EFFECTS OF INSULIN ON MURINE LEP EXPRESSION IN ADIPOCYTES

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Leptin is a recently discovered hormone, encoded by the *obese (ob/Lep)* gene, which is expressed predominantly in adipose tissue. It is involved in regulating the size of adipose tissue mass and signalling nutritional status. Insulin has been demonstrated to be among a number of factors that regulate *Lep* mRNA levels *in vivo*, although it remains unclear whether it exerts its effects by a direct, or indirect mechanism(s).

An RNase protection assay was designed to gain further insight into the effects of insulin on the steady-state levels of *Lep* mRNA in adipose tissue, both *in vivo* and *in vitro*. As expected, a subcutaneous injection of insulin into overnight fasted mice significantly increased *Lep* mRNA levels within four hours. However, in an insulin-responsive adipose tissue explant model, a significant change in *Lep* mRNA levels could only be demonstrated at supraphysiological concentrations of insulin.

As an alternative model, reporter-gene plasmids were constructed containing 0.5kb, or 1.0kb of the murine *Lep* gene 5' flanking region, with or without a 200bp segment containing exon 1 and part of intron 1, (p0.5lep-Luc, p0.7lep-Luc, p1.0lep-Luc and p1.2lep-Luc). Transient transfection of p0.5lep-Luc or p0.7lep-Luc, in both 3T3-F442A preadipocytes and adipocytes, significantly repressed luciferase activity compared to a promoterless control, whereas p1.0lep-Luc and p1.2lep-Luc significantly increased luciferase activity. Transient transfection with p1.2lep-Luc elicited a concentration-dependent down-regulation of luciferase activity in response to chronic insulin treatment in preadipocytes, and initial studies showed no significant effect in adipocytes, although a trend towards a concentration-dependent increase was seen.

In conclusion, insulin, administered acutely or chronically, has little or no direct effect on *Lep* mRNA levels, in the murine adipocyte cell models used in this study.
ACKNOWLEDGEMENTS

I would like to thank my supervisors Dr. Torben Lund and Professor Tom Rademacher for all their guidance and support throughout this project. I am especially grateful to Christina Madsen for performing the flow cytometric experiments, and to Dr. Nick Marshall for his assistance and support, particularly regarding the culture of the 3T3-F442A cell line and for the use of his luminometer. Special thanks also to Dr. Martin Payne-Smith, Dr. Suzanne Thomas, Dr. Ian Locke, Dr. Kevin Lawrence and Dr Bhawan Brar for their expert help in various aspects of this study, and to all other members of the lab, especially Debbie, for their assistance and friendship, which made my time there so enjoyable and helped to keep me sane.

Finally, I would like to extend my thanks to my family and friends for all their support and encouragement over the years, most of all to my husband, Alan.

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DECLARATION

All the work presented in this thesis is the work of Katharine Bankhead. Contributions by other researchers are acknowledged in the text.
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ABBREVIATIONS

Ab antibody
ACTH adrenocorticotropic hormone
ADD1/SREBP1 adipocyte determination differentiation dependent factor/sterol regulatory element binding protein 1
AgRP agouti-related protein
Amp' ampicillin resistance gene
α-MSH alpha melanocyte-stimulating hormone
α-MSHR alpha melanocyte-stimulating hormone receptor
AR adrenergic receptor
ATP adenosine triphosphate
BAT brown adipose tissue
BBB blood-brain barrier
BMI body mass index
bp base pairs
BSA bovine serum albumin
°C degrees Celsius
C/EBPα CCAAT/enhancer binding protein
cAMP cyclic 3', 5'-adenosine monophosphate
CART cocaine- and amphetamine-regulated transcript
CCK cholecystokinin
cDNA complementary DNA
CHO Chinese Hamster Ovary
Ci Curie
CNTF ciliary neurotrophic factor
C- carboxy- (COOH-)
CPE carboxypeptidase E
cpm counts per minute
CRH corticotropin-releasing hormone
CSF cerebrospinal fluid
CTP cytidine triphosphate
dATP deoxyadenosine triphosphate
db diabetes (leptin receptor/Lepr) gene
dCTP  deoxyctydine triphosphate
DEPC  diethyl pyrocarbonate
dH2O  distilled water
DHEA-S  dehydroepiandrosterone
DHT  5α-dihydrotestosterone
DMEM  Dulbecco's modified Eagle medium
DMSO  dimethyl sulphoxide
DNA  deoxyribonucleic acid
DNase  deoxyribonuclease
DTT  dithiothreitol
E. coli  Escherichia coli
ECL  enhanced chemiluminescence
EDTA  ethylene diaminetetra-acetic acid
EGF  epidermal growth factor
erlk-  extracellular signal regulated protein kinase-
   fatty
FACS  fluorescence activated cell sorter
FAS  fatty acid synthase
fat  fat gene
FCS  foetal calf serum
FGF  fibroblast growth factor
GAPDH  glyceraldehyde-3-phosphate dehydrogenase
G-CSF  granulocyte colony-stimulating factor
GDP  guanosine diphosphate
GLP-1  glucagon-like peptide
Glut  glucose transporter
GM  granulocyte/macrophage
GnRH  gonadotropin releasing hormone
GPDH  L-glycerol 3-phosphate dehydrogenase
GSK-  glycogen synthase kinase-
GTE  glucose/tris-HCl/EDTA buffer
GTP  guanosine triphosphate
HBS  hepes buffered saline
HBSS  Hank's balanced salt solution
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid</td>
</tr>
<tr>
<td>HI-FCS</td>
<td>heat-inactivated foetal calf serum</td>
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<tr>
<td>hGH</td>
<td>human growth hormone</td>
</tr>
<tr>
<td>HPA</td>
<td>hypothalmic-pituitary-adrenal</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl 1-methylxanthine</td>
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<tr>
<td>I.C.V.</td>
<td>intracerebroventricular</td>
</tr>
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<td>I.P.</td>
<td>intraperitoneal</td>
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<td>IAA</td>
<td>isoamyl alcohol</td>
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<td>IGF-1</td>
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<td>INF-</td>
<td>interferon-</td>
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<td>IPTG</td>
<td>isopropylthio-β-D-galactoside</td>
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<td>IRE</td>
<td>insulin response element</td>
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<td>IRS-</td>
<td>insulin receptor substrate-</td>
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<tr>
<td>Jak</td>
<td>Janus kinase</td>
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<td>kb</td>
<td>kilobase pair</td>
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<td>kDa</td>
<td>kilodalton</td>
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<td>LB-medium</td>
<td>Luria-Bertani medium</td>
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<td>Lep</td>
<td>leptin (obese/ob) gene</td>
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<td>Lepr</td>
<td>leptin receptor (diabetes/db) gene</td>
</tr>
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<td>LH</td>
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<td>LIF</td>
<td>leukaemia inhibitory factor</td>
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<td>LPS</td>
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<tr>
<td>mA</td>
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<td>MAP</td>
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<td>melanocortin-4 receptor</td>
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<tr>
<td>MCH</td>
<td>melanin-concentrating hormone</td>
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<tr>
<td>MEA</td>
<td>mops-EDTA/acetate</td>
</tr>
<tr>
<td>MEKK</td>
<td>MAP/ERK kinase kinase</td>
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<td>MOPS</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>N-</td>
<td>amino- (NH₂-)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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</tr>
<tr>
<td>NIDDM</td>
<td>non-insulin-dependent diabetes-mellitus</td>
</tr>
<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide(s)</td>
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<tr>
<td>NTPs</td>
<td>nucleotide triphosphates</td>
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<td>NTS</td>
<td>nucleus tractus solitarius</td>
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<td>NZO</td>
<td>New Zealand obese</td>
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<td>obese (leptin./Lep) gene</td>
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<td>PPARγ</td>
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<td>PTB</td>
<td>phosphotyrosine binding</td>
</tr>
<tr>
<td>QTL</td>
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<td>Ras</td>
<td>Rous avian sarcoma</td>
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<td>rpm</td>
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<td>RT</td>
<td>reverse transcriptase</td>
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<td>rUTP</td>
<td>uridine 5' -triphosphate</td>
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<td>s.c.</td>
<td>subcutaneous</td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>src homology containing sequence</td>
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<tr>
<td>SHP2</td>
<td>src homology-phosphatase 2</td>
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<td>SNS</td>
<td>sympathetic nervous system</td>
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<td>SOCS</td>
<td>suppressor-of-cytokine-signalling</td>
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<td>SPAP</td>
<td>secretable placental alkaline phosphatase</td>
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<td>sodium chloride/ sodium citrate buffer</td>
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<td>STAT</td>
<td>signal transducer and activator of transcription</td>
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<td>Taq</td>
<td><em>Thermus aquaticus</em></td>
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<tr>
<td>TBE</td>
<td>tris-borate/EDTA</td>
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<tr>
<td>TE</td>
<td>tris/EDTA</td>
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<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetra methyl ethylenediamine</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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<tr>
<td>TH</td>
<td>thyroid hormone</td>
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<tr>
<td>TNF-α</td>
<td>tumour necrosis factor alpha</td>
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<tr>
<td>TRH</td>
<td>thyroid releasing hormone</td>
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<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
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<tr>
<td>tRNA</td>
<td>transfer ribonucleic acid</td>
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<tr>
<td>TSH</td>
<td>thyroid stimulating hormone</td>
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<tr>
<td>tub</td>
<td><em>tubby</em> gene</td>
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<tr>
<td>Tween 20</td>
<td>polyoxyethylene sorbitan monolaurate</td>
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<tr>
<td>TZDs</td>
<td>thiazolidinediones</td>
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<td>UCP</td>
<td>uncoupling protein</td>
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<td>USF</td>
<td>upstream nuclear factor</td>
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<td>U.V.</td>
<td>ultraviolet</td>
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V : volt
VMH : ventromedial hypothalamus
v/v : volume by volume
WAT : white adipose tissue
w/v : weight by volume
X-Gal : 5-Bromo-4-chloro-3-indolyl-β-D-galactoside
1 INTRODUCTION
1.1 The Control of Body Weight - Historical Background

There is considerable interest in determining the mechanisms involved in the control of body weight and energy homeostasis, especially with regard to the investigation and treatment of disorders such as anorexia nervosa and obesity. The latter for instance, is associated with a number of diseases, including type II diabetes (non-insulin dependent diabetes mellitus - NIDDM), hypertension, dyslipidaemia, cardiovascular disease, osteoarthritis and some cancers, as well as lower life expectancy (Pi-Sunyer, 1993). Furthermore obesity is rapidly increasing in prevalence (Kopelman, 2000), to such an extent that the World Health Organization has declared it a global epidemic.

Energy homeostasis can be defined as the balance between food intake and energy expenditure, and it is clear that under normal circumstances this balance is robustly controlled. Considerable resistance to short-term changes in body weight has been demonstrated, both in rodents (Cohn and Joseph, 1962; Harris et al., 1986), and in humans (Leibel et al., 1995; Sims and Horton, 1968), whereby conditions of forced under- or over-feeding are accompanied by appropriate compensatory changes in appetite and energy expenditure to regain the original body composition. In the long term, it has been calculated that the 11kg gained over forty years by an average adult female corresponds to an error of a mere 350mg of food per day, despite a total food intake of twenty tons during this time (Hervey, 1969). Studies such as these have led to the set-point theory of body-weight, whereby it is postulated that individuals have a predetermined, preferred body weight, which is maintained through a feedback control mechanism (reviewed in Harris, 1990). This theory may help to explain the failure of many long-term weight loss programs (Leibel et al., 1995), and is consistent with the mounting evidence that body weight is a highly heritable trait (Bouchard and Perusse, 1988; Stunkard et al., 1990).

Important control centres for energy homeostasis are known to reside in the hypothalamic region of the brain. As long ago as 1840, an association between obesity and hypothalamic injury was described (reviewed in Bray, 1979). Further research, including studies of tumours, surgery, and chemically- and electrolytically-induced lesions within this region, has revealed that the hypothalamus contains centres of major importance with respect to hunger and food seeking behaviour, as well as in the termination of feeding (reviewed in Bray, 1979).
The feedback mechanism of set-point theory is proposed to comprise of an afferent signalling molecule that reports body weight status to the hypothalamus, which then mediates efferent signals that modulate food intake and energy expenditure. The existence of such a circulating satiety factor, signalling to the hypothalamus, was postulated following a series of key parabiosis experiments in rodents. In these studies the circulatory systems of two animals are connected to allow the exchange of humoral factors. Initial studies using ventromedial hypothalamic (VMH)-lesioned hyperphagic, obese rats, parabiosed with control, lean rats resulted in the death of the lean rat, apparently by starvation (Hervey, 1959). This was interpreted as the presence of an excessive amount of a satiety factor produced by the resistant VMH-lesioned rat, causing inappropriate satiety in the lean animal. Similar results were obtained when the genetically obese, db/db (diabetes) mouse was parabiosed with a lean counterpart; implying that this genetic model of obesity was similarly resistant to the effects of the satiety factor, and hence might have defects in its hypothalamic signalling pathway (Coleman and Hummel, 1969). Conversely, another genetic model of obesity, the ob/ob (obese) mouse, when parabiosed with normal, lean controls, suppressed its usually excessive weight gain (Coleman, 1973; Hausberger, 1958). It was postulated therefore, that this obese model lacked the circulating satiety factor, which, when supplied by the normal animal during parabiosis, normalized feeding behaviour in the ob/ob mouse.

1.2 Rodent Models of Obesity

The recent elucidation of the defects that cause obesity in several spontaneously obese rodent models, especially the monogenic mouse models of agouti, fat, tubby, obese and diabetes, has been instrumental in further advancing our understanding of the physiological mechanisms involved in the control of body weight.

Novel signalling pathways, such as the central melanocortin and leptin systems have been discovered, which have opened up whole new fields of research and have identified novel targets for drug intervention. A brief summary of the aetiology of these models is described below.

1.2.1 The agouti gene

Several mutations occur at the murine agouti locus. Heterozygous mice carrying the dominant allele, lethal yellow (A^l), have a phenotype which includes obesity, all-yellow
coat colour, hyperinsulinaemia, insulin resistant hyperglycaemia, increased somatic growth and increased tumour susceptibility, (reviewed in Herberg and Coleman, 1977). The wild-type *agouti* gene encodes a novel 131-amino-acid protein with a consensus signal peptide. The *A^y* phenotype is the result of a chromosomal rearrangement producing a chimeric gene that is expressed in an unregulated manner, in almost all tissues (Bultman et al., 1992). The *agouti* protein (Agouti) ordinarily functions in melanocytes to antagonize the interaction between alpha melanocyte-stimulating hormone (α-MSH) and its receptor (α-MSHR, or MC1-R (melanocortin-1 receptor)), so influencing hair colouration (Lu et al., 1994). In addition, it has also been shown to be an antagonist of the melanocortin-4 (MC4) receptor, a related MSH-binding receptor expressed in the brain (although ordinarily its tissue-specificity prevents this interaction) (Lu et al., 1994). Targeted deletion of the MC4 receptor in mice results in a syndrome with many of the characteristics of the *agouti* obesity syndrome. This suggests that antagonism of the MC4 receptor by the ubiquitously expressed Agouti in the *A^y* mouse is the primary cause of obesity (Huszar et al., 1997). This finding, and the discovery of the endogenous antagonist for central MC receptors – the *agouti*-related protein (AgRP) (Ollmann et al., 1997), has led to the elucidation of an important pathway in the regulation of energy homeostasis both in rodents and humans, known as the central melanocortin system (reviewed in Cone, 1999). Cases of human obesity, caused by mutations in the genes involved in the central melanocortin system have been identified, and are in fact, not uncommon in the morbidly obese population. For example, mutations in MC4R (Farooqi et al., 2000; Hinney et al., 1999; Vaisse et al., 2000; Vaisse et al., 1998; Yeo et al., 1998) and pro-opiomelanocortin (POMC - the precursor to α-MSH and other anorexigenic peptides) (Krude et al., 1998) have all been detected.

1.2.2 The *fat* gene

Homozygous *fat/fat* mice develop hyperinsulinaemia, hyperproinsulinaemia and late onset obesity (Coleman and Eicher, 1990). The *fat* mutation has been shown to be a mutation in the gene encoding carboxypeptidase E (CPE), a prehormone-processing enzyme. The single Ser202Pro *fat* mutation results in lack of carboxypeptidase E enzymatic activity in pancreatic islets and the pituitary (Naggert et al., 1995), and has been shown to be causes by an altered, destabilized structure, which causes the protein to be rapidly degraded instead of secreted (Varlamov et al., 1996). The mechanism by
which this loss in activity leads to obesity is still unresolved, however it is thought that lack of CPE activity causes prohormones, such as POMC, to fail to be sorted into secretory vesicles to be correctly processed (Cool et al., 1997). Wild-type CPE is also responsible for processing Substance P (Perloff et al., 1998) and cholecystokinin 8 (CCK 8) (Cain et al., 1997) in the brain. There is speculation that the lower levels of these fully processed peptides in the brains of fatfat mice may also contribute to the obesity syndrome, (reviewed in Weigle and Kuijper, 1996). Cpe defects in humans have yet to be identified, although a similar syndrome caused by prohormone convertase 1 (PC-1) deficiency has been reported in one individual (Jackson et al., 1997).

1.2.3 The tubby (tub) gene

Mice homozygous for the tubby (tub) mutation develop late-onset obesity, insulin resistance, retinal degeneration and neurosensory hearing loss (Coleman and Eicher, 1990). The wild-type gene gives rise to a 6.3kb transcript, expressed predominantly in eye, testis and brain (particularly hippocampus, hypothalamus and cortex). The product is thought to be a membrane bound regulator of transcription, involved in the maintenance and function of neuronal cells (Santagata et al., 2001). The tub mutation is a G→T transversion in a donor splice site, which results in the inclusion of an intron in the transcript. It is thought that the mutation leads to obesity through apoptosis in, and gradual loss of function of, hypothalamic nuclei (Kleyn et al., 1996; Noben-Trauth et al., 1996) and review (Weigle and Kuijper, 1996).

1.2.4 The obese (ob/Lep) and diabetes (db/Lepr) genes

These two most characterized models of obesity have almost identical phenotypes including infertility, early-onset severe obesity, insulin resistance, hyperinsulinaemia, hyperglycaemia, decreased activity and cold intolerance. (Bray and York, 1979; Herberg and Coleman, 1977). Due to such striking phenotypic similarity, it was proposed that the two genes involved must be related in some way. Indeed, the parabiosis studies discussed earlier, suggested that obese mice (ob/ob) lacked a blood-borne signalling molecule, and diabetes mice (db/db) were unable to respond to this molecule (Coleman, 1973). The positional cloning of these two genes verified this early work, and it has been subsequently shown that the wild-type obese (ob) gene encodes the hormone leptin, and the wild-type diabetes gene (db) encodes the leptin receptor. Since the discovery of
leptin, it has become convention to refer to the ob gene as Lep, and the db gene as Lepr. The leptin receptor is referred to as LEPR. This convention has been observed for the remainder of this study. Additionally, the ob/ob mouse is now conventionally referred to as Lep\textsuperscript{ob}/Lep\textsuperscript{ob} and the db/db mouse, Lepr\textsuperscript{db}/Lepr\textsuperscript{db}. However, for simplicity these obese models will still be referred to as ob/ob and db/db in this study.

Both these genes and their encoded proteins are described in more detail in section 1.3.

1.2.5 OTHER MODELS

Other rodent models include the fatty (fa/fa) and Koletsky f/f rat obesity syndromes show similar characteristics to the db/db mouse model (Bray and York, 1979). db, fa and f map to syntenic chromosome regions (Truett et al., 1991; Yen et al., 1977), and like the db/db mouse, fa/fa and f/f rats possess mutated leptin receptors (Iida et al., 1996a; Phillips et al., 1996; Takaya et al., 1996).

Polygenic rodent models of obesity also exist, and include the BSB (Warden et al., 1993) and New Zealand obese (NZO) (Bielschowsky and Bielschowsky, 1953), mouse strains, as well as a dietary-induced model derived from a cross between the dietary-fat sensitive AKR/J mouse strain and the dietary-fat resistant SWR/J strain (West et al., 1994). Quantitative trait loci (QTL) analysis of these mouse models is yielding new candidate genes which contribute to the obese phenotype, (reviewed in Chagnon and Bouchard, 1996; and Fisler and Warden, 1997).

Additionally, transgenic and knock-out mouse models that show modified body fat content are also being created, and are adding to our knowledge of the diverse systems involved in controlling body weight homeostasis, (reviewed in Chagnon and Bouchard, 1996; and Robinson et al, 2000).

1.3 CLONING AND CHARACTERIZATION OF LEPTIN AND THE LEPTIN RECEPTOR

1.3.1 CLONING OF LEPTIN

The obese (ob/Lep) gene, cloned in mouse and human in 1994 (Zhang et al., 1994), is a unique gene with no significant homology to any other sequences. Lep encodes a 167 amino acid, 16kDa secreted protein termed leptin, from the Greek word leptos, meaning thin. It is expressed primarily in adipocytes (Maffei et al., 1995a; Zhang et al., 1994), and to a lesser extent in placenta (Masuzaki et al., 1997), gastric epithelium (Bado et al.,
1998) and skeletal muscle (Wang et al., 1998). The gene is highly conserved throughout evolution, and there is an 84% identity between the mouse and human amino acid sequence (Zhang et al., 1994).

Two naturally occurring cDNA variants have been isolated both in mouse and human. They differ by the inclusion of a glutamine at codon 49, immediately 3’ to a splice acceptor. The glutamine codon (CAG) includes a possible AG splice-acceptor, suggesting slippage in splicing occurred. The percentage of isolated cDNAs missing glutamine at codon 49 is 30% in both mouse and human (Zhang et al., 1994). The significance of this alternate splice variant is unknown.

**1.3.2 Mutations in the Lep Gene**

Two separate mutations resulting in the ob/ob phenotype have been identified. The original C57BL/6J ob/ob mouse is characterized by a C→T transversion mutation in the first base of codon 105 of the Lep gene. This causes a change from arginine to a stop codon and results in the formation of a truncated, non-functional protein product. The other mutation, in the strain SM/CKc+Dac ob21/ob21, was initially proposed to affect the promoter region of the Lep gene, due to the fact that a restriction length polymorphism exists within this region, and that no detectable RNA is expressed, (Zhang et al., 1994). It is now known that the mutation is the result of the insertion of a retroviral-like transposon in the first intron of the Lep gene. Aberrant splicing occurs, and as a consequence no mature mRNA is produced (Moon and Friedman, 1997).

Mutations in the human Lep gene are rare. Early analysis, including single-strand conformation polymorphism screening on large samples of obese individuals from several different ethnic origins, failed to show any mutations in the coding region of the human Lep gene (Maffei et al., 1996; Niki et al., 1996). However, linkage analysis has indicated an association between extreme obesity and markers close to the Lep gene locus - although this of course does not rule out the possibility of other genes in the region being responsible (Clement et al., 1996; Reed et al., 1996). To date only five obese individuals from just two families have been shown to be homozygous for loss-of-function mutations in the coding region of the Lep gene. These patients exhibit many of the characteristics displayed by the ob/ob mouse, including hyperphagia, elevated plasma-insulin concentrations, low sympathetic tone and impaired reproductive
function. Cortisol levels and body temperature are normal however (Montague et al., 1997; Strobel et al., 1998). These data have led to the conclusion that human obesity is not usually caused by defects in the production of leptin, in fact most cases of human obesity are characterized by an increase in leptin production, indicating a resistance to the presumed effects of leptin (see section 1.5).

1.3.3 Structure of Leptin

The N-terminal signal and amino acid sequences of leptin from mouse, rat, human, *Sminthopsis crassicaudata* (a marsupial), and chicken are illustrated in Figure 1.1, alongside a depiction of its secondary structure. The areas of high homology lie in the regions of the predicted alpha helices. The glutamine 49 residue, absent in 30% of all human and mouse cDNAs is shown highlighted in red. Note the substitution of a tyrosine for glutamine 49 in the marsupial, and the complete absence of codon 49 in the chicken published sequence. Arginine 105, mutated in the original C57BL/6J *ob/ob* mouse model, is highlighted in yellow and the resulting truncation of helix C is also illustrated.

A search of a 3-dimensional structure database suggested that leptin shared homology with the helical cytokine family (Madej et al., 1995). This was verified when the crystal structure was deduced from a recombinant form of human leptin (leptin E100), (Zhang et al., 1997). The structure revealed a four-helix bundle protein with significant homology to members of the long-chain helical cytokine family, including granulocyte colony-stimulating factor (G-CSF), leukaemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF) and human growth hormone (hGH). Figure 1.2 shows a ribbon diagram representation of human leptin. The N- and C-termini have been labelled, as have the alpha-helices. Helical cytokines signal through receptors of the gp130 class, using members of the Janus kinase/Signal Transducer and Activator of Transcription (Jak/STAT) pathway. It was therefore hypothesized that leptin would also mediate its signal by this process (Madej et al., 1995). The cloning of the leptin receptor verified this hypothesis (see section 1.3.4). Leptin has a number of diverse functions, which are mediated by signalling through the leptin receptor. A brief description of the leptin receptor and its signalling capabilities are therefore presented, before the function and regulation of leptin is detailed.
Figure 1.1  Sequence alignment of leptin from various species

Schematic diagram of the sequence alignment and secondary structure of leptin from both wild-type and C57B1/6J ob/ob mice, and other species, showing a high degree of homology between species. Alpha-helices labelled A-E. Boxes indicate highly conserved regions, letters in red show variants within these regions. Grey shaded areas show other invariant sequences. Red shaded area indicates glutamine codon 49. Yellow shaded letter indicates arginine codon 105 in helix C.

Protein sequences were converted from the NCBI Entrez Nucleotides database sequences (U18812, D45862, D63710, AF159713 and AF012727 respectively). Adapted from Zhang et al., 1997.
Figure 1.2 Ribbon diagram of human leptin-E100

Ribbon diagram of a recombinant form of human leptin, showing labelled alpha helices (A-E). Adapted from Zhang et al., 1997.
1.3.4 CLONING OF LEPTIN RECEPTOR

The leptin receptor (LEPR) is a single membrane-spanning receptor of the class I cytokine receptor family, most related to the gp130 signal-transducing component of the interleukin-6 (IL-6), G-CSF, and LIF receptor. It was identified by binding studies in mouse choroid plexus and was cloned from a choroid plexus expression library (Tartaglia et al., 1995). Five murine splice-variant isoforms are known to be expressed, four of which differ in the lengths of their cytoplasmic tails (LEPR-a – LEPR-d). The fifth lacks the transmembrane domain and is predicted to encode a soluble receptor, known as LEPR-e (Lee et al., 1996) (see Figure 1.3).

LEPR-a and LEPR-b, known as the short and long forms respectively, are highly conserved between mouse and human (Lee et al., 1996). LEPR-b possesses two putative Jak binding domains, and is the main signalling isoform of the receptor (see section 1.3.6), (Baumann et al., 1996; Lee et al., 1996). Roles for the alternate receptor isoforms are discussed in section 1.3.7. Figure 1.3 shows a diagrammatical depiction of the various isoforms of the murine leptin receptor including the conserved extracellular domain, transmembrane domain and intracellular domain containing putative Jak binding domain motifs. Also shown is the truncated LEPR-b receptor encoded by the \(db/db\) mouse (see section 1.3.5).

The receptor is present in a wide range of tissues in an isoform-specific manner. LEPR-b is predominantly expressed in various parts of the hypothalamus, including the arcuate, ventromedial, paraventricular and ventral premammillary nuclei (Mercer et al., 1996). This is consistent with the proposed centrally mediated effects of leptin. Other areas of the brain expressing this long-form receptor include the cortex, hippocampus, and thalamus. LEPR-b is also expressed in tissues of the reproductive system (placenta, uterus, ovary), (Ghilardi et al., 1996; Kawai et al., 1999), tissues and cells of the immune system (lymph nodes, spleen, CD4+ T cells), (Ghilardi et al., 1996; Lord et al., 1998), and others (pancreatic beta islets, heart, kidney, liver, skin and jejunum) (Emilsson et al., 1997; Fei et al., 1997; Frank et al., 2000; Morton et al., 1998). This may have implications in the direct effects of leptin in these tissues. LEPR-a is abundantly expressed in most tissues tested to date, including kidney, lung, pancreas, adipose, liver, hypothalamus, choroid plexus, ovary and uterus (Ghilardi et al., 1996).
The other splice isoforms are less well characterized, but are not highly expressed (Fei et al., 1997).

1.3.5 Mutations in the Lepr Gene

The C57Bl/KsJ db/db mouse possesses a mutation in the leptin receptor gene (Chen et al., 1996; Lee et al., 1996) consistent with the earlier hypothesis by Coleman et al that this mouse model is unable to respond to the blood-borne molecule that is absent in ob/ob mice (Coleman, 1973), (see section 1.1). The Lepr mutation is a G→T mutation residing in an intronic sequence which forms a consensus splice donor site. As a consequence, the exon unique to LEPR-a is inserted, resulting in the formation of the LEPR-a receptor isoform instead of LEPR-b (Chen et al., 1996; Lee et al., 1996), (see Figure 1.3). The other splice variants are unaffected by the mutation in the db/db mouse indicating that the LEPR-b splice variant is the critical isoform for leptin function in energy homeostasis.

Mutations in the human Lepr gene have been identified in members of just one family. Homozygous individuals exhibit early-onset obesity and fail to enter puberty. A G→A substitution occurs in the splice donor site of exon 16 resulting in an mRNA that lacks this exon. The coding sequence for the entire transmembrane and intracellular domains and part of the extracellular domain of the receptor are consequently missing (Clement et al., 1998). Unlike the great similarities between the mouse ob/ob and db/db syndromes, mutation in the human leptin receptor results in the additional defects of growth retardation and hypothyroidism which are absent in patients with human leptin mutations. Population-based surveys have shown little (Chagnon et al., 1997; Chagnon et al., 2000), or no association between human leptin receptor DNA sequence variants and adiposity (Francke et al., 1997; Norman et al., 1996), suggesting, as with the case of leptin, that human obesity is not commonly caused by defects in this gene.
Figure 1.3  Splice isoforms of the murine leptin receptor

Schematic diagram of the murine leptin receptor isoforms; • denotes a consensus stop codon; * indicates the G→T mutation in db/db mice; Box 1 and Box 2 (red boxes) are putative motifs for JAK binding domains; TM (yellow box) is a putative motif for a transmembrane domain. Adapted from Lee et al., 1996.
1.3.6 **LEPTIN RECEPTOR SIGNAL TRANSDUCTION**

Leptin binds to the leptin receptor with nanomolar affinity (Tartaglia et al., 1995). Both leptin-dependent (Nakashima et al., 1997; White et al., 1997), and leptin-independent (Devos et al., 1997; White and Tartaglia, 1999) homodimerization of the leptin receptor has been observed, and there is evidence that oligomerization can also occur (Devos et al., 1997). Leptin-dependent heterodimerization between LEPR-a and LEPR-b has also been noted, albeit at a low level (White and Tartaglia, 1999). Other members of this receptor family mediate their signal through the Jak/STAT pathway (reviewed in Ihle, 1995). The long-form leptin receptor contains two putative Jak binding domains and a putative binding motif for STAT3 (Lee et al., 1996; Stahl et al., 1995). In co-transfection studies, LEPR-b activates exogenous Jak2 (Ghilardi and Skoda, 1997), STAT-3, -5 and -6 (Ghilardi et al., 1996), STAT-1, -3 and -5B (Baumann et al., 1996), and induces transcription of reporter genes containing IL-6 responsive elements (Baumann et al., 1996). More conclusively, endogenous STATs are also activated in vitro, in brown and white adipose tissue (Siegrist-Kaiser et al., 1997), and specifically, STAT3 is activated in oocytes (Matsuoka et al., 1999), pancreatic islets, and the pancreatic β-cell line RINm5F (Morton et al., 1999).

**In vivo**, in ob/ob and normal mice, but not db/db mice, leptin administration induces STAT3 activation in the hypothalamus (Vaisse et al., 1996). Additionally in the periphery, activation of STAT1 in brown adipose tissue (Siegrist-Kaiser et al., 1997), and STAT5 DNA binding activity in the jejunum (Morton et al., 1998) has been observed. LEPR-a, the short form receptor, which replaces LEPR-b in db/db mice is unable to activate the STAT pathway (Baumann et al., 1996; Ghilardi et al., 1996).

Evidence now exists to show that both the long and short form leptin receptors have signalling capabilities through the box 1 Jak binding domain in vitro (see Figure 1.3). This involves activation of Jak2 and the MAP (mitogen-activated protein) kinase pathway. Signal transduction through the LEPR-a receptor is notably weaker than that observed for LEPR-b, and in contrast to LEPR-b does not go on to activate STAT3 (Bjorbaek et al., 1997). Murakami et al have also reported that Chinese Hamster Ovary (CHO) cells expressing LEPR-a can mediate the expression of immediate early genes in
vitro in response to leptin (Murakami et al., 1997). The functional role of LEPR-a in vivo is currently unknown, although several are postulated (see section 1.3.7).

The intracellular molecules, SHP-2 (src homology-phosphatase 2) and SOCS-3 (suppressor-of-cytokine-signalling) have been implicated in leptin-mediated signal transduction, and may be involved in negatively regulating leptin signalling (Bjorbaek et al., 1998a; Carpenter et al., 1998) thus contributing to leptin resistance (see section 1.5).

1.3.7 ADDITIONAL ROLES OF THE LEPTIN RECEPTOR

In addition to mediating the central and peripheral effects of leptin (see section 1.4), it is thought that the leptin receptor also plays an important role in leptin transport and clearance.

The soluble LEPR-e leptin receptor isoform may function as a plasma leptin-binding protein. Several leptin-binding proteins have been isolated from serum. One of these, based on the protein’s molecular mass and anti-leptin receptor antibody specificity, is hypothesized to be LEPR-e (Li et al., 1998; Sinha et al., 1996b). This protein is absent in db^Pas/db^Pas mice, the leptin receptor of which is truncated at amino acid 281, abating expression of all receptor isoforms. Furthermore, the soluble receptor when exogenously expressed, binds as a dimer to both mouse and human leptin with high affinity (Liu et al., 1997). Distinct roles for the free and bound forms of leptin are being elucidated, perhaps being involved in limiting the action of leptin by the inhibition of binding to the full-length membrane-bound receptor (Brabant et al., 2000; Liu et al., 1997).

Transport of leptin across the blood-brain barrier (BBB) has also been suggested to be attributable to the leptin receptor. In vitro and in vivo studies demonstrate that the transport of leptin across the BBB is a specific, saturable and temperature-dependent process, (Banks et al., 1996; Golden et al., 1997), probably occurring in brain microvessels (Golden et al., 1997) and the epithelium of the choroid plexus (Devos et al., 1996; Lynn et al., 1996). The leptin transporter has been suggested to be LEPR-a, the short-form receptor, which is expressed at extremely high levels at the BBB (Bjorbaek et al., 1998b). Indeed LEPR-a has been demonstrated to perform this task in an in vitro model system (Hileman et al., 2000). However, the CSF leptin levels in Koletsky (f/f) obese rats (which do not express detectable levels of any of the leptin
receptor isoforms) are comparable to levels seen in lean controls. A LEPR independent transport mechanism is therefore also likely to exist (Wu-Peng et al., 1997).

Clearance of leptin from the plasma may also involve the leptin receptor. LEPR isoforms mediate the internalization and lysosomal degradation of leptin via a coated pit-dependent mechanism in vitro (Barr et al., 1999; Uotani et al., 1999). As leptin receptors are present in the kidney, a role for the leptin receptor in the renal clearance of leptin (see section 1.6.9) has also been suggested, although evidence to support this has yet to be reported.

1.4 THE ROLE OF LEPTIN

Leptin serves a variety of functions in mammalian physiology. It is primarily involved in the control of food intake and energy expenditure, but also has important roles in reproduction, and the immune response as well as other functions such as mitogenesis and angiogenesis. Evidence suggests that some of these roles have evolved as part of the neuroendocrine response to starvation (Ahima et al., 1996). Leptin's effects on these systems are depicted diagrammatically in Figure 1.4 and Figure 1.5, and are discussed below.

1.4.1 ROLE IN ENERGY HOMEOSTASIS

Leptin’s main role involves the regulation of body fat content by modulating food intake and energy expenditure. Daily intraperitoneal (I.P.) administration of recombinant leptin to ob/ob mice decreases food intake and normalizes body fat content in a dose-dependent manner. Other metabolic defects in the ob/ob mouse are also rectified, including an increase in body temperature, metabolic rate and locomotor activity, and a decrease in serum concentrations of glucose and insulin (Campfield et al., 1995; Halaas et al., 1995; Pellemounter et al., 1995; Stephens et al., 1995; Weigle et al., 1995). Central (intracerebroventricular (I.C.V.)) administration of leptin to the lateral ventricle (Campfield et al., 1995; Hwa et al., 1996) or third ventricle of the brain (Stephens et al., 1995), produces even more potent effects, in keeping with the hypothesis that leptin exerts most of its effects centrally.

The reduction of body fat resulting from leptin therapy is not solely due to a dramatic reduction in food intake, as control pair-fed animals fail to lose a similar amount of weight (Halaas et al., 1995). Nor are the reductions in blood glucose, insulin, and levels
of the hypothalamic orexigen, neuropeptide Y (NPY) following leptin administration to rodents primarily a consequence of weight loss. These reductions have been shown to occur before a significant loss in body fat content, and are more pronounced than the decreases found in control, pair-fed mice (Pelleymounter et al., 1995; Schwartz et al., 1996a; Stephens et al., 1995).

Leptin therapy in a patient with congenital leptin deficiency has also resulted in a dramatic reduction in food intake and body fat content (Farooqi et al., 1999) providing evidence for similar physiological roles of leptin in both rodents and humans.

At physiological concentrations, leptin also functions in normal, lean mice to reduce body fat mass, in a dose-dependent manner (Halaas et al., 1997). However, leptin treatment does not decrease food intake or correct other metabolic defects in db/db mice, consistent with the defect in the leptin receptor and resistance to the effects of leptin (Campfield et al., 1995; Halaas et al., 1995). In fact, leptin resistance is observed in most cases of rodent (Halaas et al., 1997), and human obesity (Heymsfield et al., 1999), (see section 1.5).

The mechanisms by which leptin acts to decrease food intake, increase energy expenditure and alter glucose and lipid metabolism are becoming elucidated, and they involve several pathways acting in parallel. A summary of some of the effects mediated by leptin that contribute to these overall physiological changes are detailed in the following sections.

1.4.1.1 FOOD INTAKE

It is known that leptin can interact with, and alter the expression of, several neuropeptides known to have orexigenic or anorexigenic effects, thereby affecting appetite. For instance, the orexigenic molecule NPY potently stimulates food intake and lowers energy expenditure (Billington et al., 1991; Stanley et al., 1986). Leptin has been shown to decrease the elevated levels of NPY in the hypothalamic arcuate nucleus of ob/ob mice in vivo and in vitro (Schwartz et al., 1996a; Stephens et al., 1995). Furthermore, double knockout, NPY^{+/−} ob/ob mice are less obese than ob/ob controls, exhibiting reduced food intake and increased energy expenditure, suggesting that NPY is an important effector molecule in leptin signalling (Erickson et al., 1996).
### Neuropeptide Y (NPY)
- **Site of Expression**: Arcuate Nucleus
- **Principle Receptor(s)**: Y1/Y5 in the PVN
- **Effects of**: Increases food intake, reduces SNS outflow to BAT, leads to obesity
- **Levels in Rodent Models of Obesity**: Increased expression in ob/ob and db/db mice. Attenuated by leptin administration in ob/ob mice
- **Levels in Fasting Rodents**: Increased leptin and insulin administration attenuates this
- **Transgenic Knock-Out Models**: NPY knockout (KO) mice exhibit normal food intake and body weight. Double mutant ob/ob, NPY KO mice exhibit a less severe obese phenotype than ob/ob however.

### Melanin-Concentrating Hormone (MCH)
- **Site of Expression**: Lateral Hypothalamus
- **Principle Receptor(s)**: MCH-R (SLC-1) in the hypothalamus
- **Effects of**: Transiently and moderately increases food intake compared to NPY, does not lead to obesity
- **Levels in Rodent Models of Obesity**: Increased expression in ob/ob mice
- **Levels in Fasting Rodents**: Mildly increased leptin administration attenuates this
- **Transgenic Knock-Out Models**: MCH KO mice are mildly anorexic and have an inappropriately increased metabolic rate, despite reduced amounts of both leptin and arcuate nucleus POMC mRNA.

### Agouti-related peptide (AgRP)
- **Site of Expression**: Arcuate Nucleus
- **Principle Receptor(s)**: MC3R and MC4R in the hypothalamus
- **Effects of**: Increases food intake and body weight
- **Levels in Rodent Models of Obesity**: Increased expression in ob/ob and db/db mice. Attenuated by leptin administration in ob/ob mice
- **Levels in Fasting Rodents**: Increased leptin administration attenuates this
- **Transgenic Knock-Out Models**: Agrp KO mice exhibit normal food intake and body weight.

### a-MSH (derived from POMC)
- **Site of Expression**: Arcuate Nucleus
- **Principle Receptor(s)**: MC3R and MC4R in the hypothalamus
- **Effects of**: Moderately decreases food intake and body weight
- **Levels in Rodent Models of Obesity**: Decreased POMC expression in ob/ob and db/db mice. Increased by leptin administration in ob/ob mice
- **Levels in Fasting Rodents**: POMC decreased
- **Transgenic Knock-Out Models**: Pomc KO mice develop hyperphagia and obesity.

### CRH
- **Site of Expression**: PVN
- **Principle Receptor(s)**: CRH 2α R in the hypothalamus
- **Effects of**: Decreases food intake and body weight
- **Levels in Rodent Models of Obesity**: Decreased POMC expression in ob/ob mice. Increased by leptin administration in ob/ob mice
- **Levels in Fasting Rodents**: Decreased leptin administration attenuates this
- **Transgenic Knock-Out Models**: CRH KO mice exhibit normal food intake and body weight.

### CART
- **Site of Expression**: Arcuate Nucleus and PVN
- **Principle Receptor(s)**: 
- **Effects of**: Decreases food intake and body weight
- **Levels in Rodent Models of Obesity**: Decreased POMC expression in ob/ob. Increased by leptin administration in ob/ob mice
- **Levels in Fasting Rodents**: Decreased leptin administration attenuates this
- **Transgenic Knock-Out Models**: ?

