INHIBITION OF ADIPOGENESIS BY THE c-MYC ONCOPROTEIN.

by

VICTORIA J. HEATH

This thesis is submitted in part fulfilment of the Degree of Doctor of Philosophy in the University of Glasgow

The Beatson Institute for Cancer Research,
CRC Beatson Laboratories,
Bearsden, Glasgow.

Faculty of Medicine,
University of Glasgow,
Glasgow.

April, 1997.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

ABSTRACT.

Enforced expression of c-*myc* in many cell types is associated with inhibition of terminal differentiation. However, the mechanism by which this occurs remains unclear. In order to address this issue we therefore exploited the ability of c-Myc to block differentiation in the 3T3-L1 cell line, a well characterised *in vitro* model of adipogenesis.

Analysis of 3T3-L1 lines constitutively expressing an avian c-*myc* transgene revealed an inability to undergo morphological changes or accumulate cytoplasmic triglyceride, which was dependent upon the integrity of the Myc leucine zipper. In order to define the point at which adipogenesis was inhibited we analysed patterns of gene expression during the differentiation programme. This study demonstrated that the Myc block was associated with repression of the late stage markers C/EBPα, PPARγ2, aP2 and SCD1. C/EBPα and PPARγ2 are key transcriptional regulators of adipogenesis that co-ordinate the expression of genes required for lipid metabolism, including aP2 and SCD1. Hence, repression of these factors provides a molecular basis for the inability of Myc-expressing cell lines to undergo morphological differentiation. Interestingly, a 3T3-L1 clone that had spontaneously lost the ability to differentiate displayed a similar profile of gene expression. However, subtle differences indicated that this block occurred *via* a different mechanism.

Surprisingly, low levels of c-Myc were sufficient to inhibit 3T3-L1 differentiation and this was not associated with transformation or the capacity to undergo apoptosis. Additionally, the continued presence of c-Myc did not alter the ability of the 3T3-L1 line to undergo a number of defined cell cycle events during the early phases of the differentiation programme. Hence, inhibition of adipogenesis was unlikely to reflect altered cell cycle control.

It had been proposed that c-Myc inhibits adipogenesis by preventing entry into a differentiation-specific growth arrest (G_D) that is both irreversible and a prerequisite for terminal differentiation. However, we were unable to characterise the G_D state by either conventional criteria or by analysis of factors known to mediate cell cycle withdrawal in other differentiation systems. Indeed, irreversible cell cycle exit was only apparent in mature adipocytes, suggesting this was a consequence of terminal differentiation rather than the driving force. Thus, c-Myc did not inhibit adipogenesis by precluding entry into G_D.

Finally, treatment with 10% foetal calf serum (FCS) was sufficient to rescue Myc-mediated inhibition of adipogenesis but had no effect on the differentiation-defective clone. Abrogation of the Myc block was associated with restoration of the late stage markers, accounting for the ability of the Myc-expressing lines to accumulate lipid in the presence of FCS. Whilst the active component(s) present in FCS have yet to be fully defined, it is likely that growth hormone may be partly responsible for this phenomenon.

We therefore conclude that c-Myc inhibits adipogenesis by repressing the expression of master transcription factors. However, this effect is dependent upon external factors, since FCS rescues the phenotype.

For Mum, Dad and Nigel.

ACKNOWLEDGEMENTS.

I would like to express my extreme gratitude to all of the following who in one way or another have helped me out during the course of this awesome (?awful) task:

My supervisors, Sam Crouch and Dave Gillespie, for all their help and encouragement during the last three (and abit!) years.
My advisor, Chris Bartholomew, who bravely stepped in after I frightened the first one away.
All members of R1 and R11, both past and present, but especially Billy "It's Only a Game" Clark, Val "Don't Cross the Green Line" Fincham, Dewi "Cack" Owens, Val "Where's the Pub Quiz" Brunton, Debbie (who pretends to be the sensible one but seems to spend all her spare time hanging upside down from a rock. Personally, I'd much rather go shopping), Gerhard Burger and Burky (not one but *two* Germans with a sense of humour!), Liz for helpful discussions (about what's on at the cinema) and Anne for the kind provision of lipstick (which reminds me, you haven't provided any recently.....).
All those at the Beatson Institute who keep the place running but rarely get any thanks for it: Bill in the Stores who knows what you want even if you don't, Liz Gordon in the library, the computer whiz kids Iain and Peter, Anne and Laraine in the front office, Noel "To the Rescue" Wigglesworth, the tissue culture goddesses Mary and Marion, Don who mends stuff and all members of the wash-up whose misfortune it is to clear up after us.
All of my drinking buddies, including Dewi and Burky (again!), Dave, Lorraine, Chris, the dreaded Vivien Tunnock's Tea Cake, Gareth/Gary and Lenny (who can't help being a light-weight, it's an enzyme deficiency.....).
The staff of the Brewery Tap, who must have seen millions of Beatson students pass through their doors (or is that pass out??).
Foofi for taking me to the pictures every week and for giving me a nice place to live.
Grant for making cups of rosie.
Joolie Congealing, Anthony, Jane, Tiny, Hils, Pierre, Teddy and Fred just for being my mates.
Mum, Dad and Bruv, whose generosity made it possible for me to stay in Glasgow while I wrote up (which no doubt was to their advantage as well as mine.....).
And finally, I would like to thank Kevin for running up his trans-Atlantic phone bill to astronomical heights over the last few months (spending money not usually being considered one of his strong points) and making encouraging noises during yet another PhD-induced tantrum. See you in Baltimore!

This project was funded by the Medical Research Council and the CRC Beatson Laboratories.

Declaration.

I declare that all the work in this thesis was performed personally unless otherwise acknowledged.

CONTENTS.

LIST OF FIGURES.

ABBREVIATIONS.

ADD1	:	adipocyte determination and differentiation factor 1.
AMP	:	adenosine 5'-monophosphate.
ATP	:	adenosine 5'-triphosphate.
BAT	:	brown adipose tissue.
BCIP	:	5-bromo-4-chloro-3-indolyl phosphate.
bHLH	:	basic region, helix-loop-helix.
bp	:	base pair (s).
BrdU	:	bromodeoxyuridine.
c-	:	cellular.
cAMP	:	cyclic AMP.
cDNA	:	complementary deoxyribonucleic acid.
C/EBP	:	CCAAT/enhancer binding protein.
CHOP	:	C/EBP homologous protein.
CKI	:	cyclin-dependent kinase inhibitor.
cpm	:	counts per minute.
CTP	:	cytosine 5'-triphosphate.
CUP	:	C/EBP undifferentiated protein.
DCS	:	donor calf serum.
DEPC	:	diethylpyrocarbonate.
dH_2O	:	de-ionised water.
DAB	:	diaminobenzidine.
DEX	:	dexamethasone.
DMSO	:	dimethyl sulphoxide.
DNA	:	deoxyribonucleic acid.
dNTP	:	3'-deoxyribonucleoside 5'-triphosphate.
DTT	:	dithiothreitol.
ECL	:	enhanced chemiluminescence.
ECM	:	extracellular matrix.
EDTA	:	ethylenediaminetetra-acetic acid, disodium salt.
FAAR	:	fatty acid activated receptor.
FCS	:	foetal calf serum.
g	:	gram (s).
GADD	:	growth arrest and DNA damage.
GAPDH	:	glyceraldehyde-3-phosphate dehydrogenase.
GDP	:	guanosine 5'-diphosphate.
GLUT	:	glucose transporter.
GTP	:	guanosine 5'-triphophate.
HEPES	:	N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulphonic acid).
IGF-1	:	insulin-like growth factor-1.
IRS-1	:	insulin receptor substrate-1.
Inr	:	Initiator element.
IPTG	:	Isopropyl-β-D-thiogalactoside.
JAK	:	janus kinase.
kb	:	kilobase (s).
kDa	:	kilo Dalton (s).
LZ	:	leucine zipper.

μ	:	micro.
m	:	milli.
M	:	molar.
mA	:	milliamps.
MAPK	:	mitogen activated protein kinase.
MEL	:	murine erythroleukaemia.
MEM	:	modified Eagle medium.
MIX	:	methyl isobutylxanthine.
MOPS	:	3-(N-morpholino) propanesulphonic acid.
mRNA	:	messenger ribonuleic acid.
n	:	nano.
NBT	:	nitroblue tetrazolium.
°C	:	degrees Celsius.
ODC	:	ornithine decarboxylase.
ODx	:	optical density (x = wavelength).
PAGE	:	polyacrylamide gel electrophoresis.
PBS	:	phosphate-buffered saline.
PPAR	:	peroxisome proliferator activated receptor.
RNA	:	ribonucleic acid.
RNAse	:	ribonuclease.
rpm	:	revolutions per minute.
RXR	:	retinoid X receptor.
SCD1	:	stearoyl CoA desaturase 1.
SDS	:	sodium dodecyl sulphate.
SSC	:	sodium chloride, sodium citrate.
SSPE	:	sodium chloride, sodium phosphate, ethylenediaminetetra-acetic acid.
STAT	:	signal transducer and activator of transcription.
TEMED	:	tetramethylenediamine.
TNFα	:	tumour necrosis factor α.
Tris	:	2-amino-2-(hydroxymethyl) propane-1,3-diol.
UCP	:	uncoupling protein.
UV	:	ultraviolet.
V	:	volts.
v-	:	viral.
v/v	:	volume for volume.
WAT	:	white adipose tissue.
w/v	:	weight for volume.
WT	:	wild-type.

Throughout this thesis genes are indicated by italics (e.g. *myc*) whilst proteins are unitalicised (e.g. Myc).

INTRODUCTION.

INTRODUCTION.

CHAPTER 1: Adipocyte Development.

1.1 Biological Function of Adipose Tissue.

The primary role of white adipose tissue (WAT) is to store energy in the form of triglycerides during times of caloric excess, and to mobilise this reserve during periods of energy deprivation. Mature adipocytes possess all the necessary enzymes required for *de novo* lipogenesis and lipolysis, and these processes are maintained under tight hormonal control (Stryer, 1988). In contrast, brown adipose tissue (BAT) has a somewhat specialised function, the dissipation of stored energy as heat, which is achieved by uncoupling mitochondrial electron transport from the production of ATP (Darnell *et al*, 1990). This response, known as non-shivering thermogenesis, is of particular importance in neonates, cold-adapted animals and hibernating vertebrates. Finally, there is currently a large body of evidence accumulating to suggest that adipose may also perform an endocrine function (McGarry, 1995). This is discussed in Section 1.6.

1.2 Adipocyte Development *in vivo*.

The adipocyte lineage derives from a multipotent embryonic stem cell of mesodermal origin, that can also give rise to cartilage and muscle cell precursors. Preadipocytes appear relatively late in embryogenesis, undergoing massive expansion and differentiation during the neonatal period when the acquisition of an energy reserve becomes essential to survival (Ailhaud *et al*, 1992). Some species, including humans, have macroscopic WAT deposits at birth, whilst in rodents WAT does not develop until the post-natal period. Mature adipocytes represent between one and two-thirds of the total WAT cell population, the remainder comprising the stromal vascular fraction, which in addition to blood and endothelial cells, contains the preadipocyte population. These preadipocytes retain

the potential to differentiate in response to external cues throughout the life-span of the animal.

BAT is present in most mammalian species at birth and is thought to be a specific organ, distinct from WAT. However, the developmental relationship between the two tissues is not clear. The existence of distinct precursor cells is generally assumed, but a common progenitor can not be ruled out. Alternatively, it has been suggested that BAT may be the default pathway of adipocyte development and that WAT arises by transdifferentiation of pre-existing BAT depots (Ailhaud *et al*, 1992).

1.3 Cell Culture Models of Adipogenesis.

The study of adipocyte development has been greatly facilitated by the availability of established preadipocyte cell lines. These can be broadly sub-divided into two classes: (1) multipotent stem cell lines and (2) lines committed to the adipocyte lineage.

1.3.1 *Multipotent Stem Cell Lines.*

Multipotent cell lines, such as 10T1/2 (Reznikoff *et al*, 1973) and Balb/c 3T3 (Sparks *et al*, 1986), have not undergone the commitment phase, but may be induced to do so. Thus, treatment of 10T1/2 cells with 5-azacytidine, an inhibitor of DNA methylation, generates a number of stable, determined lineages: preadipocytes, premyocytes and prechondrocytes (Taylor and Jones, 1979; Konieczny and Emerson, 1984). Additionally, transfection of 10T1/2 cells with large segments of hypomethylated genomic DNA derived from 5-azacytidine treated cells was found to result in myogenic conversion at a frequency suggesting that a single gene was responsible for commitment to this lineage (Lassar *et al*, 1986). This seminal work led to the isolation of the MyoD family of basic helix-

loop-helix (bHLH) transcription factors, thought to be master regulators of muscle development (reviewed in Olson and Klein, 1994). However, this approach has so far proven unsuccessful in the identification of genes that control adipocyte determination.

1.3.2 *Preadipocyte Cell Lines.*

Preadipocyte cell lines have passed through the commitment phase and when appropriately stimulated can differentiate into cells resembling mature adipocytes. The properties of some of these cell lines are described below. Whilst these lines display many similar characteristics, their responsiveness to both positive and negative regulators of adipogenesis varies widely. This probably reflects stage-specific differences which relate to the point in development at which the lines were derived.

A. 3T3-L1 and 3T3-F442A Cell Lines. These cell lines are probably the most extensively characterised cell culture models for preadipocyte development. They were originally selected from the murine embryonic Swiss 3T3 M cell line for their ability to spontaneously accumulate triglyceride (Green and Kehinde, 1974; Green and Kehinde, 1976). The differentiation defective 3T3-C2 cell line was also derived from the same source. Prior to differentiation these cell lines resemble the fibroblastic preadipocyte cells found in WAT stroma. Although both the 3T3-L1 and F442A lines can differentiate spontaneously, protocols have been developed that induce rapid adipogenesis at high frequency (Student *et al*, 1980). For the 3T3-L1 cell line, the most widely used agents are dexamethasone, supra-physiological levels of insulin and methyl isobutylxanthine (MIX), a cyclic AMP (cAMP) phosphodiesterase inhibitor, whilst 3T3-F442A cells are responsive to insulin and growth hormone.

B. Ob17 Cell Line. The Ob17 preadipocyte cell line was derived from the stromal cells of epididymal fat pads from C57/BL/6J *ob/ob* (genetically obese) mice (Negrel *et al*, 1978). Various sub-lines with high adipogenic capacity have since been derived from Ob17, (e.g. Ob1771), which can be induced to differentiate in serum-free medium containing insulin, growth hormone, triiodothyronine and either agents that elevate intracellular cAMP concentrations or glucocorticoid.

C. TA1 Cell Line. This cell line was derived from 10T1/2 cells treated with 5-azacytidine in order to pass through the commitment phase (Chapman *et al,* 1984). Differentiation is induced with dexamethasone or indomethacin, a prostaglandin synthesis inhibitor.

D. HIB-1B Cell Line. The HIB-1B cell line represents a model for BAT development. Originally derived from a cultured murine brown cell tumour (hibernoma), this line differentiates in response to insulin, triiodothyronine, MIX, hydrocortisone and indomethacin (Ross *et al*, 1992). The unique feature of this cell line is that, once differentiated, it is competent to express the uncoupling protein (UCP/thermogenin), which is responsible for uncoupling respiration from oxidative phosphorylation. Expression of UCP is induced by β-adrenergic agents.

1.3.3 *Validity of Cell Culture Models for the Study of Adipogenesis.*

Considerable evidence exists to suggest that preadipocyte cell lines do, indeed, represent valid models for adipogenesis. Sub-cutaneous injection of 3T3-F442A cells into Balb/c athymic mice, at anatomical sites usually devoid of adipose, results in the formation of apparently normal fat pads at the site of injection (Green and Kehinde, 1979). Additionally, detailed histological and electron microscopic studies of mature 3T3-L1 cells revealed structural and ultra-structural features indistinguishable from adipocytes *in situ* (Novikoff *et al*, 1980). Fully

differentiated cells from these lines are also found to mimic the metabolic properties of WAT-derived adipocytes. Hence, they display appropriate hormone sensitivity and responsiveness to metabolic effectors, and express the full range of proteins required for effective lipid metabolism. In addition, studies employing primary preadipocytes from a variety of sources suggest that such cells are responsive to the same group of effectors that initiate adipogenesis in established cultures (Ramsay *et al*, 1989a; Ramsay *et al*, 1989b). Finally, with the advent of transgenic and nullizygous animal technology, it is likely that many of the conclusions drawn from cell culture systems will be verified *in vivo*.

1.4 Modulators of Adipogenesis.

Efficient differentiation of the preadipocyte cell lines is achieved by treatment with external inducers. The most commonly used factors are described below, whilst the reader is referred to reviews by Smyth *et al* (1993) and Cornelius *et al* (1994) for a comprehensive list of agents that both positively, and negatively, affect adipogenesis.

1.4.1 *Insulin, Insulin-like Growth Factor-1 and Growth Hormone.*

Whilst growth hormone and high levels of insulin are known to promote adipogenesis, it is now apparent that both hormones exert many of their effects through the insulin-like growth factor-1 (IGF-1) receptor, and that physiological doses of IGF-1 can substitute for insulin and growth hormone in some cell lines (Smith *et al*, 1988). Preadipocytes possess large numbers of IGF-1 receptors, but relatively few insulin receptors. Thus, when present at supra-physiological levels, insulin is able to bind to the IGF-1 receptor, and so mimic the effects of IGF-1. In contrast, growth hormone acts by initiating an autocrine/paracrine loop, whereby signalling *via* the growth hormone receptor stimulates increased expression and

secretion of IGF-1, which subsequently binds and signals through its own receptor (Zezulak and Green, 1986; Kamai *et al*, 1996).

The IGF-1 receptor is a member of the receptor tyrosine kinase family, which signal through the Ras pathway in order to transmit extracellular signals to the nucleus (see Figure 1). Ligand stimulated activation of the IGF-1 receptor results in the activation of Ras, a small GTP/GDP binding protein, which in turn stimulates a cascade of serine/threonine kinases, including Raf-1 and the mitogen activated protein kinase (MAPK), ultimately influencing the expression of a number of target genes (Khosravi-Far and Der, 1994; Maruta and Burgess, 1994). Evidence for an involvement of the Ras signal transduction pathway in adipogenesis comes from several sources. Liao and Lane (1995) demonstrated that over-expression of a phosphotyrosine phosphatase, HA2, in 3T3-L1 preadipocytes effectively blocked differentiation, whilst treatment with the phosphatase inhibitor, vanadate, reversed this effect. Additionally, over-expression of a constitutively active Ras allele in the 3T3-L1 system resulted in differentiation in the absence of inducing hormones (Benito *et al*, 1991), as did constitutive activation of Raf-1, (Porras *et al*, 1994). Conversely, blocking Raf-1 function with a dominant negative allele inhibited differentiation (Porras *et al*, 1994).

An important function of insulin in adipocyte differentiation is the facilitative uptake of glucose by specific transport proteins. Whilst Ras mediates the insulin-stimulated translocation of the ubiquitous transporter, GLUT1, it has no effect on GLUT4, the major glucose transport protein active during adipogenesis (Hausdorff *et al*, 1994; van den Berghe *et al*, 1994). Insulin-stimulated glucose uptake is now thought to be mediated by phosphatidylinositol 3-kinase (Okada *et al*, 1994), which is linked to insulin receptor activation *via* the insulin receptor substrate 1 (IRS-1) protein (Holman and Cushman, 1994). However, recent studies suggest that whilst

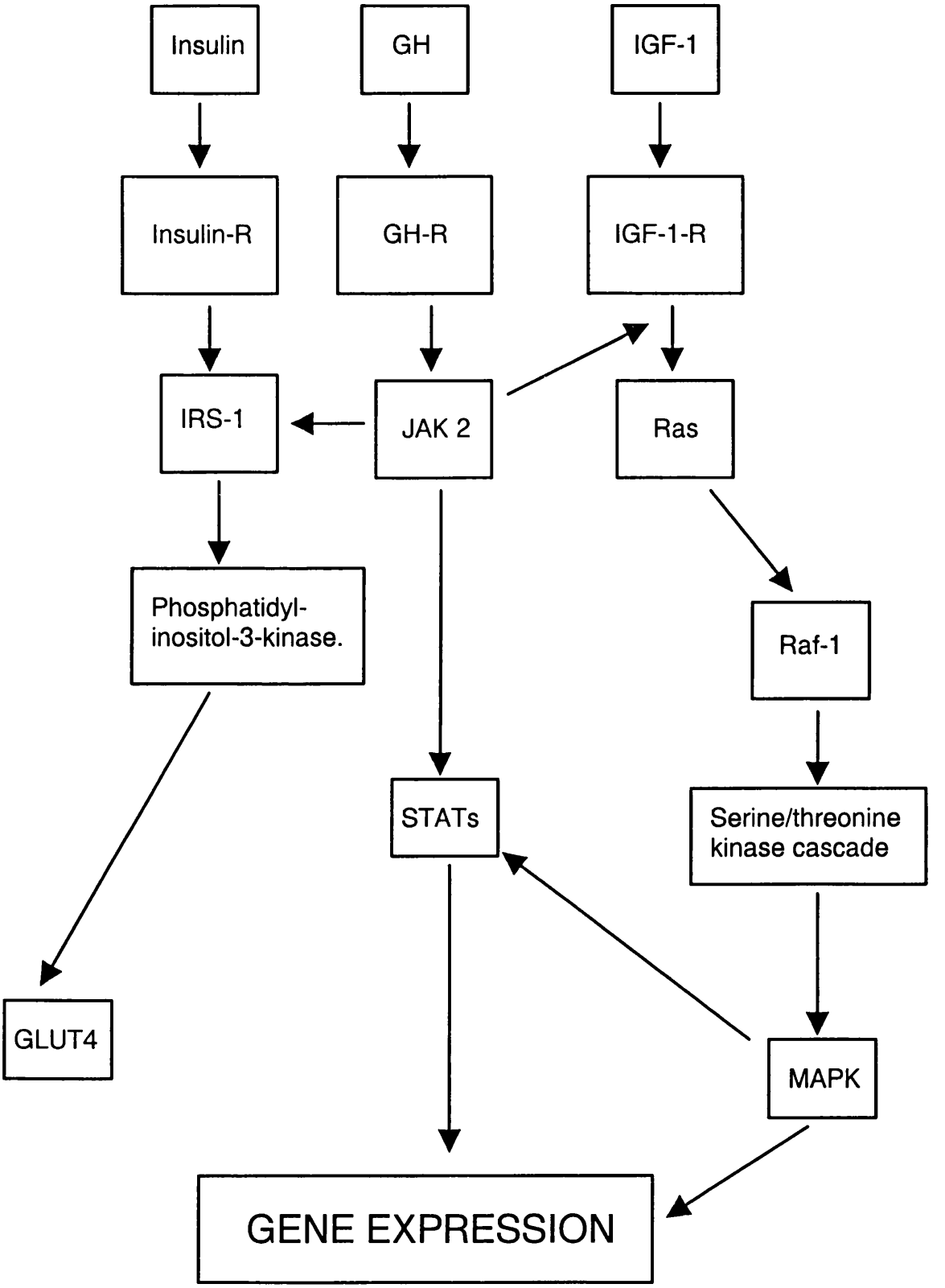


Figure 1. Signal Transduction Pathways Implicated in Adipogenesis.

Ras and phosphatidylinositol 3-kinase activity are necessary to mediate the various effects of insulin, they are not in themselves sufficient to promote the full adipogenic response, suggesting a requirement for other signal transduction pathways (Wiese *et al*, 1995).

This hypothesis is further supported by studies using growth hormone. 3T3-F442A preadipocytes require growth hormone for differentiation when grown in serum supplemented conditions, as shown by immunodepletion studies. However, in this system IGF-1 can not substitute for growth hormone (Nixon and Green, 1984). Additionally, the ob1771 cell line has a requirement for growth hormone that can not be met by IGF-1. This suggests that growth hormone functions by more than one mechanism. Indeed, the growth hormone receptor is a member of the cytokine receptor superfamily, which has recently been shown to signal *via* a novel route, the so called JAK/STAT pathway (Figure 1). Members of the Janus (JAK) kinase family of soluble tyrosine kinases associate with the cytokine receptor and are rapidly activated by tyrosine phosphorylation following ligand binding. The activated JAKs then phosphorylate members of the Signal Transducers and Activators of Transcription (STAT) family of transcription factors, which translocate into the nucleus, where they activate transcription of specific target genes (Ihle and Kerr, 1995; Watanabe and Arai, 1996). Growth hormone binding to its receptor has been shown to activate JAK2 (Argetsinger *et al*, 1993), and STATs 1, 3 and 5 (Ram *et al*, 1996). With respect to the role of growth hormone in promoting adipogenesis, it is also of interest to note that there may be considerable cross-talk between the Ras and JAK/STAT signalling pathways (Ihle, 1996; Winston and Hunter, 1996) and that JAK2 may also be involved in IRS-1 activation (Argetsinger *et al*, 1995).

1.4.2 *Glucocorticoid and cAMP.*

Differentiation of ob1771 preadipocytes is potentiated by glucocorticoid. Glucocorticoids stimulate the expression of the enzyme, phospholipase A2, resulting in the release of arachidonic acid for prostaglandin biosynthesis. Increased levels of prostaglandins in turn lead to an increase in the intracellular levels of the second messengers, cAMP and Ca^{2+}, which presumably act *via* the protein kinase A and C pathways respectively (Vassaux *et al*, 1992). However, in some cell lines, including TA1, arachidonic acid and its metabolites inhibit adipogenesis (Serrero *et al*, 1992). In this case, treatment with dexamethasone is thought to induce an inhibitor of phospholipase A2, whilst indomethacin inhibits the activity of cyclo-oxygenase, the enzyme which catalyses the initial breakdown of arachidonic acid. In addition, both dexamethasone and MIX, which increases cAMP levels by inhibiting the cAMP phosphodiesterase, elevate the expression of two transcription factors thought to be involved in the early stages of the differentiation programme: the CCAAT/enhancer binding proteins (C/EBP) δ and β, respectively (Yeh *et al*, 1995a). The role of these factors in adipogenesis is discussed in Chapter 2. Finally, cAMP is also implicated in the positive regulation of at least one gene involved in lipid metabolism, the fatty acid binding protein, aP2/422 (Yang *et al*, 1989).

1.4.3 *Fatty Acids and Peroxisome Proliferators.*

Treatment of preadipocytes with long chain fatty acids stimulates the expression of several genes involved in lipid metabolism (Distel *et al*, 1992; Abumrad *et al*, 1993) and induces differentiation. More recently, it has been demonstrated that peroxisome proliferators, a diverse class of amphipathic compounds, including certain herbicides and hypolipidaemic drugs, can induce adipogenesis in the 3T3-L1 cell line with varying degrees of efficacy (Chawla and Lazar, 1994). Natural fatty acids and the peroxisome proliferators appear to function by a common

mechanism (Tontonoz *et al*, 1995a; MacDougald and Lane, 1995a), *viz.* stimulation of members of a sub-group of the nuclear hormone receptor family of transcription factors, the peroxisome proliferator activated receptors (PPARs). Two PPARs are implicated in adipogenesis thus far: PPARγ2 (Tontonoz *et al*, 1994a) and the fatty acid activated receptor (FAAR/PPARδ/Nuc1), which was recently cloned by Amri and co-workers (1995). These factors are discussed in Chapter 2.

1.4.4 *Inhibitors of Adipogenesis.*

The adipogenic programme may be inhibited by a variety of substances, including growth factors, cytokines, and the product of the c-*myc* oncogene (Smyth *et al*, 1993). The mode of action of many of these anti-adipogenic agents remains unclear. However, in the case of growth factors, inhibition of differentiation is likely to be an indirect consequence of stimulating proliferation. Both epidermal growth factor (EGF) and transforming growth factor α (TGFα) are able to inhibit adipogenesis *in vitro* and studies with TGFα transgenic mice demonstrate a 50% reduction in total body fat when compared with non-transgenic controls (Luetteke *et al*, 1993). Similarly, sub-cutaneous injection of EGF into neonatal rats decreased fat pad weight due to a reduced differentiation potential of the adipocyte precursors (Serrero and Mills, 1991).

The cytokine, tumour necrosis factor α (TNFα), is able to inhibit differentiation of the TA1 cell line in the presence of inducing agents (Torti *et al*, 1985). In addition, treatment of mature adipocytes with TNFα suppresses the expression of a sub-set of fat-specific genes and is associated with marked delipidation and morphological changes, a phenomenon known as dedifferentiation. The genes affected by TNFα appear to be predominantly targets of the transcription factor, C/EBPα, whilst the expression of other genes such as lipoprotein lipase and malic enzyme is not obviously deregulated (Weiner *et al*, 1991) It is now clear that at least some of the

inhibitory effects of TNFα may be mediated by c-*myc* (Ninomiya-Tsuji *et al*, 1993). The role of the Myc oncoprotein as a negative regulator of adipogenesis is described in detail in Chapter 3.

1.5 The Differentiation Programme.

Adipose cell differentiation is a multistep process, the key features of which are outlined below and summarised in Figure 2.

1.5.1 *Determination.*

As described in Section 1.3.1, 5-azacytidine treatment of multipotent stem cell lines results in the generation of three determined lineages, including preadipocytes (Taylor and Jones, 1979). This suggests that demethylation is an important trigger for differentiation, and various mechanisms are proposed to account for this activity. Firstly, hypomethylation may alter the chromatin structure and so unmask putative *cis*-acting regulatory elements. Alternatively, based on the 10T1/2 model for myogenic determination, expression of a unique transcription factor may commit the mesodermal stem cell to the adipocyte lineage. Transfection of genomic DNA derived from 3T3-F442A or human fat cells into the differentiation defective 3T3-C2 cell line resulted in adipogenic conversion when cultured in the presence of insulin (Chen *et al*, 1989), suggesting the presence of a sequence(s) capable of initiating the differentiation programme. This activity was found to reside in two non-identical sequences of 1.2 and 2.0kb (Colon-Teicher *et al*, 1993). However, the mechanism by which they exert their effect, or the identity of any putative gene products, remains unclear.

Postulating that regulatory genes should be expressed early in the differentiation programme, Sadowski and co-workers (1992) isolated five cDNAs that were expressed in the correct temporal pattern during induced differentiation of 3T3-L1

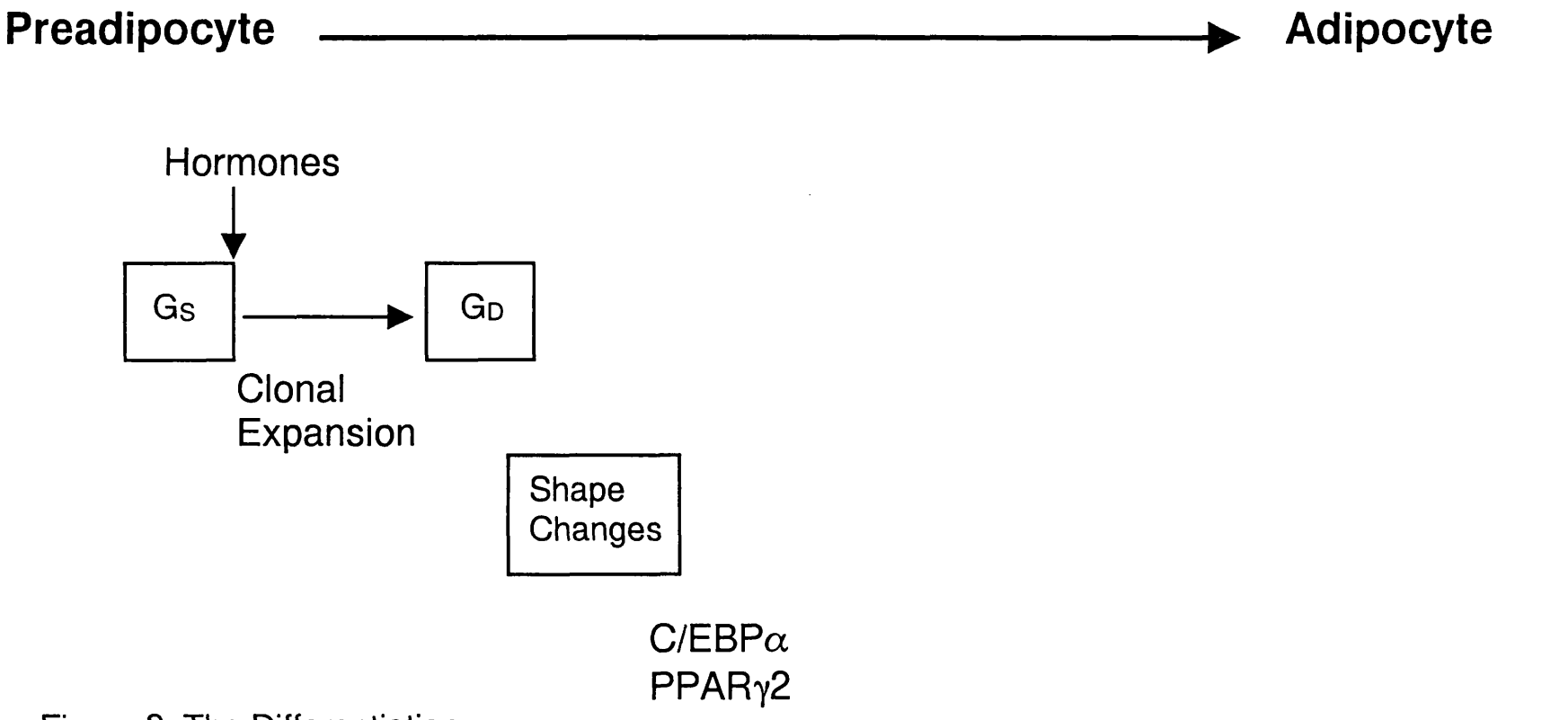


Figure 2. The Differentiation Programme.

cells. However, these still require further characterisation in order to establish a role, if any, in the commitment process. Other candidates for the "master regulator" of adipogenesis include a number of previously described transcription factors. Under appropriate conditions both C/EBPα and PPARγ2 can convert fibroblastic cell lines with no overt adipogenic capacity (e.g. NIH-3T3) to the adipocyte phenotype (Freytag *et al*, 1994; Tontonoz *et al*, 1994b). In addition, the adipocyte determination and differentiation factor, ADD1, cloned by Spiegelman's laboratory (Tontonoz *et al*, 1993) and the HNF/*forkhead*-like factor involved in the early expression of lipoprotein lipase (Enerback *et al*, 1992) are also proposed to be involved in adipocyte commitment. The role of each of these factors during adipogenesis is described fully in Chapter 2.

1.5.2 *Growth Arrest at Confluence: Cell-Cell and Cell-Extracellular Matrix Communication.*

Once determined, preadipocytes in culture proliferate until cell-cell contact at confluence inhibits further mitosis and the cells enter the G_0 phase of the cell cycle. This appears to be a prerequisite for subsequent differentiation and is associated with the expression of several "early" marker genes: lipoprotein lipase (Cornelius *et al*, 1988), FAAR (Amri *et al*, 1995) and the α2 chain of Type VI collagen (Dani *et al*, 1989). It is likely that these effects are mediated by cell-cell or cell-extracellular matrix (ECM) interactions, as described in other model systems. Thus, muscle cell precursors in *Xenopus laevis* require to interact in groups of at least 100 in order to induce the expression of MyoD (Holt *et al*, 1994)-the so-called "community effect"-whilst P19 embryonal carcinoma cells are induced to differentiate by cellular aggregation (Skerjanc *et al*, 1994). However, with respect to adipogenesis, it is of interest to note that gap-junctional communication between cells is lost as differentiation progresses, and that down-regulation of Connexin-43 is observed prior to the expression of certain fat-specific genes (Smas and Sul, 1995).

The influence of ECM components on differentiation and development is well documented (Adams and Watt, 1993). Laminin mediates the tissue-specific expression of β-casein in mammary epithelia (Streuli *et al*, 1995) and this occurs in the absence of cell-cell interaction (Streuli *et al*, 1991). In contrast, fibronectin inhibits the differentiation of both keratinocytes (Adams and Watt, 1989) and 3T3-F442A preadipocytes (Spiegelman and Ginty, 1983). In the case of adipogenesis, it was found that this block could be relieved by cytochalasin D, an agent which disrupts actin filaments (Spiegelman and Ginty, 1983). During the early stages of adipocyte differentiation, there are alterations in the expression of various ECM components and in the ultrastructure of the actin cytoskeleton (Smas and Sul, 1995). It is therefore likely that such alterations are involved in initiating or transducing signals for adipose differentiation, for example *via* integrin signalling pathways (Hynes, 1994).

Preadipocyte factor 1 (Pref-1) is a recently cloned transmembrane protein that may be involved in maintaining the preadipocyte phenotype (Smas and Sul, 1993). This protein is expressed exclusively in preadipocytes and is down-regulated as differentiation progresses. Interestingly, its expression is elevated in the 3T3-C2 differentiation defective clone, and enforced expression in 3T3-L1 preadipocytes inhibited differentiation (Smas and Sul, 1993). This protein is characterised by large amounts of N-linked glycosylation and the presence of six tandem EGF-like repeats in the extracellular domain. This motif is found in a number of molecules involved in protein-protein interactions, including ECM components (Thiery and Boyer, 1992) and the Drosophila proteins, Notch and Delta, which govern cell fate decisions (Fehon *et al*, 1990). It is therefore possible that Pref-1 may inhibit adipogenesis *via* ECM interactions, which are disrupted as differentiation commences due to alterations in the overall composition of the matrix.

1.5.3 *Mitotic Clonal Expansion.*

Treatment of the determined preadipocyte culture with external modulators of adipogenesis results in the initiation of one or more rounds of mitosis, which appear to be required for subsequent differentiation (Bernlohr *et al*, 1985). Clonal expansion is also observed in the L6E9 myoblast line following treatment with IGF-1 (Engert *et al*, 1996), suggesting that in general proliferation precedes differentiation. However, certain mitogens may stimulate post-confluent mitosis without inducing differentiation. This suggests that DNA replication is necessary, but not sufficient, for adipogenesis. The function of this clonal expansion is not clear, but it is possible that replication and accompanying changes in chromatin structure may make *cis*-acting elements accessible to transcription factors, which either activate or derepress genes involved in the acquisition of the adipocyte phenotype.

1.5.4 *The Differentiation-Specific Growth Arrest (G_D).*

Following clonal expansion, preadipocytes enter a unique growth arrested state, designated G_D, which is thought to be permissive for terminal differentiation (Scott *et al*, 1982). G_D can be distinguished from other forms of growth arrest by a number of criteria (Wille and Scott, 1982). Cells arrested at confluence (G_S) or due to nutrient (e.g. amino acid) deprivation (G_N) retain the ability to respond to serum mitogens and are unable to undergo adipogenesis, whilst cells arrested in G_D have the ability to differentiate in the absence of further DNA synthesis and are unresponsive to serum stimulation. However, G_D cells are responsive to the mitogenic effects of MIX, whilst cells arrested in the G_S or G_N state remain unaffected by this treatment. It has been proposed that each of these arrest states represents a distinct topographical stage in what is more typically referred to as the G_0 phase of the cell cycle, since they can be readily interconverted in the absence of further genome replication (Wille and Scott, 1982).

The relationship between growth arrest and differentiation has been extensively studied in the A31T6 preadipocyte model (Wang *et al*, 1994). A31T6 cells that have attained the G_D arrest state, but do not yet display the adipocyte phenotype, can be induced to re-enter the cell cycle by treatment with MIX (Scott *et al*, 1983). However, as differentiation proceeds this proliferative potential is progressively lost and the growth arrest becomes irreversible. This suggests that G_D may initially be a relatively plastic state and that cells lodged in this phase of the cell cycle may either progress to terminal differentiation or else remain in a "non-terminally" differentiated state, depending on the external cues (Yun and Scott, 1983). The molecular basis for the differentiation-specific growth arrest is not fully understood. However, as described in Chapter 2, the larger isoform of C/EBPα is reported to possess anti-mitotic activity (Umek *et al*, 1991) and so may therefore function in a manner analogous to MyoD during myogenesis (Crescenzi *et al*, 1990).

1.5.5 *Morphological Changes.*

As differentiation progresses, alterations in cellular morphology become apparent: the spindle-shaped preadipocyte converting to a rounded cell type that rapidly enlarges as a consequence of cytoplasmic triglyceride accumulation. Changes in the actin cytoskeleton are involved in this phenomenon. In preadipocytes, actin filaments are present in a well defined stress fibre pattern which becomes highly disorganised as the adipocyte morphology is attained. Indeed, treatment with cytochalasin D is sufficient to rescue adipogenesis in cells blocked by growth on fibronectin (Spiegelman and Ginty, 1983). Expression of actin and tubulin decrease during differentiation (Spiegelman and Farmer, 1982) and this precedes both morphological changes and the expression of adipocyte-specific genes.

1.5.6 *Co-ordinate Changes in Gene Expression.*

The terminal stages of adipocyte differentiation are initiated by the induction of C/EBPα and PPARγ2, both of which are implicated in directing the co-ordinate expression of many fat-specific genes (see Chapter 2). Acquisition of the adipocyte phenotype involves changes in expression of a large number of genes (Sidhu, 1979). These encode proteins involved in lipid and carbohydrate metabolism, hormone signalling, secretory molecules and components of the ECM and cytoskeleton. A comprehensive list of these proteins has been compiled by Cornelius *et al* (1994).

1.5.7 *Triglyceride accumulation.*

A classical feature of fully differentiated adipocytes is the accumulation of cytoplasmic triglyceride droplets. A key intermediate of triglyceride biosynthesis is glycerol-3-phosphate (Stryer, 1988). However, adipocytes lack the kinase required to phosphorylate endogenous glycerol and so utilise glucose in order to initiate the biosynthetic pathway. Glucose is taken up by the adipocyte where it enters the glycolytic pathway. At the branch point of glycolysis, where fructose 1,6-bisphosphate is cleaved to form dihydroxyacetone phosphate and glyceraldehyde-3-phosphate, an isomerase reaction occurs which favours the formation of glyceraldehyde-3-phosphate. However, during *de novo* triglyceride biosynthesis, the dihydroxyacetone phosphate is instead converted to glycerol-3-phosphate by the action of the enzyme, glycerophosphate dehydrogenase (GPD). Glycerol-3-phosphate then undergoes a number of acylation reactions to form phosphatidate, which is hydrolysed then re-acylated to form triacylglycerol, the major form of lipid stored by the adipocyte.

1.5.8 *Terminal Differentiation.*

Maintenance of the terminally differentiated state is thought to be a consequence of continued expression of C/EBPα and is likely to result from combined functional effects: namely, the capacity to inhibit mitosis and transactivation of fat-specific genes. Sustained expression of C/EBPα results from autoactivation due to a C/EBP binding site in the proximal promoter (Lin and Lane, 1992). Agents such as TNFα cause dedifferentiation and this is associated with the loss of C/EBPα expression (see Section 1.4.4). Additionally, treatment of fully differentiated 3T3-L1 cells with either insulin or glucocorticoid results in both reduced expression and activity of the C/EBPα protein (MacDougald *et al*, 1994; MacDougald *et al*, 1995a). However, the physiological relevance of this observation remains unclear.

1.6 Obesity.

Adipocytes represent the major energy reserve of higher species and as such presumably evolved to aid survival during periods of nutrient deprivation. However, the lifestyle now prevalent in most western societies has rendered the requirement for such an energy reserve physiologically obsolete. In consequence, the availability of high-fat foodstuffs and a sedentary lifestyle are reflected in an increased incidence of obesity and its attendant syndromes, most notably cardio-vascular disease and non-insulin dependent diabetes.

Whilst the aetiology of obesity is complex and likely to be multi-factorial, it is becoming increasingly apparent that in some cases a genetic component potentiates individuals towards this disease. Recent advances in our understanding of the molecular biology of obesity have arisen from the cloning and characterisation of a number of genes associated with murine models of obesity. These include the genetic lesions associated with the *obese* (Zhang *et al*, 1994), *diabetes* (Tartaglia *et al*, 1995), *tubby* (Kleyn *et al*, 1996; Noben-Trauth *et al*, 1996) and *fat* (Naggert *et*

al, 1995) mice. At least one of these genes, *obese* (leptin), is a target of C/EBPα in mature adipocytes (MacDougald *et al*, 1995b; Hwang *et al*, 1996). Whilst the precise biological function of the obesity genes requires further investigation, a picture is emerging whereby the adipose secretes peptides, such as leptin, in response to feeding. These are targeted to specific receptors, including the product of the *diabetes* gene, within the hypothalamus which has been proposed to function as the "satiety" centre of higher organisms (Flier, 1995). In this manner, adipose is therefore functioning as an endocrine tissue, initiating a feedback loop in order to regulate food intake. Hence, the molecular biology of obesity has, in recent months, become an area of intense activity. Assuming that the observations made with murine models can be extrapolated to the human disease, the ultimate goal will be the introduction of therapeutics designed to specifically combat what is fast becoming one of the major health problems of the late twentieth century.

CHAPTER 2: Transcriptional Regulation of Adipogenesis.

2.1 Introduction.

As described in Chapter 1, acquisition of the adipocyte phenotype is associated with dramatic changes in gene expression. This phenomenon is largely mediated by the activity of *trans*-acting factors which either activate or derepress the transcription of adipocyte-specific genes. Several transcription factors implicated in adipogenesis have been cloned and characterised and these are discussed in detail below.

2.2 The CCAAT/Enhancer Binding Protein (C/EBP) Family.

The C/EBP proteins are members of the basic leucine zipper (bLZ) family of transcription factors (Hurst, 1994), which are characterised by a C-terminal DNA binding domain rich in basic amino acids, positioned immediately upstream of a protein dimerisation motif. The prototypic family member, C/EBPα, was originally characterised as a protein with the ability to bind both the CCAAT pentanucleotide present in the proximal promoter of many genes (Graves *et al*, 1986) and the enhancer core homology region of certain animal viruses (Johnson *et al*, 1987). Whilst the consensus DNA binding site is reported to be ATTGCGCAAT, a broad range of substitutions is tolerated (Osada *et al*, 1996). Protein dimerisation is mediated by the leucine zipper, which comprises five heptad repeats with a leucine residue at every seventh position, that forms an amphipathic α-helical array (Johnson and McKnight, 1989). Protein-protein interactions occur *via* the association of these α-helices to form a parallel, double stranded, coiled-coil structure, stabilised primarily by hydrophobic interactions. Following dimerisation, DNA binding is proposed to occur by the "scissors-grip" model (Vinson *et al*, 1989). In addition to forming homodimers, C/EBP proteins are also able to heterodimerise with other family members and with unrelated bLZ proteins,

including those of the ATF/CREB class (Vallejo *et al*, 1993). This creates functional diversity, as does the presence of highly divergent transactivation domains located in the N-terminus of the molecule (Friedman and McKnight, 1990). C/EBP proteins are expressed in a variety of cell types, where they function as mediators of both differentiation and stress responses. The role of individual C/EBP proteins during adipogenesis is discussed in Sections 2.2.1-2.2.4.

2.2.1 *C/EBPα.*

C/EBPα was originally characterised as a heat-stable protein present in rat liver (Landschulz *et al*, 1988), capable of binding two *cis*-regulatory elements (Section 2.2). A single 2.7kb mRNA gives rise to multiple isoforms (Ossipow *et al*, 1993), the 42-kDa and 30-kDa proteins being the most abundant. These differ at the N-terminus and are generated as alternative translation products by leaky ribosomal scanning (Calkhoven *et al*, 1994), initiation of the 30-kDa form occurring at an internal methionine codon located in a more favourable context than that for the 43-kDa form (Kozak, 1991). Expression of C/EBPα is limited to tissues displaying a high lipogenic capacity (Birkenmeier *et al*, 1989), including WAT, BAT, liver, lung and intestine, and whilst not strictly adipocyte-specific is probably the best characterised of all the adipogenic transcription factors.

Evidence for a role in adipogenesis comes from several sources. Expression of C/EBPα was seen to increase during induced differentiation of the 3T3-L1 cell line (Christy *et al*, 1989), due to transactivation of the *c/ebpα* gene (Christy *et al*, 1991). Subsequently, C/EBP binding sites were identified in the promoters of certain adipocyte-specific genes that are expressed in a temporal pattern suggestive of co-ordinate regulation by C/EBPα during differentiation. Indeed, co-transfection studies confirmed the ability of C/EBPα to both bind and transactivate the promoters of several of these genes, including the fatty acid binding protein,

aP2/422 (Herrera *et al*, 1989), stearoyl-CoA desaturase 1 [SCD1] (Christy *et al*, 1989) and GLUT 4 (Kaestner *et al*, 1990). Mutation of the C/EBP binding site in these promoter constructs specifically abolished this effect. However, whilst persuasive, such experiments do not in themselves provide direct evidence for a role for C/EBPα during adipogenesis. Thus, an alternative strategy was pursued.

Adopting an antisense approach, two separate groups were able to demonstrate that specific inhibition of C/EBPα was associated with a concomitant block to differentiation in 3T3-F442A and 3T3-L1 cells (Samuelsson *et al*, 1991; Lin and Lane, 1992). Expression of an antisense C/EBPα vector in these cell lines was associated with the loss of aP2/422, SCD1 and GLUT4 expression and an inability to accumulate cytoplasmic triglyceride. Interestingly, early markers such as lipoprotein lipase were unaffected, suggesting that C/EBPα is required for the terminal stages of differentiation. Expression of a sense C/EBPα construct in cell lines already harbouring the antisense vector was able to rescue the adipocyte phenotype.

Other workers sought to establish a role for C/EBPα in the differentiation process by transfection into preadipocytes. Assuming that the protein was necessary for adipogenesis, it was proposed that ectopic over-expression of C/EBPα in the undifferentiated cell type would result in adipogenic conversion. However, such studies were hampered by the anti-mitotic activity of C/EBPα, and stable cell lines expressing the protein could not be propagated. This was partially overcome by the use of a C/EBPα/oestrogen receptor fusion protein which was only active in the presence of β-oestradiol (Umek *et al*, 1991). However, contrary to expectation, activation of the fusion protein blocked mitosis but did not result in differentiation. Adipogenesis could only be achieved in the presence of external modulators. In contrast, Freytag and Geddes (1992) demonstrated that enforced expression of

C/EBPα in 3T3-L1 cells generated small foci that could not be propagated but underwent spontaneous adipogenic conversion. The conversion rate was extremely low (~12%) but provided the first indication that C/EBPα is sufficient for 3T3-L1 adipogenesis. Further studies employing an isopropyl β-D-thiogalactoside (IPTG) inducible C/EBPα vector in 3T3-L1 cells confirmed this finding (Lin and Lane, 1994). However, the most conclusive evidence that C/EBPα is a key factor in adipocyte differentiation comes from studies in cell lines that usually display no adipogenic capacity. Ectopic retroviral expression of C/EBPα was shown to promote the differentiation programme in a variety of murine fibroblastic cell lines, (Freytag *et al*, 1994), and this also occurred in the absence of inducing agents.

As described in Section 1.5.8, C/EBPα appears to have multiple roles in adipogenesis. Possibly the most intriguing of these is the ability to inhibit mitosis. This anti-mitotic function has been mapped to the N-terminal 12-kDa portion of the molecule, since over-expression of the 30-kDa isoform, which lacks this domain, in 3T3-L1 cells does not inhibit proliferation (Lin *et al*, 1993). Since C/EBPα is induced at the cessation of clonal mitotic expansion, it has been proposed that it may be required for cells to enter the differentiation-specific growth arrest, G_D (see Section 1.5.4). Whilst the mechanism by which C/EBPα exerts this effect remains unclear, various possibilities exist, with analogies being drawn from the myogenic lineage. Both MyoD (Halevy *et al*, 1995) and Myogenin (Andres and Walsh, 1996) are proposed to mediate cell cycle withdrawal during myogenesis *via* the induction of the cyclin-dependent kinase inhibitor (CKI), p21. It is therefore of interest to note that expression of p21 was induced during growth arrest of a human fibrosarcoma cell line conditionally over-expressing C/EBPα (Timchenko *et al*, 1996). Whether C/EBPα employs such a mechanism during adipogenesis remains unclear. However, in 3T3-L1 cells, C/EBPα was found to induce the expression of the growth arrest and DNA damage gene, *gadd*45, with similar kinetics to aP2/422

and SCD1 (Constance *et al*, 1996), suggesting a way in which mitotic arrest and fat-specific gene expression could be coupled. Another candidate is the product of the retinoblastoma susceptibility gene, pRb. This protein is both induced and bound by MyoD (Martelli *et al*, 1994) and its growth suppressive activity is thought to be required for myogenesis (Gu *et al*, 1994). Myocytes lacking pRb accumulate in the S and G2 phases of the cell cycle and display defective muscle-specific gene expression (Novitch *et al*, 1996). Similarly, lung fibroblasts derived from *rb* null mice are unable to undergo adipogenesis (Chen *et al*, 1996a). In this system, pRb was found to influence terminal differentiation of wild type cells through direct interaction with C/EBP family members. However, work by Hendricks-Taylor and Darlington (1995) suggests that C/EBPα inhibits proliferation in many cell types in the absence of pRb.

Conclusions drawn regarding the role of C/EBPα during *in vitro* adipogenesis have been substantiated *in vivo* using nullizygous mouse models. Mice homozygous for a targeted deletion of the *c/ebpα* gene display abnormalities in both WAT and BAT (Wang *et al*, 1995), and have a reduced capacity to accumulate lipids. In addition, hyperproliferation is observed in the lung and liver, supporting a role for C/EBPα in cell cycle withdrawal (Flodby *et al*, 1996). However, the major feature of these mice is their inability to synthesise and store liver glycogen and, in concert with a number of other metabolic defects, they rapidly become hypoglycaemic and die within 8 hours *post-partum*. Since C/EBPα is known to transactivate a number of liver-specific genes in addition to those expressed during the terminal stages of adipogenesis, it has therefore been proposed that this transcription factor acts to co-ordinate the expression of genes essential for integrative metabolic processes (Darlington *et al*, 1995) and hence is a critical regulator of global energy homeostasis (Yeh and McKnight, 1995).

2.2.2 *C/EBPβ.*

C/EBPβ was cloned by Cao and co-workers (1991) in an attempt to identify factors that may be involved in adipogenesis, but was later found to be identical to a number of previously characterised proteins, including liver activatory protein [LAP] (Descombes *et al*, 1990), NF-IL6 (Akira *et al*, 1990) and IL-6DBP (Poli *et al*, 1990). As described above for C/EBPα, two isoforms of C/EBPβ are derived from a single mRNA species by leaky ribosomal scanning, LAP (32-kDa) and a 20-kDa form referred to as the liver inhibitory protein [LIP] (Descombes and Schibler, 1991). Whilst LAP and LIP possess identical bLZ domains, LIP lacks the N-terminal transactivation domain. As such, LIP is incapable of transactivating target genes harbouring C/EBP sites and so may be regarded as a dominant negative inhibitor of other C/EBP family members. It is proposed that LIP functions either as an inactive homodimer that competitively occupies the binding site, or else heterodimerisation generates complexes with impaired transactivation potential. Interestingly, the LAP:LIP ratio increases during adipogenesis, suggesting that LIP may be required to modulate the effects of LAP in the earlier stages of the differentiation programme.

C/EBPβ is expressed in a number of cell types with high lipogenic or metabolic capacity, and functions in a variety of biological processes including haemopoiesis (Muller *et al*, 1995; Wall *et al*, 1996) and the acute phase response (An *et al*, 1996; Estes *et al*, 1995). In the 3T3-L1 cell line, C/EBPβ is induced by MIX during clonal expansion, the levels declining thereafter (Yeh *et al*, 1995a). This temporal expression pattern and the presence of a specific binding site in the C/EBPα promoter suggested that C/EBPβ may induce the expression of C/EBPα, possibly in combination with C/EBPδ (Figure 3). Hence, C/EBPβ is implicated in a cascade of regulation during adipogenesis, relaying the effects of external modulators to the terminal phases of the differentiation programme (Yeh *et al*, 1995a).

