture was pipetted into 10ml of Luria Broth/Ampicillin(100µg/ml) and grown to OD_{600}=1 at 37°C. 200µl of the culture was then added to 200µl of 2x SDS load buffer. Peptide expression was induced by the addition of 0.1mM IPTG to the bacterial culture. 200µl samples were taken every 1 hour for up to 3 hours and added to 200µl of 2x SDS load buffer to analyse the induction. The samples were then boiled at 100°C for 5 minutes and then approximately 60µl of each sample was added to a 1.5mm thick SDS Polyacrylamide minigel. This was run as of 2.6.1 and Coomassie Stained as in 2.6.2.

2.8.3 Purification of GST-Tagged Peptides

50ml of Luria Broth/Ampicillin(100µg/ml)/1% glucose was inoculated from the glycerol stocks of the pGEX-6P-1::N100 and pGEX-6P-1::N262 BL21 strains and grown overnight at 37°C. This was then poured into a 500ml of Luria Broth/Ampicillin(100µg/ml) and grown to OD_{600}=1 at 37°C. IPTG was added to 0.1mM and the culture was left shaking at room temperature overnight. The cells were then centrifuged at 3000rpm for 15 minutes in a Beckman Benchtop Centrifuge. The Medium was then poured off and the centrifuge bottle was weighed. 10ml of STE buffer, with 25mM glucose; 5mM dTT; 50mM benzamadine, was added per gram of cells and they were resuspended by vortexing. The cells were lysed via passing them through the French Press twice. A 1/10 volume of STE; 10% Triton X-100 was added to the solution to the final volume of 1% and the solution was left rocking at 4°C for 30 minutes. The solution was then spun in a Sorvall RC-5B Refrigerated Superspeed Centrifuge in an SS34 rotor at 10,000rpm for 15 minutes at 4°C. 1ml of Glutathione Agarose beads was added to every 30ml of the supernatant with 1% Sodium Azide and the mix was left rocking overnight at 4°C. The tubes were then spun at 500rpm to bring down the beads, the supernatant was aspirated and the beads were transferred to a BioRad econo-column. The beads were then washed with 10 times bed volume STE with 5mM dTT; 50mM benzamadine and the peptide was eluted in five 1ml aliquots of STE with 10mM reduced glutathione. The amount of peptide eluted, in each sample, was assessed by running on a SDS Polyacrylamide minigel and comparing protein levels to BSA standards via coomassie staining as in 2.6.2.

2.8.4 Antibody Generation

Antibodies were raised in lop-eared and New Zealand White Rabbits. The first immunisation contained 1ml complete freunds adjuvant, 200µg of antigen and PBS to a

final volume of 2ml. The mix was emulsified and 200µl was injected subcutaneously into 5 sites each in 2 rabbits. The second immunisation occurred 3 weeks later with 1ml incomplete freunds adjuvant, 200µg of antigen and PBS to a final volume of 2ml. The mix was emulsified and injected as above and the rabbits were left for 2.5 weeks until the third immunisation. The third and all subsequent immunisations were performed with a emulsion of 200µg of antigen, 100µg of MDP and PBS to 2ml. Following the course of injections and test bleeds the rabbits were bled out and the antibodies were purified.

2.8.5 Affinity Purification of Antibodies

A $2cm^2$ piece of nitrocellulose membrane was soaked in 2ml of STE containing 100-200µg of the desired peptide and sealed in a plastic bag. This was left rocking at room temperature for 2 hours for the peptide to attach to the membrane. The membrane was then taken from the solution and washed gently for 5 minutes, 4 times, in PBST. The membrane was then placed in a plastic bag and 3-4ml of serum was added to the bag and sealed. The association of the antibodies was allowed to proceed overnight, rocking at 4°C. The membrane was then washed as above. Elution of the bound antibodies was achieved via placing the rolled-up membrane in a screw-cap microcentrifuge tube and adding 900µl of 0.1M Glycine, pH 2.5 and rotating it slowly at room temperature for 30 minutes. Following removal of the membrane from the tube 100µl of 1M Tris, pH 8 was added to neutralise the solution. Sodium Azide was also added to 0.02% and 900µl of the total solution was stored at -80°C and the rest at 4°C.

2.9 Northern Analysis

2.9.1 RNA Extraction

Cells were harvested from the dish by trypsinisation. Cells were washed twice and the pellet frozen at –80°C until required. Total RNA was extracted using the RNAzol kit (Tel-test). Cells were resuspended in RNAzol for 5 minutes and then disrupted by manual pipetting. The RNA is extracted by the addition of 100µl of chloroform and centrifugation at 12,000 rpm for 10 minutes. The top layer was removed and placed into a new tube and subjected to precipitation with 2 volumes of isopropanol. Precipitated RNA was then washed with 70% ethanol and then air dried at room temperature. RNA was dissolved in autoclaved distilled water.

2.9.2 Northern Transfer

15µg of RNA was dissolved in 20mM MOPS buffer, 5mM sodium acetate, 30% formamide, 10% formaldehyde and 7µl of gel loading buffer and resolved on a 1% agarose gel at a constant voltage of 70V. The agarose gel was prepared by dissolving 1.5g of agarose into 108ml of water, followed by the addition of 15ml of 10 x MOPS buffer and 27ml of formaldehyde before the gel was poured into the gel cast. Gels were electrophoresed in 1x MOPS buffer at a limiting voltage of 70V. Following gel electrophoresis, the gel was washed twice in RNAse free water. RNA was transferred to nitrocellulose (Amersham) overnight by capillary action. The nitrocellulose was then air dried before RNA was crosslinked by UV light at 120,000 microjoules.

2.9.3 Radioactively Labelling DNA Probe

The P^{32}-ATP labelled probe was made using megaprime kit (Amersham). Primer was annealed to the DNA template by adding approximately 150ng of DNA template to 5µl of

primer solution in a final volume of 26µl with MQ water, followed by heating of the reaction at 95°C for 5 minutes. The reaction was allowed to cool at room temperature before the DNA was radioactively labelled by the addition of the following constituents in the following order:

4 µl dCTP

4 µl dTTP

4 µl dGTP

5 µl Reaction buffer

5 µl α-P^{32}dATP

The reaction was started following the addition of 2µl of Klenow and incubated at 37°C for 15 minutes. Radiolabelled DNA probe was purified from excess radioactive nucleotide by centrifugation through Q-spin columns. The resultant probe was the denatured by heating at 95°C for 2 minutes and then cooled on ice for 5 minutes.

2.9.4 Hybridisation and washing

Membranes were prehybridised in Ultrahyb (Amersham) from 2 hours to overnight. Radioactively labelled DNA probe was added to the hybridisation mixture at a concentration of 10ng probe/ml of hybridisation buffer and incubated overnight. Membranes were washed twice for 5 minutes in 2 x SSC, 0.1% SDS at 42°C, and twice for 15 minutes in 0.1x SSC at 42°C. Membranes were exposed to Kodak Imaging Screen and developed and quantitated using a Biorad Molecular Imager® FX.

2.10 Bacterial Manipulation Methods

2.10.1 Calcium Chloride Plasmid Transformation

45µl of *E. coli* cells were thawed on ice. 1ng of DNA was added to the cells and the mix was incubated on ice for 30 minutes, then placed in a 42°C water bath for 2 minutes and then on ice for 2 minutes. 1ml of BHIB was then added to the cells and placed at 37°C for 30 minutes. The cells where then plated on L+AMP plates and incubated at 37°C overnight.

2.10.2 Plasmid Preparation from Bacterial Cells

Plasmids were prepared utilising Qiagen Miniprep, Midiprep and Maxiprep kits following manufacturers instructions.

CHAPTER 3

THE ROLE OF c-MYC IN ES CELL DIFFERENTIATION

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THE ROLE OF c-MYC IN ES CELL DIFFERENTIATION

3.1 Endogenous c-Myc Expression in ES Cells and ESEBs

3.1.1 Introduction

The Myc family are essential regulators of early mouse embryogenesis as has been demonstrated by expression and knockout studies. Both c-*myc* and N-*myc* RNA are widely expressed during embryogenesis (Downs *et al.*, 1989) and the knockouts of c-*myc* (Davis *et al.*, 1993) and N-*myc* (Sawai *et al.*, 1993) leads to early embryonic lethality, at 10.5dpc and 11.5dpc, respectively. *Max* knockout mice die in early post-implantation, prior to gastrulation, demonstrating that the disruption of the entire Max network, which includes Myc function, is essential for the differentiation of the pluripotent cell lineages into the three primary germ layers. These mice were also 50 – 70% smaller than wild-type and *Max* +/- heterozygous embryos. It was also suggested that the embryos, in early development, utilised a maternal store of Max protein as was demonstrated by the high levels of Max protein in the unfertilised egg and the 0.5dpc embryo (Shen-Li *et al.*, 2000). Malynn *et al.*, 2000 demonstrated that functional compensation between the c- and N-Myc proteins can occur during embryogenesis and this compensation may be attributed to the fact that c- and N-*myc* knockouts survive as long as they do.

As Myc proteins appear to be essential for differentiation in the embryo they would be expected to be involved with the differentiation of the ICM *in vitro* equivalent, embryonic stem cells. Both ES and EPL cells can be differentiated into embryoid bodies. While this system lacks spatial organization, it is a trusted *in vitro* model for the differentiation of

ICM cells into nascent mesoderm (Rathjen *et al.*, 1999; Lake *et al.*, 2000). Thus, Northern and Western analysis was employed to elucidate the expression of c-Myc RNA and protein as ES cells differentiate into ES Embryoid bodies (ESEBs). ESEBs were used instead of EPLEBs as they differentiate into all three primary germ layers and

3.1.2 Analysis of ESEB Differentiation

To analyse the kinetics of ES cell differentiation, ES Embryoid Bodies (ESEBs) were prepared as described in Section 2.3.7. The cells were harvested daily, the RNA was prepared for Northern Analysis as described in section 2.9. The nylon membrane was probed for various differentiation markers such as *Rex1*, *Fgf5*, *Oct4* and *Brachyury* to verify differentiation kinetics as seen in Lake *et al.*, 1999. This result is shown in figure 3.1(a). *GAPDH* was utilised as a loading control as its expression is known to be constant across the differentiation of ESEBs (Rathjen *et al.*, 1999). *Rex1*, a marker of the ICM (Rogers *et al.*, 1991; Rathjen *et al.*, 1999; Lake *et al.*, 2000) is down-regulated as the primitive ectoderm marker *Fgf5* is up-regulated (Hebert, *et al.*, 1991; Rathjen *et al.*, 1999; Lake *et al.*, 2000). Down-regulation of *Oct4* demonstrates that there is a loss of pluripotency (Schöler *et al.*, 1990; Schöler, 1991; Rathjen *et al.*, 1999) and is associated with up-regulation of *Brachyury*, a marker for nascent mesoderm (Herrmann, 1991). *Ornithine decarboxylase* (*odc*) is a known target gene of c-Myc (Bello-Fernandez *et al.*, 1993) and its RNA expression is confined to day 1 of ESEB differentiation.

3.1.3 Expression of c-Myc Protein During ESEB Differentiation

To investigate the expression pattern of c-Myc protein during differentiation of ES cells Western Analysis was employed to detect c-Myc expression across the differentiation of ES embryoid bodies. Embryoid bodies were formed by growing the ES cells in suspension

in the absence of LIF (2.3.7). Every day cell samples were taken and protein was prepared as described in 2.5.1. The anti-c-Myc (N262) antibody (2.4.10), that recognises the first 262 N-terminal amino acids of c-Myc (raised against human but detects both human and mouse), was used to detect c-Myc. Cdk2 protein levels were utilised as a loading control as it has been demonstrated in our laboratory that its expression is constant during ESEB differentiation (E. Stead, PhD Thesis).