### Table 1.1 Principle neuropeptides involved in appetite regulation

*(after Kalra et al., 1999; Spiegelman and Flier, 2001)*
mRNA levels of the orexigenic antagonist of central melanocortin receptors, agouti-related protein (AgRP), are similarly decreased by leptin in the arcuate nucleus (Mizuno and Mobbs, 1999; Wilson et al., 1999), as are levels of melanin-concentrating hormone (MCH) and galanin in other areas of the hypothalamus (Huang et al., 1999; Sahu, 1998).

Leptin also modulates the anorexigenic arm of the central melanocortin pathway by restoring levels of POMC mRNA in the arcuate nucleus of fasted normal or ob/ob mice (Schwartz et al., 1997; Thornton et al., 1997). Other hypothalamic anorexigenes, are also regulated by leptin. Corticotropin-releasing hormone (CRH) mRNA and protein production, as well as cocaine- and amphetamine-regulated transcript (CART) mRNA, and glucagon like peptide-1 (GLP-1) are also increased in response to leptin in fasted rodents and ob/ob mice (Goldstone et al., 2000; Kristensen et al., 1998; Schwartz et al., 1996c; Uehara et al., 1998). The principle orexigenic and anorexigenic neuropeptides are summarised in Table 1.1. Leptin's modulation of food intake is not solely restricted to the hypothalamic region of the brain. Recent findings implicate it in potentiating the effects of the satiety signal cholecystokinin (CCK) in the nucleus tractus solitarius (NTS) of the hindbrain (Emond et al., 1999), and it has been shown to modulate the serotonin signalling pathway located in the caudal brain stem (Calapai et al., 1999), (also reviewed in Kalra et al., 1999; and Spiegelman and Flier, 2001).

1.4.1.2 ENERGY EXPENDITURE

Leptin's role in modulating energy expenditure appears to be mediated by β3-adrenergic receptors on brown and white adipocytes, increasing thermogenesis and lipolysis (reviewed in Giacobino, 1996). In ob/ob mice, leptin administration raises core temperature and metabolic rate (Hwa et al., 1996), and in rats it increases peripheral norepinephrine turnover in brown adipose tissue, leading to an increase in thermogenesis (Collins et al., 1996), as well as increasing mRNA levels for uncoupling proteins (UCP-1, UCP-2 and UCP-3) in brown adipose tissue (Scarpace et al., 1997; Scarpace et al., 1998). Leptin is also capable of increasing sympathetic outflow and raising plasma levels of catecholamines (Haynes et al., 1997; Satoh et al., 1999), and this is apparently centrally-mediated through the VMH (Satoh et al., 1999).

Thyroid hormone (TH) has also been implicated in mediating the central effects of leptin on energy expenditure. Administration of TH increases thermogenesis in many tissues, whereas thyroidectomy in rats leads to a reduction in basal metabolic rate. The effects of
TH are partly mediated by β-adrenergic receptors (Silva, 1995). Fasting reduces TH levels primarily through reducing the expression of thyroid releasing hormone (TRH) in the PVN, and reducing the expression of thyroid stimulating hormone (TSH) in the anterior pituitary. Systemic administration of leptin prevents the fasting-induced reduction in pro-TRH levels and partially corrects the reduction in TH (Ahima et al., 1996; Legradi et al., 1997). See Orban et al., 1998 for a review on this area.

1.4.1.3 INSULIN REGULATION AND GLUCOSE HOMEOSTASIS

Hyperinsulaemia and hyperglycaemia are part of the obesity syndrome observed in ob/ob and db/db mice (Bray and York, 1979; Herberg and Coleman, 1977). Furthermore, when ob/ob mice are treated with leptin, changes in glycaemia precede the decrease in body weight (Pelleymounter et al., 1995), and a reduction in plasma insulin concentration is also seen, that is not solely due to the concomitant decrease in weight (Campfield et al., 1995). Leptin treatment in normal fed mice also decreases insulin secretion (Kulkarni et al., 1997). These findings, together with the observation that the long-form leptin receptor (LEPR-b) is expressed in pancreatic islet cells (Emilsson et al., 1997) suggest that leptin may have a role in regulating insulin secretion, possibly directly. Data indicating that leptin gene expression is modulated by insulin (see section 1.6.3) provide evidence to suggest that leptin and insulin are involved in a negative feedback loop, making up part of an adipoinsular axis.

Leptin's direct effects on insulin secretion in vitro is contentious however. The majority of studies indicate that leptin is able to directly inhibit glucose-stimulated insulin secretion from pancreatic islets and insulin-secreting cell lines (Kulkarni et al., 1997; Ookuma et al., 1998; Pallett et al., 1997; Roduit and Thorens, 1997; Seufert et al., 1999b). Other studies suggest that leptin can decrease hypersecretion of insulin to normal levels in islets obtained from ob/ob mice (Chen et al., 1997; Emilsson et al., 1997; Kieffer et al., 1997). In contrast, some studies report no effect of leptin on either basal- or glucose-stimulated insulin secretion in normal rodents (Chen et al., 1997; Leclercq-Meyer et al., 1996; Leclercq-Meyer and Malaisse, 1998), whereas Fehmann et al showed inhibition of both basal and glucose-stimulated insulin secretion by leptin (Fehmann et al., 1997). Furthermore Poitout et al only observed an inhibiting effect when insulin secretion was stimulated with both glucose and 3-isobutyl 1-methylxanthine (IBMX) (Poitout et al., 1998). Conversely, others report increased
insulin secretion upon leptin stimulation \textit{in vitro} (Shimizu et al., 1997a; Tanizawa et al., 1997).

Insulin gene expression has also been reported to be affected by leptin treatment. Leptin inhibits the activity of the rat insulin I gene promoter \textit{in vitro} in glucose-stimulated INS-1 beta-cells, and in \textit{ob/ob} islets, and reduces the levels of preproinsulin mRNA \textit{in vivo} in \textit{ob/ob} mice (Seufert et al., 1999a). Insulin mRNA levels have also been reported to be suppressed by leptin in \textit{bTC6} cells and rat pancreatic islets (Kulkarni et al., 1997; Pallett et al., 1997). Glucose- and GLP-1 stimulated preproinsulin mRNA expression is also reduced by leptin in cultured human pancreatic islets (Seufert et al., 1999b).

Leptin effects on glucose homeostasis are also conflicting. Glucose uptake has been shown to be blocked by leptin in cultured rat adipocytes (Muller et al., 1997) and human adipocytes (Zhang et al., 1999), while no effect, either acute or chronic, was observed in similar adipocyte cell models or skeletal muscle preparations by others (Furnsinn et al., 1998; Ranganathan et al., 1998; Zierath et al., 1998). By comparison, leptin has been shown to increase glucose uptake in \textit{C}_{2}\textit{C}_{12} myotubes (Berti et al., 1997). \textit{In vivo}, intravenous (I.V.) or intracerebroventricular (I.C.V.) administration of leptin causes an increase in glucose uptake in various peripheral tissues, excluding white adipose tissue, and an increase in whole body glucose utilization (Kamohara et al., 1997; Minokoshi et al., 1999; Sivitz et al., 1997), suggesting central effects of leptin could play a major role in the regulation of glucose homeostasis. Recent reports have suggested that leptin-induced activation of the sympathetic nervous system (SNS) contributes to this phenomenon (Haque et al., 1999; Mizuno et al., 1998).

Leptin modulates a whole host of other actions of insulin in various tissues, including insulin-stimulated phosphorylation of insulin receptor substrate-1 (IRS-1), association of the adapter molecule growth factor receptor-bound protein 2 with IRS-1, gluconeogenesis, the activity of IRS-1-associated phosphatidylinositol 3-kinase and glycogen synthase, lipogenesis, inhibition of isoproterenol-induced lipolysis, and protein kinase A activation. (Cohen et al., 1996; Muller et al., 1997; Rossetti et al., 1997). For a more in depth review of this rapidly expanding body of research, see Fruhbeck and Salvador, 2000.
1.4.1.4 Fat Metabolism

Normal dieting leads to decreases in both lean and body fat mass, whereas leptin-induced weight loss is associated only with loss of fat mass in both humans and rodents (Farooqi et al., 1999; Halaas et al., 1995). Additionally, the decrease in body fat mass induced by leptin therapy is not accompanied by the increase in free fatty acids or ketones that normally occur through the well-characterized adrenergic-mediated activation of lipolysis during starvation (Shimabukuro et al., 1997). It has been observed that leptin has a direct effect on white adipose tissue by instigating a novel form of lipolysis, whereby fatty acid oxidation is proposed to occur intracellularly (Wang et al., 1999a). A similar phenomenon has also been observed in other tissues, including the pancreas, liver and skeletal muscle (Shimabukuro et al., 1997). Further evidence for this effect of leptin include the observation that expression of enzymes involved in long chain fatty acid oxidation become upregulated, and lipogenic enzymes are downregulated in rats receiving leptin gene therapy (Zhou et al., 1997; Zhou et al., 1999). In vitro, leptin can also downregulate lipogenic enzymes in preadipocytes (Bai et al., 1996), and increases glycerol release from mature adipocytes (Siegrist-Kaiser et al., 1997).

1.4.2 Role in Reproduction

Nutritional status has, for a long time, been known to play an important role in many aspects of reproductive function, presumably due to the high energy costs of sustaining pregnancy and lactation. Leptin has been implicated as a signalling link between adipose tissue energy reserves and the reproductive axis, as discussed in the following sections. More in-depth reviews on this area of research are available elsewhere (Caprio et al., 2001; and Holness et al., 1999).

1.4.2.1 Puberty and Fertility

Recent findings support a role for leptin in triggering the onset of puberty. In girls, body weight is a better predictor of onset of puberty than is age (Frisch, 1972), and it has been shown that leptin levels rise immediately prior to the onset of puberty in both girls and boys (Garcia-Mayor et al., 1997; Mantzoros et al., 1997a). Levels of leptin-binding activity have also been observed to fall during puberty (Quinton et al., 1999). It has also been observed that children with loss-of-function mutations in the Lep or Lepr genes fail to enter puberty (Clement et al., 1998; Montague et al., 1997; Strobel et al., 1998). With
twelve months of leptin therapy, a leptin deficient nine-year old child began nocturnal pulsatile secretion of gonadotropins, characteristic of early puberty (Farooqi et al., 1999). Furthermore, premature onset of puberty can be induced in wild-type mice by repeated injections of leptin (Ahima et al., 1997; Chehab et al., 1997), and the female transgenic skinny mouse (which possesses no apparent adipose tissue, but overexpresses leptin), exhibits accelerated puberty (Yura et al., 2000).

Leptin also plays a role in fertility. \textit{ob/ob} mice are infertile (Bray and York, 1979), and exogenous leptin treatment can stimulate their reproductive endocrine system (Barash et al., 1996), and correct their infertility (Chehab et al., 1996; Mounzih et al., 1997), whereas caloric restriction alone does not (Chehab et al., 1996). Furthermore, short-term fasting of wild-type mice results in an ovulatory delay of several days, which can be reversed by leptin injections alone (Ahima et al., 1996).

In humans, the situation is less clear. Low body fat content seen in trained distance runners, ballet dancers and patients with anorexia nervosa is often associated with infertility (De Souza and Metzger, 1991). Increasing leptin levels during weight gain in anorexia nervosa patients occurs in parallel to increasing levels of luteinizing hormone (Ballauff et al., 1999), yet the amenorrhea associated with low body fat content in highly trained athletes is linked to the loss of the diurnal rhythm of leptin secretion, rather than low leptin levels \textit{per se} (Laughlin and Yen, 1997). Contrary evidence from two patients with lipoatrophic diabetes, displaying complete atrophy of subcutaneous and visceral adipose tissue, suggests however that it is possible for puberty and fertility to progress normally despite low serum leptin levels in humans (Andreelli et al., 2000).

The mechanism by which leptin regulates the secretion of gonadotropin-releasing hormone (GnRH) and the activation of the hypothalamic-pituitary-gonadal axis to initiate puberty and maintain fertility is currently unknown. However, the arcuate nucleus and ventromedial hypothalamus are important areas for controlling GnRH release and sexual behaviour (Styne, 1994), and both express high levels of the long form leptin receptor (Mercer et al., 1996). I.C.V. administration of leptin antibodies reduces pulsatile luteinizing hormone (LH) release (Carro et al., 1997a), and \textit{in vitro}, leptin is able to directly stimulate the release of GnRH from median eminence arcuate nuclei explants (Yu et al., 1997). Recent studies suggest that leptin mediates an effect on GnRH secretion \textit{in vitro} via the anorectic peptide, CART (Lebrethon et al., 2000). The
long form receptor and direct effects of leptin have also been observed in the pituitary (Yu et al., 1997), and the gonads (Spicer and Francisco, 1997; Tena-Sempere et al., 1999; Zachow and Magoffin, 1997), suggesting that peripheral effects of leptin may also contribute to these phenomena.

1.4.2.2 PREGNANCY AND FOETAL DEVELOPMENT

The levels of leptin rise during pregnancy, particularly during the second and third trimesters (Chien et al., 1997; Hardie et al., 1997; Masuzaki et al., 1997). This rise is due in part to an increase in adipose tissue mass (Chien et al., 1997), but also due to the production of leptin in the placenta (Hoggard et al., 1997; Masuzaki et al., 1997; Senaris et al., 1997). In mice, the placenta also increases production of the soluble form of the leptin receptor (LEPR-e), which is proposed to increase the half-life of leptin in the circulation (Gavrilova et al., 1997). Leptin has been detected in cord blood, indicating that leptin is synthesized and released from foetal adipocytes (Sivan et al., 1997), and leptin has been reported to be expressed in other foetal tissues including cartilage/bone and hair follicles (Hoggard et al., 1997).

Several roles for the increased leptin levels during pregnancy have been suggested. Due to its effects on the reproductive endocrine system, it is feasible to assume that it may aid in the maintenance of the correct hormonal balance to support pregnancy, especially in periods of short-term food deprivation, when leptin levels normally drop (Considine and Caro, 1999).

Other possibilities include leptin acting to partition nutrients in the placenta, or since leptin is related to the helical cytokine family (Madej et al., 1995), it could act as a foetal growth factor. The presence of leptin receptors in placental and other foetal tissues, suggest that leptin could function in an autocrine or paracrine manner to regulate growth and development (Hoggard et al., 1997). The high levels of leptin and its receptor in foetal bone especially, imply a role in foetal bone development (Hoggard et al., 1997), and haematopoiesis (see section 1.4.3).

Evidence does, however, suggest that leptin may not be necessary to sustain a successful pregnancy, in rodents at least. ob/ob females require exogenous leptin treatment only for conception, although discontinuation of leptin treatment postpartum results in the failure to lactate (Chehab et al., 1996), suggesting a potential role in lactation also. It has also
been shown that maternal leptin is transferred via the offspring’s stomach to its blood, and so could potentially aid in the regulation of neonatal food intake (Casabiell et al., 1997).

1.4.3 ROLE IN HAEMATOPOIESIS AND THE IMMUNE RESPONSE

Nutritional deprivation results in a decrease in immune function, perhaps as an adaptive response to avoid the energy expenditure involved in large-scale clonal expansion (Chandra, 1991). Both ob/ob and db/db mice have impaired cellular immunity (reviewed in Bray and York, 1979), suggesting a role for leptin in immune function. This is supported by various lines of evidence. Firstly, bone marrow preadipocytes differentiated in vitro secrete leptin (Laharrague et al., 1998). Secondly, leptin receptors can be detected on many cells of haematopoietic lineage including blast cells, promyelocytes, promonocytes, monocytes and macrophages (Bennett et al., 1996; Cioffi et al., 1996; Gainsford et al., 1996). Thirdly, leptin expression is regulated by inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and IL-1 (Grunfeld et al., 1996b; Kirchgessner et al., 1997; Sarraf et al., 1997) (see section 1.6.7).

Further evidence comes from the observation that leptin stimulates proliferation of haematopoietic stem cell populations in vitro resulting in murine granulocyte/macrophage (GM) colony formation (Umamoto et al., 1997), and yolk sac and foetal liver cell proliferation (Mikhail et al., 1997), increased myelopoiesis, erythropoiesis and lymphopoiesis (Bennett et al., 1996), and proliferation of human monocytes (Santos-Alvarez et al., 1999). Leptin has also been shown to modulate cytokine production in cultures of murine peritoneal macrophages. Leptin treatment favours a proinflammatory T-cell response by inhibiting IL-4 secretion, and increasing IL-2 and interferon gamma (INF-γ) secretion in a murine mixed lymphocyte reaction (Lord et al., 1998).

In vivo, studies have also shown that administration of leptin can reverse the reduction in the delayed-type hypersensitivity response exhibited by 48-hour fasted mice (Lord et al., 1998), and can up-regulate phagocytosis and the production of proinflammatory cytokines in ob/ob mice (Loffreda et al., 1998).

It is the long form leptin receptor (LEPR-b) that is implicated in the function of haematopoietic cells, since ectopic expression of the LEPR-a in the factor-dependent
hematopoietic cell line, BaF3, cannot mimic the proliferative responses of leptin observed in cells ectopically expressing LEPR-b (Gainsford et al., 1996; Ghilardi and Skoda, 1997). However, GM colony formation in response to leptin has been observed, albeit at a reduced level, in bone marrow cell cultures of \( \text{db/db} \) mice, suggesting leptin receptor isoforms other than LEPR-b may also play a role in haematopoiesis (Umemoto et al., 1997).

For a more in-depth review of this topic, see Faggioni et al., 2001.

### 1.4.4 Other Functions

Other functions of this pleiotropic hormone are continually being elucidated. It has been shown to activate pituitary-adrenocortical function, not only through enhanced pituitary adrenocorticotropic hormone (ACTH) release, but also as a consequence of a direct stimulatory effect on adrenocortical cells (Malendowicz et al., 1998).

It has a mitogenic capacity, both \textit{in vitro} and \textit{in vivo}, for diverse cell types such as endothelial cells, monocytes, lung epithelial cells, pancreatic beta cells and keratinocytes (Bouloumie et al., 1998; Frank et al., 2000; Islam et al., 1997; Santos-Alvarez et al., 1999; Tanabe et al., 1997; Tsuchiya et al., 1999), and is also angiogenic (Bouloumie et al., 1998; Sierra-Honigmann et al., 1998). Moreover anti-apoptotic activity for myeloid leukaemia cells has also been observed (Konopleva et al., 1999), as has a centrally-mediated role in the inhibition of bone formation (Ducy et al., 2000). It is also capable of overriding the fasting-induced inhibition of growth hormone secretion in rats (Carro et al., 1997b).

The central and peripheral actions of leptin are depicted diagrammatically in Figure 1.4 and Figure 1.5.
Figure 1.4 Central leptin-mediated pathways
Leptin mediates its central effects on satiety and energy expenditure through pathways such as the well characterized neuropeptide Y (NPY) and central melanocortin pathways. In the arcuate nucleus of the hypothalamus, leptin acts on two populations of neurons. The first population expresses the orexigenic neuropeptides, NPY and agouti-related protein (AgRP), whilst the second expresses the anorexigenic neuropeptides, pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART). POMC is processed to produce alpha-melanocyte stimulating hormone (α-MSH), a melanocortin receptor-4 (MC4-R) agonist. Leptin inhibits expression of the orexigens, and stimulates expression of the anorexigens. This, coupled with the lack of antagonism on MC4-R by AgRP, leads to the modulation of thyroid releasing hormone (TRH) neurons in the paraventricular nucleus and melanin concentration hormone (MCH) neurons within the lateral hypothalamus, as well as potentially other effects. This leads to changes in endocrine and autonomic function, as well as satiety. Adapted from Spiegelman and Flier, 2001.
Figure 1.5 Central and peripheral effects of leptin
Schematic diagram showing the major functions of leptin, mediated both centrally and peripherally (see text for details).
1.5 The Concept of Leptin Resistance

The finding that most cases of obesity, both genetically and environmentally produced, are characterized not with an absence of leptin, but rather with increased levels (Maffei et al., 1995b) (see section 1.6.1), implies that obesity is usually associated with leptin resistance. Leptin treatment of non-leptin-defective obese animals, and clinical trials with leptin in obese individuals have, on the whole, not met with a great deal of success (Halaas et al., 1997; Heymsfield et al., 1999), substantiating this hypothesis.

Leptin resistance could theoretically occur at several points in the pathway. Firstly, defective transport of leptin across the blood-brain barrier could occur. Leptin levels in cerebrospinal fluid of obese individuals are much lower than expected for the corresponding levels in serum, consequently the CSF/serum ratio is lower in obese than in lean individuals (Caro et al., 1996; Schwartz et al., 1996b). As demonstrated by Banks et al and Golden et al, (Banks et al., 1996; Golden et al., 1997), leptin transport across the BBB is a specific, saturable and temperature-dependent process (see section 1.3.7). Defective transport across the BBB has been hypothesized to be the cause of leptin resistance in the NZO and the diet-induced mouse, since these polygenic model of obesity are resistant to peripheral effects of leptin, but respond to centrally administrated leptin (Banks, 2001; Halaas et al., 1997; Van Heek et al., 1997).

Receptor down-regulation is another point at which leptin resistance could occur, and it is observed with increasing serum leptin levels in obese mouse models (Lynn et al., 1996; Malik and Young, 1996), although there is, as yet, no evidence for this in humans (Considine et al., 1996a).

Post-receptor defects are also possible causes of leptin resistance, as characterized by the agouti mouse model. This model is completely resistant to peripherally administered leptin and considerably resistant to leptin when centrally administered, suggesting that the central melanocortin pathway is an important downstream pathway mediated by leptin (Halaas et al., 1997). The fact that up to 5% of extremely obese individuals have defects in genes involved in the central melanocortin pathway implies that leptin resistance in humans could also be caused by post-receptor defects (Farooqi et al., 2000; Hinney et al., 1999; Vaisse et al., 1998; Yeo et al., 1998). The downstream signalling
molecules SHP2 and SOCS-3 have also been implicated as potential targets at which leptin resistance could occur (Bjorbaek et al., 1998a; Carpenter et al., 1998).

As well as potential genetic defects leading to leptin resistance, there is also speculation that the inhibitory effect of glucocorticoids on leptin action in vivo may also contribute to the phenomenon of leptin resistance (Ur et al., 1996; Zakrzewska et al., 1997), (see section 1.6.4).

1.6 Factors Governing Leptin Levels

Since leptin has been shown to play vital roles in numerous physiological pathways, there has been intense interest in the factors that govern its levels - both at the mRNA level and plasma levels of the protein.

1.6.1 Body Fat Content

In steady-state conditions of plentiful food and normal energy expenditure, Lep mRNA and leptin levels in rodents and humans are highly correlated with body mass index (weight in kilograms, divided by height in metres, squared - kg/m² (BMI)), and more specifically, with body fat content. This is true over a wide range of body weights, including normal weight, obese and anorexic; and is shown in adults, juveniles and even neonates (Considine et al., 1996b; Frederich et al., 1995a; Grinspoon et al., 1996; Hassink et al., 1997; Hassink et al., 1996; Maffei et al., 1995b; Matsuda et al., 1997).

Despite being strongly correlated with body fat content, there is still considerable variation between individuals after adjusting for adiposity. For example, females have higher levels of leptin than males (Hassink et al., 1996; Matsuda et al., 1997; Ostlund et al., 1996; Rosenbaum et al., 1996). Since there are adipose tissue depot-specific differences in levels of leptin expression (Masuzaki et al., 1995; Trayhurn et al., 1995b), sex-specific differences in adipose tissue distribution, and sex-specific hormone levels (see section 1.6.6) are thought to contribute to this phenomenon. (Roemmich et al., 1998; Rosenbaum et al., 1996). Variations in Lep mRNA and leptin levels in steady-state conditions still exist however, even when gender is accounted for (Considine et al., 1996b; Maffei et al., 1995b).
1.6.2 **Nutritional Status**

As would be expected for a hormone involved in energy homeostasis, and similarly to other factors involved in this system - (e.g. the glucose transporter, Glut-4 (Sivitz et al., 1989), and fatty acid synthase (FAS) (Paulauskis and Sul, 1988)) - leptin is under physiological control by the nutritional state. In the short-term, \( Lep \) mRNA and serum leptin levels have been shown to decrease rapidly in response to fasting in lean rodents, which can be reversed following *ad libitum* re-feeding (Becker et al., 1995; Frederich et al., 1995b; MacDougald et al., 1995b; Saladin et al., 1995; Thompson, 1996; Trayhurn et al., 1995b; Zheng et al., 1996a). In contrast, the elevated levels of \( Lep \) mRNA in rodent obesity models, such as \( ob/ob \) mice, and \( fa/fa \) rats, are insensitive to short-term fasting (Cusin et al., 1995; Trayhurn et al., 1995b).

In humans, \( Lep \) mRNA and leptin levels have also been shown to be sensitive to fluctuations in food intake. Kolaczynski et al observed declines in both these parameters during 36 and 60 hours of fasting, which were rectified rapidly upon refeeding, and Considine et al found that the 10% reduction in body weight achieved by obese subjects over an 8-12 week period, was associated with a 53% reduction in serum leptin. Others, including Boden et al and Weigle et al, have also found a decrease in leptin levels upon fasting that is disproportionate to the reduction in adipose mass (Boden et al., 1996; Considine et al., 1996b; Kolaczynski et al., 1996a; Weigle et al., 1997). However, Vidal et al did not identify a decrease in human abdominal subcutaneous adipose tissue \( Lep \) mRNA after a five-day hypocaloric diet (1996).

Voluntary overfeeding in humans also influences leptin levels. Twelve hours of massive overfeeding resulted in a 40% increase in serum leptin levels, which was maintained even after an overnight fast; and prolonged overfeeding (over five weeks) was associated with higher leptin levels than would be expected, taking into account the resulting increase in body fat content (Kolaczynski et al., 1996c). Short-term, post-prandial changes in leptin levels in humans are not observed however (Considine et al., 1996b; Dagogo-Jack et al., 1996).

These data signify that leptin production is under dual control, where factors other than the size of the adipose tissue mass are involved in regulation during short- to mid-term fluctuations in energy intake and expenditure. Since the role of leptin has been hypothesized to be part of the neuroendocrine response to starvation (Ahima et al.,
1996), the role of the physiological axes that are affected in this state, in the regulation of \textit{Lep} mRNA and serum leptin levels, are in the process of being characterized fully. Some of the most important, and best-characterized pathways, and their significance in feedback control are discussed in the following sections.

1.6.3 **Insulin and Glucose**

Since insulin levels closely parallel changes in the nutritional state, the role of insulin in leptin regulation is intensively studied. Insulin alone can mimic the response to re-feeding in fasted rodents, suggesting that the increase in plasma insulin levels subsequent to re-feeding may mediate the upregulation of \textit{Lep} expression (Mizuno et al., 1996b; Saladin et al., 1995; Zheng et al., 1996a). In addition, Zheng et al found that insulin upregulated \textit{Lep} mRNA levels in epididymal and perirenal adipose tissue depots, but not levels in subcutaneous adipose tissue (1996). Together with data reported by Vidal et al (1996), (see section 1.6.2), it would appear that \textit{Lep} expression in subcutaneous adipose tissue is less responsive than in other depots.

Studies performed in streptozotocin-treated rats also points to the role of insulin in \textit{Lep} gene regulation. In the time taken for rats to become diabetic with streptozotocin-treatment, \textit{Lep} mRNA levels decrease substantially, and after four hours of insulin treatment levels increase to a maximum of around 50% of normal, control levels. However, this response is meagre compared to the over-correction of levels of other insulin-responsive genes, such as FAS and Glu4 (Becker et al., 1995; MacDougald et al., 1995b).

Most short-term (60-300 minute) hyperinsulinaemic clamps (either with euglycaemia or hypoglycaemia); or hyperglycaemic clamps, have no effect on leptin levels in humans however, irrespective of body composition (normal weight or obese) or diabetic status (Dagogo-Jack et al., 1996; Kolaczynski et al., 1996b; Ryan and Elahi, 1996), although a hyperinsulinaemic clamp did significantly elevate leptin levels after 4 hours in one study (Utriainen et al., 1996). Chronic effects of insulin have been observed – after 6 hours of hyperinsulinaemia, or during the final 24-hours of a 72-hour hyperglycaemic clamp, plasma leptin levels have been shown to rise significantly (Kolaczynski et al., 1996b; Malmstrom et al., 1996).
Comparison of these studies is, however, complicated by the different experimental designs employed, with regard to fed and fasted states and taking into account the diurnal rhythm of plasma leptin levels (see section 1.6.8).

There is still considerable debate as to whether insulin has a direct effect on increasing \( \text{Lep} \) mRNA levels \textit{in vitro} however, and if so, to what extent. This is complicated by the variety of model systems used and quantification methods employed to address the issue. In suspension culture, primary rat adipocytes have been shown to modestly increase \( \text{Lep} \) mRNA levels in response to physiological concentrations of insulin within 24 hours (Saladin et al., 1995; Slicker et al., 1996). Using the rather unusual technique of primary ceiling culture of rat adipocytes, which relies on the proliferation of differentiated adipocytes, Yoshida et al observed a concentration-dependent increase in \( \text{Lep} \) mRNA levels after 24 hours of treatment with insulin, with a large (21.4-fold) increase with 100nM insulin (1996). Physiological concentrations of insulin significantly increased the steady-state levels of \( \text{Lep} \) mRNA in 3T3-F442A and 3T3-L1 adipocytes (Leroy et al., 1996; Rentsch and Chiesi, 1996), and some experiments suggest that removal of insulin from the tissue culture medium of 3T3-F442A adipocytes, and human or rat preadipocytes differentiated \textit{in vitro}, results in a decrease in \( \text{Lep} \) mRNA levels, which can be restored back to control levels by the re-addition of insulin (Leroy et al., 1996; Mitchell et al., 1997; Wabitsch et al., 1996). In line with these observations, Maffei et al found that during the differentiation of 3T3-F442A cells, \( \text{Lep} \) mRNA was not detectable in late postconfluent cells not exposed to insulin (Maffei et al., 1995a). Reporter gene studies also point to insulin regulating the transcription of the \( \text{Lep} \) gene (see section 1.7.2).

Other groups, again using varied approaches, have observed no effect of insulin on \( \text{Lep} \) expression, including work performed in primary rat adipocytes (Murakami et al., 1995), rat explant culture (Reul et al., 1997) and 3T3-L1 adipocytes (Kosaki et al., 1996; MacDougald et al., 1995b) with concentrations of up to 100nM and culture periods of up to 24 hours.

Investigations into insulin's effects on rodent leptin secretion have resulted in more consistent data however, with a rapid increase in leptin secretion observed with insulin treatment, in primary rat adipocytes (Gettys et al., 1996; Hardie et al., 1996b; Slicker et
al., 1996), 3T3-F442A adipocytes (Leroy et al., 1996) and rat explant cultures (Barr et al., 1997; Fain and Bahouth, 2000).

Studies in primary human adipocytes and explants, derived from various depots, suggest that insulin has no acute, direct effect on human Lep mRNA levels, (Halleux et al., 1998; Kolaczynski et al., 1996b), although a two-fold induction was observed after 72 hours in culture with 100nM insulin by Kolaczynski et al (Kolaczynski et al., 1996b). The trophic effect of insulin was offered as an explanation of this phenomenon however, rather than a metabolic effect. Leptin secretion was observed to increase following this increase in Lep mRNA levels (Kolaczynski et al., 1996b). Other groups have also reported an increase in human leptin secretion following insulin treatment in vitro (Nolan et al., 1996; Wabitsch et al., 1996).

Since plasma glucose levels are also affected by changes in the nutritional state, it has been hypothesized that glucose uptake and/or metabolism may contribute to the regulation of leptin. The distinction between the effects of glucose or insulin in in vivo studies is difficult to observe however, since hyperinsulinaemic clamps must be accompanied by euglycaemic clamps, otherwise hypoglycaemia will occur. Similarly hyperglycaemic clamps result in raised insulin levels. However, certain in vivo results do point to a role of glucose in leptin regulation. For instance, Lep mRNA levels have been observed to be more closely related to plasma glucose than insulin in mice (Mizuno et al., 1996a), and the decrease in plasma leptin during caloric restriction in humans is better correlated with the decrease in plasma glucose than with changes in insulin levels (Dubuc et al., 1998).

In vitro experiments addressing this hypothesis have found that inhibiting glucose transport or glycolysis in rat adipocyte model cultures, inhibits leptin release and decreases Lep mRNA levels (Mueller et al., 1998).

The pathway(s) by which glucose potentially exerts its effects on leptin expression is in the process of being elucidated. Glucosamine, an end product of the hexosamine biosynthetic pathway, which has been shown to be a cellular 'sensor' of energy availability (Hawkins et al., 1997) increases Lep mRNA levels in 3T3-L1 preadipocytes (Wang et al., 1998), and more recently, transgenic mice overexpressing the rate-limiting enzyme for hexosamine synthesis (glutamine: fructose-6-phosphate amidotransferase) in muscle and fat, thereby elevating intracellular UDP-N-acetylglucosamine levels, have
been produced. These mice are hyperleptinaemic compared to normal controls after an overnight fast (McClain et al., 2000).

1.6.4 GLUCOCORTICOIDS

Adrenalectomy attenuates obesity in rodent models, which can be reversed by glucocorticoid treatment (Freedman et al., 1986). This appears to be mediated by NPY (Sainsbury et al., 1997). Additionally, the administration of leptin to lean, adrenalectomized rats results in a greater decrease in body weight, food intake and Lep mRNA levels, than in control lean rats, and this can be attenuated by glucocorticoid treatment. This has led to the suggestion that glucocorticoids may act to limit the effects of leptin (Zakrzewska et al., 1997). It has also been reported that leptin can activate pituitary-adrenocortical function, not only through enhanced pituitary ACTH release, but also as a consequence of a direct stimulatory effect on adrenocortical cells (Malendowicz et al., 1998). The effect of glucocorticoids on leptin regulation has therefore been extensively studied.

In rats, short-term treatment with doses of hydrocortisone high enough to provoke a catabolic response including reduced food intake and decreased body weight, cause a dose-dependent increase in Lep mRNA levels (De Vos et al., 1995). In the same study, dexamethasone and triamcinolone were also shown to raise Lep mRNA levels.

In humans, short-term glucocorticoid treatment also raises plasma leptin levels, across a wide age spectrum, and this is not impaired in obese individuals (Dagogo-Jack et al., 1997; Kiess et al., 1996; Larsson and Ahren, 1996; Miell et al., 1996). However, it should be noted that Tataranni et al failed to observe an effect with methylprednisolone in a small sample of lean subjects (Tataranni et al., 1997).

Raised Lep mRNA levels in subcutaneous abdominal, and gluteal adipose tissue after glucocorticoid treatment have been observed (Papaspyrou-Rao et al., 1997) although another study disputes this finding - in subcutaneous abdominal tissue at least (Kolaczynski et al., 1997). Furthermore, the concomitant rise in insulin levels caused by glucocorticoid-induced insulin resistance, does not correlate with the raised leptin levels in human studies, indicating that insulin is not responsible for mediating this effect (Dagogo-Jack et al., 1997; Larsson and Ahren, 1996).
Most *in vitro* studies in rats have confirmed that glucocorticoids have a direct effect on *Lep* expression. Glucocorticoids such as dexamethasone, cortisone and hydrocortisone rapidly increase *Lep* mRNA levels in, and release leptin from, cultured adipocytes (Murakami et al., 1995; Slieker et al., 1996). However, Rentsch et al observed a marginal inhibitory effect in the differentiated 3T3-L1 cell line model (1996). Reporter gene studies also point to glucocorticoids regulating the transcription of the *Lep* gene (see section 1.7.2).

Human *Lep* expression and leptin secretion *in vitro* is also increased in response to glucocorticoids, in adipocytes differentiated *in vitro*, subcutaneous adipocytes and visceral adipose tissue explants (Considine et al., 1997; Halleux et al., 1998; Wabitsch et al., 1996).

### 1.6.5 The Sympathetic Nervous System and Thyroid Hormone

The fasting-induced reduction in sympathetic nervous system (SNS) activity and leptin’s effects on increasing thermogenesis in brown adipose tissue and lipolysis in white adipose tissue have been well-documented (see section 1.4.1.2). To ensure proper regulation of this system, and to complete the feedback loop, activation of the SNS and the resulting increase in catecholamines has a negative effect on the expression of *Lep* mRNA and circulating levels of leptin.

Cold exposure (4°C) for as little as two hours down-regulates *Lep* gene expression and circulating leptin levels in rodents (Hardie et al., 1996b; Trayhurn et al., 1995a). This has been shown to be mediated through the sympathetic nervous system, and in particular the β3-adrenoreceptor (β3-AR), as subcutaneous administration of norepinephrine, the β-adrenoreceptor agonist, isoprenaline, and specific β3-AR agonists, including CL 316,243, Ro 16-8714, BRL 35135A and ZD2079, produce similar effects in both mice and rats (Mantzoros et al., 1996; Moinat et al., 1995; Trayhurn et al., 1995a; Trayhurn et al., 1996). Leptin levels in CL 316,243-treated β3-AR knockout mice do not decrease, providing further evidence for a specific effect on β3-ARs (Mantzoros et al., 1996).

In humans, adrenergic inhibition of leptin secretion also occurs. The β-AR agonists isoproterenol (Donahoo et al., 1997), and isoprenaline (Pinkney et al., 1998a) have comparable, suppressing effects on circulating leptin levels in humans.
The observed decrease in leptin production has been shown to be a direct effect *in vitro*. Rat adipocytes and 3T3-L1 adipocytes show reduced *Lep* mRNA levels and leptin secretion when treated with the beta-adrenergic agonist, isoprenaline; or selective β3-AR agonists (Gettys et al., 1996; Hardie et al., 1996a; Kosaki et al., 1996; Slieker et al., 1996), and antagonists to the β1- and β2-ARs fail to reverse this effect (Gettys et al., 1996). β-AR activation leads to stimulation of the Gs protein-coupled pathway, activation of protein kinase A (PKA) and ultimately to an increase in cellular cyclic 3',5'-adenosine monophosphate (cAMP). Cholera toxin and cAMP analogues have also been shown to decrease *Lep* mRNA levels and leptin secretion *in vitro*, providing further evidence for a direct effect of the stimulation of the sympathetic nervous system on leptin production (Gettys et al., 1996; Slieker et al., 1996).

However, leptin secretion has also been reported to be decreased in a non-specific manner at lower temperatures (Peino et al., 2000). As *Lep* mRNA levels were unaffected in this *in vitro* human adipocyte model, and growth hormone secretion was similarly reduced, it was concluded that this effect was probably a mechanical, rather than a biological phenomenon.

Thyroid hormones increase the basal metabolic rate and increase thermogenesis, and have a permissive role on the effects of catecholamines on β-ARs. Since levels are also suppressed in the response to starvation (Ahima et al., 1996), the role of thyroid in the modulation of leptin levels has therefore been investigated.

*Lep* mRNA in epididymal and retroperitoneal, but not subcutaneous or mesenteric adipose tissue, and serum leptin levels, have been shown to be elevated in hypothyroid rats, with no evidence for the alteration of hormone stability (Fain et al., 1997; Leonhardt et al., 1999), and levels have been shown to be corrected with triiodothyronine (T3) or thyroxine (T4) administration to hypothyroid rats, by levels greater than would be expected from the corresponding changes in body weight (Escobar-Morreale et al., 1997; Fain et al., 1997).

Induced short-term hyperthyroidism by administration of thyroxine or TSH fails to modulate leptin levels in normal rodents however, suggesting a role in the normalization of leptin levels, rather than elevation *per se* (Leonhardt et al., 1999).
In vitro data in rodent cell culture is conflicting however. T3, but not T4, has been reported to increase Lep mRNA levels and secretion of leptin in 3T3-L1 adipocytes (Yoshida et al., 1997), whereas it has been reported that T3 enhances the decrease in Lep mRNA and secreted leptin levels cultured with CL 316,243 in rat adipocytes, yet potentiates the effects of insulin to increase levels (Fain and Bahouth, 1998a).

In humans, the role of thyroid hormones in the regulation of leptin levels is also far from being elucidated fully. Inducing a hyperthyroid state in normal men or women, by T3 treatment for one week fails to modulate serum leptin levels (Kristensen et al., 1999; Mantzoros et al., 1997c), even though other recorded parameters indicate that a 'functional hyperadrenergic' state is achieved by this treatment (Mantzoros et al., 1997c). These studies are in agreement with the animal studies described above (Leonhardt et al., 1999), and human studies that report no difference in serum leptin levels in hyperthyroid patients before and after treatment to regain the euthyroid state (Ozata et al., 1998). Indeed, several studies have concluded that serum leptin levels are not correlated with thyroid status at all (Corbetta et al., 1997; Sesmilo et al., 1998; Sreenan et al., 1997). However, other studies report decreased serum leptin levels in patients with hypothyroidism, independent of BMI (Diekman et al., 1998; Valcavi et al., 1997; Yoshida et al., 1998), which increase with TH treatment to restore euthyroidism (Diekman et al., 1998; Valcavi et al., 1997). Opposing findings were shown by Leonhardt et al and Pinkney et al, where serum leptin levels were observed to be elevated in hypothyroid patients and decreased with normalization of thyroid status, (Leonhardt et al., 1998; Pinkney et al., 1998b). In vitro, a direct role for thyroid hormone in the modulation of human leptin production has been reported by Kristensen et al, who showed that T3 is able to decrease Lep mRNA and leptin secretion from human adipocytes (1999).

In conclusion, the effects of thyroid hormone on modulating leptin levels in rodents and humans are clearly not yet well defined. For further discussion of this area of research, see Flier et al., 2000 and Orban et al., 1998.

1.6.6 REPRODUCTIVE HORMONES

Leptin's effects on the hypothalamic-pituitary-gonadal axis have been discussed in section 1.4.2. Studies have been conducted to investigate whether sex-steroids influence
*Lep* gene expression or serum leptin levels as part of a hypothalamic-pituitary-gonadal-adipose tissue axis.

In mice, a gender-specific difference in serum leptin levels has been observed where levels in females are higher than in males. This has been shown to be independent of the gender-specific differences in body fat content, using the quantitative method of carcass analysis of total body lipid (Frederich et al., 1995a).

The same gender-specific differences in serum leptin levels have also been shown to exist in several human studies, and are also independent of the gender-specific differences in body fat content, using various different measures of body fat content (Havel et al., 1996; Ma et al., 1996; Ostlund et al., 1996; Rosenbaum et al., 1996). Two earlier studies found no evidence for sex-specific differences in leptin levels after adjusting for body fat content however, although the accuracy of the method of determination used by Considine et al (bioelectric impedance) is limited (Considine et al., 1996b; Maffei et al., 1995b).