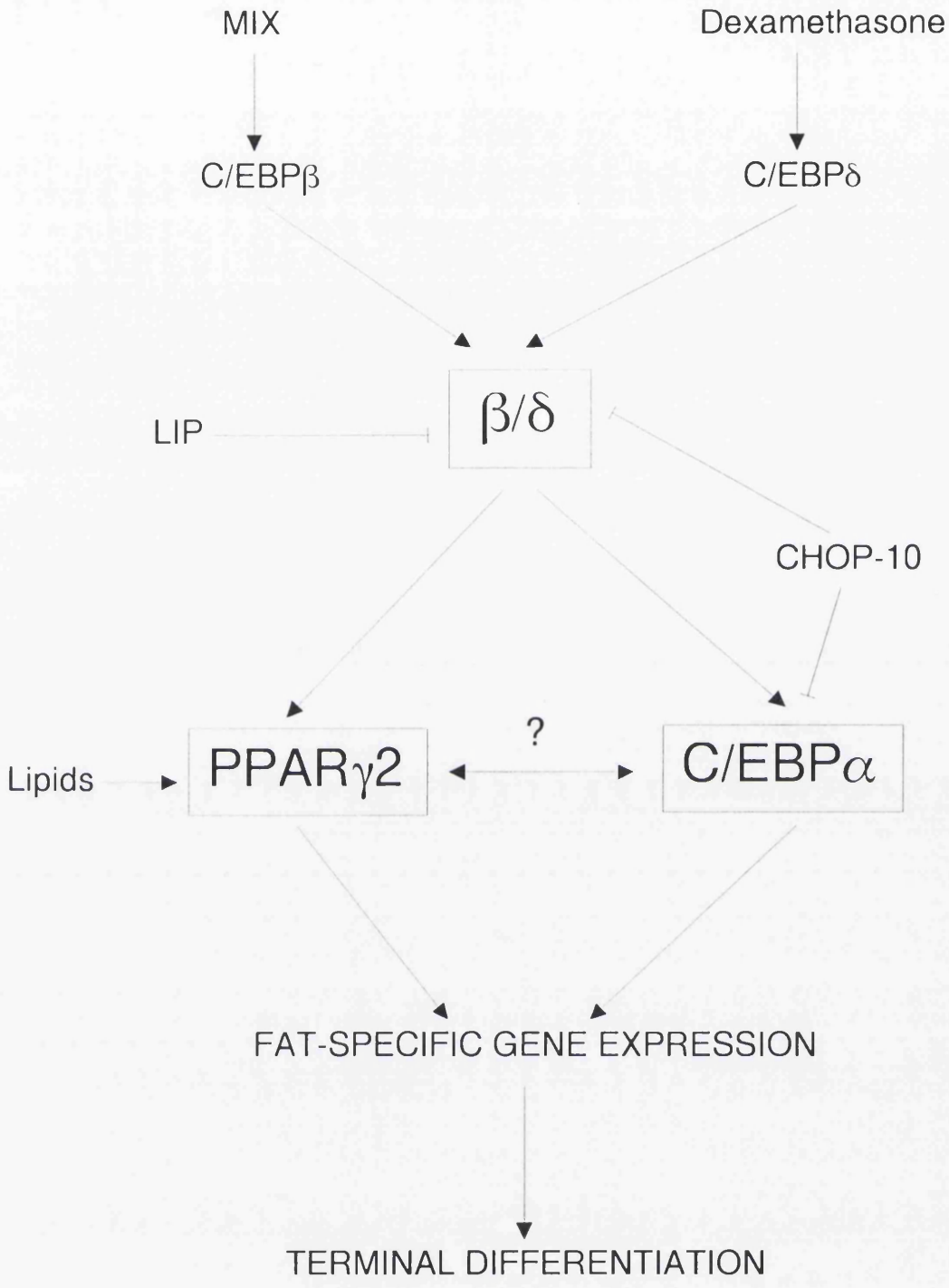


Figure 3. Interaction of C/EBP and PPAR Families During Adipogenesis.

A role for C/EBPβ in adipogenesis has also been demonstrated by transfection experiments. Retroviral expression of LAP in 3T3-L1 cells resulted in differentiation in the absence of inducers (Yeh *et al*, 1995a), whilst over-expression of LIP inhibited adipocyte conversion. Previously, work by Freytag and co-workers (1994) had suggested that retroviral expression of C/EBPβ in a range of fibroblastic cell lines did not result in spontaneous differentiation. However, Yeh *et al* (1995) were able to demonstrate that ectopic over-expression of C/EBPβ in the NIH-3T3 cell line was adipogenic, but only in the presence of hormones. This finding was confirmed by the conditional expression of C/EBPβ in NIH-3T3 cells (Wu *et al*, 1995). Interestingly, in this system, C/EBPβ did not induce the expression of C/EBPα, despite acquisition of the adipocyte phenotype, and a similar finding was reported by Yeh *et al* (1995). This suggests that C/EBPβ may transactivate additional genes required to confer tissue specificity. Indeed, Wu *et al* (1995) demonstrated that conditional over-expression of C/EBPβ in NIH-3T3 cells specifically induced the expression of the adipocyte specific transcription factor, PPARγ2.

Since C/EBPβ is highly expressed during the clonal expansion phase which precedes terminal differentiation, it is possible that this protein could also play a role in proliferation. Growth hormone treatment of the 3T3-F442A cell line induces the expression of the immediate early response genes, c-*fos* and c-*jun*, and this appears to be a consequence of increased C/EBPβ DNA binding activity (Clarkson *et al*, 1995). However, the C/EBPβ nullizygous mouse is characterised by increased proliferation, particularly within the myeloid and lymphoid compartments (Screpanti *et al*, 1995). Finally, C/EBPβ has been found to interact directly with the cell cycle regulator pRb, both in myeloid cells (Chen *et al*, 1996b) and lung fibroblasts (Chen *et al*, 1996a-see Section 2.2.1), and this is associated with increased DNA binding and transactivation potential. Whether this represents

a growth suppressive activity or is unrelated to the ability of pRb to mediate cell cycle withdrawal remains to be determined.

2.2.3 *C/EBPδ*

C/EBPδ was originally isolated as a protein expressed transiently during the early stages of adipogenesis (Cao *et al*, 1991) and was later found to be specifically induced by dexamethasone (Yeh *et al*, 1995a). The protein is present as a single species of 29-kDa and is implicated in the transactivation of C/EBPα. Like other family members, C/EBPδ is expressed in a variety of tissues and is involved in the acute phase response (Ramji *et al*, 1993). However, the precise role of C/EBPδ in adipogenesis is not well characterised. Retroviral expression in 3T3-L1 cells results in a modest differentiation potential (Yeh *et al*, 1995a), whilst over-expression in NIH-3T3 cells is not adipogenic. However, co-expression of C/EBPβ and δ in this cell line has a synergistic effect, both on adipocyte conversion and expression of PPARγ2 (Wu *et al*, 1996). Since PPARγ2 is known to possess two C/EBP binding sites (Zhu *et al*, 1995), it is likely that C/EBPβ and δ act in concert to induce the expression of this transcription factor during adipogenesis (Figure 3).

2.2.4 *CHOP-10/GADD153.*

The C/EBP homologous protein, CHOP-10, was cloned from a 3T3-L1 cDNA library by its ability to bind C/EBPβ (Ron and Habener, 1992) and was later found to be the murine homologue of the growth arrest and DNA damage gene, *gadd*153 (Fornace *et al*, 1989). As such, the activity of CHOP-10 is well documented in the stress response (Wang *et al*, 1996; Guyton *et al*, 1996). Additionally, chromosomal translocations of CHOP-10 are observed in a number of malignancies (Sanchez-Garcia and Rabbitts, 1994), most notably myxoid liposarcoma (Crozat *et al*, 1993).

Whilst considered a member of the C/EBP family by virtue of sequence similarity, CHOP-10 is unique in that the basic DNA binding region contains helix-breaking amino acid substitutions (Ron and Habener, 1992), rendering it incapable of site-specific DNA binding. However, the protein retains the capacity to heterodimerise with other C/EBPs and so acts as a dominant negative inhibitor. In certain cases CHOP/C/EBP dimers may be directed to non-canonical binding sites (Barone *et al*, 1994), although the biological relevance of this finding remains unclear. CHOP-10 has been shown to inhibit differentiation of the 3T3-L1 cell line, and this phenotype may be reversed by over-expressing C/EBPα (Batchvarova *et al*, 1995). However, it now seems likely that the key role of CHOP-10 during adipogenesis is as a glucose sensor. CHOP-10 is induced by low concentrations of glucose (Carlson *et al*, 1993), whilst maintaining glucose at high levels throughout the differentiation programme abrogates this effect. This suggests that CHOP-10 may only operate when cells are stressed, for example by nutrient deprivation, and that under normal conditions it is not a physiological regulator of adipogenesis.

2.3 The Peroxisome Proliferator-Activated Receptor (PPAR) Family.

The PPAR proteins are members of the nuclear hormone receptor superfamily (Calkhoven and Ab, 1996), which link lipophilic extracellular signals to gene expression. These transcription factors are characterised by a conserved DNA binding domain, containing two zinc finger motifs, and a divergent ligand binding and dimerisation domain. Increased transactivation of target genes is mediated by specific binding of endogenous ligand. PPARs were first isolated by virtue of their activation by peroxisome proliferators (Isseman and Green, 1990), a diverse range of compounds, classically associated with hepatic peroxisomal proliferation and induction of the β-oxidation pathway in rodent models. Three family members have since been cloned from *Xenopus* (Dreyer *et al*, 1992), which differ in their pharmacological responses to the various activators, suggesting distinct biological

functions. PPARs bind to a specific response element, the PPRE, comprising direct repeats of the sequence AGGTCA, separated by a single nucleotide, the so-called DR-1 motif (Dreyer *et al*, 1992). DNA binding requires heterodimerisation with members of the retinoid X receptor (RXR) family (Kliewer *et al*, 1992) and is associated with the transactivation of several enzymes involved in lipid metabolism (Tugwood *et al*, 1992; Castelein *et al*, 1994).

2.3.1 *PPARγ2.*

Whilst C/EBPα is enriched in adipose, it is also expressed in a variety of other cell types, suggesting that it is unlikely to be the sole determinant of adipocyte-specific gene expression during terminal differentiation. Indeed, the portion of the aP2/422 gene which confers tissue specificity in transgenic mice lacks a consensus C/EBP binding site (Ross *et al*, 1990). The active region was mapped to a 518bp enhancer element, located approximately 5kb upstream of the transcriptional initiation site (Graves *et al*, 1991) and was found to comprise five protein recognition sites, designated adipose regulatory elements (AREs). Two of these domains, ARE6 and 7, were bound by an adipocyte-specific factor, ARF6 (Graves *et al*, 1992). Binding of ARF6 was both necessary and sufficient for adipocyte-specific gene expression and transactivation of ARE6/7 reporter constructs occurred in adipocytes but not preadipocytes (Graves *et al*, 1992). Mutation of the ARE6 site abolished this effect.

The ARF6 target sequence was found to share sequence homology with the DR-1 site bound preferentially by RXR/PPAR heterodimers. Subsequently, Tontonoz and co-workers (1994a) cloned the murine homologue of *Xenopus* PPARγ from an adipocyte cDNA library, adopting a polymerase chain reaction (PCR) approach to identify clones with sequence similarity to RXR and PPAR family members. This adipocyte-specific form was referred to as PPARγ2. Simultaneously, other

laboratories cloned a second isoform, PPARγ1 (Kliewer *et al*, 1994; Zhu *et al*, 1993). These isoforms were found to arise from a single gene (Zhu *et al*, 1995), containing two promoters that generate two alternatively spliced gene products, differing at the N-termini. PPARγ1 is expressed at low levels in many tissues, whilst PPARγ2 is present predominantly in adipose and is expressed with similar kinetics to C/EBPα during differentiation. Subsequently, characterisation of ARF6 purified from the HIB-IB cell line demonstrated that it was a heterodimer, composed of PPARγ2 and RXRα (Tontonoz *et al*, 1994c).

PPARγ2 was originally classified as an "orphan" receptor since the natural ligand was unknown. As described above, synthetic activation of the PPARs could be achieved by a number of structurally unrelated chemicals, including fatty acid analogues. Indeed, treatment of U2OS osteosarcoma cells conditionally over-expressing PPARγ2 with eicosanoids resulted in increased transactivation potential (Yu *et al*, 1995). This effect was blocked by indomethacin, which inhibits prostaglandin biosynthesis. Subsequently, the endogenous ligand was shown to be a prostaglandin J_2 metabolite (Forman *et al*, 1995; Kliewer *et al*, 1995), thus linking fatty acid stimulation to adipocyte differentiation.

As described for C/EBP family members, a role for PPARγ2 in directing adipocyte differentiation was demonstrated by retroviral expression in NIH-3T3 cells (Tontonoz *et al*, 1994b). In this case, the differentiation potential of PPARγ2-expressing cells was stimulated by treatment with various PPAR activators. However, co-expression of PPARγ2 with C/EBPα resulted in differentiation in the absence of external stimulants. This synergy suggests that PPARγ2 and C/EBPα may co-operate to orchestrate the events associated with terminal differentiation. Indeed, co-expression of these transcription factors in myoblasts is sufficient to cause transdifferentiation to the adipocyte phenotype (Hu *et al*, 1995). As

described in Section 2.2.1, expression of both PPARγ2 and C/EBPα during the differentiation programme appears to be under the control of C/EBPβ and δ, and there is currently some speculation that PPARγ2 and C/EBPα may also cross-regulate one another. In addition, PPARγ2 and C/EBPα share a common set of target genes, including aP2/422 and phosphoenolpyruvate carboxykinase (Tontonoz *et al*, 1995b). Hence, a picture emerges (Figure 3) whereby a cascade of regulatory signals is channelled towards the activation of C/EBPα and PPARγ2, thereby culminating in the co-ordinate expression of genes required to execute the final stages of the adipogenic programme.

2.3.2 *Fatty Acid Activated Receptor (FAAR).*

It is well established that fatty acids influence the early stages of adipogenesis (Abumrad *et al*, 1991; Abumrad *et al*, 1993). In an attempt to understand this phenomenon, Amri and co-workers (1995) cloned a protein from an ob1771 library that appeared to mediate the transcriptional effects of fatty acids. This protein was referred to as the fatty acid activated receptor (FAAR), but was later found to be identical to human Nuc1 and PPARδ (Kliewer *et al*, 1994). Like other PPAR family members, FAAR binds to DR-1 elements as a heterodimer with RXR proteins. FAAR is expressed in a wide range of tissues, including adipose, and whilst undetectable in growing ob1771 cells, is strongly induced at confluence. This expression is augmented by treatment with palmitate. Ectopic expression of FAAR in the differentiation defective cell line, 3T3-C2, conferred fatty-acid responsiveness on both a lipid binding protein, ALBP, and the lipid transporter, FAT (Amri *et al*, 1995), and there is some evidence to suggest that FAAR may also regulate the expression of lipoprotein lipase in response to lipids (Amri *et al*, 1996). However, Schoonjans *et al* (1996) have recently demonstrated that upregulation of lipoprotein lipase in adipose is mediated by PPARγ2, whilst in liver, tissue-specific expression is directed by PPARα. Since the gold standard of adipogenic potential

appears to be the ability to convert fibroblastic cell lines to the adipocyte phenotype, it is therefore of interest to note that in a recent study both PPARα and γ2 were able to induce differentiation of NIH-3T3 cells in response to various PPAR activators, whilst ectopic expression of FAAR was without effect (Brun *et al*, 1996). In contrast, FAAR had been previously reported to mediate the conversion of myoblasts to adipocytes following fatty acid treatment (Teboul *et al*, 1995). Clearly, the role of FAAR in adipocyte differentiation requires further investigation.

2.4 Adipocyte Determination and Differentiation Factor 1 (ADD1).

Transcription factors of the basic region-helix-loop-helix (bHLH) class have been implicated in cell fate determination in a number of lineages, including MyoD in myogenesis, SCL in haemopoiesis and the achaete-scute complex in neurogenesis (Jan and Jan, 1993). In addition to the basic DNA binding domain, these proteins are characterised by a protein dimerisation motif (HLH), typified by two amphipathic α helices joined by an intervening theta form loop, and the ability to bind the canonical E-box sequence, CANNTG. Reasoning that adipocyte determination may also be mediated by such a factor, a protein was cloned from a rat adipocyte library *via* interaction with the E-box motif of the fatty acid synthase gene (FAS). This protein was found to be a novel member of the bHLH-leucine zipper (bHLH-LZ) family and was designated adipocyte determination and differentiation factor 1 [ADD1] (Tontonoz *et al*, 1993). ADD1 is expressed at low levels in several tissues, including WAT, but is found predominantly in BAT. Both 3T3-F442A preadipocytes and 10T1/2 cells express low levels of the protein, which is seen to increase dramatically following hormonal induction of adipogenesis, suggesting that ADD1 may function in a manner analogous to MyoD, which is also present in the undifferentiated cell type. ADD1 is able to transactivate the promoters of two adipocyte-specific genes, FAS (Tontonoz *et al*, 1993) and

lipoprotein lipase (Kim and Spiegelman, 1996). Retroviral expression of ADD1 in NIH-3T3 cells had a modest adipogenic effect, which could be augmented by co-expression with PPARγ2 (Kim and Spiegelman, 1996), whilst expression of a dominant negative form of ADD1 in the 3T3-L1 cell line repressed adipogenesis. The human homologue of ADD1 was independently identified as the sterol response element binding protein [SREBP1] (Wang *et al*, 1993), that binds the motif ATCACCCCAC (Yokoyama *et al*, 1993), and is implicated in the transcriptional regulation of cholesterol homeostasis. This suggests that ADD1 may play multiple roles in lipid metabolism.

2.5 HNF-3/*Forkhead.*

Lipoprotein lipase is one of the earliest genes to be induced during adipogenesis. Differentiation-linked expression was found to be conferred by two *cis*-regulatory elements, LP-α and LP-β, (Enerback *et al*, 1992), which share sequence homology to the binding site for members of the HNF-3/*forkhead* family of transcription factors, which are implicated in tissue-specific gene expression and developmental processes (Clevidence *et al*, 1993; Kaestner *et al*, 1993). Indeed, a HNF-3/*forkhead*-like binding activity was detected in 3T3-F442A cells and this was found to increase during differentiation. Lipoprotein lipase is induced in preadipocytes as they reach confluence and appears to be independent of the action of the adipogenic hormones. At this time, the α2 chain of Type IV collagen is also being expressed and a dense extracellular matrix (ECM) is deposited. During liver differentiation, HNF-3 expression and subsequent transactivation of the albumin gene promoter requires stimulation by ECM-associated collagen (Liu *et al*, 1991). By analogy, it is therefore proposed that the early phases of adipocyte differentiation are mediated by similar interactions and this explains why growth arrest at confluence is an absolute requirement for the differentiation programme to proceed efficiently.

CHAPTER 3: c-Myc: a Multifunctional Transcriptional Regulator.

3.1 Introduction.

The c-*myc* proto-oncogene is the prototypic member of a highly related gene family, which is strongly implicated in the genesis of a wide range of human malignancies. The gene itself encodes an unstable nuclear phosphoprotein comprising several domains consistent with a role in transcriptional regulation and is thought to function in a variety of biological processes, including the inhibition of cellular differentiation.

3.2 Myc Family Proteins.

c-*myc* was originally identified as the cellular homologue of a gene transduced by the transforming avian retrovirus, MC29 (Vennstrom *et al*, 1982). Subsequently, two further *myc* family members were isolated from amplified regions detected in naturally occurring tumours. These were designated N- and L-*myc* and were associated with neuroblastoma (Schwab *et al*, 1983) and small cell lung carcinoma (Nau *et al*, 1985) respectively. All *myc* family members share a characteristic three exon structure, whereby exons two and three constitute a single open reading frame, whilst exon one is essentially non-coding (Kelly and Siebenlist, 1986). This first exon is thought to regulate gene expression. The protein itself comprises several distinct domains and is a member of the basic helix-loop-helix leucine zipper (bHLH-LZ) family of transcription factors (Torres *et al*, 1992), which also includes ADD1 (Tontonoz *et al*, 1993) and USF (Gregor *et al*, 1990).

As described in Chapter 2, the basic region mediates sequence-specific DNA binding, whilst the HLH and LZ motifs provide a dimerisation interface. Like ADD1 (Section 2.4), Myc family members bind to the sequence CACGTG (Blackwell *et al*, 1990; Prendergast and Ziff, 1991) and protein dimerisation is a

pre-requisite for this activity. Whilst Myc is able to bind DNA as a homodimer *in vitro*, this is not thought to occur *in vivo* where the physiologically relevant form is a heterodimer with a second, but unrelated, bHLH-LZ protein, Max (Blackwood and Eisenman, 1991). Max is a stable nuclear phosphoprotein, constitutively expressed in a wide range of cell types and during diverse cellular conditions. Two major forms are generated by alternative splicing, p21 and p22, which differ only by a nine amino acid insertion N-terminal to the basic region in p22. These are able to form both homo and heterodimers with intrinsic DNA binding capacity but the physiological relevance of the two isoforms is not yet known.

In addition to the C-terminal bHLH-LZ motif, Myc proteins also contain an N-terminal domain with transactivating potential. This region is characteristically proline and glutamine-rich, interspersed with acidic regions, and when fused to the DNA binding domain of the yeast protein GAL4 is able to stimulate expression of a reporter gene construct (Kato *et al*, 1990).

3.3 Myc Functions as a Transcription Factor.

3.3.1 *Myc as an Activator.*

Transactivation is a property of the Myc/Max heterodimer, and requires the Max bHLH-LZ domain but *both* the Myc bHLH-LZ and N-terminal regions (Amati and Land, 1994), suggesting that Max may be required solely for DNA binding. Indeed, Max contains no obvious transactivation domain and so is likely to be transcriptionally inert (Kato *et al*, 1992). Since these regions of Myc are essential for its growth regulatory properties (Stone *et al*, 1987), it appears that dimerisation, DNA binding and transactivation are fundamental to Myc activity. That the Myc/Max complex is the biologically active form has been verified by genetic complementation studies (Amati *et al*, 1993). In addition, using synthetic promoter constructs containing the CACGTG binding site, it has been demonstrated that the

Myc/Max complex can activate transcription in both mammalian and yeast cells (Kretzner *et al*, 1992; Amati *et al*, 1992; Crouch *et al*, 1993). However, Max homodimers repress this activity, suggesting that the relative Myc:Max ratio may also influence the ability of Myc to promote gene expression.

Whilst c-Myc is known to function in a variety of biological phenomena (Section 3.6), the identification of Myc target genes has thus far proven somewhat limited. Candidates include ornithine decarboxylase [ODC] (Bello-Fernandez *et al*, 1993; Wagner *et al*, 1993), α-prothymosin (Eilers *et al*, 1991), p53 (Roy *et al*, 1994) and ECA39 (Benvenisty *et al*, 1992), all of which contain one or more CACGTG motifs downstream of the transcription initiation site (Vastrik *et al*, 1994). Target genes most recently identified include an RNA helicase (Grandori *et al*, 1996), the eukaryotic initiation factor 4E (Jones *et al*, 1996) and the cell cycle phosphatase, cdc25 (Galaktionov *et al*, 1996). However, the physiological relevance of many of these genes for Myc function is not entirely clear.

3.3.2 *Myc as a Repressor.*

The search for direct targets of c-Myc transcriptional activity led investigators to conclude that Myc may also repress gene expression (Kaddurah-Daouk *et al*, 1987). Genes repressed by c-Myc include C/EBPα (Li *et al*, 1994), collagen (Yang *et al*, 1991), Cyclin D1 (Philipp *et al*, 1994), CHOP-10 (Chen *et al*, 1996), LFA-1 (Inghirami *et al*, 1990) and c-Myc itself (Grignani *et al*, 1990; Crouch *et al*, 1990). Additionally, N-Myc has been shown to down-regulate neural cell adhesion molecule (N-CAM) expression in neuroblastoma cells (Akeson and Bernards, 1990). In some cases, repression apparently occurs *via* an alternative binding site within the promoter of susceptible genes, known as the initiator element [Inr] (Smale and Baltimore, 1989) and may involve interaction with proteins other than Max.

3.4 Alternative Myc Binding Proteins.

Several alternative Myc binding partners have recently been described. A number of these are factors involved in transcriptional initiation and include the TATA box binding protein [TBP] (Maheswaran *et al*, 1994), TFII-I (Roy *et al*, 1993) and YY1 (Shrivastava *et al*, 1993; Shrivastava *et al*, 1996). TFII-I is thought to be involved in the Myc-mediated repression of gene expression *via* the Inr (Roy *et al*, 1993; Phillip *et al*, 1994). Additionally, the Myc transactivation domain contains a region capable of binding *in vitro* to the cell cycle regulator, pRb (Rustgi *et al*, 1991). However, work by Gu *et al* (1994) suggests that *in vivo* Myc may associate with the pRb family member, p107, and that this results in suppression of Myc-associated transactivation (Beijersbergen *et al*, 1994). Interaction of Myc with TBP overlaps this p107 binding site (Hateboer *et al*, 1993). Other proteins found to associate with c-Myc include AP-2 (Gaubatz *et al*, 1995), α-tubulin (Alexandrova *et al*, 1995), and the novel proteins Nmi (Bao and Zervos, 1996) and BIN1 (Sakamuro *et al*, 1996).

3.5 Alternative Max Binding Proteins.

Like Myc, Max also has the ability to associate with other proteins, most notably a family of novel bHLH-LZ factors, designated Mad1 (Ayer *et al*, 1993), Mxi1/Mad2 (Zervos *et al*, 1993), Mad3 and Mad4 (Hurlin *et al*, 1995a). These proteins dimerise specifically with Max and as such compete with equal affinity to Myc for Max association and recognition of the consensus binding site. As reported for Max/Max homodimers, Mad/Max complexes also repress gene expression (Wu *et al*, 1996). This activity appears to be dependent on the co-repressor, mSin3, which binds to the paired α-helical (PAH) region located at the N-terminus of the various Mad proteins (Ayer *et al*, 1995; Schreiber-Agus *et al*, 1995). Since expression of the Mad proteins is reciprocal to that of c-Myc during a number of cellular processes, it has been proposed that Mad family function may be to antagonise Myc

activity. Hence, whilst Myc positively regulates proliferation and apoptosis, Mad1-4 may be required to promote differentiation and cell survival (Amati and Land, 1994).

3.6 Biological Functions of Myc.

3.6.1 *Proliferation.*

Whilst present at low or undetectable levels in quiescent and differentiated cells, expression of c-*myc* correlates strongly with cellular proliferation. Myc expression is induced upon mitogenic stimulation in a manner that is independent of *de novo* protein synthesis (Kelly *et al*, 1983). Hence, c-*myc* is classified as an immediate early response gene. The rapid induction of Myc is a critical event in the G_0-G_1 transition and it has been demonstrated that conditional activation of a c-Myc-oestrogen receptor fusion protein is sufficient to mediate cell cycle entry in the absence of exogenous growth factors (Eilers *et al*, 1991). However, in contrast to other genes of this class (e.g. c-*fos*; c-*jun*) which are only expressed transiently during this phase, Myc is required at a basal level throughout the cell cycle (Hann *et al*, 1985), suggesting a role in the maintenance of proliferation. In contrast, Mad proteins are detected in quiescent cells whilst Max is constitutively expressed regardless of the cell cycle status. Myc presumably exerts its effects on proliferation by modulating genes required during the cell cycle. For example, ODC, the rate limiting enzyme of polyamine biosynthesis, and α-prothymosin are both required for S phase. Additionally, Myc may activate the expression of Cyclins A and E (Jansen-Durr *et al*, 1993) and more recently has been shown to positively regulate the expression of cdc25 (Galaktionov *et al*, 1996).

3.6.2 *Transformation.*

As described in Section 3.2, c-*myc* was originally characterised as the cellular homologue of the transforming component of MC29. The viral gene is composed

predominantly of sequences derived from the coding regions of chicken c-*myc*, suggesting that loss of the first exon correlates with deregulated expression. Indeed, this appears to be the case in Myc-associated malignancies where inappropriate expression of an essentially normal protein results in oncogenesis. Hence, DNA rearrangements generally lead to quantitative rather than qualitative differences (Cole, 1986). The transforming potential of Myc *in vitro* requires the co-operation of a second activated oncogene, such as Ha-*ras* (Land *et al*, 1983), consistent with the multi-step model of carcinogenesis (Vogelstein and Kinzler, 1993). Thus, in most primary cell lines Myc requires another oncogene for transformation, whilst in established cell lines Myc can act alone. In contrast, high levels of Max (Prendergast *et al*, 1992) or the Mad family proteins (Lahoz *et al*, 1994) can suppress Myc-mediated transformation.

3.6.3 *Apoptosis.*

Apoptosis (programmed cell death) is a physiologically relevant cell suicide mechanism, distinct from necrosis, whereby cells are triggered to die by a range of stimuli that induce the expression of a novel set of "death genes" (White, 1996). Paradoxically, whilst Myc is intimately associated with proliferation, it is also able to induce apoptosis under sub-optimal growth conditions (Evan *et al*, 1992). This activity is blocked by a discrete class of cytokine (Harrington *et al*, 1994). Since Myc-induced apoptosis has the same requirements as gene activation (Section 3.3.1) it is likely that Myc is modulating the expression of apoptotic genes. A possible candidate is ODC (Packham and Cleveland, 1994), the activity of which ultimately results in the generation of reactive oxygen species, thought to be key modulators of apoptosis.

3.7 The Role of Myc in Development and Cellular Differentiation.

3.7.1 *Development.*

The expression pattern of *myc* family genes during development is well documented (Downs *et al*, 1989; Schmid *et al*, 1989) and correlates with the types of tumour induced by deregulated expression of each Myc protein. Whilst c-*myc* is expressed at all stages and in many tissues during development, expression of N- and L-*myc* is restricted to specific stages and cell types. Surprisingly, the correlation between proliferation and expression of c-*myc* is not strong in the early stages of embryogenesis, whilst during organogenesis c-*myc* is readily detected in tissues with a high proliferative capacity. There are exceptions to this general rule, however. Expression of c-*myc* is maximal in cells of mesodermal origin but low in endoderm and ectoderm, even though all three tissues are actively proliferating. Additionally, c-*myc* has been detected in both proliferating and post-mitotic lens cells (Harris *et al*, 1992) and lens development is unaffected by enforced expression of a *myc* transgene (Morgenbesser *et al*, 1995). In contrast, expression of N- and L-*myc* is often associated with the onset of differentiation and appears not to correlate that well with proliferative potential.

The role of Myc family members in development has been investigated using nullizygous mouse technology. Mice homozygous for a targeted deletion of c-*myc* die between day 9.5 and 10.5 of gestation (Davis *et al*, 1993) and display retarded development. Abnormalities included defects of the heart, pericardium and neural tube. This suggests that c-*myc* may not be required for the early stages of development, including embryonic stem cell proliferation, but is essential for survival after day 10.5. Work by three separate groups showed that N-*myc* null mice are also embryonic lethal (Stanton *et al*, 1992; Charron *et al*, 1992; Sawai *et al*, 1993). These mice die between day 11.5 and 12.5 of development and display deficiencies in normal organogenesis. In contrast, L-*myc* null mouse are viable,

surviving well into adulthood with no obvious abnormalities (Hatton *et al*, 1996). Thus it seems likely that Myc proteins have some overlapping functions during normal development, and so may be able to substitute for one another at a given stage. However, individual proteins also seem to possess discrete functions which cannot be fulfilled in their absence by other family members. In contrast, Max is essential for survival at an early stage, null mutations being embryonic lethal at day 3.5 to 6.5 (Henriksson and Luscher, 1996). This suggests that Max function is unique and cannot be compensated for by another protein. Whilst the effects of targeted deletions within *mad* family genes have yet to be reported, the expression pattern during development is well established, with *mad* mRNAs being detected almost exclusively in differentiating cell types (Vastrik *et al*, 1995; Chin *et al*, 1995; Hurlin *et al*, 1995a)

The expression pattern of *myc* genes in adult tissues has also been determined (Zimmerman *et al*, 1986). Expression of c-*myc* is relatively generalised and corresponds to tissues that are actively proliferating, for example during liver (Makino *et al*, 1984) and muscle (Izumo *et al*, 1988) regeneration. In addition, c-*myc* is also highly expressed in cells that are undergoing apoptosis, such as mammary tissue during involution (Strange *et al*, 1992). In contrast, expression of N- and L-*myc* is restricted to a subset of differentiated tissues, as are the *mad* genes.

3.7.2 *Differentiation.*

Rapid down-regulation of c-*myc* expression is observed in many cell lines following exposure to differentiation inducers. These include murine erythroleukaemia (MEL) cells (Lachman and Skoultchi, 1984), HL60 promyelocytic cells (Westin *et al*, 1982; Reitsma *et al*, 1983), U937 monoblastic cells (Einat *et al*, 1985) and primary keratinocytes (Dotto *et al*, 1986). This generally occurs prior to growth arrest and initiation of terminal differentiation.

However, in some lines, such as MEL (Lachman and Skoultchi, 1984) and L6E9 myoblasts (Endo and Nadal-Ginard, 1986), *myc* down-regulation is biphasic, with a transient decrease being followed by a second peak of activity which declines later. In contrast, in the F9 teratocarcinoma cell line down-regulation of c-*myc* is delayed until the terminal stages of differentiation (Campisi *et al*, 1984). That *myc* down-regulation is necessary for differentiation has been confirmed by a number of anti-sense experiments. Expression of a *myc* anti-sense construct in MEL cells resulted in accelerated differentiation (Prochownik *et al*, 1988), whilst a similar approach in the HL60 cell line suppressed expression of the endogenous c-*myc* gene, reduced proliferation and potentiated monocytic differentiation (Yokoyama and Imamoto, 1987; Holt *et al*, 1988). In F9 cells expression of anti-sense *myc* resulted in differentiation indistinguishable from that induced by retinoic acid (Griep and Westphal, 1988). However, over-expression of a sense construct generated F9 cell lines that were resistant to induced differentiation. Indeed, over-expression of Myc proteins has been shown to inhibit differentiation in a number of cell types, including MEL (Coppola and Cole, 1986), U937 (Larsson *et al*, 1988), primary quail myoblasts (Falcone *et al*, 1985) and preadipocytes (Freytag, 1988).

In contrast to c-*myc*, there appears to be little regulation of *max* expression during differentiation. However, in erythroid (Dunn *et al*, 1994; Delgado *et al*, 1995) and epithelial cells (Martel *et al*, 1995) there is some evidence for fluctuations in the levels of Max. Additionally, differentiation in many haematopoietic cell lines is associated with a switch from Myc/Max to Mad/Max complexes (Zervos *et al*, 1993; Ayer and Eisenman, 1993; Larsson *et al*, 1994), suggesting that Mad function may be required for differentiation. Indeed, increased expression of Mad proteins also accompanies epidermal cell differentiation (Gandarillas and Watt, 1995; Hurlin *et al*, 1995b).

3.8 c-Myc is a Negative Regulator of Adipogenesis.

Constitutive over-expression of a murine c-*myc* cDNA in the 3T3-L1 cell line was found to be sufficient to inhibit adipogenesis, as judged by an inability to accumulate cytoplasmic triglyceride and repression of the adipocyte-specific gene, pyruvate carboxylase (Freytag, 1988). The ability of c-*myc* to inhibit 3T3-L1 differentiation correlated with the level of transgene expression and the phenotype could be rescued by the expression of a c-*myc* anti-sense construct in lines already harbouring the *myc* transgene. This suggests that inhibition of adipogenesis was specifically due to enforced expression of Myc. A series of transfection studies using the human c-*myc* mutants previously described by Stone *et al* (1987) demonstrated that inhibition of 3T3-L1 differentiation required those domains of the protein already deemed necessary for Myc-mediated transactivation (Freytag *et al*, 1990). It was therefore proposed that the ability of Myc to inhibit adipogenesis arose as a direct consequence of its activity as a transcription factor. To investigate whether Myc controls the expression of cellular genes in the 3T3-L1 system, Yang and co-workers (1991) screened Myc-expressing cell lines to identify genes that were differentially expressed. As a result, they found that four clones were repressed in lines expressing high levels of Myc. DNA sequence analysis revealed that these were the genes for pro-α 1 (I), pro-α 2 (I) and pro-α 3(VI) collagen and a gene of unknown identity. Interestingly, expression of pro-α 2 (I) collagen mRNA was restored in c-*myc* anti-sense cell lines. The effect of Myc on these genes was shown to require *cis*-elements in the collagen promoter, suggesting that Myc-mediated repression occurs at the level of transcription. Later, it was shown that enforced expression of Myc in 3T3-L1 cells was also associated with inhibition of C/EBPα (Freytag and Geddes, 1992). It was therefore proposed that loss of C/EBPα expression was a critical determinant of the ability of Myc to inhibit differentiation. Indeed, expression of a *c/ebpα* construct in lines already over-expressing c-*myc* was sufficient to rescue adipogenesis. This did not involve

suppression of the *myc* gene, suggesting that the decision to differentiate may be dependent on the relative levels of the two proteins.

TNFα is well known to block adipogenesis and it appears that the inhibitory effects of this cytokine are mediated at the level of C/EBPα (see Section 1.4.4). Additionally, TNFα has been shown to induce the expression of c-*myc* in both preadipocytes and adipocytes (Ninomiya-Tsuji *et al*, 1993) and this correlates with the TNF-mediated reduction in adipocyte-specific gene expression. Conditional expression of c-*myc* in TA1 cells mimicked the effects of TNFα, whilst *myc* antisense constructs impaired TNF activity. Hence, a pathway may be envisaged whereby inhibition of adipogenesis by TNFα is mediated by the activity of the Myc oncoprotein. Whilst there is no evidence to support a role for c-*myc* in adipose development *in vivo*, it is of interest to note that in certain pathological conditions increased expression of TNFα is associated with WAT wastage (Torti *et al*, 1985). It is therefore tempting to speculate that this may involve c-Myc. However, up-regulation of TNFα is also a classical feature of obesity, where it is associated with the development of insulin resistance and diabetes (Hotamisligil *et al*, 1996).

3.9 Models for the Myc-Mediated Block to Adipogenesis.

A number of models designed to clarify the role of c-Myc in differentiation control are outlined below:

3.9.1 *Myc Mediates Continued Proliferation.*

Proliferation and differentiation are generally considered to be alternative and mutually exclusive pathways. Thus, Myc may inhibit differentiation by fostering continued cell cycle progression. Indeed, many Myc-associated malignancies manifest as immature cell types with increased proliferative potential. Transgenic mice in which expression of c-*myc* is directed to the lymphoid compartment display

deregulated cell cycle control in the pre-B cell population and impaired B cell maturation (Langdon *et al*, 1986). Additionally, lymphoid tumours were apparent in many of the animals (Adams *et al*, 1985). Hence, it is possible that inhibition of differentiation may be a direct consequence of abnormal proliferation.

3.9.2 *Myc Prevents Cells from Arresting in G_D.*

As described in Section 1.5.4, arrest in the G_D state following mitotic clonal expansion is a pre-requisite for adipogenesis. It has therefore been proposed that Myc may inhibit 3T3-L1 differentiation by preventing cells from entering the G_D state (Freytag, 1988). Whilst 3T3-L1 cells over-expressing c-*myc* retained the ability to arrest at confluence and undergo DNA replication in response to the adipogenic hormones, they differed from the parental line in that they did not lose responsiveness to serum mitogens following the hormone treatment. Thus, cells expressing high levels of Myc entered S phase in response to 30% serum whilst untransfected 3T3-L1 cells were refractory to this treatment. Interestingly, confluent monolayers of 3T3-L1 cells that had not been exposed to the differentiation inducers were also responsive to the mitogenic effects of 30% serum. This suggests that hormone treatment is necessary for the 3T3-L1 cell line to achieve the G_D arrest state and that the presence of high levels of Myc somehow interferes with this process.

3.9.3 *Myc Modulates the Activity of Key Regulators of Adipogenesis.*

Myc has been shown to inhibit the expression of a number of key factors involved in differentiation, including MyoD and myogenin in myogenesis (Miner and Wold, 1991) and C/EBPα in adipogenesis (Freytag and Geddes, 1992). The precise mechanism by which Myc exerts these effects is not clear, but studies of the C/EBPα promoter have shed some light on this phenomenon. The 5' proximal promoter region is found to contain a consensus Myc/Max binding site (Vasseur-

Cognet and Lane, 1993a). Interestingly, this is located in a 30bp segment characterised by a high density of negative elements, including that for the novel factor, C/EBPα undifferentiated protein (CUP), which is thought to repress expression of the *c/ebpα* gene in inappropriate cell types (Vasseur-Cognet and Lane, 1993b). Thus, Myc/Max complexes may repress C/EBPα *via* the CACGTG site, possibly in association with other factors such as CUP. However, recent work by Li and co-workers (1994) suggests that Myc-mediated repression requires the Inr element of the C/EBPα promoter. Examination of deletion mutants suggested that this activity also required the presence of a distinct region of the Myc protein, designated Myc box II. It is therefore of interest to note that a previous study had concluded that Myc Box II mutants were incapable of inhibiting adipogenesis (Freytag *et al*, 1990). Finally, a somewhat controversial study suggests that Myc may also affect gene expression *via* the C/EBP binding site (Hann *et al*, 1994). An interesting feature of the c-*myc* gene is that it encodes two alternatively translated isoforms (Hann and Eisenman, 1984), designated Myc1 and 2. Hann and co-workers demonstrated that Myc1 was able to bind and transactivate *via* the C/EBP site of the Rous sarcoma virus EFII enhancer, whilst Myc2 bound without effect. Since Myc2 is the predominant form of the protein it is possible that repression of C/EBPα arises as a consequence of competitive binding for the C/EBP site by Myc/Max and other C/EBP family members. Whilst the physiological relevance of Myc1-mediated transactivation is not clear it should be noted that this form is preferentially translated as cultures attain high cell densities and growth arrest. It is therefore possible that Myc1 may be involved in the positive modulation of C/EBPα driven adipogenesis. However, this remains to be formally proven.

Initial Aims of the Project.

The primary objective of this project was to study the process of cellular differentiation. The c-*myc* proto-oncogene has been shown to block differentiation in a variety of cell types. Since the 3T3-L1 preadipocyte cell line has proven a valid *in vitro* model of adipogenesis, it was therefore proposed to exploit the ability of c-*myc* to inhibit differentiation in this system in order to gain further insight into the processes that might regulate induced adipogenesis, with particular emphasis being placed on the role of adipogenic transcription factors. Additionally, it was anticipated that such studies might also increase our understanding of the mechanism/s by which c-*myc* inhibits differentiation *in vitro*.

MATERIALS AND METHODS.

MATERIALS AND METHODS.

CHAPTER 4: Materials.

4.1 Tissue Culture.

Supplier: *European Collection of Animal Cell Cultures, Salisbury, Wiltshire, England.*

3T3-L1 preadipocyte cell line.

Supplier: *Gibco Europe Life Technologies Ltd., Paisley, Scotland.*

10X Dulbeccos MEM.

200mM glutamine.

100mM sodium pyruvate.

7.5% (w/v) sodium bicarbonate.

2.5% (w/v) trypsin.

Geneticin sulphate (G418).

Supplier: *Beatson Institute Central Services.*

Sterile dH_2O.

Penicillin (7.5 mg/ml).

Streptomycin (10 mg/ml).

Amphotericin B (250µg/ml).

Sterile PBS-EDTA.

Sterile PBS.

Sterile glycerol.

Sterile 1N NaOH.

Sterile glassware and pipettes.

Supplier: *Advanced Protein Products, Brierley Hill, West Midlands, England.*

Foetal calf serum.

Supplier: *ICN Biomedicals, Thame, Oxon, England.*

Donor calf serum.

Supplier: *A/S Nunc, Roskilde, Denmark.*

Tissue culture flasks.

Nunc cryotubes.

Chamber slides.

Supplier: *Becton Dickinson Labware, Plymouth, Devon, England.*

Tissue culture dishes.

Supplier: *Costar Corporation, Cambridge, Massachusetts, USA.*

Tissue culture plates.

Supplier: *Fisher Scientific International, Loughborough, Leicestershire, England.*

Dimethyl sulphoxide (DMSO).

4.2 Plasticware.

Supplier: *Becton Dickinson Labware, Plymouth, Devon, England.*

Falcon tubes.

Supplier: *Bibby-Sterilin Ltd., Stone, Staffordshire, England.*

20ml universal and 5ml bijou tubes.

Supplier: *Du Pont Co., Wilmington, Delaware, USA.*

Sorvall ultracentrifuge tubes.

Microfuge tubes, pipette tips and other plasticware were obtained from the Beatson Institute Store.

4.3 Bacterial Culture.

Supplier: *Beatson Institute Central Services.*

L-broth, prepared as outlined in Sambrook *et al* (1989).

Sterile glassware.

Supplier: *Difco, Detroit, Michigan, USA.*

Bacto-agar.

Supplier: *Sigma Chemical Co. Ltd., Poole, Dorset, England.*

Ampicillin.

Kanamycin.

Supplier: *Bibby-Sterilin Ltd., Stone, Staffordshire, England.*

Petri dishes.

4.4 Nucleotides, Polynucleotides and DNA.

Supplier: *Amersham International plc, Amersham, Buckinghamshire, England.*

[α-^{32}P] dCTP ~ 3000 Ci/mmol.

Supplier: *Sigma Chemical Co. Ltd., Poole, Dorset, England.*

Salmon sperm DNA.

Supplier: *Boehringer Mannheim UK, Lewes, East Sussex, England.*

Random hexanucleotide mix.

Unlabelled nucleotides.

4.5 Chemicals.

All chemicals (AnalaR grade) not individually listed were obtained from either *BDH Chemicals Ltd., Poole, Dorset, England* or *Fisher Scientific International, Loughborough, Leicestershire, England.*

Supplier: *Sigma Chemical Co. Ltd., Poole, Dorset, England.*

Bromophenol blue.	Ethidium bromide.
Dithiothreitol (DTT).	Propidium iodide.
Polyvinylpyrrolidone	Ficoll.
Bromodeoxyuridine (BrdU).	Tween 20.
Oil red O.	Nitroblue tetrazolium (NBT).
1-Methyl-3-isobutylxanthine (MIX).	Paraformaldehyde.
Insulin.	Diaminobenzidine (DAB).

Dexamethasone (DEX). Ponceau S.

Bovine serum albumin Fraction V. β-mercaptoethanol.

3-(N-morpholino) propane sulphonic acid (MOPS).

5-bromo-4-chloro-3-indolyl phosphate (BCIP).

Tetramethylethylene diamine (TEMED).

Supplier: *ICN Biomedicals, Thame, Oxon, England.*

Bovine pituitary growth hormone.

Supplier: *Premier Beverages, Adbaston, Staffordshire, England.*

"Marvel" non-fat dried milk powder.

Supplier: *James Burrough Ltd., Witham, Essex, England.*

Ethanol.

Supplier: *Cinna/Biotecx Laboratories Inc., Houston, Texas, USA.*

RNAzol B.

Supplier: *New Bruswick Scientific, Haverhill, Suffolk, England.*

3-methyl-1-butanol.

Supplier: *Fluka Chemika-Biochemika AG, Buchs, Switzerland.*

Formamide.

Supplier: *Boehringer Mannheim UK Ltd., Lewes, East Sussex, England.*

Caesium chloride.

Supplier: *Bethesda Research Laboratories, Life Technologies, Inc., USA.*

Agarose, ultrapure electrophoresis grade.

4.6 Antisera.

Supplier: *Santa Cruz Biotechnology Inc., Santa Cruz, California, USA.*

C/EBPα antisera: Rabbit IgG polyclonal (14AA).

C/EBPβ antisera: Rabbit IgG polyclonal (C-19).

Mad1 antisera: Rabbit IgG polyclonal (C-19).

p21 antisera: Rabbit IgG polyclonal (C-19).

Supplier: *Promega Ltd., Southampton, Hampshire, England.*

Goat anti-rabbit IgG (Fc) alkaline phosphatase conjugate.

Supplier: *Amersham International plc, Amersham, Buckinghamshire, England.*

Donkey anti-rabbit IgG (whole molecule) horseradish peroxidase conjugate.

Supplier: *DAKO Ltd., High Wycombe, Buckinghamshire, England.*

BrdU antisera: Mouse IgG monoclonal (Bu20a).

The sources of antisera obtained as gifts are listed where quoted.

4.7 Enzymes and Enzyme Inhibitors.

All DNA modifying enzymes and their buffers, except those listed below, were obtained from: *Bethesda Research Laboratories, Gibco Ltd. Paisley, Scotland.*

Supplier: *Boehringer Mannheim UK, Lewes, East Sussex, England.*

RNAse A.

Supplier: *NBL Gene Sciences Ltd., Cramlington, Northumberland, England.*

Calf intestinal alkaline phosphatase.

Klenow Polymerase.

Supplier: *Sigma Chemical Co. Ltd., Poole, Dorset, England.*

Diethylpyrocarbonate (DEPC).

4.8 Gels and Columns.

Supplier: *Severn Biotech Ltd., Kidderminster, Worcestershire, England.*

Design-a-Gel 30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide solution.

Supplier: *Pharmacia Ltd., Milton Keynes, Buckinghamshire, England.*

NICK columns.

4.9 Kits.

Supplier: *Amersham International plc, Amersham, Buckinghamshire, England.*

ECL western blotting detection kit.

Supplier: *Vector Laboratories Ltd., Peterborough, Cambridgeshire, England.*

VectaStain ABC mouse IgG peroxidase immunostaining kit.

Supplier: *QIAGEN Ltd., Dorking, Surrey, England.*

RNeasy total RNA kit.

QIAshredder cell lysate homogeniser.

4.10 Membranes, Paper and X-ray Film.

Supplier: *Amersham International plc, Amersham, Buckinghamshire, England.*

Hybond N+ nylon membranes.

Supplier: *Sartorius AG, Gottingen, Germany.*

Nitrocellulose Extra blotting membrane.

Collodion dialysis bags.

Supplier: *Vernon-Carus Ltd., Preston, Lancashire, England.*

Gauze swabs.

Supplier: *Whatman International Ltd., Maidstone, Kent, England.*

3MM filter paper.

Supplier: *Eastman Kodak Co., Rochester, New York, USA.*

X-ray film (X-OMAT AR).

Kodak Gold print film ISO 100.

Supplier: *Fuji Photo Film Co., Tokyo, Japan.*

X-ray film (RX).

4.11 Markers.

Supplier: *Bethesda Research Laboratories, Gibco Ltd., Paisley, Scotland.*

Pre-stained protein markers: range 3-46 kDa and 14-200 kDa.

ϕX174 RF DNA / *Hae* III fragments.

λ DNA / *Hind* III fragments.

RNA ladder.

4.12 Water.

De-ionised water (dH_2O) for general use and for the preparation of solutions and buffers was obtained from a Millipore MilliRO 15 system. Water for enzymatic reactions and recombinant DNA work (RQH_2O) was further purified by reverse osmosis on a Millipore MilliQ system to 18Ωcm.

CHAPTER 5: Methods.

5.1 Cell Culture and Induction of Differentiation.

5.1.1 *Serial Passage of the 3T3-L1 Cell Line.*

The 3T3-L1 preadipocyte cell line was maintained in culture as an adherent monolayer, according to the protocol of Dr. E.K. Parkinson (Beatson Institute). After dissociation with 0.25% trypsin in PBS-EDTA, cultures were seeded at a density of 10^4 cells per 90mm tissue culture dish in 10mls 1X Dulbeccos MEM supplemented with 10% donor calf serum, 0.3% sodium bicarbonate, 1mM sodium pyruvate, 2mM glutamine and 6.5mM NaOH in an atmosphere of 5% CO_2 in air at 37°C. Sub-confluent monolayers were passaged every 7 days, with fresh cultures being established every 2 months from frozen stocks.

5.1.2 *Culture of Rat-1 Cells.*

Rat-1 cells for control transfections were obtained from Dr. D.H. Crouch (Beatson Institute) and grown in 1X Dulbeccos MEM, supplemented with 10% foetal calf serum, 0.37% sodium bicarbonate, 1mM sodium pyruvate and 2mM glutamine. After trypsinisation, cultures were seeded at a density of $2x10^5$ cells per $25cm^2$ tissue culture flask one day prior to transfection and maintained in an atmosphere of 5% CO_2 in air at 37°C.

5.1.3 *Cell Storage and Recovery.*

The cell lines described in this study were stored frozen in liquid nitrogen. Cultures of growing cells were harvested by trypsinisation and centrifugation at 1000rpm for 5 minutes in a MSE benchtop centrifuge. Cell pellets were resuspended to a final concentration of 10^6 cells/ml in the appropriate growth medium containing 10% (v/v) DMSO and 20% serum and aliquotted into 2ml cryotubes. These were placed in a polystyrene container at -70°C for 24 hours, after

which time they were deposited in liquid nitrogen for long-term storage. Cultures were recovered by transferring cryotubes directly from liquid nitrogen into water at 37°C. Once thawed, the cells were diluted in 5ml of pre-warmed growth medium, and centrifuged for 5 minutes at 1000rpm in a MSE benchtop centrifuge. Cells from one vial were resuspended in 5ml of fresh growth medium and seeded into a 25cm^2 tissue culture flask.

5.1.4 *Mycoplasma Testing.*

Cell lines were regularly tested for the presence of *Mycoplasma* contamination using the method of Chen (1977). This was performed in house by Mary Freshney and Marion Lacey.

5.1.5 *Induction of Differentiation.*

Cultures of 3T3-L1 preadipocytes were grown to confluence, and maintained in this state for 4 days prior to induction of differentiation. At induction (day 0), the growth medium was aspirated and replaced with fresh medium supplemented with 0.5mM methyl isobutylxanthine (MIX), 0.25μM dexamethasone (DEX) and 10μg/ml bovine pancreatic insulin. After 48 hours (day 2) cultures were changed to growth medium containing 10μg/ml insulin, and subsequently fed every 2 days until day 10, at which time maximal differentiation was judged to have occurred.

5.1.6 *Markers of Differentiation.*

A. Morphology. Throughout the differentiation process, cultures were viewed by phase contrast microscopy to assess the number of cells that had attained the rounded and enlarged morphology typical of mature adipocytes. Preadipocytes were characteristically fibroblastic in appearance.

B. Oil red O Staining. Cultures were washed with PBS prior to fixation with 0.66% (w/v) paraformaldehyde in PBS for 10 minutes at room temperature. Fixed

cells were washed twice with PBS, followed by a brief rinse in 60% isopropanol. A 0.25% stock solution of oil red O in isopropanol, prepared as described by Bancroft and Cook (1984), was diluted appropriately prior to use and monolayers stained for 10 minutes at room temperature. Excess stain was removed by several washes with PBS, and the stained monolayers viewed by phase contrast microscopy. Where appropriate, stained cultures were photographed using an Olympus OM-21 camera mounted onto the microscope. Processing of all print film was performed by Boots the Chemist, Sauchiehall Street, Glasgow.

5.1.7 *Calcium Phosphate Transfection.*

Cell lines stably expressing the avian *myc* gene constructs were generated by calcium phosphate precipitation (Graham and van der Eb, 1973). One day prior to transfection, cells were seeded at a density of $2x10^5$ per $25cm^2$ flask and incubated in a humid atmosphere of 5% CO_2 in air at 37°C. On the following day, 10μg CsCl-purified plasmid DNA was mixed with 240μl 0.2X SSC (20X is 3M NaCl, 0.3M sodium citrate, pH 7), 35μl 2M calcium chloride and 275μl 2X HEPES-buffered saline [HeBS](280mM NaCl, 10mM KCl, 1.5mM disodium hydrogen orthophosphate dihydrate, 12mM glucose, pH 7.05 +/- 0.05), and incubated at room temperature for 15 minutes. After this time, the resultant precipitate was transferred to the exponentially growing cultures seeded on day 1 and incubated in a humid atmosphere of 5% CO_2 in air at 37°C for 3-4 hours. To increase the efficiency of transfection the cells were then subjected to a glycerol shock. After aspirating the medium and washing the monolayer with fresh growth medium, cultures were incubated with 1ml 1X HeBS/15% glycerol for 4 minutes at room temperature. This solution was removed by aspiration and, after rinsing with growth medium, the cultures were fed with 5ml fresh growth medium and reincubated under the standard culture conditions until the monolayers had attained approximately 80% confluence. At this point the cells were trypsinised and

reseeded into 90mm tissue culture dishes in 10ml growth medium, supplemented with 1mg/ml geneticin (G418). The antibiotic-containing medium was replaced every 2 days and after approximately 2 weeks G418 resistant colonies were apparent. Single colonies were picked for further investigation.

5.1.8 *Picking Colonies after Selection.*

A solution of sterile 2.5 % low melting point agarose in serum free growth medium was briefly microwaved at full power, cooled to 37°C, and 7mls carefully pipetted into the tissue culture dish. Once set, pre-marked colonies were picked through the agarose using a Pasteur pipette and each seeded into a single well of a 24 well tissue culture plate by gentle aspiration. Once 80% confluent, clones were subcultured in the usual manner.

5.1.9 *Single Cell Cloning.*

Single cell cloning was undertaken by plating cells at a dilution of 10 cells/ml in a 90mm tissue culture dish and the resultant colonies picked as described in 5.1.8. Plating at low dilution in this manner ensures that colonies will have arisen from a single cell and so represent a homogeneous population.

5.1.10 *Growth Curves.*

Cultures were initially seeded at a density of $5x10^4$ cells per 35mm dish and incubated under standard growth conditions. At various time points (generally days 1-6), cells were trypsinised and resuspended in 1ml of growth medium. Cell counts were then performed manually using a haemocytometer and the time point plotted against the log number of cells in each culture.