Western analysis utilising this antibody to detect c-Myc was a relatively insensitive process as the antibody detected c-Myc protein very weakly. Thus the results seen in this Chapter are of very long exposures (greater than 1 hour) of the membrane to film. Across the differentiation of ES cells into EBs, seen in figure 3.1(a), there were two prominent bands, of different sizes, detected. One of these protein species migrated with a predicted molecular weight of approximately 65kDa and another with a predicted molecular weight of approximately 50kDa. The 65kDa band is detected in ES cells, its level decreases in day 1 and day 2 ESEBs and remains at this lower level until day 4. This suggests that full-length c-Myc was expressed at its highest level in ES cells and down-regulated following differentiation. The 50kDa band appeared one day after withdrawal of LIF and expression peaked on day 2 and continued to be detected at low levels for the rest of the time course. This expression profile closely resembles the appearance of *Fgf5*, suggesting that as ES cells differentiate into the *in vitro* equivalent of primitive ectoderm the 50kDa protein species was up-regulated. This band is down-regulated before *brachyury* appears at day 4, demonstrating that its expression is not associated with mesoderm.

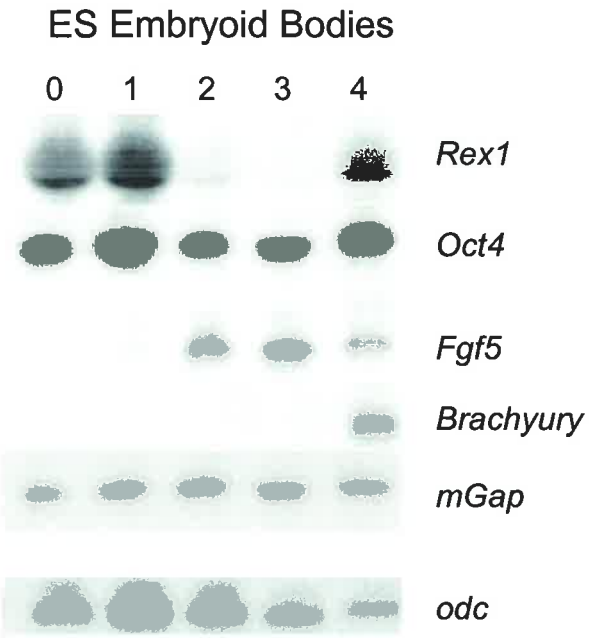
The Western result demonstrated that during the differentiation of ES cells into embryoid bodies there may be a change in the translational control of the c-Myc protein. The upper

Figure 3.1

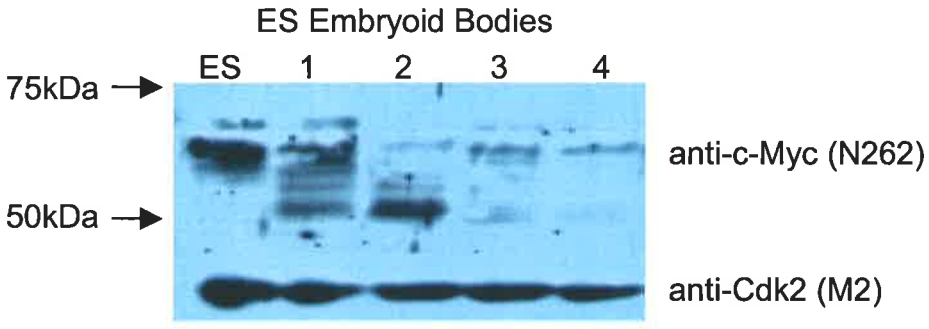
(a) Northern analysis was utilised to assess the differentiation of ES embryoid bodies and the expression of the c-Myc target gene, *odc*. Specific probes were used to detect *Rex1*, *Oct4*, *Fgf5* and *Brachyury* to assess the timing of differentiation events. *mGap* expression was used as a loading control as its levels are constant across differentiation.

(b) Western analysis was utilised to analyse the expression of c-Myc protein across the differentiation of ES embryoid bodies. The anti-c-Myc (N262) antibody (2.4.10) was used to detect c-Myc and the anti-Cdk2 (M2) was used to detect Cdk2. Cdk2 expression levels were used as a loading control.

(a)


ES Embryoid Bodies
0
1
2
3
4
Rex1
Oct4
Fgf5
Brachyury
mGap
odc

(b)


ES Embryoid Bodies
ES
1
2
3
4
75kDa
50kDa
anti-c-Myc (N262)
anti-Cdk2 (M2)

band at approximately 65kDa is predicted to be the full-length c-Myc2, which is the ATG-initiated, full length, c-Myc. There are a number of possible explanations for the appearance of the lower band. This band may be a non-specific interaction. Alternatively, it may be an intermediate breakdown product caused by increased degradation of Myc during differentiation. This assumption is supported by the presence of the bands that are detected in between the prominent bands. The 50kDa band could also be the 100aa N-terminal truncated c-MycS protein, the translation of which is initiated at an ATG downstream of the suboptimal c-Myc ATG and has been shown to run on Polyacrylamide gels at approximately 50kDa (Spotts *et al.*, 1997). If this is true, the levels of c-MycS may be higher than they are represented in this western as the N262 antibody is raised against the first 262 N-terminal amino acids there may be only a sub-population of polyclonal antibodies that detect epitopes in the 100-262aa region. This means that the real levels of MycS protein may be understated in comparison to c-Myc2.

If the lower band is in fact c-MycS this suggests that there is possibly some type of Myc translational regulation during differentiation, where changes in the Myc protein are made. It appears from this analysis that full-length c-Myc expression is at its highest levels in *Rex1* positive ES cells and is down-regulated rapidly as the cells differentiate into *Fgf5* positive cells. Associated with this differentiation there is also an up-regulation of the smaller protein species, closely related to when *Fgf5* RNA is expressed at its highest levels.

3.1.4 Conclusion

From this analysis it is evident that c-Myc protein levels are modulated during ES cell differentiation. There clearly is a down-regulation of c-Myc2 protein as pluripotent, *Oct4*

positive, cells differentiate into non-pluripotent lineages. Preceding this differentiation there is an up-regulation of the 50kDa c-Myc band, possibly associated with a concurrent up-regulation of *Fgf5* expression. This indicates that high c-Myc expression appears to be associated with rapidly dividing cells pluripotent cells and must be down-regulated in order for the cells to differentiate into non-pluripotent cell lineages. As it is known that MycS up-regulation is commonly associated with periods of rapid growth (Spotts *et al.*, 1997) it may be deduced that c-MycS is associated with the rapid cell divisions of primitive ectoderm cells, demonstrated by Snow (1977).

3.2 c-Myc Over-Expression During ES Cell Differentiation

3.2.1 Introduction

Down-regulation of c-Myc is widely considered to be a prerequisite for the transition of a dividing cell to a non-dividing differentiated cell (Lin *et al.*, 2000; Xu *et al.*, 2001). This may be attributed to the fact that as cells differentiate they remodel their cell cycle and adopt the appropriate characteristics of the new cell type. It has been demonstrated in our laboratory that as ES cells differentiate into EPL cells their cell cycles accelerate from approximately 12.3 hours to approximately 8.1 hours. The differentiation of ES and EPL cells as EBs is also associated with the remodelling of the cell cycle and a slowing of cell cycle times (Stead, personal communication). This remodelling of the cell cycle is characterised by the establishment of periodic expression of cell cycle regulators not present in ES cells. Cdk2 and Cyclin E and A kinase activity becomes restricted to their discreet cell cycle phases and Cdk inhibitors, p21 and p27, are up-regulated (E. Stead and R. Faast, personal communication).

From this information it may be infered that Myc down-regulation may be essential to enable the transition from ES and EPL cells into the differentiated cell types in embryoid bodies. This hypothesis has been tested in ES cells by MacLean-Hunter *et al.*, 1994 where they demonstrated that the tumour-derived RLF/L-Myc is able to delay the differentiation of ES cells as embryoid bodies.

3.2.2 Expression of Human c-Myc in ES cells

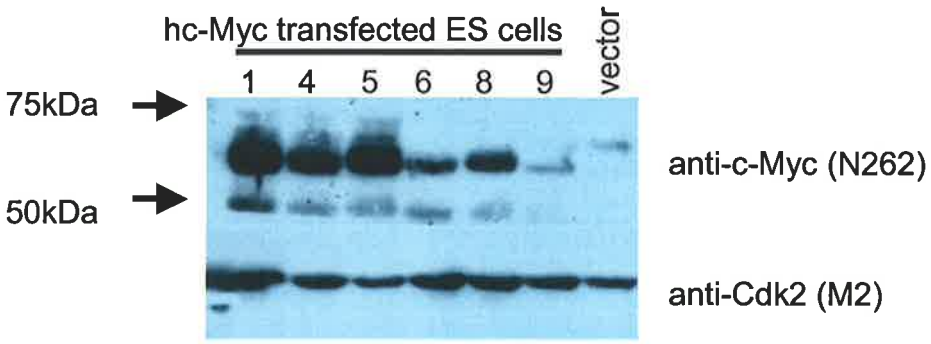
To analyse the effect of over-expression of c-Myc on ES cells an expression vector was constructed to enforce human c-Myc expression in ES cells. The 1395bp full-length human c-Myc was excised from pSRαMSVTKNEO::c-Myc (2.4.13) using EcoRI and cloned into pEF-IRES-puro6 (2.4.13 and Appendix 1) containing an EF-1α promoter, which allows constitutive transcription in ES cells. This construct was linearised via a PvuI site in the Ampicillin resistance portion of pEF-IRES-puro6, and transfected into ES cells, as described in Section 2.3.3. pEF-IRES-puro6 alone was also transfected to create a negative control cell line for experiments. Following the selection in 1μg/ml puromycin individual colonies were picked and expanded. These were subsequently separated with SDS-PAGE and transferred to nitrocellulose. An anti-c-Myc (N262) antibody (2.4.10) was used to specifically detect the c-Myc protein by Western Analysis.

3.2.2.1 CMES c-Myc Protein Expression

Figure 3.2 demonstrates that of the puromycin resistant clones, clones 1, 4, 5, 6 and 8 express Myc at much higher levels than in the vector control and HL60 cells. Interestingly, the Myc-expressing ES cell lines also appear to express the smaller protein sub-type detected in the differentiation experiment described in 3.1. This may be the c-MycS

Figure 3.2

Embryonic stem cells were transfected with the pEF-IRES-puro6::hc-Myc construct and western analysis was performed on the several puromycin resistant clones. c-Myc expression was detected by the anti-c-Myc (N262) antibody (2.4.10) in clone number 1, 4, 5, 6, 8 and 9. A cell line that was transfected with the pEF-IRES-puro6 vector alone was included in the analysis as a negative control. The anti-Cdk2 (M2) was used to detect Cdk2, the expression of which is used as a loading control.


hc-Myc transfected ES cells
1
4
5
6
8
9
vector
75kDa
50kDa
anti-c-Myc (N262)
anti-Cdk2 (M2)

protein, as the downstream translation initiation sites are still present in the over-expressed Myc.

3.2.2.2 CMES Morphology

The c-Myc over-expressing ES cells, termed CMES for c-Myc ES, appear morphologically different to the untransfected ES cells and those expressing the vector alone. Severalx colonies appear rounder and more dome-like (Figure 3.3). There are two potential reasons why Myc may cause these morphological differences. The first is that Myc, to some extent, prevents spontaneous differentiation, and this prevents the appearance of the flattened out, differentiated colonies in the culture. The second possibility is that Myc is causing a down-regulation genes and proteins involved with cell adhesion such as collagen, fibronectin, intcgrins and actin (Coller *et al.*, 2000; Frye *et al.*, 2003; Shiio *et al.*, 2003). Consequently the cells may have decreased attachment to the collagen matrix on the culture dish. This would appear to mimic the effect that Myc reintroduction has on *myc*-null Rat1 cells, where attachment to the culture dish is greatly reduced (Shiio *et al.*, 2003). This effect is most apparent in figure 3.3(a), where the two right most colonies are attached but appear to be embryoid body-like.

