UNIVERSITY OF SOUTHAMPTON

FACULTY OF MEDICINE, HEALTH AND LIFE SCIENCES

School of Biological Sciences

Leptin and Peroxisome Proliferator Activated Receptor Alpha: Understanding their Contribution Towards Normalising the Programmed Phenotype in the Peripheral Tissues of IUGR Offspring

by

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ABSTRACT

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LEPTIN AND PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR ALPHA: UNDERSTANDING THEIR CONTRIBUTION TOWARDS NORMALISING THE PROGRAMMED PHENOTYPE IN THE PERIPHERAL TISSUES OF IUGR OFFSPRING

By Emma Sian Garratt

In a rat model of intrauterine growth restriction (IUGR) induced by maternal global undernutrition, adult offspring are obese with associated metabolic disturbances. These metabolic abnormalities are all augmented by feeding a high calorie postnatal diet and reversed by neonatal leptin treatment. Evidence is now accumulating which indicates that altered epigenetic regulation and gene expression may underpin the relationship between the early life environment and metabolic disturbances in adult life. Therefore, to determine the mechanism responsible for the alterations in energy balance in the IUGR rat, this study investigated the effect of maternal diet, neonatal leptin treatment and a postnatal high fat diet on the expression and DNA methylation of genes involved in energy balance in the liver and adipose tissue of adult offspring. These genes included the peroxisome proliferator activated receptors (PPARs) and their target genes; acyl-coA oxidase (AOX), carnitine palmitoyl transferase-1 (CPT-1) and lipoprotein lipase (LPL).

Real time PCR indicated that the expression of several key genes involved in energy balance, including PPARα, PPARγ and their target genes, was not altered by maternal diet or postnatal diet in the liver or adipose tissue of these offspring. However, in adipose tissue, neonatal leptin treatment resulted in an increase in the expression of most genes tested, including PPARα, PPARγ and their target genes. The increased PPARγ and LPL would facilitate the uptake of fatty acids into the adipocyte, whilst the upregulation of PPARα and its target genes AOX and CPT-1, not normally expressed in adipocytes, would direct fatty acids taken up towards the β-oxidation pathway instead of storage. This would imply that the fat cell had transformed from a fat storing cell to a fat metabolising cell. Gene expression data therefore indicated that the phenotypic changes induced by neonatal leptin treatment, i.e. the reduced weight gain, could be due to increased expression of PPARα, PPARγ and their target genes in adipose tissue: Furthermore, the effects of this are persistent, due to the specific period of leptin administration during neonatal development. There was, however, no evidence of altered DNA methylation in the promoter regions measured which could account for these persistent effects.

To investigate mechanisms underlying the regulation of the PPARα promoter by leptin, the rat PPARα promoter was mapped, cloned and characterised. As part of this process, six alternatively spliced variants were identified; one from adipose tissue (P1), two from the liver (P2, P3), one from the heart (P4) and two from the kidney (P5, P6). These transcripts were found to differ in their 5'untranslated region due to tissue specific promoter usage and alternative transcription start sites. The liver and adipose specific promoters were cloned and characterised using a reporter gene strategy. They were shown to differ in their basal activity, response to known activators of transcription and to neonatal leptin treatment. The regulation of the PPARα promoter by leptin was investigated and shown to function via a non-canonical mechanism requiring both signal transducer and activators of transcription (Stat3) and specificity protein-1 (Sp1), which act at a unique region of the liver specific P2 promoter. The adipose specific P1 promoter was shown to be unresponsive to leptin treatment. Furthermore, real time PCR with primers specific to the P1 and P2 PPARα transcripts indicated that the increased PPARα expression seen in leptin treated offspring was due to an increase in the P2 specific transcript, not the P1 transcript. This indicated that the neonatal leptin treatment facilitated a selective switch in promoter usage to increase the expression of PPARα and its target genes in a tissue in which they are not normally expressed, thus inducing an altered metabolism within the adipocytes of these offspring.

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DECLARATION OF AUTHORSHIP

I, Emma Garratt declare that the thesis entitled Leptin and Peroxisome Proliferator Activated Receptor α: Understanding their contribution towards normalising the programmed phenotype in the peripheral tissues of IUGR Offspring, and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

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- where I have consulted the published work of others, this is always clearly attributed;
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Signed: …Emma Garratt……..

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Thank you

ABBREVIATIONS

5’ RNA ligase mediated rapid amplification of cDNA ends	5’RLM RACE
11 beta-hydroxysteroid dehydrogenase	11βHSD
Acetyl coA carboxylase	ACC
Activator protein 1	AP1
Acyl-coA oxidase	AOX
Adenosine triphosphate	ATP
Ad libitum	AD
Agouti-related peptide	AgRP
Alpha-melanocyte stimulating hormone	α-MSH
Analysis of variance	ANOVA
AMP-activated protein kinase	AMPK
Arcuate nucleus of the hypothalamus	ARH
ATP-citrate lyase	ACL
Beckwith Wiedemann syndrome	BWS
Calf Intestinal alkaline phosphatase	CIP
cAMP response element binding protein	CREB
Carnitine palmitoyl transferase-1	CPT-1
CCAAT-enhancer-binding proteins	C/EBP
Chicken ovalbumin upstream promoter transcription factor	Coup TF
Chromatin immumoprecipitation	ChIP
Clofibric acid	CFA
Cocaine and amphetamine-related transcript	CART
Copy-deoxyribonucleic acid	c-DNA
Coronary heart disease	CHD
CREB binding protein	CBP
Cycle threshold	Ct
Cyclophillin	CYC
Deoxyribonucleic acid	DNA
Developmental origins of health and disease	DOHaD
Dexamethasone	DEX
Differentially methylated region	DMR
Dihydrofolate reductase gene	DHFR
DNA methyl transferases	DNMT
Downstream promoter element	DPE
Dorsomedial hypothalamic nucleus	DMH
Embryonic stem	ES
Enhancer box	E-Box

Fat mass and obesity associated	FTO
Fatty acid synthesase	FAS
GATA binding protein	GATA
Global undernutrition	UN
Glucocorticoid receptor	GR
Glucocorticoid response elements	GRE
Glucokinase	GK
Heterochromatin protein 1	HP1
Hepatocyte nuclear factor	HNF
High Fat	HF
Histone deacetylase	HDAC
Histone acetyl transferase	HAT
Hormone sensitive lipase	HSL
Hypothalamic-pituitary-adrenal	HPA
Initiator element	INR
Insulin-like growth factor	IGF
Interleukin	IL
Intracytoplasmic sperm injection	ICSI
Internal ribosome entry site-dependent	IRES
Intracisternal A particle	IAP
Intrauterine growth retardation	IUGR
In vitro fertilisation	IVF
Janus kinase	JAK
Lateral hypothalamic area	LHA
Leptin	L
Leptin receptor	LR
Ligand independent activation domain	AF-1
Lipoprotein lipase	LPL
Loss of imprinting	LOI
Luria broth	LB
Malic enzyme	ME
Maternal low protein	MLP
Methylation sensitive PCR	MSP
Methyl binding domain	MBD
Methyl CpG binding proteins	MBD
Micro RNAs	miRNA
Motif ten elements	MTE
Neuropeptide Y	NPY
Nonesterified fatty acid	NEFA
Nuclear factor 1	NF-1

Nuclear factor kappa-light-chain-enhancer of activated B cells	NFκB
Nucleosome remodelling and histone deacetylase	NuRD
Obese gene	OB
Octamer 1	OCT1
Open reading frame	ORF
Paraventricular nucleus	PVH
Peroxisome proliferator activated receptor	PPAR
Peroxisome proliferator response element	PPRE
Perfluorodecanoid acid	PFDA
Phosphoenolpyruvate carboxykinase	PEPCK
Phosphoinositide 3-kinase	PI3K
Polycomb group proteins	PcG
Polymerase chain reaction	PCR
Postnatal day	PND
PPAR gamma coactivator 1	PGC-1
Predictive adaptive response	PAR
Primordial germ cells	PGCs
Proliferating cell nuclear antigen	PCNA
Pro-opiomelanocortin	POMC
Protein tyrosine phosphatase 1B	PTP1B
Retinoic acid receptor	RAR
Retinoid X receptor	RXR
Ribonucleic acid	RNA
5'RNA ligase mediated rapid amplification of cDNA ends	5'RLM RACE
s-adenosyl-L-homocysteine	SAH
s-adenosyl-l-methionine	SAM
SH-2 domain containing phosphatases	SHP2
Signal transducer and activator of transcription	STAT
Specificity protein 1	P1
Stat3 binding element	SBE
Sterol regulatory binding protein	SREBP
Suppressors of cytokine signalling 3	SOCS3
TATA binding protein	TBP
TBP associated factor 4	TAF 4
TFIIB recognition elements	BRE
Thiazolidinedione	TZD
Thyrotrophin releasing hormone	TRH
Tissue inhibitor of metalloproteinase 1	TIMP-1
Tobacco acid pyrophosphatase	TAP
Transcriptional repression domain	TRD

Transcription start site	TSS
Transcription factor	TF
Triacylglycerol	TAG
Trichostatin A	TSA
Tumour necrosis factor α	TNF-α
Tyrosine	TYR
Tyrosine aminotransferase	TAT
Undernutrition	UN
Untranslated region	UTR
Upstream ATG	uATG
Upstream ORF	uORF
White adipose tissue	WAT
X Core promoter element 1	XCPE1

Chapter 1

Introduction

1.0 Introduction

1.1 Origins of obesity

Western society has an obesity epidemic. Obesity is a disorder of energy balance associated with a cluster of disturbances termed the metabolic syndrome, which includes; insulin resistance, dyslipidemia and hypertension. Thus obesity is a high risk factor for chronic disease, including type II diabetes and cardiovascular disease [1,2]. The incidence of obesity has risen dramatically in recent years, for example in the US prevalence has increased nearly 8% in just 7 years, with 1 in 3 Americans now being obese [2] and worryingly, one third of pregnant women are now obese [3]. Statistics show that this increase in obesity is also reflected in children; in the US 9 million children between the ages of 6-19 are overweight and in the UK this figure reaches nearly 1 million. This represents levels three times that of 1980 and the incidence in this age range is increasing by 1% each year, the adverse effects of which will manifest in later life [4]. Startlingly, obesity related metabolic disturbances have even been identified in 5 year old children and the incidence of type II diabetes has increased dramatically in adolescents in recent years [5]. Consequently, obesity has reached epidemic levels worldwide, in children as well as adults, with 300 million people thought to be obese and the levels still increasing.

The rapid increase in obesity can not be exclusively due to genetic factors, but can also be attributed to changes in lifestyle factors due to the increasing prevalence of a westernised lifestyle. This includes increased energy intake due to larger portions and energy rich foods and reduced energy expenditure due to a more sedentary lifestyle. Interestingly, evidence from human epidemiological studies and animal models has clearly shown that the origins of obesity and its associated metabolic disturbances can be programmed by the maternal environment in utero and this has led to the acceptance of the 'developmental origins of health and disease' hypothesis [6-8].

1.2 The developmental origins of health and disease hypothesis

The basic concept of the developmental origins of health and disease (DOHaD) hypothesis is that environmental constraints such as poor maternal nutrition, acting during critical periods of early development, can induce metabolic disturbances in the developing offspring which result in an increased risk of non-communicable chronic diseases in adult life. These diseases include type II diabetes and coronary heart disease (CHD).

1.2.1 Programming of the adult phenotype by maternal undernutrition

Retrospective human epidemiological studies in the UK provided the first evidence for the DOHaD hypothesis. In 1986, Barker and colleagues made the original observation that reduced fetal growth, as a result of adverse intrauterine environment such as maternal undernutrition, was associated with an increased risk of CHD in adulthood [9-11]. This reduced fetal growth manifests as a smaller birth size [12], but growth restriction does not need to be severe, as birth sizes within the normal range have been shown to have a continuous relationship with disease risk [11,13]. Since these original studies by Barker, the association between maternal undernutrition and risk of CHD in adult life has been extensively replicated in other cohorts throughout the world, in both men and women, including the US [14], India [15] and Europe [16].

One important retrospective epidemiological study which has generated much information on the effect of maternal undernutrition on adult disease risk is the Dutch Hunger Winter [17]. During the harsh winter of 1944-1945, a severe food shortage affected the west Netherlands as a result of German occupation and frozen waterways preventing the passage of food. As a result, the scarce food supplies were rationed. Consequently, at the height of the famine, daily calorie intake for adults was less than 1000 kcal per day, whereas both before and after the famine, the population was well nourished. Individuals were classed as being exposed to the famine if they consumed less than 1000 kcal per day for a 13 week period, furthermore exposures were classed in to early, mid or late gestation. As a result, this famine provided a unique opportunity to study the effects of maternal undernutrition at different periods of gestation on the health of adult offspring [17].

When exposure to the famine was during early gestation, an increased incidence of atherogenic lipid profiles was evident in men and women when compared to unexposed individuals. This included; lower plasma HDL, lower apolipoprotein concentrations and higher total cholesterol, LDL-cholesterol and apolipoprotein B concentrations [18]. There was also an increased incidence of abdominal obesity in 50yr old women, which is known to be associated with metabolic disease. Therefore, this indicated a disruption to bodyweight control, which could be due to either endocrine or adipocyte dysfunction [6]. However, this increased prevalence of obesity was not found in 50yr old men, which indicates that the mechanisms underlying the predisposition to obesity were more likely to involve central endocrine abnormalities rather than peripheral dysregulation of adipocytes [17]. Furthermore, abdominal obesity was not apparent in men or women exposed in mid or late gestation [6,17]. An increase in the prevalence of CHD was also identified in those exposed to famine during early gestation [19], which again was absent in those exposed during mid or late gestation [19]. Furthermore, as famine exposure during late gestation did not affect central obesity, lipid profiles or CHD, it is possible that the transition from the undernutrition to adequate nutrition in individuals exposed during early gestation played a role in the onset of these disturbances. In addition, as individuals exposed to the famine in early gestation had a normal birth weight despite the

metabolic disturbances in adulthood, this indicated that size at birth was not always predictive of adult disease [18].

When exposure to the famine was during late gestation, an increased occurrence of impaired glucose tolerance was apparent, indicating alterations to insulin-glucose metabolism in these individuals. Furthermore, in contrast to those subjected to famine in early gestation, those exposed during late gestation were found to be smaller at birth than unexposed individuals [20]. Data also indicated that increased blood pressure in adults exposed to the famine was associated with this smaller size at birth [21]. In addition, increased blood pressure also correlated with a low protein to carbohydrate ratio in the maternal diet during the third trimester, indicating that the balance of macronutrients may play a role in formation of disease [21].

Extensive studies into this cohort have therefore demonstrated that maternal undernutrition during gestation results in a variety of phenotypes associated with metabolic syndrome and that the adult phenotype varies according to the timing of the nutrient restriction during development [17]. These studies have therefore informed us of the different organs and metabolic systems which are susceptible to maternal nutrition at differing periods of gestation. In addition to this, analysis of many other epidemiological studies have led to the conclusion that a smaller birth size within the normal range is associated with an increased risk in adulthood of; obesity [6,22], impaired insulin metabolism and non-insulin dependent diabetes [23,24], dyslipidemia [18] and hypertension [25,26], which all contribute to an increased risk of coronary heart disease. Furthermore, a study of males born in Helsinki has shown that the adverse long term effects of inadequate prenatal nutrition and subsequent small birth size are augmented by increased catch up growth during early childhood [27]. This finding has been replicated in other cohorts, for example, IUGR offspring whom experienced rapid catch up growth had an increased risk of obesity at the age of two [28].

1.2.2 Programming of the adult phenotype by maternal overnutrition

In addition to maternal undernutrition, fetal overnutrition due to maternal adiposity is also associated with altered fetal growth and development, which results in an increased risk of chronic disease such as obesity in adult life [3]. This has been termed the 'fetal overnutrition' hypothesis [29]. It is thought that maternal obesity programs alterations in metabolism, appetite or neuroendocrine function due to an increased nutrient transfer across the placenta [5]. For example, in a prospective study of 7000 women in Australia, maternal BMI in pregnancy was found to have a positive association with offspring BMI at the age of 14 [29]. Furthermore, in a retrospective cohort from Finland, children born to mothers with high BMI were found to have higher BMI themselves throughout childhood than those children born to normal weight mothers. In addition, offspring who were overweight at the age of 31 were more likely to be born from women whom were

overweight before pregnancy [30]. This worrying fact indicates that obese women tend to produce obese offspring, thus amplifying the incidence of obese individuals throughout subsequent generations [31].

Therefore two opposing fetal environments; nutrient deficiency and nutrient surplus, converge towards a common phenotype, which may indicate similar underlying mechanisms. Consequently, a U shaped curve can be used to describe the relationship between birth weight and the risk of chronic disease in adulthood[32], with those at risk being the smaller and larger babies. All these findings from human epidemiological studies indicate that environmental constraints, which include both maternal undernutrition and overnutrition, can alter physiology and metabolism to induce an increased risk of chronic disease in postnatal life, including obesity, insulin resistance, dyslipidemia and hypertension. In addition, adequate nutrition in postnatal life can augment this disease risk. Furthermore, the timing and nature of the nutritional constraint can affect the resulting phenotype, indicating that different systems such as glucose metabolism and lipid metabolism are sensitive to maternal undernutrition at different periods of gestation.

Although retrospective studies have been of utmost importance in identifying the consequences of maternal undernutrition, these studies are limiting for obvious reasons. As a result, prospective studies are now emerging which investigate the effects of the maternal environment on the development of offspring and the association with adult disease risk. These studies have the advantage of being able to overcome the short fallings of retrospective studies, such the inability to look at underlying mechanisms, absent or incomplete data and the lack of appropriate measurements and information on the maternal environment. One such study is the Southampton women's survey [33]. This study was set up to determine the effects of the maternal environment before and during pregnancy on fetal growth throughout gestation, postnatal growth and also on risk factors for chronic disease such as type II diabetes and CVD. Unlike the retrospective studies discussed in this report, this study benefits from pre-pregnancy maternal measurements, which can therefore be related to fetal growth, development and disease risk. In addition, numerous samples and measurements are being taken throughout the study, including; anthropometric measurements, DXA scans for bone and body composition, blood and urine before and during pregnancy and buccal swabs from offspring at different stages of postnatal life. Furthermore, a wealth of information is being collected on maternal diet, lifestyle and illnesses before and during pregnancy. However, a major disadvantage of this study and others like it is that they extend over a very long period of time; therefore a complete set of data will not be available until all children in the cohort have reached the study endpoint. However, it is hoped that the outcomes from this unique experiment will enable the development of strategies to enable optimal growth and development of the offspring and thus minimise the risk of chronic disease in adult life [33].

1.2.3 Developmental plasticity and predictive adaptive responses

The term 'Fetal Programming' has been used to describe the developmental alterations which underpin the increased risk of disease in later life. These 'alterations' are a consequence of the environmental cue, such as altered maternal nutrition or increased glucocorticoid exposure, acting during a sensitive window of early development [13]. This developmental window extends throughout early development from conception to the neonatal period and is associated with developmental plasticity [34]. Plasticity results from an increased vulnerability due to tissue growth and differentiation, which occurs at different times in different cells, tissues and organs. Subsequently those cells undergoing rapid growth are more at risk. As a result, adverse environmental cues can alter physiology and metabolism and lead development down alternative and irreversible paths, resulting in the production of alternative phenotypes from one genotype [12]. The timing of plasticity can also differ according to species, for example in humans adipose tissue is formed during fetal life, whereas in rodents adipogenesis occurs after birth. Subsequently, the effects of environmental constraints on programming will depend not only on the nature of the constraint, but also on the stage of gestation and species.

The response an organism makes to the adverse environmental cue during this critical period of development is crucial for its future fitness. It has been proposed that organisms respond to such environmental constraints by making what has been termed a predictive adaptive response (PAR) [12], whereby the organism predicts the future postnatal environment and makes changes in physiology and metabolism to adapt to this. The PAR therefore leads the organism down an alternative developmental path which provides it with an increased chance of survival if the adverse conditions persist in the future. If however, the postnatal environment doesn't match that which it predicted, then there is an increased risk of disease (figure 1.1) [12]. Therefore, appropriate PARs increase the chance of survival to reproduce, whereas inappropriate PARs carry an increased risk of disease in later life [35].

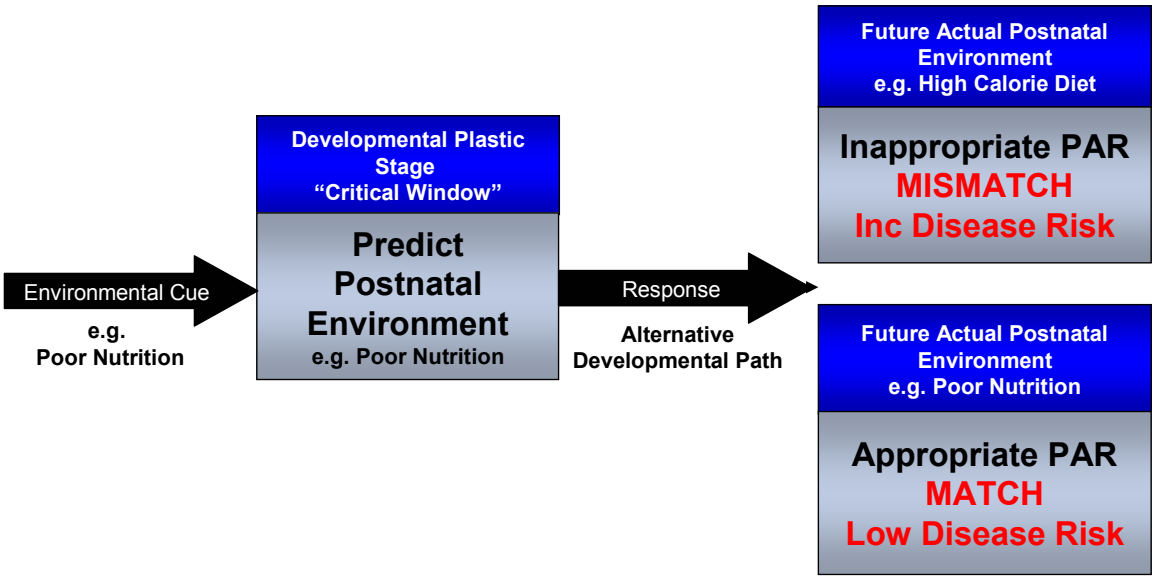


Figure 1.1 The relationship between predictive adaptive responses and the risk of disease in later life. The organism responds to an environmental cue (e.g. poor nutrition) during the plastic stage of development by predicting the future environment and altering its developmental trajectory to enable improved fitness in the predicted environment (i.e. poor nutrition). When the future environment matches that which it predicted, it has maximal fitness and chance of survival and the PAR is said to be appropriate. If the predicted environment does not match that predicted (i.e. high calorie diet), the developmental path undertaken leads the organism to reduced fitness and an increased risk of disease and the PAR is said to be inappropriate [35].

1.2.4 Animal models of programming

Studying the molecular mechanisms underlying developmental programming in humans is limited due to obvious ethical considerations as well as social and environmental variations. For these reasons, animal models of programming have been developed using animals such as mice and rats. These models enable subjects to be carefully chosen, environments controlled and diets manipulated, whilst tissues and measurements can be collected throughout gestation and beyond. As mechanisms which alter fetal growth play a main role in developmental programming, such as maternal nutrition or stress, these have therefore provided the main cue for inducing alternative phenotypes in these animals. Applying these nutritional and endocrine insults during early development to rodents results in offspring with similar phenotypes to that identified in humans, such as; reduced birth weight, increased blood pressure, obesity and impaired glucose tolerance [13]

(figure 1.2). These animal models have produced a wealth of data and have been invaluable in understanding the underlying mechanisms linking maternal nutrition to risk of obesity and metabolic disease in later life in the offspring.

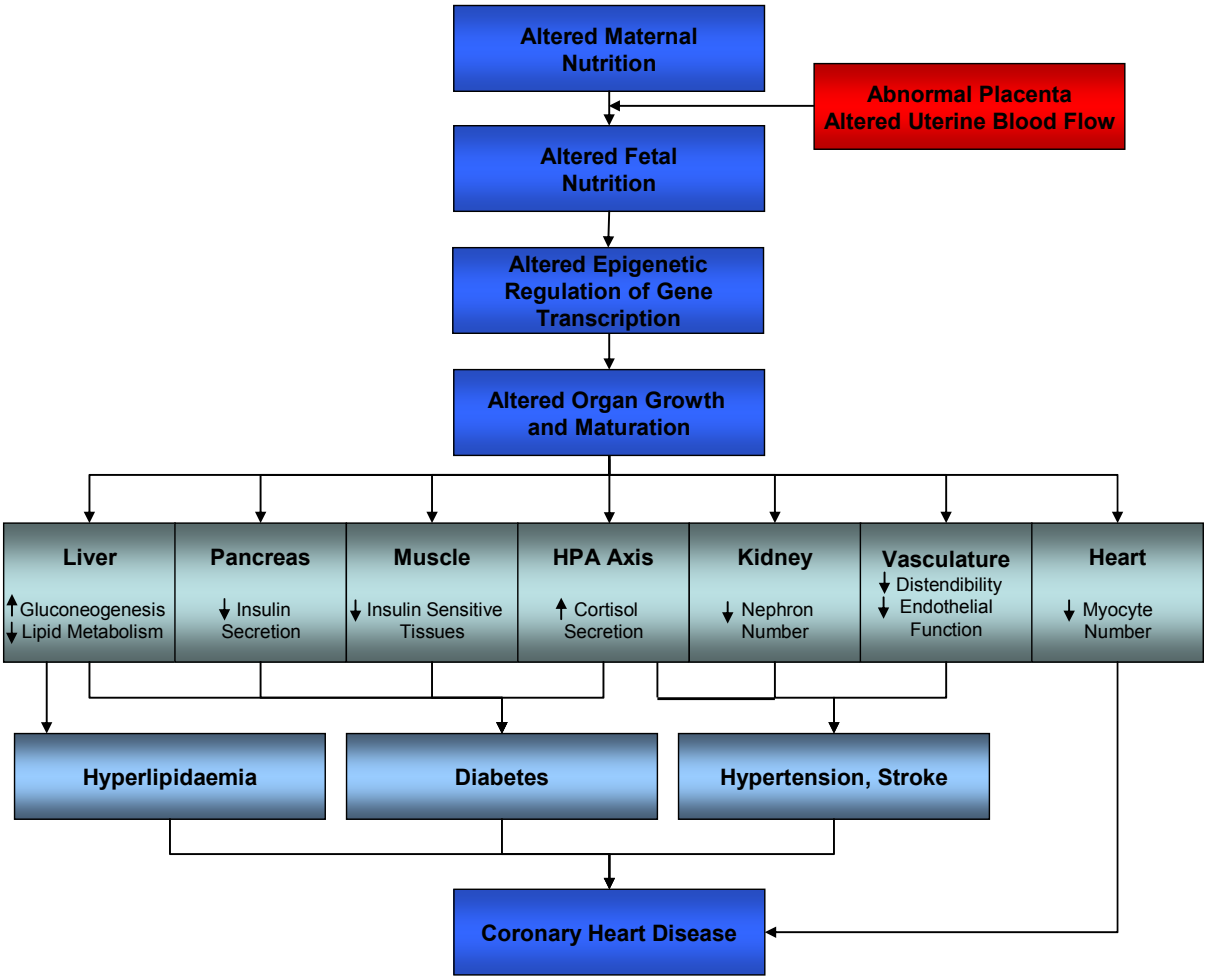


Figure 1.2 The relationship of an altered fetal environment with adult disease. Adapted from [13]. Altered maternal nutrition is reflected in the fetal nutrition. This can induce altered epigenetic regulation of genes which therefore affects gene transcription. The effects of altered gene transcription will depend on the genes affected, but may include, for example, increased expression of genes of gluconeogenesis. This can contribute to the development of diabetes, which is a risk factor for CHD.

1.2.5 Nutritional models of programming

Many different nutritional models of programming have been developed and these can differ in both the content and timing of the altered diet. A variety of different diet options are employed, some of which vary in the overall level of energy, such as undernutrition and overnutrition and some of which differ in the ratio of specific nutrients, such as a low protein or high fat. Furthermore, different postnatal diets, in particular high fat, are often employed to determine the effects of catch up growth at different stages of postnatal development. Diets can also be supplemented with additional nutrients, to determine the effects on disease risk in an attempt to prevent or even reverse the effects of altered nutrition during development. In addition, studies can also differ according to the timing of nutrient manipulation, which enables the effects on differing developing organs and metabolic or neuroendocrine systems to be determined. Two contrasting models of programming include a model of global undernutrition and a well characterised maternal low protein diet (MLP), which program offspring with phenotypes displaying some similarities, but also some differences. Furthermore, models of maternal overnutrition are now being investigated.

1.2.5.1 Maternal global undernutrition

A rat model employing a maternal global undernutrition (30% of *ad libitum*) throughout gestation was developed [8] to produce offspring with a phenotype consistent with the metabolic disturbances of human intrauterine growth retardation (IUGR) [6,25,26,36]. The resulting IUGR offspring were significantly smaller at birth compared to control offspring and adult offspring from dams fed this diet exhibited hyperinsulinemia, hyperleptinemia and increased systolic blood pressure compared to control offspring. Offspring had significantly reduced locomotor behaviour [37], were hyperphagic and consequently developed obesity [8]. Furthermore, all these metabolic abnormalities were augmented by feeding a post weaning hypercaloric diet (30% fat), which supports human epidemiological data [27,28] and indicates the importance of the postnatal environment in exacerbating disease risk. The combination of obesity, hyperphagia and hyperleptinemia indicated that the offspring were leptin resistant, as normally leptin functions to decrease appetite, whilst the hyperinsulinemia indicated insulin resistance [8]. It was hypothesised that the increased insulin and leptin concentrations mediated all the disturbances identified in these rats. Currently, the mechanisms which underlie the alterations to development in these rats have not been determined, for example, no work has been undertaken to identify alterations in the expression of key genes involved in energy balance in metabolic organs, such as adipose or liver tissue.

Similar models of global undernutrition have been utilised, which differ in both the severity and duration of maternal undernutrition; for example, in a model of 50% *ad libitum* maternal undernutrition during the first 2 weeks of gestation, adult female offspring exhibited marked

adiposity at postnatal day (PND) 53 despite similar food intake to controls [38]. A mouse model employing a maternal diet 70% *ad libitum* from 18.5 post conception (p.c.) to delivery, produced offspring that were born small but exhibited rapid catch up growth by PND 10, developed increased adiposity, increased leptin concentrations, impaired glucose metabolism and abnormal lipid profiles compared to controls. These offspring had increased weight gain on a high fat diet compared to controls despite a similar energy intake and therefore did not exhibit hyperphagia [39]. Thus variations on the extent of global undernutrition all seem to induce a similar phenotype prone to obesity and its metabolic disturbances, although some differences in phenotype are apparent. All these features indicate that offspring from these rodent models of global undernutrition have adapted their metabolism to allow for a poor postnatal supply of nutrients by increasing their long term storage of energy and are thus predisposed to obesity upon a nutrient rich or even standard postnatal diet.

1.2.5.2 Maternal low protein diet

The maternal low protein (MLP) diet, in which there is a disproportionately low amount of protein compared to carbohydrate, results in offspring with low birth weight presenting with metabolic disorders in later life including dyslipidemia, impaired glucose homeostasis, insulin resistance, diabetes and hypertension [40,41]. Abnormalities found in the adult offspring of dams fed a MLP diet include increased corticosteroid activity [40] and altered hepatic glucose output [42]. Studies have uncovered that the increased corticosteroid activity may induce the altered hepatic output in these offspring due to increased activity of the rate limiting enzyme of gluconeogenesis phosphoenolpyruvate carboxykinase (PEPCK) and reduced activity of glucokinase (GK), a key enzyme of glycolysis, in the liver of adult offspring [43,44]. The MLP diet also results in reduced islet size and beta-cell proliferation in the pancreas of offspring [45] and an impaired insulin response to glucose when fed a high sugar diet, which predisposes to later glucose intolerance [46]. In agreement with human studies, obesity is heightened in programmed offspring who experience catch up growth [7]. Furthermore, it has been shown that restricting nutrition between conception and implantation followed by a control diet can induce a programmed phenotype characterised by smaller size at birth and hypertension in adult offspring, thus emphasising the sensitivity of the periconceptual period [47]. This confirms that the timing effects of nutrient restriction are important in determining later risk of disease [7] and confirms findings in Dutch Hunger Winter studies [17]. However, the phenotype of MLP offspring can vary according to the exact composition of the diet [48,49]. For example, the Southampton model produces offspring which develop hypertension that persists into adulthood [48], whilst the Hope farm MLP model does not [49]. This indicates that even small changes in similar diets can induce different programming effects and underlines the importance of the balance of nutrients on risk of adult disease [50], which is in agreement with data from the Dutch Hunger Winter [21].

Much work has been undertaken in MLP offspring investigating the mechanisms underlying altered lipid and carbohydrate metabolism and the programming of hypertension. Much of this work has involved looking at gene transcription, as persistent alterations in the expression of genes can provide a link between the environment, alterations in metabolic systems and the adult phenotype [51]. As such, the MLP model has been extensively investigated for alterations in the transcription of genes in the liver, which could be playing a causative role in the altered metabolic systems. This has generally involved a candidate gene approach, in which specific genes are targeted, such as transcription factors and their target genes involved in lipid metabolism and glucose metabolism. As such, much work has focused on the glucocorticoid receptor (GR), peroxisome proliferator activated receptors (PPARs) and their target genes. The role of PPARs in programming of lipid metabolism will be discussed later in this report.

The evident role of glucocorticoids in programming both glucose intolerance and hypertension has instigated interest in determining expression levels of the glucocorticoid receptor (GR) in programmed offspring. Glucocorticoids mediate their action through expression of the GR and glucocorticoid activity is determined by the level of GR expression. Activated GR translocates to the nucleus to control expression of a wide variety of genes involved in glucose metabolism [52], such as PEPCK, GK (involved in glycolysis) and Na/K-ATPase α1- and β1- subunits (involved in sodium reabsorption in the kidney) [52]. In MLP offspring, expression of GR mRNA and protein levels were found to be twice that of controls in the brain, lung, liver and kidney in fetal and neonate tissue and this increased to 3 times that of controls in adult life. Also increased was expression of Na/K-adenosine triphosphate α1- and β1-subunit mRNA. In addition to this, GR expression was reduced in the hypothalamus, thus reducing negative feedback to the hypothalamic-pituitary-adrenal (HPA) axis. As such, alterations in GR expression may result in increased sensitivity to glucocorticoids and consequently increase the expression and activity of glucocorticoid sensitive enzymes in protein restricted offspring [53].

Another factor which controls glucocorticoid activity is the expression of the enzyme 11β hydroxysteroid dehydrogenase type 2 (11βHDS2). This converts cortisol (corticosterone in the rat) into its inactive form, cortisone (11-dehydrocorticosterone in the rat) in aldosterone target tissues and prevents glucocorticoids activating the mineralocorticoid receptor in such tissues [54]. 11βHSD is found in fetal tissues such as lung, adrenal, colon, kidney and is expressed in high levels in the placenta [54]. It plays a crucial role by limiting the amount of maternal glucocorticoids exposed to the fetus in the placenta [55]. In the MLP model, 11βHSD2 expression [52] and activity [41] have been shown to be down-regulated by gestation d20 in the placenta. This reduction in 11βHSD2 activity is thought to play a major role in the reduced growth, hypertension and glucose intolerance found in this model of programming, by permitting maternal glucocorticoids to cross the placenta. The changes in 11βHSD2 and GR expression may therefore both contribute to increased glucocorticoid activity in specific tissues seen in programmed adult offspring [52].

Work is also focusing on global changes in gene transcription, with gene expression arrays increasingly being employed to determine the global expression patterns within tissues. This method enables the identification of specific sets of genes or pathways which have been altered by maternal or postnatal diet. For example, in MLP offspring, an expression array of genes in the placenta identified both upregulated genes, such as negative regulators of cell growth and metabolism and those involved in epigenetic modifications, whilst it also identified genes which were downregulated, including genes of nucleotide metabolism. This implicates both reduced growth and epigenetic alterations the placenta of PR offspring [56].

Most work in MLP offspring on energy metabolism has centred on the liver and little on adipose tissue despite the wealth of data implicating an increased risk of obesity and altered lipid profiles [32,57]. However, one study [58] has used an expression array to determine alterations in gene expression in adipose tissue in offspring of rats fed a protein restricted diet throughout pregnancy and lactation. These offspring develop increased visceral adiposity in adult life, are insulin resistant, diabetic and are hypertensive in adult life compared to controls. The array identified that the maternal diet altered gene expression of several genes associated with carbohydrate metabolism, adipocyte differentiation and lipid metabolism, such as upregulated genes of lipogenesis (fatty acid synthase (FAS) and stearoyl-CoA desaturase) and adipocyte differentiation (CCAAT-enhancer-binding protein β) [58]. Contrastingly, another study has failed to identify any changes in the ability of preadipocytes isolated from MLP rats to store fat or divide [59]. This is, however, in agreement with data from the Dutch Hunger Winter study [6,17], which implies that changes seen in fat storage are not due to a programming effect on the adipocytes directly, but due to neuroendocrine changes [59].

1.2.5.3 Maternal and postnatal high fat diet

Human epidemiological studies indicate that maternal obesity can induce an increased risk of chronic disease in offspring. As such, animal models have been developed employing maternal overfeeding or obesity to induce an altered phenotype in the offspring. The findings in these animal models support human data, for example, offspring from mice fed a high fat before and during pregnancy and lactation, which are fed a high fat diet from weaning, have an increased risk of non alcoholic fatty liver disease and obesity [31]. At 7 weeks, offspring were heavier than controls despite similar calorie intake, whilst at 15 weeks abdominal fat mass was increased compared to control groups. Furthermore, offspring from dams fed a HF diet, which were fed a HF postnatal diet or chow diet had increased expression of genes of fatty acid synthesis, such as sterol regulatory element binding transcription factor 1c (SREBP1c), Fas and ATP citrate lyase (Acl). They also had increased expression of genes involved in TAG synthesis, such as diacylglycerol O-acyltransferase (Dgat1). The effects on gene expression were exacerbated by the high fat diet.

Given that these genes were also affected in the chow offspring, it follows that these gene expression profiles were set up prior to the commencement of the postnatal diet, therefore indicating a persistent effect on gene expression. This study indicates that high fat nutrition during development and postweaning life exacerbates the effects of developmental HF feeding alone and thus contradicts the PAR hypothesis [31]. In another study, mice fed an obesegenic diet before conception and during pregnancy and lactation, resulted in adult offspring which were hyperphagic, had decreased locomotor activity, increased adiposity, hypertension and diabetes indicates altered metabolic and cardiovascular function [60]. Therefore, both an excess and lack of nutrients during the early stages of development result in adaptations which result in a similar phenotype, which favours obesity.

1.2.6 Programming by excess glucocorticoid exposure

IUGR infants have been shown to have elevated placental cortisol levels [61] and glucocorticoids have been shown to have important roles in regulating fetal growth, promoting gluconeogenesis and increasing blood pressure [52], all of which are altered in programmed humans and rats. Normally, exposure to glucocorticoids occurs during late gestation [53]. It has been shown that administration of excess glucocorticoids using dexamethasone (dex) during late gestation, results in a similar adult phenotype to the nutrition models i.e. reduced size at birth, hyperglycaemia and hyperinsulinemia offspring [62]. This indicates that programming by undernutrition and glucocorticoid exposure may involve similar mechanisms, mediated in part by glucocorticoids.

In agreement with the MLP model, the offspring of rats administered dex during last week of pregnancy, have increased expression of GR and PEPCK mRNA and activity of PEPCK in the liver, thus leading to glucose intolerance [62]. These alterations in gene expression are not seen if the dex administration was during the first stages of gestation [62], in keeping with observations from the Dutch Hunger Winter of impaired glucose tolerance in individuals exposed to the famine during late gestation [20]. These data could imply that exposure to glucocorticoids, be it due to maternal undernutrition/stress or glucocorticoid administration in utero, are responsible for impaired glucose tolerance in later life and that this is mediated by increased GR expression and reductions in 11βHSD2 expression in the placenta [52]. This in turn results in over expression of target genes, including PEPCK [62], which may contribute to altered glucose metabolism.

1.3 PPARα, leptin and their role in developmental programming

Evidence from human studies and animal models has clearly indicated that programmed offspring have an increased risk of developing dyslipidemia and obesity in adult life. Two factors which play key roles in the regulation of energy balance are that of the nuclear hormone receptor PPARα and the adipokine leptin. Evidence is emerging identifying alterations in PPARα and leptin regulation during development, which may account for some of the mechanisms underlying adult disease in programmed offspring, whilst leptin may also be employed as a potential mechanism for reversing disease. Given the emphasis on PPARα and leptin in this report as a whole, the following sections will discuss their regulation, function and signalling. Furthermore evidence of their role in development and their alteration in programmed offspring will be discussed.

1.4 Peroxisome proliferator activated receptor alpha

1.4.1 Nutritional programming and peroxisome proliferator activated receptors

The finding that developmental programming can result in dyslipidemia in both humans [18] and animal models [57], implies that regulatory factors and/or key genes involved in lipid metabolism may be altered by environmental constraints during development, resulting in an altered metabolism and an inability to maintain lipid homeostasis in later life. As a result of this, interest has developed in the potential role of peroxisome proliferator activated receptors (PPARs), a family of nuclear hormone receptors which control the expression of a variety of key genes involved in lipid metabolism, including those involved with transport, cellular uptake, catabolism and storage of fatty acids. They include PPARα, PPARγ2 and PPARβ.

1.4.2 PPAR functions

Knock out mice which lack functional PPARα protein were initially developed to study PPARα function. These mice were found to be viable, of healthy appearance and fertile, thus arguing strongly against a role for PPARα in development [63]. However, it was found that they were unable to respond to peroxisome proliferators, failed to induce several key enzymes of β-oxidation and subsequently accumulate lipid in the liver, thus implicating PPARα as a key mediator of lipid metabolism [63]. Consequently, these mice have helped to confirm the role of PPARα in controlling the transcriptional regulation of many genes involved in lipid homeostasis, specifically genes involved in mitochondrial β-oxidation, peroxisomal β-oxidation, uptake of fatty acids and lipoprotein metabolism [64-66]. Furthermore, the role of PPARα in inflammation has been discovered using these animal models [67]. Contrastingly, PPARγ knock out mice die at E10, as it interferes

with the development of the trophoblast, indicating a crucial role for PPARγ in development [68]. However, adipose specific disruption of PPARγ results in mice with reduced white adipose tissue (WAT) mass due to the presence of hypertrophic adipocytes in reduced numbers. These mice also have reduced plasma leptin, increased plasma free fatty acids and triglycerides, have a fatty liver and are insulin resistant [69].

It has since been established that PPARα and PPARγ have key roles in directing either the oxidation or storage of fatty acids to maintain plasma lipid profiles. PPARα functions to promote fatty acid uptake and β-oxidation in liver [70], by regulating the expression of key rate limiting enzymes of fatty acid oxidation, including acyl-coA oxidase (AOX) [71], which catalyses the rate limiting step of peroxisomal β-oxidation and carnitine palmitoyl transferase-1 (CPT-1) [72], an enzyme which controls the transport of fatty acids into mitochondria for oxidation. PPARγ on the other hand promotes adipocyte differentiation, the uptake of fatty acids and their storage in adipose tissue [73]. PPARγ achieves this by up regulating the expression of genes such as lipoprotein lipase (LPL), which hydrolyses lipids in lipoproteins to release fatty acids for uptake [74], PEPCK, which is involved in glycerolneogenesis and stearoyl-CoA desaturase-1 (SCD-1), which catalyzes the rate-limiting step in the synthesis of monounsaturated fatty acids. Interestingly, PPARγ has been shown to mediate the actions of the insulin sensitizing thiazolidinediones (TZD's). These actions include increasing the lipid storing potential of adipocytes to protect against lipotoxicity of liver and muscle, increasing the expression of insulin sensitising adipokines such as adiponectin and inhibiting the expression of insulin desensitizing adipokines such as TNFα [75]. Less is known about the functions of PPARβ, however it appears to have roles in fatty acid catabolism, adaptive thermogenesis and embryo implantation [76].

1.4.3 PPAR expression in programmed offspring

Interestingly, a study has revealed that the expression of two of these PPARs is altered in the liver and adipose tissue of offspring from dams fed a low protein diet throughout gestation [57]. PPARα expression was found to be up regulated by 69% in the liver, whereas PPARγ2 expression was shown to be 59% lower in the adipose tissue of these offspring and this was associated with increased plasma triacylglycerol (TAG) and nonesterified fatty acid (NEFA) concentrations [57]. Since this initial study, altered PPAR expression has been repeatedly found in similar models of programming [77-79]. Thus, given the functions of these proteins, reduced PPARγ expression implies reduced fatty acid uptake and TAG synthesis for storage within adipocytes, whilst increased hepatic PPARα expression would lead to increased fatty acid uptake and β-oxidation in the liver. This implies a causative role for both PPARα and PPARγ in the dyslipidemia found in these programmed offspring.

1.4.4 PPAR expression during development and in the adult

The 3 PPAR isotypes possess distinct expression patterns during development and in the adult, which is consistent with their physiological function [80,81]. In the rat expression of PPARα is detected late in development around day E13.5 in all the tissues in which it will be expressed in adult life. In the adult, expression of PPARα is consistent with tissues required for fatty acid oxidation i.e. the liver, heart, kidney, intestine and skeletal muscle [80]. Similar expression profiles are found for PPARα in developing and adult mice [82], whilst in humans, PPARα is predominantly expressed in the liver kidney, heart, skeletal muscle and large intestine [83]. Expression of PPARγ in the developing embryo can be detected in brown adipose tissue from E18.5 when tissues are beginning to form. In agreement with its role in lipid storage, PPARγ expression in adult rodents is predominantly in adipose tissue, however it is also found in brown adipose tissue, the intestine, liver and lymphoid tissues [80]. In humans, PPARγ expression is found in adipose tissue, liver and heart [84]. PPARβ is expressed from an early stage in the developing embryo with expression as early as E8.5 peaking at E13.5 then decreasing to adult levels [81]. In contrast to PPARα and PPARγ, the expression of PPARβ is ubiquitous, with transcripts present in most tissues in the adult, often at high levels with particular abundance in the NS [80]. In humans, PPARβ also has a ubiquitous expression, with high expression in the placenta [85].

The expression of PPARs is therefore initiated at a time of intense tissue differentiation in the developing embryo, making each isoform susceptible to the effects of maternal undernutrition during pregnancy. It is therefore possible that maternal undernutrition could somehow cause a reprogramming of PPAR regulation, which impacts on their control of lipid homeostasis in postnatal life leading to dyslipidemia.

1.4.5 Regulation of PPAR expression

To understand how PPARs may be involved in the mechanisms underlying programming it is important to understand the factors dictating their regulation. Given the emphasis on PPARα in this report, a detailed focus is therefore directed predominantly to this specific isoform.

One mechanism which could account for the increased expression of PPARα in programmed offspring, is an increase upstream signaling mechanisms. Several diverse factors have been found to alter the expression of PPARα, including; positive regulation by glucocorticoids, peroxisome proliferators, fatty acids and other nuclear hormone receptors and negative regulation by insulin, growth hormone and the cytokine interleukin 6 (IL6). In addition, PPARα has also been shown to positively regulate its own expression. Interestingly, several of these factors have been found to be altered by maternal undernutrition and thus provide a plausible mechanism for altered PPARα

expression in these offspring. However, in most cases, the exact mechanisms underlying the regulation of the PPARα promoter have not been studied.

The effects of glucocorticoids on PPARα expression are well documented. In primary rat hepatocytes, mRNA levels of PPARα are increased by both natural and synthetic glucocorticoids and this increase is prevented upon use of a glucocorticoid antagonist [86]. In conjunction with the finding that the mineralocorticoid aldosterone has no effect on PPARα expression, this implies that the effects of glucocorticoids on PPARα expression are mediated specifically by GR, not the mineralocorticoid receptor. Furthermore, the use of the protein synthesis inhibitor cyclohexamide and an inhibitor of RNA synthesis actinomycin D, indicate that the effects on PPARα mRNA levels are at the transcriptional level of control, using pre-existing GR protein [86]. The stimulatory effect of the synthetic glucocorticoid dex on PPARα expression has also been confirmed in vivo in male rats [87]. Furthermore, dex induction of PPARα is inhibited if insulin is added at the same time and conversely increased if fatty acids are added to the dex [87]. Another group has shown that hepatic PPARα expression is rapidly increased in the rat after immobolisation stress. It was demonstrated that glucocorticiods mediated this response as specific antagonists inhibited their action [88].

All these results clearly indicate that glucocorticoids play a key role in the regulation of PPARα expression in rodents, although the exact mechanisms are unknown. Given that both glucocorticoids [40] and hepatic GR [77] are increased in programmed rodents, they therefore provide a mechanism which could account for the induction of hepatic PPARα expression and thus a means of linking glucocorticoids with altered lipid metabolism in protein restricted offspring. In addition, reduced actions of insulin in the programmed offspring as a result of insulin resistance could also contribute to the increased levels of PPARα mRNA.

PPARα has been found to exhibit diurnal rhythmic expression. It was originally proposed that glucocorticoids mediated this effect, as PPARα mRNA levels follow normal diurnal variations in corticosterone at both the mRNA and protein level [88]. However, the use of adrenalectomised mice has shown that this circadian rhythm of PPARα is not due to glucocorticoids, but instead mediated by CLOCK, as the circadian rhythm of PPARα expression in the liver was absent in CLOCK mutant mice. This was further verified by showing direct activation of PPARα by CLOCK via an EBOX located within the second intron of the PPARα gene [89].

It is well established that PPARα expression is induced by its own ligands, i.e. fatty acids and peroxisome proliferators. For example, treatment of rats with the strong peroxisome proliferator fenofibrate results in increased PPARα at both the mRNA and protein levels [90]. Using a rat hepatoma cell line, it was shown that oleic acid and the peroxisome proliferators perfluorodecanoid acid (PFDA) and Wy14,643 all cause a rapid increase in PPARα mRNA. Treating cells with actinomycin D before Wy14,653 prevented the induction of PPARα, indicating that effects were at

the transcriptional level [91]. Furthermore, the use of cyclohexamide did not prevent upregulation by Wy14,643, indicating therefore that the upregulation was independent of protein synthesis and thus PPARα mediated. Consequently, this is thought to be a form of positive autoregulation of transcription by PPARα. This is a common feature of nuclear hormone receptors and has been shown for the androgen receptor [92], thyroid receptor β1 and retinoic acid receptor β2 [93].

Interestingly, it has been shown that hPPARα can regulate its own expression through an alternative nuclear hormone response element, namely the hepatocyte nuclear factor-4 response element (HNF-4) instead of via a PPRE [94]. In addition to positive autoregulation, PPARα expression can also be regulated by other nuclear hormone receptors, including negative regulation by chicken ovalbumin upstream promoter-transcription factor II (coup TFII) [94] and positive regulation by a key mediator of lipid metabolism, HNF-4, via a HNF-4RE within the human PPARα promoter [94]. This effect has been confirmed using an HNF-4 overexpression microarray [95] and an HNF-4 knock out mouse model [96].

Under normal conditions, PPARα maintains lipid homeostasis by controlling fatty acid uptake and peroxisomal β-oxidation in tissues such as the liver. As glucocorticoids and fatty acids play key roles in regulating the expression and activation of PPARα, it stands to reason that the expression of PPARα is altered under conditions of stress and altered nutrition, such as found in programmed offspring, fasting or consuming a high fat diet. Thus, under conditions of stress, the hypothalamic-pituitary-adrenal axis (HPA axis) is activated, resulting in the release of glucocorticoids from the adrenal cortex, which functions to mobilize energy. In addition, activation of the sympathetic nervous system results in the release of catecholamines which stimulate fatty acid mobilization from adipose tissue [88]. The increased glucocorticoid thus induces PPARα in the liver to increase expression of enzymes involved in fatty acid β-oxidation. Consequently, it has been shown in the fasting state that PPARα is needed in order to provide energy from released fatty acids from adipose tissue, as PPARα null mice which have been fasted for 24hr exhibit lipid accumulation in the liver due impaired β-oxidation of fatty acids [97]. Likewise PPARα knockout mice fed a high fat diet exhibit a similar phenotype [97].

In contrast to dex, fatty acids and CLOCK, which all act in a positive manner on PPARα expression, growth hormone (GH) exposure has been shown to cause a 50% reduction in PPARα basal levels in primary rat hepatocytes [98]. Furthermore, regulation of PPARα in inflammation by C/EBP has been shown to be involved in the transcriptional repression of human PPARα by the cytokine Il-6 in HepG2 cells as part of its role in mediating the acute phase response [99].

Given the role of PPARγ in adipose differentiation and fatty acid storage, it is unsurprising that like PPARα, the expression of PPARγ is controlled by both hormonal and nutritional status. As such, it is up regulated in the presence of a high fat diet [100], obesity, insulin and glucocorticoids [101] and

reduced upon fasting and insulin resistance. Thus both PPARα and PPARγ are; controlled by insulin and glucocorticoids, are sensitive to alterations in environmental stimuli, such as availability of food and stress and all these have all been shown to be affected in offspring programmed by maternal nutrition.

1.4.6 The structure and activation of PPARs

In order for PPARs to regulate the transcription of downstream target genes, they must first be activated. Activation of PPAR and target genes is mediated by specific structural domains, which share a similar structure to that of other members of this nuclear hormone receptor superfamily. PPARs are composed of 3 domains derived from 6 translated exons; 1 exon encodes an NH_3-terminal A/B domain, 2 exons encode a highly conserved DNA binding domain (DBD), one encodes a hinge region and finally two exons encode the ligand binding domain (LBD) [102,103].

The NH terminal domain possesses little sequence identity between the PPAR isotypes and is considered to be the domain responsible for the various PPAR functions. This domain can function as a ligand independent activation domain and can be subjected to post translational modifications which alter PPAR activity, such as phosphorylation [104].

In keeping with their central role in lipid metabolism, PPARs act as lipid sensors activated by the very molecules whose fate they control. The natural ligands of PPARs are therefore fatty acids and their derivatives, which activate PPARs via the LBD. The LBD is not as conserved as the DBD between isotypes, so whilst some ligands are able to activate all 3 PPAR isoforms to varying degrees, many are isotype specific. Medium and long chain unsaturated fatty acids such as such as arachidonic acid (C20:4) and linoleic acid (C18:2) can activate all 3 PPAR isoforms, whereas saturated fatty acids such as lauric acid (C12:0) are poor activators of PPAR generally [105]. Derivatives of fatty acids which can activate PPARs include eicosanoids (derived from arachidonic acid) which can form both prostaglandins and leukotreines. These can be specific to particular isotypes of PPAR, for example the prostaglandin15-Deoxy-Δ12,14-PGJ2 is specific to PPARγ and Leukotriene B4 (LTB4), which is involved in inflammation is a specific ligand for PPARα [106]. Synthetic PPAR ligands have also been identified and for PPARα these were identified as chemicals which cause peroxisome proliferation in the rat liver [107]. This process results in increased peroxisome size and number, induction of peroxisomal and non peroxisomal enzymes and consequently increased peroxisomal β oxidation. Such chemicals include fibrates (hypolipidemic drugs), herbicides and phthalate plasticizers. For PPARα, activators include the hypolipidemic agents fenofibrate [90], clofibric acid (CFA) and Wy-14643 [105]. Synthetic activators of PPARγ include the hypoglycaemic agents thiazolidinediones (TZD), which include the antidiabetic drugs rosiglitazone and troglitazone.

The PPAR DBD is highly conserved in PPARs. It is composed of 2 zinc fingers, each encoded by one exon. These zinc fingers recognise and bind to a specific hexanucleotide sequence (AGGTCA) in target gene promoters. However, as with other nuclear receptors, binding to the specific DNA response element requires binding as dimers and in the case of PPAR, binding is as a heterodimer with the 9-*cis*-retinoic acid receptor (RXR) [108]. Consequently, PPAR:RXR has been shown to bind a DR1 element [109], which is composed of 'a direct repeat of 2 core recognition motifs AGGTCA spaced by 1 nucleotide' [108]. However, other steroid receptors including thyroid hormone receptor (TR), all-*trans*-retinoic acid receptor (RAR) and vitamin D receptor (VDR) are able to heterodimerise with RXR and bind to DR1 elements and thus may compete with PPARs. However, the peroxisome proliferator response element (PPRE) definition as a DR1 was further defined to have 3 main properties which discriminate it from other DR1 response elements; firstly an imperfect core DR1 sequence, adenylate as the spacer nucleotide and finally an extended 5' half site (AACT). In addition to this, it has been shown that PPAR binds the 5' extended half site and hexamer, whilst RXR binds the 3' hexamer. It is these characteristics that permit selectivity for PPAR:RXR binding as opposed to other RXR heterodimers [110]. Thus the consensus sequence of PPRE is 5'-AACTAGGNCAAAGGTCA-3'.

As PPARs are often co-expressed in the same tissues [80] and can be activated by the same ligands, other factors must determine isotype selectivity. PPREs can be strong, intermediate or weak and it is the conformity of consensus and the 5' extension which aids isotype selectivity by determining which PPAR can bind a given PPRE. For example, an unconserved 5' extension is unimportant for PPARγ binding and it can therefore bind strongly to weak PPREs. On the other hand, PPARα binding requires a conserved 5' extension, i.e. a strong PPRE. A second level of isoform selectivity is determined by the RXR binding partner and PPAR's can heterodimer with RXRα, RXRβ and RXRγ. Weak PPREs to which PPARγ can preferentially bind, favour RXRα binding and strong PPREs to which PPARα can bind favour RXRγ binding. Therefore, ability of a given PPAR isotype to bind a PPRE depends on both the DNA sequence of PPRE and the RXR isoform present in that cell type [108]. However, generally the differing RXR isoforms are all expressed in the tissues in which PPARs are expressed [80].

In the inactive state, PPARα:RXR heterodimers are bound to co-repressors such as the nuclear co-repressor (N-CoR). Ligand binding induces a conformational change within the LBD which causes co-repressors to disassociate and allows PPARα to interact with co-activator proteins, such as steroid receptor co-activator-1 (SRC1), CREB binding protein (CBP)/p300 and PPAR Gamma co-activator 1 (PGC-1) [111]. These co-activator proteins are able to modify chromatin structure due to their intrinsic histone acetylation activity and thus direct transcription of target genes (figure 1.3).

PPAR activation of target genes and thus impact on lipid metabolism may therefore be affected by several factors involved in the regulation and activation of PPARα, some of which have been found

to be altered in animal models of programming; such as regulation of mRNA transcription by hormonal factors such as glucocorticoids and the availability of ligand by stress and diet. In addition to this, the presence of accessory molecules (repressors and activators), RXR isoforms, of competitors for RXR binding and finally posttranslational modifications, such as phosphorylation may also influence PPARα activity. It is possible that these factors may be altered in programmed offspring, but this is currently unknown. However, as PPAR target genes have also been shown to be upregulated alongside increased PPARα in programmed offspring [57], it is probable that the alterations to PPARα expression alone are responsible and not those affecting activity, such as availability of accessory proteins and binding partner. The influence of epigenetic factors on transcription of PPARs is discussed later in this report.

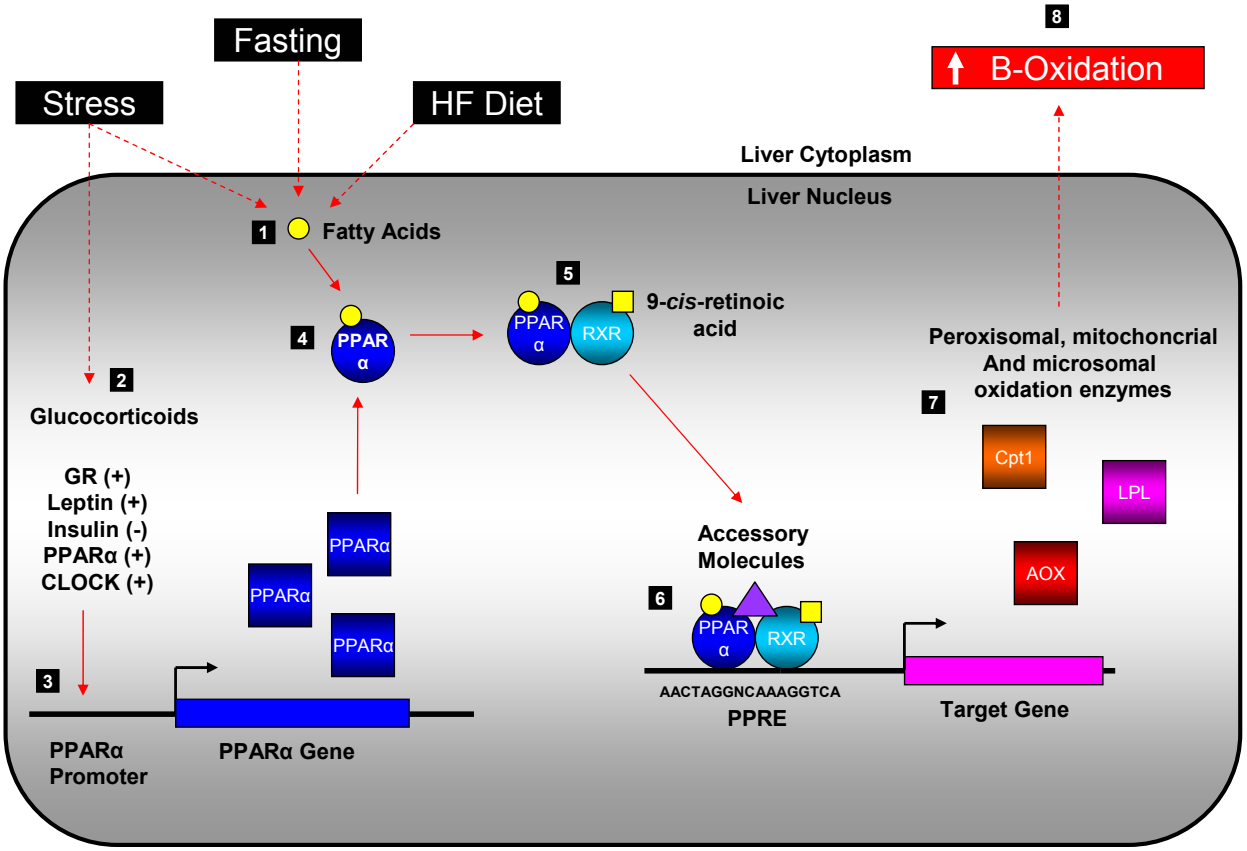


Figure 1.3 Mechanisms of PPARα expression and activation. **1)** Stress, for example due to maternal undernutrition, fasting or a high fat diet, brings about in an increase in plasma free fatty acids. **2)** Stress results in increased glucocorticoid levels and consequently GR expression. **3)** Several factors are known to upregulate (+) or downregulate (-) PPARα gene expression by interacting with as yet undetermined response elements within the promoter region, resulting in altered levels of PPARα protein within the nucleus. **4, 5)** Fatty acids bind to the LBD to activate PPARα and induce a conformational change in LBD. **6)** Accessory proteins bind which facilitate an active chromatin conformation. **7)** Altered transcription of PPARα target genes, e.g. AOX. **8)** The end result is altered lipid metabolism, e.g. an increased rate of fatty acid peroxisomal and mitochondrial β-oxidation.

1.5 Leptin

1.5.1 Leptin function in bodyweight regulation

Over the past 14 years, evidence has accumulated implicating a central role for the hormone leptin in the regulation of bodyweight and studies using both ob/ob and db/db mice have been central to understanding this role. ob/ob mice have a complete lack of leptin, they are infertile, obese and present with hyperinsulinemia and hyperglycemia. However, when these mice are administered leptin, glucose and insulin concentrations are normalised and they lose weight as a result of a reduction in food intake and increased energy expenditure [112,113]. Similarly, the db/db mouse is obese and diabetic and in this model, mutations in the leptin receptor render it non-functional. Therefore an inability to propagate the leptin signal is responsible for the adverse phenotype in the db/db mouse [114]. Thus it has emerged that leptin is an anti obesity hormone, which is involved in the regulation of bodyweight and energy expenditure [112,113].

Central and peripheral administration of leptin to both ob/ob and db/db/ mice have revealed that leptin exerts its effects on feeding and energy balance by acting through leptin receptor signalling on the hypothalamus [115]. The hypothalamus acts as the feeding centre of the central nervous system, which receives signals from the periphery relating to the nutritional, energy and environmental status via satiety and adiposity signals. Leptin is an adipokine produced mainly in adipose tissue [116] and leptin concentrations correlate with adipose tissue mass; the more adipose tissue present, as occurs in obesity, the higher the circulating leptin levels [117]. Therefore the circulating leptin acts as a peripheral sensor of fat mass and signals to the hypothalamus the nutritional status of the body. Leptin prevents the excess storage of adipose tissue by acting on long form receptors within the arcuate nucleus of the hypothalamus (ARH) [118,119]. These receptors act predominantly by a janus kinase / signal transducer and activator of transcription (Jak/Stat) pathway and alter activity within these neurons which relay the signal to reduce food intake and increase energy expenditure (figure 1.4) [112]. However, leptin receptors (LR) are also found in peripheral tissues including adipose tissue and pancreatic β cells, indicating that they are also direct targets of leptin [120,121]. In both humans and rodents, leptin levels are often elevated in obesity indicating they have become leptin resistant [117,122]. This is thought to be due to either receptor or post receptor defects which prevent the transmission of the leptin signal, or the inability of leptin to be transported across the blood brain barrier.

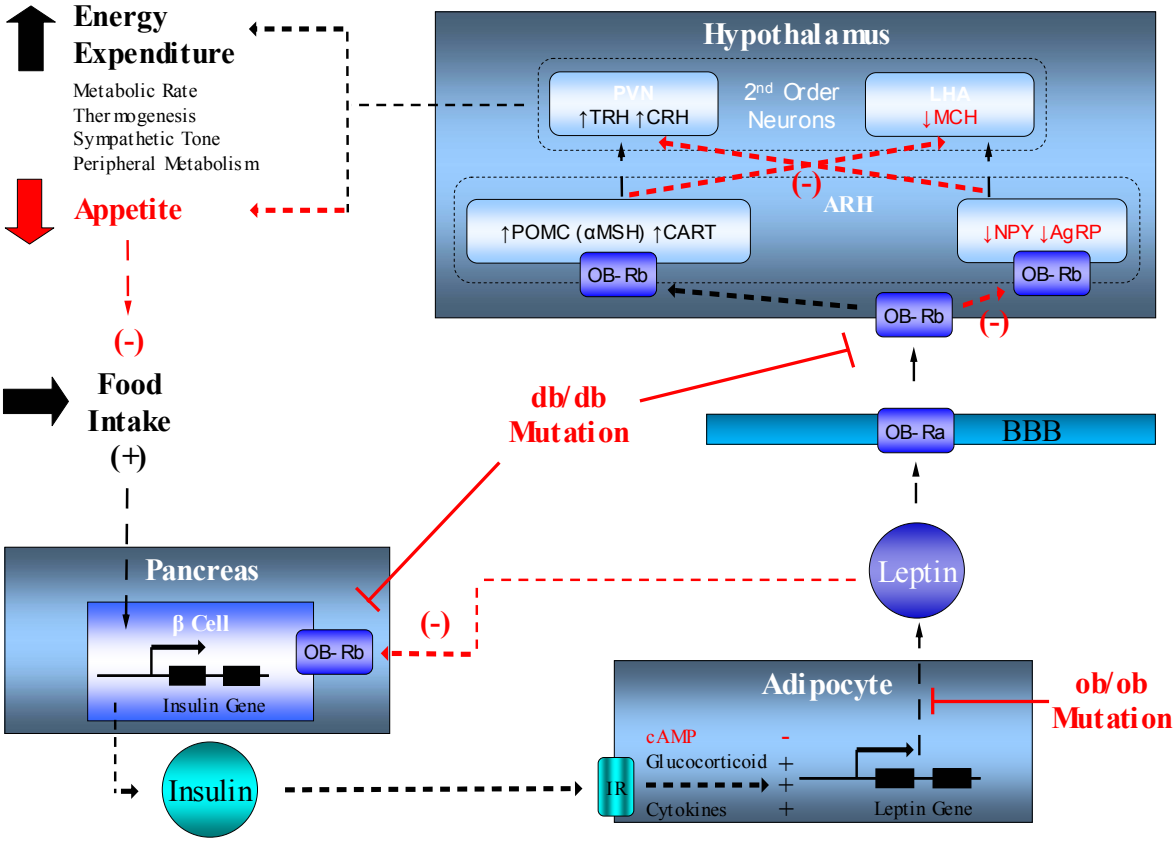


Figure 1.4 Central and peripheral actions of leptin on energy balance. Leptin is able transverse the BBB via a transport mechanism using the short form of the leptin receptor. Leptin induces a negative energy balance by acting on leptin receptors on two populations of neurones within the ARH [123]; the pro-opiomelanocortin (POMC) and cocaine and amphetamine-related transcript (CART) containing neurons and the neuropeptide Y (NPY) and agouti related protein (AgRp) containing neurons. Leptin induces an increase in the expression of CART and POMC which inhibit appetite (anorexigenic) and decreases the expression of the appetite enhancing peptides NPY [123] and AgRP (orexigenic) [124]. Therefore in the absence of leptin, the appetite enhancing peptides dominate. These first order neurons relay the leptin signal to second order neurons involved in bodyweight regulation, including the paraventricular nucleus (PVN), the lateral hypothalamic area (LHA) and the dorsomedial hypothalamic nucleus (DMH) [124]. For example, alpha-melanocyte stimulating hormone (α-MSH) is a melanocortin derived from the cleavage of POMC, which activates MC3/4 receptors in PVN to relay its inhibitory effect on feeding by inducing thyrotropin releasing hormone (TRH) and corticotrophin releasing hormone (CRH) [123]. AgRP blocks effect of αMSH on MC4 receptors resulting in increased feeding, therefore the balance of the two peptides determines the overall outcome. NPY acts on Y1/5R in the second order neurons to exert its effects on appetite [2]. NPY and AgRP suppress sympathetic output to decrease energy expenditure, whereas αMSH stimulate sympathetic output. This is mediated by the effects of catecholamines on adrenergic receptors of peripheral cells.

1.5.2 Leptin, insulin and the adipoinsular axis

Given their important roles in maintaining an energy balance, it is not surprising that a close relationship exists between leptin and insulin. Adipocytes possess insulin receptors on their surface, whilst pancreatic β cells express the functional long form of the leptin receptor [125]. A feedback loop exists between leptin produced by adipocytes and insulin produced by pancreatic islets and this interaction has been termed the adipoinsular axis (figure 1.4) [120]. Within this feedback loop, leptin expression is induced by the action of insulin on adipocytes to increase fat mass as part of its anabolic role [126,127] and increasing leptin levels in response to this signal suppresses insulin secretion from β cells in a form of negative feedback [125,128]. In addition, leptin levels also follow that of insulin in response to food intake; fasting normally results in reduced leptin mRNA which permits feeding, whilst food intake increases leptin mRNA to inhibit feeding. Furthermore, fasting rats given an insulin injection have increased leptin mRNA, indicating that the increased leptin found after food intake is due to the action of insulin on adipocyte [126]. Thus the adipoinsular axis is thought to maintain a set point for adipose stores, i.e. if adipose stores are low, reduced leptin facilitates increased insulin synthesis and secretion, whilst if adipose stores are high, the increased leptin synthesis exerts a negative feedback on insulin secretion. Thus alterations in the levels either of these hormones as occurs in programmed offspring may influence the activity of the other. For example, it has been proposed that the hyperinsulinemia identified in programmed offspring may be due to dysregulation of this adipoinsular axis as a result of leptin resistance [120].

1.5.3 Leptin in early development

It is well known that IUGR induced by maternal undernutrition is associated with an increased risk of obesity in later life in both humans and rodents [8,22,28,39]. As leptin plays a key role in the regulation of bodyweight, this could indicate altered leptin function in IUGR offspring. In support of this hypothesis, expression profiles indicate that leptin is expressed throughout gestation and early postnatal life and in addition, evidence suggests that leptin plays an important role in early growth and development of both rodents and humans throughout this important time. Furthermore, alterations in leptin levels have been identified in both human and rodent IUGR offspring. Interestingly, administration of leptin during specific phases of early development has been shown to have beneficial effects on the regulation of bodyweight in adult offspring. Consequently, these studies all indicate that alterations in leptin levels during early development could therefore play a key role in the mechanisms underlying fetal programming of obesity and its associated metabolic disturbances.

1.5.3.1 Leptin concentrations in early development in humans

The importance of leptin function during early development in humans can be inferred by its expression profile in both the placenta and fetal tissues. In the human placenta, leptin enhancer elements specific to the placenta enhance the transcription of leptin [129]. This source probably contributes greatly to fetal leptin levels during gestation, as the high leptin levels in the offspring drop after birth [130]. Furthermore, placental leptin may contribute to maternal levels during gestation, as maternal fat mass does not correlate with leptin during pregnancy. It is thought that this placental source of leptin may function to mobilise fat to sustain growth of the fetus [131]. Serum leptin can be detected from 18 weeks gestation, a time which coincides with the start of adipose development [132]. At 34 weeks gestation, leptin levels rise dramatically, a period of exponential adipose development [132] after which levels drop around birth. This large increase in maternal leptin alongside an increased requirement for nutrients indicates either maternal leptin resistance or an alternative and unknown role. Leptin is also expressed in the mammary epithelium and therefore leptin is found in human breast milk [133]. Furthermore it is found at levels associated with maternal plasma leptin levels and thus reflects maternal obesity [134]. Interestingly, epidemiological studies indicate that breast feeding may be associated with a reduced risk of obesity in later life [135] and this may be in part due to the presence of leptin in breast milk.

Leptin levels in the cord blood of newborns correlate positively with birth weight [136,137]. IUGR newborns therefore have significantly reduced serum leptin compared to normal and this correlates with fat mass [132,138]. This indicates that fetal growth relates to circulating leptin levels [137]. However, other studies have shown that leptin levels are associated with reduced placental leptin, as leptin levels drop after birth [130]. Interestingly, although leptin is reduced in IUGR offspring at birth, in later life these individuals develop hyperleptinemia compared to normal birth weight individuals of the same obesity [139], i.e. low birth weight individuals have higher leptin levels in adulthood than would be expected by their obesity, implying some leptin resistance or adipocyte dysfunction [139].

1.5.3.2 Leptin concentrations in early development in rodents

Similarly to humans, both leptin and leptin receptor have been identified in murine fetal tissues and the placenta, indicating a key role for leptin in growth and development [140]. For example, administration of leptin to pregnant rats in the second trimester of gestation results in reduced skeletal muscle growth and adipose tissue mass in adult offspring, which is unrelated to altered food intake [141]. In addition, fetal pancreatic islet cells are stimulated to proliferate by leptin, indicating that islet cell mass may be partly determined by leptin at birth [142]. Leptin is also present in the maternal milk, indicating that maternal milk is important for neonatal leptin concentrations

[133]. Furthermore, the placenta may also have a role in leptin transport as it expresses a short isoform of the leptin receptor [143]. Interestingly, expression of this leptin receptor is markedly upregulated in the 3rd trimester of gestation in the rat and accordingly placental passage of leptin from the dam to the fetus increases ten fold during this time. This is therefore a significant source of leptin for the fetus [143]. Thus rodents experience a similar increase in leptin during the last stage of pregnancy as has been identified in humans.

Interestingly, dex induced growth retardation in the rat prevents the increase in transplacental leptin passage [143]. This indicates that programmed offspring which exhibit upregulated glucocorticoids [40] may be hypoleptinemic at birth due to reduced passage of leptin from the mother to the fetus. In agreement with this, IUGR induced by maternal glucocorticoids in the 3rd trimester has been found to cause fetal hypoleptinemia, although in this study, this was due to reduced placental expression of leptin. Placental transport of leptin was not measured. These offspring like human IUGR offspring, went on to develop hyperleptinemia, hyperinsulinemia and developed hypertension in later life [144]. Hypoleptinemia in IUGR rodents agrees with findings in humans [132]. It is possible that some of the metabolic and physiological disturbances which occur during fetal programming may be in part due to this hypoleptinemia [122]. The leptin resistance that results from hypoleptinemia could be interpreted as an adaptive resistance programmed from the intrauterine environment to maximise catch up growth. Leptin resistance in combination with an improved nutritional status would therefore play a causative role in the development of obesity, by altering the regulation of systems involved in growth and bodyweight regulation.

Unsurprisingly, treating programmed offspring with leptin can have beneficial effects on adult phenotype. For example, offspring from dams fed a maternal low protein diet have reduced placental 11βHSD2 activity [41,52]. This reduction in 11βHSD2 activity is thought to play a major role in the reduced growth, hypertension and glucose intolerance found in this model of programming, by permitting maternal glucocorticoids to cross the placenta. It has been shown that leptin treatment during the 3rd trimester of gestation and lactation can prevent this down regulation of 11βHSD2 and suggests that leptin may reduce fetal exposure to corticosterone [145]. These leptin treated offspring have a reduced susceptibility to weight gain and insulin resistance when fed a high fat postnatal diet [145].

Evidence has also shown that postnatal overnutrition alone can induce alterations in the regulation of bodyweight [146]. As both the hypothalamus and adipose tissue are still plastic in the postnatal phase in rodents, it is possible that altered leptin signalling could affect the development of these organs during this time. For example, postnatal overfeeding has been shown to induce disturbances to hypothalamic leptin signalling which persist into adult hood [147]. Giving normal rat offspring five times the normal concentration of leptin found in breast milk throughout lactation (pnd1-20) altered leptin signalling in PND 84 adult offspring, which afforded some protection against weight gain

during adulthood. Leptin treated offspring ate fewer calories, weighed less and had a lower fat content than controls, regardless of whether they were fed high fat or a normal fat postnatal diet. These leptin treated offspring fed a high fat diet had increased POMC signalling compared to control offspring fed a high fat diet. Furthermore, leptin treatment in offspring fed a high fat diet prevented the reduction in hypothalamic OB-Rb found in control rats fed a high fat diet. In addition, Socs-3 mRNA was reduced in all leptin treated offspring. Both these features indicate an increase in leptin sensitivity in leptin treated offspring and indicates that the leptin treatment resulted in a PAR, which provided some protection to high fat induced leptin resistance in later life [147].

1.5.4 The neonatal leptin surge

The extent of leptins role in the development of energy balance systems in rodents has been revealed following the discovery that there is a significant, but transient surge in plasma leptin during early postnatal development (PND 7-10), with levels reaching up to ten times that of normal [148,149]. This is a period of development in rodents where food intake is maximised for growth and development. As such, studies indicate that during this period rodents exhibit increased feeding [148] and increase their bodyweight whilst maintaining a low fat mass [148,149]. The presence of these features in combination with increased leptin concentrations is therefore contrary to its anorexigenic role and was originally thought to imply leptin resistance [148]. However, emerging studies have revealed that unlike in adults, leptins role at this time is not in energy balance [150]. For example, in adult rats, leptin challenge induces normal hypothalamic signalling, however leptin challenge between PND 7-21 fails to influence NPY POMC and AgRP and CART mRNA [150]. In fact, leptin is unable to have an effect on energy balance until the 3rd postnatal week, indicating that during this early postnatal period, leptin has a role which differs to that in adults [150]. It is now known that this surge in leptin is in fact a developmental signal [124].

The source of the leptin surge is still controversial. Two options have been proposed, both of which have supporting evidence; increased expression of leptin in adipose tissue or the maternal milk supply. Several studies have found that adipose leptin mRNA increases up to ten times normal levels during the surge period [148,149]. However, if adipose tissue is the source, it is unknown what induces this transient change. It is known that insulin and glucocorticoids have a positive effect on leptin expression, but it is unlikely they are the source of the surge as insulin levels were unchanged during one study and adult corticosteroid levels were not yet in place [149]. On the other hand, plasma leptin concentration in pups is greatly reduced when they are removed from their mothers for more than 24 hours [151], however, in another study, depriving neonatal mice of food did not affect the leptin surge [149].

1.5.5 The role of leptin in the formation of hypothalamic feeding circuits

Further studies into the significance of the leptin surge have uncovered an exciting role for leptin. Rather than the hypothalamus being insensitive to leptin during the first two weeks of life as first thought, it appears that leptin has a neurotrophic role instead. Experiments have shown that the period of the leptin surge coincides exactly with the formation of feeding/bodyweight circuits in the arcuate nucleus of the hypothalamus and that these centres remain immature in the absence of leptin [124]. In ob/ob mice, ARH projections are permanently disrupted and have a reduced density of fibres innervating the PVH, DMH and LHA. However, if these mice are given leptin treatment to replicate the normal surge, fibre innervation is permanently restored to normal levels. Thus the neonatal leptin surge acts as a peripheral developmental signal' [124] for the formation of the hypothalamic feeding circuits which it goes on to regulate in adult life. This concept of hormones acting as signals to direct hypothalamic development and activity is well established [152]. Interestingly, if leptin is given to given to adult ob/ob mice, projections remain disrupted, indicating that leptins neurotrophic role is restricted to a specific period in early postnatal life [124]. Therefore, the timing of formation of these circuits is restricted to a critical period which coincides with a surge in leptin concentrations [124]. Furthermore, maturation of feeding circuits in the 3rd week of life coincides with the ability of leptin to resume its normal role in the regulation of food intake and bodyweight [153].

1.5.6 Alterations to the leptin surge and hypothalamic circuitry in programmed offspring

Given the inherent link between leptin and nutrition, it was hypothesised that maternal undernutrition and postnatal nutrition may affect the leptin surge. As such, alterations in leptin levels during development would therefore have long lasting consequences on the formation of feeding circuits in the hypothalamus, which impact the regulation of energy balance in adults. This would therefore implicate developmental leptin concentrations in the development of adult obesity. Several key studies have explored this hypothesis and identified alterations in the leptin surge in response programmed offspring [39,151]. They have identified changes to the leptin surge and to specific hypothalamic wiring and gene expression as a result of maternal undernutrition, thus providing a link between maternal undernutrition, leptin and a propensity towards an obese phenotype in adulthood.

In a mouse model of maternal undernutrition (50% *ad libitum*), a postnatal high fat diet induces marked weight gain in adult mice despite the same calorie intake as controls. These mice exhibit hyperleptinemia, impaired glucose metabolism, hyperinsulinemia and dyslipidemia [39]. It has been shown in these neonates that the leptin surge has premature onset and peak, which appears to stem from alterations in adipose leptin expression. It was proposed that this increase in adipose leptin

was a result of the undernutrition induced hyperinsulinemia. Interestingly, control mice given leptin administration to match the premature surge also exhibited marked increased weight gain from weaning when fed a high fat diet, despite a similar calorie intake to controls. They also exhibited hyperleptinemia levels at 17 weeks age. Therefore in this study, maternal undernutrition induced a premature leptin surge, which was coupled to the development of obesity and its associated metabolic disturbances upon feeding a high fat postnatal diet. This study therefore indicates that the timing of the surge is critical for bodyweight regulation [39].