The positive results could imply therefore that sex-hormones play a role in determining overall levels of leptin expression in both rodents and humans, although gender-specific differences in leptin clearance, adipose-tissue distribution or other genetic factors could also explain the differences.

Further evidence in favour of sex-steroid influences on circulating leptin levels comes from a number of experiments. In men, serum levels of testosterone show a strong negative correlation with levels of leptin (Behre et al., 1997; Jockenhovel et al., 1997; Nystrom et al., 1997). Furthermore, testosterone treatment of hypogonadal men decreases their abnormally high levels of leptin (Behre et al., 1997; Jockenhovel et al., 1997), without affecting the percentage of body fat (Jockenhovel et al., 1997). Rosenbaum et al and Shimizu et al’s studies reported a gradation of serum leptin levels where levels were ranked pre-menopausal women>post-menopausal women>men, after body fat adjustment (Rosenbaum et al., 1996; Shimizu et al., 1997b), possibly implying a role for oestrogen in stimulating leptin levels and/or testosterone in decreasing serum leptin levels.

In rats, castration results in an increase in *Lep* mRNA levels in perirenal adipose tissue, (but conversely decreases levels slightly in subcutaneous adipose tissue), whereas
ovariectomy decreases levels in subcutaneous and retroperitoneal white adipose tissue depots, but increases expression in mesenteric adipose tissue (Machinal et al., 1999; Shimizu et al., 1997b). Serum leptin levels are also reduced in ovariectomized rats, and this can be reversed with oestradiol treatment (Shimizu et al., 1997b).

In vitro, no gender specific differences have been observed in Lep mRNA levels in human subcutaneous adipocytes after adjusting for fat mass, (Considine et al., 1996b), and in in situ hybridization experiments, no difference between mRNA levels was observed in subcutaneous adipocytes from normal weight males and females, although mRNA levels are higher in adipose tissue from obese females compared to obese males (Lonnqvist et al., 1995).

Leptin secretion is however, significantly higher in organ cultures of human omental fat derived from females than males (Casabiell et al., 1998; Pineiro et al., 1999). In this model system, oestradiol was shown to stimulate leptin secretion in female tissue, but did not affect secretion in male tissue (Casabiell et al., 1998). Testosterone had no effect on secretion in either gender, whereas other androgens, including dihydrotestosterone (DHT) (the active metabolite of testosterone), the non-aromatizable androgen stanozolol, androstenedione and dehydroepiandrosterone-sulphate (DHEA-S) all slightly decreased secretion in female samples (Pineiro et al., 1999). There is no evidence for gender-based differences in the number of steroid receptors in omental fat, and since modulation of leptin secretion in male omental fat was observed with other factors, the responsiveness of this tissue was not in doubt (Pineiro et al., 1999). This leaves no real explanation for the unresponsiveness of male omental fat to sex-steroid treatment.

The strong negative correlation between testosterone and leptin levels, without convincing evidence to suggest that testosterone influences leptin production directly, could imply that testosterone is capable of modulating leptin production via an indirect mechanism. The effect of testosterone on stimulating catecholamine-induced lipolysis in adipocytes, thought to be through increasing the number of cell-surface beta-adrenergic receptors and the activity of adenylate cyclase, may help to account for this (Xu et al., 1990).

In rats, DHT has a direct effect at the mRNA level, where it slightly decreases Lep mRNA levels in perirenal and subcutaneous adipocytes in vitro, apparently mediating its
effects through androgen receptors at the level of transcription (Machinal et al., 1999). Direct effects of 17β-oestradiol have also been observed in cultured rat adipocytes, where leptin secretion (Machinal et al., 1999; Slierer et al., 1996), and Lep mRNA levels (Machinal et al., 1999; Murakami et al., 1995) increase approximately 2-fold. The Lep gene promoter contains a consensus sequence of the oestrogen-responsive element, and in certain cell types, reporter gene plasmids containing the leptin promoter show a response to oestrogen, signifying a direct effect on transcription therefore (O'Neil et al., 2001).

Overall, a strong case can be brought forward to suggest that sex-steroids do indeed influence circulating leptin levels, albeit to a small degree, with androgens exerting an inhibiting, and oestrogen exerting a stimulating effect on leptin production. However, results from a study of cord blood leptin in new born humans showed increased levels in females after adjustment for body weight, without a difference in levels of sex-steroids (Matsuda et al., 1997). This implies that other factors, possibly genetic differences between the sexes, account for some of this dimorphism in serum leptin levels.

1.6.7 CYTOKINES

Infection associated anorexia and the role of cytokines, especially TNF-α, in inducing weight loss and increasing energy expenditure, have, together with the findings of leptin's effects on stimulating the immune system, led to the investigation of cytokine regulation of leptin production.

Lipopolysaccharide (LPS) treatment, a model of gram negative infections, increases Lep mRNA and circulating leptin levels in hamsters and mice despite an inversely proportional inhibition of food intake (Grunfeld et al., 1996b; Sarraf et al., 1997). This effect can be mimicked by mediators of the host response to LPS, notably the pro-inflammatory cytokines TNF-α and IL-1 (Grunfeld et al., 1996b; Kirchgessner et al., 1997; Sarraf et al., 1997). Further evidence attributing the response to TNF-α and IL-1 comes from reports that IL-1β knockout mice, unlike wild-type mice, do not increase levels of leptin when administered with LPS (Faggioni et al., 1998). Counterinflammatory cytokines such as IL-4 and IL-10, which have no anorectic effect in mice, have no effect on leptin levels in vivo (Sarraf et al., 1997).
In humans, serum leptin levels have been shown to be significantly positively correlated with TNF-α, after adjustment for BMI in normal controls and NIDDM patients (Mantzoros et al., 1997b), and levels have been shown to be elevated in cancer patients treated for 12 hours with TNF-α (Zumbach et al., 1997), or for one day with IL-1 (Janik et al., 1997). In contrast, levels are not elevated in AIDS- or HIV-infected- patients (Grunfeld et al., 1996a; Yarasheski et al., 1997), nor has acute experimental endotoxemia in humans been shown to alter circulating leptin levels (Bornstein et al., 1998).

The acute rise in circulating leptin levels in response to pro-inflammatory cytokines is apparently not a direct effect however, since it has been observed in vitro that pro-inflammatory cytokines, including IL-1β, TNF-α, IL-6 and IL-11 have been shown to actually decrease \textit{Lep} mRNA levels (Granowitz, 1997; Kirchgessner et al., 1997; Zhang et al., 2000).

It has been suggested that induction of \textit{Lep} levels in vivo by pro-inflammatory cytokines could occur via secondary mediators, such as TGF-β, which has been shown to directly increase steady-state levels of \textit{Lep} mRNA (Granowitz, 1997). In addition, it is believed that cytokines such as TNF-α increase the release of leptin into the medium from a preformed pool of leptin, since the increase in leptin secretion observed with TNF-α from 3T3-L1 adipocytes and human adipocytes is insensitive to cycloheximide (an inhibitor of \textit{de novo} protein synthesis), but is inhibited by brefeldin A (an inhibitor of secretion) (Kirchgessner et al., 1997; Zhang et al., 2000).

### 1.6.8 Diurnal Rhythm and Pulsatility

Leptin secretion and \textit{Lep} mRNA expression in rodents are also regulated in a diurnal fashion. Levels rise during the dark feeding phase, and fall to basal levels during the day (Ahima et al., 1996; Saladin et al., 1995; Xu et al., 1999). Although it is unclear whether this rhythm is entirely dependent on food intake, it is apparent that nocturnal feeding contributes to the maintenance of peak levels during the night, since overnight fasting abolishes this rise (Ahima et al., 1996). Furthermore, restricting food availability to the light cycle shifts the timing of peak \textit{Lep} mRNA expression and leptin secretion forward (Xu et al., 1999).
Leptin secretion in humans follows a similar diurnal rhythm, with a distinct nocturnal peak. In terms of feeding behaviour, this is in marked contrast to the situation in rodents, as humans are normally daytime feeders (Sinha et al., 1996a).

Secretion has been shown to be pulsatile, with a periodicity of around 45 minutes. This pulsatility remains intact in obese individuals - increased pulse height explains the higher serum leptin levels observed in this group (Licinio et al., 1997; Sinha et al., 1996c). Circadian variation in leptin levels is absent in amenorrheic athletes and anorexia nervosa patients however (Balligand et al., 1998; Laughlin and Yen, 1997).

Normal fluctuations in the secretion of human leptin are strongly negatively correlated with ACTH and cortisol levels (Licinio et al., 1997), and do not appear to correlate with food intake and resulting glucose and insulin levels (Sinha et al., 1996a). However, in a study of normal weight males where either meal timing was shifted or jet-lag was simulated, it was suggested that the diurnal rhythm of secretion was entrained to meal pattern rather than the circadian clock (Schoeller et al., 1997).

### 1.6.9 Leptin Clearance

A change in the rate of leptin clearance from plasma could also contribute to plasma leptin levels. The mean plasma half-life of leptin in both normal weight and obese humans has been calculated to be 25 minutes, (Klein et al., 1996), analogous to that seen for other peptide hormones of comparable molecular weight (Bennett and McMartin, 1979). This suggests that increased plasma leptin levels observed in obese patients is caused by an increase in production of leptin, rather than defects in leptin clearance.

Studies of patients with renal insufficiencies and end-stage renal failure, together with data obtained from rat nephrectomies, clearly show that the kidney contributes to the clearance of leptin from plasma (Cumin et al., 1997a; Cumin et al., 1997b; Iida et al., 1996b; Merabet et al., 1997; Sharma et al., 1997). Clearance through lung and liver tissue (both of which express leptin receptors) has not been observed (Jensen et al., 1999).

In rodents, a role for increased levels of the soluble leptin receptor in increasing the plasma half-life of leptin has been observed, in pregnancy (see section 1.4.2.2) and in rodent models of obesity (Huang et al., 2001), thus contributing to increased levels of plasma leptin. This however, has not been observed in humans.
1.6.10 OTHERS

Various other factors have been observed to have an effect on leptin regulation. Perhaps with significance to the role of the transcription factor, peroxisome proliferator-activated receptor gamma (PPARγ), in adipocyte differentiation and Lep gene expression, the thiazolidinediones (TZDs) – exogenous ligands of PPARγ - suppress rodent Lep gene expression, both in vivo and in vitro, and suppress Lep promoter activity (De Vos et al., 1996; Kallen and Lazar, 1996). In humans, TZDs suppress leptin production in vitro, but have no effect on plasma leptin levels in vivo, although the effect of TZDs on the improvement in insulin sensitivity and reducing plasma insulin concentrations must be taken into account when interpreting these results (Nolan et al., 1996). The endogenous ligand of PPARγ (15-deoxy-delta(12,14) prostaglandin J2), also inhibits Lep expression (Sinha et al., 1999).

Physical activity has been shown to have an effect on leptin regulation, and this is implied to be linked to free fatty acid levels (Hickey et al., 1997; van Aggel-Leijssen et al., 1999). Free fatty acids have been observed to decrease Lep mRNA levels and leptin production in cell lines and rat primary adipocytes, although human in vivo studies show no effect (Hennes et al., 1997; Rentsch and Chiesi, 1996; Shintani et al., 2000).

IGF-1 (Boni-Schnetzler et al., 1999; Dagogo-Jack et al., 1998; Reul et al., 1997) and growth hormone (Gill et al., 1999; Houseknecht et al., 2000; Nystrom et al., 1997) have also been implicated in regulating leptin levels (Zheng et al., 1996b). Finally, no autocrine effect of leptin on its own regulation has been observed (Slieker et al., 1996).

1.7 THE LEVELS AT WHICH LEPTIN REGULATION OCCURS

1.7.1 PROTEIN

The secretory pathways of the adipocyte are not well characterized, and currently there is conflicting evidence as to whether a hormone responsive intracellular pool of leptin exists, or whether the majority of leptin trafficking is via the constitutive secretory pathway, and as an example, that the primary acute insulin effect on leptin secretion is to increase leptin protein content (Barr et al., 1997; Bradley and Cheatham, 1999; Bradley et al., 2001).
When epitope-tagged leptin is expressed in pituitary AtT-20 cells, which serves as a model of both regulated and constitutive protein secretion, leptin behaves as a regulated protein, and is responsive to 8-Br-cAMP (Chavez and Moore, 1997). Bradley et al observed an increase in leptin secretion, without a concurrent increase in Lep mRNA levels, in response to insulin in rat adipocytes, which was not affected by the inhibitor of transcription, actinomycin D (Bradley and Cheatham, 1999), and Russell et al determined that adipocytes from obese patients contain preformed stores of leptin, which are acutely regulated by insulin (Russell et al., 2001). However, later studies by Bradley et al indicate that the majority of leptin trafficking occurs via a constitutive secretory pathway and that the primary acute insulin effect on leptin secretion is to increase leptin protein content (Bradley and Cheatham, 1999). In support of this, other studies have shown that actinomycin D inhibits leptin secretion in vitro (Hardie et al., 1996a; Machinal et al., 1999).

Circulating leptin levels could also theoretically be regulated by degradation/clearance and bioavailability, since leptin is present in both free and bound forms (see sections 1.3.7 and 1.6.9).

### 1.7.2 mRNA

Given that serum leptin levels are thought to be mainly regulated at the level of protein production, the control of this process has been investigated.

The majority of evidence points to regulation being at the level of transcription. Actinomycin D, injected prior to refeeding prevents the increase in Lep mRNA levels in rat adipose tissue in vivo (Thompson, 1996) and also in vitro, actinomycin D prevents Lep expression and leptin secretion (Hardie et al., 1996a; Leroy et al., 1996; Machinal et al., 1999; Reul et al., 1997). However, Fain and Bahouth have observed converse findings, in that actinomycin D markedly reduces the decline in leptin release and Lep mRNA levels in vitro, but suggest that this could be due to a process involving mRNA stabilization (Fain and Bahouth, 1998b).

With regard to Lep mRNA levels being regulated at the level of transcription, the 5'-flanking regions of rodent and human Lep genes have been characterized, and have been shown to contain several potentially relevant response elements including Sp1, CCAAT/enhancer binding protein (C/EBP) - a family of transcription factors known to
transcriptionally activate a number of adipocyte genes during adipogenesis (Lane et al., 1999) and elements responsive to glucocorticoids (Drouin et al., 1989). In vitro reporter gene assays have demonstrated that the *Lep* promoter activity is activated by C/EBPα (de la Brousse et al., 1996; He et al., 1995; Hwang et al., 1996; Miller et al., 1996), Sp1 (Mason et al., 1998), adipocyte determination differentiation dependent factor/sterol regulatory element binding protein 1 (ADD1/SREBP1) (Kim et al., 1998), oestradiol (O'Neil et al., 2001), and by glucocorticoids and insulin (see below). *Lep* promoter activity appears also to be elevated in primary rat adipocytes derived from *fa/fa* rats compared to lean controls, and is under the control of an unidentified factor binding to a novel sequence motif designated LP1 (Mason et al., 1998).

Recently it has been reported that a small section of the rat *Lep* promoter, consisting of an Sp1 and LP1 site, when present as a tandem repeat, confers insulin-responsiveness to the basic promoter of a reporter gene construct in both hepatocytes and adipocytes, consistent with the role of the Sp1 response element in the insulin responsiveness of the FAS and ATP citrate lyase promoters (Fukuda and Iritani, 1999; Fukuda et al., 1996; Fukuda et al., 1997).

With regard to glucocorticoid regulation of *Lep* expression, De Vos and colleagues found an increase in *Lep* promoter activity by dexamethasone in a reporter gene assay, but this was not mediated via a consensus glucocorticoid-response element, thus suggesting a non-classical mechanism of transcriptional activation that remains to be elucidated (De Vos et al., 1998).

1.8 The Role of Insulin and Its Signalling Pathways

Given the evidence for a role of insulin in the regulation of leptin levels, this next section will summarize the key components of the insulin signal transduction pathways and their significance in energy homeostasis and particularly in gene expression, with emphasis on the situation in adipose tissue.

Insulin is a small polypeptide hormone, consisting of an A-chain and a B-chain, linked by two disulphide bonds. It is synthesized as preproinsulin, and undergoes proteolytic cleavage in pancreatic β-cells, where it is stored and eventually secreted in response to high blood glucose levels (reviewed in Endocrinology, 2000: Eds; DeGroot and Jameson). The effects of insulin on glucose homeostasis are largely mediated through
stimulation of glucose uptake into skeletal muscle and adipose tissue, increased glycogen synthesis in liver and muscle, increased fat synthesis in adipose and liver, and a concomitant reduction in hepatic glucose output. These effects of insulin on metabolism occur through acute effects, e.g. the modulation of enzyme activity, such as glycogen synthase and pyruvate dehydrogenase dephosphorylation (Denton et al., 1989; Lawrence, 1992), acetyl-CoA carboxylase phosphorylation (Borthwick et al., 1990), and translocation of the glucose transporter, Glut-4 (Cushman and Wardzala, 1980; Suzuki and Kono, 1980), but also through longer-term effects on gene expression by the control of DNA transcription and translation (O'Brien and Granner, 1991; Proud and Denton, 1997).

Until quite recently, the mechanisms by which insulin mediated its signal were relatively unknown. Presently, however, a vast body of knowledge is accumulating on the various signal transduction pathways that exist for mediating the insulin signal.

1.8.1 INSULIN SIGNALLING

A simplified diagram representing the signalling pathways utilized by insulin is depicted in Figure 1.6, and is briefly described below.

1.8.1.1 RECEPTOR ACTIVATION

The initial step in the insulin-signalling pathway is the high affinity binding of insulin to its receptor. The insulin receptor is located at the cell surface of most cell types (reviewed in Kahn et al., 1981) and is a heterotetrameric tyrosine kinase, consisting of two extracellular insulin binding α-subunits and two transmembrane β-subunits, linked by disulphide bonds (Jacobs and Cuatrecasas, 1981). It is highly evolutionarily conserved (Muggeo et al., 1979). Upon insulin binding, tyrosine residues on the intracellular part of the β-subunits become autophosphorylated as a result of the activation of the tyrosine kinase domain (Kasuga et al., 1983; Kasuga et al., 1982). The tyrosine kinase activity of the insulin receptor is essential for subsequent signalling. Studies using monoclonal antibodies which inhibit the tyrosine kinase activity of the insulin receptor (Morgan and Roth, 1987), and mutant receptors lacking kinase activity (Chou et al., 1987; Ebina et al., 1987), show impaired postreceptor effects including glucose transport, p70 S6 kinase (p70 S6K) activation, glycogen synthesis and thymidine uptake. Also, naturally occurring mutations of the insulin receptor which
inhibit tyrosine kinase activity are associated with severe insulin resistance (Moller et al., 1990; Odawara et al., 1989).

1.8.1.2 Adapter Molecules

The activated insulin receptor recruits and phosphorylates a number of proteins which serve as adapter molecules, whose role it is to associate with, and activate a large number of signalling proteins. The use of adapter molecules is thought to alleviate the stoichiometric restraints imposed by the receptor, they are also used by other receptor signalling pathways, perhaps enabling cross-talk between systems therefore, and they enable the intracellular signalling complex to dissociate from the endocytic pathway of the activated receptor (reviewed in White, 1998). These adapter molecules possess pleckstrin homology (PH) domains and phosphotyrosine binding (PTB) domains through which they associate with the activated receptor. Once tyrosine phosphorylated by the insulin receptor, they recruit other signalling molecules, which bind through specific phosphotyrosine recognition domains known as SH2 and SH3 (Src homology) domains. These adapter molecules include members of the insulin receptor substrate (IRS) family, (Lavan et al., 1997a; Lavan et al., 1997b; Sun et al., 1991; Sun et al., 1995), Shc (Src homology containing sequence) (Pelicci et al., 1992), Gab 1 (Hosomi et al., 1994) and others, such as the tissue-specific substrates pp120 (liver) (Perrotti et al., 1987), 442(ap2) and pp115 (adipose) (Bernier et al., 1988; Hresko et al., 1988; Yamauchi et al., 1995).

The 131 kDa adaptor molecule, IRS-1 is by far the best characterized. It possesses multiple potential tyrosine phosphorylation sites, which serve as specific recognition sites for proteins bearing SH2 domains (Sun et al., 1991). Binding of SH2-proteins to IRS-1 serves several purposes. Firstly the SH2-proteins may be activated (e.g. phosphatidylinositol 3-kinase (PI 3-K), SHP2 and Grb-2). Also IRS-1 may serve to juxtapose various signalling molecules, target molecules to specific intracellular sites, and in addition the complex is free to move from the receptor (White, 1998). Other members of the insulin receptor substrate family include IRS-2, -3 and -4 (Lavan et al., 1997a; Lavan et al., 1997b; Sun et al., 1995). Through mice gene knockout studies, a degree of functional overlap between IRS-1 and IRS-2, and between IRS-1 and IRS-3 has been observed (Araki et al., 1994; Bruning et al., 1997; Smith-Hall et al., 1997; Tamemoto et al., 1994). Further roles of IRS-3 and IRS-4 are still being determined. The
IRS proteins are not phosphorylated by epidermal or platelet derived growth factors (EGF and PDGF), although IRS-1 and IRS-2 can be phosphorylated through activation by insulin-like growth factor 1 (IGF-1), growth hormone (GH), IL-4 and other cytokines (Argetsinger et al., 1995; Isakoff et al., 1995; Izumi et al., 1987; Ridderstrale et al., 1995; Sun et al., 1995; Wang et al., 1995).

The Shc family is comprised of members with molecular weights of approximately 50kDa (Pelicci et al., 1992). Shc becomes tyrosine phosphorylated in response to several growth factors, including insulin (Skolnik et al., 1993). Like IRS-1, it can associate with the SH2-domain of Grb-2, and activates the Ras/MAP (Rous avian sarcoma/ mitogen-activated protein) pathway (Skolnik et al., 1993), (see section 1.8.1.4).

The two main arms of the insulin-signalling pathway resulting from IRS recruitment to the insulin receptor, begin with the activation of PI 3-kinase and Ras. PI 3-kinase generates phosphatidylinositol intermediates - mainly phosphatidylinositol 3,4,5-trisphosphate (PIP_3), whereas Ras activation induces the well-characterized MAP-kinase (mitogen-activated protein kinase) cascade. Overlap between the two pathways exists however, with recent evidence implying that PI 3-kinase, as well as protein kinase Cζ, are involved in insulin activation of MAP kinase in adipocytes (Sajan et al., 1999).

1.8.1.3 PI 3-KINASE CASCADE

Several different classes of PI-kinases exist. The class 1a PI 3-kinases are thought to be the most important in insulin signalling, due to their rapid recruitment to signalling complexes after insulin stimulation, and their ability to generate PIP_3. They exist as heterodimers, comprising of a regulatory subunit and a catalytic subunit, known commonly as p85 and p110 respectively, although several isoforms of varying sizes exist (reviewed in Shepherd et al., 1998). The association of p85 with tyrosine phosphorylated IRS-1 activates PI 3-K (Giorgetti et al., 1993; Myers et al., 1992), as does association with phosphorylated YMXM-motifs (Rordorf-Nikolic et al., 1995). The regulatory subunit's major functions are to recruit the p110 catalytic subunit to the cell membrane, where its substrate is located, and to induce its catalytic activity (Giorgetti et al., 1993; Kelly and Ruderman, 1993; Nave et al., 1996; Ricort et al., 1996).

PIP_3, which is generated by p110, can interact with, and modulate a large number of molecules, mainly through pleckstrin homology domains. The serine/threonine kinase, protein kinase B (PKB or akt), and PKC isoforms are recruited to the cell membrane and
are activated through this process, with at least PKB activation involving phosphorylation by phosphoinositide-dependent protein kinase-1 (PDK-1) (Alessi and Cohen, 1998). PIP$_3$ can bind to PH domains in both PDK-1 and PKB, and it is thought that this binding not only co-localizes these enzymes, but also induces a conformational change in PKB which gives PDK-1 access to phosphorylate a key residue on PKB, and hence activate it. PKB is known to directly and acutely modulate key enzymes of metabolism, such as its ability to inhibit glycogen synthase kinase-3 (GSK-3) (Cross et al., 1995; Cross et al., 1997), and activate p70 S6K (Kohn et al., 1998). Inhibition of GSK-3 leads to the activation of glycogen synthesis, and GSK-3 is also involved in the regulation of several intracellular signalling pathways including the rate of transcription of certain genes (Lochhead et al., 2001), whereas p70 S6K is involved the regulation of protein synthesis (Proud and Denton, 1997) (see section 1.8.2).

Growing evidence also implicates PKB and the PKC isoforms $\zeta$ and $\lambda$ in the translocation of the glucose transporter, Glut 4, to the membrane in response to insulin (Bandyopadhyay et al., 1999; Hill et al., 1999; Wang et al., 1999b).

1.8.1.4 MAP KINASE CASCADE

Certain isoforms of MAP-kinase - Erk-1 and Erk-2 (extracellular signal regulated protein kinases) - are activated by insulin via Ras and Raf-1. This well characterized pathway (Morrison and Cutler, 1997; Robinson and Cobb, 1997), involves the protein Grb-2, which is recruited to the insulin receptor via adaptor proteins including members of the IRS family and Shc. The guanine nucleotide exchange factor, Sos, is tightly bound to Grb-2, and promotes the exchange of GDP for GTP on the GTP hydrolysing protein, Ras, when these are brought into conjunction on the plasma membrane after Grb-2 recruitment.

Ras contributes to the activation of Raf-1, a member of the MEKK family (MAP/ERK kinase kinase) (also known as MAP-kinase kinase kinase (MAP-KKK)). Activation is thought to involve the translocation of this molecule to the plasma membrane, involving phosphatidic acid. This brings it into juxtaposition with other components of the signalling complex where activation via Ras can occur (Rizzo et al., 2000; Roy et al., 1997). PI 3-kinase is also thought to play a role in the interaction between Ras and Raf-1, in adipocytes at least, demonstrating potential cross-talk between these pathways (Suga et al., 1997).
Raf-1 then in turn activates a member of the MEK family (also known as MAPKK). MEKs are dual specificity kinases, which can phosphorylate and activate MAP kinase isoforms on threonine and tyrosine residues. The MAP kinase isoforms activated by insulin through MEK are Erk-1 and Erk-2. Activation of these MAP kinases then results in the activation of pp90-ribosomal S6 kinase (pp90\textsuperscript{S6}) (Sturgill et al., 1988), and transcription factors such as c-jun and c-fos (Bernstein et al., 1994), (reviewed in Denton and Tavare, 1995).

1.8.2 **REGULATION OF TRANSLATION BY INSULIN**

A body of evidence is accumulating to show the involvement of insulin in the control of translation, both at a global level of increased translation, and also at the level of specific mRNAs (reviewed in Proud and Denton, 1997). For instance, insulin stimulates global translation by the activation of the initiation factor eIF2B and the subsequent release of GDP from eIF2, which in its GTP-bound form recruits the initiator Met-tRNA to the ribosome (Price and Proud, 1994). This is likely to occur by the dephosphorylation of eIF2B (Singh et al., 1996; Welsh and Proud, 1993) as a consequence of insulin-stimulated GSK-3 inhibition.

Peptide-chain elongation is also activated by insulin, again by dephosphorylation and ensuing activation, this time, of the elongation factor eEF2. This is understood to involve an insulin-stimulated decrease in eEF2 kinase activity, with a role for p70S6K being postulated in this process (Redpath et al., 1996).

The translation of specific types of mRNA are also regulated by insulin. For example, translation of mRNAs rich in 5\textsuperscript{\prime} secondary structure is thought to be facilitated by the formation of the eIF4F complex, which contains the ATP-dependent RNA helicase, eIF4A. eIF4F complex formation is prevented by the association of one of its constituents - eIF4E - with 4E-binding proteins (4E-BPs). Insulin, through phosphorylation of 4E-BPs, prevents the association between 4E-BP and eIF4E, thus enabling eIF4F complex formation to proceed (Proud and Denton, 1997). Ornithine decarboxylase is a well-characterized protein whose production is regulated in this manner (Manzella et al., 1991).

Finally, synthesis of certain proteins involved in translation has been shown to be upregulated by insulin. These mRNAs contain a sequence of pyrimidine bases at their 5\textsuperscript{\prime}
end, e.g. eEF2 (Levenson et al., 1989), although the mechanism by which insulin controls their increased translation has yet to be fully elucidated.

1.8.3 REGULATION OF GENE EXPRESSION BY INSULIN

The steady-state levels of mRNA of a large number of genes are known to be regulated by insulin. Insulin is known to modify the steady-state levels of mRNA by both transcriptional and post-transcriptional control.

1.8.3.1 CONTROL OF TRANSCRIPTION

Insulin is known to regulate numerous genes at the transcriptional level (reviewed in O'Brien 1996; and O'Brien, 2001).

Insulin administration to streptozotocin-diabetic mice increases FAS and glycerol-3-phosphate acyltransferase mRNA levels in liver (Paulauskis and Sul, 1989), and at a direct level increases the rate of synthesis of FAS mRNA in 3T3-L1 adipocytes (Paulauskis and Sul, 1988), and FAS promoter activity in reporter gene constructs (Moustaid et al., 1993). Glucose has however, been observed to be necessary for insulin's effect on FAS transcription in vitro, since replacing glucose in the medium with lactose abolishes this response (Paulauskis and Sul, 1988), which is in agreement with the regulation of the enzyme's activity by insulin (Giffhorn-Katz and Katz, 1986) and suggests that an increase in glycolysis is likely to be necessary for FAS expression.

Conversely, the expression of other genes are inhibited by insulin. For instance, mRNA levels of the serine protease, adipsin, progressively decrease with exposure to insulin in 3T3-F442A adipocytes (Lowell and Flier, 1990), and insulin abolishes the induction of basal- and cAMP- or glucocorticoid- stimulated expression of phosphoenolpyruvate carboxykinase (PEPCK) in hepatocytes (Sasaki et al., 1984). At a direct transcriptional level, insulin can also prevent expression of reporter genes containing the PEPCK promoter stably transfected into a hepatoma cell line (O'Brien et al., 1990).

The molecular mechanisms of transcriptional regulation by insulin are not fully understood. However, research into a number of insulin-responsive genes has revealed several distinct consensus insulin response sequences (reviewed in O'Brien, 2001).
The E-box DNA binding motif has been implicated as an insulin-response element. DNase I footprinting and gel mobility shift assays have revealed nuclear factors from liver and adipocytes that specifically complex with this sequence on the FAS promoter (Moustaid et al., 1994). Moreover, tandem repeats of this sequence, linked to the SV40 promoter in a reporter gene construct confers insulin responsiveness on luciferase activity (Moustaid et al., 1994). Gel shift competition and supershift assays implicate the involvement of upstream nuclear factor (USF1 and USF2) binding (Wang and Sul, 1997). Cotransfection of USF expression vectors with FAS-promoter reporter genes in 3T3-L1 adipocytes increases insulin-stimulated activity, whereas cotransfection with USF dominant negative mutants inhibits insulin-stimulated activity (Wang and Sul, 1997).

However, the Sp1 site overlapping the E-box in the FAS proximal promoter has also been implicated as a glucose/insulin response element, and it is suggested that there is competition between these two sites (Fukuda et al., 1997). Insulin is thought to mediate its effects on FAS promoter activity via the PI 3-kinase pathway (Wang and Sul, 1998). Wortmannin and LY294002, specific inhibitors of PI 3-kinase, prevent insulin-stimulation of the FAS promoter and also endogenous FAS mRNA levels, whereas inhibitors of MAP kinase and p70 S6 kinase (PD98059 and rapamycin respectively) have little effect (Wang and Sul, 1998). Furthermore, cotransfection with an expression vector of the constitutively active p110 subunit of PI 3-kinase results in elevated FAS promoter activity regardless of insulin, whereas a dominant negative p85 subunit expression vector abolishes the insulin response (Wang and Sul, 1998). Overexpression of protein kinase B/akt (PKB/akt), a kinase downstream of PI 3-kinase, also increases FAS promoter activity in the absence of insulin, to levels comparable to insulin-stimulated levels, and expression of a kinase dead dominant negative form of this enzyme, abolishes insulin-stimulated FAS promoter activity (Wang and Sul, 1998).

A different cis-acting element is involved in insulin-mediated suppression of glucocorticoid-induced PEPCK, insulin-like growth factor binding protein-1 (IGFBP-1) expression, and others (O'Brien R et al., 2001; O'Brien et al., 1995). This insulin-response element (IRE) provides a binding site for subclasses of the winged helix/forkhead (Fox) family of transcription factors – i.e. members of the forkhead
transcription factor (FKHR) and hepatic nuclear factor-3 (HNF-3) subclasses. It has been shown that overexpression of FKHRL1 (a member of the FKHR transcription factor family) stimulates promoter activity in constructs containing PEPCK or IGFBP-1 IREs, and that insulin inhibits this. (Hall et al., 2000). The mechanism of action of this response involves signalling via PI 3-K, involving PKB and other as yet unspecified kinases (Gabbay et al., 1996; Nakae et al., 2000; Sutherland et al., 1995). Phosphorylation of FKHR by insulin and the consequent stimulation of nuclear export of this factor has been put forward as the model by which insulin inhibits transcription of these genes (Nakae et al., 2000).

In another case, GABP, a member of the Ets family of transcription factors has been shown to interact with the defined insulin-response element of the prolactin promoter in GH cells (Ouyang et al., 1996), and may be regulated via the MAP kinase pathway.

Insulin is also involved in the regulation of the C/EBP family of transcription factors, both at the level of transcription in adipocytes (suppressing expression of C/EBPα and transiently inducing expression of C/EBPβ and δ), and through dephosphorylation of C/EBPα (MacDougald et al., 1995a). Furthermore, the insulin-response element-A (IRE-A) located in the upstream region of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (Nasrin et al., 1990) binds to an IRE-A binding protein, which, in other genes has been shown to bind to sequences overlapping with the binding sequence for C/EBPα (Buggs et al., 1998).

### 1.8.3.2 Control of mRNA Stability

The steady-state levels of mRNA can also be altered by message stabilization. There have been several reports of insulin's role in mRNA stability as well as altering the rate of transcription. For instance, insulin has been observed to both increase the stability and rate of transcription of glycerol 3-phosphate dehydrogenase (GPDH) (Bhandari et al., 1991) and Glut 1 (Maher and Harrison, 1990) mRNAs, but decreases the stability of glycogen synthase kinase-3 alpha mRNA (Rao et al., 1995). The mechanism by which insulin mediates altered mRNA stability is currently unknown.
Figure 1.6  Insulin signalling pathways
Schematic diagram of the main signalling pathways employed by insulin, including the well characterized MAP kinase and PI3-kinase pathways (see text for details and abbreviations).
1.9 AIMS

Leptin has been shown to play vital roles in numerous physiological pathways especially involving energy homeostasis, hence determining the factors governing its regulation would greatly increase our understanding of these physiological processes.

As insulin levels closely parallel changes in the nutritional state, this aspect of Lep regulation was to be investigated. The in vivo effects of insulin on Lep mRNA levels have been extensively studied in rats, however only minimal research has been carried out in mice. Given that the majority of rodent models of obesity are murine, insight into the regulation of Lep expression in mice would enable more meaningful comparisons between the lean and obese states, as well as adding to the general body of knowledge of insulin regulation of Lep expression.

The initial aim of the present study was therefore to build on these results, through a more extensive study of the effects of insulin in fasted mice, comparing against the effects of re-feeding, and against fed controls. Since many of the murine models of obesity are available on the C57BL/6J background, lean C57BL/6J mice were selected for use in these experiments.

Additionally, in light of the inconsistencies in the reports of a direct effect of insulin on the regulation of the Lep gene in rodents, this project aimed to add to the current knowledge of this effect, by the employment of mouse adipocyte model systems. The use of an in vitro model could also be utilized to dissect further the signal transduction pathways involved in a direct effect of insulin on Lep mRNA levels.

In order to quantify relative levels of Lep mRNA in response to insulin, an RNase protection assay was to be developed and optimized. Lep promoter activity was also to be examined, and several in vitro adipocyte models would be investigated for suitability of use.
2 MATERIALS AND METHODS
All chemicals were dissolved in double distilled water (Milli-Q Plus 185, Millipore UK Ltd., Watford, Herts UK) and were autoclaved unless otherwise specified (see Appendix). RNA solutions were DEPC treated (see Appendix). Unless supplied sterile, all reagents used in tissue culture were filtered through 0.2μm acrodisc filters. Unless otherwise stated, centrifugation was carried out in a bench top microfuge at 4°C.

2.1 MATERIALS

2.1.1 CHEMICAL REAGENTS

General laboratory chemicals were all of analytical or molecular biology grade, and were purchased from Merck Ltd., Poole, Dorset, England or Sigma Chemical Company, St. Louis, MO, USA, unless otherwise stated.

Agarose was obtained from Gibco BRL, Life Technologies Ltd., Paisley, Scotland.

Acrylamide solutions – denaturing, Gene-PAGE 6% (19:1 acrylamide: bisacrylamide), and non-denaturing, 30% Acryl/Bis solution (37.5:1) were obtained from Amresco, Solon, OH, USA.

Marvel dried skimmed milk was obtained from Premier Beverages, Adbaston, Stafford, England.

Phenol was obtained from Fisher Scientific UK Ltd., Loughborough, Leics, England.

5x reporter lysis buffer and luciferase assay reagent were obtained from Promega Corp, Madison, WI, USA.

Ultima Gold scintillation fluid was obtained from Packard Biosciences B.V., Groningen, the Netherlands.

Photographic developing and fixing chemicals were obtained from X-OGRAPH Ltd., Tetbury, U.K.

2.1.2 BIOLOGICAL REAGENTS

Restriction endonucleases, DNA modification enzymes, T4 DNA ligase, DNase I, RNasin and their optimized 10x buffers were all obtained from Promega Corp, Madison, WI, USA except Pfu, which was from Stratagene, La Jolla, Ca, USA and RNase Cocktail, from Ambion Inc, Austin, TX, USA.

dNTPs and rNTPs were obtained from Promega Corp, Madison, WI, USA.

Protein A/G Plus-Agarose was obtained from Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA.
Radioactive isotopes, [α-32P]CTP, [γ-32P]dATP, [α-35S]dATP, (all 3000Ci/mmol, 10mCi/ml), were purchased from Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Bucks, England.

DNA markers; λHindIII, φX174HaeIII and 100bp ladder, were obtained from MBI Fermentas, Sunderland, Tyne and Wear, England.

Rainbow coloured protein molecular weight markers were purchased from Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Bucks, England.

Human insulin for in vivo studies (Actrapid 100IU/ml) was obtained from Novo-Nordisk, UK.

Bovine insulin for in vitro studies was obtained from Calbiochem-Novabiochem Corp., La Jolla, California, USA.

2.1.3 Buffers

The composition of buffers used in this body of work can be found in the appendix.

2.1.4 Consumables

DE-81 ion-exchange membranes and 3MM paper were obtained from Whatman International, Maidstone, Kent, England.

Ponyvial scintillation vials were obtained from Packard Biosciences B.V., Groningen, the Netherlands.

Hybond N+ and C membranes were obtained from Amersham Biotech UK Ltd., Little Chalfont, Bucks, England.

General laboratory plastic ware was purchased from Merck Ltd., and Eppendorf, Cambridge, U.K.

Gilson pipette tips were obtained from Elkay Products Ltd., Boston, MA, USA.

0.2μm acrodisc filters were obtained from Gelman Sciences, Ann Arbor, MI, USA.

Clear 96 well microtitre plates and sterile 10ml pipettes were obtained from Bibby Sterilin Ltd., Stone, Staffs, England.

White 96 well microtitre plates were obtained from Zeptogen Ltd., Ruislip, Middx, England.

Polaroid black and white print film (type 667) was purchased from Polaroid, Cambridge, MA, USA.

Kodak X-Omat R autoradiographic film was obtained from Eastman Kodak Co. Rochester, NY, USA.
2.1.5 Kits

T7 Sequenase v2.0 manual sequencing kit, Enhanced Chemiluminescence system (ECL) and Rediprime II random prime labelling kit were purchased from Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Bucks, England.

Qiagen mini and midi-plasmid preparation kits were obtained from Qiagen Ltd., Crawley, West Sussex, England.

Hybaid Recovery DNA Purification Kit II was obtained from Hybaid Limited, Teddington, Middlesex, England.

BCA Protein Assay Reagent and Coomassie Plus Protein Assay Reagent were purchased from Pierce and Warriner (UK) Ltd., Chester, England.

2.1.6 Bacterial Culture

*Escherichia coli* (*E. coli*) strain JS5:

\[F^- \text{araD139} \Delta(\text{ara-leu})7696\ \Delta(\text{lac})X74 \text{galU galK hsdR2} (r_{+}k^-m_k^+){\text{mcrB1 rpsL (Strf)}}\]

was a kind gift from Dr Pamjit Jat, Ludwig Institute, UCL, UK.

Yeast extract, tryptone and bacto-agar were obtained from Difco Laboratories, Detroit, MI, USA.

2.1.7 Tissue Culture

The following cell lines were used in this study:

The immortalized mouse embryonic cell line, NIH-3T3 was a kind gift from Dr. J. Newton, Dept. of Molecular Pathology, UCL, UK.

The preadipocyte cell line 3T3-F442A, derived from NIH-3T3 cells which when confluent, differentiates into adipocytes, was a kind gift from Dr. A. Holder, The Babraham Institute, Cambridge, UK.

Freshly prepared murine primary adipocytes were a kind gift from Prof. P. Maclean, Dept. of Molecular Pathology, UCL, UK.

Foetal calf serum, high glucose Dulbecco's modified Eagle medium (DMEM) (4500mg/l), trypsin (25%), Hank’s Balanced Salt Solution (HBSS), penicillin/streptomycin (10000 IU/ml, 10000μg/ml) and Geneticin G-418 sulphate were from Gibco BRL, Life Technologies Ltd., Paisley, Scotland.

Gene Pulser cuvettes (0.4cm electrode gap) were purchased from Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts, England.
Flasks were from Nalgene Nunc International, Poole, Dorset, England.

2.1.8 ANIMALS

C57BL/6J mice were purchased from Harlan UK, Bicester, Oxon, England.