3.2.3 The Effect of c-Myc Over-Expression on ES Cell Differentiation

3.2.3.1 ESEB Differentiation in the Presence of Puromycin

Initial investigation into the differentiation of ES cells over-expressing c-Myc was performed by differentiating ES cells into ESEBs as described in 2.3.7. Two different CMES lines, CMES-6 and –8 were selected and maintained in the presence of 1μg/ml puromycin for the period of the time course to ensure c-Myc expression. In this experiment high levels of cell death were observed and thus, low amounts of cells were harvested.

Figure 3.3

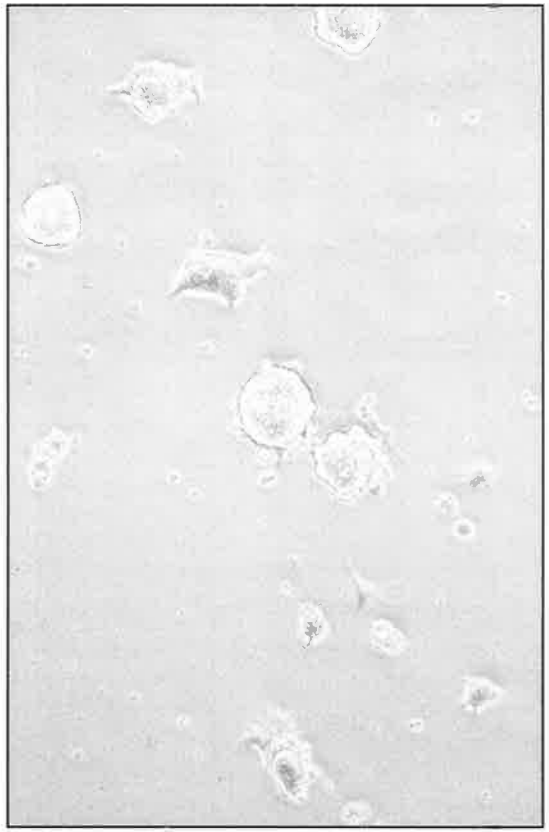
Morphology of ES cells which over-express human c-Myc. CMES lines 6 and 8 are shown in (a) and (b), respectively. The morphology of the vector alone cell line is shown in (c).

(c)

CMES 11 (Vector Alone)

(b)

CMES 8

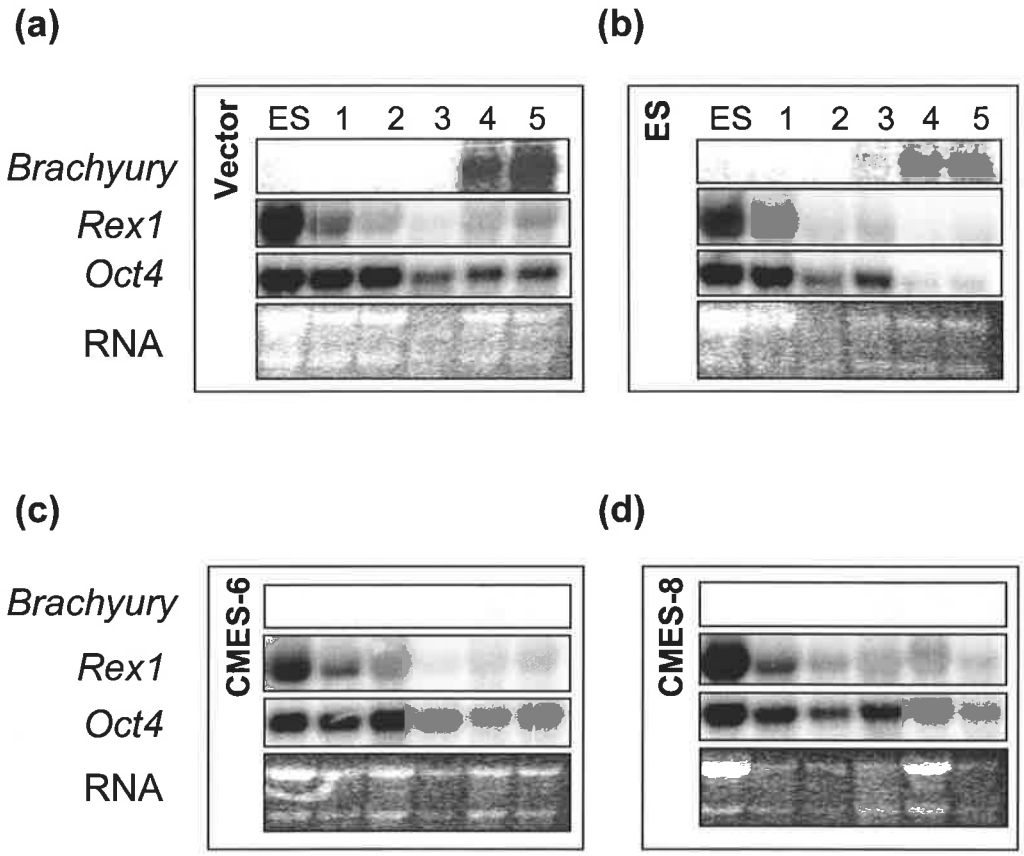


(a)

CMES 6

Figure 3.4

CMES lines 6 (c) and 8 (d) were differentiated into ES embryoid bodies in the presence of puromycin and northern analysis was used to analyse any differences in differentiation kinetics in comparision to vector alone ES cells (a) and untransfected cells (d). Differentiation markers *Brachyury*, *Rex1* and *Oct4* were used to assess differentiation kinetics and the Ethidium Bromide stained agarose gel demonstrates the relative RNA loading in each lane.


(a)
(b)
Brachyury
Rex1
Oct4
RNA
Vector
ES 1 2 3 4 5
ES
ES 1 2 3 4 5
(c)
(d)
Brachyury
Rex1
Oct4
RNA
CMES-6
CMES-8

Northern Analysis (2.9) was performed on the cells to allow detection of differentiation markers and assess the effect of Myc over-expression on kinetics of differentiation (Figure 3.4). In this experiment ES and vector alone cells were differentiated as controls.

In this experiment, *Rex1* expression decreased with the same kinetics in the CMES cell lines 6 and 8 and the ES and vector alone controls. It was high in ES cells and down-regulated in day1 ESEBs indicating the ES cells had differentiated at the normal rate. *Oct4* expression in the CMES cell lines also decreased with similar kinetics to the parental ES cells and vector alone cells. Interestingly *Brachyury* expression was not detected in either of the Myc over-expressing cell lines indicating that nascent mesoderm was not being formed in the Myc over-expressing cells even by day 5 in ESEB differentiation. Down-regulation of *Oct4* indicates a loss of pluripotency in the CMES cells nearing the end of the time course indicating that there is possibly a transition into another non-pluripotent cell type.

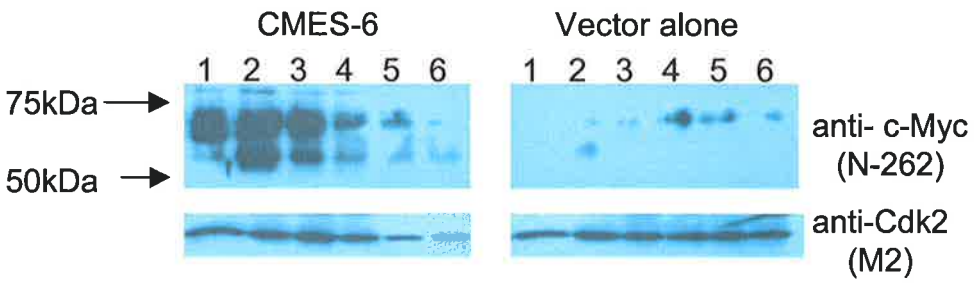
3.2.3.2 ESEB Differentiation in the Absence of Puromycin

As the addition of puromycin to the medium caused high levels of cell death in the CMES cell lines during ESEB differentiation, these experiments were repeated in the absence of puromycin. In these experiments there was negligible cell death in comparison to the untransfected ES and vector alone cells. Western analysis (2.6.4) utilising the anti-c-Myc (N262) antibody (2.4.10) was employed to investigate the expression of Myc protein in the CMES-6 and vector alone cell lines (Figure 3.5(a)). This indicated that Myc protein is expressed at high levels in the CMES-6 cell line when compared to D3ES cells, but was down-regulated at days 4, 5 and 6 of differentiation. Down-regulation is, most likely, the result of increased c-Myc ubiquitin-mediated degradation during ESEB differentiation as

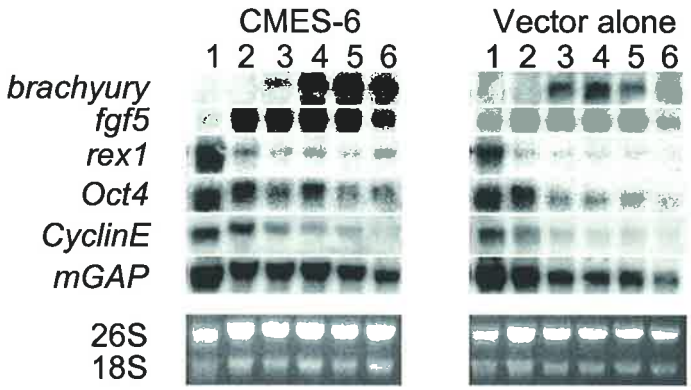
Figure 3.5

CMES-6 and vector alone ES cells were differentiated into ES embryoid bodies in the absence of puromycin. Western analysis (a), utilising the anti-c-Myc (N262) antibody (2.4.10), was used to analyse the expression of the c-Myc protein during differentiation. Northern analysis (b), detected the expression of the differentiation markers *Brachyury*, *Fgf5*, *Rex1* and *Oct4*, the loading control *mGap* and the c-Myc target *Cyclin E.*

(a)


CMES-6
Vector alone
1 2 3 4 5 6
1 2 3 4 5 6
75kDa
50kDa
anti- c-Myc
(N-262)
anti-Cdk2
(M2)

(b)


CMES-6
Vector alone
1 2 3 4 5 6
1 2 3 4 5 6
brachyury
fgf5
rex1
Oct4
CyclinE
mGAP
26S
18S

has been demonstrated by Cartwright *et al.*, 2003. It may also be due to the fact the EF-1α promoter is not maintained at a high level during ESEB differentiation (Cartwright, unpublished data)

Interestingly, the 50kDa band, previously described in 3.1, appears to be differentially expressed in Myc over-expressing cells, shown in figure 3.5, in a similar fashion to what was seen for ES cells as shown in figure 3.1(b). A peak in expression of the 50kDa band can be seen in day 2 CMESEB differentiation, reminiscent of when it is at its highest in ES cells. Cdk2 protein levels are utilised as a loading control in this experiment.

Northern Analysis was utilised to analyse the behaviour of CMES-6 during differentiation into ESEBs (figure 3.5(b)). These cells differentiated in much the same way as the vector alone cells, with the exception of *Brachyury* expression, which is up-regulated one day later in CMES-6 EBs than ES cells expressing the vector alone. This indicates that nascent mesoderm forms at day 4 in CMES cells and in day 3 in non-expressing cells in each case correlating to the reduction of Myc protein levels (Figure 3.5(a)). As the ES cells maintained in puromycin (3.2.3.1) were expected to retain c-Myc protein expression and *Brachyury* was not detected when these cells differentiated, this suggests that the down-regulation of Myc is important for the formation of mesoderm. *Cyclin E* RNA was also detected in this northern analysis and its levels were found to only be modestly up-regulated in the c-Myc over-expressing ES cells. *GAPDH* was detected at relatively constant levels in figure 3.5(b) demonstrating that the expression patterns seen in differentiation markers is a true representation of their kinetics.