In a second study, it has been shown that maternal undernutrition during gestation and lactation (50% *ad libitum*) also affects the postnatal leptin surge in rats [151]. In these offspring, growth is reduced in UN offspring until PND 30. In contrast to the study in mice, the leptin surge in these offspring is not premature, but is instead severely blunted. This reduced leptin surge is associated with a subsequent reduction in hypothalamic neuropeptide expression specific to POMC from PND 14-30. In addition, hypothalamic projections specific to POMC are affected from the ARC to PVN at PND 21. Normal levels of leptin and POMC levels were resumed by PND 30 in all offspring. Despite these changes, offspring in this study do not become overweight on a chow diet, indicating that a combination of both maternal undernutrition and postnatal over nutrition may be required to induce an obese phenotype. However, it may also be that these offspring at PND 30 are too young to exhibit the effects of maternal undernutrition. Therefore, this study indicates that the strength of the leptin surge is affected by maternal undernutrition, which induces alterations in hypothalamic wiring [151].

These studies therefore indicate that both the timing and size of the leptin surge may be affected by maternal undernutrition, although it is not known if the different effects on the leptin surge are species or diet specific. However, it could be implied from these data that the early leptin surge and blunted leptin surge seen in these studies result in inadequate leptin levels at a critical period of development. Clearly in both models, early life nutrition affects the leptin surge and the formation or regulation of hypothalamic feeding circuits and thus impacts on the regulation of body weight in adult life. It is not currently known if the leptin surge has affects on the peripheral organs involved in bodyweight regulation such as adipose tissue.

The human brain in neonates is more mature than that of the neonate rodent. Information on human brain development is limited, but work with non human primates has indicated that the development of hypothalamic feeding projections occurs in the last trimester of gestation [154]. Therefore the development of hypothalamic feeding circuits is likely to occur during the last trimester of gestation in humans as opposed to neonatal life. As a consequence, development would be under direct control of the maternal environment. This finding indicates that leptin levels at the end of gestation may play a role in circuit formation, rather than postnatal leptin concentrations [153]. This is consistent with the finding that in humans, leptin concentration is

increased at the later stages of gestation and is reduced at birth, which could be equivalent to the rodent neonatal leptin surge [132].

1.5.7 Neonatal leptin treatment and the reversal of developmental programming

One study has illustrated the importance of leptin in fetal programming using the previously described model of maternal undernutrition (30% *ad libitum*) [8]. The aim of the study was to determine if neonatal leptin treatment could prevent the postnatal obesity and its associated metabolic disturbances found in programmed offspring fed a high fat postnatal diet. These offspring were given neonatal leptin treatment during the proposed timing of leptin surge (d3-13) and its effects investigated in adult female offspring. Results showed that the leptin administration normalised all the phenotypic and metabolic features of the programmed offspring, i.e. it abolished the hyperphagia, reduced fat mass and body weight, normalised insulin and leptin concentrations and normalised locomotor behaviour [122]. The outcome of the study therefore indicates that the phenotype induced by programming may be "reversed" by leptin treatment given during the later stages of developmental plasticity.

Previous studies have shown that maternal undernutrition induces alterations in the neonatal leptin surge [39,151]. Unfortunately, in this study it is unknown if the neonatal leptin surge or hypothalamic wiring were altered in the programmed or leptin treated offspring. It is, however possible that the undernutrition in these offspring induced neonatal hypoleptinemia. If this were the case, the restoration of the leptin surge with neonatal leptin treatment would be interpreted as a high nutritional state, thus enabling the offspring to readjust from their earlier adverse developmental trajectory [122]. In support of this data, it has been shown that giving leptin deficient mice neonatal leptin treatment induces a long term decrease in food consumption [124]. Interestingly, not all leptin-induced fat mass reduction is a result of reduced feeding; leptin-induced weight loss in ob/ob mice results in both reduced fat mass and feeding, but pair fed ob/ob mice do not lose as much weight. Therefore leptin treatment has effects on adipocyte mass other than by reducing feeding [155], indicating that other systems may also be altered as a result of the neonatal leptin treatment in this study. Furthermore, offspring subjected to maternal undernutrition in this study were cross fostered onto *ad libitum* fed dams. This indicates that the maternal milk supply is not enough to prevent the development of obesity and its associated metabolic abnormalities and strengthens the argument that favours adipose leptin expression as the source of leptin surge.

1.6 Epigenetics and developmental programming

Epigenetic modifications affect the control of gene expression by altering the structure of DNA without altering the genetic code. They are also heritable due to their ability to be passed on through cell division. Epigenetic systems include DNA methylation, histone modifications and micro RNA (miRNA), which work cooperatively with the overall effect of exposing or shielding regulatory regions of genes (promoters) to factors which control and direct transcription. Thus epigenetic modifications which expose these regions allow transcription to occur if the correct regulatory complexes are available, whilst those which shield these regions inhibit transcription by sterically preventing regulatory proteins from binding.

As the epigenome is set up in early development, during the window in which environmental constraints such as maternal diet are able to influence developmental trajectories, altered epigenetic regulation is therefore one mechanism which could underpin the persistent changes in gene expression identified in programmed offspring. It is therefore important to understand the nature of epigenetic modifications to recognise how early life constraints may impact on them.

1.6.1 Methylation of DNA

DNA Methylation is the most common covalent modification of DNA found in vertebrate animals and is stably inherited through cell division. It is an epigenetic event in which the dinucleotide 5'-Cytosine-p-Guanine-3' has a methyl group (CH_3) added at the carbon 5 position of the cytosine. Although 60-90% of dinucleotides are methylated [156], their distribution throughout the genome is uneven and two percent of the genome comprises short (1000-1500bp) CpG rich stretches of DNA termed CpG islands which are located within the regulatory regions of genes.

The main function of DNA methylation is to silence transcription (table 1.1) and as such, regulatory DNA which is unmethylated is associated with transcription and that which is methylated with a lack of transcription. CpG islands are usually unmethylated and therefore associated genes have the ability to be expressed. As such, these CpG islands are usually found at regulatory regions of constitutively expressed genes such as house keeping genes [157]. Exceptions to this occur for some tissue specific genes, for example the POMC gene whereby methylation of the regulatory CpG islands is tissue specific [158] and imprinted genes whereby methylation of CpG islands ensures transcriptional silencing of the associated parental allele [159]. However, hypomethylation of a gene promoter does not guarantee that it will be expressed, just that it has the ability to be expressed, as gene expression is dependent on multiple factors such as chromatin conformation and the availability of transcription factors. In agreement with this, some tissue specific genes with CpG islands are hypomethylated in all tissues.

Table 1.1 Main functions of DNA methylation. The main repressive functions of DNA methylation are indicated.

Process	Description
Gene repression	Repress expression of mRNA [77] and miRNA [160]
X chromosome inactivation	Methylation of CpG islands ensures silencing of X linked genes in female embryos [161] e.g. phosphoglycerate kinase 1 (PGK-1) (together with genomic imprinting, accounts for <10% CpG in genome)
Genomic imprinting	Only the paternal or maternal allele of a gene is expressed. The CpG island of the non expressing gene is heavily methylated thereby irreversibly silencing the gene [159]. e.g. H19 is maternally expressed and IGF-2 is paternally expressed.
Silencing of retroviruses	8% of genome is composed of endogenous retroviruses (junk DNA) which have integrated into human genome
Silencing transposons	Silencing promoters of these GC rich mobile genetic elements contained within introns (>40% of genome) accounts for the majority of methylated CpG [162].

Unsurprisingly, given the role of epigenetics in gene regulation, aberrant epigenetic modifications (epimutations) have been implicated in several disorders, including ageing, cancer and congenital disorders. For example, loss of imprinting (LOI) can result in the silencing of both parental alleles or biallelic expression of imprinted genes, such as occurs in Angelman's syndrome, Prader-Willi syndrome and Beckwith Wiedemann syndrome (BWS) [159]. In addition a gradual loss of global methylation is a feature of cellular senescence and when this occurs in gene promoters it may result in aberrant gene transcription and lead to cancer [163]. Contrastingly, de novo DNA hypermethylation at CpG islands of tumour suppressor genes will repress their expression and also lead to cancer, as has been identified for the breast cancer 1 (BRCA1) gene [164].

1.6.2 DNA methyltransferases

Methyl groups, which are largely supplied by the diet, are transferred to nucleotides by a group of enzymes called DNA methyltransferases (Dnmts) using the donor s-adenosyl-l-methionine (SAM) (figure 1.5). Three families of Dnmt have been identified, Dnmt1 (comprised of Dnmt1o, Dnmt1s and Dnmt1p), Dnmt2 and Dnmt3 (comprised of Dnmt3a, Dnmt3b and Dnmt3l). They are classed as either de novo Dnmts, which methylate unmethylated DNA and therefore involved in establishing methylation patterns, or maintenance Dnmts, which methylate hemi-methylated DNA and function to propagate established methylation patterns through mitosis.

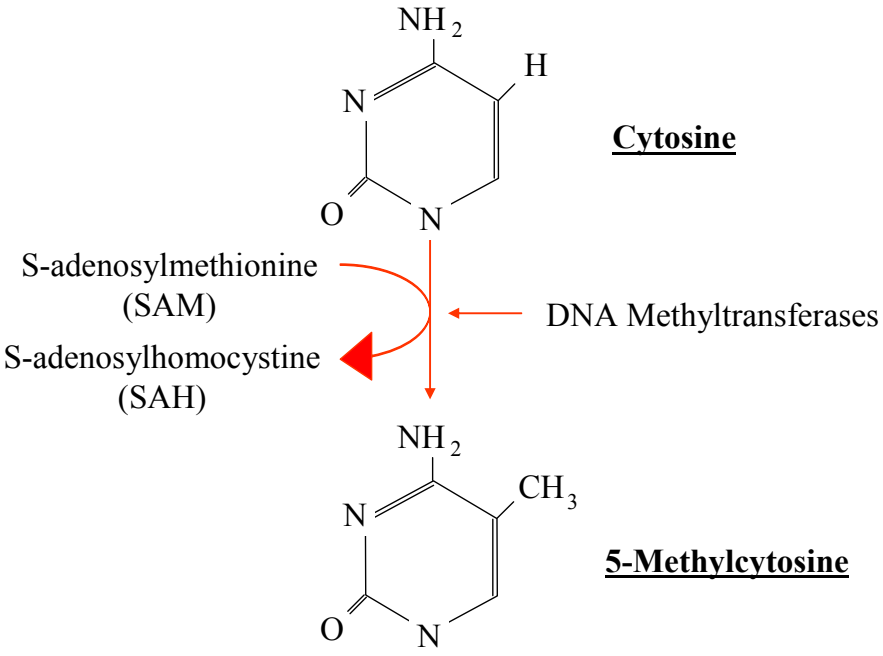


Figure 1.5 Formation of 5-methylcytosine. The methyl group is covalently added to the carbon 5 position of cytosine of CpG by a family of enzymes called DNA methyltransferases, resulting in 5-me-Cytosine. It is an active process and requires the donor SAM which is recruited by the methyl transferase. The products of the reaction are methylated cytosine and s-adenosyl-L-homocysteine (SAH).

1.6.3 Maintenance methylation

In somatic cells, the methylation status of DNA is maintained by Dnmt1s, which acts predominantly as a maintenance DNA methyltransferase due to its 5-30 fold preference for hemi-

methylated DNA [165]. This enables methylation patterns to be faithfully replicated on the daughter strand during mitosis, thus maintaining the heritability of the methylation blueprint laid down during development. The role of Dnmt1s has been confirmed using Dnmt1 null embryonic stem (ES) cells, which can grow normally until stimulated to differentiate whereby they undergo apoptosis [166] and mice embryos with mutant Dnmt1 which lose 95% of methylation and die mid gestation [166]. Furthermore, in these mutant embryos, inactive X chromosomes become reactivated and LOI occurs [167]. Dnmt1 also has sex specific promoters, resulting in an oocyte specific isoform (Dnmt1o), which is also expressed in the early embryo and an isoform specific to spermatocytes (Dnmt1p) [168]. Deletion of Dnmt1o results in loss of imprinting, suggesting that it maintains methylation of imprinted loci [169].

1.6.4 Epigenetic reprogramming and de novo methylation

DNA methylation patterns are established early in development and during this time the dynamic epigenome undergoes two major phases of reprogramming, throughout which epigenetic modifications are erased and reset [159]. This is therefore a sensitive period for the epigenome which can be highly susceptible to dysregulation by environmental factors such as maternal undernutrition.

The first event which occurs is the demethylation of primordial germ cells (PGCs), which is followed by remethylation during gametogenesis whereby parental imprinting patterns are reset [170]. This reprogramming of the epigenome also serves to reset any epimutations that have developed in parental genomes and therefore prevents their transmission to the subsequent generation. Secondly, after fertilisation of the egg, the epigenome undergoes a wave of demethylation to produce a pluripotent zygote, followed by locus specific de novo methylation after the fifth division [171]. One exception to the second wave of reprogramming are the imprinted genes whose methylation is set up during gametogenesis and do not undergo demethylation [172]. The methylation status of the genome is therefore resumed by implantation.

1.6.5 Demethylation of the parental genomes in the embryo

Demethylation of the maternal and paternal genomes in the zygote during preimplantation development occurs by contrasting mechanisms. Maternally derived chromosomes show a gradual reduction in methylation over several cell divisions, due to a lack of maintenance methylation following DNA replication, therefore indicating a passive demethylation [173,174]. This is proposed to be due to exclusion of Dnmt1o from the nucleus in the initial three divisions [170]. Conversely, it has been shown that demethylation of the paternally derived genome after fertilization precedes any

replication, thus inferring an active mechanism [174,175]. In agreement with this, immunofluorescence studies have provided evidence that completion of demethylation of the male derived genome is within 4hr of fertilization [176]. It is not known why demethylation of the male and female genome differs, but it has been hypothesised that demethylases are unable to access the maternal genome [175].

Exact mechanisms of active demethylation are currently under debate, but 3 potential mechanisms have been speculated; the complete removal of the methyl-CpG, removal of the methyl group and its subsequent substitution with hydrogen and finally the excision of the methylated cytosine base [177]. The search is on to identify the demethylase(s) and once this is achieved the mechanism will become clearer. One potential demethylase, identified as methyl CpG binding protein 2 (MBD2), was claimed to demethylate DNA [178], but this has been contested [177], as it has been found that demethylation of the paternal genome occurred normally in fertilized oocytes deficient in MBD2 [176].

1.6.6 De novo methylation

The highly coordinated remethylation of the genome is performed largely by the de novo DNA methyl transferases Dnmt3a and Dnmt3b using unmethylated DNA as their substrate [179]. In mice, Dnmt3a and Dnmt3b knockouts block de novo methylation and are lethal to the early embryo [179]. In contrast, deletion of these genes does not effect maintenance methylation, thus providing evidence they are the main de novo DNA methyltransferases crucial for early development [179]. In addition, Dnmt3b is also mutated in patients with ICF syndrome, in which satellite DNA of pericentric regions loses its methylation [168]. Furthermore, loss of Dnmt3b causes demethylation of X inactivated CpG islands. Normally, these regions are methylated shortly following implantation, thus indicating a role for Dnmt3b in this particular de novo methylation [168]. Another member of this family, Dnmt3l has no methyltransferase activity, but is instead involved in recruiting other members of the Dnmt3 family to promoters. It has been shown that this isoform is necessary for establishing maternal imprints in the female germ line [180].

Further changes to the methylation status of genes also occur later in development during tissue differentiation [181]. This involves the do novo methylation and demethylation of specific genes which creates cells with their own epigenetic fingerprint, thus permitting a gene expression profile appropriate for the particular cell type and time of development. These epigenetic alterations therefore occur at different times depending on the tissue involved and the role of the gene. For example, genes may be demethylated during tissue differentiation such as leptin [182], whilst others may undergo methylation during the later stages of development, such as the Hox genes [183].

1.6.7 What directs de novo methylation?

A major unanswered question in the field of epigenetics is what directs de novo methylation to specific sequences during development. Several theories exist, including both the exclusion and targeting of Dnmt and the direction of methylation by non coding RNA. For example, sequence specific targeting of Dnmt to DNA could occur by sequence specific protein repressor complexes such as transcription factors and histone modifying enzymes [184]. Alternatively, it has been hypothesised that the accessibility of response elements at critical periods of development leaves them exposed and accessible to Dnmts, which would result in de novo methylation, whereas steric hindrance due to presence of protein factors such as transcription factors may exclude Dnmt from the DNA and therefore prevent methylation [185]. In agreement with this theory, Sp1 response elements, which are normally present in multiple copies in CpG islands have been hypothesized to play a role in directing the hypomethylation of CpG islands [186]. It has been revealed that Sp1 response elements at the periphery of a CpG island in the promoter of the housekeeping gene adenine phosphoribosyltransferase are crucial in order to maintain its hypomethylation, thus providing protection from methylation [186]. Using transgenic mice and embryonic stem cells, mutation and deletion of Sp1 sites in the CpG island were shown to prevent Sp1 binding, which led to the methylation of the CpG island [186].

1.6.8 Histone modifications

Collectively the complex of DNA and its associated histone proteins are termed chromatin and the structure of chromatin around promoter regions of genes affects the ability of the gene to be transcribed. Modifications to amino acids on the N-terminal tails of histones protruding from the nucleosome core can induce both an open or closed chromatin structure and these affect the ability of transcription factors to access promoter regions to activate transcription. These covalent modifications include; acetylation, methylation, phosphorylation and ubiquitination and the combination of histone modifications have been collectively termed the histone code [187] (figure 1.6). Methylation of some residues is associated with transcriptional repression, such as methylation of histone 3 lysine 9 (H3 K9) [188] and others with transcriptional activation, such as methylation of histone 3 lysine 4 (H3 K4) [189]. Methylation is performed by histone methyltransferases (HMTs), which can transfer up to three methyl groups to lysine residues with differing effects on gene activity; for example, tri methylation of H3 K9 is associated with silent heterochromatin and monomethylated H3 K27 is found throughout euchromatin. Acetylation, which occurs solely at lysine residues such as H3 K9 is associated with transcriptional activation [190]. This modification is performed by histone acetylases (HATs) and histone deacetylases (HDAC) can remove the acetylation mark.

Other important regulators of chromatin conformation include the polycomb group (PcG) and trithorax group (trxG) proteins, which have key roles in developmental gene regulation [191]. They are recruited to response elements near proximal promoters to direct histone modifications, which induce both an active chromatin structure (trxG) and an inactive chromatin structure (PcG). Trithorax group proteins methylate H3 K4 to induce an active chromatin conformation [191], whilst PcG proteins direct the methylation of H3 K27 to induce a repressive chromatin conformation. The effects of PcG proteins are however reversible, as removal of PcG during development leads to gene activation. PcG proteins have been implicated in regulation of developmental transcription factors, genomic imprinting and X chromosome inactivation [161].

Thus in addition to DNA methylation recruiting protein complexes to induce histone modifications, modifications to histones themselves may also induce alterations to DNA methylation, owing to their interaction with protein complexes such as HDAC, HMT and MBD which may recruit Dnmts. Thus DNA methylation can bring about histone modifications and histone modifications can influence DNA methylation [192]. However, as histone modifications generally can be viewed as more short term and dynamic, the additional brace of DNA methylation serves to stabilise gene silencing.

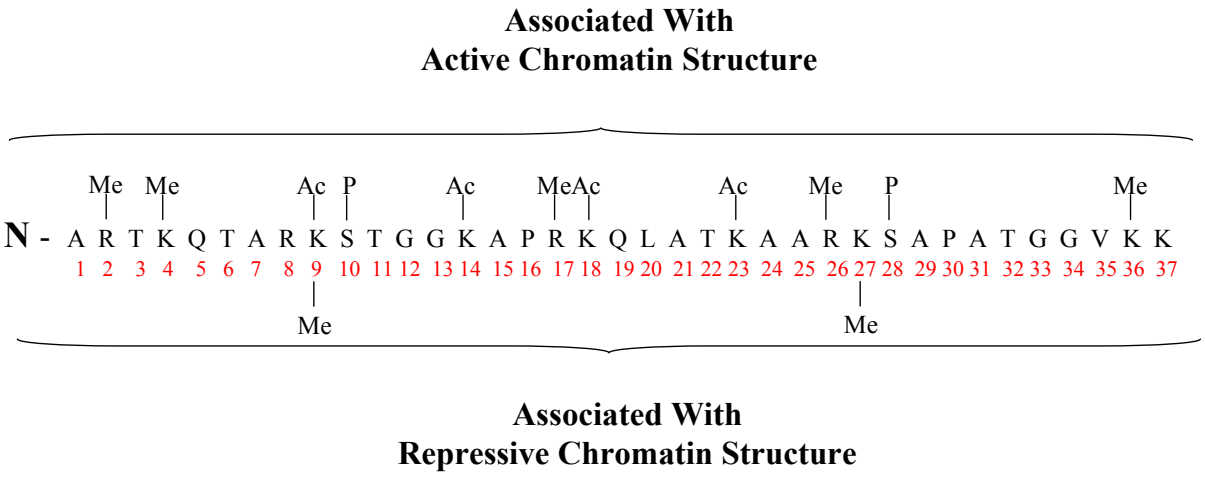


Figure 1.6 Common modifications of histone 3 Common histone modifications to lysine tails of histone 3 are shown, indicating both modifications associated with a repressive chromatin structure and those associated with an active chromatin structure.

1.6.9 DNA methylation and repression of gene expression

Methyl-CpG can lead to the repression of gene transcription by one of two contrasting mechanisms; firstly a direct mechanism in which the methyl group physically prevents transcription factor access to its target DNA sequence and secondly from providing a binding site for methyl CpG binding proteins (MBDs), which in turn recruit repressive protein complexes that facilitate a closed chromatin structure. It is considered that the latter indirect method is the most prevalent mechanism [157].

1.6.9.1 DNA methylation and transcription factor binding

Many transcription factor response elements possess CpGs within their response elements and the methylation status of these CpGs may affect transcription factor binding and therefore influence gene transcription. As such, increasing examples of DNA methylation physically interfering with transcription factor binding are emerging. For example, binding assays have shown that methylation of CpGs within the DNA binding site for the transcription factor MLTF can prevent its binding at the adenovirus major late promoter and inhibit gene expression in vitro and this is effect is dependent on the exact position of the CpG within the response element [193]. Methylation of an E2F response element in the POMC promoter prevents its binding and contributes its tissue specific expression [158]. CpG methylation within the specificity protein 1 (sp1) response element has also been shown to reduce protein binding by 95% [194] and it has even been found that methylation just outside its response element can prevent Sp1 binding and thus inhibit transcription [195]. Clearly the effects of methylation of CpGs within response elements vary for the same transcription factor within different promoters and therefore need to be characterised on an individual basis to determine if and how DNA binding is affected by cytosine methylation.

1.6.9.2 DNA methylation and methyl DNA binding proteins

MBD proteins are a family of proteins expressed in most somatic tissues which can bind me-CpG. To date they include; MeCP1, MeCP2, MBD2 and Kaiso. They serve to mediate the methylation signal by linking DNA methylation to alterations in chromatin structure [196] and exert their repressive natures by specifically binding me-CpG via their methyl CpG binding domain (MBD) and recruiting histone modifying proteins via their transcriptional repression domain (TRD). The histone modifying proteins recruited include both histone deacetylases (HDAC) and histone methyltransferases (HMT).

The most extensively studied methyl DNA binding protein is MeCP2, which can bind to a single me-CpG via its MBD [197]. MeCP2 is composed of a single polypeptide chain which contains a both a MBD and a TRD. The TRD recruits a co-repressor complex which includes mSin3A, HDAC1 and HDAC2 [198], which enable the deacetylation of H3 and H4, the formation of a more closed chromatin structure and thus transcriptional repression (figure 1.7). Although deacetylase inhibitors such as trichostatin A (TSA) remove most repression [199], some repression remains, thus indicating other factors are involved. Subsequently, chromatin immumoprecipitation (ChIP) and co-immumoprecipitation studies have provided evidence that MeCP2 also recruits a HMT which add methyl groups specifically to H3 K9, a repressive modification, therefore adding a further brace to the closed chromatin structure [200].

MeCP1 is a protein complex which requires at least 12 me-CpGs to bind DNA [201]. As such, it can repress heavily methylated promoters, but its suppression of poorly methylated promoters is weak [196]. MeCP1 complex contains MBD2 which facilitates the DNA binding activity of this complex [202] and the nucleosome remodelling and histone deacetylase (NuRD) co-repressor complex [203]. Conversely, Kaiso binds methylated DNA via zinc finger motifs to repress transcription. It has also been identified as part of the MeCP1 protein complex and requires two adjacent methyl-CpGs to bind methylated DNA [204].

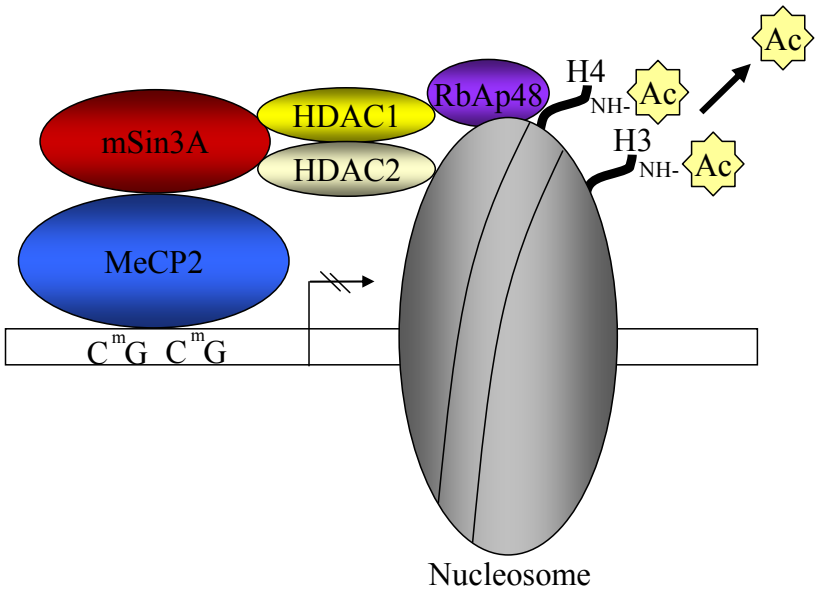


Figure 1.7 **Mechanism of repressive action by MeCP2.** The MeCP2 MBD binds to me-CpG, whereas the TRD recruits a co-repressor complex which includes mSin3A, HDAC1 and HDAC2, which enable the deacetylation of histones, the formation of a more closed chromatin structure and transcriptional repression (adapted from [205]).

1.6.10 Micro RNA and epigentics

Recently, interest has also arisen in micro RNAs (miRNA) as an epigenetic mechanism with the potential to be implicated in programming. miRNA are small untranslated RNAs about 22bp long involved in posttranscriptional repression of target genes by translational repression and mRNA degradation [160]. Over 1000 human miRNAs exist and each miRNA may regulate in the region of 100 target genes, thus these molecules are an important determinant of the transcriptome. Intriguingly, miRNAs may be both controlled by epigenetic mechanisms [160] and induce epigenetic alterations in target genes [206]. Using a Dnmt1 and Dnmt3b double knock out cell line, it has been shown that DNA methylation has an important role in the regulation of around 10% of miRNA [160]. Furthermore, the use of HDAC inhibitors has revealed that histone modifications can regulate miRNA expression [206]. The ability of miRNA to induce epigenetic modifications is owing to their regulation of factors involved in the processes of DNA methylation [207] and histone modification (e.g. HDAC4) to induce a closed chromatin conformation [206]. These recent findings imply that like normal genes, miRNA have the potential to be affected by environmental constraints during development, however due to their ability to regulate 100s of target genes and their ability to induce epigenetic modifications, any alterations in miRNA expression during development could have profound effects on numerous biological systems.

1.7 Evidence implicating epigenetic alterations in developmental programming

Each cell type possesses its own epigenetic "fingerprint" which is laid down in early development to determine the array of genes which will be expressed. There is a growing body of evidence giving substance to the hypothesis that an adverse environment during critical periods of development, which stretch from the periconceptual period to postnatal life, may induce aberrant epigenetic marks which alter this epigenetic fingerprint and underpin the gene expression changes identified in programmed offspring. Thus, the stable propagation of these epimutations contributes to the altered phenotype and associated metabolic disorders.

Emerging studies in rodents are discovering that throughout early life, the epigenome is vulnerable to environmental insults including not only maternal diet, but also postnatal diet, social behaviour and stress. Furthermore, despite the main body of work focusing on rodents, data is now emerging identifying alterations in human epigenome as a result of similar developmental constraints. These studies are unravelling the complex epigenetic alterations which bring about the potential for altered gene expression. They include altered expression of Dnmts, altered DNA methylation of specific CpGs, altered transcription factor binding or binding of MBD and altered histone modifications. These elegant studies show that the epigenetic alterations induced can occur in genes involved in a variety biological processes, include both imprinted and non imprinted genes,

occur in a variety of tissues and may be induced at different time points throughout gestation and postnatal life. Interestingly, it is emerging that these marks may be reversible, thus providing a means to develop therapies to intervene and prevent the onset of adult disease. The following studies represent some key landmarks that have contributed to the current knowledge of epigenetic programming.

1.7.1 Evidence of altered epigenetic regulation in rodents

1.7.1.1 Epigenetic alterations arising from maternal behaviour and stress

Interest in the role of epigenetic modifications in programmed offspring arose from one key paper. In rodents, maternal care during the first week of life is associated with an altered stress response in adult offspring. Weaver and colleagues provided evidence that the ability of maternal behavior to alter this stress response was due to altered epigenetic regulation of the GR promoter in the hippocampus [208]. It was shown that offspring subjected to high levels of maternal care had increased expression of GR. This was found to be due to hypomethylation of a CpG within an NGF1-A response element in the promoter. Furthermore, the alteration in methylation was shown to be specific, as the methylation status of an alternative CpG within the NGF1-A response element remained unchanged. In line with the reduced methylation, increased binding of NGF1-A and increased acetylation of H3K9 were also found, consistent with inducing a transcription competent chromatin conformation. Thus the increased GR expression which resulted from these alterations acted as a negative feed back on the HPA axis to dampen the response to stress in the offspring. Weaver and colleagues therefore provided evidence that specific and permanent epigenetic changes can occur through maternal behavioral programming which affect the stress response of adult offspring [208]. Interestingly, later studies showed that all the epigenetic alterations identified in offspring subjected to high levels of maternal care could be reversed by methionine supplementation to adult offspring, indicating that the epigenetic marks are reversible even in terminally differentiated cells of the hippocampus [209].

1.7.1.2 Epigenetic alterations arising from maternal nutrition

The first evidence to show that maternal diet can affect the epigenetic status of genes in the offspring was that of the agouti mouse model. In A^{vy}/a mice, the phenotype of the mouse is altered depending on the expression of the agouti gene, which regulates production of fur colour. These phenotypes range from yellow mice which are obese, through a range of mottled mosaics, to black pseudo agouti mice which are lean and healthy. Expression of the agouti gene in these mice is controlled by an intracisternal A Particle (IAP) promoter and maximal expression results in mice

with yellow fur and an unhealthy obese phenotype. However, expression of the agouti gene is affected by methylation status of the IAP promoter and when methylated, agouti gene expression is switched off resulting in a black pseudoagouti fur colour and mice which are lean and healthy. Further studies indicated that giving methyl supplements to the mother throughout pregnancy induced hypermethylation of the IAP promoter in offspring, resulting in reduced gene expression and offspring which displayed the pseudoagouti phenotype [210]. Although this study is not relevant to natural conditions due to control of the agouti gene by a foreign promoter, it is a good model system in which the effects of maternal factors such as diet on epigenetic status can be studied.

Another example which showcases the ability of nutrition to modify developmental fate via modification of the epigenome is that of the honeybee [211]. Development of the honeybee into a queen is determined by prior nutrition; royal jelly fed female larva become queens, whereas those fed a lesser substance become sterile workers. siRNA directed knockdown of Dnmt3 in hatched larvae was shown to have effects similar to that of royal jelly feeding, i.e. induced an altered developmental trajectory such that the honeybee became a fertile queen rather than a sterile worker, thus implicating altered de novo methylation in the honeybee development. By analysing the methylation status of specific genes, it was shown that average methylation levels were higher in control larvae than siRNA treated larvae or queens. Furthermore, alterations to DNA methylation were found to be specific to particular CpGs rather than a global effect. In agreement with altered methylation, an expression array identified altered gene expression in siRNA knockdown larvae and queens compared to control larvae. Thus here, the fertile queen, induced by natural royal jelly feeding or laboratory induced Dnmt3 knockdown, is associated with reduced capacity to methylate specific genes during a critical period of larval development, facilitating altered gene expression and an alternative phenotype [211]. The honeybee example although not relevant to humans, is a good example of the mechanism of early nutrition affecting epigenetics and altering the adult phenotype.

1.7.1.3 Epigenetic alterations to GR and PPARs arising from maternal nutrition

Evidence is accumulating implicating altered DNA methylation in nutritional models of programming in rodents. Interest in epigenetic modification has concentrated on genes whose expression has previously been shown to be altered in these offspring, such as GR and PPARα. For example, in the liver of day 34 MLP offspring, the PPARα promoter was found to be hypomethylated [77], consistent with previous reports of increased expression in this tissue [57]. In addition, methylation of the GR exon 1_{10} promoter was also shown to be reduced and corresponded to an increase in gene expression. Furthermore, this hypomethylation was also shown to be gene specific and not a general effect, as methylation of the PPARγ promoter remained unchanged in response to maternal diet. Interestingly, this work also identified that supplementing the MLP diet

with folic acid specifically prevented the methylation and expression changes in PPARα and GR, thus implicating a reduced supply of folic acid and subsequent altered 1-carbon metabolism to alterations in DNA methylation in programmed offspring [77].

Further to this, a more detailed analysis of the methylation status of the PPARα promoter in the same offspring has since been undertaken. Pyrosequencing of 16 CpGs in the promoter region showed that the hypomethylation seen in PR offspring was specific to individual CpGs rather than a global effect, as the methylation of many CpGs remained unchanged by maternal diet. Mean methylation of all these 16 CpG was shown to be 26% lower in PR compared to control and feeding a PRF diet prevented all individual CpG methylation changes seen in PR offspring. Furthermore, the individual changes in methylation were also found in day 80 offspring, indicating that the changes were persistent. Further to this, CpGs with altered methylation were shown to coincide with potential binding sites for transcription factors such as Sp1 whose DNA binding ability has previously been found to be affected by methylation and could therefore play a key role in altered PPARα gene expression [212]. However, although DNA methylation levels in the PPARα promoter were found to be low overall, they had a negative correlation with gene expression, indicating that these subtle changes were enough permit changes in gene expression.

To understand how the altered methylation of the GR promoter results in altered transcriptional activity, a detailed investigation was undertaken to characterise the mechanisms involved and this elegant study uncovered a series of alterations which could facilitate a chromatin structure permissive for increased gene expression [213]. Lillycrop and colleagues identified reduced methylation of the GR exon 1_{10} promoter in day 34 offspring from dams fed a MLP diet, which corresponded to an 84% increase in GR expression and a 16% increase in the expression of its target gene PEPCK. Evidence was provided that this reduction in methylation was linked to altered maintenance methylation, as Dnmt1 expression was also reduced by 17% indicating a reduced maintenance methylation in these cells and consequently binding of Dnmt1 to the GR promoter was reduced. In contrast, Dnmt3a and Dnmt3b expression were unchanged indicating alterations in de novo methylation were not involved. The MLP offspring were also found to have reduced expression of MeCP2 and reduced binding of MeCP2 to the GR promoter, providing a link to histone structure. Unsurprisingly, chromatin immunoprecipitation (ChIP) studies indicated increased amount of histone modifications associated with open chromatin structure and reduced histone modifications involved with the repression of transcription. Thus a complete picture of the mechanisms underlying altered GR expression was uncovered. Interestingly, it was shown that supplementing the maternal diet with folic acid prevented the reduction in Dnmt1 expression [213]. This supports the theory that Dnmt1 expression is reduced due to impaired carbon-1 metabolism and suggests that the protein restricted diet causes a reduction in Dnmt1 expression during development which is associated with reduced maintenance methylation of specific genes in specific tissues [213]. Interestingly, evidence to support the gene specificity of altered Dnmt1

methylation has emerged, indicating that it represses transcription in promoters with E2F binding sites [184], which explains why methylation of all genes is not altered. Furthermore, this study also shows that when epimutations affect the expression of transcription factors, effects can be relayed on to target genes which affect the metabolic activity of a cell therefore influencing the adult phenotype.

1.7.1.4 Epigenetic alterations to other genes implicated in acquisition of the programmed phenotype

Other biological aspects of programming have been investigated for epigenetic alterations, such as the mechanisms underlying the programming of hypertension. For example, activation of the AT_{1b} receptor in the adrenal gland stimulates aldosterone production which results in increased blood pressure. In the adrenal gland of MLP offspring, bisulfite sequencing and pyrosequencing have confirmed that there is significant under-methylation of 3 CpG sites within the AT_{1b} Angiotensin receptor proximal promoter. Interestingly, 1 of these CpG sites falls within Sp1 consensus site and may therefore affect protein binding. This altered methylation is accompanied by increased expression of the AT_{1b} gene prior to the 1st week of life which persists at least to week 12. Thus increased expression of this receptor due to reduced methylation may contribute to the formation of hypertension in later life [214].

The IUGR rat is also associated with an increased risk of hypertension in later life and IUGR offspring in both rat and humans have decreased glomeruli number and therefore impaired renal function. A study has found that the p53 promoter is hypomethylated in the kidney of IUGR neonates in the rat, specifically methylation of regions which have previously been shown to affect levels of p53 mRNA. Again hypomethylation was specific to certain CpGs and led to increased expression of its protein and apoptotic target genes, resulting in increased apoptosis. As apoptosis has a fundamental role in nephrogenesis which peaks late gestation (when the IUGR was induced), it is probable that the altered methylation of p53 contributed to the reduced glomeruli number and the reduced glomerular filtration rate found in these offspring [215].

Pdx1 is a transcription factor involved in β cell differentiation and reductions in Pdx1 can result in type II diabetes. One interesting study has tracked the sequence of epigenetic modifications which occur over time to repress the expression of the Pdx1 gene in the β-cells of a rodent model of IUGR [216]. It has shown that the course of development was associated with a cascade of epigenetic modifications which led to the permanent silencing of Pdx1 in adult offspring. In the fetus, repressive protein complexes were recruited to the Pdx1 promoter resulting in the deacetylation of histones. This was associated with reduced binding of the transcription factor USF-1, an activator of Pdx1 gene transcription, to the Pdx-1 promoter compared to controls. After birth, this was

followed by alterations in histone methylation associated with gene repression and deterioration of glucose homeostasis. At this point, no DNA methylation is apparent in the Pdx1 promoter. In adulthood, when offspring have developed type II diabetes, the Pdx1 promoter is methylated and the Pdx1 gene permanently silenced. Thus the progressive silencing of this gene by a "self propagating epigenetic cycle" [216], plays a causative role in the onset of diabetes in this IUGR rodent model [216].

1.7.1.5 Epigenetic alterations arising from postnatal nutrition

In addition to programming of the epigenome by maternal undernutrition, studies have identified that neonatal overnutrition can alter the epigenome in the hypothalamus and adipose tissue of rodents. For example, rats from small litters bred to develop obesity exhibit hyperinsulinemia, hyperglycemia and hyperleptinemia and have hypermethylation of the POMC promoter compared to controls. Activity of the POMC promoter has previously been shown to be affected by DNA methylation [217]. It has been shown that overnutrition induced increased methylation of two CpGs in the POMC promoter, one of which is located in an NFκB response element and the other just upstream a Sp1 binding site which has previously been shown to be crucial for leptin mediated activation of POMC. It is known that methylated CpG upstream of Sp1 response elements can effect Sp1 binding to DNA [195] and methylation of this CpG was found to be inversely associated with POMC expression [185]. As a result, POMC expression was not increased in these rats despite hyperleptinemia. It explains the inability of the hyperleptinemia to prevent obesity, as the increased leptin would normally increase POMC expression to reduce feeding. The alterations in methylation were also gene specific, as methylation of the hypothalamic NPY promoter was unaffected by overnutrition. Thus this study has shown that overfeeding can specifically induce hypermethylation of the POMC gene in the hypothalamus during a period of plasticity and thus induce hypothalamic leptin resistance which contributes to the altered phenotype [185].

Furthermore, it has recently been shown that the leptin promoter is subject to altered DNA methylation by postnatal high fat feeding. Rats fed a high fat diet for 11 weeks become obese with associated hyperleptinemia. Analysis of the leptin promoter from adipose tissue in these rats found hypermethylation of a specific CpG in the leptin promoter, which was associated with reduced leptin expression [218]. As leptin is an adipokine which mediates its effects body wide, alterations in its methylation and therefore expression could have effects on many biological processes, with a particular emphasis on bodyweight regulation.

Thus these two studies indicate that the postnatal environment is still able to alter the epigenome in tissues which maintain some developmental plasticity, such as adipose tissue and the

hypothalamus, implicating postnatal life as a mediator of an altered adult phenotype, with particular relevance to obesity and its related metabolic disorders.

1.7.2 Evidence of altered epigenetic regulation in humans

1.7.2.1 Epigenetic alterations arising from childhood abuse

In rodents, maternal behaviour is able to induce epigenetic alterations to the promoter of GR in offspring, which induces an altered HPA response to stress that persists throughout life. Intriguingly, recent work in humans has uncovered similar epigenetic alterations to the GR promoter in the hippocampus of suicide victims with a history of childhood abuse. In these individuals, reduced GR expression was found specific to the 1_F splice variant and this was associated with increased methylation of specific CpGs in the GR promoter. Furthermore, ChIP and patch methylation reporter gene experiments indicated that CpG methylation which mimicked that identified in child abuse suicide victims, resulted in reduced NGF1-A binding to its response element and a reduced ability to induce GR transcription. These experiments therefore imply that mechanisms underlying HPA stress responses in rodents and humans as a result of care in early life share a common pathway, based around epigenetic modification and expression of GR in the hippocampus [219].

1.7.2.2 Epigenetic alterations arising from maternal nutrition

Evidence is now emerging in humans implicating altered DNA methylation as a consequence of nutritional constraints during development. The first evidence of altered DNA methylation in humans as a result of altered nutrition came from the Dutch Hunger Winter cohort in which alterations in both imprinted [220,221] and non imprinted genes [221] were identified 60 years after the initial famine exposure. For example, 5 CpGs within the DMR of the maternally imprinted insulin-like growth factor II (IGF2) gene were analysed using Sequenom technology and found to have reduced methylation compared to controls. Although differences were small (5.2% average of all 5 CpGs), they were significant [220]. Furthermore, this effect was shown to be specific to famine exposure in the periconceptual period, a period in which the epigenome is especially susceptible to environmental effects. Interestingly these individuals had a normal birth weight. Contrastingly, alterations in DNA methylation were not found in those individuals of low birth weight exposed in late gestation, indicating that the effect on DNA methylation was specific to the timing of exposure [220]. These results also indicate that birth weight is not a reliable indicator of altered epigenetic regulation. A later study using the same cohort, found that alterations in DNA methylation induced by famine exposure were not specific to imprinted genes, but also found in non imprinted genes

involved in growth and metabolism including; leptin, interleukin 10, tumor necrosis factor (TNF) and the fat mass and obesity associated gene (FTO) [221]. Here, alterations in the methylation status were found to be dependent on the timing of exposure and in some cases the sex of the individual. For example, both hypomethylation (IGF2) and hypermethylation (IL10, leptin) were associated with periconceptual exposure, whereas leptin hypermethylation was also identified in men exposed during late gestation. Again, changes in methylation status were small (<3%), but significant [221].

1.7.2.3 Epigenetic alterations arising from assisted reproductive technologies

Children who are conceived by assisted reproductive technologies (ART) including in vitro fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) account for 1-3% of births in the western world [222]. These children have an increased risk of congenital disorders arising from LOI of imprinted genes [223]. For example, ART is associated with increased incidence of; Angelman syndrome, Prader-Willi syndrome and Beckwith Wiedemann syndrome (BWS) [223]. This indicates that the procedures associated with these technologies, such as gonadotrophin treatment, embryo retrieval, embryo culture and embryo replacement may induce epigenetic alterations in imprinted genes. For example, BWS LOI occurs due to hypermethylation of the H19 gene (which is normally maternally transcribed) and leads to aberrant activation of IGF2 (which is normally paternally transcribed). In addition to this, hypomethylation of Lit1 DMR has been identified, which is normally methylated on the maternal allele [224]. These findings in ART children, which indicate the vulnerability of the periconceptual period, are consistent with studies culturing mouse embryos in which both culture and embryo transfer were found to be associated with aberrant expression of imprinted genes in day 9.5 concepti, in particular that of H19 and the biallelic expression of Lit1 due to hypomethyalation of the maternal allele [225]. Furthermore, analysis of 700 genes covering 1536 CpG in children conceived from both ART and natural conception showed that IVF was associated with higher average DNA methylation in cord blood and reduced overall methylation in the placenta compared to controls, with associated alterations in gene expression. Furthermore, these differences were identified in not only imprinted genes, but also non imprinted genes. Thus, like the differences in methylation identified in the Dutch Hunger Winter, changes in DNA methylation were small but statistically significant [222].

Results from all these studies clearly indicate that the periconceptual period is most susceptible period for the induction of epigenetic alterations, especially in humans. In addition, overwhelming evidence indicates that the alterations in CpG methylation are specific to particular CpGs and not a global effect. These studies therefore raise the question as to how methylation changes are not only targeted to specific genes during development, but also to specific cytosines. As such, the mechanisms by which alterations in epigenetic modifications are targeted needs to be elucidated.

Two mechanisms have been proposed for how the environment may lead to alterations in DNA methylation during development [226]; the first mechanism is by modifying the availability of methyl donors or the activity of Dnmt1, resulting in an inability to faithfully replicated methylation blueprint onto the daughter strand. An example of the maternal environment potentially effecting Dnmt1 is that of altered GR and PPARα DNA hypomethylation, which is prevented by increasing maternal folic acid [77]. The second theory concerns preventing de novo methylation. If signalling pathways are activated during development as a result of an environmental constraint, this would result in regulatory factors binding to promoter regions, thus protecting the promoter area from DNA methylation. This region would therefore remain unmethylated with the ability to be expressed if stimulated to do so in later life. An example of this could involve the effects of increased GR signalling as a consequence of maternal behaviour [208].

1.8 Methods for measuring DNA methylation

Many methods are available to measure DNA methylation levels and these differ in the cost, ease of use, sensitivity, scale and level of throughput. As such, these methods range from the simple, which measure average DNA methylation over a stretch of DNA, to methods which look at individual CpG methylation of the 'whole genome'. Therefore the method chosen will depend on the cost, level of detail and throughput required. The simple methods are useful for an initial look at DNA methylation of a known region of DNA. For example, methylation sensitive PCR is a semi-quantitative method which utilises methylation sensitive restriction enzymes to differentiate between methylated and unmethylated sequences in DNA. Subsequent real time PCR will only amplify amplicons containing undigested methylated DNA, whilst digested unmethylated DNA is unable to be amplified. Alternatively, more sensitive quantitative methods are available which can look at the methylation status of individual CpGs within a stretch of DNA, for example Pyrosequencing or Sequenom analysis. Although more costly, these methods are highly sensitive and thought to be the gold standard for measuring DNA methylation of specific CpGs within a short stretch of DNA. Pyrosequencing, for example, uses bisulfite converted DNA and PCR of the region of interest to introduce a C-T SNP which depends on the methylation status of the CpG; methylated C remains a 'C', whereas unmethylated C becomes a 'T'. This SNP is accurately quantified in a sequencing by synthesis reaction. On a larger scale, whole genome technology now exists which can determine the methylation status of the "whole genome", although it is very costly to use. For example, ChIP-ChIP can be performed in which antibodies (e.g. for methylated cytosine) or protein complexes (e.g. MBD2) are used to capture methylated DNA from sonicated chromatin, whilst the unmethylated DNA is washed away. The captured methylated DNA can then be applied to an array for samples to be identified. Alternatively, enriched methylated samples may be sequenced and then mapped (ChIP-Seq), for example using the Illumina Genome Analyzer and Solexa sequencing.

1.9 Thesis aims

There are two main aims to this report:

i) In a rat model of programming induced by a maternal global undernutrition, offspring have a reduced birth weight compared to controls and adult offspring present with; hyperphagia, reduced locomotor behaviour, high blood pressure, hyperleptinemia, hyperinsulinemia and are obese. These disturbances are all augmented by a calorie rich postnatal diet and reversed by neonatal leptin treatment [122]. The molecular mechanisms underlying the metabolic disturbances in these IUGR offspring are not known, but it is hypothesised that they may involve similar mechanisms to those found in the PR offspring, i.e. altered DNA methylation and mRNA expression of key transcription factors and enzymes involved in carbohydrate and lipid metabolism. Furthermore, it is hypothesised that the leptin treatment may act during the neonatal period to prevent the programmed phenotype of IUGR offspring by altering the regulation of key genes involved in energy balance.

AIM: To investigate the DNA methylation and gene expression of key genes involved in energy balance in the adipose tissue and liver of IUGR offspring in response to maternal diet, postnatal high fat diet and neonatal leptin treatment using real time PCR and methylation sensitive PCR

ii) Previous work within this laboratory has identified altered PPARα mRNA expression in the liver of PR offspring in response to a protein restricted maternal diet. Furthermore, these changes have been associated with alterations in DNA methylation of the PPARα promoter [77,212]. However, exact mechanisms underlying these changes in gene expression and methylation are unknown. Furthermore, mechanisms underlying regulation of PPARα by factors such as glucocorticoids and leptin at the promoter level have been largely unexplored. Nuclear hormone receptors such as the PPARs have been shown to possess multiple tissue specific transcripts and this has been shown to be the case for both the mouse and human PPARα orthologues. However, to date, it is not known if such variant transcripts are present in the rat. It is hypothesised that the rat PPARα gene may also possess alternative transcript variants, which may play a key role in the tissue specific regulation of PPARα gene expression and may therefore be differentially regulated by maternal undernutrition.

AIM: To identify the structure of the rat PPARα gene using 5'RLM RACE
To characterise the structure of the rat PPARα promoter
To use a reporter gene strategy to investigate the mechanisms underlying the regulation of PPARα transcription by factors known to induce its expression, including leptin.
To analyse the methylation status of the PPARα promoter(s) using pyrosequencing

Chapter 2

Materials and Methods

2.0 Materials and methods

2.1 Materials

Table 2.1 Reagents and chemicals used in experiments

Reagent/Chemical	Supplier
3-isobutyl-1-methylxanthine	Sigma-Aldrich, Dorset, UK
1 Kb DNA ladder	Invitrogen, Paisley UK
AciI restriction enzyme and buffer	New England Biolabs, Herts, UK
Agar	Sigma-Aldrich, Dorset, UK
Ampicillin, sodium salt	Sigma-Aldrich, Dorset, UK
Annealing buffer (Pyrosequencing)	Biotage AB, Kungsgatan, Sweeden
Binding buffer (Pyrosequencing)	Biotage AB, Kungsgatan, Sweeden
Calcium chloride	Sigma-Aldrich, Dorset, UK
Chloroform	Fisher, Leicestershire, UK
Clofibric acid	Sigma-Aldrich, Dorset, UK
CG methyltransferase (M.SssI) and buffer	New England Biolabs, Herts, UK
Denaturation solution (Pyrosequencing)	Biotage AB, Kungsgatan, Sweeden
Deoxynucleotide triphosphates (dNTP's)	Promega, Southampton, UK
Dexamethasone	Sigma-Aldrich, Dorset, UK
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich, Dorset, UK
Dulbecco's modified Eagle's medium	Sigma-Aldrich, Dorset, UK
EcoRI restriction enzyme	Promega, Southampton, UK
Ethanol	Fisher, Leicestershire, UK
Ethylenediaminetetraacetic acid disodium salt (EDTA)	Sigma-Aldrich, Dorset, UK
Ficoll 400	Sigma-Aldrich, Dorset, UK
Fetal calf serum	Sigma-Aldrich, Dorset, UK
GC solution	Roche, Burgess Hill, UK
Glacial acetic acid	Fisher, Leicestershire, UK
Glucose	Sigma-Aldrich, Dorset, UK
HEPES (Salt free)	Sigma-Aldrich, Dorset, UK
HindIII restriction enzyme	Promega, Southampton, UK
HinP1I restriction enzyme	New England Biolabs, Herts, UK
HotStar Taq	Qiagen, West Sussex, UK
HpaII methyltransferase and buffer	New England Biolabs, Herts, UK

HpaII Restriction enzyme and buffer	Promega, Southampton, UK
Insulin from porcine pancreas	Sigma-Aldrich, Dorset, UK
Isopropanol	Fisher, Leicestershire, UK
IPTG	Melford Laboratories, Ipswich, UK
Leptin (Recombinant Murine)	Insight Biotech, Wembley, UK
L-Glutamine	Sigma-Aldrich, Dorset, UK
Luria broth (LB) base	Sigma-Aldrich, Dorset, UK
Lysozyme (from chicken egg white)	Sigma-Aldrich, Dorset, UK
M-MLV reverse transcriptase	Sigma-Aldrich, Dorset, UK
Orange G	Sigma-Aldrich, Dorset, UK
Penicillin streptomycin 100× Solution	Sigma-Aldrich, Dorset, UK
Pfu DNA polymerase	Promega, Southampton, UK
Phenol	Fisher, Leicestershire, UK
Phosphate buffered saline (PBS)	Oxon, UK
Poly(ethylene glycol) 6000 (PEG)	Sigma-Aldrich, Dorset, UK
Potassium acetate (KAc)	Sigma-Aldrich, Dorset, UK
Propan-2-ol	Fisher, Leicestershire, UK
Proteinase K	Promega, Southampton, UK
Random primers	Promega, Southampton, UK
RNAse A	Sigma-Aldrich, Dorset, UK
SacI restriction enzyme	Promega, Southampton, UK
Sodium acetate (NaAc)	Sigma-Aldrich, Dorset, UK
Sodium chloride (NaCl)	Fisher, Leicestershire, UK
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich, Dorset, UK
Sodium hydroxide (NaOH)	Sigma-Aldrich, Dorset, UK
Sodium phosphate dibasic (Na_2HPO_4)	Sigma-Aldrich, Dorset, UK
Stat3 inhibitor	Merck Chemicals Ltd, Notts, UK
Streptavidin sepharose HP beads	GE Healthcare Life Sci, Bucks, UK
Sucrose	Sigma-Aldrich, Dorset, UK
SYBR Green JumpStart taq ready mix	Sigma-Aldrich, Dorset, UK
T4 DNA ligase	Promega, Southampton, UK
Taq DNA polymerase – Hot Start	Promega, Southampton, UK
TRIReagent	Sigma-Aldrich, Dorset, UK
Tris	Fisher, Leicestershire, UK
Triton® X-100	Sigma-Aldrich, Dorset, UK
Trypsin-EDTA	Sigma-Aldrich, Dorset, UK
Wash buffer (Pyrosequencing)	Biotage AB, Kungsgatan, Sweeden
X-Gal	Melford Laboratories, Ipswich, UK
XhoI restriction enzyme	Promega, Southampton, UK

2.1.1 Sterilization

Water

All water used in experiments was obtained from a Purite Neptune (Purite Ltd Oxon, UK) and autoclaved at 3 bar, 121^{o}C for 15 minutes.

DEPC treated water

DEPC treated water was used for all RNA/cDNA related work. DEPC was added to a final concentration of 0.1% in deionised water (Purite Neptune, Purite Ltd Oxon, UK) and left 16hr. The solution was subsequently autoclaved to degrade DEPC at 3 bar, 121^{o}C for 15 minutes.

Sterilization of solutions

Where mentioned, all solutions were autoclaved at 3 bar, 121^{o}C for 15 minutes. Heat labile solutions including calcium chloride and antibiotics were filter sterilized using Millipore 0.2µM filters.

2.1.2 Bacterial cell culture

Luria broth culture medium

20g LB base/ litre dH_2O, sterilized by autoclaving.

LB agar

20g LB base/litre dH_2O, 1.5% Agar, sterilized by autoclaving. LB Agar plates were prepared by pouring 25ml of cooled autoclaved LB agar into 90mm Petri dishes and allowed to cool. Plates were then dried at 37^{o}C, sealed and stored at 4^{o}C until use.

Media supplements / antibiotics

Ampicillin stocks were made to a concentration of 100mg/ml and filter sterilized through a 0.2µM filter. Stocks were aliquoted and stored at -20^{o}C. These stocks were used to inoculate culture media (LB and LB Agar) after autoclaving, at a final concentration of 100µg/ml.

To prepare agar supplemented with ampicillin for blue / white colour screening plates, 5% w/v X-Gal in Di-methyl Formamide was prepared and used to supplement the autoclaved agar to a final concentration of 0.02% and 100µM IPTG was prepared and filter sterilized and added to a final concentration of 0.5µM.

2.1.3 DNA purification

TNES

50mM Tris pH 7.5, 400mM NaCl, 100mM EDTA, 0.5% SDS, stored room temperature

RNAse A (DNAse free)

RNAse A 1mg/ml in 10mM Tris pH7.5, 15mM NaCl, boiled for 15 minutes and cooled slowly to room temperature. Stocks are aliquoted and stored at -20^{o}C.

Mini prep solution I

50mM glucose, 25mM Tris.Cl pH 8.0, 10mM EDTA pH 8.0. Autoclaved, stored 4^{o}C.

Mini prep solution II

0.2N NaOH freshly diluted from 10N stock, 1% SDS. Stored at room temperature.

Mini prep solution III

60ml 5M KAc, 11.5ml glacial acetic acid, 28.5ml dH_2O, resulting in a solution that is 3M with respect to potassium and 5M with respect to acetate. Stored at room temperature.

Maxi prep sucrose-tris buffer

25% sucrose, 50mM Tris pH 8.0, stored room temperature

Maxi prep 3×triton buffer

1.5ml Triton X-100, 7.5ml 1M Tris pH 8.0, 18.75ml 0.5M EDTA, 22.5ml dH_2O. Stored at room temperature.

Maxi prep peg buffer

10mM Tris pH 8.0, 1mM EDTA, 1M NaCl, 20% PEG 6000. Autoclaved and stored at room temperature.

2.1.4 Agarose gel electrophoresis

1×Tris acetate EDTA buffer (TAE)

0.04M Tris Acetate, 0.001M EDTA

Agarose gel

0.8%, 1% or 1.5% in 1×TAE

DNA loading solution

20% Ficoll 400, 0.25% Orange G in 1×TAE buffer

2.1.5 Cell culture

Phosphate buffered saline

Prepared from tablets according to manufacturers instructions and autoclaved at 3 bar, 121^{o}C for 15 minutes.

3T3-L1 media

10% foetal calf serum, 2mM L-Glutamine in Dulbecco's modified Eagle's medium (DMEM).

3T3-L1 differentiation media

3T3-L1 media supplemented with 0.5mM 3-isobutyl-1-methylxanthine, 0.25µM dex, 175nM insulin.

HepG2 complete media

DMEM supplemented with 10% fetal bovine serum, 2mM glutamine, 10U/ml penicillin and 100ug/ml streptomycin.

No adds media

DMEM only.

2×HBS

A stock solution of 10×HBS (8.18% NaCl, 5.94% Hepes, 0.2% $Na_2 HPO_4$) was diluted to 2× with dH_2O. The 2× solution was then adjusted to pH 7.12 exactly using NaOH. This was then filter sterilized and stored at 4^{o}C.

HepG2 cell treatments

Leptin stock solution 1mg/ml in dH_2O, aliquoted and stored -20^{o}C. Diluted in HepG2 complete media for appropriate concentration.

CFA stock solution 100mM in 100% ethanol, made fresh. Diluted in HepG2 complete media for appropriate concentration.

Dex stock solution 1mg/ml in 100% ethanol, stored -20^{0}C. Diluted in HepG2 complete media for appropriate concentration.

Stat3 Inhibitor 500μM in dH_2O, stored -20°C. Diluted in HepG2 complete media for appropriate concentration.

2.2 Methods

2.2.1 Animal methods

All rat work was performed by the Liggins Institute, University of Auckland, New Zealand. Rats used in the study were from an established model of fetal programming by maternal undernutrition [227].

Virgin Wistar rats age 100 ± 5 days were time mated and after mating housed in the same room in individual cages with free access to water, 12 hour light 12 hour dark cycle at a constant temperature of 25°C. Animals were allocated to either the undernutrition group (UN) (diet 30% of *ad libitum*) throughout gestation or the standard diet *ad libitum* (AD) throughout gestation. Recordings were made of maternal weight and food intake until pregnancy ended. At birth all pups were weighed. To ensure standardised nutrition of pups until weaning, litter size was adjusted to 8 pups per litter. Pups from the UN group were cross-fostered onto dams from the AD group. At postnatal day 3 female pups from both AD and UN groups were randomly selected to receive either saline or recombinant rat leptin (L) (2.5μg/g/day) for a total of 10 days by subcutaneous injection (n=16/group). From treatment period and until weaning, all animals were maintained on *ad libitum* diet. At weaning, saline or leptin treated AD and UN offspring were weight matched and placed on either standard rat chow (C) or a high fat (HF) diet (Research Diets Inc no 12451, 45% kcal as fat). At postnatal day 170, rats were fasted overnight and killed by halothane anaesthesia followed by decapitation, resulting in 8 treatment groups with 8 female adult offspring per group (figure 2.1).

All animal work was approved by the Animal Ethics Committee of the University of Auckland. Samples of liver and adipose tissue were provided snap frozen.

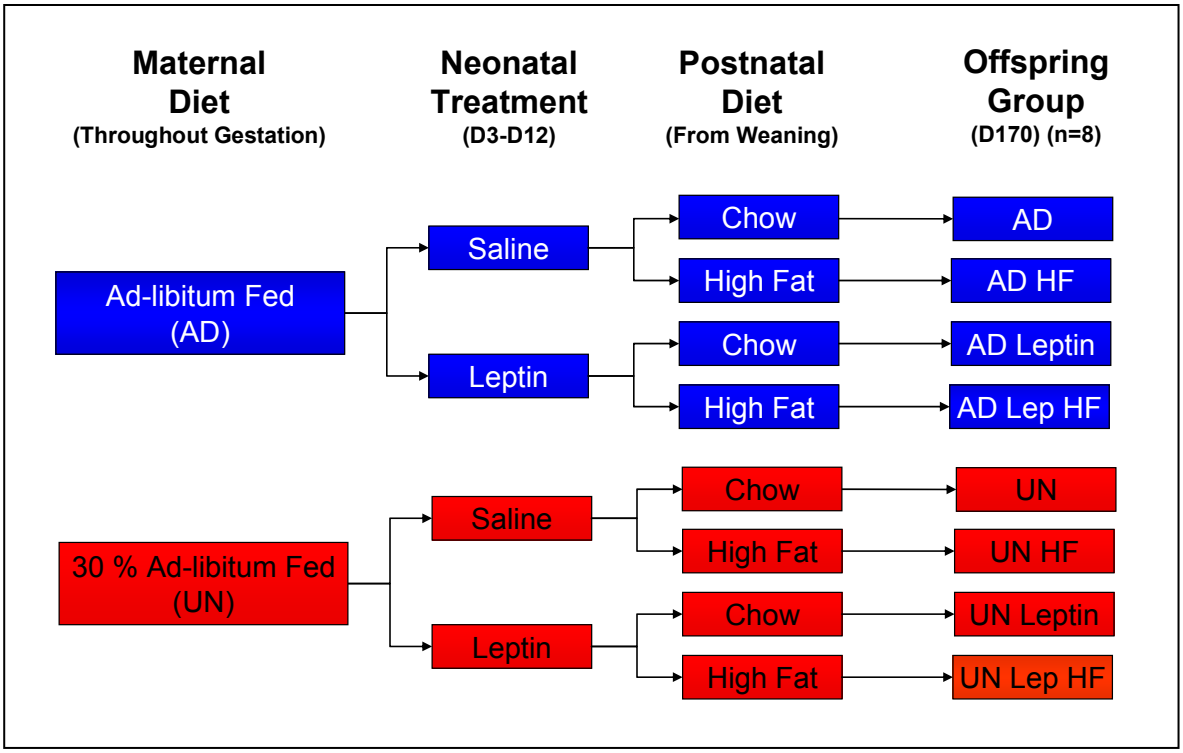


Figure 2.1 **Design of rat study.** Maternal and postnatal diet groups, treatments and timings are shown.

2.2.2 Isolation and digestion of genomic DNA

High salt genomic DNA isolation from liver tissue

30mg of snap frozen tissue was ground under liquid nitrogen and incubated with 500µl TNES and 2.5µl 20mg/ml proteinase K at 55°C for 16 hours. 500µl 2.6M NaCl was added to make a final concentration of 1.5M and shaken vigorously for 15 seconds. Samples were centrifuged at 12,000rpm for 5 min and the supernatant removed to a new tube. An equal volume of ethanol was added to the supernatant. Precipitated genomic DNA was spooled into 500µl sterile water. Quality and quantity of genomic DNA was checked on the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, USA. All DNA had a 260/280 ratio of >1.7. Genomic DNA was stored at -20°C.

Isolation of genomic DNA from adipose tissue

30mg of snap frozen adipose tissue was ground under liquid nitrogen. Isolation of DNA was then performed using the Wizard SV Genomic DNA Purification System according to manufacturer's instructions (Promega, Southampton, UK). DNA was eluted in sterile water. Quality and quantity of genomic DNA was checked on the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, USA). All DNA had a 260/280 ratio of >1.7. Genomic DNA was stored at -20°C.

Digestion of genomic DNA

All genomic DNA for use in methylation sensitive real time PCR was cut with the methylation sensitive restriction endonucleases AciI (5'-C▼CGC) and Hinp1I (G▼CGC). 500ng genomic DNA was incubated with 5U of each enzyme at 37°C for 24 hours in a total volume of 25µl. The DNA was then analysed for digestion on a 0.8% agarose gel and then stored at -20°C.

2.2.3 Agarose gel electrophoresis of DNA/RNA

All DNA/RNA used in experiments was checked for integrity, or for analysis of digestion on a 0.8% agarose gel (genomic DNA), a 1% agarose gel (plasmid DNA/RNA) or a 1.5% agarose gel (PCR product) in 1× TAE buffer at 100v for 40 minutes.

2.2.4 Isolation of RNA from liver, adipose, muscle and kidney tissue

50-100mg of snap frozen tissue was ground under liquid nitrogen and 1ml TRI Reagent added. Samples were then spun 12,000rpm at 4°C 10 minutes and supernatant removed to a clean tube. The lipid layer was also removed from adipose samples. Extraction of total cellular RNA was then performed according to manufacturer's instructions. All RNA was resuspended in DEPC treated water. Quality and quantity of RNA was checked on the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, USA) and diluted to 500ng/µl. All RNA had a 260/280 ratio of >1.7. RNA was stored at -70°C.

2.2.5 cDNA synthesis

1µg total RNA was used as a template for cDNA synthesis and incubated with 500ng random primers, 1µl 10mM dNTP's and 200U M-MLV Reverse Transcriptase according to manufacturer's instructions. cDNA was stored at -70°C until use.

2.2.6 Real time PCR

Primers for real time PCR

Real time PCR primers (table 2.2) were designed using Beacon Designer 2.0 software (Premier Biosoft, CA, USA). For expression, primers were designed to span intron-exon boundaries or exons and for methylation specific PCR primers were designed within CpG islands (figure 2.2).

All primers were supplied by Invitrogen (Paisley, UK), made to a concentration of 100µM and stored at -20°C.

Real time PCR

cDNA was amplified for quantification using 12.5µl SYBR Green JumpStart Taq Ready Mix, 1µl cDNA and forward and reverse primers to a final concentration of 0.2µM and made to a final volume of 25µl with DEPC treated water. For methylation sensitive real time PCR, 25ng digested genomic DNA was used instead of cDNA. Real time PCR was performed using the DNA Engine Opticon 2 Real Time PCR Detection System (MJ Research, MA, USA). Cycling conditions were as follows; initial denaturation of 94°C 2 minutes, followed by 40 cycles of 94°C 30s, anneal at a primer specific temperature (table 2.2) 1 minute and extension at 72°C 1 minute. DNA samples were normalised to PPARγ2 which contains no AciI or HinP1I restriction sites and cDNA samples were normalised to either 18s (adipose tissue samples) or cyclophillin (Cyc) (liver tissue samples) and quantified using the ΔCt method [228].

Statistical analysis of real time PCR

Statistical comparisons of gene expression between data groups were by a general linear model using maternal diet, leptin treatement and post-weaning diet as fixed factors with Bonferonni's *post hoc* analysis. For female and male liver expression analysis, a retrospective statistical power calculation was performed unsing an online statistical power calculator tool: www.dssresearch.com/toolkit/spcalc/power_a2.asp.

Table 2.2 Sequence and anneal temperatures of methylation sensitive PCR and real time PCR primers

Gene	Forward Primer (5'→3')	Reverse Primer (5'→3')	Temp
	Methylation-Sensitive PCR		
LR	CATTTCCCACATCTGCTGCTG	CCCGAATGTCCTGCGTCCTG	68.1
AOX	ACAATGAACCCTTTCCCGAACG	AGGAGAGCAAGGTAGAAGGTCAAG	60
Leptin	GACCCGTCCTTAAACTACC	TGCCTGCCCCTCTTATAG	60
PPAR α	TGTGTCTCGTTCTGAACCG	TCCACCCACCTCACTGTC	52.9
PPAR γ2	GTCTCTGCTCTGGTAATTC	AAGGCTTGTGGTCATTGAG	60
GR	CGTCTTGTTCCACCCACT	CCTTGCAGTTGCCGACAG	61
11βHSD2	GCGTGCCAATAGGCTCACA	TGGCTAGATCCGCTTTCGAA	60
PPAR γ1	CGACTGTGAGGAGCAAGG	CCCAGGTCTCTTCTTCAG	60
	mRNA Expression		
Cyc	TTGGGTCGCGTCTGCTTCGA	GCCAGGACCTGTATGCTTCA	60
CPT-1	ACCACTGGCCGAATGTCAAG	AGCGAGTAGCGCATGGTCAT	60
LPL	ACTCGCTCTCAGATGCCCTAC	ATGTCCACCTCCGTGTAAATCAAG	60
LR	ATGCTGTGCAGTCACTCAGTG	CAACTCCTTCCATAAATACTGGG	60
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGTAGCG	60
AOX	CCAATCACGCAATAGTTCTGG	CGCTGTATCGTATGGCGAT	60
Leptin	CACACACGCAGTCGGTATCC	AGGTCTCGCAGGTTCTCCAG	60
PPARα	CGGGTCATACTCGCAGGAAAG	TGGCAGCAGTGGAAGAATCG	60
PPARαP1	CGCAGCCTCAGCCAAGAC	CTCTGTGTCCACCATGTTGAATG	60
PPARαP2	CTGGGTCCTCTGGTTGTC	GCCTTGAACTTCAGTCTTGG	60
PPARγ2	TATGCTGTTATGGGTGAAAC	TGGTAATTTCTTGTGAAGTGCTC	60
GR	GGAGAATTATGACCACACTCAAC	GCAGTAGGTAAGGACATTCTCAA	58
11βHSD2	TGGCCACTGTGTTGGATTT	ATCGGCCACTACCATGTTG	60
GR 1 10	TGACTTCCTTCTCCGTGACA	GGAGAATCCTCTGCTGCTTG	65
Dnmt1	QIAGEN Quantitect primer assay	QT00493577	55
Dnmt3a	QIAGEN Quantitect primer assay	QT00429380	55
PEPCK	AGCTGCATAATGGTCTGG	GAACCTGGCGTTGAATGC	53
IR	ATGCCACCAATCCTTCCGTTCC	GTCCTCCGCCTGCCTCTCC	52.5
HSL	ACGGAGATTGAGGTGCTATC	AGGTGAGATGGTAACTGTGAG	52.5

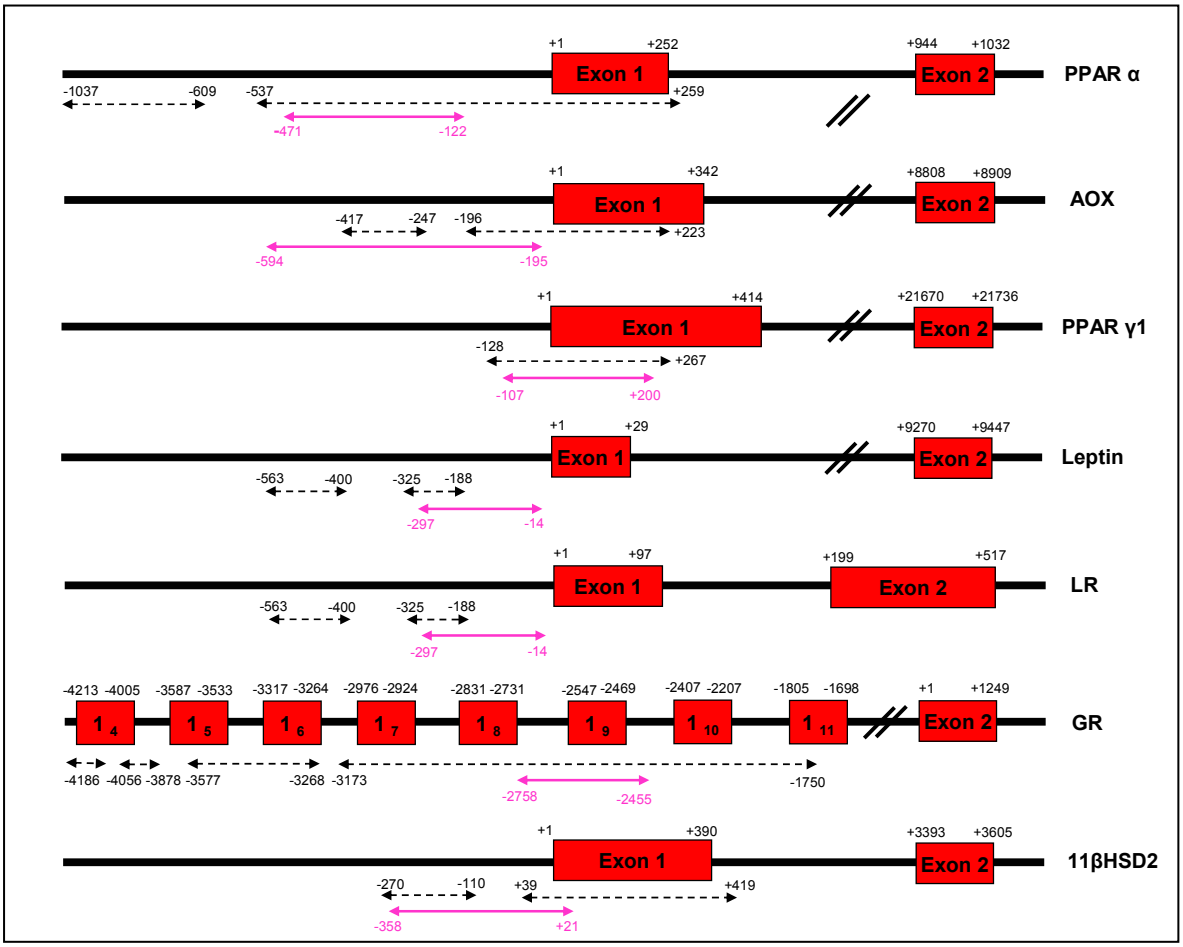


Figure 2.2 A schematic diagram showing the location of MSP amplicons relative to CpG islands for all MSP amplicons. The first 2 exons are shown for each gene in red. The position of methylation sensitive PCR amplicons and CpG islands relative to exons are shown by pink arrows and black dashed arrows respectively.

2.2.7 Gene cloning

Ligation

Insert DNA was ligated to vector DNA at a insert:vector molar ratio of 3:1 using 3U T4 DNA ligase in a total volume of 10µl. Ligations were incubated at 4°C overnight. Ligations without insert DNA were included as a negative control.

Preparation of competent DH5α cells

DH5α cells were made competent just prior to transformation to enhance their ability to take up DNA. A single colony of DH5α cells was picked from an LB agar plate and used to inoculate 10ml LB. This was incubated for 16hrs at 37°C with agitation to bring cultures to the stationery phase. An aliquot of cells were then removed and diluted 1:100 with fresh LB and incubated at

37°C with agitation until cultures had reached an OD_{600} of 0.6 whereby cells had reached the exponential phase. Cultures were then centrifuged at 3,000rpm 5 min at 4°C and pellets were resuspended in 5ml ice cold filter sterile 100mM $CaCl_2$. Cells were then centrifuged again at 3,000rpm for 5 min and resulting cell pellets resuspended in 500µl ice cold filter sterile 100mM $CaCl_2$. Competent DH5α cells were kept on ice until needed.

Transformation into DH5α bacterial cells

All plasmid constructs prepared were transformed into competent DH5α cells. 2µl ligated plasmid DNA or 0.5µl complete plasmid DNA were added to pre-chilled eppendorf tubes and kept on ice. Next, 100µl competent DH5α cells were added to the DNA and samples mixed well. DNA/cell mixtures were incubated on ice for 30 minutes and then subjected to a heat shock 2 minutes at 42°C to allow the cells to take up the DNA. Cells were then subjected to a further incubation on ice for 20 minutes. Next 400µl LB was added to the samples which were subsequently incubated at 37°C for 60 minutes with shaking. Cells were then centrifuged at 3,000rpm 5 minutes and all but the last 100µl supernatant discarded. Cell pellets were then resuspended in the remaining LB and spread over LB agar plates supplemented with 100µg/ml ampicillin. The use of ampicillin in the medium enables transformants to be distinguished from non-transformants, as only the cells which have successfully taken up plasmids containing the Amp^r gene will survive. Plates were incubated overnight inverted at 37°C and then sealed and stored at 4°C until needed.

Preparation of glycerol stocks

Single colonies were picked from agar plates and used to inoculate 10ml LB. This was incubated at 37°C overnight with agitation. 500µl of LB was then added to 500µl of a 50% LB/50% Glycerol solution and mixed well. Glycerol stocks were stored at -80°C.

Purification of plasmid DNA

Glycerol stocks were streaked out onto agar plates containing 100µg/ml ampicillin. Colonies were allowed to grow overnight at 37°C. Single colonies were then picked and used to inoculate 10ml LB containing 100µg/ml ampicillin. For small scale plasmid preparations, cultures were grown overnight at 37°C with agitation. For large scale plasmid preparations, cultures were grown to an DO_{600} of 0.6, then used to inoculate 500ml LB containing 100µg/ml ampicillin and allowed to grow overnight at 37°C with agitation.

For small scale plasmid DNA preparations, 10ml cultures were centrifuged at 3,500 rpm for 10 min at 4°C. Cell pellets were resuspended in 100µl of solution I and incubated at room temperature for 5 minutes. Next, 200µl of solution II was added, mixed well and incubated on ice for 5 minutes. 150µl solution III was then added, mixed well and incubated on ice for 15 minutes. Cell lysates were then centrifuged at 12,000 rpm for 10 minutes and the supernatant removed to a clean microfuge tube. 500µl of a 50:50 phenol:chloroform mix was added to the supernatant and mixed

well. This was then centrifuged at 12,000 rpm 5 minutes and the top aqueous layer removed. The DNA was then precipitated using 1/10th volume of 3M NaAc pH5.2 and 2 volumes of ethanol. This was incubated at -20^{o}C for 20 minutes and then centrifuged at 12,000 rpm for 15 minutes. The resulting DNA pellet was resuspended in 50µl dH_2O. To remove RNA from manual DNA preparations, RNAse A was added to a final concentration of 10µg/ml and incubated at 37^{o}C for 60 minutes. DNA was then cleaned again using the phenol-chloroform and ethanol precipitation procedure. When DNA was needed for DNA sequencing submission, plasmid DNA was purified using QIAprep Spin Miniprep Kit (Qiagen, Crawley, UK) according to manufacturer's instructions.

Large scale preparations of plasmid DNA were prepared for cell culture experiments. 500ml cell cultures were centrifuged at 3,000 rpm at 4^{o}C for 20 minutes. Cell pellets were resuspended in 4ml sucrose tris buffer and 100µl of 40 mg/ml lysozyme and incubated on ice for 15 minutes. Next, EDTA was added to a final concentration of 10mM and incubated on ice for 15 minutes. ½ volume of 3×triton buffer was then added to the cell lysate, mixed well and incubated on ice for 60 minutes. This was then centrifuged at 18,000 rpm for 90 minutes at 4^{o}C. The supernatant was removed to a clean tube and NaCl added to a final concentration of 0.5M. Next, an equal volume of phenol:chloroform mixture added, mixed well and then centrifuged at 4,000 rpm for 10 minutes. The top aqueous layer was then removed to a clean tube and an equal volume of chloroform added and mixed well. This was then centrifuged at 3,500 rpm for 10 minutes and the top aqueous layer removed. PEG was added to a concentration of 10% w/v and this was dissolved at 37^{o}C for 20 minutes. This solution was then incubated at 4^{o}C overnight. The next day, this was centrifuged at 12,000 rpm for 20 minutes and the resulting pellet re-dissolved in 500µl 0.1M tris pH 8.0 and RNase. This was incubated at 37^{o}C for 30 minutes. Next, 500µl PEG buffer was added and incubated on ice for 60 minutes. This was then centrifuged at 12,000 rpm for 15 minutes and the resulting pellet re-dissolved in 400µl of 10mM tris pH 8.0, 0.5M NaCl. The resulting DNA solution was then subjected to phenol:chloroform extraction and ethanol precipitation as previously described. All DNA preparations were checked for integrity on a 1% agarose gel and for concentration and purity 2µl was measured on the NanoDrop.

2.2.8 5' RNA ligase mediated rapid amplification of cDNA ends (5' RLM RACE)

RACE procedure

5' ends of RNA were amplified using a 5'RLM RACE kit (Ambion, Austin, USA) according to manufacturer's instructions. Briefly, 10µg total RNA from liver, adipose, muscle and kidney were incubated with calf intestinal alkaline phosphatase (CIP) for 1hr at 37^{o}C to remove free 5' phosphates from any degraded / fragmented RNA or contaminating DNA. RNA was then cleaned by adding 15µl ammonium acetate, 115µl nuclease free water and 150µl phenol:chloroform. This was vortexed and centrifuged at 12,000rpm for 5 minutes. The top aqueous layer containing the

RNA was precipitated by adding 150µl isopropanol and then chilled on ice for 10 minutes. The RNA was pelleted by centrifuging at 12,000rpm for 20 minutes and the resulting pellet washed in 70% ethanol. The RNA pellet was next resuspended in 11µl nuclease free water and incubated with tobacco acid pyrophosphatase (TAP) for 1hr at 37^{o}C to remove the CAP structure from all the intact RNA. The TAP treated RNA was then incubated with 5' RACE adapter and T4 RNA ligase for 1hr at 37^{o}C. 2µl of ligated RNA was used in a standard reverse transcriptase reaction.