5.1.11 *Geimsa Staining.*

To assess plating or transfection efficiency cultures were stained using Geimsa. Following fixation in methanol for 10 minutes at room temperature, cells were stained with Geimsa (diluted 1:10 with dH_20) for 1 hour. Excess stain was removed by washing in tap water and the number of colonies in each culture counted manually.

5.1.12 *Growth in Soft Agar.*

Following trypsinisation, cells were resuspended at a final density of $5x10^3$ in growth medium supplemented with 50μg/ml streptomycin, 37μg/ml penicillin, 1.25ng/ml amphotericin B and 0.35% bacto-agar, in a *total* volume of 3ml. This suspension was carefully pipetted onto a pre-prepared baseplate, comprising 5mls 0.7% bacto-agar in growth medium set in a 60mm bacteriological Petri dish. Once set, dishes were incubated in a sealed box for 2 weeks in an atmosphere of 5% CO_2 in air at 37°C, being refed twice weekly with 2mls growth medium/0.35% bacto-agar.

5.2 Recombinant DNA Techniques.

5.2.1 *Restriction Digests and Agarose Gel Electrophoresis.*

Generally, 1μg of DNA for plasmid analysis and 10μg for fragment isolation were digested in a volume sufficiently large enough to dilute the restriction endonuclease 10-fold. Digests were carried out using 5 units of enzyme per μg plasmid DNA, except when partial digestion was required, in which case the enzyme was diluted 10-fold in 1X reaction buffer (supplied as a 10X stock with the enzyme) prior to use. All digests were incubated according to the recommendations of the supplier, generally 1 hour at 37°C, the reaction being stopped by the addition of 1/6th. volume of gel-loading buffer (0.25% bromophenol blue, 40% sucrose). After digestion, the resultant fragments were separated in non-denaturing agarose gels

and visualised by UV transillumination. Gels were prepared by dissolving 1-1.5% (w/v) electrophoresis grade agarose in 1X TAE buffer (40mM Tris-acetate, 20mM sodium acetate, 2mM EDTA, pH 7.4), containing 5μg/ml ethidium bromide. After microwaving to dissolve the agarose, molten gels were cooled to 50°C, and poured into an appropriate gel former. Gels were run in 1X TAE at 100V for 5 minutes to run the samples into the gel, then subsequently at 40-50V. To estimate the size of fragments resolved by electrophoresis, molecular weight markers derived from restriction digests of viral genomes were included for comparison. For large fragments, a *Hind* III digest of bacteriophage λ was used, whilst for smaller fragments a *Hae* III digest of the phage ϕX174 was the marker of choice.

5.2.2 *Purification of DNA Fragments from Agarose Gels.*

After electrophoresis, the band of interest was excised from the gel and purified by passing through siliconised glasswool. This was achieved by placing a plug of glasswool into the bottom of a 0.5ml microfuge tube, which had previously been pierced at the base with a sterile needle. This tube was then inserted into a 1.5ml screw top microfuge tube, the gel slice macerated and placed within the smaller tube and the entire assembly spun at full speed in a microfuge for 2-3 minutes. This forces the liquid from the gel, which collects in the larger tube. The small tube was then discarded and the DNA precipitated from the flow-through with 1/10th. volume 3M sodium acetate and 2 volumes ice cold ethanol. Following incubation on dry ice for 15 minutes, the DNA was pelleted by spinning in a microfuge at full speed for 5 minutes, and resuspended in 10-20 μl TE (10mM Tris-HCl pH 7.4, 1mM EDTA pH 8). The purified DNA was quantified by agarose gel electrophoresis and comparison with known quantities of ϕX174 *Hae* III marker.

5.2.3 *Phosphatase Treatment of Vector DNA.*

To prevent religation, digested vector DNA was treated with phosphatase to remove the 5' phosphate group. 1μg vector DNA was digested to completion in a total reaction volume of 50μl and treated with 1 unit calf intestinal phosphatase for 1 hour at 37°C. This reaction was stopped by the addition of 6μl 10% SDS and 6μl 10X STE (100mM Tris-HCl pH 8, 1M NaCl, 10mM EDTA) and subsequent incubation at 68°C for 15 minutes. Following extraction with 1 volume TE-saturated phenol, the DNA was precipitated with 3M sodium acetate and ethanol, in the presence of 2μg carrier tRNA. The phosphatase treated vector was resuspended in TE to a final concentration of 10ng/μl.

5.2.4 *Ligation of DNA Fragments into Vector DNA.*

Phosphatase treated vector DNA was mixed with a 3-fold excess of purified fragment DNA, in the presence of 1 unit T4 DNA ligase. The reaction was incubated at 4°C overnight.

5.2.5 *Preparation of Competent Bacterial Cells.*

The TG1 strain of *Escherichia coli* was obtained from Beatson Institute glycerol stocks. A growing culture was established by inoculating 5ml L-broth with a loopful of TG1 and incubating overnight at 37°C in an orbital shaker at 225 rpm. The following day 2.5ml of this culture was used to inoculate 250ml L-broth and incubated under the above conditions until the culture was judged to be in exponential growth (OD_{600}~0.3). At this point the cells were harvested by centrifugation in a DuPont Sorvall RC-5B centrifuge at 5000rpm for 10 minutes. The bacterial pellet was resuspended in 100ml 0.1M NaCl/5mM magnesium chloride and spun for 10 minutes at 5000rpm. The resultant pellet was resuspended in 50ml 0.1M calcium chloride/5mM magnesium chloride and incubated on ice for 20 minutes, with occasional gentle mixing. Following centrifugation at 5000rpm

for 10 minutes, the bacterial cells were resuspended in 15ml 0.1M calcium chloride/5mM magnesium chloride and used immediately for transformation. Alternatively, long term stocks were established by the addition of 5ml 60% glycerol/0.1M calcium chloride/5mM magnesium chloride. This solution was aliquoted into snap-top microfuge tubes and stored at -70°C.

5.2.6 *Transformation of Bacterial Cells with Plasmid DNA.*

TG1 competent cells were slowly thawed on ice and 100µl aliquots placed into pre-chilled 2058 tubes (Falcon). Approximately 1ng of plasmid DNA or 10µl ligation mix were added to the cells and gently mixed by stirring with a pipette tip. After incubation on ice for 45 minutes, the cells were heat-shocked at 42°C for 90 seconds and placed on ice for a further 2 minutes. 400µl of L-broth was then added to the cells and this mixture incubated at 37°C for 1 hour in an orbital shaker at 225rpm, to allow time for the cells to express the appropriate antibiotic resistance gene. After this time, 100µl of the cell suspension was spread onto L-broth plates containing 1.5%(w/v) bacto-agar and supplemented with the appropriate antibiotic at a concentration recommended by Sambrook *et al* (1989). Plates were air-dried for 5 minutes and incubated in an inverted position overnight at 37°C.

5.2.7 *Small Scale Preparation of Plasmid DNA.*

A single bacterial colony from an agar plate was picked using a sterile toothpick and transferred to 5ml of L-broth containing the appropriate antibiotic. This culture was incubated overnight at 37°C with shaking at 225rpm, and 1.5ml transferred to a microfuge tube and centrifuged at full speed for 5 minutes. After aspirating the supernatant, the bacterial pellet was resuspended in 100µl of solution I (50mM glucose, 25mM Tris-HCl pH 8, 10mM EDTA). The cells were then lysed by the addition of 200µl of freshly prepared solution II (0.2M NaOH, 1% SDS), followed by gentle inversion of the tube. To precipitate the chromosomal DNA, 150µl of

solution III (3M sodium acetate, pH 4.8) was added, the solution mixed thoroughly by inversion and placed on ice for 5 minutes. The precipitated chromosomal DNA and protein was removed by centrifugation for 5 minutes at full speed and the supernatant transferred to a fresh tube. Nucleic acid was precipitated from the supernatant by the addition of an equal volume of isopropanol and incubation at room temperature for 10-30 minutes. Following centrifugation for 5 minutes at full speed, the pellet was resuspended in 100µl TE/3M sodium acetate and the nucleic acids reprecipitated by the addition of 2 volumes of ethanol and incubation on dry ice for 10 minutes. The DNA was recovered by centrifugation at full speed for 5 minutes and the resulting pellet dissolved in 75µl TE. Typically 5µl of this mixture was then used for analysis by restriction digest, in the presence of DNAse-free RNAse at a final concentration of 1µg/µl.

5.2.8 *Large Scale Preparation of Plasmid DNA.*

A 5ml culture of bacteria, prepared as described above, was used to inoculate 500ml of L-broth containing the appropriate antibiotic and incubated overnight at 37°C with shaking. The cells were harvested by centrifugation in a DuPont Sorvall RC-5B centrifuge at 5000rpm at 4°C for 10 minutes and the pellet thoroughly resuspended in 20ml solution I (see above). Following the addition of 40ml of freshly prepared solution II (see above), the cell lysate was placed on ice for 5 minutes. After this time, 30ml solution III (see above) was added, the solution mixed thoroughly and placed on ice for a further 10 minutes to precipitate the protein and genomic DNA. Following centrifugation at 5000rpm at 4°C for 10 minutes to pellet the flocculant, the supernatant was transferred to a fresh tube by filtration through a double layer of gauze and the nucleic acids precipitated with a equal volume of isopropanol for 15 minutes at room temperature. The nucleic acid was recovered by centrifugation as described above. After being air-dried for 5 minutes, the pellet was re-dissolved in 7.7ml TE containing 8.47g CsCl and 0.35ml

of 10mg/ml ethidium bromide solution. Samples were transferred to screw-cap ultracentrifuge tubes and spun at 40,000rpm for greater than 40 hours in a Du Pont Sorvall OTD Combi ultracentrifuge. Bands corresponding to closed circular plasmid DNA were removed from the tubes using a syringe and serially extracted with 3-methyl-1-butanol to remove the ethidium bromide. Purified DNA was precipitated with 3M sodium acetate and ethanol as previously described, air dried and resuspended in 500µl of TE. If the plasmid DNA was to be used to transfect mammalian cell lines, it was further purified by dialysis overnight against 1 litre TE in a collodion bag.

5.2.9 *Storage of Bacterial Glycerol Stocks.*

Stationary bacterial cultures in liquid medium were mixed with an equal volume of glycerol, chilled on ice, then stored at -70°C in plastic cryotubes. Cultures were re-established by inoculation of 5ml of L-broth medium, containing the appropriate antibiotic, with a sterile loopful of the glycerol stock.

5.2.10 *Quantitation of Nucleic Acid Concentrations.*

Nucleic acids were quantified with respect to their absorbance of UV light. 5µl of sample was added to 500µl of de-ionised water and the absorbance of the solution measured at 260nm and 280nm in a quartz cuvette, using de-ionised water as a blank. The concentration of the solution was calculated on the basis that an optical density of 1 corresponds to a concentration of 50µg/ml for plasmid or genomic DNA and 40µg/ml for RNA. The ratio of the readings OD_{260}/OD_{280} was used as an estimate of the purity of the nucleic acid. Samples with ratios between 1.8 and 2.0 were taken as being sufficiently pure for all the techniques undertaken in this study.

5.3 Northern Blot Analysis.

5.3.1 *Preparation of Total Cellular RNA.*

Total cellular RNA was isolated using RNAzol B. Monolayers were cultured in 150cm^2 flasks, then lysed with 10ml RNAzol solution, before being transferred to a 2059 Falcon tube. Following the addition of 1ml chloroform and incubation on ice for 5 minutes, the suspension was centrifuged for 15 minutes at 10,000rpm in a pre-chilled Sorvall RC-5B centrifuge. Exactly 5ml of the upper phase was removed and an equal volume of isopropanol added. Tubes were incubated on ice for 30 minutes, then centrifuged as described above. The resultant pellet was resuspended in 5ml 75% ethanol and spun at 7500rpm for 8 minutes. Residual ethanol was removed with a pulled Pasteur pipette, the pellet air dried for 5 minutes and resuspended in 25-50µl DEPC-treated Milli-Q water. The RNA concentration was determined as described in Section 5.2.10. For the growth hormone experiments, total RNA was prepared using the QIAGEN RNeasy kit, as advised by the manufacturers.

5.3.2 *Electrophoresis and Transfer of RNA.*

RNA was denatured by suspension in 5 volumes of fresh sample buffer (750µl formamide, 240µl 37% formaldehyde, 150µl 10X MOPS [0.2M 3-(N-morpholino) propane sulphonic acid, 50mM sodium acetate, 5mM EDTA, pH 7], 200µl 50% glycerol and 7µl 2.5% bromophenol blue) and heating at 65°C for 15 minutes. Following the addition of 1/25th. volume 1mg/ml ethidium bromide, 10µg RNA per lane was size fractionated by electrophoresis through 1.25% (w/v) agarose gels in 1X MOPS, containing 2.2M formaldehyde, using 1X MOPS as the gel running buffer. 10µg RNA ladder, treated as described above, was included for size comparison. Gels were generally run overnight at 30V and the buffer circulated by a peristaltic pump (Pharmacia). After electrophoresis, gels were washed in water (2 x 30 minutes), then transferred overnight to Hybond N+ membranes by capillary

blotting using 20X SSC (3M NaCl, 0.3M sodium citrate, pH 7). The RNA was fixed to the membrane by exposure to UV light in a Stratagene 1800 crosslinker, after which the filters were washed in 2X SSC, sealed in polythene bags and stored at 4°C until required.

5.3.3 *Preparation of Random-Primed Radio-Labelled Probes.*

The probes used in this study for Northern blot analysis were as follows:

The avian c-*myc* probe was the 500bp *Cla*I fragment of fpGV-WT, described in Section 6.4, which corresponds to the C-terminus of the protein.

The FAAR probe was the 1.5kb *Bam* HI fragment of pSGFAAR, (kindly provided by Dr. Ez-Zoubir Amri, University of Nice, France), corresponding to the murine FAAR cDNA (Amri *et al,* 1995).

CHOP-10 corresponded to the 710bp *Bam* HI/*Acc*I fragment of Chop-10 pBS (a gift from Dr. David Ron, Skirball Institute of Biomolecular Medicine, New York, USA). This probe encompassed almost the entire murine CHOP-10 coding sequence (Ron and Habener, 1992).

Probes for C/EBPα, β and δ were kindly provided by Dr. Steve McKnight, Tularik Inc., South San Francisco, California, USA. C/EBPα corresponded to the 900bp *Nco*I fragment of MSV/EBPα, whilst C/EBP β and δ were the 1.5kb *Bam* HI/*Eco* RI fragment of MSV/EBPβ and the 1kb *Bam* HI/*Eco* RI fragment of MSV/EBPδ respectively. All three represent cDNA clones, C/EBPα being of rat origin (Landschulz *et al*, 1988), whilst C/EBPβ and δ were isolated from a 3T3-L1 cDNA library (Cao *et al*, 1991).

The PPARγ2 probe was the 520bp *Stu*I fragment of PPARγ2/SPORT, corresponding to a portion of the murine cDNA (Tontonoz *et al*, 1994a) and was the gift of Dr. Peter Tontonoz, Dana-Farber Cancer Institute, Boston, Massachusetts, USA.

The aP2 probe was the 700bp *Pst*I fragment of aP2/pGEM and corresponded to the murine cDNA. This was kindly provided by Dr. M. Daniel Lane, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA.

The SCD1 probe was the 800bp *PstI/Eco* RI fragment of PC4 (Ntambi *et al*, 1988), corresponding to a murine cDNA clone. This was also the gift of M. Daniel Lane.

All probes were radio-labelled with [α–^{32}P] dCTP by random priming (Sambrook *et al*, 1989). Typically, 50-75ng purified fragment DNA in a total volume of 8µl were denatured by boiling for 10 minutes. Following incubation on ice for 5 minutes, the DNA was mixed with 5µl random hexanucleotide/cold dNTP mix (prepared according to the manufacturers instructions), 1.85 MBq [α–^{32}P] dCTP, 2 units of Klenow polymerase and incubated at 37°C for 30 minutes. Labelled probes were separated from unincorporated nucleotides by purification on NICK columns as described in the manufacturers instructions. Prior to use in hybridisation reactions, all double-stranded probes were denatured by heating to 100°C for 10 minutes and placed on ice for a further 5 minutes.

5.3.4 *Hybridisation.*

Nylon membranes were pre-hybridised in a solution of 50% formamide, 5X SSPE (20X SSPE is 3.6 M NaCl, 0.2 M sodium phosphate, 20 mM EDTA, pH 7.4), 5X Denhardt's reagent (prepared as described in Sambrook *et al,* 1989), 0.5 % (w/v) SDS and 200µg/ml sonicated salmon sperm DNA at 42°C for at least 4 hours.

Radio-labelled probe was added at a concentration of 1 x 10^6 cpm/ml and the incubation continued for a further 16 to 24 hours. The blots were then washed thoroughly (2 x 20 minutes in 2X SSC/0.1% SDS at room temperature, followed by 30 minutes in 0.1X SSC/0.1% SDS at 65°C), and exposed to Kodak X-OMAT film with intensifying screens at -70°C.

5.3.5 *Removal of Radio-Labelled Probe from Nylon Membranes.*

In order that nylon membranes could be re-hybridised with a series of different probes, bound probe was removed by washing in boiling 0.1% (w/v) SDS for 30 minutes at room temperature, with shaking. Filters were then re-sealed into polythene bags and stored at 4°C until required. At no point during this procedure were the blots allowed to dry out as this causes the probe to become irreversibly bound to the nylon membranes.

5.4 Western Blot Analysis.

5.4.1 *Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).*

The separation of proteins for immuno-blot analysis were undertaken using the discontinuous buffer system (described in Sambrook *et al*, 1989). A solution of 30% (w/v) acrylamide/0.8% (w/v) bisacrylamide was diluted to the desired concentration of acrylamide (generally 7.5-12.5%) in 4X gel buffer (1.5M Tris-HCl pH 8.8, 0.4% (w/v) SDS) and water to give a final volume of 50ml. This solution was polymerised by the addition of 250µl 10% (w/v) ammonium persulphate and 100µl TEMED and cast to within 3cm of the top of two glass plates separated by 2mm spacers within an ATTO gel forming apparatus (ATTO Corp., Japan). To aid polymerisation and to prevent the formation of bubbles, the gel was overlaid with water-saturated butanol, which was rinsed away with dH_2O once the gel had set. A stacking gel was then prepared, containing 0.125M Tris-HCl pH 6.8, 0.1% (w/v) SDS, 0.005% (w/v) ammonium persulphate and 0.002% TEMED. This was poured

on top of the resolving gel, a comb inserted and the stack allowed to polymerise. Protein samples were prepared by lysing cell monolayers in SDS-PAGE sample buffer (0.08M Tris-HCl pH6.8, 10% glycerol, 5% SDS and 40µl/ml β-mercaptoethanol), followed by sonication, and equivalent amounts of protein loaded in each lane. Pre-stained molecular weight markers were included for size comparison. SDS-PAGE gels were run at 200V for 2 hours in 1X TG running buffer (50mM Tris-HCl, 1% (w/v) glycine and 0.25% (w/v) SDS). Once electrophoresis was complete, the separated proteins were transferred to nitrocellulose filters by semi-dry electro-blotting for 1 hour at 200mA using layers of Whatman 3MM paper pre-soaked in transfer buffer (60mM Tris.HCl, 50mM glycine, 1.7mM (w/v) SDS and 20% (v/v) methanol), in a blotter made to our specifications by Don McBean (Beatson Institute). To assess the efficiency of transfer and the relative loading per lane, filters were stained with Ponceau S solution for 2-3 minutes, and destained with dH_20 prior to Western blot analysis.

5.4.2 *Western Blot Analysis.*

Filters were probed by either of the following methods:

A. Enhanced Chemi-Luminescence (ECL). Blots were first blocked overnight at 4°C with shaking in TBST (10mM Tris-HCl, 150mM NaCl, 0.1% (v/v) Tween 20), containing 5% (w/v) non-fat milk powder, and subsequently incubated in this solution with an appropriate dilution of the primary antibody, raised against the protein of interest, for 1 hour at room temperature with shaking. The filters were thoroughly washed in TBST (1 x 15 minutes, followed by 4 x 5 minutes), before incubation with a horseradish peroxidase conjugated secondary antibody, diluted 1:5000 in TBST/5% (w/v) milk, for 1 hour at room temperature. Following washing (as described above), the extent of reactivity against the protein of interest was determined using the ECL Western blotting detection system (Amersham) and exposure to Fuji RX film.

B. Alkaline Phosphatase Detection. This method is essentially as described for the ECL detection, but with some minor modifications. Blocking, antibody incubation and washes were all performed in TBST containing 2.5% (w/v) milk, and filters were subjected to 4 x 5 minute washes only. After the final wash, filters were rinsed in TBST, and subjected to several 2 minute washes in AP buffer (0.1M Tris-HCl, 0.1M NaCl, 5mM magnesium chloride, pH 9.5). Reactivity was then determined by the addition of a solution containing 16mg NBT and 8mg BCIP in 50ml AP buffer. A positive reaction was indicated by the formation of an insoluble purple deposit. The reaction was stopped by rinsing in dH_20 and stained filters stored in the dark at room temperature to prevent fading.

5.5 Cell Cycle Analysis.

5.5.1 *Propidium Iodide Staining and Flow Cytometry.*

Monolayers were washed with 7.5ml pre-warmed PBS-EDTA, then treated with 5ml trypsin for 1 minute. After this time, the trypsin was transferred to a 15ml conical tube and the monolayer reincubated for a further 2-3 minutes. The cells were resuspended in 10ml growth medium, added to the conical tube and thoroughly dispersed by pipetting. Following centrifugation at 1000rpm for 5 minutes in an MSE benchtop centrifuge, the cell pellet was resuspended in 10ml ice cold PBS, and recentrifuged as described above. The resultant pellet was resuspended in 1ml cold PBS, then 9ml ice cold 70% ethanol added whilst vortexing. At this point the fixed cells could be stored at 4°C until required. To stain the cells, the ethanol was removed by centrifugation as before and the pellet resuspended in 0.5-2ml PBS, containing 250μg/ml RNAse A and 10μg/ml propidium iodide, and left for 30 minutes at room temperature. Samples were analysed using a fluorescence-activated flow cytometer (Becton Dickinson FACScan). Equal numbers of events were examined for each population of cells and the cell cycle distribution estimated using the MODFIT-LT programme.

5.5.2 *In Situ BrdU Staining.*

Monolayers cultured in chamber slides were treated with BrdU to a final concentration of 10μM for the required length of time, then stained using the VectaStain immunocytochemistry kit. Following incubation with BrdU, the cells were fixed with methanol:acetone (1:1 ratio) for 10 minutes at room temperature and air-dried. After fixation, the slides were washed in PBS/0.25% Tween 20 for 5 minutes, followed by a 10 minute wash in PBS. The DNA was denatured by treating with 1M HCl for 15 minutes at 60°C, after which time the slides were washed 3 times in PBS for a total time of 10 minutes. The slides were next incubated with anti-BrdU antiserum, diluted 1:30 in PBS/0.5%BSA, for 1-3 hours at room temperature. Following washing in PBS/0.05% Tween 20 (3 x 10 minutes), the slides were incubated with the biotinylated secondary antibody (supplied in the kit) for 1 hour at room temperature. The slides were washed as described above, and incubated with the avidin-biotin complex (ABC-supplied in kit). Following this incubation, the slides were washed as previously described, and stained with DAB solution (10mg DAB and 10μl hydrogen peroxide in 16.7ml PBS) for precisely 7.5 minutes, after which time the reaction was stopped by washing with PBS. A positive reaction was indicated by brown nuclear staining.

RESULTS.

RESULTS.

CHAPTER 6: Generation and Preliminary Characterisation of 3T3-L1 Cell Lines Constitutively Expressing Avian c-*myc*.

6.1 Introduction.

The primary objective of this project was to investigate the ability of the c-*myc* proto-oncogene to inhibit cellular differentiation. We therefore adopted the strategy of generating stable cell lines constitutively expressing c-*myc* and examined the consequence of this enforced expression on the ability of cells to undergo a well-defined differentiation programme. For this purpose the murine 3T3-L1 preadipocyte cell line was selected since an earlier study had demonstrated that this line was susceptible to a Myc-mediated differentiation block (Freytag, 1988).

6.2 The 3T3-L1 Preadipocyte Cell Line has the Ability to Differentiate into Cells Resembling Mature Adipocytes.

The 3T3-L1 preadipocyte cell line is probably one of the best characterised *in vitro* models of adipogenesis. In the undifferentiated state, 3T3-L1 cells are typically fibroblastic (Figure 4A), with a bipolar appearance and little lipogenic capacity. However, as adipogenesis proceeds the cells adopt an enlarged, rounded appearance and triglyceride droplets are seen to accumulate within the cytoplasm. These eventually coalesce and displace the nucleus to one side, giving rise to the characteristic "signet ring" morphology. This stored lipid may be visualised histologically with oil red O which specifically stains triglycerides a deep red colour (Figure 4B). Oil red O staining therefore provides a quick and reliable method by which to assess the ability of a culture to undergo terminal differentiation.

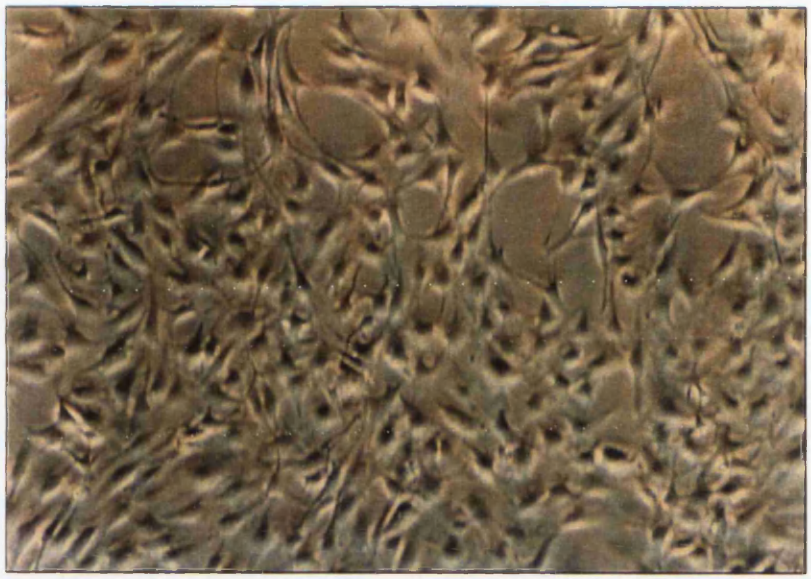
Figure 4.

Figure 4. Morphological Features of the 3T3-L1 Cell Line.

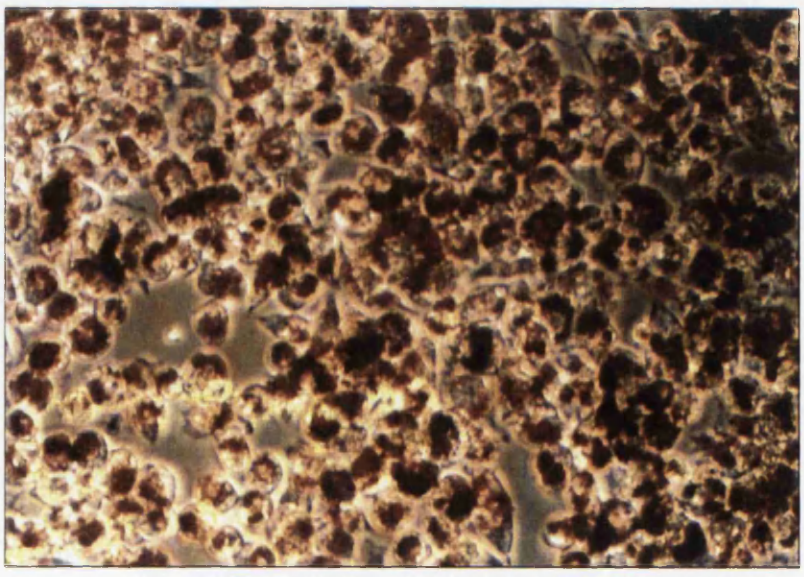
Panel A. Sub-confluent culture of 3T3-L1 preadipocytes viewed by phase contrast light microscopy (x100 magnification).

Panel B. 3T3-L1 adipocytes following staining with oil red O viewed by phase contrast light microscopy (x100 magnification).

A.



B.



6.3 Requirements for the Efficient Differentiation of the 3T3-L1 Cell Line.

The key premise of our investigation was that enforced expression of c-*myc* in the 3T3-L1 cell line would inhibit adipogenesis. We therefore felt that it would be prudent to optimise the differentiation conditions for our cells prior to transfection. In this way, we could be confident that any effects observed following gene transfer arose from constitutive expression of the c-*myc* transgene and did not reflect an inherent deficit in the ability of the 3T3-L1 cells to undergo adipogenesis.

6.3.1 *Spontaneous Differentiation of the 3T3-L1 Cell Line is an Inefficient Process Which is Enhanced by Hormone Treatment.*

Once a culture of 3T3-L1 cells had attained confluence, it could be maintained in this state for long periods without differentiating. When viewed microscopically, occasional mature adipocytes were observed and it was assumed that these had arisen spontaneously. However, these cells represented less than 5% of the total population, suggesting that spontaneous differentiation is an inefficient process. In contrast, treatment of the same culture with a cocktail of adipogenic hormones rapidly induced differentiation. Examination of the monolayer at regular intervals by phase contrast microscopy indicated that morphological changes started to occur approximately three days post-induction, with cells assuming a more rounded appearance. Cytoplasmic triglyceride accumulation was first observed at day 4 and increased thereafter, reaching a maximum at day 10. Following induced differentiation, cells resembling mature adipocytes represented approximately 80-90% of the total cell population. Thus, hormonal treatment greatly enhanced the ability of the 3T3-L1 cell line to undergo adipogenesis.

The induction protocol used was essentially that described by Freytag (1988). On day 0 of the differentiation programme, 3T3-L1 monolayers were treated with growth medium supplemented with 0.25μM dexamethasone, 0.5mM MIX and

10μg/ml bovine pancreatic insulin for 48 hours. After this time (day 2), the hormone cocktail was removed and replaced with fresh growth medium containing 10μg/ml insulin. This medium was changed every two days until day 10, at which point differentiation was judged to be complete. The contribution made by each hormone to the adipogenic process was also assessed. Replicate 3T3-L1 cultures were treated with the hormones, either alone or in combination, and the ability of each culture to undergo adipogenesis assayed by oil red O staining at day 10. As expected, induction with the full complement of hormones resulted in maximal differentiation, whilst cultures treated with unsupplemented growth medium were essentially devoid of mature adipocytes (Figure 5). Treatment with MIX alone resulted in impaired differentiation (~50%) when compared to cultures induced by the hormone cocktail. In contrast, cells exposed to dexamethasone or insulin alone did not differentiate and were indistinguishable from the uninduced cultures. However, supplementing MIX with either insulin or dexamethasone had a synergistic effect and restored the differentiation capacity to that observed with all three hormones. Surprisingly, treatment with dexamethasone and insulin in combination was still without effect. Thus, induced differentiation of the 3T3-L1 cell line required MIX and at least one other hormone for optimal conversion.

6.3.2 *Cellular Density at Induction is a Critical Determinant of Differentiation in the 3T3-L1 Cell Line.*

In the course of this work it became apparent that the state of the 3T3-L1 culture prior to induction was also critical. Hence, pre-confluent monolayers or cultures that had just attained confluency did not differentiate as readily as those which had been maintained at confluence for several days before hormone treatment. This requirement for sustained confluency is demonstrated in Figure 6. Cells were seeded at a range of densities and differentiation induced once the culture

Figure 5.

Figure 5. Assessment of the Adipogenic Potential of Dexamethasone, Methyl Isobutylxanthine and Insulin.

Differentiation was induced in replicate 3T3-L1 cultures by the following treatments:

Growth medium (Uninduced).

Growth medium supplemented with 0.25μM dexamethasone, 0.5mM MIX and 10μg/ml insulin (DEX+MIX+Insulin).

Growth medium supplemented with 0.25μM dexamethasone (DEX).

Growth medium supplemented with 0.5mM MIX (MIX).

Growth medium supplemented with 10μg/ml insulin (Insulin).

Growth medium supplemented with 0.25μM dexamethasone and 0.5mM MIX (DEX+MIX).

Growth medium supplemented with 0.25μM dexamethasone and 10μg/ml insulin (DEX+Insulin)

Growth medium supplemented with 0.5mM MIX and 10μg/ml insulin (MIX+Insulin).

Following differentiation, cultures were stained with oil red O at day 10 and the ability to differentiate determined relative to that of DEX+MIX+Insulin.

Hormone Treatment.	Ability to Differentiate.
Uninduced	–
Dex + MIX + Insulin	+
Dex	–
MIX	+/-
Insulin	–
Dex + MIX	+
Dex + Insulin	–
MIX + Insulin	+

Figure 6.

Figure 6. Effect of Cellular Density on 3T3-L1 Differentiation.

3T3-L1 cells were trypsinised and seeded into 60mm tissue culture dishes at the following densities:

A. $1x10^3$

B. $5x10^3$

C. $1x10^4$

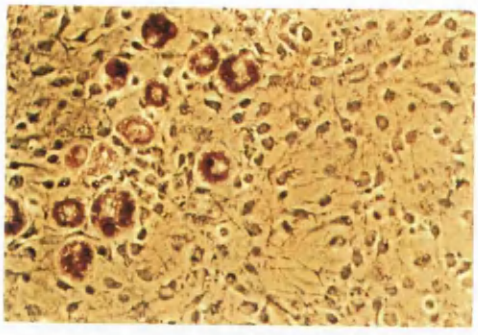
D. $5x10^4$

E. $1x10^5$

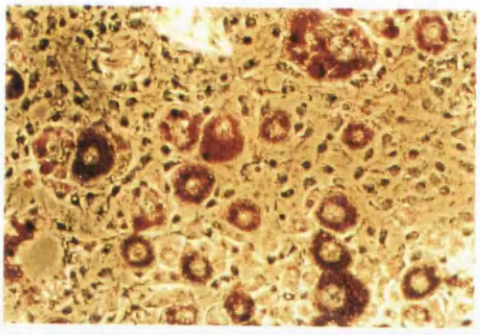
F. $5x10^5$

Once the cells in culture F had been at confluence for 2 days, all cultures were subjected to the standard differentiation protocol. At day 10 monolayers were stained with oil red O and viewed by phase contrast light microscopy (x100 magnification) in order to assess the degree to which each culture had differentiated.

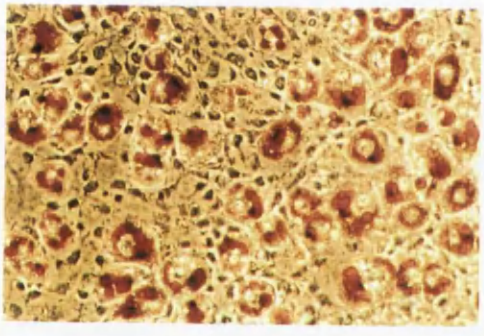
A.



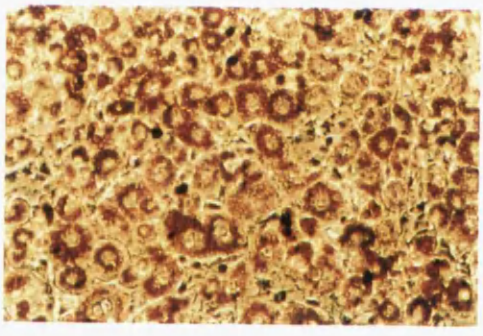
B.



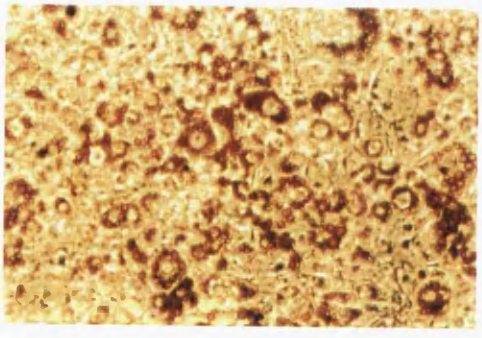
C.



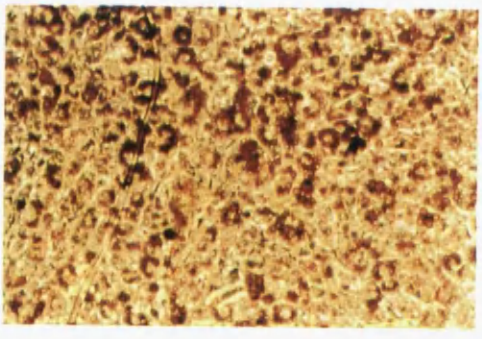
D.



E.



F.



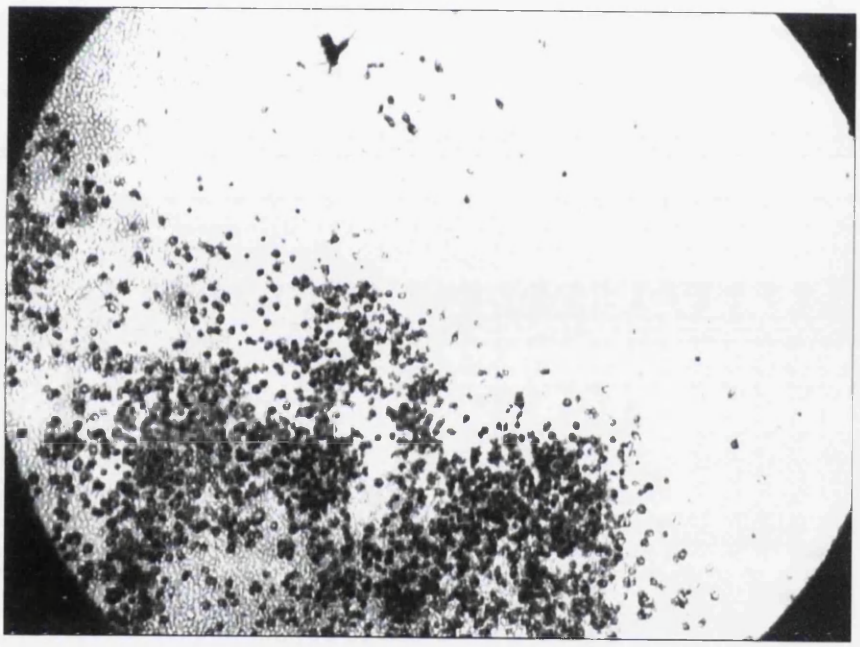
seeded at the highest density had been confluent for two days. Following staining with oil red O at day 10, it was apparent that cultures which had been sub-confluent at induction contained few mature adipocytes (Figure 6A and B), whilst those which had attained confluence at day 0 were able to differentiate to a greater extent (Figure 6C and D). However, maximal differentiation was observed where monolayers had been maintained at confluence prior to induction (Figure 6E and F).

A similar conclusion was drawn from a second experiment in which a post-confluent 3T3-L1 monolayer was wounded by scraping a strip of cells from the centre of the dish. The cells were allowed to recover for 24 hours, during which time the preadipocytes at the edge of the wound migrated into the space generating a culture composed of three distinct populations: pre-confluent, confluent and post-confluent fibroblasts. The ability of each of these sub-populations to undergo adipogenesis was then assessed by subjecting the culture to the standard differentiation protocol. Oil red O staining was performed at day 10, the striking feature of which was the presence of a macroscopic band of unstained cells running across the dish. Figure 7 shows the boundary between the stained and unstained cells, as viewed under low power light microscopy. Using a higher magnification it was apparent that the culture at the centre of the dish was composed almost entirely of fibroblasts, and that these presumably represented the cells that had migrated into the wound. In contrast, the culture on either side of the wound was fully differentiated. Taken together, these findings suggest that the efficiency of induced differentiation in the 3T3-L1 system is greatly enhanced in post-confluent monolayers. In order to ensure optimal conditions, it was therefore proposed that all cultures should be maintained in a post-confluent state for a period of four days prior to induction of differentiation.

Figure 7.

Figure 7. Effect of Wounding the Monolayer Prior to Differentiation.

A post-confluent 3T3-L1 monolayer was treated as described in the text, subjected to the differentiation protocol and stained with oil red O at day 10. The figure shows the border between the stained and unstained cells by light microscopy (x40 magnification).



The molecular basis for this phenomenon is not entirely clear but may reflect the need to establish cell-cell or cell-ECM interactions (see Section 1.5.2). However, cell-cell communication may not be paramount since the 3T3-F442A cell line can be induced to differentiate in suspension culture (Pairault and Green, 1979). Indeed, it has been suggested that gap-junctional communication is lost as adipogenesis commences (Smas and Sul, 1995). At present the issue of ECM interactions remains unresolved since attempts to induce premature differentiation of pre-confluent cells on reconstituted ECM were unsuccessful (data not shown). A second possibility is that post-confluent cells produce a secreted factor which conditions the medium, resulting in competence to differentiate once a certain threshold level is attained. However, conditioned medium harvested from 3T3-L1 preadipocytes was unable to stimulate differentiation of pre-confluent monolayers (data not shown). Finally, there appears to be a requirement for growth arrest prior to induction. Pre-confluent cultures were found to be impaired in their ability to undergo the differentiation programme and it is possible that this arose as a consequence of continued proliferation. Thus, maintenance at confluence may be required for full cell cycle withdrawal.

6.3.3 *The 3T3-L1 Cell Line does not Harbour a Differentiation-Defective Sub-Population.*

It was noted that induced differentiation of the 3T3-L1 line generally resulted in approximately 80-90% of the total cell population acquiring the characteristic adipocyte morphology. Two possibilities were proposed to account for this observation. Firstly, the ability of individual cells within a culture to undergo the differentiation programme may be stochastic, dependent upon chance rather than adipogenic potential *per se*. Alternatively, the residual fibroblastic fraction could represent a sub-population that had lost the ability to undergo adipogenesis. To test these hypotheses, 3T3-L1 cells were differentiated as usual then trypsinised

and reseeded at low density. Since adipocytes are post-mitotic they could not be propagated and were rapidly lost from the culture. In contrast, the fibroblastic cells proliferated readily generating a culture composed almost entirely of the progeny of those cells that originally failed to differentiate. Once post-confluent, this culture was then subjected to the standard differentiation protocol. Oil red O staining at day 10 revealed that as before 80-90% of the population had undergone adipogenesis. Since this procedure could be repeated several times, with the same result, we therefore concluded that the 3T3-L1 line used in our study did not harbour a sub-population of cells that were refractory to induced differentiation. Hence, adipogenesis is likely to be a stochastic process.

6.4 Construction of Avian c-*myc* Expression Vectors.

Avian wild type c-*myc*, and the c-*myc*Δ7 and c-*myc*Δ10 LZ deletion mutants were the kind gift of Dr. D.H. Crouch (Beatson Institute) and have been described in detail elsewhere (Crouch *et al*, 1990). The wild type c-*myc* was essentially an artificial cDNA composed of exons 2 and 3 of the genomic sequence, fused *via* a 66bp fragment derived from v-*myc* in order to eliminate intron 2. However, the construct retains the exon 2 splice acceptor site. The LZ mutants were generated by oligonucleotide directed mutagenesis of the wild type cDNA, which introduced premature stop codons into the sequence (Figure 8A). The positions of these termination codons were selected to progressively truncate the protein from the extreme C-terminus. Hence, c-MycΔ7 has a deletion of seven amino acids including leucine 4 of the LZ motif, whilst c-MycΔ10 lacks ten residues including leucines 4 and 3A (Figure 8A). Unlike leucine 4, leucine 3A is not part of the canonical heptad repeat. However, it is conserved in Myc proteins from all species and occurs in the 3 position of a potential 3,4 hydrophobic repeat where it is likely to perform an essential function in the formation and stabilisation of the coiled-coil structure.

Figure 8.

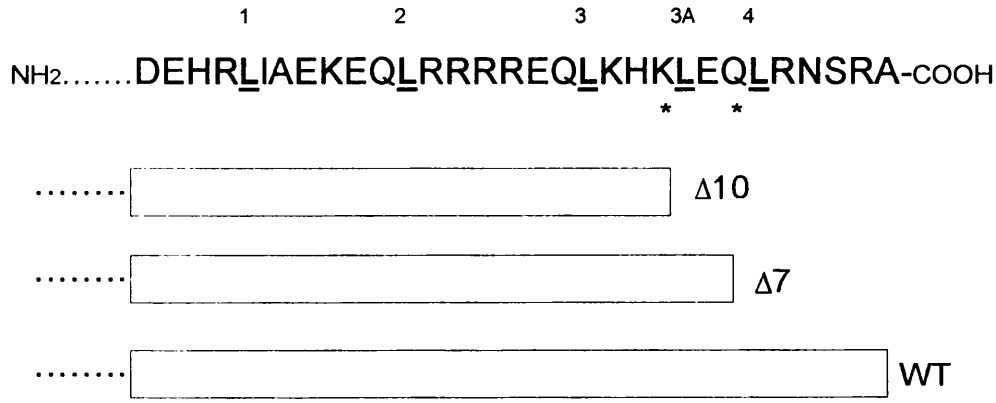
Figure 8. Generation of Avian c-*myc* Expression Vectors.

Panel A depicts the extreme C-terminus of avian c-Myc, with the position of the leucine residues highlighted (**L**). The relative position of the premature stop codons introduced into the c-*myc*Δ7 and c-*myc*Δ10 sequences are also indicated (*) and the resultant truncated proteins represented by open blocks (reproduced from Crouch *et al*, 1990).

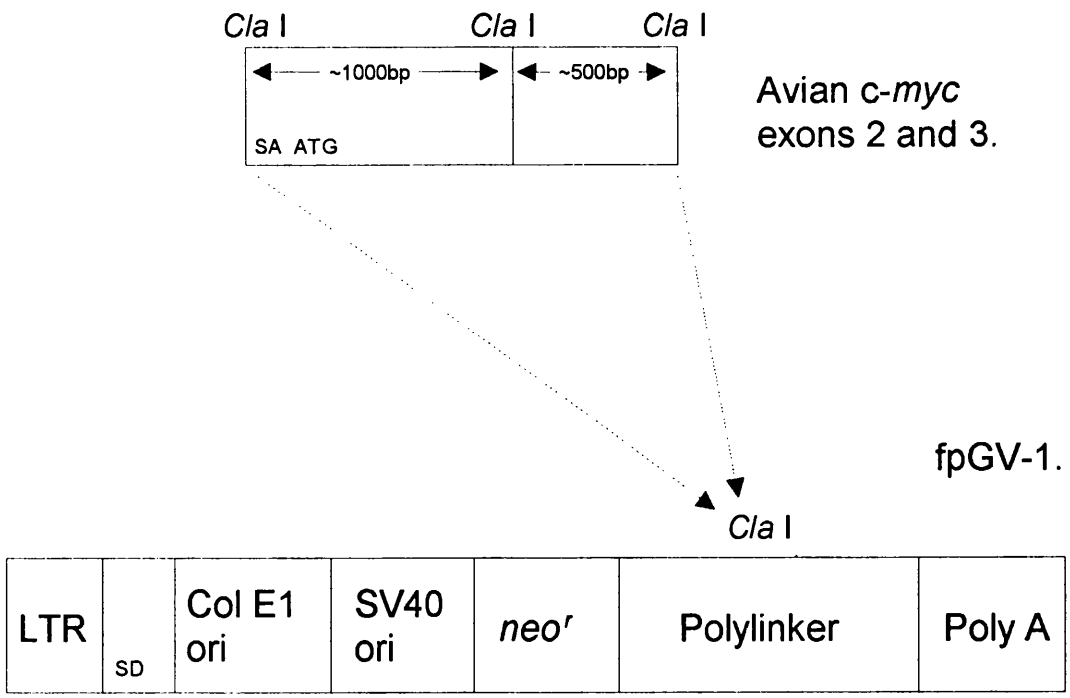
Panel B outlines the strategy for sub-cloning the avian c-*myc* sequences from RCAN into fpGV-1.

Abbreviations: SA splice acceptor site; ATG translation start site; LTR long terminal repeat; SD splice donor site; *neo*^{r} neomycin phosphotransferase gene; PolyA polyadenylation signal.

A.


1
2
3
3A
4
NH2.....DEHRLIAEKEQLRRRREQLKHKLEQLRNSRA-COOH
*
*
.....
Δ10
.....
Δ7
.....
WT

B.


Cla I
Cla I
Cla I
~1000bp
~500bp
SA ATG
Avian c-myc
exons 2 and 3.
fpGV-1.
Cla I
LTR
SD
Col E1
ori
SV40
ori
neor
Polylinker
Poly A

When originally constructed by Crouch and co-workers, these artificial c-*myc* cDNAs were cloned into the replication competent avian retrovirus, RCAN (Hughes *et al*, 1987). However, this vector was felt to be unsuitable for high-level expression in mammalian cells since it is composed of elements designed for optimal activity in cells of avian origin. In addition, it lacks a selectable marker. Thus, for the purposes of the present study the c-*myc* sequences were sub-cloned into an alternative vector, fpGV-1 (DeClue and Martin, 1989), a linear map of which is shown in Figure 8B. Inserted sequences were placed under the control of the Moloney murine leukaemia virus long terminal repeat (LTR) and transfected cells selected by virtue of a co-linear *neo* resistance marker, which confers kanamycin resistance in *E. coli* and G418 resistance in eukaryotic cells. fpGV-1 is approximately 5kb in size and contains both the Col E1 and SV40 origins of replication, a splice donor site and polyadenylation signal. In addition, the polylinker harbours unique *Bam* HI (3658), *Eco* RI (3676) and *Cla* I (4332) restriction sites. The sub-cloning strategy is outlined in Figure 8B. In brief, the c-*myc* sequences were excised from RCAN as a 1.5kb partial *Cla* I fragment and ligated into the *Cla* I site of fpGV-1. The orientation of the insert was then mapped by restriction digest and the region across the LZ sequenced to confirm the identity of each cloned fragment (data not shown).

6.5 Generation of 3T3-L1 Cell Lines Constitutively Expressing Avian c-*myc*.

Wild type c-*myc* (fpGV-WT) or empty vector (fpGV-1) were introduced into the 3T3-L1 preadipocytes by calcium phosphate precipitation and stable transfectants selected with G418. Following antibiotic selection, resistant colonies were pooled and assessed for their ability to undergo induced differentiation. As judged by oil red O staining at day 10, both the parental 3T3-L1 and vector control cell lines differentiated fully, indicating that fpGV-1 did not interfere with the ability of 3T3-L1 cells to differentiate. In contrast, the cell line harbouring the c-*myc*

transgene displayed a modest impairment in differentiation capacity (~60%, data not shown), suggesting that the transfected gene was exerting an inhibitory effect. In order to enhance this effect, we therefore subjected both the fpGV-WT and fpGV-1 pooled lines to single cell cloning procedures. This resulted in the generation of six fpGV-WT clones designated WT2-8 and seven fpGV-1 cell lines, a representative clone (fpGV5) being used as a control for subsequent experiments. Preadipocyte cell lines harbouring c-*myc*Δ7 and c-*myc*Δ10 were also generated essentially as described above. However, they differ from the WT lines in that they each represent a single colony picked after antibiotic selection. Six cell lines were established per construct.

6.6 3T3-L1-WT Clones Fail to Undergo Induced Differentiation.

Following cloning, the 3T3-L1-WT cell lines were assayed for their ability to differentiate under the standard conditions. As judged by morphology and oil red O staining at day 10, both the parental 3T3-L1 and fpGV5 cell lines differentiated fully. However, hormone treatment failed to induce any of the morphological features of adipogenesis in the WT clones (Figure 9), which retained the fibroblastic appearance of the 3T3-L1 preadipocyte. Hence, the presence of an avian c-*myc* transgene was sufficient to inhibit cellular differentiation in the 3T3-L1 system.

Whilst cytoplasmic triglyceride accumulation provides an excellent visual index of adipocyte differentiation, we decided to confirm our observations by examining a molecular marker of terminal differentiation. For this purpose we selected C/EBPα, since expression of this protein correlates well with the adipocyte phenotype. In addition, earlier work in this field had suggested that c-Myc and C/EBPα were reciprocally regulated during adipogenesis (Freytag and Geddes, 1992) and that C/EBPα is a putative target for Myc-mediated transcriptional

Figure 9.

Figure 9. Morphology of the WT Cell Lines at Day 10 of the Differentiation Programme.

To investigate the ability of the WT clones to undergo adipogenesis, cultures were subjected to the standard differentiation protocol and stained with oil red O at day 10. Morphology and accumulation of cytoplasmic triglyceride were assessed by phase contrast light microscopy (x100 magnification).

A. 3T3-L1

B. fpGV5

C. WT2

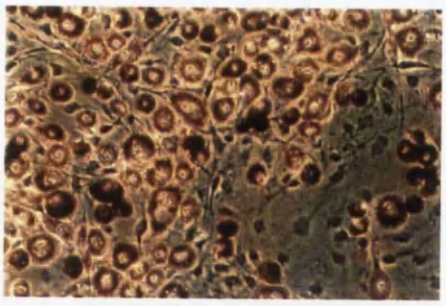
D. WT5

E. WT6

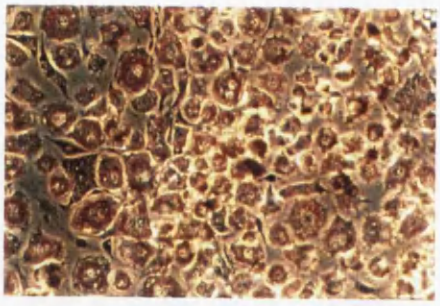
F. WT7

G. WT8

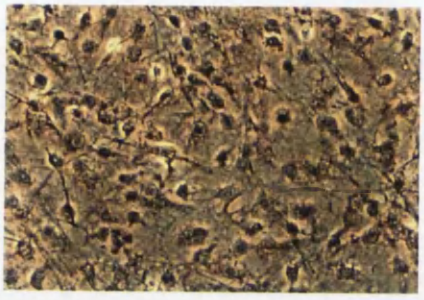
A.



B.

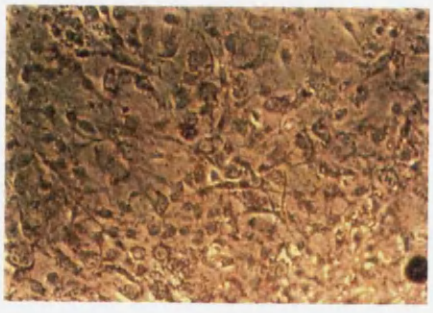


C.

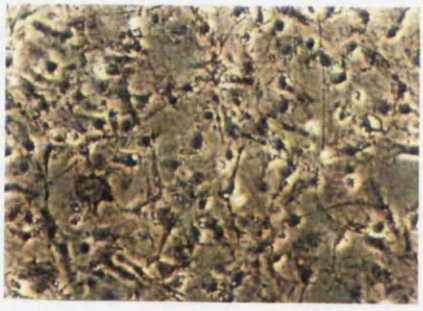


D.

E.



F.



G.

repression (Li *et al*, 1994). Following induced differentiation, lysates were prepared at day 10 and expression of C/EBPα assessed by Western blot analysis using the 14AA polyclonal antibody (Santa Cruz). This antibody was raised against a peptide corresponding to amino acids 253-265 of the rat sequence and was known to cross-react with both the 43-kDa and 30-kDa isoforms of C/EBPα. As expected, C/EBPα was not present in fpGV5 protein extracts at day 0 of the differentiation programme (Figure 10). However, at day 10 both the 43-kDa and 30-kDa isoforms were apparent. In contrast, neither isoform was detected in day 10 lysates prepared from the WT clones, indicating that cell lines harbouring fpGV-WT were indeed impaired in their ability to undergo terminal differentiation.

6.7 3T3-L1-WT Clones Express Low Levels of Avian c-Myc.

In order to verify that the transfected c-*myc* gene was expressed in the WT clones, Northern blot analysis of total cellular RNA was performed. We predicted that transcription of fpGV-WT would generate two major mRNA species, one of approximately 6.5kb corresponding to virtually the entire sequence of the construct and a second of approximately 2.1kb, representing the spliced avian c-*myc* mRNA. Whilst preliminary analysis of a Rat-1 cell line transfected with fpGV-WT confirmed this expectation, a number of splice variants or intermediates were also detected (Figure 11). This observation may reflect rearrangements of the construct which frequently occur when transfected DNA is integrated into the cellular genome. The c-*myc* probe employed in this study represented the 500bp *Cla* I fragment of fpGV-WT (Figure 8B) which corresponds to the C-terminal portion of the avian protein, a region which is highly conserved between species. However, this probe did not hybridise to any transcripts within the 3T3-L1 and fpGV5 samples. It is thought that this finding reflects the fact that the RNA was prepared once the cultures had attained

Figure 10.

Figure 10. Western Blot Analysis of C/EBPα Expression in the WT Cell Lines at Day 10 of the Differentiation Programme.