3.2.3.3 EPLEB Differentiation

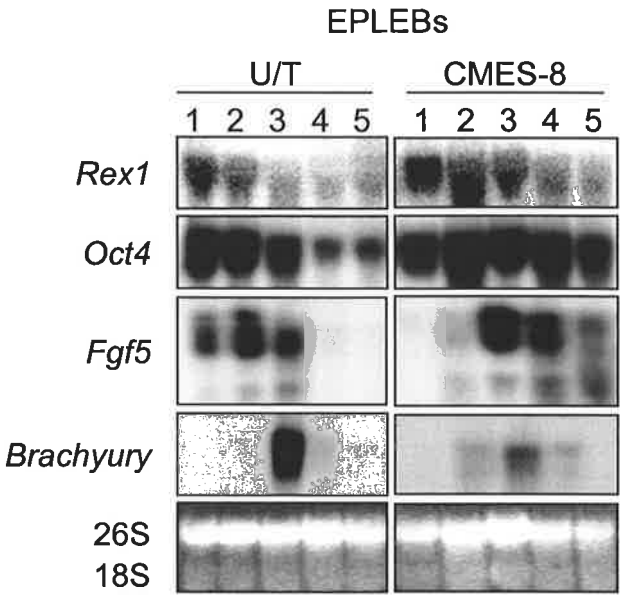
As over-expression of c-Myc appeared to delay ESEB differentiation it was of interest to analyse the effect during EPLEB differentiation, where predominantly mesoderm is formed (Lake *et al.*, 2000). To investigate this, CMES cell line 8 and untransfected ES cells were differentiated in the absence of puromycin into EPL cells as described in 2.3.5 and both were grown as embryoid bodies as described in 2.3.8. Embryoid bodies were harvested daily, as described in 2.3.9 and Northern Analysis was performed as described in 2.9. The levels of gene expression of *Rex1*, *Oct4*, *Fgf5* and *Brachyury* were assessed to analyse differentiation (figure 3.6). This figure demonstrates that whilst the CMES cells lose ES cell-like characteristics, as assessed by *Rex1* expression, albeit later than untransfected cells, they appear not to lose pluripotency, as assessed by *Oct4* expression, for the 5 days EPLEB formation was analysed. This result is supported by the observation that *Brachyury*, the nascent mesoderm marker, is barely detected in differentiating CMES cells, indicating that these cells lose the ability to form mesoderm, at high levels. This result also indicates that there is a delay in the differentiation of CMES cells to lose ES cell characteristics and become cells equivalent to primitive ectoderm, as *Fgf5* expression appears later in CMES cells when compared to untransfected cells. Interestingly, *Fgf5* is down-regulated when there is still a high level of *Oct4* meaning that these cells retain pluripotency but express, neither *Rex1* or *Fgf5*. This means the precise nature of the cell type differentiated is difficult to define.

3.2.4 Investigation in the Requirement for LIF in c-Myc Over-Expressing ES Cells

Maintenance of ES cells in culture is dependent on the presence of the cytokine, LIF, in the culture medium (Nichols *et al.*, 1990). The LIF titration assay is a tool to assess the level of LIF required to maintain an undifferentiated, pluripotent, ES cells. This technique utilises a

Figure 3.6

CMES-8 and vector alone ES cells were differentiated in to EPL embryoid bodies. Northern analysis detected the expression of the differentiation markers *Fgf5*, *Rex1* and *Oct4*. Loading was assessed via visualisation of the Ethidium Bromide stained RNA in an agarose gel.


EPLEBs
U/T
CMES-8
1 2 3 4 5
1 2 3 4 5
Rex1
Oct4
Fgf5
Brachyury
26S
18S

staining procedure to detect activity of the Alkaline Phosphatase enzyme as a measure of pluripotency (Hanel *et al.*, 1990; Pease *et al.*, 1990).

3.2.4.1 CMES Cells LIF Titrations

Typically ES cells in culture are maintained in 1000 units/ml of LIF to help prevent the loss of pluripotency. However, Dr Gavin Chapman has shown, that this can be reduced to 40 units/ml without significant loss of ES cell characteristics (G. Chapman, PhD Thesis). Thus, to assess the differences in the level of LIF requirement of c-Myc over-expressing cells and non-over-expressing cells, CMES and vector alone, were grown for 6 days in 40, 30, 20, 15, 10, 7.5, 5, 2, 1, 0.5 and 0 units/ml of ESGRO LIF, as described in 2.3.6.1. The cells were plated out at 500 cells per well and grown for 6 days so the culture would not reach confluency and the colonies would be big and easy to identify. Following this the cells were stained for alkaline phosphatase activity as a marker of pluripotency (Hanel *et al.*, 1990; Pease *et al.*, 1990). This experiment was performed in quadruplicate and the colonies in each well were counted and scored blind as:

- round*, ES-like morphology and alkaline phosphatase positive**,
- flattened, non-ES-like morphology and alkaline phosphatase positive, or
- flattened, non-ES-like morphology and alkaline phosphatase negative.

* Colonies scored as round had no cells with a flattened diamond-shaped morphology.

** Alkaline Phosphatase positive was scored as when more than 50% of the cells in the colony were purple.

The number of each of the colony type was then averaged across the quadruplicates and the standard error of the mean was calculated.

Figure 3.7 shows the staining of the cells in decreasing LIF concentration and the results of the counting and scoring of colonies. It can be seen from the graph in figure 3.7(b) that the loss of ES-like colony formation in decreasing LIF concentration is reduced slightly by c-Myc over-expression. The effect is moderate but repeatable and significant. The graph demonstrates that ES cell-like colonies are rarely seen in the vector alone wells after 6 days in 7.5 units/ml of LIF. Conversely, ES cell-like colonies are seen approximately at 20% and 40% in CMES-6 and CMES-8, respectively, at 7.5 units/ml and ES cell-like colonies approach 0% at 2 units/ml. Figure 3.7(c) demonstrates that the percentage of pluripotent cells in low LIF concentrations (below 7.5 units/ml) is higher in c-Myc over-expressing populations. This result, however, does not give strong evidence as to whether the difference in maintenance of pluripotency between the vector alone and the c-Myc lines is a result of overexpression of c-Myc or some by-product of clonal selection of colonies. As only 2 cell lines were used it is unclear if the slightly significant result is a result of the chance that vector alone is more dependent on LIF.

3.2.4.2 c-MycER LIF Titrations

As LIF titrations on the CMES cell lines showed only a slight decrease in the level of LIF required to maintain ES colony characteristics it was expected that other c-Myc over-expressing ES cell lines may show a similar effect. To test this a D3ES cell line over-expressing c-MycER (2.2.6) was analysed by LIF titration. The c-MycER fusion acts as an inducible c-Myc system. The ER-fusion, in the absence of ligand, binds heat shock proteins, which result in the cytoplasmic localisation, and thus inactivation, of the protein. The addition of the synthetic ligand, 4-hydroxytamoxifen (4-OHT), results in the disassociation of the heat shock proteins, the exposure of the nuclear localisation sequence of c-Myc and nuclear translocation, and thus c-Myc activation (Littlewood *et al.*, 1995).

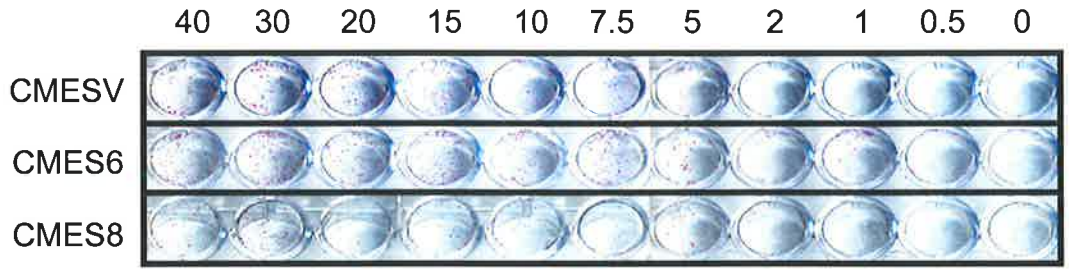
Figure 3.7

LIF titration analysis was performed on CMES-6, CMES–8 and vector alone cells. The cells were grown for 6 days in 40, 30, 20, 15, 10, 7.5, 5, 2, 1, 0.5 and 0 units/ml of LIF and then were stained for alkaline phosphatase activity. A photo of the stained colonies is shown in (a). The colonies were then counted and scored as:

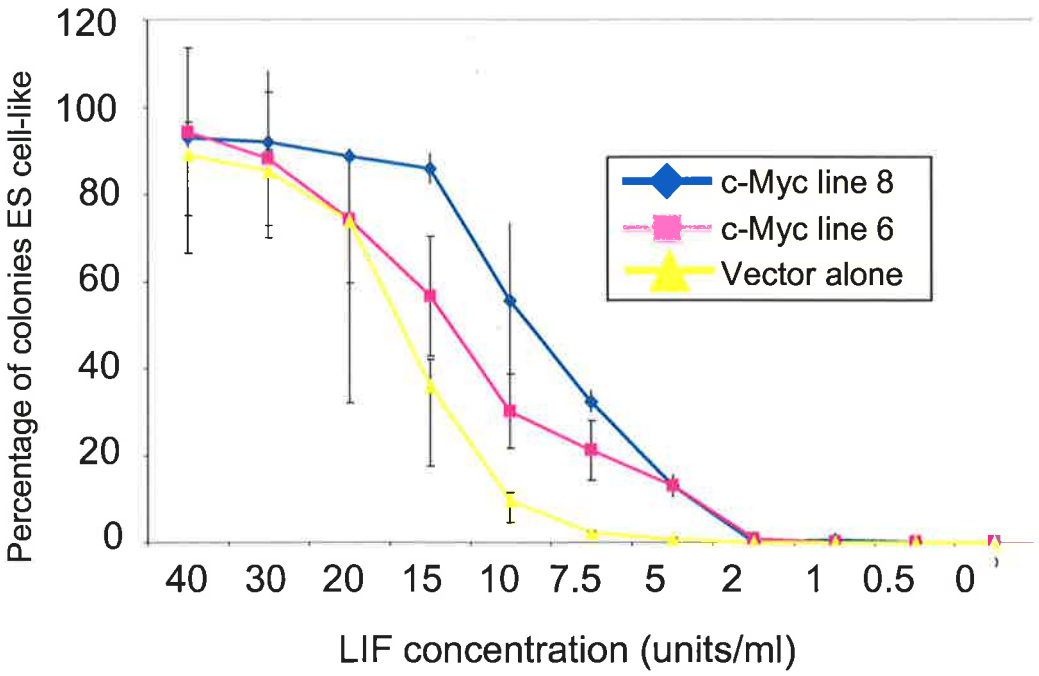
- round, ES-like morphology and alkaline phosphatase positive,
- flattened out, non-ES-like morphology and alkaline phosphatase positive,
- or flattened out non-ES-like morphology and alkaline phosphatase negative.

The results were represented graphically in (b) and (c). The graph in (b) represents the colonies scored as ES-like, as described in the first dot-point above. The graph in (c) represents the colonies scored as alkaline phosphatase positive and results from the addition of number of colonies scored as the first two dot-points above.