PCR

In order to amplify the 5' region of PPARα mRNA generated from the 5'RLM RACE, nested gene specific reverse primers for PPARα were designed to use with the standard forward 5' RACE primers. These primers were designed so that the 5' end of the transcript would be amplified without giving too large a product (figure 2.3). A forward gene specific primer was also designed to ensure that the mRNA population being investigated contained the transcript of interest. Primers were evaluated using Netprimer software (Premier Biosoft Int, Palo Alto, USA). All primers were supplied by Invitrogen (Paisley, UK), made to a concentration of 100µM and stored at -20^{o}C. For primer sequences, see (table 2.3).

2µl liver 5'RLM RACE cDNA and 5µl of adipose, kidney and muscle 5'RLM RACE cDNA were amplified using 1.25U Promega Taq DNA polymerase with $MgCl_2$ to a final concentration of 1.5mM, forward and reverse primers to a final concentration of 0.4µM and dNTP's at a final concentration of 0.2µM each in a total volume of 50µl. 2µl or 5µl outer PCR product was used as template for the nested PCR with the same conditions. PCR was performed using the PCR Express PCR machine DNA (Thermo Fisher Scientific, Epsom UK). Cycling conditions for both outer and nested PCR were as follows; initial denaturation of 94^{0}C 3 minutes, followed by 35 cycles of 94^{o}C 30s, anneal at a 60^{o}C 30 sec and extension at 72^{o}C 1 minute 30 sec. Resulting PCR products from the inner PCR reaction were gel extracted using QIAquick Gel Extraction Kit (Qiagen, Crawley, UK) and 2µl measured on the NanoDrop. Purity of bands was checked by agarose gel electrophoresis.

Cloning RACE PCR products using Promega pGEM T-Easy vector system

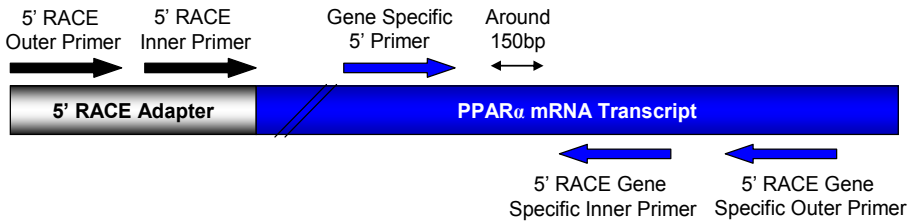
25ng of each purified Liver (P2 and P3) and Adipose (P1) RACE PCR product was ligated with 50ng pGem T-Easy cloning vector (Promega, Southampton, UK) for a 3:1 insert:vector molar ratio. This cloning vector has single 3'-T overhangs (figure 2.4) and PCR products generated by Taq DNA Polymerase have a single deoxyadenosine added to the 3'-ends, allowing easy ligation to the pGem T-Easy vector. In addition, the vector contains the Amp^{r} gene. Ligations were transformed into competent DH5α cells and plated out onto XGal-IPTG-ampicillin agar plates. Successful ligation of inserts into this vector interrupts the coding region of β-galactosidase and this allows recombinants to be identified by colour screening; positive colonies are white, whilst negative colonies blue. Positive white colonies were picked and grown in 10ml LB supplemented with

100µG/ML ampicillin at 37°C overnight with agitation. These cultures were used to prepare small scale plasmid preparations. The pGem T-Easy vector has EcoRI restriction sites either side of the cloned insert within the multiple cloning region (figure 2.4). To confirm presence of the RACE insert in the vector, 500ng of all DNA preparations were digested with 12U of the restriction endonuclease EcoRI in a total volume of 10µl at 37°C for 60 minutes (figure 2.5). Positive clones for liver and adipose samples were sequenced by university of Dundee sequencing service using a T7 sequencing primer (Dundee, UK). The muscle and kidney RACE PCR products were sequenced directly by the University of Dundee sequencing service using the gene specific reverse inner primer. In addition to this, a PPARα heart RACE PCR product prepared by Dr Jo Slater-Jefferies using the same primers was also sequenced directly by the University of Dundee.

Table 2.3 Gene specific primer sequences for PPARα 5' RLM RACE

Primer	Sequence (5' → 3')
Gene Specific Forward	TTCGTGGAGTCCTGGAACTGAA
Gene Specific Reverse Outer	AGCCTTCACATGCGTGGACT
Gene Specific Reverse Inner	TGACTGAGGAGGGGCTGGAA

A



B

GAACTGTCCGCTACTTCGAGTCCCCTTGAGCGCCGTGTGCCGGCTCTGAACATTGGCGTT

Exon 1 CGCAGCTGTTTTGTGGGCTGGAGGGTTCGTGGAGTCCTGGAACTGAAGCGACGCTGGGTC

CTCTGGTTGTCCCCTTGAGGGGAGGGCACACGAGCGGGGACATCGGGGCGCTCCCTTCCC

ACAGCGTGGTGCATTTGGGCGTAACTCACCGGGAGGCGTTTCCTGAGACCCTCGGGGATC

TTAGAGGCGAGCCAAGACTGAAGTTCAAGGCCCTGCCTTCCCTGTGAACTGACATTTGTG

Exon 2 ACTGGTCAAGCTCAGGACACAAGACGTTGTCATCACAGATTGGTGCTCTGTGGCCCGCCT

GGCCACAACCATTCAACATGGTGGACACAGAGAGCCCCATCTGTCCTCTCTCCCCACTTG

Exon 3 AAGCAGATGACCTGGAAAGTCCCTTATCTGAAGAATTCTTACAAGAGATGGGAAACATTC

AAGAGATTTCTCAGTCCCTCGGAGAGGAGAGTTCCGGAAGCTTTAGTTTTGCGGACTACC

AGTACTTAGGGAGCTGTCCAGGCTCGGAGGGCTCTGTCATCACAGACACCCTCTCTCCAG

Exon 4 CTTCCAGCCCCTCCTCAGTCAGCTGCCCTGCTGTCCCCACCAGTACAGATGAGTCCCCTG

GCAATGCACTGAACATCGAGTGTCGAATATGTGGGGACAAGGCCTCAGGATACCACTATG

GAGTCCACGCATGTGAAGGCTGCAAGGGCTTCTTTCGGCGAACTATTCGGCTAAAGCTGG

Exon 5 CGTACGACAAGTGTGATCGAAGCTGCAAGATTCAGAAAAAGAACCGGAACAAATGCCAGT

ACTGCCGTTTCCACAAGTGCCTGTCCGTCGGGATGTCACACAATGCAATCCGTTTTGGAA

Figure 2.3 Locations of primers for PPARα 5' RLM RACE. (**A**) 5' RLM Race Nested PCR Design. (B) First 5 exons of PPARα mRNA Transcript (Ensembl Transcript ID: ENSRNOT00000030082) showing location of gene specific RACE primers. Exons 1-5 are indicated in alternating blue and black. Forward GS primer is shown in green highlight, reverse GS primers in yellow highlight. Primers were designed to give as small a product as possible, but far enough into sequence to ensure that RACE products would amplify. EcoRI restriction enzyme site is shown in blue highlight.

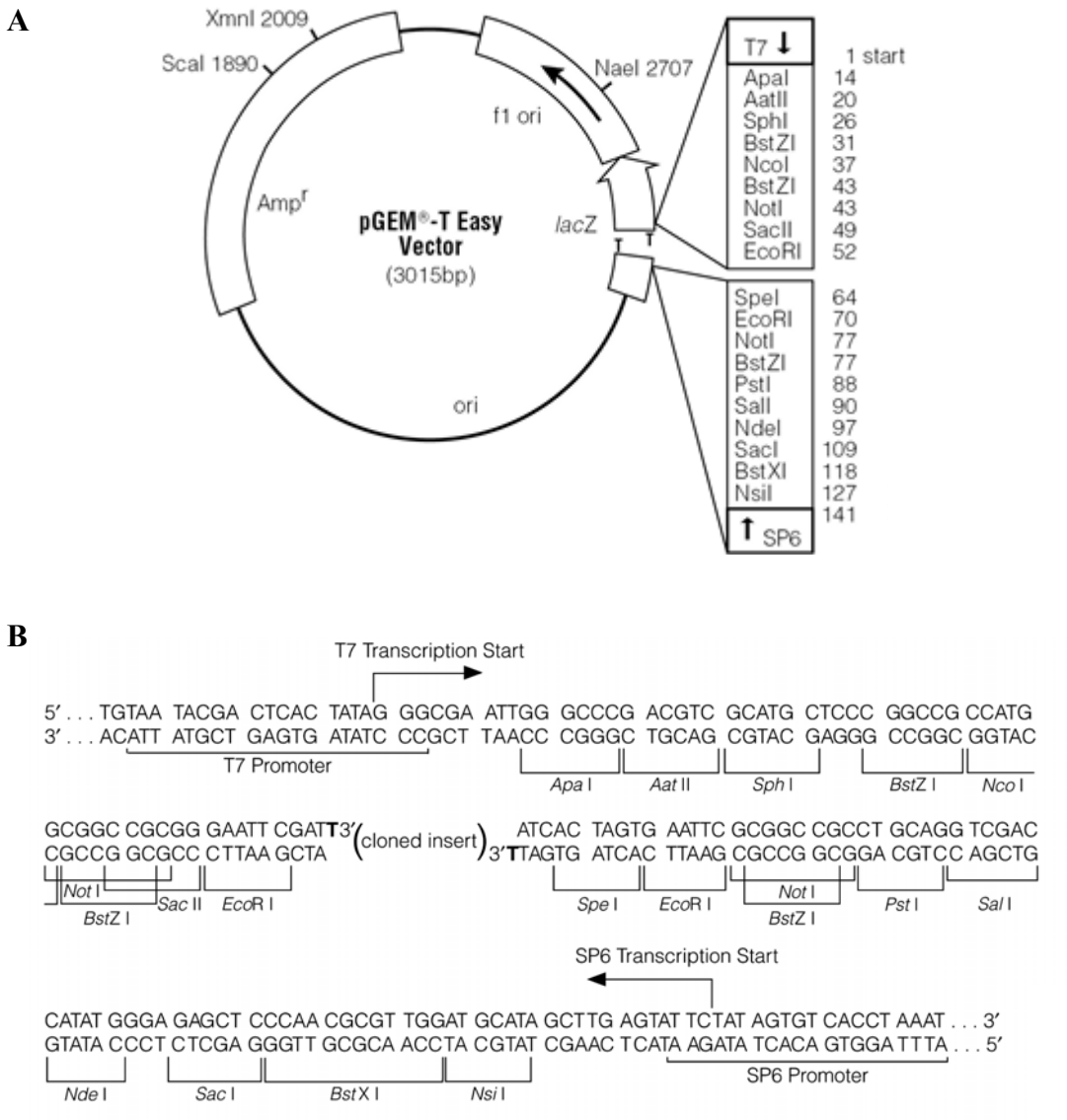


Figure 2.4 Circular map and multiple cloning region of the pGem T-Easy plasmid. (**A**) pGem T-Easy vector circle map showing ampicillin resistance, β- Galactosidase coding region and multiple cloning region. (B) Detailed view of multiple cloning region including 3'-T overhangs, T7 promoter and location of EcoRI restriction sites.

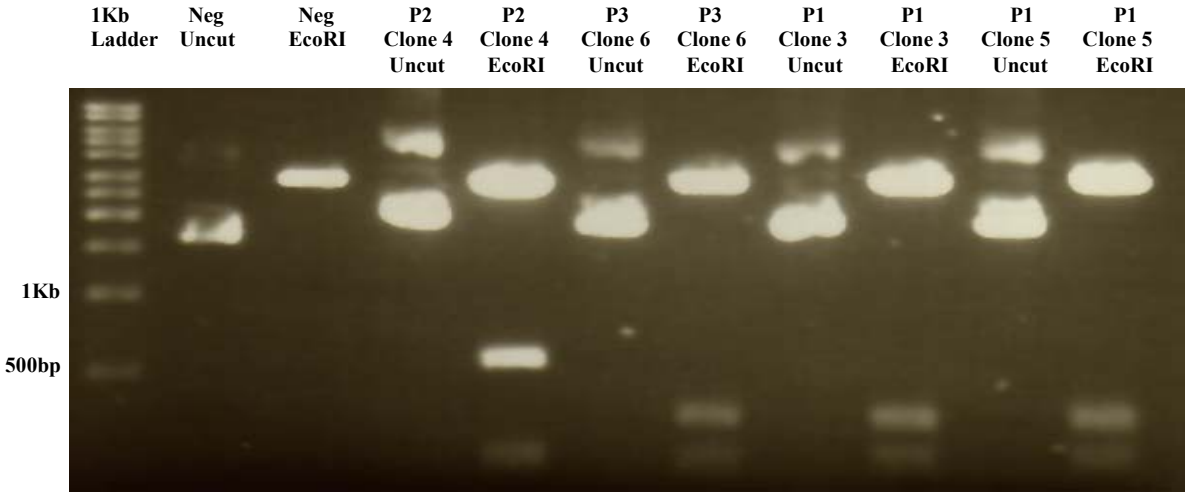


Figure 2.5 Digests of PPARα liver and adipose 5' RACE PCR - pGEM T-Easy clones with EcoRI. A selection of RACE product-pGEM T-Easy clones were digested with EcoRI, whereby successful recombinants will drop out the appropriate sized insert (Liver P2 800bp, Liver P3 450bp and Adipose P1 450bp). Digests are shown for each clone without and with EcoRI. As each insert also contains an EcoRI site, approximate sizes of insert fragments are; P2 550bp+ 250bp, P3 250bp+200bp, P1 250bp+200bp.

2.2.9 pGL3 Basic constructs

PPARα promoter constructs

Primers were designed to amplify 1-1.5Kb of upstream sequence from the transcription start site for the adipose specific transcript (P1), liver specific transcript (P2) and the liver specific transcript (P3). Primers were designed to contain a SacI/XhoI restriction site at the 5' end of the forward primer and a HindIII restriction site at the 5' end of the reverse primer (figure 2.6) to allow cloning of PCR fragments into the pGL3 Basic reporter vector (Promega, Southampton, UK) (figure 2.7). Use of 2 different restriction sites allows easy cloning into the pGL3 reporter vector in the correct orientation. In addition, primers contained an extra 2 bases at the 5' end of each primer to permit efficient digestion of amplified promoters for cloning. Primers were evaluated using Netprimer software (Premier Biosoft Int, Palo Alto, USA). All primers were supplied by Invitrogen (Paisley, UK), made to a concentration of 100µM and stored at -20^{o}C. For primer sequences see (table 2.4).

The pGL3 basic reporter vector contains a modified gene for firefly luciferase, but lacks any functional promoter or enhancer sequences. Presence of the Amp^{r} gene within the vector allows transformants to be identified from non-transformants. Firefly luciferase catalyses a bioluminescent reaction, in which luciferin is converted to oxyluciferin, producing light in the process. Insertion of PPARα regulatory DNA into the multiple cloning region of the vector allows its activity to be assessed in transfected eukaryotic cells by measuring luminescence.

5µg of the pGL3 Basic vector was digested with 25U each of either XhoI and HindIII or SacI and HindIII in a total volume of 50µl at 37^{o}C for 60 minutes. Digested vector was checked on an agarose gel for completion and gel extracted using QIAquick Gel Extraction Kit (Qiagen, Crawley, UK). DNA was quantified using the NanoDrop.

100ng rat genomic DNA (female liver sample 1.0.0) was used as a template for PPARα promoter PCR. DNA was amplified using 1.25U Pfu DNA polymerase (P1, P2) and Taq DNA polymerase (P3) with and forward and reverse primers to a final concentration of 0.4µM and dNTP's at a final concentration of 0.2µM each and 10µl GC solution, in a total volume of 50µl. PCR was performed using the PCR Express PCR machine DNA (Thermo Fisher Scientific, Epsom UK). Cycling conditions for PCR were as follows; initial denaturation of 94^{0}C 2 minutes, followed by 40 cycles of 95^{o}C 45s, anneal at a 60^{o}C 45 sec and extension at 72^{o}C 5 minutes. Resulting PCR products were gel extracted using QIAquick Gel Extraction Kit (Qiagen, Crawley, UK).

All gel extracted PCR products were digested with 10U each of either XhoI and HindIII, or SacI and HindIII in a total volume of 25µl at 37^{o}C for 60 minutes. Digested PCR product was cleaned using QIAquick PCR Cleanup Kit (Qiagen, Crawley, UK). Purity of products was checked by agarose gel electrophoresis (figure 2.8) and 2µl product was measured on the NanoDrop.

Digested pGL3 Basic vector was ligated with the digested PPARα promoter fragments at a molar ratio of 1:3. These ligations were then transformed into DH5α cells. Individual colonies were picked and grown in 10ml LB supplemented with 100µg/ml ampicillin at 37^{o}C overnight with agitation. These cultures were used to prepare small scale plasmid preparations. To confirm presence of the PPARα promoter inserts in the pGL3 Basic vector, 500ng of all DNA preparations were digested with 6U each of either XhoI and HindIII or SacI and HindIII in a total volume of 10µl at 37^{o}C for 60 minutes (figure 2.9). Positive clones were sequenced by university of Dundee sequencing service using a pGl3 Basic sequencing primer (GL primer 2 see figure 2.8B). Subsequent reporter constructs were named P1- pGL3 (adipose specific), P2-pGL3 (liver P2 specific) and P3-pGL3 (liver P3 specific).

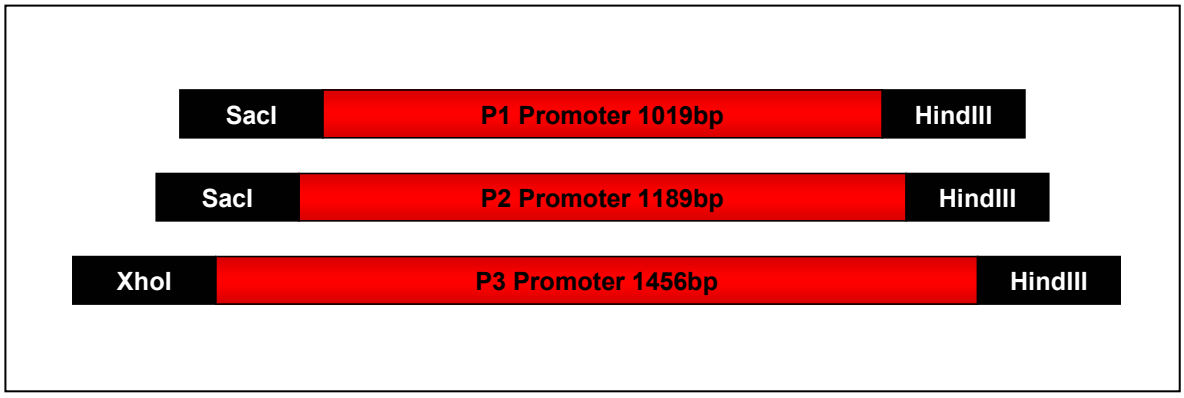
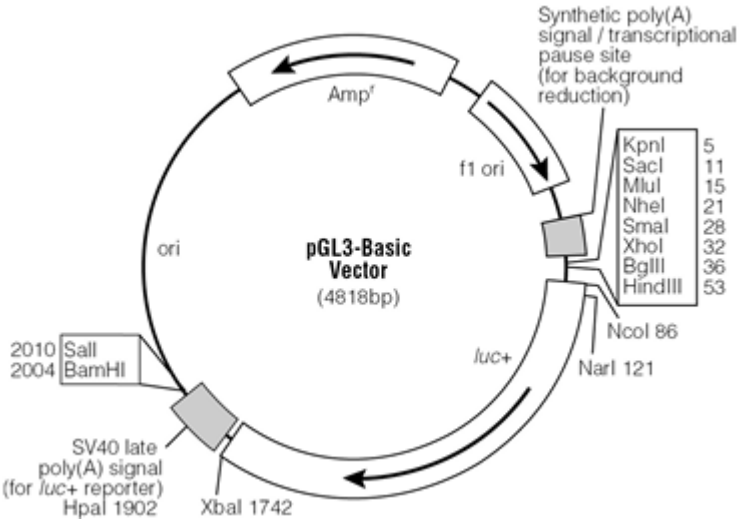


Figure 2.6 Pictorial representation of the PPARα promoter constructs showing the 5' and 3' restriction sites for cloning. The P2 promoter construct (1189bp) and P1 promoter construct (1019bp) have a 5' SacI restriction site (GAGCTC) and a 3' HindIII restriction site (AAGCTT). The P3 promoter construct (1456bp) has a 5' XhoI restriction site (CTCGAG) and a 3' HindIII restriction site (AAGCTT).

Table 2.4 Sequences of the PPARα promoter cloning primers

Primer	Forward Primer Sequence 5'→3'	Reverse Primer Sequence 5'→3'
P1, P2	ATGAGCTCAGCAGCGTCCTGAGGCGTT	
P1		ATAAGCTTACCTGAGGCTGCGCTCCG
P2		ATAAGCTTGTGCCCTTCCTAGCGTGT
P3	ATAAGCTTGGAGTCTTCCTTCTGGTT	ATACTCGAGTCTGCGTGGGTGTCTAAT

A



B

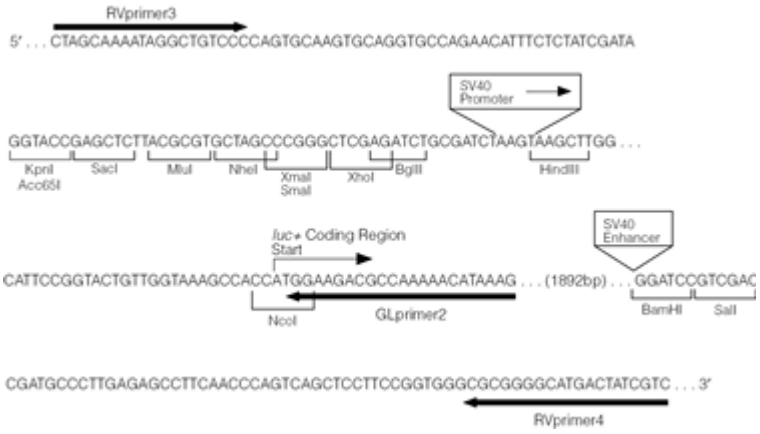


Figure 2.7 Circular map and multiple cloning region of the pGL3 basic reporter vector. (**A**) Circular map showing location of multiple cloning region, luciferase gene and Amp^r. (**B**) Detailed view of pGL3 Basic multiple cloning region showing location of the GL primer 2 and cloning restriction sites.

Figure 2.8 Digested and gel extracted pGL3 basic vector and PPARα promoter fragments. Digested gel extracted DNA is shown for pGL3 Basic (4818bp) and the PPARα promoter fragments; P1 (1019bp), P2 (1189bp), P3 (1456bp).

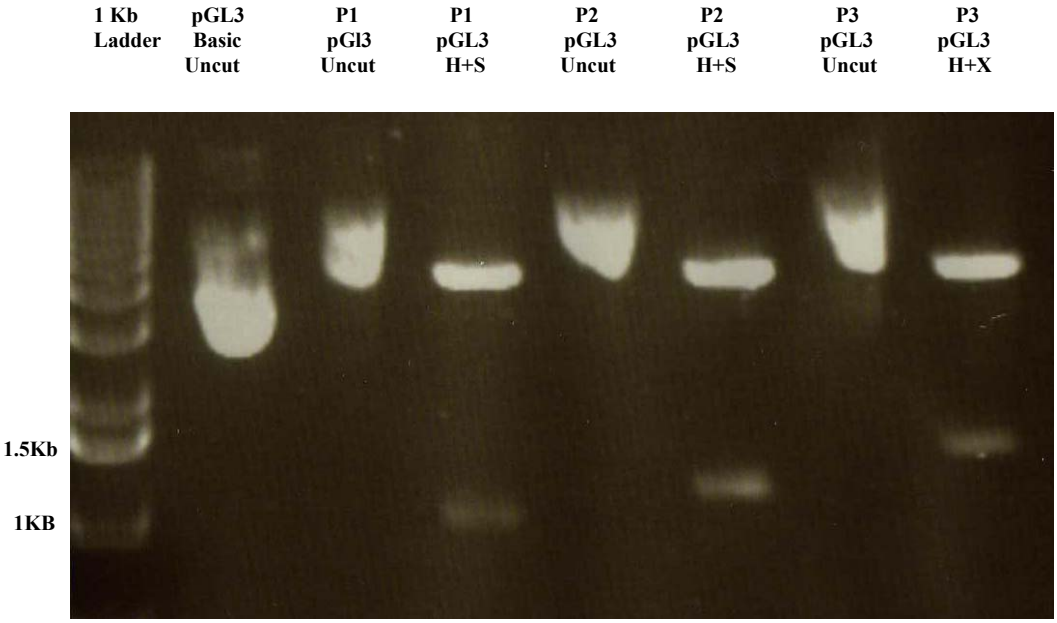


Figure 2.9 Digests of PPARα promoter-pGL3 basic clones to drop out promoter insert. Clones for P1-pGL3, P2-pGL3, P3-pGL3 are shown with and without restriction enzymes to drop out insert (P1+P2 HindIII and SacI, P3 HindIII and XhoI). Correct size inserts are shown for P1 1019bp, P2 1189bp and P3 1456bp.

PPARα promoter-pGL3 5' deletion mutants

PPARα promoter-pGL3 5' deletion constructs were generated using exactly the same procedure as the normal PPARα promoter-pGL3 constructs, except 50ng PPARα P2-pGL3 construct was used as the DNA template for PCR. Primers were designed to amplify the P2 promoter using the same reverse primer, but using forward primers which generated products which progressively lost 200bp from the 5' end of the promoter (figure 2.10, 2.11, table 2.5). Primers were evaluated using Netprimer software (Premier Biosoft Int, Palo Alto, USA). All primers were supplied by Invitrogen (Paisley, UK), made to a concentration of 100µM and stored at -20°C. Subsequent constructs were named according to their size; A 198bp, B 462bp, C 598bp, D 845bp, E 1096bp (figures 2.12, 2.13).

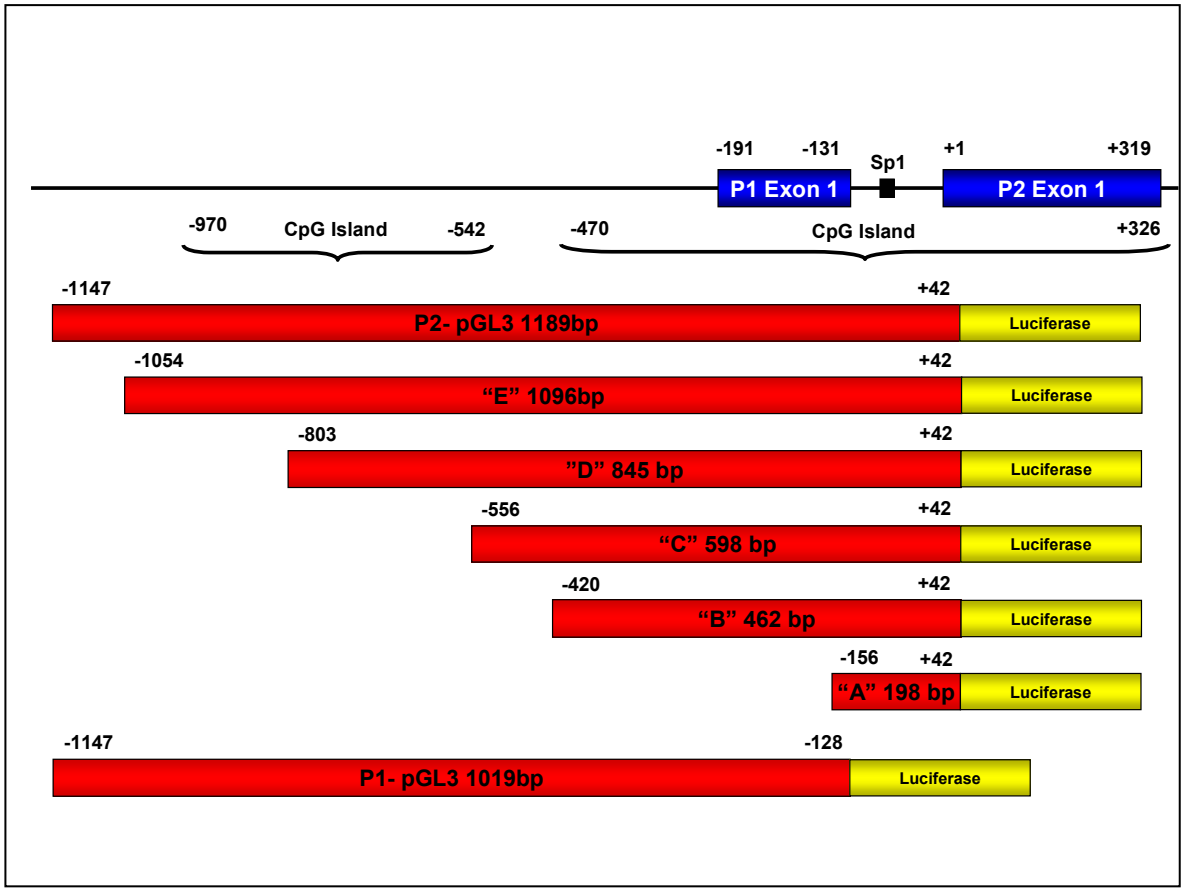


Figure 2.10 Schematic representation of the PPARα promoter 5' deletion mutant pGL3 Basic constructs. PPARα deletion promoter constructs are shown relative to the P2 transcription start site (+1). The red bars represent the promoter deletion mutant and the yellow bars represent pGL3 Basic. Location of promoters is also shown relative to P2 exon 1, P1 exon 1, CpG islands and Sp1 consensus sequence within region of P2 promoter which is not present in P1 promoter.

Figure 2.11 Location of primers used for PPARα promoter 5' deletion mutants. The original PPARα promoter cloning primers are shown in yellow highlight. The reverse primer for PPARα promoter 5' deletion mutants is shown in blue highlight and is the same as the original P2 reverse primer. Forward primers for deletion mutants are shown in red and the size of the PCR product is indicated. P2 transcription start site is shown in green and P1 start site is shown in pink. The 2 CpG islands are shown in bold and underlined.

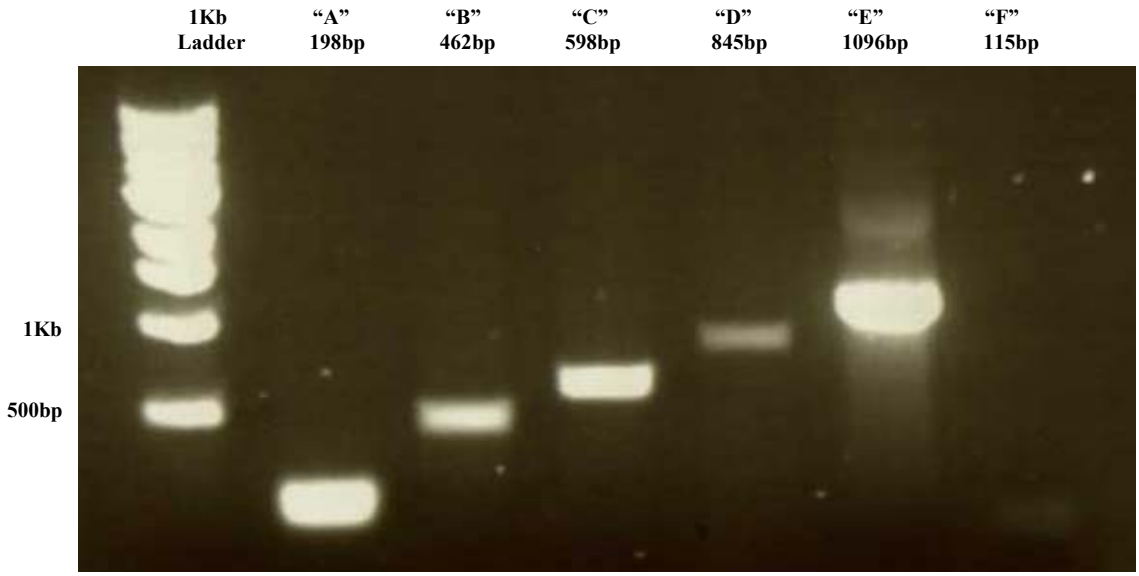


Figure 2.12 Gel extracted PPARα promoter 5' deletion mutant PCR products. Gel extracted PCR products of the correct size are shown for deletion mutant A (198bp), B (462bp), C (598bp), D (845bp), E (1096bp) and F (115bp).

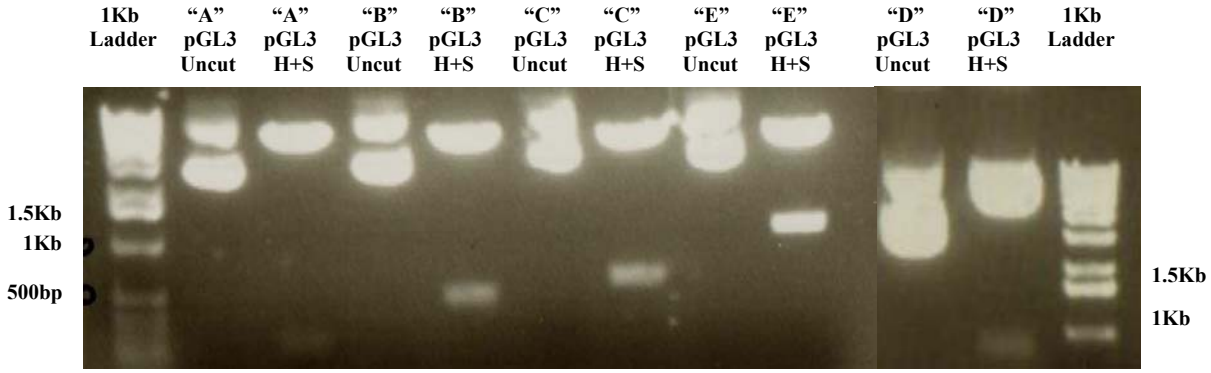


Figure 2.13 Digests of PPARα promoter 5' deletion mutant-pGL3 basic clones to drop out the promoter insert. Clones for the deletion mutants A-E are shown with and without HindIII + SacI restriction enzymes to drop out the promoter insert. Correct size inserts are shown for Deletion mutant A (198bp), B (462bp), C (598bp), D (845bp) and E (1096bp).

Table 2.5 Sequences of the PPARα promoter deletion mutant primers

Primer	Primer Sequence 5'→3'
PPARα Del Mut A For	GCAGCCTCAGGTGCCCAG
PPARα Del Mut B For	TGTGTCTCGTTCTGAAC
PPARα Del Mut C For	CTTCACCTAGCCAGCCTC
PPARα Del Mut D For	CATCCTGGGGCTCTGAAG
PPARα Del Mut E For	GCTGTCAGTTGGCAGAGT
PPARα Del Mut F For	GACAGTGAGGTGGGTGGA

Production of P2-pGL3 Sp1 mutants

Primers were designed to mutate the Sp1 consensus site within the unique region of the PPARα P2 promoter. Two sets of mutagenic primers were designed; the first set to change the internal CG of the Sp1 consensus sequence GGGCGG to an AT and the second to change it to an EcoRI restriction site (GAATTC). Primers were designed using the QuikChange® Primer Design Program (Stratagene, Texas, USA). All primers were supplied by Invitrogen (Paisley, UK), made to a concentration of 1µg/µl and stored at -20^{o}C. For primer sequences see table 2.6.

The Stratagene QuikChange method of mutagenesis was used to mutate the Sp1 site. 30ng and 50ng of the PPARα P2-pGL3 construct was used as a template for Sp1M QuikChange PCR. DNA was amplified using 1.5U Pfu DNA polymerase with and forward and reverse primers to a final concentration of 0.4µM, dNTP's at a final concentration of 0.2µM each and 10µl GC solution in a total volume of 50µl. PCR was performed using the PCR Express PCR machine DNA (Thermo Fisher Scientific, Epsom UK). Cycling conditions for PCR were as follows; initial denaturation of 95^{0}C 2 minutes, followed by 18 cycles of 95^{o}C 30s, anneal at a 60^{o}C 1 min and extension at 72^{o}C 4 minutes, followed by a final extension at 72^{o}C for 5 minutes. Resulting PCR products (figure 2.14) were digested with the restriction enzyme DpnI at 37^{o}C for 1 hour. This enzyme digests the sequence G^{me}A$^{\blacktriangledown}$TC found on the parental strands of the plasmid, leaving the newly formed strands intact. Next, 4µl of digested PCR product was used to transform competent DH5α cells. Individual colonies were picked from the plates and grown in 10ml LB supplemented with 100µg/ml ampicillin at 37^{o}C overnight with agitation. These cultures were used to prepare small scale plasmid preparations. To confirm mutation of the Sp1 site in the EcoRI mutant, 500ng of plasmid was digested with 6U of EcoRI at 37^{o}C for 60 minutes. As the pGL3 vector does not contain an EcoRI restriction site, the Sp1M AT mutants should not cut with the restriction enzyme, whereas the EcoRI positive mutants should (figure 2.15). Sp1M RI and Sp1M AT clones were sequenced by university of Dundee sequencing service using the GL primer 2 pGl3 Basic Reverse Sequencing Primer (5'CTTTATGTTTTTGGCGTCTTCCA-3').

Table 2.6 Sequences of PPARα P2 SP1 mutagenesis primers. EcoRI site is shown in blue highlight and mutated sequence is in bold and underlined.

Primer	Primer Sequence 5'→3'
SP1M AT For	CAGGTGCCCAGGG**AT**GGGAGGGCACGCG
SP1M AT Rev	CGCGTGCCCTCC**AT**CCCTGGGCACCTG
SP1M RI For	GCCTCAGGTGCCCAGG**AATTC**GAGGGCACGCGCGAGG
SP1M RI Rev	CCTCGCGCGTGCCCTC**GAATTC**CTGGGCACCTGAGGC

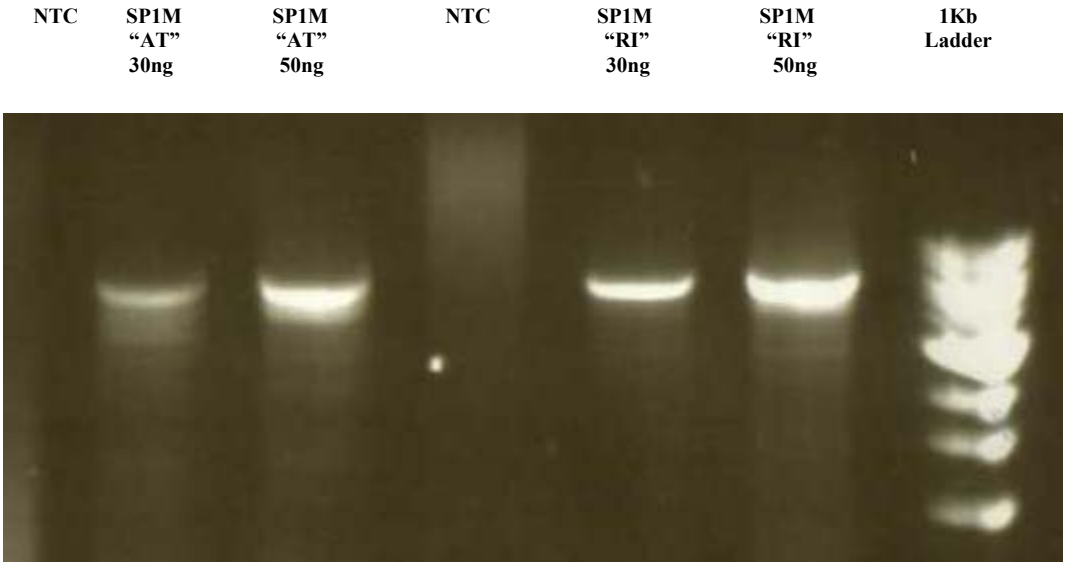


Figure 2.14 PPARα P2 SP1M PCR products. PCR products are shown for PPARα P2 SP1M PCR, for the "AT" mutant and "RI" mutant, both 30ng and 50ng PCR reactions.

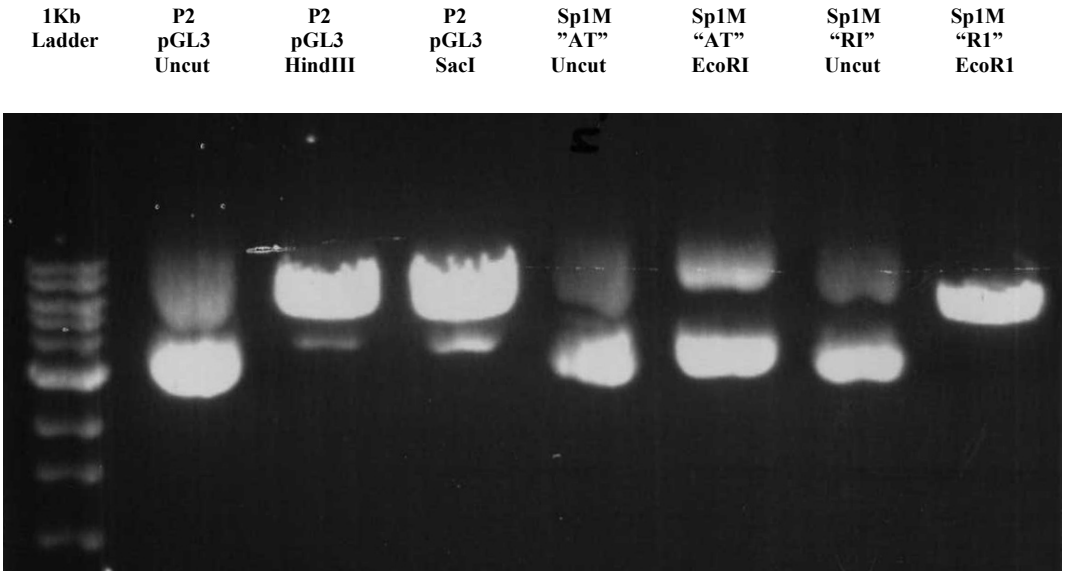


Figure 2.15 Digests of PPARα P2 Sp1M-pGL3 basic clones. Sp1M AT and R1 are shown with and without EcoRI restriction enzyme. P2-pGl3 has no EcoRI restriction sites. "AT" mutant is unable to cut with EcoRI, but the Sp1M RI construct, which has an EcoRI site instead of Sp1 site is able to be cut, indicating that mutagenesis has been successful.

Methylation of PPARα promoter-pGL3 constructs with CpG and HpaII methyltransferases

The PPARα P1-pGL3, P2-pGL3, P3-pGL3 and pGL3 Basic constructs were all methylated using CpG methyltransferase (M.SssI) which methylates the cytosine within the dinucleotide 5'-CG-3' and HpaII methyltransferase which methylates the internal cytosine of the sequence 5'-CCGG-3'. Briefly 5µg of promoter construct were methylated with either 4U of SssI methyltransferase, 4U of HpaII methyltransferase or 1µl water (mock methylated). These were incubated with 160µM SAM and either 1× NEB buffer 2 (M.SssI methyltransferase), or 1×NEB HpaII Methylase buffer (HpaII methyltransferase). Reactions took place in a total volume of 100µl for 3 hours at 37^{o}C, after which enzymes were heat inactivated at 65^{o}C for 15 minutes.

The differentially methylated constructs were then cleaned by phenol chloroform extraction and ethanol precipitation as previously described. 2µl of each construct was measured on the NanoDrop, made to a concentration of 500ng/µl and then 500ng of this was digested with HpaII restriction enzyme in Promega buffer A buffer at 37^{o}C for 30 minutes. This methylation sensitive restriction enzyme cuts the sequence 5'-C$^{\blacktriangledown}$CGG-3' only if it is unmethylated. If methylated (5'-CC^{me}GG-3') it will not cut. After digestion, all differentially methylated and digested constructs were analysed for methylation by agarose gel electrophoresis against an equivalent amount of uncut plasmid (figure 2.16).

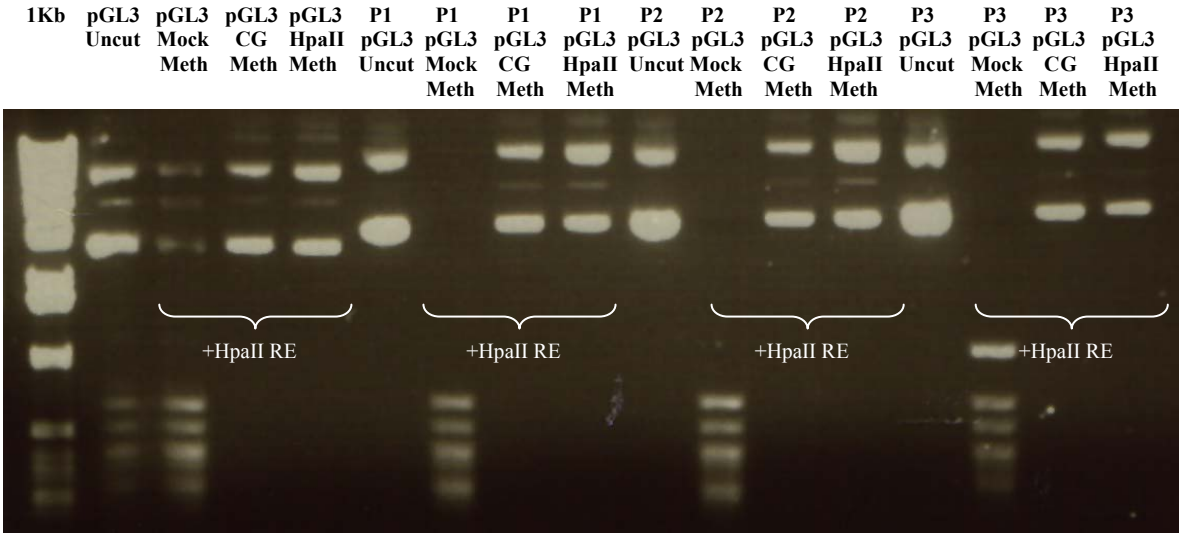


Figure 2.16 Digests of methylated PPARα-pGL3 constructs. P1-pGL3, P2-pGL3, P3-pGL3 and pGL3 Basic were methylated with either M.SssI or HpaII methyltransferase and then digested with the HpaII methylation sensitive restriction enzyme. As expected, all mock methylated constructs were digested to completion with the HpaII restriction enzyme, whereas the CG and HpaII methylated constructs were completely protected from digestion by methylation.

2.2.10 Cell culture

Maintenance of murine 3T3-L1 pre-adipocyte cells

Mouse 3T3 L1 cells were cultured in 3T3-L1 media in 75cm^2 tissue culture flasks (Greiner Bio One, Glos, UK) at 37^{o}C with 5% CO_2 in a humidified atmosphere. 80% confluent cultures were washed in PBS, 1ml trypsin-EDTA added and incubated 2-5minutes to detach cells from base of flask. 10ml DMEM was then added to neutralise trypsin and cells diluted 1:50 and added to a new flask.

Differentiation of murine 3T3-L1 pre-adipocyte cells

3T3-L1 cells at passage 6-9 were stimulated to differentiate into adipocytes as follows. Cells were detached as described above and added in 3T3 media to 9.6cm^2 6 well culture plates (Greiner Bio One, Glos, UK). At 2 days post confluence (day 0), 3T3-L1 media was replaced with 3T3-L1 differentiation media. 48 hours later (day 2) media was removed and replaced with 3T3-L1 media containing 175nM insulin. Another 48 hours later (day 4), media was again removed and replaced with 3T3-L1 media containing 175nM insulin. At day 6 media was removed and replaced with 3T3-L1 media.

Treatment of 3T3 cells

3T3 cells undergoing differentiation were treated with murine leptin at either 0ng/ml or 10ng/ml in 3T3-L1 media at both day 2 and day 8 and incubated for 0hr, 2hr, 6hr, 24hr and 48hr at 37^{o}C 5% CO_2. All samples were performed in duplicate. At each time point cells were washed in PBS and scraped into 1ml ice cold PBS, spun at 3,000 rpm 5 minutes and supernatant removed. Cell pellets were either stored at -70^{o}C or used to prepare RNA straight away.

Maintenance of HepG2 cells

The human hepatoma cell line HepG2 were cultured in HepG2 complete media in 75cm^2 tissue culture flasks at 37^{o}C with 5% CO_2 in a humidified atmosphere. 80% confluent cultures were washed in PBS, 1ml trypsin-EDTA added and incubated 2-5minutes to detach cells from base of flask. 10ml DMEM was then added to neutralise trypsin and cells diluted 1:50 in HepG2 complete media and added to a new flask.

Preparation of DNA for cell culture

For cell culture, plasmid DNA was precipitated in 1/10th volume of 3M NaAc pH5.2 and 2 volumes of 100% ethanol. This was incubated at -20^{o}C for 20 minutes and then centrifuged at 12,000rpm for 15 minutes. The DNA pellet was then resuspended under sterile conditions in dH_2O to a concentration of 1µg/µl and stored at -20^{o}C. For use in transfection experiments, DNA was diluted to 500ng/µl.

Transfection of HepG2 cells

Transfection of HepG2 cells with PPARα promoter fragments was by the calcium phosphate method [229]. All transfections were performed in triplicate. The day before transfection, cells were seeded onto 6 well culture plates at a density of 2×10^5 cells / well. On the day of transfection, 1µg of promoter-pGL3 construct was added to 12.4µl $CaCl_2$ and made to 100µl with dH_2O. This was added drop wise to an eppendorf containing 100µl 2×HBS and then incubated at room temperature for 20 minutes. After incubation samples were then added drop wise to the cells. Cells were incubated for 5 hours then washed twice in no adds media. 2ml complete DMEM was then added to cells with or without treatments and incubated for a further 24 hours (see below).

Transfection of HepG2 cells - treatments

Cells transfected with PPARα P1-pGL3, P2-pGL3, P3-pGL3 and pGL3 basic were initially treated with concentration gradients of CFA, leptin and dex as follows; i) CFA treatment, 20µM, 40µM, 60µM, 80µM, 100µM. ii) leptin treatment 10ng/ml, 50ng/ml, 100ng/ml, 500ng/ml, 1000ng/ml. iii) dex 1nM, 10nM, 100nM, 1µM, 10µM.

Cells transfected with the PPARα P2-pGL3, P1-pGL3 and pGL3 basic were treated with Stat3 inhibitor as follows; 1nM, 10nM, 100nM, 1μM, 10μM. 1 hour after Stat3 inhibitor treatment leptin was added to cells (1000ng/ml). Cells were then incubated for 24hours.

Cells transfected with the PPARα Sp1M were treated with 1000ng/ml leptin.

Luciferase assay

Luciferase assays were performed on all cells transfected with pGL3 Basic-promoter constructs, using the luciferase assay system (Promega, Southampton, UK). Media was aspirated from transfected cells and then washed in 2ml ice cold PBS. PBS was removed and cells scraped into 100μl 1× reporter lysis buffer using a cell scraper and removed to an eppendorf tube. Cells were subjected to 2 freeze thaw cycles by submerging in liquid nitrogen and thawing at 37^{o}C in a waterbath. Cells were then centrifuged at 12,000rpm for 30 seconds to pellet cell debris and supernatant transferred to a new tube. Samples and luciferase assay reagent were allowed to reach room temperature before use in the assay. To measure luminescence of samples, 50μl of luciferase assay reagent was transferred to a luminometer tube and 5μl of sample added and mixed well. The tube was then placed in the luminometer (Turner Designs 20-20, Promega, UK) and a reading initiated. For all assays, the luminometer was set to 100% sensitivity, a 2 second delay before reading and a 10 second measurement read for luciferase activity. Each sample was measured 3 times in succession.

Protein assay

Protein levels within transfected cell lysates were determined using BioRad protein assay reagent (BioRad, Hemel Hempstead, UK) according to manufacturer's instructions. All samples were measured in duplicate. Briefly, 2μl sample was added to a microtitre plate, followed by 250μl 1× protein assay reagent. In addition, BSA was added to separate wells to create a standard curve. Protein concentrations of samples and standards were read at 570nm and absolute amounts of protein in unknown samples calculated. Results from luciferase assays were normalized to protein levels within cell lysates.

Statistical analysis of transfections

All samples were transfected in triplicate. Statistical comparisons of luciferase activity between promoter constructs and treatments were determined by 1 way or 2 way ANOVA (as appropriate) followed by Bonferroni *post hoc* analysis using the PRISM software package.

2.2.11 Methylation analysis by pyrosequencing

Bisulfite conversion of DNA

Bisulfite conversions were performed using the EZ DNA methylation gold Kit (Cambridge Biosciences, Cambridge, UK) according to manufacturers instructions. 2µg of genomic DNA was used in all bisulfite conversions. Resulting bisulfite DNA was stored at -20^{o}C and used within 2 months.

Primer design for bisulfite PCR

Prior to designing PCR primers for bisulfite PCR, the sequence of the region of interest was first changed for assay design purposes; all CpGs within the sequence were converted to YG, then all C's were converted to T. This represents the changes which would have occurred to the DNA during the bisulfite conversion process. Bisulfite PCR primers and sequencing primers (table 2.7) were then designed using PSQ Assay Design software version 1.0.6 (Biotage AB, Kungsgatan, Sweden). Primers were designed to cover as much of the CpG island within the PPARα promoter as possible (without themselves containing a CpG within their sequence) (figure 2.17-2.20). All standard primers were supplied by Invitrogen (Paisley, UK) and 5'-biotin labelled primers by Biomers (Ulm, Germany). Primers were made to a concentration of 100µM and stored at -20^{o}C.

Bisulfite PCR

2µl Bisulfite DNA was amplified using 1.25 units HotStar Taq, 200µM of each dNTP, 0.2µM of forward and reverse primers (one of which is 5' biotin labelled) and made to a final volume of 50µl with DEPC treated water. PCR was performed using the PCR Express PCR machine DNA (Thermo Fisher Scientific, Epsom UK). Cycling conditions were as follows; initial denaturation of 95^{o}C 15 minutes, followed by 45 cycles of 94^{o}C 30s, anneal at a previously optimised primer specific temperature (table 2.7) 1 minute and extension at 72^{o}C for 1 minute. This was followed by a final extension at 72^{o}C for 10 minutes. 10µl of all PCR products were run on a 1.5% agarose gel to confirm the presence of a single band of the correct size.

Pyrosequencing

All solutions were allowed to reach room temperature before starting the pyrosequencing procedure. First, the biotinylated PCR Products were immobilized to Streptavidin beads. To achieve this, 10µl of PCR product (previously optimized) was added to a 96-well plate and the volume made up to 40µl with dH_2O. Next, 38µl binding buffer and 2µl Streptavidin beads were added to each well, the plate sealed and mixed for 5 minutes on a plate shaker. Next, the PCR product was made single stranded and cleaned. To accomplish this, the PCR plate was placed onto the plate holder of a vacuum prep workstation (Biotage AB, Kungsgatan, Sweden). The beads were then captured onto the vacuum prep tool ensuring that all solution was removed. The captured beads were next washed in 70% ethanol and then the strands separated in denaturation

solution. The beads were subsequently washed in wash buffer then the vacuum was switched off and the beads were released into a PSQ plate (Biotage AB, Kungsgatan, Sweden) containing 12µl of 0.3µM sequencing primer in annealing buffer. To anneal the primers to the newly single stranded biotin labeled PCR product, the plate was heated with the samples at 80˚C for 2 minutes then allowed to cool slowly to room temperature. Nucleotides, enzymes and substrate were added to nucleotide and reagent dispensing tips which were loaded into the tip holder and a tip test performed. Samples were then analyzed by pyrosequencing using the Pyromark MD Pyrosequencer (Biotage AB, Kungsgatan, Sweden). To check that the bisulfite conversion had gone to completion, a quality control was included in the pyrosequencing assay which checks that any cytosine not followed by a guanine had been 100% converted to thymine.

Table 2.7 Sequences of the PPARα pyrosequencing PCR and sequencing primers

Primer	Sequence (5' → 3')	Anneal
290-475 Forward	GAGAATTGTTTAGGGTTTTTTAGG	58.5
290-475 Reverse (5'-Biotin)	CCCTATCCACCCACCTCACTA	
290-380 Sequencing	AGGGATTTAGTAGGGGA	
395-475 Sequencing	GGTTTTAAGATAGGGGTGA	
475-540 Forward (5'- Biotin)	GGGTGATTTTGGGTAGTTTTT	56.4
475-540 Reverse	TCCCTATCCACCCACCTCACTA	
475-540 Sequencing	CCACCCACCTCACTAT	

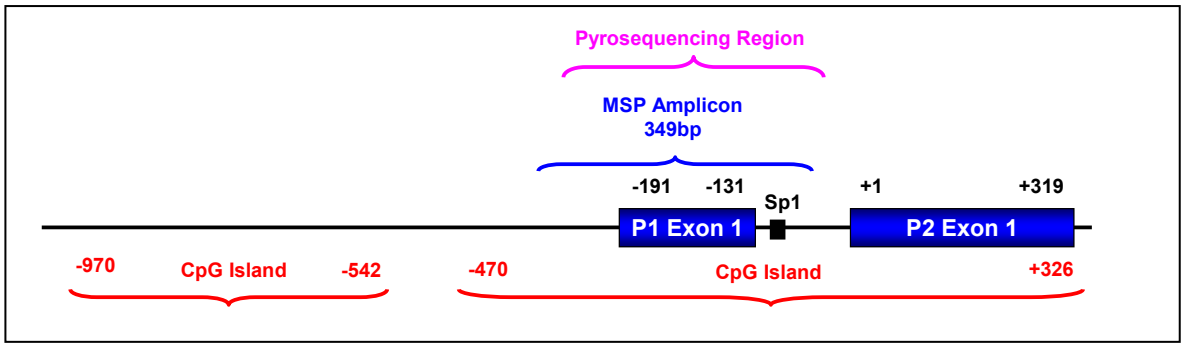


Figure 2.17 Diagram showing the location of the PPARα pyrosequencing amplicons. The pyrosequencing amplicon is shown relative to the P1 and P2 transcription start sites, MSP primers and the CpG islands found within the PPARα promoter.

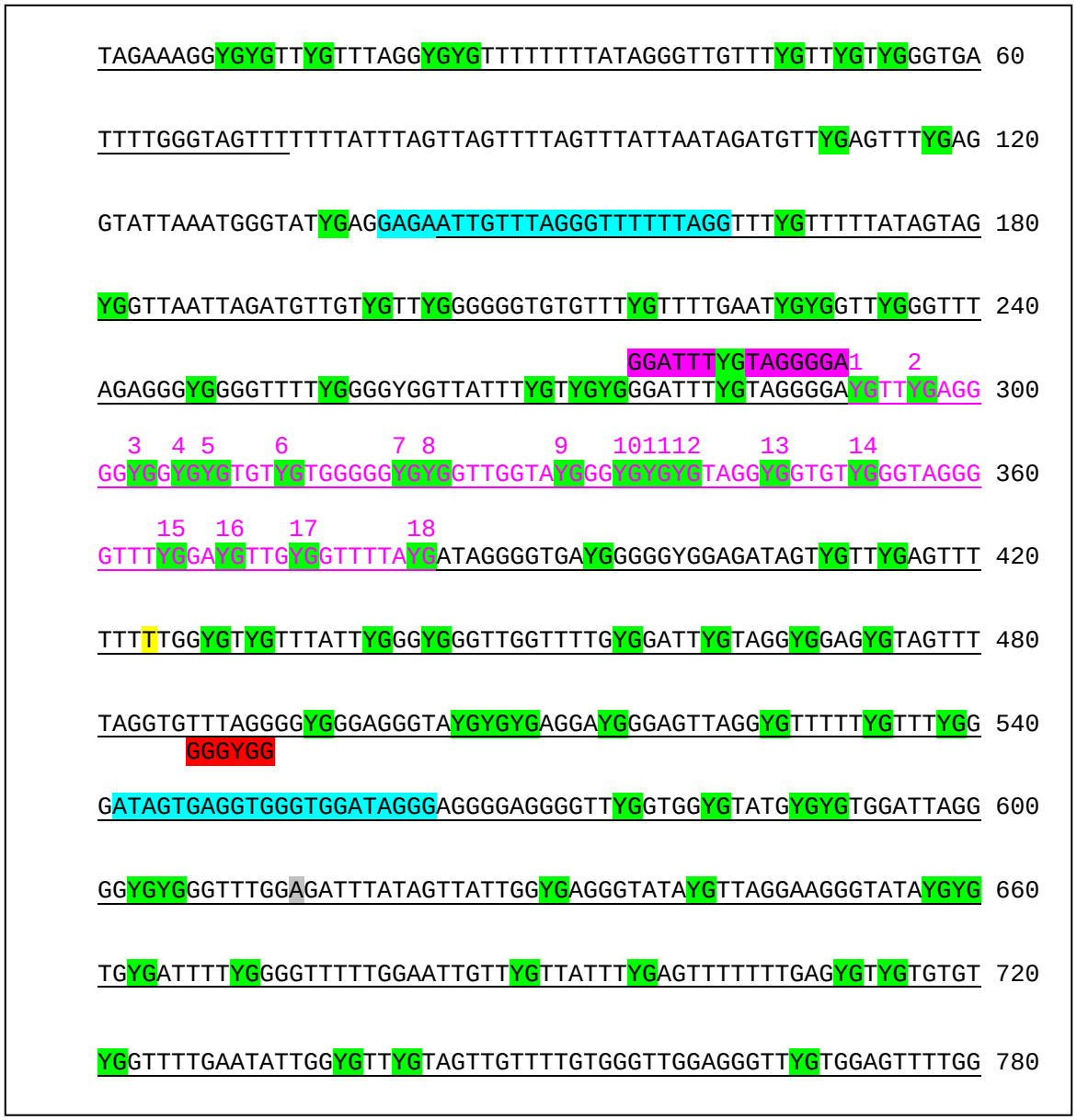


Figure 2.18 PPARα promoter sequence showing the location of the 290-380 pyrosequencing amplicon. Diagram shows the location of the PPARα 290-380 PCR amplicon and sequencing primers on bisulfite converted DNA. Forward and reverse primers are shown in blue highlight and sequencing primer in pink highlight. The region to be sequenced is indicated by pink text. CpG islands are underlined, whilst CpGs are highlighted in green and numbering of the CpGs is shown above the sequence. The Sp1 consensus within the "unique region" is shown in red highlight. The P2 transcription start site is highlighted in grey and the P1 transcription start site is highlighted in yellow.

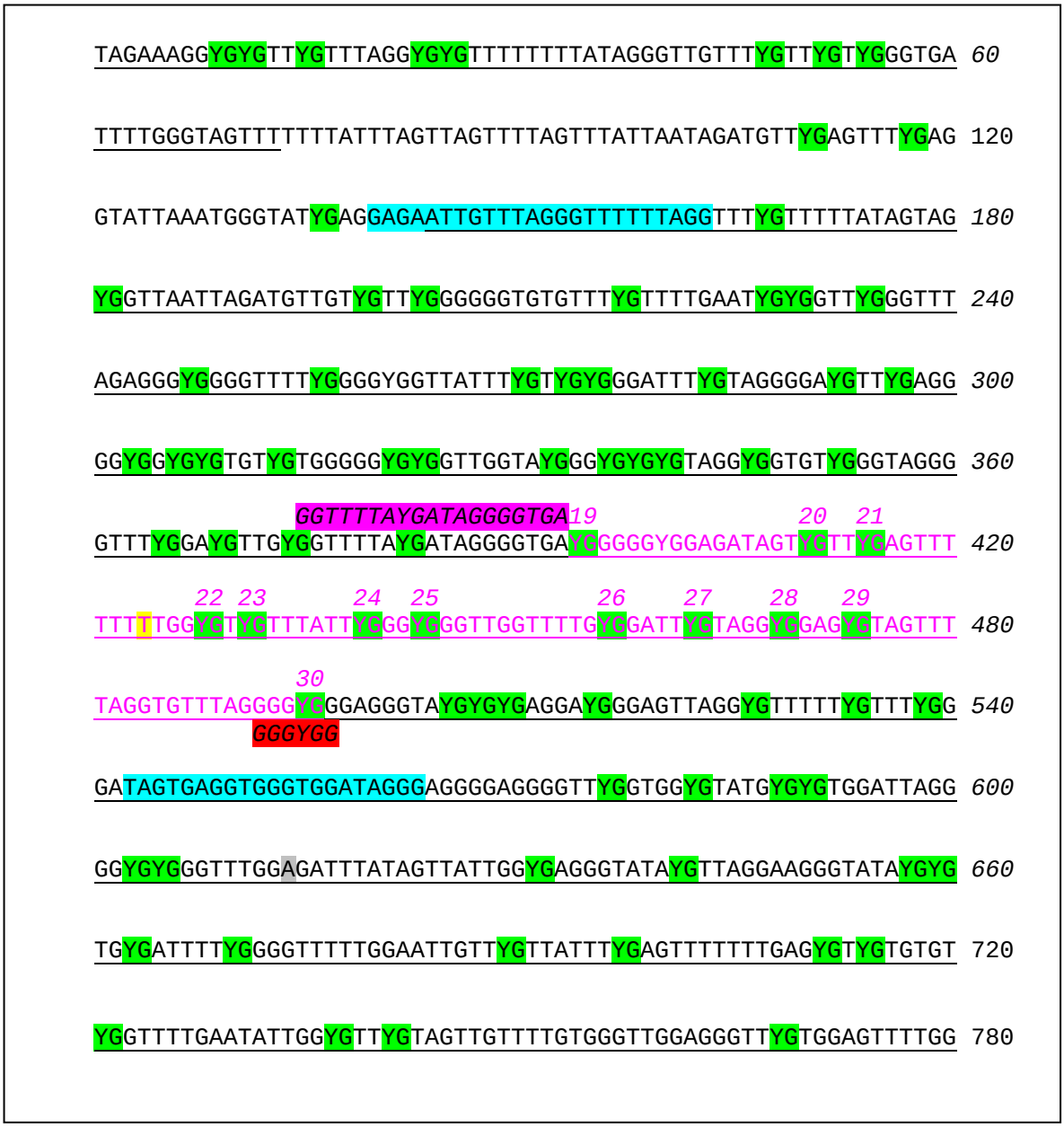


Figure 2.19 PPARα promoter sequence showing the location of the 395-475 pyrosequencing amplicon. Diagram shows the location of the PPARα 395-475 PCR amplicon and sequencing primers on bisulfite converted DNA. Forward and reverse primers are shown in blue highlight and sequencing primer in pink highlight. The region to be sequenced is indicated by pink text. CpG islands are underlined, whilst CpGs are highlighted in green and numbering of the CpGs is shown above the sequence. The Sp1 consensus within the "unique region" is shown in red highlight. The P2 transcription start site is highlighted in grey and the P1 transcription start site is highlighted in yellow.

Chapter 3

Gene Expression and Methylation in the Liver of Female and Male IUGR Rats

3.1 Introduction

3.1.1 Liver function in energy homeostasis

The liver is a complex metabolic organ composed not only of hepatocytes (80%), but also of a variety of cells, including Kupffer cells, stellate cells and endothelial cells. Hepatocytes are central to energy homeostasis and perform many vital metabolic processes involving carbohydrate, protein and lipid metabolism. All these processes are under the strict control of either the anabolic hormone insulin or the catabolic hormone glucagon depending on the metabolic status. However, in addition to insulin and glucagon, catecholamines and glucocorticoids can also exert metabolic control of the liver in response to stress.

One of the main functions of the liver is to maintain blood glucose concentrations within strict parameters. In the fed state, insulin synthesis is stimulated to promote the storage of energy. As a consequence, excess dietary glucose is removed from the blood and converted to glycogen for storage in the liver until needed (glyconeogenesis) and fatty acids are synthesized and esterified with glycerol for storage as TAG in adipose tissue (lipogenesis). Conversely in times of energy demand such as during fasting, glucagon synthesis is stimulated which opposes the effects of insulin. Here, catabolic processes are stimulated along with the production of glucose in order to supply vital glucose to the brain and fatty acids and ketones are produced for tissues which do not rely on glucose. Glucagon achieves this by increasing the production of glucose in the liver by promoting glycogen breakdown (glycogenolysis) and converting non-carbohydrate sources into glucose (gluconeogenesis). Glucagon also stimulates the production of fatty acids and ketones by stimulating fatty acid oxidation and ketogenesis (figure 3.1). In addition during times of stress or if glycogen stores have been depleted, the HPA axis is activated to release glucocorticoids which have an anti-insulin effect, thereby promoting gluconeogenesis, lipolysis and fatty acid oxidation in the liver. Furthermore, the catecholamine epinephrine is also stimulated by stress and directly stimulates catabolic processes to increase blood glucose levels.

Alterations in carbohydrate and lipid metabolism occur in metabolic disorders such as insulin resistance. When insulin signaling is defective, glucagon becomes the predominant active hormone as it is unopposed by insulin and therefore inappropriately promotes catabolic processes and glucose production, which can lead to hyperglycemia. If untreated, this can also lead to ketoacidosis and hypertriglyceridemia (figure 3.1). In a rat model of programming, protein restricted offspring have been shown to be insulin resistant with an altered hepatic glucose output, indicating that the regulation of hepatic metabolic processes is disturbed [42]. These offspring also have increased corticosteroid activity, which may have a causative role in the altered carbohydrate metabolism and indicates a “stressed” phenotype [40]. Normally, gluconeogenesis only occurs during

fasting when glycogen stores have run out, however in PR offspring there is increased expression of hepatic GR and its target gene PEPCK [57]. It is probable that these gene expression changes may result in increased gluconeogenesis and contribute to the altered hepatic glucose output. Furthermore, these offspring also exhibit increased PPARα expression in the liver along with increased expression of its target genes AOX and CPT1, resulting in inappropriate fatty acid β-oxidation. It has been shown that the changes in gene expression of the transcription factors GR and PPARα are due to alterations in the methylation status of CpG dinucleotides in their promoter [77]. Evidence is now accumulating which indicates that epigenetic alterations in gene promoters in programmed offspring may underpin the relationship between early life environment and an altered phenotype in adult life.

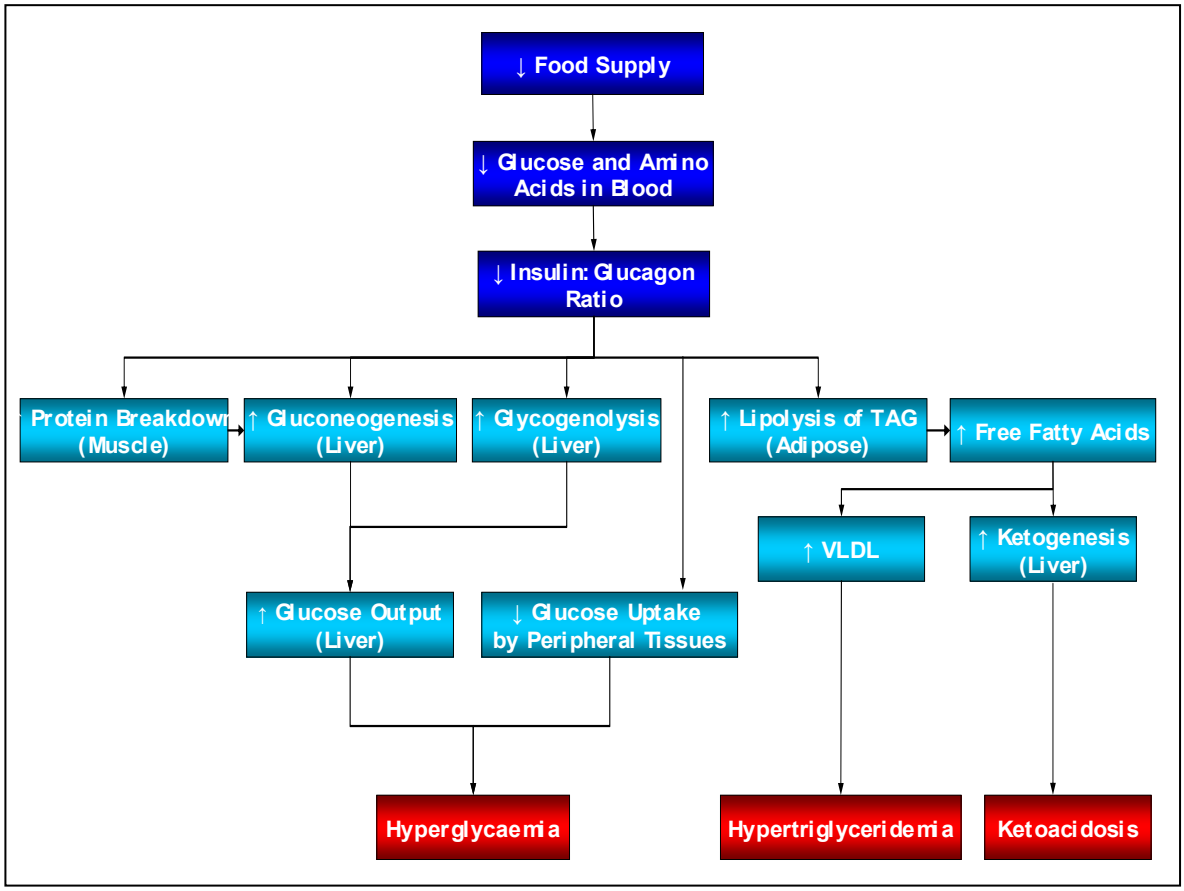


Figure 3.1 The effect of a reduced insulin:glucagon ratio on energy metabolism. The effect of a reduced insulin:glucagon ratio on the control of metabolic processes in the liver is shown along with the disturbances which may occur if left untreated.

3.1.2 Aims

In rodents, the PR model of programming results in offspring with low birth weight presenting with metabolic disorders in later life including insulin resistance, diabetes and hypertension [48]. This

model has been intensively studied for alterations in the epigenetic regulation of transcription factors and enzymes which may affect glucose and lipid metabolism in the liver. As a result, such studies have identified specific epigenetic changes and alterations in gene expression of important hepatic transcription factors and enzymes associated with gluconeogenesis and fatty acid oxidation, including GR, PPARα and their target genes [77,213].

Another animal model of programming by maternal nutrition is the IUGR rat [227]. The offspring have low birth weight and present with hyperphagia, high blood pressure, hyperleptinemia, hyperinsulinemia (indicating they are insulin resistant) and become obese. Furthermore, all these disorders are augmented by a calorie rich postnatal diet and reversed by neonatal leptin treatment [122]. Currently, knowledge of the molecular mechanisms underpinning the metabolic disturbances have not been investigated in the IUGR rat, but may involve similar mechanisms to those found in the liver of PR offspring, i.e. altered carbohydrate and lipid metabolism. In addition, the mechanism of leptin action in the reversal of metabolic disturbances is unknown, but may also involve peripheral effects on key hepatic transcription factors and enzymes involved in energy metabolism.

To gain a better understanding of the programming of energy balance in the IUGR rats and the effect of neonatal leptin treatment, this study uses liver tissue from the same rat model of global undernutrition used by Vickers and colleagues [122] to investigate the expression and methylation status of several key genes in the liver associated with glucose and lipid metabolism. Effects are investigated in response to maternal diet, postnatal diet and neonatal leptin treatment in both male and female offspring using real time PCR and methylation sensitive real time PCR.

Data presented in this chapter shows that the mechanisms underlying altered carbohydrate metabolism in the livers of PR offspring are not present in the liver of IUGR offspring and that leptin treatment has largely no effect on expression of these genes. Results indicate that programming occurs by alternative mechanisms in the IUGR rat.

3.2 Results

3.2.1 The expression of genes involved in energy balance in the liver of adult female offspring differ in their response to maternal diet, postnatal diet and neonatal leptin treatment.

In order to ascertain if the expression of genes involved in energy balance in the liver of female offspring are affected by maternal diet, postnatal diet or neonatal leptin treatment, total RNA was prepared from the liver of adult female offspring from the IUGR rat study (n=8 / diet-treatment group) and used to prepare copy DNA (cDNA). Real time polymerase chain reaction (PCR) was performed with validated gene specific primers using the cDNA as a template.

Genes studied included those involved in gluconeogenesis; GR, PEPCK and 11βHSD-2 and also key genes involved in fatty acid metabolism; PPARα, AOX, CPT-1 and LPL. Resulting gene expression data were normalised to CYC cDNA and analysed by the ΔCT method [228]. Results for each group were converted to % of the control group (AD Chow 100%) and a one way analysis of variance (ANOVA) performed followed by Bonferroni's post hoc test. Graphs showing differences in mRNA expression for each gene with statistical analysis can be found in figures 3.2 to 3.4. All results are described relative to the control group only. In addition, a 3 way ANOVA was performed for all genes measured to analyse single factor and interaction effects on gene expression. The results of the 3 way ANOVA are shown in table 3.1.

Analysis of PPARα expression by real time PCR demonstrated that its expression was significantly reduced by maternal diet and this effect was dependent on offspring being fed a chow diet (39.76% p=0.001) (figure 3.2a). This reduction in expression was prevented if the offspring were given neonatal leptin treatment. There were no other effects of neonatal leptin treatment, maternal diet or postnatal diet on PPARα expression. The expression of the PPARα target genes AOX and CPT-1 differed to that of PPARα. For AOX gene expression, there a significant effect of neonatal leptin treatment on AD offspring and this was dependent on their being fed a HF diet (151.0%, p=0.05). There were no other effects of neonatal leptin treatment, maternal diet or postnatal diet on AOX expression (figure 3.2b). For CPT-1, there was no effect of any diet or neonatal leptin treatment on gene expression (figure 3.2c). These data show that differences in PPARα gene expression are not passed onto its target genes in the liver of these offspring.

The expression of LPL was not altered by maternal diet, postnatal diet or neonatal leptin treatment (figure 3.3). Conversely, examination of GR and its target gene PEPCK revealed that they were both significantly affected by maternal diet, but the response depended on the postnatal diet. For GR expression, there was a significant effect of maternal diet on offspring fed a chow diet (45.53%

p=0.001) (figure 3.4a), whereas for PEPCK expression, the effect was only evident in offspring fed a high fat diet (50.81% P=0.05) (figure 3.4b). This effect was not evident if the offspring were given neonatal leptin treatment. There were no other effects of diet or neonatal leptin treatment on the expression of GR or its target gene PEPCK. There was also no significant effect of any treatment on the expression of 11βHSD-2 (figure 3.4c).

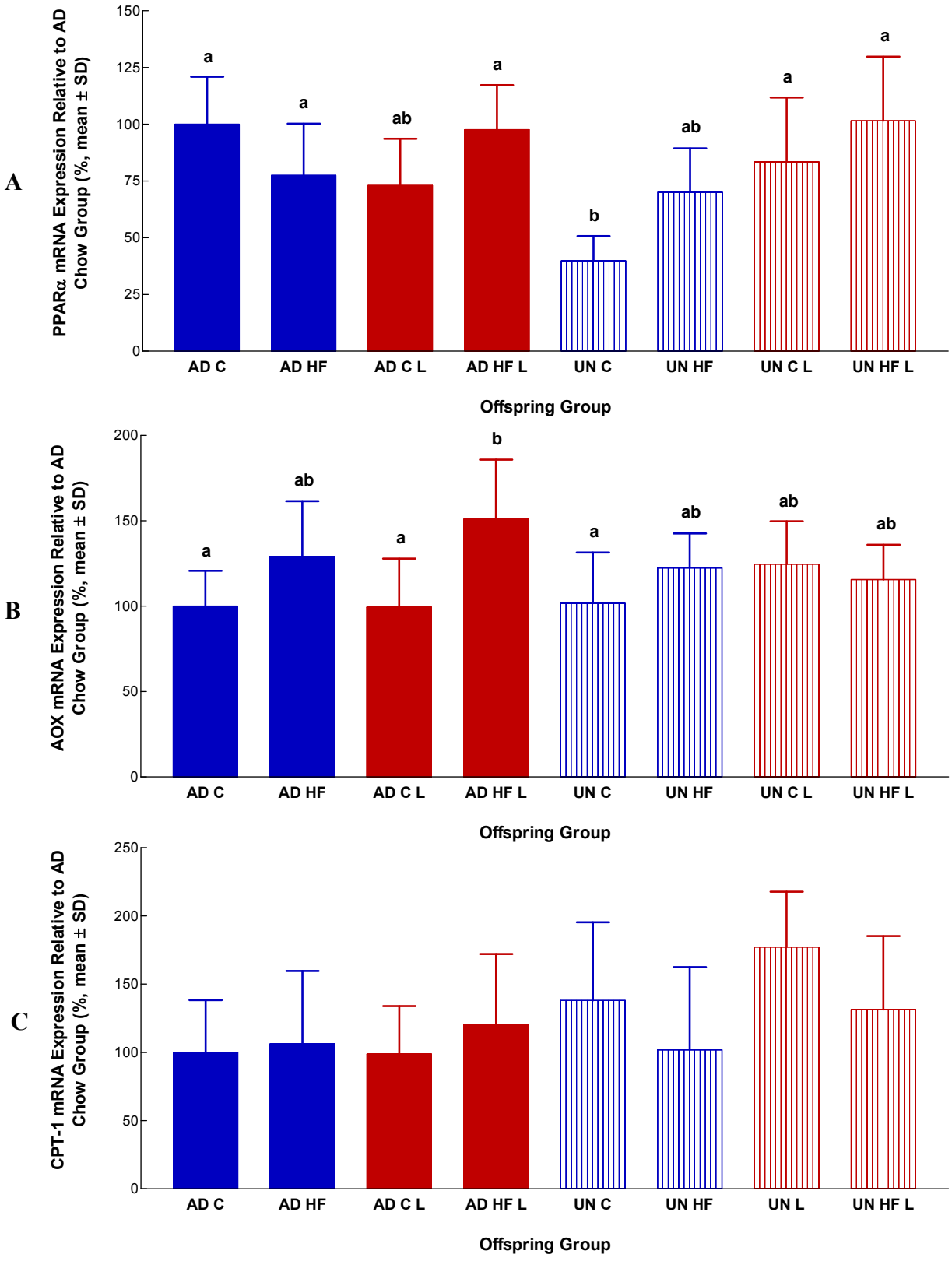


Figure 3.2 The expression of PPARα and its target genes CPT-1 and AOX in the liver of adult female offspring differ in their response to maternal diet, postnatal diet and neonatal leptin treatment. Real time PCR data for expression of PPARα (A), CPT-1 (B), AOX (C) are shown in response to maternal diet (ad libitum [AD] and undernutrition [UN]), postnatal diet (Chow [C] and High Fat [HF]) and neonatal leptin treatment (leptin [L]) (n=8/group). Values with different letters are significantly different ($p<0.05$) by Bonferroni's post hoc test.