Protein samples were prepared at day 10 of the differentiation programme and equivalent amounts subjected to SDS-PAGE and Western blotting. Expression of C/EBPα was assessed using the 14AA polyclonal antibody (1:1000) and the alkaline phosphatase detection method. The position of the 43-kDa and 30-kDa isoforms of C/EBPα are indicated.

Negative Control: fpGV5 (day 0)

Positive Control: fpGV5 (day 10)

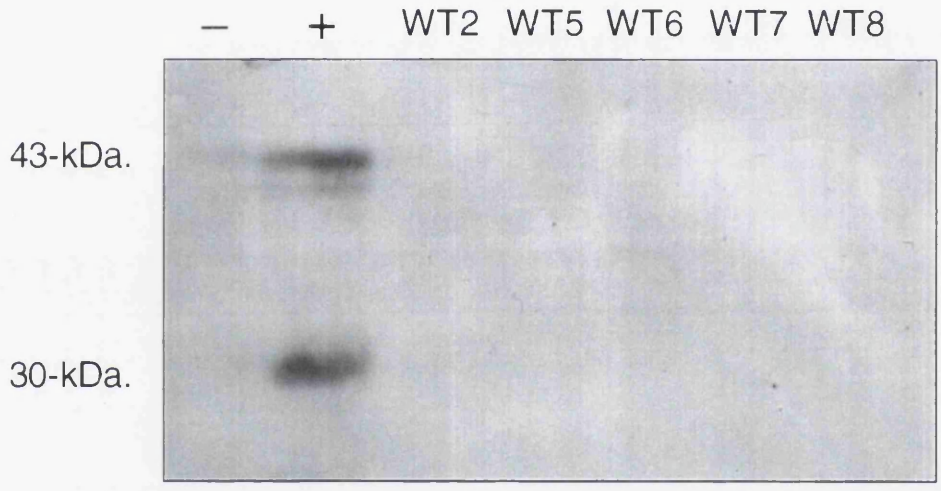

–
+
WT2
WT5
WT6
WT7
WT8
43-kDa.
30-kDa.

Figure 11.

Figure 11. Northern Blot Analysis of c-*myc* mRNA in the WT Cell Lines.

Panel A. Equivalent amounts of total cellular RNA were resolved by gel electrophoresis and subjected to Northern blot analysis, using the 500bp *Cla* I fragment of fpGV-WT as a probe. The position of the 6.5kb and 2.1kb transcripts are indicated.

Lane 1: 3T3-L1

Lane 2: fpGV5

Lane 3: Rat-1-WT

Lane 4: WT2

Lane 5: WT5

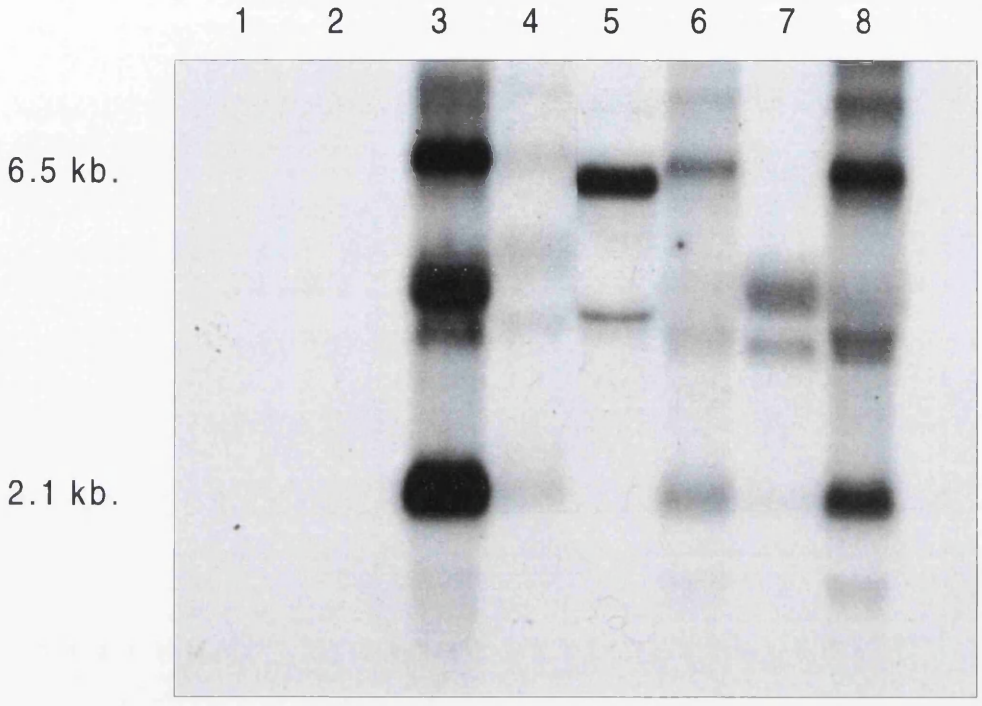
Lane 6: WT6

Lane 7: WT7

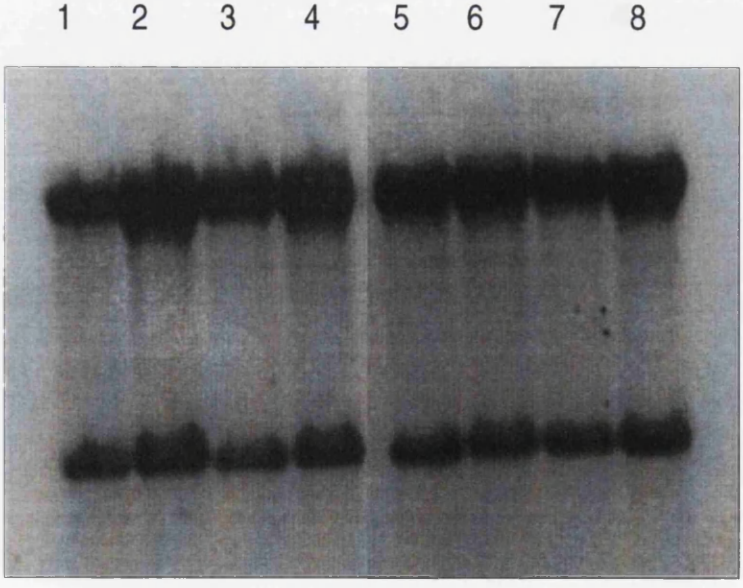
Lane 8: WT8

Panel B. Equivalence of loading and integrity of the RNA are demonstrated by ethidium bromide staining of the gel prior to transfer.

A.



B.



confluence, at which time expression of the endogenous c-*myc* gene would probably be beneath the limits of detection for Northern blotting. Alternatively, poor cross-species hybridisation could also account for this observation. In contrast, analysis of the WT clones suggested that all of these lines expressed a number of mRNA species that specifically hybridised to the c-*myc* probe (Figure 11). However, only WT2, WT6 and WT8 were found to express the subgenomic 2.1kb avian c-*myc* mRNA.

In contrast, endeavours to detect the avian c-Myc protein within the WT cell lines proved somewhat disappointing. Immunoprecipitation following metabolic labelling and immunocytochemisty were both negative (data not shown), whilst Western blot analysis was generally inconclusive, despite employing a range of primary antibodies and detection methods. To ensure that the fpGV-*myc* constructs were indeed functional, control transfections were performed using the Rat-1 cell line. Stable lines were generated by calcium phosphate precipitation and selection with G418 as described in the Materials and Methods. Antibiotic resistant colonies were then pooled and expression of the transgene assessed by Western blot analysis using the polyclonal antibody, 237-6 (the generous gift of D.A.F. Gillespie, Beatson Institute) This antibody was raised against the full length human c-Myc protein but had previously been shown by our laboratory to cross-react with avian c-Myc. Wild type c- Myc, c-MycΔ7 and c-MycΔ10 were all readily detected in the Rat-1 lysates and could be distinguished from one another on the basis of size (Figure 12A). In contrast, a representative Myc Western blot using lysates prepared from the WT cell lines is shown in Figure 12B, extremely low levels of the protein being detected in the WT2, WT6 and WT8 samples. That the WT clones apparently expressed such low amounts of the exogenous protein was somewhat surprising, since in other systems inhibition of differentiation generally correlates with over-expression of c-Myc. Thus, in the

Figure 12.

Figure 12. Western Blot Analysis of c-Myc Expression in the Transfected Cell Lines.

Panel A. Protein samples were prepared from Rat-1 lines harbouring the fpGV-*myc* constructs and equal amounts subjected to SDS-PAGE and Western blot analysis using the 237-6 polyclonal antibody (1:1000) and the ECL detection method.

WT: Rat-1-WT

Δ7: Rat-1-Δ7.

Δ10: Rat-1-Δ10

fpGV: Rat-1-fpGV

Panel B. Protein samples were prepared from 3T3-L1 cell lines harbouring the fpGV-WT construct and equivalent amounts subjected to SDS-PAGE and Western blot analysis using the 237-6 polyclonal antibody (1:1000) and the ECL detection method.

Lane 1: Rat-1-WT

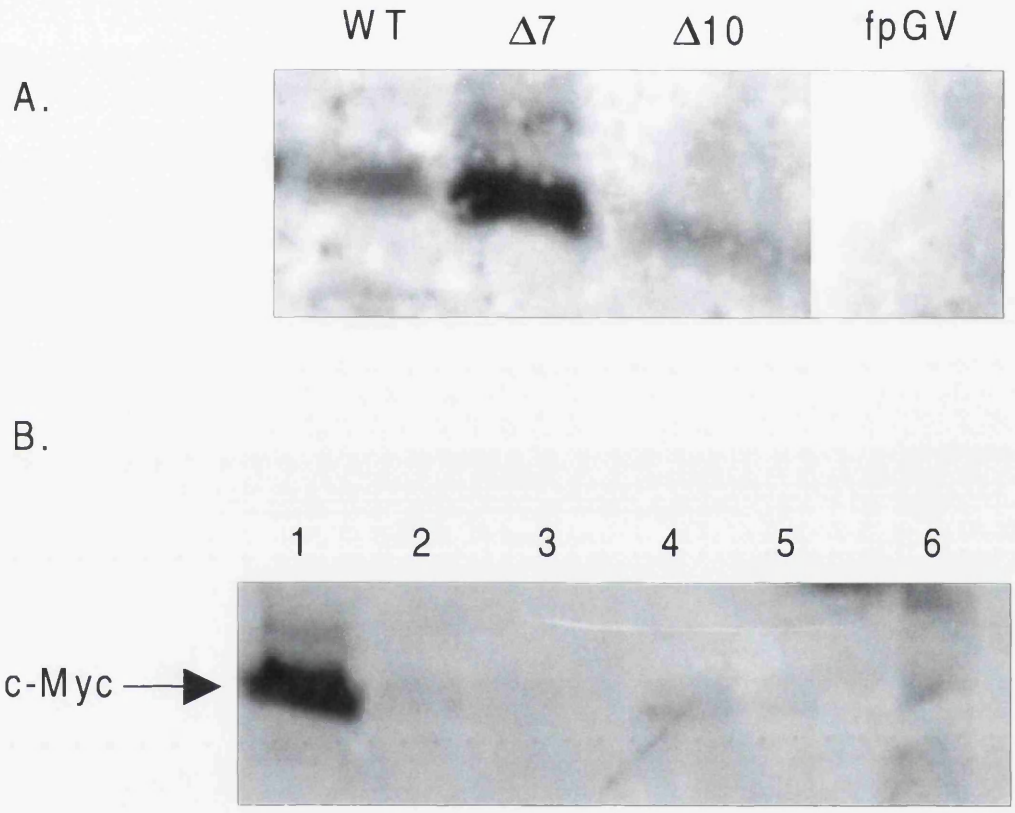
Lane 2: WT2

Lane 3: WT5

Lane 4: WT6

Lane 5: WT7

Lane 6: WT8


WT
Δ7
Δ10
fpGV
A.
B.
1
2
3
4
5
6
c-Myc

HL60 cell line the endogenous c-*myc* gene is greatly amplified and myelopoiesis can only occur if expression of c-Myc is down-regulated at induction of the differentiation programme (Westin *et al*, 1982). However, previous studies also failed to detect expression of exogenous c-Myc in the 3T3-L1 cell line by a variety of methods (Freytag, 1988; Freytag and Geddes, 1992), but were able to demonstrate the presence of the mRNA and a specific biological effect. It therefore appears likely that in contrast to other well-documented systems, inhibition of 3T3-L1 adipogenesis requires relatively low levels of Myc protein. This is supported by additional experiments in which we exploited the LacSwitch IPTG inducible vector system (Stratagene) in order to conditionally express avian c-*myc* in 3T3-L1 cells. The promoter of this vector was found to be sufficiently leaky that low levels of c-*myc* mRNA were expressed even in the absence of the inducer and this correlated with an impaired ability of transfected cells to undergo the differentiation programme (data not shown).

6.8 High Level Expression of Avian c-Myc in the 3T3-L1 Cell Line May be Cytotoxic.

Whilst expression of exogenous c-Myc was low or undetectable in the majority of the WT clones, a single cell line (WT3) expressed large amounts of the protein (Figure 13A). This clone was characterised by an abnormal morphology (Figure 13B), in which the cells were enlarged and displayed an irregular cytoplasm. Additionally, the line had a reduced growth rate when compared to 3T3-L1 and fpGV5 controls (Figure 14) suggesting that high level expression of avian c-Myc in the 3T3-L1 system could be cytostatic or even cytotoxic. Myc "toxicity" has previously been described in a number of immortalised cell lines (Facchini *et al*, 1994; Wurm *et al*, 1986). Additionally, NIH-3T3 cell lines transfected with human c-*myc* were characterised by high level expression of the c-*myc* mRNA but low levels of the protein (Ray *et al*, 1989), suggesting that expression of the

Figure 13.

Figure 13. Western Blot Analysis of c-Myc Expression in the WT3 Cell Line.

Panel A. Equivalent amounts of protein were subjected to SDS-PAGE and Western blot analysis using the 237-6 polyclonal antibody (1:1000) and the alkaline phosphatase detection method.

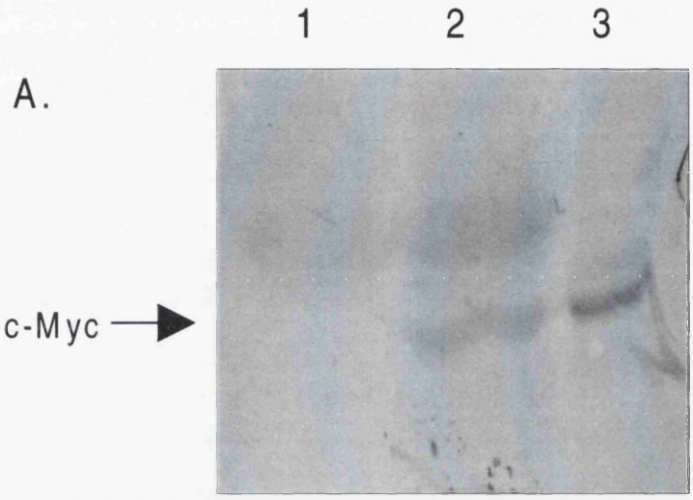
Lane 1: fpGV5

Lane 2: WT3

Lane 3: Rat-1-WT

Panel B. Morphology of WT3 cell line. Phase contrast light microscopy (x100 magnification).

A.



B.

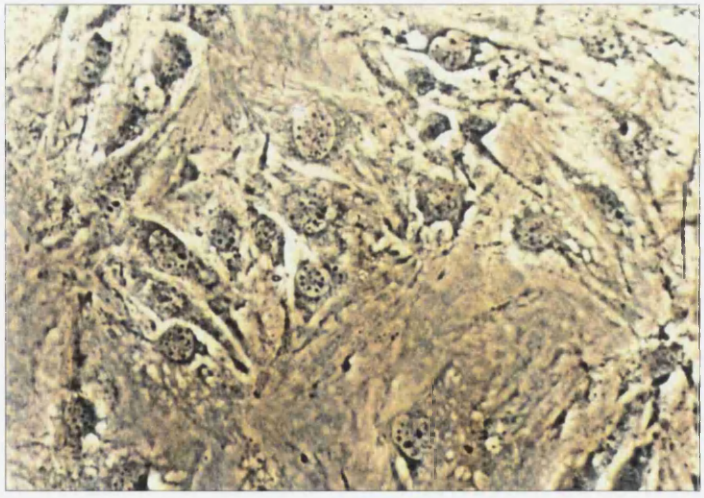
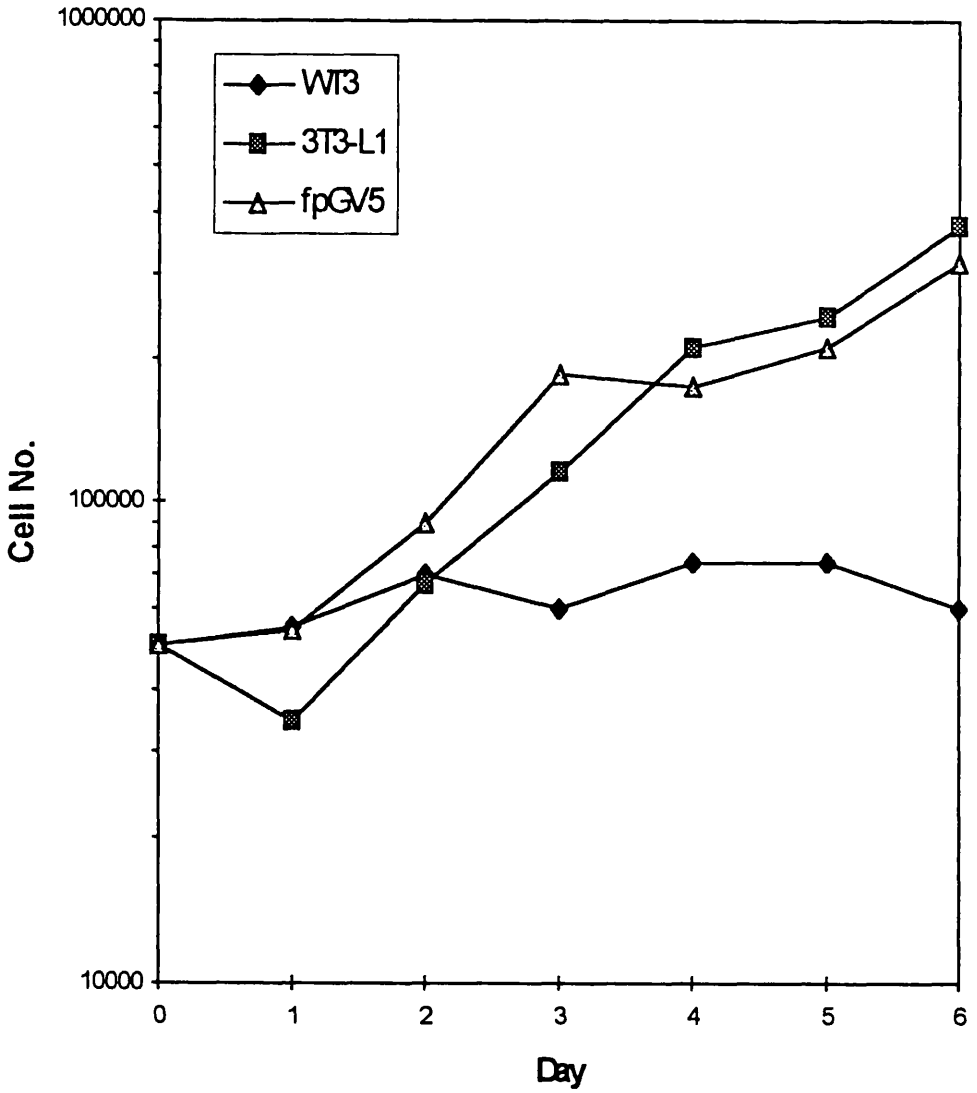


Figure 14.

Figure 14. Growth Rate of the WT3 Cell Line.

The growth rates of the individual cell lines were determined as described in Materials and Methods and a representative plot shown.


1000000
100000
10000
Cell No.
WT3
3T3-L1
fpGV5
0
1
2
3
4
5
6
Day

transgene may be subject to post-transcriptional control. If high level expression of c-Myc in cell lines such as 3T3-L1 is indeed toxic then a situation may be envisaged in which selection pressure favours clones expressing low levels of the protein. This could explain the low abundance of avian c-Myc within the majority of the 3T3-L1-WT clones.

6.9 Enforced Expression of Avian c-Myc Does Not Transform the 3T3-L1 Cell Line.

The Myc-mediated block to differentiation is often associated with cellular transformation (Gonos and Spandidos, 1993). In order to determine whether enforced expression of the avian c-*myc* transgene also caused transformation in the 3T3-L1 line we examined a number of well-defined parameters of this phenomenon. With the exception of WT3, the Myc-expressing clones were morphologically indistinguishable from the parental 3T3-L1 cell line and fpGV5. They retained the property of contact inhibition, since they were able to arrest at confluence (Figures 42 and 43, Chapter 9) and did not form foci. None of the clones were found to proliferate in soft agar (data not shown), suggesting that growth was dependent upon attachment to a substrate and could not occur independently of such anchorage. Finally, when compared to 3T3-L1 and fpGV5, the WT clones did not display an increased growth rate (Figure 15). These observations are in agreement with those of Freytag (1988), who demonstrated that constitutive expression of murine c-*myc* in the 3T3-L1 cell line was not associated with cellular transformation.

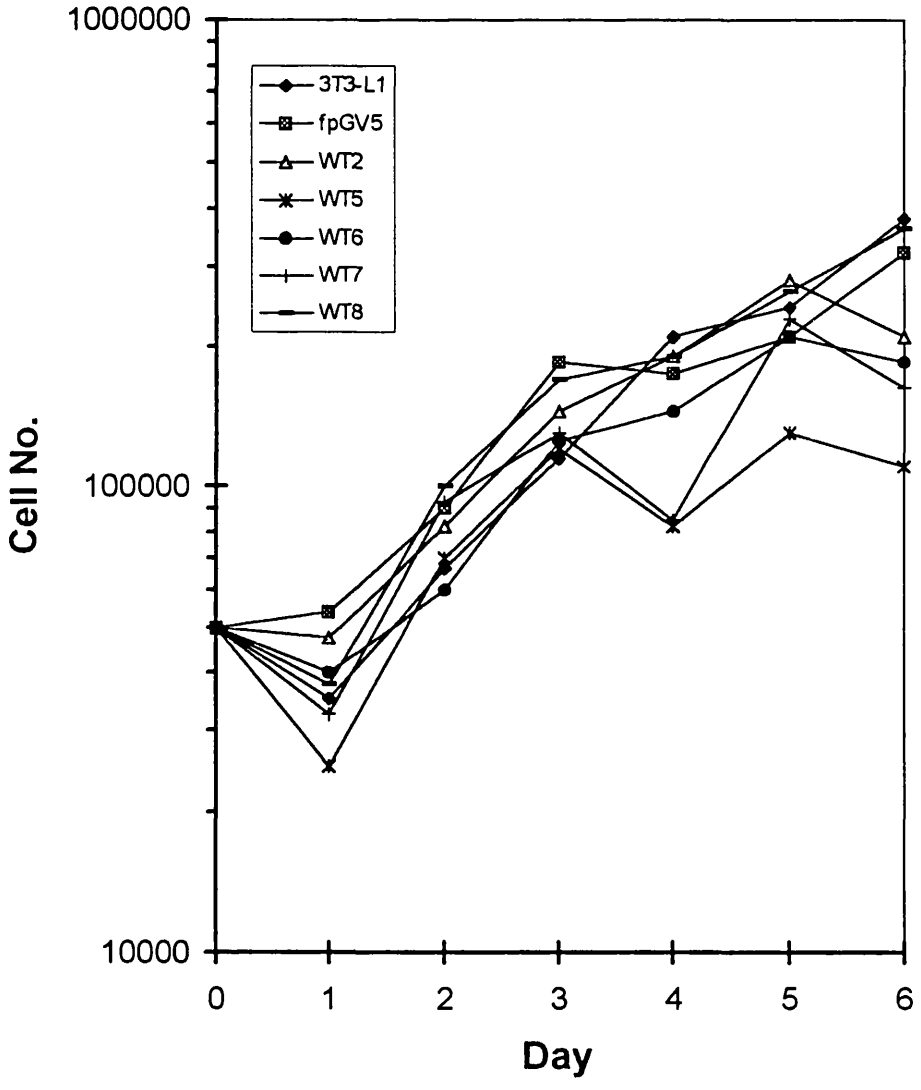
6.10 The WT Cell Lines Do Not Undergo Apoptosis in Low Serum Conditions.

Since over-expression of avian c-*myc* in the 3T3-L1 system did not result in transformation we decided to examine an additional biological function of c-Myc,

Figure 15.

Figure 15. Growth Characteristics of the WT Cell lines.

The growth rates of the individual cell lines were determined as described in Materials and Methods and a representative plot shown.


1000000
100000
10000
Cell No.
3T3-L1
fpGV5
WT2
WT5
WT6
WT7
WT8
0
1
2
3
4
5
6
Day

namely the ability to mediate programmed cell death in unfavourable growth conditions. Exponentially growing cultures were changed to medium containing 0.2% donor calf serum and the appearance of the cells monitored by light microscopy over a period of several hours. Cultures that are actively undergoing apoptosis are characterised by a number of morphological features (Martin *et al*, 1994). Upon receipt of the apoptotic signal, adherent cells round up and detach from the substrate. The nuclear DNA becomes condensed, possibly as a result of fragmentation by specific nucleases, and the nucleus rapidly disintegrates. In addition, the plasma membrane undergoes "blebbing" resulting in the formation of the characteristic apoptotic bodies. However, examination of the WT clones failed to reveal any of these features even after 24 hours incubation indicating that they were resistant to apoptosis during serum depletion. Thus, it was concluded that constitutive expression of avian c-*myc* does not potentiate apoptosis in the 3T3-L1 cell line under sub-optimal growth conditions. However, a recent study using dominant negative mutants of c-*myc* demonstrated that over-expression of the Myc HLH-LZ domain in 3T3-L1 cells readily induced apoptosis in a manner that appeared to be independent of Max (Kohlhuber *et al*, 1995). It should be noted, however, that these workers did not address the ability of the wild type protein to induce apoptosis in this system.

6.11 Deletion of Ten Amino Acids from the LZ Domain Impairs the Ability of c-Myc to Inhibit Adipogenesis.

It was previously demonstrated by our laboratory that whilst both v-Myc and c-Myc were able to block differentiation in the primary quail myoblast system, a deletion mutant, c-MycΔ7, was not inhibitory (La Rocca *et al*, 1994). This mutation deletes seven residues from the extreme C-terminus of the protein, including one leucine of the heptad repeat. However, the protein retained the ability to bind Max and was able to transform the cells. Since these experiments

had not been repeated in another model differentiation system, we therefore felt that it would be of interest to examine the ability of c-MycΔ7 to inhibit 3T3-L1 adipogenesis. Clones harbouring the mutant were generated as described in Section 6.5 and subjected to the standard differentiation protocol. Surprisingly, in contrast to the results obtained in the quail myoblasts, c-MycΔ7 was able to fully inhibit adipocyte differentiation as judged by oil red O staining at day 10. A representative clone, Δ7B, is shown in Figure 16. However, 3T3-L1 cell lines constitutively expressing c-MycΔ10, which differs from c-MycΔ7 by the deletion of a further three amino acids, were not impaired in their ability to undergo induced differentiation (Figure 16). As expected, attempts to detect the mutant proteins in 3T3-L1 lysates by Western blot analysis were equivocal but the c-*mycΔ7* and *c-mycΔ10* mRNAs were demonstrated in representative clones by Northern blotting (Figure 17). We therefore concluded that whilst the ability of c-Myc to inhibit adipogenesis could tolerate the loss of seven amino acids from the LZ domain of the molecule, larger deletions rendered the protein inactive.

6.12 The 3T3-L1 Cell Line Predominantly Expresses p21 Max.

It is widely accepted that the biologically active form of c-Myc constitutes a heterodimer with a second bHLH-LZ factor designated Max (Amati and Land, 1994). However, it was recently reported that functional Max is not expressed in the PC12 pheochromocytoma cell line, due to synthesis of a mutant *max* transcript (Hopewell and Ziff, 1995). In this respect, it is of interest to note that ectopic expression of c-*myc* in PC12 cells had previously been shown to cause cellular transformation and inhibit nerve growth factor induced differentiation (Maruyama *et al*, 1987). Taken together, these results suggest that Myc may be able to function independently of Max in the PC12 system. Since the status of Max in a variety of cell lines, including 3T3-L1, was not known we decided to analyse the expression of this protein by Western blotting. As depicted in Figure 18A (data

Figure 16.

Figure 16. Morphology of the c-MycΔ7 and c-MycΔ10 Cell Lines at Day 10 of the Differentiation Programme.

Cell lines were subjected to the standard differentiation protocol and the ability to accumulate cytoplasmic triglyceride assessed by oil red O staining at day 10. Phase contrast light microscopy (x100 magnification).

A. 3T3-L1

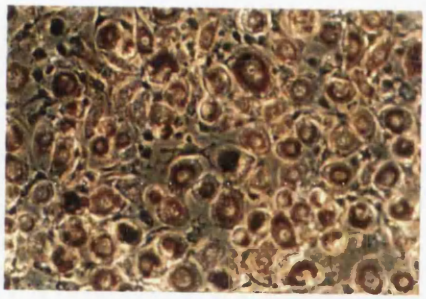
B. fpGV5

C. WT6

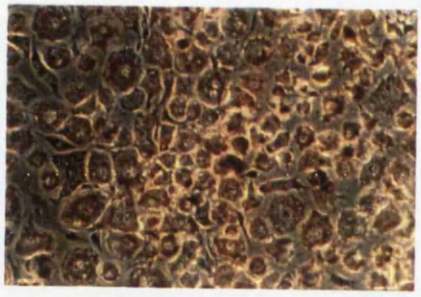
D. Δ7B

E. Δ10C

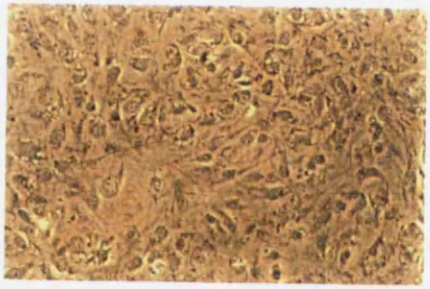
A.



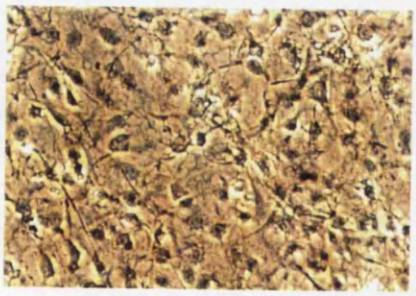
B.



C.



D.



E.

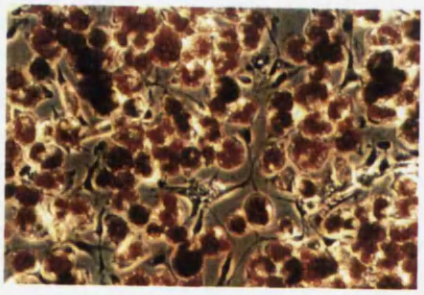


Figure 17.

Figure 17. Northern Blot Analysis of c-*myc* mRNA in the c-MycΔ7 and c-MycΔ10 Cell Lines.

Panel A. Equivalent amounts of total cellular RNA were resolved by gel electrophoresis and subjected to Northern blot analysis using the 500bp *Cla* I fragment of fpGV-WT as a probe. The position of the 6.5kb and 2.1kb transcripts are indicated.

Lane 1: 3T3-L1

Lane 2: fpGV5

Lane 3: Rat-1-WT

Lane 4: WT6

Lane 5: Δ7B

Lane 6: Δ10C

Panel B. Equivalence of loading and integrity of the RNA are demonstrated by ethidium bromide staining of the gel prior to transfer.

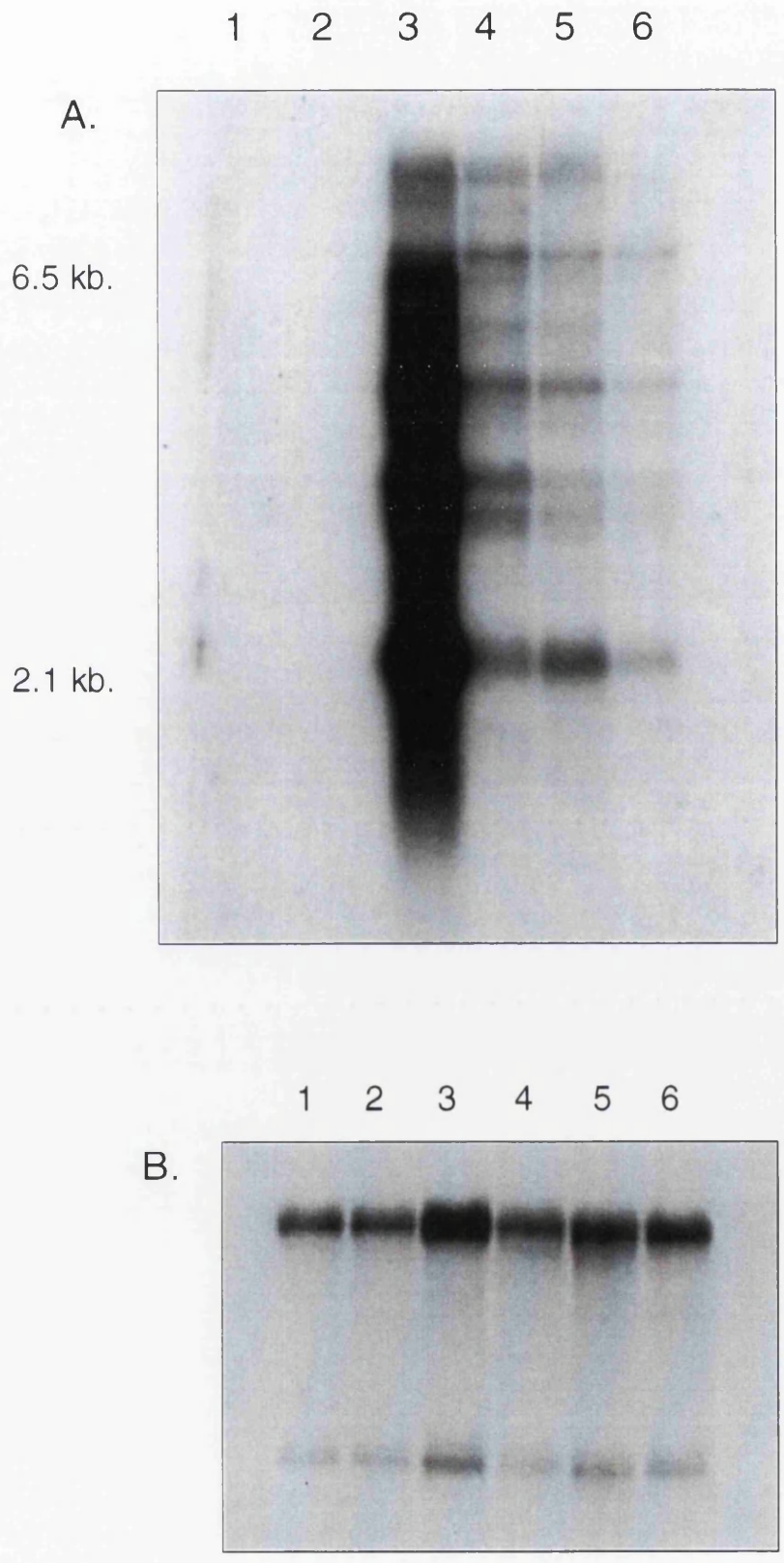

1 2 3 4 5 6
A.
6.5 kb.
2.1 kb.
1 2 3 4 5 6
B.

Figure 18.

Figure 18. Western Blot Analysis of Max Expression in the 3T3-L1 Cell Line.

Panel A. Equivalent amounts of protein were subjected to SDS-PAGE and Western blot analysis using the 1.2FB polyclonal antibody (1:1000) and the ECL detection method. The positions of the p21 and p22 isoforms of Max are indicated.

Lane 1: 3T3-L1

Lane 2: QT35

Lane 3: Q8

Lane 4: MH2

Lane 5: Rat-1

Lane 6: U937

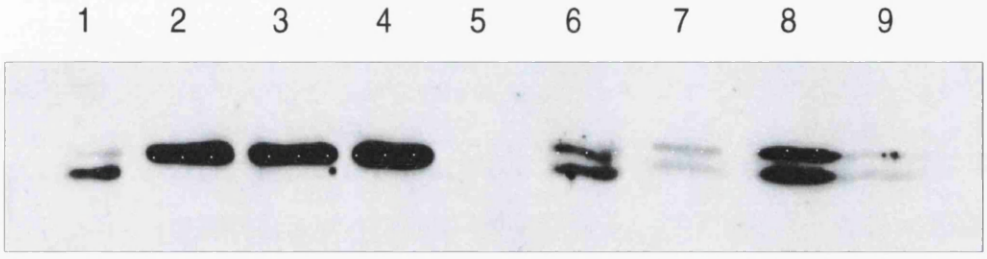
Lane 7: HL60

Lane 8: CHO

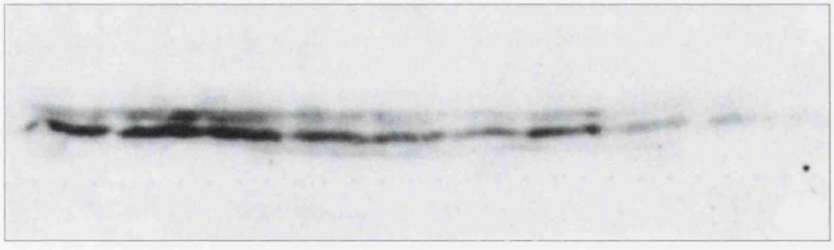
Lane 9: HeLa

Panel B. Protein samples were prepared from the 3T3-L1 cell line on the days indicated and equivalent amounts subjected to SDS-PAGE and Western blot analysis using the 1.2FB polyclonal antibody (1:1000) and the ECL detection method. The positions of the p21 and p22 Max isoforms are highlighted.

A.



B.



generously provided by W. Clark, Beatson Institute), protein samples were prepared from a number of cell lines and Max detected using the rabbit polyclonal antiserum, 1.2FB (the kind gift of Trevor Littlewood, Imperial Cancer Research Fund, London). Interestingly, the expression pattern of the two Max isoforms was found to vary widely between the cell lines studied. Hence, in avian cell lines (e.g. QT35; Q8; MH2) the p22 isoform predominated, whilst lines derived from human (HeLa; U937; HL60), hamster (CHO) and rodent (Rat-1) sources expressed both forms. In contrast, the 3T3-L1 cell line was characterised by the predominant expression of the p21 isoform, and this pattern was retained throughout the differentiation programme (Figure 18B).

6.13 Expression of Mad1 is Not Associated with 3T3-L1 Adipogenesis.

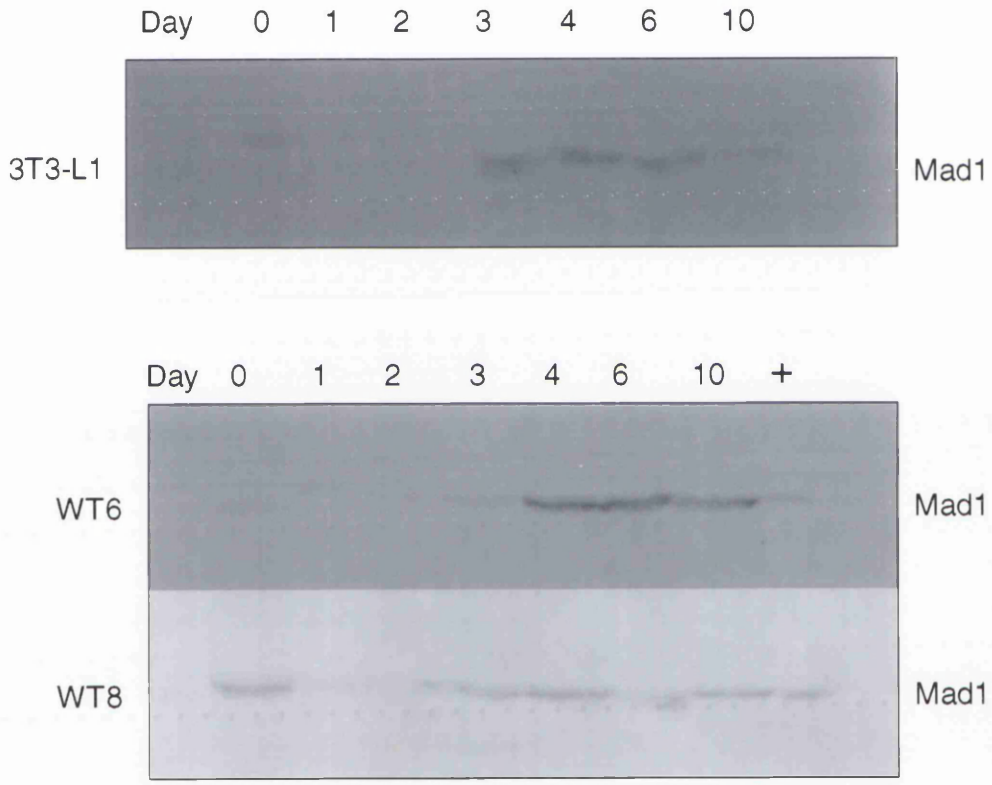
In a number of cell types, differentiation is associated with a switch from Myc/Max to Mad/Max complexes (Larsson *et al*, 1994; Hurlin *et al*, 1995b). Since expression of the Mad family during induced differentiation of the 3T3-L1 cell line has not previously been studied, we felt that it would be of interest to analyse the expression patterns of these proteins during adipogenesis. Protein lysates were prepared at various time points and subjected to Western blot analysis using a commercially available polyclonal Mad1 antiserum, C-19 (Santa Cruz). This antibody was raised against a synthetic peptide corresponding to residues 203-221 of human Mad1 but cross-reacts with Mad1 of murine origin. In the 3T3-L1 cells, Mad1 was found to be expressed in uninduced (day 0) samples (Figure 19). However, treatment with the hormone cocktail resulted in a rapid down-regulation of the protein (days 1 and 2). Following removal of the inducing hormones, high levels of Mad1 were detected in day 3, 4 and 6 lysates, whilst in the day 10 sample the protein appeared to be down-regulated. This expression pattern is highly suggestive of a role in growth arrest, since the protein was absent in lysates derived from the clonal expansion phase but was detected at time points

Figure 19.

Figure 19. Western Blot Analysis of Mad1 Expression During the Differentiation Programme.

Equivalent amounts of protein prepared from the 3T3-L1, WT6 and WT8 cell lines on the days indicated were subjected to SDS-PAGE and Western blot analysis using the C-19 polyclonal antibody (1:250) and the alkaline phosphatase detection method.

Positive control (+): 3T3-L1 (day 4)


Day 0 1 2 3 4 6 10
3T3-L1
Mad1
Day 0 1 2 3 4 6 10 +
WT6
Mad1
WT8
Mad1

in which the cells were no longer proliferating. This proposal is strengthened by analysis of Mad1 expression in two representative Myc-expressing clones, WT6 and WT8. Whilst these cell lines were unable to differentiate, the pattern of Mad1 expression was very similar to that observed in the 3T3-L1 cells (Figure 19). Thus, in the 3T3-L1 cell line Mad1 is unlikely to be associated with terminal differentiation but may play a role in cell cycle exit. Unfortunately, the commercial antibodies currently available for detection of Mxi1, Mad3 and Mad4 were extremely unreliable and, in the absence of appropriate positive controls, Western blot analyses were equivocal. Thus, determination of the expression patterns of these proteins during 3T3-L1 differentiation will require further investigation.

6.14 The WT Cell Lines Provide a Model System in Which to Study the Effects of Enforced Expression of c-Myc on Cellular Differentiation.

In summary, we have generated a number of 3T3-L1 preadipocyte cell lines constitutively expressing avian c-Myc. Whilst the protein is present at extremely low levels, this appears to be sufficient to block induced differentiation as judged by loss of expression of the key regulator of adipogenesis, C/EBPα, and an inability to accumulate cytoplasmic triglyceride. This function requires regions at the extreme C-terminus of the molecule, since the deletion mutant c-MycΔ10 had lost the capacity to inhibit differentiation. Enforced expression of c-Myc in the 3T3-L1 cell line was not associated with cellular transformation or the ability to undergo apoptosis in low serum. Thus, it appears that the WT cell lines represent a model system in which to examine a single biological function of c-Myc, *viz* the capacity to block differentiation.

CHAPTER 7: Enforced Expression of c-*myc* in the 3T3-L1 System Inhibits the Terminal Stages of the Differentiation Programme.

7.1 Introduction.

Whilst constitutive expression of c-*myc* in the 3T3-L1 cell line had previously been shown to inhibit differentiation (Freytag, 1988; Freytag and Geddes, 1992), the precise point at which Myc exerted this effect had not been determined. In order to address this issue, we decided to examine the expression patterns of a number of molecular markers representative of the various stages of the differentiation programme. By identifying genes that were differentially expressed, it was anticipated that we should be able to define a point where the Myc-mediated block to adipogenesis was likely to occur.

7.2 The 3T3-L1 Cell Line May Spontaneously Lose the Ability to Differentiate.

It has previously been demonstrated that the 3T3-L1 cell line may spontaneously lose the ability to differentiate following serial passage at high density or single cell cloning procedures and that this trait is stably inherited by the clonal progeny (Green and Kehinde, 1975). Since our aim was to define key regulatory points in the adipogenic programme, we felt that in addition to studying the Myc-mediated block it would also be of interest to investigate the properties of a cell line that had spontaneously lost the ability to differentiate. The line chosen for this purpose was derived by single cell cloning from the same batch of 3T3-L1 cells as fpGV5 and the WT cell lines and was designated Def (differentiation defective).

7.3 Strategy for the Molecular Analysis of Adipogenesis.

As shown in Figure 2, adipogenesis can be subdivided into a number of discrete phases each of which is associated with the expression of a particular set of genes. In order to carry out a detailed analysis of gene expression during induced

differentiation, we obtained Northern probes for a number of these markers from other workers in the field (described in Materials and Methods) and where appropriate Western blot analyses were also performed. To simplify the analysis of the Myc-expressing cell lines two representative clones, WT6 and WT8, were chosen. These had previously been used to examine the expression of Mad1 (Figure 19). In addition, since the differentiation profiles of the control cell lines, 3T3-L1 and fpGV5, were found to be highly similar, only data relating to the 3T3-L1 cell line is presented.

7.4 Expression of the C/EBP Family During Induced Differentiation.

As described in Chapter 2, several members of the C/EBP family of bLZ transcription factors are implicated in adipocyte differentiation. These are thought to participate in a cascade of regulation culminating in the expression of C/EBPα, a key regulator of the terminal stages of adipogenesis.

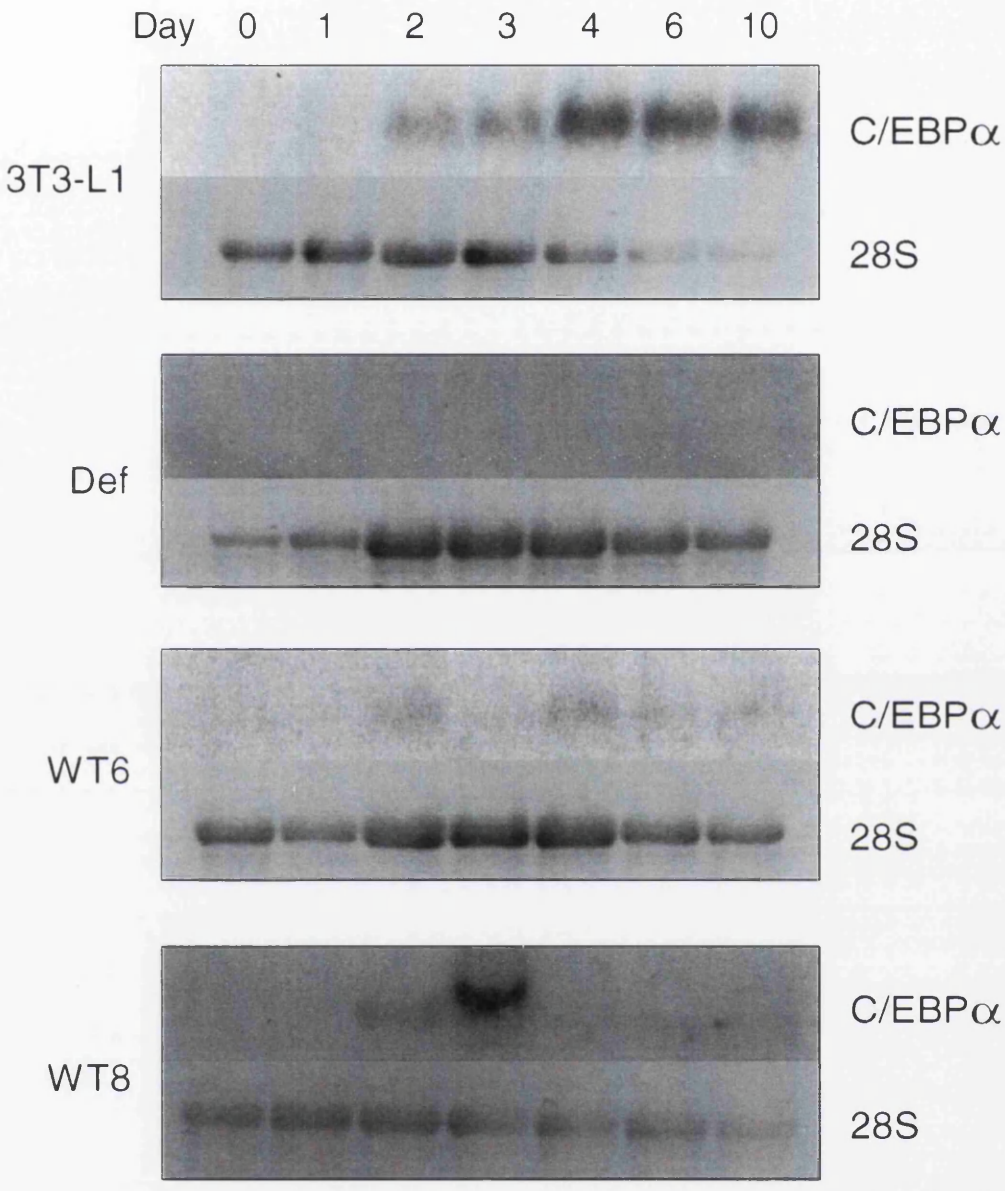
7.4.1 *C/EBPα*

We had previously shown by Western blot analysis that in contrast to 3T3-L1 and fpGV5, neither isoform of C/EBPα could be detected in day 10 lysates derived from the WT cell lines (Figure 10). This was also found to be the case following induced differentiation of Def (data not shown). Since repression of gene expression may occur at a post-transcriptional level, we decided to examine the expression of the *c/ebpα* mRNA during adipogenesis. Total cellular RNA was prepared at various time points and subjected to Northern blot analysis using a *c/ebpα* specific probe. In the 3T3-L1 cell line, the 2.7kb *c/ebpα* mRNA was first detected at day 2 and increased thereafter (Figure 20). This pattern of expression was in agreement with previous observations and correlated well with expression of the protein during 3T3-L1 adipogenesis (Christy *et al*, 1989). In contrast, the *c/ebpα* transcript could not be detected at any time point in the Def cell line.

Figure 20.

Figure 20. Northern Blot Analysis of *c/ebpα* mRNA During the Differentiation Programme.

Total cellular RNA was prepared on the days indicated and equal amounts subjected to Northern blot analysis using the 900bp *Nco* I fragment of MSV/EBPα as a probe. Equivalence of loading is demonstrated by the 28S signal.


Day 0 1 2 3 4 6 10
3T3-L1
C/EBPα
28S
Def
C/EBPα
28S
WT6
C/EBPα
28S
WT8
C/EBPα
28S

However, examination of WT6 and WT8 indicated that very low levels of *c/ebpα* mRNA were present and that this occurred in a temporal pattern similar to that observed in the 3T3-L1 line. Thus, it appears that repression of *c/ebpα* in the WT and Def cell lines may occur by different mechanisms. The data derived from the analyses of *c/ebpα* mRNA and protein expression in the WT cell lines may be explained in a number of ways. Firstly, if c-Myc represses the *c/ebpα* promoter, as has previously been suggested (Li *et al*, 1994), it is possible that the low level of c-Myc present in the WT clones was insufficient to fully inhibit transcription. However, reducing the *c/ebpα* mRNA below a certain threshold level may still be sufficient to block adipogenesis. Alternatively, *c/ebpα* may be subject to post-transcriptional control in the Myc-expressing cell lines, possibly at the level of translation or protein stability. Obviously, further investigation will be required to clarify this issue.

A major problem encountered when embarking on this analysis was the selection of an appropriate loading control, since genes traditionally employed are differentially expressed during adipogenesis. Hence, actin and tubulin are seen to decrease (Spiegelman and Farmer, 1982) whilst expression of GAPDH is upregulated as differentiation proceeds (Cornelius *et al*, 1994). It was therefore fortuitous that the *c/ebpα* probe was found to cross-hybridise to the 28S ribosomal RNA (Figure 20). Equivalence of loading and integrity of the RNA preparations were therefore assessed by examination of this 28S signal and ethidium bromide staining of the gel prior to transfer (not shown).

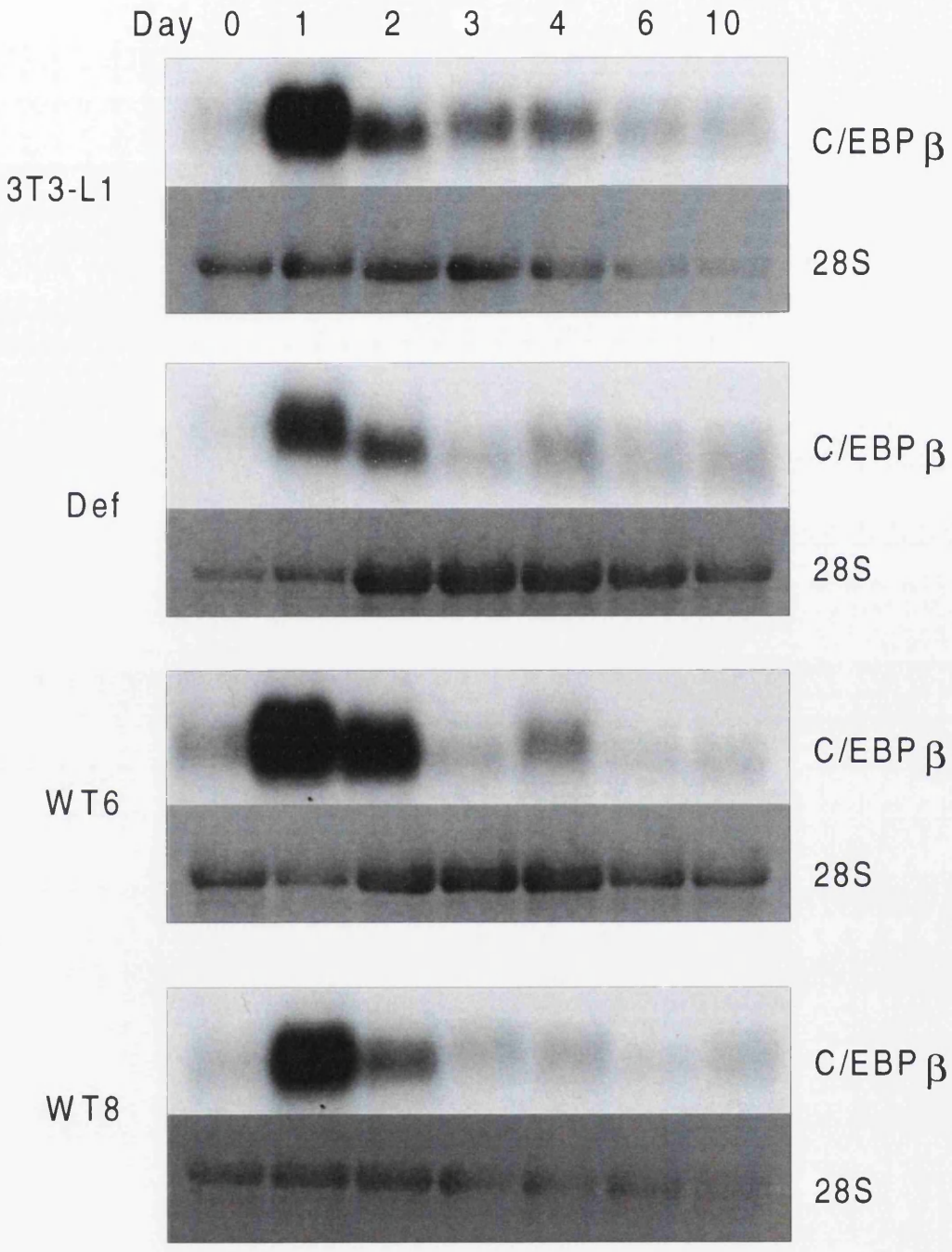
7.4.2 *C/EBPβ*

Northern blot analysis of *c/ebpβ* expression during the differentiation programme is shown in Figure 21. In the 3T3-L1 cell line, the 1.5kb *c/ebpβ* transcript was present throughout adipogenesis but was obviously up-regulated between days 1

Figure 21.