2.1.9 DNA

Table 2.1 Plasmids

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<td>GFP expressing plasmid, with neomycin resistance gene</td>
<td>Dr. Suzanne Thomas, Dept. Molecular Pathology, UCL, UK</td>
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Mouse genomic DNA was a kind gift from Dr. T. Lund, Dept. Molecular Pathology, UCL, UK.

Oligonucleotides were synthesized by Genosys Biotechnologies Inc, The Woodlands, TX, USA. Commercially available sequencing primers were purchased from Promega Corp, Madison, WI, USA.
In-house designed oligonucleotides, shown 5’ to 3’ were as follows;

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### 2.1.10 ANTIBODIES

Table 2.3 Antibodies

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2.2 DNA METHODOLOGY

2.2.1 RESTRICTION ENDONUCLEASE DIGESTS

Small-scale restriction endonuclease digests were carried out to characterize a newly isolated plasmid. Digests were generally performed on 1μg of plasmid, in a 20μl volume, for at least 1 hour using the enzyme's manufacturer supplied buffers and conditions. Large scale digests (100-200μg DNA), of both plasmids and PCR products, were carried out in a 100μl volume for a minimum of 2 hours.

2.2.2 PHOSPHATASE TREATMENT

5' phosphate groups on restriction endonuclease digested plasmid DNA were removed to prevent recircularization of the plasmid during ligation. 10 units of calf intestinal alkaline phosphatase per 100pmoles of 5' phosphate groups were incubated for 30 minutes in 1x reaction buffer, supplied by the enzyme's manufacturer. The enzyme was heat-inactivated at 65°C for 10 minutes, and then the DNA was phenol/chloroform extracted and ethanol precipitated.

2.2.3 PHENOL/CHLOROFORM EXTRACTION

Contaminating protein was removed from DNA samples by the addition of an equal volume (usually 500μl) of DNA purpose (TE-equilibrated) phenol/chloroform/isoamyl alcohol. The mix was vortexed for 30 seconds and then microfuged for 5 minutes. After recovery of the aqueous (top) layer, traces of phenol were removed with a further extraction with an equal volume of chloroform alone.

2.2.4 PRECIPITATION OF DNA

DNA was mixed with 0.1 volumes of 3M sodium acetate (pH 5.6) and 2.5 volumes of 100% ethanol or alternatively with 0.6 volumes of propan-2-ol alone. The precipitated DNA was stored at -20°C for 1 hour before being pelleted in a microfuge for 15 minutes. When small quantities of DNA (less than 500ng) were precipitated, 5μl of glycogen solution (20mg/ml) was added before centrifugation, to act as a carrier, and to aid in visualizing the pelleted DNA. The pellet was briefly washed in 70% ethanol, air dried, and resuspended in a suitable volume of 1x TE.
2.2.5 LIGATION

A 10 molar excess of DNA fragment was added to 100ng of restriction endonuclease digested plasmid. This was incubated in 1x ligase buffer (supplied by the enzyme’s manufacturer), with 2 units of T4 DNA ligase, overnight at 16°C. A control reaction of digested plasmid alone, was included to aid in interpretation of the subsequent transformation.

2.2.6 PLASMID DNA EXTRACTION FROM E.COLI

2.2.6.1 SMALL SCALE EXTRACTION - MINIPREP

1.5ml of culture was pelleted in a microfuge for 5 minutes. The pellet was resuspended in 100µl ice cold GTE buffer (see appendix) and lysed with 200µl E. coli lysis buffer for 5 minutes at room temperature. Cellular debris and chromosomal DNA were precipitated with 150µl 3M potassium acetate (pH 4.8) on ice for 5 minutes, and pelleted out in a microfuge for 15 minutes. The supernatant was phenol/chloroform extracted, ethanol precipitated, and resuspended in 50µl of 1x TE containing 20µg/ml of RNase A.

2.2.6.2 LARGE SCALE EXTRACTION - MAXIPREP

Plasmid DNA was recovered from 500ml of culture according to the alkaline lysis and polyethylene glycol (PEG) precipitation method (Sambrook and Fritsch, 1989). Briefly, pelleted bacteria were resuspended in 10ml GTE. 1ml of lysozyme solution was added with 20ml E. coli lysis buffer, and cells were allowed to lyse for 10 minutes at room temperature. 15ml of 3M potassium acetate (pH 4.8) was added to precipitate cellular debris and chromosomal DNA. After 10 minutes on ice, the debris was pelleted in a centrifuge at 14,000g for 15 minutes. 0.6 volumes of propan-2-ol was added to the supernatant, and plasmid DNA was allowed to precipitate out for 10 minutes at room temperature before being pelleted at 14,000g for 15 minutes. The pellet was 70% ethanol washed and resuspended in 3 ml of 1x TE (pH8.0).

High molecular weight RNA was precipitated out of the solution by the addition of 3ml of ice-cold 5M LiCl and immediate centrifugation at 14,000g for 10 minutes. The DNA in the supernatant was reprecipitated with propan-2-ol, pelleted and resuspended in 500µl of 1x TE (pH8.0) containing 20µg/ml RNase A. Contaminating RNA was digested for 30 minutes at room temperature. Plasmid DNA was then precipitated by the addition of 500µl PEG solution and pelleted in a microfuge for 5 minutes. The pellet
was resuspended in 400μl of 1x TE and extracted with an equal volume of DNA purpose phenol/chloroform, and once with an equal volume of chloroform. Finally, DNA was precipitated by the addition of 100μl 10M ammonium acetate and 2 volumes of ethanol. After centrifugation the pellet was 70% ethanol washed and resuspended in 500μl 1x TE.

2.2.7 RT-PCR

1.5μM PPARγ reverse primer and 250ng of total RNA, in a final volume of 15μl, were annealed by heating to 70°C for 10 minutes and then chilling on ice for 5 minutes. First strand synthesis was then performed by incubation for 60 minutes with 1x first strand buffer, 40 units of RNase inhibitor, 200μM dNTPs, 4mM sodium pyrophosphate and 30 units of MMTV reverse transcriptase in a final volume of 25μl. One-fifth of the resulting solution was then used as template in a normal PCR reaction.

2.2.8 PCR

For amplification of specific DNA fragments, primers were designed which shared at least 17 nucleotides of homology to the template. Restriction enzyme sites at the 5' ends were included in most primers for ease in subsequent cloning steps.

Reactions were carried out in a Biometra UNO-Thermoblock PCR machine, using the following reaction conditions;

50ng template DNA, 0.2μM forward primer, 0.2μM reverse primer, 250μM dNTPs, 2.5units Pfu, 1x Pfu reaction buffer; in 100μl final volume overlaid with paraffin oil.

Cycles; 
1x 94°C, 5 min;
30x 94°C, 1 min; n°C, 45 sec; 72°C 1 min;
1x 72°C, 5 min;
Hold 4°C.

The annealing temperature (n°C) for each specific set of primers is shown in Table 2.4.

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<th>Primer Set</th>
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<td>50°C</td>
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</table>

2.2.9 COLUMN PURIFICATION OF DNA

DNA was column purified from agarose gels or PCR reactions using the Hybaid Recovery DNA Purification Kit II. Briefly, sample was added to 400μl binding buffer in a spin filter. If agarose was present, the filter was placed at 55°C for 5 minutes to melt it. The filter was then spun in a microfuge for 30 seconds and the spin through discarded. Bound DNA was washed by spinning 500μl of wash buffer through the filter, and was finally eluted in 25μl of elution buffer.

2.2.10 SEQUENCING PLASMID DNA

Plasmid DNA was sequenced using the T7 Sequenase v2.0 kit, according to the manufacturer's instructions with slight modifications. Initially, 1μg DNA was denatured by incubation for 30 minutes with 0.2N NaOH and 0.2mM EDTA in a final volume of 25μl. The single stranded DNA was then ethanol precipitated at -80°C for 30 minutes. After centrifugation, the pelleted DNA was resuspended in 1x T7 Sequenase reaction buffer and 0.5pmol of sequencing primer in a 10μl volume and was annealed by heating the solution to 65°C for 2 minutes and allowing it to cool gradually to room temperature. Labelling was performed by the addition of 0.5μl [α-35S]dATP, 6.5mM DTT, 2μl labelling mix (diluted 1:1) and 8 units of T7 Sequenase DNA polymerase in a volume of 15.5μl. The reactions were terminated by adding 3.5μl of the labelling mix to 2μl each, of the four dideoxy termination mixes (A, C, G and T) for 3 minutes at 37°C, and then adding 4μl stop solution. 2.5μl of each of the four reactions was run, denatured, on 6% denaturing acrylamide gels, adjacent to each other (see section 2.2.12).
2.2.11 AGAROSE GEL ELECTROPHORESIS

DNA was resolved, according to size and charge, on 0.8-1.5% agarose gels. Molten gel was prepared by heating an agarose, 1x TAE solution in the microwave and cooling to 60°C. Ethidium bromide, a fluorescent DNA intercalator, was added to a final concentration of 5μg/ml. The gel solution was set in a gel casting tray with combs and placed in an electrophoresis tank in 1x TAE running buffer. DNA samples and appropriate size markers containing 1x loading dye, were loaded into the wells formed by the comb. The gel was electrophoresed at 80V/cm until the leading dye had migrated to within 1cm of the end of the gel. DNA was visualized under ultraviolet light on a transilluminator, and captured on Polaroid film with a Polaroid MP.4 land camera, Polaroid, Cambridge, MA, USA.

2.2.12 POLYACRYLAMIDE GEL ELECTROPHORESIS

Sequence reactions and RNase protected fragments were resolved by denaturing polyacrylamide gel electrophoresis. 6% denaturing acrylamide solution containing 0.1% ammonium persulphate and 0.4μl/ml TEMED was poured between 2 glass sequencing plates, separated by 0.4mm spacers at each side. A suitable comb was then inserted (standard shark tooth sequencing comb for sequence reactions or square toothed comb for RNase protected fragments), and the gel allowed to polymerize. The gel was pre-run for 1 hour at 1500 volts in 1x TBE. Samples were denatured at 100°C for 2 minutes, cooled on ice, and then separated on the gel for up to 5 hours in 1x TBE at 1500 volts. The gel was transferred to 3MM Whatman paper, covered in Clingfilm, and dried on a Genevac CVPI00 vacuum drier, Genevac Ltd., Ipswich, Norfolk, England, before being exposed to Kodak X-Omat R autoradiographic film. Unless otherwise stated, exposures were performed at room temperature for 35S and at -80°C for 32P.

2.2.13 ESTIMATION OF DNA CONCENTRATION

Purified DNA solutions were diluted in distilled water by a factor of 100. The OD260 of the solutions were measured in a quartz cuvette. The nucleic acid concentration (μg/ml) was calculated by multiplying the absorbance reading by a factor of 50, taking into account the dilution factor. The purity of the sample was estimated from the ratio of
OD$_{260}$/OD$_{280}$, a value greater than 1.7 indicating low protein contamination and sufficient purity. Agarose gel electrophoresis of DNA samples was also performed, against standards of known DNA concentration.

2.2.14 DNA SEQUENCE ANALYSIS

DNA sequence analysis was conducted using FASTA and SignalScan software, supported by BIDS.

2.2.15 KINASE END-LABELLING OF DNA

The DNA marker, 100bp ladder, was end-labelled with [$\gamma$-³²P]dATP to allow visualization by autoradiography. Briefly, 2µg of DNA was incubated in 1x buffer (supplied by manufacturer), 3 units of T4 polynucleotide kinase and 10µCi of [$\gamma$-³²P]dATP in a 10µl final volume at 37°C for 30 minutes. Heat inactivation of the enzyme was then carried out at 65°C for 10 minutes.

2.3 RNA METHODOLOGY

2.3.1 DISSECTION AND STORAGE OF TISSUE

Tissue was obtained from animals immediately after sacrifice and was snap frozen in liquid nitrogen and stored at -80°C.

2.3.2 RNA EXTRACTION

Total RNA was prepared using the one step guanidinium thiocyanate, phenol/chloroform method (Chomczynski and Sacchi, 1987), with additional purification steps. Tissue was homogenized from frozen in a Polytron, or cells immediately lysed in their flask, in 3ml of solution D (see appendix) and 300µl 2M NaAc (pH 4.1), and phenol/chloroform extracted using RNA purpose phenol/chloroform. After propan-2-ol precipitation, the pellet was resuspended in 500µl solution D, phenol/chloroform extracted and propan-2-ol precipitated. Two further ethanol precipitations (with 3M NaAc pH5.6) were carried out, between which the pellet was resuspended in 200µl DEPC dH$_2$O. RNA was stored at -20°C in the final ethanol precipitation solution.
2.3.3 Estimation of RNA Concentration

Purified RNA solutions were diluted in distilled water by a factor of 100. The OD$_{260}$ of the solutions were measured in a quartz cuvette. The nucleic acid concentration (µg/ml) was calculated by multiplying the absorbance reading by a factor of 40, taking into account the dilution factor. The purity of the sample was estimated from the ratio of OD$_{260}$/OD$_{280}$, a value greater than 1.7 indicating low protein contamination and sufficient purity.

2.3.4 RNA Formaldehyde Agarose Gel Electrophoresis

The integrity of prepared total RNA samples was determined by resolution of the two discrete bands of ribosomal RNA (28S and 18S) on an agarose gel. Typically, a 1.5% agarose gel containing 1x MEA and 18% v/v formaldehyde solution was prepared. 3µg of total RNA in 5µl DEPC dH2O was added to 20µl of RNA loading buffer and was denatured at 80°C for 3 minutes and on ice for 2 minutes. Samples were fractionated on a gel at 100 volts for one hour in 1x MEA running buffer. RNA was visualized under ultraviolet light, and photographed as described in section 2.2.11.

2.3.5 cDNA Radiolabelling

Radiolabelled cDNA was prepared using the Rediprime II random prime labelling kit, according to the manufacturer’s instructions, using 50µCi [α-³²P]CTP. Briefly, DNA (in a volume of 45µl) was denatured at 100°C for 5 minutes, and placed on ice for 5 minutes. The DNA was then added to the supplied reaction tube and 5µl of [α-³²P]CTP was added. The mix was incubated for 10 minutes at 37°C, and the reaction was stopped by the addition of 5µl of 0.2M EDTA.

2.3.6 Riboprobe Radiolabelling

1.5µg of riboprobe template was in vitro transcribed at either 4°C or 37°C by T7 RNA polymerase in a volume of 25µl. Reactions contained 1x transcription buffer, 0.5mM each of rATP, rUTP and rGTP, 40mM DTT, 20 units of T7 RNA polymerase and 40 units of RNasin. 2µM rCTP and 50µCi [α-³²P]CTP were added to make probes of high specific activity (leptin, PPARγ and FAS riboprobes). 10 µM rCTP and 5µCi [α-³²P]CTP was used to make a probe of lower specific activity (β-actin riboprobe).
After 1 hour of incubation, an additional 20 units of T7 RNA polymerase was added and incubated for a further 1 hour. DNA template was then removed by the addition of 50 units of RNase free DNase I and incubation for 15 minutes. Unincorporated nucleotides were removed by precipitation of the sample in 50μl solution D, 5μl glycogen solution and 100μl propan-2-ol. The washed pellet was resuspended in 1ml DEPC dH2O. 2μl of sample was added to 2ml of scintillation fluid in a scintillation vial, and counts per minute were measured on a Beckman LS5000CE scintillation counter.

2.3.7 MEASURING RADIONUCLEOTIDE INCORPORATION BY THE DE-81 FILTER ABSORPTION METHOD

A time course of incorporation of [α-32P]CTP into RNA was performed using the DE-81 method as described (Sambrook and Fritsch, 1989). plep200-Blue and pTRI-β-actin-Mouse (see section 3.2.2.1 and Table 2.1) were in vitro transcribed at 4°C and 37°C (see section 2.3.6). Duplicate 1μl samples were removed every 15 minutes, spotted onto two separate DE-81 ion-exchange membranes and allowed to air dry. One of each pair of membranes was washed four times in ice-cold 5% trichloroacetic acid/20mM sodium pyrophosphate, to remove unincorporated nucleotides, and allowed to air dry. Washed and unwashed membranes were placed in scintillation vials with 2ml of scintillation fluid and counted for 30 seconds on a Beckman LS5000CE scintillation counter. The percentage of incorporated radionucleotides was calculated as follows:

\[
\frac{\text{cpm in washed membrane}}{\text{cpm in unwashed membrane}} \times 100 = \text{percentage incorporated}
\]

2.3.8 NORTHERN BLOTTING

Total RNA was fractionated by formaldehyde agarose gel electrophoresis (see section 2.3.4) and transferred to Hybond N+ membranes by capillary blotting in 20xSSC overnight. Membranes were U.V. crosslinked (U.V. Stratalinker 2400, Stratgene), gently washed in 2x SSC and air-dried. Blots were prehybridized in 25ml of prehybridization buffer for 1 hour at 65°C. Hybridization was performed in 25ml of hybridization buffer plus probe (the entire amount from one reaction (see sections 2.3.5 and 2.3.6)) overnight at 65°C. Washes were carried out for 30 minutes in the following solutions at 65°C, 2x
SSC, 1% SDS; 1x SSC, 1% SDS; 0.5x SSC, 1% SDS. The membranes were then sealed in Clingfilm and autoradiographed. Blots were stripped of riboprobe in boiling 0.5% SDS and allowed to cool to room temperature.

2.3.9 RNase Protection Assay

RNase protection assays (RPAs) were carried out as described (Sambrook and Fritsch, 1989), with modifications. Briefly, 50,000 cpm each of leptin, PPARγ and FAS riboprobes, and 5,000 cpm of β-actin riboprobe were added to total RNA (usually 10 μg) and 20 μl RPA hybridization buffer, in a final volume of 25 μl. Samples were denatured at 80°C for 3 minutes and were immediately placed at 42°C to hybridize overnight. 200 μl RPA digestion buffer and 2 μl RNase Cocktail (RNase A/T1) were added to each sample for 30 minutes to digest any unhybridized, single stranded RNA. The RNase Cocktail was inactivated, and the protected fragments (RNA-RNA hybrids) precipitated, in 300 μl solution D, 5 μl glycogen solution and 500 μl propan-2-ol at -20°C for 1 hour. The precipitates were pelleted in a microfuge and were 70% ethanol washed. Pellets were resuspended in 8 μl of RNA loading buffer, denatured and resolved on 6% denaturing acrylamide gels (see section 2.2.12). The radioactive protected fragments were quantified on a Bio-Rad GS-250 molecular imager with phosphor analyst v1.1 software (Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts, England). Control samples containing yeast tRNA were treated with RPA digestion buffer with or without RNase Cocktail.

2.4 Bacterial Methodology

2.4.1 Growth Conditions

For the isolation of single colonies, *E. coli* JS5 were cultured overnight, on inverted LB-agar plates containing 25 μg/ml tetracycline. Large-scale preparations of *E. coli* were grown in suspension culture. Flasks of LB medium containing 25 μg/ml tetracycline were inoculated with a single colony using a sterile metal loop, and were incubated overnight in an orbital shaker. Bacteria transformed with ampicillin-resistant plasmids were cultured in the same manner, with the medium supplemented with 75 μg/ml ampicillin.
2.4.2 **Preparation of Competent Cells**

A 5ml overnight culture of a single JS5 *E. coli* colony was subcultured in 100ml of LB medium until an OD$_{550}$ of 0.4 was reached. At this stage the cells are in exponential growth phase. Cells were chilled on ice for 5 minutes and centrifuged at 2500g in a Beckman J-6B centrifuge for 5 minutes at 4°C. The pelleted cells were gently resuspended in 40ml of ice-cold buffer I and were left on ice for 5 minutes. Cells were centrifuged again for 5 minutes at 2500g, and resuspended in 2ml of ice-cold buffer II. After 15 minutes on ice, the cells were aliquotted into 50µl aliquots, snap frozen in liquid nitrogen, and stored at -80°C.

2.4.3 **Transformation of Plasmid DNA into Competent Cells**

Frozen competent *E. coli* cells (50µl aliquots) were thawed on ice. 50ng DNA, either supercoiled DNA, or half a ligation reaction, was added to the cells, gently mixed and left on ice for 45 minutes. The cells were heat shocked by immersion in a 42°C waterbath for 2 minutes and briefly cooled on ice. The cells were then incubated, in 1ml of LB medium for 1 hour at 37°C, shaking, to allow the expression of the ampicillin resistance gene, β-lactamase. Finally the cells were pelleted in a microfuge for 1 minute, resuspended in 100µl of LB medium, plated onto LB-agar plates containing ampicillin and tetracycline, and incubated overnight. For blue/white selection of recombinant plasmids, LB-agar plates were coated with 100µl 100mM IPTG and 40µl 50mg/ml X-Gal, 30 minutes prior to plating out the bacteria.

2.4.4 **Long Term Storage**

Overnight *E. coli* cultures supplemented with 1x Hogness buffer were stored indefinitely at -80°C in 1ml aliquots.

2.5 **Tissue Culture Methodology**

2.5.1 **Growth Conditions**

All manipulations were carried out using sterile techniques in a class II microbiological safety cabinet (Medical Air Technology Ltd., Manchester, UK). Cells/adipose explants were cultured in medium containing high glucose (4500mg/l) DMEM supplemented with 10% (v/v) foetal calf serum (FCS), 10 IU/ml penicillin and 10µg/ml streptomycin.
(standard medium), in an incubator (WTB Binder Labortechnik GmbH, Tuttlingen, Germany), with a humidified atmosphere containing 5% CO₂ at 37°C. Growth medium was replaced every 2-3 days.

2.5.2 PASSAGING

Cells were passaged at 70% confluence, whilst still in exponential growth phase. The cells were detached from the flasks by incubation with 0.1% (v/v) trypsin in HBSS for 10 minutes at 37°C. Two volumes of growth medium were added to dilute out the trypsin, and the cells were pelleted at 145g in a Beckman J-6B centrifuge for 5 minutes. The cells were then seeded in new flasks at a density of 1000 cells/ml.

2.5.3 DIFFERENTIATION

Differentiation of confluent 3T3-F442A preadipocytes was accelerated by the addition of 100nM insulin, or by the substitution of heat-inactivated FCS for FCS (HI-FCS medium), and further culture for 10 days (FCS was heat-inactivated at 55°C for 30 minutes). The level of differentiation was determined under phase contrast or fluorescent microscopy, by visualization of the formation of lipid droplets (aided by Oil Red O or Nile Red staining), or by the level of activity of the adipocyte marker, L-glycerol 3-phosphate dehydrogenase (GPDH).

2.5.4 OIL RED O STAINING

1% paraformaldehyde fixative was added to cells for 10 minutes at room temperature. The stock solution of Oil Red O was diluted to 0.6mg/ml in water, 5 minutes before use and was filtered. The cells were washed and then covered in the diluted Oil Red O solution for 20 minutes. Cells were washed in tap water, covered in 50% (v/v) glycerol and visualized under a light microscope.

2.5.5 NILE RED STAINING

Nile Red staining of adipocytes was carried out according to the method of Smyth and Wharton, (Smyth and Wharton, 1992). Briefly, paraformaldehyde fixative was added to cells for 10 minutes at room temperature. The cells were washed and left covered in 1x PBS. The stock solution of Nile Red was diluted to 10μg/ml in 1x PBS 20 minutes before use. An equal volume of Nile Red solution was added to the cells in 1x PBS.
Staining was visualized on a TMS-F inverted microscope (Nikon Europe B.V., Badhoevedorp, The Netherlands), under U.V. light 5 minutes after the addition of Nile Red.

2.5.6 Flow Cytometric Analysis

Cells were trypsinized, washed and resuspended in 1% paraformaldehyde fixative for 10 minutes at room temperature, before finally being resuspended in 1xPBS/1mM EDTA at a density of 1x10^6 cells/ml. A working solution of Nile Red (see section 2.5.5) was added to the cells at a 1:2 ratio (Nile Red:cells).

Flow cytometric analysis was performed by Christina Madsen (Department of Immunology, Windeyer Institute of Medical Sciences, UCL). After five minutes of stain incubation, 10,000 cells were passed through a Coulter Epics Elite cell sorter with a 75μm flow cell rate. Results were analysed using WinMDI 2.8 (http://facs.scripps.edu/software.html).

2.5.7 Calcium Phosphate Transient Transfection

NIH-3T3 and F442A-3T3 preadipocytes were transfected using the calcium phosphate method, with glycerol shock. 1μg reporter gene plasmid and 0.5μg pCMV-SPAP were mixed with 248mM CaCl_2 (50μl final volume). The solution was added dropwise, vortexing, to 50μl 2x HBS. After 10 minutes at room temperature, the precipitate was added to one well of cells at 70% confluence (24-well plate), (quantities were scaled up according to the number of wells of cells to be transfected). After 6 hours in the incubator, the media was removed. Cells were glycerol shocked by the addition of 200μl 15% (v/v) glycerol in 1x HBS for 45 seconds at room temperature, followed by extensive washes with PBS. Cells were incubated for a further 42 hours in DMEM containing 0.1% (w/v) bovine serum albumin (BSA). Any other treatments were performed 18 hours after glycerol shock.

2.5.8 Calcium Phosphate Stable Transfection

Calcium phosphate transfection and glycerol shock was carried out as described in section 2.5.6, using 5μg reporter gene plasmid and 1μg pCDNA3EGFP-N1 per well (6-well plate). 48 hours post-transfection, media was changed to include 800μl/ml Geneticin G-418 sulphate. Geneticin treatment was continued for 10 days, with
replacement of media every 2-3 days. Resistant colonies were purified by limiting dilution.

2.5.9 ELECTROPORATION

Trypsinized cells were resuspended in full growth media at a density of either $2.5 \times 10^6$ or $5 \times 10^6$ cells/ml. 500μl of cell suspension was mixed with either 6μg or 12μg of reporter plasmid and either 3μg or 6μg of pCMV-SPAP, added to a Gene Pulser cuvette (0.4cm electrode gap) and left to cool on ice. Cells were subjected to 6 pulses of 800 volts and 25μF in an Gene Pulser II electroporator (Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts, England). After the first three pulses, the cuvette was flicked gently to mix the cells. The time constant was typically 0.8msec during the final pulse. Cells were left to return to room temperature for 30 minutes before being resuspended in 6ml full growth media. Cells were then aliquotted into 3-4 wells of a 24-well plate. Surviving cells were allowed to adhere for a minimum of 3 hours before a medium change to DMEM containing 0.1% (w/v) BSA. Cells were then incubated for a further 44 hours, any other treatments were added 24 hours after electroporation.

2.5.10 LUCIFERASE ASSAY

48-hours post-transfection, cells were lysed in 75μl 1x reporter lysis buffer for 15 minutes at room temperature. 30μl lysate (NIH-3T3 and F442A-3T3 preadipocytes) or 60μl lysate F442A-3T3 adipocytes were assayed in white 96-well microtitre plates with 30μl luciferase assay reagent in an Anthos Lucyl microplate luminometer, using L1 Com 2.6 software (Anthos Labtec instruments, Wals, Austria).

2.5.11 ALKALINE PHOSPHATASE ASSAY

48-hours post-transfection, media was taken from cells and was heat-inactivated for 30 minutes at 65°C. Suspended debris was pelleted in a microfuge. 200μl supernatant was added to 100μl 3x alkaline phosphatase assay buffer in a clear 96-well microtitre plate and incubated until an OD$_{405}$>0.4 was reached. OD$_{405}$ was recorded on a LabSystems multiskan RC microtitre plate reader using genesis software v1.87 (Life Sciences (UK) Ltd.). Serial dilutions of FCS were used to create a standard curve. Units of activity in cell supernatants were expressed as a relative percentage of FCS.
2.5.12 LONG TERM STORAGE

Stocks of $10^6$ cells/ml in growth medium supplemented with 10% (v/v) DMSO were frozen slowly overnight in cryotubes, in a polystyrene box at -80°C, and were then stored indefinitely in liquid nitrogen.

2.6 PROTEIN METHODOLOGY

2.6.1 PROTEIN EXTRACTION (FOR IMMUNOPRECIPITATION)

3T3-F442A adipocytes, grown in 6 well plates, were washed in ice-cold 1x PBS and then lysed in 200μl ice-cold lysis buffer (see appendix) for 10 minutes on ice. Cell lysate was centrifuged for 15 minutes to pellet cellular debris. The protein concentration of the supernatant was determined using the BCA protein assay (see section 2.6.3), and the extract stored at -80°C.

2.6.2 PROTEIN EXTRACTION (FOR GPDH ASSAY)

3T3-F442A adipocytes, grown in 24 well plates, were washed in ice-cold 1x PBS and resuspended in 200μl ice-cold resuspension buffer (see appendix). Cells were scraped off the plate into microfuge tubes and were lysed by sonication at 1.8A for 5 seconds. Cell debris was pelleted out in a microfuge for 30 minutes. Due to 2-mercaptoethanol in the resuspension buffer, protein concentration of the supernatant was determined using the Coomassie Plus protein assay. The extract was stored at -80°C.

2.6.3 QUANTIFICATION OF PROTEIN CONCENTRATION

The total protein content of cell lysates was determined using the BCA protein assay, or Coomassie Plus protein assay according to the manufacturer’s instructions. Briefly, duplicate 10μl samples were dispensed into clear 96-well microtitre plates, alongside duplicate BSA standards (from 0.2mg/ml to 20 mg/ml). 200μl of assay buffer was added to each well, and the plate incubated for approximately 30 minutes. The OD$_{560}$ (BCA) or OD$_{595}$ (Coomassie) after this time was recorded on a LabSystems multiskan RC microtitre plate reader using genesis software v1.87 (Life Sciences (UK) Ltd.). The protein content of the samples was calculated from the standard curve for BSA.
2.6.4 IMMUNOPRECIPITATION

1ml of cell lysate containing 1.5mg of total protein was mixed with 30µl Protein A/G Plus-Agarose and 10µl (1:100 dilution) of anti-insulin receptor beta antibody. The slurry was incubated at 4°C for 1.5 hours on a revolving carrousel. The slurry was pelleted at 2500rpm for 2 minutes, and was washed once in ice-cold immunoprecipitation lysis buffer, revolving, for 5 minutes. The spins and washes were repeated twice more with ice-cold 1x PBS. The pellet was resuspended in 50µl of 2x Laemelli SDS loading buffer. Samples were immediately denatured and loaded onto an 8% SDS-acrylamide gel.

2.6.5 SDS-PAGE Gel Electrophoresis

Immunoprecipitates were resolved on SDS-acrylamide gels as described (Sambrook and Fritsch, 1989). Using the mini-PROTEAN II electrophoresis system (Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts, England), an 8% SDS acrylamide resolving gel was poured and topped with 5% acrylamide stacking gel. Samples were denatured at 100°C for 5 minutes and placed on ice for 2 minutes before loading. The gel was run at 80 volts, until the dye front was just running off the bottom of the gel. 5µl of denatured Rainbow coloured protein molecular weight marker in 1x Laemelli SDS loading buffer was run in parallel as a standard for determining the molecular mass of the proteins detected.

2.6.6 Western Blotting

Proteins were transferred from an SDS-acrylamide gel to a Hybond C membrane in 1x transfer buffer using a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad Laboratories Ltd., Hemel Hempstead, Herts, England), run at 200mA for 1.5 hours at 4°C. Non-specific binding sites on the membrane were blocked overnight at 4°C in Western blocking buffer. Primary antibody (anti-insulin receptor beta or anti-phosphotyrosine) was incubated with the membrane in Western blocking buffer at a 1:1000 dilution for 1.5 hours. 3, five-minute washes in Western wash buffer were followed by the addition of HRP-conjugated secondary antibody (1:2000 dilution in blocking buffer) for 1 hour. 5, five-minute washes in Western wash buffer were then performed. Protein-antibody conjugates were detected using the Enhanced Chemiluminescence (ECL) system according to the manufacturer's instructions, and exposure to autoradiographic film. The membranes were stripped for re-probing in 1M
glycine pH 2.9 for 30 minutes. Bands were quantified by densitometry on a Biorad GS-670 Densitometer.

2.6.7 GPDH ASSAY

GPDH activity in adipocytes was determined spectrophotometrically, according to the method of Kozak and Jensen (Kozak and Jennsen, 1974), by measuring the change in rate of oxidation of NADH at 25°C. 40μl of GPDH assay buffer and 1μg of adipocyte protein extract in a final volume of 50μl was added to 50μl 0.4mM dihydroxyacetone. The change in absorption at 340nm was measured for a maximum of 5 minutes on a ThermoMax microplate reader (Molecular Devices Corp, USA). The rate of enzyme activity was expressed simply in mOD/min during the linear phase of the reaction.

2.7 ANIMAL METHODOLOGY

2.7.1 HOUSING AND CARE OF ANIMALS

All procedures were carried out according to Home Office regulations. Mice were housed up to 10 to a cage. Animals had free access to water and standard chow (4% fat, 20.5% protein, 35.8% starch, 7.7% sugars (w/w) - Rodent Maintenance diet 3 pellet form (RM3), Special Diet Services, Witham, Essex, U.K.), unless otherwise stated, and were subjected to standard dark/light cycles. Mice were sacrificed by asphyxiation with CO₂ or cervical dislocation.

2.7.2 IN VIVO FASTING/ RE-FEEDING/ INSULIN ADMINISTRATION PROTOCOL

8-week-old male C57BL/6J mice were housed in groups of 5-8. Food was removed from the cages 24 hours before treatment, at 10 o’clock in the morning, except for the ad libitum fed control group, which were allowed to eat ad libitum throughout the entire 28 hour procedure. After the overnight fast, animals were given subcutaneous injections of insulin (0.1IU in 50μl 1x PBS) or vehicle alone (1x PBS) and were either allowed to re-feed ad libitum (re-fed), or remained fasted (injections were performed by Prof. T. Rademacher). Re-fed animals received either standard chow, or in the case of the high carbohydrate diet, received white bread soaked in 60% sucrose. After 4 hours, the animals were anaesthetized with halothane, exsanguinated by cardiac puncture and then immediately killed. Tissue was removed immediately for RNA extraction.
2.7.3 **MEASUREMENT OF BLOOD GLUCOSE**

Blood glucose levels were measured from a spot of freshly drawn blood on a Bayer Diagnostics Glucometer, model 4, according to the manufacturer's instructions.

2.8 **STATISTICAL ANALYSIS**

Results are presented graphically as mean ± standard error of the mean (SEM). Significance between groups was assessed using the Student's unpaired t-test in Microsoft Excel. F-tests were first performed to establish whether to assume equal or unequal variance in the t-test. Significance was accepted if a p value of less than 0.05 was returned (95% confidence limits).
3 DEVELOPMENT OF AN RNASE PROTECTION ASSAY TO DETERMINE THE STEADY-STATE LEVELS OF Lep mRNA IN MURINE ADIPOSE TISSUE
3.1 INTRODUCTION

The aim of this study was to determine the effects of insulin on the steady-state levels of Lep mRNA in murine adipose tissue, and to determine whether it exerts its effects by a direct mechanism, by observing any changes in the relative level of Lep mRNA in a suitable in vitro model system. Since Lep mRNA is a relatively rare transcript in adipose tissue, an RNase protection assay was first developed and optimized to achieve this aim. This is a sensitive and accurate technique, and in addition, enables quantification of several different mRNA species concurrently, including internal controls.

The results in this chapter describe the design and development of a specific RNase protection assay, to accurately determine the relative steady-state levels of Lep mRNA in murine epididymal adipose tissue.

3.2 RESULTS

3.2.1 DESIGN OF RNASE PROTECTION ASSAY

Several elements, key to the design of the RNase protection assay used in this body of work are described in this section. The identification of these design elements explain the rationale behind the choice of riboprobes and source of adipose tissue used in this particular assay.

3.2.1.1 DESIGN OF RIBOPROBE TEMPLATES

When designing riboprobe templates for an RNase protection assay, a number of criteria must be considered. The riboprobes that are generated from them must be highly sequence-specific; suitable internal control riboprobes must be designed; and the riboprobes, and their derived protected transcripts, must be clearly distinguishable from each other when resolved by polyacrylamide gel electrophoresis.

3.2.1.1 SEQUENCE SPECIFICITY

To avoid cross-hybridization of the riboprobe to other RNA species, the section of gene used to create the riboprobe template must be unique. This can be ensured by running a standard sequence database search, such as FASTA, when deciding on the section of gene to be used. It is possible, however, that genes sharing sequence homology exist, that are not yet in the public domain. For this reason it is important to test the generated
riboprobes in Northern blots, to ensure that they hybridise with only one RNA species, of the expected size and tissue distribution.

3.2.1.2 CONTROLS

As well as a riboprobe for the gene of interest (in this case, \textit{Lep}), a riboprobe that hybridises to a control RNA species also must to be included in each sample in the assay. This internal control should not be responsive to any of the experimental treatments being used (in this case, insulin) and serves to normalize the amount of target mRNA in each sample. House-keeping genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and members of the actin family are often used for this purpose and riboprobes templates for these genes are available commercially.

It is also useful to include positive control riboprobes, of genes that are known to be responsive to the experimental treatments used. This serves to ensure that the model system and conditions used, are appropriate.

3.2.1.3 RIBOPROBE SIZE

Protected transcripts are resolved by polyacrylamide gel electrophoresis (PAGE). It is therefore important to design riboprobe templates, for the target and control RNA species, that will produce riboprobes and protected transcripts of lengths readily distinguishable from each other.

A clear and easily analysed, ‘ladder’ of probes can be resolved on a gel, by designing larger riboprobe templates for the least abundant RNA species being measured, and smaller riboprobe templates for the most abundant RNA species. This strategy has two benefits. Firstly, the greater number of counts per minute emitted from larger riboprobes increases the sensitivity of detection, which is of benefit for rare transcripts. Secondly, by designing the smallest riboprobes for the most abundant RNA species, the ‘smearing’ of the protected transcript band, which often occurs when high concentrations of nucleic acid are resolved by PAGE, does not interfere with the detection of any transcripts underneath.

3.2.1.2 CHOICE OF RNA SOURCE

\textit{Lep} mRNA is synthesized in various adipose depots (Masuzaki et al., 1995; Ogawa et al., 1995; Trayhurn et al., 1995b). In this study, \textit{Lep} mRNA levels were to be measured solely from epididymal adipose tissue, for the following reasons: firstly, \textit{in vivo} studies
indicate that steady-state levels of Lep mRNA from rodent epididymal adipose tissue are responsive to refeeding and insulin (Saladin et al., 1995; Trayhum et al., 1995b), and moreover, the rat Lep gene in this depot shows a greater response to insulin than perirenal or subcutaneous adipose tissue - consistent with reports that rodent epididymal adipose tissue is highly insulin-responsive (Santos et al., 1991; Zheng et al., 1996a). As further added advantages, studies have shown that Lep mRNA is expressed at higher levels in this depot than in other rodent adipose tissue depots (Ogawa et al., 1995; Trayhum et al., 1995b; Zheng et al., 1996a), and epididymal adipose tissue is a discrete and easy-to-dissect organ.

Previous reports have focussed on Lep mRNA levels in rats. By studying responses in mice, not only would this add to our understanding of Lep gene regulation in rodent species, but it would also be more appropriate for comparison against ob/ob mice and other mouse models of obesity. The original ob/ob mutation was found in mice on the C57BL/6J background, and hence C57BL/6J mice were chosen for these studies.

3.2.2 CONSTRUCTION OF PLASMID DNA RIBOPROBE TEMPLATES

With the above design considerations in mind, plasmids were constructed containing riboprobe templates for Lep; an internal control, β-actin; and the positive controls, fatty acid synthase (FAS) and peroxisome proliferator activated receptor gamma (PPARγ).

The sequence specificity of the regions of the genes to be used as the riboprobe templates was assessed by FASTA. A description of the primers, and details of the PCR reaction conditions used to amplify the riboprobe template sequences can be found in chapter two – tables 2.2 and 2.4, and section 2.2.8. Cloning was carried out as described in section 2.2, and is shown schematically in Figure 3.1. Positive clones were identified by restriction endonuclease digestion and were sequenced in both directions to ensure fidelity in the PCR reaction using T3 and T7 primers. The plasmids, and the rationale behind the choice of template sequence are detailed below.

3.2.2.1 LEP

Initially, a plasmid containing an Lep riboprobe template, generating a 216 nucleotide (nt) protected transcript (approximately 200nt and therefore designated plep200-Blue), was designed and constructed to measure changes in the steady-state levels of Lep mRNA in response to insulin. Murine genomic DNA was PCR amplified using primers
specific for the *Lep* gene (see Table 2.2). The PCR product was restriction endonuclease digested with *SacI/SalI* and cloned into *SacI/SalI* digested pBluescript II SK (+).

Later on in the course of the study, to increase the sensitivity of detection of *Lep* mRNA, and to incorporate positive control probes into the assay, a plasmid containing a template to produce a 402nt protected transcript was also constructed (approximately 400nt and therefore designated plep400-Blue). To achieve this, murine genomic DNA was PCR amplified using primers specific for the *Lep* gene (see Table 2.2). The PCR product was restriction endonuclease digested with *SacI/SalI* and cloned into *SacI/SalI* digested pBluescript II SK (+).

As only the cDNA sequence of murine *Lep* was available at the time, and PCR was performed using genomic DNA, both probes were designed using sequence from the 3' untranslated region of the gene. This ensured that the amplified sequences did not span any intron/exon boundaries.

### 3.2.2.2 INTERNAL CONTROL - β-ACTIN

Some commonly used internal controls, such as GAPDH, are known to be insulin-responsive and were therefore rejected for this assay (Alexander et al., 1988). Since we were in possession of a commercially available murine β-actin riboprobe template, and that this gene had been used as an internal control in similar experiments (Cusin et al., 1995; Rentsch and Chiesi, 1996), β-actin was chosen to act as the internal control. pTRI-β-actin-Mouse is purchased as an *XbaI/HindIII* linearized plasmid. Since β-actin is an abundant RNA species, the template sequence was shortened, to produce a smaller riboprobe and protected transcript (see section 3.2.1.1.3). Digestion of pTRI-β-actin-Mouse with *DdeI* generated a template that produced a riboprobe of 195nt and a protected transcript size of 131nt. The smaller size of this riboprobe ensured easy analysis of the ‘ladder’ of probes resolved by PAGE.

### 3.2.2.3 POSITIVE CONTROLS - FAS AND PPARγ

For the *in vitro* studies, positive controls were also employed in the assays. Fatty acid synthase (FAS) mRNA levels are known to be directly inducible by insulin in adipocytes (Paulauskis and Sul, 1988). A plasmid containing a riboprobe template to produce a 301nt protected transcript of the murine FAS gene was designed and constructed. Murine genomic DNA was PCR amplified using primers specific for the FAS gene (see Table 2.2). The PCR product was restriction endonuclease digested with...
SacI/SalI and cloned into SacI/SalI digested pBluescript II SK (+). The plasmid was designated pFAS-Blue.