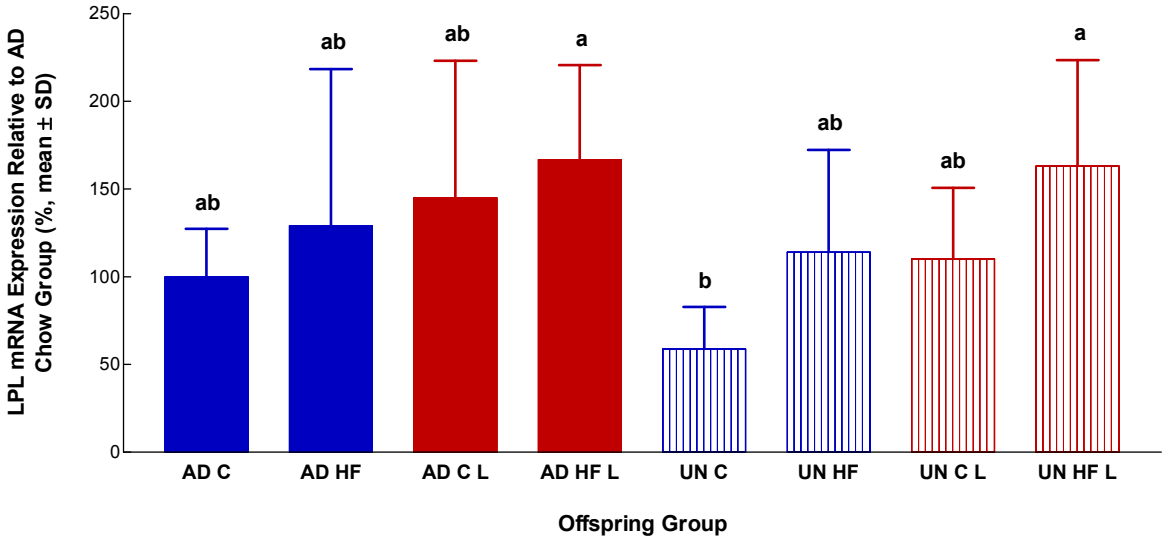


Figure 3.3 The expression of LPL in the liver of adult female offspring is not altered by maternal diet, postnatal diet or neonatal leptin treatment. Real time PCR data for expression of LPL are shown in response to maternal diet (ad libitum [AD] and undernutrition [UN]), postnatal diet (Chow [C] and High Fat [HF]) and neonatal leptin treatment (leptin [L]) (n=8/group). Values with different letters are significantly different ($p<0.05$) by Bonferroni's post hoc test.

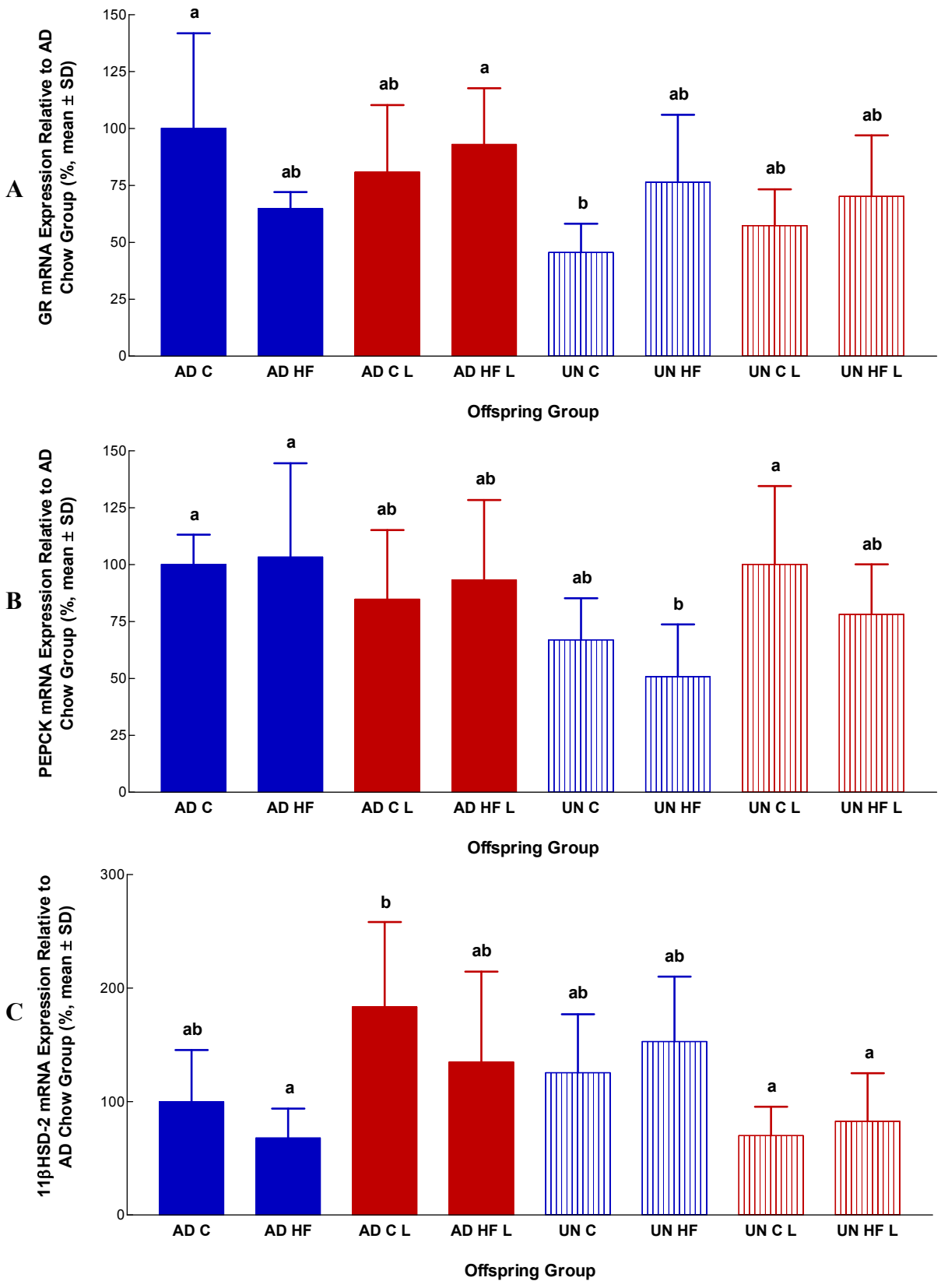


Figure 3.4 The expression of GR and its target gene PEPCK and 11βHSD-2 in the liver of adult female offspring differ in their response to maternal diet and postnatal diet, but are not altered by neonatal leptin treatment. Real time PCR data for expression of GR (A), PEPCK (B), 11βHSD-2 (C), are shown in response to maternal diet (ad libitum [AD] and undernutrition [UN]), postnatal diet (Chow [C] and High Fat [HF]) and neonatal leptin treatment (leptin [L]) (n=8/group). Values with different letters are significantly different ($p<0.05$) by Bonferroni's post hoc test.

Table 3.1 Table showing tests of between subject effects on the mRNA expression of genes measured in the liver of female offspring. Single factor effects (MD, T, PND) and interaction effects (MD*T, MD*PND, T*PND, MD*T*PND) are shown. Significant effects ($P<0.05$) are indicated in red. There were significant single factor effects for all genes except 11βHDS2. There were significant effects of MD for PPARα, CPT-1, GR and PEPCK. There were significant effects of T for PPARα and LPL. There were significant effects of PND for PPARα, AOX and LPL. There were significant interaction effects for PPARα (MD*T, MD*PND, MD*T*PND), CPT-1 (MD*PND), AOX (MD*PND), GR (MD*PND, MD*T*PND), PEPCK (MD*T) and 11βHDS2 (MD*T, MD*PND). There were no significant interaction effects for LPL.

Variable	Anova	Maternal Diet (MD)	Treatment (T)	Postnatal Diet (PND)	MD* T	MD* PND	T* PND	MD* T* PND
PPARα	0.000	0.019	0.003	0.027	0.001	0.042	0.122	0.010
CPT-1	0.039	0.018	0.109	0.289	0.273	0.033	0.907	0.615
AOX	0.006	0.589	0.186	0.002	0.856	0.017	0.788	0.067
GR	0.002	0.001	0.580	0.430	0.901	0.013	0.264	0.015
PEPCK	0.006	0.005	0.231	0.369	0.005	0.092	0.979	0.702
11BHSD2	0.000	0.313	0.654	0.456	0.000	0.030	0.567	0.973
LPL	0.013	0.119	0.003	0.010	0.765	0.340	0.874	0.933

3.2.2 The methylation of GR and PPARα in the liver of adult female offspring is not altered by maternal diet or postnatal diet. The methylation status of GR but not PPARα is affected by neonatal leptin treatment.

In order to ascertain if the methylation status of the promoter regions of key mediators of energy balance in the liver of female offspring is affected by maternal diet, postnatal diet or neonatal leptin treatment, methylation sensitive PCR (MSP) was performed. Genomic DNA was prepared from the liver of female offspring from the IUGR rat study (n=8 / diet-treatment group). This DNA was digested with methylation sensitive restriction enzymes (AciI or Hinp1I) and real time PCR was performed with validated gene specific primers using the digested genomic DNA as a template. Resulting data were normalised to PPARγ2 which has no AciI or Hinp1I sites. Results were converted to % of the control group (AD Chow 100%) and a 1 way ANOVA performed followed by Bonferroni's post hoc test. Graphs showing differences in methylation for both genes with statistical analysis can be found in figure 3.5. All results are described relative to the control group only. In addition, a 3 way ANOVA was performed for both genes to analyse single factor and interaction effects on DNA methylation. The results of the 3 way ANOVA are shown in table 3.2.

MSP was performed on the PPARα and GR promoters. Resulting data showed that there were no significant differences in DNA methylation for the PPARα promoter, however, there was a trend for increased methylation in AD offspring irrespective of postnatal diet, but this failed to reach significance (figure 3.5a). For GR there was a significant increase in promoter methylation in leptin treated AD offspring which was dependent on offspring being fed a chow diet (285.3% p=0.05) (figure 3.5b). There was also a trend for increased methylation in AD offspring fed a HF diet, but this failed to reach significance. For both GR and PPARα, leptin treatment and postnatal diet had no effect on methylation in UN offspring.

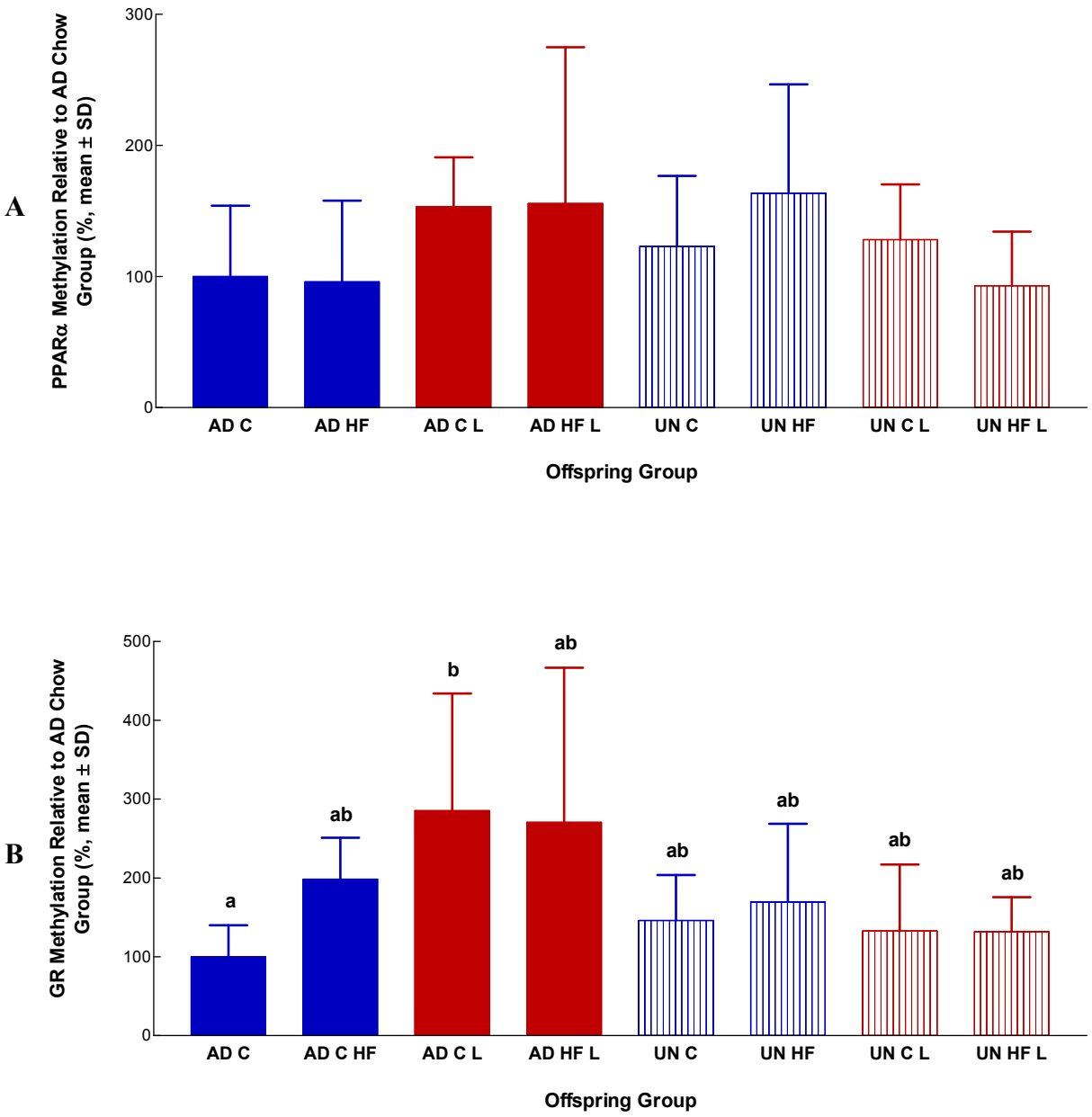


Figure 3.5 The methylation status of the PPARα and GR promoters in the liver of adult female offspring is not altered by maternal diet or postnatal diet, but GR methylation is altered by neonatal leptin treatment. Real time PCR data for methylation of PPARα (A) and GR (B) are shown in response to maternal diet (ad libitum [AD] and undernutrition [UN]), postnatal diet (Chow [C] and High Fat [HF]) and neonatal leptin treatment (leptin [L]) (n=8/group). Values with different letters are significantly different ($p<0.05$) by Bonferroni's post hoc test.

Table 3.2 Table showing tests of between subject effects on DNA methylation for genes measured in liver of female offspring. Single factor effects (MD, T, PND) and interaction effects (MD*T, MD*PND, T*PND, MD*T*PND) are shown. Significant effects (P<0.05) are indicated in red. For PPARα, there were no significant single factor effects, whereas for GR there were single factor effects (MD). For both PPARα and GR, there were significant interaction effects (MD*T).

Gene	Anova	Maternal Diet (MD)	Treatment (T)	Postnatal Diet (PND)	MD* T	MD* PND	T* PND	MD* T* PND
PPARα	0.215	0.966	0.484	0.957	0.011	0.916	0.312	0.230
GR	0.006	0.011	0.054	0.315	0.005	0.559	0.193	0.399

3.2.3 The expression of genes involved in energy balance in the liver of adult male offspring is not altered by maternal diet or postnatal diet. The effect of neonatal leptin treatment is gene specific and dependent on maternal and postnatal diet.

In order to ascertain if the expression of genes involved in energy balance in the liver of male offspring are affected by maternal diet, postnatal diet or neonatal leptin treatment, total RNA was prepared from the liver of adult male offspring from the IUGR rat study (n=8 / diet-treatment group) and used to prepare cDNA. Real time polymerase chain reaction was performed with validated gene specific primers using the cDNA as a template.

Genes studied were the same as those studied in the liver of female offspring; GR, PEPCK, 11βHSD-2, PPARα, AOX, CPT-1 and LPL. Resulting gene expression data were normalised to CYC cDNA and analysed by the ΔCT method [228]. Results were converted to % of the control group (AD Chow 100%) and a 1 way analysis of variance (ANOVA) performed followed by Bonferroni's post hoc test. Graphs showing differences in mRNA expression for each gene with statistical analysis can be found in figures 3.6 to 3.8. All results are described relative to the control group only. In addition, a 3 way ANOVA was performed for all genes measured, to analyse single factor and interaction effects on gene expression. The results of the 3 way ANOVA are shown in table 3.3.

Results showed that there was no effect of a maternal diet or postnatal diet on the expression of any of the genes studied in the liver of male offspring. In addition, the majority of genes tested were not significantly altered by neonatal leptin treatment. However, there was a significant effect of neonatal leptin treatment for 2 of the genes tested. For PEPCK, there was a significant effect of leptin treatment in UN offspring fed a chow diet (42.0% p=0.05) (figure 3.6b). Conversely for CPT-1, leptin treatment significantly affected UN offspring given a HF diet (49.91% p=0.05) (figure 3.7c). These data indicate that an effect of leptin treatment on gene expression in this tissue is dependent on maternal UN diet.

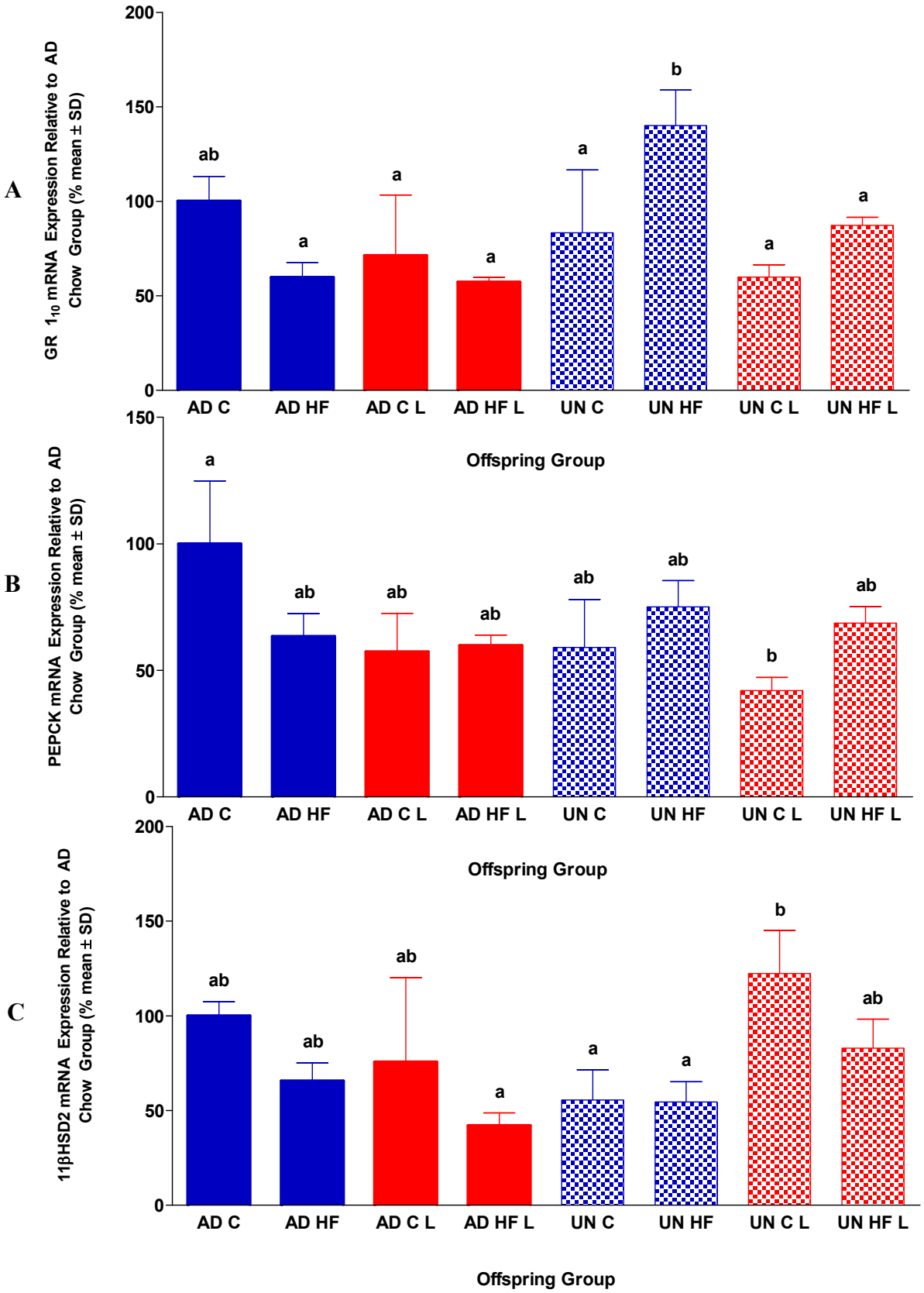


Figure 3.6 The expression of GR1$_{10}$, PEPCK and 11βHSD-2 in the liver of adult male offspring is not altered by maternal diet or postnatal diet. Expression of PEPCK is altered by neonatal leptin treatment and the effect is dependent on both maternal and postnatal diet. Real time PCR data for expression of GR1$_{10}$ (A), PEPCK (B) and 11βHSD-2 (C) are shown in response to maternal diet (ad libitum [AD] and undernutrition [UN]), postnatal diet (Chow [C] and High Fat [HF]) and neonatal leptin treatment (leptin [L]) (n=8/group). Values with different letters are significantly different ($p<0.05$) by Bonferroni's post hoc test.

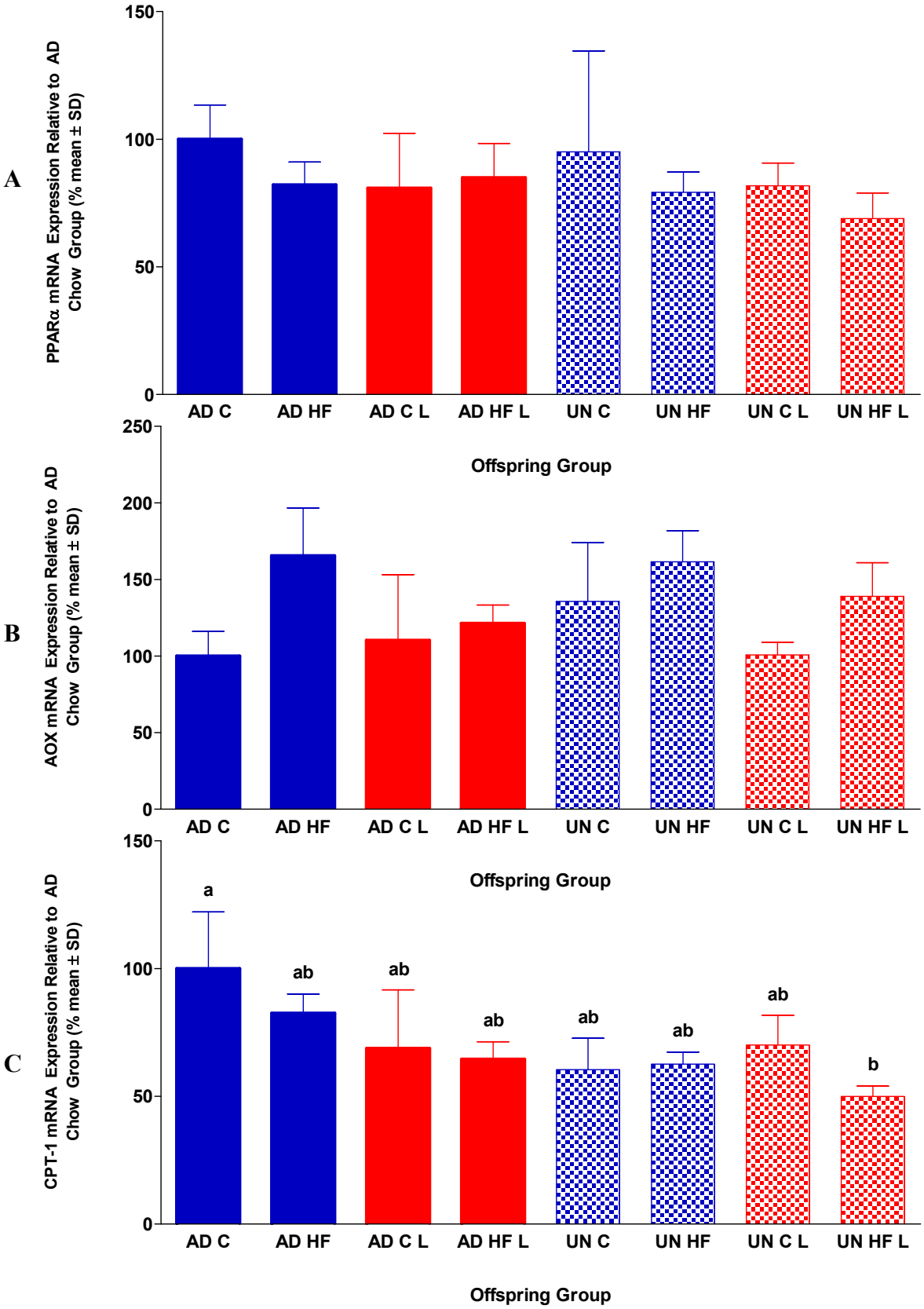


Figure 3.7 The expression of PPARα and its target gene AOX in the liver of adult male offspring is not altered by maternal diet, postnatal diet or neonatal leptin treatment. Expression of CPT-1 is altered by neonatal leptin treatment and the effect is dependent on both maternal and postnatal diet. Real time PCR data for expression of PPARα (A) and AOX (B) and CPT-1 (C) are shown in response to maternal diet (ad libitum [AD] and undernutrition [UN]), postnatal diet (Chow [C] and High Fat [HF]) and neonatal leptin treatment (leptin [L]) (n=8/group). Values with different letters are significantly different ($p<0.05$) by Bonferroni's post hoc test.

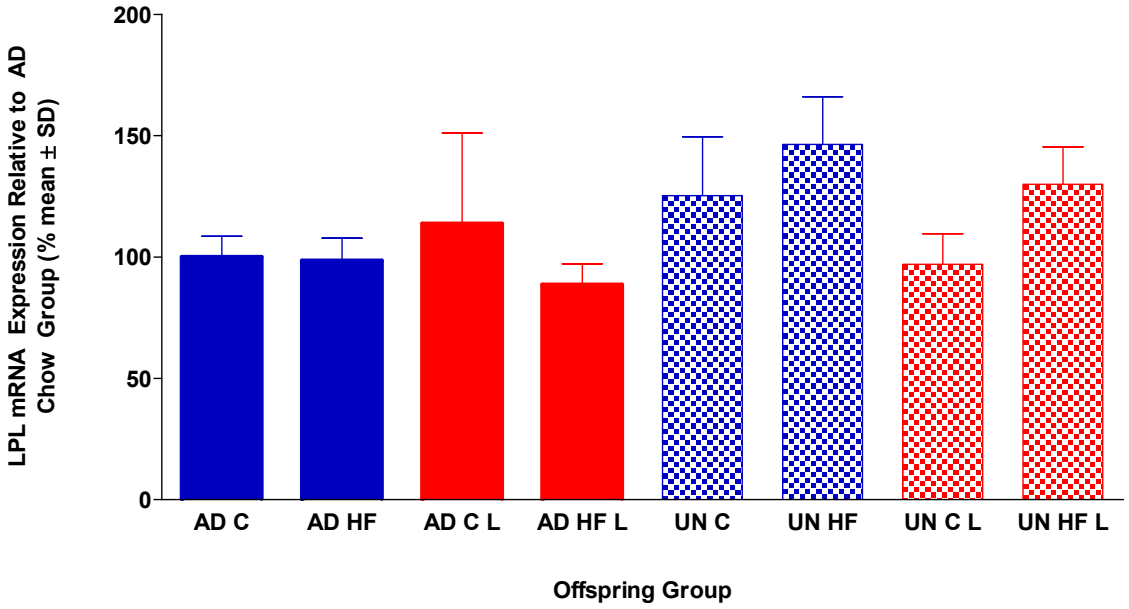


Figure 3.8 The expression of LPL in the liver of adult male offspring is not altered by maternal diet, postnatal diet or neonatal leptin treatment. Real time PCR data for expression of LPL are shown in response to maternal diet (ad libitum [AD] and undernutrition [UN]), postnatal diet (Chow [C] and High Fat [HF]) and neonatal leptin treatment (leptin [L]) (n=8/ group).

Table 3.3 Table showing tests of between subject effects on the mRNA expression of genes measured in the liver of male offspring. Single factor effects (MD, T, PND) and interaction effects (MD*T, MD*PND, T*PND, MD*T*PND) are shown. Significant effects ($P<0.05$) are indicated in red. There were no significant single factor or interaction effects for PPARα. There were significant single factor effects for CPT-1 (MD), AOX (PND), LPL (MD), GR_{110} (MD,T) and 11βHSD2 (PND). There were significant interaction effects for LPL (MD*PND), GR_{110} (MD*PND), 11βHSD2 (MD*T) and PEPCK (MD*PND).

Variable	Anova	Maternal Diet (MD)	Treatment (T)	Postnatal Diet (PND)	MD*T	MD*PND	T*PND	MD*T*PND
PPARα	0.637	0.464	0.204	0.178	0.818	0.634	0.426	0.546
CPT-1	0.047	0.015	0.076	0.175	0.116	0.899	0.754	0.224
AOX	0.082	0.445	0.084	0.009	0.648	0.810	0.421	0.203
LPL	0.030	0.010	0.268	0.455	0.190	0.032	0.746	0.334
GR_{110}	0.000	0.012	0.001	0.351	0.161	0.000	0.923	0.085
11βHSD2	0.001	0.394	0.212	0.005	0.000	0.464	0.320	0.298
PEPCK	0.036	0.250	0.026	0.782	0.460	0.015	0.110	0.354

3.3 Discussion

The regulation of energy metabolism in the liver is under strict hormonal control. This hormonal action is mediated by specific transcription factors which regulate the expression of key enzymes involved in metabolic pathways such as glucose metabolism. For example, under conditions of stress glucocorticoids mediate their catabolic effects via the glucocorticoid receptor. GR in turn regulates the expression of key enzymes such as PEPCK and this in turn could alter the rate of gluconeogenesis, which will impact blood glucose levels. Alterations in the regulation of transcription factor expression will therefore impact on their respective metabolic pathways and have consequences on overall energy homeostasis.

In offspring programmed by a protein restricted maternal diet, metabolic control mechanisms are disturbed, as indicated by the presence of disorders such as impaired glucose tolerance and dyslipidemia [40,42]. In the liver of PR offspring, studies have shown that energy metabolism favours gluconeogenesis and fatty acid β-oxidation, as a result of reduced gene expression of 11βHSD2, increased expression of GR, PPARα and their target genes. In addition to this, methylation changes have been identified in the promoters of PPARα and GR in these offspring which favour the altered gene expression [77,213]. Differential methylation of key transcription factors in the liver is therefore implicated in the altered metabolic state of these offspring. In agreement with this, emerging evidence has indicated the causative role of alterations in the expression of other genes in programmed rats due to altered DNA methylation [214,215].

In another established model of programming, the IUGR rat, it has been shown that neonatal leptin treatment can prevent all metabolic abnormalities, i.e. hyperphagia, high blood pressure, hyperinsulinemia, hyperleptinemia and obesity [122]. If mechanisms of programming are similar to that identified in PR offspring and aberrant methylation of key transcription factors underwrites the metabolic abnormalities, this may imply that leptin treatment is able to prevent or counteract the methylation changes. The aim of this study was to determine if there were similar changes in the expression and methylation of key genes involved in energy metabolism in the liver of male and female IUGR offspring as identified in PR offspring and in addition to determine if neonatal leptin treatment could prevent any gene expression and methylation changes. To answer these questions we measured the expression and methylation status of genes involved in energy balance in the liver from the IUGR offspring [122] using real time PCR.

The work in this chapter demonstrated that in the liver of both male and female offspring, the expression of key transcription factors and enzymes involved in gluconeogenesis and fatty acid β-oxidation were not generally altered by maternal undernutrition, a postnatal high fat nutrition or by neonatal leptin treatment. In keeping with this, there was little evidence of any altered DNA methylation in the promoters of the transcription factors GR and PPARα.

In female offspring, gene expression was mostly unchanged by maternal diet, postnatal diet and neonatal leptin treatment. However, for most of the genes tested, data had a large spread which could potentially mask the presence of any significant changes. However, despite this, a few significant changes in gene expression were identified, namely of PPARα, GR and PEPCK. For these genes, expression was reduced by UN maternal diet and this reduction was dependent on the postnatal diet. Interestingly, these changes were prevented by neonatal leptin treatment. Furthermore, any significant changes which were identified in the transcription factors PPARα and GR were not passed onto their target genes. For example, the effect of maternal diet on GR gene expression in offspring fed a chow diet was not passed on to PEPCK. In fact alterations occurred in PEPCK gene expression, which were independent of GR. In addition, alterations in PPARα expression were not passed onto AOX and again, AOX had alterations in gene expression which were independent of PPARα. This implies that alternative signalling mechanisms were affecting PEPCK and AOX expression, for example the alterations in PEPCK could have been mediated by glucagon, whilst alterations in AOX could be a result of leptin receptor signalling. Alternatively, it is possible that the methylation of their own promoter regions was altered, thus affecting gene expression and this warrants further investigation. In male offspring, significant differences in gene expression were found for only 2 of the genes studied; PEPCK and CPT-1. These alterations in gene expression were dependent on maternal diet (UN), postnatal diet and neonatal leptin treatment, but as shown in females, were not associated with changes in the expression of the transcription factors GR and PPARα. This implies again that either alternative signaling mechanisms were causing the differences in gene expression or differential methylation of their own promoter regions. The failure of transcription factor gene differences to be passed on to their target genes in both males and female offspring is contrasting to that found in PR offspring in which target gene expression mirrored that of GR and PPARα. The lack of significant changes in gene expression was not due to low sample numbers, as a retrospective statistical power calculation confirmed that an N of 8 per dietary group provided statistical power of at least 70% for detecting a 50% difference between groups, with a probability of $P<0.05$ for all of the genes measured.

Methylation of the PPARα and GR promoters were investigated in the female offspring. The amplicons used were the same as those used to measure methylation in the PR offspring. Unsurprisingly, no methylation changes were identified for PPARα and GR in offspring groups where gene expression had not changed. However, the few significant alterations in gene expression found in PPARα and GR were not accompanied by methylation changes, in fact, no methylation changes were identified what so ever for PPARα. The only significant alteration in methylation identified was an increase in methylation of the GR promoter in AD offspring given neonatal leptin treatment. Although this change corresponded to a small decrease in corresponding gene expression, as would be expected, this did not reach significance. Overall, trends in methylation between the different offspring groups were not representative of gene expression changes. However, the MSP amplicons do not cover the whole promoter regions of PPARα and

GR. It is therefore possible that methylation is altered in different locations of the PPARα and GR promoters to that measured.

Overall, none of the gene expression and methylation changes seen in the liver of PR offspring were found in the same genes in the liver of IUGR offspring. These results indicate that alternative mechanisms may exist in the programming by severe maternal nutrition as opposed to a protein restriction. This could be expected, as the 70% diet reduction in the IUGR rat represents a much more extreme situation than the mild protein restriction of PR offspring. It is therefore plausible that in IUGR rats different metabolic pathways within the liver and other tissues types are affected compared to those altered in the PR offspring. This would therefore involve epigenetic alterations in different transcription factors and enzymes. The metabolic disturbances found in PR offspring are consistent with altered hepatic glucose production by gluconeogenesis and increased fatty acid β-oxidation. Given the role of adipose tissue in the metabolic processes associated with IUGR disturbances, in particular obesity and the ability of the adipokine leptin to reverse these disturbances, it is therefore possible that in IUGR offspring, metabolic disturbances may be a consequence of altered lipid metabolism in adipose tissue.

Research into the effect of leptin treatment on genes involved in energy balance in peripheral tissues in adult rats may give clues as to the genes which are affected by maternal diet in the IUGR rat. It has been shown that leptin given to ob/ob mice not only depletes adipose lipid, but also depletes liver lipid indicating that fat metabolism is affected in both tissue types [230]. A hepatic microarray on leptin treated ob/ob mice has identified lipogenic genes which are down regulated by leptin including fatty acid synthase (FAS), ATP-citrate lyase (ACL) and stearoyl CoA desaturase-1 (SCD1) [230]. Furthermore leptin was also shown to regulate genes involved with carbohydrate metabolism including glucose-6-phosphatase and fructose bisphosphatase 1, two gluconeogenic enzymes [230]. In addition to this, it has also been shown that leptin treatment in adult rats also effects gene expression in adipose tissue. For example, leptin has been found to suppress the adipogenic expression of the lipogenic enzymes ACL and FAS [231], whilst in another study it increased expression of PPARα and its target genes, CPT-1 and AOX within adipose tissue [232].

Findings in this report have not identified how neonatal leptin treatment may act to reverse metabolic disturbances in programmed offspring, however data indicates that leptin may act by normalising the expression of genes involved in energy balance which are affected by undernutrition. The finding that leptin regulates several other genes of lipid metabolism in both the liver and adipose tissue of adult rats [230,231], indicates which genes may be altered by neonatal leptin treatment and possibly by maternal undernutrition in the IUGR rat. It would therefore be interesting to determine if regulation of any of these lipid and carbohydrate metabolism genes were altered not only by neonatal leptin treatment but also by maternal undernutrition in the IUGR offspring.

Chapter 4

Gene Expression and Methylation in Adipose Tissue of Female IUGR Rats

4.1 Introduction

4.1.1 Adipose tissue, obesity and developmental programming

White adipose tissue (WAT) is the body's largest endocrine organ. It is composed predominantly of adipocytes (33-66%), however a variety of cells are also present including fibroblasts, vascular cells, macrophages, preadipocytes and monocytes [233]. Like the liver, adipose tissue plays a fundamental role in whole body energy homeostasis. As such, it has three main roles; it houses the major energy reserve of the body TAG, it releases fatty acids and glycerol according to energy demands and it secretes adipokines which have autocrine, paracrine and endocrine effects on carbohydrate and lipid metabolism.

An excessive increase in WAT mass results in obesity, which is associated with metabolic disorders such as dyslipidemia, insulin resistance, glucose intolerance and hypertension [233]. These disorders are also found in IUGR offspring whom have experienced rapid catch up growth during early life [27,28,39,122] and collectively are associated with an increased risk of type II diabetes and CV disease. An understanding of adipose tissue metabolic function and formation is therefore necessary in order to recognise which pathways could be altered in the developmental programming of obesity and its related disorders and elucidate how they could be intervened.

4.1.2 Storage and mobilisation of energy stores

WAT maintains an overall energy balance by sensing and responding to external signals, which result in the storage of excess energy in the form of TAG during times of nutritional affluence, or by the mobilisation of energy during times of nutritional deprivation. The size of WAT mass is therefore determined by the balance between storage and mobilisation, i.e. lipogenesis and lipolysis, which are predominantly under strict hormonal control by the regulatory hormones insulin and glucagon [234].

In the fed state, high blood glucose levels stimulate insulin secretion which binds to its receptor on adipocytes to induce lipogenesis and the storage of excess energy as TAG. This is achieved in a number of ways. Firstly, insulin signalling increases glucose transporters in the adipocyte cell membrane which stimulate glucose uptake into the adipocytes. The glucose is needed to provide glycerol-3-phosphate from glycolysis for the synthesis of TAG, as adipose tissue does not express glycerol kinase. In addition, the glucose can also be oxidised to acetyl CoA for fatty acid synthesis and insulin increases the expression and activation of several key enzymes central to this process, including fatty acid synthetase (FAS) and acetyl CoA carboxylase (ACC). Insulin also increases

LPL activity, which facilitates the hydrolysis of triglycerides in lipoproteins, allowing FA to be taken up into the adipocyte. Fatty acids can then be esterified to form TAG for storage until needed. In addition to these roles, the increased insulin to glucagon ratio also results in the dephosphorylation and inactivation of a major enzyme of lipogenesis, hormone sensitive lipase (HSL), thereby inhibiting lipolysis and the release of FA from adipocytes.

Conversely, during times of energy demand such as fasting, lipogenesis is inhibited by an increased glucagon to insulin ratio and lipolysis takes place. Glucagon inhibits lipogenesis by inactivating ACC by phosphorylation, whereas it stimulates lipolysis by phosphorylating and activating HSL which cleaves TAG. The end result is the mobilisation of TAG to fatty acids and glycerol, which can be transported to the liver whereby fatty acids are oxidised for energy and glycerol can be used in gluconeogenesis [235]. The action of glucagon on adipocytes therefore results in net lipolysis in adipose tissue, providing FA to other metabolic tissues in times of energy demand. Furthermore, in times of stress such as exercise and cold, glucocorticoids and epinephrine are also able to stimulate lipolysis within adipocytes.

If energy intake exceeds expenditure over a long period of time, it results in the excessive growth of adipose tissue and obesity develops, which is associated with a loss of adipocyte function. Here, adipocytes become enlarged and insensitive to external signals, with altered regulation, expression and activation of key transcription factors and enzymes involved in the metabolic pathways, resulting in inappropriate lipogenesis and lipolysis [235]. For example, in obesity, excess FA are inappropriately released from adipocytes and taken up in tissues such as the liver and muscle resulting in lipotoxicity and the stimulation of gluconeogenesis which can both lead to insulin resistance. As the activating effects of glucagon on HSL are unopposed in insulin resistance, this only serves to worsen the condition.

4.1.3 Adipokines and obesity

Adipose tissue functions as an important endocrine organ by producing an array of adipokines (hormones and cytokines). Important adipokines secreted by WAT include leptin, adiponectin, retinol binding protein 4 (RBP4), resistin and the pro-inflammatory cytokines TNFα and IL6. They can act not only on adipose tissue but also on other key metabolic organs such as the liver, muscle, pancreas and brain and have varying functions in these tissues, including modulating carbohydrate and lipid metabolism and the immune response [235]. Unsurprisingly, adipokine profiles are altered in obesity and this can have profound effects on carbohydrate and lipid metabolism and thus the insulin sensitivity of the organs they target [236].

Some of these adipokines, such as leptin and adiponectin have insulin sensitising roles. Adiponectin, for example, is thought to enhance insulin sensitivity due to several key roles in carbohydrate and lipid metabolism; It decreases hepatic gluconeogenesis, activates AMPK in liver and muscle to increase FA oxidation, reduces TAG storage, enhances insulin signalling by phosphorylating IRS in the liver and muscle [235] and also increases adipose and muscle glucose uptake [236]. Adiponectin release is stimulated by insulin, but is also increased in response to the insulin sensitising thiazolidinediones (TZD's) indicating that it is in part regulated by PPARγ. As such, adiponectin levels are reduced in insulin resistance, glucose intolerance, hyperlipidemia, type 2 diabetes and obesity [236].

The expression of some adipokines, however, is associated with insulin resistance. For example, RBP4 decreases insulin sensitivity by modulating glucose homeostasis and levels are increased in HF feeding, obesity and the metabolic syndrome. Resistin acts on adipose tissue and skeletal muscle to antagonise insulin actions, e.g. by increasing gluconeogenic enzymes and reducing AMPK activity [235], resulting in glucose intolerance and insulin resistance. Furthermore, the pro-inflammatory cytokines such as tumour necrosis factor α (TNF-α) and interleukin 6 (IL6) are proposed to increase insulin resistance in obesity, in part by increasing the release of FFA from adipocytes and by reducing adiponectin synthesis [236].

4.1.4 Adipogenesis

Initiation of adipogenesis during development occurs at different periods in humans and rodents. In humans, WAT is not produced until the third trimester and is subsequently present at birth, whereas in rodents WAT is not detected until after birth [233]. WAT mass is dynamic and can vary in humans and rodents according to the global energy status due to its ability to increase not only adipocyte size by increasing TAG storage (hypertrophy), but also by increasing cell numbers (hyperplasia). As a result, in addition to early development, adipogenesis can occur continuously throughout adult life and WAT has the novel capacity for unlimited growth.

In vitro models of adipocyte differentiation such as the pre-adipocyte cell line 3T3-L1 derived from 17-19 day old Swiss 3T3 mouse embryos have been central to deciphering the process of differentiation and the main factors involved in the process. Pre-adipocytes are derived from mesenchymal precursors and adipogenesis occurs when these fibroblastic pre-adipocytes are stimulated to differentiate by hormones, the effects of which are mediated by a cascade of transcription factors in a highly coordinated process [233].

Prior to differentiation, pre-adipocytes in culture first undergo cell contact inhibition and growth arrest. Next, upon hormonal stimulation with factors including insulin, glucocorticoids and cAMP,

clonal expansion occurs, whereby cells re enter the cell cycle and go through at least two rounds of cell division. This is then followed by growth arrest and terminal differentiation. Three main classes of transcription factors play fundamental roles in orchestrating adipose differentiation; CCAAT-enhancer-binding proteins (C/EBP), PPARγ2 and sterol regulatory binding protein (SREBP) (ADD-1 in rodents) [234] (figure 4.0). PPARγ2 plays a particularly important role in differentiation and has been termed "the master regulator". Its expression is induced early in differentiation, whereby it induces exit from cell cycle and in combination with C/EBPα induces transcription of adipose specific genes, for example LPL, FAS, fatty acid binding protein [234]. In all, around 100 proteins are altered at the transcriptional level during terminal differentiation [233], leading the adipocyte to take on the spherical, lipid filled characteristics of a terminally differentiated mature adipocyte [234].

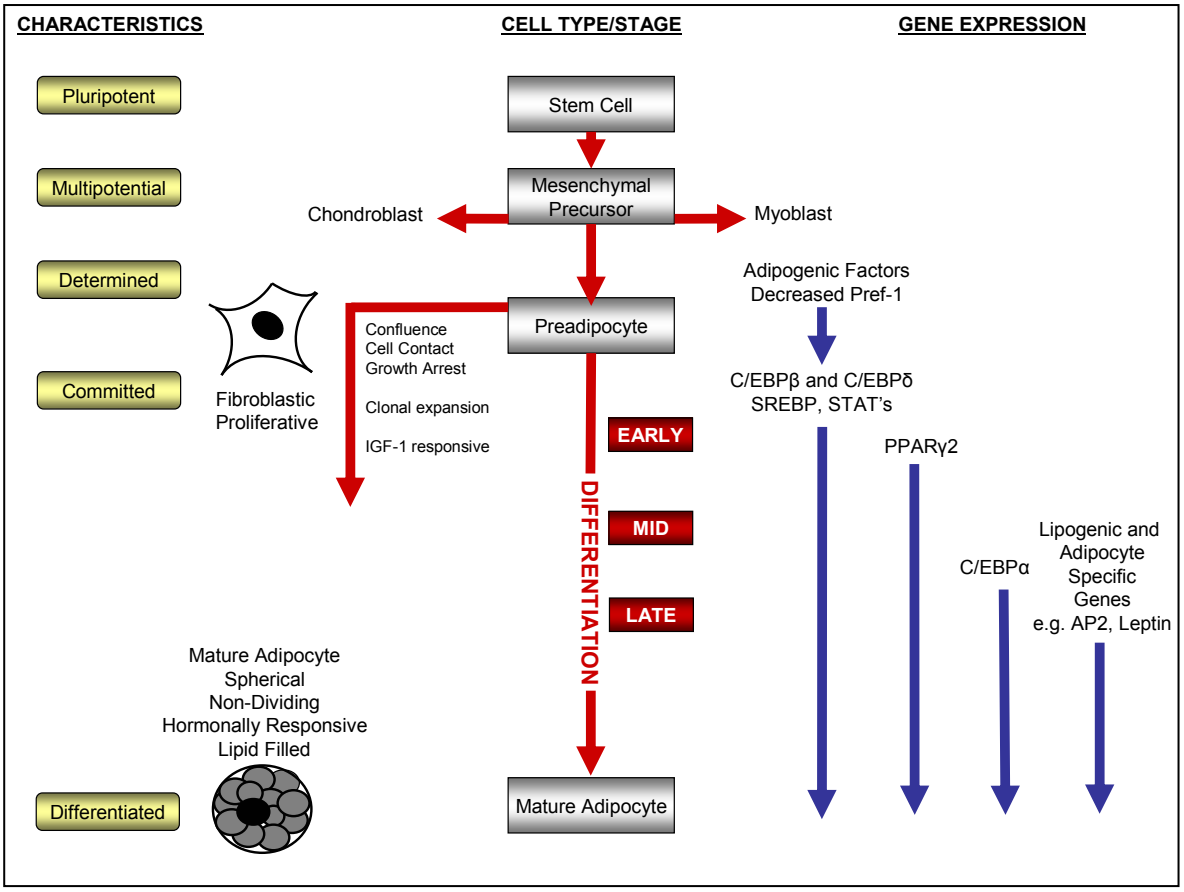


Figure 4.0 The involvement of adipogenic transcription factors in adipocyte differentiation (adapted from [233]). Fibroblastic, proliferative, pre-adipocytes express preadipocyte factor-1 (Pref-1), which is markedly down regulated upon initiation of differentiation. During clonal expansion, the first transcriptional event known is induction of C/EBPδ and β, which in vitro are induced by hormones such as glucocorticoids, cAMP and insulin. SREBP1 is also expressed early during differentiation and this coordinates transcription of genes of lipid, fatty acid and cholesterol metabolism. In addition, Stats are implicated during the early stages of differentiation; Stat's 1,3 and 5 are induced strongly and this is probably due to the effect of cytokines and peptide hormones such as GH on cytokine receptors found on adipocytes. C/EBPβ, C/EBPδ and SREBP1 all subsequently induce expression of the master regulator of adipose differentiation, PPARγ2. In addition GR can also induce PPARγ2 expression indirectly via C/EBP's and secondly by inducing PPARγ2 directly. PPARγ2 induces the expression of key adipocyte genes such as LPL, fatty acid synthetase (FAS), acyl CoA synthesase and PEPCK, all of which facilitate uptake and storage of fatty acids. C/EBPα is induced in later stages of differentiation (by C/EBP β and δ) and it functions to stabilize the mature phenotype in part by maintaining PPARγ expression and also by inducing other adipose specific genes [234].

4.1.5 De-differentiation of adipocytes

An interesting and useful feature of adipocytes is that anti-adipogenic factors can inhibit or even reverse differentiation. Such factors include TNFα which can induce de-differentiation via down regulating PPARγ and C/EBPα expression [237] and retinoic acid which can inhibit the early stages of differentiation [235]. A key paper has indicated that leptin can exert fat reducing effects in adult rodents via inducing adipocyte de-differentiation. Hyperleptinemia was induced in normal adult rats which resulted in the rapid disappearance of all visible body fat within 7 days owing to a 95% reduction in adipocyte TAG content and the gene profile of a de-differentiated adipocyte [232]. However, as the rats regained weight two months after leptin levels returned to normal, this ability to de-differentiate the adipocyte was not permanent [238]. Normally, calorie reduction results in the reduction of fat in adipocytes through lipolysis, providing FA for oxidation in the liver, as occurs in sympathetic mediated lipolysis. However, in leptin treated rats there was no corresponding release of free fatty acid or ketones alongside glycerol as would be expected. It further was shown in isolated adipocytes that the leptin treatment caused a significant reduction in the mRNA of leptin, PPARγ2 and FAS, key adipogenic genes. Contrastingly they found an increase in the mRNA of PPARα and its target genes, CPT-1 and AOX, enzymes of fatty acid β-oxidation not normally expressed in adipocytes [232]. Furthermore, in hyperleptinimic rats, researchers also found increased UCP-1 and UCP-2 mRNA, indicating that the energy from oxidation of FA was released as heat [238] and down regulated adipocyte markers, including ACC, TNFα and leptin, whilst the preadipocyte specific gene pref-1 was up regulated [238]. It was therefore concluded that the leptin treatment had reduced the expression of key adipogenic factors and induced fatty acid oxidation within the adipocytes, resulting in their transformation from fat storing cells into de-differentiated fat burning cells, with the PPAR's playing a key regulatory role [232,238].

Subsequent studies have shown that the oxidative effects of leptin were not centrally mediated on the adipocyte (sympathetic stimulation), as norepinephrine stimulated lipolysis resulted in normal FFA release from adipocytes [232]. The leptin receptor (OB-Rb) is expressed on adipocytes [121] and evidence indicates that the effects of leptin were mediated directly on the adipocyte, as the lipolytic effects were shown to be absent in adipocytes from OB-Rb deficient fa/fa rats [232]. It was also shown that PPARα is absolutely required to facilitate this novel form of lipolysis, as in PPARα null mice, hyperleptinemia reduced food intake, but not fat pad weight. Furthermore CPT-1 expression did not increase, indicating the need for PPARα to facilitate the oxidative effects of hyperleptinemia in these adult mice [239].

This novel oxidation function of leptin has also been identified in liver, skeletal muscle and the pancreas [240]. For example, in the pancreas fatty acid oxidation was shown to be increased due to upregulated AOX and CPT1 and a reduction in the enzymes of lipogenesis including ACC. These alterations in gene expression did not occur in islets from OB-Rb deficient fa/fa rats indicating a

direct effect [241]. The role of leptin in these tissues was proposed to be anti-diabetic by preventing non-adipocyte lipotoxicity [240] and to maintain the TAG content of these cells within a strict range [73].

4.1.6 Aims

Interest in the developmental programming of disease by altered epigenetic regulation has focused predominantly on pathways involved in programming of hypertension, energy metabolism and glucose intolerance in the metabolically active liver tissue. These studies have identified specific DNA methylation and gene expression changes of important transcription factors, such as GR and PPARα, which are involved in key metabolic pathways such as gluconeogenesis and fatty acid metabolism respectively [77,213]. Little work has focused on the programming of energy balance in adipose tissue, which could play a central causative role in the development of adult obesity and its associated metabolic disturbances commonly found in IUGR rodents and humans.

It has been shown in a rat model of global undernutrition that neonatal leptin treatment to reproduce the natural leptin surge during development reverses the metabolic disturbances found in programmed IUGR offspring, including obesity [122]. Work in this thesis has not identified any relevant changes in the expression or methylation of genes involved in energy balance in the liver due to maternal diet or neonatal leptin treatment. It is possible that the mechanisms underlying programming and reversal by leptin involves alterations in key adipocyte genes, such as transcription factors involved in adipogenesis and lipid metabolism. Interestingly, recent work has shown that leptin can induce de-differentiation of adipose cells in adult rats as a result of down-regulating key adipocyte genes including PPARγ and upregulating genes of lipolysis such as PPARα [232]. Taken together, these two studies imply that leptin may act during the later stages of developmental plasticity to reverse the programmed phenotype of offspring from undernourished dams in part by altering the regulation of key genes involved in energy balance to prevent the storage of excess adipose tissue. This, however, has not yet been tested.

To gain a better understanding of the programming of energy balance in adipose tissue and the molecular mechanisms induced by neonatal leptin treatment at the level of the adipocyte, this study uses adipose tissue from the same rat model of global undernutrition used by Vickers and colleagues [122]. It investigates the expression and methylation status of several key genes associated with adipocyte function and energy balance in response to maternal diet, postnatal diet and neonatal leptin treatment using real time PCR and methylation sensitive real time PCR. Furthermore, it investigates the impact of the timing of leptin treatment in differentiating adipocytes on the expression of several key genes involved in energy balance.

Data presented in this chapter shows that there is no effect of maternal diet or postnatal diet on the expression of any of the genes tested. There was, however a significant effect of neonatal leptin treatment, which increased the expression of many of the genes involved in energy balance tested. Results indicate that neonatal leptin treatment may induce a novel form of lipolysis in adipocytes which persists into adult hood to protect against HF feeding. However, unlike mechanisms underlying altered gene expression in the livers of PR offspring [77,213] methylation sensitive PCR indicates that methylation changes in the regions measured were not responsible for the leptin-induced changes in gene expression. Furthermore, work with 3T3-L1 adipocytes indicates that leptin treatment is able act on the adipocyte to alter gene expression of key genes involved in energy balance during differentiation.

4.2 Results

4.2.1 The expression of genes involved in energy balance in the adipose tissue of adult female offspring is not altered by maternal diet or postnatal diet, but is altered by neonatal leptin treatment

In order to ascertain if the expression of genes involved in energy balance in adipose tissue of female offspring is affected by maternal diet, postnatal diet or neonatal leptin treatment, total RNA was prepared from adipose tissue of adult female offspring from the IUGR rat study (n=8 / diet-treatment group) and used to prepare cDNA. Real time PCR was performed with validated gene specific primers using the cDNA as a template. Genes studied were those involved in adipocyte differentiation and fatty acid storage (IR, GR, 11βHSD2, PPARγ2 and LPL), lipolysis (PPARα, AOX, CPT-1 and HSL) and body weight regulation (Leptin and its receptor). Resulting gene expression data were normalised to 18s cDNA and analysed by the ΔCT method [228]. Results were converted to % of the control group (AD Chow 100%) and a 1 way ANOVA performed followed by Bonferroni's post hoc test. Graphs showing differences in mRNA expression for each gene with statistical analysis can be found in figures 4.1 to 4.5. All results are described relative to the control group only. In addition, a 3 way ANOVA was performed for all genes measured, to analyse single factor and interaction effects on gene expression. The results of the 3 way ANOVA are shown in table 4.1.

Results showed that there were no significant differences in gene expression between AD and UN offspring, or between AD and UN offspring fed a chow diet or a high fat diet in any of the genes measured. There was therefore no significant effect of a maternal diet or postnatal diet on the expression of genes involved in energy balance in the adipose tissue of female offspring.

Analysis of PPARα expression by real time PCR demonstrated that its expression was significantly increased by neonatal leptin treatment in offspring fed a chow diet from both AD dams (303.8% p=0.05) and UN dams (317.3% p=0.05). In addition, PPARα expression was significantly increased by neonatal leptin treatment in offspring fed a high fat diet from both AD dams (478.5% p=0.001) and UN dams (391.6% p=0.001) (figure 4.1a). The expression of PPARα's target genes AOX and CPT-1 were affected by neonatal leptin treatment in a similar manner. AOX expression was increased in leptin treated offspring fed a chow diet from both AD dams (530.2% p=0.01) and UN dams (466.5% p=0.05) (figure 4.1c). In addition to this, AOX expression was increased in leptin treated offspring fed a high fat diet from AD dams (728.6% p=0.001) and UN dams (565.2% p=0.001). For CPT-1, the increase in gene expression was limited to leptin treated offspring fed a high fat diet from both AD dams (350.6% p=0.001) and UN dams (248.8% p=0.01). Although there was a trend for increased CPT-1 expression in leptin treated offspring fed a chow diet in AD

and UN offspring, this did not reach significance (figure 4.1b). These data indicate that PPARα may be responsible for the changes in CPT-1 and AOX expression.

Analysis of PPARγ2 by real time PCR demonstrated that its expression was significantly increased in all offspring given neonatal leptin treatment; for those offspring fed a chow diet from both AD dams (324.5% p=0.01) and UN dams (349.7% p=0.001) and also for offspring fed a high fat diet from both AD dams (340.1% p=0.001) and UN dams (279.2% p=0.5) (figure 4.2a). The expression of PPARγ2's target gene LPL was also affected by neonatal leptin treatment, but only in those offspring fed a high fat diet from both offspring from AD dams (441.9% p=0.001) and UN dams (414.6% p=0.01) (figure 4.2b). Although there was a trend for increased expression of LPL in offspring fed a chow diet, this did not reach significance. Like PPARα, these data indicate that PPARγ2 may be responsible for the changes seen in the expression of its target gene, LPL.

For GR, there was a trend for increased expression in all leptin treated offspring, however this only reached significance in offspring fed a chow diet from AD dams (1412% p=0.05) and those fed a high fat diet from UN dams (1954% p=0.001) (figure 4.3a). However, expression of the GR exon1_{10} specific transcript was significantly increased in all leptin treated offspring. Expression was increased in leptin treated offspring fed a chow diet from both AD dams (420.7% p=0.05) and UN dams (309.6% p=0.05) and leptin treated offspring fed a high fat diet from both AD dams (408.4% p=0.001) and UN dams (328.8% p=0.01) (figure 4.3b). For 11βHSD2, expression was also increased in offspring given neonatal leptin treatment (figure 4.3c). Expression was significantly increased in leptin treated offspring from AD dams fed both a chow (452.4% p=0.001) and high fat (579.2% p=0.001) diet, but for UN offspring the increase was only significant in leptin treated offspring fed a high fat diet (352.4% p=0.05). Expression of 11βHSD2 was increased in leptin treated UN offspring fed a chow diet, but this failed to reach significance.

The expression of HSL was not significantly altered by neonatal leptin treatment in any of the offspring groups (figure 4.4a). There was however, a trend for reduced expression of HSL in AD offspring regardless of postnatal diet but this failed to reach significance. For IR, there was a trend for reduced expression in all leptin treated offspring, but this only reached significance in AD offspring fed a HF diet (51.5% p=0.05) (figure 4.4b).

Analysis of the effects of neonatal leptin treatment on leptin expression revealed that there was a trend for increased leptin expression in all offspring when compared to the control group, but this only reached significance in offspring from AD dams fed a high fat diet (723.1% p=0.001) (figure 4.5a). For leptin receptor there was also a trend for increased expression in all leptin treated offspring, but this only reached significance in UN offspring fed a high fat diet (766.1% p=0.001) (figure 4.5b).

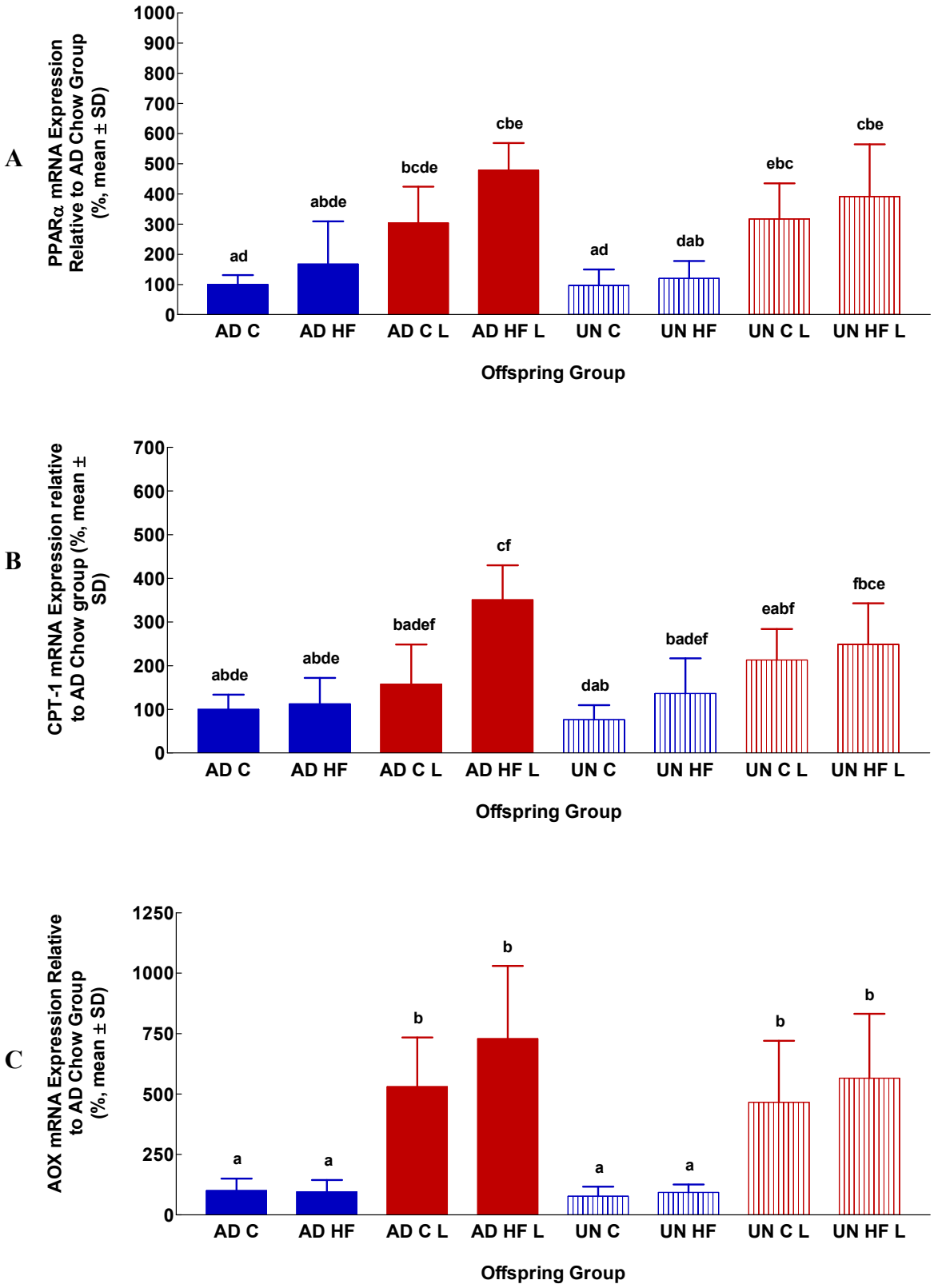


Figure 4.1 The expression of PPARα and its target genes CPT-1 and AOX in adipose tissue of adult female offspring is altered by neonatal leptin treatment but not maternal diet or postnatal diet. Real time PCR data for expression of PPARα (**A**), CPT-1 (**B**), AOX (**C**) are shown in response to maternal diet (ad libitum [AD] and undernutrition [UN]), postnatal diet (Chow [C] and High Fat [HF]) and neonatal leptin treatment (leptin [L]) (n=8/group). Values with different letters are significantly different ($p<0.05$) by Bonferroni's post hoc test.

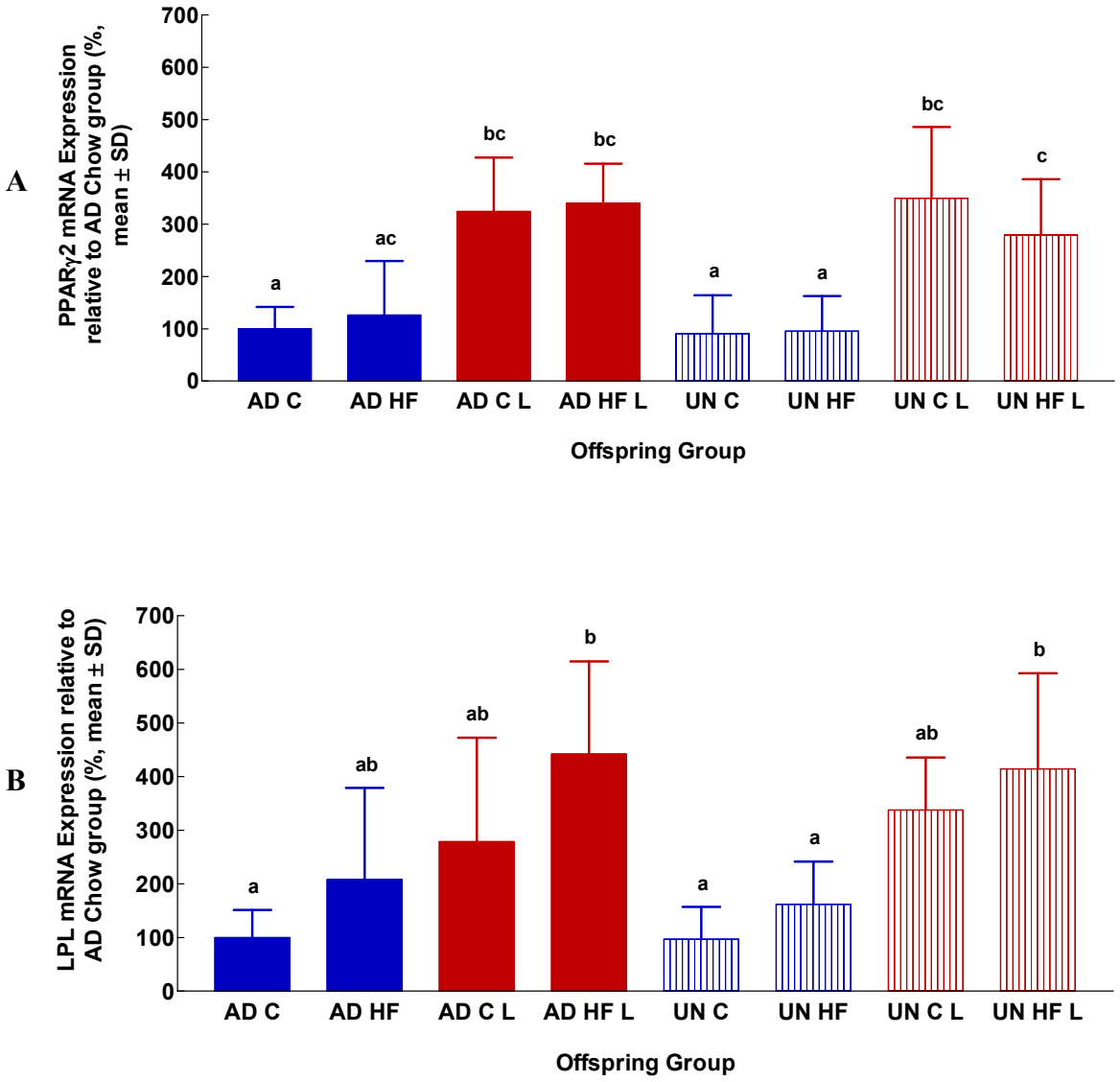


Figure 4.2 The expression of PPARγ2 and its target gene LPL in adipose tissue of adult female offspring is altered by neonatal leptin treatment but not maternal diet or postnatal diet. Real time PCR data for expression of PPAR γ2 (**A**) and LPL (**B**) are shown in response to maternal diet (ad libitum [AD] and undernutrition [UN]), postnatal diet (Chow [C] and High Fat [HF]) and neonatal leptin treatment (leptin [L]) (n=8/group). Values with different letters are significantly different ($p<0.05$) by Bonferroni's post hoc test.

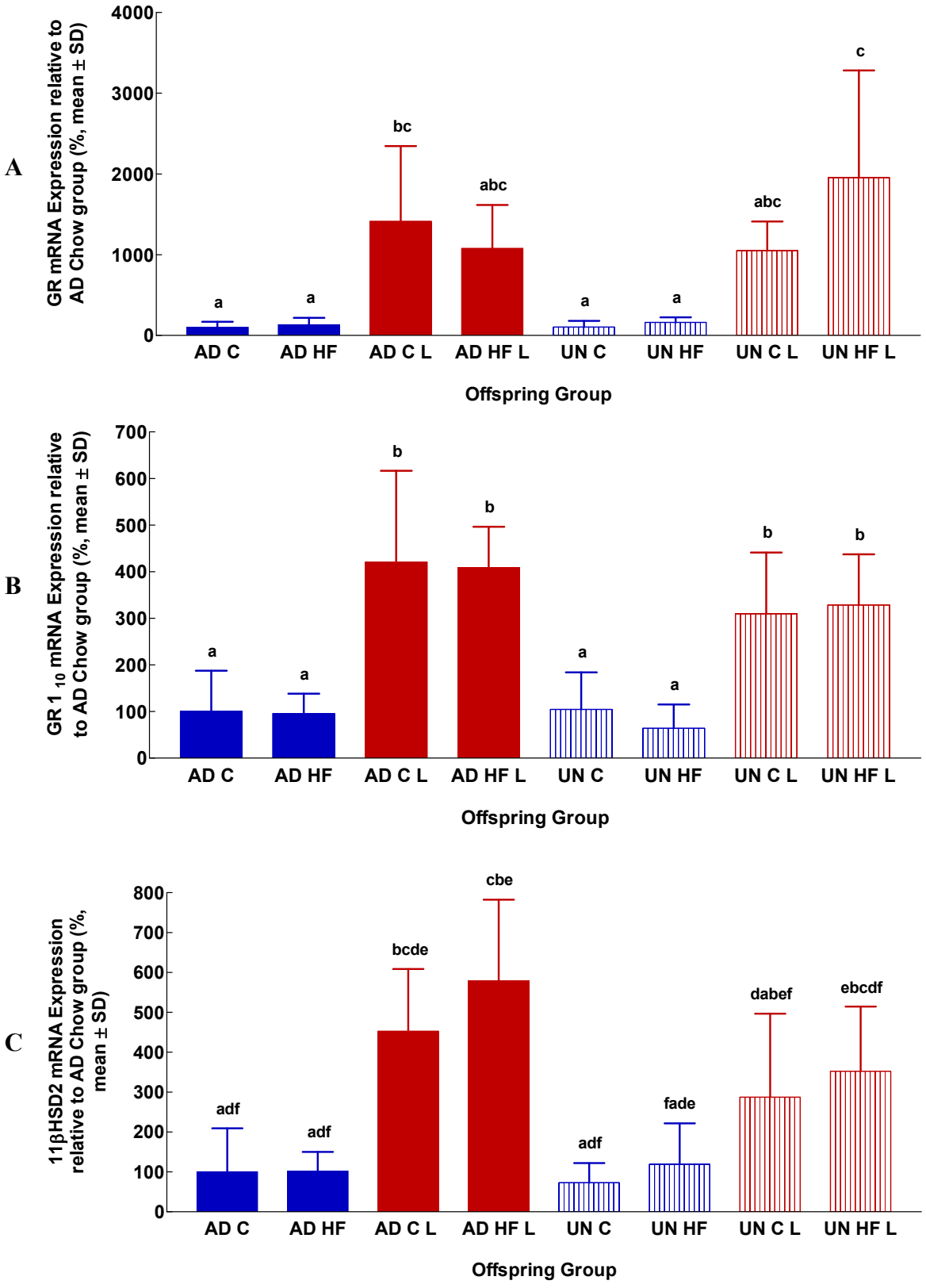


Figure 4.3 The expression of GR, GR 1_{10} and 11βHSD2 in adipose tissue of adult female offspring is altered by neonatal leptin treatment but not maternal diet or postnatal diet. Real time PCR data for expression of GR (**A**), GR 1_{10} (**B**) and 11βHSD2 (**C**)are shown in response to maternal diet (ad libitum [AD] and undernutrition [UN]), postnatal diet (Chow [C] and High Fat [HF]) and neonatal leptin treatment (leptin [L]) (n=8/group). Values with different letters are significantly different ($p<0.05$) by Bonferroni's post hoc test.

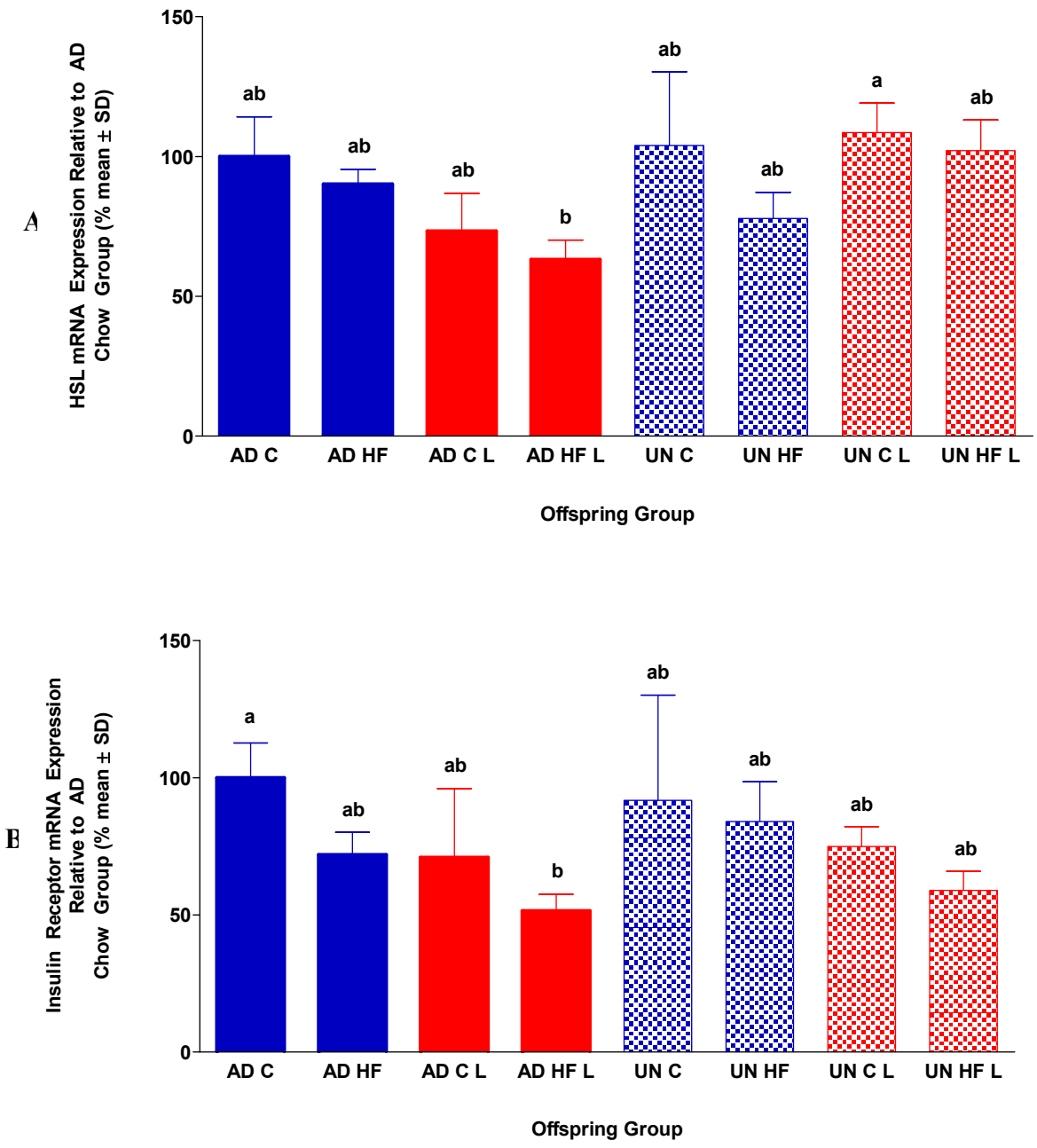


Figure 4.4 The expression of HSL in adipose tissue of adult female offspring is not affected by maternal diet, postnatal diet or neonatal leptin treatment. The expression of Insulin Receptor is effected by leptin treatment and this effect is dependent on maternal diet and postnatal diet. Real time PCR data for expression of HSL (A) and Insulin Receptor (B) are shown in response to maternal diet (ad libitum [AD] and undernutrition [UN]), postnatal diet (Chow [C] and High Fat [HF]) and neonatal leptin treatment (leptin [L]) (n=8/group). Values with different letters are significantly different ($p<0.05$) by Bonferroni's post hoc test.

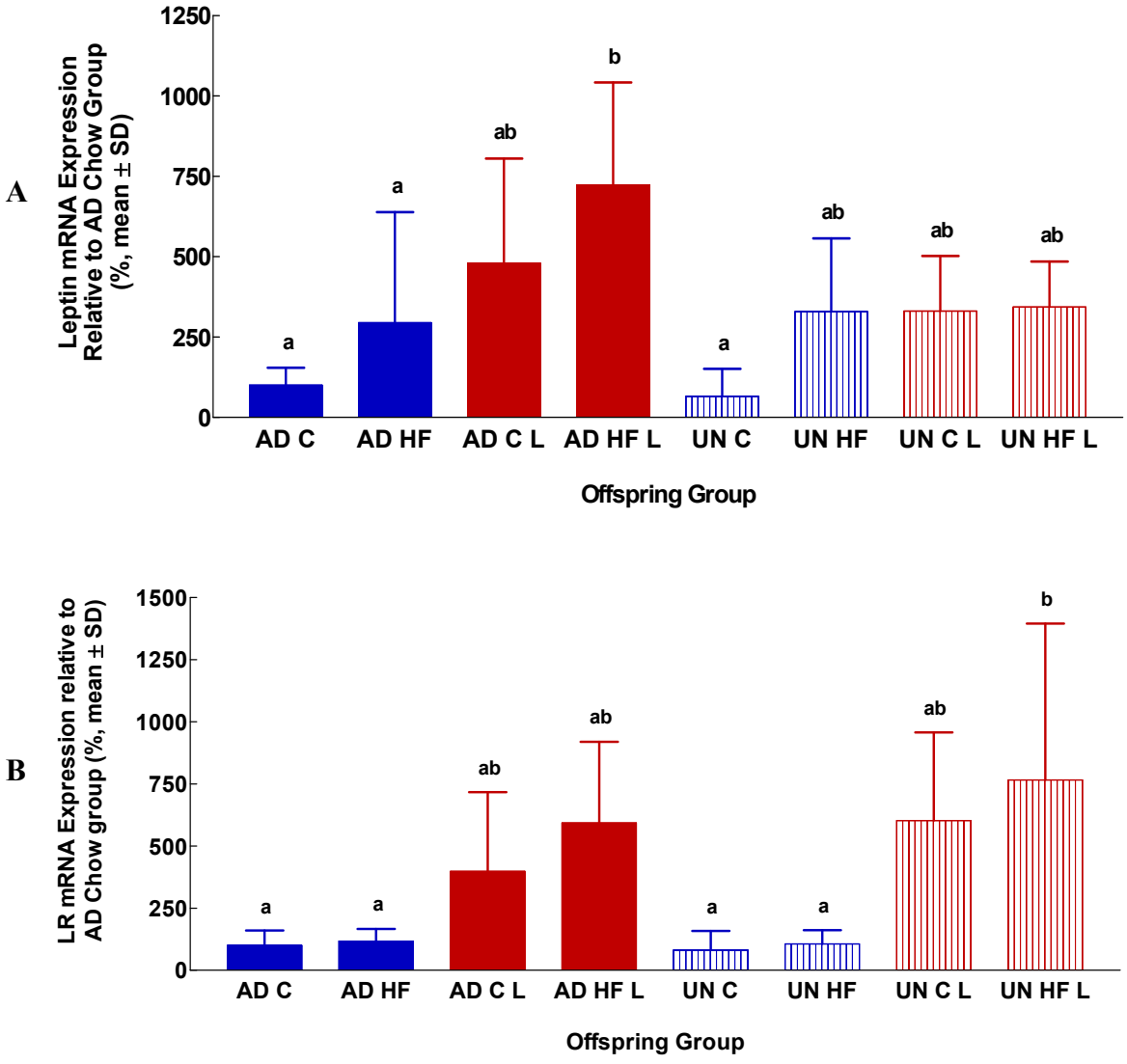


Figure 4.5 The expression of leptin and its receptor in adipose tissue of adult female offspring is altered by neonatal leptin treatment but not maternal diet or postnatal diet. effect of leptin treatment is dependent on maternal diet. Real time PCR data for expression of leptin (**A**) and leptin Receptor (**B**) are shown in response to maternal diet (ad libitum [AD] and undernutrition [UN]), postnatal diet (Chow [C] and High Fat [HF]) and neonatal leptin treatment (leptin [L]) (n=8/group). Values with different letters are significantly different ($p<0.05$) by Bonferroni's post hoc test.