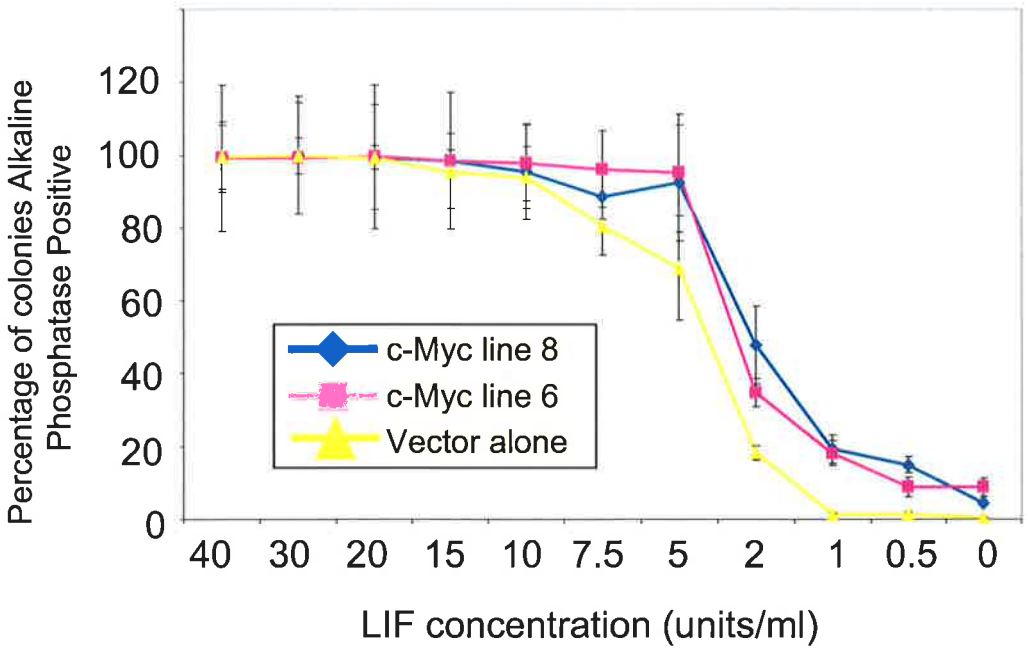
(a)


40 30 20 15 10 7.5 5 2 1 0.5 0
CMESV
CMES6
CMES8

(b)


Percentage of colonies ES cell-like
120
100
80
60
40
20
0
c-Myc line 8
c-Myc line 6
Vector alone
40 30 20 15 10 7.5 5 2 1 0.5 0
LIF concentration (units/ml)

(c)


Percentage of colonies Alkaline Phosphatase Positive
120
100
80
60
40
20
0
c-Myc line 8
c-Myc line 6
Vector alone
40 30 20 15 10 7.5 5 2 1 0.5 0
LIF concentration (units/ml)

The c-MycER in these cells is under the control of the pCAG promoter, which has been shown to not be shut down during ESEB differentiation (Niwa *et al.*, 1998).

LIF titration analysis was undertaken on MycER ES cells in quadruplicates of cells in the presence and absence of 100nM 4-OHT, as described in 2.3.6. The colonies were scored as described above (3.2.4.1), the standard error of the mean was calculated and all the results were collated into the graph shown in Figure 3.8. The graph demonstrates that whilst cells in both the presence and absence of 4-OHT lose ES morphological colony characteristics, alkaline phosphatase expression is maintained for the 6 days of the experiment in the absence of LIF and the presence of 4-OHT. This suggests that c-Myc must be down-regulated in order for the cells to lose pluripotency and differentiate. Interestingly, less than 40% of colonies demonstrate ES cell-like morphology in both the absence and presence of 4-OHT in 40 units/ml of LIF. It is unclear why these cells have an increased dependence on LIF in the cells not maintained in 4-OHT.

3.2.4.3 c-MycT58ER Cells LIF Titrations

To confirm that c-Myc over-expression prevents loss of pluripotency, LIF titrations were performed on another c-MycER cell line (2.2.6). The c-MycT58ER cell line over-expresses a human c-MycER fusion with Threonine 58 mutated to Alanine. This c-Myc mutant does not get targeted for ubiquitin-mediated degradation and thus is not down-regulated during differentiation of ESEBs (Cartwright *et al.*, 2003). LIF titrations were performed as described above (3.2.4.1) and the results are shown in the graph in figure 3.9. This demonstrated that in the absence of LIF and the presence of 4-OHT approximately 60% of colonies retain ES-like characteristics, as evaluated via a rounded colony formation and

Figure 3.8

LIF titration analysis was performed on MycER cells in the presence and absence of 4-OHT. The cells were grown for 6 days in 1000, 40, 30, 20, 15, 10, 7.5, 5, 2, 1, 0.5 and 0 units/ml of LIF and then were stained for alkaline phosphatase activity. The colonies were then counted and scored as:

- round, ES-like morphology and alkaline phosphatase positive,
- flattened out, non-ES-like morphology and alkaline phosphatase positive,
- or flattened out non-ES-like morphology and alkaline phosphatase negative.

The results were represented graphically. The bar graph represents the colonies scored as ES-like, as described in the first dot-point above. The line graph represents the colonies scored as alkaline phosphatase positive and results from the addition of number of colonies scored as the first two dot-points above.

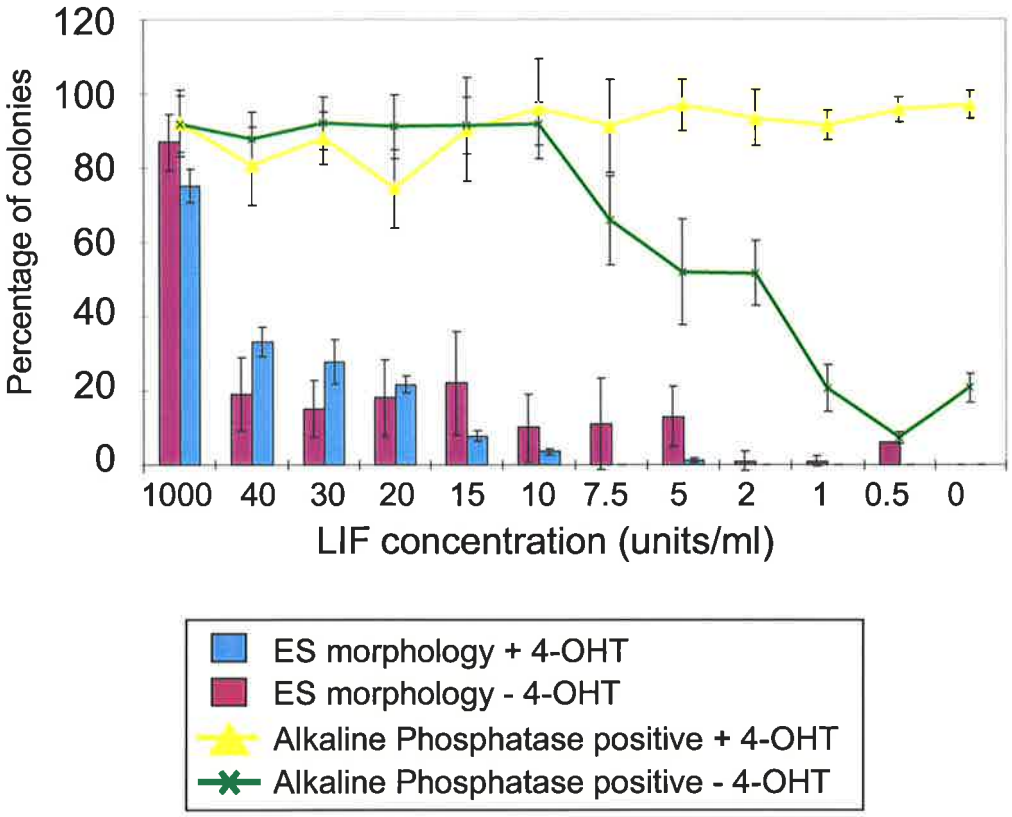
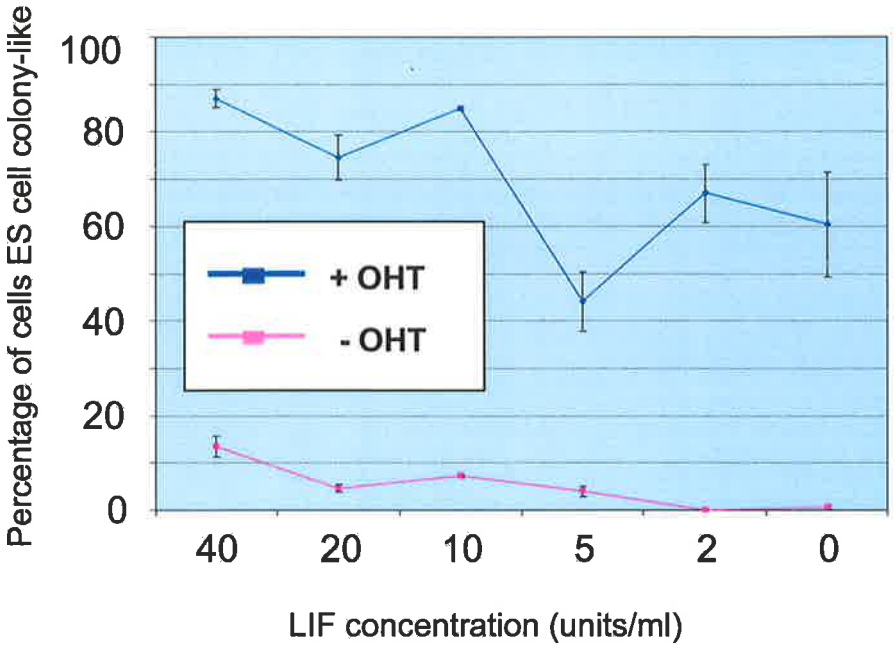

120
100
80
60
40
20
0
Percentage of colonies
1000
40
30
20
15
10
7.5
5
2
1
0.5
0
LIF concentration (units/ml)
ES morphology + 4-OHT
ES morphology - 4-OHT
Alkaline Phosphatase positive + 4-OHT
Alkaline Phosphatase positive - 4-OHT

Figure 3.9

LIF titration analysis was performed on MycT58ER cells in the presence and absence of 4-OHT. The cells were grown for 6 days in 40, 30, 20, 15, 10, 7.5, 5, 2, 1, 0.5 and 0 units/ml of LIF and then were stained for alkaline phosphatase activity. The colonies were then counted and scored round, ES-like morphology and alkaline phosphatase positive and the results were represented graphically.


Percentage of cells ES cell colony-like
100
80
60
40
20
0
+ OHT
- OHT
40
20
10
5
2
0
LIF concentration (units/ml)

alkaline phosphatase staining. Interestingly, in the absence of 4-OHT, a large percentage of the cells in 40 units/ml LIF did not form ES cell-like colonies.

3.2.5 Conclusion

The c-Myc over-expression experiments discussed in this Chapter collectively suggest that c-Myc may prevent the differentiation of pluripotent ES cells into other cell types. This is because during both ESEB and EPLEB differentiation, constitutive c-Myc expression maintains pluripotency and reduces levels of non-pluripotent cell formation. This indicates that in order for pluripotent cells to differentiate, there must be a down-regulation of c-Myc. This may be intrinsically linked to the role of Myc as a cell cycle regulator which drives the rapid cell cycles in pluripotent cells but must be reduced in order for slower cycling cells to form.

CHAPTER 4

PRODUCTION OF ANTI-c-MYC ANTIBODIES

CHAPTER 4
PRODUCTION OF ANTI-c-MYC ANTIBODIES

4.1 Introduction

The modulation of c-Myc translation and the appearance of the smaller c-Myc protein subtypes is a characteristic of various cell types (Spotts *et al.*, 1997). The smaller MycS protein has been characterised in various studies and has been shown to be able to promote cell cycle progression and apoptosis in immortalised rodent cells (Xiao *et al.*, 1998) and up-regulated in rapidly dividing cells, such as NIH3T3, BHK and MEL cells (Spotts *et al.*, 1997).

The western analysis of c-Myc shown in Chapter 3 demonstrated that during the differentiation of ES cells into embryoid bodies, a 50kDa band was detected by the anti-c-Myc (N262) antibody (2.4.10). The size of the band was an indicator that there may be an up-regulation the c-MycS subtype during embryoid body differentiation. To elucidate if this band seen in ES cell differentiation is in fact c-MycS an antibody was raised to recognise the 100 most N-terminal amino acids of full-length human c-Myc. This antibody, theoretically, would not detect MycS, which is identical to full length c-Myc except that it lacks the 100 most N-terminal amino acids. Thus, if the 50kDa band is not detected by the antibody, it would indicate that the band seen by the anti-c-Myc (N262) antibody (2.4.10) is c-MycS.