Peroxisome proliferator-activated receptor gamma (PPARγ) is a member of the peroxisome proliferator-activated receptor subfamily of nuclear hormone receptors, and it plays a key role in adipogenesis and adipocyte gene expression (Tontonoz et al., 1994). The murine PPARγ gene (mPPARγ) encodes two isoforms, PPARγ1 and PPARγ2, derived from alternative promoter usage and differential mRNA splicing. PPARγ2 possesses an additional 30 amino acids at its N-terminus (Zhu et al., 1995). Both isoforms of mPPARγ mRNA have been shown to be upregulated by insulin both in vivo and in vitro (Rieusset et al., 1999; Vidal-Puig et al., 1996). A single RNase protection assay riboprobe that can hybridise to, and differentiate between both isoforms has been described (Vidal-Puig et al., 1996), and a plasmid was constructed to contain this riboprobe template. To achieve this, PPARγ specific cDNA (see section 2.2.7) was PCR amplified using primers specific for PPARγ cDNA (see Table 2.2). The PCR product was restriction endonuclease digested with SacI/SalI and cloned into SacI/SalI digested pBluescript II SK (+). This plasmid was designated pPPARγ-Blue.

Linear DNA, either blunt-ended or with a 5’ overhang, is needed for use as a template for in vitro transcription. Two PvuII sites (blunt ended), flanking the multiple cloning site, are present in pBluescript SK+, and were used to generate linear DNA riboprobe templates from the four constructed plasmids (see Figure 3-1). (The remaining portion of plasmid would not interfere with the transcription reaction, and thus it was not deemed necessary to gel purify the linear DNA fragments containing the riboprobe templates away from the rest of the pBluescript SK+ plasmid).

Equal molar concentrations of digested plasmid DNA from plep400-Blue, pFAS-Blue and pPPARγ-Blue were pooled, and were used to synthesize all three riboprobes concurrently, by the method described in section 2.3.6. This ensured that the specific activity for all three probes would be identical, and thus the protected transcripts generated could be directly compared to one another.

Figure 3-2, lanes 2-5 show each separate plasmid, plep200-Blue, plep400-Blue, pFAS-Blue and pPPARγ-Blue, digested with SalI and SacI. The resulting two bands in each
lane represent the pBluescript SK+ vector backbone and the inserted gene sequence. The sizes of these inserted gene sequences correspond to the sizes of the RNase protection assay protected transcripts. Lane 6 shows the pooled, PvulII digested, plep400-Blue, pFAS-Blue and pPPARγ-Blue plasmids. The three plasmid DNA fragments of sizes 764nt, 663nt and 635nt are the templates for lep400, FAS and PPARγ riboprobes, respectively. As can be seen, the templates are of equimolar concentration. Lane 7 shows pTRI-β-actin-mouse digested with Ddel.
pBluescript II SK(+) and the purified PCR amplified gene fragment were digested with SalI and SacI, and were ligated to generate a plasmid containing the riboprobe template. Positive clones were identified by restriction endonuclease digestion, and were sequenced in both directions. The resulting plasmid was linearized with PvuII to generate the final riboprobe template. The X in pXBlue denotes the various inserted gene sequences.
Amp' fl (+) ori

pBluescript II SK (+)
(2961bp)
lacz
SacI

San
CoIE1 ori
PvuII

PCR AMPLIFIED
GENE FRAGMENT

Insert gene fragment

SalI/SacI digest

Amp' fl (+) ori

PxBlue

SalI

Plasmid Containing the
Riboprobe Template

ColE1 ori

PvuII

PvuII digest

DNA Riboprobe Template

In vitro transcription using
T7 RNA polymerase

Antisense Strand Riboprobe

Size of Protected Transcript
Figure 3.2 Photograph of agarose gel showing plasmid DNA riboprobe templates

2 μg of each of the riboprobe template plasmids were digested with specific restriction endonucleases (shown above) and were resolved on a 1% agarose gel. The sizes of the inserted gene sequences, which correlate with the protected transcript sizes (SacI/SalI digests) are shown on the left, and the sizes of the pooled riboprobe templates (PvuII digest) are shown on the right.

DNA markers: λ = λ.HindIII, φX = φX174 HaeIII.
3.2.3 Verification of Specificity of Riboprobes

In order to verify that the designed riboprobes hybridized specifically to their respective mRNA species, RNase protection assays were carried out to confirm that they generated protected transcripts of the expected sizes. For the less well characterized *Lep* gene, Northern blot analysis was also carried out using probes from the designed riboprobe templates to ensure that they only recognised *Lep* mRNA (see section 3.2.3.2). An investigation into the tissue-specificity of the *Lep* riboprobes was also carried out.

3.2.3.1 Size of Riboprobes and Protected Transcripts

RNase protection assays were initially performed with epididymal adipose tissue to verify the sizes of the generated riboprobes, and their protected transcripts. The assay was performed with individual riboprobes, and in the combinations to be used in the optimized assays. The individual riboprobe samples acted as references to determine which protected transcripts corresponded to which mRNA species in the samples containing pooled riboprobes.

Table 3.1 presents the theoretical sizes of the riboprobes and protected transcripts produced from each of the designed riboprobe templates, and Figure 3.3 and Figure 3.4 illustrate autoradiographs of these transcripts.

<table>
<thead>
<tr>
<th>PLASMID</th>
<th>SIZE OF RIBOPROBE (nt)</th>
<th>SIZE OF PROTECTED TRANSCRIPT(S) (nt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>plep200-Blue</td>
<td>419</td>
<td>216</td>
</tr>
<tr>
<td>plep400-Blue</td>
<td>605</td>
<td>402</td>
</tr>
<tr>
<td>pFAS-Blue</td>
<td>504</td>
<td>301</td>
</tr>
<tr>
<td>pPPARγ-Blue</td>
<td>474</td>
<td>273 and 185</td>
</tr>
<tr>
<td>pTRI-β-actin-mouse</td>
<td>195</td>
<td>131</td>
</tr>
</tbody>
</table>

Figure 3.3 illustrates the autoradiograph from the assay performed using the lep200 and β-actin riboprobes. Both the β-actin and lep200 protected transcripts were of the
expected sizes (216nt and 131nt respectively) and were clearly distinguishable from each other when both riboprobes were used in combination.

The lep200 and β-actin undigested riboprobes were resolved as a pooled sample in this experiment. However, in addition to the fact that the sizes of the two riboprobes were as expected, it was possible to determine that the β-actin riboprobe was the smaller of the two, since the protected transcript for *Lep* was larger than it.

This assay also illustrates the positive and negative controls for RNase treatment that are always used. "Y+" indicates the positive control - a 10µg yeast tRNA sample, treated in an identical way to the other samples, i.e. incubated with the riboprobes and digested with RNases. Since the riboprobes do not hybridise to any yeast tRNA species, all the sample RNA and riboprobes are digested. "Y-" indicates the negative control. Here the 10µg sample of yeast tRNA is incubated with the riboprobes, but is not subjected to RNase digestion. The resulting intact, resolved riboprobes illustrate that no contaminating ribonuclease activity is present in any of the steps of the assay.

Figure 3.4 illustrates the autoradiograph from a similar assay, performed using the lep400, FAS, PPARγ and β-actin riboprobes. The lanes containing the pooled riboprobes show that the riboprobes and their protected transcripts are all clearly distinguishable from each other. In this experiment, the undigested riboprobes and protected transcripts resolved on the gel in the correct size order relative to one another. However, when the radiolabelled DNA marker (100bp ladder) was used to estimate their sizes, the apparent sizes of the larger riboprobes (over 400nt) were not as expected. This was probably due to differences in the rates of migration of DNA and RNA. It was therefore not possible to verify the exact sizes of these particular riboprobes. However, since they produced protected transcripts of the correct size, and their sizes relative to each other corresponded with the order of their theoretical sizes, it was assumed they had been produced as full-length probes from their respective templates.

Finally, small amounts of undigested riboprobe were sometimes observed along with the protected transcripts, indicating that the amount of riboprobe added was sometimes slightly in excess of the amount RNases (see section 3.2.5). Unfortunately, the undigested β-actin riboprobe was of a similar size to the protected transcript of the PPARγ1 isoform, and could potentially interfere with the measurement of this species.
Since quantification of PPARγ1 was not necessary for our purposes, in subsequent assays PPARγ1 levels were not to be quantified.
Figure 3.3 Autoradiograph of RNase protection assay, illustrating the sizes of the lep200 and β-actin riboprobes and protected transcripts.

An RNase protection assay was performed using 20μg samples of epididymal adipose tissue total RNA, demonstrating the sizes of the lep200 and β-actin undigested riboprobes and protected transcripts. Samples were resolved on a denaturing 6% polyacrylamide gel, and were exposed overnight onto autoradiographic film. Also shown are the controls (riboprobes + yeast tRNA +/- RNase (Y+, Y-)). DNA marker: 100bp ladder.
Figure 3.4  Autoradiograph of RNase protection assay, illustrating the sizes of the lep400, FAS, PPARγ and β-actin riboprobes and protected transcripts

An RNase protection assay was performed using 10µg samples of epididymal adipose tissue total RNA, demonstrating the sizes of the lep400, FAS, PPARγ, and β-actin undigested riboprobes and protected transcripts. Samples were resolved on a denaturing 6% polyacrylamide gel, and were exposed overnight onto autoradiographic film.

Also shown are the controls (riboprobes + yeast tRNA +/-RNase (Y-, Y+)). DNA marker: 100bp ladder.
undigested riboprobes

protected transcripts

605nt
leptin undigested riboprobe

504nt
FAS undigested riboprobe

474nt
PPARγ undigested riboprobe

195nt
β-actin undigested riboprobe

100bp
β-actin
PPARγ
FAS
leptin

Y-
Y+
β-actin
PPARγ
FAS
leptin pooled

402nt
leptin protected transcript

301nt
FAS protected transcript

273nt
PPARγ2 protected transcript

185nt
PPARγ1 protected transcript

131nt
β-actin protected transcript
3.2.3.2 Sequence and Tissue Specificity - Northern Blot Analysis

To further ensure the sequence specificity of the designed Lep riboprobes, Northern blot analysis was carried out on epididymal adipose tissue total RNA. As a verification of tissue specificity, the analyses also included liver total RNA control samples (Figure 3.5). Equal quantities of adipose tissue and liver total RNA were resolved on the gel, which was verified by visualization of ethidium bromide stained 18S and 28S ribosomal RNA bands prior and subsequent to membrane transfer (data not shown).

A single RNA species, corresponding to the size of murine Lep mRNA (4.1kb) (Trayhurn et al., 1995b), was found to hybridise with the lep200 riboprobe in adipose tissue total RNA (Figure 3.5a). No hybridization was detected in an equal loading of liver total RNA. It was observed that the riboprobe hybridized non-specifically to the 28S and 18S rRNA species in both adipose and liver RNA however, even after high stringency washes. This served to further verify that equal amounts of adipose and liver RNA had been resolved on the gel. However, as the Lep mRNA species resolves close to the 28S RNA band, this non-specific hybridization was undesirable for the detection of Lep mRNA.

For this reason, to ensure sequence specificity of the lep400 probe, a cDNA probe was utilized. Figure 3.5b shows the single, ~4.1kb RNA species which was found to hybridise to the lep400 probe in RNA from adipose tissue, but not from liver.

The absence of detectable hybridization to any other specific RNA species in adipose or liver RNA firstly confirmed that the sections of the Lep cDNA sequence chosen to make the Lep probes were indeed unique to Lep - for RNA species present in these tissues at least. Secondly, the detection of the ~4.1kb RNA species, corresponding to Lep mRNA, only in adipose tissue, helped to verify previous reports as to the tissue specificity of Lep expression (Frederich et al., 1995b; Trayhurn et al., 1995b; Zhang et al., 1994).
10μg samples of liver and epididymal adipose tissue total RNA were used in Northern blots using a) the lep200 riboprobe and b) the lep400 cDNA probe, to illustrate that the sequence of the Lep gene used in these designed probes were unique to Lep in these tissues; and to confirm reports of the tissue specificity of Lep expression.

28S and 18S refer to the positions of the 28S and 18S ribosomal RNA.
In order to further verify that the lep200 riboprobe was specific for \textit{Lep} mRNA, an RNase protection assay was performed on total RNA derived from various tissues. Figure 3.6 illustrates the resulting autoradiograph from this experiment. The lep200 riboprobe did not produce a detectable protected transcript in any tissue type except adipose, whereas the control protected transcript, \(\beta\)-actin, was visible in the majority of tissue samples (the absence of detectable \(\beta\)-actin protected transcripts in heart and soft muscle is due to the riboprobe being specific for the non-muscle isoform, or isoactin, of \(\beta\)-actin).

This result is consistent with reports of the adipose tissue-specificity of \textit{Lep} mRNA (Frederich et al., 1995b; Trayhurn et al., 1995b; Zhang et al., 1994), and together with the results from section 3.2.3.2, also confirms that the lep200 riboprobe does indeed only recognise \textit{Lep} mRNA in adipose tissue.

Levels of \(\beta\)-actin mRNA were shown to vary considerably from tissue to tissue in this experiment, and levels of \(\beta\)-actin in adipose tissue were relatively high. In order to accurately quantify the amount of the less abundant \textit{Lep} mRNA species relative to \(\beta\)-actin in future experiments, the specific activity of the \(\beta\)-actin riboprobe was subsequently reduced by using 10-fold less \([\alpha^{32}\text{P}]\)CTP and supplementing the labelling reaction with 5-fold more non-radioactive CTP (see section 2.3.6).
Figure 3.6 Autoradiograph of RNase protection assay illustrating the tissue specificity of murine \textit{Lep} expression

An RNase protection assay was performed using 10\(\mu\)g samples of total RNA from a range of tissue types (see above) to demonstrate the tissue specificity of the lep200 riboprobe. Samples were resolved on a denaturing 6\% polyacrylamide gel, and were exposed overnight onto autoradiographic film. DNA marker: 100bp ladder.
3.2.4 OPTIMIZATION OF PRODUCTION OF RIBOPROBES

To accurately quantify mRNA levels in an RNase protection assay, an excess of riboprobe to target mRNA species is required. Transcription must therefore be optimized to ensure that sufficient quantities of riboprobe are produced. It is also essential that the majority of riboprobes produced are full-length. This is for the obvious reason that a range of riboprobe sizes will generate a range of sizes of protected transcripts, which cannot therefore be accurately quantified. Lowering the temperature of the transcription reaction can increase the proportion of full-length RNA transcripts produced. This is thought to be due to a decreased likelihood of the polymerase being displaced by secondary structure or by a string of one specific nucleotide, when it is progressing at a slower rate. The slower rate of transcription at lower temperatures could lower the yield of riboprobe however.

It was therefore important to investigate conditions required for the optimal production of sufficient quantities of full-length riboprobes.

3.2.4.1 THE EFFECT OF TEMPERATURE OF TRANSCRIPTION ON THE YIELD AND QUALITY OF RIBOPROBE

The yield, and quality, of the lep200 and β-actin riboprobes, generated during a two-hour labelling reaction at 37°C and 4°C, was assessed by autoradiography (Figure 3.7). Subjective analysis of the autoradiograph indicated that the amount of riboprobe generated was not adversely affected by transcription at the lower temperature of 4°C for the lep200 riboprobe, and may have even been more optimal for the β-actin riboprobe. Furthermore, with both probes, discrete bands of premature transcripts (see arrows in Figure 3.7) were observed to a much lesser extent at 4°C than at 37°C. These specifically-sized, prematurely-terminated transcripts are likely to be caused by termination of transcription at specific sequences within the riboprobe template sequence, as discussed in section 3.2.4.
Figure 3.7  Autoradiograph of lep200 and β-actin riboprobes, illustrating the effect of temperature on the quantity and quality of riboprobe production

lep200 and β-actin riboprobes were produced during a two-hour labelling reaction at 4°C and 37°C. For the β-actin riboprobe, the labelling was not supplemented with non-radioactive CTP. 1μl and 2μl aliquots of the reaction mix were resolved in adjacent lanes by denaturing polyacrylamide gel electrophoresis, and the gel was exposed for five minutes onto autoradiographic film at room temperature.
3.2.4.2 The Effect of Temperature on the Kinetics of Radionucleotide Incorporation into Riboprobe

To gain an insight into the rate of transcription at the two temperatures, the rate of incorporation of $[\alpha^{32}\text{P}]\text{CTP}$ into riboprobe during transcription was investigated using the DE-81 filter absorption method. The $\text{lep200}$ riboprobe was arbitrarily chosen for this two-hour labelling experiment.

Figure 3.8 shows that the percentage of radionucleotide incorporation into the $\text{lep200}$ riboprobe was similar after two hours, at both $37^\circ\text{C}$ and $4^\circ\text{C}$ (around 80% incorporation). This is in agreement with the subjective analysis described in section 3.2.4.1, indicating that similar quantities of riboprobe are produced after two hours of labelling at the two temperatures. However, as predicted, the rate of incorporation was dramatically reduced at $4^\circ\text{C}$, to such an extent that two hours was the minimum time required to incorporate a similar percentage of radionucleotide as at $37^\circ\text{C}$ during just 30 minutes.

The rate of radionucleotide incorporation was affected by factors other than just temperature however. The decrease in rate during the first hour, and subsequent increase in rate after the addition of extra T7 RNA polymerase after this time, was clearly apparent at both temperatures (see arrow, Figure 3.8). This is due to the fact that T7 RNA polymerase has a limited lifespan of activity, and hence rate of transcription gradually reduces over time (i.e. over the first hour).

Furthermore, at a certain stage during nucleotide incorporation, the decrease in concentration of free nucleotides becomes a rate-limiting factor. This is the most likely explanation for the plateau of incorporation, at around 80%, observed at $37^\circ\text{C}$ within the first hour. A similar percentage of incorporation was not reached at $4^\circ\text{C}$ until the final time point, thus explaining why the reaction appeared to remain in the linear phase for the duration of the experiment at $4^\circ\text{C}$.

Therefore, since lowering the temperature of the transcription reaction to $4^\circ\text{C}$ increased the quality of both riboprobes produced (i.e. the proportion of full-length transcripts); and a two-hour labelling reaction was required to produce optimal quantities of riboprobe at this temperature, these conditions were adopted for use in subsequent experiments, for all riboprobes.
The rate of incorporation of $[\alpha^{32}\text{P}]\text{CTP}$ into the lep200 riboprobe using T7 RNA polymerase was determined at both 37°C and at 4°C over a two-hour period, using the DE-81 filter absorption method. Extra T7 RNA polymerase was added after the first hour of the transcription reaction.
3.2.5 Determination of Optimal Amount of Total RNA and Riboprobe to Be Used in Assay

In order to accurately quantify levels of mRNA by RNase protection, it is essential that the molar concentration of riboprobe is in excess of the target mRNA species. Titration experiments were therefore performed using increasing amounts of total RNA and constant, standard, amounts of riboprobe, in order to determine the point at which riboprobe was no longer in excess of the target mRNA species.

The total concentration of RNA in each assay sample (i.e. input RNA plus riboprobe) must not exceed the capacity of nuclease enzyme activity however, since any remaining undigested RNA interferes with the quantification of the assay. Thus, the resulting autoradiographs from these experiments were examined to ensure that the final concentration of RNA (total RNA plus riboprobe) used in the assays was sufficiently low enough to be completely digested.

5 x 10^6 cpm of each probe per sample was used for the lep200, lep400, PPARγ and FAS riboprobes, and since the specific activity of the β-actin riboprobe was lower, 7500 cpm of probe per sample was used for this riboprobe.

Figure 3.9 shows the resulting autoradiograph from an experiment using the lep200 and β-actin riboprobes. The maximum amount of total RNA used in this experiment was only 20μg. Figure 3.10a and b show the radioactivity emitted from each of the resolved protected transcripts, measured by phosphorimaging. Within the accuracy of the experiment, the amount of radioactivity in the protected transcripts was directly proportional to the amount of RNA added to the assay.

Figure 3.11a and b illustrate similar results from an experiment using pooled lep400, PPARγ and FAS riboprobes and the β-actin probe. A linear correlation between the amount of each of the specific mRNA protected transcripts and input total RNA was observed between 10μg and 30μg of total RNA. At 40μg of total RNA, saturation in hybridization was beginning to be observed with the FAS, PPARγ and β-actin riboprobes, but not with the lep400 riboprobe. The absence of undigested riboprobe in any of the samples also confirmed that the maximum total amount of RNA (40μg total RNA plus riboprobes) did not exceed the capacity of nuclease enzyme activity (data not shown).
The linearity in signal between 10μg and 30μg of total RNA for all four mRNA species meant that within this range, the amount of Lep, PPARγ2 and FAS mRNA relative to β-actin remained constant, indicating that the amount of probe was in excess for each mRNA species. Thus these conditions could be used to accurately determine changes in the relative levels of Lep, PPARγ2 and FAS mRNA in adipose tissue.

Therefore, on the basis of these results, in subsequent assays, 10μg samples of epididymal adipose tissue total RNA were used to determine the relative levels of Lep, PPARγ2 and FAS mRNA in adipose tissue. The standard amounts of riboprobe (5 x 10⁴ cpm of probe for the lep200, lep400, FAS and PPARγ riboprobes, and 7500 cpm of probe for the β-actin riboprobe) were also adopted for the developed assay.
Figure 3.9 Autoradiograph illustrating the increase in counts of lep200 and β-actin protected transcripts with increasing amounts of total RNA, at a constant amount of riboprobe

An RNase protection assay was performed using increasing amounts of epididymal adipose tissue total RNA. Samples were resolved on a denaturing 6% acrylamide gel, and were exposed overnight onto autoradiographic film. Also shown are the controls (riboprobes + yeast tRNA +/- RNase (Y+, Y-)). DNA marker: 100bp ladder.
Figure 3.10  Graphs illustrating the increase in counts of lep200 and β-actin protected transcripts with increasing amounts of total RNA, at constant amounts of riboprobe

An RNase protection assay was performed using samples of the indicated amounts of epididymal adipose tissue total RNA (5µg, n=1; 10µg and 20µg, n=2) and standard amounts of riboprobe (see below). Samples were resolved on a denaturing 6% polyacrylamide gel, and were exposed overnight onto autoradiographic film. The resulting polyacrylamide gel was dried and phosphorimaged for 4 hours.

a) Increase in phosphorimager counts of Lep protected transcripts with increasing amounts of epididymal adipose tissue total RNA, using 5x 10⁶ cpm of lep200 riboprobe.

b) Increase in phosphorimager counts of β-actin protected transcripts with increasing amounts of epididymal adipose tissue total RNA using 7500 cpm of β-actin riboprobe.
Figure 3.11  Graphs illustrating the increase in counts of lep400, PPARγ2, FAS and β-actin protected transcripts with increasing amounts of total RNA, at constant amounts of riboprobe

An RNase protection assay was performed using the indicated amounts of epididymal adipose tissue total RNA and standard amounts of riboprobe (see below). Samples were resolved on a denaturing 6% polyacrylamide gel, and were exposed overnight onto autoradiographic film. The resulting polyacrylamide gel was dried and phosphorimaged for 4 hours.

a) Increase in phosphorimager counts of Lep, PPARγ2 and FAS protected transcripts with increasing amounts of epididymal adipose tissue total RNA, using 5x $10^4$ cpm of lep400, PPARγ and FAS riboprobes.

b) Increase in phosphorimager counts of β-actin protected transcripts with increasing amounts of epididymal adipose tissue total RNA using 7500 cpm of β-actin riboprobe.
3.3 DISCUSSION

In order to determine changes in the steady-state levels of Lep mRNA in murine adipose tissue in response to insulin in vivo and in vitro, a specific and sensitive RNase protection assay was designed, created and optimized. This assay method has the advantages of being more sensitive and more suitable for multi-probe analysis than Northern blot analysis, and is less prone to errors through contamination than RT-PCR analysis. The assay is sensitive, since complementary RNA-RNA hybrids are very stable. In addition, because the added RNases remove all non-hybridizing RNA molecules, the assay also has a very low background. It is also a highly specific assay method, since even single base pair differences will be cleaved by the high concentration of RNases present in the reaction mix.

The assay was optimized to detect Lep mRNA in murine epididymal adipose tissue, since this particular adipose depot had been reported to express relatively high levels of Lep mRNA in rodents (Ogawa et al., 1995; Trayhurn et al., 1995b; Zheng et al., 1996a). Furthermore, in vivo studies have reported that rat epididymal adipose Lep mRNA levels have a greater response to insulin than Lep mRNA levels from other depots (Zheng et al., 1996a).

With certain design considerations in mind, plasmids were constructed containing riboprobe templates for Lep mRNA. In addition, to act as positive controls for the insulin-response assay conditions, riboprobe templates were constructed for the insulin-responsive mRNAs, FAS and PPARγ. The sizes of the probes were designed to be clearly distinguishable from each other, and from their respective protected transcripts when resolved by denaturing PAGE.

Levels of the Lep, FAS and PPARγ mRNA species were to be quantified relative to the internal control mRNA, β-actin. However, β-actin mRNA was found to be an abundant transcript in epididymal adipose tissue, and Lep mRNA was shown to be a rare transcript. In order to more accurately measure Lep mRNA levels relative to β-actin therefore, steps were taken to reduce the number of counts emitted from the resulting β-actin protected transcripts. Firstly, the purchased β-actin riboprobe template was shortened in order to produce protected transcripts that would emit fewer counts. Making the β-actin riboprobe smaller than any of the other probes had the added
advantage of preventing any potential smearing of its more abundant protected transcripts when resolved by PAGE, interfering with the analysis of other protected transcripts underneath. In addition to shortening the β-actin riboprobe, and more effectively reducing the amount of counts emitted from its protected transcripts, the specific activity of the labelled probe was reduced by using 10-fold less [α-32P]CTP and supplementing the labelling reaction with 5-fold more competing, non-radioactive CTP.

Each designed riboprobe was confirmed to be sequence-specific. This was shown by the fact that each probe produced protected transcripts of the expected size in RNase protection assays using epididymal adipose tissue. More extensive analysis with the two Lep riboprobes showed that, in Northern blot analysis, a single mRNA species was detected in epididymal adipose tissue, corresponding to the size of murine Lep mRNA (4.1kb). Neither this species, nor any other, was detected in liver total RNA samples. In addition, in an RNase protection assay using total RNA from a more extensive range of tissue types, the lep200 riboprobe was shown to only generate detectable adipose tissue-specific protected transcripts.

Several labelling conditions were investigated to empirically determine optimal conditions to gain high yields of full-length riboprobes for the assay. Lowering the temperature of the labelling reaction for the β-actin and lep200 riboprobes, from 37°C to 4°C, decreased the proportion of prematurely-terminated transcripts formed. However, a study of the rate of incorporation of radionucleotide into the lep200 riboprobe indicated that for maximal yield, two hours of labelling was the minimum time required at this temperature, due to a decrease in the rate of transcription. Since these conditions were subjectively shown to be optimal for both the lep200 and β-actin riboprobes, it was assumed that these conditions would be suitable for all the other riboprobes without further testing.

A standard amount of riboprobe, (5 x 10^4 cpm for the lep200, lep400, FAS and PPARγ probes, and 7500 cpm for the β-actin probe), was shown to be in excess of epididymal adipose tissue levels of all the mRNA species tested, in samples of total RNA up to 30μg (20μg for the lep200 probe, since this was the maximum amount investigated). It was therefore assured that even if fairly large errors in total RNA quantification or pipetting occurred, and thus initial starting amounts of RNA varied considerably, the
levels of *Lep*, FAS and PPARγ2 mRNA relative to β-actin could still be accurately
determined.

The studies presented in this chapter therefore confirm that a highly specific, sensitive
and accurate RNase protection assay has been developed to determine changes in the
relative levels of *Lep* mRNA in murine epididymal adipose tissue.
4 THE EFFECT OF INSULIN ON MURINE 
Lep mRNA LEVELS In Vivo
4.1 INTRODUCTION

Consistent with its proposed role in energy homeostasis, rodent Leptin mRNA levels have been shown to be responsive to changes in basic nutritional status in vivo. A reduction in adipose tissue Leptin mRNA levels occurs after fasting, which can be reversed following short-term re-feeding in both rats and mice (Becker et al., 1995; MacDougald et al., 1995b; Saladin et al., 1995; Trayhurn et al., 1995b; Zheng et al., 1996a). Insulin alone has been shown to mimic this response to re-feeding in fasted rats, suggesting that the increase in plasma insulin levels subsequent to re-feeding may mediate the upregulation of Leptin expression (Saladin et al., 1995; Zheng et al., 1996a).

This phenomenon has been observed in other genes involved in energy homeostasis, demonstrating a rapid responsiveness to fluctuations in the nutritional state. For example, the levels of rat adipose tissue mRNA for the insulin-sensitive glucose transporter, Glut-4, are dramatically decreased after eight hours of fasting, and are rapidly restored after re-feeding. In fact, levels actually overshoot, by two-fold, the original, control levels following eight hours of re-feeding (Sivitz et al., 1989). The observation that streptozotocin-treated, insulin-deficient rats also have low adipose tissue Glut-4 mRNA levels, which can be restored by insulin administration, also points to the phenomenon being caused by the availability of insulin rather than circulating glucose levels, since glucose levels decline during fasting, yet conversely, are elevated in the diabetic state (Sivitz et al., 1989). Similarly, mRNA levels of fatty acid synthase (FAS), and other key enzymes of fatty acid and triacylglycerol synthesis are regulated in this manner (Paulauskis and Sul, 1988; Sul et al., 1984).

Given that the majority of rodent models of obesity are murine, it was of interest to study the effects of insulin on Leptin expression in the mouse, in part to enable meaningful comparisons between lean and obese states. At the outset of this study, these effects had not been extensively studied. Mizuno et al had shown that after 30 minutes, Leptin mRNA levels increased approximately two-fold by the addition of insulin in 7-hour-fasted CBA mice, but had not compared against re-feeding, or against fed controls, as had been the case with previous experiments in rats (Mizuno et al., 1996b; Saladin et al., 1995; Zheng et al., 1996a).
The initial aim of the present study was therefore to build on these results, through a more extensive study of the effects of insulin injection in fasted mice, comparing against the effects of re-feeding and against fed controls. Since many of the murine models of obesity are available on the C57BL/6J background, lean C57BL/6J mice were selected for use in these experiments.

4.2 Results

4.2.1 The Effect of Fasting, Re-feeding and Insulin on Lep mRNA Levels In Vivo

A protocol for fasting, re-feeding and insulin administration similar to that used by Saladin et al in their experiment in rats was employed (Saladin et al., 1995) (Table 4.1), although in addition, the effects of a high-sucrose diet was investigated (see section 2.7.2). The Lep mRNA levels in epididymal adipose tissue from mice in these groups were subsequently measured with the developed RNase protection assay, using as probes, the lep200 and β-actin riboprobes.

Table 4.1 Protocol of fasting, re-feeding and insulin treatment

<table>
<thead>
<tr>
<th>GROUP</th>
<th>24 HOUR PROCEDURE</th>
<th>FURTHER 4 HOUR PROCEDURE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed</td>
<td><em>ad libitum</em> chow fed</td>
<td><em>ad libitum</em> chow fed</td>
</tr>
<tr>
<td>Fasted</td>
<td>fasted</td>
<td>saline injection + continued fast</td>
</tr>
<tr>
<td>Fasted, chow re-fed</td>
<td>fasted</td>
<td>saline injection + <em>ad libitum</em> chow re-fed</td>
</tr>
<tr>
<td>Fasted, insulin</td>
<td>fasted</td>
<td>0.1 IU insulin injection + continued fast</td>
</tr>
<tr>
<td>Fasted, chow re-fed + insulin</td>
<td>fasted</td>
<td>0.1 IU insulin injection + <em>ad libitum</em> chow re-fed</td>
</tr>
<tr>
<td>Fasted, sucrose re-fed</td>
<td>fasted</td>
<td>saline injection + <em>ad libitum</em> high sucrose diet re-fed</td>
</tr>
</tbody>
</table>

As shown in Figure 4.1, a 28-hour fast reduced Lep mRNA levels approximately four-fold compared to the *ad libitum* fed control mice (fed vs. fasted) (p<0.001), consistent with the findings of Trayhum et al in mice (Trayhum et al., 1995b), and similar to the studies performed in rats (Becker et al., 1995; MacDougald et al., 1995b; Saladin et al., 1995; Zheng et al., 1996a).
Simply allowing fasted mice to *ad libitum* re-feed on standard chow for four hours substantially upregulated Lep mRNA levels, to levels approximately 8.5 fold higher than those observed in the fasted group (fasted vs. fasted, chow re-fed) (p<0.005). These levels were also observed to be twice as high as the never-fasted, *ad libitum* fed, control group (fasted, chow re-fed vs. fed) (p<0.001), in contradiction to the results of Trayhurn et al, who observed levels in mice not significantly different from control (Trayhurn et al., 1995b). However, in that study, samples were taken later, after six hours of re-feeding. Also, reports from studies in rats are highly variable, ranging from levels rising to almost twice as high as fed controls (Saladin et al., 1995), through levels comparable to fed controls (MacDougald et al., 1995b; Thompson, 1996), to levels slightly lower than controls, all within a similar time-frame (3-6 hours) (Becker et al., 1995).

In keeping with the previous reports in rodents (Mizuno et al., 1996b; Saladin et al., 1995; Zheng et al., 1996a), insulin administration caused a substantial rise in the relative levels of murine Lep mRNA after four hours. Levels were 6.5-fold higher than the fasted controls (fasted vs. fasted, insulin), (p<0.005). This was not significantly different from either *ad libitum* fed controls, or from the chow re-fed group (fed; fasted, chow re-fed).

Insulin administration in addition to *ad libitum* re-feeding for four hours, also increased Lep mRNA levels. Levels were comparable to those of the fed control and the insulin-treated group (fasted, chow re-fed + insulin vs. fed; fasted, chow re-fed + insulin vs. fasted, insulin). Levels were also significantly increased compared to the fasted controls (fasted, chow re-fed + insulin vs. fasted) (p<0.05). In addition, however, it was observed that Lep mRNA levels in the re-fed plus insulin treated mice were statistically lower than levels in re-fed only mice (p<0.005) (for clarity, this significance is not indicated on the graph).

Unexpectedly, it was found that *ad libitum* re-feeding fasted mice on a high sucrose diet for four hours failed to induce an increase in the steady-state levels of Lep mRNA compared to fasted controls (fasted vs. fasted, sucrose re-fed).

Figure 4.2 shows a representative autoradiograph of an RNase protection assay illustrating epididymal adipose tissue Lep and β-actin mRNA levels in mice, after these various procedures.
Figure 4.1 Graph illustrating relative Lep mRNA levels in mice after fasting, re-feeding and insulin-treatment

Groups of mice were subjected to various regimes of ad libitum feeding, overnight fasting, and short-term re-feeding and/or insulin-administration (see Table 4.1). Immediately after sacrifice, epididymal adipose tissue samples were taken. An RNase protection assay to measure relative Lep mRNA levels was performed using 10µg samples of epididymal adipose tissue total RNA. The lep200 and β-actin riboprobes were used. Samples were resolved on a denaturing 6% acrylamide gel, were exposed overnight onto autoradiographic film, and then phosphorimaged for four hours. The relative mRNA level of the fed control was arbitrarily assigned a value of 1.

Student’s t-tests were performed to test for significant differences between control and experimental groups. (* = p<0.05, ** = p<0.005 compared to fasted control; † = p<0.001, compared to fed control).
Figure 4.2  Autoradiograph of RNase protection assay illustrating Lep mRNA levels in mice after fasting, re-feeding and insulin-treatment

An RNase protection assay was performed using 10µg samples of total RNA from mice subjected to the various treatments indicated, using the lep200 and β-actin riboprobes. Samples were resolved on a denaturing 6% polyacrylamide gel, and were exposed overnight onto autoradiographic film. Also shown are the controls (riboprobes + yeast tRNA +/-RNase (Y+, Y-)). DNA marker: 100bp ladder.
4.2.2 BLOOD GLUCOSE LEVELS AFTER FASTING, RE-FEEDING AND INSULIN-ADMINISTRATION

The blood glucose levels of each animal were measured in all groups of mice immediately after sacrifice (insulin levels were not measured). The results are shown in Figure 4.3.

Fasting, as expected, reduced blood glucose levels significantly compared to control ad libitum fed mice. Levels fell from 10.8mM to 6.6mM (fed vs. fasted) (p<0.001). No further decrease was observed in the fasted, insulin-treated mice (fasted vs. fasted, insulin), presumably because the effects of insulin on blood glucose levels are acute, and would not be observable after a further four hours, and also since compensatory mechanisms would prevent a deleterious drop in blood glucose levels.

Nor did administered insulin influence levels of blood glucose after four hours of ad libitum chow feeding, since levels were similar with or without insulin-administration (fasted, chow re-fed vs. fasted, chow re-fed + insulin). These levels were significantly higher than fasted levels (p<0.001), but had not reached normal, control levels within the four-hour period – on average they were 14.4% and 17.8% lower than ad libitum fed control levels respectively (p<0.01).

Ad libitum re-feeding on the high sucrose diet, on the other hand, caused blood glucose levels to return to normal, control fed levels within four hours (fed vs. fasted, sucrose re-fed).

Blood glucose levels, four hours after the different procedures, therefore changed according to the fed and fasted states, as expected. However, changes in glucose levels did not reflect the changes in Lep mRNA levels in all groups. For instance, whilst low blood glucose levels were observed in both the fasted and insulin-treated mice, the insulin-treated mice had significantly higher Lep mRNA levels. Furthermore, the sucrose re-fed mice had significantly elevated blood glucose levels compared to the fasted mice, yet Lep mRNA levels were similarly as low as the fasted group.

These results, especially concerning the fasted, insulin-treated mice, therefore demonstrate that the blood glucose levels observed in ad libitum fed mice are not required for the regulation of Lep mRNA levels in vivo by insulin.
Figure 4.3  

Graph illustrating blood glucose levels in mice after fasting, re-feeding and insulin-treatment

Blood glucose levels in *ad libitum* fed, fasted, re-fed and insulin-treated mice (see Table 4.1) were measured immediately after sacrifice using a Bayer Diagnostics glucometer, model 4. Student’s t-tests were performed to test for significant differences between control and experimental groups.

(* = p<0.001 compared to fasted controls; † = p<0.01 compared to fed controls).
4.2.3 **Body Weight after Fasting, Re-feeding and Insulin-Administration**

Since *Lep* mRNA and plasma leptin levels have been shown to be correlated with body weight (Considine et al., 1995; Considine et al., 1996b; Frederich et al., 1995a; Frederich et al., 1995b; Maffei et al., 1995b), the change in body weight and *Lep* mRNA levels, following fasting, re-feeding and insulin-administration, was determined in small groups of mice, and were compared to *ad libitum* fed control levels.

Figure 4.4 and Table 4.2 show the body weights observed in this experiment. The mice within each group were weighed together not individually, thus weights are shown as the average weight of mice in each group but are without standard error measurements.

The resulting weight changes were in good agreement with results described by Ahima et al (Ahima et al., 1996). 24-hours of fasting reduced body weight by between 6.5% and 9.8%, (fasted vs. fasted, insulin respectively). An additional four hours of fasting reduced body weight still further in both the saline and insulin-treated mice (4.5% and 2% respectively), whereas re-feeding fasted animals for four hours increased body weight by between 1.3% and 3.3%, depending on the re-fed diet and presence/absence of insulin. The amount of weight regained within four hours of re-feeding standard chow were similar to those shown by Ahima et al, where precise measurements of chow were recorded.

Mice with continual access to chow were weight stable over the initial 24 hours, but lost a small amount of weight during the subsequent four hours. This can be explained by the normal feeding behaviour of mice. The initial 24-hour period began in the light-cycle (see section 2.7.2), when mice with continual access to food do not normally eat. Any loss in body weight during this time would be regained once feeding began again during the dark cycle (in the remaining part of the 24-hour period). The additional four hours of the experiment were conducted again during the light-cycle, hence the small reduction in body weight observed here is in keeping with the notion that the mice were not feeding during this time.
The average weight of mice in each group of *ad libitum* fed, fasted, re-fed and insulin-treated mice (see Table 4.1) were measured at the beginning of the treatment period (0 hours) and after 24 and 28 hours.

### Table 4.2  Average body weights of mice subjected to fasting, re-feeding and insulin treatment

<table>
<thead>
<tr>
<th>GROUP</th>
<th>AVERAGE WEIGHT (g)</th>
<th>AVERAGE WEIGHT CHANGE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 HOURS</td>
<td>24 HOURS</td>
</tr>
<tr>
<td>fed (n=3)</td>
<td>25.20</td>
<td>25.23</td>
</tr>
<tr>
<td>fasted (n=4)</td>
<td>24.25</td>
<td>22.68</td>
</tr>
<tr>
<td>fasted, chow re-fed (n=3)</td>
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<td>21.60</td>
</tr>
<tr>
<td>fasted, insulin (n=4)</td>
<td>24.23</td>
<td>21.85</td>
</tr>
<tr>
<td>fasted, chow re-fed + insulin (n=3)</td>
<td>25.07</td>
<td>23.23</td>
</tr>
<tr>
<td>fasted, sucrose re-fed (n=3)</td>
<td>24.03</td>
<td>20.97</td>
</tr>
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</table>
At the end of this experiment, epididymal adipose tissue from the animals in each group was pooled to determine average *Lep* mRNA levels. These were found to be comparable to those observed in the more extensive study described in section 4.2.1, except for the chow re-fed + insulin group, which had slightly higher values (data not shown).