4.2.2 The expression of DNA methyl transferases in the adipose tissue of adult female offspring in programmed rats is not significantly altered by maternal diet, postnatal diet or neonatal leptin treatment

In order to determine if the expression of the DNA methyltransferases 1 and 3a are affected by maternal diet, postnatal diet or neonatal leptin treatment in adipose tissue of female offspring, total RNA was prepared from adipose tissue of female offspring from the IUGR rat study (n=8 / diet-treatment group) and used to prepare cDNA. Real time PCR was performed with validated primers specific for Dnmt1 and Dnmt3a using the cDNA as a template. Resulting gene expression data were normalised to 18s cDNA and analysed by the ΔCT method [228]. Results were converted to % of the control group (AD Chow 100%) and a 1 way ANOVA performed followed by Bonferroni's post hoc test. Graphs showing differences in mRNA expression with statistical analysis can be found in figure 4.6. All results are described relative to the control group only. In addition, a 3 way ANOVA was performed to analyse single factor and interaction effects on gene expression. The results of the 3 way ANOVA are shown in table 4.1.

Analysis of Dnmt1 expression revealed that its expression was not significantly altered by maternal diet, postnatal diet or neonatal leptin treatment (figure 4.6a). However, some trends were evident in leptin treated offspring; there was a trend for increased expression in AD offspring, but this failed to reach significance and similarly there was a trend for reduced expression in UN offspring but again this failed to reach significance. Dnmt3a expression was also not significantly altered by maternal diet, postnatal diet or neonatal leptin treatment, although there was a trend towards reduced Dnmt3a expression in all UN offspring (figure 4.6b).

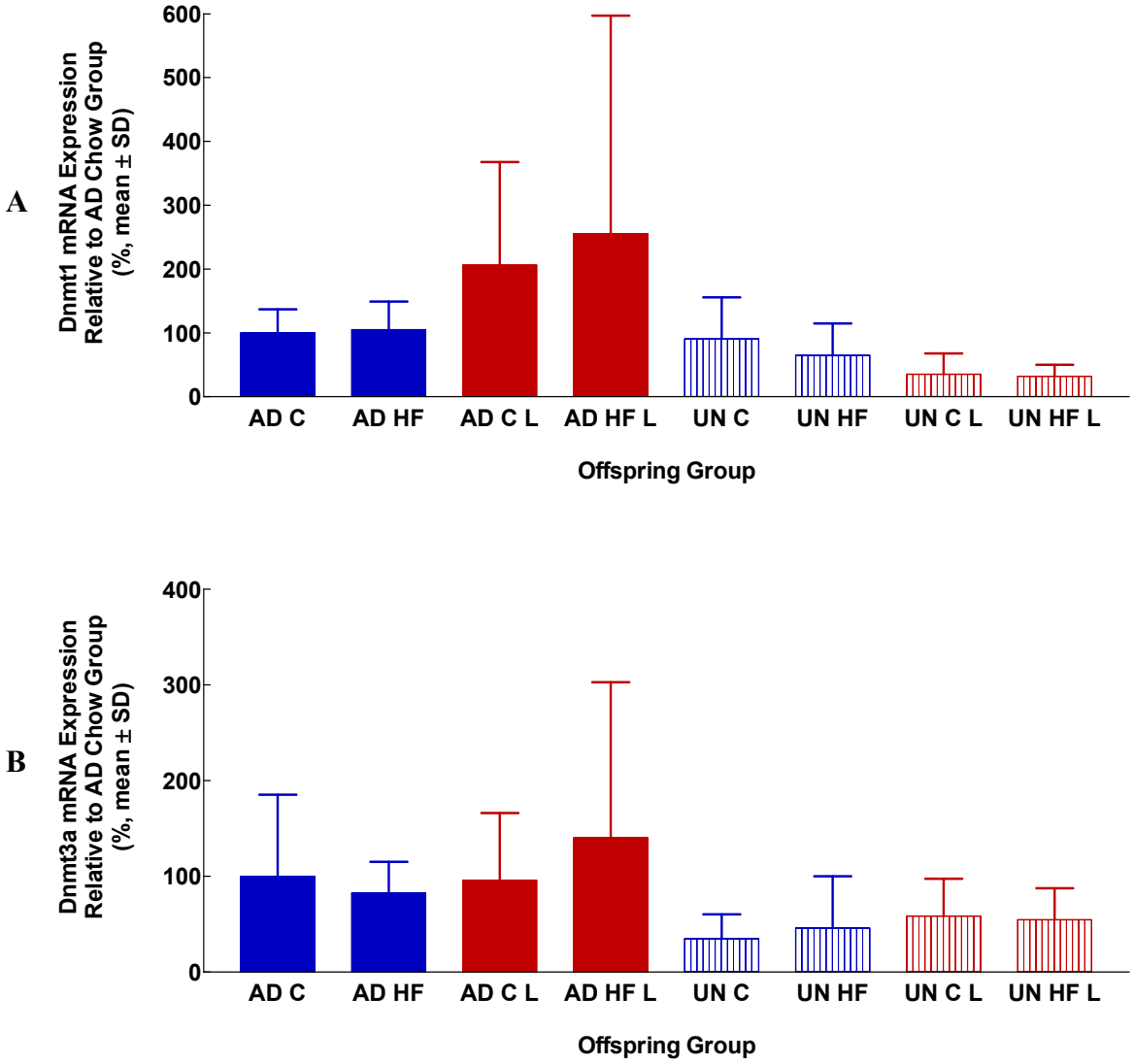


Figure 4.6 The expression of DNA methyltransferases in adipose tissue of adult female offspring is not significantly altered by neonatal leptin treatment, maternal diet or postnatal diet. Real time PCR data for expression of Dnmt1 (**A**) and Dnmt3a (**B**) are shown in response to maternal diet (ad libitum [AD] and undernutrition [UN]), postnatal diet (Chow [C] and High Fat [HF]) and neonatal leptin treatment (leptin [L]) (n=8/group).

Table 4.1 Table showing tests of between subject effects on the mRNA expression of genes measured in the adipose tissue of female offspring. Single factor effects (MD, T, PND) and interaction effects (MD*T, MD*PND, T*PND, MD*T*PND) are shown. Significant effects ($P<0.05$) are indicated in red. There were significant single factor effects for all genes. There were significant effects of MD for 11βHDS2, HSL, leptin, Dnmt1 and Dnmt3a. There were significant effects of T for all genes except HSL, Dnmt1 and Dnmt3a. There were significant effects of PND for PPARα, CPT-1, LPL, IR, HSL and leptin. There were significant interaction effects for CPT-1 (T*PND, MD*T*PND), 11βHSD2 (MD*T), HSL (MD*T), leptin (MD*T) and Dnmt1 (MD*T).

Variable	Anova	Maternal Diet (MD)	Treatment (T)	Postnatal Diet (PND)	MD* T	MD* PND	T* PND	MD* T* PND
PPARα	0.000	0.298	0.000	0.006	0.847	0.223	0.184	0.642
CPT-1	0.000	0.540	0.000	0.000	0.543	0.152	0.043	0.009
AOX	0.000	0.231	0.000	0.146	0.339	0.704	0.176	0.568
PPARγ2	0.000	0.451	0.000	0.809	0.965	0.287	0.396	0.522
LPL	0.000	0.905	0.000	0.008	0.587	0.392	0.657	0.770
GR	0.000	0.439	0.000	0.355	0.498	0.078	0.499	0.091
GR_{110}	0.000	0.059	0.000	0.734	0.152	0.971	0.644	0.554
11βHSD2	0.000	0.010	0.000	0.114	0.013	0.908	0.338	0.480
IR	0.030	0.601	0.003	0.018	0.794	0.421	0.993	0.564
HSL	0.006	0.016	0.358	0.053	0.003	0.637	0.468	0.456
Leptin	0.000	0.043	0.000	0.007	0.044	0.528	0.431	0.247
LR	0.000	0.304	0.000	0.238	0.231	0.946	0.350	0.912
Dnmt1	0.035	0.004	0.262	0.864	0.025	0.576	0.653	0.884
Dnmt3a	0.198	0.008	0.296	0.679	0.793	0.808	0.568	0.348

4.2.3 The methylation of genes involved in energy balance in the adipose tissue of adult female offspring in programmed rats is not altered by maternal diet, postnatal diet or neonatal leptin treatment

In order to ascertain if the methylation status of promoter regions of genes involved in energy balance is affected by maternal diet, postnatal diet or neonatal leptin treatment in adipose tissue of female offspring, genomic DNA was prepared from adipose tissue of female offspring from the IUGR rat study (n=8 / diet-treatment group). This DNA was digested with methylation sensitive restriction enzymes (AciI or Hinp1I) and real time PCR was performed with validated gene specific primers using the digested genomic DNA as a template. Promoters studied were those from genes involved in adipocyte differentiation and fatty acid storage (GR, 11βHSD2 PPARγ1), fatty acid metabolism (PPARα, AOX) and body weight regulation (Leptin and its receptor). Resulting data were normalised to PPARγ2 which has no AciI or Hinp1I sites. Results were converted to % of the control group (AD Chow 100%) and a 1 way ANOVA performed followed by Bonferroni's post hoc test. Graphs showing differences in methylation for each gene with statistical analysis can be found in figures 4.7 to 4.9. All results are described relative to the control group. In addition, a 3 way ANOVA was performed for all genes measured, to analyse single factor and interaction effects on DNA methylation. The results of the 3 way ANOVA are shown in table 4.2.

There were no significant effects of maternal diet or postnatal diet on the methylation status of any of the genes studied. There were also no significant effects of neonatal leptin treatment on the methylation of any of the genes except for an increase in methylation of the PPARα promoter in UN offspring fed a high fat diet (109.27% p=0.001) (figure 4.8a). Furthermore, no trends in methylation status were apparent with any of the treatment groups.

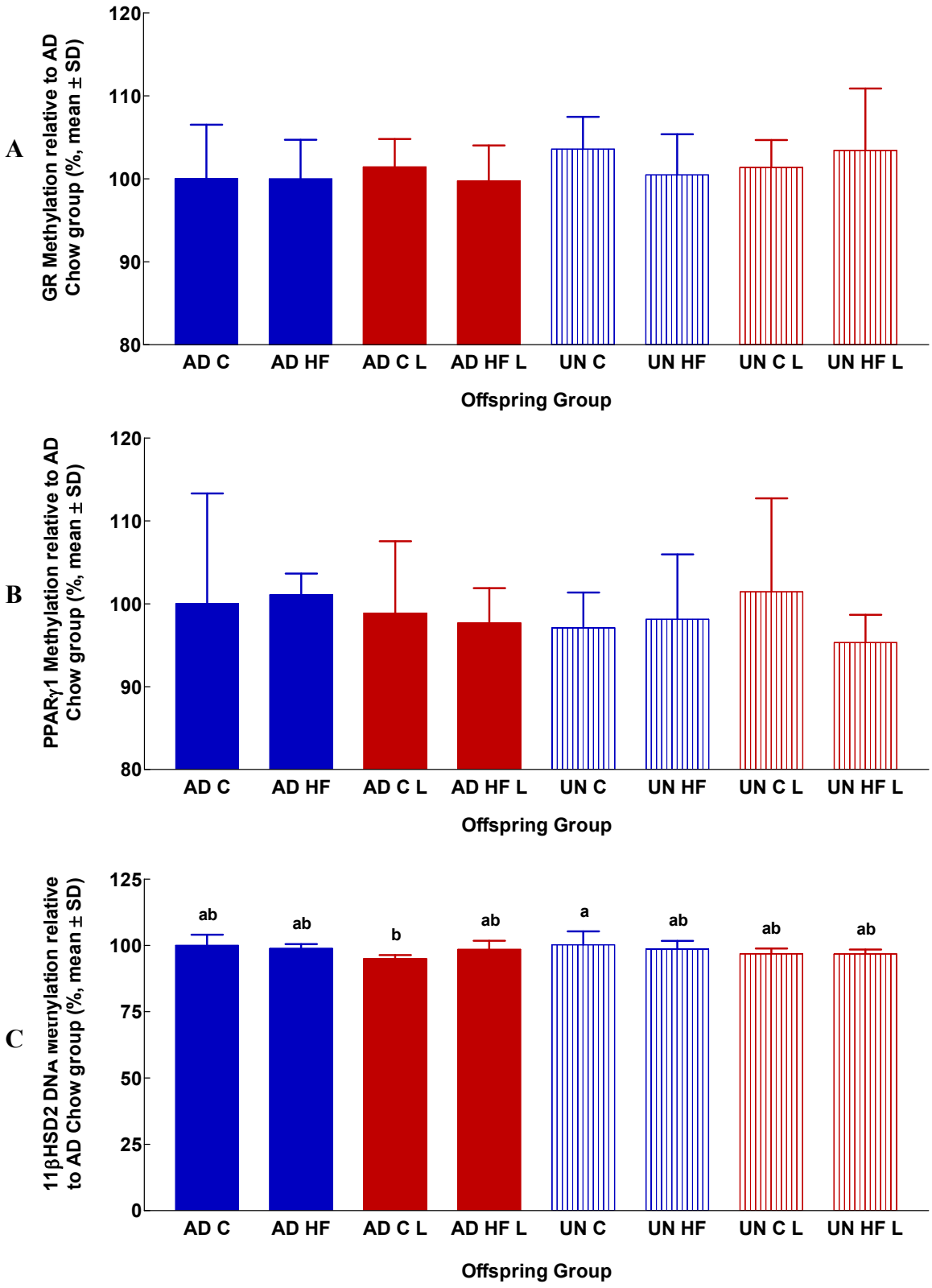


Figure 4.7 The methylation of the GR, PPARγ1 and 11βHSD2 promoters in adipose tissue of adult female offspring are not altered by maternal diet, postnatal diet or neonatal leptin treatment. Real time PCR data for methylation of GR (**A**), PPARγ1 (**B**) and 11βHSD2 (**C**)are shown in response to maternal diet (ad libitum [AD] and undernutrition [UN]), postnatal diet (Chow [C] and High Fat [HF]) and neonatal leptin treatment (leptin [L]) (n=8/group). Values with different letters are significantly different ($p<0.05$) by Bonferroni's post hoc test.

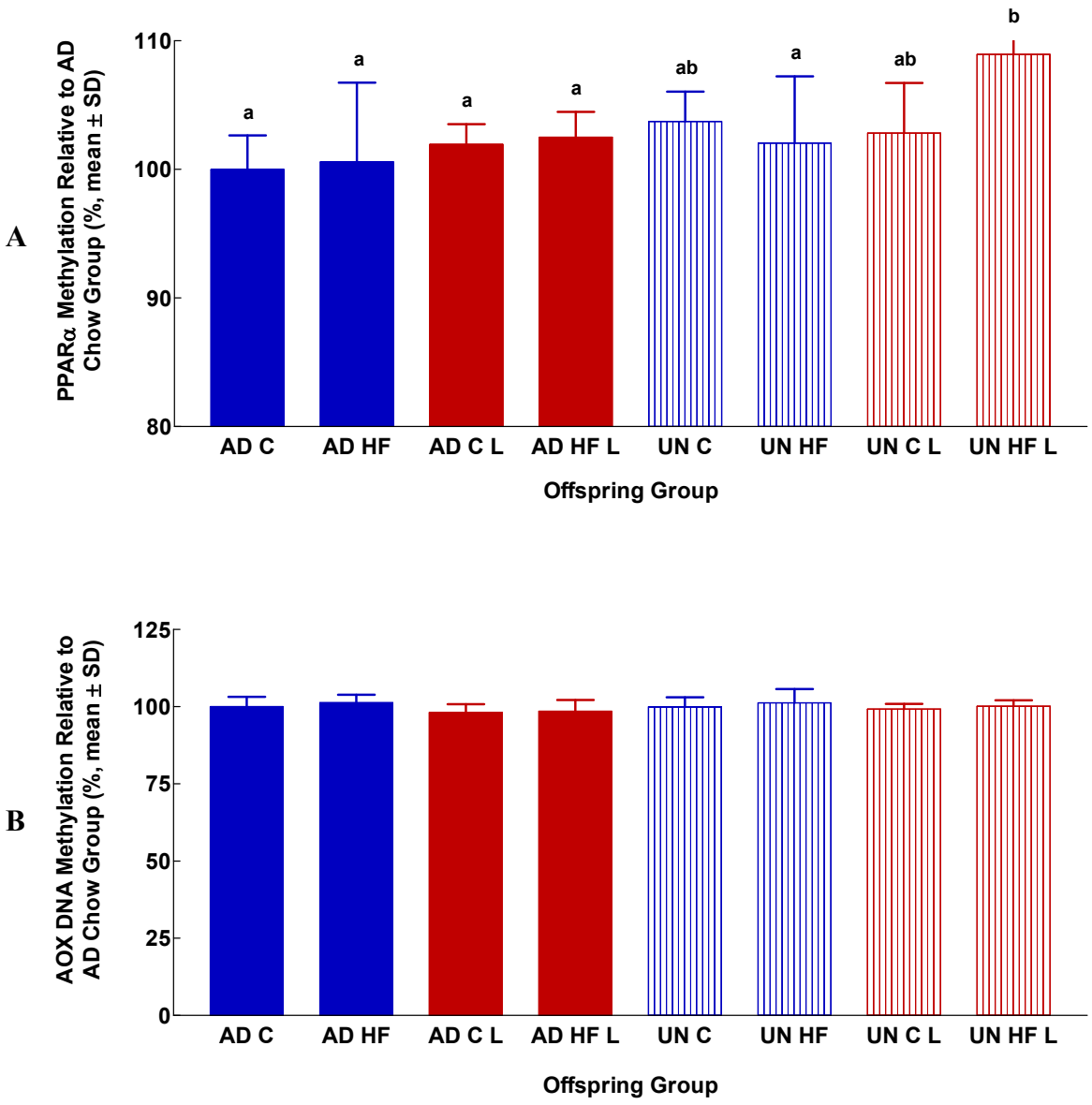


Figure 4.8 Methylation of the PPARα promoter in adipose tissue of adult female offspring is affected by neonatal leptin treatment but not maternal diet or postnatal diet. the methylation of the AOX promoter is not altered by maternal diet, postnatal diet or neonatal leptin treatment. Real time PCR data for methylation of PPARα (**A**) and AOX (**B**) are shown in response to maternal diet (ad libitum [AD] and undernutrition [UN]), postnatal diet (Chow [C] and High Fat [HF]) and neonatal leptin treatment (leptin [L]) (n=8/group). Values with different letters are significantly different ($p<0.05$) by Bonferroni's post hoc test.

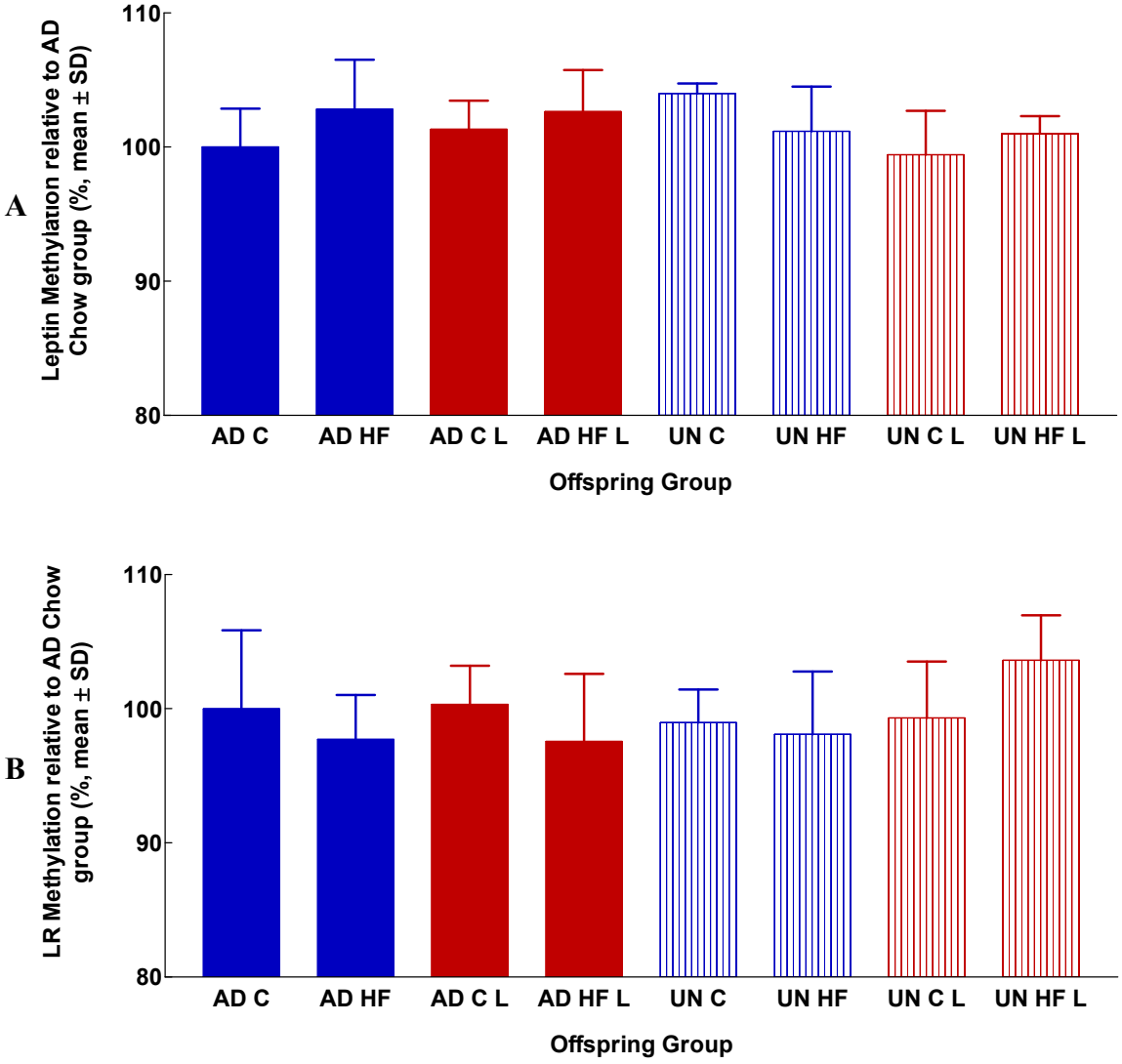


Figure 4.9 Methylation of the leptin and leptin receptor promoters in adipose tissue of adult female offspring are not altered by maternal diet, postnatal diet or neonatal leptin treatment. Real time PCR data for methylation of leptin (**A**) and LR (**B**) are shown in response to maternal diet (ad libitum [AD] and undernutrition [UN]), postnatal diet (Chow [C] and High Fat [HF]) and neonatal leptin treatment (leptin [L]) (n=8/group).

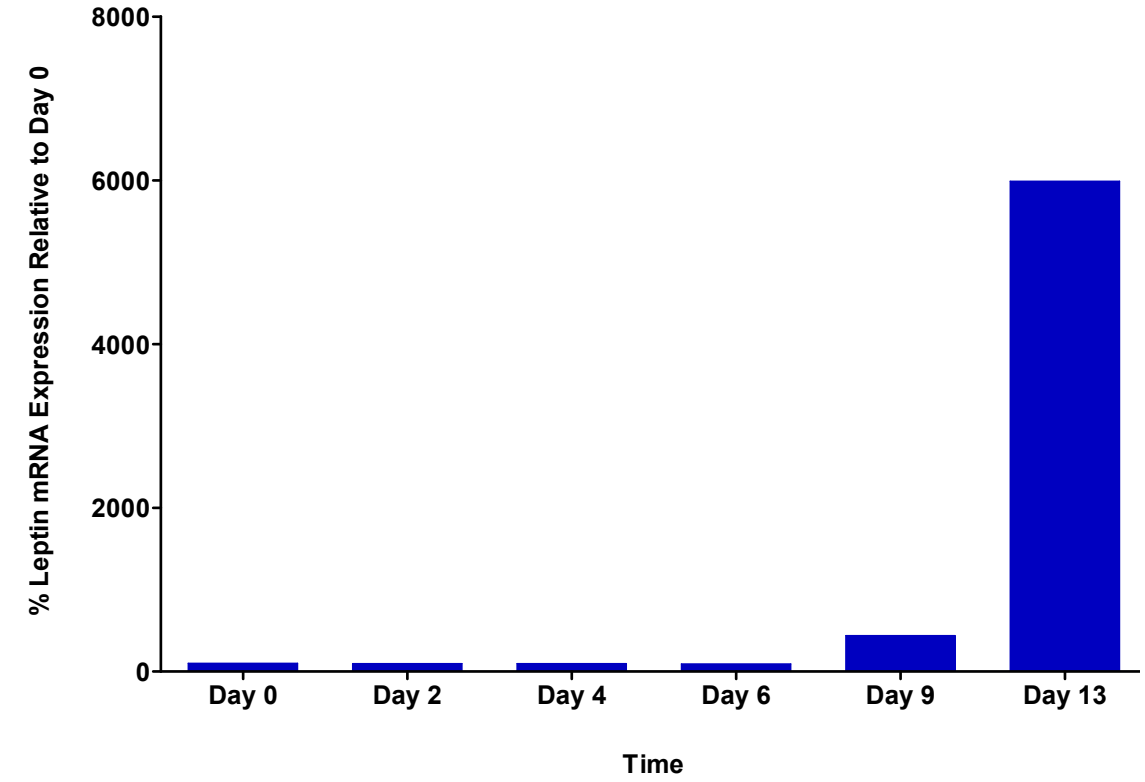
Table 4.2 Table showing tests of between subject effects on DNA methylation for genes measured in adipose tissue of female offspring. Single factor effects (MD, T, PND) and interaction effects (MD*T, MD*PND, T*PND, MD*T*PND) are shown. Significant effects (P<0.05) are indicated in red. There were no significant single factor or interaction effects for GR, LR or PPARγ1. For PPARα, there were significant single factor effects (MD, T) and interaction effects (T*PND, MD*T*PND). For AOX and 11βHSD2, there were only significant single factor effects (T). For leptin, there were significant interaction effects (MD*T, MD*T*PND).

Variable	Anova	Maternal Diet (MD)	Treatment (T)	Postnatal Diet (PND)	MD* T	MD* PND	T* PND	MD* T* PND
PPARα	0.000	0.001	0.009	0.133	0.564	0.368	0.038	0.037
AOX	0.359	0.437	0.044	0.224	0.364	0.888	0.708	0.882
GR	0.669	0.146	0.711	0.598	0.935	0.900	0.499	0.194
11βHSD2	0.026	0.970	0.001	0.866	0.989	0.200	0.054	0.307
Leptin	0.049	0.681	0.216	0.317	0.045	0.067	0.322	0.046
LR	0.147	0.312	0.163	0.703	0.189	0.053	0.275	0.190
PPARγ1	0.812	0.493	0.712	0.524	0.459	0.544	0.255	0.550

4.2.4 Leptin undergoes a surge in expression during differentiation of 3T3-L1 pre-adipocytes

In order to determine if leptin treatment to differentiating 3T3-L1 adipocytes is able to induce the expression of genes found to be upregulated in response to neonatal leptin treatment in IUGR offspring and to determine if the effect of leptin treatment differs according to the phase of the leptin surge, the period of the leptin surge during the differentiation of 3T3-L1 cells first needs to be determined. Therefore 3T3 cells were stimulated to differentiate and total RNA prepared from 3T3-L1 cells at different stages of differentiation (day 0 to day 13), which was used to prepare cDNA. Real time PCR was then performed with validated primers specific for leptin using the cDNA as a template. Resulting expression data were normalised to CYC cDNA and analysed by the ΔCT method [228]. Data at each time point was expressed as a percentage of treatment day 0, which was set to 100%. A graph showing leptin expression alongside the appearance of the adipocytes throughout the stages of differentiation is shown in figure 4.6.

Results indicated that leptin expression did not alter between differentiation day 0 and 6 (figure 4.10a). By day 9, leptin levels had increased to levels 400% that of undifferentiated cells. Leptin expression then further increased dramatically up to day 13 resulting in levels 6000% that of undifferentiated cells, at which point the adipocytes showed morphological features of fully differentiated adipocytes (figure 4.10b).



A

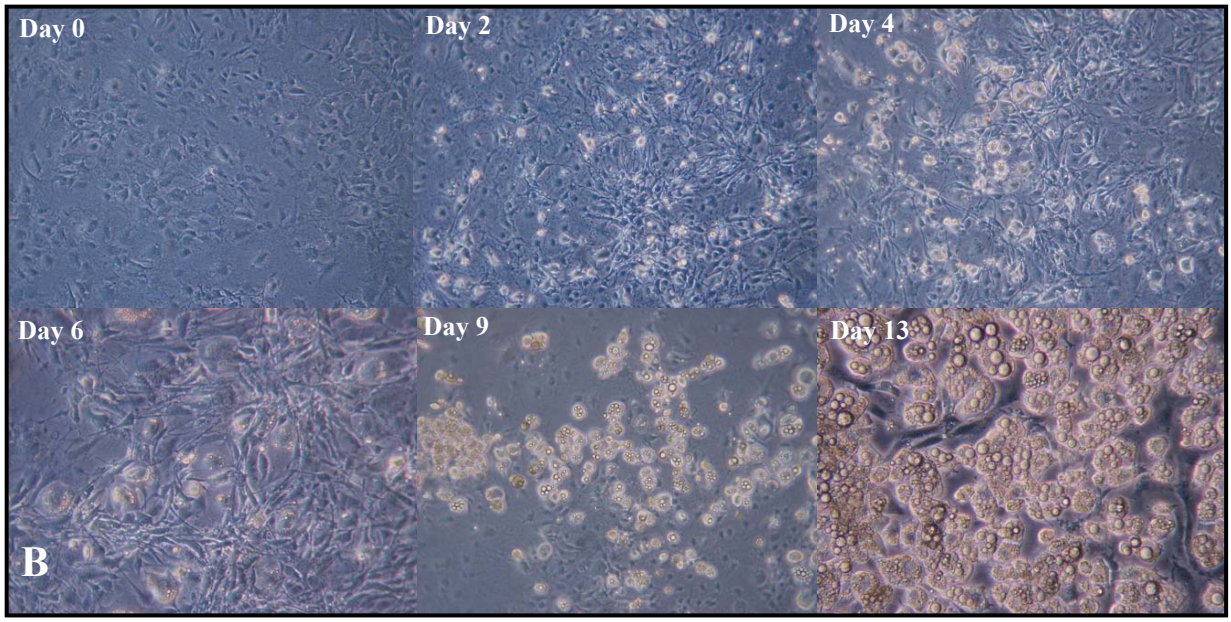


Figure 4.10 Appearance of 3T3-L1 adipocytes and expression profile of leptin mRNA during differentiation. A) Endogenous leptin expression in 3T3-L1 pre-adipocytes undergoing differentiation from undifferentiated (Day 0) to fully differentiated (Day 13) B) Appearance of adipocytes during the same differentiation period.

4.2.5 Endogenous leptin treatment alters the expression of specific genes involved in energy balance before and during the differentiation of 3T3-L1 adipocytes

In order to ascertain if leptin treatment can induce an increase in the expression of key genes involved in energy balance in differentiating adipocytes, 3T3-L1 pre-adipocytes were stimulated to differentiate into adipocytes and treated with leptin (10ng/ml). Leptin treatment was given in two separate experiments at differentiation day 2 (prior to the leptin surge) and day 8 (start of the leptin surge), to determine if the timing of treatment relative to the leptin surge in adipocytes was important for the gene response. mRNA expression was measured in 4 genes; PPARα, leptin, GR and 11βHSD2. Therefore, to determine the effect of leptin treatment on the expression profile of these genes, total RNA was prepared from 3T3-L1 cells at different timepoints after the onset of leptin treatment for both day 2 and day 8 (0, 2, 6, 24, 48hr) and used to prepare cDNA. Real time PCR was performed with validated primers using the cDNA as a template. Resulting gene expression data were normalised to CYC cDNA and analysed by the ΔCT method [228]. Data from each timepoint is expressed as a percentage of day 0, which was set to 100%. Graphs showing representative replicates of gene expression profiles for all genes can be found in figures 4.11 to 4.18. Results are described as a mean of 2 replicates.

At differentiation day 2, endogenous leptin expression showed a slight dip in expression over the following 48 hours (figure 4.11). Up to this time point, there was no effect of exogenous leptin treatment on the expression of leptin. However, after 48 hours, mean leptin levels in control cells remained dipped at 88% compared to time 0, whereas in leptin treated cells, mean expression increased to 173%. PPARα expression in the untreated cells underwent a steady increase in expression throughout the 48 hour period from day 2, reaching a mean of 2468% compared to 0hr (figure 4.12). The effect of leptin treatment on PPARα gene expression was only apparent at 24 hours after treatment, reaching a mean of 2933% compared to untreated (2009%) at 24hr. However this increase in expression was lost at 48 hours, returning to mean levels of 1613% at 48 hours compared to 2468% in controls. Mean GR gene expression showed a steady increase to 225% and 302% original levels in untreated cells over the 24 and 48 hours respectively. Leptin treated cells showed a similar expression profile and GR gene expression was only mildly increased at 24 and 48 hrs after treatment with leptin compared to untreated, with mean values of 295% at 24hr and 335% at 48hr (figure 4.13). Expression of 11βHSD2 in untreated cells showed no variation in expression throughout the time course, except for a spike in expression at 6hrs to a mean of 750% compared to untreated value of 106%. At the end of the 48 hours, the expression of 11BHSD2 in untreated cells was 196% compared to time 0, whereas for leptin treated cells, this mean was slightly higher at 217% (figure 4.14).

Expression profiles were next measured in the same genes undergoing differentiation from day 8. Untreated cells showed a dip in leptin expression over the 24hr period with mean values of 54%

compared to time 0. After this leptin expression increased over the next 24hr with levels reaching a mean of 250% compared to 0hr at 48hr (figure 4.15). In leptin treated cells, again, there was a dip in expression but only over the first 6 hours. After this, mean leptin expression increased reaching 187% at 24hr and 1061% at 48hr post treatment. Mean PPARα expression at day 8 remained fairly constant over the first 24hr and then increased to 815% at 48hr in untreated cells (figure 4.16). In the leptin treated cells, this increase in expression occurred earlier at 24hrs with mean levels of 700% compared to time 0, which dropped off to 74% at 48hr. Contrastingly, mean GR expression in untreated differentiating cells remained constant for the first 6hr and then showed a reduction in expression at 24hr to 15% original levels, then dropping further to a mean of 8% at 48 hr (figure 4.17). The leptin treated cells followed a similar pattern of expression, but expression dropped at an earlier time point, dropping at 6hr to mean levels of 18% and then dropping further to 3% at 48 hours. For 11βHSD2 expression untreated cells showed a steady increase in mean 11βHSD2 expression over the time course resulting in levels of 434% compared to 0hr at 48hr (figure 4.18). Treatment with leptin resulted in a similar steady increase in gene expression, but with overall higher levels at all time points, resulting in mean expression levels of 280% at 24hr and 634% at 48hr.

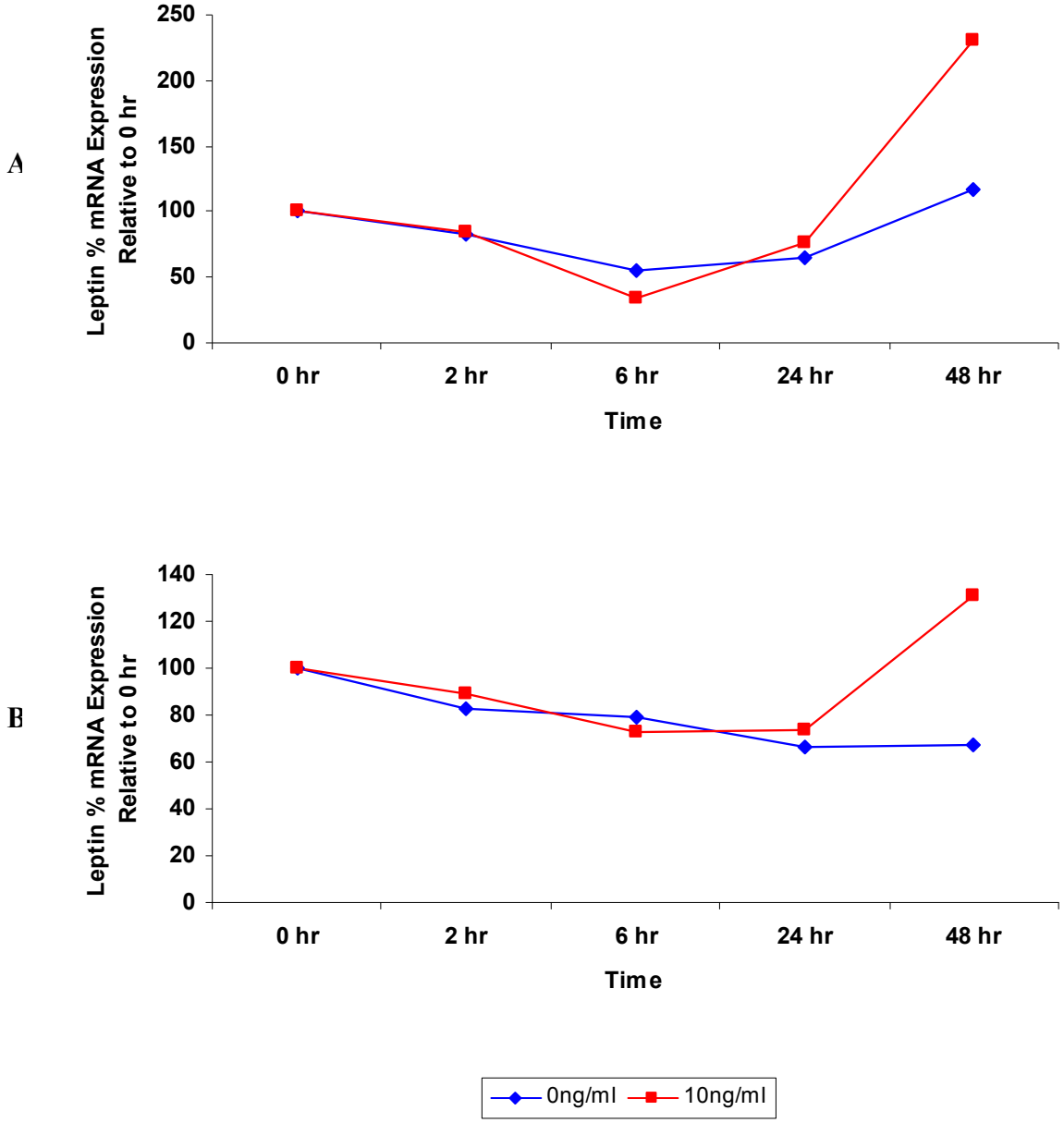


Figure 4.11 Expression profile of leptin in 3T3-L1 pre-adipocytes undergoing differentiation at day 2 treated with exogenous leptin. Real time PCR data for expression of leptin are shown in response to leptin treatment at 0ng/ml and 10ng/ml. Treatment starts at differentiation day 2 (0hr) and results are taken over a 48 hour time course. Replicates are shown in separate graphs (**A** and **B**).

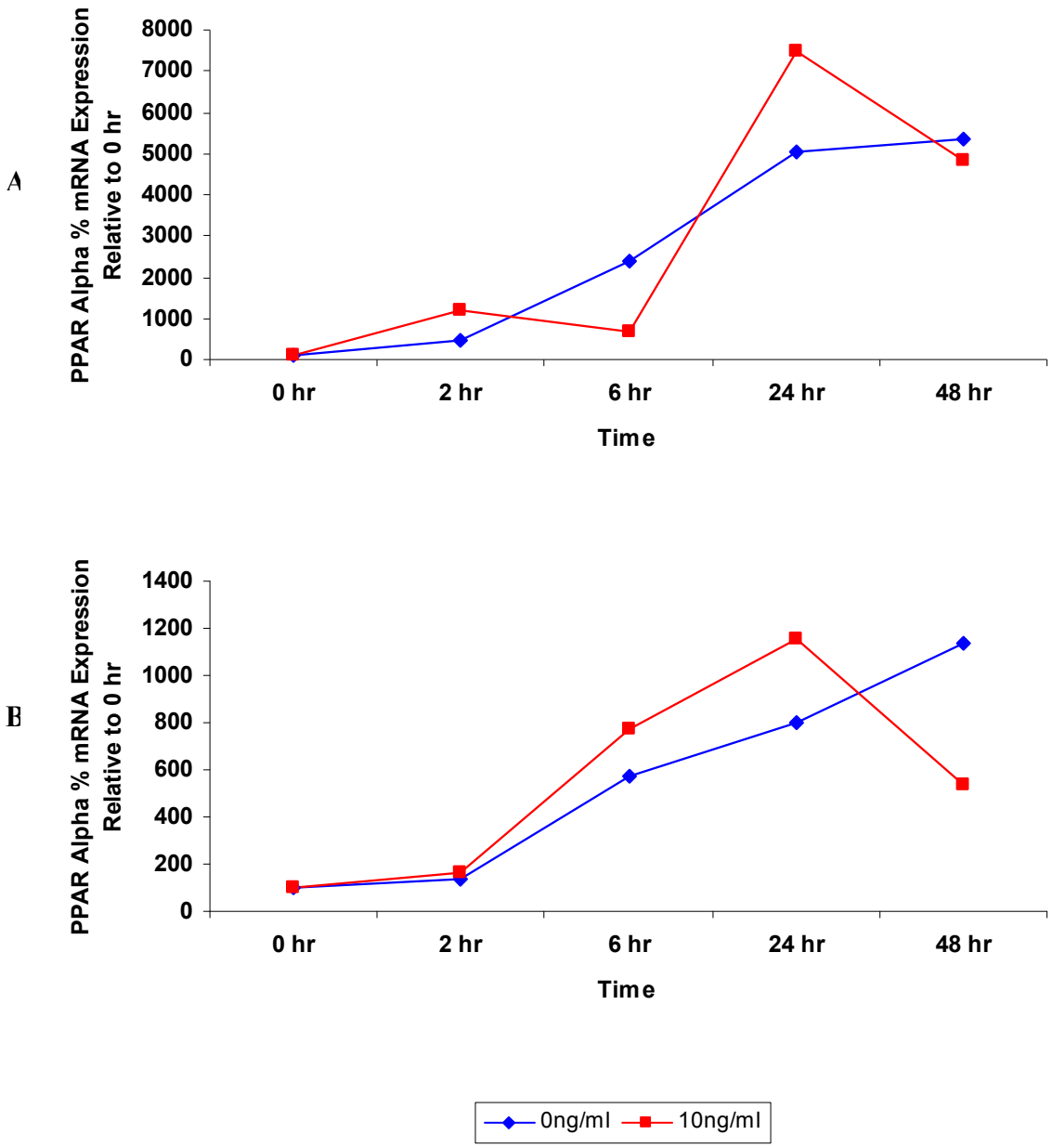


Figure 4.12 Expression profile of PPARα in 3T3-L1 pre-adipocytes undergoing differentiation at day 2 treated with exogenous leptin. Real time PCR data for expression of PPARα are shown in response to leptin treatment at 0ng/ml and 10ng/ml. Treatment starts at differentiation day 2 (0hr) and results are taken over a 48 hour time course. Replicates are shown in separate graphs (**A** and **B**).

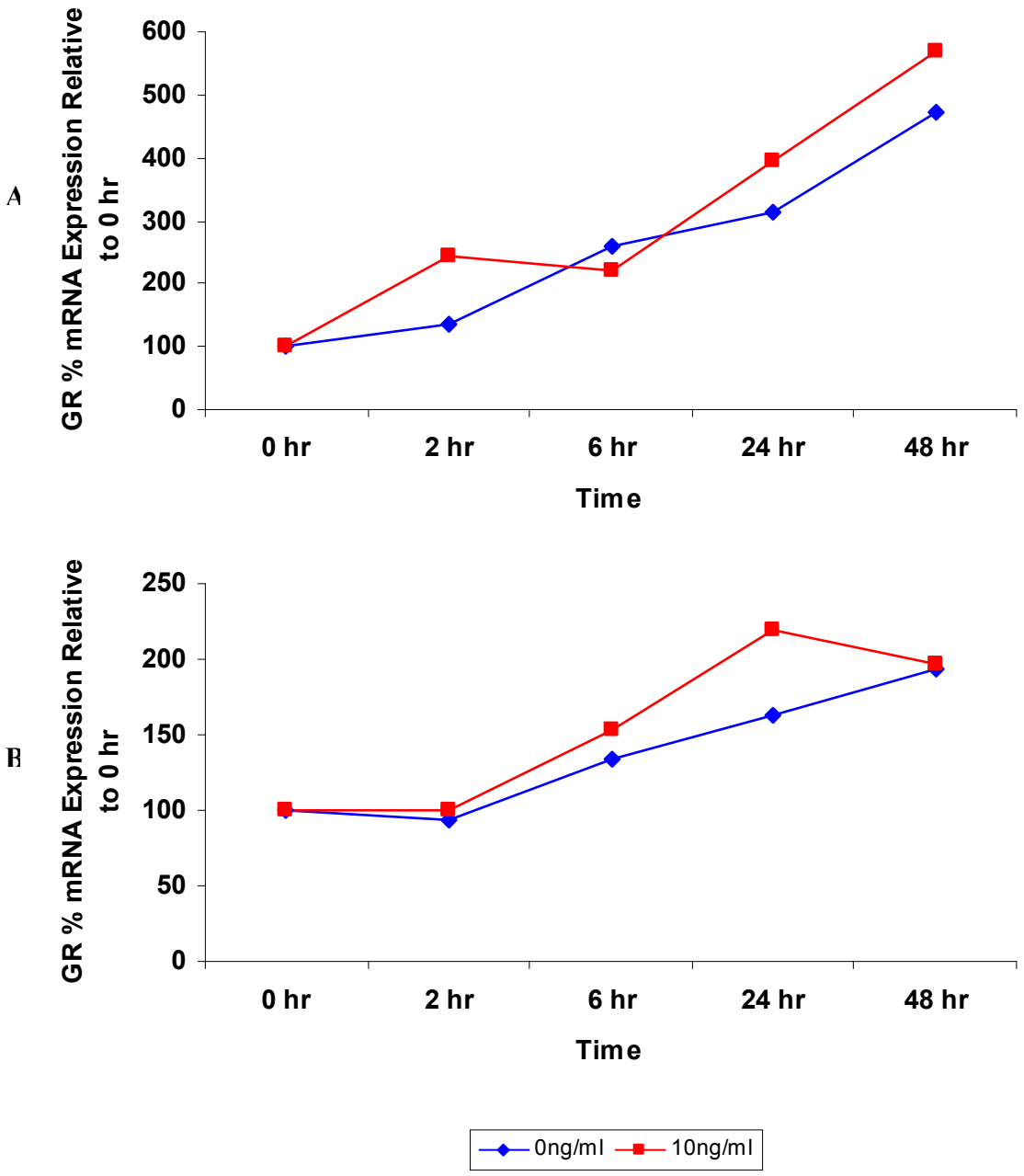


Figure 4.13 Expression profile of GR in 3T3-L1 pre-adipocytes undergoing differentiation at day 2 treated with exogenous leptin. Real time PCR data for expression of GR are shown in response to leptin treatment at 0ng/ml and 10ng/ml. Treatment starts at differentiation day 2 (0hr) and results are taken over a 48 hour time course. Replicates are shown in separate graphs (**A** and **B**).

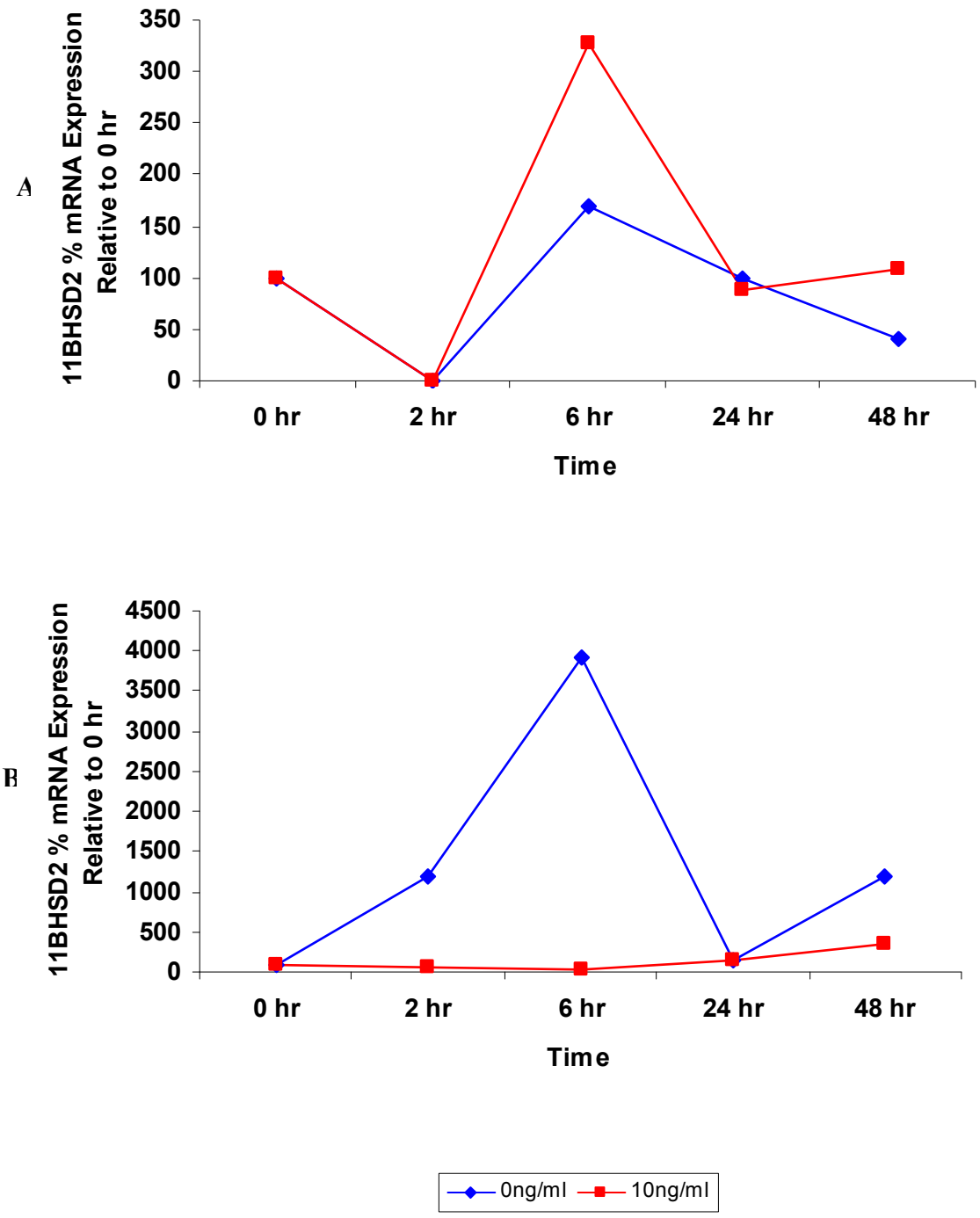


Figure 4.14 Expression profile of 11βHSD2 in 3T3-L1 pre-adipocytes undergoing differentiation at day 2 treated with exogenous leptin. Real time PCR data for expression of 11βHSD2 are shown in response to leptin treatment at 0ng/ml and 10ng/ml. Treatment starts at differentiation day 2 (0hr) and results are taken over a 48 hour time course. Replicates are shown in separate graphs (**A** and **B**).

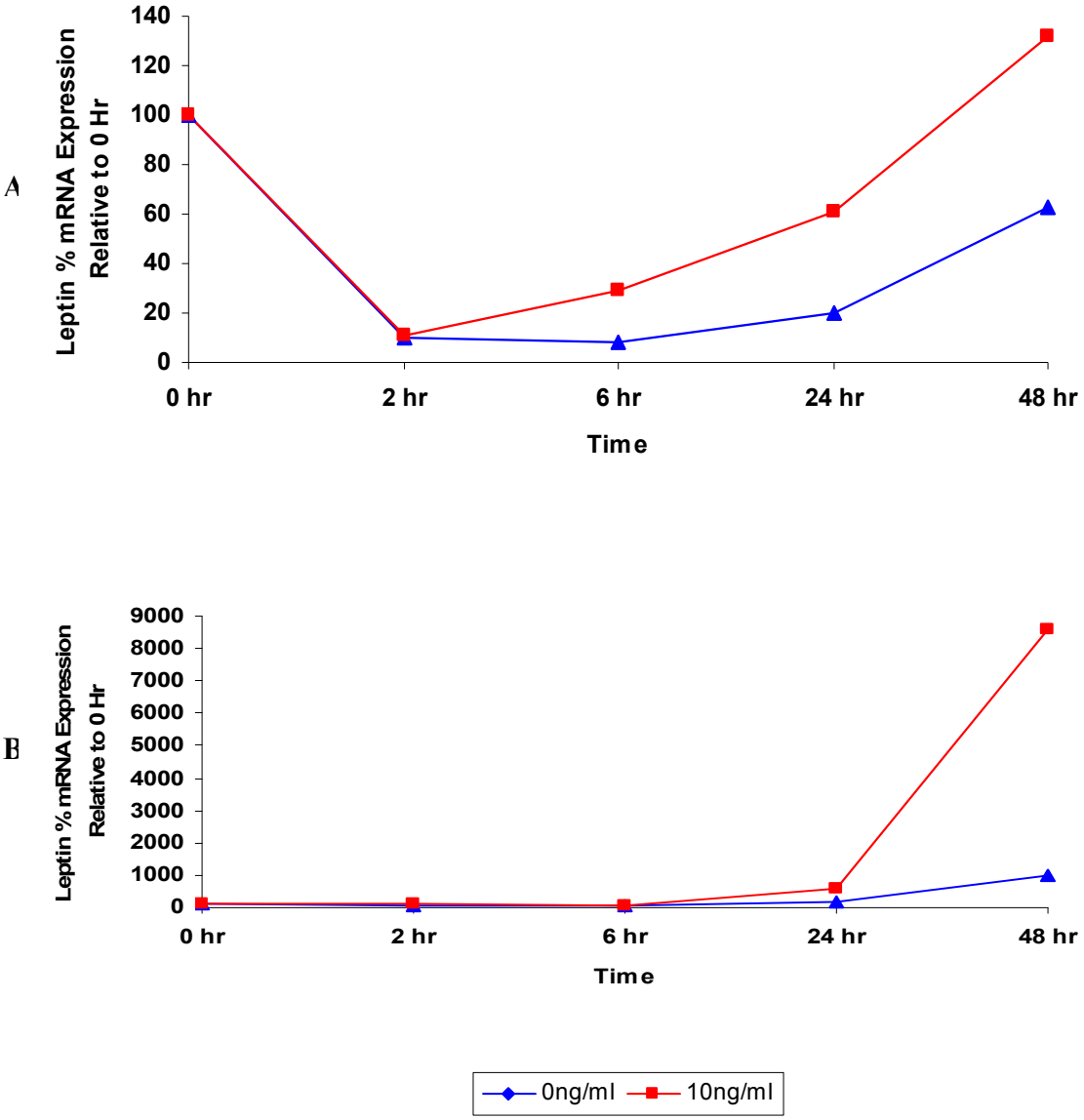


Figure 4.15 Expression profile of leptin in 3T3-L1 pre-adipocytes undergoing differentiation at day 8 treated with exogenous leptin. Real time PCR data for expression of leptin are shown in response to leptin treatment at 0ng/ml and 10ng/ml. Treatment starts at differentiation day 8 (0hr) and results are taken over a 48 hour time course. Replicates are shown in separate graphs (**A** and **B**).

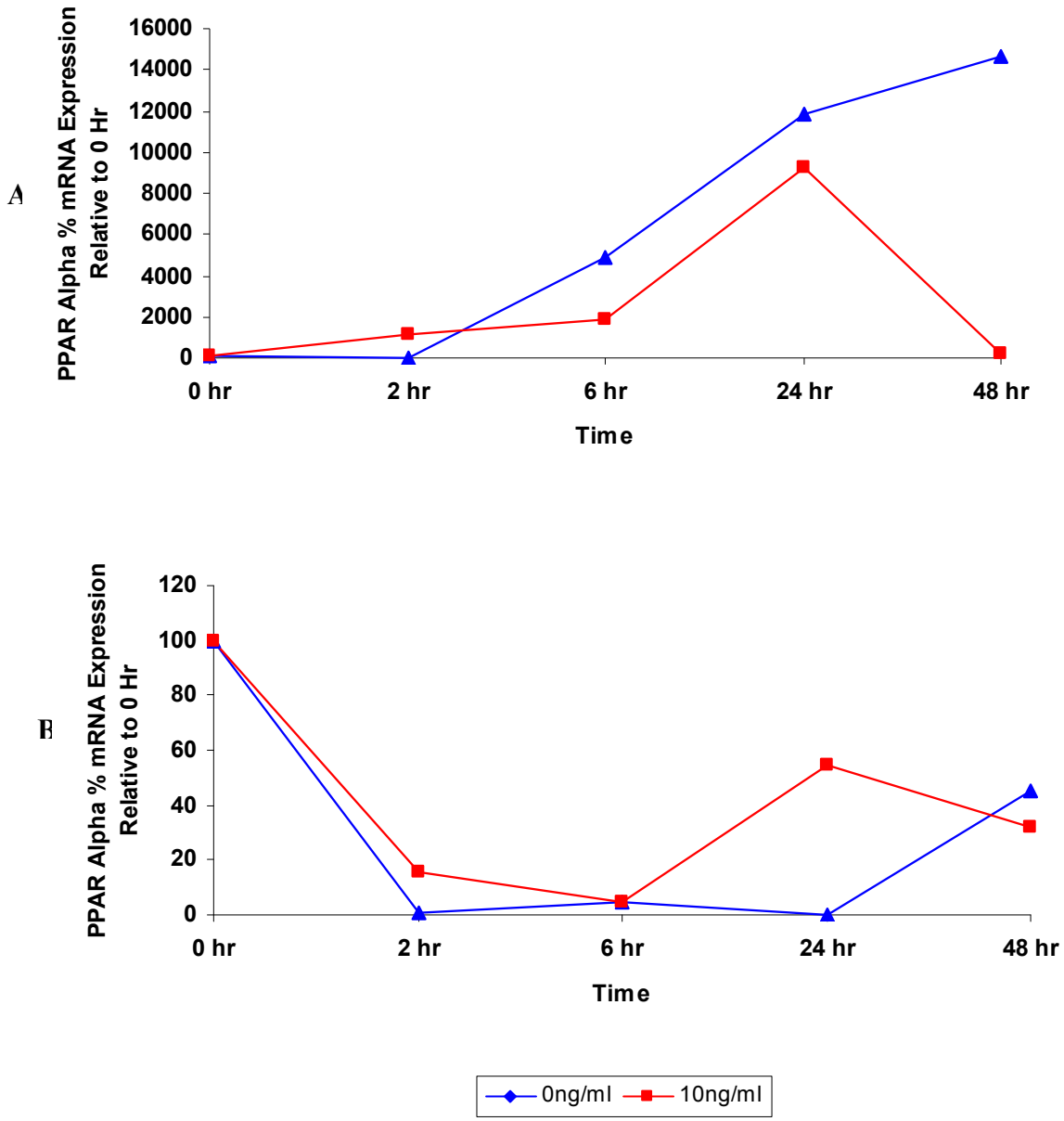


Figure 4.16 Expression profile of PPARα in 3T3-L1 pre-adipocytes undergoing differentiation at day 8 treated with exogenous leptin. Real time PCR data for expression of PPARα are shown in response to leptin treatment at 0ng/ml and 10ng/ml. Treatment starts at differentiation day 8 (0hr) and results are taken over a 48 hour time course. Replicates are shown in separate graphs (**A** and **B**).

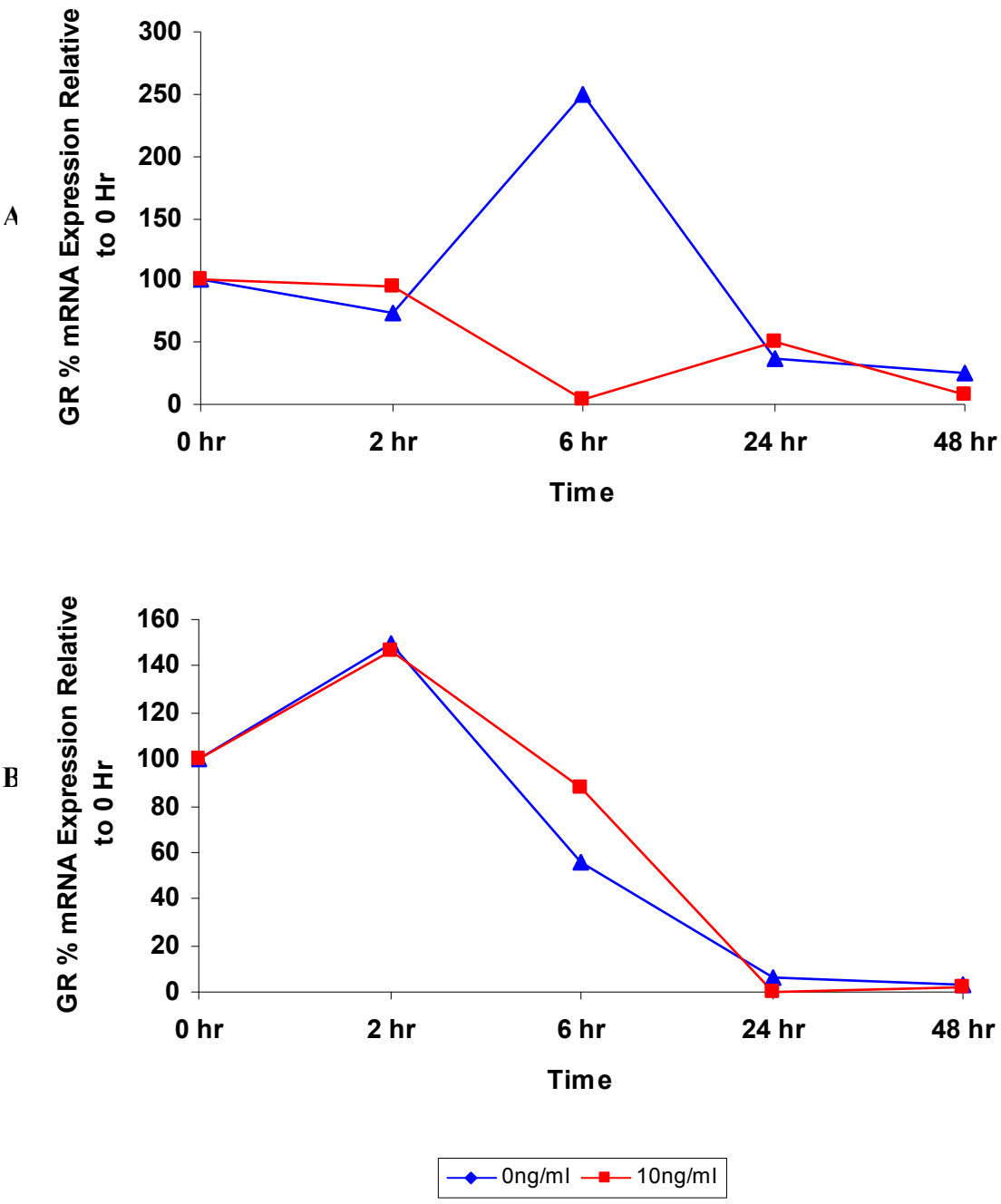


Figure 4.17 Expression profile of GR in 3T3-L1 pre-adipocytes undergoing differentiation at day 8 treated with exogenous leptin. Real time PCR data for expression of GR are shown in response to leptin treatment at 0ng/ml and 10ng/ml. Treatment starts at differentiation day 8 (0hr) and results are taken over a 48 hour time course. Replicates are shown in separate graphs (**A** and **B**).

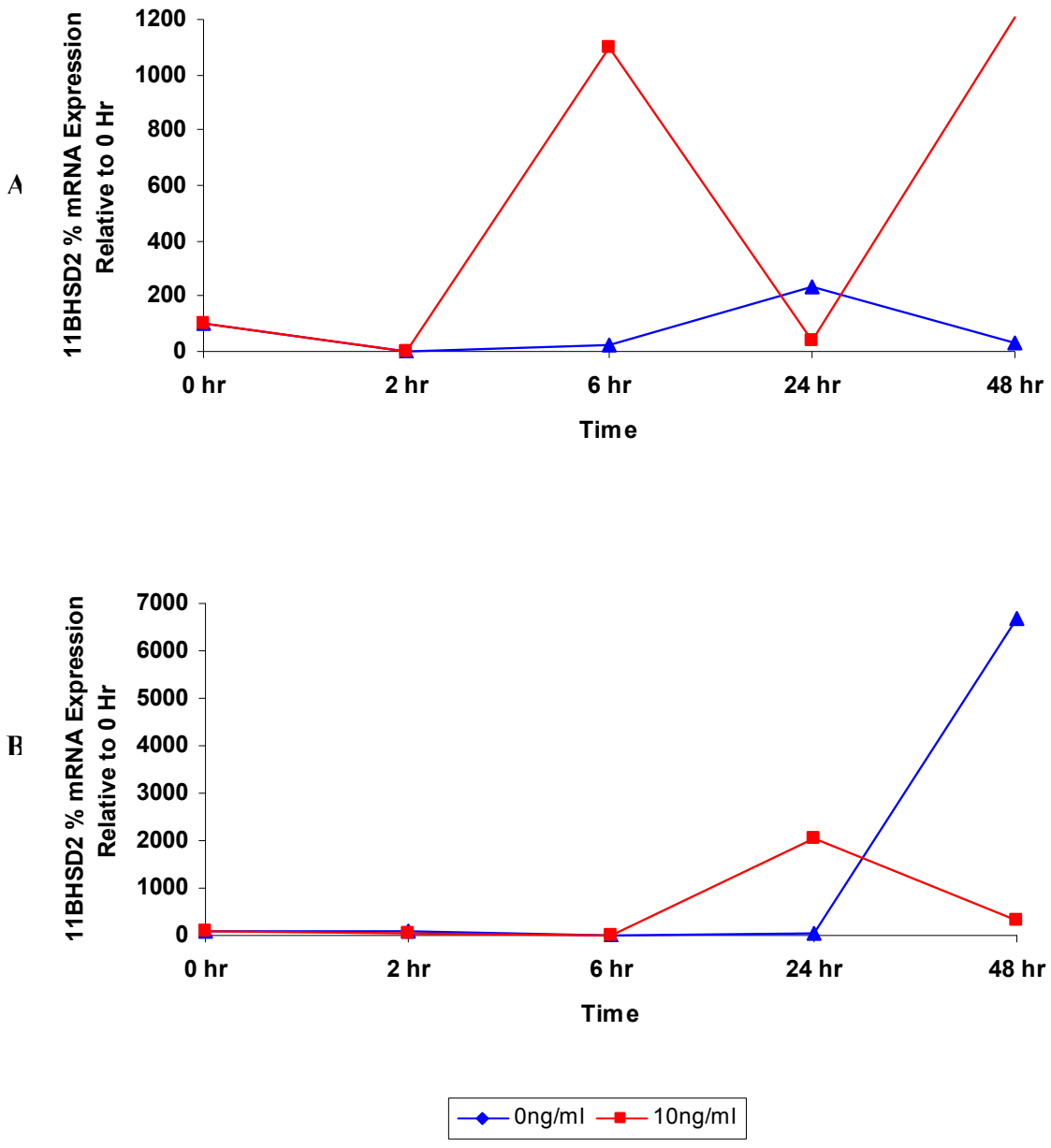


Figure 4.18 Expression profile of 11βHSD2 in 3T3-L1 pre-adipocytes undergoing differentiation at day 8 treated with exogenous leptin. Real time PCR data for expression of 11βHSD2 are shown in response to leptin treatment at 0ng/ml and 10ng/ml. Treatment starts at differentiation day 8 (0hr) and results are taken over a 48 hour time course. Replicates are shown in separate graphs (**A** and **B**).

4.3 Discussion

Evidence is accumulating which implicates altered DNA methylation and expression of key transcription factors and their target genes as causative factors underlying the metabolic disturbances associated with developmental programming [77,213,216]. For example, alterations in the epigenetic regulation of genes involved with energy homeostasis such as GR and PPARα have been identified in the liver of MLP offspring [77,213]. These mechanisms have not been investigated in an alternative animal model, the IUGR rat, an established model of programming involving a maternal 30% global undernutrition. These offspring are born small for their gestational age with reduced body fat, indicating altered adipose tissue development. They are also predisposed to become obese in adulthood upon nutritional affluence, have hyperphagia, increased systolic blood pressure and present with hyperinsulinemia and hyperleptinemia [122].

Work in the previous chapter of this report failed to identify any of the gene expression and methylation changes found in the liver of PR offspring [57,77] in the liver of IUGR offspring. It is therefore probable, that different genes, pathways or tissues are affected by maternal undernutrition in the IUGR rat, resulting in the altered capacity to store fat and the associated metabolic disturbances. Given the key role of adipocytes in the altered metabolism and the role of adipokines such as leptin in fetal growth, adipose tissue is strongly implicated as a causative factor in the development of obesity in the IUGR offspring. This would indicate that in IUGR rats, adipocyte function may be lost and this could be a result of altered methylation and expression of adipokines, transcription factors and their target genes involved in energy metabolism.

Previous work using this IUGR rat model of undernutrition has found that neonatal leptin treatment can prevent all the metabolic and physiological effects found in programmed offspring, by reducing weight gain [122]. Work in the previous chapter of this report could not find any relevant alterations in hepatic transcription factors and their target genes in IUGR offspring which could account for these changes induced by leptin. However, it has been shown in normal adult rats that leptin treatment can induce weight loss by directly acting on adipocytes to alter the expression of key genes involved with lipid metabolism, such as PPARα and its target genes [232]. Therefore, given that in adipose tissue, leptin has previously been shown to affect the expression of genes of lipogenesis [231] and lipolysis [232], it is possible that neonatal leptin treatment acts to alter signaling pathways to reduce lipogenesis and increase lipolysis in adipose tissue, to normalise the programmed phenotype.

The aim of this chapter was to determine if maternal or postnatal diet altered the expression and methylation of key genes of lipid metabolism in the adipose tissue of adult offspring from the IUGR rat study and to determine if leptin treatment was able to prevent any associated gene expression and methylation changes. To address these questions, the expression and methylation

status of a variety of genes involved in lipid metabolism were measured in the adipose tissue from IUGR offspring using real time PCR.

This study demonstrated in adipose tissue that the expression of key genes involved in energy homeostasis, including PPARα, PPARγ2 and their target genes, were not altered by maternal diet or postnatal nutrition. In agreement with this, the DNA methylation status of their respective promoters was also unchanged by diet. In contrast, neonatal leptin treatment resulted in a persistent increase in mRNA expression for many of the genes studied and this effect tended to be amplified in response to a HF postnatal diet, consistent with reduced weight gain in these offspring. There was, however, no evidence of altered DNA methylation in the promoters of offspring given neonatal leptin treatment. Analysis of the effect of leptin treatment on differentiating 3T3-L1 adipocytes in vitro conformed that leptin could increase the expression of several of these genes by acting directly on the adipocyte.

Unlike the MLP model which found increased GR and PPAR Alpha expression in liver of MPL offspring [57,77], there were no significant effects of maternal diet on the expression or methylation of any of the genes measured in adipose tissue. This may well be due to the timing effects of maternal undernutrition, because in rats, adipose tissue undergoes differentiation after birth. As these offspring were cross fostered onto AD dams at birth, the nutritional constraint may be outside the plastic stage of adipocytes, which would therefore be unaffected by maternal diet. It is also possible however, that different pathways and therefore genes were modified by maternal diet compared to MLP offspring. However, despite this, some interesting alterations in gene expression were apparent in the leptin treated offspring.

The expression of PPARα and its target genes AOX and CPT-1 were increased in response to neonatal leptin treatment and this increase was heightened in response to a high fat diet. As fatty acids are known to regulate PPARα expression by auto-regulation, it follows that PPARα would be up-regulated by HF feeding. Furthermore, the similar expression profiles of these three genes mean it is likely that PPARα is responsible for the differences in expression of CPT-1 and AOX. Thus in adipose tissue, the effect of neonatal leptin treatment in offspring fed a high fat diet is the permanent increase in expression of key genes involved in fatty acid metabolism not normally highly expressed in adipocytes. This could indicate an increase in fatty acid oxidation within adipose tissue and is in agreement with a previous report on the transient effects of leptin treatment in the adipose tissue of adult rats [232]. However, in this rat model the effects of neonatal leptin treatment are persistent and this is most likely to be due to the epigenetic plasticity which is still present in the neonate.

There was also a strong effect of leptin treatment on the expression of PPARγ2 in offspring fed both a chow and high fat postnatal diet, with increased expression compared to control offspring.

The expression of its target gene LPL had a similar expression profile, with a significant effect of leptin on offspring fed a high fat diet. As PPARγ2 controls the expression of LPL, this indicates that it may well be responsible for its changes in expression. Thus the leptin treatment resulted in a permanent increase in the expression of key adipocyte genes which function in adipogenesis, lipogenesis and transport of fatty acids into adipocytes. A previous report of leptin treatment in adult rats found reduced expression of PPARγ2, LPL and other adipogenic genes which contributed towards the de-differentiation of adipocytes [232]. This study did not find a reduction in PPARγ2 or LPL expression in response to leptin treatment, but this may be a consequence of the timing of leptin administration, as in this study the first two weeks of neonatal life is a critical time for the development of adipose tissue. Furthermore, GR expression was vastly increased in all leptin treated offspring and as GR can both directly and indirectly induce PPARγ2 expression [234], this could be responsible for preventing PPARγ2 down regulation.

GR is known to have at least 11 transcript variants which have tissue specific expression, but it is not known which transcripts are expressed in the adipose tissue of rats [242]. Results from this study indicated that there were differences in the expression of GR transcript variants in response to the treatments. The expression of GR_{110}, which is thought to be expressed in most tissues, was increased in all leptin treated offspring, whereas the expression of total GR was more strongly induced by leptin treatment, indicating an alternative transcript(s) may be responsible for the increase in total GR in adipose tissue. The increase in GR expression in all leptin treated offspring could also contribute to the increase in PPARα and 11βHDS-2 expression seen in the same offspring. Increased 11βHDS-2 expression in leptin treated offspring is consistent with the finding that in MLP rats, leptin treatment can prevent the placental reduction in 11βHSD-2 to increase conversion of cortisone to inactive corticosterone [145].

Leptin treatment resulted in a trend towards a reduction in the expression of IR. This would be consistent with a reduction in insulin signaling in the leptin treated adipose tissue and could potentially result in the reduction in expression of lipogenic genes. It has been found in other studies that leptin can reduce the expression of key lipogenic genes in adipose tissue such as FAS and ACL [231]. It is therefore possible that these genes are suppressed in neonatal leptin treated offspring. It would therefore be invaluable to look at the expression of such genes in future experiments.

The expression of leptin receptor was increased in all leptin treated offspring, but due to the variability in data, this did not reach significance, apart from UN offspring fed a high fat diet. An increase in leptin receptor signalling would be consistent with effects of leptin being peripherally mediated on the adipocyte and would implicate increased leptin sensitivity and thus increased signalling mechanisms such as Jak/Stat on target genes in leptin treated offspring. Several lines of evidence indicate that the expression of leptin receptor could play a key role in the programming of

obesity, with reduced expression contributing to leptin resistance and increased expression protecting from postnatal HF induced obesity.

Firstly, leptin receptor expression in the hypothalamus is reduced in normal offspring fed a postnatal HF diet, indicating HF diet induced central leptin resistance. However, there is no effect of a HF diet in these offspring given leptin treatment from PND1-20 and in these offspring OB-Rb expression is increased [147]. This indicates that at the hypothalamic level neonatal leptin treatment helps prevent HF induced leptin resistance, by restoring OB-Rb expression and therefore leptin sensitivity [147]. This has also found to be the case in adipose tissue [243]. It has been shown that adipocyte specific OB-Rb is down regulated to virtually undetectable levels in the DIO rat, with an associated reduction in Stat3 activation and an increase in SOCS3 expression. All these features imply adipose specific leptin resistance. Reintroduction of adipocyte specific OB-Rb into these rats normalises Stat3 signalling and prevents obesity [243].

Furthermore, in a separate study it has been shown that adipose specific disruption of leptin signalling is responsible for increased weight gain in HF feeding even in the presence of normal hypothalamic leptin signalling. Mice with adipose specific knockout of OB-Rb have increased fat mass due to hypertrophy and associated metabolic disturbances particularly upon HF feeding, as found in IUGR offspring. This obesity occurs in the presence of normal hypothalamic OB-Rb expression and pair matched food intake to wild type mice. In addition, these rats have altered adipose gene expression, including that of FAS TNFα and leptin. This study indicates the importance of adipose specific leptin receptor signalling on the regulation of bodyweight and that leptin resistance at the adipocyte is important for onset of obesity and associated metabolic disturbances [244].

Interestingly, it is possible that insulin could mediate the down regulation of adipose specific OB-Rb in the programmed offspring, as in rats, DIO can be prevented by blocking insulin signalling [243]. In agreement with this hypothesis, leptin treated IUGR offspring had a trend for down regulated insulin receptor compared to saline treated offspring, implying reduced insulin signalling in leptin treated offspring.

In this study, IUGR rats have hyperleptinemia in conjunction with obesity, implying leptin resistance. Collectively, all the above studies on OB-Rb imply that leptin resistance at the adipocyte due to reduced OB-Rb signalling is a causative factor in HF induced obesity and that by restoring OB-Rb, such as occurs with neonatal leptin treatment, obesity resulting from HF diet can be prevented. This theory is consistent with results from this study, whereby leptin receptor expression is up regulated in the adipocytes of leptin treated offspring whom have reduced weight gain compared to saline treated offspring, particularly on a HF postnatal diet. Therefore increased OB-Rb signalling in the adipose tissue and not the hypothalamus may play the main role in

preventing the diet induced obesity found in these offspring, by increasing leptin sensitivity at the adipocyte. Increased OB-Rb expression may also be responsible for the other changes seen in gene expression in adipose tissue of IUGR rats, such as the PPAR's. It should be mentioned, however, that OB-Rb expression was not reduced in the saline treated IUGR offspring. However, it may be that in these offspring signalling downstream of OB-Rb may have been affected, such as altered Socs-3 expression. It would therefore be interesting to determine if Socs-3 was altered in adipocytes, or if OB-Rb and other factors such as Socs-3 were affected in the hypothalamus of the programmed IUGR rat.

The expression of leptin was also increased in all leptin treated offspring, but did not reach significance apart from in AD offspring fed a high fat diet. Increased leptin expression without leptin resistance could indicate increased leptin action in these offspring and could be in part responsible for reversing some of the metabolic disturbances in IUGR offspring. For example, leptin reduces insulin expression as part of the adipoinsular axis [125] and through central mechanisms. Increased leptin action could therefore facilitate the reduction in insulin concentrations found in leptin treated IUGR offspring.

It would have been of value to determine if the expression of leptin was altered in adipose tissue throughout the first two weeks of life, in particular if leptin expression was reduced in UN offspring. Previous reports have shown that the neonatal leptin surge originates from leptin expression in adipose tissue [148,149] and that the surge is responsible for the formation of hypothalamic feeding circuits which are central to whole body energy balance in adult life [124]. Furthermore, in the DIO rat, disrupted projections are associated with an increased propensity towards an obese phenotype [245] and leptin treatment during the neonatal period can restore the formation of feeding circuits in leptin deficient Ob/Ob mice [124]. Therefore, if leptin expression was absent or blunted in adipose tissue of UN offspring compared to controls, with associated alterations in plasma leptin concentration, this would indicate neonatal hypoleptinemia and probable altered hypothalamic appetite circuitry and would be consistent with previous reports that a 50% maternal undernutrition results in a blunted leptin surge and immature hypothalamic projections [151]. Unfortunately, the neonatal leptin concentrations were not measured in the IUGR rat study and it is unknown if hypothalamic feeding circuits were affected. However, it is possible that the neonatal leptin treatment prevented the effects of programming by acting at the level of the hypothalamus to maintain a leptin surge in UN offspring, possibly blunted in UN offspring due to reduced adipose leptin expression, thereby ensuring protection against later obesity and its complications. Clearly additional rat studies would be needed to answer these questions.

Evidence has indicated that alterations in the expression of genes in programmed rats are in part due to altered epigenetic regulation, for example GR and PPARα [77,213-215]. Therefore it was speculated that this could be the mechanism by which leptin treatment causes long term changes in

gene expression in this study. For example, it is possible that the alterations in leptin expression could be due to altered methylation, because it is known that the leptin promoter is sensitive to alterations in methylation status [246,247] and in adipose tissue, the leptin promoter has been shown to be hyper methylated in response to HF feeding, with an associated with reduction in leptin expression [218]. This study did not, however, find any relevant alterations in DNA methylation of the genes tested in response to neonatal leptin treatment. Despite this, it may well be that a change in the methylation status had occurred in the leptin treated offspring in genes such as GR, PPARα and leptin, but was not observed due to technical limitations of the MSP assay. Methylation primers are optimal at 150-200bp and some CpG islands span up to 2000bp, therefore in many cases, such as for PPARα and GR, only a small proportion of the CpG island could be studied (figure 2.2). There are also limitations as to where MSP primers can work efficiently, for example primers must avoid areas of secondary structure and this is common in CpG islands. It was therefore not possible to cover complete CpG islands with one set of primers, leaving areas outside the amplicons which have not been studied, which may be epigenetically altered. In addition, MSP is a crude method to assess DNA methylation. For example, just one unmethylated CpG within a highly methylated amplicon could prevent a PCR product and thus be classed as unmethylated. It would therefore be useful to employ additional methods such as pyrosequencing to investigate DNA methylation of promoter regions, as this can determine the methylation status of individual CpGs. Another explanation for the lack of change in DNA methylation is that upstream signaling factors may be epigenetically modified instead.

The timing of the neonatal leptin treatment in the IUGR rat study was designed to mimic the normal leptin surge, which is crucial for the formation of hypothalamic feeding circuits and thus energy regulation in later life [124]. Results showed that the neonatal leptin treatment resulted in a persistent increase in the expression of several genes involved in energy balance in the adipose tissue of female IUGR offspring, which was associated with reduced weight gain upon HF feeding. Evidence from other rodent studies suggest that this effect of leptin could be the result of a direct effect on the adipocytes, rather than an indirect effects via the hypothalamus [121,232]. Interestingly, studies with mice subjected to maternal undernutrition have found that the neonatal leptin surge has a premature onset in programmed offspring compared to controls [39]. Thus alterations in the timing of the natural leptin surge may have long term consequences on energy metabolism. In order to confirm that the neonatal leptin treatment could have acted peripherally on adipocytes to alter the expression of genes involved in energy balance in the IUGR rat study, pre-adipocytes undergoing differentiation were treated with leptin and gene expression measured. Furthermore, to determine if the timing of leptin treatment relative to the stage of differentiation affected the response of the same genes, the effect of leptin treatment was assessed following treatment before differentiation (day 2) and at the start of differentiation (day 8).