Generation of this antibody would also provide a useful tool for other future experiments, especially when used in combination with the N262 antibody. Its use in dual western

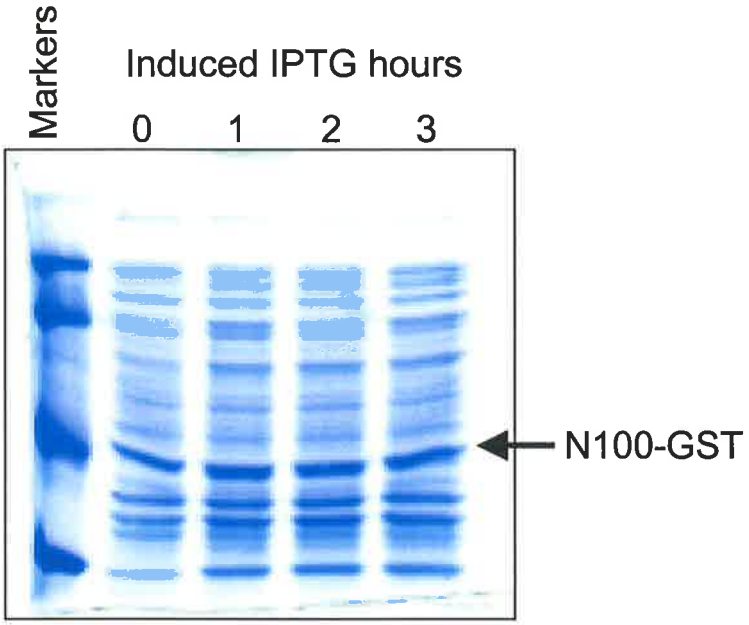
analysis would demonstrate where full length c-Myc and c-MycS are expressed, enabling elucidation of the differential regulation of the translation of c-Myc protein.

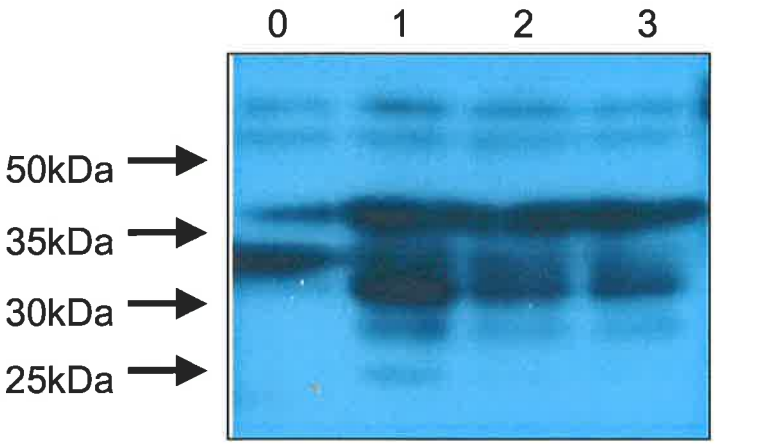
4.2 Production of Human c-Myc Peptide for Antibody Production

Expression of the N-terminal 100 amino acids (henceforth termed MycN100) of human c-Myc was performed via cloning the first 300bp of the human c-Myc cDNA sequence into the pGEX-6P-1 (Amersham Pharmacia Biotech). The 300bp region was amplified via a PCR reaction utilising the primers, cMyc100NAbR and AbMycF (2.4.12) and cloned via an EcoRI site into the vector. c-MycN100::pGEX-6P-1 was then transformed into BL21 *E. coli* bacteria and selected using ampicillin as described in 2.8.1. Test inductions of the GST tagged MycN100 were performed as 2.8.2 and are shown in Figure 4.1. The predicted size of peptide is 37-46kDa reflecting the molecular weight of GST which is 26.5kDa and the predicted molecular weight of the N100 amino acids is between 11 and 20kDa. The reason for the variation in size of this peptide is because full length c-Myc runs approximately 20kDa higher than c-MycS (Spotts *et al.*, 1997) but the actual weight of the peptide is 11306.3Da [calculated by the ExPASy Molecular Biology Server ProtParam Tool (http://kr.expasy.org/cgi-bin/protparam)]. There is a strong band seen in figure 4.1(a) migrating at approximately 40kDa however the induction in IPTG was not greatly enhanced. To confirm that this band did indeed represent the MycN100 peptide, western analysis was performed using an anti-c-Myc (N262) antibody (2.4.10). Figure 4.1(b) demonstrates that the band seen in the inductions in figure 4.1(a) is in fact a Myc peptide. The equal loading of the western is confirmed by the non-specific 'doublet' band seen migrating above 50kDa.

Figure 4.1

Test induction of the c-MycN100-GST peptide were performed on BL21 cells transformed with the c-MycN100::pGEX-6P-1 plasmid. IPTG was added to the LB and samples of the bacteria were taken every hour for three hours. The samples were added to load buffer, boiled and were subjected to SDS-PAGE. The gel was coomassie stained (a) to enable detection of the induced peptide. To confirm that the band seen on the coomassie stained gel was c-MycN100-GST the same samples were subjected to western analysis (b), utilising the anti-c-Myc (N262) antibody (2.4.10), was performed.





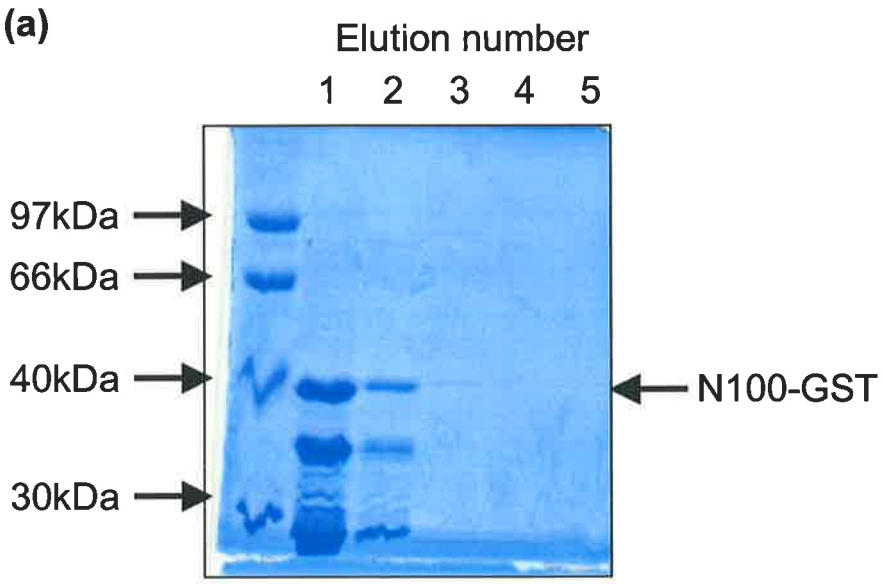
Anti-c-Myc Antibody (Santa Cruz)

The induction and purification of the GST tagged protein was performed as described in 2.8.3. 10µl of purified protein was run on a SDS PAGE and the purified peptide can be seen migrating at about 40kDa. When compared to BSA standards, c-MycN100GST is estimated to be present in excess of 10µg in 10µl (>1µg/µl) as can be seen in figure 4.2. There are also 2 lower bands seen on the gel. The upper species or band may be a truncated form of the peptide and the lower band, based on its size, may be GST alone. 150µl of antigen was utilised for the injection into rabbits for polyclonal antibody production as described in 2.8.4. Two rabbits, rabbit 54 and 55, were used to generate antibodies of which, only serum from rabbit 54 appeared to detect c-Myc in ES cells. Data from rabbit 55 is not shown.

The positive control used when testing the antibodies was protein from a D3ES cell line over-expressing full-length human c-MycER (constructed and provided by Dr Peter Cartwright). Figure 4.3(a) demonstrates that the whole crude serum used at a 1/2000 dilution in 5% skim milk detected a band just below the 105kDa marker via Western Analysis (2.6.4). It has shown that the MycER is detected at approximately 100kDa by the anti-c-Myc (N262) antibody (2.4.10) (Cartwright *et al.*, 2003). Interestingly, in both the ES and MycER ES cells a band could be seen at approximately 70kDa, which is where mouse c-Myc migrates on SDS PAGE. Affinity purification of the serum against the c-MycN100GST peptide was then performed as described in 2.8.5 and the purified antibodies were used to detect c-Myc in the same protein samples used in 4.3(a). The blot shown in 4.3(b) demonstrates that at a 1/1000 dilution the N100 antibody detects a band in the c-MycER D3ES cells just below the 105kDa marker, which is predicted to be the over-expressed c-MycER protein. In both samples a band at approximately 70kDa is detected, which is probably the full length mouse c-Myc. Comparison of 4.3(a) and (b) demonstrates

Figure 4.2

Affinity purification of c-MycN100-GST was performed and the five 1ml samples eluted by reduced glutathione were subjected to SDS-PAGE and coomassie stained (a). BSA standards (4 and 8 μg) were also run on a gel and coomassie stained (b) to allow approximate estimation of the amount of c-MycN100-GST eluted in each sample.