Figure 21. Northern Blot Analysis of *c/ebpβ* mRNA During the Differentiation Programme.

Total cellular RNA was prepared at the time points indicated and equal amounts subjected to Northern blot analysis using the 1.5kb *Bam* HI/*Eco* RI fragment of MSV/EBPβ as a probe. Equivalence of loading is demonstrated by the 28S signal.


Day 0 1 2 3 4 6 10
3T3-L1
C/EBP β
28S
Def
C/EBP β
28S
WT6
C/EBP β
28S
WT8
C/EBP β
28S

and 4. Since expression of *c/ebpβ* was previously shown to be induced by MIX (Yeh *et al*, 1995a), this observation probably represents a consequence of hormonal stimulation of the differentiation pathway. However, a similar pattern of expression was observed in the WT and Def cell lines, indicating that inhibition of adipogenesis was not associated with repression of the *c/ebpβ* gene at the level of transcription.

Since we had previously demonstrated that repression of C/EBPα did not necessarily equate with inhibition of transcription in 3T3-L1 cells constitutively expressing c-*myc* (Section 7.4.1), we decided to examine the expression of C/EBPβ by Western blot analysis. Protein samples were prepared at various time points and C/EBPβ detected using the polyclonal antibody, C-19 (Santa Cruz), raised against amino acids 258-276 of the rat protein. This antiserum is able to recognise both the LAP (31-kDa) and LIP (20-kDa) isoforms of C/EBPβ. In the 3T3-L1 cell line expression of LAP was first apparent at day 1 of the differentiation programme but was elevated in the day 2 lysate (Figure 22), the levels declining thereafter. The LIP isoform was found to exhibit a similar pattern of expression. As described above, it is likely that the increased expression of both isoforms during hormone treatment (days 1 and 2) was a consequence of MIX stimulation. LIP and LAP were also found to be upregulated in the WT cell lines during the first two days of the differentiation programme (Figure 22). However, expression during the later stages appeared to be reduced when compared to the 3T3-L1 cell line although this was not a consistent finding and may represent blot to blot variation. In contrast, LIP and LAP were barely detectable in the Def cell line. In order to make a direct comparison of the cell lines, expression of C/EBPβ in day 2 lysates was therefore examined on the same filter (Figure 23). Whilst Def displayed reduced levels of LAP when compared to the other cell lines, the major deficit appeared to be in the expression of LIP, suggesting that *c/ebpβ* may be subject to post-transcriptional

Figure 22.

Figure 22. Western Blot Analysis of C/EBPβ Expression During the Differentiation Programme.

Protein samples were prepared at the time points indicated and equivalent amounts subjected to SDS-PAGE and Western blot analysis using the C-19 polyclonal antibody (1:500) and the ECL detection method. The positions of the LIP and LAP isoforms of C/EBPβ are indicated.

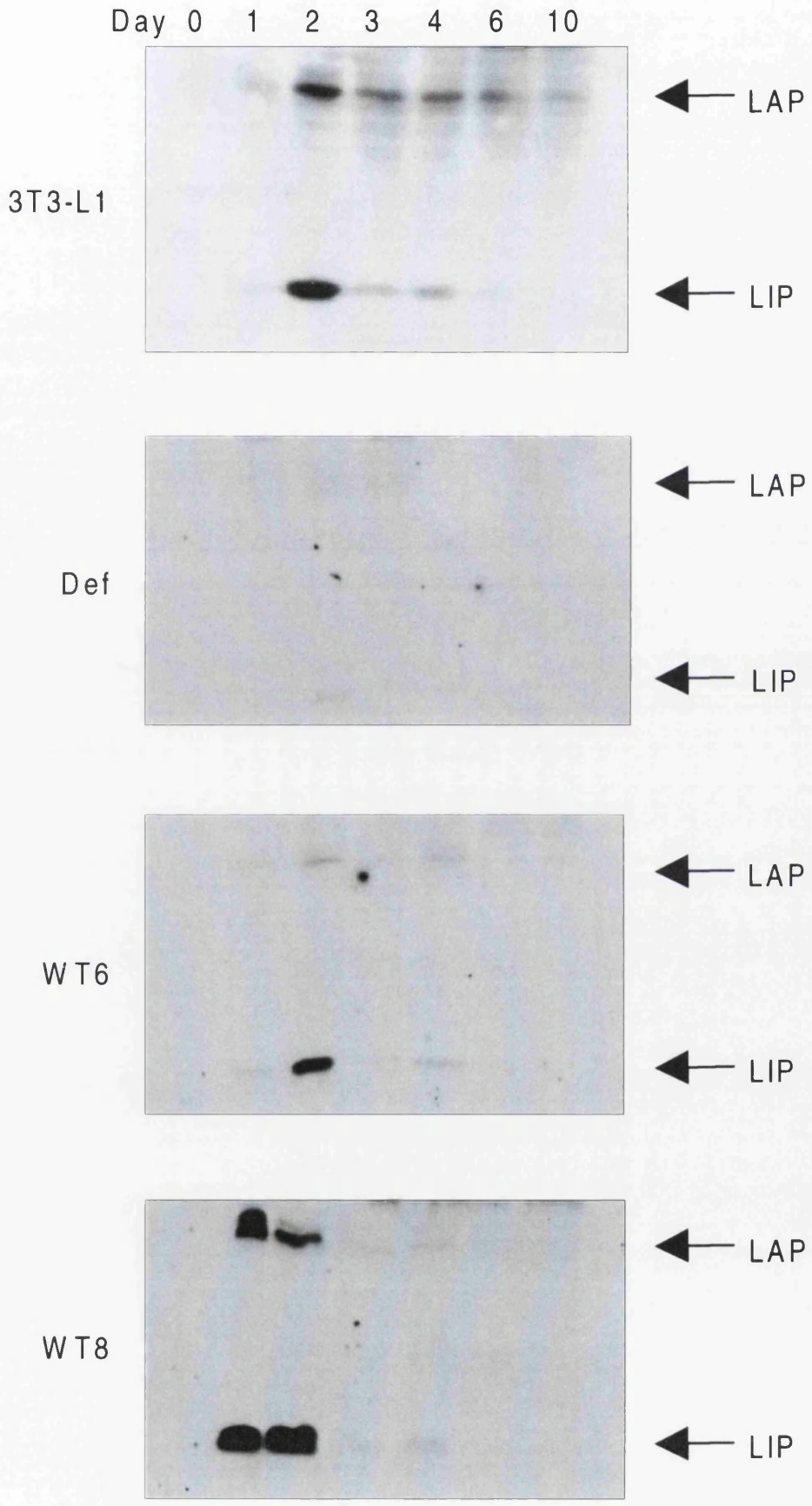

Day 0 1 2 3 4 6 10
3T3-L1
LAP
LIP
Def
LAP
LIP
WT6
LAP
LIP
WT8
LAP
LIP

Figure 23.

Figure 23. Western Blot Analysis of C/EBPβ Expression at Day 2 of the Differentiation Programme.

Protein samples were prepared at day 2 of the differentiation programme and equal amounts subjected to SDS-PAGE and Western blot analysis using the C-19 polyclonal antibody (1:500) and the alkaline phosphatase detection method. The positions of the LIP and LAP isoforms of C/EBPβ are indicated.

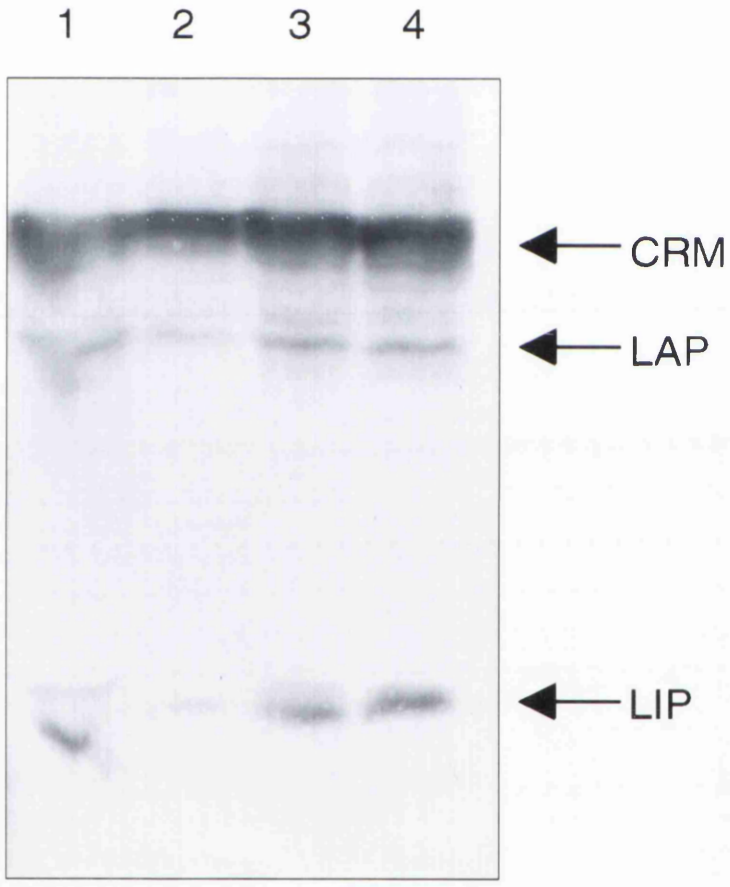
Lane 1: 3T3-L1

Lane 2: Def

Lane 3: WT6

Lane 4: WT8

Equivalence of loading is indicated by the cross-reacting material (CRM).


1
2
3
4
CRM
LAP
LIP

control in this cell line. In contrast, there was little difference in the expression of either isoform between the Myc-expressing cell lines and 3T3-L1.

7.4.3 *C/EBPδ.*

Since expression of C/EBPδ was previously shown to be induced by dexamethasone (Yeh *et al*, 1995a), it was not surprising that Northern hybridisation with a *c/ebpδ* specific probe gave a similar result to that observed with *c/ebpβ.* Hence during induced differentiation of the 3T3-L1 cell line expression of the *c/ebpδ* mRNA was low at day 0 but rapidly increased following hormone treatment, declining to basal levels by day 6 (Figure 24). A similar pattern of expression was observed in the WT6, WT8 and Def clones. Thus, it appears unlikely that the *c/ebpδ* gene is a target for repression during 3T3-L1 adipogenesis.

7.4.4 *CHOP-10/GADD153.*

Northern blot analysis of *chop-10* mRNA levels during induced differentiation of the 3T3-L1 line suggested that expression of this gene correlated with growth arrest (Figure 25). Hence, the 1.1kb mRNA was strongly expressed at confluence and from day 3 onwards, but was reduced during the clonal expansion phase (days 1 and 2). This pattern of expression was reminiscent of the Mad1 protein during the differentiation programme (Figure 19). In addition to a role in growth arrest, CHOP-10 has also been implicated as a dominant negative inhibitor of C/EBP family function (Ron and Habener, 1992) and constitutive expression in the 3T3-L1 cell line was sufficient to inhibit differentiation (Batchvarova *et al*, 1995). CHOP-10 therefore represents an appealing target for up-regulation during the inhibition of adipogenesis. However, examination of *chop-10* expression in the WT and Def cell lines did not support this proposal (Figure 25). Indeed, it has previously been demonstrated that enforced expression of c-*myc* in the Rat-1 cell line was

Figure 24.

Figure 24. Northern Blot Analysis of *c/ebpδ* mRNA During the Differentiation Programme.

Total cellular RNA was prepared on the days indicated and equal amounts subjected to Northern blot analysis using the 1.5kb *Bam* HI/*Eco* RI fragment of MSV/EBPδ as a probe. Equivalence of loading is demonstrated by the 28S signal.

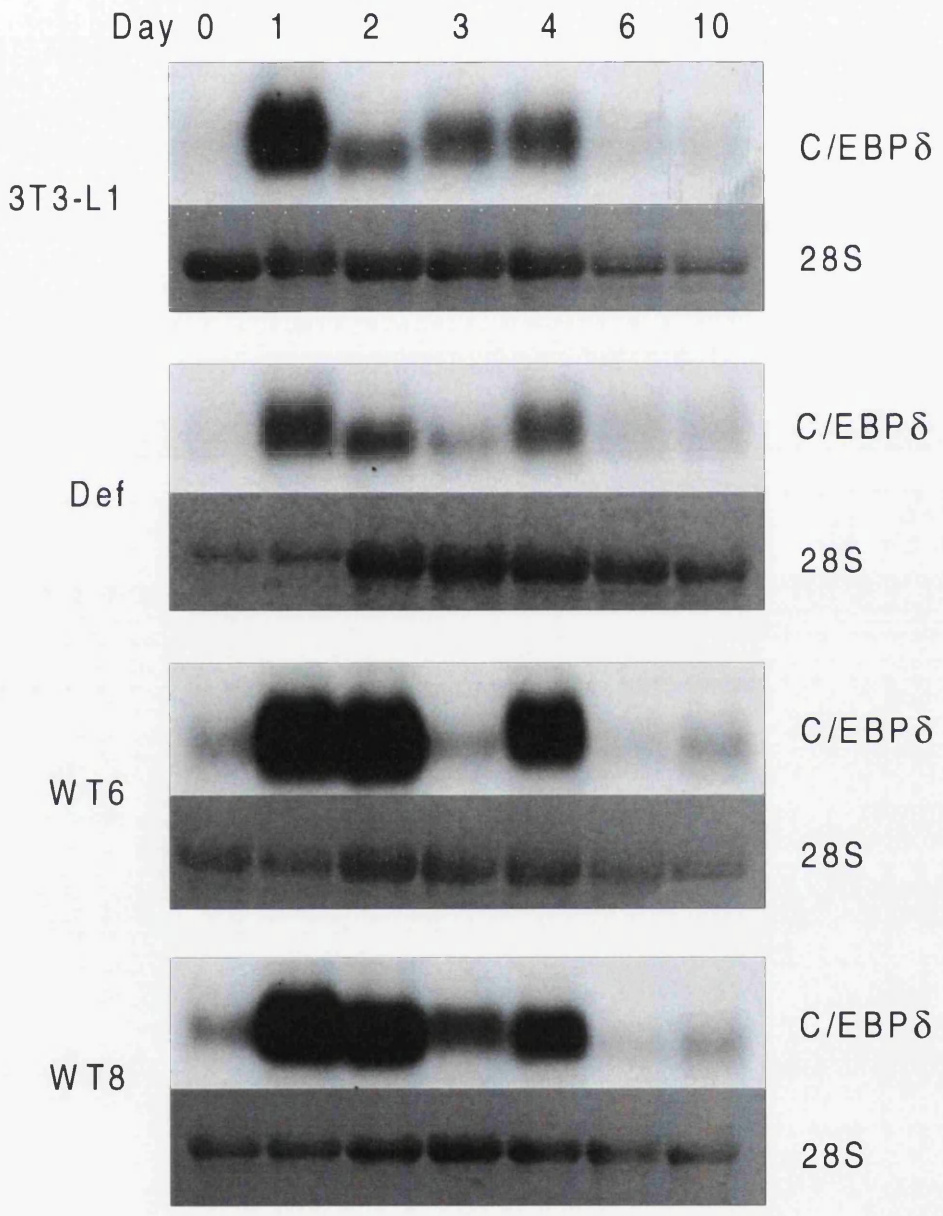
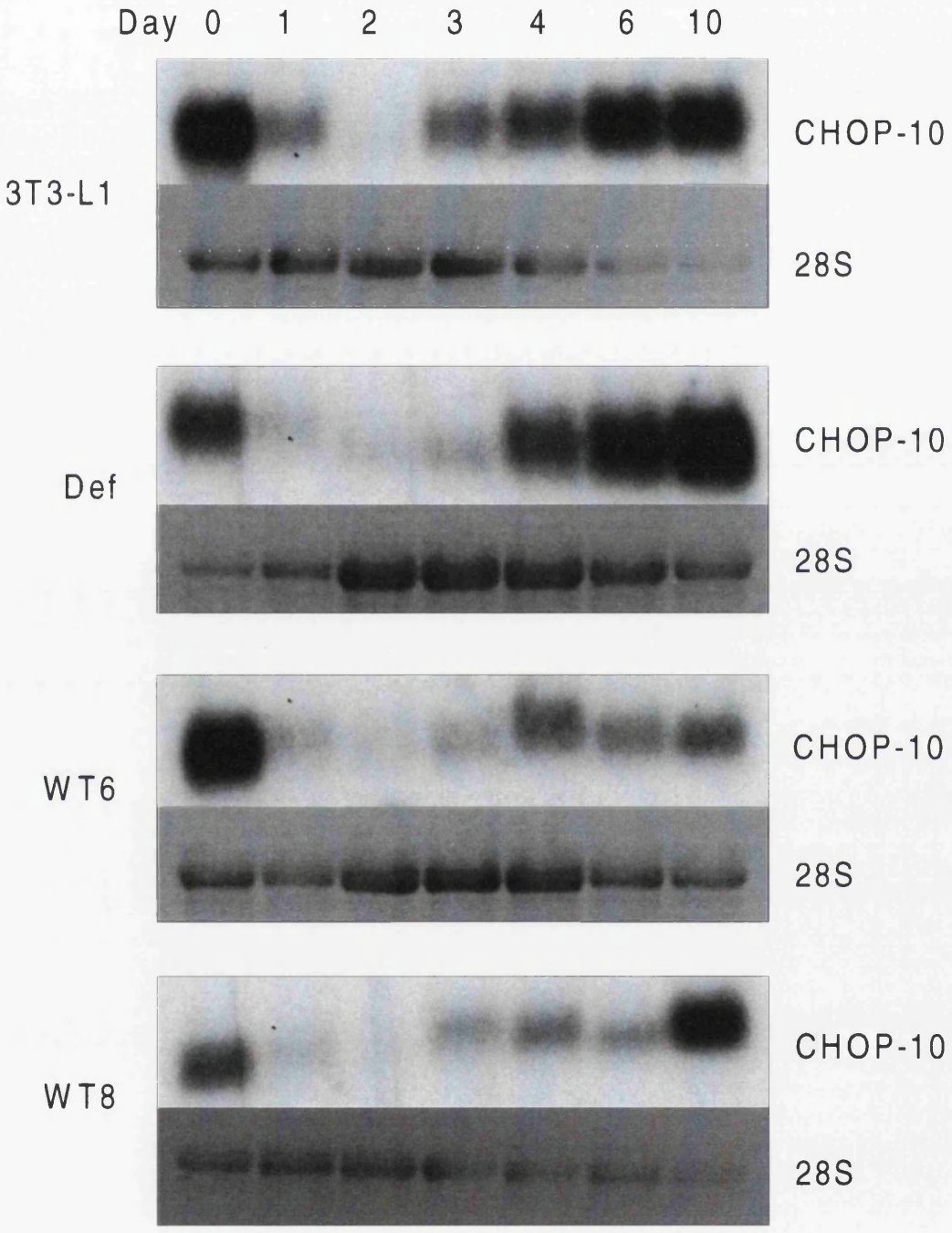

Day 0 1 2 3 4 6 10
3T3-L1
C/EBPδ
28S
Def
C/EBPδ
28S
WT6
C/EBPδ
28S
WT8
C/EBPδ
28S

Figure 25.

Figure 25. Northern Blot Analysis of *chop-10* mRNA During the Differentiation Programme.

Total cellular RNA was prepared at the time points indicated and equal amounts subjected to Northern blot analysis using the 710bp *Bam* HI/*Acc* I fragment of Chop-10 pBS as a probe. Equivalence of loading is demonstrated by the 28S signal.


Day 0 1 2 3 4 6 10
3T3-L1
CHOP-10
28S
Def
CHOP-10
28S
WT6
CHOP-10
28S
WT8
CHOP-10
28S

associated with the repression of CHOP-10 (Chen *et al*, 1996), although this was obviously not the case in the 3T3-L1 cell line.

7.5 Expression of the PPAR Family During Induced Differentiation.

As described in Chapter 2, the PPAR family of transcription factors are implicated in mediating the cellular response to fatty acids during adipogenesis. In addition, PPARγ2 is thought to be a key regulator of the terminal stages of the differentiation programme.

7.5.1 *FAAR*.

Expression of the *faar* mRNA was examined in the 3T3-L1 cell line by Northern blot analysis. In agreement with a previous report (Amri *et al*, 1995), the 3.1kb mRNA was present at confluence and increased following hormonal induction, remaining at a fairly invariant level throughout the differentiation programme (Figure 26). A similar pattern was observed in the WT and Def cell lines, suggesting that this gene was unaffected by the differentiation block.

7.5.2 *PPARγ2*.

In accordance with previous reports (Tontonoz *et al*, 1994a), expression of the 2kb *pparγ2* mRNA in the 3T3-L1 cell line was first detected at low levels in the day 1 sample and increased thereafter, reaching a maximum at day 4 (Figure 27). High levels of expression were retained throughout the terminal stages of the differentiation programme. In contrast, *pparγ2* mRNA was not detected at any time point in the Myc-expressing or Def cell lines. Hence, *pparγ2* is a target for repression during inhibition of preadipocyte differentiation.

Figure 26.

Figure 26. Northern Blot Analysis of *faar* mRNA During the Differentiation Programme.

Total cellular RNA was prepared at the time points indicated and equal amounts subjected to Northern blot analysis using the 1.5kb *Bam* HI fragment of pSGFAAR as a probe. Equivalence of loading is demonstrated by the 28S signal.

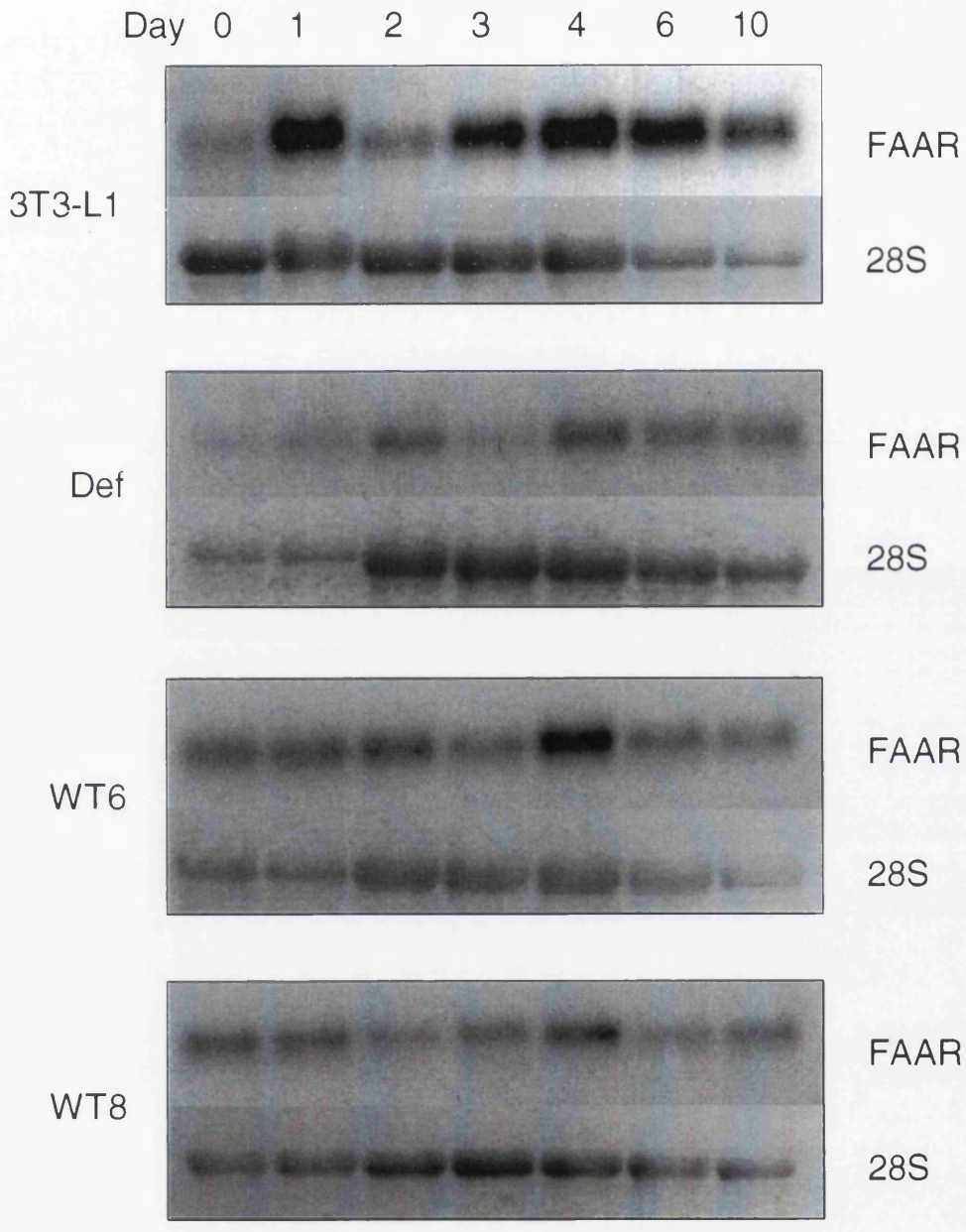
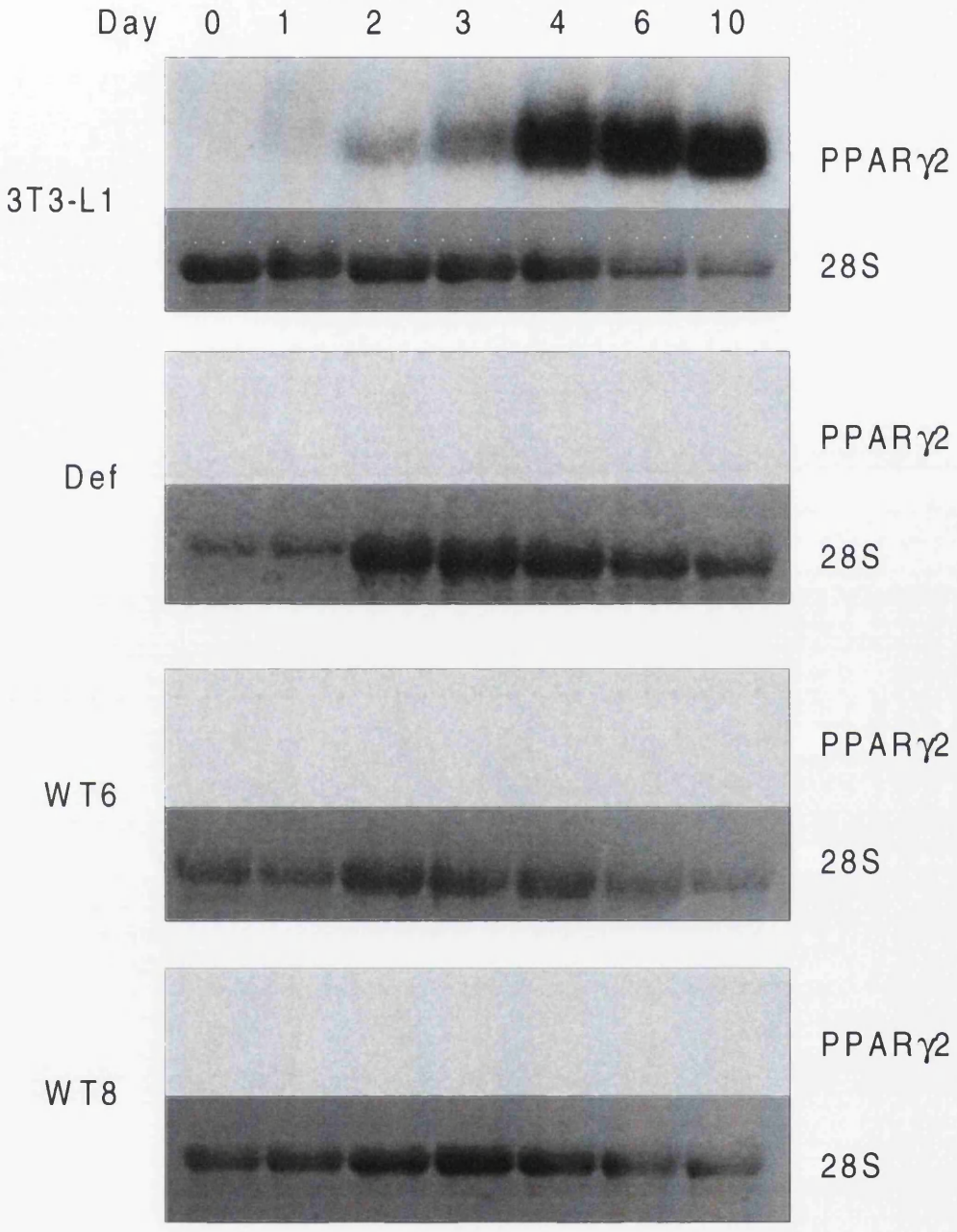

Day 0 1 2 3 4 6 10
3T3-L1
FAAR
28S
Def
FAAR
28S
WT6
FAAR
28S
WT8
FAAR
28S

Figure 27.

Figure 27. Northern Blot Analysis of *pparγ2* mRNA During the Differentiation Programme.

Total cellular RNA was prepared on the days indicated and equal amounts subjected to Northern blot analysis using the 520bp *Stu* I fragment of PPARγ2/SPORT as a probe. Equivalence of loading is demonstrated by the 28S signal.


Day 0 1 2 3 4 6 10
3T3-L1
PPARγ2
28S
Def
PPARγ2
28S
WT6
PPARγ2
28S
WT8
PPARγ2
28S

7.6 Expression of Genes Required for Lipid Metabolism.

Acquisition of the adipocyte phenotype is associated with the co-ordinate expression of a number of genes involved in lipid metabolism (Cornelius *et al*, 1994). We therefore elected to examine the expression patterns of two of these genes: *ap2*, which encodes a fatty acid binding protein and *scd1*, which encodes the enzyme stearoyl CoA desaturase 1. As determined by Northern blot analysis, expression of these genes was restricted to the terminal stages of the differentiation programme in the 3T3-L1 cell line (Figure 28), consistent with a role in lipogenesis. In contrast, neither gene could be detected in the WT and Def cell lines. This observation therefore provides a molecular basis for the inability of these cell lines to accumulate cytoplasmic triglyceride following induction of the differentiation programme.

7.7 The WT and Def Cell Lines are Unable to Participate in the Terminal Stages of the Differentiation Programme.

By conducting a detailed analysis of the expression patterns of a number of markers during induced differentiation, we have been able to define a point at which adipogenesis may be blocked. Our observations are summarised in Figure 29 and demonstrate that inhibition of differentiation is manifest at the terminal stages of the programme in both a cell line that had spontaneously lost the ability to differentiate and lines constitutively over-expressing the c-Myc oncoprotein. Hence, expression of the key adipogenic transcription factors, C/EBPα and PPARγ2, is deregulated which results in an inability to express genes required for lipid metabolism. However, subtle differences detected in the expression patterns of C/EBPα and C/EBPβ between these cell lines suggests that repression may not occur by a common mechanism.

Figure 28.

Figure 28. Northern Blot Analysis of *ap2* and *scd1* mRNAs During the Differentiation Programme.

Total cellular RNA was prepared at the time points indicated and equal amounts subjected to Northern blot analysis using either the 700bp *Pst* I fragment of aP2/pGem or the 800bp *Pst* I/*Eco* RI fragment of PC4. Equivalence of loading is demonstrated by the 28S signal.

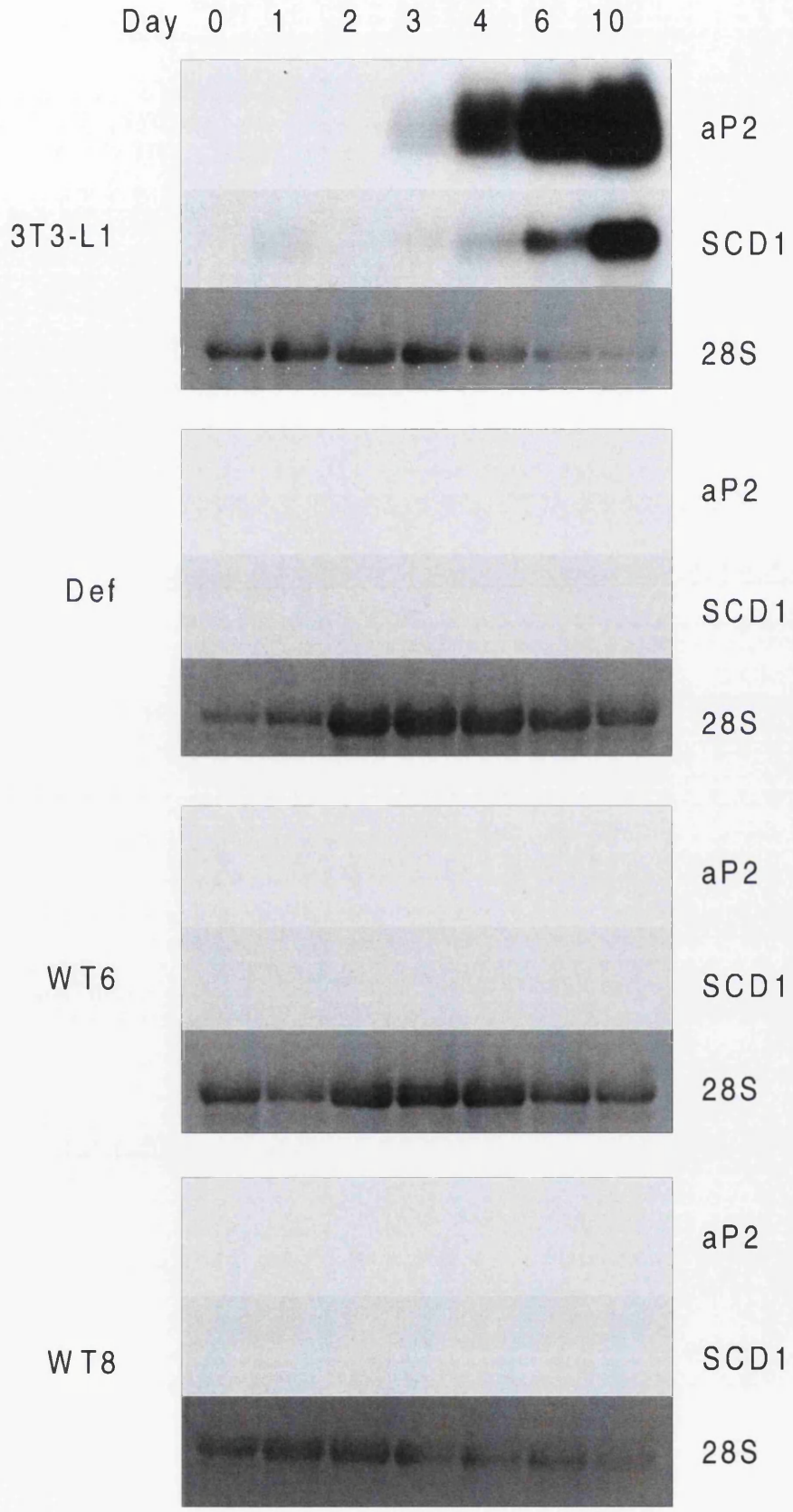

Day 0 1 2 3 4 6 10
3T3-L1
aP2
SCD1
28S
Def
aP2
SCD1
28S
WT6
aP2
SCD1
28S
WT8
aP2
SCD1
28S

Figure 29.

Figure 29. Profiles of Gene Expression During the Differentiation Programme.

The molecular markers analysed in this study were sub-divided into early, intermediate and late gene classes and the expression patterns in the Def and WT clones compared with those observed in the 3T3-L1 cell line. Inverted arrows indicate reduced levels of expression, whilst the asterisk indicates where gene expression could not be detected. Where no difference was observed the box is left blank.

GENE CLASS	Def	WT6	WT8
EARLY:			
CHOP-10 mRNA			
FAAR mRNA			
INTERMEDIATE:			
C/EBPβ mRNA			
Protein	↓		
C/EBPδ mRNA			
LATE:			
C/EBPα mRNA	*	↓	↓
Protein	*	*	*
PPARγ2 mRNA	*	*	*
aP2 mRNA	*	*	*
SCD1 mRNA	*	*	*

CHAPTER 8: The Myc-Mediated Block to Adipogenesis Is Abrogated by Foetal Calf Serum.

8.1 Introduction.

The differentiation protocol employed in this study was routinely carried out in the presence of 10% donor calf serum (DCS). However, reference to the literature suggested that many workers used foetal calf serum [FCS] (Student *et al*, 1980; Smulson *et al*, 1995; Benjamin *et al*, 1994). We therefore felt it would be of value to assess whether the type of serum employed during the differentiation programme was a critical determinant of adipogenic capacity. Duplicate 3T3-L1 cultures were induced to differentiate in the presence of either 10% DCS or 10% FCS and the ability to accumulate triglyceride assessed by oil red O staining at day 10. In accordance with our previous observations (Section 6.3.1), induction in the presence of 10% DCS resulted in 80-90% of the total population attaining the characteristic adipocyte morphology. However, differentiation in the presence of 10% FCS increased this number by an additional 5-10% (Figure 30A), indicating that FCS may be slightly more effective at promoting adipogenesis than DCS. In light of this finding, we therefore modified the standard differentiation protocol by substituting FCS for DCS at induction.

8.2 Induced Differentiation in the Presence of 10% FCS Rescues the Myc-Mediated Block to Adipogenesis.

Whilst induction in the presence of FCS led to a modest increase in the number of 3T3-L1 adipocytes, implementation of the revised differentiation protocol had a startling effect on the adipogenic capacity of the Myc-expressing cell lines, WT6 and WT8. As described in Section 6.6, induction in the presence of 10% DCS was not associated with acquisition of the adipocyte phenotype in

Figure 30.

Figure 30. Morphology at Day 10 of the Differentiation Programme in the Presence of 10% FCS.

Cultures were induced to differentiate in the presence of either 10% DCS or 10% FCS and the ability to accumulate cytoplasmic triglyceride assessed at day 10 of the programme by oil red O staining. Phase contrast light microscopy (x100 magnification).

A. 3T3-L1

B. Def

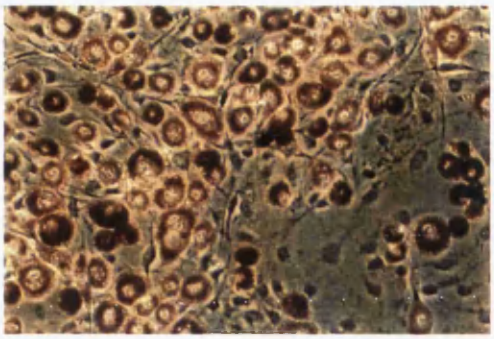
C. WT6

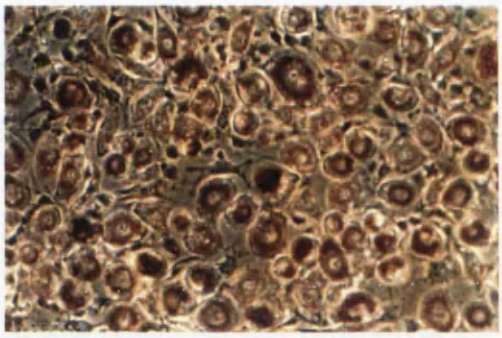
D. WT8

10% DCS

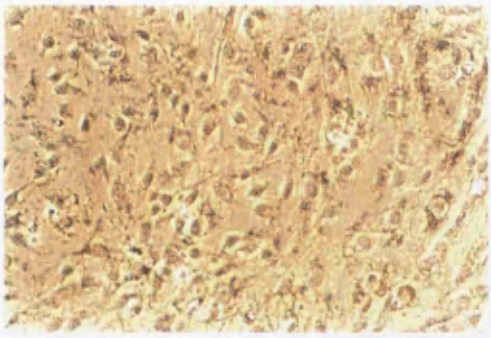
10% FCS

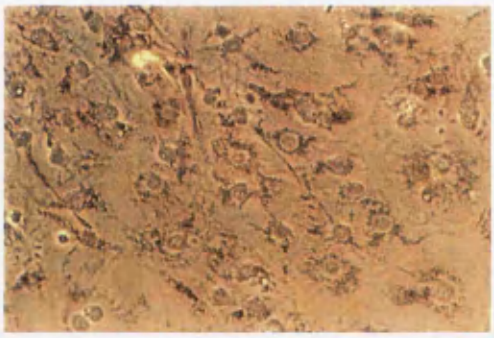
A.



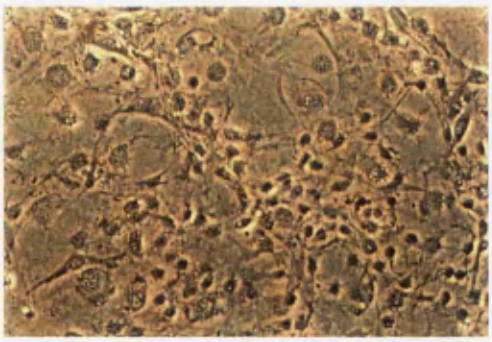


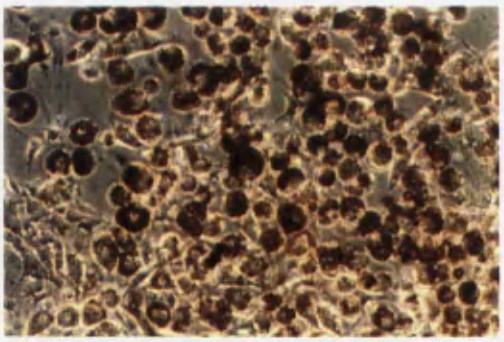
B.



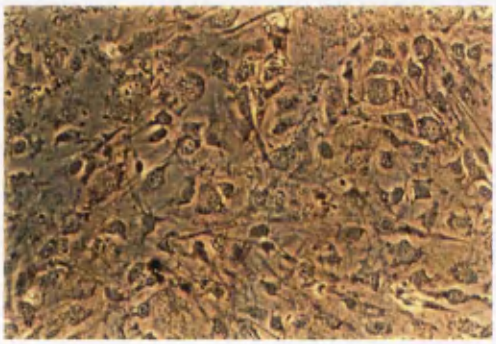


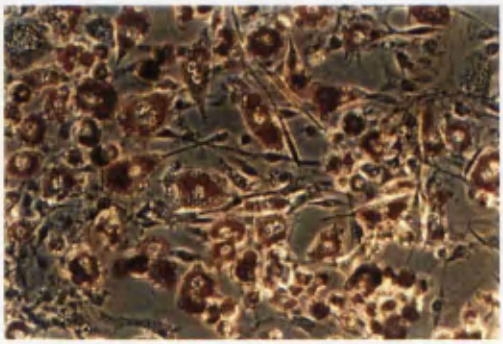
C.





D.





these clones, presumably as a consequence of the enforced expression of c-*myc*. However, treatment of WT6 and WT8 with the hormone cocktail in the presence of 10% FCS resulted in approximately 60-80% of the total cell population undergoing adipogenesis as judged by oil red O staining at day 10 (Figure 30C and D). This phenomenon was not unique to WT6 and WT8, since the remaining WT clones could also be induced to differentiate by this treatment (data not shown). Additionally, the differentiation block described in the c-MycΔ7 cell lines (Section 6.11) was abrogated in the presence of FCS (data not shown). In contrast, the differentiation defective cell line, Def, was unresponsive to the adipogenic effects of FCS (Figure 30B), although this treatment was associated with an abnormal morphology. Thus, it was concluded that the Myc-mediated block to adipogenesis could be rescued by FCS, whilst a cell line that had spontaneously lost the ability to differentiate was refractory to this treatment. These findings therefore indicate that inhibition of 3T3-L1 adipogenesis may occur by more than one mechanism.

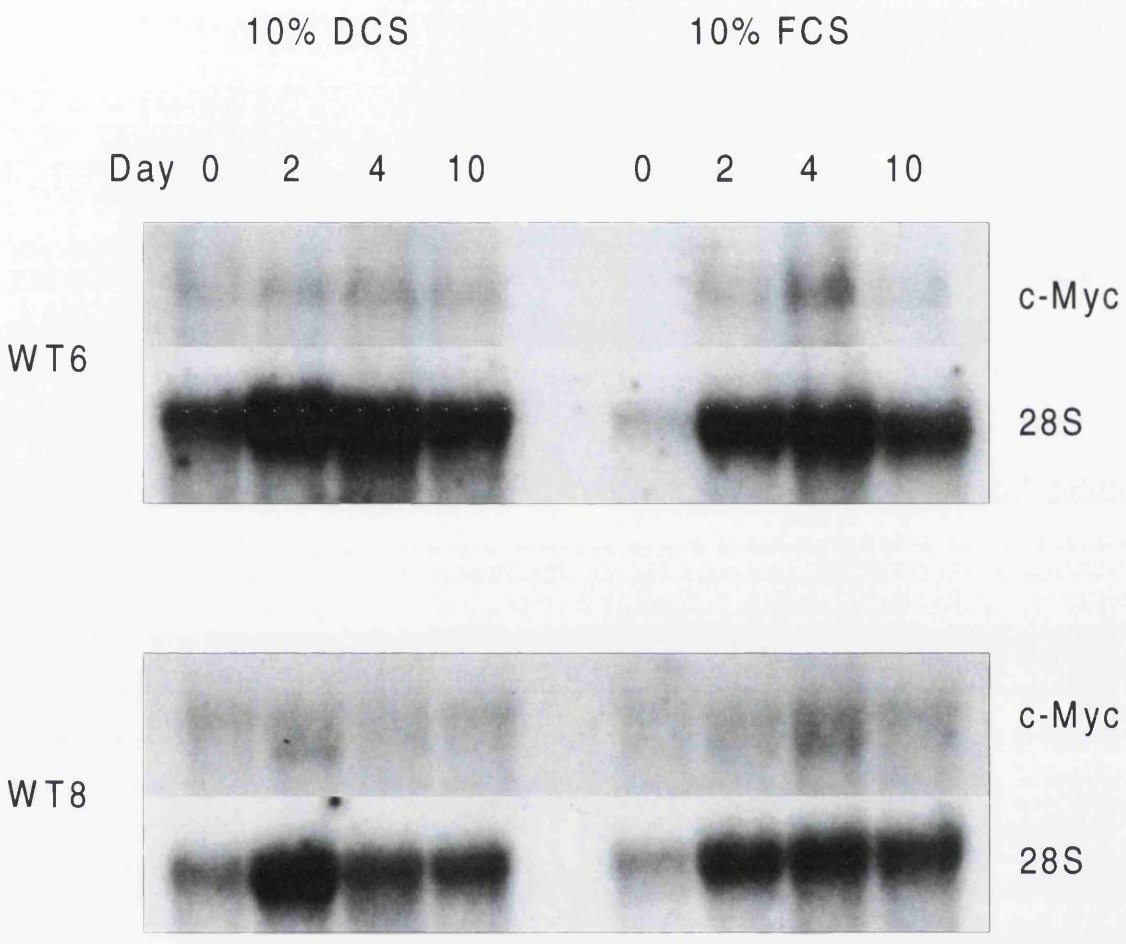
8.3 Differentiation is Not Associated with Down-Regulation of the Avian c-*myc* Transgene in the WT Cell Lines.

One possible explanation for the observed effect of FCS on the ability of Myc-expressing cell lines to differentiate is that expression of the avian c-*myc* transgene was down-regulated during this treatment. To address this issue, total cellular RNA was prepared from the WT6 and WT8 clones at several points following induction of differentiation in either 10% DCS or 10% FCS. Equal quantities were then subjected to Northern blot analysis using the c-*myc* specific hybridisation probe. As can be seen in Figure 31, expression of the avian c-*myc* 2.1kb mRNA was similar at all time points in both DCS and FCS, demonstrating that relief of the Myc-mediated block was not associated with down-regulation of the transgene. Conversely, up-regulation of c-*myc* was not

Figure 31.

Figure 31. Northern Blot Analysis of the Avian c-*myc* 2.1kb mRNA in the WT6 and WT8 Cell Lines Following Differentiation in 10% FCS.

Total cellular RNA was prepared at the times indicated following induction of the differentiation programme in the presence of either 10% DCS or 10% FCS. Equal amounts of RNA were subjected to Northern blot analysis using the 500bp *Cla* I fragment of fpGV-WT as a probe. Equivalence of loading is demonstrated by the 28S signal.


10% DCS
10% FCS
Day 0 2 4 10
0 2 4 10
WT6
c-Myc
28S
WT8
c-Myc
28S

observed in the Def cell line (data not shown), reinforcing the proposal that spontaneous loss of differentiation capacity occurs independently of c-Myc.

8.4 FCS Restores the Expression of Late Stage Markers in the WT Cell Lines.

Since treatment of the WT cell lines with FCS restored the ability to accumulate cytoplasmic triglyceride, we speculated that this phenomenon would also be associated with re-expression of the late stage markers. We therefore examined profiles of gene expression during the differentiation programme in the presence of FCS and compared them to those obtained using DCS. For simplicity we limited the study to those time points felt to be most informative (days 0, 2, 4 and 10) and made a direct comparison by probing samples on the same filter. Northern blot analysis of the 3T3-L1 cell line under the two conditions indicated that expression of the late stage markers was accelerated in the presence of 10% FCS (Figure 32). However, it should be noted that our previous data clearly demonstrate that these genes are induced between days 2 and 4 in 10% DCS (Chapter 7) and that these findings were in agreement with previous observations. In contrast, treatment of the WT lines with 10% FCS resulted in the restoration of those genes found to be repressed in the presence of 10% DCS (Figures 33 and 34), namely *c/ebpα*, *pparγ2*, *ap2* and *scd1*. Additionally, Western blot analysis of C/EBPα expression using the 14AA polyclonal antibody demonstrated that this protein was present in lysates prepared from WT6 and WT8 at day 10 of the differentiation programme following treatment with 10% FCS (Figure 35). This was in stark contrast to our previous observations (Figure 10). This analysis therefore provides a molecular basis for the morphological changes detected in cell lines constitutively expressing c-*myc* following induced differentiation in the presence of 10% FCS.

Figure 32.

Figure 32. Northern Blot Analysis of Late Gene Expression in the 3T3-L1 Cell Line Following Induced Differentiation in the Presence of 10% FCS.

Total cellular RNA was prepared at the times indicated following induction of the differentiation programme in the presence of either 10% DCS or 10% FCS. Equal amounts of RNA were subjected to Northern blot analysis using the probes previously described. Equivalence of loading is demonstrated by the 28S signal.

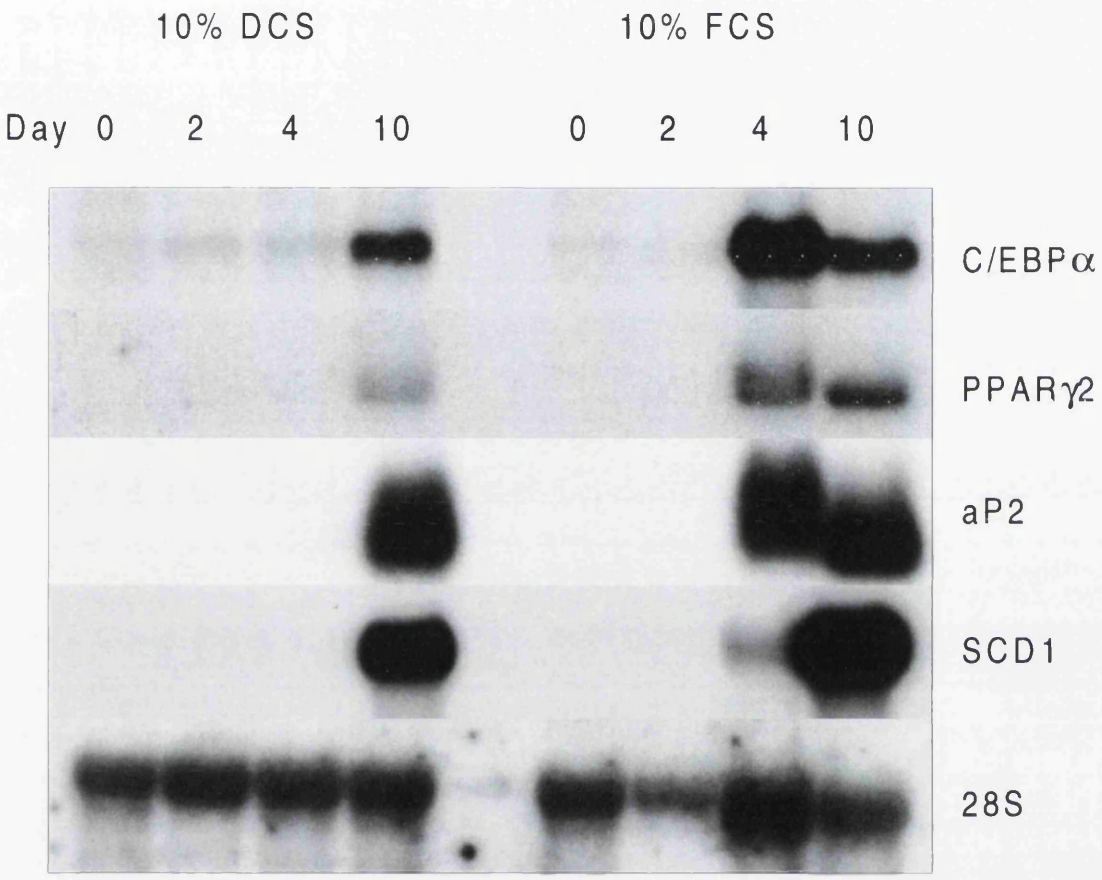

10% DCS
10% FCS
Day 0 2 4 10 0 2 4 10
C/EBPα
PPARγ2
aP2
SCD1
28S

Figure 33.

Figure 33. Northern Blot Analysis of Late Gene Expression in the WT6 Cell Line Following Induced Differentiation in the Presence of 10% FCS.

Total cellular RNA was prepared at the times indicated following induction of the differentiation programme in the presence of either 10% DCS or 10% FCS. Equal amounts of RNA were subjected to Northern blot analysis using the probes previously described. Equivalence of loading is demonstrated by the 28S signal.

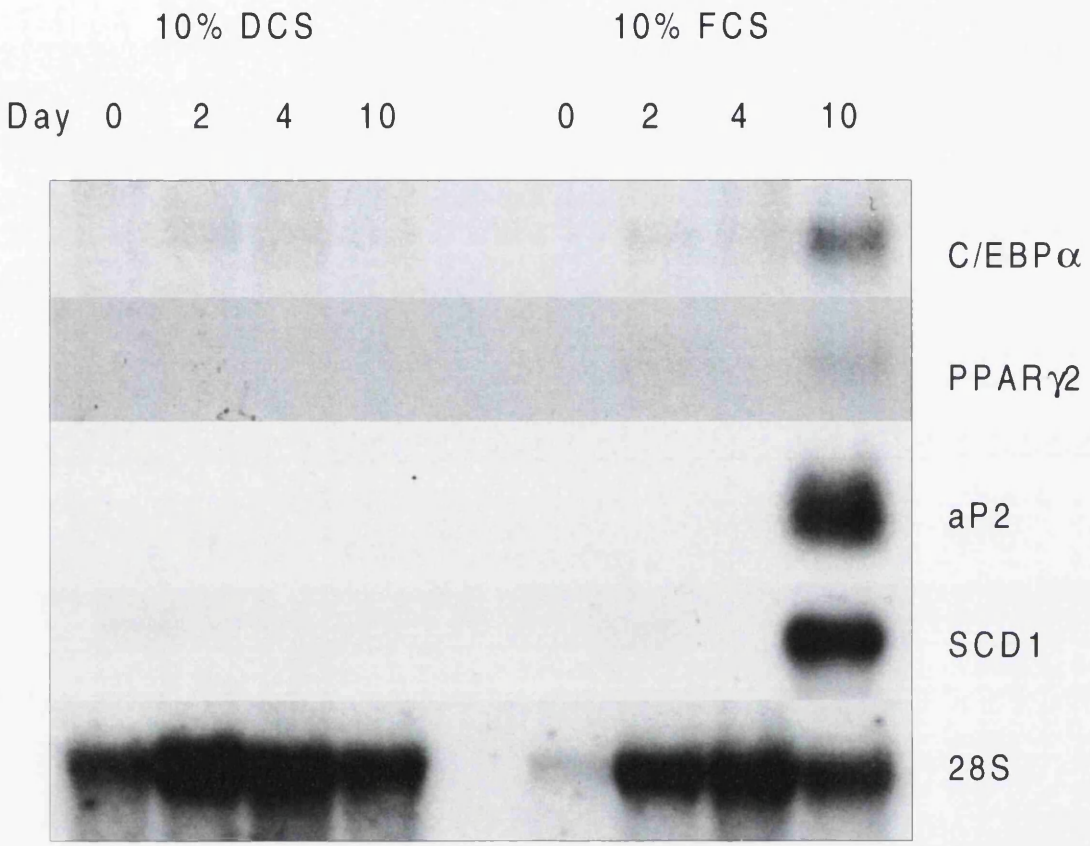

10% DCS
10% FCS
Day 0 2 4 10 0 2 4 10
C/EBPα
PPARγ2
aP2
SCD1
28S

Figure 34.