In normal 3T3 cells undergoing differentiation, leptin was shown to undergo a surge in expression from day 6, peaking at day 13, consistent with previous reports that leptin undergoes a surge in

expression throughout differentiation [248]. The genes tested for responsiveness to leptin treatment were PPARα, GR, 11βHSD-2 and leptin, which were found to be increased in response to neonatal leptin treatment in the adipose tissue of adult female offspring from the IUGR rat study. Leptin treatment to adipocytes in vitro resulted in increased expression of leptin and PPARα at both day 2 and day 8, whereas a small increase in GR expression was only found when the cells were treated with leptin on day 2 and a small increase in 11βHSD-2 when cells were treated with leptin on day 8. In addition, the effects on PPARα dropped off at 48hrs, indicating that the cells may need continual treatment with leptin, which has a short half life in order to mimic the effects found in the rat study. These experiments therefore indicate that the effects of neonatal leptin treatment could have occurred by direct effects on adipocytes, with both neonatal leptin treatment and leptin treatment to differentiating adipocytes resulting in increased gene expression of the same genes. Furthermore, results indicate that apart from GR and 11B-HSD-2, the timing of leptin treatment to differentiating adipocytes did not affect the pattern of gene expression. However, the strength of all gene responses in 3T3 cells at the later time points may have been strengthened with daily leptin treatment. It remains to be determined the exact mechanisms by which leptin is able to alter expression of these genes.

Work in this chapter has failed to identify any significant alterations in gene expression as a result of maternal diet in IUGR offspring and therefore none of the gene expression changes previously identified in MLP offspring. Investigations were focused on key lipolytic and genes of β-oxidation due to their history being epigenetically altered in MLP offspring and the ability of leptin to induce their expression in adipocytes of adult rats. However, it is likely that the genes altered by maternal diet may be involved in lipogenesis. For example, in a similar nutritional model to this study, utilising a 50% maternal undernutrition, it has been shown in 9mth male rats, that SPEBP1c, LPL, FAS were all up-regulated by maternal diet, indicating a propensity towards increased fatty acid uptake and lipogenesis [249]. Evidence from leptin treated adult rats [232] and leptin treated DIO rats [243], indicates that leptin treatment not only increases FA oxidation, but also reduces lipogenesis within the adipocyte. Therefore, due to the finding that leptin can down-regulate some of these lipogenic genes e.g. FAS [232], SREBP [249], ACC [243], it would be worthwhile to measure the expression of more key adipocyte genes such as Pref-1, C/EBP, SREBP, FAS as well as adipokines, as this would help to give a broader representation of the metabolic state of the adipocytes in response to maternal undernutrition. Leptin is also known to increase UCP-1 and UCP-2 mRNA [243]. It would therefore be interesting to see if UCP-2 was increased in this rat study which could facilitate dissipation of energy from β-oxidation as heat as found in islets [241].

In conclusion, it could be hypothesised that in the IUGR rat, neonatal leptin treatment acts directly on the adipocyte resulting in reduced insulin signalling, increased leptin signaling and consequently an increased uptake of fatty acids and β-oxidation of fatty acids within the adipocyte. It is likely that leptin treatment also down-regulates key enzymes of lipogenesis, but this has not yet been

demonstrated in this study. As the effects of leptin treatment were only transient in adult rats [232] and long term in this study, results indicate that it was the specific period of leptin treatment responsible for these differences. This may be due to not only central hypothalamic effects, but also direct peripheral effects on the adipocyte. It remains to be elucidated if epigenetic alterations are responsible for the persistent changes in gene expression and which genes in the signalling cascade are affected. For example, are epigenetic alterations responsible for the changes in PPARα expression itself, or is the altered leptin receptor signalling responsible for the many changes in gene expression? It is therefore important to determine how leptin effects the expression of these genes and identify how the effects could persist through to adulthood.

Chapter 5

Identification of Alternative PPARα Transcripts and Characterisation of Promoter Regions

5.1 Introduction

5.1.1 Nuclear hormone receptors and their regulation

Nuclear hormone receptors such as PPAR's, which are activated by hormones, control the expression of specific sets of genes within specific cell types, at specific times. For example, PPARα directs the expression of an array of genes involved in fatty acid oxidation in tissues such as the liver and skeletal muscle and this activity is up regulated during times of stress. The expression and activation of such receptors can therefore significantly impact the biochemical properties of the cell in which they are expressed. Gene expression of nuclear receptors must therefore be under tight control in order to ensure that they are transcribed in correct tissues and that the timing is appropriate. Many different factors are involved in determining when and where a gene is transcribed and also at what level the gene is expressed. These factors can act at the transcriptional, post transcriptional or translational level.

As a consequence of their tissue specific expression profiles, nuclear hormone receptors frequently have a complex genomic organisation, as this enhances their capacity for both transcriptional and translational regulation. The step with most regulatory control is gene transcription, i.e. determining which genes are 'switched on' in a given cell type at a given time and this is controlled primarily by the gene promoter and regulatory elements within it. Nuclear hormone receptors tend to have multiple promoters and thus transcriptional start sites, which increases the capacity for tissue specific transcriptional regulation by providing diversity in the factors they respond to [250]. Quite often these promoters have a complex secondary structure due to a high GC content, have multiple Sp1 response elements and lack a recognizable TATA box. Furthermore, as a result of alternative promoter usage and alternative splicing, these genes commonly produce multiple transcript variants which differ in the 5'untranslated region (UTR). Some nuclear hormone receptor genes may possess many 5'UTR variants, for example GR [242], whilst others may have just a few such variants, such as PPARγ [84]. The 5'UTR of these transcripts may differ in length, GC content, secondary structure, the presence of upstream AUG (uATG) initiation codons and upstream open reading frames (uORF). All these features can affect selection of translation initiation codon and translation efficiency and represent another important component of gene regulation [251].

5.1.2 Genomic organisation of nuclear receptors

A classic example of a nuclear hormone receptor with a complex expression profile is the glucocorticoid receptor (GR type II) which mediates the effects of the steroid hormone cortisol and

is involved in controlling physiological processes such as metabolism, differentiation and the stress response. GR is expressed in most tissues at vastly differing levels, with both basal and stimulated expression in these tissues under tight control. Subsequently, this receptor has a complex genomic structure to provide the additional levels of gene regulation to accommodate these needs [250].

For example, rat GR levels in a given tissue are a result of the differential expression of 11 transcript variants which differ solely in the 5'UTR, specifically the first exon as a result of alternative promoter use. All these different first exons lie within a large CpG island rich in Sp1 sites [242] and differ in the response elements present, providing the variety to cope with the many factors involved in the basal and stimulated tissue specific GR expression [250]. As a result, some of these variants have broad patterns of expression, whilst some are more tissue specific. Similarly, the mouse orthologue of GR has at least 4 promoters and variant first exons in the 5'UTR, whilst the human GR has 7 alternative first exons. These alternative first exons exhibit high homology with those in the rat, indicating that it is probable that transcriptional regulation mechanisms are similar between species [250].

Other examples of nuclear hormone receptors with an array of alternative transcripts include the retinoic acid receptor (RAR) γ [252] and the growth hormone receptor [103]. The RARγ gene has 7 alternative transcripts, 5 of which differ in the 5'UTR and 2 which differ in protein coding region [252], whilst the human growth hormone receptor has 8 variants all of which differ in their 5'UTR [103]. Clearly, alternative 5'UTR transcripts are common to nuclear hormone receptors and represent an important feature of their complex regulation.

5.1.3 Genomic organisation of PPARγ gene in humans and mice

The PPARγ gene has been fully characterised in humans and mice, but not in the rat. Like other nuclear hormone receptors, it has been revealed that the PPARγ gene produces tissue specific variant transcripts as a result of alternative promoter usage and splicing of the 5'UTR. In addition, for the PPARγ isoform this not only results in 5'UTR transcript variants but also in a fully functional variant protein. Furthermore, the mouse and human orthologues of PPARγ demonstrate remarkable similarity in their promoter and 5'UTR as well as their coding regions, indicating that regulation at the level of gene transcription may be similar in both species.

Alternative promoter usage and splicing of the human PPARγ gene, produces 3 5'UTR variants; PPARγ1, PPARγ2 [84] and PPARγ3 [253] (figure 5.1). Two of these variants, PPARγ1 and PPARγ3, differ solely in the 5'UTR, whilst the hPPARγ2 transcript, which harbors an in-frame upstream initiation codon in its 5'UTR, produces a variant protein which is 28 amino acids longer at the N-terminal end. As all 3 PPARγ transcripts share the same coding exons 1-6, the remainder of the

protein has the same structural organisation in all 3 transcripts. The hPPARγ gene has distinct promoters which are responsible for tissue specific gene expression. The hPPARγ1 promoter is GC rich, containing multiple Sp1 sites but no TATA box. Its corresponding transcript is the predominant form and is most widely expressed, found mainly in adipose tissue, the large intestine and to a lesser extent in the liver, kidney and macrophages. In contrast, the structure of both the hPPARγ2 and γ3 promoters differs to that of γ1 such that they contain a potential TATA like element and are not GC rich [84,253]. Furthermore, hPPARγ2 and γ3 have similar expression profiles with hPPARγ2 expression found in adipose tissue [84] and hPPARγ3 in adipose tissue and the large intestine [253].

For the mPPARγ gene, only 2 variants have been identified so far, PPARγ1 and PPARγ2 and these differ in both their 5'UTR and coding region, exactly like their human orthologues. As the PPARγ2 protein is 30 amino acids longer at the 5'end due to a uATG in the 5'UTR, PPAR γ1 and γ2 result in different, but fully functional proteins. The mPPARγ1 promoter region, like that of hPPARγ1 is very GC rich with a CpG island containing several Sp1 sites. Again, similar to that of hPPARγ2, the mPPARγ2 promoter is not CG rich and has an upstream TATA like element near the transcription start site [254].

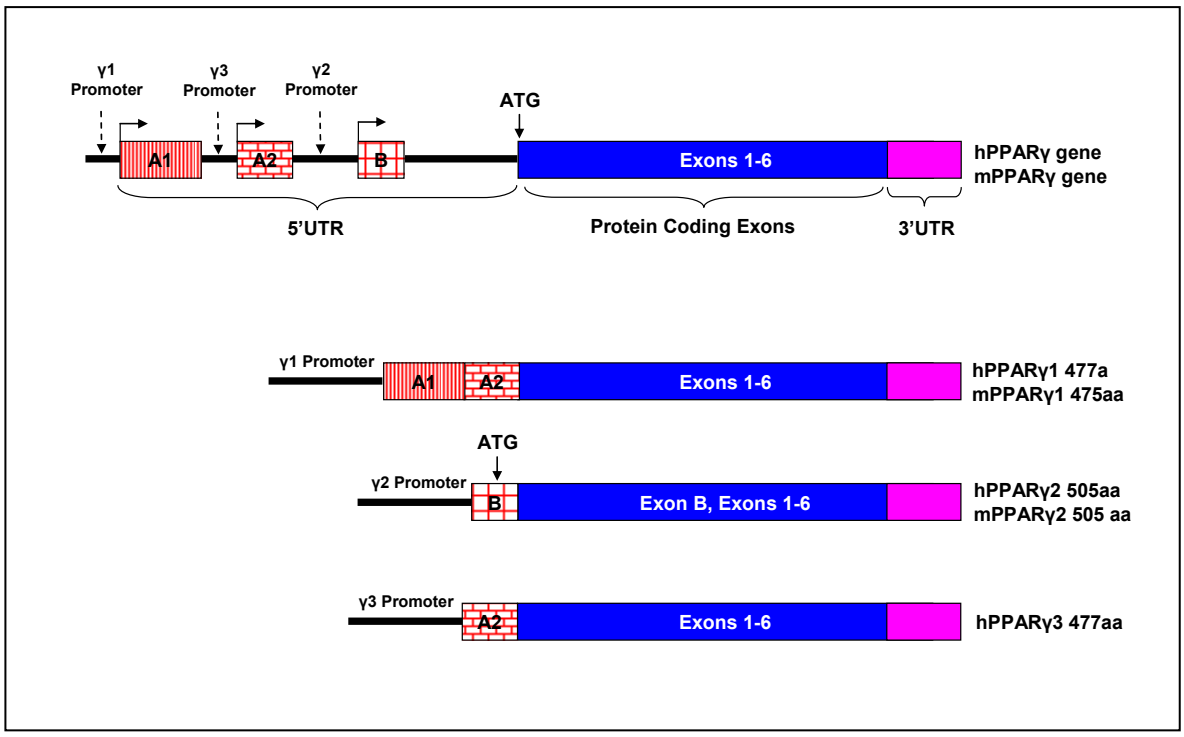


Figure 5.1 The genomic organisation of the human and mouse PPARγ genes (adapted from [253]). Differential use of promoters and alternative splicing mechanisms give rise to 3 hPPARγ variants (hPPARγ 1-3) and 2 mPPARγ variants (mPPARγ1-2). 5'UTR exons are shown in red, coding exons in blue and 3'UTR exons in pink. Human and mouse PPARγ show remarkable homology in their promoter, 5'UTR and coding regions. PPARγ2 results in a longer protein at the N terminal for both human and mouse forms due to an upstream in frame initiation codon.

5.1.4 Genomic organisation of PPARδ in different species

In contrast to PPARγ, the expression profile of PPARδ is ubiquitous and similar to GR, total transcript levels vary between the many tissues in which it is expressed. Detailed investigations into the structure of the mouse, human and rat PPARδ have been undertaken [76,85,255] and in all except the rat, multiple 5'UTR variants have been identified. Interestingly, studies have uncovered that translational control may be an important part of regulation of both the human and mouse PPARδ due to the structural complexity of the 5'UTR. It has been proposed that this form of translational regulation may be responsible for the 'temporal and spatial' fine tuning of PPARδ protein levels.

hPPARδ has a very complex genomic organisation (figure 5.2). Differential promoter usage and alternative splicing of the hPPARδ gene result in 11 different transcripts which vary solely in the 5'UTR. Many of these variants share the same starting exon and these transcripts are expressed at higher levels than the others. The promoter region for this major starting exon is CG rich and contains a CpG island and consistent with this no TATA box is present [85]. Interestingly, all the transcript variants differ in both their length and the amount of uORF in their 5'UTR, with amounts varying from 4-14 uORF in total. Coupled transcription / translation in a reticulocyte system has uncovered that both those transcripts with long 5'UTRs and those with more uORF had a reduced translational efficiency compared to those with shorter simpler 5'UTR, indicating an extra level of gene regulation for PPARδ compared to PPARγ. In addition to the 5'UTR variants, researchers have also identified a further hPPARδ variant, which differs in the coding region. This is a 3' truncated splice variant which lacks some of the LBD and consequently is non functional [85]. As such, expression of this variant protein may be another level of gene regulation which effects the overall expression of functional protein.

mPPARδ has a similar genomic organisation to that of hPPARδ (figure 5.3). Four variant transcripts have been identified which differ in the 5'UTR as a result of differential promoter usage and alternative splicing. The major variant, expressed in most tissues, has high homology with the main human transcript, as does the upstream regulatory region which contains a CpG island. This main transcript has a long 5'UTR containing multiple Kozak compatible uORF and like the human orthologue was shown to have a reduced translation efficiency compared to other less complex transcripts. Being the predominantly expressed transcript, a possible IRES mode of transcriptional initiation was speculated [76].

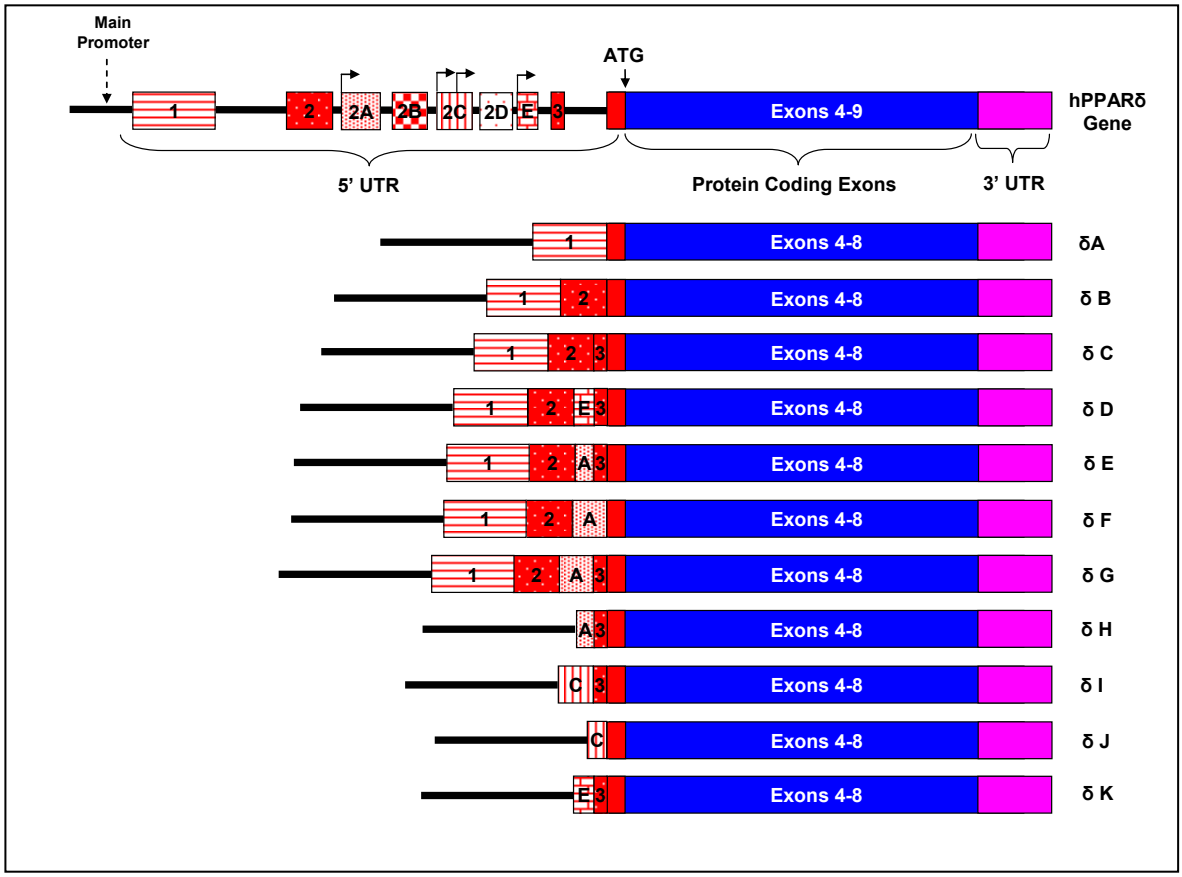


Figure 5.2 The genomic organisation of the human PPARδ gene. Differential use of promoters and alternative splicing mechanisms give rise to 11 hPPARγ variants (hPPARδ A-K). 5'UTR exons are shown in red, coding exons in blue and 3'UTR exons in pink. Transcripts differ in their 5'UTR, but not the coding region.

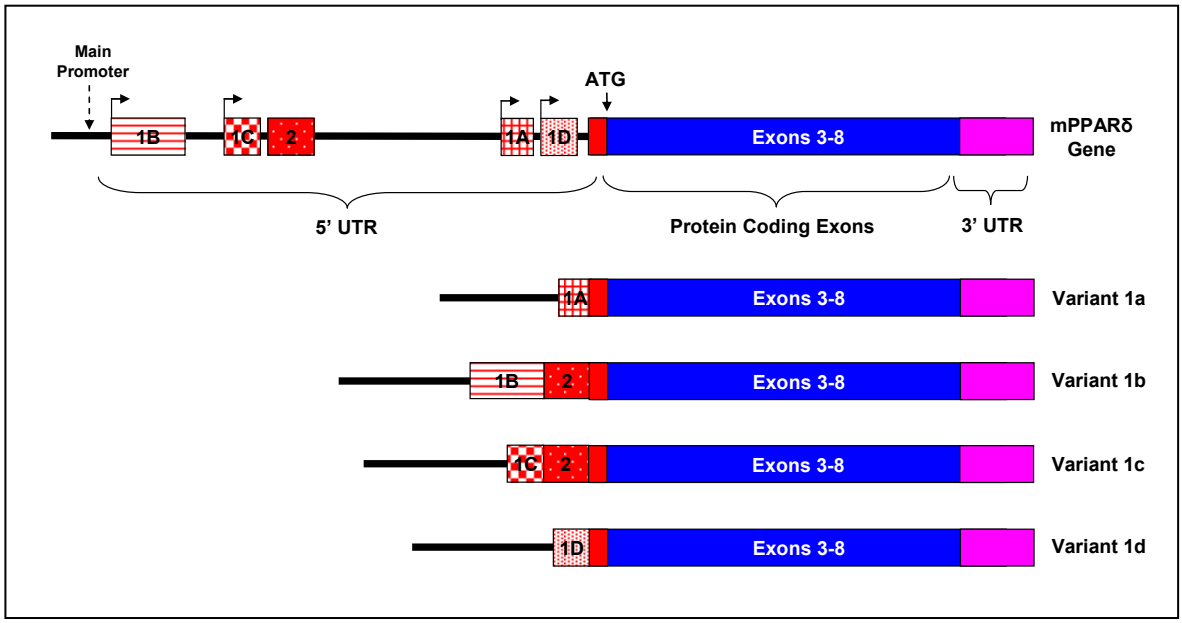


Figure 5.3 The genomic organisation of the mouse PPARδ gene. Differential use of promoters and alternative splicing mechanisms give rise to 4 mPPARγ variants (Variant 1a-1c). 5'UTR exons are shown in red, coding exons in blue and 3'UTR exons in pink. Transcripts differ in their 5'UTR, but not the coding region.

In contrast to human and mouse PPARδ, only 1 transcript has been identified so far for the rat orthologue. This work was however limited to a rat brain cDNA library and as such more tissue specific transcripts may exist [255]. Both the rat 5'UTR and regulatory regions are very similar to the predominant transcripts and promoters of the human and mouse PPARδ genes and like the other PPAR isoforms, the coding exons are conserved in all species (figure 5.4). However, work to examine the structure of the 5'UTR has not been not undertaken and therefore it is not known if like the mouse and human orthologues, translational efficiency impacts rat PPARδ protein levels [255].

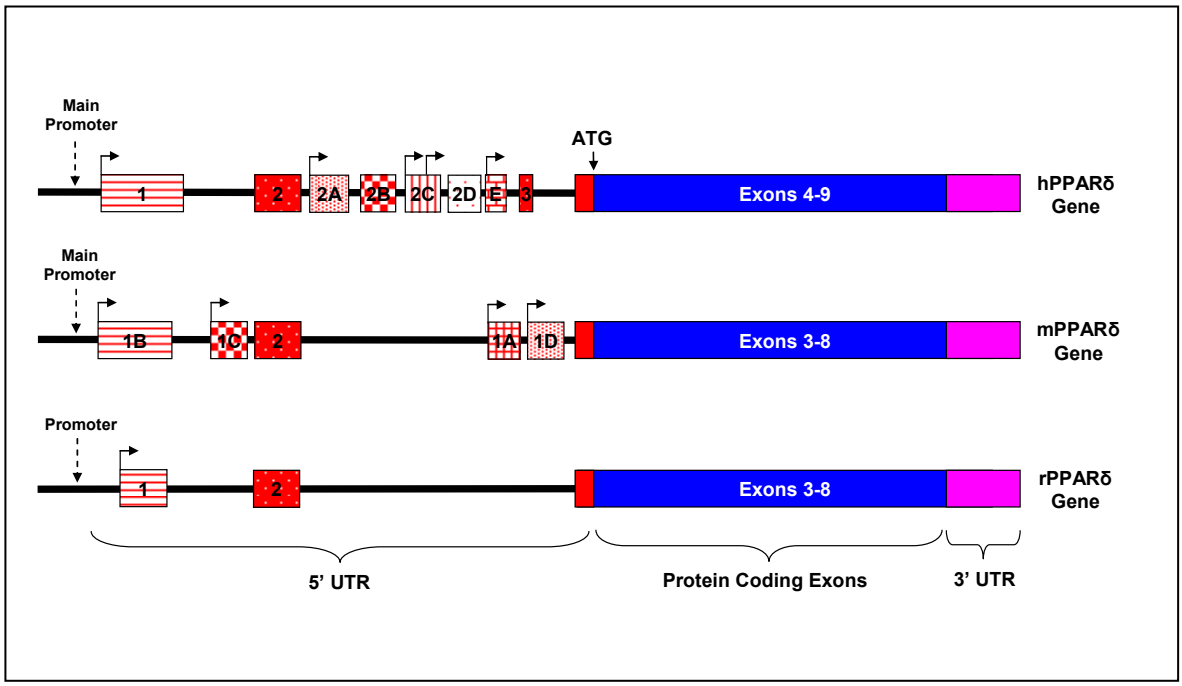


Figure 5.4 A comparison of the genomic organisation of the human, mouse and rat PPARδ genes. Human, mouse and rat PPARδ have similar organisation of their promoter and predominant 5'UTR exons. 5'UTR exons are shown in red, coding exons in blue and 3'UTR exons in pink. All coding exons are shared between species.

5.1.5 Genomic organisation of PPARα gene in different species

PPARα has a tissue specific expression profile and levels of receptor vary according to metabolic needs of the cell. As such, it is probable that gene regulation of PPARα will be complex. Unsurprisingly, studies into the genomic organisation of the PPARα gene indicate the presence of multiple 5'UTR variants in both the human and mouse orthologues and data indicates that these may play a role in not only the transcriptional regulation of gene expression but possibly the translational control as well. The rat PPARα gene, however, has not been studied since its identification. Despite this, data gathered confirms that all 3 species share not only the same

coding exons, but some 5'UTR exons also. Furthermore, all 3 PPARα genes have 5' regulatory regions which are highly GC rich and contain large CpG islands, indicating that regulation of transcription may be similar between species.

Initially, the human PPARα gene was shown to be composed of 8 exons which formed just 1 transcript. The 5'UTR was composed of 3 exons; exon 1a, exon 2a and the 5'end of exon 3, the coding exons were composed of the remainder of exon 3 and exons 4-8, whilst the 3'UTR was composed of the 3'end of exon 8 [256]. Subsequent to this, a further study has revealed that human PPARα has a much more complex genomic organisation [103]. This study demonstrated that PPARα is composed of 12 exons due to the identification of an extra 4 exons within the 5'UTR. Consequently it was revealed that 7 exons form the 5'UTR; exons A, 1A, B, 1B, 2A, 2B and the 5'end of exon 3, whilst the remainder of the gene was exactly as previously reported [103] (figure 5.5). Interestingly, Ensembl indicates the presence of a further 2 exons in the human PPARα 5'UTR (gene ID: ENSG00000186951), one of which is conserved in both the mouse and rat PPARα 5'UTR (exon X). However, in total a further 6 transcripts have been identified which differ purely in the 5'UTR, due to the use of 4 distinct promoter regions, several transcriptional start sites and alternative splicing. Furthermore like PPARδ, the regulatory region for the predominant transcript was shown to be TATAless, GC rich and contain several putative Sp1 sites. It was found to contain putative response elements for transcription factors such as such as activator protein 1 (AP1), Stat, OCT1, TCF II,GATA binding protein 2 (Gata2), Coup II, CEBPβ, HNF3, CCAAT, CREB and NF1 [103].

One other splice variant of human PPARα has been identified in which exon 6 is spliced out. This results in a premature stop codon and consequently a truncated hPPARα protein which has no LBD (termed PPARα2). Akin to the PPARδ truncated variant, this PPARα variant does not code a functional receptor protein [257]. It was further shown that PPARα2 also exists in other human tissues in which PPARα is normally expressed including the kidney, muscle and heart. Furthermore, PPARα2 was found to represent 30% of all human PPARα in liver samples and therefore thought to contribute towards reduced functional PPARα expression [258]. Further to this, this truncated version of PPARα was also found in mouse and rat, but at extremely low levels which were barely detectable [257].

The mouse PPARα gene extends over 30kb and is composed of 9 exons. Four comprise the 5'UTR; exon 1a, 1b, 2 and the 5'end of exon 3, the coding exons, like the human orthologue are composed of the 3'end of exon 3 and exons 4-8, whilst the 3' UTR is composed of the 3'end of exon 8. Initially, just 2 transcripts were identified in the mouse which differed solely in the 3' end of exon 1b. This exon was shown to harbor 2 major and 2 minor transcription start sites all within a 10bp range [102]. Subsequent to this a second variant (variant 2 NM_001113418) has been identified which differs in the 5'UTR, specifically the use of an alternative first exon (exon 1a)

(figure 5.6). Therefore, in contrast to hPPARα, there is not considerable diversity in the mouse PPARα 5'UTR. However, the exons present in the mouse 5'UTR are all present in the human PPARα 5'UTR. Furthermore, like the human version of PPARα, the 5' flanking promoter region has no TATA box or CCAAT sequences and is very GC rich, containing several putative Sp1 sites [102], indicating that regulatory mechanisms may be similar between species.

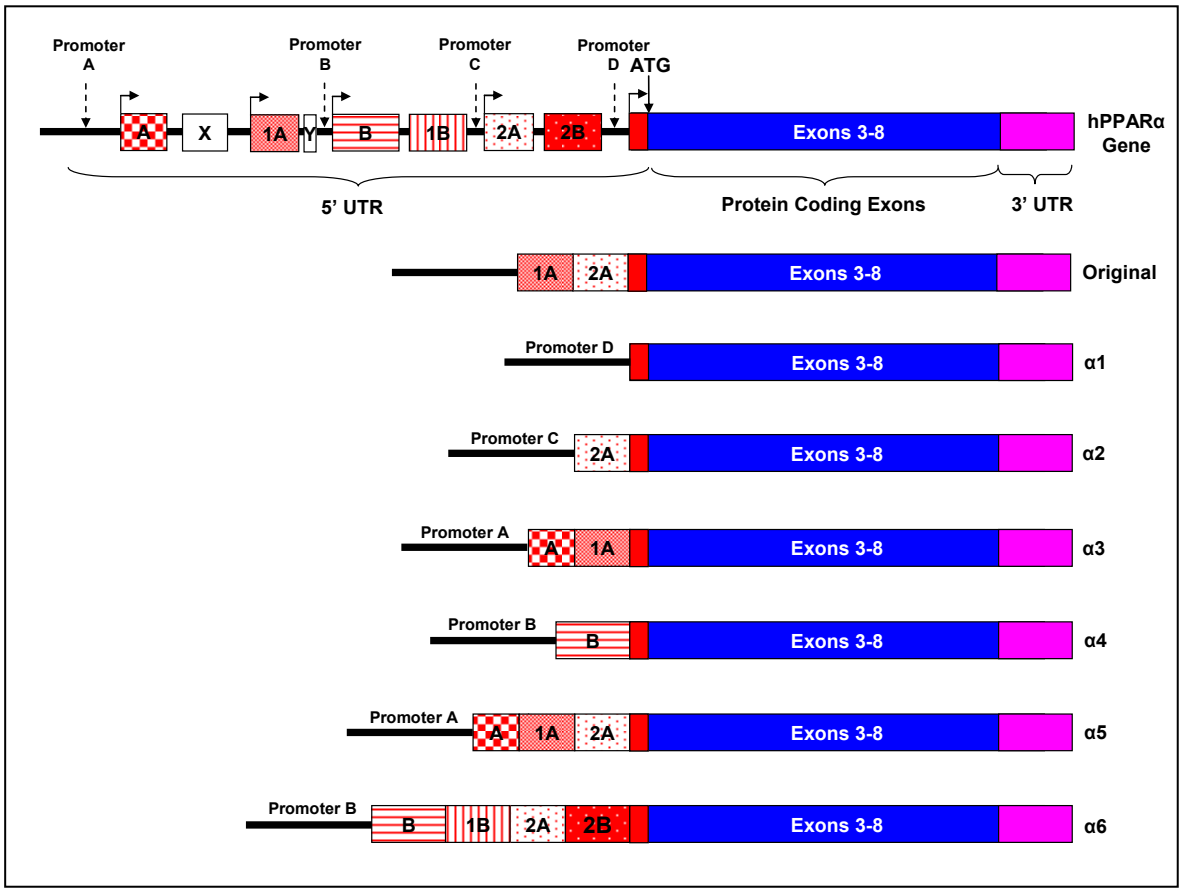


Figure 5.5 The genomic organisation of the human PPARα gene. Differential use of promoters and alternative splicing mechanisms give rise to 7 hPPARα variants (hPPARα 1-6). 5'UTR exons are shown in red, coding exons in blue and 3'UTR exons in pink. Transcripts differ in the 5'UTR, but not the coding region. Exons marked X and Y represent the additional exons identified in Ensembl PPARα gene (ENSG00000186951).

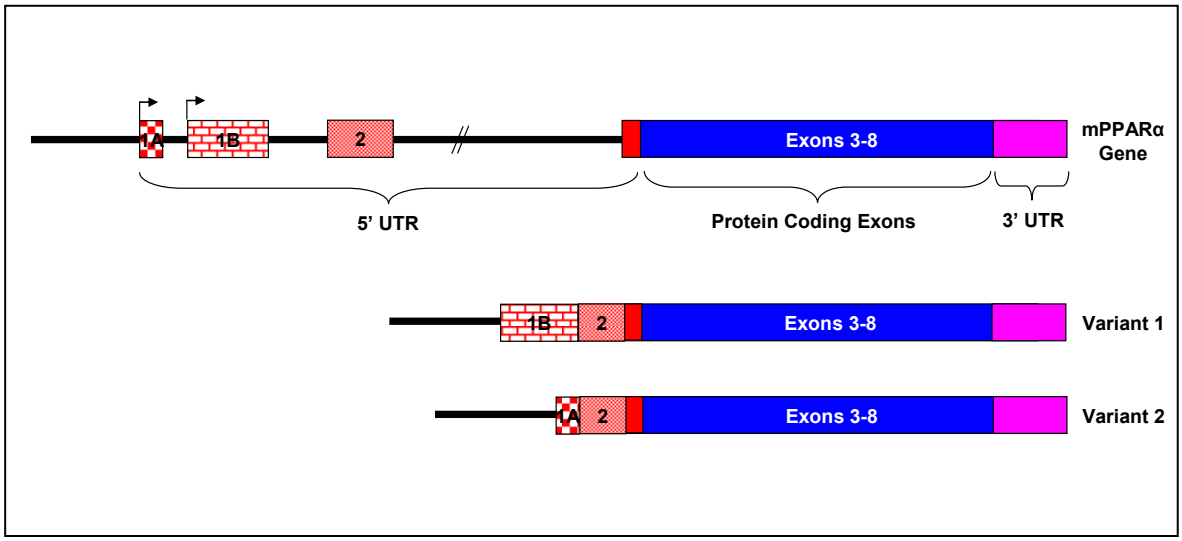


Figure 5.6 The genomic organisation of the mouse PPARα gene. Differential use of 2 promoters and alternative splicing mechanisms give rise to 2 mPPARα variants (Variant 1 and 2). 5'UTR exons are shown in red, coding exons in blue and 3'UTR exons in pink. Transcripts differ in their 5'UTR, but not the coding region.

Since its identification in 1992, the genomic organisation of the rat PPARα gene has not been investigated. Rat PPARα was identified from a Sprague-Dawley rat liver cDNA library (accession M88592). The 77 Kb gene was shown to comprise 8 exons, resulting in just 1 transcript of 2020bp. Three exons were shown to form the 5'UTR; exon 1, 2 and the 5' end of exon 3, the coding regions were found to be composed of the 3' end of exon3 and exons 4-8 and the 3'UTR contained the 3' end of exon 8 [105] (figure 5.7). In common with the other isoforms, coding regions of rat PPARα have high homology with the human and mouse orthologues (figure 5.8) and the exons present in the 5'UTR are present in both the human and mouse orthologues. The promoter region of PPAR in the rat has not been characterised, but in common with human and mouse promoters, the upstream regulatory sequence of the rat gene is highly GC rich.

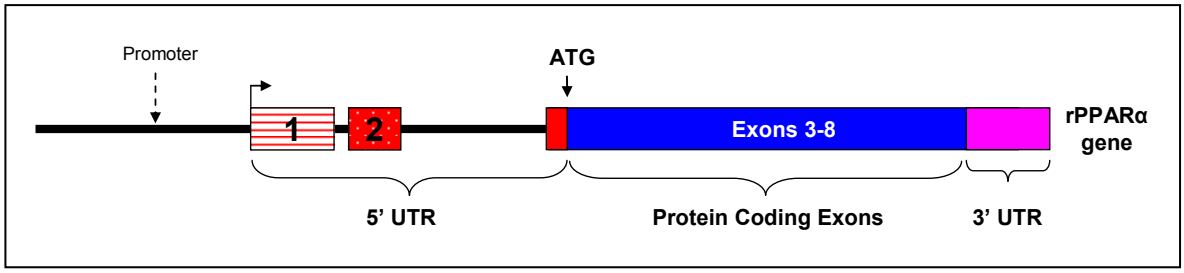


Figure 5.7 The genomic organisation of the rat PPARα gene. Genomic organisation of the rat PPARα gene, showing the untranslated and coding region organisation. 5'UTR exons are shown in red, coding exons in blue and 3'UTR exons in pink.

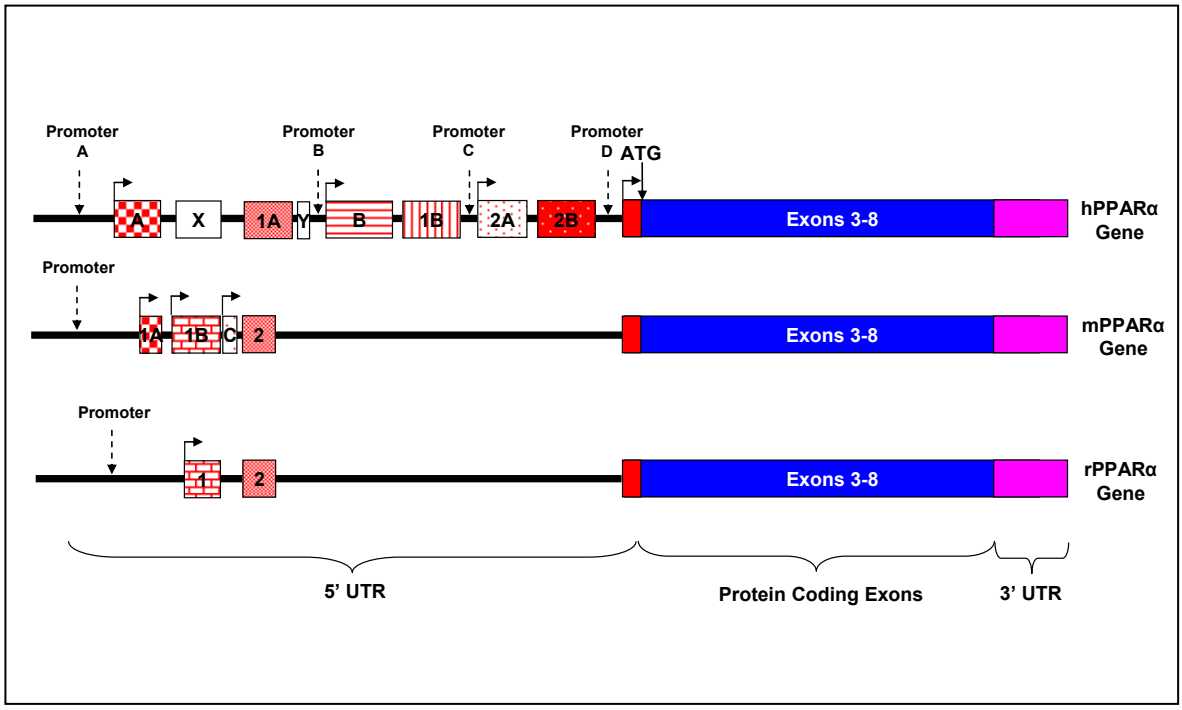


Figure 5.8 A comparison of the genomic organisation of the human, mouse and rat PPARα genes. Human PPARα currently has a more complex 5'UTR organisation than mouse and rat. 5'UTR exons are shown in red, coding exons in blue and 3'UTR exons in pink. Some 5'UTR exons are conserved between species. All coding exons are shared between species.

5.1.6 Aims

Previous work within this laboratory and also that identified in this report has indicated that PPARα mRNA expression can be modified by maternal diet, postnatal diet, or neonatal leptin treatment and that this altered regulation is tissue dependent. Furthermore, some of these changes have been associated with alterations in DNA methylation of the PPARα promoter [77,212]. However, exact mechanisms underlying these changes in gene expression and methylation have not been elucidated. In addition to this, although it is known that factors such as glucocorticoids may regulate the expression of PPARα, the mechanisms underlying regulation at the promoter level have been largely unexplored.

Nuclear hormone receptors such as the PPAR's have been shown to possess complex genomic organisations which create additional means of transcriptional and translational regulation to cope with their varied expression profiles. Characterisation of PPARα gene in humans has already been undertaken and multiple transcripts with diversity in the 5'UTR have been identified. However, to date, it is not known if such variant transcripts are present in the rat. As the recent work with human PPARα uncovered more transcripts than originally identified, this may also be true for the rat orthologue.

It is therefore important to identify the structure of the rat PPARα gene to understand not only the basic tissue specific regulation of PPARα gene expression, but also to provide a basis for further understanding its role in programming and subsequent disease risk, by identifying regulatory regions and response elements which are important for basal and stimulated gene transcription. For example, identification and characterisation of the complex GR gene structure has helped to clarify the exact mechanisms by which maternal grooming leads to altered stress response in pups, i.e. via specific methylation differences within response elements of tissue specific GR promoters.

To address these PPARα issues and further our understanding of the mechanisms contributing to PPARα gene regulation, the PPARα gene and its 5' regulatory region needed to be characterised. Work in this chapter identifies and characterises the 5'UTR of PPARα in the rat. Six alternatively spliced variants were identified which differ in their 5'UTR due to tissue specific differential promoter usage. Five novel exons were identified and flanking promoter regions of adipose and liver specific transcripts were identified, cloned and characterised.

5.2 Results

5.2.1 5' RLM RACE indicates that rat PPARα has multiple tissue specific transcript variants

In order to fully characterise the rat PPARα gene, a 5'RLM RACE was initially performed to enable identification of any 5'UTR transcript variants and their transcription start sites. This would in turn enable potential promoter regions to be located. Gene specific RACE primers were designed against exon 4 of the rat PPARα gene which is located downstream of the ATG initiation codon in exon 3 (ensemble gene ID ENSRNOG00000021463) (figure 2.3). This positioning of the GS RACE primers thus ensures that any 5'UTR variants would be included in the results. To determine if PPARα has tissue specific transcripts and promoters, the 5'RLM RACE was performed on tissue from liver, kidney, heart and muscle in which PPARα expression is high and adipose tissue where expression is low. All tissues used for this procedure were taken from control offspring from the IUGR rat study.

7 PCR products of varying size were successfully identified from the RACE procedure in total (figures 5.9-5.10); 2 different sized PCR products from the liver (723bp and 463bp), 1 from adipose tissue (465bp), 2 from kidney (591 and 473bp), 1 from muscle (400bp) and 1 from the heart (approx 481bp data not shown). A third faint band of ~1.2kb was also present in the liver PCR reaction, but as it disappeared with a temperature gradient, it was therefore presumed non-specific. As the size of the PCR product represents the length of the 5'UTR up to the GS reverse primer in the coding region of exon 4, the difference in PCR product size indicates the presence of variant transcripts which may differ in all or part of their 5'UTR sequence and possibly part of their coding sequence.

The presence of multiple PCR products of different sizes in both the liver and kidney indicates the presence of multiple PPARα transcripts in these tissues, whilst the presence of just 1 PCR product in adipose tissue, muscle and heart indicates that PPARα transcription in these tissues primarily arises from one start site. However, it is possible that variant RACE transcripts of the same size are present as a single band in these tissues.

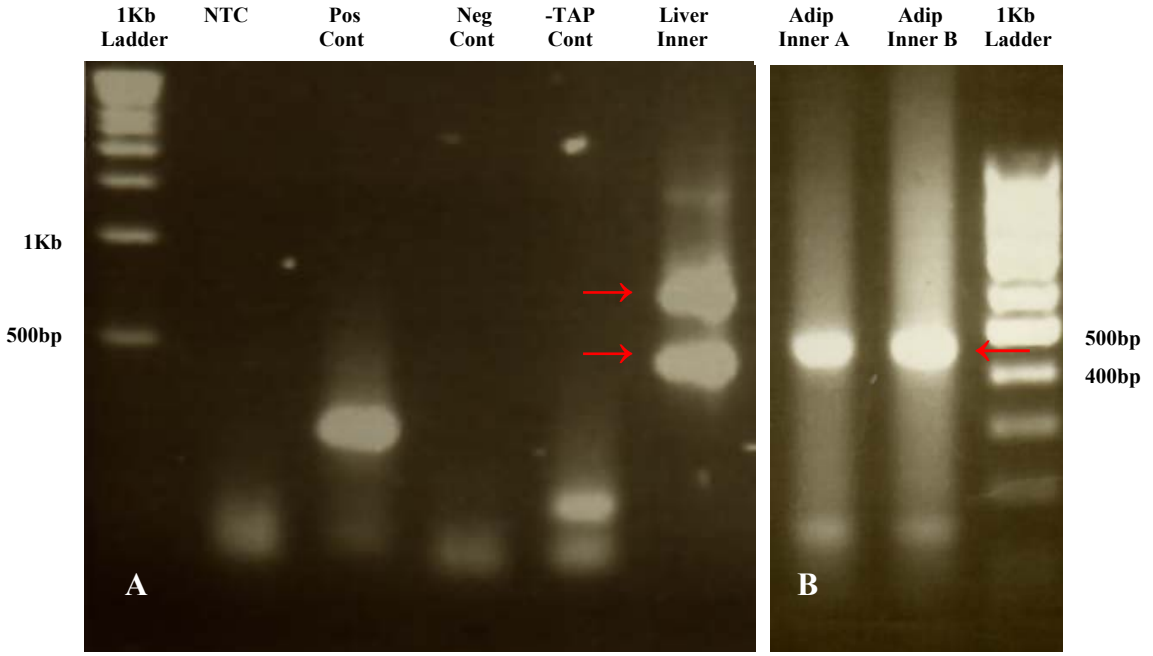


Figure 5.9 5' RLM RACE PCR indicates that the liver expresses multiple PPARα transcripts whilst adipose tissue expresses just one PPARα transcript. Agarose gel showing PPARα RACE PCR results: (A) Positive control (mouse thymus RNA with control primers) indicates that both the 5'RACE and PCR have been successful, whilst the –TAP control indicates that the RACE products are specific to de-capped RNA. The 5'RACE performed on liver indicates 2 different PCR products (723bp and 463bp). (B) Adipose 5'RACE indicates just 1 PCR product (465bp).

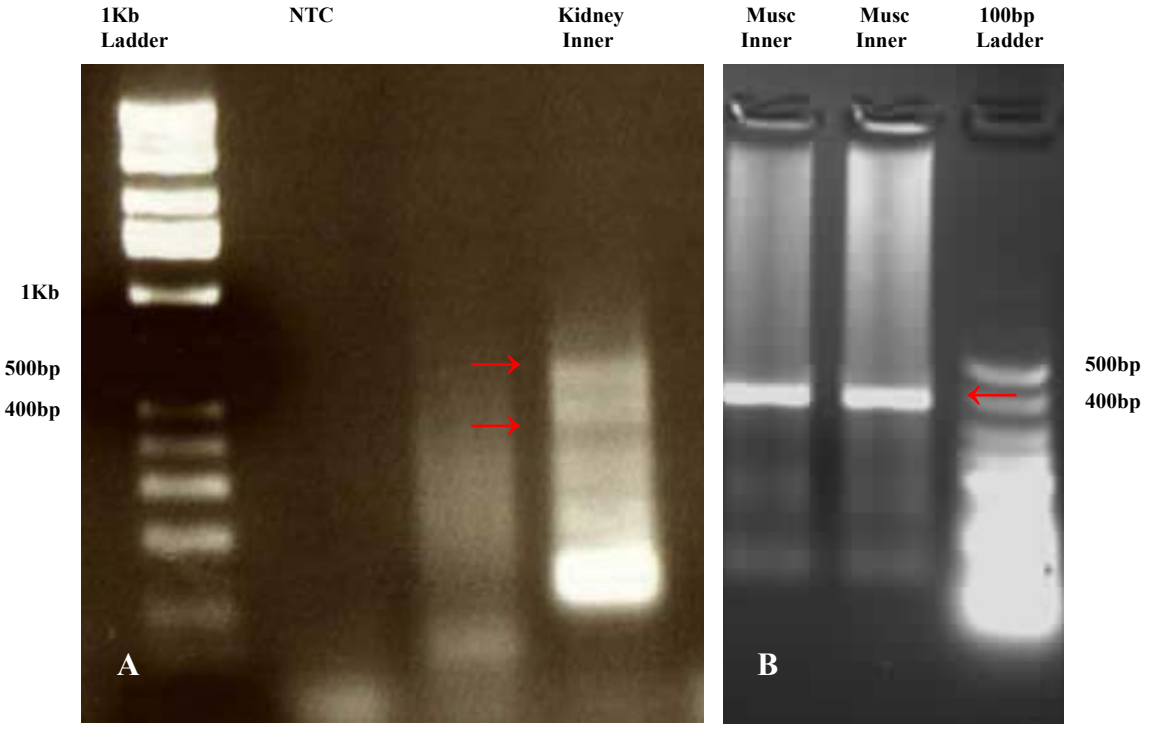


Figure 5.10 5' RLM RACE PCR indicates that the kidney expresses multiple PPARα transcripts whilst muscle expresses just one PPARα transcript. Agarose gel showing PPARα RACE PCR results: (A) 5'RACE performed on the kidney indicates 2 different PCR products (591bp and 473bp). (B) Muscle 5'RACE indicates just 1 PCR product (400bp).

5.2.2 Sequencing of the PPARα 5' RLM RACE products identifies multiple novel 5'UTR exons and six tissue specific 5'UTR variant transcripts

To identify the sequence of the 7 alternative PPARα 5'RLM RACE transcripts, PCR products were either cloned into the pGEM T-easy vector and the resulting clones sequenced (2 clones for adipose and 1 clone for the liver), or the PCR products were sequenced directly (heart, kidney and muscle). All the transcript sequences were then compared to the published genomic sequence of rat PPARα (Ensemble gene ID ENSRNOG00000021463) to identify the exact genomic location of exons. All sequences were analysed up to exon 4 which contained the gene specific reverse 5'RACE PCR primer.

Prior to this study, the rat PPARα 5'UTR was reported to be composed of exon 1, exon 2 and the 5'end of exon 3 (accession M88592), however none of the transcripts identified by 5'RLM RACE matched this sequence exactly. All transcripts were, however found to contain Ensembl exon 3 and 4 at the 3' end, confirming that all PCR products were specific to PPARα. Unfortunately, although a PCR product was generated for PPARα in the muscle, the corresponding sequence could not be matched to the PPARα genomic sequence, therefore no further work was undertaken on the muscle RACE transcript.

All 6 transcripts differed from each other at the 5' end and comparisons to the genomic sequence revealed that they had different transcription start sites owing to the presence of unique first exons. The PPARα transcripts were subsequently termed P1-6 and a schematic diagram indicating the genomic location of the PPARα 5'UTR exons, the organisation of the alternative 5'UTR and their sequences can be found in figures 5.11-5.14. In total 8 exons were found to encode the PPARα 5'UTR covering a genomic region of 45,000bp. Exons were named according to their genomic location (1A, 1B, 1C, 1D, 1E, 2, 2A and 3). Five of these exons were novel (1A, 1B, 1C, 1D, 2A), 2 were a modified version of exon 1 (now termed 1E) and the remainder were exactly the same sequence as identified by Ensembl (exons 2 and 3).

The 5'UTR of the adipose specific transcript (P1) is encoded by exons 1C, 2 and 3. The transcription start site for the this transcript is 257bp upstream of the Ensembl published start site and gave rise to a distinct unique first exon of 60bp termed exon 1C. This exon is spliced onto the published Ensembl exon 2, which is subsequently spliced onto Ensemble exon 3. The liver specific transcripts were termed P2 and P3. P2 is exactly the same as the Ensembl published sequence, except the first exon 1E (previously exon 1) is 67 bp longer than the original exon 1 at the 5' end resulting in a 318bp exon. This exon is spliced onto Ensembl exon 2, which is spliced onto Ensembl exon 3. For P3, the first exon is a 145bp novel exon located several Kb downstream from exon 2 and has been termed exon 2A. This exon is spliced directly onto Ensembl exon 3 and is the only transcript to be absent of exon 2. The heart specific variant P4 is similar to P2 such that

it contains exon 1E, 2 and 3. However, the transcription start site is located 262bp downstream of the P2 TSS within exon 1E. The P5 and P6 transcripts were identified in the kidney. P5 is composed of exons 1A, 1B, 2 and 3. This is the only transcript to have 2 novel exons identified within its 5'UTR. Exon 1A is located 3068bp upstream of the Ensembl published TSS and forms a short 94bp exon. This exon is spliced on to novel exon 1B another short exon of 92bp which is 2015bp upstream of the Ensembl TSS. Exon 1B is spliced to Ensembl exon 2. Finally, P6 is composed of exons 1D, 2 and 3. This novel first exon is located at the 3' end of exon 1C and is a short exon of 68bp which ends just upstream of exon 1E. Exon 1D is spliced to Ensembl exon 2.

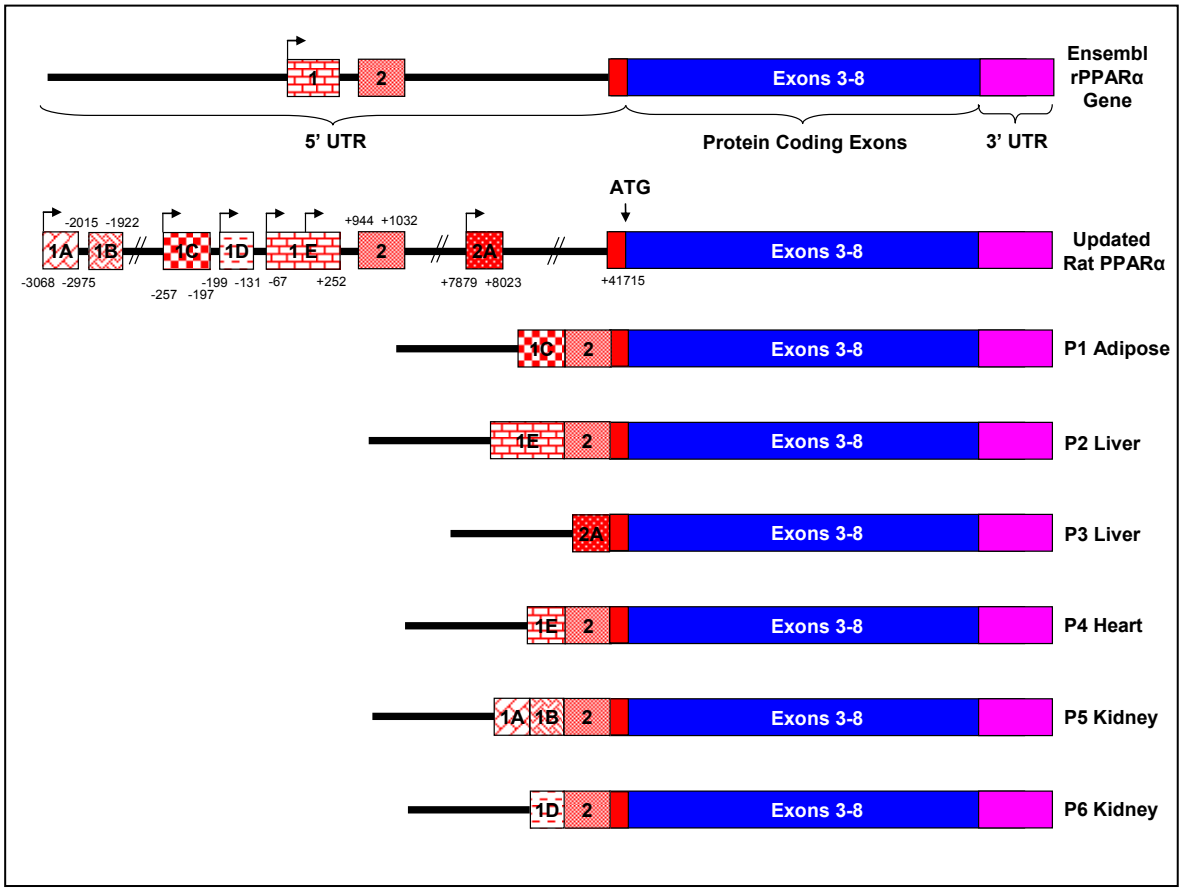


Figure 5.11 A schematic diagram showing the genomic organisation of the rat PPARα gene. Location of PPARα 5'UTR Exons on the genomic sequence are shown for the original Ensembl PPARα transcript and the updated transcripts; the adipose specific transcript (P1), liver specific transcripts (P2 and P3), the heart specific transcript (P4) and the kidney specific transcripts (P5 and P6). All exon positions are indicated relative to the Ensembl transcription start site. Ensembl exon 3 which contains the translation ATG start codon[105] is present in all transcripts. Non-coding 5'UTR exons are shown in red, coding exons in blue and 3'UTR exons in pink. Transcripts differ in the 5'UTR.

A Liver P2

AGACCCACAGCCACTGGCGAGGGCACACGCTAGGAAGGGCACACGCGTGCGACTTTCGGGGCCCCTG
GAACTGTCCGCTACTTCGAGTCCCCTTGAGCGCCGTGTGCCGGCTCTGAACATTGGCGTTCGCAGCT
GTTTTGTGGGCTGGAGGGTTCGTGGAGTCCTGGAACTGAAGCGACGCTGGGTCCTCTGGTTGTCCCC
TTGAGGGGAGGGCACACGAGCGGGGACATCGGGGCGCTCCCTTCCCACAGCGTGGTGCATTTGGGCG
TAACTCACCGGGAGGCGTTTCCTGAGACCCTCGGGGATCTTAGAGGCGAGgtaactcga.....gtt
ctatagCCAAGACTGAAGTTCAAGGCCCTGCCTTCCCTGTGAACTGACATTTGTGACTGGTCAAGCT
CAGGACACAAGACGTTGTCATCACAGgtaagaggg.....ctcctacagATTGGTGCTCTGTGGCCC
GCCTGGCCACAACCATTCAACATGGTGGACACAGAGAGCCCCATCTGTCCTCTCTCCCCACTTGAAG
CAGATGACCTGGAAAGTCCCTTATCTGAAGAATTCTTACAAGAGATGGGAAACATTCAAGAGATTTC
TCAGTCCCTCGGAGAGGAGAGTTCCGGAAGCTTTAGTTTTGCGGACTACCAGTACTTAGGGAGCTGT
CCAGGCTCGGAGGGCTCTGTCATCACAGgtaagggct.....ctttctctgtagACACCCTCTCTCC
AGCTTCCAGCCCCTCCTCAGTCAGCTGCCCTGCTGTCCCCACCAGTACAGATGAGTCCCCTGGCAAT
GCACTGAACATCGAGTGTCGAATATGTGGGGACAAGGCCTCAGGATACCACTATGGAGTCCACGCAT
GTGAAGGCTGCAAG

B Liver P3

AGAGACTTGGAGACGCGGGAACCAGAAGGAAGACTCCCGGCAGGACTTCCTTGACTGAGAAAGTACAA
TCCTACAATGAATTGTGACCTTTGGAGGAATGAATGAACTTAGAGGGGGCTTTCCGGATGGGAGAAAG
CATCTTCTGgtaggtatt.....ctcctacagATTGGTGCTCTGTGGCCCGCCTGGCCACAACCATTC
AACATGGTGGACACAGAGAGCCCCATCTGTCCTCTCTCCCCACTTGAAGCAGATGACCTGGAAAGTCC
CTTATCTGAAGAATTCTTACAAGAGATGGGAAACATTCAAGAGATTTCTCAGTCCCTCGGAGAGGAGA
GTTCCGGAAGCTTTAGTTTTGCGGACTACCAGTACTTAGGGAGCTGTCCAGGCTCGGAGGGCTCTGTC
ATCACAGgtaagggct.....ctttctctgtagACACCCTCTCTCCAGCTTCCAGCCCCTCCTCAGTC
AGCTGCCCTGCTGTCCCCACCAGTACAGATGAGTCCCCTGGCAATGCACTGAACATCGAGTGTCGAAT
ATGTGGGGACAAGGCCTCAGGATACCACTATGGAGTCCACGCATGTGAAGGCTGCAAG

Figure 5.12 Sequence of exons at the 5' end of the PPARα P2 and P3 transcripts. The sequences of 5'RLM RACE transcripts are shown for (A) Liver P2 and (B) Liver P3. Exons are shown in differing colours; red indicates alternative first exons for each transcript. Partial introns are shown in lower case, with GT-AG donor and acceptor splice sites bold and underlined. Published ATG translation start codon is shown in yellow highlight[105]. The original Ensembl TSS is indicated by grey highlight within the P2 first exon. The outer GS RACE primer is underlined.

A Kidney P5

ACACAATAGGAAGCTTTGCAGATCAGCAGAGGGCAAGCCAGTGACCCTCAGAGGCCTTGGGTGGTTCC
TGGAGAGGACTTACATAAGTCACGAG**gt**aaacata.....gtctggt**ag**ACCTGAGTTAGCAGCAGAA
CACTCTGAATAAGGAGGCAACATTCCTGCACGTGGACTCTGGTCTTTGGAGCTTGGGACCTGCTGTAC
GCCAG**gt**aactaag.....gttctat**ag**CCAAGACTGAAGTTCAAGGCCCTGCCTTCCCTGTGAACTG
ACATTTGTGACTGGTCAAGCTCAGGACACAAGACGTTGTCATCACAG**gt**aagaggg.....ctcctac
agATTGGTGCTCTGTGGCCCGCCTGGCCACAACCATTCAAC**ATG**GTGGACACAGAGAGCCCCATCTGT
CCTCTCTCCCCACTTGAAGCAGATGACCTGGAAAGTCCCTTATCTGAAGAATTCTTACAAGAGATGGG
AAACATTCAAGAGATTTCTCAGTCCCTCGGAGAGGAGAGTTCCGGAAGCTTTAGTTTTGCGGACTACC
AGTACTTAGGGAGCTGTCCAGGCTCGGAGGGCTCTGTCATCACAG**gt**aagggct.....ctttctctg
t**ag**ACACCCTCTCTCCAGCTTCCAGCCCCTCCTCAGTCAGCTGCCCTGCTGTCCCCACCAGTACAGAT
GAGTCCCCTGGCAATGCACTGAACATCGAGTGTCGAATATGTGGGGACAAGGCCTCAGGATACCACTA
TGGAGTCCACGCATGTGAAGGCTGCAAG

B Kidney P6

AGGTGCCCAGGGGCGGGAGGGCACGCGCGAGGACGGGAGCCAGGCGTCCCCCGTCCCGGGACAGTGAG
gtgggtgga.....gttctat**ag**CCAAGACTGAAGTTCAAGGCCCTGCCTTCCCTGTGAACTGACATT
TGTGACTGGTCAAGCTCAGGACACAAGACGTTGTCATCACAG**gt**aagaggg.....ctcctac**ag**ATT
GGTGCTCTGTGGCCCGCCTGGCCACAACCATTCAAC**ATG**GTGGACACAGAGAGCCCCATCTGTCCTCT
CTCCCCACTTGAAGCAGATGACCTGGAAAGTCCCTTATCTGAAGAATTCTTACAAGAGATGGGAAACA
TTCAAGAGATTTCTCAGTCCCTCGGAGAGGAGAGTTCCGGAAGCTTTAGTTTTGCGGACTACCAGTAC
TTAGGGAGCTGTCCAGGCTCGGAGGGCTCTGTCATCACAG**gt**aagggct.....ctttctctg**ag**AC
ACCCTCTCTCCAGCTTCCAGCCCCTCCTCAGTCAGCTGCCCTGCTGTCCCCACCAGTACAGATGAGTC
CCCTGGCAATGCACTGAACATCGAGTGTCGAATATGTGGGGACAAGGCCTCAGGATACCACTATGGAG
TCCACGCATGTGAAGGCTGCAAG

Figure 5.13 Sequence of exons at the 5' end of the PPARα P5 and P6 transcripts. The sequences of 5'RLM RACE transcripts are shown for (A) Kidney P5 and (B) Kidney P6. Exons are shown in differing colours; red indicates alternative first exons for each transcript. Partial introns are shown in lower case, with GT-AG donor and acceptor splice sites bold and underlined. Published ATG translation start codon is shown in yellow highlight[105]. The outer gene specific RACE primer is underlined.

Figure 5.14 Sequence of exons at the 5' end of the PPARα P1 and P4 transcripts. The sequences of 5'RLM RACE transcripts are shown for (A) Adipose P1 and (B) Heart P4. Exons are shown in differing colours; red indicates alternative first exons for each transcript. Partial introns are shown in lower case, with GT-AG donor and acceptor splice sites bold and underlined. Published ATG translation start codon is shown in yellow highlight[105]. The outer gene specific RACE primer is underlined.

5.2.3 All the novel PPARα transcripts intron exon boundaries conform to the GT-AG splice site rule

Sequences at exon/intron junctions of all the novel PPARα 5'UTR exons were scrutinised to determine if their boundaries conform to the GT-AG splice site rule. This rule stipulates that the first 2 nucleotides within an intron (the 5' donor splice site) are GT (within the consensus GTAAG), whilst the last 2 nucleotides within the intron (the 3' acceptor splice site) are AG (within the consensus PyPyPyPyPyPyNCAG).

A more detailed look at the sequence of the PPARα introns within the novel exons showed that they all conformed to the GT AG splice site rule, with 5' end of introns containing the GT splice donor sequence and the 3' end containing the AG splice acceptor sequence. The remainder of the sequences at all donor and acceptor splice sites were very similar to consensus and are shown in table 5.1.

Table 5.1 Alternative PPARα 5'UTR exon/intron junctions conform to the GT-AG splice site rule. 5' (donor) and 3' (acceptor) intron splice site consensus sequences are indicated. The sequences at P1, P2, P3, P4, P5 and P6 transcript exon/intron boundaries are shown. Splice site sequences within introns are shown in lowercase; consensus sequence matches are shown in blue, mismatches are shown in red. Exons are shown in black capitals and sizes of exons are given. All transcript exon boundaries conform to the GT-AG splice site rule.

Transcript	Exon (bp)	5' Donor Splice Site Consensus 5'-gtaagt-3'	3' Acceptor Splice Site Consensus 5'-PyPyPyPyPyPyncag-3'	Exon (bp)
Adipose P1	1C (60)	CCTCAGgtgccc	cgttctatagCCAAGA	2 (87)
	2 (87)	TCACAGgtaaga	cctcctacagATTGGT	3 (247)
Liver P2	1E (318)	GGCGAGgtaact	cgttctatagCCAAGA	2 (87)
	2 (87)	TCACAGgtaaga	cctcctacagATTGGT	3 (247)
Liver P3	2A (145)	CTTCTGgtaggt	cctcctacagATTGGT	3 (247)
Heart P4	1E (76)	GGCGAGgtaact	cgttctatagCCAAGA	2 (87)
	2 (87)	TCACAGgtaaga	cctcctacagATTGGT	3 (247)
Kidney P5	1A (94)	CACGAGgtaaac	tgtctggtagACCTGA	1B (92)
	1B (92)	CGCCAGgtaact	cgttctatagCCAAGA	2 (87)
	2 (87)	TCACAGgtaaga	cctcctacagATTGGT	3 (247)
Kidney P6	1D (68)	AGTGAGgtgggt	cgttctatagCCAAGA	2 (87)
	2 (87)	TCACAGgtaaga	cctcctacagATTGGT	3 (247)

5.2.4 A comparison of the rat PPARα 5'UTR exons after 5'RACE with corresponding exons in mouse and human 5'UTR reveals more homology than previously known.

To determine if the novel rat PPARα 5'UTR exons are conserved between species, the pre and post 5'RLM RACE sequence was aligned with that of mouse (ENSMUSG00000022383) and human PPARα (ENSG00000186951) [103] using the Ensembl genomic alignment tool and the genomic location of 5'UTR exons was subsequently compared. In addition, the Clustal 2.0.12 multiple sequence alignment tool [259] was used to determine the percent homology of the common 5'UTR exons between species both before and after the RACE procedure (appendix 1).

A diagram comparing the 5'UTR genomic organisation between species pre and post 5'RACE can be found in figure 5.15. Prior to the RACE procedure, rat, mouse and human PPARα 5'UTR were known to share 3 common exons; rat exon 1, 2 and 3. Rat exon 1 is homologous to mouse exon 1B and Ensembl exon X in the human 5'UTR, but the human and mouse exons are longer at both the 5' and the 3' end. Rat exon 2 is homologous to mouse exon 2 and human exon 1A and the 3 species exon boundaries all align at both the 5' and 3' end apart from the human exon which is 83bp longer at the 3' end. All 3 species share the same exon 3 and align perfectly apart from the human exon 3 being 3 nucleotides longer at the 5' end. No additional exons were shown to exist in the rat 5'UTR. However, the mouse 5'UTR has an additional exon, 1A, which was not present in the rat transcript prior to the 5'RACE and likewise the human 5'UTR had 6 exons (A, Y, B, 1B, 2A, 2B) which were not present in the rat transcript prior to 5'RACE.

After the 5'RACE procedure, the rat, mouse and human PPARα 5'UTR's were found to share 4 common exons; rat exon 1C, 1E, 2 and 3. Exons 2 and 3 in the rat were unchanged after 5'RACE. The previously unidentified exon, 1C, found in the rat 5'UTR was shown to be homologous to the mouse exon 1A and human exon A. The 5' end of this exon is longer in the human and mouse corresponding exons, but all share the same 3' exon boundary. The RACE also found rat exon 1E to be longer at the 5'end than previously known, bringing it more inline with the mouse and human corresponding exons. After 5'RACE, all exons found in the mouse 5'UTR were shown to be present in the rat 5'UTR, whereas the human 5'UTR had 5 exons present (Y, B, 1B, 2A, 2B) which were not found in the rat 5'UTR. In addition, rat exons 1A, 1E, 1D and 2A identified by 5'RACE had no corresponding exons in the human or mouse 5'UTR.

Prior to the race procedure, the homology of the rat 5'UTR exons 1, 2 and 3 to the corresponding exons in the mouse were 94%, 94% and 92% respectively and to the corresponding human exons 64%, 75% and 77% (table 5.2). After the race procedure the homology of these 3 exons were exactly the same for mouse, whereas for the human exon X, homology to exon 1E in the rat dropped from 64% to 50%. Furthermore, the novel exon 1C in the rat identified by the RACE procedure was found to have 91% identity to the corresponding mouse exon and 65% identity to

the corresponding human exon. Subsequent to this, 1.5Kb of regulatory region upstream of the predominant exon 1E in the rat was also compared between species and found to have 88% homology to the corresponding regulatory region in the mouse and 69% homology to the equivalent human sequence.

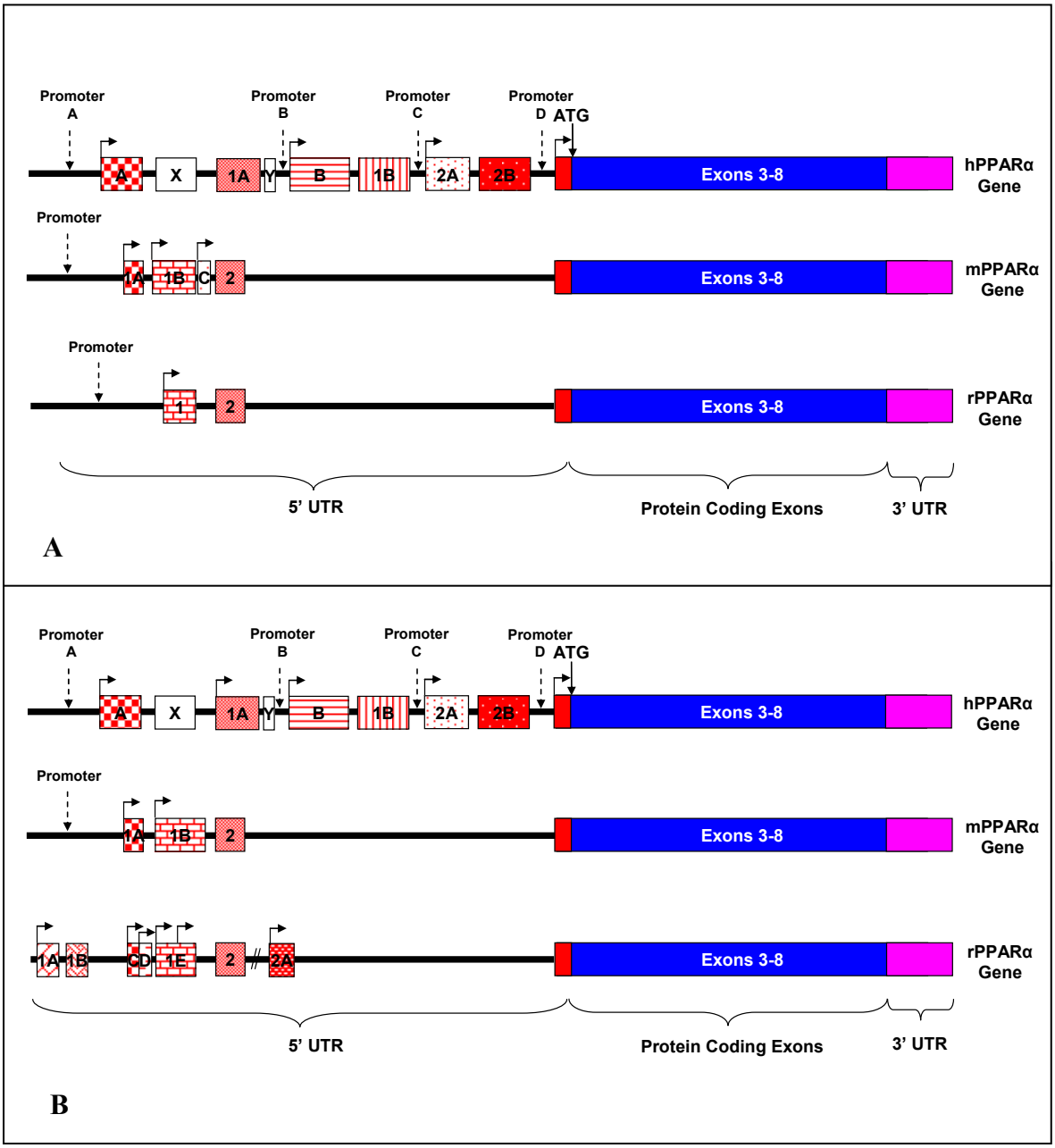


Figure 5.15 A comparison of the rat PPARα 5'UTR genomic organisation with human and mouse 5'UTR before and after 5' RLM RACE procedure. The genomic location of PPARα 5'UTR exons are shown for human, mouse and rat (A) Prior to rat PPARα RACE, (B) After rat PPARα RACE. Non-coding 5'UTR exons are shown in red, coding exons in blue and 3'UTR exons in pink. Species differ in the 5'UTR exons, whereas all coding exons are shared between species. Identification of additional 5'UTR exons by PPARα RACE in the rat brings it more inline with the genomic positioning of the more complex mouse and human 5'UTR exons - in particular the identification of exon 1C which is conserved in mouse and human and the extension of exon 1E at the 5' end.

Table 5.2 Clustal 2.0.12 sequence alignment scores for rat PPARα 5'UTR exons with corresponding human and mouse 5'UTR exons. CLUSTAL 2.0.12 multiple sequence alignment scores are shown for 5'UTR exons in the rat against corresponding human and mouse 5'UTR exons. The percentage identity between species both before and after 5'RLM RACE results are indicated.

Exon / Promoter	Alignment	Alignment	% Identity before RACE	% Identity after RACE
Rat Exon 3 v Mouse Exon 3 v Human Exon 3	Mouse	Human	78	-
	Rat	Mouse	92	92
	Rat	Human	77	77
Rat Exon 2 v Mouse exon 2 v Human exon 1A	Mouse	Human	74	-
	Rat	Mouse	94	94
	Rat	Human	75	75
Rat Exon 1E v Mouse Exon 1B v Human Exon X	Mouse	Human	52	-
	Rat	Mouse	94	94
	Rat	Human	64	50
Rat Exon 1C v Mouse exon 1A v Human exon A	Mouse	Human	71	-
	Rat	Mouse	-	91
	Rat	Human	-	65
1.5Kb Upstream Promoter of Exon 1E Rat, 1B Mouse, Human Exon X	Mouse	Human	70	-
	Rat	Mouse	88	-
	Rat	Human	69	-

5.2.5 The alternative PPARα transcripts have structurally diverse 5'UTR and P3 has the potential to produce a variant N-terminal extended protein

The structure of the 5'UTR of the alternative PPARα transcripts were analysed to determine if any had the potential to be regulated translationally and to see if any could give rise to variant proteins. To investigate the structure and the potential for translational regulation, the sequences of all 6 5'UTR (P1-P6) were compared for overall length, GC content and free folding energy, whilst to determine if alternative functional ORF were present, upstream sequences were scrutinised for uATG, surrounding Kozak sequences and termination codons.

As a consequence of the different transcription start sites and alternative splicing, the alternative transcripts have 5'UTR which differ considerably in length. The P2 transcript has the longest 5'UTR of 444bp, followed by P5 with a 5'UTR of 312bp. The remainder of the transcripts are considerably shorter and have similar lengths of 202bp, 194bp 186bp and 184 bp for P4, P6, P1 and P3 respectively. The GC content of the alternative 5'UTR's is more constant between transcripts. The highest GC content is found for the P1, P2 and P6 5'UTR with values of 62% GC for P1 and P6 and 61% GC for P2. For P3 P4 and P5 their GC content is slightly lower with values of 51%, 56% and 53% respectively.

In order to calculate the minimum free energy and the most energetically favourable conformations of the alternative 5'UTR's, DNA sequences of 5'UTR's were analysed by Zucker RNA mfold 2.3 software [260]. Diagrams showing folding structure of the alternative transcript 5'UTR's can be found in appendix 2. The minimum free energy values calculated ranged from; -169.95 Kcal/mol for P2, -101.54 Kcal/mol for P5, -74.65 Kcal/mol for P1 and -71.87 Kcal/mol for P6, to -64.18 Kcal/mol for P4 and -56.15 Kcal/mol for P3. These highly negative values indicate that all transcripts have a stable secondary structure, in particular that of P2 and P5 5'UTR's.

The alternative 5'UTR were next scrutinised for potential alternative initiator codons within their 5'UTR. Sequence analysis revealed that there were no additional initiation codons within the 5'UTR for P1, P2, P4, P5 or P6. Consequently the main ORF would be utilized and all these variants would produce the same protein (figures 5.16, 5.17, 5.23, 5.24, 5.25). However for P3, the novel exon 2A within its 5'UTR contained 4 possible ATG codons upstream of the published ATG initiator codon (figure 5.18), all positioned within 109bp of the main ORF initiation codon. For convenience, these uATG were termed uATG 1-4.

To further check if the uATG could be functional, the sequence surrounding the uATG 1-4 was analysed for Kozak sequence (GCC G/A CCATGG) [261] (table 5.3). None of the alternative uATG 1-4 or the known PPARα initiator ATG for the main ORF conform 100% to the Kozak consensus sequence. The initiator codon for the published ORF does however contain the conserved bases -3A and +4G which are associated with increased translation efficiency [262]. However, sequence

analysis of the uATG revealed that uATG1 contains the -3A, uATG 2 the -3G and uATG 4 contains the +4G base, thus indicating that initiation could occur at these uATG [251]. Sequence analysis confirms that only uATG 3 does not contain either of these conserved bases.

Analysis of each of these uATG for potential ORF revealed that uATG 1, 3 and 4 are terminated after 30bp, 45bp and 21 bp respectively and are therefore unlikely to encode a functional protein (figures 5.19, 5.21, 5.22). Conversely, uATG 2 is in frame with the published ATG codon and no termination codons were identified upstream of the main ATG. Consequently, no barrier is present to prevent the formation of alternative in frame proteins (figure 5.20). Collectively, these results indicate that uATG 2 being 87bp upstream of the published ATG codon could potentially give rise to an alternative PPARα protein with a 29 amino acid extended N terminal domain.

Table 5.3 Alternative PPARα transcript ATG initiation sites and surrounding kozak sequence. Main ORF ATG initiation site for the P1 P2 P4 P5 and P6 transcripts and potential alternative ATG initiation sites for the P3 transcript along with their surrounding sequence are shown. Consensus sequences matches are shown in blue, conserved bases associated with increased translation efficiency are underlined and sequence mismatches are shown in red. Potential strength of initiation is indicated [251].