When the body weights after 28-hours were compared to their respective *Lep* mRNA levels, and to mRNA levels observed in the more extensive study (see section 4.2.1), no correlations between short-term changes in body weight and *Lep* mRNA levels could be identified, except during fasting alone. Firstly, the similarly large percentage weight losses exhibited by both saline- and insulin-injected fasted mice, did not show a relationship with the significantly different *Lep* mRNA levels observed in these two groups. Secondly, the similarly low *Lep* mRNA levels observed in fasted and sucrose re-fed mice, did not correlate with the fact that the sucrose re-fed mice had regained almost half the body weight that they had lost during 24 hours of fasting, whereas the fasted mice continued to lose weight after the initial 24 hours. Thirdly, the chow- and sucrose re-fed mice had regained similar amounts of weight after four hours, but again, showed significantly different levels of *Lep* mRNA.
4.3 DISCUSSION

The results presented in this chapter corroborate reports from similar, previously performed studies. In the first instance, murine *Lep* mRNA levels were shown to be significantly down-regulated by fasting, which could be reversed following short-term re-feeding. This is in agreement with reports in both rats (Becker et al., 1995; Saladin et al., 1995; Thompson, 1996; Zheng et al., 1996a), and mice (MacDougald et al., 1995b; Trayhurn et al., 1995b). Comparable *Lep* mRNA levels were observed by Trayhurn et al (Trayhurn et al., 1995b) following a similar fasting regimen in mice (28% vs. 24.4% of fed, control levels respectively), indicating that the designed RNase protection assay used in the present study is capable of considerable accuracy.

The apparent 'overshoot' in mRNA levels that was observed after re-feeding, compared to control, *ad libitum* fed mice, was reminiscent of results from studies performed on other genes involved in energy homeostasis, such as FAS and Glut 4 (Paulauskis and Sul, 1988; Sivitz et al., 1989), and was comparable to the study performed in rats by Saladin et al (Saladin et al., 1995), where average *Lep* mRNA levels were almost double those of the control group. Despite high variability in levels of *Lep* mRNA within each group of mice in the experiment described in this present body of work, each individual sample in the re-fed group was higher than any sample in the control, *ad libitum* fed group, suggesting that this 'overshoot' is indeed a real phenomena, and not simply an anomaly due to high variability within groups coupled with a relatively small sample size.

Most importantly for this study, it was observed that a single subcutaneous 0.1 IU insulin injection could indeed reverse the fasting-mediated down-regulation of *Lep* mRNA levels in C57BL/6J mice, increasing to levels comparable with re-feeding alone. These results also compare favourably with results reported in rodents (Mizuno et al., 1996b; Saladin et al., 1995; Zheng et al., 1996a), and correlate with subsequent changes in serum leptin levels that occur after these procedures (Hardie et al., 1996b).

Also in agreement with a previous study in rats, standard chow re-feeding plus insulin-administration also raised *Lep* mRNA from fasted levels back to *ad libitum* fed control levels, and levels did not rise above levels observed from either condition alone (Saladin et al., 1995). This lack of an additive effect between the two treatments could
therefore indicate that insulin is responsible for the increase in \( \text{Lep} \) expression that occurs after re-feeding standard chow. However, since \( \text{Lep} \) mRNA levels observed with both treatments together were statistically lower than re-fed alone levels, further studies must be undertaken to further understand the interactions between these two treatments.

It was unclear why sucrose re-feeding did not mediate an increase from fasted \( \text{Lep} \) mRNA levels. At first glance, it may be speculated that the animals simply did not consume this unfamiliar food source, and effectively remained fasted, since precise experiments to ascertain this information were not carried out (e.g. weighing the food). This theory may be discounted however, by the following indirect evidence;

i) teeth marks were observed in the remaining food at the end of the experiment,

ii) blood glucose levels had returned to fed, control levels (whilst chow re-feeding had not resulted in a full return to these levels),

iii) the average body weight of this group had risen by 3.3% during the four hour period of re-feeding (as opposed to continued fasting, where body weight fell still further, although the re-hydrating effect of the sucrose solution undoubtedly contributed substantially to this increase in short-term body weight).

The short-term effects of a high sucrose diet are somewhat different than feeding standard chow however, which is the reason why this group was added to the experiment in the first instance. Sucrose re-feeding has been shown to rapidly increase plasma insulin levels, to levels higher than that seen in chow re-feeding (Hendley et al., 1987), but it also stimulates the sympathetic nervous system to a greater extent than chow re-feeding, as part of a process known as nutritionally-determined thermogenesis. (Young and Landsberg, 1977). It may be the case that the inhibitory effects of catecholamines on \( \text{Lep} \) expression (Trayhurn et al., 1995a; Trayhurn et al., 1996), could potentially be responsible for the inhibition of \( \text{Lep} \) expression in this circumstance. Further experiments would need to be carried out to investigate these findings.

Short-term changes in blood glucose levels were found not to correlate with changes in \( \text{Lep} \) mRNA levels in this study, simply being associated with either the fed or fasted state. This indicates that normal levels of blood glucose are not required in the short-
term regulation of the *Lep* gene by insulin in mice. This is consistent with the results of Saladin et al, where hyperglycaemic and euglycaemic, hyperinsulinaemic clamps showed no effect of glucose on *Lep* mRNA levels in rats (Saladin et al., 1995). In a longer-term study in humans, serum leptin and insulin levels were shown to decrease significantly during weight-loss, whereas glucose levels did not change (Considine et al., 1996b). However a wider role for glucose in the regulation of *Lep* gene expression may exist. Mizuno et al found that *Lep* mRNA levels in mice after glucose administration were more closely correlated with plasma glucose concentrations than to the concomitant rise in plasma insulin concentrations (Mizuno et al., 1996a), and Mueller et al report that inhibition of glucose transport, glucose metabolism or glycolysis can inhibit insulin-stimulated increases in *Lep* mRNA levels *in vitro*, thus suggesting a permissive role for glucose in this instance (Mueller et al., 1998).

Although a more relevant comparison of the weights of the epididymal fat pads of the different groups of mice was not performed in this study, the fact that whole body weight changes were observed as would be expected following fasting and re-feeding, and did not correlate with changes in *Lep* mRNA levels in all instances, suggested that short-term changes in body weight also do not contribute considerably to the regulation of the *Lep* gene *in vivo*. This is in agreement with other studies, in humans, which report that acute changes in leptin levels after fasting or forced overfeeding, are not related to the degree of adiposity (Kolaczynski et al., 1996a; Kolaczynski et al., 1996c). It must be borne in mind however, that re-hydration and the weight of food rather than adipose mass accumulation *per se* are mostly responsible for the short-term body weight fluctuations reported in the present study.

In conclusion, therefore, these findings illustrate that acute (four-hour) insulin administration reverses the fasting-mediated reduction in epididymal adipose tissue *Lep* mRNA levels in mice, in a similar manner to that observed previously. This response is independent of short-term body weight changes and of blood glucose levels. Other changes in the nutritional state (i.e. occurring from re-feeding), are not required to contribute to this response, since insulin can restore levels to those observed with re-feeding alone. However, as was observed in the sucrose re-fed group, certain factors could potentially be involved in inhibiting this process.
5 The Effect of Insulin on Murine Lep mRNA Levels *In Vitro* – Using an Adipose Tissue Explant Model
5.1 INTRODUCTION

Although the effect of insulin on \textit{Lep expression in vivo} is clear, a debate continues as to whether insulin exerts a direct effect on \textit{Lep expression in adipocytes}. Various rodent \textit{in vitro} model systems have been implemented to clarify this issue, but have returned with conflicting evidence, complicated by the use of different culture and quantification methods. Some studies report that physiological concentrations of insulin increase the steady-state levels of \textit{Lep} mRNA directly in rat adipocytes (Saladin et al., 1995; Slieker et al., 1996; Yoshida et al., 1996) and in the adipocyte cell line models, 3T3-L1 (Rentsch and Chiesi, 1996) and 3T3-F442A (Leroy et al., 1996). However, others see no effect, even at relatively high concentrations of insulin, e.g. in primary rat adipocytes (Murakami et al., 1995), rat explant culture (Reul et al., 1997) and 3T3-L1 adipocytes (Kosaki et al., 1996; MacDougald et al., 1995b).

It is more generally accepted that insulin can increase leptin secretion \textit{in vitro} however, in primary rat adipocytes (Gettys et al., 1996; Hardie et al., 1996b; Slieker et al., 1996), 3T3-F442A adipocytes (Leroy et al., 1996) and rat explant cultures (Barr et al., 1997; Fain and Bahouth, 2000), although whether this constitutes an effect on \textit{de novo} synthesis, or an effect on stored leptin release, or both, is still debatable (Barr et al., 1997; Bradley and Cheatham, 1999; Bradley et al., 2001).

The direct effect of insulin on \textit{Lep} mRNA levels in murine adipocytes had not been reported at the onset of the present study - although subsequently studies on immortalized cell lines have been done, (Kosaki et al., 1996; Leroy et al., 1996; Rentsch and Chiesi, 1996). To begin to address this gap in the existing knowledge of rodent \textit{Lep} regulation, the effect of insulin on murine \textit{Lep} mRNA levels was investigated \textit{in vitro}.

5.2 RESULTS

5.2.1 BASAL \textit{LEP} AND FAS mRNA LEVELS IN 3T3-F442A ADIPOCYTES

The 3T3-F442A adipocyte cell line was initially investigated as a model system to observe the effects of insulin on murine \textit{Lep expression in vitro}. Firstly, to assess basal levels of expression, a time course of differentiation of 3T3-F442A cells from confluent preadipocytes to adipocytes was performed over ten days, using standard differentiation procedures (see section 2.5.3). \textit{Lep}, PPARγ2 and FAS mRNA levels, relative to β-actin
were assayed at different time points, using the lep400, PPARγ2 and FAS riboprobes. Lep mRNA levels were undetectable at all time points during this ten-day study (even after several days of autoradiographic exposure of the RNase protection assay), whereas PPARγ2 and FAS mRNA levels were easily detectable, and had noticeably increased over the course of the experiment (Figure 5.1).

The undetectable levels of Lep mRNA using this model system supports previous findings that low levels of Lep mRNA are observed in adipocyte cell lines compared to adipose tissue (MacDougald et al., 1995b; Murakami et al., 1995). MacDougald et al suggest that this is possibly because adipocytes in a tissue context are subject to humoral or other influences - not present in a cell culture environment - that upregulate Lep expression. The lack of detection of Lep mRNA in the present study, may suggest that the designed RNase protection assay is not sensitive enough to detect such low levels.

5.2.2 Basal Lep mRNA Levels in Murine Primary Adipocytes

Lep mRNA levels were also assayed in murine primary adipocyte cultures, using the lep200 riboprobe. Levels of Lep mRNA in 10µg total RNA from freshly prepared primary adipocytes were barely detectable using this system, and were below the levels of sensitivity of the developed RNase protection assay (data not shown).

5.2.3 Basal Lep and FAS mRNA Levels in Adipose Tissue Explants

Small sections of epididymal adipose tissue in culture were next investigated as an in vitro model system for this study. Adipose tissue explants consist of more than one cell type and maintain interactions between cells, hence this model is considered more physiological than disaggregated adipocytes.

mRNA levels in these explants, cultured for eight hours, displayed low, but detectable levels of Lep mRNA. Levels were observed to be considerably lower than those determined at the beginning of the culture period however. Levels of PPARγ2 mRNA were also low (data not shown).

Following this result, a time course experiment was carried out to further delineate the decline in Lep mRNA levels in adipose tissue explant culture. Figure 5.2a illustrates the Lep, FAS and β-actin mRNA levels from the autoradiograph of the RNase protection assay from this experiment, and Figure 5.2b and Figure 5.2c show graphically the fall in
the relative levels of *Lep* mRNA during this time — *Lep* mRNA levels were virtually undetectable after 22 hours in culture.

The half-life of murine *Lep* mRNA has been calculated to be approximately two hours (Leroy et al., 1996; Thompson, 1996). If the theoretical levels of *Lep* mRNA degrading in this manner are plotted alongside the experimental data, the values correspond remarkably well, at least up to 8 hours (Figure 5.2b and Figure 5.2c, experimental vs. theoretical). This is particularly evident in Figure 5.2c, which has been plotted on a semi-logarithmic scale. This data suggests that *Lep* expression is inhibited or substantially reduced in these *in vitro* culture conditions, and is being degraded in accordance with its observed half-life, suggesting that factors responsible for the normal regulation of *Lep* gene expression are absent in the culture conditions used. It is unclear from this data whether *Lep* mRNA levels stabilize after 8 hours in culture, since levels are at the limit of the sensitivity of the assay.

This degradation could also help to explain why *Lep* mRNA levels were barely detectable in the primary adipocytes, since these have a relatively long preparation time.

A decline in the relative levels of FAS and PPARγ2 mRNA in culture were also observed, whilst β-actin levels appeared to be unaffected (Figure 5.2a). The low levels of PPARγ2 mRNA observed (data not shown), led us to abandon the use of this positive control in future studies, since FAS alone could perform this function.

Subsequently, others too have reported lower levels of *Lep* mRNA in primary adipocytes and explants in culture, compared to *in vivo* levels (Fain and Bahouth, 2000; Machinal et al., 1999; Reul et al., 1997).
Figure 5.1  PPARγ2 and FAS mRNA levels during the differentiation of 3T3-F442A adipocytes

Confluent 3T3-F442A preadipocytes were cultured in standard medium plus 100nM insulin for 10 days. Total RNA was extracted from individual samples at various time points throughout the culture period. An RNase protection assay was then performed with 10μg samples of total RNA from each time point, using the lep400, PPARγ, FAS and β-actin riboprobes. Samples were resolved on a denaturing 6% polyacrylamide gel, and were exposed overnight onto autoradiographic film. PPARγ2 and FAS mRNA levels, relative to β-actin were then determined by phosphorimaging (4 hour exposure). The relative mRNA level in preadipocytes (Day 0) was arbitrarily assigned a value of 1. (Lep mRNA levels were undetectable).
Figure 5.2 The effect of time in culture on relative *Lep* and FAS mRNA levels in adipose tissue explants

Adipose tissue explants were prepared, and cultured for 22 hours in standard medium. Total RNA was extracted at the various time points throughout the culture period. An RNase protection assay was then performed with 10µg of total RNA from each sample time point, using the lep400, FAS and β-actin riboprobes. Samples were resolved on a denaturing 6% polyacrylamide gel, and were exposed overnight onto autoradiographic film. *Lep* and FAS mRNA levels, relative to β-actin were then determined by phosphorimaging (4 hour exposure). The relative mRNA level at time 0 was arbitrarily assigned a value of 1 (or 100 for the semi-logarithmic graph).

a) Autoradiograph of RNase protection assay, illustrating *Lep*, FAS and β-actin mRNA levels.

b) Graph illustrating the reduction in relative *Lep* mRNA levels. The theoretical levels of *Lep* mRNA undergoing degradation according to its half-life (2 hours) have also been plotted.

c) Semi-logarithmic graph illustrating the reduction in relative *Lep* mRNA levels. The theoretical levels of *Lep* mRNA undergoing degradation according to its half-life (2 hours) have also been plotted.
a) 

Lep
FAS
β-actin

b) 

Time in culture (hours)

- relative Lep mRNA level

0 2 4 8 10 12 14 16 18 20 22

0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1

C % experimental theoretical

132
5.2.4 THE EFFECT OF INSULIN ON PREVENTING THE DECREASE IN mRNA LEVELS IN CULTURE

The suggestion that factors absent from the existing culture conditions might account for the dysregulation of gene expression in adipocyte culture, led to the hypothesis that insulin might be this missing factor in the case of Lep and FAS expression, and therefore supplementing the medium with insulin could potentially prevent the decrease in Lep mRNA levels in cultured adipose tissue explants over time. To test this hypothesis, adipose tissue explants were cultured for eight hours, in the presence or absence of 100nM insulin. Both Lep, and the insulin-responsive positive control, FAS, mRNA levels were determined, and compared to levels at time zero, and after four hours in culture without insulin.

Figure 5.3 shows the levels of Lep mRNA determined by RNase protection assay, and Figure 5.4 illustrates the resulting levels of FAS mRNA.

The marked reduction in Lep mRNA levels in culture was not prevented by incubation with 100nM insulin, although it appeared that it may modestly slow this reduction, and/or stimulate Lep mRNA levels after eight hours (8 hours vs. 8 hours +100nM insulin). However, only single samples were analysed during this experiment, and due to the fact that Lep levels were at the limits of detection after eight hours, further experiments were required to confirm this.

Conversely, the reduction in FAS mRNA levels over time in culture was wholly prevented by insulin - in fact, levels were raised compared to levels at time zero, confirming that the culture system was indeed responsive to insulin (Figure 5.4).
Adipose tissue explants were prepared and cultured for up to eight hours in standard medium. One sample was also cultured in standard medium supplemented with 100nM insulin for the entire eight-hour period. Total RNA was extracted from the samples at the various time points throughout the culture period. An RNase protection assay was then performed with 10µg of total RNA from each sample, using the lep400, FAS and β-actin riboprobes. Samples were resolved on a denaturing 6% polyacrylamide gel, and were exposed overnight onto autoradiographic film. Lep mRNA levels, relative to β-actin were determined by phosphorimaging (4 hour exposure). The relative mRNA level at time 0 was arbitrarily assigned a value of 1.

a) Autoradiograph of RNase protection assay, illustrating Lep and β-actin mRNA levels.

b) Graph illustrating the relative Lep mRNA levels over time in culture, in the absence or presence of 100nM insulin.
a) *Lep*

β-actin

<table>
<thead>
<tr>
<th>0</th>
<th>4</th>
<th>8</th>
<th>8</th>
<th>Hours (+ insulin)</th>
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b) Relative *Lep* mRNA level

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<th>Time in culture (hours)</th>
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<td>0</td>
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- 0 nM insulin
- 100 nM insulin
Figure 5.4 The effect of 100nm insulin on relative FAS mRNA levels in adipose explant culture

In the same RNase protection assay described in Figure 5.3, FAS mRNA levels, relative to β-actin were determined by phosphorimaging (4 hour exposure). The relative mRNA level at time 0 was arbitrarily assigned a value of 1.

a) Autoradiograph of RNase protection assay, illustrating FAS and β-actin mRNA levels.

b) Graph illustrating the relative FAS mRNA levels over time in culture, in the absence or presence of 100nM insulin.
a) FAS

b) β-actin

0 4 8 8 Hours
(+insulin)

0nM insulin 100nM insulin

Time in culture (hours)

relative FAS mRNA level
5.2.5 THE CONCENTRATION-DEPENDENT EFFECTS OF INSULIN ON LEP AND FAS mRNA LEVELS

To investigate more extensively whether insulin has a direct effect on Lep mRNA levels, the effect of insulin concentration on the steady-state levels of both Lep and FAS mRNA was determined. The effects of insulin after four hours, as had been explored in the in vivo study (see chapter four) were investigated. Epididymal adipose explants were cultured for four hours in standard medium, before exposure to a range of insulin concentrations for an additional four hours.

Although a general increase in steady-state Lep mRNA levels was observed in response to increasing concentrations of insulin, this was only statistically significant (p<0.05) at supraphysiological concentrations over 1μM, whereas at relatively physiological concentrations (10nM) no such response was detectable (Figure 5.5). However, even at the highest concentration of insulin tested - 10μM - levels of Lep mRNA were only around one-third of levels detected in an RNA sample from freshly prepared adipose tissue explants (data not shown).

Likewise, FAS expression in response to insulin followed a similar pattern, with significant increases occurring only at supraphysiological concentrations (Figure 5.6). The lack of a considerable response to insulin at lower concentrations was in contradiction to the previous finding (see section 5.2.4) that showed that explants cultured continuously with 100nM insulin exhibited FAS mRNA levels twice as high as compared to without insulin. The shorter incubation time in the present study (four hours, compared to eight hours in the latter experiment) is potentially the cause of this discrepancy.

To determine if a longer exposure to insulin could result in a more pronounced effect on FAS and Lep mRNA levels, the experiment was repeated in a smaller study. Samples were incubated for eight hours in standard medium, before exposure overnight (sixteen hours) to a range of insulin concentrations (Figure 5.7).

The resulting RNase protection assay revealed that Lep mRNA levels remained at the limits of detection of the assay after this time, even when the explants were incubated with the highest concentration of insulin (1μM) (data not shown).
FAS expression in response to insulin was found to be more pronounced after this length of time in culture (Figure 5.7). At 1nM insulin, the steady-state levels of FAS mRNA were almost twice those observed in the control sample incubated without insulin. Maximal expression occurred at 100nM.
Figure 5.5 The concentration-dependent effect of insulin on relative Lep mRNA levels in adipose explant culture

Adipose tissue explants were prepared and cultured for four hours in standard medium. Insulin was then added to achieve the final concentrations indicated, and the samples were cultured for a further four hours. Total RNA was extracted from individual samples and an RNase protection assay was then performed with 10μg of total RNA from each sample, using the lep400, FAS and β-actin riboprobes. Samples were resolved on a denaturing 6% polyacrylamide gel, and were exposed overnight onto autoradiographic film. Lep mRNA levels, relative to β-actin were then determined by phosphorimaging (4 hour exposure). The relative mRNA level at 0nM insulin was arbitrarily assigned a value of 1.

Student’s t-tests were performed to test for significant differences between control and experimental groups.

(* = p<0.05 compared to 0nM insulin).
Figure 5.6 The concentration-dependent effect of insulin on relative FAS mRNA levels in adipose explant culture

In the same RNase protection assay described in Figure 5.5, FAS mRNA levels, relative to β-actin were determined by phosphorimaging (4 hour exposure). The relative mRNA level at 0nM insulin was arbitrarily assigned a value of 1.

Student's t-tests were performed to test for significant differences between control and experimental groups.

(* = p<0.05 compared to 0nM insulin).
Figure 5.7 The concentration-dependent effect of insulin on relative FAS mRNA levels in longer-term adipose explant culture

Adipose tissue explants were prepared and cultured for eight hours in standard medium. Insulin was then added to achieve the final concentrations indicated, and the samples were cultured for a further sixteen hours (overnight). Total RNA was extracted from individual samples and an RNase protection assay was then performed with 10μg of total RNA from each sample, using the lep400, FAS and β-actin riboprobes. Samples were resolved on a denaturing 6% polyacrylamide gel, and were exposed overnight onto autoradiographic film. FAS mRNA levels, relative to β-actin were determined by phosphorimaging (4 hour exposure). The relative mRNA level at 0nM insulin was arbitrarily assigned a value of 1. (n=1).

(Lep mRNA levels were undetectable).
a) FAS
β-actin

0 1 10 100 1000 Insulin (nM)

b) Relative FAS mRNA level

0 1 10 100 1000 Insulin concentration (nM)
5.2.6 THE CONCENTRATION-DEPENDENT EFFECTS OF DEXAMETHASONE ON Lep mRNA LEVELS

The increased mRNA levels of the insulin-responsive control (FAS), with insulin, allayed concerns of the explants culture’s insulin-responsiveness. However, the very low levels of Lep mRNA observed in vitro compared to in vivo, possibly implied that Lep expression was inherently dysregulated in this culture system.

To address this concern, Lep expression was studied in response to the synthetic glucocorticosteroid, dexamethasone. Studies have reported glucocorticosteroids to be positive regulators of Lep expression in rodents and humans, both in vivo (De Vos et al., 1995; Kolaczynski et al., 1997) and in vitro, (Murakami et al., 1995; Slieker et al., 1996; Wabitsch et al., 1996).

Using similar periods of incubation as reported in the literature (Murakami et al., 1995; Slieker et al., 1996), epididymal adipose tissue explants were incubated for eight hours in standard medium, before exposure overnight (sixteen hours) to a range of dexamethasone concentrations.

After the full 24-hour culture period, Lep mRNA levels in the adipose explants cultured without dexamethasone were virtually undetectable, and were below the sensitivity of the assay system. However, levels were detectable in samples incubated with all concentrations of dexamethasone tested. Maximal expression was observed at the lowest level of dexamethasone tested (10nM) (Figure 5.8). This data correlates well with previous findings of the effective concentration range of dexamethasone on both leptin secretion and Lep expression in vitro (Murakami et al., 1995; Slieker et al., 1996).

Dexamethasone treatment raised Lep mRNA to approximately one-third of the levels observed in vivo (i.e. 0 hours culture), in agreement with similar experiments performed in primary rat adipocytes (Fain and Bahouth, 1998b).

Since Lep mRNA levels were virtually undetectable even with high concentrations of insulin after 24 hours in culture, the results with dexamethasone verified that Lep expression was not inherently dysregulated in longer-term (24-hour) culture of adipose explants.
Adipose tissue explants were prepared and cultured for eight hours in standard medium. Dexamethasone was then added, to achieve the final concentrations indicated, and the samples were cultured for a further sixteen hours (overnight). Total RNA was extracted from individual samples and an RNase protection assay was then performed with 10μg of total RNA from each sample, using the lep400 and β-actin riboprobes. Samples were resolved on a denaturing 6% polyacrylamide gel, and were exposed overnight onto autoradiographic film. Lep mRNA levels, relative to β-actin were determined by phosphorimaging (4 hour exposure). The relative mRNA level at time 0 was arbitrarily assigned a value of 1. (Lep mRNA levels in samples cultured without dexamethasone were undetectable).

Student’s t-tests were performed to test for significant differences between groups treated with dexamethasone. No significant difference was found (n=3).
5.3 DISCUSSION

With confirmation that insulin reverses the fasting-mediated down-regulation of *Lep* mRNA levels *in vivo* in mice, several *in vitro* model systems were investigated to determine any direct effect of insulin on *Lep* gene expression. These models included the 3T3-F442A cell line and murine epididymal adipose tissue cultures.

It was noted that the steady-state levels of *Lep* mRNA in *in vitro* culture conditions were considerably lower than those observed in total RNA samples from freshly isolated adipose tissue. This phenomenon has been observed by other researchers (MacDougald et al., 1995b; Murakami et al., 1995), and suggests that the culture conditions lack factors necessary for appropriate regulation of gene expression. When investigated further in adipose tissue explants, it was observed that *Lep* mRNA levels decreased over time in culture, in a manner suggestive of inhibited or substantially reduced *de novo* mRNA synthesis, coupled with mRNA degradation in accordance with its proposed half-life (Leroy et al., 1996; Thompson, 1996). Supplementing the culture medium with 100nM insulin could not prevent this decrease in *Lep* mRNA levels over eight hours, although it may have had a small effect on stabilizing and/or stimulating mRNA levels.

In contrast, 100nM insulin was sufficient to raise FAS mRNA levels above those observed at the beginning of the culture period. The upregulation of this insulin-responsive gene, confirmed that this particular adipose tissue model system was indeed responsive to insulin, but suggested that insulin may not play a major role in the direct regulation of the *Lep* gene.

Leading on from this small study, the effects of a range of concentrations of insulin were studied, to further investigate any effects of insulin on the steady-state levels of *Lep* mRNA. A four-hour exposure to physiological concentrations of insulin did not affect *Lep* mRNA steady-state levels, although they were raised in response to supraphysiological levels (1-10μM). The response to such high concentrations of insulin could however, be mediated via insulin-growth factor receptor 1 (IGF-1), which insulin has an affinity to at high concentrations.

Furthermore, in contrast to the initial finding that during an eight-hour exposure, FAS expression was responsive to 100nM insulin, a four-hour exposure to this and lower, more physiological, concentrations of insulin did not significantly affect FAS mRNA
levels. Likewise to *Lep* expression, supraphysiological concentrations of insulin did, however, significantly increase FAS mRNA steady-state levels. The shorter treatment time (four hours instead of eight) was likely to be the reason for this discrepancy with the earlier findings.

A longer exposure to insulin (sixteen hours) was necessary to observe FAS expression responsiveness to physiological concentrations of insulin, confirming that the explants could retain their insulin-sensitivity in culture. This is in agreement with *in vivo* findings of the differing kinetics of the nutritional-responsiveness of the FAS and *Lep* genes. Fasted rat FAS mRNA levels in adipose tissue only begin to rise after 4-8 hours of re-feeding, and are maximal after 16 hours, compared to *Lep* mRNA levels, which begin to rise much earlier, within 30 minutes, and are maximal at 8 hours (Iritani et al., 2000).

Insulin at any concentration tested did not appreciably raise the low levels of *Lep* mRNA observed after this length of time in culture, since levels were found to be at the limits of detection of the assay. This, of course, cannot rule out the possibility that insulin may have a small effect on raising *Lep* mRNA levels over a longer culture period. However a more sensitive assay system would be necessary to investigate this further.

Finally, in light of the rapid decline in *Lep* mRNA levels in adipose tissue explants in culture, the effect of another factor known to affect *Lep* expression was investigated, in an effort to determine if *Lep* expression was inherently dysregulated in this particular culture system. Relatively low concentrations of dexamethasone (10nM) stimulated a maximal increase in *Lep* mRNA levels in overnight cultures of adipose explants, whereas without dexamethasone, *Lep* mRNA levels were virtually undetectable. This response was in contrast to that observed with insulin, which could not increase *Lep* mRNA levels above the limits of detection of the assay after this length of time in culture, and required supraphysiological concentrations to achieve it in short-term culture. Dexamethasone failed, however, to restore *Lep* mRNA to *in vivo* levels, again suggesting that other factors, not present in the culture system, are necessary for normal *Lep* expression.

In conclusion therefore, the extremely low levels of *Lep* mRNA observed *in vitro*, even in long-term culture in the presence of high concentrations of insulin, together with the evidence from the previous *in vivo* experiments - that *Lep* mRNA levels are raised
significantly in response to insulin within four hours - infers that insulin does not have a major, acute, direct, effect on murine Lep expression. The possibility that insulin exerts a small effect still remains, however the RNase protection assay used here is not sensitive enough to detect this.

In addition to its low sensitivity for Lep mRNA detection in vitro, the many steps involved in the RNase protection assay was found to be a disadvantage. In order to minimise the number of mice involved in each experiment, only enough RNA could be extracted from each sample to perform the assay once. This meant that any loss of sample occurring during the complicated assay process, prevented full interpretation of the experiment. These problems unfortunately meant that the insulin-responsiveness assays were never repeated in their entirety, although similar trends could be inferred from other incomplete experiments. Additionally, the assay system was complicated by relatively large variations in Lep mRNA levels from samples subjected to the same conditions. This may have been due to an inadequately homogenous starting material.

The results from chapter four, and other researchers (Barr et al., 1997) have reported large variations in Lep mRNA and leptin levels between different animals. It is therefore plausible that, although the epididymal adipose tissues from all mice were pooled before the explants were prepared, that high levels in some tissue compared to others could result in some samples receiving more than an average number of explants from these particular tissues. If Lep mRNA levels in primary adipocytes had been detectable by this assay system, they would have constituted better, more homogenous, tissue samples.

To further investigate whether insulin has a direct effect on Lep mRNA levels, albeit a small one, a more sensitive assay procedure was developed. The development, optimization and findings from this assay system are described in chapter six.
6 Initial Investigations Into The Effect of Insulin on Reporter Gene Constructs Containing the Murine Lep Promoter in the 3T3-F442A Cell Line
6.1 INTRODUCTION

Due to the low Lep expression levels and therefore insufficient sensitivity of the adipose explant in vitro RNase protection assay to detect any small effect of insulin, an alternative model was sought to investigate the direct effects of insulin on Lep gene expression in adipocytes.

As previously discussed in section 1.8.3, insulin can affect the steady-state levels of mRNA at both the transcriptional and post-transcriptional level. To date, evidence coming from studies utilizing actinomycin D to inhibit total gene expression suggests that insulin may regulate Lep expression at the transcriptional level, rather than post-transcriptionally (Hardie et al., 1996a; Leroy et al., 1996; Machinal et al., 1999; Reul et al., 1997).

Reporter gene assays, which determine promoter activity, are a convenient method for studying regulation of gene expression at the transcriptional level. They have been used to identify cis-acting DNA sequences and trans-acting factors responsible for constitutive and regulated expression, including nutritional and hormonal regulation. (section 1.8.3.1 lists a number of genes that have been studied in this manner in adipocyte cell models).

At the outset of this study, the effect on insulin on Lep promoter activity in adipocytes had not been reported, thus to determine whether insulin exerts an effect on Lep gene expression at the transcriptional level, constructs were generated containing sequences corresponding to regulatory regions of the murine Lep gene, upstream of a reporter gene. These constructs were used in transient transfection studies in a suitable adipocyte cell line to assess potential effects of insulin on promoter activity.

6.2 RESULTS

6.2.1 DESIGN OF ASSAY

6.2.1.1 CHOICE OF ADIPOCYTE MODEL SYSTEM

Both primary cultures of adipocytes, and adipocyte cell lines have been employed in transfection studies (see sections 1.8.3.1 and 6.2.6).
Primary adipocytes are more physiologically relevant than immortalized cell lines, in that they have the correct complement of chromosomes, possess large quantities of lipid, and are highly responsive to insulin. However, the isolation procedure is complicated, and can be quite harsh, depending on the batch of collagenase used, leading to variations in insulin responsiveness, (Dr. P. Maclean, personal communication; and Williams et al., 1995). Furthermore, due to the fact that primary adipocytes are terminally differentiated, only transient transfections can be carried out in this model system. Several studies on the \textit{Lep} promoter have nevertheless been performed in both rat and mouse primary adipocytes (de la Brousse et al., 1996; Fukuda and Iritani, 1999; Hollenberg et al., 1997; Mason et al., 1998; Miller et al., 1996).

As an alternative, a number of preadipose cell lines exist which, with appropriate stimulation, differentiate from fibroblast-like precursors into adipocytes. These cells acquire many of the biochemical and morphological characteristics of mature adipocytes, including high expression of enzymes related to fatty acid and triglyceride synthesis, accumulation of triglyceride, and increased sensitivity to certain hormones, including insulin (reviewed in Green, 1979). Examples of these cell lines are 3T3-L1 (Green and Kehinde, 1974), 3T3-F442A (Green and Kehinde, 1976), Ob17 (Negrel et al., 1978) and TA1 (Chapman et al., 1984). Cell lines have the advantage over primary cells, in that they are much easier to manipulate, can be maintained indefinitely and can be used to generate stable cell lines.

After consideration of these factors, it was decided that a cell line would be adopted for use in this investigation. This would allow for studies to determine the effects of insulin on \textit{Lep} promoter activity by both transient and stable transfection. Comparisons between responses in preadipocytes and adipocytes could also be made.

The 3T3-F442A cell line was considered an appropriate choice. Like other adipocyte cell lines, differentiated 3T3-F442A cells exhibit many of the morphological and biochemical characteristics of adipocytes, including insulin responsiveness (Green and Kehinde, 1976; Kuri-Harcuch and Green, 1977; Wise and Green, 1979). As an additional advantage, 3T3-F442A preadipocytes have the potential to give rise to discrete, well differentiated, fat pads when injected subcutaneously into BALB/c (athymic) nude mice, (Green and Kehinde, 1979). This ability, despite the fact that the cell line is abnormal in being immortalized and aneuploid, proves its authenticity as a
genuine adipocyte precursor, and hence is a suitable choice for use in an *in vitro* model system. The ability to form fat pads *in vivo* also poses the possibility that stable cell lines generated to express a reporter gene under the control of the *Lep* promoter, could be analysed in an *in vivo* tissue context in this manner. Mandrup et al have already utilized this method to express a reporter gene from *Lep* promoter constructs (Mandrup et al., 1997).

### 6.2.1.2 Choice of Reporter Gene

The expression of *Lep* mRNA in primary culture or in cell lines has previously been reported to be extremely low, with some estimating levels of approximately 1% of those observed *in vivo* (MacDougald et al., 1995b; Murakami et al., 1995). Indeed, *Lep* mRNA could not be detected in 3T3-F442A adipocytes using the RNase protection assay described in this thesis (see section 5.2.1), perhaps because the developed assay was not sensitive enough or due to variations in culture conditions compared to other researcher’s conditions, therefore affecting expression. Due to the fact that the transfection efficiency in adipocytes by conventional methods, such as calcium phosphate precipitation, is additionally very low (Meunier-Durmort et al., 1996), it was clear that an extremely sensitive reporter gene assay was required to analyse *Lep* promoter activity. Luciferase was chosen to be the reporter gene for this reason - as for example, it is typically 100-fold more sensitive than chloramphenicol acetyltransferase (CAT) (Alam and Cook, 1990). To increase sensitivity further, a luciferase vector including an SV40 enhancer was utilized (pGL3-Enhancer vector, Promega, U.K.).

### 6.2.1.3 Choice of *Lep* Gene Regulatory Sequence

Regulatory sequences of the murine *Lep* gene were chosen to include the maximum amount of published 5' flanking sequence available at the time (~1.0kb), and a smaller proportion of this (~0.5kb) (He et al., 1995).

In similar studies, both Miller et al (Miller et al., 1996), and de la Brousse et al (de la Brousse et al., 1996), included the untranslated first exon in their human and murine promoter constructs (+1 to +30, and +1 to +25 respectively, where +1 represents the transcription initiation site), and thus for ease of comparison, it was decided that this exon would also be included in the constructs generated in the current work.

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Additionally, the first ~160bp of intron 1 (+26 to +183), were also incorporated, in combination with these two differing lengths of regulatory sequence. Four different sections of the Lep gene regulatory sequence were therefore selected for study, and are shown schematically in Figure 6.1.

6.2.2 CONSTRUCTION OF Lep-LUCIFERASE PLASMIDS

The four different lengths of regulatory sequences of the murine Lep gene were selectively amplified by PCR from mouse genomic DNA. A description of the primers, and details of the PCR reaction conditions used can be found in chapter two – tables 2.2 and 2.4, and section 2.2.8. The resulting PCR products were approximately 0.5kb (-535 to +25), 0.7kb (-535 to +183), 1.0kb (-1009 to +25) and 1.2kb (-1009 to +183) in length (see Figure 6.1). These DNA fragments were cloned into the promoterless pGL3-Enhancer vector, upstream of the luciferase gene. The cloning strategy for the generation of these Lep promoter-luciferase vectors is shown in Figure 6.1.

The resulting plasmids were designated p0.5lep-Luc, p0.7lep-Luc, p1.0lep-Luc and p1.2lep-Luc, to reflect the sizes of the inserted regulatory sequences. All constructs were sequenced in both directions, to ensure the fidelity of the PCR amplification (see Figure 6.1). An agarose gel of the plasmids digested with BgIII and HindIII is illustrated in Figure 6.2. The resulting bands in each lane represent the pGL3-Enhancer vector backbone and the inserted Lep regulatory sequence.
Figure 6.1 Cloning strategy for the construction of Lep-luciferase plasmids

a) Primers lepprom1 for, lepprom2 for, lepprom1 rev and lepprom2 rev were used in combinations to generate PCR products from the Lep regulatory region, including exon 1 and start of intron 1, from mouse genomic DNA. The PCR products were purified, restriction endonuclease digested with BgII and HindIII, and cloned into pGL3-Enhancer. Positive clones were identified by restriction endonuclease digestion, and were sequenced in both directions. Sequence primers included the primers used in the PCR amplification, and also lepprom3 for and rev, and GLprimer 2 (shown in blue). Numbers in red are relative to the transcriptional initiation site (+1). Luciferase is shown in the diagram of the promoter to show the position of GLprimer 2. The X in pXlep-Luc denotes the varying sizes of the inserted regulatory sequences.

b) Schematic diagram illustrating the positions, relative to the transcriptional initiation site (+1), of known regulatory element sequences in the Lep proximal promoter region, including the TATA box (red) and the C/EBP (pink) and SP-1 (green) response elements (He et al., 1995). The LP1 site identified by Mason et al is also shown (blue) (Mason et al., 1998).
a) PCR amplification of *Lep* gene regulatory sequences

b) 

```plaintext
-110 -100 -90 -80 -70 -60 -50 -40 -30 -20 -10 -1+1

CCGCTGGTGAGGGCCAGTGCTGCAGGGACTGGGGCTGGCCGGACAGTTGCGCAAGTGGCACTGGGGCAGTTATAAGAGGGGCAGGCAGGCATGGAGCCCCGGAGGGAG
```
Figure 6.2 Photograph of agarose gel showing Lep-luciferase plasmids

2μg of each Lep-luciferase reporter gene plasmid was restriction endonuclease digested with BglII and HindIII and was resolved on a 1% agarose gel. The sizes of the inserted gene sequences, which correlate with the different sized Lep gene 5' regulatory regions are shown on the right.

DNA markers: λ = λ.HindIII, 100bp = 100bp ladder.
6.2.3 **Optimization of Differentiation of the 3T3-F442A Cell Line**

Lep mRNA is expressed late in the differentiation process (Mitchell et al., 1997; Rentsch and Chiesi, 1996), and levels are thought to correlate with the amount of triglyceride stored within the cell, consistent with a role in sensing energy stores (Hamilton et al., 1995; Maffei et al., 1995a; Zhang et al., 2002). It was felt to be important therefore, to ensure that differentiation of the 3T3-F442A cell line was optimized to produce a high proportion of lipid-containing cells.

Different methods exist to stimulate the conversion of confluent preadipocytes into adipocytes, and different methods can be used to determine the degree of differentiation within the cell population. For this reason, experiments were undertaken to find a suitable method for determining differentiation, and also optimal conditions for 3T3-F442A differentiation.

A previous study has suggested that adipocyte cell lines, during non-selective serial cultivation, can gradually lose their susceptibility to convert to adipocytes with high frequency (Green and Kehinde, 1976). This phenomenon was not, however, observed in this study. Nevertheless low-passage number cell stocks were used for experimentation at all times.

6.2.3.1 **Time Course of Differentiation Using Standard Methods**

Initial experiments used established methods to determine the degree of differentiation of 3T3-F442A cells. The activity of the adipocyte marker, L-glycerol 3-phosphate dehydrogenase (GPDH) in cell lysates was determined over a period of three weeks of culture, and morphological analysis of Oil Red O stained cells was conducted after 10 and 21 days. The cells were cultured in standard medium (DMEM containing 10% foetal calf serum (FCS)), and at confluence (Day 0), differentiation was accelerated by the addition of 100nM insulin.

The GPDH activity of four-day differentiated adipocytes had increased significantly compared to preadipocyte levels (Day 0) (p<0.05), and as shown in Figure 6.3, began to peak at day 6, reaching a maximum after 11 days in culture. This is in good agreement with previous studies by Wise and Green (Wise and Green, 1979). Further culture did not significantly increase GPDH levels.
Microscopic analysis of the morphology of the cells at this time, aided by Oil Red O staining, showed that lipid accumulation was not a uniform process however. Only small clusters of differentiated cells, possessing numerous lipid droplets were observed, surrounded by the majority of cells clearly not containing visible stores of lipid. At 21 days, it was observed that the diameters of these clusters had increased (i.e. more cells had accumulated triglyceride). An example micrograph of Oil Red O stained 10-day differentiated adipocytes is shown in Figure 6.4.

As a control, the fibroblastic cell line, NIH-3T3, was treated in an identical manner for up to ten days. This cell line is the parental cell line of the 3T3-F442A cell line, and is representative of the fibroblast phenotype. As expected, NIH-3T3 cells did not visibly undergo the morphological conversion to adipocytes, and GPDH activity remained undetectable at all time points tested (data not shown).