Figure 34. Northern Blot Analysis of Late Gene Expression in the WT8 Cell Line Following Induced Differentiation in the Presence of 10% FCS.

Total cellular RNA was prepared at the times indicated following induction of the differentiation programme in the presence of either 10% DCS or 10% FCS. Equal amounts of RNA were subjected to Northern blot analysis using the probes previously described. Equivalence of loading is demonstrated by the 28S signal.

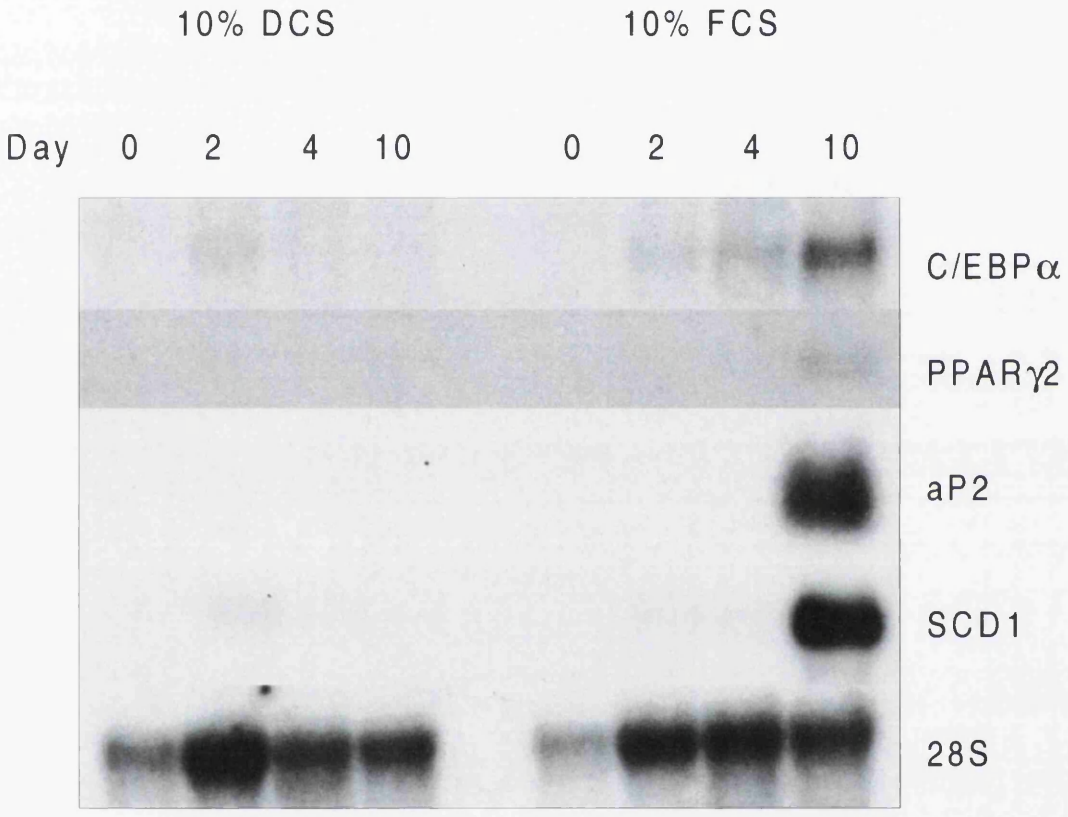

10% DCS
10% FCS
Day 0 2 4 10 0 2 4 10
C/EBPα
PPARγ2
aP2
SCD1
28S

Figure 35.

Figure 35. Western Blot Analysis of C/EBPα Expression Following Induction of the Differentiation Programme in the Presence of 10% FCS.

Protein samples were prepared at days 0 and 10 of the differentiation programme and equivalent amounts subjected to SDS-PAGE and Western blot analysis using the 14AA polyclonal antibody (1:500) and the alkaline phosphatase detection method. The positions of the 43-kDa and 30-kDa isoforms of C/EBPα are highlighted.

Lane 1: 3T3-L1 day 0

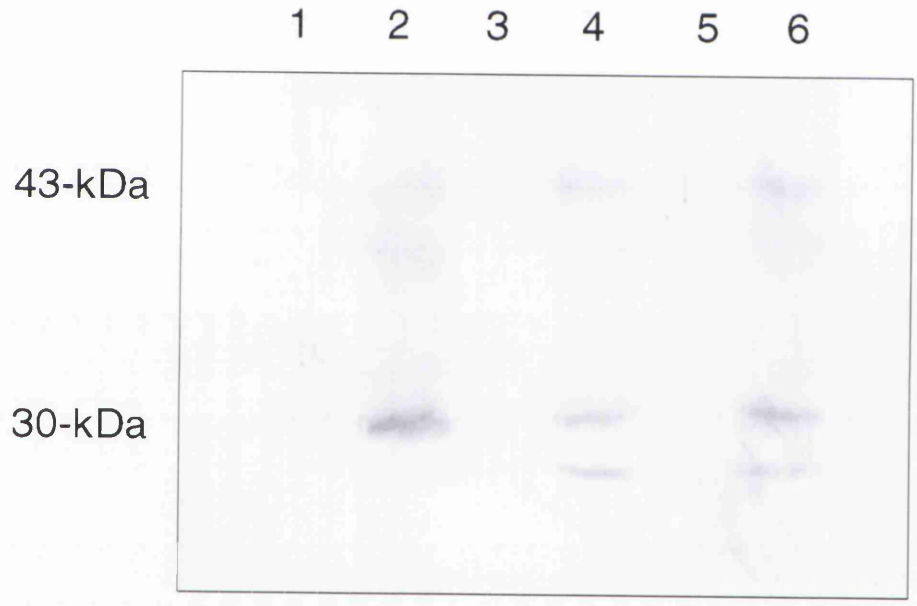
Lane 2: 3T3-L1 day 10

Lane 3: WT6 day 0

Lane 4: WT6 day 10

Lane 5: WT8 day 0

Lane 6: WT8 day 10


1
2
3
4
5
6
43-kDa
30-kDa

8.5 Each Cell Line is Responsive to a Different Concentration of FCS.

It was clear from the data presented above that treatment with 10% FCS was sufficient to over-come the inhibition of 3T3-L1 adipogenesis imposed by constitutive expression of c-*myc*. Since the Myc-mediated differentiation block was manifest in 10% DCS, we wondered whether the FCS effect could be titrated out by dilution with DCS. Replicate cultures were induced to differentiate in 10% DCS, 10% FCS or a mixture of the two (see Figure 36 for details) and the adipogenic capacity determined by staining with oil red O at day 10. Stained cultures were then viewed macroscopically and the degree of differentiation compared with that observed in the presence of 10% FCS. This data is tabulated in Figure 36. The 3T3-L1 cell line was found to be maximally responsive to a mixture of 2% FCS:8% DCS, whilst WT8 required at least 4% FCS for optimal effect. In contrast, WT6 could only differentiate in the presence of high concentrations of FCS (8%). We therefore concluded that the three cell lines differ in their ability to respond to the adipogenic effects of FCS.

8.6 The FCS Effect May be Mediated by Growth Hormone.

A possible explanation for the FCS effect on 3T3-L1 differentiation is that a critical serum component(s) is either absent from DCS or enhanced in FCS. As described in Chapter 1, growth hormone is a well characterised positive modulator of adipogenesis and thus presents as a plausible candidate for this factor. Indeed, the concentration of this hormone is elevated in FCS (Figure 37). We therefore postulated that supplementing DCS with growth hormone should mimic the effects of FCS in the WT cell lines. Replicate cultures were induced to differentiate in the presence of 10% DCS containing bovine pituitary growth hormone at a range of concentrations (50-500ng/ml) and the ability to differentiate compared with cultures induced in the presence of 10%

Figure 36.

Figure 36. Assessment of the Adipogenic Capacity of Serum Mixtures.

Replicate cultures were induced to differentiate in the presence of 10% DCS, 10% FCS or a mixture of the two types of sera and the ability to differentiate assessed by oil red O staining at day 10. A cross indicates where differentiation was equivalent to that observed in 10% FCS.

Serum / Line	10% DCS	9% DCS 1% FCS	8% DCS 2% FCS	7% DCS 3% FCS	6% DCS 4% FCS	5% DCS 5% FCS	4% DCS 6% FCS	3% DCS 7% FCS	2% DCS 8% FCS	1% DCS 9% FCS	10% FCS
3T3-L1			+	+	+	+	+	+	+	+	+
WT6									+	+	+
WT8					+	+	+	+	+	+	+

Figure 37.

Figure 37. Comparison of the Hormone Composition of FCS and DCS.

The table depicts the average concentrations of a number of hormones present in FCS and DCS. Information kindly provided by Gibco Life Technologies Inc.

Hormone.	Units.	Foetal Calf Serum	Donor Calf Serum
Growth Hormone	ng/ml	131	26
Insulin	uIU/ml	4.3	5.4
Oestradiol	pg/ml	13.8	32.9
Progesterone	ng/ml	0.03	1.65
Testosterone	ng/ml	0.4	1.05
T4	µg/dl	14.8	9.2
T3	ng/ml	1.2	1.9

FCS. This data is summarised in Figure 38, which shows the morphology of the three cell lines at day 10 of the differentiation programme following treatment with 10% DCS supplemented with 150ng/ml growth hormone. In the 3T3-L1 cell line the effect of growth hormone on adipogenic capacity was found to be concentration independent, since 50ng/ml was as effective as 500ng/ml (data not shown), although the number of cells attaining the differentiated phenotype was slightly less than that observed with 10% FCS. A similar result was obtained with WT8 and this was also associated with restoration of expression of the late stage markers (Figure 39). However, unlike the parental 3T3-L1 cell line, the adipogenic capacity of WT8 appeared to be concentration dependent with maximal differentiation being observed at 150ng/ml growth hormone. In contrast, WT6 could only be induced to differentiate in the presence of 10% FCS and was refractory to even high concentrations of growth hormone.

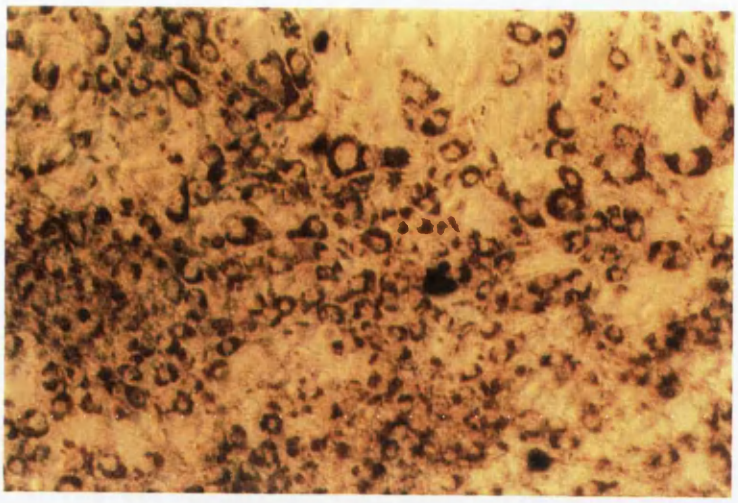
That the WT clones demonstrated opposing responses to this treatment was somewhat surprising since they had behaved in a similar manner when exposed to 10% FCS. However, the results of the serum mixing experiment (Figure 36) demonstrated that the two cell lines had different requirements regarding serum stimulation. Interestingly, treatment of the remaining WT cell lines with 10% DCS supplemented with 150ng/ml growth hormone uncovered a range of responses (data not shown), suggesting that more than one serum factor may be involved in abrogation of the Myc-mediated block to adipogenesis. As expected, the differentiation defective clone, Def, was unaffected by growth hormone (data not shown).

Figure 38.

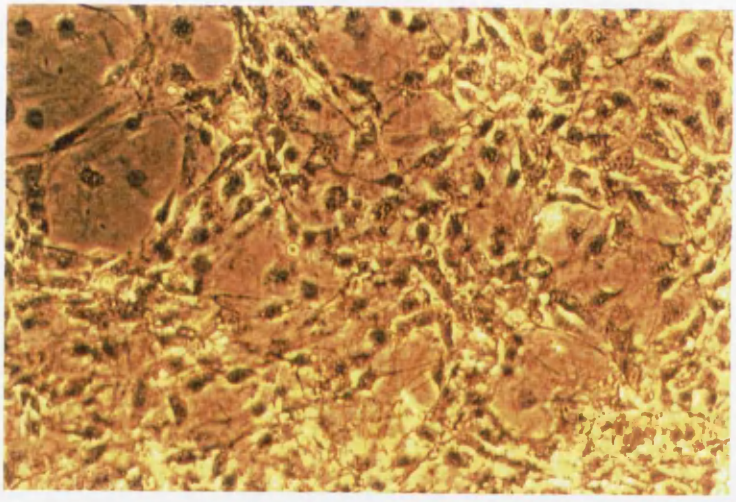
Figure 38. Morphology at Day 10 of the Differentiation Programme Following Treatment with Growth Hormone.

Cells were induced to differentiate in the presence of 10% DCS supplemented with 150ng/ml bovine pituitary growth hormone and the ability to accumulate cytoplasmic triglyceride assessed by oil red O staining at day 10. Phase contrast light microscopy (x100 magnification).

3T3-L1



WT6



WT8

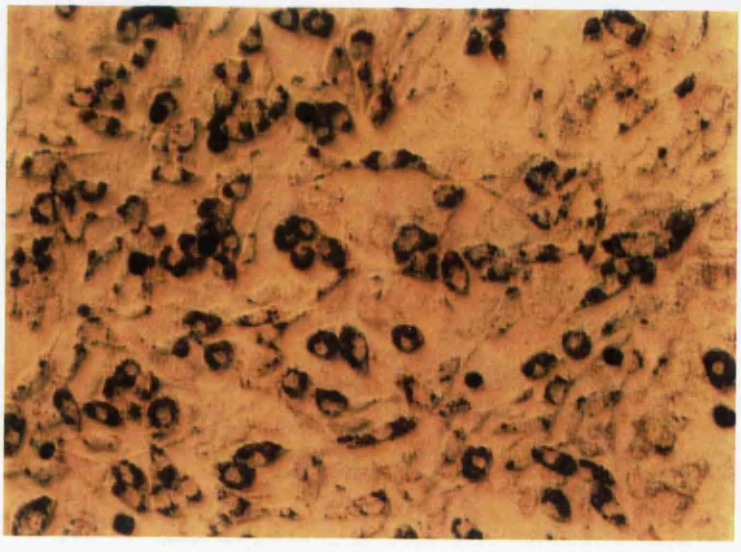
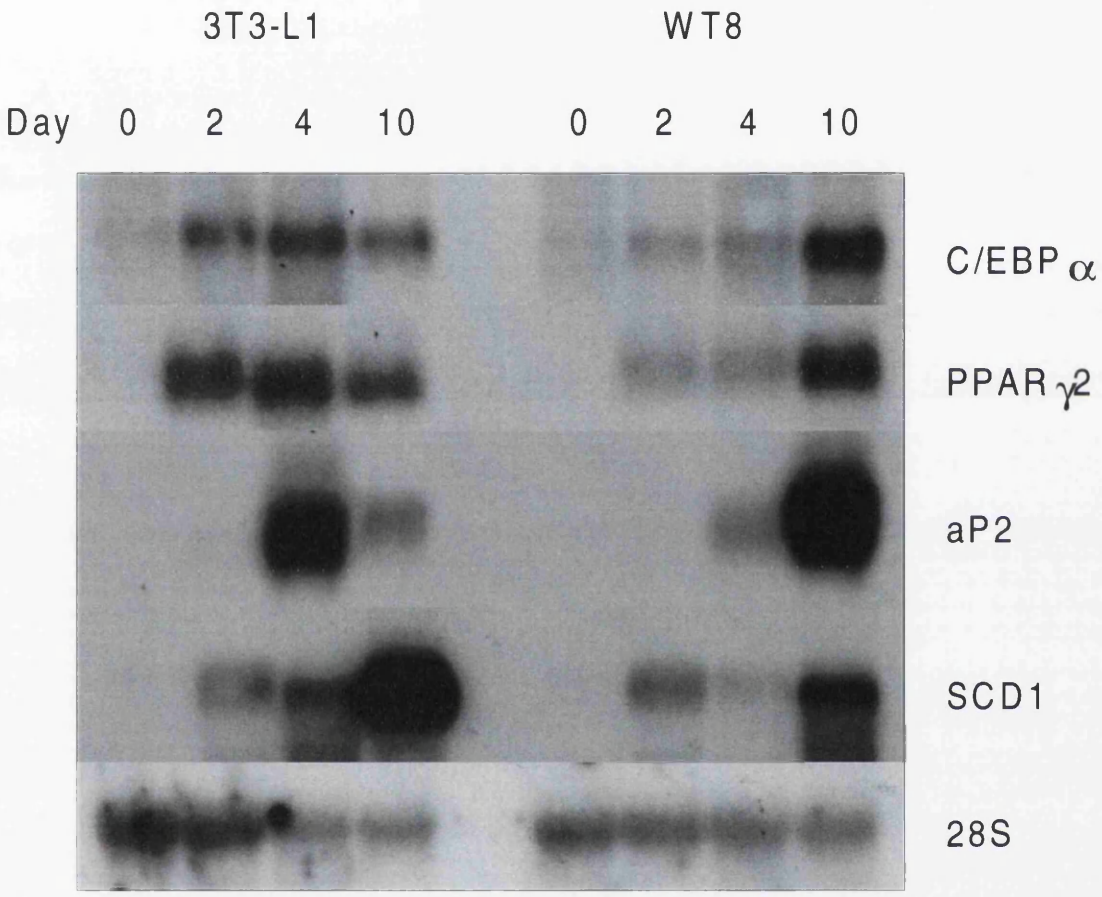


Figure 39.

Figure 39. Northern Blot Analysis of Late Stage Markers Following Differentiation in the Presence of Growth Hormone

Cells were induced to differentiate in the presence of 10% DCS supplemented with 150ng/ml bovine pituitary growth hormone and total cellular RNA prepared at the times indicated. Equal amounts were then subjected to Northern blot analysis using the probes previously described. Equivalence of loading is demonstrated by the 28S signal.


3T3-L1
WT8
Day 0 2 4 10 0 2 4 10
C/EBP α
PPAR γ2
aP2
SCD1
28S

8.7 Summary.

The ability of c-Myc to inhibit 3T3-L1 differentiation appears to be dependent on external factors, since treatment with 10% FCS was able to relieve the Myc block. This phenomenon was associated with the restoration of a number of gene markers which were previously found to be repressed in cell lines constitutively over-expressing c-*myc*. However, FCS was unable to induce adipogenesis in a cell line that had spontaneously lost the ability to differentiate, suggesting that inhibition of adipocyte differentiation may occur *via* more than one mechanism. The way in which FCS abrogates the inhibitory effects of c-Myc has yet to be fully characterised but is likely to involve the activity of growth hormone.

CHAPTER 9: Enforced Expression of c-*myc* in the 3T3-L1 Cell Line is Not Associated with Cell Cycle Alterations.

9.1 Introduction.

Proliferation and differentiation are generally thought to be mutually exclusive events. Hence, whilst expression of c-*myc* correlates positively with proliferative potential, it is also associated with inhibition of differentiation in a number of cell types. A simple explanation for this observation is that c-*myc* inhibits cellular differentiation by promoting continued cell cycle progression. With respect to the 3T3-L1 preadipocyte system, it is well documented that a number of cell cycle events are required to occur prior to acquisition of the differentiated phenotype. These include growth arrest at confluence (G_S), mitotic clonal expansion in response to the inducing agents and a second arrest (G_D) following hormone withdrawal. It is therefore possible that enforced expression of c-*myc* in the 3T3-L1 cell line inhibits adipogenesis by perturbing one or more of these critical events. In order to address this issue we therefore examined a number of cell cycle parameters following induction of the differentiation programme.

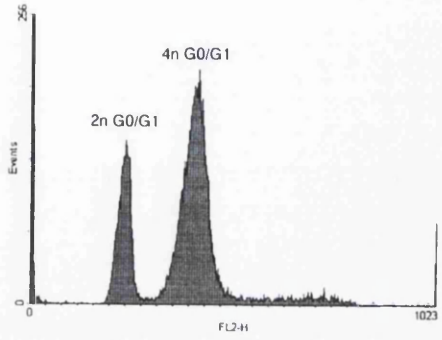
9.2 Enforced Expression of c-*myc* in the 3T3-L1 Cell Line is Not Associated With Cell Cycle Alterations Following Induction of the Differentiation Programme.

In order to gain an insight into cell cycle behaviour during adipogenesis, replicate cultures were induced to differentiate in the presence of 10% DCS, under which condition differentiation was blocked in the Myc-expressing clones, and samples taken at various time points. Cell cycle profiles for each cell line were then generated by flow cytometry following staining with propidium iodide. Surprisingly, analysis of the 3T3-L1 cell line clearly indicated that it was composed of two distinct sub-populations displaying different ploidy (Figure 40),

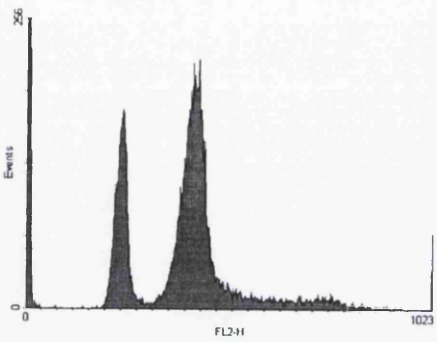
Figure 40.

Figure 40. Cell Cycle Analysis of the 3T3-L1 Cell Line Following Induction of the Differentiation Programme.

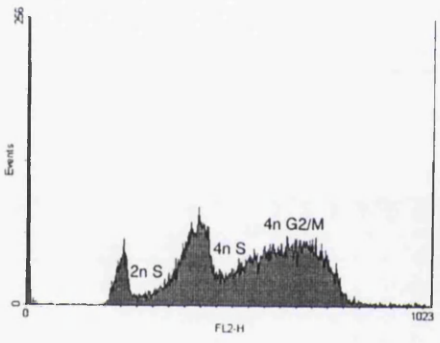
Replicate cultures were induced to differentiate in the presence of 10% DCS and samples prepared at the times indicated (see Materials and Methods). Stained samples were then subjected to flow cytometry and equal numbers of events examined at each time point. The x axis of the profile represents the DNA content as determined by propidium iodide staining, the fluorescence activity of the dye being proportional to the amount of cellular DNA. The cell number is indicated on the y axis.



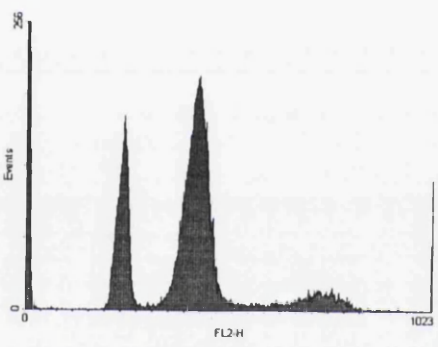
0 hours



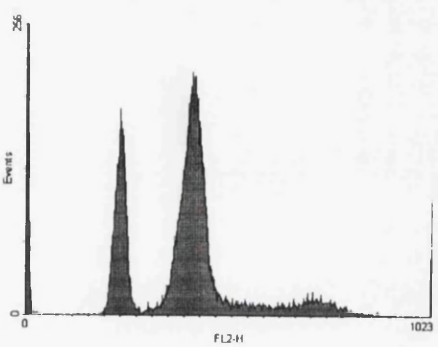
12 hours



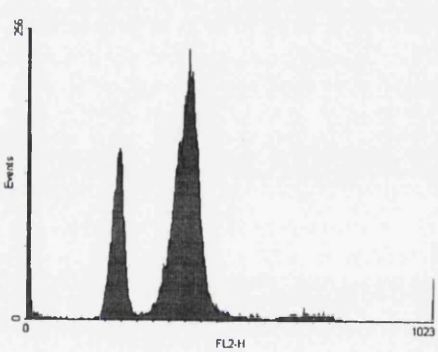
24 hours



48 hours



72 hours



96 hours

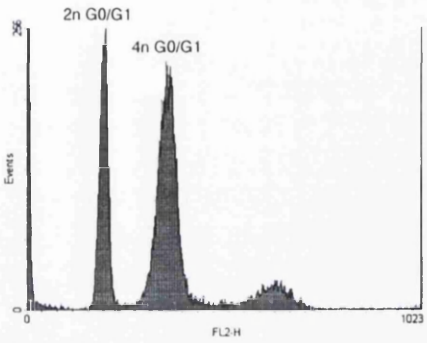
one being diploid (2n) and the other tetraploid (4n). Whilst the mechanism by which this phenomenon arose is not entirely clear, it is well documented that the 3T3-L1 cell line has an unstable karyotype (ATTC, Catalogue of Cell Lines and Hybridomas), although this is generally associated with aneuploidy resulting from chromosome loss. The 3T3-L1 cell cycle profile indicated that both the 2n and 4n sub-populations were arrested at confluence (0 hours, Figure 40). However, subsequent treatment with the adipogenic hormones resulted in both populations making a fairly synchronous entry into S phase following a lag period of 24 hours. After 48 hours the majority of these cells had re-entered G_0 despite the continued presence of the inducing agents suggesting that hormonal stimulation was associated with a single round of mitosis. In contrast, hormone withdrawal followed by refeeding did not result in further cell cycle progression and the cells remained in G_0 throughout the remaining time points. Hence, although the 3T3-L1 line was found to harbour cells of more than one ploidy, their cell cycle behaviour in response to external cues was indistinguishable.

The cell cycle profile of the differentiation defective clone, Def, indicated that this cell line was also composed of the two sub-populations (Figure 41). Like the parental 3T3-L1 cell line, these cells were growth arrested at confluence but responded to the inducing agents by entering S phase at 24 hours. However, in contrast to the 3T3-L1 cells, a slight increase in the G_2/M tetraploid population at the 48 and 72 hour time points suggested that a small percentage of the cells continued to cycle following hormone withdrawal. It is therefore possible that the block to adipogenesis manifest in this cell line may arise as a consequence of continued proliferation following the clonal expansion phase of the differentiation programme, resulting in an inability to fully withdraw from the cell cycle. However, further experimental work will be required to confirm these observations.

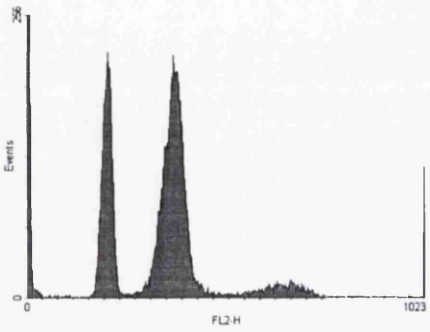
Figure 41.

Figure 41. Cell Cycle Analysis of the Def Cell Line Following Induction of the Differentiation Programme.

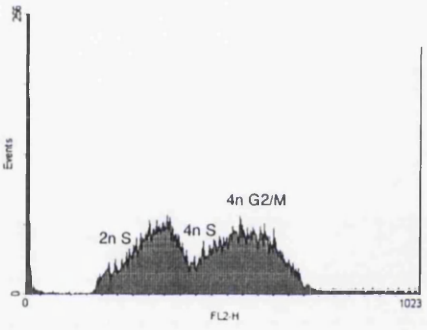
Replicate cultures were induced to differentiate in the presence of 10% DCS and samples prepared at the times indicated (see Materials and Methods). Stained samples were then subjected to flow cytometry and equal numbers of events examined at each time point. The x axis of the profile represents the DNA content as determined by propidium iodide staining, the fluorescence activity of the dye being proportional to the amount of cellular DNA. The cell number is indicated on the y axis.



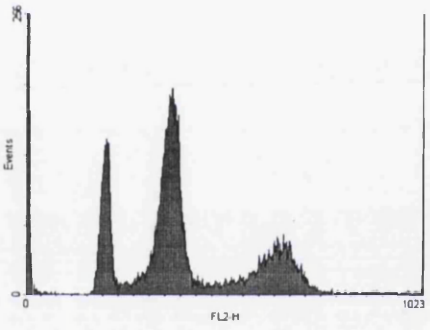
0 hours



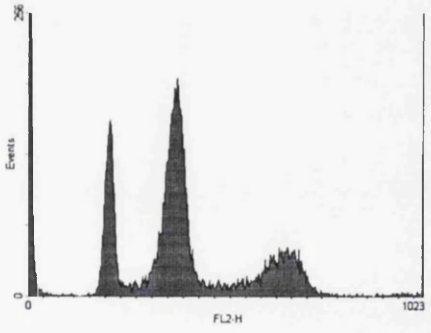
12 hours



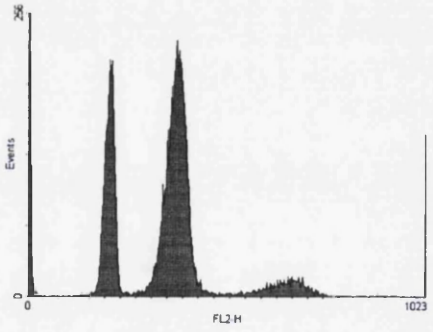
24 hours



48 hours



72 hours



96 hours

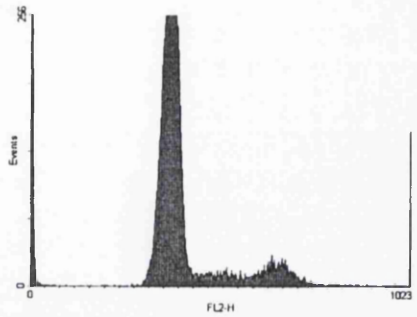
In contrast to the 3T3-L1 and Def cell lines, the Myc-expressing clones, WT6 and WT8, were composed of a single population representing the tetraploid (4n) cell type (Figures 42 and 43). Two possibilities therefore exist to explain the difference in ploidy. Firstly, generation of the WT cell lines from the original 3T3-L1 stock may have resulted in the preferential selection of the tetraploid lineage whilst Def retained both populations. Alternatively, the 3T3-L1 line was originally tetraploid and the diploid population arose spontaneously following the generation of the WT cell lines. In this case, it is likely that the diploid population evident in the Def cell line arose as an independent event. To address this issue, it would therefore be of interest to investigate the ploidy of other cell lines generated during the course of this study and compare them with the master stock of 3T3-L1 cells originally obtained from the ECACC.

As observed with the 3T3-L1 and Def cells, the Myc-expressing lines were found to be growth arrested at confluence (Figures 42 and 43) confirming that they retained the property of contact inhibition and were not transformed (see Section 6.9). Following treatment with the inducing hormones, the cells were seen to enter S phase at 24 hours demonstrating that enforced expression of c-*myc* in these cell lines did not alter their ability to undergo mitotic clonal expansion and that this phenomenon occurred with similar kinetics to that described for the 3T3-L1 cells. In addition, as previously seen in the 3T3-L1 system, hormonal stimulation was associated with a single round of mitosis followed by cell cycle exit. Subsequent serum stimulation at hormone withdrawal did not result in further mitoses, although the WT6 line did display a slight increase in the G_2/M population at 48 and 72 hours when compared to 3T3-L1 and WT8. We therefore concluded that constitutive expression of c-*myc* in the 3T3-L1 preadipocyte line did not result in any gross alterations in cell cycle behaviour following induction of the differentiation programme.

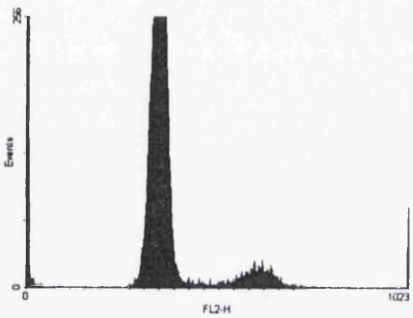
Figure 42.

Figure 42. Cell Cycle Analysis of the WT6 Cell Line Following Induction of the Differentiation Programme.

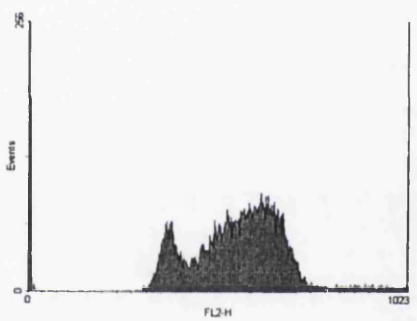
Replicate cultures were induced to differentiate in the presence of 10% DCS and samples prepared at the times indicated (see Materials and Methods). Stained samples were then subjected to flow cytometry and equal numbers of events examined at each time point. The x axis of the profile represents the DNA content as determined by propidium iodide staining, the fluorescence activity of the dye being proportional to the amount of cellular DNA. The cell number is indicated on the y axis.



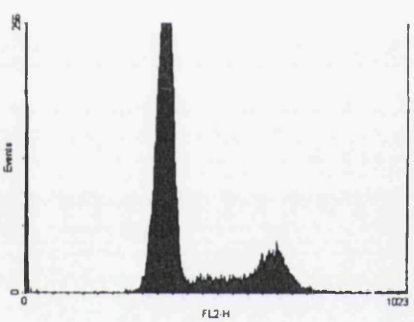
0 hours



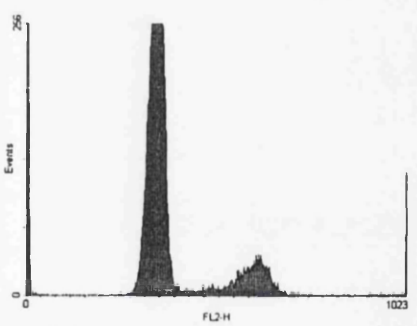
12 hours



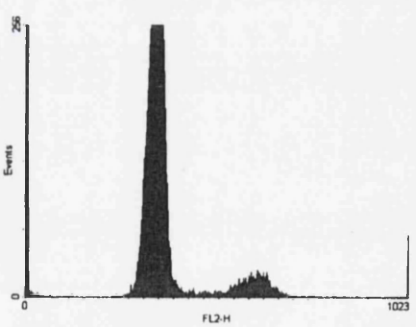
24 hours



48 hours



72 hours

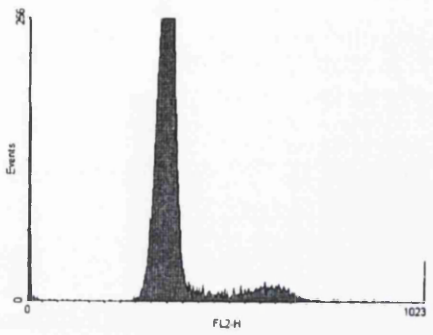


96 hours

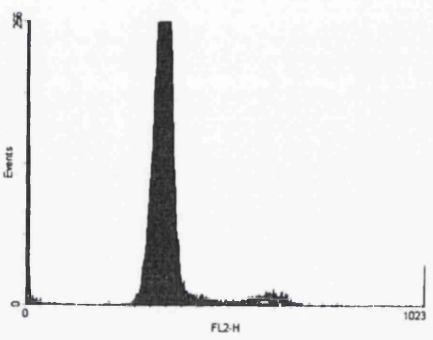
Figure 43.

Figure 43. Cell Cycle Analysis of the WT8 Cell Line Following Induction of the Differentiation Programme.

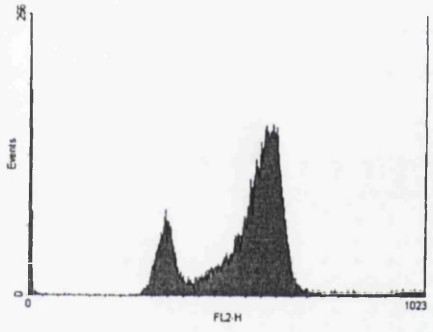
Replicate cultures were induced to differentiate in the presence of 10% DCS and samples prepared at the times indicated (see Materials and Methods). Stained samples were then subjected to flow cytometry and equal numbers of events examined at each time point. The x axis of the profile represents the DNA content as determined by propidium iodide staining, the fluorescence activity of the dye being proportional to the amount of cellular DNA. The cell number is indicated on the y axis.



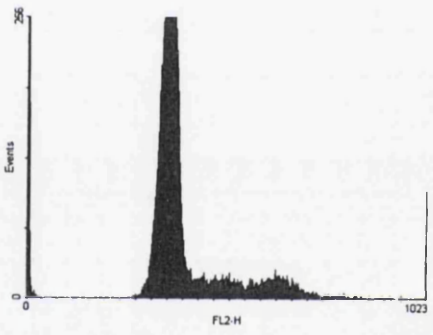
0 hours



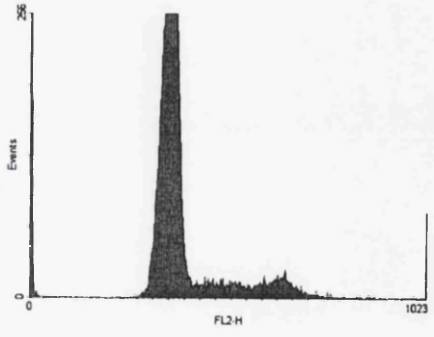
12 hours



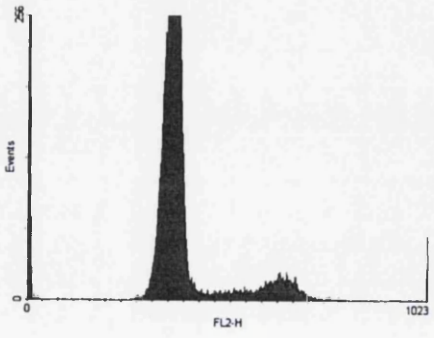
24 hours



48 hours



72 hours



96 hours

9.3 The G_D Arrest State Occurs Late in the Differentiation Programme.

Previous work had suggested that enforced expression of c-*myc* may inhibit 3T3-L1 adipogenesis by preventing entry into the G_D arrest state (Freytag, 1988). Whilst the nature of this growth arrest is poorly defined, it may be distinguished from cell cycle exit at confluence (G_S) by a number of biochemical criteria. Thus cells which have attained the G_D state are reported to respond to the mitogenic effects of MIX, whilst G_S cells are unaffected by such treatment (Wille and Scott, 1982). In contrast, only G_S cells are responsive to mitogenic stimulation with 30% serum (Freytag, 1988).

Following mitotic clonal expansion both the 3T3-L1 and Myc-expressing cell lines were seen to undergo cell cycle arrest (Figures 40, 42 and 43). However, in the 3T3-L1 line arrest at 72 hours was associated with morphological changes consistent with on-going adipogenesis whilst the WT clones retained the typical preadipocyte morphology. Thus, despite displaying similar cell cycle profiles the cell lines could be distinguished visually. To determine whether cultures attained the G_S or G_D state following clonal expansion, 72 hour (day 3) cultures were treated with either 0.5mM MIX or 30% DCS for a further 24 hours and the ability to undergo DNA synthesis assessed by *in situ* BrdU incorporation. In order to assess the basal level of DNA replication, control cultures were either left untreated or exposed to 10% DCS. As shown in Figure 44, these control cultures displayed relatively low levels of BrdU incorporation. However, treatment with either MIX or 30% serum resulted in a massive increase in the number of cells undergoing S phase in all of the cell lines studied. Hence, contrary to expectation, the biochemical criteria employed in this experiment could not distinguish individual growth arrest states.

Figure 44.

Figure 44. *In Situ* BrdU Labelling Following Induction of the Differentiation Programme.

Following induction of the differentiation programme replicate day 3 cultures were treated as follows:

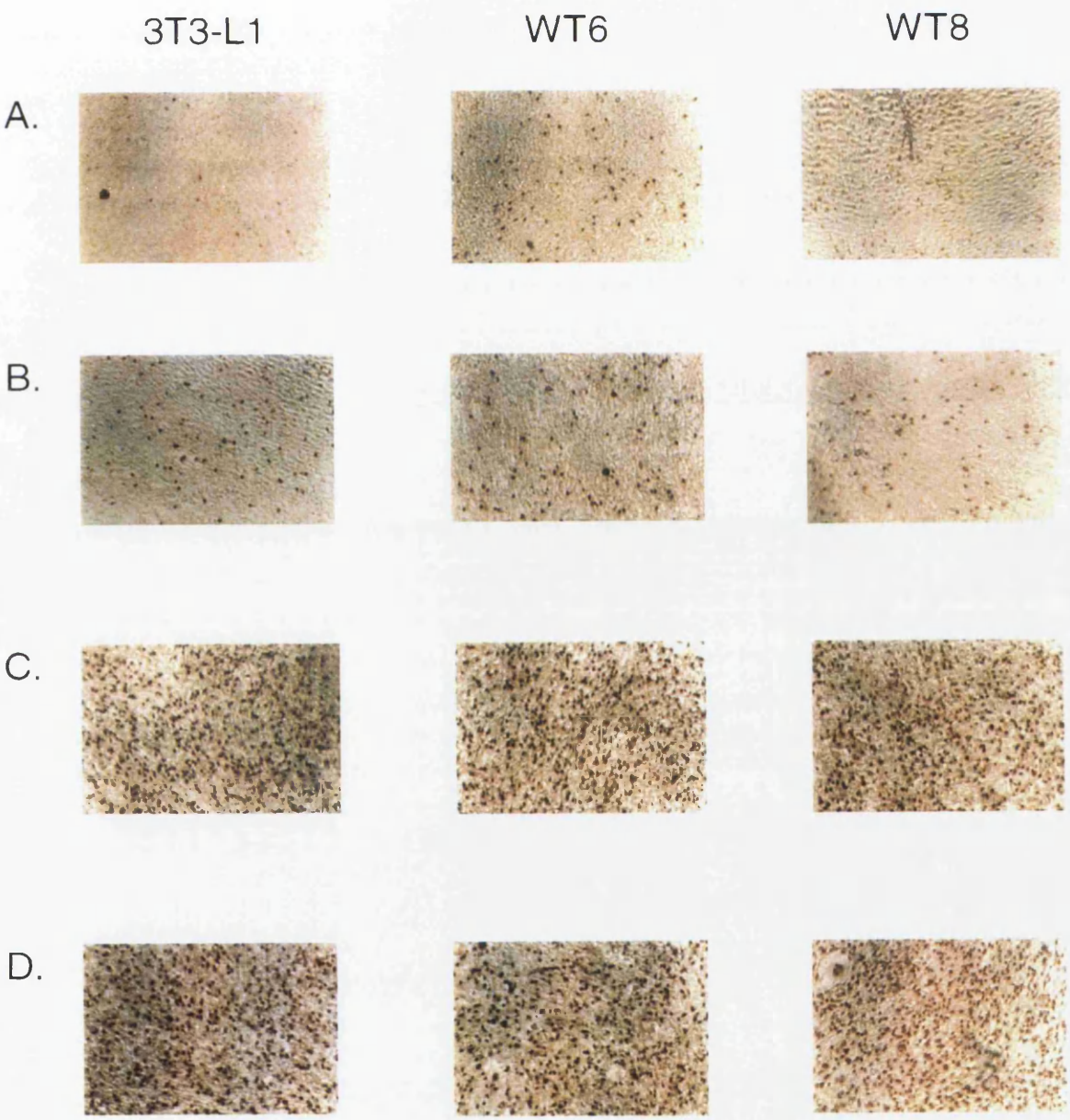
A. No treatment.

B. Growth medium supplemented with 10% DCS.

C. Growth medium supplemented with 10% DCS and 0.5mM MIX.

D. Growth medium supplemented with 30% DCS.

Cultures were incubated for 24 hours then treated with 10μM BrdU for 6 hours after which time they were fixed and stained as described in Materials and Methods. Light microscopy (x40 magnification).


3T3-L1
WT6
WT8
A.
B.
C.
D.

However, it is clear that at some point in the differentiation programme the 3T3-L1 cell line loses its responsiveness to 30% DCS since day 10 adipocytes were unable to incorporate BrdU following serum stimulation (data not shown). In contrast, day 10 cultures of Myc-expressing cells retained the ability to undergo DNA synthesis in response to 30% DCS. Loss of mitogenic responsiveness at the late stages of the differentiation programme was also found to correlate with a reduced plating efficiency (Figure 45). Replicate cultures were induced to differentiate in the presence of either 10% DCS or 10% FCS and at the times indicated samples trypsinised and reseeded at low density. Following incubation under standard growth conditions for 7 days, these cultures were then stained with Geimsa and the number of colonies on each dish counted. As seen in Figure 45, day 0, 1, 2 and 3 cultures retained their plating efficiency regardless of cell type and serum condition. However, in the 3T3-L1 cell line day 10 cultures had an obviously reduced plating efficiency. This appeared to be associated with terminal differentiation since the colonies were composed entirely of fibroblasts, indicating that mature adipocytes could not be passaged. In addition, WT6 and WT8 displayed a similar impairment when exposed to 10% FCS and *in situ* BrdU labelling demonstrated that Myc-expressing adipocytes were no longer responsive to the mitogenic effects of 30% DCS (data not shown). In contrast, the plating efficiency of the differentiation defective cell line was unaffected by either treatment, presumably reflecting the inability to undergo terminal differentiation. Hence, the late stages of 3T3-L1 adipogenesis appear to be associated with an irreversible cell cycle withdrawal which is not observed under conditions in which differentiation is blocked.

Figure 45.

Figure 45. Plating Efficiency Following Induction of the Differentiation Programme.

Replicate cultures were induced to differentiate in the presence of either 10% DCS or 10% FCS. At the time points indicated, monolayers were trypsinised and reseeded at a concentration of 10^3 cells per 60 mm tissue culture dish and incubated under the standard growth conditions for 7 days. Cultures were then stained with Geimsa and the number of colonies counted manually. These values are presented opposite.

Panel A: Differentiation in 10% DCS.

Panel B: Differentiation in 10% FCS.

A.

Cell Line / Day	3T3-L1	Def	WT6	WT8
0	183	203	212	150
1	173	215	199	158
2	191	227	197	152
3	175	245	203	179
10	97	222	223	183

B.

Cell Line / Day	3T3-L1	Def	WT6	WT8
0	169	179	157	191
1	177	241	164	184
2	187	235	177	189
3	169	171	159	208
10	73	166	91	107

9.4 Inhibition of Adipogenesis is Not Associated with Aberrant Expression of the Cyclin-Dependent Kinase Inhibitors, p21 and p27.

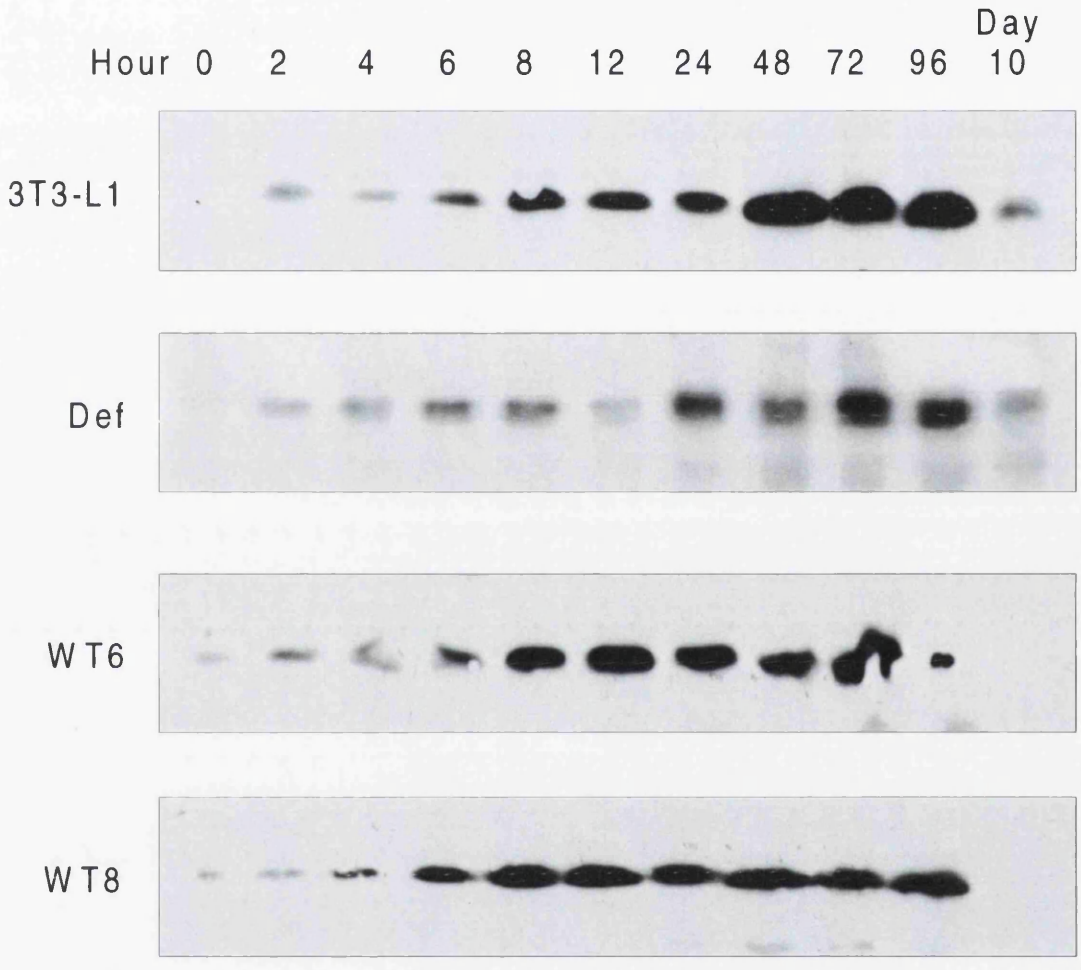
Since the G_D growth arrest could not be defined biochemically in our system, we decided to examine the expression of factors implicated in the negative regulation of the cell cycle machinery. Cellular proliferation is largely mediated by the sequential assembly and activation of cyclin/cyclin-dependent kinase (cdk) complexes (Pines, 1994). However, this network is also subject to negative regulation by cdk inhibitory proteins (CKIs), including p21, p27 and p16 (Sherr and Roberts, 1995). Expression of p21 has been implicated in the terminal differentiation of several cell types (Missero *et al*, 1996; MacLeod *et al*, 1995; Billon *et al*, 1996) and it is likely that this protein is required to initiate the cell cycle withdrawal characteristic of the differentiated phenotype (Andres and Walsh, 1996). In addition, it was recently reported that p21 may be regulated by C/EBPα (Timchenko *et al*, 1996). Since C/EBPα is a key regulator of adipogenesis which has also been reported to display anti-mitotic activity, we postulated that this property may require the activity of p21.

We therefore examined the expression of p21 in the 3T3-L1 cell line following induction of the differentiation programme. Protein samples were prepared at various time points and subjected to Western blot analysis using the C-19 polyclonal antibody (Santa Cruz), which was raised against residues 146-164 of the human protein. As shown in Figure 46, p21 could not be detected at confluence but was rapidly induced within 2 hours of initiating the differentiation programme. This expression increased steadily until 48 hours after which point high levels of p21 were maintained until day 10. Since C/EBPα is only expressed from day 2 (48 hours) of the adipogenic programme, the kinetics of p21 expression therefore suggest that it is unlikely to be a target for C/EBPα-mediated transactivation in the 3T3-L1 system. A similar pattern of expression was also

Figure 46.

Figure 46. Western Blot Analysis of p21 Expression During the Differentiation Programme.

Protein samples were prepared at the times indicated and equal amounts subjected to SDS-PAGE and Western blot analysis using the C-19 polyclonal antibody (1:250) and the ECL detection method.


Hour 0 2 4 6 8 12 24 48 72 96
Day 10
3T3-L1
Def
WT6
WT8

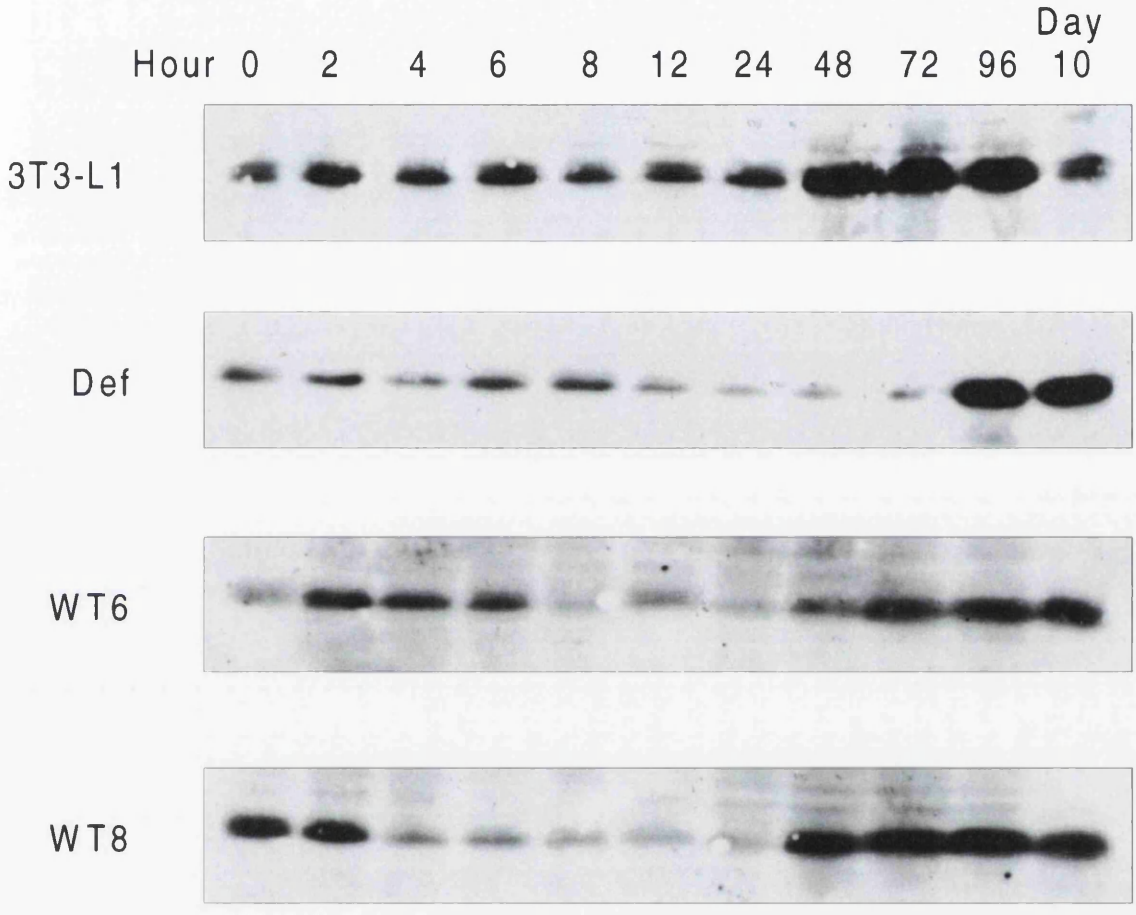
observed in the Myc-expressing cell lines and the differentiation defective clone (Figure 46). Whilst p21 was not apparent at day 10 in WT6 and WT8, it should be noted that this was not a consistent finding and low levels of the protein were generally evident at this time point. It therefore appears unlikely that p21 is involved in the irreversible cell cycle withdrawal associated with acquisition of the adipocyte phenotype.

In addition to p21, we also examined the expression pattern of p27 during 3T3-L1 differentiation since it was previously shown that the p27 mRNA was induced following clonal expansion (Vaziri and Faller, 1996). Lysates were prepared at various times and subjected to Western blot analysis using a p27-specific polyclonal antibody raised against a recombinant murine protein (the generous gift of S. Coats, Amgen, CA, USA). Expression of p27 during 3T3-L1 differentiation was found to be very similar to that observed with p21, with the exception that the protein was readily detected at confluence (Figure 47). As for p21, the highest level of expression was observed between 48 and 96 hours (day 4), with a down-regulation in the fully differentiated sample (day 10). In contrast, reduced levels of the protein were detected in WT6 between 8 and 24 hours, with high levels being maintained thereafter, whilst in WT8 p27 was low between hours 4 and 24 (Figure 47). The differentiation defective cell line was found to express reduced amounts of the protein between hours 12 and 72. The functional significance of these findings is not clear but may relate to the clonal expansion phase (see Figures 41, 42 and 43). However, the fact that p27 was detected in all the cell lines during the late stages of the differentiation programme suggests that this protein is not responsible for the irreversible cell cycle withdrawal.

Figure 47.