(a)
Elution number
1 2 3 4 5
97kDa
66kDa
40kDa
30kDa
N100-GST

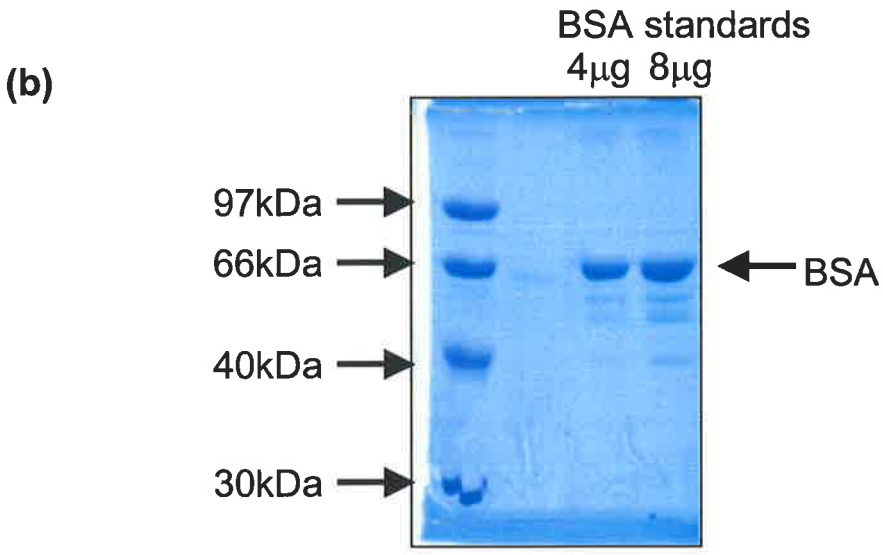
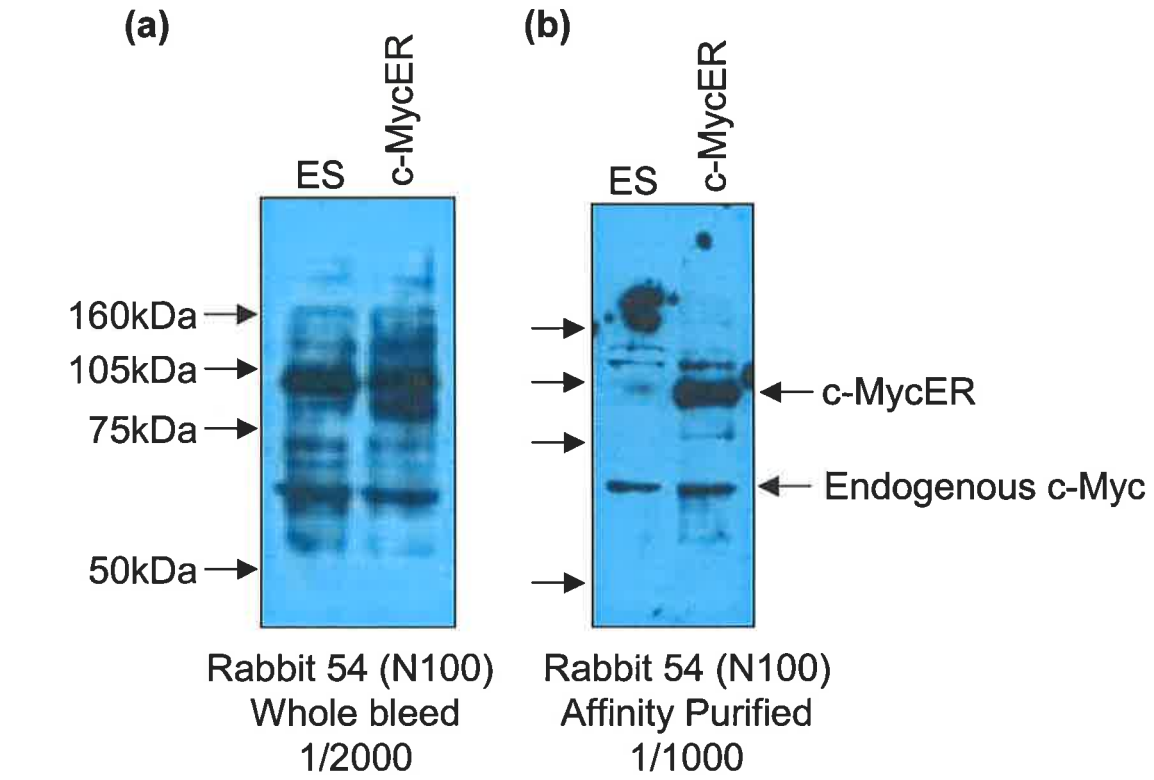
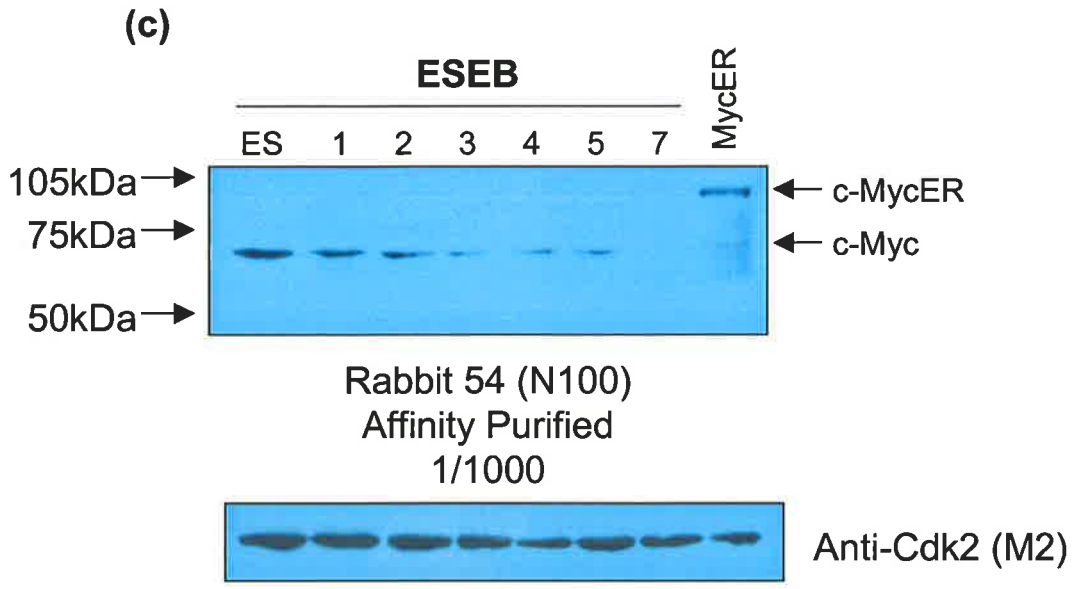

(b)
BSA standards
4μg 8μg
97kDa
66kDa
40kDa
30kDa
BSA

Figure 4.3

Following inoculation of Rabbit 54 with the c-MycN100-GST peptide serum was harvested and this was used to perform western analysis at a dilution of 1/2000 on ES and MycER whole cell extracts (a). Affinity purification of the serum to c-MycN100-GST was then performed and this was used to perform western analysis at a dilution of 1/1000 on ES and MycER whole cell extracts (b). Affinity purified antibodies were then used to detect c-Myc expression during ESEB differentiation (c). MycER extracts were utilised as a positive control and detection of Cdk2, by the anti-Cdk2 (M2) antibody, was used as a loading control.


(a)
ES
c-MycER
160kDa
105kDa
75kDa
50kDa
Rabbit 54 (N100)
Whole bleed
1/2000
(b)
ES
c-MycER
c-MycER
Endogenous c-Myc
Rabbit 54 (N100)
Affinity Purified
1/1000


(c)
ESEB
ES
1
2
3
4
5
7
MycER
105kDa
75kDa
50kDa
c-MycER
c-Myc
Rabbit 54 (N100)
Affinity Purified
1/1000
Anti-Cdk2 (M2)

a significant reduction in the background and the strong band seen at approximately 105kDa is greatly reduced following affinity purification. As expected, in both of the experiments no bands are detected at approximately 50kDa, where c-MycS would be expected to migrate.

4.3 Analysis of Full-Length c-Myc Protein Expression During ESEB Differentiation

As the affinity-purified antibody generated from rabbit 54 (henceforth termed Ab54) was able to detect mouse and human c-Myc in ES cells it was of interest to analyse the kinetics of full-length c-Myc during ESEB differentiation. As was demonstrated in Chapter 3, the 50kDa band was absent in ES cells but was detected in day 1 and 2 ESEBs, thus it was of interest to see if this band would be detected using the Ab54 in these days. Confirmation that this band was not detected in these cells would give validity to the argument that this band is c-MycS.

ES Embryoid bodies were prepared as described in 2.3.7 and cells were harvested daily for whole cell protein extracts as described in 2.5.1. Western blot, utilising Ab54, was performed to detect c-Myc across ESEB differentiation. MycER D3ES cells were used as a positive control for this experiment and the result is shown in figure 4.3(c). This experiment demonstrated that whilst a clear band is seen at approximately 70kDa, which I have previously designated as full-length c-Myc2, there is no 50kDa band detected at any stage of ESEB differentiation. The expression of the 70kDa band is consistent with the western analysis, utilising anti-c-Myc (N262) antibody (2.4.10), shown in Figure 3.1.3. This experiment was repeated with the same samples used in figure 4.3(c) but unfortunately the batch of the anti-c-Myc (N262) antibody (2.4.10) from Santa Cruz did

not detect c-Myc at endogenous expression levels in ES cells. This meant that the expression of c-MycS (or full length c-Myc) in these samples was not detected by this antibody. The expression of full length c-Myc was confirmed by the Ab54 antibody and it was shown that the levels of the protein were gradually down-regulated as differentiation proceeded and failed to be detected by day 7 ESEB. The most significant down-regulation of the protein, however, appears to occur at day 3 ESEB. This is the day when *brachyury* is typically up-regulated suggesting that c-Myc2 needs to be down-regulated at this stage of differentiation. This expression profile is similar to that shown in section 3.3.1, but there are some differences that would need to be addressed via comparison of the Ab54 with a N262 western result, on the same samples.

4.4 Conclusion

Coupled with the results of the western analysis from Chapter 3, creation of the Ab54, anti-c-Myc antibody provided evidence that there is an up-regulation of c-MycS during the differentiation of ESEBs. The results in this Chapter support the evidence in Chapter 3 for the appearance of c-MycS during periods of rapid cell division. The up-regulation of c-MycS demonstrated in Chapter 3 appears to be associated with the up-regulation of *Fgf5*. Interestingly, it has been shown in our laboratory that EPL cells, which are *Fgf5* positive, have more rapid cell cycle times than ES cells (Stead *et al.*, 2002). This evidence indicates that up-regulation of c-MycS is associated with the rapid proliferation of cells equivalent of primitive ectoderm, which is also known to rapidly proliferate within the embryo (Snow, 1977; Poelmann, 1980; Lawson *et al.*, 1991).

CHAPTER 5

FINAL DISCUSSION

CHAPTER 5
FINAL DISCUSSION

5.1 Introduction

The Myc family of proteins are activators of proliferation, apoptosis and cell growth (Ryan and Birnie, 1996). Members of this family are widely expressed during development of the mouse embryo (Downs *et al.*, 1989; Queva *et al.*, 1998) and knockouts of Myc genes, typically, undergo abnormal development and early embryonic leathality (Davis *et al.*, 1993; Sawai *et al.*, 1993; Hatton *et al.*, 1996). The *in vitro* model of the pluripotent cells of the ICM, ES cells, are a useful tool for the investigation of molecular regulators of the dynamic process of embryogenesis. Utilising this tool, it was the purpose of this investigation to elucidate possible roles of c-Myc in regulating the changes in pluripotent cells that underlie their differentiation.

5.2 Changes in c-Myc Expression During ES Cell Differentiation

Embryonic stem cells undergo very rapid cell division (12.3 hours) in comparison to somatic cells which have cell cycle times in excess of 24 hours. ES cell cycles are characterised by high levels of, non-cell cycle regulated, Cyclin E-associated kinase activity (Stead *et al.*, 2002). Upon signals to differentiate into non-pluripotent cell lineages, ES cells down-regulate their Cyclin E-associated kinase activity and restore more somatic cell-like cell cycle structure (Stead, personal communication). A possible up-stream regulator of these rapid cell cycles in pluripotent cells is c-Myc, which has roles in the activation of Cyclin E and normal embryogenesis. Thus it was of great interest to analyse the kinetics of c-Myc protein expression during the differentiation of ES cells.

Two different sized bands are detected by western analysis utilising the anti-c-Myc (N262) antibody (2.4.10). The bands that migrated slower had a predicted molecular weight of 65kDa on denaturing SDS-PAGE. This band would be expected to be, full-length, c-Myc2, which is down-regulated as ESEB differentiation proceeds. Its expression closely follows the expression of *Rex1*, indicating that c-Myc2 expression be associated with pluripotent ICM cells. The second band detected migrated at the predicted molecular weight of 50kDa. There are several different possible identities of this band. It may be the result of a non-specific interaction with a protein that is differentially regulated across this stage of ES cell differentiation. It may also be an intermediate breakdown product of full-length c-Myc resulting from proteolysis of the protein. The other possibility is that this band is the c-MycS protein, which arises from a down-stream translational start site in c-Myc. The shorter c-MycS proteins have been demonstrated to be up-regulated in various cell types at periods when there is rapid cell division (Spotts *et al*, 1997). This band is detected at its highest levels in days 2 and 3 of ESEB differentiation, which correlates with when *Fgf5* is detected and before *Brachyury* is detected at day 4. This would be expected to be a period of rapid cell division, as it has been shown in our laboratory that, *Fgf5* positive, EPL cells have cell cycle times of 8.1 hours, in comparison to the 12.3 hour cell cycle times of *Rex1* positive ES cells (Stead *et al.*, 2002). This suggests that c-Myc2 is high in ES cells and c-MycS may be up-regulated in the more rapidly dividing cells. Previously, it has been shown in embryoid bodies and the embryo that following gastrulation there is a deceleration in the cell cycle. In the embryo, the cells of the primitive ectoderm cycle in 5-7 hours and the mesoderm cells cycle in 22.2 hours (Snow, 1977). In Embryoid bodies this stage of differentiation is associated with a more tightly regulated, more somatic cell-like

cell cycle (Stead, personal communication). Potentially regulation of c-Myc is an up-stream effector of this process.