This data confirmed that the conditions used could stimulate the differentiation of 3T3-F442A preadipocytes into adipocytes as expected, with maximal expression of GPDH occurring after around 10 days. However, due to the fact that only a small proportion of cells had accumulated visible lipid stores (judged by Oil Red O staining), these conditions were not considered optimal for the needs of this particular study. Furthermore, since the GPDH assay quantifies the level of differentiation of the whole cell population, a technique was sought to measure the accumulation of lipid at an individual cell level, which could then be used to determine optimal differentiation conditions for a high level of adipocyte conversion and consequent lipid accumulation.
Figure 6.3  
GPDH activity during the differentiation of 3T3-F442A adipocytes

3T3-F442A cells were cultured in 24-well plates in standard medium and 100nm insulin for up to three weeks post-confluence. At the time points indicated, each well of cells were resuspended in resuspension buffer and lysed by sonication. Samples were stored at -80°C until the end of the experiment. The GPDH activity of all samples was then measured in 1µg samples of cell lysate, according to the method of Kozak and Jensen (Kozak and Jensen, 1974) (n=4).

Student’s t-tests were performed to test for significant differences between control (Day 0) and later timepoints. Significant differences were found from day 4 onwards.
Figure 6.4 Photomicrograph of Oil Red O stained 10-day differentiated 3T3-F442A adipocytes

Post-confluent 3T3-F442A cells were cultured in 6-well plates in standard medium and 100nM insulin for ten days. The cells were fixed, stained with Oil Red O and then covered in 50% (v/v) glycerol. The stained cells were visualized and photographed under a light microscope. Clusters of triglyceride containing, Oil Red O stained, adipocytes were observed, but the majority of cells only showed low level staining.
6.2.3.2 Flow Cytometric Analysis of Adipocyte Differentiation

Flow cytometric analysis can be used to quantitatively provide information on the accumulation of cytoplasmic triglyceride on a single-cell basis. The hydrophobic fluorescent dye, Nile Red, which emits both red and yellow-gold fluorescence when dissolved in lipid, has been utilized in this process (Greenspan et al., 1985; Smyth and Wharton, 1992). Red fluorescence is emitted when the stain is dissolved in amphipathic lipids, such as cell membrane, and yellow-gold fluorescence is emitted in neutral lipids, such as triglyceride (see Figure 6.10). A procedure to analyse the triglyceride content of 3T3-F442A adipocytes was therefore developed based on these assays (see section 2.5.6).

Initial experiments showed no difference in side-scatter (SSC-H) - a measure of the granularity of the cells, or forward scatter (FSC-H) - a measure of cell size, in the absence or presence of the fluorescent dye Nile Red, in either preadipocytes or 10-day differentiated adipocytes (Figure 6.5). This indicated that the presence of the dye did not alter the morphology of the cells.

The majority of preadipocytes formed a relatively homogeneous population characterized by small size and low granularity. 90% of the preadipocyte population was arbitrarily gated to represent a core, undifferentiated population (see Figure 6.5, preadipocytes, lower left quadrants). This meant that 10% of the preadipocyte population exhibited a more extensive spread of size and granularity characteristics. After a 10-day differentiation period, half of the cell population remained in the core undifferentiated population quadrant, although the spread here was greater than that observed in the preadipocyte population. This helped to quantitatively confirm earlier observations that a large proportion of 10-day differentiated cells do not display morphological features of mature adipocytes (e.g. they do not accumulate substantial triglyceride droplets). Around 40% of the cells in the 10-day differentiated population had significantly changed morphology within this time however (i.e. 50% of cells were now outside of the lower left quadrant, compared to 10% in the preadipocyte population). The main shift in morphology in the differentiated population was an increase in granularity (side-scatter), making up around one-third of the total cell population (see Figure 6.5, adipocytes, lower right quadrants). However, a small
percentage of cells displayed increased size (upper left quadrant) and many showed increased size and granularity (upper right quadrant).
Table 6.5 Flow cytometric dot-plots comparing 3T3-F442A preadipocytes and adipocytes in the absence or presence of Nile Red

<table>
<thead>
<tr>
<th>3T3-F442A PREADIPOCYTES</th>
<th>3T3-F442A ADIPOCYTES</th>
</tr>
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<tbody>
<tr>
<td>- Nile Red</td>
<td></td>
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<td></td>
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Figure 6.5 Flow cytometric dot-plots comparing 3T3-F442A preadipocytes and adipocytes in the absence or presence of Nile Red

Flow cytometric analysis of 3T3-F442A preadipocytes and adipocytes (post-confluent 3T3-F442A cells cultured in 6-well plates in standard medium and 100nm insulin for ten days) (10,000 cells in each analysis) was performed, plotting side-scatter (SSC-H) – a measure of granularity, against forward-scatter (FSC-H) – a measure of size, in the absence or presence of Nile Red. The gated quadrants were placed in such a way as for the lower left quadrant to represent an arbitrary 90% of the preadipocyte population. The percentage figure given in each quadrant indicates the percentage of the total cell population falling within this region.
The associated fluorescence of the cell populations was analysed, and the results from the three different channels analysed (FL-1, FL-2, FL-3) are illustrated in Figure 6.6 and Table 6.1.

Fluorescence emitted by the Nile Red-stained cells, and detected in the FL-2 and FL-3 channels, was relatively high in the preadipocyte population and increased by only a moderate amount after the 10-day differentiation period (increase in mean fluorescent intensity for the total cell populations was 78% and 60.8% respectively). Fluorescence in the FL-1 channel was an order of magnitude lower than that which was observed in the other two channels. However, it was considerably higher in the adipocyte population than in the preadipocyte population, increasing by 270.7%, although there was an extensive spread of values. Since the FL-1 channel represents yellow fluorescence, this result suggested that the analysis was indeed detecting an increase in triglyceride accumulation in the cells.

Two- and three-dimensional analysis of the Nile Red-stained preadipocyte and adipocyte cell populations, plotting side-scatter against FL-1, and forward-scatter (see Figure 6.7a and b), revealed an overall increase in yellow fluorescence with increasing side-scatter. An increase in fluorescence was not discernable when FL-2 or FL-3 were plotted against side-scatter. The adipocytes emitting the highest intensity of fluorescence in FL-1 (above $10^2$), were all small (low forward-scatter values on Figure 6.7b), (region 1, Figure 6.7a), and the majority (80%) were of the highest side-scatter reading (Figure 6.7a and b, region 2). However, only a small fraction of the adipocyte cell population (0.6%) fell into region 1, with fluorescent intensities greater than $10^2$, again correlating with observations that the majority of 10-day differentiated cells did not accumulate large amounts of triglyceride.

Since the increase in yellow fluorescence was supposedly detecting the presence of triglyceride stores within individual cells and that the increase in yellow fluorescence increased with increasing side-scatter, this indicated that the increased granularity of the cells was caused by the accumulation of lipid droplets. This suggested that analysis of the differentiation process can be achieved without the need for Nile Red staining and fluorescence measurements, simply using increased side-scatter as a measure of triglyceride accumulation.
The results from this experiment confirmed observations that the differentiation process is far from a uniform process under these conditions. It shows that the proliferating preadipocyte population does not form a distinct, homogenous population, since approximately 10% already display characteristics more like the majority of the 10-day differentiated population. Also, the 10-day differentiation process results in an even more heterogeneous population, and the analysis quantitatively confirms earlier morphological observations (see section 6.2.3.1), that a large proportion of cells in the differentiated population do not exhibit adipocyte characteristics. It was again made clear therefore, that the conditions used to induce differentiation were not sufficient for our needs. A different method to induce differentiation was therefore sought, to greatly increase the proportion of lipid-containing cells within the population. Also, although flow cytometric analysis was useful as a quantitative measure of the asynchrony in lipid accumulation in adipocytes, difficulties were encountered in extracting the fragile, lipid-laden cells. Moreover, as the cells acquired more lipid, and hence became less dense, they became increasingly more difficult to pellet during the centrifugation process. It was observed that a small proportion of the most differentiated cells remained in the supranatant, and thus this sub-set of cells was under-represented in the final population analysed.

Table 6.1  Mean fluorescent intensities of 3T3-F442A preadipocytes and adipocytes, stained with Nile Red

<table>
<thead>
<tr>
<th>CHANNEL</th>
<th>MEAN FLUORESCENT INTENSITY</th>
<th>% INCREASE IN MFI</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>3T3-F442A PREADIPOCYTES</td>
<td>3T3-F442A ADIPOCYTES</td>
</tr>
<tr>
<td>FL-1</td>
<td>1.84</td>
<td>6.82</td>
</tr>
<tr>
<td>FL-2</td>
<td>56.6</td>
<td>100.75</td>
</tr>
<tr>
<td>FL-3</td>
<td>36.35</td>
<td>58.44</td>
</tr>
</tbody>
</table>
Flow cytometric analysis of Nile Red stained 3T3-F442A preadipocytes and adipocytes (post-confluent 3T3-F442A cells cultured in 6-well plates in standard medium and 100μM insulin for ten days) (10,000 cells in each analysis) was performed, plotting number of events against fluorescent intensity (FL-1, FL-2 and FL-3). The percentage figure given on each histogram indicates the mean fluorescent intensity of the cell population.
Figure 6.7  Flow cytometric dotplots comparing granularity, size and FL-1 fluorescent intensity characteristics in 3T3-F442A preadipocytes and adipocytes in the presence of Nile Red

a) Flow cytometric analysis of Nile Red-stained 3T3-F442A preadipocytes and adipocytes (post-confluent 3T3-F442A cells cultured in 6-well plates in standard medium and 100nM insulin for ten days) (10,000 cells in each analysis) was performed, plotting side-scatter (SSC-H) – a measure of granularity, against yellow fluorescence (FL-1). Cells in the adipocyte population with fluorescent intensity greater than 100, were gated as region 1 (R1), and cells within this region, with maximum side-scatter, (representing 80% of this sub-population) were gated as region 2 (R2).

b) Flow cytometric analysis of Nile Red-stained 3T3-F442A preadipocytes and adipocytes (post-confluent 3T3-F442A cells cultured in 6-well plates in standard medium and 100nM insulin for ten days) (10,000 cells in each analysis) was performed, plotting side-scatter (SSC-H) – a measure of granularity, against forward-scatter (FSC-H) – a measure of size, and yellow fluorescence (FL-1). Cells in the adipocyte population with fluorescent intensity greater than 10^2, and with maximum side-scatter values (gated as region 2 (R2) in the 2-D plot in (a)) is denoted.
6.2.3.3 USE OF HEAT-INACTIVATED FOETAL CALF SERUM

During the course of investigating alternative conditions for optimal differentiation of the 3T3-F442A cell line, replacing foetal calf serum with heat-inactivated FCS (HI-FCS) was tested. Even without supplementing the medium with 100nM insulin, this resulted in a high proportion of adipocytes accumulating triglyceride. After conversion for ten days in culture, numerous, large clusters of lipid-containing cells were visible to such an extent, that they had begun to merge with neighbouring clusters. These clusters were clearly visible to the naked eye due to their opacity. Difficulties were encountered in the flow cytometric analysis of these cells however, as they proved problematic to disaggregate fully. GPDH activity in cells differentiated by this method was assayed however. Differentiating cells in HI-FCS resulted in a significant rise in GPDH activity compared to differentiating in FCS (p<0.005), ranging from an increase of approximately 2.5-fold to over 7-fold, depending on the presence or absence of 100nM insulin in the culture medium (see Figure 6.8). However, whereas insulin significantly increased GPDH activity in cells cultured in FCS, no such effect was observed with HI-FCS.

This data therefore showed that HI-FCS was capable of promoting the differentiation of preadipocytes to adipocytes, and could greatly accelerate the accumulation of lipid within these cells. Despite being unable to accurately quantify this conversion by flow cytometry, this extremely high level of differentiation, clearly visible even to the naked eye, was far greater than that found using any other method in the course of these studies. It was therefore felt that these conditions were unlikely to be greatly improved upon in the time allocated, and hence for all further experiments, differentiation was accelerated by the use of HI-FCS for ten days, ensuring clusters of differentiated cells had begun to merge with neighbouring clusters at this time.
Figure 6.8  GPDH activity in 3T3-F442A adipocytes cultured in FCS or HI-FCS

3T3-F442A cells were cultured in 24-well plates in medium containing either 10% foetal calf serum (FCS) or 10% heat-inactivated foetal calf serum (HI-FCS), with or without 100nM insulin for ten days post-confluence. Cells were resuspended in resuspension buffer and were lysed by sonication. The GPDH activity of all samples was then measured in 1µg samples of cell lysate, according to the method of Kozak and Jensen (Kozak and Jensen, 1974) (n=4).

Student’s t-tests were performed to test for significant differences between experimental conditions.

(* = p<0.05 compared to FCS, 0nM insulin; ** = p<0.005 compared to FCS, 0nM insulin).
6.2.4 **Optimization of Electroporation into 3T3-F442A Adipocytes**

Before the basal activity and potential effects of insulin on *Lep* promoter activity could be determined in 3T3-F442A adipocytes, a suitable method for transfection had to be identified. Transient transfection of adipocytes by conventional methods such as calcium phosphate precipitation, is known to be inefficient (Meunier-Durmort et al., 1996). Electroporation methods have successfully been used to transf ect primary rat adipocytes with plasmid DNA, with high efficiency and without substantial loss of cell viability or insulin responsiveness (Quon et al., 1993). This method was therefore adapted for 3T3-F442A adipocytes in the present study.

Initially, the parameters used by Quon et al for the optimal electroporation of primary rat adipocytes were tested (Quon et al., 1993). These authors employed six successive pulses of low capacitance and high voltage (6x 25μF and 800V), with charge reversal after three pulses. Each pulse however, was shown to decrease cell survival. To minimize the degree of cell death, the initial tests described here used only four pulses. Charge reversal was not carried out. Similarly to Quon et al, these conditions were compared to an alternate set of parameters, namely one pulse of high capacitance and low voltage (1x 960μF, 180V).

Figure 6.9a shows the basal luciferase activity of 3T3-F442A adipocytes electroporated with both the p.12lep-Luc construct and the green fluorescent protein (GFP) expressing plasmid, pCDNA3EGFP-N1.

The optimal parameters used for the electroporation of primary rat adipocytes, namely low capacitance and high voltage (4x 25μF, 800V), resulted in a significantly higher basal luciferase activity compared to the alternative parameters of high capacitance and low voltage (1x 960μF, 180V) (p<0.005).

Fluorescent microscopic analysis furthermore revealed that a greater number of GFP positive cells was observed after transfection using 4x 25μF, 800V than with the alternative parameters, indicative of a higher transfection efficiency (data not shown).

Although some cell death was observed (estimated to be no more than 50%), those that remained proved to be viable, and capable of adhering to the tissue culture flasks for the duration of the experiment.
To further test for the optimal uptake of DNA in 3T3-F442A adipocytes, a range of voltages, from 500-1500V, was investigated, using a constant capacitance of 25\(\mu\)F, this time for six pulses. Figure 6.9b clearly demonstrates that six pulses at 800 volts and 25\(\mu\)F resulted in maximal luciferase activity. These parameters also resulted in the greatest number of green fluorescing cells (data not shown). Identical conditions did not result in high levels of luciferase activity, or a significant number of GFP positive cells, in a control sample of 3T3-F442A preadipocytes (800V preadips).

Although the use of heat-inactivated foetal calf serum dramatically increased the proportion of lipid-containing cells in the 3T3-F442A adipocyte cell population, a small proportion of cells without lipid still remained. To ensure accurate analysis of Lep promoter activity in adipocytes, it was therefore important to ensure that the cells being transfected were predominantly lipid-containing cells.

Two lines of evidence suggested that this cell-type was predominantly being transfected. Firstly, fluorescent microscopic analysis of preadipocytes co-transfected with pCDNA3EGFP-N1 by electroporation, revealed very few GFP positive cells, whereas in adipocytes, a large number of lipid-droplet containing cells were clearly expressing GFP. An example fluorescent micrograph of GFP expressing 3T3-F442A adipocytes is illustrated in Figure 6.10. Secondly, when 3T3-F442A preadipocytes were electroporated for six pulses at 25\(\mu\)F and 800V, they failed to exhibit substantial levels of luciferase activity (Figure 6.9b, 800v preadips), indicating that these parameters are not optimal for this undifferentiated cell-type.

From fluorescent microscopic analysis, it was clear however, that the transfection efficiency of electroporation into adipocytes was still relatively low. Although transfection efficiency was never accurately determined, comparisons with other transfection methods (calcium phosphate precipitation ± glycerol shock, and liposomes) consistently showed electroporation to be the most efficient method (data not shown).

Although true comparisons of luciferase activity between the two sets of experiments described here is not possible, the raw luciferase data from these experiments indicated that six pulses resulted in substantially higher levels of luciferase activity compared to four pulses. Furthermore, due to the fact that these optimal conditions are identical to the optimal parameters found empirically by Quon et al for primary rat adipocytes (Quon et
al., 1993), it was decided that these conditions would be employed in all further experiments.
Figure 6.9  Basal luciferase activity in 3T3-F442A adipocytes electroporated with p1.2lep-Luc and pCDNA3EGFP-N1

a)  $1.25 \times 10^6$ 3T3-F442A adipocytes (post-confluent 3T3-F442A cells cultured in 175cm$^2$ flasks in HI-FCS medium for 10 days) were co-transfected by electroporation with 6μg of p1.2lep-Luc and 3μg of the internal control, pCDNA3EGFP-N1. Cells were plated on 3 wells of a 24-well plate, and samples were assayed 48 hours after electroporation. Conditions of electroporation were four pulses at low capacitance and high voltage (4x 25μF, 800V), compared to one pulse at high capacitance and low voltage (1x 960μF, 180V), (n=3). (Luciferase activity was compared by calculating relative levels, arbitrarily assigning the mean of the 4x 25μF, 800V group to a value of 1).

Student's t-tests were performed to test for significant differences between the two conditions.

(* = p<0.005 compared to 1x 960μF, 180V).

b)  $1.25 \times 10^6$ 3T3-F442A adipocytes (post-confluent 3T3-F442A cells cultured in 175cm$^2$ flasks in HI-FCS medium for 10 days) or preadipocytes (pre-confluent) were co-transfected by electroporation with 6μg of p1.2lep-Luc and 3μg of the internal control, pCDNA3EGFP-N1. Cells were plated on 4 wells of a 24-well plate, and samples were assayed 48 hours after electroporation. Conditions of electroporation were six pulses at low capacitance (25μF) over a range of voltages (500-1500V), (n=4). (Luciferase activity was compared by calculating relative levels, arbitrarily assigning the mean of the adipocyte, 800V group to a value of 1).

Student's t-tests were performed to test for significant differences between the control (adipocyte, 800V) and other experimental conditions.

(* = p<0.01, ** = p<0.05 compared to adipocytes at 800V).
Figure 6.10  Fluorescent confocal micrograph of 3T3-F442A adipocytes electroporated with pCDNA3EGFP-N1, and stained with Nile Red

Fluorescent confocal micrograph of 3T3-F442A adipocytes (post-confluent 3T3-F442A cells cultured in 175cm² flasks in HI-FCS medium for 10 days) electroporated with pCDNA3EGFP-N1. Differentiated cells, with neutral lipid droplets fluorescing yellow due to Nile Red staining, can be seen expressing GFP (green fluorescence). Background red fluorescence is caused by Nile Red stained amphipathic lipids.
6.2.5 Basal Luciferase Activity Conferred by the Lep-Luciferase Plasmids in 3T3-F442A Preadipocytes and NIH-3T3 Cells

Lep mRNA is expressed predominantly in adipocytes, but not in preadipocytes or most other cell types, (see section 1.3.1), (Maffei et al., 1995a; Zhang et al., 1994). It was therefore of interest to assess the tissue specificity of the Lep gene regulatory sequences in the two distinct forms of the 3T3-F442A cell type, and in a control, non-adipose cell type. Basal activity conferred by the luciferase constructs containing the different lengths of Lep regulatory sequence were initially determined in the non-adipocyte cell types, 3T3-F442A preadipocytes and NIH-3T3 fibroblasts. These were transfected using the calcium phosphate precipitation method, which was determined to be the most efficient protocol in these cell lines (data not shown).

Basal levels of luciferase activity in 3T3-F442A preadipocytes transiently transfected with the luciferase plasmids containing the short Lep regulatory sequences (p0.7lep-Luc and p0.5lep-Luc) were significantly lower than activity in cells transfected with the promoterless control, pGL3-Enhancer (p<0.005), (Figure 6.11a), and were virtually undetectable. This was a somewhat unexpected result. As Lep mRNA is expressed at very low levels in preadipocytes, the basal promoter activity of all constructs was expected to be low in this cell type. Such a significant repression, below levels observed with a promoterless control was, however, completely unanticipated. This repressive phenomenon has not been reported in other studies, where reporter constructs containing shorter or longer sequences of the Lep promoter have been studied (de la Brousse et al., 1996; Fukuda and Iritani, 1999; He et al., 1995; Mason et al., 1998; Miller et al., 1996). Constructs with this precise length of promoter have not been studied however, and it is possible that repressor elements existing within this additional sequence may be responsible for this phenomenon.

Transient transfection of the luciferase plasmids containing the long Lep regulatory sequences (p1.2lep-Luc and p1.0lep-Luc) into 3T3-F442A preadipocytes resulted in significantly higher basal relative luciferase activity compared to cells transfected with pGL3-Enhancer (p<0.005), (Figure 6.11b). Activity was 18- and 22-fold higher than control levels respectively. Similar results (10-15 fold), were documented by Miller et al, with luciferase constructs containing 217bp or ~3kb of the human Lep 5' regulatory region plus exon 1, transiently transfected into 3T3-L1 preadipocytes (Miller et al.,
1996). As Lep mRNA is expressed at very low levels in preadipocytes, this relatively high level of basal luciferase activity observed in Miller and co-worker’s, as well as our own studies, was also an unanticipated result.

Differences in the levels of luciferase activity between p0.7lep-Luc and p0.5lep-Luc, and also between pl.2lep-Luc and p1.0lep-Luc, were not significant, indicating that the ~160bp of intron 1 sequence had no effect on basal levels of luciferase activity in 3T3-F442A preadipocytes.

To test basal luciferase activity conferred by the constructs in a cell line of non-adipocyte lineage, the plasmids were also transiently transfected into NIH-3T3 fibroblasts. NIH-3T3 fibroblasts transiently transfected with the constructs generated qualitatively similar results to those found with 3T3-F442A preadipocytes.

Activity conferred by the constructs containing the short sequences of the Lep regulatory region was significantly lower than that seen with pGL3-Enhancer (p<0.005), (Figure 6.12a), although compared to 3T3-F442A preadipocytes, this activity was quantitatively greater.

Activity conferred by the constructs containing the long sequences of the Lep regulatory region was significantly higher than that conferred by the pGL3-Enhancer control (approximately 60-fold, p<0.005) (Figure 6.12a and b). Again, compared to 3T3-F442A preadipocytes, this activity was quantitatively greater.

As in preadipocytes, differences in levels of luciferase activity between p0.7lep-Luc and p0.5lep-Luc, and between pl.2lep-Luc and p1.0lep-Luc in NIH-3T3 fibroblasts, were not statistically significant, indicating that the ~160bp of intron 1 sequence also had no effect on basal levels of luciferase activity in this cell type.

The qualitatively comparable levels of luciferase activity conferred by the long and short promoter constructs between these two cell types fits well with their assumed phenotypic similarity. However, these results are in contrast to those reported by Miller et al (1996). In their experiments, CV-1 and COS cells were utilized as control fibroblastic, non-adipocyte lineage cell lines. Luciferase activity was negligible in these cells for both lengths of regulatory sequence tested.
Figure 6.11  Basal relative luciferase activity conferred by *Lep*-luciferase constructs in 3T3-F442A preadipocytes

Each well of 70% confluent 3T3-F442A preadipocytes (24-well plate format) was co-transfected by calcium phosphate precipitation with 1μg of reporter gene plasmid and 0.5μg of the internal control, pCMV-SPAP and glycerol shocked. Assays were carried out 48 hours after transfection, and luciferase levels relative to alkaline phosphatase were determined.

The data shown are the pooled results from two separate experiments (n=6), using different plasmid DNA preparations.

a) Basal relative luciferase activity in 3T3-F442A preadipocytes transiently transfected with p0.7lep-Luc and p0.5lep-Luc constructs compared to pGL3-Enhancer (n=12).

b) Basal relative luciferase activity in 3T3-F442A preadipocytes transiently transfected with p1.2lep-Luc and p1.0lep-Luc constructs compared to pGL3-Enhancer (n=12).

Due to differences in the levels of activity between the short promoter constructs (p0.7lep-Luc and p0.5lep-Luc) and the long promoter constructs (p1.2lep-Luc and p1.0lep-Luc), the results are shown on two separate graphs with appropriate Y-axis scales, both compared to the activity found using pGL3-Enhancer (arbitrarily assigned a relative value of 1).

Student's t-tests were performed to test for significant differences between the control and experimental conditions.

(• = p<0.005, compared to pGL3-Enhancer).
Figure 6.12 Basal relative luciferase activity conferred by the *Lep*-luciferase constructs in NIH-3T3 fibroblasts

Each well of 70% confluent 3T3-NIH fibroblasts (24-well plate format) was co-transfected by calcium phosphate precipitation with 1μg of reporter gene plasmid and 0.5μg of the internal control, pCMV-SPAP and glycerol shocked. Assays were carried out 48 hours after transfection, and luciferase levels relative to alkaline phosphatase were determined.

The data shown are the pooled results from two separate experiments (n=6), using different plasmid DNA preparations.

a) Basal relative luciferase activity in NIH-3T3 fibroblasts transiently transfected with *p0.7lep*-Luc and *p0.5lep*-Luc constructs compared to pGL3-Enhancer (n=12).

b) Basal relative luciferase activity in NIH-3T3 fibroblasts transiently transfected with *p1.2lep*-Luc and *p1.0lep*-Luc constructs compared to pGL3-Enhancer (n=12).

Due to differences in the levels of activity between the short promoter constructs (*p0.7lep*-Luc and *p0.5lep*-Luc) and the long promoter constructs (*p1.2lep*-Luc and *p1.0lep*-Luc), the results are shown on two separate graphs with appropriate Y-axis scales, both compared to the activity found using pGL3-Enhancer (arbitrarily assigned a value of 1).

Student's t-tests were performed to test for significant differences between the control and experimental conditions.

(∗ = p<0.005 compared to pGL3-Enhancer).
6.2.6 **Basal Luciferase Activity Conferred by the Lep-Luciferase Plasmids in 3T3-F442A Adipocytes**

Basal luciferase activity of the Lep-luciferase constructs was next assessed in 3T3-F442A adipocytes, and was found to follow a similar pattern to that observed in preadipocytes and NIH-3T3 fibroblasts (Figure 6.13).

Basal luciferase activity was significantly lower in 3T3-F442A adipocytes transiently transfected with p0.7lep-Luc, compared to the promoterless control (p< 0.05), consistent with the results from preadipocytes and NIH-3T3 cells. Activity was approximately 3-fold lower than that obtained with pGL3-Enhancer. No luciferase activity could be detected using the p0.5lep-Luc construct.

Electroporation of p1.2lep-Luc and p1.0lep-Luc plasmid DNA into 3T3-F442A adipocytes resulted in statistically significantly higher basal relative luciferase activity compared to cells transfected with the promoterless control plasmid, pGL3-Enhancer (p<0.01, p<0.05 respectively). Activity was 10.8-fold and 13.8-fold higher than control levels, respectively. However, the relative levels of luciferase were less than observed in the NIH-3T3 fibroblasts.

In support of these observations, two studies have reported similar activities in alternate adipocyte model systems - mouse and rat primary adipocytes respectively. de la Brousse et al recorded activity 15-20-fold greater than promoterless control levels, using a murine promoter construct containing 458bp of 5' regulatory sequence and exon 1 (de la Brousse et al., 1996). Miller's study (Miller et al., 1996) reported luciferase activity up to 15-fold higher than the promoterless control, using the 217bp and ~3kb human Lep regulatory region constructs. Moreover, in agreement with the results presented here, in Miller's study, basal luciferase activity in adipocytes and in preadipocytes using these two constructs was similar.

Differences in levels of luciferase activity between p1.2lep-Luc and p1.0lep-Luc were not significant, suggesting that the ~160bp of intron 1 had no effect on basal levels of luciferase activity in 3T3-F442A adipocytes, again consistent with the findings in 3T3-F442A preadipocytes and NIH-3T3 fibroblasts.

The results therefore were qualitatively similar to those observed in preadipocytes and NIH-3T3 fibroblasts, namely increased luciferase activity conferred by the constructs.
containing the longer *Lep* regulatory sequences, and lower activity conferred by the constructs containing the shorter *Lep* regulatory sequences.

For further studies on the effects of insulin on *Lep* promoter activity, the p1.2lep-Luc construct was initially selected. There were several reasons for this choice. Firstly, the exceedingly low basal levels of activity from the constructs containing the short *Lep* regulatory sequences ruled them out for immediate subsequent study. Next, since other studies, using different lengths of *Lep* regulatory sequence, had reported similar basal levels of luciferase activity as for the constructs containing the long *Lep* regulatory sequences, these plasmids were felt to be more representative of *Lep* promoter activity than the shorter-length constructs. Lastly, as basal activity was not significantly different between p1.2lep-Luc and p1.0lep-Luc in any cell type tested, it was felt prudent to initially select p1.2lep-Luc, so as to include the ~160bp of intron 1, which although found to be unnecessary for basal expression, could potentially contain important response elements in terms of a response to insulin.

If time had allowed, the effects of insulin on the other constructs described in this study, would also have been assessed.
Basal relative luciferase activity conferred by Lep-luciferase constructs in 3T3-F442A adipocytes

2.5x 10^6 3T3-F442A adipocytes (post-confluent 3T3-F442A cells cultured in 175cm^2 flasks in HI-FCS medium for 10 days) were co-transfected by electroporation with 12µg of reporter gene plasmid and 6µg of the internal control, pCMV-SPAP. Cells were plated on 4 wells of a 24-well plate. Assays were carried out 48 hours after transfection, and luciferase levels relative to alkaline phosphatase were determined.

a) Basal relative luciferase activity in 3T3-F442A adipocytes transiently transfected with p0.7lep-Luc and p0.5lep-Luc constructs compared to pGL3-Enhancer (n=4).

b) Basal relative luciferase activity in 3T3-F442A adipocytes transiently transfected with p1.2lep-Luc and p1.0lep-Luc constructs compared to pGL3-Enhancer (n=4).

Due to differences in the levels of activity between the short promoter constructs (p0.7lep-Luc and p0.5lep-Luc), and the long promoter constructs (p1.2lep-Luc and p1.0lep-Luc), the results are shown on two separate graphs with appropriate Y-axis scales, both compared to the activity found using pGL3-Enhancer (arbitrarily assigned a value of 1).

Student’s t-tests were performed to test for significant differences between the control and experimental conditions.

(* = p<0.05, ** = p<0.01 compared to pGL3-Enhancer).

n/d = not detected.
6.2.7 **The Effect of Insulin on Luciferase Activity Conferred by p1.2LEP-Luc in 3T3-F442A Preadipocytes**

Since the p1.2lep-Luc plasmid conferred similar levels of basal luciferase activity in both preadipocytes and adipocytes, the effects of insulin in both distinct forms of the cell line were investigated. Preadipocytes do not possess large numbers of insulin receptors, and are not highly responsive to insulin (Reed and Lane, 1980; Rubin et al., 1978; Saad et al., 1994), thus act here as a negative control.

24-hours after transfection by calcium-phosphate precipitation, the media was changed to include a range of insulin concentrations, and the cells were cultured for a further 24-hours. Unexpectedly, a concentration-dependent decrease in relative luciferase activity was observed in preadipocytes in response to insulin (Figure 6.14). This was statistically significant at the lowest, physiological concentration, of insulin tested (1nM) \((p<0.05)\), suggesting that even though there are only low numbers of insulin receptors on preadipocytes, this phenomenon is mediated through the insulin receptor. However, at the higher concentrations, additional inhibition via the involvement of the IGF-1 signalling system (which insulin is capable of signalling through at higher concentrations), could not be ruled out.

Insulin had no effect on the promoterless control, pGL3 plasmid (data not shown).
Each well of 70% confluent 3T3-F442A preadipocytes (24-well plate format) was co-transfected by calcium phosphate precipitation with 1μg of p1.2lep-Luc and 0.5μg of the internal control, pCMV-SPAP and glycerol shocked. 24-hours after transfection, the cells were exposed to media containing the indicated insulin concentration, and were incubated for a further 24-hours before assaying. Luciferase levels relative to alkaline phosphatase were determined.

Values are expressed relative to 0nm control levels, arbitrarily assigned to a value of 1.0 (n=12).

The data shown are the pooled results from two separate experiments (n=6), using the same plasmid DNA preparations.

Student’s t-tests were performed to test for significant differences between the control and experimental conditions.

(* = p<0.05, ** = p<0.005 compared to 0nm control).
6.2.8 **The Effect of Insulin on Luciferase Activity Conferred by p1.2Lep-Luc in 3T3-F442A Adipocytes**

The effect of insulin on *Lep* promoter activity was next determined in 3T3-F442A adipocytes transiently transfected with the p1.2lep-Luc construct.

24-hours after transfection by electroporation, the media was changed to include a range of insulin concentrations, and the cells were cultured for a further 24-hours (Figure 6.15). No significant increase in luciferase activity was observed at any concentration of insulin investigated, compared to basal levels, although a concentration-dependent trend of increased *Lep* mRNA levels was observed. Maximum activity was observed at supraphysiological concentrations of insulin (100nM), with only a small response being observed at physiological concentrations (1nM).

Since the sample size was small (n=3), and relatively high variation in response within groups was observed, these results can only be used as an initial determination of the effect of insulin on *Lep* promoter activity in adipocytes. However, it suggests that chronic (24-hour) insulin treatment at physiological concentrations, may have a small, positive effect on *Lep* mRNA levels *in vitro*.

6.2.9 **Insulin Responsiveness of 3T3-F442A Adipocytes**

The culture conditions used for 3T3-F442A adipocytes are well established, and the cells' insulin responsiveness is well documented (Green, 1979; Spiegelman and Green, 1980). Nevertheless, as a positive control, it was desirable to prove in some manner, by conventional means, that the cells were indeed insulin responsive.

An attempt was made at producing a reporter gene construct containing a known insulin responsive promoter (the rat fatty acid synthase promoter), that could be used as a positive control to ensure the 3T3-F442A adipocytes were responding appropriately to insulin treatment. Unfortunately, the PCR amplification of this promoter proved to be problematical, possibly due to its high GC content. The generation of this construct was never completed due to time constraints.

In order to prove to some extent that the cells were insulin responsive, the tyrosine phosphorylation of the beta subunit of the insulin receptor (IRβ) after insulin stimulation was determined. Overnight serum-starved 3T3-F442A adipocytes were subjected to a five-minute insulin treatment and were then lysed. Insulin receptors were
immunoprecipitated from the cell lysate with an anti-IRβ antibody (IR.CT), and were subsequently Western blotted. Figure 6.16 shows a representative Western blot of immunoprecipitated protein, a) probed first with the anti-phosphotyrosine antibody, (PY20) and then b) stripped and reprobed with a second anti-IRβ antibody (Rosl). An approximately 92kDa band, corresponding to IRβ was present in the lanes of the blot that were immunoprecipitated with anti-IRβ antibody. Negative control lanes, where protein was immunoprecipitated with a non-specific antibody (n/s ab) or with no antibody at all (-ab), do not possess this band.

The extent of tyrosine phosphorylation of IRβ in response to insulin was determined by densitometry, where the density of the bands corresponding to total IRβ from the blot using the anti-IRβ antibody were used to normalize potential loading differences between lanes of the initial blot using the anti-phosphotyrosine antibody.

Insulin increased the level of tyrosine phosphorylated IRβ in 3T3-F442A adipocytes. 10nM insulin produced an approximately 4-fold increase in tyrosine phosphorylation, whereas 100nM insulin increased phosphorylation 3-fold, consistent with previously published results (Kasuga et al., 1982).

Table 6.2 presents the densitometry data from both the blots, and the fold-increase in tyrosine phosphorylated IRβ with respect to the control (0nM insulin). Although lower concentrations of insulin were not tested in this experiment, it was concluded that the adipocytes were indeed sensitive to relatively low concentrations of insulin in the culture conditions used throughout this study, as expected.
1.25x 10^6 3T3-F442A adipocytes (post-confluent 3T3-F442A cells cultured in 175cm^2 flasks in HI-FCS medium for 10 days) were co-transfected by electroporation with 6μg of p1.2lep-Luc and 3μg of the internal control, pCMV-SPAP. Cells were divided equally between 3 wells of a 24-well plate. 24-hours after electroporation the cells were exposed to media containing the indicated insulin concentration, and were incubated for a further 24-hours before assaying. Luciferase levels relative to alkaline phosphatase were determined.

Values are expressed relative to 0nM control levels, arbitrarily assigned to a value of 1.

Student’s t-tests were performed to test for significant differences between experimental conditions. No significant difference was found between any of the experimental values compared to each other or to the 0nM control (n=3).
Overnight serum starved 3T3-F442A adipocytes (post-confluent 3T3-F442A cells cultured in 6-well plates in HI-FCS medium for 10 days) were exposed to the indicated concentration of insulin for five minutes before cell lysates were prepared, and the beta subunit of the insulin receptor (IRβ) was immunoprecipitated using the anti-IRβ antibody, IR.CT.

a) Western blot of IR.CT anti-IRβ immunoprecipitated protein from insulin stimulated 3T3-F442A adipocytes probed with an anti-phosphotyrosine antibody (PY20).

b) Same Western blot, stripped and reprobed with a second anti-IRβ antibody (Ros1).

**Figure 6.16 Representative Western blot of tyrosine phosphorylated insulin receptor beta**

**Table 6.2 Densitometric analysis of tyrosine phosphorylated insulin receptor beta in 3T3-F442A adipocytes, in response to insulin**

<table>
<thead>
<tr>
<th>INSULIN CONCENTRATION</th>
<th>0nm</th>
<th>10nm</th>
<th>100nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>PY20 (phosphotyrosine)</td>
<td>0.8</td>
<td>5.1</td>
<td>5.3</td>
</tr>
<tr>
<td>Ros-1 (beta subunit of IR)</td>
<td>1.3</td>
<td>2.1</td>
<td>2.7</td>
</tr>
<tr>
<td>Fold increase (w.r.t. 0nm)</td>
<td>1</td>
<td>3.9</td>
<td>3.2</td>
</tr>
</tbody>
</table>

(densitometric values are raw data).
6.2.10 The Effect of Dexamethasone on Luciferase Activity Conferred by p1.2LEP-Luc in 3T3-F442A Adipocytes

Results presented in this body of work (chapter five), and by others (De Vos et al., 1995; Murakami et al., 1995; Slieker et al., 1996), report that steady-state levels of Lep mRNA and serum leptin levels are increased by glucocorticoid treatment both in vitro and in vivo.

Although no exact consensus sites for members of the steroid/thyroid superfamily are present in the murine leptin gene proximal promoter (Hollenberg et al., 1997), elements are present that are known be recognised by the glucocorticoid receptor (Drouin et al., 1989). Furthermore, deletion mapping of the human leptin promoter has revealed cis-elements involved in transcriptional activation by glucocorticoids that do not correlate with known consensus sequences (De Vos et al., 1998).

Therefore, as a further control for the responsiveness of the cells, the effects of the synthetic glucocorticoid, dexamethasone, on Lep promoter activity, was investigated in 3T3-F442A adipocytes, transiently transfected with the p1.2lep-Luc plasmid. Again, due to time restraints, this experiment was only performed once, in triplicate. 24-hours after transfection by electroporation, the cells were transferred to medium containing a range of dexamethasone concentrations, and were cultured for a further 24-hours. No significant increase in luciferase activity could be demonstrated at any concentration of dexamethasone, although a trend towards maximal activity (two-fold greater than control) at around 10nM was observed (Figure 6.1). This level of activity compares favourably with the results from the reporter gene studies of De Vos et al, who demonstrated a 2.5-fold increase in the activity of the human Lep promoter with 1μM dexamethasone.

The effective concentration range shown in the present study is also comparable to that observed in the adipose explant culture model described in chapter five and is in accordance with other previous findings (Murakami et al., 1995; Slieker et al., 1996), although the extent of the increase was higher in the case of the explants and primary adipocyte cultures, compared to the promoter activity observed here, in 3T3-F442A adipocytes. However, to make any firm conclusions about the glucocorticoid responsiveness of the murine Lep promoter, the experiment requires repetition.
Figure 6.17 The effect of dexamethasone on relative luciferase activity conferred by p1.2lep-Luc in 3T3-F442A adipocytes

1.25x 10^6 3T3-F442A adipocytes (post-confluent 3T3-F442A cells cultured in 175cm^2 flasks in HI-FCS medium for 10 days) were co-transfected by electroporation with 6µg of p1.2lep-Luc and 3µg of the internal control, pCMV-SPAP. Cells were divided equally between 3 wells of a 24-well plate. 24-hours after electroporation the cells were exposed to media containing the indicated dexamethasone concentration, and were incubated for a further 24-hours before assaying. Luciferase levels relative to alkaline phosphatase were determined.

Values are expressed relative to 0nM control levels, arbitrarily assigned to a value of 1.

Student's t-tests were performed to test for significant differences between experimental conditions. No significant difference was found between any of the experimental values compared to each other or to the 0nM control (n=3).
6.3 DISCUSSION

In order to investigate any small effect of insulin on murine \( Lep \) expression, and to explore an alternative, more sensitive, model system, the mouse adipocyte cell line 3T3-F442A was employed in a series of transfection studies. Although cell culture models lack the paracrine and intracellular interactions found between different cell types \textit{in vivo}, cell lines provide simplified models, which are well characterized and widely used. 3T3-F442A adipocytes have been successfully used in a variety of studies to investigate insulin’s effects on adipocyte functions, (e.g. Alexander et al., 1988; Leroy et al., 1996; Lowell and Flier, 1990; Wise and Green, 1979).

The 3T3-F442A cell line was chosen in preference to primary cell culture methods or alternative cell lines to enable both transient and stable transfections to be performed, and to compare responses in preadipocytes and adipocytes. In addition, by using this model system, the possibility existed of analysing clones stably expressing \( Lep \) promoter-reporter constructs in an \textit{in vivo} context, since 3T3-F442A preadipocytes injected into BALB/c mice form well-differentiated fat pads (Green and Kehinde, 1979).