Figure 47. Western Blot Analysis of p27 Expression During the Differentiation Programme.

Protein samples were prepared at the times indicated and equal amounts subjected to SDS-PAGE and Western blot analysis using the p27 polyclonal antibody (1:5000) and the ECL detection method.


Hour 0 2 4 6 8 12 24 48 72 96
Day 10
3T3-L1
Def
WT6
WT8

9.5 Summary.

Examination of cell cycle profiles during induced differentiation suggested that inhibition of adipogenesis was not associated with an inability to arrest at confluence or undergo mitotic clonal expansion in response to hormonal stimulants. Additionally, preadipocytes did not appear to enter an irreversible growth arrest state prior to undergoing morphological differentiation as had previously been postulated, since cells which were actively undergoing shape changes retained the ability to respond to mitogens and did not display a reduced cloning efficiency. However, adipocytes lost these properties at an ill-defined point late in the differentiation programme and permanently withdrew from the cell cycle. This phenomenon was not observed in cell lines which were unable to differentiate. Attempts to distinguish this growth arrest by biochemical and molecular criteria proved somewhat disappointing but obviously represent an area for future study.

DISCUSSION.

DISCUSSION.

CHAPTER 10: Inhibition of Adipogenesis by the c-Myc Oncoprotein.

10.1 Introduction.

The primary objective of this project was to investigate the ability of the c-Myc oncoprotein to inhibit the process of cellular differentiation. For this purpose we employed the 3T3-L1 cell line, a well-characterised *in vitro* model of adipocyte development, since enforced expression of c-*myc* in this system is associated with loss of differentiation capacity. Whilst differentiation-control remains a poorly understood facet of c-Myc function, several models have been proposed to account for the ability of this protein to inhibit adipogenesis (Section 3.9). These models are therefore discussed in detail below, where they are placed within the context of observations made during the course of this study.

10.2 Enforced Expression of c-*myc* in the 3T3-L1 Cell Line Does Not Result in Deregulated Growth Control.

Proliferation and differentiation are generally regarded as mutually exclusive events. It is therefore possible that c-Myc inhibits differentiation indirectly as a consequence of continued proliferation. However, whilst constitutive expression of an avian c-*myc* transgene in the 3T3-L1 cell line was clearly associated with a block to adipogenesis, there was no evidence to suggest that deregulated growth control had also occurred. The Myc-expressing WT clones remained contact inhibited, could not grow in suspension and did not exhibit an increased growth rate relative to the control cultures. Indeed, a single clone (WT3) that expressed high levels of the exogenous protein was severely retarded in its ability to grow under standard culture conditions. These findings were not peculiar to the avian protein since previous studies had concluded that neither murine (Freytag, 1988) nor human c-Myc (Freytag *et al*, 1990) were able to transform the 3T3-L1 cell line. However,

enforced expression of these proteins was associated with a differentiation block. That constitutive expression of c-*myc* in the 3T3-L1 cell line was not associated with deregulated growth control was confirmed by flow cytometric analysis following induction of the differentiation programme. In this experiment, the cell cycle profiles of the Myc-expressing clones were found to be highly similar to that observed with the parental 3T3-L1 line. Thus, maintenance at confluence resulted in an accumulation of cells in the G_0 phase of the cell cycle. Following treatment with the inducing hormones, a synchronous entry into S phase was detected in all lines at 24 hours with the cells undergoing a single round of mitosis before rearresting in G_0 at 48 hours, despite the continued presence of the hormone cocktail. Additionally, removal of the hormones by changing the medium was not associated with further cell divisions. Hence, the continued presence of c-*myc* in the WT6 and WT8 clones was not associated with abnormal cell cycle behaviour during the differentiation programme.

From our observations, it is apparent that differentiation control and cellular transformation are separable functions of the c-Myc protein and it is therefore unlikely that the Myc-mediated block to adipogenesis occurs indirectly as a consequence of deregulated proliferation. A similar conclusion was drawn from a recent study by our laboratory using the primary quail myoblast system (La Rocca *et al*, 1994). In this cell line, enforced expression of c-*myc* resulted in both cellular transformation and a blockade of the myogenic programme. In contrast, the leucine zipper deletion mutant, c-MycΔ7, was unable to inhibit myogenesis. However, this protein retained both the ability to bind Max and transformation potential. This suggested that Myc-mediated differentiation control and transformation may occur *via* distinct pathways and two models were proposed to account for these observations. Firstly, the threshold level of Myc/Max required to elicit cellular transformation may differ from that needed to block differentiation. Alternatively,

Myc may inhibit myogenesis by a Max-independent pathway. The latter model is particularly attractive in the light of the recent isolation of alternative Myc binding partners (Section 3.4). A possible candidate for this activity is YY1 (Lee *et al*, 1994), which is able to simultaneously control c-*myc* and the muscle-specific gene, α-actin. Dissociation of differentiation from transformation was also observed when another oncogene, Ha-*ras*, was over-expressed in the 23A2 myoblast cell line. In this system, Ha-*ras* was found to utilise separate signal transduction pathways in order to both elicit transformation and inhibit myogenesis (Weyman *et al*, 1997). Thus, transformation required the activity of the MAPK pathway, whilst differentiation control occurred *via* an as yet unidentified route.

In contrast to the Myc-expressing cell lines, flow cytometric analysis of the differentiation-defective clone, Def, suggested that it may continue to cycle following hormone withdrawal. That this cell line underwent the clonal expansion phase at all was somewhat surprising since we had consistently failed to detect a significant increase in cell number following hormone stimulation, suggesting that it may be unresponsive to this treatment. However, maintenance at confluence was often associated with increased cellular debris in the medium, particularly in the presence of 10% FCS, which might arise as a consequence of cell death. It is therefore possible, but not proven, that in the differentiation defective cell line proliferation in response to the inducing agents may be balanced by death, thereby resulting in no net increase in cell number. How this would relate to the inability of the cell line to undergo adipogenesis is not, however, entirely clear.

A possible molecular mechanism for the differentiation block manifest in the Def cell line has, however, arisen from a recent study in which rapamycin was used to inhibit adipogenesis (Yeh *et al*, 1995b). The effect of this immunosuppressant on the 3T3-L1 cell line was found to be very similar to the phenotype of our clone

which had spontaneously lost the ability to differentiate. Hence, rapamycin-mediated inhibition of differentiation resulted in an inability to accumulate cytoplasmic triglyceride, presumably as a consequence of the impaired expression of both isoforms of C/EBPα. However, of greater interest with respect to the Def cell line, rapamycin did not alter the expression of C/EBPδ but did cause a reduction in the levels of the C/EBPβ protein, particularly the LIP isoform. Additionally, the clonal expansion phase was characterised by no net increase in cell number as compared to untreated controls. Rapamycin is thought to act by binding to specific intracellular receptors termed FKBPs, which are members of a family displaying peptidyl *cis-trans*-isomerase activity (Siekierka *et al*, 1989). This enzymatic activity is lost following ligand binding and rapamycin-FKBP complexes are then able to bind and inhibit a variety of secondary proteins involved in signal transduction pathways, including p70 S6 kinase (Chung *et al*, 1992) and the 110-kDa subunit of phosphatidylinositol-3-kinase (Kunz *et al*, 1993). It was therefore postulated by Yeh and co-workers (1995b) that the insulin/IGF-1 signal transduction pathway represented an attractive candidate for inhibition by rapamycin during 3T3-L1 differentiation. Thus, it is possible that the Def cell line is unable to undergo induced differentiation as a consequence of impaired insulin signalling. Interestingly, a novel FKBP has recently been identified in the 3T3-L1 cell line that is expressed predominantly during clonal expansion, suggesting a specialised function during this phase of the differentiation programme (Yeh *et al*, 1995c). Since the enzymatic activity of this protein was also found to be inhibited by rapamycin, it will doubtless be of great interest to determine the physiological ligands of the FKBPs as such studies might shed light on novel factors that control the differentiation process.

10.3 Initiation of the Terminal Stages of the Differentiation Programme Does Not Require Entry into a Specific Growth Arrested State.

As described in detail in Section 1.5.4, it is widely assumed that following clonal expansion preadipocyte cell lines enter a specific growth arrest state, designated G_D, which is thought to be both permissive for the terminal stages of the differentiation programme and irreversible. Thus, it is possible that constitutive expression of c-*myc* inhibits adipogenesis by preventing entry into this G_D arrest state. To test this hypothesis we therefore examined cell cycle behaviour following the clonal expansion phase.

Flow cytometric analysis at day 3 of the differentiation programme demonstrated that both the Myc-expressing clones and the parental 3T3-L1 cell line were arrested in the G_0 phase of the cell cycle. However, at this time the 3T3-L1 cells were obviously undergoing morphological changes consistent with terminal differentiation whilst the WT lines retained the fibroblastic appearance characteristic of the preadipocyte stage. We therefore reasoned that if terminal differentiation did indeed require a specialised form of growth arrest, this would be apparent at day 3. It had previously been demonstrated that the G_D state can be distinguished from arrest at confluence (G_S) on the basis of a differential response to mitogens. Hence, whilst G_D cells undergo DNA synthesis in response to 0.5mM MIX, stimulation with 30% serum is without effect. Conversely, G_S cells are only able to undergo mitosis in response to 30% serum. In order to determine which of these arrest states the 3T3-L1 and WT clones had entered following clonal expansion we treated day 3 cultures with either 0.5mM MIX or 30% DCS and assessed the subsequent ability to synthesise nuclear DNA by *in situ* BrdU labelling. Surprisingly, all three cell lines were found to incorporate high levels of BrdU in response to both treatments. Hence, the biochemical criteria employed in this experiment were unable to distinguish separate growth arrest states. This result

was somewhat unexpected since the study of Freytag (1988) had previously demonstrated that following clonal expansion 3T3-L1 cells constitutively expressing murine c-*myc* underwent DNA synthesis in response to 30% serum, whilst untransfected controls remained growth arrested. The inability of the 3T3-L1 cell line to respond to the mitogenic effects of high levels of serum was found to require prior treatment with the hormone cocktail since uninduced cultures replicated in the presence of 30% serum. Conversely, the ability to undergo mitosis following hormone treatment required c-*myc* since co-expression of a c-*myc* antisense construct abrogated this effect. On the basis of these findings, Freytag therefore proposed that hormone treatment/clonal expansion was necessary for cells to enter a differentiation-specific growth arrest state and that the continued presence of c-*myc* somehow interfered with this process thereby resulting in an inability to undergo adipocyte differentiation. However, it has been shown that enforced expression of v-*myc* in C2C12 myoblasts did not prevent cell cycle withdrawal following initiation of the myogenic programme (Crescenzi *et al*, 1994). As described for adipocyte differentiation, this growth arrest, designated "commitment", is thought to be both irreversible and necessary for subsequent muscle-specific gene expression. Hence, in this system v-*myc* could inhibit the terminal stages of the differentiation programme without affecting the ability to exit the cell cycle.

Since we were unable to define the G_D arrest state biochemically within our system, we decided to examine the expression of proteins known to play a role in growth arrest. It is well documented that the large isoform of C/EBPα possesses anti-mitotic activity and it was recently suggested that this function may require induction of the CKI, p21(Timchenko *et al*, 1996), which was previously implicated in the growth arrest mediated by myogenic transcription factors during *in vitro* differentiation. In addition, high levels of this protein have been detected in

a variety of post-mitotic, terminally differentiated cell types (Parker *et al*, 1995). Postulating that entry into the G_D arrest state may also require the activity of p21, we therefore examined the expression of this protein during 3T3-L1 adipogenesis. Assuming that p21 did indeed represent a *bona fide* target for C/EBPα-mediated transactivation, we predicted that it would be expressed in a similar temporal pattern to other C/EBPα regulated genes but would be absent in the Myc-expressing clones, which did not express either isoform of the C/EBPα protein. However, contrary to these expectations p21 was found to be expressed prior to C/EBPα during differentiation of the 3T3-L1 cell line and a similar pattern of expression was observed in the WT clones. Additionally, the differentiation defective cell line, which also lacked C/EBPα, expressed p21 in a manner indistinguishable from that detected in the 3T3-L1 line. Thus, it is unlikely that p21 mediates the anti-mitotic effects of C/EBPα in the 3T3-L1 system or that it is involved in the differentiation-specific growth arrest. Similarly, expression of another CKI, p27, was unaltered by either Myc-mediated or spontaneous inhibition of differentiation. However, since such studies did not address the ability of these CKIs to inhibit their specific cell cycle targets, we cannot exclude the possibility that functional differences may exist between the various cell lines. Indeed, c-Myc has been shown to abrogate the activity of p27 by sequestration into an inactive form (Vlach *et al*, 1996). In addition to p21 and p27, a number of other growth arrest-associated genes are differentially expressed during 3T3-L1 differentiation (Shugart *et al*, 1995). One of these, *gas*6, is expressed towards the end of the clonal expansion phase, making it a plausible candidate for the mediator of the G_D arrest. It would therefore be of interest to examine the expression of this gene under conditions in which adipogenesis is blocked, for example by the constitutive expression of c-*myc*.

What is clear from our study, however, is that acquisition of the adipocyte phenotype was associated with a reduced plating efficiency and fully differentiated cells were no longer able to respond to 30% DCS. This loss of mitogenic responsiveness occurred at some point between day 3 and 10 of the differentiation programme and followed expression of the fat-specific markers aP2 and SCD1. On the basis of these findings we suggest that irreversible growth arrest is not so much a driving force of the adipogenic programme as a consequence of it. It is therefore possible that the anti-mitotic effects of C/EBPα are required to maintain fully differentiated adipocytes in the quiescent state rather than to initiate terminal differentiation. In this regard, it is of interest to note that the growth arrest and DNA damage gene, GADD45, was found to be expressed during the terminal stages of 3T3-L1 differentiation with similar kinetics to the fat-specific genes (Constance *et al*, 1996). Transactivation studies using *gadd45* promoter constructs indicated that expression of this gene was positively regulated by C/EBPα and that this activity could be antagonised by co-expression with c-Myc. Hence, GADD45 represents a putative target for Myc-mediated repression late in the differentiation programme. Interestingly, GADD45 and p21 have been found to directly interact with one another and this is synergistic for growth arrest (Kearsey *et al*, 1995). Thus, the differentiation-specific cell cycle withdrawal may require the interaction of two or more negative regulators of cell growth. In this regard, the tumour suppressor pRb has been implicated in cell cycle exit during myogenesis (Zacksenhaus *et al*, 1996) and MEL cell differentiation (Zhuo *et al*, 1995). A recent study using the C2C12 myoblast system concluded that maintenance of this growth arrest following terminal differentiation required the activity of both pRb and the CKI, p18 (Franklin and Xiong, 1996). Since lung fibroblasts derived from pRb null mice were unable to undergo induced adipogenesis (Chen *et al*, 1996a), this protein might also be involved in establishing the growth arrest state

characteristic of mature adipocytes. Clearly the relationship between terminal differentiation and cell cycle withdrawal represents an area ripe for further study.

10.4 Enforced Expression of c-*myc* in the 3T3-L1 Cell Line Results in Repression of the Key Adipogenic Transcription Factors, C/EBPα and PPARγ2.

Analysis of a number of markers representative of the various stages of the adipogenic programme demonstrated that the Myc-mediated block to 3T3-L1 differentiation was associated with impaired expression of the late genes C/EBPα, PPARγ2, aP2 and SCD1. This phenotype was very similar to that previously described when adipocyte differentiation was blocked by the cytokine TNFα, which is hardly surprising since it is now believed that TNFα exerts its effects on adipogenesis indirectly *via* induction of c-*myc* (Ninomiya-Tsuji *et al*, 1993). Since C/EBPα and PPARγ2 are thought to act in concert in order to co-ordinate the expression of genes required for lipid metabolism, such as aP2 and SCD1, these observations provide a molecular mechanism for the inability of the WT cell lines to accumulate cytoplasmic triglyceride. We therefore propose that constitutive expression of c-*myc* in the 3T3-L1 cell line inhibits differentiation by repressing expression of the key adipogenic transcription factors, C/EBPα and PPARγ2, thereby resulting in an inability to induce fat-specific gene expression and triglyceride biosynthesis. Indeed, repression of master transcriptional regulators may prove to be a common theme in oncogene-mediated differentiation control. Thus, inhibition of myogenesis by enforced expression of c-*myc* in primary quail myoblasts was associated with the down-regulation of both MyoD and myogenin (La Rocca *et al*, 1994), whilst co-expression of c-*myc* in NIH-3T3 cells suppressed MyoD and myogenin-initiated terminal differentiation (Miner and Wold, 1991). Additionally, constitutive expression of a wide range of activated oncogenes,

including v-*ras*, v-*src* and v-*jun*, in primary quail myoblasts was found to severely impair the expression of myogenin (Russo *et al*, 1997).

Whilst our observations define a point at which c-Myc is repressing adipogenesis, they do not in themselves provide an explanation for the mechanism by which this occurs. Expression of C/EBPα and PPARγ2 during the differentiation programme is thought to require the activity of C/EBPβ and C/EBPδ, which are themselves induced during the clonal expansion phase. However, Northern blot analysis clearly demonstrated that expression of the C/EBPβ and δ mRNAs was not altered in the WT cell lines. Additionally, both the LAP and LIP isoforms of C/EBPβ were found to be expressed in response to MIX during clonal expansion. However, since we did not determine whether these proteins were still functional, it is possible that their activity was somehow impaired by the continued presence of c-Myc. Studies using the C/EBPβ homologue, NF-IL6, have demonstrated that the ability of this factor to activate target genes is greatly enhanced by site-specific phosphorylation at serine 105 (Trautwein *et al*, 1993). Phosphorylation *in vitro* could be achieved by a variety of kinases including MAPK and protein kinase C but did not alter the ability of the protein to bind DNA. It was subsequently demonstrated that C/EBPβ exists in a transcriptionally inert form which requires phosphorylation for derepression (Kowenz-Leutz *et al*, 1994). The inhibitory activity was found to reside in the N-terminus of the molecule, where two regions (CR5 and CR7) appear to mask the transactivation domain. This masking effect could, however, be relieved by phosphorylation and it is interesting to note that serine 105 is located within the CR5 domain. Hence, inhibition of such site-specific phosphorylation might restrain the transcriptional activity of C/EBPβ during 3T3-L1 differentiation. However, it is not clear how c-Myc might exert such an effect. An alternative explanation is that enforced expression of c-Myc inhibits the DNA binding activity of C/EBP family proteins. Both the C/EBPα and PPARγ2 promoters contain

consensus C/EBP binding sites and it is assumed that C/EBPβ and δ regulate gene expression *via* these sequences. As described in Section 3.9.3, it has recently been demonstrated that the Myc/Max complex has the capacity to bind a consensus C/EBP recognition site located within the EFII enhancer of the Rous sarcoma virus LTR (Hann *et al*, 1994). It is therefore possible that c-Myc inhibits C/EBPβ/δ mediated transactivation of C/EBPα and PPARγ2 by competitive binding at the C/EBP binding site. However, v-Myc-mediated inhibition of C/EBPα and C/EBPβ-dependent transactivation during myelopoiesis was found to require only the N-terminal portion of the oncoprotein, suggesting that repression did not involve DNA binding (Mink *et al*, 1996). What is intriguing, however, is the observation that the alternatively translated product of c-*myc*, Myc1, may also bind the C/EBP site (Hann *et al*, 1994). However, in contrast to Myc2, this interaction was associated with increased transactivation potential rather than repression. Since all studies conducted thus far on the ability of c-Myc to inhibit adipogenesis have utilised the Myc2 protein, it would therefore be of great interest to determine the effect of Myc1 on the differentiation programme.

It is widely assumed that the biologically active form of c-Myc comprises a heterodimer with Max, and that it is Max which directs the ability of the complex to specifically bind the E-box sequence. However, recent evidence had suggested that the Myc-mediated inhibition of PC12 cell differentiation occurred in the absence of functional Max. Since the Max status of the 3T3-L1 cell line was unknown, we decided to analyse the expression of this protein during adipogenesis. Western blot analysis demonstrated that Max was readily detectable in this system, with the p21 isoform predominating. Whilst the PPARγ2 promoter remains to be fully characterised, a canonical Myc/Max binding site has been identified in the proximal promoter of the C/EBPα gene. The possibility therefore exists that Myc-mediated repression of C/EBPα occurs through this site. Indeed, this Myc/Max

binding motif is located in a region of the promoter enriched in negative regulatory elements, including the site for CUP which is proposed to inhibit expression of C/EBPα in the preadipocyte stage. The case for repression by the Myc/Max complex is strengthened by the observation that deletion of residues from the LZ domain of c-Myc imposed radical effects on the capacity of this protein to inhibit differentiation. As judged by cytoplasmic triglyceride accumulation, the removal of seven amino acids from the extreme C-terminus of the molecule, including leucine 4 of the heptad repeat, did not alter the ability to block adipogenesis. However, deletion of a further three residues completely abolished the inhibitory activity. This mutant (c-MycΔ10) lacked both leucine 4 and an additional leucine residue, 3A, which is thought to participate in interactions which stabilise the zipper domain. Since we had previously demonstrated that c-MycΔ10 lacks the ability to bind Max (Crouch *et al*, 1993), an obvious explanation for our data is that inhibition of adipogenesis requires interaction with Max *via* an intact LZ motif. However, our findings cannot exclude the possibility that c-Myc inhibits 3T3-L1 differentiation by a Max-independent pathway, which also requires the extreme C-terminus of the protein.

The formation of Myc/Max heterodimers is regulated primarily by the availability of the c-Myc protein. However, our analysis of the Myc antagonist, Mad1, in the 3T3-L1 system clearly demonstrated that high levels of this protein were present in the WT clones, whilst expression of the exogenous c-Myc was barely detectable. If Mad1 was indeed in excess of c-Myc in these cells then it might be envisaged that the equilibrium would be pushed in favour of Mad/Max complex formation, resulting in loss of Myc/Max binding at the E-box. However, enforced expression of c-Myc was obviously associated with a block to 3T3-L1 differentiation, suggesting that this function may not require the presence of Max or binding to the consensus sequence. In this regard, it was recently shown that c-Myc has the

capacity to bind an additional DNA sequence, designated the initiator (Inr) element, and that binding at this site represses gene expression (Mai and Martensson, 1995). Interestingly, this activity required a conserved region in the N-terminus of c-Myc, Myc Box II (Li *et al*, 1994), which was previously demonstrated to be necessary for the Myc-mediated inhibition of adipogenesis (Freytag *et al*, 1990). Since the core promoter of the C/EBPα gene contains an Inr element, Li and co-workers (1994) therefore postulated that repression in the presence of c-Myc occurred *via* this site. Thus, they were able to demonstrate that enforced expression of c-*myc* in the 3T3-L1 cell line resulted in a concentration-dependent inhibition of a C/EBPα promoter construct. Since this construct lacked the E-box site and repression depended solely upon the integrity of the Inr sequence, it was concluded that c-Myc inhibited C/EBPα gene expression by a novel mechanism.

In addition to Max, c-Myc is able to interact with a number of additional factors (Section 3.4). Two of these proteins, YY-1 and TFII-I, are known to exert their transcriptional effects through the Inr of target genes. It is therefore of interest to note that interaction of c-Myc and TFII-I was associated with repression of core promoter constructs harbouring an Inr element (Roy *et al*, 1993). However, TFII-I may also activate such constructs when associated with the transcription factor, USF (Roy *et al*, 1991). Like c-Myc, USF is a member of the bHLH-LZ family of transcriptional regulators, with the capacity to bind both the E-box and Inr sequences. Since positive modulation of C/EBPα gene expression has been shown to require the activity of USF (Timchenko *et al*, 1995), it is therefore possible that c-Myc and USF have opposing functions during adipogenesis. Indeed, c-Myc was found to repress the USF mediated transactivation of C/EBPα promoter constructs in 3T3-L1 cells (Li *et al*, 1994). Since USF is able to inhibit cellular transformation arising from the co-operation of c-Myc and Ras (Luo and Sawadogo, 1996), it would therefore be of great interest to determine whether co-

expression of USF in the WT clones could also abrogate the Myc-mediated block to adipogenesis.

10.5 Myc-Mediated Inhibition of Adipogenesis is Abrogated by Foetal Calf Serum.

Possibly the most unexpected finding of this study was the observation that differentiation in the presence of 10% FCS was sufficient to over-ride the inhibitory effects of c-*myc* in the 3T3-L1 cell line. Analysis of genetic markers of adipogenesis indicated that this treatment restored the expression of C/EBPα, PPARγ2, aP2 and SCD1 and this was associated with accumulation of cytoplasmic triglyceride in both the WT and Δ7 cell lines. However, acquisition of the adipocyte phenotype did not affect expression of the avian c-*myc* 2.1kb mRNA species during the differentiation programme. This was surprising since it is widely assumed that down-regulation of c-*myc* is a prerequisite for differentiation to proceed in a variety of cell types. However, it was previously demonstrated that inhibition of U-937 monoblast differentiation by enforced expression of v-*myc* could be overcome by co-stimulation with interferon-γ, and that this was accomplished without altering either the expression or sub-cellular localisation of the v-Myc protein (Oberg *et al*, 1991). It is well documented that interferon-γ exerts its effects on gene expression *via* activation of the JAK/STAT signal transduction pathway (Ihle and Kerr, 1995). Interestingly, it has now become apparent that these factors are also involved in adipocyte biology (Darnell, 1996). Indeed, expression of STAT 1 and STAT 5 was found to be markedly reduced during a TNFα-mediated differentiation block in the 3T3-L1 cell line (Stephens *et al*, 1996). Since the ability of TNFα to inhibit adipogenesis is thought to be mediated in part by c-Myc, it is therefore possible that the STAT family of transcription factors represent another target for repression by this oncoprotein.

In contrast to TNFα, growth hormone stimulation positively modulates the activity of the JAK/STAT pathway during adipogenesis (Section 1.4.1). As the adipogenic effects of growth hormone have been well documented and FCS contains obviously elevated levels of this factor when compared with DCS, we therefore postulated that growth hormone might be the component of FCS responsible for abrogating the effects of c-Myc during 3T3-L1 differentiation. To test this hypothesis, we supplemented DCS with purified bovine pituitary growth hormone and assessed the ability to induce differentiation in the WT cell lines. Surprisingly, whilst WT8 differentiated in a manner akin to that previously observed in the presence of 10% FCS, growth hormone treatment was without effect in the WT6 cell line. However, WT6 and WT8 had displayed a similar response following induction in 10% FCS. It should be noted, however, that when we induced differentiation in the presence of DCS/FCS mixtures WT6 required much higher levels of FCS than WT8 in order to differentiate. To fully assess the effect of growth hormone we therefore examined the ability of the remaining WT clones to differentiate in response to DCS supplemented with this factor. From this experiment it was determined that growth hormone could elicit a range of responses in the Myc-expressing cell lines, whilst treatment with 10% FCS resulted in a fairly uniform pattern of differentiation. One possible explanation for these results is that the individual WT cell lines express different numbers of growth hormone receptors on the cell surface. Alternatively, growth hormone signalling might be impaired in cell lines such as WT6. However, at present we cannot distinguish between these possibilities. Since adipogenesis requires the interaction of a large number of signal transduction pathways, we therefore suggest that the effects of FCS on the differentiation capacity of 3T3-L1 cell lines constitutively expressing c-*myc* will probably involve the activity of more than one serum component. Whether growth hormone is actually required to mediate this effect will require further investigation.

10.6 Conclusions.

In summary, we have generated a number of preadipocyte cell lines which constitutively express c-*myc* and as a result are no longer able to undergo induced differentiation. Inhibition of adipogenesis was not found to be associated with either cellular transformation or abnormal cell cycle behaviour during the differentiation programme. However, repression of fat-specific gene expression and the inability to accumulate cytoplasmic triglyceride were shown to arise as a consequence of the inhibition of the key adipogenic transcription factors, C/EBPα and PPARγ2. The mechanism by which c-Myc inhibits expression of these genes remains unclear. However, treatment with 10% FCS was found to over-ride these effects and restore the ability of Myc-expressing cell lines to differentiate. Thus the ability of c-Myc to inhibit adipogenesis in the 3T3-L1 cell line may be modulated by external factors.

10.7 Future Prospects.

The ability to inhibit cellular differentiation is probably one of the least understood biological functions of the c-Myc oncoprotein. However, the lines generated during the course of this study will doubtless provide a useful tool with which to investigate this phenomenon further. We have thus far established a point at which c-Myc blocks adipogenesis but the mechanism by which this occurs remains unclear, although reference to the literature indicates that several possibilities exist. To address this issue it will therefore be vital to determine whether Myc-mediated inhibition of 3T3-L1 differentiation is a Max-dependent or Max-independent phenomenon and which of the putative DNA binding sites is actually required to exert this effect. Secondly, further investigation is necessary to establish the mode of action of FCS during abrogation of the Myc block. Since FCS restores expression of genes known to be repressed by the continued presence of c-Myc, these studies may also shed light on factors that either positively or negatively

regulate gene expression during adipogenesis. Finally, the issue of whether irreversible growth arrest is a prerequisite for adipocyte development and the identification of differentiation-specific cell cycle regulators warrants further study. Indeed, the recent observation that the "irreversible" growth arrest manifest in both mature myotubes and adipocytes may be over-come by the adenovirus E1A protein (Crescenzi *et al*, 1995) suggests that such studies will be relevant not just to differentiation in particular but to growth control in general. It is therefore anticipated that by investigating the ability of c-Myc to inhibit adipogenesis we will further our current understanding of both the mode of action of this important oncoprotein and the processes that drive terminal differentiation.

REFERENCES.

REFERENCES.

Abumrad, N.A., El-Maghrabi, M.R., Amri, E-Z., Lopez, E. and Grimaldi, P.A. (1993) Cloning of a rat adipocyte protein implicated in binding or transport of long-chain fatty acids that is induced during preadipocyte differentiation. *J. Biol. Chem.* **268**, 17665-17668.

Abumrad, N.A., Forest, C.C., Regen, D.M. and Sanders, S. (1991) Increase in membrane uptake of long-chain fatty acids early during preadipocyte differentiation. *Proc. Natl. Acad. Sci. USA* **88**, 6008-6012.

Adams, J.C. and Watt, F.M. (1993) Regulation of development and differentiation by the extracellular matrix. *Development* **117**, 1183-1198.

Adams, J.C. and Watt, F.M. (1989) Fibronectin inhibits the terminal differentiation of human keratinocytes. *Nature* **340**, 307-309.

Adams, J.M., Harris, A.W., Pinkert, C.A., Corcoran, L.M., Alexander, W.S., Cory, S., Palmiter, R.D. and Brinster, R.L. (1985) The c-*myc* oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature* **318**, 533-538.

Ailhaud, G., Grimaldi, P. and Negrel, R. (1992) Cellular and molecular aspects of adipose tissue development. *Annu. Rev. Nutr.* **12**, 207-233.

Akeson, R. and Bernards, R. (1990) N-*myc* down regulates neural cell adhesion molecule expression in rat neuroblastoma. *Mol. Cell. Biol.* **10**, 2012-2016.

Akira, S., Isshiki, H., Sugita, T., Tanabe, O., Kinoshita, S., Nishio, Y., Nakajima, T., Hirano, T. and Kishimoto, T. (1990) A nuclear factor for IL-6 expression (NF-IL6) is a member of the C/EBP family. *EMBO J.* **9**, 1897-1906.

Alexandrova, N., Niklinski, J., Bliskovsky, V., Otterson, G.A., Blake, M., Kaye, F.J. and Zajac-Kaye, M. (1995) The N-terminal domain of c-Myc associates with α-tubulin and microtubules in vivo and in vitro. *Mol. Cell. Biol.* **15**, 5188-5195.

Amati, B., Brooks, M.W., Levy, N., Littlewood, T.D., Evan, G.I. and Land, H. (1993) Oncogenic activity of the c-Myc protein requires dimerization with Max. *Cell* **72**, 233-245.

Amati, B., Dalton, S., Brooks, M.W., Littlewood, T.D., Evan, G.I. and Land, H. (1992) Transcriptional activation by the human c-Myc oncoprotein in yeast requires interaction with Max. *Nature* **359**,423-426.

Amati, B. and Land, H. (1994) Myc-Max-Mad: a transcription factor network controlling cell cycle progression, differentiation and death. *Curr. Opin. Genet. Dev.* **4**, 102-108.

Amri, E-Z., Bonino, F., Ailhaud, G., Abumrad, N.A. and Grimaldi, P.A. (1995) Cloning of a protein that mediates transcriptional effects of fatty acids in preadipocytes. *J. Biol. Chem.* **270**, 2367-2371.

Amri, E-Z., Teboul, L., Vannier, C., Grimaldi, P-A. and Ailhaud, G. (1996) Fatty acids regulate the expression of lipoprotein lipase gene and activity in preadipose and adipose cells. *Biochem. J.* **314**, 541-546.

An, M.R., Hsieh, C-C., Reisner, P.D., Rabek, J.P., Scott, S.G., Kuninger, D.T. and Papaconstantinou, J. (1996) Evidence for posttranscriptional regulation of C/EBPα and C/EBPβ isoform expression during the lipopolysaccharide-mediated acute phase response. *Mol. Cell. Biol.* **16**, 2295-2306.

Andres, V. and Walsh, K. (1996) Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis. *J. Cell Biol.* **132**, 657-666.

Argetsinger, L.S., Campbell, G.S., Yang, X., Witthuhn, B.A., Silvennoinen, O., Ihle, J.N. and Carter-Su, C. (1993) Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase. *Cell* **74**, 237-244.

Argetsinger, L.S., Hsu, G.W., Myers, M.G.Jr., Billestrup, N., White, M.F. and Carter-Su, C. (1995) Growth hormone, interferon-γ, and leukemia inhibitory factor promoted tyrosyl phosphorylation of insulin receptor substrate-1. *J. Biol. Chem.* **270**, 14685-14692.

Ayer, D.E. and Eisenman, R.N. (1993) A switch from Myc:Max to Mad:Max heterocomplexes accompanies monocyte/macrophage differentiation. *Genes Dev.* **7**, 2110-2119.

Ayer, D.E., Kretzner, L. and Eisenman, R.N. (1993) Mad: a heterodimeric partner for Max that antagonises Myc transcriptional activity. *Cell* **72**, 211-222.

Ayer, D.E., Lawrence, Q.A. and Eisenman, R.N. (1995) Mad:Max transcriptional repression is mediated by ternary complex formation with mammalian homologs of yeast repressor Sin3. *Cell* **80**, 767-776.

Bancroft, J.D. and Cook, H.C. (1984) *Manual of Histological Techniques.* Churchill Livingstone, London, 132-133.

Bao, J. and Zervos, A.S. (1996) Isolation and characterisation of Nmi, a novel partner of Myc proteins. *Oncogene* **12**, 2171-2176.

Barone, M.V., Crozat, A., Tabaee, A., Philipson, L. and Ron, D. (1994) CHOP (GADD153) and its oncogenic variant, TLS-CHOP, have opposing effects on the induction of G_1/S arrest. *Genes Dev.***8**, 453-464.

Batchvarova, N., Wang, X-Z. and Ron, D. (1995) Inhibition of adipogenesis by the stress-induced protein CHOP (Gadd153). *EMBO J.* **14**, 4654-4661.

Beijersbergen, R.L., Hijmans, E.M., Zhu, L. and Bernards, R. (1994) Interaction of c-Myc with the pRb-related protein p107 results in inhibition of c-Myc-mediated transactivation. *EMBO J.* **13**, 4080-4086.

Bello-Fernandez, C., Packham, G. and Cleveland, J.L. (1993) The ornithine decarboxylase gene is a transcriptional target of c-Myc. *Proc. Natl. Acad. Sci. USA* **90**, 7804-7808.

Benito, M., Porras, A., Nebreda, A.R. and Santos, E. (1991) Differentiation of 3T3-L1 fibroblasts to adipocytes induced by transfection of *ras* oncogenes. *Science*, **253**, 565-568.

Benjamin, W.B., Pentyala, S.N., Woodgett, J.R., Hod, Y. and Marshak, D. (1994) ATP citrate-lyase and glycogen synthase kinase-3β in 3T3-L1 cells during differentiation into adipocytes. *Biochem. J.* **300**, 477-482.

Benvenisty, N., Leder, A., Kuo, A. and Leder, P. (1992) An embryonically expressed gene is a target for c-Myc regulation via the c-Myc-binding sequence. *Genes Dev.* **6**, 2513-2523.

Bernlohr, D.A., Bolanowski, M.A., Kelly, T.J. and Lane, M.D. (1985) Evidence for an increase in transcription of specific mRNAs during differentiation of 3T3-L1 preadipocytes. *J. Biol. Chem.* **260**, 5563-5567.

Billon, N., van Grunsven, L.A. and Rudkin, B.B. (1996) The CDK inhibitor $p21^{WAF1/Cip1}$ is induced through a p300-dependent mechanism during NGF-mediated neuronal differentiation of PC12 cells. *Oncogene* **13**, 2047-2054.

Birkenmeier, E.H., Gwynn, B., Howard, S., Jerry, J., Gordon, J.I., Landschulz, W.H. and McKnight, S.L. (1989) Tissue-specific expression, developmental regulation, and genetic mapping of the gene encoding CCAAT/enhancer binding protein. *Genes Dev.* **3**, 1146-1156.

Blackwell, T.K., Kretzner, L., Blackwood, E.M., Eisenman, R.N. and Weintraub, H. (1990) Sequence-specific DNA binding by the c-Myc protein. *Science* **250**, 1149-1151.

Blackwood, E.M. and Eisenman, R.N. (1991) Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. *Science* **251**, 1211-1217.

Brun, R.P., Tontonoz, P., Forman, B.M., Ellis, R., Chen, J., Evans, R.M. and Spiegelman, B.M. (1996) Differential activation of adipogenesis by multiple PPAR isoforms. *Genes Dev.* **10**, 974-984.

Calkhoven, C.F. and Ab, G. (1996) Multiple steps in the regulation of transcription factor level and activity. *Biochem. J.* **317**, 329-342.

Calkhoven, C.F., Bouwman, P.R.J., Snippe, L. and Ab, G. (1994) Translation start site multiplicity of the CCAAT/enhancer binding protein α mRNA is dictated by a small 5' open reading frame. *Nucleic Acids Res.* **22**, 5540-5547.

Campisi, J., Gray, H.E., Pardee, A.B., Dean, M. and Sonenshein, G.E. (1984) Cell-cycle control of c-*myc* but not c-*ras* expression is lost following chemical transformation. *Cell* **36**, 241-247.

Cao, Z., Umek, R.M. and McKnight, S.L. (1991) Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev.* **5**, 1538-1552.

Carlson, S.G., Fawcett, T.W., Bartlett, J.D., Bernier, M. and Holbrook, N.J. (1993) Regulation of the C/EBP-related gene *gadd*153 by glucose deprivation. *Mol. Cell. Biol.* **13**, 4736-4744.

Castelein, H., Gulick, T., Declercq, P.E., Mannaerts, G.P., Moore, D.D. and Baes, M.I. (1994) The peroxisome proliferator activated receptor regulates malic enzyme gene expression. *J. Biol. Chem.* **269**, 26754-26758.

Chapman, A.B., Knight, D.M., Dieckmann, B.S. and Ringold, G.M. (1984) Analysis of gene expression during differentiation of adipogenic cells in culture and hormonal control of the developmental program. *J. Biol. Chem.* **259**, 15548-15555.

Charron, J., Malynn, B.A., Fisher, P., Stewart, V., Jeannotte, L., Goff, S.P., Robertson, E.J. and Alt, F.W. (1992) Embryonic lethality in mice homozygous for a targeted disruption of the N-*myc* gene. *Genes Dev.* **6**, 2248-2257.

Chawla, A. and Lazar, M.A. (1994) Peroxisome proliferator and retinoid signaling pathways co-regulate preadipocyte phenotype and survival. *Proc. Natl. Acad. Sci. USA* **91**, 1786-1790.

Chen, C., Nussenzweig, A., Guo, M., Kim, D., Li, G.C. and Ling, C.C. (1996) Down-regulation of gadd153 by c-*myc* in rat fibroblasts and its effect on cell growth and radiation-induced apoptosis. *Oncogene* **13**, 1659-1665.

Chen, P-L., Riley, D.J., Chen, Y. and Lee, W-H. (1996a) Retinoblastoma protein positively regulates terminal adipocyte differentiation through direct interaction with C/EBPs. *Genes Dev.* **10**, 2794-2804.

Chen, P-L., Riley, D.J., Chen-Kiang, S. and Lee, W-H. (1996b) Retinoblastoma protein directly interacts with and activates the transcription factor NF-IL6. *Proc. Natl. Acad. Sci. USA* **93**, 465-469.

Chen, S., Teicher, L.C., Kazim, D., Pollack, R.E. and Wise, L.S. (1989) Commitment of mouse fibroblasts to adipocyte differentiation by DNA transfection. *Science* **244**, 582-585.

Chen, T.R. (1977) *In situ* detection of Mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. *Exp. Cell Res.* **104**, 255-262.

Chin, L., Schreiber-Agus, N., Pellicer, I., Chen, K., Lee, H-W., Dudast, M., Cordon-Cardo, C. and dePinho, R.A. (1995) Contrasting roles for Myc and Mad proteins in cellular growth and differentiation. *Proc. Natl. Acad. Sci. USA* **92**, 8488-8492.

Christy, R.J., Kaestner, K.H., Geiman, D.E. and Lane, M.D. (1991) CCAAT/enhancer binding protein gene promoter: binding of nuclear factors during differentiation of 3T3-L1 preadipocytes. *Proc. Natl. Acad. Sci. USA* **88**, 2593-2597.

Christy, R.J., Yang, V.W., Ntambi, J.M., Geiman, D.E., Landshulz, W.H., Friedman, A.D., Nakabeppu, Y., Kelly, T.J. and Lane, M.D. (1989) Differentiation induced gene expression in 3T3-L1 preadipocytes: CCAAT/enhancer binding protein interacts with and activates the promoters of two adipocyte-specific genes. *Genes Dev.* **3**, 1323-1335.

Chung, J., Kuo, C.J., Crabtree, G.R. and Blenis, J. (1992) Rapamycin-FKBP specifically blocks growth-dependent activation of and signalling by the 70 kd S6 protein kinases. *Cell* **69**, 1227-1236.

Clarkson, R.W.E., Chen, C.M., Harrison, S., Wells, C., Muscat, G.E.O. and Waters, M.J. (1995) Early responses of *trans*-acting factors to growth hormone in

preadipocytes: differential regulation of CCAAT enhancer binding protein-β (C/EBPβ) and C/EBPδ. *Mol. Endocrinology* **9**, 108-120.

Clevidence, D.E., Overdier, D.G., Tao, W., Qian, X., Pani, L., Lai, E. and Costa, R.H. (1993) Identification of nine tissue-specific transcription factors of the hepatocyte nuclear factor 3/forkhead DNA-binding-domain family. *Proc. Natl. Acad. Sci. USA* **90**, 3948-3952.

Cole, M.D. (1986) The *myc* oncogene: its role in transformation and differentiation. *Annu. Rev. Genet.* **20**, 361-384.

Colon-Teicher, L., Wise, L.S., Martino, J.J., Baskin, L., Sakoulas, G., Pollack, R.E. and Chen, S. (1993) Genomic sequences capable of committing mouse and rat fibroblasts to adipogenesis. *Nucleic Acids Res.* **21**, 2223-2228.

Constance, C.M., Morgan, J.I. IV and Umek, R.M. (1996) C/EBPα regulation of the growth-arrest-associated gene *gadd*45. *Mol. Cell. Biol.* **16**, 3878-3883.

Coppola, J.A. and Cole, M.D. (1986) Constitutive c-*myc* oncogene expression blocks mouse erythroleukaemia cell differentiation but not commitment. *Nature* **320**, 760-763.

Cornelius, P., Enerback, S., Bjursell, G., Olivecrona, T. and Pekala, P.H. (1988). Regulation of lipoprotein lipase mRNA content in 3T3-L1 cells by tumour necrosis factor. *Biochem. J.* **249**, 765-769.

Cornelius P., MacDougald, O.A. and Lane, M.D. (1994) Regulation of adipocyte development. *Annu. Rev. Nutr.* **14**, 99-129.

Crescenzi, M., Crouch, D.H. and Tato, F. (1994) Transformation by *myc* prevents fusion but not biochemical differentiation of C2C12 myoblasts: mechanisms of phenotypic correction in mixed culture with normal cells. *J. Cell Biol.* **125**, 1137-1145.

Crescenzi, M., Fleming, T.P., Lassar, A.B., Weintraub, H. and Aaronson, S.A. (1990) MyoD induces growth arrest independent of differentiation in normal and transformed cells. *Proc. Natl. Acad. Sci. USA* **87**, 8442-8446.

Crescenzi, M., Soddu, S. and Tato, F. (1995) Mitotic cycle reactivation in terminally differentiated cells by adenovirus infection. *J. Cell. Physiol.* **162**, 26-35.

Crouch, D.H., Fisher, F., Clark, W., Jayaraman, P-S., Coding, C.R. and Gillespie, D.A.F. (1993) Gene-regulatory properties of Myc helix-loop-helix/leucine zipper mutants: Max-dependent transcriptional activation in yeast correlates with transforming activity. *Oncogene* **8**, 1849-1855.

Crouch, D.H., Lang, C. and Gillespie, D.A.F (1990) The leucine zipper domain of avian cMyc is required for transformation and autoregulation. *Oncogene* **5**, 683-689.

Crozat, A., Aman, P, Mandahl, N. and Ron, D. (1993) Fusion of CHOP to a novel RNA-binding protein in human myxoid liposarcoma. *Nature* **363**, 640-644.

Dani, C., Doglio, A., Amri, E-Z., Bardon, S., Fort, P., Bertrand, B., Grimaldi, P. and Ailhaud, G. (1989) Cloning and regulation of a mRNA specifically expressed in the preadipose state. *J. Biol. Chem.* **264**, 10119-10125.

Darlington, G.J., Wang, N. and Hanson, R.W. (1995) C/EBPα: a critical regulator of genes governing integrative metabolic processes. *Curr. Opin. Genet. Dev.* **5**, 565-570.

Darnell, J.E. jnr. (1996) Reflections on STAT3, STAT5 and STAT6 as fat STATs. *Proc. Natl. Acad. Sci. USA* **93**, 6221-6224.

Darnell, J., Lodish, H. and Baltimore, D. (1990) *Molecular Cell Biology* (2nd. edition). Scientific American Books Inc., New York.

Davis, A.C., Wims, M., Spotts, G.D., Hann, S.H. and Bradley, A. (1993) A null c-*myc* mutation causes lethality before 10.5 days of gestation in homozygotes and reduced fertility in heterozygous female mice. *Genes Dev.* **7**, 671-682.

DeClue, J.E. and Martin, G.S. (1989) Linker insertion-deletion mutagenesis of the v-*src* gene: isolation of host- and temperature-dependent mutants. *J. Virol.* **63**, 542-554.

Delgado, M.D., Lerga, A., Canelles, M., Gomez-Casares, M.T. and Leon, J. (1995) Differential regulation of Max and the role of c-Myc during erythroid and myelomonocytic differentiation of K562 cells. *Oncogene* **10**, 1659-1665.

Descombes, P., Chojkier, M., Lichsteiner, S., Falvey, E. and Schibler, U. (1990) LAP, a novel member of the C/EBP gene family, encodes a liver-enriched transcriptional activator protein. *Genes Dev.* **4**, 1541-1551.

Descombes, P. and Schibler, U. (1991) A liver-enriched transcriptional activator protein, LAP, and a transcriptional inhibitory protein, LIP, are translated from the same mRNA. *Cell* **67**, 569-579.

Distel, R.J., Robinson, G.S. and Spiegelman, B.M. (1992) Fatty acid regulation of gene expression. *J. Biol. Chem.* **267**, 5937-5941.

Dotto, G.P., Gilman, M.Z., Maruyama, M. and Weinberg, R.A. (1986) c-*myc* and c-*fos* expression in differentiating mouse primary keratinocytes. *EMBO J.* **5**, 2853-2857.

Downs, K.M., Martin, G.R. and Bishop, J.M. (1989) Contrasting patterns of *myc* and N-*myc* expression during gastrulation of the mouse embryo. *Genes Dev.* **3**, 860-869.

Dreyer, C., Krey, G., Keller, H., Givel, F., Helftenbein, G. and Wahli, W. (1992) Control of the peroxisomal β-oxidation pathway by a novel family of nuclear hormone receptors. *Cell* **68**, 879-887.

Dunn, B.K., Cogliati, T., Cultraro, C.M., Bar-Ner, M. and Segal, S. (1994) Regulation of murine *max* (*myn*) parallels the regulation of c-*myc* in differentiating murine erythroleukemia cells. *Cell Growth Differ.* **5**, 847-854.

Eilers, M., Schrim, S. and Bishop, J.M. (1991) The MYC protein activates transcription of the α-prothymosin gene. *EMBO J.* **10**, 133-141.

Einat, M., Resnitsky, D. and Kimchi, A. (1985) Close link between reduction in c-*myc* expression by interferon and G_0/G_1 arrest. *Nature* **313**, 597-600.

Endo, T. and Nadal-Ginard, B. (1986) Transcriptional and posttranscriptional control of c-*myc* during myogenesis: its mRNA remains inducible in differentiated

cells and does not suppress the differentiated phenotype. *Mol. Cell. Biol.* **6**, 1412-1421.

Enerback, S., Ohlsson, B.G., Samuelsson, L. and Bjursell, G. (1992) Characterisation of the human lipoprotein lipase (LPL) promoter: evidence of two *cis*-regulatory regions, LP-α and LP-β, of importance for the differentiation-linked induction of the LPL gene during adipogenesis. *Mol. Cell. Biol.* **12**, 4622-4633.

Engert, J.C., Berglund, E.B. and Rosenthal, N. (1996) Proliferation precedes differentiation in IGF-1 stimulated myogenesis. *J. Cell Biol.* **135**, 431-440.

Estes, S.D., Stoler, D.L. and Anderson, G.R. (1995) Normal fibroblasts induce the C/EBPβ and ATF-4 bZIP transcription factors in response to anoxia. *Exp. Cell Res.* **220**, 47-54.

Evan, G.I., Wyllie, A.H., Gilbert, C.S., Littlewood, T.D., Land, H., Brooks, M., Waters, C.M., Penn, L.Z. and Hancock, D.C. (1992) Induction of apoptosis in fibroblasts by c-Myc protein. *Cell* **69**, 119-128.

Facchini, L.M., Chen, S. and Penn, L.J.Z. (1994) Dysfunction of the Myc-induced apoptosis mechanism accompanies c-*myc* activation in the tumorigenic L929 cell line. *Cell Growth Differ.* **5**, 637-646.

Falcone, G., Tato, F. and Alema, S. (1985) Distinctive effects of the viral oncogenes *myc*, *erb*, *fps* and *src* on the differentiation program of quail myogenic cells. *Proc. Natl. Acad. Sci. USA* **82**, 426-430.

Fehon, R.G., Kooh, P.J., Rebay, I., Regan, C.L., Xu, T., Muskavitch, M.A.T. and Artavanis-Tsakonas, S. (1990) Molecular interactions between the protein products of the neurogenic loci *notch* and *delta*, two EGF-homologous genes in Drosophila. *Cell* **61**, 523-534.

Flier, J.S. (1995).The adipocyte: storage depot or node on the energy information superhighway?. *Cell* **80**, 15-18.

Flodby, P., Barlow, C., Kylefjord, H., Ahrlund-Richter, L. and Xanthopoulos, K.G. (1996) Increased hepatic cell proliferation and lung abnormalities in mice deficient in CCAAT/enhancer binding protein α. *J. Biol. Chem.* **271**, 24753-24760.

Forman, B.M., Tontonoz, P., Chen, J., Brun, R.P., Spiegelman, B.M. and Evans, R.M. (1995) 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 is a ligand for the adipocyte determination factor PPARγ. *Cell* **83**, 803-812.

Fornace, A.J. Jr., Nebert, D.W., Hollander, M.C., Luethy, J.D., Papathanasiou, M., Fargnoli, J. and Holbrook, N.J. (1989) Mammalian genes coordinately regulated by growth arrest signals and DNA-damaging agents. *Mol. Cell. Biol.* **9**, 4196-4203.

Franklin, D.S. and Xiong, Y. (1996) Induction of $p18^{ink4c}$ and its predominant association with CDK4 and CDK6 during myogenic differentiation. *Mol. Biol. Cell* **7**, 1587-1599.

Freytag, S.O. (1988) Enforced expression of the c-*myc* oncogene inhibits cell differentiation by precluding entry into a distinct predifferentiation state in G_0/G_1. *Mol. Cell. Biol.* **8**, 1614-1624.

Freytag, S.O., Dang, C.V. and Lee, W.M.F. (1990) Definition of the activities and properties of c-*myc* required to inhibit cell differentiation. *Cell Growth Differ.* **1**, 339-343.

Freytag, S.O. and Geddes, T.J. (1992) Reciprocal regulation of adipogenesis by Myc and C/EBPα. *Science* **256**, 379-382.

Freytag, S.O., Paielli, D.L. and Gilbert, J.D. (1994) Ectopic expression of the CCAAT/enhancer-binding protein α promotes the adipogenic program in a variety of mouse fibroblastic cells. *Genes Dev.* **8**, 1654-1663.

Friedman, A.D. and McKnight, S.L. (1990) Identification of two polypeptide segments of CCAAT/enhancer-binding protein required for transcriptional activation of the serum albumin gene. *Genes Dev.* **4**, 1416-1426.

Galaktionov, K., Chen, X. and Beach, D. (1996) Cdc25 cell-cycle phosphatase as a target of c-*myc*. *Nature* **382**, 511-517.

Gandarillas, A. and Watt, F.M. (1995) Changes in expression of members of the *fos* and *jun* families and *myc* network during terminal differentiation of human keratinocytes. *Oncogene* **11**, 1403-1407.

Gaubatz, S., Imhof, A., Dosch, R., Werner, O., Mitchell, P., Buettner, R. and Eilers, M. (1995) Transcriptional activation by Myc is under negative control by the transcription factor AP-2. *EMBO J.* **14**, 1508-1519.

Gonos, E.S. and Spandidos, D.A. (1993) Oncogenes in cellular immortalisation and differentiation. *Anticancer Res.* **13**, 1117-1122.

Graham, F.L. and van der Eb, A.J. (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**, 456-467.

Grandori, C., Mac, J., Siebelt, F., Ayer, D.E. and Eisenman, R.N. (1996) Myc-Max heterodimers activate a DEAD box gene and interact with multiple E box-related sites *in vivo*. *EMBO J.* **15**, 4344-4357.

Graves, B.J., Johnson, P.F. and McKnight, S.L. (1986) Homologous recognition of a promoter domain common to the MSV LTR and the HSV tk gene. *Cell* **44**, 565-576.

Graves, R.A., Tontonoz, P., Ross, S.R. and Spiegelman, B.M. (1991) Identification of a potent adipocyte-specific enhancer: involvement of an NF-1-like factor. *Genes Dev.* **5**, 428-437.

Graves, R.A., Tontonoz, P. and Spiegelman, B.M. (1992) Analysis of a tissue-specific enhancer: ARF6 regulates adipogenic gene expression. *Mol. Cell. Biol.* **12**, 1202-1208.

Green, H. and Kehinde, O. (1979) Formation of normally differentiated subcutaneous fat pads by an established preadipose cell line. *J. Cell. Physiol.* **101**, 169-172.

Green, H. and Kehinde, O. (1976) Spontaneous heritable changes leading to increased adipose conversion in 3T3 cells. *Cell* **7**, 105-113.

Green, H. and Kehinde, O. (1975) An established preadipose cell line and its differentiation in culture II. factors affecting the adipose conversion. *Cell* **5**, 19-27.

Green, H. and Kehinde, O. (1974) Sublines of mouse 3T3 cells that accumulate lipid. *Cell* **1**, 113-116.

Gregor, P.D., Sawadogo, M. and Roeder R.G. (1990) The adenovirus major late transcription factor USF is a member of the helix-loop-helix group of regulatory proteins and binds to DNA as a dimer. *Genes Dev.* **4**, 1730-1740.

Griep, A.E. and Westphal, H. (1988) Antisense *Myc* sequences induce differentiation of F9 cells. *Proc. Natl. Acad. Sci.* USA **85**, 6806-6810.

Grignani, F., Lombardi, L., Inghirami, G., Sternas, L., Cechova, K. and Dalla-Favera, R. (1990) Negative autoregulation of c-*myc* gene expression is inactivated in transformed cells. *EMBO J.* **9**, 3913-3922.

Gu, W., Bhatia, K., Magrath, I.T., Dang, C.V. and Dalla-Favera, R. (1994) Binding and suppression of the Myc transactivation domain by p107. *Science* **264**, 251-254.

Gu, W., Schneider, J.W., Condorelli, G., Kaushal, S., Mahdavi, V. and Nadal-Ginard, B. (1993) Interaction of myogenic factors and the retinoblastoma protein mediates muscle cell commitment and differentiation. *Cell* **72**, 309-324.