Transcript	ATG	Surrounding Sequence (Kozak Consensus GCC G/A CCATGG)	Potential Strength of Initiation
Adipose P1	Main ORF	TTCAACATGG	Good
Liver P2	Main ORF	TTCAACATGG	Good
Liver P3	uATG1	CCTACAATGA	Adequate
	uATG2	GGAGGAATGA	Adequate
	uATG3	GAATGAATGA	Weak
	uATG4	TTCCGGATGG	Adequate
Heart P4	Main ORF	TTCAACATGG	Good
Kidney P5	Main ORF	TTCAACATGG	Good
Kidney P6	Main ORF	TTCAACATGG	Good

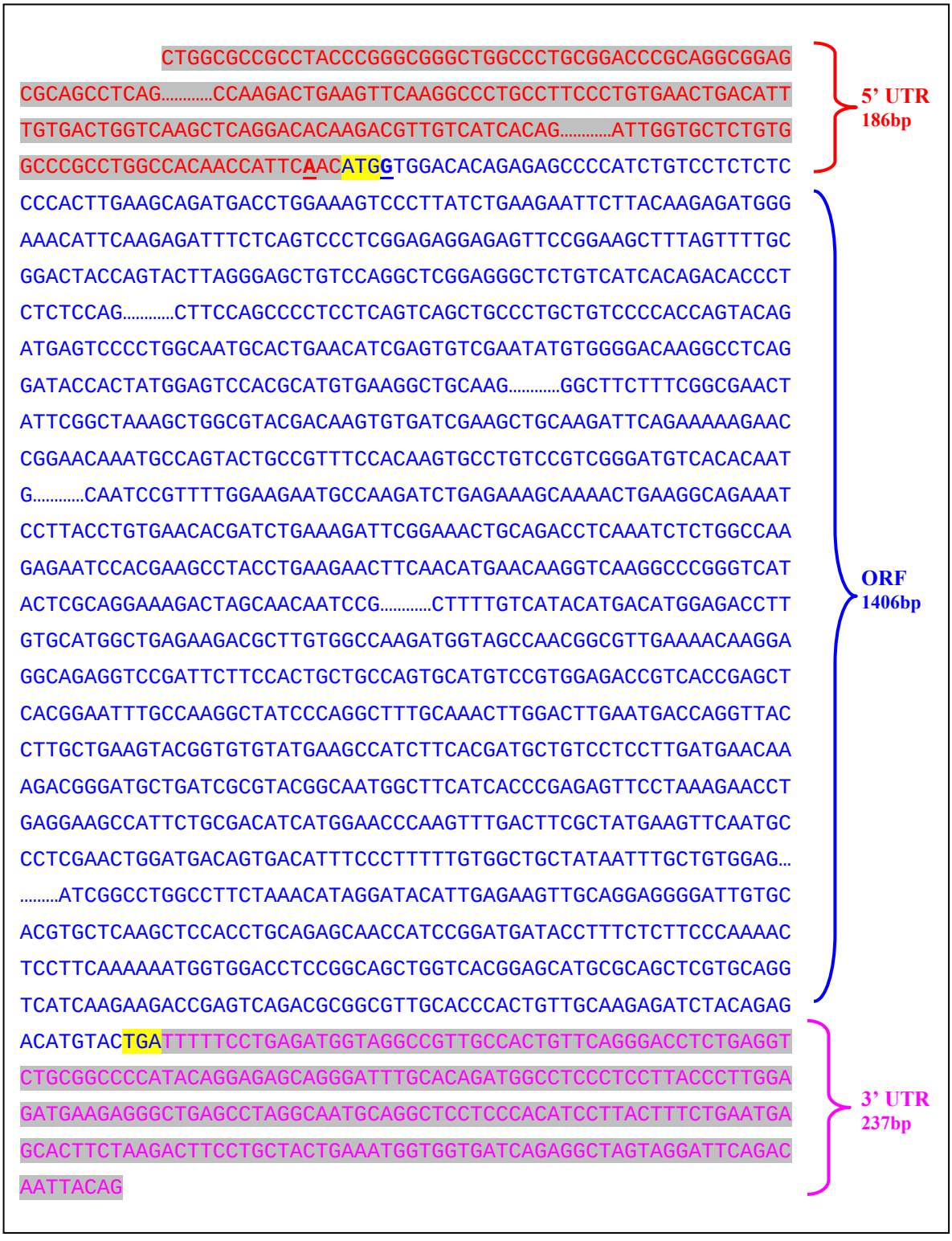


Figure 5.16 PPARα P1 transcript has no alternative initiator codons within the 5'UTR. The rat PPARα P1 transcript sequence is shown. P1 is composed of 8 exons (exon boundaries are indicated by dotted lines). The initiator codon (ATG) and terminator codon (TGA) from the main 1406bp ORF are highlighted in yellow. Highly conserved bases from the Kozak sequence (-3 and +4) are underlined and in bold. The 5'UTR is shown in red text, the ORF is shown by blue text and the 3'UTR is shown in pink text. There are no alternative ATG initiation sites within the 5'UTR for this transcript.

Figure 5.17 PPARα P2 transcript has no alternative initiator codons within the 5'UTR. The rat PPARα P2 transcript sequence is shown. P2 is composed of 8 exons (exon boundaries are indicated by dotted lines). The initiator codon (ATG) and terminator codon (TGA) from the main 1406bp ORF are highlighted in yellow. Highly conserved bases from the Kozak sequence (-3 and +4) are underlined and in bold. The 5'UTR is shown in red text, the ORF is shown by blue text and the 3'UTR is shown in pink text. There are no alternative ATG initiation sites within the 5'UTR for this transcript.

Figure 5.18 PPARα P3 transcript has four possible alternative upstream initiator codons within the 5'UTR. The rat PPARα P3 transcript sequence is shown. P3 is composed of 7 exons (exon boundaries are indicated by dotted lines). The initiator codon (ATG) and terminator codon (TGA) from the main 1406bp ORF are highlighted in yellow. Four possible initiation sites (uATG) within the unique alternative first exon for this transcript are shown in green and for convenience are termed uATG 1-4 according to their position. The main 5'UTR is shown in red text, the ORF is shown by blue text and the 3'UTR is shown in pink text.

Figure 5.19 Use of PPARα P3 transcript uATG 1 codon results in termination after 30bp. The rat PPARα P3 transcript sequence is shown. P3 is composed of 7 exons (exon boundaries are indicated by dotted lines). The initiator codon (ATG) and terminator codon (TGA) from the main 1406bp ORF are highlighted in yellow. The alternative uATG1 initiation codon and TGA termination codon are highlighted in green. The highly conserved -3 base from the Kozak sequence is underlined and in bold. The resulting 5'UTR is shown in red text, the 30bp ORF is shown by blue text and the 3'UTR is shown in pink text.

Figure 5.20 Use of PPARα P3 transcript uATG 2 codon may produce a 5' extended transcript. The rat PPARα P3 transcript sequence is shown. P3 is composed of 7 exons (exon boundaries are indicated by dotted lines). The initiator codon (ATG) and terminator codon (TGA) from the main 1406bp ORF are highlighted in yellow. The alternative uATG2 initiation codon is highlighted in green. ATG2 is in frame with the main ORF and consequently has the potential to form a 5' extended transcript. The highly conserved -3 base from the Kozak sequence is underlined and in bold. The resulting 5'UTR is shown in red text, the extended 1493bp ORF is shown by blue text and the 3'UTR is shown in pink text.

Figure 5.21 Use of PPARα P3 transcript uATG 3 codon results in termination after 45bp. The rat PPARα P3 transcript sequence is shown. P3 is composed of 7 exons (exon boundaries are indicated by dotted lines). The initiator codon (ATG) and terminator codon (TGA) from the main 1406bp ORF are highlighted in yellow. The alternative uATG3 initiation codon and TGA termination codon are highlighted in green. No highly conserved bases from the Kozak sequence are present for this ATG. The resulting 5'UTR is shown in red text, the 45bp ORF is shown by blue text and the 3'UTR is shown in pink text.

Figure 5.22 Use of PPARα P3 transcript uATG 4 codon results in termination after 21bp. The rat PPARα P3 transcript sequence is shown. P3 is composed of 7 exons (exon boundaries are indicated by dotted lines). The initiator codon (ATG) and terminator codon (TGA) from the main 1406bp ORF are highlighted in yellow. The alternative uATG3 initiation codon and TGA termination codon are highlighted in green. The highly conserved +4 base from the Kozak sequence is underlined and in bold. The resulting 5'UTR is shown in red text, the 21bp ORF is shown by blue text and the 3'UTR is shown in pink text.

Figure 5.23 PPARα P4 transcript has no alternative initiator codons within the 5'UTR. The rat PPARα P4 transcript sequence is shown. P4 is composed of 8 exons (exon boundaries are indicated by dotted lines). The initiator codon (ATG) and terminator codon (TGA) from the main 1406bp ORF are highlighted in yellow. Highly conserved bases from the Kozak sequence (-3 and +4) are underlined and in bold. The 5'UTR is shown in red text, the ORF is shown by blue text and the 3'UTR is shown in pink text. There are no alternative ATG initiation sites within the 5'UTR for this transcript.

Figure 5.24 PPARα P5 transcript has no alternative initiator codons within the 5'UTR. The rat PPARα P5 transcript sequence is shown. P5 is composed of 9 exons (exon boundaries are indicated by dotted lines). The initiator codon (ATG) and terminator codon (TGA) from the main 1406bp ORF are highlighted in yellow. Highly conserved bases from the Kozak sequence (-3 and +4) are underlined and in bold. The 5'UTR is shown in red text, the ORF is shown by blue text and the 3'UTR is shown in pink text. There are no alternative ATG initiation sites within the 5'UTR for this transcript.

Figure 5.25 PPARα P6 transcript has no alternative initiator codons within the 5'UTR. The rat PPARα P6 transcript sequence is shown. P6 is composed of 8 exons (exon boundaries are indicated by dotted lines). The initiator codon (ATG) and terminator codon (TGA) from the main 1406bp ORF are highlighted in yellow. Highly conserved bases from the Kozak sequence (-3 and +4) are underlined and in bold. The 5'UTR is shown in red text, the ORF is shown by blue text and the 3'UTR is shown in pink text. There are no alternative ATG initiation sites within the 5'UTR for this transcript.

5.2.6 Analysis of three distinct upstream regulatory regions from the multiple PPARα transcriptional start sites indicates that they differ in the presence of CpG islands, core promoter elements and transcription factor binding sites.

In order to determine potential areas of importance for regulation of both basal and simulated transcriptional PPARα activity, the upstream sequence of the multiple TSS were analysed for CpG islands, core promoter elements and transcription factor binding sites. To achieve this, first exons and 1000bp of upstream sequence from the PPARα TSS were analysed by MethPrimer software for CpG islands using the criteria; Island size > 100, GC Percent > 50.0 and Observed/Expected > 0.6. [263]. In addition, the potential regulatory regions were analysed for core promoter elements and transcription factor binding sites using the MatInspector program [264]. Only core promoter elements or relevant transcription factor binding sites with the maximum core similarity of 1.0 are shown, i.e. the highest conserved bases of a response element match the sequence exactly.

Two CpG islands were found in the upstream regulatory region of the P1/P2/P4/P6 TSS (figure5.26). These TSS were all located within a proximal 796bp CpG island positioned -470 to +326 relative to the P2 TSS, whilst the second, upstream CpG island of 430bp was found -970 to -541 relative to the P2 TSS. Furthermore, the CpG islands were found to contain a total of 12 Sp1 response elements. The P3 and P5 upstream regulatory sequences were not GC rich and consequently MethPrimer did not identify any CpG islands.

Upon scrutinizing the sequences surrounding all the PPARα TSS, no TATA box or Initiator consensus were identified. However, several other core promoter elements were identified within the upstream regulatory regions of the P1 P2 P5 and P4 TSS; 3 perfect TFIIB Recognition Elements (BRE), 2 Motif Ten Elements (MTE) and 4 X Core Promoter Element 1 (XCPE1). However, none of these core promoter elements identified were located in their normal positions relative to the TSS (table5.4). In addition no core promoter elements were found in the regulatory regions for the P3 and P5 transcripts.

Several putative transcription factor binding sites were identified by the MatInspector software in the upstream regulatory region of the P1, P2, P4 and P6 TSS, including that for; hepatocyte nuclear factor 3 (HNF3) (a perfect sequence score), nuclear factor 1 (NF-1), Chicken ovalbumin upstream promoter transcription factor (Coup TF), activator protein 1 (AP1), Stat, cAMP response element binding protein (CREB), PPAR/RXR, VDR/RXR, Myc/Max, sterol regulatory element binding protein (SREBP), E2F and nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) (figure 5.27). Some of these factors were also found in the P3 regulatory region; Stat, Stat 3, PPAR/RXR, VDR/RXR, NF-1 whilst additional response elements were also identified; CCAAT, Octamer 1 (OCT 1), GATA binding protein 2 (Gata 2), HNF1α, HNF4, CCAAT/enhancer-binding protein (C/EBP) andGATA1 (figure 5.28). For the P5 regulatory region, putative response

elements for Stat3, OCT1, HNF4, PPAR/RXR, HNF1 CEBPβ were identified as found previously and in addition a CREB2 response element was also identified (figure 5.29).

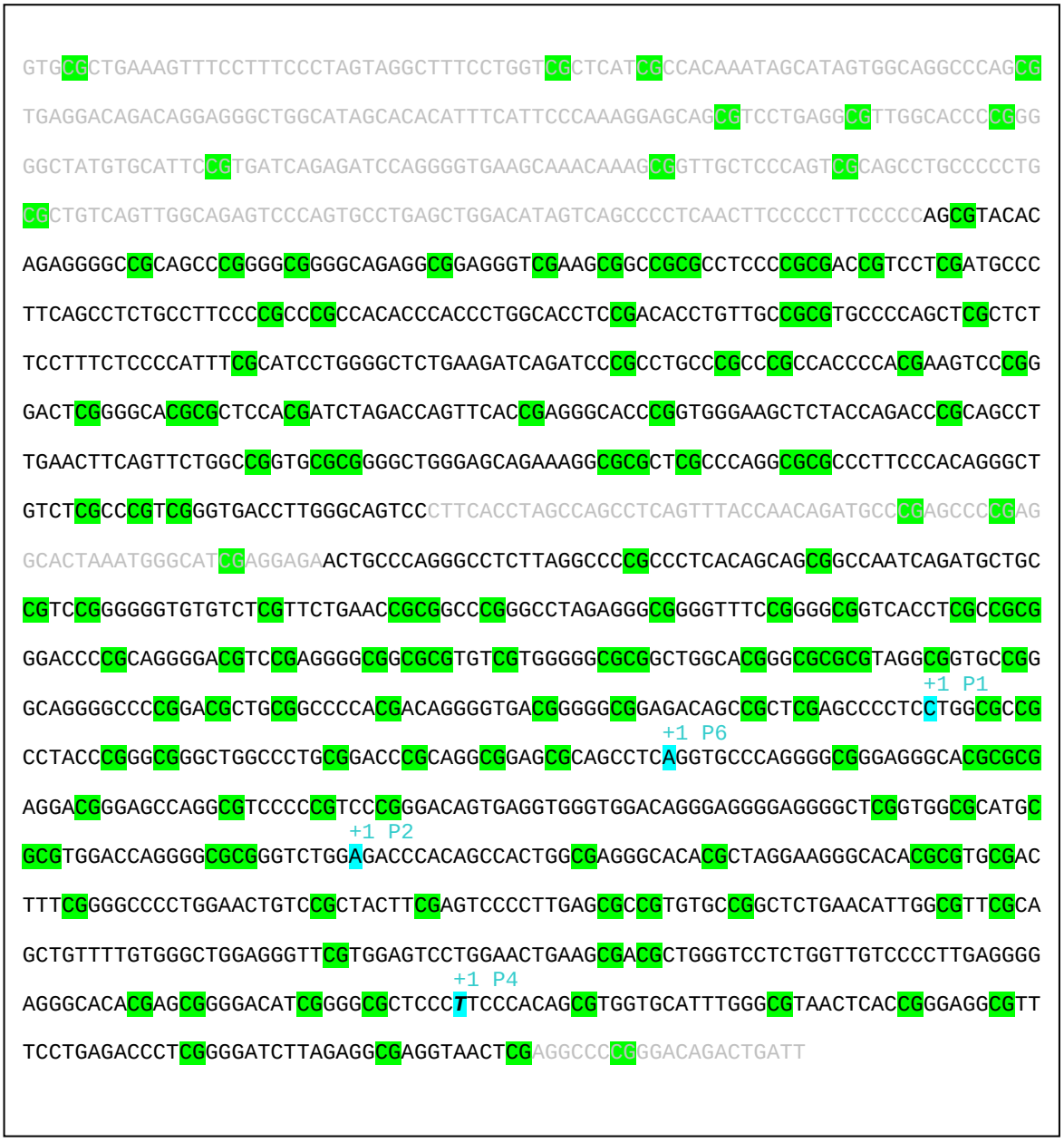


Figure 5.26 Two CpG islands are located in the P1/P2/P4/P6 upstream regulatory sequence. The upstream regulatory sequence of the P1/P2/P4/P6 TSS is shown. CpGs are highlighted in green, CpG islands are indicated in black, whilst non CpG island areas are shown in grey. Two CpG islands are present a 430bp island and a 796bp island. Transcript TSS are indicated in blue.

Table 5.4 Positions of core promoter elements within the P1/P2/P4/P6 regulatory region of PPARα as identified by MatInspector software. The consensus sequence and normal position relative to TSS +1 are shown. The number of core promoter elements identified and their actual positions relative to the nearest TSS are indicated.

Core Promoter Element	Consensus	Position relative to TSS +1	N° of Sites	Position Found
BRE	G/C-G/C-G/A-C-G-C-C	-37 to -32	3	-13 to -7 of P2 TSS -105 to -99 P1 TSS -724 to -717 of P1 TSS
TATA box	T-A-T-A-A/T-A-A-A/G	-31/-30	0	
Inr	C/T-C/T-A-N-A/T-C/T-C/T	-2 to +4	0	
MTE	C-G/C-A-A/G-C-G/C-G/C-A-A-C	+18 to +27	2	-18 to +3 to P6 TSS or +41 to +60 to P1 TSS -423 to -402 to P1 TSS
DPE	A/G-G-A/T-T/C-G/A/C-T	+28 to +33	0	
XCPE1	G/A/T-G/C-G-T/C-G-G-G/A-A-G/C_{+1}-A/C	-8 to +2	4	+11 to +21 to P6 TSS -152 to -142 to P1 TSS -263 to -253 to P1 TSS -576 to -565 to P1 TSS
XCPE2	A/C/G-C-C/T-C-G/A-T-T-G/A-C-C/A_{+1}-C/T	-9 to +2	0	

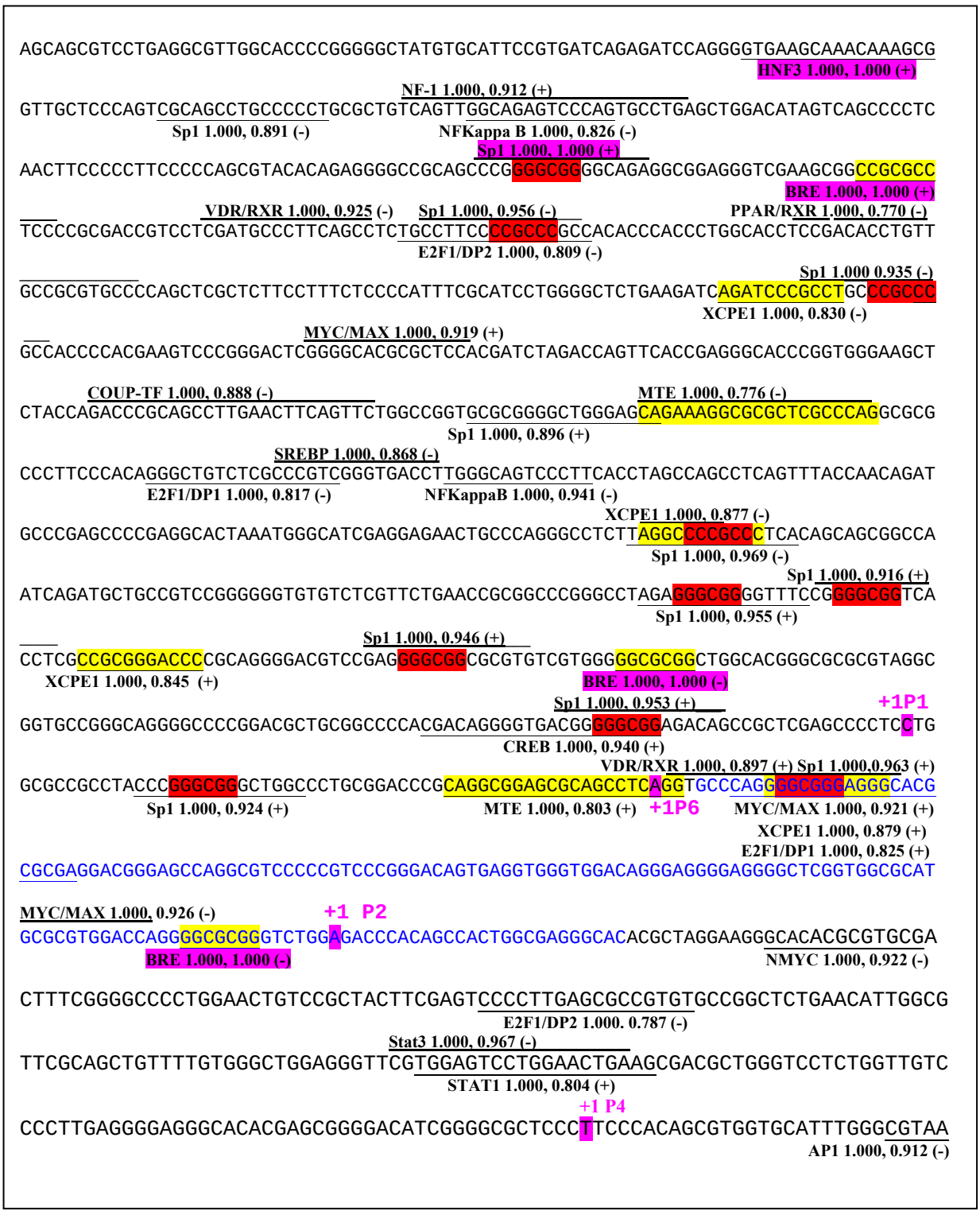


Figure 5.27 Sequence of the PPARα P1/P2/P4/P6 upstream regulatory region showing potential transcription factor binding sites and core promoter elements. The sequence of the P1/P2/P4/P6 Upstream Regulatory Region is shown. TF binding sites and core promoter elements are underlined. In addition Sp1 binding sites are highlighted red and core promoter elements in yellow. All sites are scored and marked with the strand they are located on (+ or -). The core similarity of the sequence is the first number. The maximum core similarity of 1.0 is only reached when the highest conserved bases of a matrix match exactly in the sequence and only those sites with a score of 1.0 are shown. The second figure is the matrix similarity which takes into account all bases over the whole matrix length. A perfect match has a score of 1.00 and a "good" match has a score of >0.80. Perfect sequences are highlighted in purple. TSS are indicated.

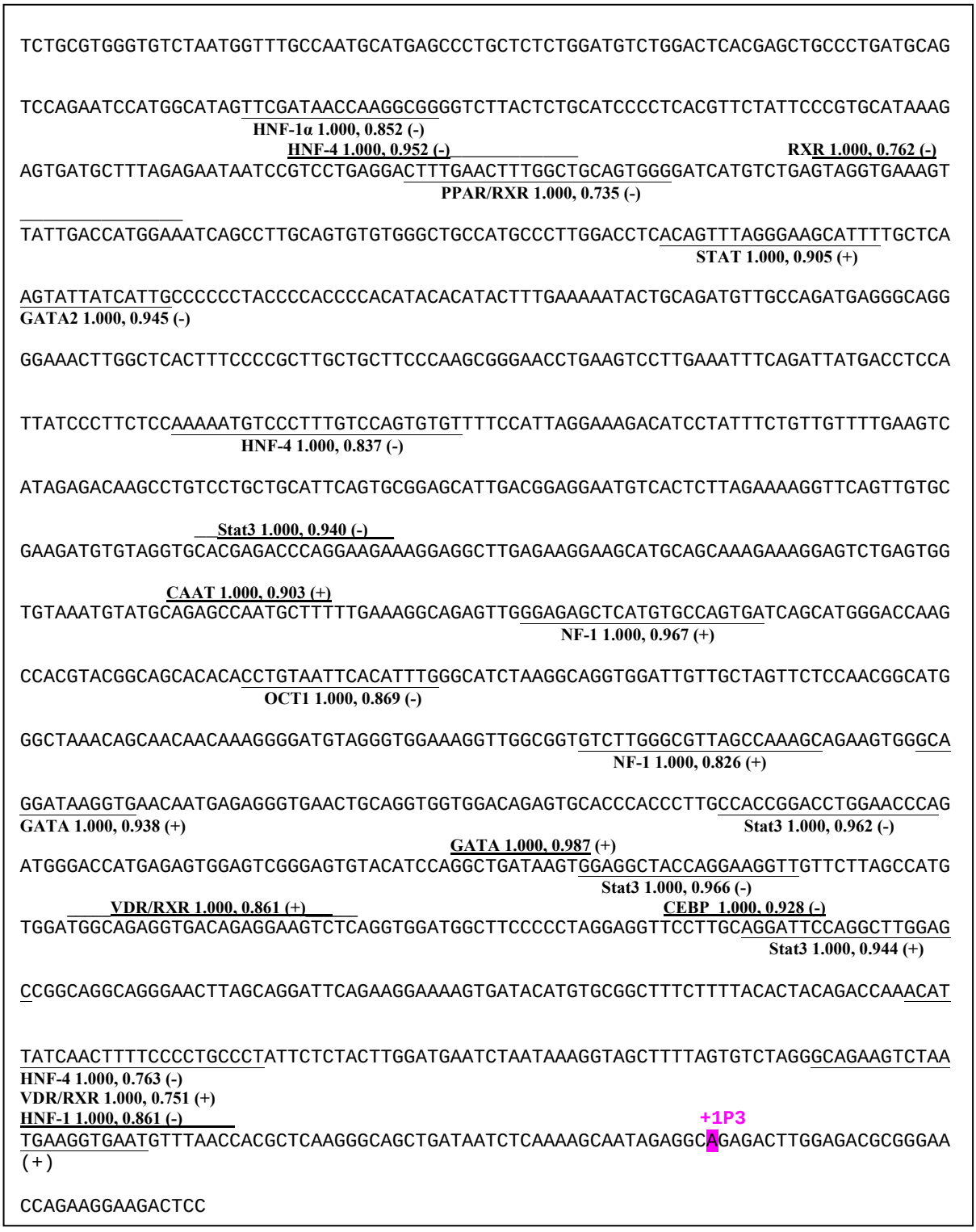


Figure 5.28 Sequence of the PPARα P3 upstream regulatory region showing potential transcription factor binding sites and core promoter elements. The sequence of the P3 Upstream Regulatory Region is shown. TF binding sites are underlined. There are no Sp1 binding sites or core promoter elements present. All sites are scored and marked with the strand they are located on (+ or -). The core similarity of the sequence is the first number. The maximum core similarity of 1.0 is only reached when the highest conserved bases of a matrix match exactly in the sequence and only those sites with a score of 1.0 are shown. The second figure is the matrix similarity which takes into account all bases over the whole matrix length. A perfect match has a score of 1.00 and a "good" match has a score of >0.80. P3 TSS is indicated.

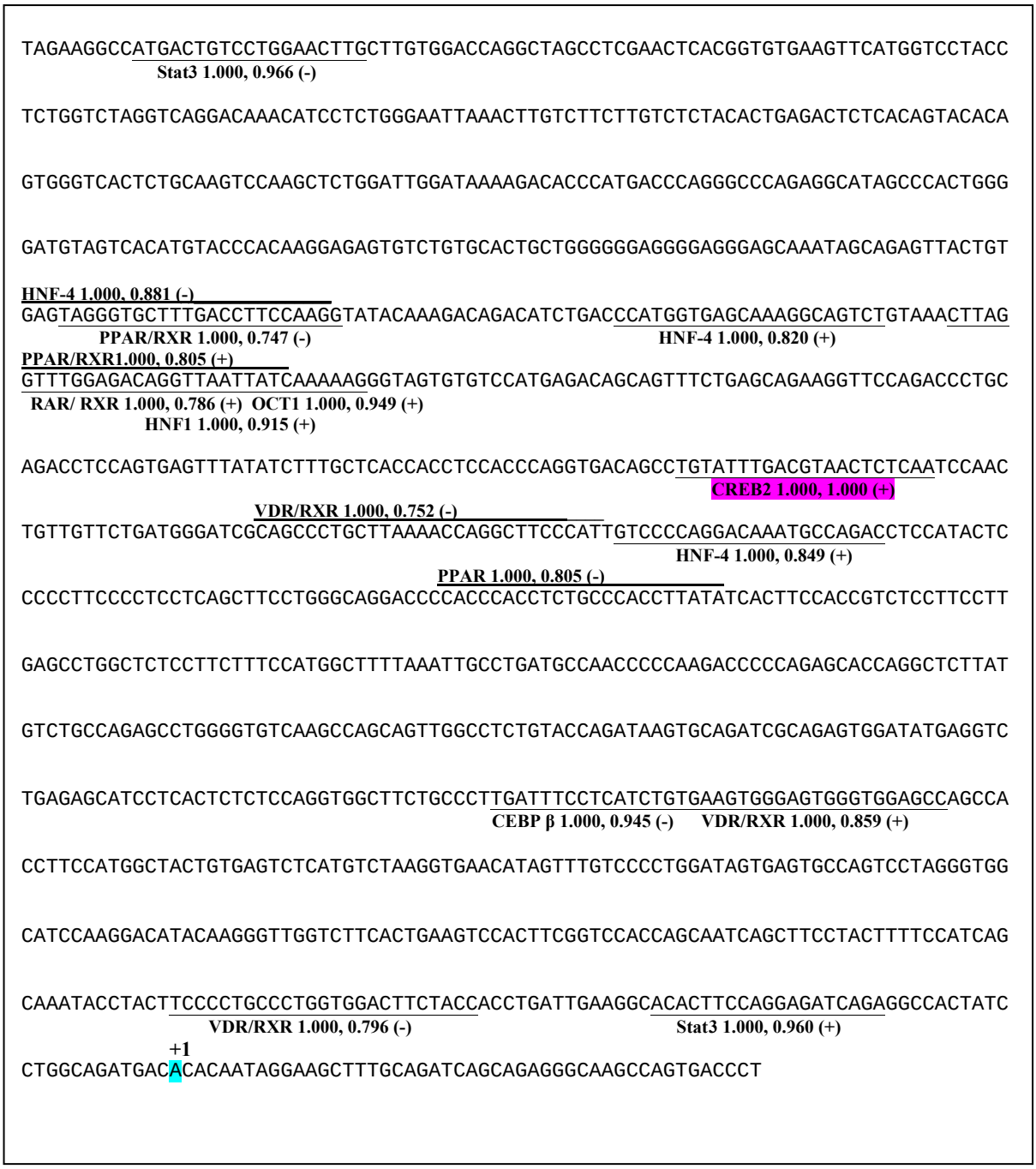


Figure 5.29 Sequence of the PPARα P5 upstream regulatory region showing potential transcription factor binding sites and core promoter elements. The sequence of the P5 Upstream Regulatory Region is shown. TF binding sites are underlined. There are no Sp1 binding sites or core promoter elements present. All sites are scored and marked with the strand they are located on (+ or -). The core similarity of the sequence is the first number. The maximum core similarity of 1.0 is only reached when the highest conserved bases of a matrix match exactly in the sequence and only those sites with a score of 1.0 are shown. The second figure is the matrix similarity which takes into account all bases over the whole matrix length. A perfect match has a score of 1 and a "good" match has a score of >0.80. Perfect sequences are highlighted in purple. P5 TSS is indicated.

5.2.7 The PPARα P2 and P1 promoters are active in HepG2 Cells, but the P3 promoter region is inactive

Promoter regions corresponding to the liver specific and adipose specific PPARα mRNA transcripts were termed according to their transcript ID (P1-3). These promoter regions were designed to extend from the region ~50bp downstream of their respective transcription start sites to at least 1Kb upstream. This resulted in two partially overlapping promoters for P1 and P2 (figure 5.30) and a distinct downstream promoter for P3 as previously shown (figure 5.28). A diagram indicating the positions of promoters relative to each other is given in figure 5.31.

To investigate the activity of the alternative PPARα transcript promoters, they were cloned into the pGL3 Basic luciferase reporter vector and used in transient transfections. Initially, 1µg and 2µg of the PPARα-pGL3 luciferase reporter constructs (P1–pGL3, P2-pGL3 and P3-pGL3) were transfected into HepG2 cells. In addition, the promoter-free pGL3 basic vector was transfected as a control.

Results show that the activity of the P3-pGL3 construct is similar to that of the promoter-less pGL3 Basic vector with luciferase activities of 20.9 and 34.1 for 1µg and 2µg of transfected plasmid respectively (figure 5.32). Conversely, the activity of P1-pGL3 is higher at 81.28 for 1µg and 172.5 for 2µg plasmid. In addition, activity of the P2-pGL3 construct is highest with 147.6 for 1µg and 284.0 for 2µg plasmid. These results indicate that the P3 promoter region is inactive in HepG2 cells, whilst the P1 and P2 promoters are both active in HepG2 cells, with P2 having the highest activity.

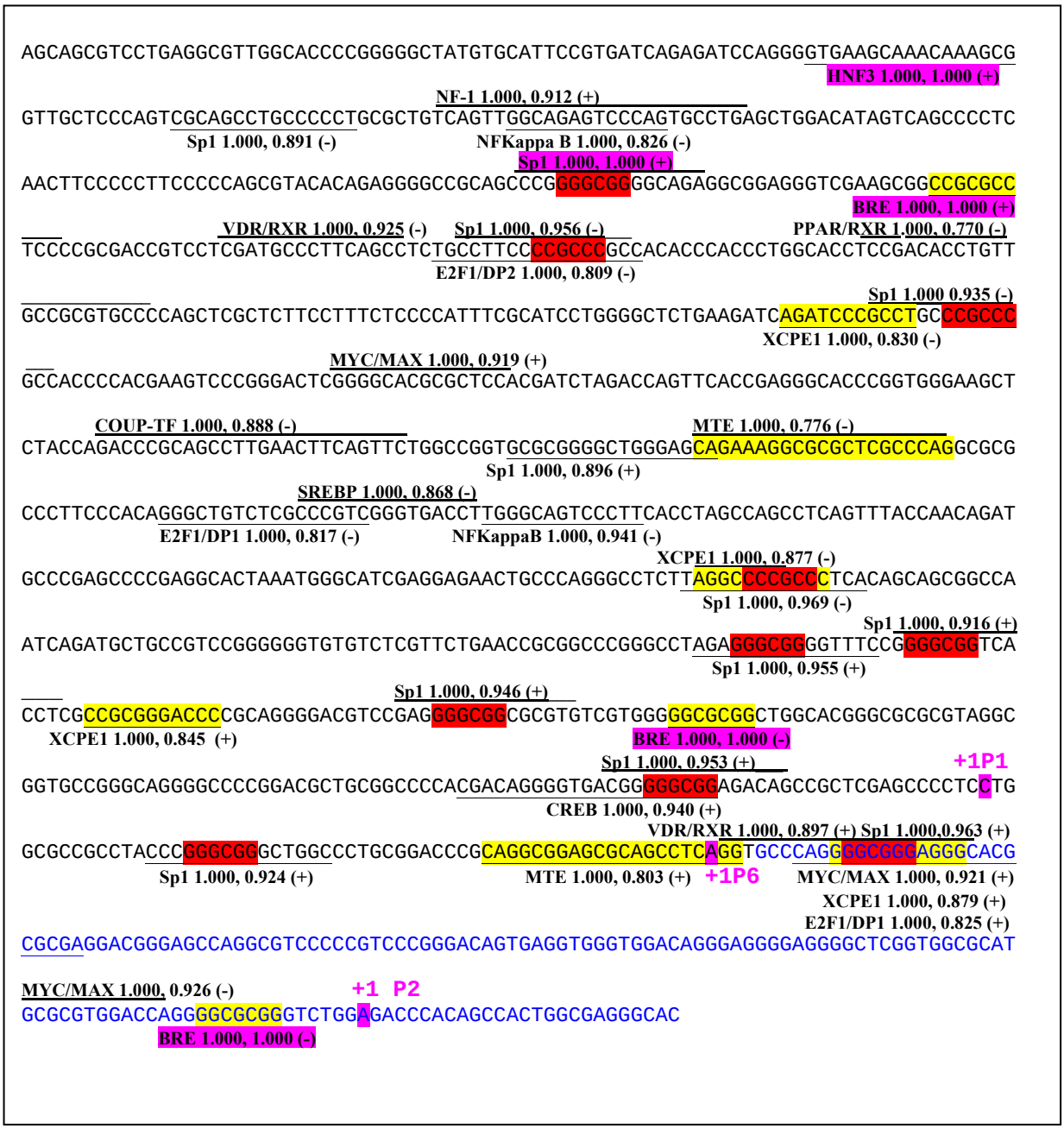


Figure 5.30 Sequence of the overlapping PPARα P1 and P2 cloned promoter regions showing potential transcription factor binding sites and core promoter elements. The sequence of the P2 and P1 cloned promoter regions are shown. The region unique to the P2 promoter is shown in blue text and transcription start sites are indicated in pink. TF binding sites and core promoter elements are underlined. In addition Sp1 binding sites are highlighted red and core promoter elements in yellow. All sites are scored and marked with the strand they are located on (+ or -). The core similarity of the sequence is the first number. The maximum core similarity of 1.0 is only reached when the highest conserved bases of a matrix match exactly in the sequence and only those sites with a score of 1.0 are shown. The second figure is the matrix similarity which takes into account all bases over the whole matrix length. A perfect match has a score of 1.00 and a "good" match has a score of >0.80. Perfect sequences are highlighted in purple.

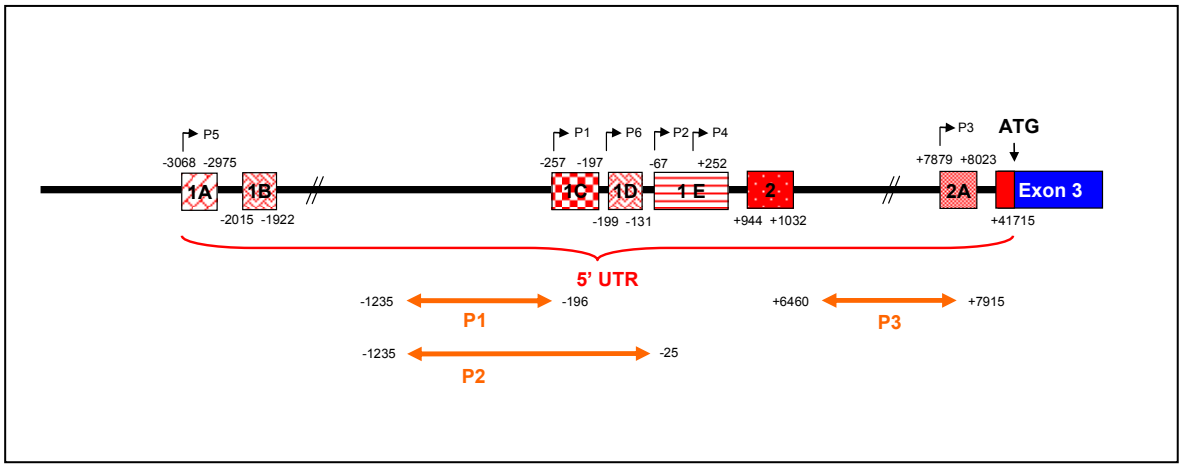


Figure 5.31 Schematic diagram showing relative locations of PPARα P1, P2 and P3 cloned promoters and their positioning relative to Ensembl transcription start site and 5'UTR exons. Location of PPARα 5'UTR Exons on the genomic sequence are shown in red. All positions are shown relative to the Ensembl transcription start site (+1). Cloned promoter locations are shown in orange for P1, P2 and P3, along with their exact positions relative to Ensembl transcription site.

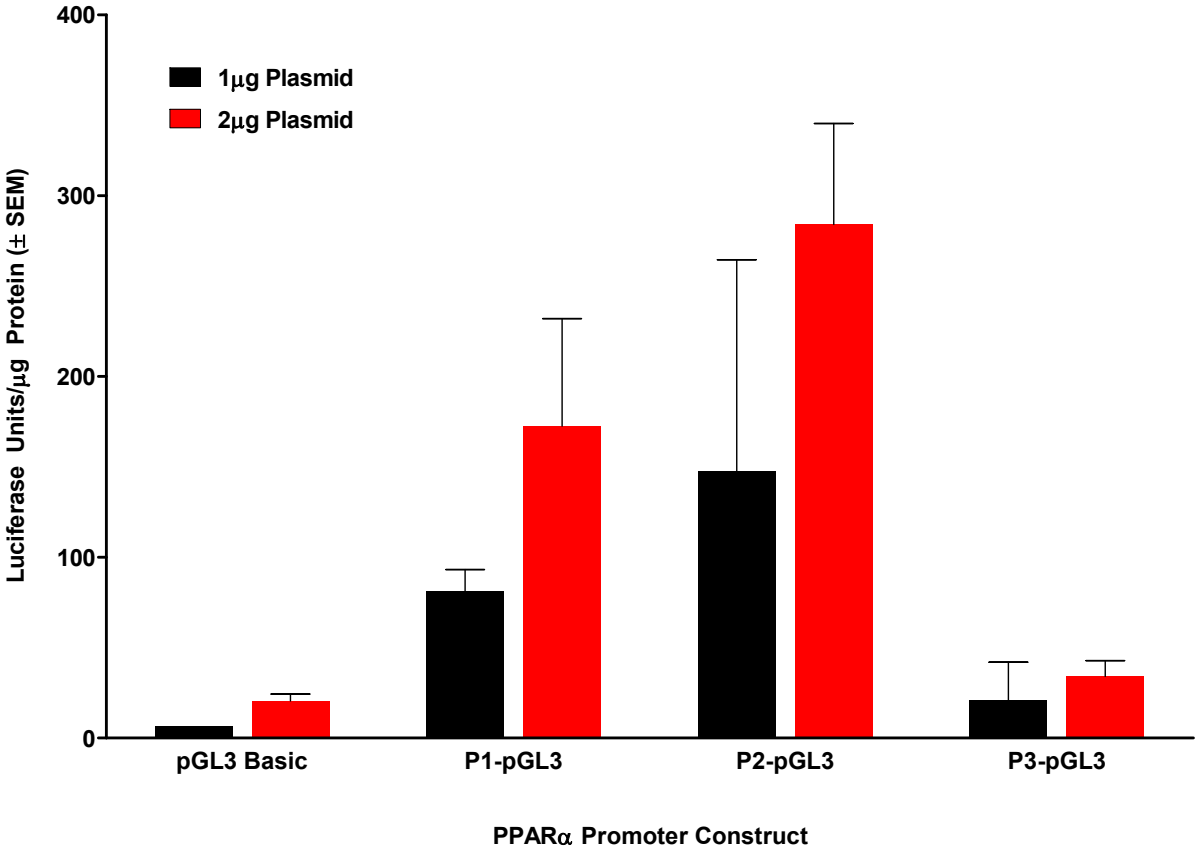


Figure 5.32 The PPARα P1 and P2 promoters are active in HepG2 cells, but the P3 promoter is inactive. Activities of 1µg and 2µg of the alternative PPARα promoters (P1, P2 and P3) and control (pGL3 Basic empty vector) in HepG2 cells are shown (n=2).

5.2.8 The alternative PPARα promoter constructs respond differently to factors known to activate PPARα transcription in HepG2 cells

It has previously been shown that PPARα gene expression is induced by factors including dex [86] and CFA [93]. To investigate the activity of the alternative PPARα promoters in response to such treatments, the PPARα-pGL3 luciferase reporter constructs (P1, P2 and P3) were transfected into HepG2 cells and subjected to treatment for 24 hours with increasing concentrations of either dex or CFA. In addition, the promoter-free pGL3 basic vector was transfected as a control. The responses of the promoters P1-3 to the treatments can be seen in figures 5.33-5.34.

Results showed that the pGL3 basic vector, which contains no promoter or enhancer sequences, was inactive in both experiments and did not respond to either treatment. In addition, the p3-pGL3 construct was also inactive and unresponsive to treatment in both experiments, with levels consistently similar to that of the pGL3-Basic control. There was therefore no effect of dex or CFA on the activity of the control or P3 promoter in HepG2 cells.

As expected, the P1 and P2 promoters were active in both transfections. Both the P1-pGL3 and P2-pGL3 promoter constructs were able to respond to dex treatment (figure 5.33). Dex increased activity of the P2-pGL3 construct from 3.36 in untreated cells to 6.73 at 10μM ($p=0.001$), whilst it increased the activity of P1-pGL3 from 2.54 in untreated cells to 5.8 at 1μM ($p=0.01$) and 8.2 at 10μm ($p=0.001$). CFA treatment also increased the activity of the P2 promoter from 8.53 in untreated cells, to 27.32 at 80μM ($p=0.001$) and 48.69 at a concentration of 100μM (48.69 $p=0.001$) (figure 5.34). There was a drop in activity of the P1 promoter from 24.56 in untreated cells, to 8.59 at a concentration of 60μM ($p=0.001$). There were no other changes in P1 activity in response to leptin treatment. Therefore, leptin treatment was able to increase the activity of the P2 construct but not that of the P1 construct.

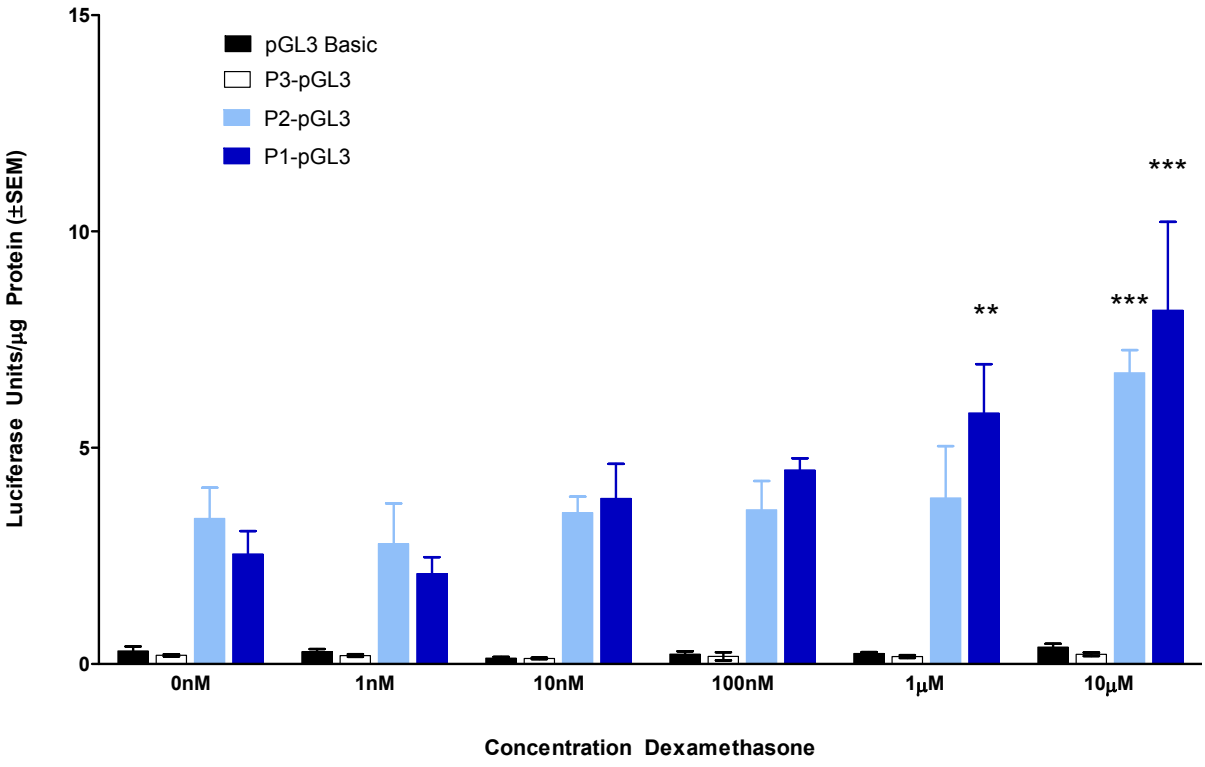


Figure 5.33 Dexamethasone activates the PPARα P1 promoter and the P2 promoter, but not the P3 promoter in HepG2 cells. Activities of the alternative PPARα promoters (P1, P2 and P3) and control (pGL3 Basic empty vector) are shown in response to increasing concentrations of the synthetic glucocorticoid dex (n=3). ** $p<0.001$, *** $p<0.001$ relative to untreated (0nM) (Bonferoni's Test).

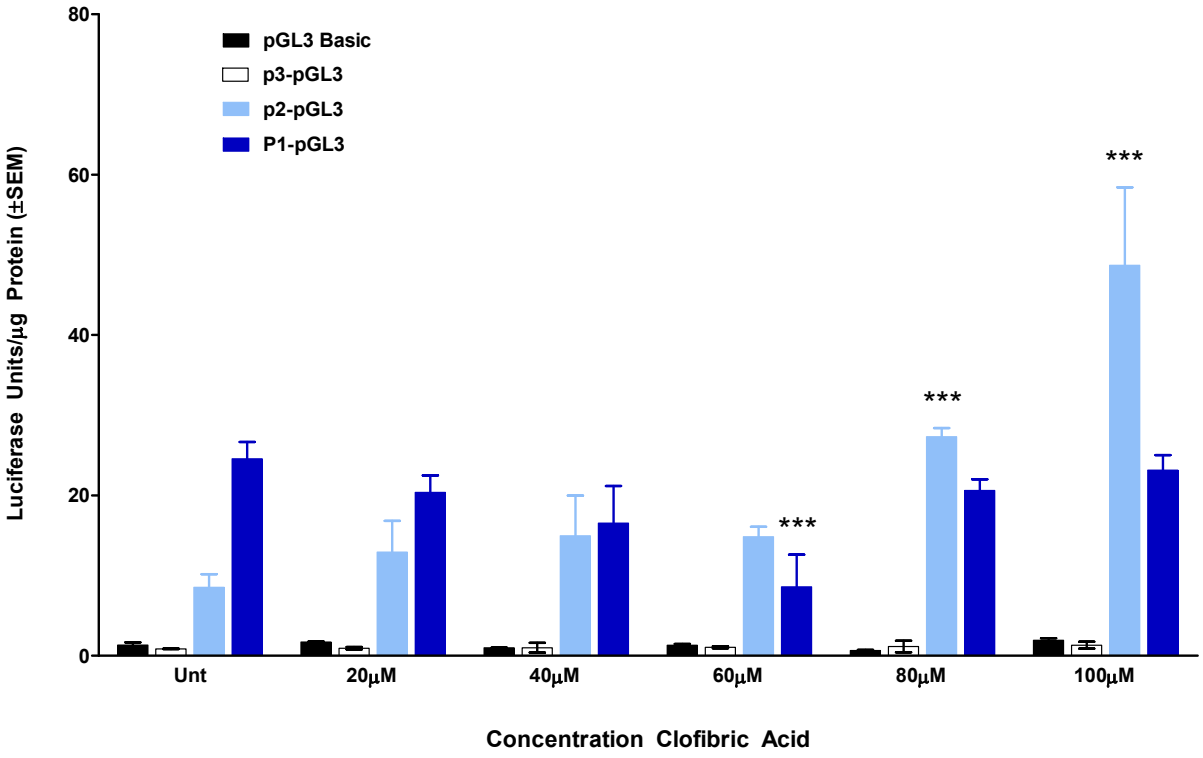


Figure 5.34 Clofibric acid activates the PPARα P2 promoter, but not the P3 or P1 promoters in HepG2 cells. Activities of the alternative PPARα promoters (P1, P2 and P3) and control (pGL3 Basic empty vector) are shown in response to increasing concentrations of the PPARα agonist CFA (n=3). *** $p<0.001$ relative to untreated (0µM) (Bonferoni's Test).

5.2.9 PPARα P2 promoter deletion constructs indicate locations of main basal activity

To further characterise the PPARα P2 promoter, a series of P2-pGL3 promoter deletion constructs were made, which progressivly lost ~200bp sequence from the 5' end of the promoter. These promoter constructs were named according to their size (figure 2.11). To investigate the activity of these P2 deletion promoters, they were transfected into HepG2 cells. The responses of the promoters P1-3 to the treatments can be seen in figure 5.35. A list of the binding sites present in each deletion construct can be found in table 5.5.

Results indicated that there were no significant differences in activity for any of the promoter constructs. Despite this, results indicated that the 845, 589 and 462 5' deletion promoter constructs had the highest promoter activity with levels of 132.59, 159.45 and 161.75 respectively, while in comparison the larger constructs 1096, P2 and P1 were less active with values of 55.38, 100 and 76.89 respectively indicating the possible presence of repressive elements in the 1096 5' deletion construct. The least activity was found in the smallest 198bp construct which contained only the unique region of the P2 promoter, with a value of 36.83.

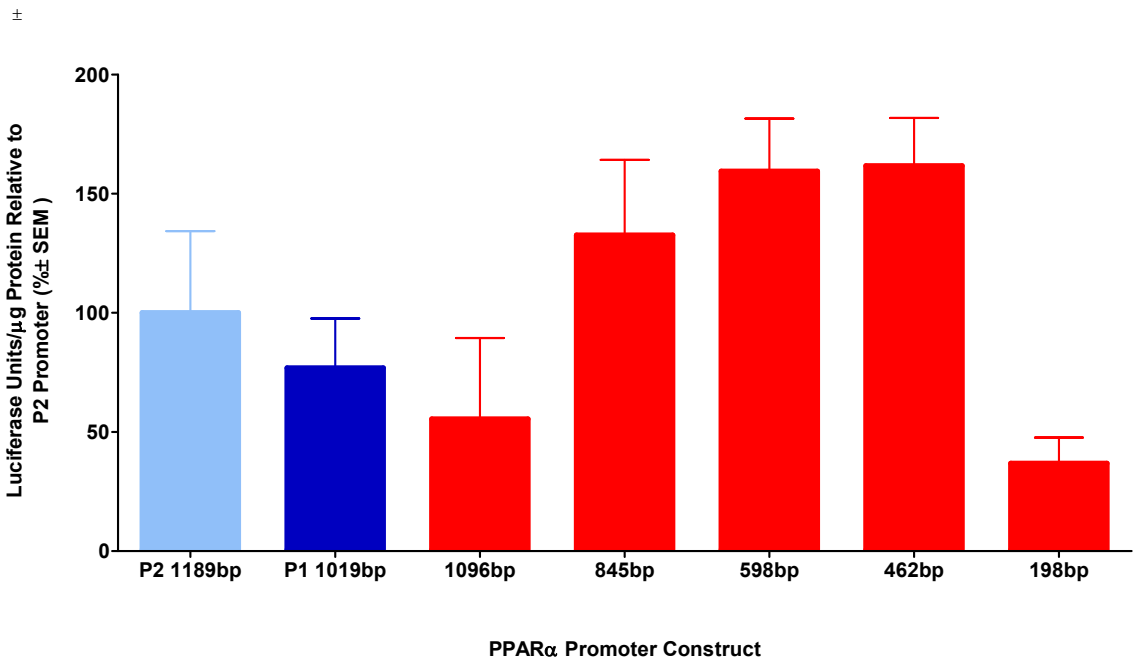


Figure 5.35 PPARα P2 promoter 5' deletion constructs indicate locations of main basal activity. Activities of the PPARα P1 promoter, P2 promoter and the 5' promoter deletion constructs and are shown (n=3)**.** The activities of all promoters are shown relative to the P2 promoter which is set at 100.

Table 5.5 Location of core promoter elements and transcription factor binding sites within the deletion constructs. Table indicates the presence of both core promoter elements and transcription factor response elements specific to the different deletion constructs and the number of sites present. Deletion constructs are listed with the smallest at the top and increase in size at the 5' end as they are listed. Sites listed are in addition to those presented in the shorter constructs.

Deletion Fragment	Element Present	Number Present
P2 Unique Region	BRE	1
	VDR/RXR	1
	Sp1	1
	Myc/Max	2
	E2F/DP1	1
462bp	MTE	1
	XCPE1	1
	BRE	1
	CREB	1
	Sp1	5
598bp	XCPE1	1
	Sp1	1
845bp	MTE	1
	XCPE1	1
	Sp1	2
	Myc/Max	1
	SREBP	1
	NFκB	1
	CoupTF	1
	E2F/DP1	1
1096bp	BRE	1
	Sp1	2
	NF1	1
	PPAR/RXR	1
	VDR/RXR	1
	NFκB	1
	E2F/DP1	1
P2	Sp1	1
	HNF3	1

5.3 Discussion

PPARα is a nuclear hormone receptor which has a diverse range of functions. For example, it not only plays a key role in energy homeostasis by controlling important metabolic processes such as fatty acid metabolism and lipoprotein metabolism [108], but also functions in the control of the inflammatory response [67]. Consequently, the effects of PPARα expression are seen on a variety of target genes, in a variety of tissues and at specific times and levels according to particular metabolic and physiological conditions. For example, the effects of PPARα expression and activation are mediated on several enzymes which are key regulators of fatty acid β-oxidation in specific tissues such as the liver, skeletal muscle and heart and this activity is crucial in the liver during fasting, to provide an alternative energy source for the peripheral tissues. In addition to this, evidence is also accumulating indicating a lipolytic role for PPARα in adipose tissue under specific hormonal conditions [232], when normally PPARα expression in this tissue is low. Furthermore, as part of its anti-inflammatory role, PPARα has cell specific effects on the expression and signalling of inflammatory genes [67]. This function and tissue specific expression of PPARα means that control of PPARα expression needs to be highly regulated in each tissue and cell type to ensure that its expression is appropriate to the metabolic and physiological status. Nuclear hormone receptors usually have complex gene regulation to accommodate these needs and this is typically mediated by differential promoter usage and alternative splicing to produce multiple 5'UTR transcript variants. These variants can provide the versatility, flexibility and fine tuning needed to maintain a tightly controlled tissue specific expression profile and this has been shown to be the case for the PPAR family [103].

It is well established that PPARα expression is controlled by hormones such as glucocorticoids and insulin in addition to its own ligands [87,93] and more recently by other nuclear hormone receptors [94-96]. Evidence also exists in rats that PPARα expression is epigenetically altered by a protein restricted maternal diet in the livers of offspring [77,212]. In addition, work in this report has identified that PPARα expression is altered by neonatal leptin treatment in a tissue specific manner. To date, the exact mechanisms underlying the regulation of PPARα transcription by these factors have not been investigated. Furthermore, the organisation of the rat PPARα gene 5'UTR has not been studied since its initial identification in 1992 and consequently is currently thought to consist of just one transcript, the regulatory region of which has not been characterised. Given that human PPARα and to a lesser extent mouse PPARα both have a complex genomic organisation, it is probable that this is the case for the rat PPARα gene. To understand how the above factors can affect gene expression, in particular that of leptin and in order to understand how tissue specific regulation is controlled, any PPARα transcript variants and their corresponding regulatory regions first need to be identified and characterised. This will enable the mechanisms underlying regulation of PPARα to be studied, which will be of utmost importance in understanding the role of PPARα in health and disease.

In this chapter, the 5'UTR of the rat PPARα gene has been characterised. Subsequently, six PPARα mRNA variants have been identified, termed P1-P6 which differ in their 5'UTR due to tissue specific differential promoter usage and alternative splicing of exons. In addition to this, the study identified five distinct novel exons within the 5'UTR. The 5'UTR of the six transcripts were shown to differ in structure; length, GC content, free folding energy and presence of uATG and uORF. To study the regulation of PPARα gene expression, the rat PPARα promoters which drive expression of the adipose and liver specific transcripts were mapped, cloned and characterised. They were shown to differ in the presence of core promoter elements and transcription factor binding sites and subsequently differed in both their basal activity and response to known activators of transcription.

The first stage in identifying the location of the PPARα promoter was to locate the genes transcription start site and this was achieved using an established method which reliably amplifies full length capped 5' ends of mRNA, the 5'RLM RACE. Previously, rat PPARα was not known to have any mRNA variants and only three exons were found to encode the 5'UTR [105]. However in this study six transcript variants were identified using the RACE procedure and they were identified from a variety of tissues which are known to vary in PPARα expression levels; two transcripts were identified in liver (P2 and P3), one in adipose tissue (P1), one in the heart (P4) and finally two in the kidney (P5 and P6). Furthermore, no transcript was identified in more than one tissue. Altogether five distinct new exons were identified, bringing the total number of exons forming the 5'UTR to eight. Each variant transcript was shown to possess a novel first exon and this high number of first exons implies that PPARα regulation by transcriptional control is important and that alternative promoters play a central role in its tissue specific gene regulation.

The presence of transcript variants is common not only to PPARα, but also to the other members of the PPAR family. The PPARα 5'UTR variants identified in different species have much in common with those found for PPARδ, i.e. multiple transcript variants with limited similarity between species. This is in contrast to PPARγ, which has very few transcript variants that are highly conserved between species. However, subsequent to the 5' RACE procedure, the genomic organisation of human, mouse and rat PPARα was found to have more homology than previously shown, in particular the finding of a 5' extended exon 1B in the rat P2 transcript and novel exon 1A in the rat P1 transcript, which are also present in both the human and mouse 5'UTR. In addition to this, the rat P1 and P2 PPARα transcript variants are very similar with those identified in the mouse; the rat P2 transcript is equivalent to the mouse variant 1 and the newly identified P1 transcript is equivalent to the mouse variant 2.

Although the different human and rat PPARα 5'UTR do share some common exons, none of the 5'UTR variants identified in rat have been found in human PPARα. However, it is possible that exons/5'UTR identified in the rat may be present in the human PPARα organisation but are as yet

unidentified, as the human transcripts were all identified in the liver, whereas the rat P1, P4, P5 and P6 transcripts were identified in either adipose, heart or kidney. Conversely, seven alternative 5'UTR transcripts were identified for human PPARα in the liver, whereas only 2 were identified in rat liver (P2 and P3). However, it is possible that more rat PPARα transcript variants exist, but were missed in the RACE procedure. If the initial PCR product contained different 5' RACE transcripts of the same size, then a population of different transcripts would have been transformed into bacterial cells. As such, many clones would need to be sequenced to determine if this was the case. As only one liver clone and two adipose clones were sequenced, it is possible that other liver and adipose specific transcripts exist. This is also the case for the 5'RACE PCR products which were sequenced, as the PCR product could be composed of a population of different 5'RACE transcripts of the same size. To determine if any transcripts have been missed, all the PCR products should have been cloned and then a larger amount of clones sequenced.

Another limitation of the 5'RLM RACE is that the full length transcripts were not able to be sequenced, thus any transcripts differing in the coding region from exon 5 onwards would be missed by this procedure. As such, the presence of exons 5-9 is presumed in the transcript variants. For this reason, it could not be determined if an equivalent of the human truncated variant lacking exon 6 [257] was present in the rat tissues. To determine if the remainder of exons are present, a PCR could be performed to amplify the cDNA using a 5' primer specific to the unique regions of each 5'UTR. This could then be sequenced to determine the exons present in the remainder of each transcript. A final limitation of the RACE is its inability to inform of the relative abundance of each transcript in the different tissues. For example, two transcripts were identified in the liver, but it is unknown if they are expressed at differing or similar levels. This information could be determined by using an RNase protection assay.

The finding that the six PPARα transcripts have differing 5'UTR is of importance to its gene regulation, as the presence of variant 5'UTR is thought to be an evolutionary mechanism to increase the scope of both translational control and transcriptional control. For example, the presence of a long 5'UTR, high GC content and secondary structure, of uATG and uORF are all associated with reduced translation efficiency of the main ORF [251]. As all of these factors were shown to be present in one or more of the alternative PPARα 5'UTR, this consequently implies that translational regulation of PPARα may be affected.

Whilst the relatively high GC content of the 5'UTR was fairly consistent between transcripts, the length of the 5'UTR varied from 184bp for the P3 transcript to 312bp for the P5 transcript and 444bp for the P2 transcript. This was mirrored in the minimum free energy values of the transcripts, i.e. the longer 5'UTR's were associated with more negative free energy values. The 5'UTRs form a natural secondary structure according to their minimum free energy. The more negative the minimum free energy, the more energy is released when forming base pairs and the

more stable the secondary structure. Therefore the transcripts with longer 5'UTR, such as P2 and P5 were predicted to have the strongest strong secondary structures, which can potentially impede translation by normal mechanisms. This is not a problem when individual secondary structures such as hairpin loops within the 5'UTR have values <30Kcal/mol, as they can be "melted" by the ribosome during the normal scanning process [265]. However, as all the PPARα transcripts contain hairpins with stabilities greater than this, it indicates that the structure of all the transcripts may impede ribosomal movement along the 5'UTR by the scanning mechanism [265]. Therefore, the minimum free energy values and potential stable folding patterns of the PPARα 5'UTRs indicate that they may all be subject to translational regulation.

The P3 transcript stood out from the rest of the PPARα transcripts, because it had a particularly complex 5'UTR structure. Although it had the shortest 5'UTR and the lowest GC content and the least negative minimum free energy value, this was still at a level which could possibly impede the initiation of translation [265]. Of particular interest this transcript differed from all the other PPARα transcripts due to the presence of four uATG the 5'UTR, the position and context of which could inhibit translation. According to the scanning method of translation initiation, translational initiation will commence at the first AUG from the 5' end within a context that favours initiation [266]. Furthermore, initiation codons do not have to conform 100% to Kozak sequence to enable initiation of translation, but the efficiency of translation is vastly increased by the presence of highly conserved bases at -3 (G/A) and +4 (G) [261]. To have one of these conserved bases can result in adequate initiation, whereas two present will result in good initiation [251]. Of the four uATG's present in P3 5'UTR, three were found to have an adequate Kozak consensus which could therefore initiate translation.

Of these three uATG codons, one has the potential to produce a protein with a 29 amino acid extended N-terminal. This would be akin to that of PPARγ2 which has an extended N terminal of 30 amino acids and 28 amino acids in mice and humans respectively compared to the predominant PPARγ1 protein. Furthermore, the P3 transcript has much in common with PPARγ2; its promoter is GC rich, its 5' UTR is very short containing just 1 exon in the 5'UTR and the N terminal extension is of a similar length. Experiments have shown that the PPARγ2 N terminal extension houses an N terminal ligand independent activation domain (AF-1), which mediates 5-6 times activation than PPARγ1 under ligand depleted conditions and this activity can be augmented by insulin treatment. Furthermore, this ligand independent activation domain in the N terminal is found in several other nuclear hormone receptors, including GR, oestrogen receptor and thyroid hormone receptor [104]. It is therefore possible that the P3 may contain an AF-1 domain, which may function under specific metabolic conditions. To determine if P3 could give rise to a larger protein, a western blot could be performed on the same liver tissue used in the 5'RLM RACE.

Two of the uATG in the P3 5'UTR also have the potential to form short uORF which terminate prior to the *Bona fide* ATG codon. It is well accepted that the presence of uATG and uORF can interfere with translation of the main ORF by forming a ribosome initiation complex at the uATG [262] and this therefore implies that the translational regulation of the P3 transcript could be further affected. For example, two alternate BRCA1 transcripts which differ in the first exon of their 5'UTR possess 5'UTR of very different lengths. The long 5'UTR was shown to have reduced translation efficiency compared to the variant with shorter 5'UTR and further analysis showed that this was due the presence of uORF and stable secondary structures impeding translation [267]. Another example of translational inhibition by the 5'UTR is that of suppressor of cytokine signalling-1 (Socs-1). Here, two uATG in the 134bp 80% GC rich 5'UTR were found to repress gene expression due to translation initiation at these upstream ATG sites. Despite the presence of several hairpin loops, translation was found to be by the normal cap dependent scanning mechanism [268]. It is therefore possible that translation of the P3 transcript may occur by the normal scanning mechanism, but this may be a modified version to cope with the uATG. For example; leaky scanning could occur, whereby not all ribosomes recognize the uATG, or reinitiation may take place, whereby the ribosomes can reinitiate at the *Bona fide* ATG after the short uORF.

In some genes, the presence of inhibitory factors such as secondary structures and uORF is associated with an alternative form of translational initiation, IRES, a cap independent mechanism of translational initiation common during periods of stress [251,269]. This type of translational initiation occurs for the AML1/RUNX1 gene which is controlled by two distinct promoters [270]. Here, one transcript has a very long 1631bp 5'UTR containing CpG islands, 15 uATG and several short uORF. Consequently, this transcript is not translated well and uses an alternative IRES mechanism. Conversely, the shorter transcript has a 452bp UTR that contains two uATG and is translated well by cap dependent mechanisms [270], which is in contrast to Socs-1 regulation. Furthermore, the presence of uATG and uORF in the 5'UTR is also common to both human and mouse PPARδ and the use of IRES has been postulated for both of these genes [76,85]. It has been found that hPPARδ variants with longer 5'UTR are translated with reduced efficiency compared to those with shorter 5'UTR and researchers found an inverse relationship between translation efficiency and uATG number [85]. Likewise, the main mPPARδ transcript possesses a long 548bp 5'UTR containing 8 uAUG codons, all of which form uORFs. Furthermore, this transcript was predicted to have a highly negative minimum free energy of -207.5Kcal/mol. This transcript was found to be regulated at the translational level as experiments indicated that the longer 5'UTR attenuated translational efficiency [76].

Clearly when comparing the 5'UTR of all these different genes, it is not always clear by the structural features present if they will affect normal mechanisms of translation, for example as indicated by the differing Socs-1 and AML1/RUNX examples which have similar structural features but differing translational mechanisms. Results indicate that all the PPARα transcripts

possess secondary structures which have the potential to impede translation, which may prevent normal scanning mechanisms. It is possible that these secondary structures could be overcome by ribosome shunting, whereby the ribosome disassociates at the secondary structure then re-associates just downstream of it, or alternatively the IRES mode of translational initiation could be employed. In addition, the presence of uATG in the P3 5'UTR may be overcome by leaky scanning or reinitiation. Further experiments need to be performed on all the transcripts using a coupled transcription translation system to determine if translational regulation is affected the structure of their 5'UTR. For the P3 transcript, it would be interesting to mutate the uATG to see if any repressive effects on translation were removed. It would be also be interesting to determine if IRES occurs for any of the transcripts and this could be determined using bicistronic constructs.

To functionally characterise the regulatory regions controlling the PPARα transcripts, a reporter gene strategy was employed which could test the activity of each regulatory region and it response to known activators of transcription. The promoter regions controlling the liver (P2 and P3) and adipose (P1) specific transcripts were therefore determined according to the position of their respective transcription start sites. This resulted in two partially overlapping GC rich promoters for the P1 and P2 transcripts and a distinct downstream promoter for the P3 transcript. The heart (P4) and kidney (P5, P6) transcripts did not have their corresponding promoters cloned as they were identified towards the end of the study. The resulting basal and stimulated activity of the variant PPARα promoters differed and computational analysis of the promoter regions by MatInspector identified the presence of several putative core promoter elements and interesting transcription factor binding sites which could be responsible for the differences in promoter activity.

Activity of the P1 and P2 promoters was high throughout experiments, indicating that they were fully functioning promoters, but despite this no TATA or Inr elements were identified in proximal regions. However, several potential core promoter elements were identified by MatInspector in the P2/P1 promoters which may direct initiation of transcription, including several potential BRE, MTE and XCPE1 sites, but none were located in their normal positions relative to the transcription start sites. Furthermore, it is unlikely that the BRE and MTE core promoter elements are functional as normally BRE act in conjunction with a TATA box and likewise MTE with an Inr element [271]. The P1/P2 promoters did however possess the characteristics of typical GC rich promoters common to nuclear hormone receptors; i.e. the absence of TATA and Inr elements and the presence of CpG islands containing multiple Sp1 response elements. As such it is possible that the XCPE1 element maybe functional, as these elements typically occur in CpG island containing TATA-less promoters and frequently act in conjunction with activators such as Sp1 [272]. However, regardless of whether Sp1 acts in conjunction with XCPE1, it is probable that Sp1 plays a key role in directing transcription initiation at the P1/P2 promoters, as Sp1 can interact directly with TATA binding protein (TBP) and TBP associated factor 4 (TAF 4) to direct basal transcription machinery [273]. Furthermore, Sp1 could act synergistically with the many Sp1 sites found throughout the promoter

region. This could occur either between closely positioned Sp1 sites or by looping out the connecting DNA between distant Sp1 sites. This ability of Sp1 to act synergistically is, however, promoter dependent and would need to be tested [273]. Further experiments would therefore be needed to test the role of all these core promoter elements in directing transcription, for example by mutating the putative sites and ascertaining the effect on transcription. Furthermore, the finding that both the human and mouse dominant promoters have similar structures which are GC rich CpG island containing regulatory regions which contain multiple Sp1 response elements and no TATA box or Inr [102,103], indicates that mechanisms of direction of transcription could be similar between species.

The P3 promoter had a contrasting structure to that of the P1 and P2 promoters, such that it did not contain any CpG islands and consequently had no Sp1 response elements present. The P3 promoter was found to be inactive in all experiments performed, indicating that it could not function as a promoter in HepG2 cells. This low activity indicates that either important proximal promoter sequences or possibly upstream elements had been missed out of the promoter region. In agreement with this, no core promoter elements were identified by MatInspector within this promoter. It would therefore necessary to clone a larger promoter fragment in order to determine promoter activity and its regulation. It is, however, also possible that this promoter is inactive as it needs stimulation by hormonal factors which were not present in this in vitro system.

It has previously been reported that PPARα transcription is induced by CFA [93] and dex [86]. Interestingly, the response of the adipose specific (P1) and liver specific (P2) promoters to these treatments differed. Indeed, both these treatments activated PPARα transcription in a dose dependent fashion, but only dex activated both the promoters, whereas CFA treatment only activated the P2 promoter.

Auto regulation of gene expression is commonly found among nuclear hormone receptors including the androgen receptor and GR [94]. Furthermore, ligands of PPARα have been reported to induce PPARα gene expression at the transcriptional level [94]. It is therefore probable that any transcriptional regulation of PPARα induced by CFA could be a form of auto regulation. When the cells were treated with 100μM CFA, the P2 promoter activity increased over 5 fold, whereas the P1 promoter activity remained unchanged. As the P2 promoter is identical to the P1 promoter apart from an extra 170bp at the 3' end, elements responsible for CFA activation of the P2 promoter must be present in this unique region. However, upon scrutinising of the unique region of the promoter, no PPRE was identified and surprisingly, a PPAR/RXR response element was identified within the shared part of the promoter region. As the P1 construct did not respond to CFA, it indicates that this response element is not functional. However, nuclear hormone receptors can sometimes bind alternative hormone response elements and experiments have unequivocally shown that human PPARα can regulate its own expression in vitro by binding a HNF-4 response element

which contains a DR1 motif [94]. Although this is not present in the unique region of the P2 promoter, a VDR/RXR site is present which contains a DR1 motif. It is thus possible that PPARα could auto regulate its own expression via this response element, which could be investigated by mutating the VDR/RXR response element. However, it should be noted that there is a potential VDR/RXR site in the shared region of the P1/P2 promoter, but as P1 did not respond to CFA treatment, it is likely that this too is not functional. Auto regulation by PPARα could be confirmed by the use of cyclohexamide which prevents protein synthesis and this could elucidate whether alterations in promoter activity are direct by PPARα, or indirectly by an unknown transcription factor.

Reporter gene experiments have also showed that the 'unique region' of the P2 promoter alone was able to direct transcription. Two BRE core promoter elements were identified within this region of promoter, but are incorrectly positioned relative to the TSS and therefore unlikely to be involved in transcription initiation. There is however an Sp1 site present in the unique region, which is in close proximity to an E2F response element. This Sp1 site may act alone to direct transcription initiation, but may also act with E2F. It has been shown that Sp1 and E2F are frequently found closely associated and these TF can act synergistically to vastly increase transcription, as has been shown for the dihydrofolate reductase gene (DHFR) [274]. It is therefore possible that the "Unique region" Sp1 site is important in initiating transcription from the P2 promoter. There are also two Myc/Max enhancer box (E-box) sites present in this region of the P2 promoter. These transcription factors generally activate gene expression and act on around 15% of all genes and may therefore function to increase transcription of the PPARα P2 promoter.

Although it is widely known that dex regulates PPARα expression via GR, the exact mechanisms and location of response elements responsible for this activation of PPARα are not known. We have shown that both the P1 and P2 promoters respond to dex treatment, indicating that response elements responsible for this activation of activity are present in the shared region of these 2 promoters. Previous experiments have shown that GR activation of PPARα promoter is direct [86]. However data from this study does not agree that dex is acting directly via GR, as MatInspector analysis did not identify any glucocorticoid response elements (GRE) within this region of the P1/P2 promoter, although this does not prove that GR can not bind to the promoter. Glucocorticoids have many effects, therefore it is possible that its actions on the PPARα promoters could be indirect via an unidentified transcription factor. For example, a putative NF-1 response element was identified by MatInspector in the P1/P2 promoter shared region. Generally an activator of transcription, NF-1 can be regulated by GR and therefore is a possible indirect means of regulation by GR in the absence of a GRE in the promoter. Interestingly, NF-1 was also identified in the promoter region of the human PPARα promoter [103]. To fully understand dex activation of the PPARa promoter, the use of cyclohexamide could again help determine if

activation of the PPARa promoter is direct by GR or indirect via another transcription factor such as NF-1.

Next, in order to identify the location of elements important to the transcriptional regulation of the PPARα P2 transcript, a series of promoter deletion constructs were made. The main area able to induce transcriptional activity was found to reside in a 462bp deletion fragment, a region of the promoter containing the unique region of the P2 promoter and an extra 364bp of 5' sequence common to the P2 and P1 promoters. Activity of this fragment was 4 fold higher than the shorter unique region promoter. This 462bp fragment contained several incorrectly positioned core promoter elements (perfect BRE, MTE, XCPE1), but more interestingly, 5 Sp1 sites. It may well be that increased transcription of this fragment is due to the synergistic effect of these extra 5 sp1 sites in conjunction with the downstream Sp1 site in the unique region of the promoter. In addition to this, a potential CREB site was identified in the 462bp promoter, which interestingly was also found in the human PPARα promoter [103]. This CREB site at the 3' end of the P1/P2 shared region could potentially be responsible for activation of PPARα under times of stress by glucagon and adrenaline signalling. This would mean that as well as activating enzymes to stimulate lipolysis, glucagon could activate a key transcription factor which directs fatty acid oxidation. The 598bp promoter construct was larger than the 462bp construct at the 5' end and had the same activity as the shorter construct. Not surprisingly, it was only found to contain an additional XCPEI and a Sp1 site. However, by adding on an extra 247bp at the 5' end, the 845bp fragment had less activity than smaller 598bp fragment, indicating the presence of possible repressive elements. In agreement with this, this region contains potential response elements for transcription factors which could act in a repressive manner; CoupTF and NFκB. For example, CoupTF has been shown to act in a negative manner on PPARα activity by binding PPRE to antagonise PPARα signalling [275]. It is therefore possible that in addition CoupTF could act on its own response element to have a negative effect on PPARα expression. In addition, the pro inflammatory NFκB can exert both positive and negative regulation of transcription. It has previously been shown that PPARα exerts some of its anti-inflammatory effects by negatively regulating/antagonising NFκB and AP1 signalling pathways [276], it is possible that in turn NFκB, which is found in most cell types, could repress PPARα gene expression by means of a response element in the PPARα promoter. The 1096bp fragment also had much less activity than the shorter fragments at the 5' end, indicating that further repressive elements may be present. Again, another NFκB site is present which may have a negative effect on transcription.

To narrow down the response elements responsible for activation of promoters by dex, experiments should be performed on the PPARα promoter deletion constructs. Clearly the transcription factors thought to be responsible for activation and repression of promoter activity would need to be mutated to determine if they are functional. It would also be interesting to look at the effect of

insulin on the promoters, as it has been shown previously that insulin can prevent activation of PPARα transcription by dex [87].

Despite the lack of basal promoter activity, analysis of the liver specific P3 promoter revealed some interesting potential transcription factor binding sites which could be involved in both the positive and negative regulation of PPARα gene expression. The P3 promoter did not respond to any treatment, but as mentioned previously, this may be because elements which could initiate transcription were missed out of the promoter. The P3 promoter was, however found to contain potential transcription factor binding sites which could be of importance in PPARa regulation, such as PPAR/RXR which could mediate auto regulation and 2 NF1 sites which could mediate the effects of dex. In addition, 3 potential HNF-4 sites were identified in the promoter. HNF-4 is a nuclear receptor which is highly expressed in the liver and involved in metabolic homeostasis and response elements for HNF-4 are frequently found in promoters of metabolic genes, including those of lipid metabolism. As PPARα is down regulated in the livers of HNF-4 liver knock out mice [96] and HNF-4 over-expression has been shown to upregulate PPARα expression [95], it is therefore possible that these response elements are responsible for activation of PPARα transcription. Furthermore, it has been shown that PPARα can bind HNF-4 response elements, therefore the 3 HNF4-RE could provide further sites for auto regulation by PPARα [94]. Lastly, a potential C/EBP response element was also identified by MatInspector in the P3 promoter region. C/EBP has been shown to be involved in the transcriptional repression of human PPARα by Il-6 in HepG2 cells [99] and may therefore act in a repressive manner on the P3 promoter. As several of the response elements identified in the P3 promoter were not found in the P2 promoter, the P3 promoter may therefore provide additional strength/fine tuning of gene expression in the liver.

Experiments determining the activity of the liver and adipose specific promoters have shown that in HepG2 cells, the adipose specific (P1) and liver specific promoter (P2) had similar basal activity, however it is known in vivo that expression of PPARα is high in liver and low in adipose tissue. Several factors could account for this. Firstly, it may be that in adipose tissue, the different array of factors present within the cell result in a lower expression of PPARα. However, the promoter regions of these transcripts were found to be very similar, although there were few differences in response elements present. It may therefore be that higher expression in the liver compared to that in adipose tissue may be due to the presence of the additional liver specific transcript (P3). For this reason, it would be very useful to determine the activity of the two liver promoters in vivo, i.e. their strengths under normal and differing metabolic conditions. Finally, one last possibility is that the liver and adipose differences in gene expression are a result of other forms of regulation, such translational regulation by 5'UTR structure.

Although the P5 promoter was not characterised in transfections, MatInspector identified some interesting potential transcription factor binding sites which may play a role regulating PPARα

transcription in the kidney. These included several RXR heterodimer response elements, two PPRE and four HNF-4 response elements which all may be involved in PPARα auto regulation.

Work presented in this chapter has identified that the rat PPARα gene has tissue specific transcripts and promoters which allow PPARα regulation to be more versatile, in keeping with its role as a nuclear hormone receptor. The alternative promoters increase the scope for transcriptional control in a tissue specific manner, for example by providing diversity in the regulatory elements present between tissues and furthermore by providing diversity in the same tissue by the presence of multiple promoters. These features enable PPARα to respond differently to different factors and metabolic conditions such as was shown for P1 and P2 promoters. The use of different promoters also results in differing 5'UTR with varying structure which may further effect PPARα gene expression by modulating translation efficiency and in addition may even result in a variant protein which is activated under differing metabolic conditions.

The work completed in this chapter will provide a basis for studying the effects of leptin treatment on the PPARα promoter. In particular how leptin treatment may alter PPARα expression, the exact mechanisms involved, which transcripts are involved and to identify how neonatal leptin treatment could induce gene expression changes which persist into adulthood.

Chapter 6

Leptin Activation of the PPARα Promoter

And

Methylation Status of the PPARα Promoter in Adult Rats in Response to Neonatal leptin Treatment

6.1 Introduction

6.1.1 Leptin receptor signalling mechanisms

The leptin receptor (OB-R) is the product of the *db* gene and a member of the class I cytokine receptor family known to stimulate transcription via activation of Stat proteins [118]. Leptin receptors typically possess three domains; an extracellular domain which permits ligand binding, a single membrane spanning domain and an intracellular domain which contains the motifs necessary for transmitting the signal. However, in both humans and rodents, alternative splicing of the leptin receptor yields six variant isoforms; OB-Ra, OB-Rb, OB-Rc, OB-Rd, OB-Re and OB-Rf. All six isoforms possess the extracellular domain, but only five possess membrane spanning domain and the intracellular cytoplasmic domain (OB-Ra-d, OB-Rf). OB-Re is the isoform which only contains the extracellular domain and this forms a soluble receptor thought to be involved in leptin transport. The remaining isoforms all possess intracellular domains of differing size and as a result are classified into either long (OB-Rb) or short (OB-Ra, OB-Rc, OB-Rd, OB-Rf). These alternative isoforms of leptin receptor therefore differ in their signalling capacities according to the motifs present in the intracellular domain [277].