Experiments were first carried out to determine the optimal procedure of differentiation and optimal method of measuring this process. Unless detailed information on the extent of \textit{in vitro} differentiation of adipocyte cell lines is required, it is usually sufficient to visually analyse cell morphology in order to confirm that differentiation has occurred (Leroy et al., 1996; Rentsch and Chiesi, 1996). This can be aided by Oil Red O or Nile Red staining, which highlights the presence of lipid-droplets. Maximal GPDH activity at this stage, also points to the fact that cells are sufficiently differentiated for most study purposes. However, lipid accumulation is an asynchronous process, and it was observed that many cells in the differentiated culture had not undergone sufficient lipid accumulation. This has also been reported in similar cell lines (Smyth and Wharton, 1992). Since \( Lep \) expression may be correlated with lipid content at the single cell level (Hamilton et al., 1995; Maffei et al., 1995a; Zhang et al., 2002), it was felt necessary for the purposes of this study, to ensure that the majority of cells contained sufficient triglyceride stores to mimic, as closely as possible, the unilocular adipocytes present \textit{in vivo}.
Many variations of culture condition protocols exist to induce differentiation in preadipocyte cell lines. A protocol already used in our laboratory was initially chosen. Maximal GPDH activity in 3T3-F442A adipocytes was observable after around ten days post-confluence, using high glucose DMEM containing 10% foetal calf serum (FCS) and 100nM insulin. Activity did not increase significantly after this point, consistent with previous reports (Wise and Green, 1979). Visual analysis of the morphology of the cells showed that lipid accumulation was not maximal after even 21 days in culture however, thus indicating that these conditions were not optimal.

A flow cytometric assay, using the fluorescent dye, Nile Red, was developed to ascertain more precisely the proportion of cells that had accumulated lipid during differentiation, and for use in further studies of optimizing differentiation procedures. In line with the observations of the visual analysis described in section 6.2.3.1, only approximately 50% of the cells analysed by flow cytometry were deemed morphologically different from the core preadipocyte morphology after ten days of differentiation, in terms of forward- and side-scatter (measures of size and granularity respectively). This is consistent with a previous report in a similar cell line using similar differentiation conditions (Smyth and Wharton, 1992).

The greatest increase in mean fluorescent intensity after differentiation was observed in the yellow fluorescent, FL-1 channel (270%). Since Nile Red emits yellow/gold fluorescence when dissolved in neutral lipids, this observation was taken to signify the increasing lipid accumulation of the adipocytes during the differentiation procedure. This increase correlated with an increase in side-scatter, suggesting that the increase in the granularity of the adipocytes was due to lipid accumulation.

Further 2-D and 3-D analysis revealed however, that the number of cells emitting the highest levels of yellow fluorescence were in the minority of the total cell population. Only 0.6% had FL-1 readings higher than 10^2 (greater than levels observed in preadipocytes). This quantitative analysis therefore confirmed the visual observation that only a small proportion of 10-day differentiated adipocytes accumulated large quantities of lipid using these differentiation conditions, and that other differentiation conditions needed to be investigated.

Moreover, a small sub-set of highly differentiated cells proved difficult to pellet in the preparation process for flow cytometric analysis, due to their low density associated
with lipid accumulation. This meant the technique had the potential to under-represent the very cells that were required in the detection of the most optimal differentiation procedure.

The replacement of heat-inactivated FCS (HI-FCS) for FCS removed the need to accurately quantify the accumulation of lipid however. Using this method of differentiation it was extremely evident, by light microscopy, that the majority of cells had accumulated lipid droplets after ten days of treatment. The clusters of differentiated cells had begun to merge with neighbouring clusters, so providing a ‘visual marker’ to determine a suitable endpoint for differentiation. This was visible even to the naked eye due to the opacity of the cells. GPDH activity was also significantly increased in these cells compared to cells differentiated with untreated FCS. Insulin had no effect on increasing GPDH activity further when using HI-FCS, perhaps due to activity being maximal already.

It is unclear why heat-inactivation of the serum should have such a profound effect on the accumulation of triglyceride, and on GPDH activity. To my knowledge no such study has been reported before. A large number of factors in serum are known to inhibit the differentiation process, including transforming growth factor β (TGF-β) (Ignotz and Massague, 1985), retinoic acid (Kuri-Harcuch, 1982), fibroblast growth factor (FGF), and platelet derived growth factor (PDGF) (Hayashi et al., 1981). It is possible that one or more of these factors is destroyed during the heat-inactivation process, allowing differentiation, and expression of adipocyte-specific markers to proceed unhindered.

With suitable differentiation conditions ascertained, a method for introducing plasmid DNA into 3T3-F442A adipocytes was investigated. High efficiency transient transfection of plasmid DNA by calcium phosphate precipitation and glycerol shock was possible in 3T3-F442A preadipocytes and NIH-3T3 cells. This method was not suitable for the transfection of adipocytes however. Electroporation of 3T3-F442A adipocytes proved to be a more efficient method of transfection than calcium phosphate precipitation or lipofection, although the proportion of cells transfected was still low. The studies showed that plasmid DNA could be taken up and expressed in 3T3-F442A adipocytes using the same conditions used for primary rat adipocytes by Quon et al (Quon et al., 1993). Cell viability was assessed by the ability of the cells to adhere to the tissue culture flask for up to 48 hours post-electroporation, but unfortunately hormone
responsiveness was not investigated due to time constraints. As the conditions for electroporation matched those for primary adipocytes exactly, it was anticipated that the cells would respond in the same way as primary cells, i.e. would retain their insulin responsiveness. If time had allowed, further optimization of this procedure would have been attempted, as well as determining hormone responsiveness.

A highly efficient liposome/viral transfection method – lipoadenofection – has also been demonstrated in adipocytes (Meunier-Durmort et al., 1996; Meunier-Durmort et al., 1997). In future experiments, it would be desirable to test this approach also, not only due to the reported higher efficiency of transfer, but also due to the minimal disruption caused to the cells during the process.

During the course of this study, the construction of stable cell lines containing the Lep-Luc plasmids was attempted, but unfortunately clones could not be identified that expressed detectable levels of luciferase, even when fully differentiated. This process was further exacerbated by the autofluorescence of this cell line, since green fluorescent protein was used as a marker for transfection.

In experiments designed to evaluate the cell type-specific, basal activity of the Lep promoter, the luciferase constructs were transfected into 3T3-F442A preadipocytes and adipocytes, and into the control fibroblastic cell line, NIH-3T3. Lep mRNA is expressed at very low levels in preadipocytes (Leroy et al., 1996; Maffei et al., 1995a; Rentsch and Chiesi, 1996), and for this reason it was hypothesized that the basal activity conferred by all constructs would be low in this cell type. However, luciferase activity conferred by the constructs containing the long Lep regulatory sequences (1.2 and 1.0kb) was easily detectable. In contrast, but unexpectedly, the constructs containing the short Lep regulatory sequences (0.7 and 0.5kb) exhibited a marked repressive effect on activity compared to the promoterless control pGL3-enhancer.

Qualitatively similar results were observed in NIH-3T3 fibroblasts with all four promoter constructs, although absolute luciferase activity was higher than that observed in preadipocytes. Exponentially growing 3T3-F442A preadipocytes are known to be phenotypically fibroblastic (Green and Kehinde, 1976), and the fact that each of the four constructs generated similar luciferase activities in both cell types was seen to confirm this fact.
Since Lep mRNA is expressed in differentiated adipocytes, basal promoter activity was expected to be higher in adipocytes than that observed in preadipocytes. Contrary to this assumption, basal luciferase activity conferred by the constructs containing the long Lep regulatory sequences in adipocytes seemed to be similar, if not lower, than levels observed in preadipocytes and NIH-3T3 fibroblasts. Basal luciferase activity conferred by the constructs containing the short Lep regulatory sequences followed the same trend of repressed activity as seen in preadipocytes and NIH-3T3 fibroblasts, compared to the promoterless control, although absolute levels of activity differed from those seen in these cell types.

Direct comparisons of luciferase activity between the cell types cannot be made, however. Firstly, since the adipocyte experiment was only performed once, it is possible that this is not a totally representative result. Several repeats of this experiment would have to be carried out before definite conclusions could be drawn. Secondly, direct comparisons between the cell types are complicated by the potential cell-type specific differences in the activity of the SV40 enhancer, present in the pGL3-Enhancer backbone of the constructs, which could contribute to the overall levels of luciferase produced.

Even without true direct comparison however, a trend emerged that in all cell types analysed, the constructs containing the long Lep regulatory sequences exhibited higher basal luciferase activity than the promoterless control, whereas the constructs containing the short Lep regulatory sequences generated lower luciferase activity than the promoterless control.

The similar activities of the constructs containing the long Lep regulatory sequences in both preadipocytes and adipocytes is comparable to data reported by Miller et al for the human Lep promoter in 3T3-L1 preadipocytes and rat primary adipocytes (Miller et al., 1996). Moreover, in these studies pGL3-Basic derived vectors were used (i.e. without the SV40 enhancer), suggesting that in the present studies, the SV40 enhancer does not display considerable cell type-specific difference in activity between these two cell types. Of course, potential species-specific differences between mouse and human promoter activities, and differences in the lengths of regulatory sequence used, must also be taken into account when interpreting this data. Miller et al explained the comparable luciferase activities of the Lep promoter in preadipocytes and adipocytes in terms of
adipocyte lineage-specific expression, since basal expression in CV-1 and COS cells was negligible (Miller et al., 1996). The results presented here are not in agreement with this hypothesis however, as the levels of activity of the long Lep promoters in NIH-3T3 cells were similar to those seen in the cell lines of adipocyte-lineage.

As the data stands, several possibilities exist to explain the expression of the constructs containing the long Lep regulatory sequences. Firstly, the basal luciferase activity conferred by the long Lep regulatory sequences could be entirely tissue-independent. Tissue-dependent elements could exist elsewhere in the Lep gene’s regulatory regions. A paper has recently been published, describing the generation of transgenic mice utilizing a 762bp murine Lep proximal promoter region to drive the expression of a reporter gene (Chen et al., 1999). Expression of the reporter gene was observed in all tissues tested, suggesting that this region of the promoter alone is not responsible for tissue-specific expression. Moreover, constitutive expression of reporter gene constructs containing either the proximal murine Lep promoter (500bp) or 6000bp of 5’ flanking region has since been observed in several diverse cell lines (Li et al., 2001). It is therefore feasible, that the regulatory regions contained in the constructs described in this thesis do not contribute to adipocyte-specific expression, and hence confer tissue-independent basal activity.

Alternatively, activity from the constructs containing the long Lep regulatory sequences could be species and/or tissue-specific, since this would explain the anomaly of Miller et al.’s control results using CV-1 or COS cells, which although of fibroblastic morphology, both originate from African green monkey kidney cells. Assessing the activity of the Lep promoter constructs in other, non-murine, cell lines, or fibroblast-like cell lines derived from different sources could go some way towards determining this.

It is also possible that the adipocyte models utilized do not sufficiently mimic in vivo adipocytes with respect to Lep expression, and hence remain preadipocytic or fibroblastic in their phenotype in this respect. This could be due to a number of factors, including the fact that adipocyte cell lines fail to accumulate the same amount of lipid as unilocular adipocytes in vivo do, or due to the absence of external factors - such as serum components or cell-cell interactions - necessary for complete differentiation, and hence the expression of relevant adipocyte-specific signal transduction components and/or transcription factors. Moreover, the rapid drop in steady-state levels of Lep
mRNA observed in adipose tissue explants in culture (see chapter five), suggests that the primary culture method employed by Miller et al, may not be an adequate adipocyte model for the study of Lep promoter activity either.

Of course, it is also worthy to note that the transfected promoter constructs are out of the context of their genomic locus, where factors regarding histone acetylation status and chromatin structure - for example - are not present, and this also could affect the appropriate expression of the reporter gene.

In contrast to the high basal luciferase activity conferred by the constructs containing the long Lep regulatory sequences compared to pGL3-Enhancer, the short promoter constructs exhibited significantly repressed activity in preadipocytes and NIH-3T3 fibroblasts. Due to the fact that the experiment in adipocytes was only performed once, it is not strictly possible to compare results from this to the findings in preadipocytes and fibroblasts - although it would appear that levels were substantially lower than control in this cell type also. It is interesting to note that the repression conferred by the 0.7kb of Lep regulatory sequence in adipocytes appeared to be substantially less severe than the repression seen in preadipocytes or fibroblasts (30% of control levels compared to 2%-4% in preadipocytes and NIH-3T3 fibroblasts). Further experiments would have to be performed to ascertain the statistical significance of this, although it is possible to speculate that because Lep mRNA is expressed to a greater degree in adipocytes than preadipocytes, the promoter activity repression observed in preadipocytes and fibroblasts may be substantially reduced in adipocytes.

Other studies, which have implemented the use of deletion mutants of the Lep promoter have not observed this repressive phenomenon, although constructs containing regulatory sequences of exactly this length have not been reported (de la Brousse et al., 1996; Fukuda and Iritani, 1999; He et al., 1995; Hollenberg et al., 1997; Li et al., 2001; Mason et al., 1998; Miller et al., 1996). An analysis of the region between the end of the short Lep constructs (-535) and -502 (being the maximum Li et al studied) yielded only a MyoD response element consensus sequence (comparison with the TRANSFAC 5.0 database (Wingender et al., 2000), located at www.gene-regulation.com). The implications of this potential response element in Lep expression requires further investigation, particularly since;
a) the higher basal levels of activity conferred by the constructs containing the long \( Lep \) regulatory sequences indicates that should such a repressor element exist within this region, its repressive effects are capable of being overridden by other downstream elements, and;

b) the lower amount of repression observed with the p0.7lep-Luc construct in adipocytes compared to preadipocytes could suggest that cell-type specific factors may also play a role in the regulation of this repressive effect.

The ~160bp sequence corresponding to the beginning of intron 1 was shown to have no effect on promoter activity for the constructs containing either the long and short \( Lep \) regulatory sequences, in preadipocytes or NIH-3T3 fibroblasts. It also did not have an effect on the constructs containing the long \( Lep \) regulatory sequences in adipocytes (comparison between the short \( Lep \) regulatory sequences was not possible from the present data).

The effects of insulin on \( Lep \) promoter activity were investigated using the p1.2lep-Luc construct. This construct contained the greatest amount of \( Lep \) regulatory sequence, including the ~160bp of intron 1, and conferred high levels of basal expression in preadipocytes and adipocytes, thus making it the most obvious candidate for initial further investigation.

Unexpectedly, insulin elicited a dose-dependent decrease in luciferase activity in preadipocytes transiently transfected with this construct. \( Lep \) mRNA is only expressed at low levels in preadipocytes, and as preadipocytes are not highly responsive to insulin (Reed and Lane, 1980; Rubin et al., 1978; Saad et al., 1994), this was an anomalous result. The repression was significant at concentrations of insulin consistent with its physiological action (1nM), implying that this direct effect of insulin was occurring through an insulin-specific signalling pathway, and not through its effects, seen at higher concentrations, on the IGF-1 pathway. However, the greater significance in repression seen at higher concentrations of insulin (100nM-10\( \mu \)M) does not rule out the possibility of additional effects of insulin on IGF-1 signalling. Future experiments detailing the response to IGF-1 would therefore be worthy of consideration. The result is in contradiction to reporter gene studies published by Li et al, which concluded that insulin had a stimulatory effect on the \( Lep \) promoter in 3T3-L1 preadipocytes (Li et al., 2001). Differences in the length of promoter sequence used, as well as differences between the
3T3-L1 and 3T3-F442A cell lines could potentially be responsible for this discrepancy, and it is therefore also worthy of further investigation.

The insulin responsiveness of p1.2lep-Luc in adipocytes was quantified only once, and hence caution must be taken in interpreting these results. Significance was not obtained at any concentration of insulin used (although it was a small sample size), but a trend emerged suggesting that physiological doses of insulin (1-10nM) were capable of increasing promoter activity to some degree, and 100nM was capable of increasing promoter activity still further.

It is possible however, that insulin responsive elements do not reside in the regions of Lep regulatory sequence that have been used during this study. Levels of a reporter gene in the adipose tissue of transgenic mice utilizing a 762bp murine Lep proximal promoter did not respond to fasting or high fat feeding, in contrast to endogenous Lep mRNA levels, suggesting that elements required for nutritional control do not reside within this region (Chen et al., 1999).

The experiment must be repeated to gain further insight into the effect of insulin on the activity of the 1.2kb fragment of the Lep proximal promoter. If insulin responsiveness were proven, it would be possible, through the use of a 5' deletion series of the promoter region to determine the location of any insulin responsive element and to conduct further analyses of them.

At present, the only insulin responsive cis-acting element(s) in the leptin promoter has been reported in the nucleotides spanning -83 to -101 of the rat promoter (Fukuda and Iritani, 1999), which correlates with the Sp1 and LP1 response elements in the mouse promoter in the same region (see Figure 6.1). The element(s) bind nuclear factors in response to insulin in the same manner as the insulin-responsive regions of the FAS and ATP citrate-lyase (ACL) promoters, suggesting a similar mechanism of action and that these genes could potentially be co-ordinately regulated (Fukuda and Iritani, 1999). Fukuda et al reported only an approximately 1.5-fold increase in transcriptional activity with this construct in response to 100nM insulin in these studies, compared with 5.5-fold in my own work with the murine promoter (bearing in mind my small sample size and high variability however). Species-, cell-type- and promoter sequence-specific differences between these experiments could contribute to this potential difference.

More recently, a more comparable study has been carried out using a 762bp murine
leptin promoter in 3T3-L1 adipocytes (Moreno-Aliaga et al., 2001). The findings from this study are more in keeping with the results presented in this thesis, with the increase in activity in response to physiological concentrations of insulin being insignificant, whereas higher concentrations significantly increased promoter activity. However work to identify the response elements involved was not conducted (Moreno-Aliaga et al., 2001).

To ascertain the insulin responsiveness of the cell line, a positive control was required. Lack of success in the PCR amplification of a sequence of the fatty acid synthase promoter to generate a reporter gene construct, led to the search for an alternative method.

The initial event in insulin signalling, the tyrosine phosphorylation of the insulin receptor beta subunit, is a relatively simple method to determine insulin responsiveness. The data presented in this thesis confirms that upon insulin stimulation, tyrosine phosphorylation of IRβ occurs in 3T3-F442A adipocytes. Although this response was not measured using 1nM insulin, a response was observed at a 10nM concentration, which is at the boundary of physiological concentrations of insulin.

As well as determining this initial event in insulin signal transduction, it would have been desirable to measure some end event, such as glucose uptake, although time did not allow for this line of investigation. The determination of the responsiveness of a suitable reporter gene construct would have been the most suitable positive control however.

As an additional control, the effect of dexamethasone on Lep promoter activity was also assessed. Although not significant, a trend was observed of increased activity conferred by the pl.2lep-Luc plasmid in response to levels of dexamethasone of 10nM and over. This is in agreement with the results described in chapter five and correlates well with previous findings of the effective concentration range of dexamethasone on both leptin secretion and Lep expression in vitro (Murakami et al., 1995; Slieker et al., 1996). The fold increase in promoter activity also compares favourably with the results from the reporter gene studies of De Vos et al, who demonstrated a 2.5-fold increase in the activity of the human Lep promoter with 1µM dexamethasone. It also goes some way towards verifying that the adipocytes were viable and responsive after the electroporation procedure.
In conclusion therefore, an assay was optimized to determine the effects of insulin on Lep promoter activity in an adipocyte cell line. A series of reporter gene constructs were generated containing different lengths of the Lep proximal promoter. Optimal differentiation and transfection methods were investigated and ascertained, as was the insulinresponsiveness of the cells. Initial findings suggested that insulin was inhibitory to Lep expression in preadipocytes, but could have a small, positive effect on Lep expression in adipocytes, but further experiments are necessary to confirm this. This latter result is in agreement with the Lep mRNA in vitro adipose explant studies performed in chapter five, and with a similar reporter gene study (Moreno-Aliaga et al., 2001). However, in comparison to the rapid and robust effects of insulin on Lep expression in vivo, the studies point to a limited direct effect of insulin on Lep expression.
7 DISCUSSION
Leptin has been shown to play vital roles in numerous physiological pathways, especially involving energy homeostasis. Therefore, there has been intense interest in the factors that govern its levels - both at the mRNA level and plasma levels of the protein. Since insulin levels closely parallel changes in the nutritional state, the work presented in this thesis addresses this aspect of research - investigating the effects of insulin on the steady-state levels of murine Lep mRNA in vivo and in vitro.

The in vivo studies presented in chapter four confirm previous studies of the effects of fasting and re-feeding on Lep mRNA levels in both rats and mice. Furthermore, they establish that a subcutaneous 0.1 IU insulin injection can reverse the fasting-induced down-regulation of Lep mRNA levels in mice within four hours, in a similar manner therefore, to previous observations in rats (Saladin et al., 1995; Zheng et al., 1996a). This similarity of regulation between rats and mice adds to the current knowledge of Lep gene regulation in rodents.

Several in vitro model systems were also investigated to establish whether insulin has a direct effect on Lep mRNA expression in adipocytes – these included an RNase protection assay in murine adipose explant cultures; and Lep promoter-reporter gene studies in the adipocyte cell line, 3T3-F442A. The majority of previous studies in rodents have focussed on rat primary adipocyte and explant models, and the murine adipocyte cell line, 3T3-L1. These previous reports lack a consensus of opinion on the direct effects of insulin on Lep mRNA levels however. Some studies report that physiological concentrations of insulin increase the steady-state levels of Lep mRNA directly in rat adipocytes (Saladin et al., 1995; Slieker et al., 1996; Yoshida et al., 1996) and in the adipocyte cell lines, 3T3-L1 (Rentsch and Chiesi, 1996) and 3T3-F442A (Leroy et al., 1996). However, others see no effect, even at relatively high concentrations of insulin, e.g. in primary rat adipocytes (Murakami et al., 1995), rat explant culture (Reul et al., 1997) and 3T3-L1 adipocytes (Kosaki et al., 1996; MacDougald et al., 1995b). The results from these different model systems are further complicated by the use of different culture and quantification methods.

By investigating the direct effect of insulin in alternative adipocyte model systems in the present study, it was anticipated that the results would help to add weight to one of the possible scenarios of insulin’s effects in vitro in rodents. Moreover, the novel use of murine adipose tissue explants, and the study of unique regions of the Lep promoter in
transient transfection reporter gene studies in 3T3-F442A adipocytes would add to current knowledge of *Lep* expression in rodents in their own right.

Through the use of the murine adipose tissue explant model, it was concluded that insulin does not play a major role in the direct regulation of *Lep* mRNA levels, either acutely or chronically, in this model system. In contrast to the in vivo findings, physiological concentrations of insulin failed to significantly raise the steady-state levels of *Lep* mRNA in short-term culture (four hours), although supraphysiological concentrations were capable of achieving this effect. Moreover, chronic exposure (overnight), even to supraphysiological concentrations of insulin, failed to reverse the steadily decreasing *Lep* mRNA levels observed in culture - *Lep* mRNA levels were undetectable after this amount of time in culture. The insulin-responsiveness of this model system was demonstrated however, by the effect of insulin on the known insulin-responsive gene, FAS. This showed a robust response to chronic exposure to physiological concentrations of insulin. Short-term insulin treatment failed to significantly raise FAS levels however, but this is in agreement with in vivo findings of the differing kinetics of nutritional-responsiveness of the FAS and *Lep* genes. Fasted rat FAS mRNA levels in adipose tissue only begin to rise after 4-8 hours of re-feeding, and are maximal after 16 hours, compared to *Lep* mRNA levels, which begin to rise much earlier, within 30 minutes, and are maximal at 8 hours (Iritani et al., 2000). The *Lep* gene’s robust response to dexamethasone also points to the fact that although *Lep* mRNA levels begin to decline in the standard culture conditions in vitro, expression is not inherently dysregulated in this model system. However the decline in *Lep* mRNA levels over time did call into question the validity of the model system, and this will be discussed in further detail in a later section.

The RNase protection assay results presented in this work point to the possibility that insulin may have a minor effect on *Lep* expression however. In the remaining time of the project therefore, a more sensitive and alternative approach was sought to address this secondary issue. An assay entailing the transient transfection of reporter genes under the control of sections of the murine *Lep* promoter into adipocytes was therefore developed. This could only be used to detect potential changes in *Lep* levels occurring at the transcriptional level however. An initial experiment regarding the effect of a 24-hour exposure of insulin on murine *Lep* promoter activity in 3T3-F442A adipocytes showed a
trend towards a concentration-dependent increase in activity, although this was not significant. Furthermore, the increase observed at physiological concentrations was not extensive. Strong conclusions cannot be drawn from this without further investigation, and therefore experiments repeating this work, and examining the effect of time on this phenomenon, (since in vivo, a robust response is observed within four hours) are necessary. However, the data correlates well with the findings from the explant model system and with a recent report by Moreno-Aliaga et al, which found that the murine Lep promoter was not responsive to physiological concentrations of insulin, and only an approximately 2-fold increase was observed with supraphysiological concentrations (Moreno-Aliaga et al., 2001). However, even though a statistically significant response to insulin was not observed using the system described in this thesis or indeed in Moreno-Aliaga’s, it does not necessarily indicate that insulin does not directly affect Lep promoter activity. Response elements sensitive to insulin might not have necessarily been present in the length of promoter regions used. With the amount of sequence available at the time, the largest section of the Lep gene’s 5’ flanking sequence used in my reporter gene constructs was approximately 1kb. Future experiments using longer stretches of sequence would be of use to help interpret the results gained using this model system.

Thus the data presented in this thesis points to the fact that insulin is not a major, direct regulator of the Lep gene, and that therefore insulin is likely to mediate its effects in vivo by an indirect/secondary mechanism. This therefore adds weight to the current body of evidence supporting this conclusion, which includes the early findings of Murakami et al, MacDougald et al, Kosaki et al and Reul et al (Kosaki et al., 1996; MacDougald et al., 1995b; Murakami et al., 1995; Reul et al., 1997). However, several factors regarding the limitations of the adipose model systems were identified during the course of these studies, and some of the findings may go someway to explaining the discrepancies in the direct effect of insulin of Lep mRNA levels reported by other researchers. The major points of discussion are the ability of the adipose tissue model systems to faithfully mimic in vivo adipocytes, and leading on from this point, to the sensitivity of the adipocytes to the culture conditions utilized.

The explant model system was shown to remain responsive to both insulin and dexamethasone for reasonably long periods of time, and hence would appear to be a
valid model system. The decline in Lep and FAS mRNA levels in standard culture medium over time however, brought this assumption into question, since it was clearly not accurately mimicking adipocytes found in an in vivo context. The findings are in agreement with others using more widely used adipocyte model systems (Fain and Bahouth, 2000; Machinal et al., 1999; Reul et al., 1997), and hence suggests that none of the commonly used adipocyte model systems satisfactorily mimic adipocytes found in an in vivo context, in terms of Lep expression. This therefore questions their ability to determine genuine changes in expression of the gene.

With regard to the 3T3-F442A cell line, it was primarily chosen because of its potential to give rise to discrete, well differentiated, fat pads when injected subcutaneously into BALB/c (athymic) nude mice (Green and Kehinde, 1979). This ability proves its authenticity as a genuine adipocyte precursor, and hence was deemed a suitable choice for use in an in vitro model system. However, Lep mRNA levels in differentiated 3T3-F442A adipocytes were also found to be low in this model system compared to in vivo adipose tissue levels, in agreement to findings in the similar cell line, 3T3-L1 (MacDougald et al., 1995b; Murakami et al., 1995). MacDougald et al suggest that this is possibly because adipocytes in a tissue context are subject to humoral or other influences - not present in a cell culture environment - that upregulate Lep expression. However, lipid accumulation in adipocyte cell lines does not approach the level of lipid accumulation found in primary adipocyte cultures, where a unilocal lipid deposit is present. Since Lep levels are thought to correlate with the amount of triglyceride stored within the cell, (Hamilton et al., 1995; Maffei et al., 1995a; Zhang et al., 2002), this also may have been the reason for low Lep expression. Hence low Lep expression and low lipid accumulation in immortalized adipocyte cell lines shows their inability to faithfully mimic the in vivo situation.

Sensitivity of the model systems to the culture conditions also challenged the suitability of the model systems used, and the ability to compare results to previous findings by other groups. Firstly, optimal differentiation of the 3T3-F442A cell line - in terms of lipid accumulation and GPDH activity - was empirically found to occur when the cells were cultured in growth medium containing DMEM plus 10% foetal calf serum - where the foetal calf serum had been heat-inactivated, (see section 6.2.3.3). Potential mechanisms by which this occurs have been discussed in chapter six. A profound
increase in lipid accumulation and GPDH activity was observed with the heat-inactivated foetal calf serum compared to normal foetal calf serum, and it is therefore possible that other adipose-specific functions could be similarly affected by this difference. The issue raises an important point in the study of adipocyte metabolism and regulation, since few, if any researchers state whether they heat inactivate serum or not. This therefore could lead to potentially conflicting evidence between similar pieces of work from different laboratories. The ability of some groups to easily detect Lep mRNA levels in adipose tissue culture, e.g. Saladin et al., 1995, compared to my own, and other groups’ observations (MacDougald et al., 1995b; Murakami et al., 1995) could therefore potentially be due to differences in culture conditions. The effect of differing culture conditions on Lep expression is therefore a case for further investigation, both in the murine adipose tissue explant model, where non-heat-inactivated foetal calf serum was used, and in the reporter gene studies, where once differentiation had occurred, insulin-responsiveness was determined in a serum-free context. This is especially relevant after a recent report has shown that the effect of insulin on Lep promoter activity in 3T3-L1 preadipocytes (adipocytes were not studied) is influenced by the presence or absence of foetal calf serum - activity being suppressed in the presence of 10% foetal calf serum (Li et al., 2001). However, the two different approaches that were undertaken in this thesis have yielded comparative results - in that insulin does not have a major effect - suggesting that the presence or absence of serum is not such an issue in adipocytes compared to preadipocytes.

In light of the findings suggesting a role of glucose in this response (see section 1.6.3), the effect of glucose levels in the culture medium is also worthy of further investigation, as is additional investigation into the effect of a high sucrose diet on Lep mRNA levels in vivo. The role of glucose in the insulin response is in the process of being elucidated. Recent progress has been made concerning the role of insulin-induced glucose metabolism as the major mediator of Lep promoter activity, rather than insulin per se, since the inhibitor of glucose uptake and metabolism, 2-deoxy-D-glucose, represses insulin-stimulated Lep promoter activity in 3T3-L1 adipocytes (Moreno-Aliaga et al., 2001). However, the mechanism by which insulin-stimulated glucose metabolism exerts its effects remains unknown. Several other genes are known to require both glucose and insulin to elicit a response and it is becoming clear that the S1 response element is involved in this process in genes such as FAS and ATP citrate lyase as well as Lep.
(Fukuda and Iritani, 1999; Fukuda et al., 1996; Fukuda et al., 1997). The model systems developed here, would make it possible for any response to insulin and/or glucose to be further dissected. This is particularly the case with the reporter gene study, whereby if a response was found to occur at the transcriptional level, the mechanism of insulin action, and any insulin-response elements identified could be easily characterized further.

Since the reporter gene study cannot detect post-transcriptional effects of insulin on Lep expression however, and the possible involvement of secretory compartments in leptin release in response to insulin, it would also be desirable to further the work on the direct effect of insulin on the levels of the secreted leptin, that have been carried out by others in our group (Kunjara et al., 2000).

In conclusion therefore, using two alternative, insulin-responsive, adipocyte model systems, it is apparent that a major, acute, direct effect of insulin on Lep expression does not occur, in comparison to the robust, acute effect observed in vivo. The possibility that insulin plays a small role in directly regulating Lep mRNA levels acutely, or affects levels chronically, cannot be ruled out however. Unfortunately the lack of sensitivity of the explant model system presented in this body of work, and time constraints in the reporter gene studies, prevented this question being adequately resolved in this study. Further research into the validity of adipocyte in vitro model systems with respect to Lep expression is also required, as is the need to determine the effect of differing culture conditions to allow meaningful comparisons between different group’s results.
APPENDIX – BUFFERS AND SOLUTIONS
### DNA Methodology

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>6x DNA loading buffer</td>
<td>0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol</td>
</tr>
<tr>
<td>1x TAE</td>
<td>40mM Tris-acetate, 1mM EDTA</td>
</tr>
<tr>
<td>1x TE</td>
<td>10mM Tris-acetate, 1mM EDTA</td>
</tr>
<tr>
<td>1x TBE</td>
<td>90mM Tris-borate, 2mM EDTA</td>
</tr>
<tr>
<td>TE saturated phenol</td>
<td>Phenol in 1x TE, <strong>not autoclaved</strong>, stored in the dark at 4°C</td>
</tr>
<tr>
<td>IAA/chloroform</td>
<td>4% (v/v) isoamyl alcohol (IAA), 96% (v/v) chloroform, <strong>not autoclaved</strong>, stored at -20°C</td>
</tr>
<tr>
<td>DNA purpose phenol/chloroform</td>
<td>1:1 TE saturated phenol: IAA chloroform, <strong>not autoclaved</strong>, stored in the dark at 4°C</td>
</tr>
<tr>
<td>GTE buffer</td>
<td>50mM glucose, 23mM Tris-HCl (pH 8), 10mM EDTA, stored at 4°C</td>
</tr>
<tr>
<td>E. coli lysis buffer</td>
<td>0.2N NaOH, 1% (w/v) SDS, <strong>made up fresh</strong>, <strong>not autoclaved</strong></td>
</tr>
<tr>
<td>Lysozyme solution</td>
<td>10mg/ml in 10mM Tris-HCl (pH8.0), <strong>made up fresh</strong>, <strong>not autoclaved</strong></td>
</tr>
<tr>
<td>PEG solution</td>
<td>1.6M NaCl, 13% (w/v) PEG 8000</td>
</tr>
</tbody>
</table>
# RNA METHODOLOGY

<table>
<thead>
<tr>
<th><strong>DEPC H₂O</strong></th>
<th>0.1% (v/v) DEPC. agitated overnight until miscible</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Deionized formamide</strong></td>
<td>Stir formamide with Amberlite MB resin (10g per 100 ml) for one hour to deionize. Filter through Whatman 3MM paper. <em>not autoclaved</em> stored in a dark bottle at 4 °C</td>
</tr>
<tr>
<td><strong>RNA purpose phenol/chloroform</strong></td>
<td>50% (v/v) water saturated phenol 50% (v/v) IAA/chloroform <em>not autoclaved</em> stored in the dark at 4 °C</td>
</tr>
<tr>
<td><strong>RNA loading buffer (for agarose gels)</strong></td>
<td>0.67xMEA 9.3% (v/v) formaldehyde 66.7% (v/v) deionized formamide 0.25% (w/v) bromophenol blue 0.25% (w/v) xylene cyanol FF <em>not autoclaved</em> stored at -20 °C</td>
</tr>
<tr>
<td><strong>RNA loading buffer (for polyacrylamide gels)</strong></td>
<td>80% (v/v) deionized formamide 10mM EDTA (pH 8.0) 1mg/ml xylene cyanol FF 1mg/ml bromophenol blue <em>not autoclaved</em> stored at -20 °C</td>
</tr>
<tr>
<td><strong>Solution D (for RNA extraction)</strong></td>
<td>25g guanidinium thiocyanate 29.3ml DEPC dH₂O 1.76ml 0.75M Na citrate 2.64ml 10% (w/v) sacrocyl 360µl 2-mercaptoethanol <em>not autoclaved</em> made up fresh</td>
</tr>
<tr>
<td><strong>20x SSC</strong></td>
<td>175.3g/l NaCl 88.2g/l sodium citrate adjusted to pH 7.0 with NaOH</td>
</tr>
<tr>
<td><strong>RPA hybridization buffer</strong></td>
<td>40mM PIPES (pH 6.4) 1mM EDTA (pH 8.0) 400mM NaCl 80% (v/v) deionized formamide</td>
</tr>
</tbody>
</table>
| Buffer I (for competent cells) | 10mM MES  
100mM RbCl₂  
10mM CaCl₂  
50mM MnCl₂  
_adjusted to pH 5.8 with KOH, stored at 4 °C |
| Buffer II (for competent cells) | 10mM Pipes (pH 6.5)  
75mM CaCl₂  
10mM RbCl₂  
15% (v/v) glycerol  
_stored at 4 °C |
| LB medium | 1% (w/v) bacto-tryptone  
0.5% (w/v) bacto-yeast extract |

**Bacterial Methodology**

| Buffer I (for competent cells) | 10mM MES  
100mM RbCl₂  
10mM CaCl₂  
50mM MnCl₂  
_adjusted to pH 5.8 with KOH, stored at 4 °C |
| Buffer II (for competent cells) | 10mM Pipes (pH 6.5)  
75mM CaCl₂  
10mM RbCl₂  
15% (v/v) glycerol  
_stored at 4 °C |
| LB medium | 1% (w/v) bacto-tryptone  
0.5% (w/v) bacto-yeast extract |
<table>
<thead>
<tr>
<th></th>
<th>1% (w/v) NaCl</th>
</tr>
</thead>
</table>
| X-Gal Stock Solution     | 50mg/ml X-Gal in DMF
                        | *not autoclaved*                                   |
|                          | *stored in the dark at -20 °C*                   |
| Hogness Buffer           | 400g/l glycerol                                  |
|                          | 8.2g/l K₂HPO₄                                    |
|                          | 4.85g/l KH₂PO₄                                   |
|                          | 1.75g/l tri-sodium citrate                       |
|                          | 2.45g/l MgSO₄                                    |
|                          | *stored at 4 °C*                                 |

**Tissue Culture Methodology**

| Paraformaldehyde fixative | 1x PBS
|                          | 1% (w/v) paraformaldehyde
|                          | *a few drops of 10N NAOH added to dissolve*
|                          | *stored at -20 °C*                               |
| Nile Red Stock Solution   | 1mg/ml Nile Red in DMSO
|                          | *not autoclaved*                                  |
|                          | *stored in the dark*                              |
| Oil Red O stock solution  | 1mg/ml Oil Red O in propan-2-ol                   |
|                          | *heated to 56 °C for 1 hour to dissolve*          |
| 2x HBS                   | 1.4 mM Na₂HPO₄                                    |
|                          | 280mM NaCl                                        |
|                          | 50mM HEPES pH7.1                                  |
|                          | *stored at 4 °C*                                  |
| Dexamethasone stock solution | 1mM dexamethasone in 95% ethanol                |
|                          | *stored at -20 °C*                                |
| Insulin stock solution   | 1mM insulin in 0.05N NaOH                         |
|                          | *stored at -80 °C*                                |
## PROTEIN METHODOLOGY

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Components</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lysis Buffer</strong></td>
<td>50mM Hepes (pH 7.4)</td>
</tr>
<tr>
<td>(for immunoprecipitation)</td>
<td>150mM NaCl</td>
</tr>
<tr>
<td></td>
<td>10mM EDTA</td>
</tr>
<tr>
<td></td>
<td>1mM Na&lt;sub&gt;3&lt;/sub&gt;VO&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>30mM NaF</td>
</tr>
<tr>
<td></td>
<td>10mM Sodium pyrophosphate</td>
</tr>
<tr>
<td></td>
<td>2.5mM benzamidine</td>
</tr>
<tr>
<td></td>
<td>1μg/ml pepstatin</td>
</tr>
<tr>
<td></td>
<td>1μg/ml leupeptin</td>
</tr>
<tr>
<td></td>
<td>1μg/ml antipain</td>
</tr>
<tr>
<td></td>
<td>0.5mM PMSF</td>
</tr>
<tr>
<td></td>
<td>1% (v/v) Triton X-100</td>
</tr>
<tr>
<td></td>
<td>made up fresh</td>
</tr>
<tr>
<td></td>
<td>not autoclaved</td>
</tr>
<tr>
<td><strong>Resuspension Buffer</strong></td>
<td>50mM Tris-HCl (pH 7.5)</td>
</tr>
<tr>
<td>(for GPDH assay)</td>
<td>1mM EDTA</td>
</tr>
<tr>
<td></td>
<td>1mM 2-mercaptoethanol</td>
</tr>
<tr>
<td></td>
<td>made up fresh</td>
</tr>
<tr>
<td></td>
<td>not autoclaved</td>
</tr>
<tr>
<td><strong>GPDH assay buffer</strong></td>
<td>100mM Triethanolamine/HCl (pH 7.5)</td>
</tr>
<tr>
<td></td>
<td>2.5mM EDTA</td>
</tr>
<tr>
<td></td>
<td>0.12mM NADH</td>
</tr>
<tr>
<td></td>
<td>0.1mM 2-mercaptoethanol</td>
</tr>
<tr>
<td></td>
<td>made up fresh</td>
</tr>
<tr>
<td></td>
<td>not autoclaved</td>
</tr>
<tr>
<td><strong>3x Alkaline phosphatase buffer</strong></td>
<td>2.85mM DEA</td>
</tr>
<tr>
<td></td>
<td>0.84mM NaCl</td>
</tr>
<tr>
<td></td>
<td>1.5mM MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
</tr>
<tr>
<td></td>
<td>15mM pNPP</td>
</tr>
<tr>
<td><strong>2x Laemmli SDS loading buffer</strong></td>
<td>100mM Tris-Cl (pH 6.8)</td>
</tr>
<tr>
<td></td>
<td>200mM DTT</td>
</tr>
<tr>
<td></td>
<td>4% (W/V) SDS</td>
</tr>
<tr>
<td></td>
<td>0.2% (w/v) bromophenol blue</td>
</tr>
<tr>
<td></td>
<td>20% (v/v) glycerol</td>
</tr>
<tr>
<td></td>
<td>DTT added just before use</td>
</tr>
<tr>
<td>Table</td>
<td>Description</td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
</tr>
<tr>
<td>Acrylamide resolving gel</td>
<td>8% (v/v) non-denaturing acrylamide solution</td>
</tr>
<tr>
<td>Acrylamide stacking gel</td>
<td>5% (v/v) non-denaturing acrylamide solution</td>
</tr>
<tr>
<td>1x PBS</td>
<td></td>
</tr>
<tr>
<td>Western blocking buffer</td>
<td></td>
</tr>
<tr>
<td>Western wash buffer</td>
<td></td>
</tr>
</tbody>
</table>
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