Guyton, K.Z., Xu, Q. and Holbrook, N.J. (1996) Induction of mammalian stress response gene GADD153 by oxidative stress: role of AP-1 element. *Biochem. J.* **314**, 547-554.

Halevy, O., Novitch, B.G., Spicer, D.B., Skapek, S.X., Rhee, J., Hannon, G.J., Beach, D. and lassar, A.B. (1995) Correlation of terminal cell cycle arrest of skeletal muscl with induction of p21 by MyoD. *Science* **267**, 1018-1021.

Hann, S.R. and Eisenman, R.N. (1984) Proteins encoded by the human c-*myc* oncogene: differential expression in neoplastic cells. *Mol. Cell. Biol.* **4**, 2486-2497.

Hann, S.R., Dixit, M., Sears, R.C. and Sealy, L. (1994) The alternatively initiated c-Myc proteins differentially regulate transcription through a noncanonical DNA-binding site. *Genes Dev.* **8**, 2441-2452.

Hann, S.R., Thompson, C.B. and Eisenman, R.N. (1985) c-*myc* oncogene protein synthesis is independent of the cell cycle in human and avian cells. *Nature* **314**, 366-369.

Harrington, E.A., Bennett, M.R., Fanadi, A. and Evan, G.I. (1994) c-Myc-induced apoptosis in fibroblasts is inhibited by specific cytokines. *EMBO J.* **13**, 3286-3295.

Harris, L.L., Talian, J.C. and Zelenka, P.S. Contrasting patterns of c-*myc* and N-*myc* expression in proliferating, quiescent, and differentiating cells of the embryonic chicken lens. *Development* **115**, 813-820.

Hateboer, G., Timmers, H.T.M., Rustgi, A.K., Billaud, M., van 't Veer, L.J. and Bernards, R. (1993) TATA-binding protein and the retinoblastoma gene product bind to overlapping epitopes on c-Myc and the adenovirus E1A protein. *Proc. Natl. Acad. Sci. USA* **90**, 8489-8493.

Hatton, K.S., Mahon, K.A., Chin, L., Chiu, F-C., Lee, H-W., Peng, D., Morgenbesser, S.D., Horner, J. and DePinho, R.A. (1996) Expression and activity of L-Myc in normal mouse development. *Mol. Cell. Biol.* **16**, 1794-1804.

Hausdorff, S.F., Frangioni, J.V. and Birnbaum, M.J. (1994) The role of p21ras in insulin-stimulated glucose transport in 3T3-L1 adipocytes. *J. Biol. Chem.* **269**, 21391-21394.

Hendricks-Taylor, L.R. and Darlington, G.J. (1995) Inhibition of cell proliferation by C/EBPα occurs in many cell types, does not require the presence of p53 or Rb, and is not affected by large T-antigen. *Nucleic Acids Res.* **23**, 4726-4733.

Henriksson, M. and Luscher, B. (1996) Proteins of the Myc network: essential regulators of cell growth and differentiation. *Adv. Cancer Res.* **68**, 109-181.

Herrera, R., Ro, H.S., Robinson, G.S., Xanthopoulos, K.G. ans Spiegelman, B.M. (1989) A direct role for C/EBP and the AP-1 binding site in gene expression linked to adipocyte differentiation. *Mol. Cell. Biol.* **9**, 5331-5339.

Holman, G.D. and Cushman, S.W. (1994) Subcellular localization and trafficking of the GLUT4 glucose transporter isoform in insulin-responsive cells. *BioEssays* **16**, 753-759.

Holt, C.E., Lemaire, P. and Gurdon, J.B. (1994) Cadherin-mediated cell interactions are necessary for the activation of MyoD in *Xenopus* mesoderm. *Proc. Natl. Acad. Sci. USA* **91**, 10844-10848.

Holt, J.T., Redner, R.L. and Nienhuis, A.W. (1988) An oligomer complimentary to c-*myc* mRNA inhibits proliferation of HL-60 promyelocytic cells and induces differentiation. *Mol. Cell. Biol.* **8**, 963-973.

Hopewell, R. and Ziff, E.B. (1995) The nerve growth factor-responsive PC12 cell line does not express the Myc dimerization partner Max. *Mol. Cell. Biol.* **15**, 3470-3478.

Hotamisligil, G.S., Peraldi, P., Budavari, A., Ellis, R., White, M.F. and Spiegelman, B.M. (1996) IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-α and obesity-induced insulin resistance. *Science* **271**, 665-668.

Hu, E., Tontonoz, P. and Spiegelman, B.M. (1995) Transdifferentiation of myoblasts by the adipogenic transcription factors PPARγ and C/EBPα. *Proc. Natl. Acad. Sci. USA* **92**, 9856-9860.

Hughes, S.H., Greenhouse, J.J., Petropoulos, C.J. and Sutrave, P. (1987) Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. *J. Virol.* **61**, 3004-3012.

Hurlin, P.J., Foley, K.P., Ayer, D.E., Eisenman, R.N., Hanahan, D. and Arbeit, J.M. (1995b) Regulation of Myc and Mad during epidermal differentiation and HPV-associated tumorigenesis. *Oncogene* **11**, 2487-2501.

Hurlin, P.J., Queva, C., Koskinen, P.J., Steingrimsson, E., Ayer, D.E., Copeland, N.G., Jenkins, N.A. and Eisenman, R.N. (1995a) Mad3 and Mad4: novel Max-interacting transcriptional repressors that suppress c-*myc* dependent transformation and are expressed during neural and epidermal differentiation. *EMBO J.* **14**, 5646-5659.

Hurst, H.C. (1994) bZIP proteins. *Protein Profile* **1**, 123-147.

Hwang, C-S., Mandrup, S., MacDougald, O.A., Geiman, D.E. and Lane, M.D. (1996) Transcriptional activation of the mouse obese (ob) gene by CCAAT/enhancer binding protein α. *Proc. Natl. Acad. Sci. USA* **93**, 873-877.

Hynes, R.O. (1994) Genetic analyses of cell-matrix interactions in development. *Curr. Opin. Genet. Dev.* **4**, 569-574.

Ihle, J.N. (1996) STATs and MAPKs: obligate or opportunistic partners in signalling. *BioEssays* **18**, 95-98.

Ihle, J.N. and Kerr, I.M. (1995) Jaks and Stats in signalling by the cytokine receptor superfamily. *Trends Genet.* **11**, 69-74.

Inghirami, G., Grignani, F., Sternas, L., Lombardi, L., Knowles, D.M. and Dalla-Favera, R. (1990) Down-regulation of LFA-1 adhesion receptors by c-*myc* oncogene in human B lymphoblastoid cells. *Science* **250**, 682-686.

Issemann, I. and Green, S. (1990) Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature* **347**, 645-650.

Izumo, S., Nadal-Ginard, B. and Mahdavi, V. (1988) Protooncogene induction and reprogramming of cardiac gene expression produced by pressure overload. *Proc. Natl. Acad. Sci. USA* **85**, 339-343.

Jan, Y.N. and Jan, L.Y. (1993) HLH proteins, fly neurogenesis, and vertebrate myogenesis. *Cell* **75**, 827-830.

Jansen-Durr, P., Meichle, A., Steiner, P., Pagano, M., Finke, K., Botz, J., Wessbecher, J., Draetta, G. and Eilers, M. (1993) Differential modulation of cyclin gene expression by MYC. *Proc. Natl. Acad. Sci. USA* **90**, 3685-3689.

Johnson, P.F., Landschulz, W.H., Graves, B.J. and McKnight, S.L. (1987) Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. *Genes Dev.* **1**, 133-146.

Johnson, P.F. and McKnight, S.L. (1989) Eukaryotic transcriptional regulatory proteins. *Annu. Rev. Biochem.* **58**, 799-839.

Jones, R.M., Branda, J., Johnston, K.A., Polymenis, M., Gadd, M., Rustgi, A., Callanan, L. and Schmidt, E. (1996) An essential E box in the promoter of the gene encoding the mRNA cap-binding protein (eukaryotic initiation factor 4E) is a target for activation by c-Myc. *Mol. Cell. Biol.* **16**, 4754-4764.

Kaddurah-Daouk, R., Greene, J.M., Baldwin, A.S Jr. and Kingston R.E. (1987) Activation and repression of mammalian gene expression by the c-Myc protein. *Genes Dev.* **1**, 347-357.

Kaestner, K.H., Christy, R.J. and Lane, M.D. (1990) Mouse insulin-responsive glucose transporter gene: characterisation of the gene and transactivation by the CCAAT/enhancer binding protein. *Proc. Natl. Acad. Sci. USA* **87**, 251-255.

Kaestner, K.H., Lee, K-H., Schlondorff, J., Hiemisch, H., Monaghan, A.P. and Schutz, G. (1993) Six members of the mouse forkhead family are developmentally regulated. *Proc. Natl. Acad. Sci. USA* **90**, 7628-7631.

Kamai, Y., Mikawa, S., Endo, K., Sakai, H. and Komano, T. (1996) Regulation of insulin-like growth factor-1 expression in mouse preadipocyte ob1771 cells. *J. Biol. Chem.* **271**, 9883-9886.

Kato, G.J., Barrett, J., Villa-Garcia, M. and Dang, C.V. (1990) An amino terminal c-Myc domain required for neoplastic transformation activates transcription. *Mol. Cell. Biol.* **10**, 5914-5920.

Kato, G.J., Lee, W.M.F., Chen, L. and Dang, C.V. (1992) Max: functional domains and interaction with c-Myc. *Genes Dev* **6**, 81-92.

Kearsey, J.M., Coates, P.J., Prescott, A.R., Warbrick, E. and Hall, P.A. (1995) Gadd45 is a nuclear cell cycle regulated protein which interacts with $p21^{cip1}$. *Oncogene* **11**, 1675-1683.

Kelly, K., Cochran, B.H., Stiles, C.D. and Leder, P. (1983) Cell-specific regulation of the c-*myc* gene by lymphocyte mitogens and platelet-derived growth factor. *Cell* **35**, 603-610.

Kelly, K. and Siebenlist, U. (1986) The regulation and expression of c-*myc* in normal and malignant cells. *Ann. Rev. Immunol.* **4**, 317-338.

Khosravi-Far, R. and Der, C.J (1994) The Ras signal transduction pathway. *Cancer and Metastasis Rev* **13**, 67-89.

Kim, J.B. and Spiegelman, B.M. (1996) ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism. *Genes Dev.* **10**, 1096-1107.

Kleyn, P.W., Fan, W., Kovats, S.G., Lee, J.J., Pulido, J.C., Wu, Y., Berkemeier, L.R., Misumi, D.J., Holmgren, L., Charlat, O., Woolf, E.A., Tayber, O., Brody, T., Shu, P., Hawkins, F., Kennedy, B., Baldini, L., Ebeling, C., Alperin, G.D., Deeds, J., Lakey, N., Culpepper, J., Chen H., Glucksmann-Kuis, M.A., Carlson, G.A., Duyk, G.M. and moore, K.J. (1996) Identification and characterisation of the mouse obesity gene *tubby*: a member of a novel gene family. *Cell* **85**, 281-290.

Kliewer, S.A., Forman, B.M., Blumberg, B., Ong, E.S., Borgmeyer, U., Mangelsdorf, D.J., Umesono, K. and Evans, R.M. (1994) Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc. Natl. Acad. Sci. USA* **91**, 7355-7359.

Kliewer, S.A., Lenhard, J.M., Willson, T.M., Patel, I., Morris, D.C. and Lehmann, J.M. (1995) A prostaglandin J_2 metabolite binds peroxisome proliferator-activated receptor γ and promotes adipocyte differentiation. *Cell* **83**, 813-819.

Kliewer, S.A., Umesono, K., Noonan, D.J., Heyman, R.A. and Evans, R.M. (1992) Convergence of 9-*cis* retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. *Nature* **358**, 771-774.

Kohlhuber, F., Hermeking, H., Graessmann, A. and Eick, D. (1995) Induction of apoptosis by the c-Myc helix-loop-helix/leucine zipper domain in mouse 3T3-L1 fibroblasts. *J. Biol. Chem.* **270**, 28797-28805.

Konieczny, S.F. and Emerson, C.P.Jr. (1984) 5-azacytidine induction of stable mesodermal stem cell lineages from 10T1/2 cells: evidence for regulatory genes controlling determination. *Cell* **38**, 791-800.

Kowenz-Leutz, E., Twamley, G., Ansieau, S. and Leutz, A. (1994) Novel mechanism of C/EBPβ (NF-M) transcriptional control: activation through derepression. *Genes Dev.* **8**, 2781-2791.

Kozak, M. (1991) Structural features in eukaryotic mRNAs that modulate the initiation of translation. *J. Biol. Chem.* **266**, 19867-19870.

Kretzner, L., Blackwood, E.M. and Eisenman, R.N. (1992) Myc and Max proteins possess distinct transcriptional activities. *Nature* **359**, 426-429.

Kunz, J., Henriquez, R., Schneider, U., Deuter-Reinhard, M., Movva, N.R. and Hall, M.N. (1993) Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G_1 progression. *Cell* **73**, 585-596.

La Rocca, S.A., Crouch, D.H. and Gillespie, D.A.F. (1994) c-Myc inhibits myogenic differentiation and *myoD* expression by a mechanism which can be dissociated from cell transformation. *Oncogene* **9**, 3499-3508.

Lachman, H.M. and Skoultchi, A.I. (1984) Expression of c-*myc* changes during differentiation of mouse erythroleukaemia cells. *Nature* **310**, 592-594.

Lahoz, E.G., Xu, L., Schreiber-Agus, N. and DePinho, R.A. (1994) Suppression of Myc, but not E1a, transformation activity by Max-associated proteins Mad and Mxi1. *Proc. Natl. Acad. Sci. USA* **91**, 5503-5507.

Land, H. , Parada, L. and Weinberg, R.A. (1983) Tumorigenic conversion of primary rat embryo fibroblasts requires at least two co-operating oncogenes. *Nature* **304**, 596-602.

Landschulz, W.H., Johnson, P.F., Adashi, E.Y., Graves, B.J. and McKnight, S.L.(1988) Isolation of a recombinant copy of the gene encoding C/EBP. *Genes Dev.***2**, 786-800.

Langdon, W.Y., Harris, A.W., Cory, S. and Adams, J.M. (1986) The c-*myc* oncogene perturbs B lymphocyte development in Eμ-*myc* transgenic mice. Cell **47**, 11-18.

Larsson, L.-G., Ivhed, I., Gidlund, M., Pettersson, U., Vennstrom, B. and Nilsson, K. (1988) Phorbol ester-induced terminal differentiation is inhibited in human U937 monoblastic cells expressing a v-*myc* oncogene. *Proc. Natl. Acad. Sci.* USA **85**, 2638-2642.

Larsson, L.-G., Pettersson, M., Oberg, F., Nilsson, K. and Luscher, B. (1994) Expression of *mad*, *mxi1*, *max* and c-*myc* during induced differentiation of hematopoietic cells: opposite regulation of *mad* and c-*myc*. *Oncogene* **9**, 1247-1252.

Lassar, A.B., Paterson, B.M. and Weintraub, H. (1986) Transfection of a DNA locus that mediates the conversion of 10T1/2 fibroblasts to myoblasts. *Cell* **47**, 649-656.

Lee, T-C., Zhang, Y. and Schwartz, R.J. (1994) Bifunctional transcriptional properties of YY1 in regulating muscle actin and c-*myc* gene expression during myogenesis. *Oncogene* **9**, 1047-1052.

Li, L.-h., Nerlov, C., Prendergast, G., MacGregor, D. and Ziff, E.B. (1994) c-Myc represses transcription *in vivo* by a novel mechanism dependent on the initiator element and Myc box II. *EMBO J.* **13**, 4070-4079.

Liao, K. and Lane, M.D. (1995) The blockade of preadipocyte differentiation by protein-tyrosine phosphatase HA2 is reversed by vanadate. *J. Biol. Chem.* **270**, 12123-12132.

Lin, F-T and Lane, M.D. (1994) CCAAT/enhancer binding protein α is sufficient to initiate the 3T3-L1 adipocyte differentiation program. *Proc. Natl. Acad. Sci. USA* **91**, 8757-8761.

Lin, F-T. and Lane, M.D. (1992) Antisense CCAAT/enhancer-binding protein RNA suppresses coordinate gene expression and triglyceride accumulation during differentiation of 3T3-L1 preadipocytes. *Genes Dev.* **6**, 533-544.

Lin, F-T., MacDougald, O.A., Diehl, A.M. and Lane, M.D. (1993) A 30-kDa alternative translation product of the CCAAT/enhancer biding protein a message: transcriptional activator lacking antimitotic activity. *Proc. Natl. Acad. Sci. USA* **90**, 9606-9610.

Liu, J-K., DiPersio, C.M. and Zaret, K.S. (1991) Extracellular signals that regulate liver transcription factors during hapatic differentiation in vitro. *Mol. Cell. Biol.* **11**, 773-784.

Luetteke, N., Lee, D.C., Palmiter, R.D., Brinster, R.L and Sangren, E.P.(1993) Regulation of fat and muscle development by transforming growth factor α in transgenic mice and cultured cells. *Cell Growth Diff.* **4**, 203-213.

Luo, X. and Sawadogo, M. (1996) Antiproliferative properties of the USF family of helix-loop-helix transcription factors. *Proc. Natl. Acad. Sci. USA* **93**, 1308-1313.

MacDougald, O.A., Cornelius, P., Liu, R. and Lane, M.D. (1995a) Insulin regulates transcription of the CCAAT/enhancer binding protein (CEBP) α, β, and δ genes in fully-differentiated 3T3-L1 adipocytes. *J. Biol. Chem.* **270**, 647-654.

MacDougald, O.A., Cornelius, P., Lin, F-T., Chen, S.S. and Lane, M.D. (1994) Glucocorticoids reciprocally regulate expression of the CCAAT/enhancer biding protein α and δ genes in 3T3-L1 adipocytes and white adipose tissue. *J. Biol. Chem.* **269**, 19041-19047.

MacDougald, O.A., Hwang, C-S., Fan, H. and Lane M.D. (1995b) Regulated expression of the obese gene product (leptin) in white adipose tissue and 3T3-L1 adipocytes. *Proc. Natl. Acad. Sci. USA* **92**, 9034-9037.

MacDougald, O.A. and Lane, M.D. (1995a) When precursors are also regulators. *Curr. Biol.* **5**, 618-621.

MacDougald, O.A. and Lane, M.D. (1995b) Transcriptional regulation of gene expression during adipocyte differentiation. *Annu. Rev. Biochem.* **64**, 345-373.

MacLeod, K.F., Sherry, N., Hannon, G., Beach, D., Tokino, T., Kinzler, K., Vogelstein, B. and Jacks, T. (1995) p53-dependent and independent expression of p21 during cell growth, differentiation and DNA damage. *Genes Dev.* **9**, 935-944.

Maheswaran, S., Lee, H. and Sonenshein, G.E. (1994) Intracellular association of the protein product of the c-*myc* oncogene with the TATA-binding protein. *Mol.Cell. Biol.* **14**, 1147-1152.

Mai, S. and Martensson, I-L. (1995) The c-Myc protein represses the λ5 and TdT initiators. *Nucleic Acids Res.* **23**, 1-9.

Makino, R., Hayashi, K. and Sugimura, T. (1984) c-*myc* transcript is induced in rat liver at an early stage of regeneration or by cycloheximide treatment. *Nature* **310**, 697-698.

Martel, C., Lallemand, D. and Cremisi, C. (1995) Specific c-*myc* and *max* regulation in epithelial cells. *Oncogene* **10**, 2195-2205.

Martelli, F., Cenciarelli, C., Santarelli, G., Polikar, B., Felsani, A. and Caruso, M. (1994) MyoD induces retinoblastoma gene expression during myogenic differentiation. *Oncogene* **9**, 3579-3580.

Martin, S.J., Green, D.R. and Cotter, T.G. (1994) Dicing with death: dissecting the components of the apoptosis machinery. *Trends Biol. Sci.* **19**, 26-30.

Maruta, H. and Burgess, A.W. (1994) Regulation of the Ras signalling network. *BioEssays* **16**, 489-495.

Maruyama, K., Schiavi, S.C., Huse, W., Johnson, G.L. and Ruley, H.E. (1987) Myc and E1A oncogenes alter the responsiveness of PC12 cells to nerve growth factor and block differentiation. *Oncogene* **1**, 361-367.

McGarry, J.D. (1995) Does leptin lighten the problem of obesity? *Curr. Biol.* **5**, 1342-1344.

Miner, J.H. and Wold, B.J. (1991) c-*myc* inhibition of MyoD and myogenin - initiated myogenic differentiation. *Mol. Cell. Biol.* **11**, 2842-2851.

Mink, S., Mutschler, B., Weiskirchen, R., Bister, K. and Klempnauer, K-H. (1996) A novel function for Myc: inhibition of C/EBP-dependent gene activation. *Proc. Natl. Acad. Sci. USA* **93**, 6635-6640.

Missero, C., Di Cunto, F., Kiyokawa, H., Koff, A., and Dotto, G.P. (1996) The absence of $p21^{Cip1/Waf1}$ alters keratinocyte growth and differentiation and promotes *ras*-tumor progression. *Genes Dev.* **10**, 3065-3075.

Morgenbesser, S.D., Schreiber-Agus, N., Bidder, M., Mahon, K.A., Overbeek, P.A., Horner, J. and DePinho, R.A. (1995) Contrasting roles for c-Myc and L-Myc in the regulation of cellular growth and differentiation *in vivo*. *EMBO J.* **14**, 743-756.

Muller, C., Kowenz-Leutz, E., Grieser-Ade, S., Graf, T. and Leutz, A. (1995) NF-M (chicken C/EBPβ) induces eosinophilic differentiation and apoptosis in a hematopoietic progenitor cell line. *EMBO J.* **14**, 6127-6135.

Naggert, J.K., Fricker, L.D., Varlamov, O., Nishina, P.M., Rouille, Y., Steiner, D.F., Carroll, R.J., Paigen, B.J. and Leiter, E.H. (1995) Hyperproinsulinaemia in obese *fat/fat* mice associated with a carboxypeptidase E mutation which reduces enzyme activity. *Nature Genetics* **10**, 135-141.

Nau, M.M., Brooks, B.J., Battey, J., Sausville, E., Gazdar, A.F., Kirsch, I.R., McBride, O.W., Bertness, V., Hollis, G.F. and Minna J.D. (1985) L-*myc*, a new *myc*-related gene amplified and expressed in human small cell lung cancer. *Nature* **318**, 69-73.

Negrel, R., Grimaldi, P. and Ailhaud, G. (1978) Establishment of preadipocyte clonal line from epididymal fat pads of *ob/ob* mouse that responds to insulin and to lipolytic hormones. *Proc. Natl. Acad. Sci. USA* **75**, 6054-6058.

Ninomiya-Tsuji, J., Torti, F.M. and Ringold, G.M. (1993) Tumor necrosis factor-induced c-*myc* expression in the absence of mitogenesis is associated with inhibition of adipocyte differentiation. *Proc. Natl. Acad. Sci. USA* **90**, 9611-9615.

Nixon, B.T. and Green, H. (1984) Growth hormone promotes the differentiation of myoblasts and preadipocytes generated by azacytidine treatment of 10T1/2 cells. *Proc. Natl. Acad. Sci. USA* **81**, 3429-3432.

Noben-Trauth, K., Naggert, J.K., North, M.A. and Nishina, P.M. (1996) A candidate gene for the mouse mutation *tubby*. *Nature* **380**, 534-538.

Novikoff, A.B., Novikoff, P.M. and Rubin, C.S. (1980) Organelle relationships in cultured 3T3-L1 preadipocytes. *J. Cell Biol.* **87**, 180-196.

Novitch, B.G., Mulligan, G.J., Jacks, T. and Lassar, A.B. (1996) Skeletal muscle cells lacking the retinoblastoma protein display defects in muscle gene expression and accumulate in the S and G_2 phases of the cell cycle. *J. Cell Biol.* **135**, 441-456.

Ntambi, J.M., Buhrow, S.A., Kaestner, K.H., Christy, R.J., Sibley, E., Kelly, T.J.Jnr. and Lane, M.D. (1988) Differentiation-induced gene expression in 3T3-L1 preadipocytes: Characterisation of a differentially expressed gene encoding stearoyl CoA desaturase. *J. Biol. Chem.* **263**, 17291-17300.

Oberg, F., Larsson, L-G., Anton, R. and Nilsson, K. (1991) Interferon γ abrogates the differentiation block in v-*myc*-expressing U-937 monoblasts. *Proc. Natl. Acad. Sci. USA* **88**, 5567-5571.

Okada, T., Kawano, Y., Sakakibara, T., Hazeki, O. and Ui, M. (1994) Essential role of phosphatidylinositol 3-kinase in insulin-induced glucose transport and antilipolysis in rat adipocytes. *J. Biol. Chem.* **269**, 3568-3573.

Olson, E.N. and Klein, W.H. (1994) bHLH factors in muscle development: dead lines and commitments, what to leave in and what to leave out. *Genes Dev.* **8**, 1-8.

Osada, S., Yamamoto, H., Nishihara, T. and Imagawa, M. (1996) DNA binding specificity of the CCAAT/enhancer binding protein transcription factor family. *J. Biol. Chem.* **271**, 3891-3896.

Ossipow, V., Descombes, P. and Schibler, U. (1993) CCAAT/enhancer binding protein mRNA is translated into multiple proteins with different transactivation potentials. *Proc. Natl. Acad. Sci. USA* **90**, 8219-8223.

Packham, G. and Cleveland, J.L. (1994) Ornithine decarboxylase is a mediator of c-Myc-induced apoptosis. *Mol. Cell. Biol.* **14**, 5741-5747.

Pairault, J. and Green, H. (1979) A study of adipose conversion of suspended 3T3 cells by using glycerophosphate dehydrogenase as differentiation marker. *Proc. Natl. Acad. Sci. USA* **76**, 5138-5142.

Parker, S.B., Eichele, G., Zhang, P., Rawls, A., Sands, A.T., Bradley, A., Olson, E.N., Harper, J.W. and Elledge, S.J. (1995) p53-independent expression of $p21^{cip1}$ in muscle and other terminally differentiating cells. *Science* **267**, 1024-1027.

Phillip, A., Schneider, A., Vasrik, I., Finke, K., Xiong, Y., Beach, D., Alitalo, K. and Eilers, M. (1994) Repression of cyclin D1: a novel function of MYC. *Mol. Cell. Biol.* **14**, 4032-4043.

Pines, J. (1994) The cell cycle kinases. *Seminars Cancer Biol.* **5**, 305-313.

Poli, V., Mancini, F.P. and Cortese, R. (1990) IL-6DBP, a nuclear protein involved in interleukin-6 signal transduction, defines a new family of leucine zipper proteins related to C/EBP. *Cell* **63**, 643-653.

Porras, A., Muszynski, K., Rapp, U.R. and Santos, E. (1994) Dissociation between activation of Raf-1 kinase and the 42-kDa mitogen-activated protein kinase/90-kDa S6 kinase (MAPK/RSK) cascade in the insulin/ras pathway of adipocytic differentiation of 3T3-L1 cells. *J. Biol. Chem.* **269**, 12741-12748.

Prendergast, G.C., Hopewell, R., Gorham, B.J. and Ziff, E.B. (1992) Biphasic effect of Max on Myc cotransformation activity and dependence on amino- and carboxy terminal Max functions. *Genes Dev.* **6**, 2429-2439.

Prendergast, G.C. and Ziff, E.B. (1991) Methylation-sensitive sequence-specific DNA binding by the c-Myc basic region. *Science* **251**, 186-189.

Prochownik, E.V., Kukowska, J. and Rodgers, C. (1988) c-*myc* antisense transcripts accelerate differentiation and inhibit G_1 progression in murine erythroleukemia cells. *Mol. Cell. Biol.* **8**, 3683-3695.

Ram, P.A., Park, S-H., Choi, H.K. and Waxman, D.J. (1996) Growth hormone activation of Stat 1, Stat 3, and Stat 5 in rat liver. *J. Biol. Chem.* **271**, 5929-5940.

Ramji, D.P., Vitelli, A., Tronche, F., Cortese, R. and Ciliberto, G. (1993) The two C/EBP isoforms, IL-6DBP/NF-IL6 and C/EBPδ/NF-IL6β, are induced by IL-6 to promote acute phase gene transcription via different mechanisms. *Nucleic Acids Res.* **21**, 289-294.

Ramsay,T.G., White, M.E.and Wolverton, C.K. (1989a) Glucocorticoids and the differentiation of porcine preadipocytes. *J. Anim. Sci.* **67**, 2222-2229.

Ramsay, T.G., White, M.E. and Wolverton, C.K. (1989b) Insulin-like growth factor 1 induction of differentiation of porcine preadipocytes. *J. Anim. Sci.* **67**, 2452-2459.

Ray, R., Thomas, S. and Miller, D.M. (1989) Mouse fibroblasts transformed with the human c-*myc* gene express a high level of the mRNA but a low level of the c-*myc* protein and are non-tumorigenic in nude mice. *Oncogene* **4**, 593-600.

Reitsma, P.H., Rothberg, P.G., Astrin, S.M., Trial, J., Bar-Shavit, Z., Hall, A., Teitelbaum, S.L and Kahn, A.J. (1983) Regulation of *myc* gene expression in HL-60 leukemia cells by a vitamin D metabolite. *Nature* **306**, 492-494.

Reznikoff, K.A., Brankow, D.W. and Heidelberger, C. (1973) Establishment and characterisation of a cloned line of C3H mouse embryo cells sensitive to post-confluence inhibition of division. *Cancer Res.* **33**, 3231-3238.

Ron, D. and Habener, J.F. (1992) CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. *Genes Dev.* **6**, 439-453.

Ross, S.R., Choy, L., Graves, R.A., Fox, N., Solevjeve, V., Klaus, S., Ricquier, D. and Spiegelman, B.M. (1992) Hibernoma formation in transgenic mice and isolation of a brown adipocyte cell line expressing the uncoupling protein gene. *Proc. Natl. Acad. Sci. USA* **89**, 7561-7565.

Ross, S.R., Graves, R.A., Greenstein, A., Platt, K.A., Shyu, H-L., Mellovitz, B. and Spiegelman, B.M. (1990) A fat-specific enhancer is the primary determinant of gene expression for adipocyte P2 *in vivo*. *Proc. Natl. Acad. Sci. USA* **87**, 9590-9594.

Roy, A.L., Carruthers, C., Gutjahr, T. and Roeder, R.G. (1993) Direct role for Myc in transcriptional initiation mediated by interactions with TFII-I. *Nature* **365**, 356-361.

Roy, A.L., Meisterernst, M., Pognonec, P. and Roeder, R.G. (1991) Cooperative interaction of an initiator-binding transcription initiation factor and the helix-loop-helix activator USF. *Nature* **354**, 245-248.

Roy, B., Beamon, J., Balint, E. and Reisman, D. (1994) Transactivation of human p53 tumor suppressor gene by c-Myc/Max contributes to elevated mutant p53 expression in some tumors. *Mol. Cell. Biol.* **14**, 7805-7815.

Russo, S., Tato, F. and Grossi, M. (1997) Transcriptional down-regulation of myogenin expression is associated with v-*ras*-induced block of differentiation in unestablished quail myoblast cells. *Oncogene* **14**, 63-73.

Rustgi, A.K., Dyson, N. and Bernards, R. (1991) Amino-terminal domains of c-*myc* and N-*myc* proteins mediate binding to the retinoblastoma gene product. *Nature* **352**, 541-544.

Sadowski, H.B., Wheeler, T.T. and Young, D.A. (1992) Gene expression during 3T3-L1 adipocyte differentiation. *J. Biol. Chem.* **266**, 4722-4731.

Sakamuro, D., Elliott, K.J., Wechsler-Reya, R. and Prendergast, G.C. (1996) BIN1 is a novel MYC-interacting protein with features of a tumour suppressor. *Nature Genetics* **14**, 69-76.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning, a Laboratory Manual*: 2nd ed., Cold Spring Harbor Laboratory Press.

Samuelsson, L., Stromberg, K., Vikman, K., Bjursell, G. and Enerback, S. (1991) The CCAAT/enhancer binding protein and its role in adipocyte differentiation: evidence for direct involvement in terminal adipocyte development. *EMBO J.* **10**, 3787-3793.

Sanchez-Garcia, I. and Rabbitts, T.H. (1994) Transcriptional activation by TAL1 and FUS-CHOP proteins expressed in acute malignancies as a result of chromosomal abnormalities. *Proc. Natl. Acad. Sci. USA* **91**, 7869-7873.

Sawai, S., Shimono, A., Wakamatsu, Y., Palmes, C., Hanaoka, K. and Kondoh, H. (1993) Defects of embryonic organogenesis resulting from targeted disruption of the N-*myc* gene in the mouse. *Development* **117**, 1445-1455.

Schmid, P., Schulz, W.A. and Hameister, H. (1989) Dynamic expression pattern of the *myc* protooncogene in midgestation mouse embryos. *Science* **243**, 226-229.

Schoonjans, K., Peinado-Onsurbe, J., Lefebvre, A-M., Heyman, R.A., Briggs, M., Deed, S., Staels, B. and Auwerx, J. (1996) PPARα and PPARγ activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. *EMBO J.* **15**, 5336-5348.

Schreiber-Agus, N., Chin, L., Chen, K., Torres, R., Rao, G., Guida, P., Skoultchi, A.I. and DePinho, R.A. (1995) An amino-terminal domain of Mxi1 mediates anti-Myc oncogenic activity and interacts with a homolog of the yeast transcriptional repressor Sin3. *Cell* **80**, 777-786.

Schwab, M., Alitalo, K., Klempnauer, K-H., Varmus, H.E., Bishop, J.M., Gilbert, F., Brodeur, G., Goldstein, M. and Trent, J. (1983) Amplified DNA with limited homology to *myc* cellular oncogene is shared by human neuroblastoma cell lines and a neuroblastoma tumour. *Nature* **305**, 245-248.

Scott, R.E., Florine, D.L., Wille, J.J.Jr. and Yun, K. (1982) Coupling of growth arrest and differentiation at a distinct state in the G_1 phase of the cell cycle: G_D. *Proc. Natl. Acad. Sci.* USA **79**, 845-849.

Scott, R.E., Yun, K. and Florine, D.L. (1983) Differential mitogenic effects of methyl isobutylxanthine and a tumor growth factor on G_1-arrested 3T3 T proadipocytes at the predifferentiation G_D state and the growth factor deficiency G_S state. *Expt. Cell Res.* **143**, 405-414.

Screpanti, I., Romani, L., Musiani, P., Modesti, A., Fattori, E., Lazzaro, D., Sellitto, C., Scarpa, S., Bellavia, D., Lattanzio, G., Bistoni, F., Frati, L., Cortese, R., Gulino, A., Ciliberto, G., Costantini, F. and Poli, V. (1995) Lymphoproliferative disorder and imbalanced T-helper response in C/EBPβ-deficient mice. *EMBO J.* **14**, 1932-1941.

Serrero, G., Lepak, N.M. and Goodrich, S.P. (1992) Prostaglandin F2α inhibits the differentiation of adipocyte precursors in primary culture. *Biochem. Biophys. Res. Commun.* **183**, 438-442.

Serrero, G. and Mills, D. (1991) Physiological role of epidermal growth factor on adipose tissue development *in vivo*. *Proc. Natl. Acad. Sci. USA* **88**, 3912-3916.

Sherr, C.J. and Roberts, J.M. (1995) Inhibitors of mammalian G_1 cyclin-dependent kinases. *Genes Dev.* **9**, 1149-1163.

Shrivastava, A., Saleque, S., Kalpana, G.V., Artandi, S., Goff, S.P. and Calame, K. (1993) Inhibition of transcriptional regulator Yin-Yang-1 by association with c-Myc. *Science* **262**, 1889-1892.

Shrivastava, A., Yu, J., Artandi, S. and Calame, K. (1996) YY1 and c-Myc associate *in vivo* in a manner that depends on c-Myc levels. *Proc. Natl. Acad. Sci. USA* **93**, 10638-10641.

Shugart, E.C., Levenson, A.S., Constance, C.M. and Umek, R.M. (1995) Differential expression of *gas* and *gadd* genes at distinct growth arrest points during adipocyte development. *Cell Growth Differ.* **6**, 1541-1547.

Sidhu, R.S. (1979) Two-dimensional electrophoretic analyses of proteins synthesised during differentiation of 3T3-L1 preadipocytes. *J. Biol. Chem.* **254**, 11111-11118.

Siekierka, J.J., Hung, S.H.Y., Poe, M., Lin, C.S. and Sigal, N.H. (1989) A cytosolic binding protein for the immunosuppressant FK506 has peptidyl-prolyl isomerase activity but is distinct from cyclophilin. *Nature* **341**, 755-757.

Skerjanc, I.S., Slack, R.S. and McBurney, M.W. (1994) Cellular aggregation enhances MyoD-directed myogenesis in embryonal carcinoma cells. *Mol. Cell. Biol.* **14**, 8451-9459.

Smale, S.T. and Baltimore, D. (1989) The "Initiator" as a transcription control element. *Cell* **57**, 103-113.

Smas, C.M. and Sul, H.S. (1995) Control of adipocyte differentiation. *Biochem. J.* **309**, 697-710.

Smas, C.M. and Sul, H.S. (1993) Pref-1, a protein containing EGF-like repeats, inhibits adipocyte differentiation. *Cell* **73**, 725-734.

Smith, P.J., Wise, L.S., Berkowitz, R., Wan, C. and Rubin, C.S. (1988) Insulin-like growth factor-1 is an essential regulator of the differentiation of 3T3-L1 adipocytes. *J. Biol. Chem.* **263**, 9402-9408.

Smulson, M.E., Kang, V.H., Ntambi, J.M., Rosenthal, D.S., Ding, R. and Simbulan, C.M.G. (1995) Requirement for the expression of poly(ADP-ribose) polymerase during the early stages of differentiation of 3T3-L1 preadipocytes, as studied by antisense RNA induction. *J. Biol. Chem.* **270**, 119-127.

Smyth, M.J., Sparks, R.L. and Wharton, W. (1993) Proadipocyte cell lines: models of cellular prolifereation and differentiation. *J. Cell Sci.* **106**, 1-9.

Sparks, R.L., Seibel-Ross, E.I., Wier, M.L. and Scott, R.E. (1986) Differentiation, dedifferentiation, and transdifferentiation of BALB/c 3T3 T mesenchymal stem cells: potential significance in metaplasia and neoplasia. *Cancer Res.* **46**, 5312-5319.

Spiegelman, B.M. and Farmer, S.R. (1982) Decreases in tubulin and actin gene expression prior to morphological differentiation of 3T3 adipocytes. *Cell* **29**, 53-60.

Spiegelman, B.M. and Ginty, C.A. (1983) Fibronectin modulation of cell shape and lipogenic gene expression in 3T3-adipocytes. *Cell* **35**, 657-666.

Stanton, B.R., Perkins, A.S., Tessarollo, L., Sassoon, D.A. and Parada, L.F. (1992) Loss of N-*myc* function results in embryonic lethality and failure of the epithelial component of the embryo to develop. *Genes Dev.* **6**, 2235-2247.

Stephens, J.M., Morrison, R.F. and Pilch, P.F. (1996) The expression and regulation of STATs during 3T3-L1 adipocyte differentiation. *J. Biol. Chem.* **271**, 10441-10444.

Stone, J., de Lange, T., Ramsay, G., Jakobovits, E., Bishop, J.M., Varmus, H. and Lee, W. (1987) Definition of regions in human c-*myc* that are involved in transformation and nuclear localization. *Mol. Cell. Biol.* **7**, 1697-1709.

Strange, R., Li, F., Saurer, S., Burkhardt, A. and Friis, R.R. (1992) Apoptotic cell death and tissue remodelling during mouse mammary gland involution. *Development* **115**, 49-58.

Streuli, C.H., Bailey, N. and Bissell, M.J. (1991) Control of mammary epithelial differentiation: basement membrane induces tissue-specific gene expression in the absence of cell-cell interaction and morphological polarity. *J. Cell Biol.* **115**, 1383-1395.

Streuli, C.H., Schmidhauser, C., Bailey, N., Yurchenco, P., Skubitz, A.P.N., Roskelley, C. and Bissell, M.J. (1995) Laminin mediates tissue-specific gene expression in mammary epithelia. *J. Cell Biol.* **129**, 591-603.

Stryer, L. (1988) *Biochemistry* (3rd. edition). W.H. Freeman and Co., New York.

Student, A.K., Hsu, R.Y. and Lane, M.D. (1980) Induction of fatty acid synthase synthesis in differentiating 3T3-L1 preadipocytes. *J. Biol. Chem.* **255**, 4745-4750.

Tartaglia, L.A., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., Richards, G.J., Campfield, L.A., Clark, F.T., Deeds, J., Muir, C., Sanker, S., Moriaty, A., Moore, K.J., Smutko, J.S., Mays, G.G., Woolf, E.A., Monroe, C.A. and Tepper, R.I. (1995) Identification and expression cloning of a leptin receptor, OB-R. *Cell* **83**, 1263-1271.

Taylor, S.M. and Jones, P.A. (1979) Multiple new phenotypes induced in 10T1/2 and 3T3 cells treated with 5-azacytidine. *Cell* **17**, 771-779.

Teboul, L., Gaillard, D., Staccini, L., Inadera, H., Amri, E-Z. and Grimaldi, P.A. (1995) Thiazolidinediones and fatty acids convert myogenic cells into adipose-like cells. *J. Biol. Chem.* **270**, 28183-28187.

Thiery, J.P. and Boyer, B. (1992) The junction between cytokines and cell adhesion. *Curr. Opin. Cell Biol.* **4**, 782-792.

Timchenko, N.A., Wilde, M., Nakanishi, M., Smith, J.R. and Darlington, G.J. (1996) CCAAT/enhancer binding protein α (C/EBPα) inhibits cell proliferation through the p21 (WAF-1/CIP-1/SDI-1) protein. *Genes Dev.* **10**, 804-815.

Timchenko, N., Wilson, D.R., Taylor, L.R., Abdelsayed, S., Wilde, M., Sawadogo, M. and Darlington, G.J. (1995) Autoregulation of the human C/EBPα gene by stimulation of upstream stimulatory factor binding. *Mol. Cell. Biol.* **15**, 1192-1202.

Tontonoz, P., Graves, R.A., Budavari, A.I., Erdjument-Bromage, H., Lui, M., Hu, E., Tempst, P. and Spiegelman, B.M. (1994c) Adipocyte-specific transcription factor ARF6 is a heterodimeric complex of two nuclear hormone receptors, PPARγ and RXRα. *Nucleic Acids Res.* **22**, 5628-5634.

Tontonoz, P., Hu, E., Devine, J., Beale, E.G. and Spiegelman, B.M. (1995b) PPARγ2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene. *Mol. Cell. Biol.* **15**, 351-357.

Tontonoz, P., Hu, E., Graves, R.A., Budavari, A. and Spiegelman, B.M (1994a) mPPARγ2: a tissue-specific regulator of an adipocyte enhancer. *Genes Dev.* **8**, 1224-1234.

Tontonoz, P., Hu, E. and Spiegelman, B.M. (1995a) Regulation of adipocyte gene expression and differentiation by peroxisome proliferator activated receptor γ. *Curr. Opin. Genet. Dev.* **5**, 571-576.

Tontonoz, P., Hu, E. and Spiegelman, B.M. (1994b) Stimulation of adipogeneis in fibroblasts by PPARγ2, a lipid-activated transcription factor. *Cell* **79**, 1147-1156.

Tontonoz, P., Kim, J.B., Graves, R.A. and Spiegelman, B.M. (1993) ADD1; a novel helix-loop-helix transcription factor associated with adipocyte determination and differentiation. *Mol. Cell. Biol.* **13**, 4753-4759.

Torres, R., Schreiber-Agus, N., Morgenbesser, S.D. and DePinho, R.A. (1992) Myc and max: a putative transcriptional complex in search of a cellular target. *Curr. Opin. Cell Biol.* **4**, 468-474.

Torti, F.M., Dieckmann, B., Beutler, B., Cerami, A. and Ringold, G.M. (1985) A macrophage factor inhibits adipocyte gene expression: an *in vitro* model of cachexia. *Science* **229**, 867-869.

Trautwein, C., Caelles, C., van der Geer, P., Hunter T., Karin, M. and Chojkier, M. (1993) Transactivation of NF-IL6/LAP is enhanced by phosphorylation of its activation domain. *Nature* **364**, 544-547.

Tugwood, J.D., Issemann, I., Anderson, R.G., Bundell, K.R., McPheat, W.L. and Green, S. (1992) The mouse peroxisome proliferator activated receptor recognises a response element in the 5' flanking sequence of the rat acyl CoA oxidase gene. *EMBO J.* **11**, 433-439.

Umek, R.M., Friedman, A.D. and McKnight, S.L. (1991) CCAAT-enhancer binding protein: a component of a differentiation switch. *Science* **251**, 288-292.

Vallejo, M., Ron, D., Miller, C.P. and Habener, J.F. (1993) C/ATF, a member of the activating transcription factor family of DNA-binding proteins, dimerizes with CAAT/enhancer-binding proteins and directs their binding to cAMP response elements. *Proc. Natl. Acad. Sci. USA* **90**, 4679-4683.

van den Berghe, N., Ouwens D.M., Maassen, J.A., van Mackelenbergh, M.G.H., Sips, H.C.M. and Krans, H.M.J. (1994) Activation of the ras/mitogen-activated protein kinase signaling pathway alone is not sufficient to induce glucose uptake in 3T3-L1 adipocytes. *Mol. Cell. Biol.* **14**, 2372-2377.

Vasseur-Cognet, M. and Lane, M.D. (1993a) Trans-acting factors involved in adipogenic differentiation. *Curr. Opin. Genet. Dev.* **3**, 238-245.

Vasseur-Cognet, M. and Lane, M.D. (1993b) CCAAT/enhancer binding protein α (C/EBPα) undifferentiated protein: a developmentally regulated nuclear protein that binds to the C/EBPα gene promoter. *Proc. Natl. Acad. Sci. USA* **90**, 7312-7316.

Vassaux, G., Gaillard, D., Ailhaud, G. and Negrel, R. (1992) Prostacyclin is a specific effector of adipose cell differentiation. Its dual role as a cAMP and Ca^{2+}-elevating agant. *J. Biol. Chem.* **267**, 11092-11097.

Vastrik, I., Kaipainen, A., Penttila, T-L., Lymboussakis, A., Alitalo, R., Parvinen, M. and Alitalo, K. (1995) Expression of the *mad* gene during celll differentiation in vivo and its inhibition of cell growth in vitro. *J. Cell Biol.* **128**, 1197-1208.

Vastrik, I., Makela, T.P., Koskinen, P.J., Klefstrom, J. and Alitalo, K. (1994) Myc proteins: partners and antagonists. *Crit. Rev. Onc.* **5**, 59-68.

Vaziri, C. and Faller, D.V. (1996) Down-regulation of platelet-derived growth factor receptor expression during terminal differentiation of 3T3-L1 preadipocyte fibroblasts. *J. Biol. Chem.* **271**, 13642-13648.

Vennstrom, B., Sheiness, D., Zabielski, J. and Bishop, J.M. (1982) Isolation and characterization of c-*myc*, a cellular homolog of the oncogene (v-*myc*) of avian myelocytomatosis virus strain 29. *J. Virol.* **42**, 773-779.

Vinson, C.R., Sigler, P.B. and McKnight, S.L. (1989) Scissors-grip model for DNA recognition by a family of leucine zipper proteins. *Science* **246**, 911-916.

Vlach, J., Hennecke, S., Alevizopoulos, K., Conti, D. and Amati, B. (1996) Growth arrest by the cyclin-dependent kinase inhibitor $p27^{kip1}$ is abrogated by c-Myc. *EMBO J.* **15**, 6595-6604.

Vogelstein, B. and Kinzler, K.W. (1993) The multistep nature of cancer. *Trends Genet.* **9**, 138-141.

Wagner, A.J., Meyers, C., Laimins, L.A. and Hay, N. (1993) c-Myc induces the expression and activity of ornithine decarboxylase. *Cell Growth Differ.* **4**, 879-883.

Wall, L., Destroismaisons, N., Delvoye, N. and Guy, L-G. (1996) CAAT/enhancer binding proteins are involved in β-globin gene expression and are differentially expressed in murine erythroleukemia and K562 cells. *J. Biol. Chem.* **271**, 16477-16484.

Wang, H., Sturtevant, D.B. and Scott, R.E. (1994) Nonterminal and terminal adipocyte differentiation of murine 3T3 T mesenchymal stem cells. In: *Cell Biology-A Laboratory Handbook* (Vol 1), Ed.Celis, J.E., Academic Press, San Diego.

Wang, N-D., Finegold, M.J., Bradley, A., Ou, C.N., Abdelsayed, S.V., Wilde, M.D., Taylor, L.R., Wilson, D.R. and Darlington, G.J. (1995) Impaired energy homeostasis in C/EBPα knockout mice. *Science* **269**, 1108-1112.

Wang, X., Briggs, M.R., Hua, X., Yokoyama, C., Goldstein, J.L. and Brown, M.S. (1993) Nuclear protein that binds sterol regulatory element of low density lipoprotein receptor promoter. *J. Biol. Chem.* **268**, 14497-14504.

Wang, X-Z., Lawson, B., Brewer, J.W., Zinszner, H., Sanjay, A., Mi, L-J., Boorstein, R., Kreibich, G., Hendershot, L.M. and Ron, D. (1996) Signals from the stressed endoplasmic reticulum induce C/EBP-homologous protein (CHOP/GADD153). *Mol. Cell. Biol.* **16**, 4273-4280.

Watanabe, S. and Arai, K-I. (1996) Roles of the JAK-STAT system in signal transduction via cytokine receptors. *Curr. Opin. Genet. Dev.* **6**, 587-596.

Weiner, F.R., Smith, P.J., Wertheimer, S. and Rubin, C.S. (1991) regulation of gene expression by insulin and tumor necrosis factor α in 3T3-L1 cells. *J. Biol. Chem.* **266**, 23525-23528.

Westin, E., Wong-Staal, F., Gelmann, E., Dalla-Favera, R., Papas, T., Lautenberger, J., Allessandra, E., Reddy, E., Tronick, S., Aaronson, S. and Gallo,

R.C. (1982) Expression of cellular homologues of retroviral *onc* genes in human hematopoietic cells. *Proc. Natl. Acad. Sci.* USA **79**, 2490-2494.

Weyman, C.M., Ramocki, M.B., Taparowsky, E.J., and Wolfman, A. (1994) Distinct signaling pathways regulate transformation and inhibition of skeletal muscle differentiation by oncogenic Ras. *Oncogene* **14**, 697-704.

White, E. (1996) Life, death and the pursuit of apoptosis. *Genes Dev.* **10**, 1-15.

Wiese, R.J., Corley-Mastick, C., Lazar, D.F. and Saltiel, A.R. (1995) Activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase is not sufficient for the hormonal stimulation of glucose uptake, lipogenesis, or glycogen synthesis in 3T3-L1 adipocytes. *J. Biol. Chem.* **270**, 3442-3446.

Wille, J.J.Jr. and Scott, R.E. (1982) Topography of the predifferentiation G_D growth arrest state relative to other growth arrest states in the G_1 phase of the cell cycle. *J. Cell. Physiol.* **112**, 115-122.

Winston, L.A. and Hunter, T. (1996) Intracellular signalling: putting JAKs on the kinase MAP. *Curr. Biol.* **6**, 668-671.

Wu, S., Pena, A., Korcz, A., Soprano, D.R. and Soprano, K.J. (1996) Overexpression of Mxi1 inhibits the induction of the human ornithine decarboxylase gene by the Myc/Max protein complex. *Oncogene* **12**, 621-629.

Wu, Z., Bucher, N.L.R. and Farmer, S.R. (1996) Induction of peroxisome proliferator-activated receptor γ during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBPβ, C/EBPδ, and glucocorticoids. *Mol. Cell. Biol.* **16**, 4128-4136.

Wu, Z., Xie, Y., Bucher, N.L.R. and Farmer, S.R. (1995) Conditional ectopic expression of C/EBPβ in NIH-3T3 cells induces PPARγ and stimulates adipogenesis. *Genes Dev.* **9**, 2350-2363.

Wurm, F.M., Gwinn, K.A. and Kingston, R.E. (1986) Inducible overproduction of the mouse c-myc protein in mammalian cells. *Proc. Natl. Acad. Sci. USA* **83**, 5414-5418.

Yang, B-S., Geddes, T.J., Pogulis, R.J., de Crombrugghe, B. and Freytag, S.O. (1991) Transcriptional suppression of cellular gene expression by c-Myc. *Mol. Cell. Biol.* **11**, 2291-2295.

Yang, V.W., Christy, R.J., Cook, J.S., Kelly, T.J. and Lane M.D. (1989) Mechanism of regulation of the 422(aP2) gene by cAMP during preadipocyte differentiation. *Proc. Natl. Acad. Sci. USA* **86**, 3629-3633.

Yeh, W-C., Bierer, B.E. and McKnight, S.L. (1995b) Rapamycin inhibits clonal expansion and adipogenic differentiation of 3T3-L1 cells. *Proc. Natl. Acad. Sci. USA* **92**, 11086-11090.

Yeh, W-C., Cao, Z., Classon, M. and McKnight, S.L. (1995a) Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. *Genes Dev.* **9**, 168-181.

Yeh, W-C., Li, T-K., Bierer, B.E. and McKnight S.L. (1995c) Identification and characterization of an immunophilin expressed during the clonal expansion phase of adipocyte differentiation. *Proc. Natl. Acad. Sci. USA* **92**, 11081-11085.

Yeh, W-C. and McKnight, S.L. (1995) Regulation of adipose maturation and energy homeostasis. *Curr. Opin. Cell Biol.* **7**, 885-890.

Yokoyama, C., Wang, X., Briggs, M.R., Admon, A., Wu, J., Hua, X., Goldstein, J.L. and Brown, M.S. (1993) SREBP-1, a basic-helix-loop-helix-leucine zipper protein that controls transcription of the low density lipoprotein receptor gene. *Cell* **75**, 187-197.

Yokoyama, K. and Imamoto, F. (1987) Transcriptional control of the endogenous MYC protooncogene by antisense RNA. *Proc. Natl. Acad. Sci. USA*. **84**, 7363-7367.

Yu, K., Bayona, W., Kallen, C.B., Harding, H.P., Ravera, C.P., McMahon, G., Brown, M. and Lazar, M.A. (1995) Differential activation of peroxisome proliferator-activated receptors by eicosanoids. *J. Biol. Chem.* **270**, 23975-23983.

Yun, K. and Scott, R.E. (1983) Biological mechanisms of phorbol myristate acetate-induced inhibition of proadipocyte differentiation. *Cancer Res.* **43**, 88-96.

Zacksenhaus, E., Jiang, Z., Chung, D., Marth, J.D., Phillips, R.A. and Gallie, B.L. (1996) pRb controls proliferartion, differentiation, and death of skeletal muscle cells and other lineages during embryogenesis. *Genes Dev.* **10**, 3051-3064.

Zervos, A.S., Gyuris, J. and Brent, R. (1993) Mxi1, a protein that specifically interacts with Max to bind Myc:Max recognition sites. *Cell* **72**, 223-232.

Zezulak, K.M. and Green, H. (1986) The generation of insulin-like growth factor-1-sensitive cells by growth hormone action. *Science* **233**, 551-553.

Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leopold, L. and Friedman, J.M. (1994) Positional cloning of the mouse *obese* gene and its human homologue. *Nature* **372**, 425-432.

Zhu, Y., Alvares, K., Qin, H., Rao, M.S. and Reddy, J.K. (1993) Cloning of a new member of the peroxisome proliferator-activated receptor gene family from mouse liver. *J. Biol. Chem.* **268**, 26817-26820.

Zhu, Y., Qi, C., Korenberg, J.R., Chen, X-N, Noya, D., Rao, M.S. and Reddy, J.K. (1995) Structural organisation of mouse peroxisome proliferator-activated receptor γ (mPPARγ) gene: alternative promoter use and different splicing yield two mPPARγ isoforms. *Proc. Natl. Acad. Sci. USA* **92**, 7921-7925.

Zhuo, S., Fan, S., Huang, S. and Kaufman, S. (1995) Study of the role of retinoblastoma protein in terminal differentiation of murine erythroleukemia cells. *Proc. Natl. Acad. Sci. USA* **92**, 4234-4238.

Zimmerman, K.A., Yancopoulos, G.D., Collum, R.G., Smith, R.K., Kohl, N.E., Denis, K.A., Nau, M.M., Witte, O.N., Toran-Allerand, D., Gee, C.E., Minna, J.D. and Alt, F.W. (1986) Differential expression of *myc* family members during murine development. *Nature* **319**, 780-783.