5.3 The Effect of Human c-Myc Over-Expression on ES Cell Differentiation

5.3.1 Introduction

As it was proposed that the down-regulation of c-Myc is essential for the differentiation of pluripotent cells into more differentiated cells it was of interest to analyse what effect over-expression of c-Myc would have on the differentiation of these cells. Experiments by MacLean-Hunter *et al* (1994) have previously demonstrated that over-expression of the tumour produced RLF/L-Myc fusion protein in ES cells, results in the delay of differentiation into embryoid bodies, suggesting that Myc family members must be down-regulated to allow normal differentiation.

5.3.2 Embryoid Body Differentiation

Generation of EF-1α promoter driven human c-Myc over-expressing ES cells, provided some early evidence that Myc down-regulation is essential for the differentiation of pluripotent cells. The one day delay in the appearance of *brachyury* expression in the absence of puromycin and inability of the cells under puromycin selection to form mesoderm, indicated that the differentiation of ES cells requires a down-regulation in c-Myc. It can be seen in figure 3.5 that the appearance of *brachyury* is associated with down-regulation of the protein levels of c-Myc on day 4 of ESEB differentiation. Interestingly, in the CMES EPLEB differentiation experiment there was a delay in the *Rex1* to *Fgf5* transition. This indicates that not only does c-Myc over-expression delay loss of pluripotency, as seen by *Oct4* expression, but it also may delay the differentiation of ES

cells to other pluripotent cell types. The low level of *brachyury* expression seen in this experiment may be attributed to a small subpopulation of cells, which are able to differentiate normally.

Interestingly, the 50kDa band also appeared in the c-Myc over-expressing cells during differentiation as ESEBs, as is seen in figure 3.5(a). This indicates that the band is a truncated form of c-Myc. It does not, however, clarify whether or not it is c-MycS or a breakdown product of c-Myc resulting from changes in the level of c-Myc degradation during differentiation.

5.3.3 The Effect of Human c-Myc Over-Expression on ES Cell LIF Dependence

Maintenance of ES cell pluripotency is dependent on the cytokine, LIF (Nichols et al., 1990). Thus if over-expression of c-Myc is expected to maintain pluripotency, it may be expected that c-Myc over-expressing ES cells may lose the requirement for LIF and thus LIF titration analysis was utilised to test this hypothesis. Initial experiments utilising CMES cells showed only a moderate retention of ES-like characteristics in decreasing LIF concentration, in comparison to vector alone cells. Also, greater than 90% of colonies after 6 days in the absence of LIF were no longer pluripotent. Whilst these experiments showed unremarkable results both of the c-MycER ES cell lines showed a more striking result, suggesting that the CMES lines may down-regulate their c-Myc expression during the period of the experiment. This may be reminiscent of the down-regulation seen in the ESEB experiment in the absence of puromycin. Whilst the c-MycER cells lose ES cell-like colony characteristics in the absence of LIF, the cells in the presence of 4-OHT retain pluripotency, as assessed by Alkaline Phosphatase activity. This tends to indicate that these cells are able to differentiate from ES cells but cannot become non-pluripotent, and thus

are possibly trapped in an early primitive ectoderm-like state. Interestingly, experiments utilising the MycT58ER over-expressing cells demonstrate that in the absence of LIF and the presence of 4-OHT for 6 days, approximately 60% of colonies were scored as ES cell colony-like. This reduction in LIF dependence for the maintenance of ES cells may be attributable to the increased stability of c-Myc containing the T58A mutation, which makes it resistant to ubiquitin-mediated degradation.

5.4 Generation of Anti-c-Myc Antibodies

Western analysis utilising the anti-c-Myc (N262) antibody (2.4.10) saw the differential expression of two bands distinct in size. I have previously suggested that the 65kDa band was c-Myc2 and there was a possibility that the 50kDa band was c-MycS. The smaller c-MycS protein is generated from translational initiation at down-stream translational start site and lacks the highly conserved MBI in the transactivation domain of the protein (Spotts *et al.*, 1997). This protein, however, has been demonstrated to be involved with the repression of genes, such as *gas1* and *gadd45* (Xiao *et al.*, 1998) and rapid proliferation of various cell types (Spotts *et al.*, 1997).

To provide evidence that the smaller band detected by the anti-c-Myc (N262) antibody (2.4.10) is c-MycS, polyclonal antibodies were generated that would detect c-Myc2 but not c-MycS. The converse experiment is not possible, as c-Myc2 and c-MycS are identical except that c-MycS lacks the N-terminal 100 amino acids. Following generation of the Ab54 antibody it was shown that it could detect both over-expressed human c-Myc from MycER cells and endogenous mouse c-Myc2 in ES cells. This antibody was then used in western analysis on ESEBs to see if the 50kDa band was detected at any stage of differentiation. As the species was not detected it provides evidence that this species is the

c-MycS protein. This strengthens the argument proposed in 5.2, that c-MycS, is involved with the rapid proliferation of *Fgf5* positive cells and that MBI is not required or detrimental to this proliferation profile.

5.5 Final Summary

The Myc family of proteins have various roles in the control of cell proliferation, cell growth, adhesion and apoptosis (Ryan and Birnie, 1996). They are important regulators of embryonic development (Downs *et al.*, 1989; Queva *et al.*, 1998; Davis *et al.*, 1993; Sawai *et al.*, 1993; Hatton *et al.*, 1996) and potent activators of the cell cycle (Jansen-Durr *et al.*, 1993; Bouchard et al., 1999; O'Hagan *et al.* 2000). As it is known that pluripotent cells of the embryo, and their *in vitro* equivalents, undergo dynamic changes in their cell cycle, it became the focus of this investigation to analyse possible roles of c-Myc in the regulation of this process.

Investigation indicated that c-Myc protein was expressed in the rapidly dividing, pluripotent ES cells and this expression was down-regulated as the cells differentiated into non-pluripotent cells. These cells exhibited slower, more tightly regulated cell cycles. It was also demonstrated that during differentiation c-Myc appeared to undergo differential translational control, where the shorter c-MycS protein appeared when *Fgf5* was high. This indicated that c-MycS expression may be associated with periods of rapid cell division in ES cell differentiation.

Over-expression studies demonstrated that for ES cells to differentiate there must be a down-regulation of c-Myc protein. This was predominantly shown to be associated with the loss of pluripotency, when cell cycle time changes were most significant. It was also

demonstrated via the reduction of LIF dependence in c-Myc over-expressing cells and the delay in the differentiation of embryoid bodies.

In conclusion, c-Myc appears to be an essential regulator in the differentiation of pluripotent cells and is a possible candidate for the regulator of the rapid cell cycle times in the pluripotent cells of the embryo, and their *in vitro* equivalents.

CHAPTER 6

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REFERENCES

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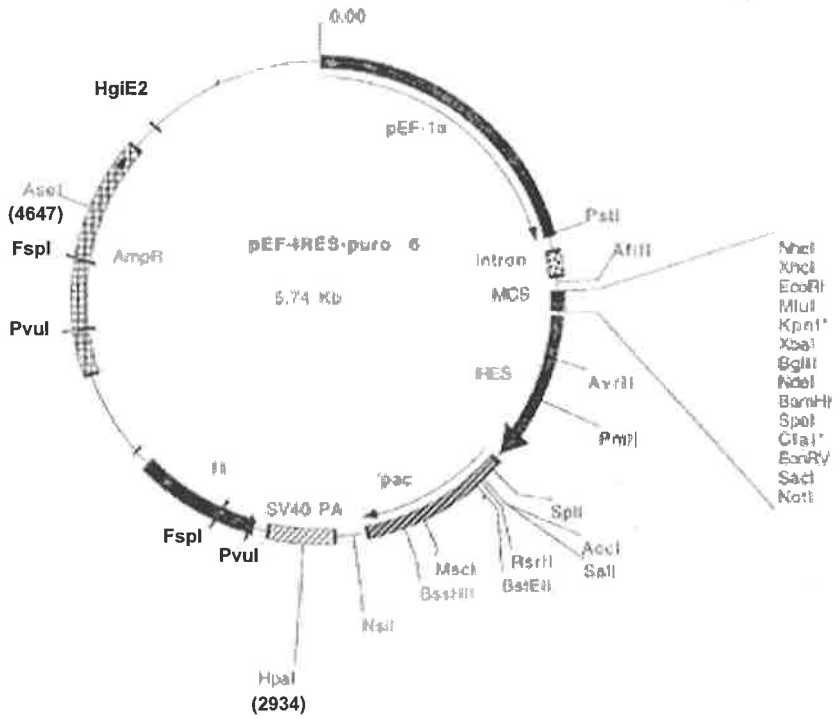
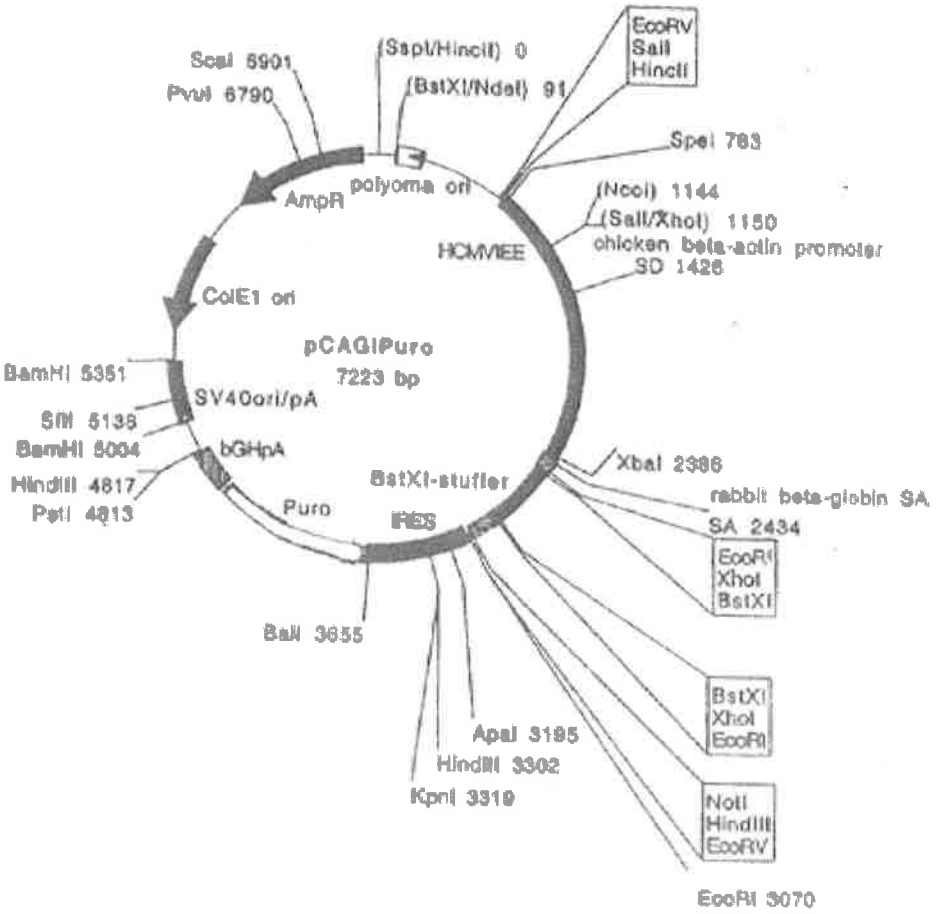
APPENDIX 1: pEF-IRES-puro6



Plasmid name: pEF-IRES-puro 6
Plasmid size: 5.74 kb
Constructed by: Dan Peel
Construction date: 9907.23
Comments/References: Made from pEF-IRES-puro 5 by removing NcoI site in MCS with exonuclease VII

*Cla and KpnI sites not unique

APPENDIX 2: pCAGIPuro



Plasmid name: pCAGIPuro
Plasmid size: 7223 bp
Constructed by: Ian Chambers & Morag Robertson
Construction date: April 1997
Comments/References: The PstI - NotI backbone and the NotI - NcoI fragment (IRES) of pCAGIZ were ligated to a PCR product from pPGKpurobpA extending from the Puro ATG to the PvuII site 3' to the bGHpA. PCR introduced a BspHI site at the ATG and mutated PvuII to PstI.