Leptin signalling has been shown to occur predominantly via the long form of the receptor, OB-Rb, which is expressed throughout peripheral tissues as well as the CNS, with particularly high expression in the hypothalamus. As OB-Rb has no intrinsic kinase activity, leptin receptor signalling pathways involve recruitment of a cytoplasmic kinase, Jak2. Jak activation results in the phosphorylation of tyrosine (tyr) residues on the intracellular domain of the receptor, in particular tyr 985 and 1138, which provide docking sites for downstream signalling molecules. However, consistent with its many physiological functions and near ubiquitous distribution, leptin participates in a number of signalling pathways mediated by Jak2, tyr 985 and 1138, including Jak/Stat, Mitogen-activated protein kinase (MAPK), Phosphoinositide 3-kinase (PI3K) and 5'-AMP-activated protein kinase (AMPK) signalling pathways [277](figure 6.1).

OB-Rb is the only isoform of the leptin receptor which contains all the intracellular motifs required for Jak Stat signalling, leptins predominant signalling mechanism. Activation of this pathway results in the recruitment of cytoplasmic kinases (Jak2) and the resulting phosphorylation of tyr residues on OB-Rb, which permit the binding and activation of Stats and subsequent transcription of target genes in the nucleus. This signalling pathway occurs in both central and peripheral tissues as a result of leptin binding. For example, leptin activates one of its key hypothalamic target genes, POMC via Stat3 signalling [278]. Furthermore, it also activates Suppressor of cytokine signalling 3 (Socs-3), a gene whose product attenuates leptin receptor mediated signalling in a well established mechanism of negative feedback, which is implicated in obesity related leptin resistance.

In peripheral tissues, some of leptins major effects on energy metabolism have been shown to occur via Stat3 signalling. For example, leptin acts via OB-Rb and Stat3 to decrease insulin synthesis in pancreatic β cells [120] and intravenous leptin increases OB-Rb mediated Stat3 phosphorylation after only 3 minutes in the liver, muscle and adipose tissue [279]. Of particular interest, is that leptin signalling via OB-Rb and Stat3 is widely implicated in adipose tissue. For example, in the DIO rat, the reduced adipocyte OB-Rb which results from HF feeding results in reduced Stat3 activation [243] and injections of leptin in mice result in increased Stat3 phosphorylation in WAT [244], whilst leptin treatment on excised fat pads activates Stats [121,279] and also in 3T3 cells [279]. Furthermore, it has been identified that the leptin mediated depletion of adipocyte fat in adult rats requires OB-Rb on adipocytes and results in increased phosphorylation of Stat3 [280]. In addition, mice with adipocyte specific Stat3 knock out have impaired leptin-induced lipolysis [281]. Interestingly, the leptin-induced depletion of adipocyte fat only occurs with normal PPARα signalling [239], implying that Stat3 is the mechanism by which OB-Rb activates PPARα. These experiments, therefore, provide overwhelming evidence that leptin signalling via Stat3 in adipose tissue plays a key role in energy metabolism, particularly in relation to its role in inducing lipolysis and reversing differentiation in adult rats.

Interestingly, leptin receptor mediated signalling of POMC, one of leptins major hypothalamic target genes, has been found to occur by a non-canonical Stat3 mechanism in the rat. The POMC promoter region which responds to leptin mediated Stat3 signalling does not contain a Stat3 binding element (SBE) and experiments found that Stat3 could not bind this promoter fragment directly, but instead Sp1 bound upon leptin treatment. Further studies uncovered that Stat3 mediated leptin signalling of POMC absolutely requires this Sp1 response element within its promoter and researchers concluded that Stat3 binds to a pre-existing Sp1/DNA complex to mediate POMC gene expression [282]. Interestingly, in the DIO rat, which has similar metabolic disturbances as the UN offspring in the IUGR rat study, methylation of a CpG just upstream of this Sp1 response site is increased and correlates with reduced POMC gene expression in response to leptin treatment [185].

The use of this novel Stat3/Sp1 mechanism of gene activation occurs in another leptin target gene, Tissue inhibitor of metalloproteinase 1 (TIMP-1). Again, no SBE was identified within the TIMP-1 promoter region which responds to leptin treatment. Here, leptin receptor signaling results in phosphorylation of Stat3 and the subsequent activation of Sp1 and its translocation to the nucleus, whereby Stat3 binds Sp1 at the Sp1 response element within the TIMP1 promoter to increase gene expression [283]. In addition to this, another cytokine has been shown to activate gene transcription via Stat3 in a similar manner; Interleukin-6 has been shown to induce transcription of the VEGF gene in a GC rich SBE free promoter, but here as with POMC, activated Stat3 translocates to the nucleus to interact directly with a pre-bound Sp1/DNA complex [284].

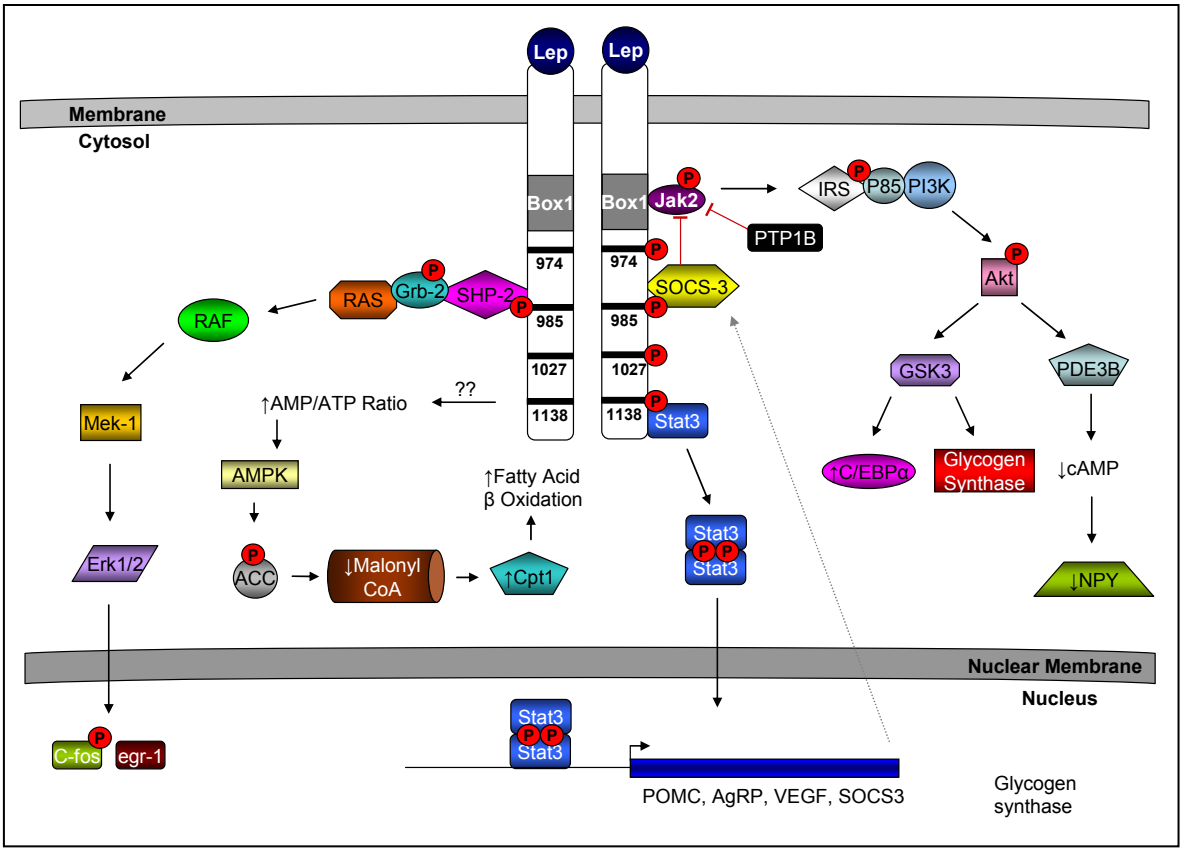


Figure 6.1 **Leptin receptor signalling mechanisms.** In the absence of ligand, OB-Rb forms a homodimer. Leptin binding induces a conformational change and results in a non-covalently associated Jak2 binding the intracellular box1 motif. Jak2 subsequently auto-phosphorylates on tyrosine residues and also phosphorylates OB-Rb tyrosine residues, including tyr 985 and tyr 1138. **Stat3:** Stat3 binds phosphorylated tyr 1138 and is itself then phosphorylated by Jak2. Stat3 homodimerises and translocates to the nucleus to bind to Stat binding element (SBE) whereby it activates transcription of target genes, e.g. POMC and SOCS3. **AMPK:** Activated AMPK phosphorylates ACC resulting in its inhibition. ACC functions to catalyse the irreversible formation of malonyl CoA from acetyl CoA, which inhibits CPT-1. AMPK also activates malonyl-CoA decarboxylase to reduce malonyl CoA levels. Thus this cascade results in the reduction of Malonyl CoA and therefore the disinhibition of CPT-1, thus increasing CPT-1 activity and fatty acid β-oxidation. **MAPK:** SHP-2 binds tyr 985 which then becomes phosphorylated and recruits Grb-2 to induce the p21 Ras-ERK pathway and expression of target genes including c-fos and c-jun. **PI3K/PDE3B/cAMP:** Leptin signalling can result in the recruitment and phosphorylation of IRS2 by Jak2. IRS2 associates with p85 to activate PI3K, which results in activation of ser/thr kinases such as phosphoinositide-dependent kinase 1 (PDK1). This in turn can activate another ser/thr kinase, Akt. Subsequent activation of PDE3B causes decreased cAMP levels and reduced PKA activation. **Leptin Resistance:** SOCS3 is a SH2 domain containing protein which binds Tyr 985 to inhibit Stat3 signalling by inhibiting Jak2 and preventing tyr phosphorylation of OB-Rb. SOCS3 also inhibits ERK signalling by competing for tyr 985. Protein tyrosine phosphatase 1B (PTP1B) de-phosphorylates Jak2 to negatively regulate leptin signalling.

The long form of leptin receptor (OB-Rb) can also activate MAPK (Ras-Raf-ERK) signalling pathways. This occurs predominantly via phosphorylation of tyr 985 which provides a docking site for SH-2 domain containing phosphatases (SHP2) which mediate downstream signalling that results in the expression of target genes such as c-fos. In addition, the MAPK pathway can be also activated by the short form of the leptin receptor (OB-Ra) by a mechanism which occurs independently of tyr 985 and involves a direct association of Jak2 with SHP-2. However, this signalling mechanism is not as efficient as the tyr 985 mediated mechanism [277]. The MAPK pathway has been identified in several peripheral tissues. For example, leptin increases MAPK phosphorylation by activation of OB-Rb in both adipose tissue and the liver [279] and phosphorylated MAPK is induced in WAT of mice but not OB-Rb KO mice [244]. In addition, leptin has been shown to activate PPAR's via an ERK-cPLA$_2$ pathway, whereby phosphorylation of cytosolic phospholipase A2 (cPLA$_2$) results in the release of unsaturated FA from membrane phospholipids in C2C12 cell, the subsequent activation of PPARα and its increased binding to the PPRE of target genes [285].

Another important signalling pathway mediated by leptin is the PI3K/PDE3B/cAMP pathway. Here, via recruiting insulin receptor substrates (IRS), leptin can act through components of the insulin signalling pathway to bring about activation of PI3K and downstream protein kinases such as PDE3B and GSK3. Activation of the PI3K/PDE3B/cAMP pathway plays an important role in mediating leptins effect to reduce food intake in the hypothalamus. Leptin signalling of NPY occurs by a Stat3 independent pathway, as experiments have found that inhibiting Stat3 signalling does not affect NPY expression. Instead, leptin exerts its negative effects on NPY in the hypothalamus by recruitment of IRS2 and activation of PI3K. Inhibiting PI3K abolished inhibition of NPY neurons by leptin and thus inhibition of food intake. Data indicates this signalling pathway also occurs in peripheral tissues. For example, IRS-1 associated PI3K activity is increased in response to leptin in adipose tissue and IRS-2 associated PI3K activity is increased in the liver [244,279]. Furthermore, in pancreatic β cells PI3K activation of phosphodiesterase 3B (PDE3B), resulting in reduced cAMP levels and Protein kinase A (PKA) activation, are thought to be involved in leptin mediated reduction of insulin secretion in pancreatic β cells [120].

Leptin is also known to activate AMPK, a serine threonine kinase which is involved in cellular energy homeostasis and whose activity is dependent on the energy status of the cell, as well as hormones and growth factors. This enzyme is activated by allosteric activation by AMP and phosphorylation by upstream kinases and its effects are mediated by the phosphorylation of downstream targets. This cascade of events results in increased catabolic processes including increased fatty acid oxidation and glucose uptake to provide energy for the cell, whilst inhibiting anabolic processes. Leptin has been found to stimulate fatty acid oxidation by activating AMPK in skeletal muscle [286] and this can occur both as a direct effect of leptin on muscle in involving an

increase in intracellular AMP concentration, or by centrally mediated sympathetic signalling via the α-adrenergic pathway [286].

It has also been shown using RTPCR and siRNA techniques specific to PPARα that leptin mediated AMPK activity has direct transcriptional effects on PPARα in skeletal muscle [287]. Subsequently, it was shown in a mouse muscle cell line that leptin activates the AMPK α2-β2-γ1 subunit which in turn translocates to the nucleus to activate expression of PPARα and therefore its target genes to induce fatty acid oxidation [288]. Furthermore, two nuclear localisation signals were identified in the α2 subunit of AMPK [288]. Researchers postulated that the effects of AMPK activation on PPARα gene transcription may be mediated by Sp1 and CRSP (Cofactor Required for Sp1 Activation) as there are many Sp1 sites in the PPARα promoter [288]. CRSp1 is a cofactor needed for Sp1 mediated transcriptional initiation by RNA polymerase II. Researchers showed that AMPK activation phosphorylates CRSP and that both Sp1 and CRSP3 RNAi experiments inhibited activation of PPARα gene expression by leptin in the C2C12 cells [288]. Therefore it is possible that the effect of leptin on PPARα transcription in adipose tissue is mediated by AMPK to increase fatty acid oxidation not only by altering the activity of specific enzymes, but also by affecting PPARα transcription. Indeed, OB-Rb signalling has been shown to increase AMPK activity in adipocytes [243]. However, leptin mediated AMPK activation does not occur in all tissues. For example, leptin treatment to isolated rat hearts does not involve activation of AMPK, despite activating fatty acid oxidation and reducing TAG within heart [289].

6.1.2 Aims

In Chapter 4 of this report, results have shown that the expression of several genes involved in energy balance is increased in response to neonatal leptin treatment in the adipose tissue adult female rats. It is not yet known how leptin mediates these changes in expression, but evidence suggests it may be due to direct peripheral effects of leptin on the adipocyte rather than central mechanisms [232]. To provide a mechanism which could account for the long term effects on gene expression, it is proposed that genes altered by neonatal leptin treatment such as PPARα are epigenetically altered themselves as a result of the leptin by an unknown mechanism, or that upstream signalling mechanisms are persistently altered such as Stat3 mediated leptin signalling, resulting in increased target gene expression within adipocytes.

Consequently, there were two principal aims for this chapter; The first aim was to identify the exact mechanism by which leptin signalling is able to affect PPARα gene expression and the second to determine if the PPARα promoter itself was epigenetically altered as a result of neonatal leptin treatment in adipose tissue of adult female offspring from the IUGR rat study. As literature suggests that leptin signalling occurs predominantly by Stat3 in adipocytes to induce lipolysis and

that this lipolysis requires OB-Rb [280], Stat3 [281] and PPARα [239] to occur, the first question was addressed by employing a reporter gene approach to investigate Stat3 signalling of the alternative PPARα promoters and secondly, methylation of individual CpGs within the PPARα promoter was investigated using a gold standard of DNA methylation assessment, pyrosequencing.

Data presented in this chapter identifies the mechanism by which leptin signalling acts on the PPARα promoter. It also characterises the methylation status of a significant region of the PPARα promoter in adipose tissue from control and leptin treated offspring of the IUGR rat study.

6.2 Results

6.2.1 The P1 and P2 PPARα promoters respond differently to leptin treatment in HepG2 cells

It has previously been shown that PPARα gene expression is induced by leptin [232,239]. In addition, as shown in Chapter 4, neonatal leptin treatment in rats may induce long term alterations in PPARα expression in adipose tissue. To investigate the activity of the alternative PPARα promoters in response to leptin, the PPARα-pGL3 luciferase reporter constructs (P1, P2 and P3) were transfected into HepG2 cells and subjected to treatment for 24 hours with a concentration gradient of leptin. In addition, the promoter-free pGL3 basic vector was transfected as a control. The responses of the promoters P1-3 to leptin treatment are shown in figure 6.2.

Results showed that the pGL3 basic vector which contains no promoter or enhancer sequences was inactive and did not respond to leptin treatment. In addition, the P3-pGL3 construct was also inactive in this experiment and unresponsive to treatment, with levels consistently similar to that of the pGL3-Basic control. There was therefore no effect of leptin treatment on the activity of the control or P3 promoter region analysed in this experiment.

As shown previously, the P1 and P2 promoters were active in HepG2 cells. However, leptin treatment was only able to increase activity of the P2 promoter. Leptin increased activity of the P2-pGL3 construct from 45.44 in untreated cells to 99.00 at a concentration of 500ng/µl ($p=0.001$) and to 125.45 at a concentration of 1000ng/ml ($p=0.001$). There was a drop in activity of the P1 promoter from 41.45 in untreated cells, to 22.62 at a concentration of 100ng/ml ($p=0.05$). There were no other changes in P1 activity in response to leptin treatment. So as seen with CFA, leptin treatment was able to increase the activity of the P2 construct but not that of the P1 construct.

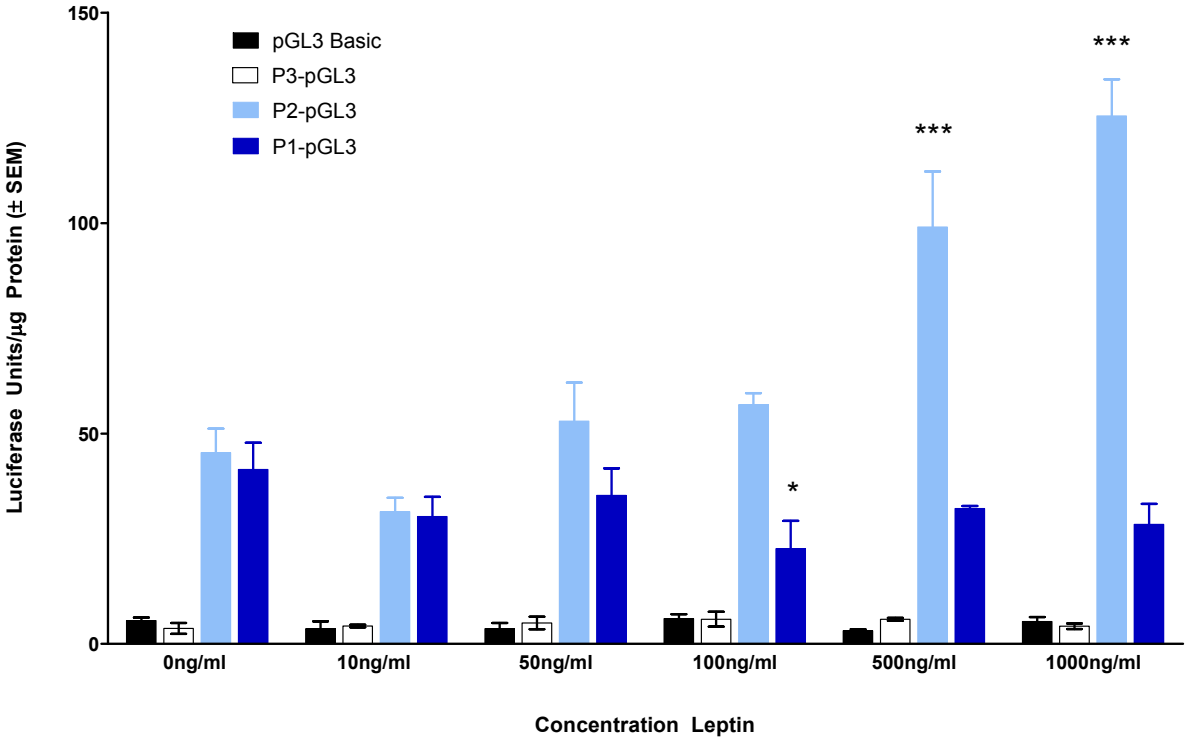


Figure 6.2 Leptin activates the PPARα P2 promoter, but not the P1 or P3 promoters in HepG2 cells. Activities of the alternative PPARα promoters (P1, P2 and P3) and control (pGL3 Basic empty vector) are shown in response to increasing concentrations of the adipokine leptin (n=3). *p<0.01, *** p<0.001 relative to untreated (0ng/ml) (Bonferroni's Test).

6.2.2 The expression of the PPARα P2 specific transcript but not the P1 specific transcript is significantly altered by neonatal leptin treatment in the adipose tissue of adult female offspring from the IUGR rat study

Work in chapter 4 showed that neonatal leptin treatment increased PPARα expression in the adipose tissue of female offspring from the IUGR rat study. In order to ascertain if this increase in PPARα expression was due to alterations in expression of the adipose specific transcript (P1), real time PCR was performed with validated gene specific primers specific to the P1 and P2 PPARα transcripts using cDNA from adipose tissue of female offspring. Resulting gene expression data were normalised to 18s cDNA and analysed by the ΔCT method [228]. Results were converted to % of the control group (AD Chow 100%) and a 1 way ANOVA performed, followed by Bonferroni's post hoc test. Graphs showing differences in mRNA expression for both the P1 and P2 transcripts with statistical analysis can be found in figure 6.3. All results are described relative to the control group only. In addition, a 3 way ANOVA was performed to analyse single factor and interaction effects on gene expression. The results of the 3 way ANOVA are shown in table 6.1.

Results showed that there were no significant differences in mRNA expression between the control (AD Chow) and UN offspring or between control offspring and offspring fed high fat diet (AD or UN) for either the P1 transcript or the P2 specific transcript. There was therefore no effect of maternal diet or postnatal diet on the expression of the P1 or P2 PPARα transcripts in the adipose tissue of female offspring. In addition, there was no effect of neonatal leptin treatment on the expression of P1 transcript (figure 6.3a). Therefore the P1 transcript was unaltered in all treatment groups.

Conversely, expression of the P2 specific transcript was increased in leptin treated offspring fed a chow diet from both AD dams (216.8% p=0.01) and UN dams (221.6% p=0.05) (figure 6.3b). In addition to this, its expression was increased in leptin treated offspring fed a high fat diet from AD dams (216.6% p=0.001) and UN dams (218.4% p=0.001). Therefore expression of the P2 specific PPARα transcript was increased in all leptin treated offspring. These data indicate that the P2 specific transcript is likely to be responsible for the changes in PPARα expression seen in Chapter 4.

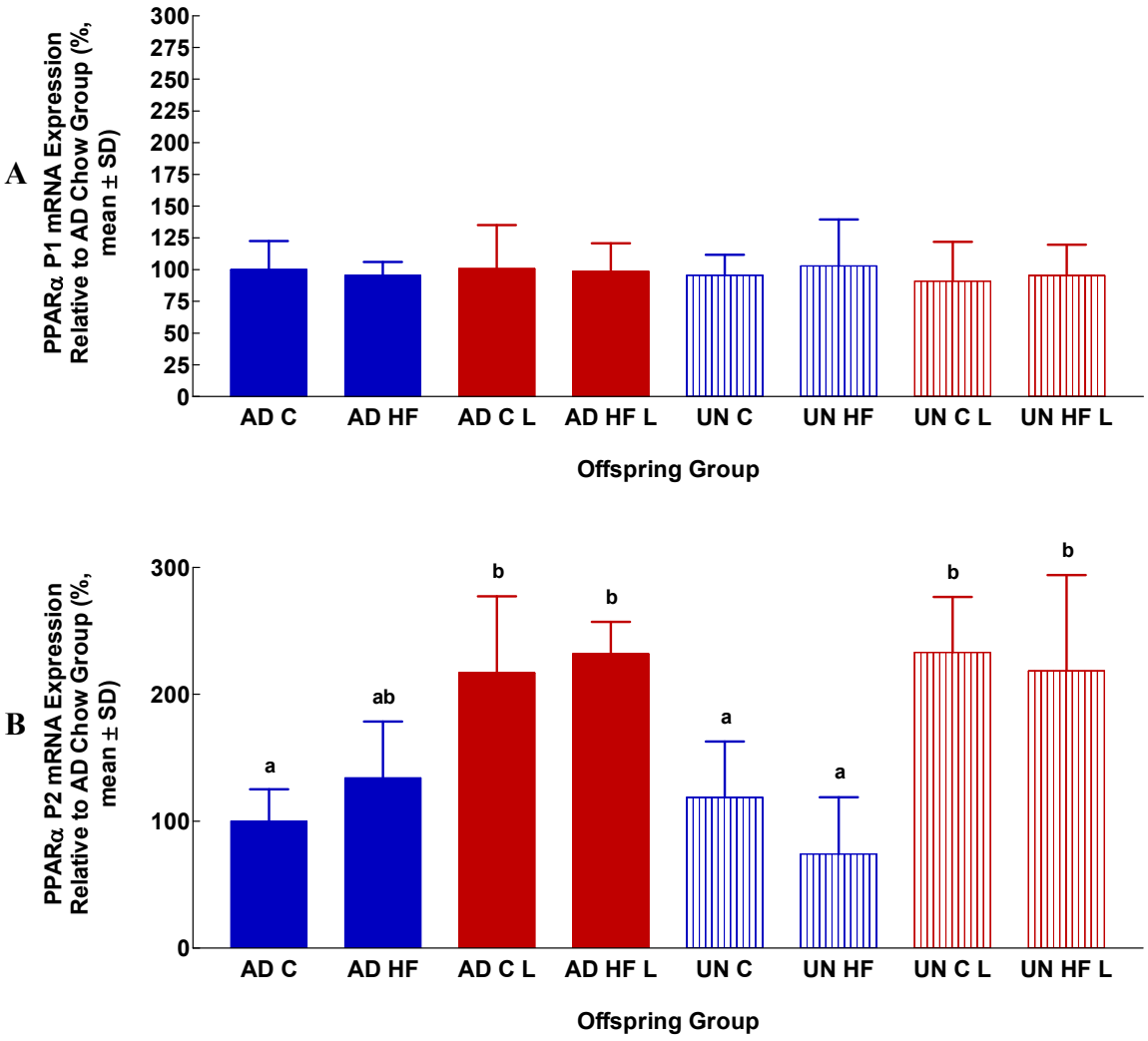


Figure 6.3 The expression of the P1 and P2 PPARα transcripts is not significantly altered by maternal diet or postnatal diet in the adipose tissue of adult female offspring. The expression of the PPARα P2 specific transcript but not the P1 specific transcript is significantly altered by neonatal leptin treatment. Real time PCR data for expression of PPARα P1 Transcript (A) and PPARα P2 Transcript (B) are shown in response to maternal diet (ad libitum [AD] and undernutrition [UN]), postnatal diet (Chow [C] and High Fat [HF]) and neonatal leptin treatment (Leptin [L]) (n=8/group). Values with different letters are significantly different ($p<0.05$) by Bonferroni's post hoc test.

Table 6.1 Table showing tests of between subject effects on the mRNA expression of PPARα P1 and P2 in the adipose tissue of female offspring. Single factor effects (MD, T, PND) and interaction effects (MD*T, MD*PND, T*PND, MD*T*PND) are shown. Significant effects (P<0.05) are indicated in red. There were no significant single factor or interaction effects for P1. There was a single factor effect for P2 (T). There were no interaction effects for P2.

Variable	Anova	Maternal Diet (MD)	Treatment (T)	Postnatal Diet (PND)	MD* T	MD* PND	T* PND	MD* T* PND
P1	0.991	0.716	0.772	0.859	0.561	0.504	0.991	0.853
P2	0.000	0.532	0.000	0.802	0.389	0.144	0.901	0.175

6.2.3 MatInspector analysis of the unique region of the liver specific P2 PPARα promoter indicates an absence of Stat3 binding elements but the presence of a potential Sp1 binding site

In order to determine potential areas of importance for regulation of PPARα transcription by leptin, the unique region of the P2 promoter, i.e. that which is not present in the P1 promoter and therefore responded to leptin treatment, was analysed for response elements which could mediate leptin signaling. This sequence had previously been analysed in chapter 5 by the MatInspector program [264] for core promoter elements and transcription factor binding sites and this is shown just for the unique figure 6.4. Only core promoter elements or relevant transcription factor binding sites with the maximum core similarity of 1.0 are shown, i.e. the highest conserved bases of a response element match the sequence exactly.

MatInspector did not identify any Stat3 binding elements (SBE) within the defined promoter regions. Several putative transcription factor binding sites were identified by the MatInspector software in the unique region of the P2 promoter; VDR/RXR, Sp1, 2 Myc/Max and E2F/DP1, whilst 3 possible core promoter elements were identified; XCPE1, BRE and MTE. As identified in chapter 5, the core promoter elements are not located in their normal positions relative to TSS. The only response element present known to signal the effects of leptin is the Sp1 consensus: 5'-(G/T)GGGCGG(G/A)(G/A)(C/T)-3'. As reports have previously shown that Stat3 can activate promoters via a Stat3/Sp1 cooperative mechanism [282,283], this analysis indicates that the P2 promoter may respond to leptin treatment via interaction of Stat3 with Sp1 at a site located in the unique region of the P2 promoter.

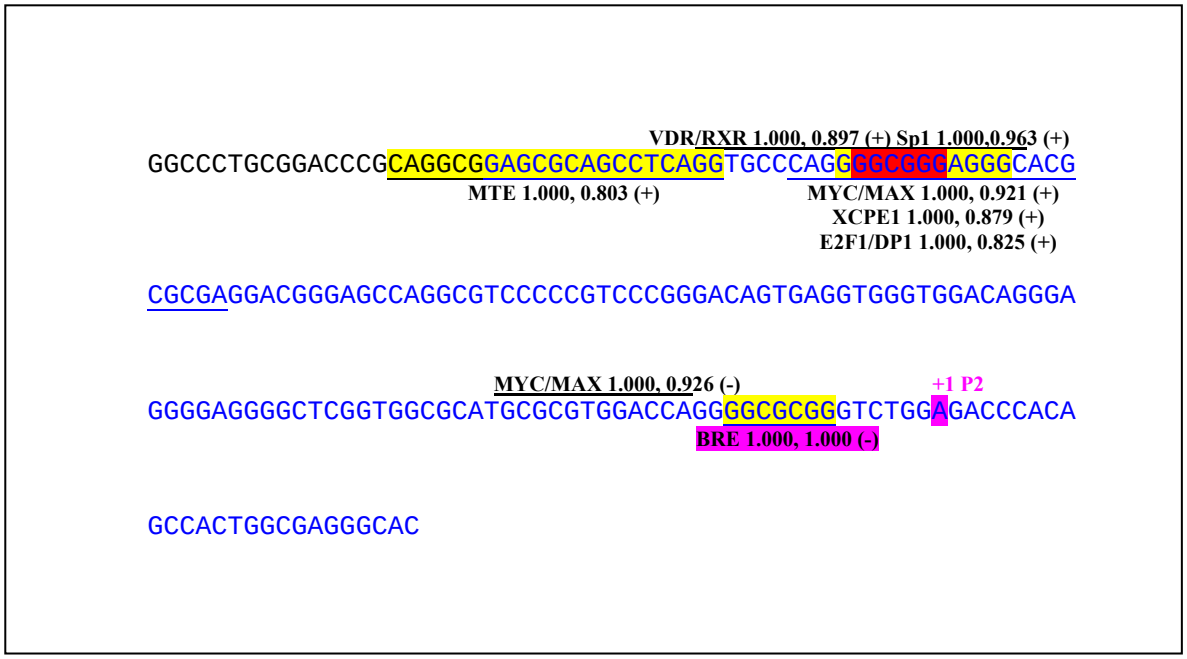


Figure 6.4 The sequence of the "unique" region of the P2 promoter indicating the location of potential core promoter elements and transcription factor binding sites. The sequence of the unique region of the P2 cloned promoter region is shown. The region unique to the P2 promoter is shown in blue text and the P2 transcription start site is indicated in pink. TF binding sites and core promoter elements are underlined. The Sp1 binding site is highlighted red and core promoter elements in yellow. All sites are scored and marked with the strand they are located on (+ or -). The core similarity of the sequence is the first number. The maximum core similarity of 1.0 is only reached when the highest conserved bases of a matrix match exactly in the sequence and only those sites with a score of 1.0 are shown. The second figure is the matrix similarity which takes into account all bases over the whole matrix length. A perfect match has a score of 1.00 and a "good" match has a score of >0.80. Perfect sequences are highlighted in purple.

6.2.4 A highly potent Stat3 inhibitor prevents activation of the liver specific P2 PPARα promoter by leptin

To verify that leptin activation of the P2 promoter is via a Stat3 signalling mechanism, a highly selective, potent Stat3 inhibitor (PpYLKTK-mts) was employed in transfections to inhibit signalling specific to Stat3. To investigate the P2 promoter response to leptin in conjunction with Stat3 inhibitor, the P2-pGL3 luciferase reporter construct was transfected into HepG2 cells and subjected to treatment for 24 hours with 1000ng/ml leptin in the presence and absence of the Stat3 inhibitor. In addition, the P1 promoter which previously did not increase in activity in response to leptin treatment and the promoter-free pGL3 basic vector were transfected as controls. The response of the promoters to leptin and Stat3 inhibitor treatments are shown in figure 6.5.

Results indicated that the control pGL3 basic vector was inactive in HepG2 cells and did not respond to treatment with leptin or Stat3 inhibitor. As previously shown, leptin treatment significantly increased P2 activity from 50.39 in untreated cells, to 121.87 in leptin treated cells (P=0.01) and this activity increased to 170.86 (P=0.001) with 1nM Stat3 inhibitor alongside the leptin treatment (figure 6.5). There was no significant difference in P2 activity in leptin treated cells with a Stat3 inhibitor concentration of 10nM, 100nM, 1μM or 10μM when compared to untreated cells. Consistent with previous results, leptin treatment did not increase activity of the P1 promoter. When Stat3 inhibitor was given in addition to the leptin treatment, there was again no significant difference in P1 promoter activity, except at a concentration of 100nM, whereby activity was reduced from 73.37 in untreated cells to 23.89 (P=0.05). These data indicate that the Stat3 inhibitor can specifically prevent activation of the P2 promoter by leptin at concentrations of 10nM or higher.

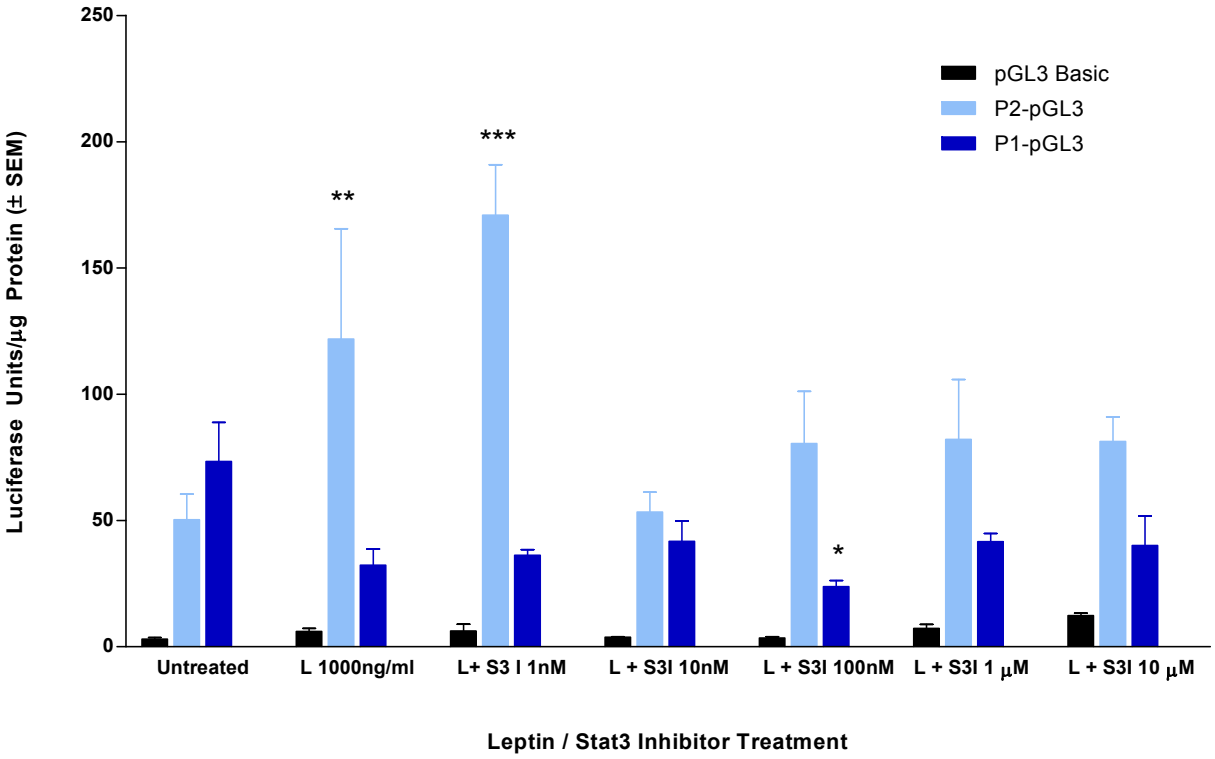


Figure 6.5 Stat3 inhibitor specifically prevents the activation of the PPARα P2 promoter by leptin. Activities of the alternative PPARα promoters (P1 and P2) and control (pGL3 Basic empty vector) are shown in response to 1000ng/ml leptin treatment (L) ± increasing concentrations of a Stat3 inhibitor (S3I) (n=3)**.** *p<0.05, ** p<0.001, *** p<0.001 relative to untreated (Bonferoni's Test).

6.2.5 Mutation of the Sp1 response element present within the unique region of the liver specific P2 PPARα promoter prevents its activation by leptin

To determine if the Sp1 site within the unique region of the P2 promoter is involved in Stat3 mediated leptin signalling (figure 6.6a), promoter mutants were prepared in which the Sp1 response element was mutated to prevent Sp1 binding (P2 Sp1M-AT and P2 Sp1M-R1). For the Sp1M-AT mutant the internal GC was mutated to AT, whilst for the R1 mutant the whole consensus sequence was converted to an EcoR1 restriction site (GAATTC). To investigate the activity of the mutant P2 promoters in response to leptin treatment, the promoter constructs P2 Sp1M-AT, P2 Sp1M-R1 and the wildtype P2-pGL3 construct were transfected into HepG2 cells subjected to treatment for 24 hours with 1000ng/ml leptin. The response of the promoters to leptin treatment is shown in figure 6.6.

Activity of the wildtype P2 promoter increased in response to 1000ng/ml leptin treatment to 241% compared to untreated cells. Although this was a clear increase in P2 activity, it did not reach significance as found in previous experiments. Similarly, activity of the P2 Sp1M-AT mutant increased to 294% in cells treated with 1000ng/ml leptin compared to untreated, but again, this did not reach significance. However, activity of the P2 Sp1M-R1 mutant promoter did not increase with 1000ng/ml leptin treatment and instead there was a small drop in activity to 73% of the activity of untreated cells. Although none of the results reached significance, the clear trends indicate that the P2 Sp1M-R1 mutant could prevent activation of the P2 promoter by leptin, whereas the AT mutant was unable to prevent activation of the PPARα P2 promoter by leptin.

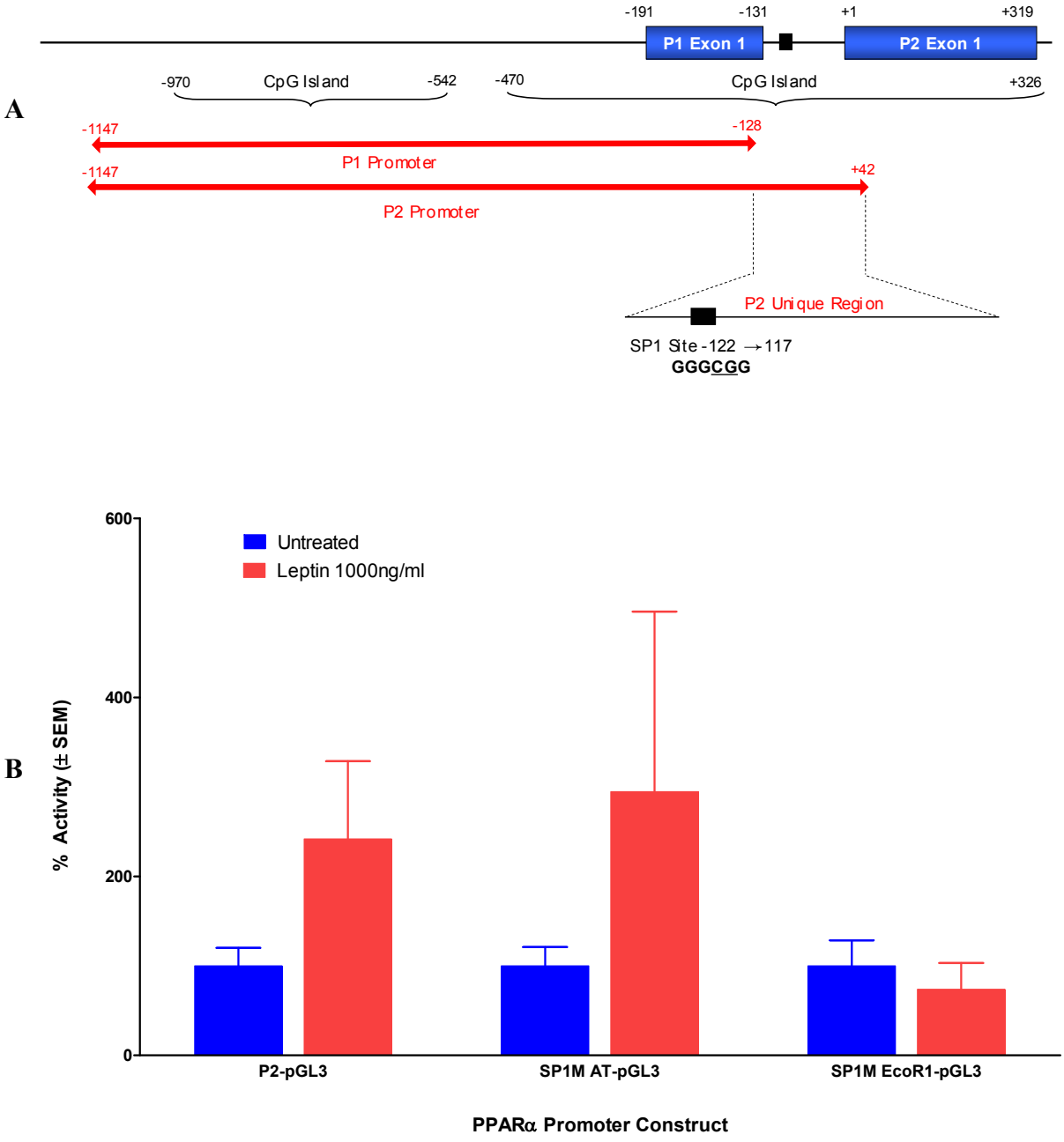


Figure 6.6 Mutation of the Sp1 response element present in the unique region of the liver specific P2 PPARα promoter prevents its activation by leptin. (A) The unique region of the P2 promoter contains an Sp1 site which could potentially by involved in the leptin mediated activation of this promoter. (B) Activities of the P2 PPARα promoter, the mutant Sp1M-AT and the Sp1M-RI P2 promoter ± 1000ng/ml leptin treatment are shown (n=3).

6.2.6 Methylation of the P1 and P2 PPARα luciferase promoter constructs abolishes all promoter activity

To determine if any of the PPARα promoter constructs are sensitive to CpG methylation, the P1-pGL3, P2-pGL3, P3-pGL3 and the pGL3 Basic control constructs were all methylated with HpaII methylase and CpG methylase. In addition, all the promoter constructs were mock methylated as a control. To investigate the effect of methylation on the activity of these promoter constructs, they were transfected into HepG2 cells and their activity assessed. The response of the promoters to each form of methylation is shown in figure 6.7.

Results showed that the pGL3 basic vector, which contains no promoter or enhancer sequences, was inactive and not significantly affected by either CpG or HpaII methylation. In addition, the P3-pGL3 construct was also inactive and unresponsive to both CpG and HpaII methylation, with levels consistently similar to that of the pGL3-Basic control. There was therefore no effect of methylation on the activity of the control or P3 promoter in HepG2 cells. Mock methylation did not significantly affect the activity of any of the promoter constructs.

The P1 and P2 promoters were both active in HepG2 cells. In addition, the activity of both the P1-pGL3 and P2-pGL3 promoter constructs was completely abolished by both HpaII and CpG methylase treatment (figure 6.7). HpaII methylation resulted in the methylation of 11 CpGs in the P1 promoter and 12 CpGs in the P2 promoter. HpaII methylase decreased activity of the P1-pGL3 construct from 58.9 in untreated cells to 0.81 ($p=0.001$), whilst it decreased the activity of P2-pGL3 from 67.46 in untreated cells to 2.49 ($p=0.001$). CpG methylation resulted in methylation of 98 CpGs in the P1 promoter and 114 CpGs in the P2 promoter. CpG methylase decreased activity of the P2-pGL3 construct from 67.46 in untreated cells to 0.62 ($p=0.001$), whilst it decreased the activity of P1-pGL3 from 58.9 in untreated cells to 0.64 ($p=0.001$). Therefore, both the P2 and P1 promoters were highly sensitive to methylation.

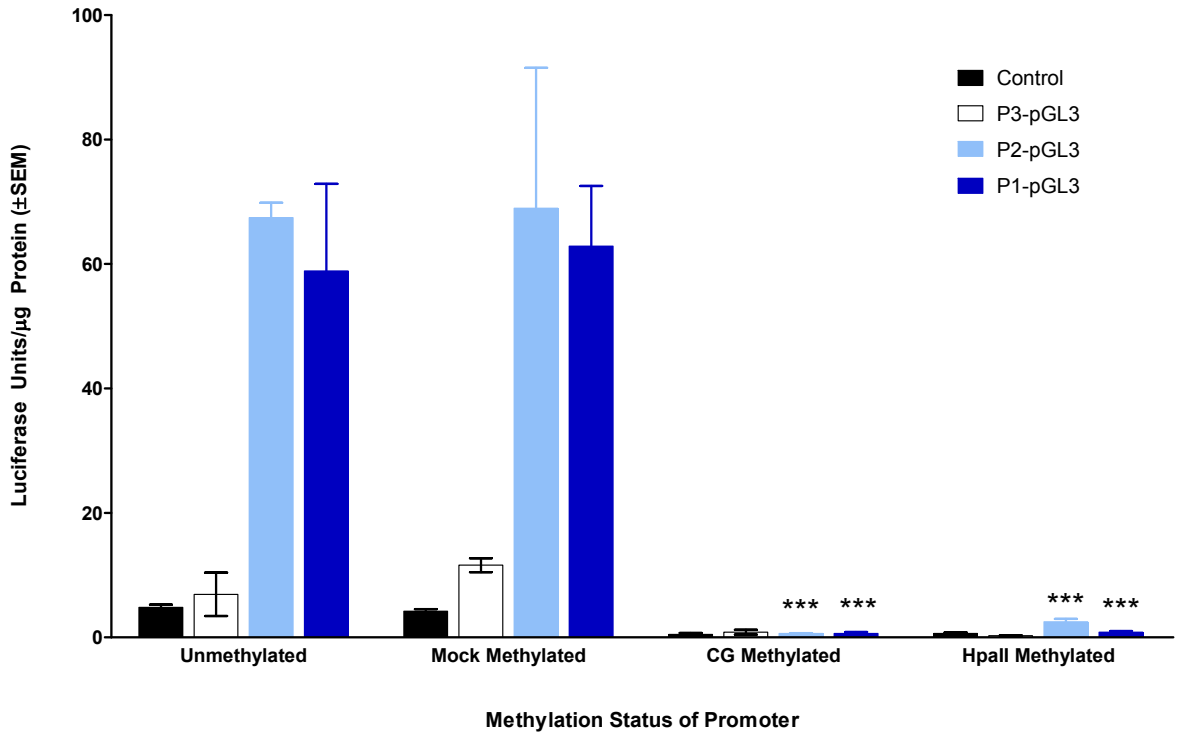


Figure 6.7 Methylation of the P1 and P2 PPARα promoter constructs by HpaII methylase and CpG methylase prevents all promoter activity. Activities of the alternative PPARα promoters (P1-pGL3 and P2-pGL3 and P3-pGL3) and control (pGL3-basic) are shown in response to methylation with CpG methylase and HpaII methylase (n=3)**.** *** $p<0.001$ relative to unmethylated (Bonferroni's Test).

6.2.7 Pyrosequencing analysis of 30 CpGs within the proximal CpG island of the PPARα P2 promoter in adipose tissue of adult female offspring fed a high fat diet indicates that all CpGs are unmethylated and unaltered by neonatal leptin treatment

In order to ascertain if the methylation status of individual CpGs within the P2 promoter were modified in response to neonatal leptin treatment in the adipose tissue of adult female offspring from the IUGR rat study, pyrosequencing was performed using genomic DNA extracted from adipose tissue of AD offspring fed a HF diet given either saline or leptin treatment (n=8 / diet-treatment group). Pyrosequencing was performed with validated bisulfite PCR and sequencing primers using bisulfite converted genomic DNA as a template. Due to technical limitations, the whole of the promoter could not be optimised for pyrosequencing. The area of the PPARα promoter successfully pyrosequenced was within the CpG island and MSP region upstream of the P2 transcription start site (TSS) and included the CpG within the Sp1 response element in the P2 unique region (figure 6.8). Bisulfite treatment controls were included in the pyrosequencing assay design and the allowed percentage of unconverted bisulfite DNA for passed quality was set at the standard 4.5%. Resulting methylation data were analysed by the students unpaired T-test. Graphs showing differences in methylation for individual CpGs within the PPARα promoter with statistical analysis can be found in figures 6.9-6.10.

Analysis of PPARα methylation by pyrosequencing indicated that methylation of the PPARα promoter was low throughout the two amplicons, with all CpGs possessing methylation levels below 10%, regardless of treatment. As the bisulfite conversion threshold was set at 4.5%, any CpGs which had methylation levels below this were too low to be measured reliably by this technique. Therefore, for this reason, although methylation was significantly affected by neonatal leptin treatment for several CpGs (CpG 7, 11, 12, 17), as all these were below the bisulfite conversion threshold they were not reliable data. Furthermore, all the remaining CpGs with methylation levels above the threshold showed no significant alterations in methylation in response to neonatal leptin treatment.

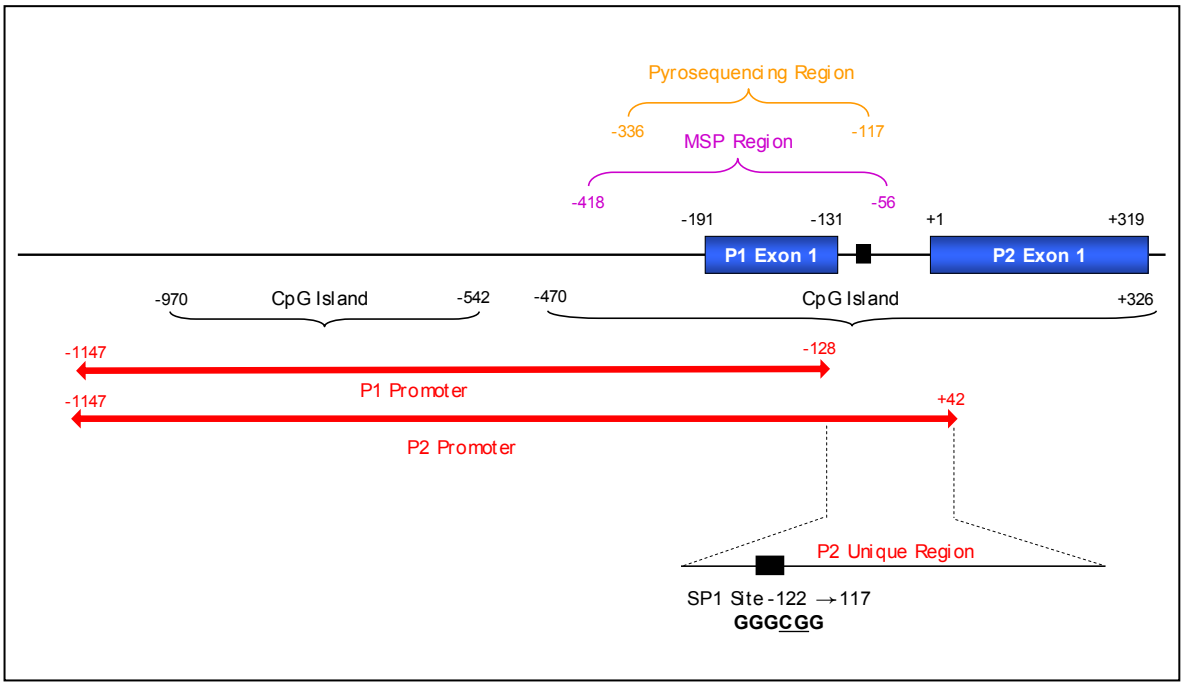


Figure 6.8 Location of the PPARα pyrosequencing region relative to P1 and P2 TSS, unique region of P2 promoter, MSP region and CpG islands. The PPARα pyrosequencing region is upstream of the P2 TSS within the PPARα MSP region and includes the Sp1 site present in the unique region of the P2 promoter.

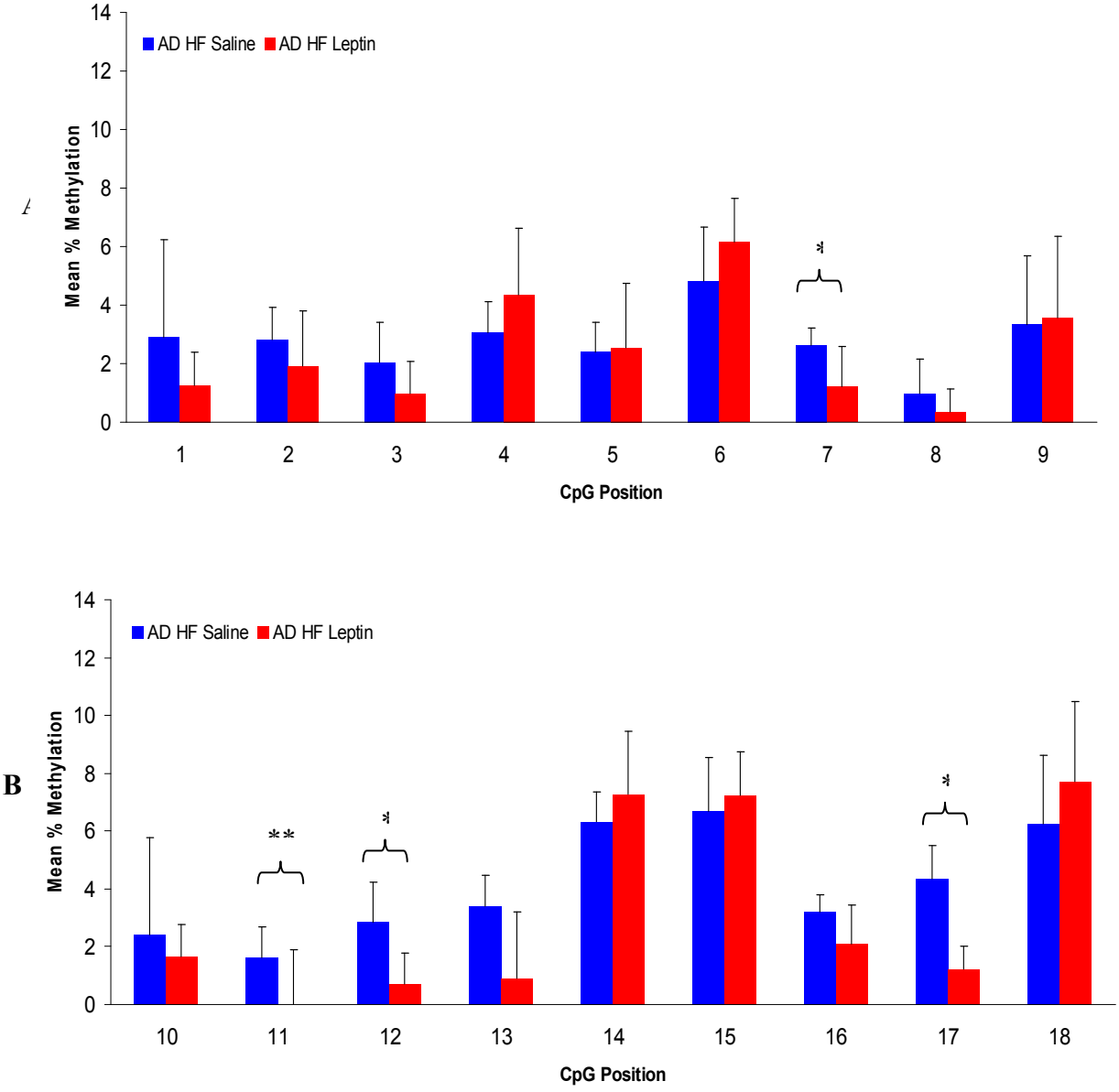


Figure 6.9 Pyrosequencing analysis Of CpGs 1-18 within the PPARα promoter in adipose tissue of adult female offspring fed a high fat diet confirms that all CpGs are unmethylated in offspring given both saline or leptin treatment. Pyrosequencing data showing mean % methylation levels ± SD for CpGs 1-9 (A) and 10-18 (B) of the PPARα promoter are shown offspring from *ad libitum* High fat [AD HF] offspring given either saline or leptin treatment (n=8/group). *p<0.05 **p<0.01 (Students unpaired T-Test).

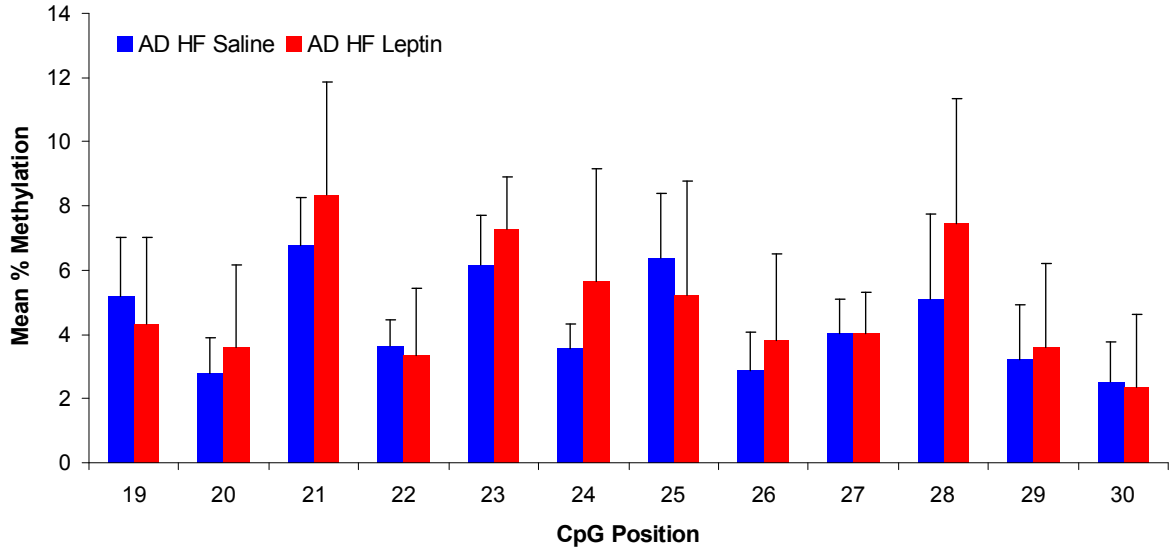


Figure 6.10 Pyrosequencing analysis of CpGs 19-30 within the PPARα promoter in adipose tissue of adult female offspring fed a high fat diet confirms that all CpGs are unmethylated in offspring given both saline or leptin treatment. Pyrosequencing data showing mean % methylation levels ± SD for CpGs 19-30 of the PPARα promoter are shown offspring from *ad libitum* High fat [AD HF] offspring given either saline or leptin treatment (n=8/group).

6.3 Discussion

In an established rat model of programming, IUGR offspring fed a high fat diet become obese and present with metabolic disturbances in adult life, including hyperphagia, hyperleptinemia and hyperinsulinism. Giving these offspring leptin treatment during a critical period of neonatal development has been shown to prevent the weight gain and associated disturbances found in these adult offspring [122]. This indicates that the early leptin treatment facilitates a reprogramming of energy balance, which results in a reduced capacity to store fat within adipocytes throughout life. Mechanisms by which the neonatal leptin treatment may influence the capacity to store fat may be mediated centrally, e.g. by reduced feeding, but it is also possible that the leptin may act directly on peripheral tissues to mediate its effects on bodyweight. This hypothesis is supported by the finding that disruption of leptin receptor signalling on adipocytes results in increased fat mass in mice when fed a high fat diet despite normal hypothalamic leptin signalling [244]. Furthermore, in adult rats leptin can induce a novel form of lipolysis in which rats are depleted of all visible body fat and this has been shown to occur via direct action of leptin on the adipocyte [232]. These studies both indicate that adipose specific leptin signalling plays an important role in leptins ability to reduce body fat.

Work in Chapter 4 of this report has shown that neonatal leptin treatment results in persistent alterations in the expression of genes involved with energy balance in the adipose tissue of adult female rats, including an increase in the expression of leptin Receptor, PPARα and it target genes CPT-1 and AOX. Upregulated leptin receptor indicates that increased leptin signalling may be responsible for these gene expression changes. In addition, as PPARα and its target genes are not normally expressed in adipose tissue, this increased expression may play a fundamental role in the reduced weight gain observed in these rodents. It was therefore hypothesised that in the IUGR rat study, the neonatal leptin treatment may have acted directly on the adipocytes to influence the expression of genes involved in energy balance such that reduced TAG storage took place. However, for this hypothesis to be valid, the leptin treatment must have acted in a manner to persistently increase the expression of such genes. Such an effect could involve the epigenetic modification of the gene promoters, for example DNA methylation. Evidence exists that PPAR's including PPARα are sensitive to methylation changes during development brought on by altered maternal nutrition [77,212]. It is therefore possible that the transcription factors themselves may be altered in response to the neonatal leptin treatment.

It remains to be determined if epigenetic alterations are responsible for the changes in PPARα expression, or if altered leptin receptor signalling is responsible. Furthermore, the exact mechanisms of the regulation of PPARα transcription by leptin are unknown. The aim of this chapter was therefore to elucidate the mechanism by which leptin is able to increase the expression of PPARα and to determine if alterations in DNA methylation of the PPARα promoter explain how

the effects persist through to adulthood. To address these questions a reporter gene strategy was employed to study the regulation of PPARα by leptin and Pyrosequencing was performed to assess the methylation status of the PPARα promoter.

Work in this Chapter showed that the alternative PPARα promoters differed in their response to leptin treatment in vitro and were also shown to be differentially regulated by neonatal leptin treatment in vivo. The mechanism underlying the regulation of PPARα promoter by leptin was investigated and was found to function via a non-canonical mechanism which involved Sp1. Furthermore, the PPARα promoter was shown to be sensitive to methylation in vitro, but in offspring from the IUGR rat study a significant region of the PPARα promoter was shown to be unmethylated in both control and leptin treated offspring.

It is known that leptin treatment can increase the expression of PPARα. For example, it has been shown in Chapter 4 that neonatal leptin treatment results in increased PPARα expression in the adipose tissue of adult offspring, whilst leptin treatment to differentiating 3T3 adipocytes also increased PPARa expression. The exact mechanisms and location of response elements responsible for this activation of PPARα by leptin are not known. To determine how leptin is able to alter PPARα promoter activity, a reporter gene strategy was performed using the liver specific (P2 and P3) and adipose specific (P1) PPARα promoters.

Interestingly, the response of the adipose specific (P1) and liver specific (P2) promoters to leptin differed. As expected, leptin treatments activated PPARα transcription in a dose dependent fashion, but only of the P2 specific promoter, whereas activity of the P1 promoter remained unchanged. As the P2 promoter is identical to the P1 promoter apart from an additional 170bp at the 3' end, response elements responsible for leptin activation of the P2 promoter must be present in this unique region. Furthermore, the pharmacological amount of leptin required to activate PPARα promoter activity is consistent with the high amounts needed to induce fatty acid oxidation in adipocytes of adult rats and also with the levels given to neonate rats to reverse programming. To confirm that this effect of leptin is direct, cyclohexamide could be employed in transfections. Furthermore the use of Actinomycin D, which prevents transcriptional elongation, could be used to confirm that leptins effects are at the transcriptional level.

Next, the mechanism by which leptin treatment affects PPARα promoter activity was investigated. Studies have indicated that leptin can induce a variety of signalling mechanisms in adipose tissue to alter target gene expression, including Jak/Stat signaling [121,244,279-281], MAPK signaling [244,279] and AMPK signaling [243]. It was therefore possible that several signalling mechanisms could be involved in activating PPARα transcription, although evidence suggests that Stat3 is the most likely mechanism to activate PPARα expression as a result of leptin mediated lipolysis [239]. It was

therefore possible that in the IUGR rats, leptin mediated Stat3 signalling was responsible for the proposed lipolysis occurring in the adipose tissue.

To determine if leptin was able to affect PPARα transcription by Stat3 signaling, the unique region of the P2 promoter sequence was initially searched for Stat3 binding elements (SBE). However, upon inspection of the unique region of this promoter, no SBE were identified. However, several papers have identified a novel transcriptional activation mechanism for leptin Stat3 in SBE free promoters, whereby Stat3 has been shown to function cooperatively with Sp1 to activate transcription without itself binding to DNA, i.e. by a Stat3/Sp1 cooperative mechanism [282,283]. Given these data, it was hypothesised this could be the case for PPARα as its promoter is very GC rich and indeed MatInspector analysis identified a Sp1 response element within the P2 promoter unique region. So it was proposed that leptin activates the liver PPARα P2 promoter via a non-canonical co-operative Stat3-Sp1 mechanism.

Firstly, the role of Stat3 in PPARα signaling was assessed by employing a Stat3 inhibitor in transfections. If Stat3 was involved in leptin signaling of PPARα, then Stat3 inhibitors should abolish the effect of leptin treatment. Indeed, results indicated that a specific Stat3 inhibitor at low concentrations prevented activation of the P2 promoter by leptin, indicating that Stat3 signaling was involved in PPARα transcription despite the lack of a SBE in the unique promoter region.

To determine whether the Sp1 consensus sequence within the P2 unique region was directly involved in the activation of PPARα promoter by Stat3 signaling, it was subjected to site directed mutagenesis to prevent any Sp1 binding to DNA, therefore preventing any Stat3 mediated activation of the promoter. Mutation of the internal GC of the Sp1 site to AT did not prevent activation by leptin. However, a more severe mutation of the Sp1 site to an EcoRI restriction site prevented activation of the PPARα P2 promoter by leptin, indicating that this site could be involved in Stat3 mediated leptin signaling. By mutating this site, the binding of several transcription factors predicted to bind by MatInspector could also be affected, but as the Stat3 inhibitor prevented activation by leptin, in conjunction with previous findings of Sp1 to mediate Stat3 activation [282-284], it is probable that that Sp1 is the transcription factor involved. Collectively, these two results indicate that leptin induces PPARα gene expression by Stat3 signaling mechanisms which act at the unique region of the PPARα P2 promoter via a non-canonical Sp1 mediated mechanism.

To absolutely confirm the role of Sp1 in Stat3 mediated leptin signaling, additional work could be performed. This should firstly confirm the role of Sp1, such as the use of Sp1 RNAi to verify its role in activation of PPARα transcription. More importantly, Stat3/Sp1 protein/DNA binding needs to be demonstrated upon activation by leptin. This could be verified by two complementary methods; firstly by electromobility shift assay (EMSA) to show that Stat3/Sp1 binds the unique

region of the PPARα promoter in leptin treated cells and secondly performing a transient ChIP using Sp1/Stat3 antibodies to show binding at promoter upon leptin activation. Furthermore, co-immunoprecipitation could show physical interaction between the two proteins.

Despite the finding that Stat3 is involved in leptin activation of PPARα, other signaling mechanisms cannot be ruled out, in particular AMPK, which has been found to induce PPARα transcription in skeletal muscle [287,288]. It is therefore possible that this signaling mechanism could occur in adipose tissue. It was suggested that AMPK induces PPARα expression via Sp1 [288], however, the finding that Stat3 inhibitor prevents activation of the PPARα promoter in this chapter argues against AMPK activation of PPARα. Furthermore, it has been shown that hyperleptinemia induced in adult mice to reduce fat mass does not alter AMPK activity in adipose tissue [239]. Another potential mechanism is MAPK signaling, which has been found in response to leptin in adipose tissue [244,279]. However no response elements which could mediate the effects of this signaling cascade, such as AP1, were identified in the unique region of the P2 promoter.

Work in Chapter 4 found that the expression of PPARα is up-regulated in the adipose tissue of adult offspring given neonatal leptin treatment. As PPARα has been shown to have tissue specific transcripts resulting from alternative promoter usage, it was hypothesised that the increase in PPARα expression seen in these leptin treated offspring would be due to an increase in the adipose specific transcript, P1. However, real time PCR with transcript specific primers showed that the increase in PPARα expression in these offspring was not due to an increase in the adipose specific P1 transcript, but due to an increase in the liver specific P2 transcript. It would have been interesting to determine if the liver specific P3 transcript was also up-regulated by neonatal leptin treatment, however RT-PCR primers could not be designed in the novel exon 2a. Expression of the remaining heart and kidney specific transcripts was not performed as they were identified at the end of the study.

These experiments have therefore shown that the effects of leptin treatment are the same in vitro as in vivo, i.e. leptin can activate PPARα gene transcription, but only via the liver specific P2 promoter. These results imply that neonatal leptin treatment during a period of adipose tissue plasticity somehow facilitates a switch from the unresponsive adipose specific P1 promoter to the responsive liver specific P2 promoter, thereby facilitating an increase in PPARα gene expression in a tissue in which it is not normally expressed.

Persistent alterations in gene expression can be explained by epigenetic mechanisms such as altered DNA methylation. In general, hypomethylation can result in increased gene expression, whilst hypermethylation can lead to reduced gene expression. Therefore, one mechanism which may explain the long term increase in PPARα gene expression in response to neonatal leptin treatment is a reduction in the methylation status of its promoter. Altered methylation of the PPARa promoter

activity in response to nutritional status is not a novel concept, as it has previously been found to be altered in response to maternal nutrition in PR rats [77,212]. Of particular interest is the methylation status of the Sp1 response element in the PPARa promoter which is involved in directing transcription of the liver specific P2 promoter by Stat3 mediated leptin signaling, as the binding of Sp1 sites to its response element have previously been shown to be sensitive to DNA methylation [194,195]. Furthermore, it has been shown that methylation of a single CpG just upstream of Sp1 site involved in Stat3 mediated POMC signaling is altered in DIO, facilitating reduced expression of POMC in response to leptin [185]. Therefore, it was hypothesised that leptin treatment may result in reduced methylation of the CpG within the Sp1 site in the unique region of the P2 promoter, facilitating the potential for increased P2 PPARα expression when stimulated.

In Chapter 4 of this report, the use of MSP to investigate methylation of the PPARa promoter in the IUGR offspring failed to show any differences in DNA methylation between treatment groups. However, this technique is a crude measure of methylation status and areas of methylation are masked by unmethylated CpGs. This is important, because evidence indicates that alterations in DNA methylation as a result of environmental stress during development such as altered nutrition tend to be specific to certain CpGs rather than a broad effect on methylation [208,212] and would therefore be missed by MSP. More sensitive methods exist that can measure methylation of a single CpG, such as pyrosequencing, a sequencing by synthesis technology which relies on the ability of bisulfite treatment to differentiate between methylated and unmethylated cytosine.

Firstly, to determine if the PPARα promoter itself is sensitive to alterations in DNA methylation, the tissue specific promoters were chemically methylated and used in a reporter gene experiments. As expected, these experiments unequivocally showed that both P1 and P2 PPAR promoter activity is sensitive to methylation of CpGs within their promoters. Next, the methylation status of individual CpGs within the PPARα promoter were assessed in response to leptin treatment in AD offspring fed a HF diet. The original intention was to pyrosequence as much of the PPARa promoter as possible. However, due to the considerable GC nature of the promoter, the region which could be sequenced was limited to the area previously analysed by MSP, but contained the CpG of interest within the Sp1 response element. However, pyrosequencing of both control and leptin treated offspring indicated that for all the CpGs measured, methylation levels were generally low and although leptin treatment did result in reduced methylation at some CpGs, any differences in methylation were not significant. However, it is quite possible that within the cell populations, the CpGs in some individual cells may have been methylated and alterations in the methylation of these CpGs by leptin treatment may have induced larger alterations in gene transcription.

Therefore all epigenetic studies performed on the PPARα promoter in this report indicate that alterations in the DNA methylation of this particular region of the PPARa promoter does not underlie the persistent increase in PPARa expression found in leptin treated offspring. However, it

cannot be ruled out that methylation of downstream CpGs could affect Sp1 binding and therefore activation of the P2 promoter, but unfortunately initial attempts to pyrosequence this region were unsuccessful. However, it is also possible that the long term changes in PPARα gene expression may be due to altered signalling upstream of PPARα, for example altered leptin receptor signalling on the adipocytes. Future work should therefore involve pyrosequencing the leptin receptor to look at its methylation status in response to leptin treatment.

In addition, it would also be interesting to determine if Polycomb group (PcG) proteins could be involved in expression of PPARα in adipose tissue. Components of the PcG complex are able to direct methylation of lysine 27 of histone 3, thus inducing a repressive state without the need for DNA methylation [191] and have been shown to be involved in both genomic imprinting and X chromosome inactivation [161]. This repression is reversible and therefore in the context of the IUGR rats, it is possible that neonatal leptin treatment somehow causes a loss of PcG repression, facilitating an increase in PPARα expression. Furthermore, the removal of PcG repression could account for the switch in promoter usage in adipose tissue if the repression was associated with the liver P2 promoter. The participation of PcG in PPARα transcription could be determined by performing a CHiP directed at methylated H3 K27 in the leptin treated and the control offspring.

The possible role of PcG proteins is further supported by the fact that PcG proteins tend to bind the promoters of genes encoding transcription factors, in particular those involved with development. Loss of PcG regulation of the PPARa promoter could therefore provide an epigenetic mechanism which could account for the maintenance of increased gene expression over time and cell divisions despite the absence of the initial stimulator, i.e. leptin.

Chapter 7

Discussion

7.0 Discussion

Over the past 20 years, evidence has accumulated from both human epidemiological data and animal studies, linking an adverse prenatal environment due to factors such as poor maternal nutrition, with an increased risk of disease in later life, including; obesity, insulin resistance, dyslipidemia and hypertension. Molecular studies have linked these altered adult phenotypes to persistent alterations in the expression and methylation of genes involved in key metabolic pathways, such as the transcription factors GR and PPARα. One study which employs a well established rat model of global undernutrition, the IUGR rat, results in offspring which are obese with associated metabolic disturbances including hyperphagia, reduced locomotor activity, hyperinsulinemia and hyperleptinemia. Intriguingly, it has been found that neonatal leptin treatment can normalise all these disturbances and reduce the excessive weight gain seen in these adult offspring.

Work in this study has focused on two key areas. Firstly, using the IUGR rat model, this study investigated the expression and DNA methylation status of genes involved in energy balance in the liver and adipose tissue of offspring in response to maternal nutrition, postnatal nutrition and neonatal leptin treatment. Secondly, given the history of altered epigenetic regulation of PPARα in programmed offspring and its central role in fatty acid metabolism, this study also mapped, cloned and characterised the PPARα promoter and investigated its regulation by leptin.

7.1 Summary of main findings

Results from this study revealed that there were no effects of maternal undernutrition or postnatal nutrition on the expression of several key genes involved in energy balance in the liver or adipose tissue. Contrastingly, neonatal leptin treatment was able to induce persistent changes to the expression of these key genes in adipose tissue, including the transcription factors PPARα, PPARγ and their target genes. However, there was no evidence of altered DNA methylation in the promoters of these genes, therefore differential DNA methylation in the measured regions did not underpin the long term effects induced by leptin treatment.

The rat PPARα promoter was mapped, cloned and characterised in order to investigate its regulation by leptin. As part of this process, six PPARα mRNA variants were identified, which differed in their 5'UTR due to differential promoter usage and transcription start sites. The 5'UTR of the six transcripts were shown to differ in their structure, length, GC content, free folding energy and presence of uATG and uORF, indicating that they may differ in both their transcriptional and translational regulation, thus allowing PPARα regulation to be more versatile. Of these transcripts, one transcript was identified in adipose tissue (P1) and two were identified in the liver (P2 and P3)

and the promoters specific to these transcripts were cloned and characterised using a reporter gene strategy. They were shown to differ in their basal activity, response to known activators of transcription, and to leptin treatment. The adipose specific P1 promoter was found to be unresponsive to leptin treatment, whilst the liver specific P2 promoter was responsive to leptin treatment, owing to the differences in their promoter regions. The mechanism underlying the activation of the P2 PPARα promoter by leptin was investigated and shown to function via a non-canonical Stat3-Sp1 mechanism acting at a region that is unique to the P2 promoter. Furthermore, the increase in PPARα expression in the leptin treated rats was found to be due to an increase in the P2 transcript, whilst the P1 transcript remained unaltered.

7.2 Discussion of main findings

Unlike offspring from dams fed a PR maternal diet, no effects on gene expression were identified as a result of maternal undernutrition in the liver or adipose tissue from IUGR offspring. However, these are two very different animal models and it is likely that different genes, pathways or tissues may have been affected by the maternal undernutrition in the IUGR rat as opposed to the PR rat. It is possible that the maternal undernutrition induced a PAR, in which offspring predicted a poor postnatal supply of nutrients and adapted their metabolism to increase their capacity to store fat, for example by altering the regulation of genes of fatty acid synthesis and storage in liver and adipose tissue. It is also possible that leptin sensitivity may have been reduced in these offspring at the adipocyte, such as altered Socs-3 signaling downstream of leptin receptor. These features would be consistent with the predisposition to obesity and its associated metabolic disturbances in IUGR offspring. Furthermore, at the level of the hypothalamus, it is possible that the IUGR neonates were hypoleptinemic, which may have resulted in an absent or premature leptin surge. If this were the case, then hypothalamic function may have been compromised due to altered hypothalamic circuitry and gene expression, which would contribute to the resulting leptin resistance. Furthermore, evidence in other animal models indicates that high fat postnatal feeding may induce epigenetic alterations in the POMC promoter to induce leptin resistance, thus this may also be present in the IUGR offspring fed a high fat postnatal diet.

This study indicated that early leptin treatment facilitated a reprogramming of energy balance, which resulted in a reduced capacity to store fat within adipocytes throughout life, despite high fat feeding. It is therefore proposed, that leptin administration during early development acted both centrally and peripherally to mediate the reversal of programming seen in leptin treated offspring. As such, neonatal leptin treatment would have replicated the normal leptin surge to facilitate a restoration of normal hypothalamic wiring and gene expression, thus restoring leptin sensitivity and preventing the onset of hyperphagia and reduced energy expenditure in adult life. It is further proposed that the leptin treatment also had persistent effects at the level of the adipocyte. Two

mechanisms are proposed for the adipose specific effects of neonatal leptin treatment on PPARα expression identified in this study:

i) Leptin treatment may have induced epigenetic alterations which affected the liver specific P2 PPARα promoter in adipose tissue. Data from this study indicates that that leptin exerts a regulatory effect on specific PPARα promoters. Normally, in adipose tissue the PPARα P1 promoter is active (but at low levels), whilst the P2 promoter is inactive. Therefore one possible explanation for the increase in P2 PPARα expression in adipose tissue is that leptin induced epigenetic alterations which resulted in a selective change in PPARα promoter activation facilitating use of the responsive P2 promoter rather than the unresponsive adipose specific P1 promoter. Although no alterations in DNA methylation were identified in the region of the PPARα promoter measured, it is still possible that during development, the PPARα P2 promoter was silenced by DNA methylation in a region downstream to that measured, thus demethylation induced by leptin treatment may have activated expression of the P2 specific transcript. Alternatively, expression of the PPARα P2 transcript may be prevented in normal circumstances due to repression by PcG proteins. Thus PcG proteins would repress specifically at the unique region of the P2 promoter to maintain its silence in adipose tissue. Thus an alternative explanation for the effect of leptin treatment, is that it caused a loss of PcG repression specific to this unique region of the P2 promoter, facilitating an increase in liver specific P2 PPARα transcript in adipose tissue, thus enabling a switch in promoter usage from the unresponsive P1 promoter to the responsive P2 promoter in a tissue in which it is not normally expressed.

ii) Alternatively, the peripheral effects on PPARα expression may have been mediated by the persistent upregulation of leptin receptor on the adipocytes to increase leptin sensitivity, which resulted in increased Jak/Stat signaling. If true, then altered epigenetic mechanisms are likely to have been responsible for the increased leptin receptor expression. It is proposed that the increased Jak/Stat signaling upregulated the expression of the PPARα P2 transcript, via a non-canonical mechanism in which Stat3 interacted with Sp1 at the unique region of the PPARα P2 promoter. To achieve this, it is proposed that leptin activated Stat3, which either translocated to the nucleus to activate PPARα transcription by binding the pre-bound Sp1, or the activated Stat3 phosphorylated Sp1, translocated to the nucleus and bound the unique region of the PPARα promoter via Sp1. This therefore would facilitate a persistent increase in the expression of the P2 PPARα transcript in a tissue in which it is not normally expressed, whilst the P1 transcript would be unaffected.

In addition to PPARα upregulation, several other genes involved in energy balance were persistently increased in leptin treated IUGR offspring. Thus one theory which could support the real time data from this study is as follows: neonatal leptin treatment induced in an increase in the expression of PPARγ2 in adipocytes, which led to a corresponding increase in the expression of its target gene, Lpl. Lpl facilitated the uptake of fatty acids into the adipocyte from the triglyceride

component of lipoproteins in the blood, which would have normally been converted back to TAG for storage. Instead, in this model the PPARα P2 transcript was upregulated, which induced increased expression of its target genes; AOX and CPT-1, key rate limiting genes involved in fatty acid metabolism not normally expressed in adipocytes. As a result, the fatty acids taken up by the adipocyte were shuttled to the β-oxidation pathway as opposed to storage pathway (figure 7.1). This model explains both the phenotypic changes (i.e. reduced weight gain) and the gene expression changes found in the leptin treated programmed adult offspring. Thus the fat cell was transformed from a fat storing cell to a fat metabolising cell. Furthermore, this is a persistent effect owing to the specific period of leptin administration during a plastic phase of early neonatal development.

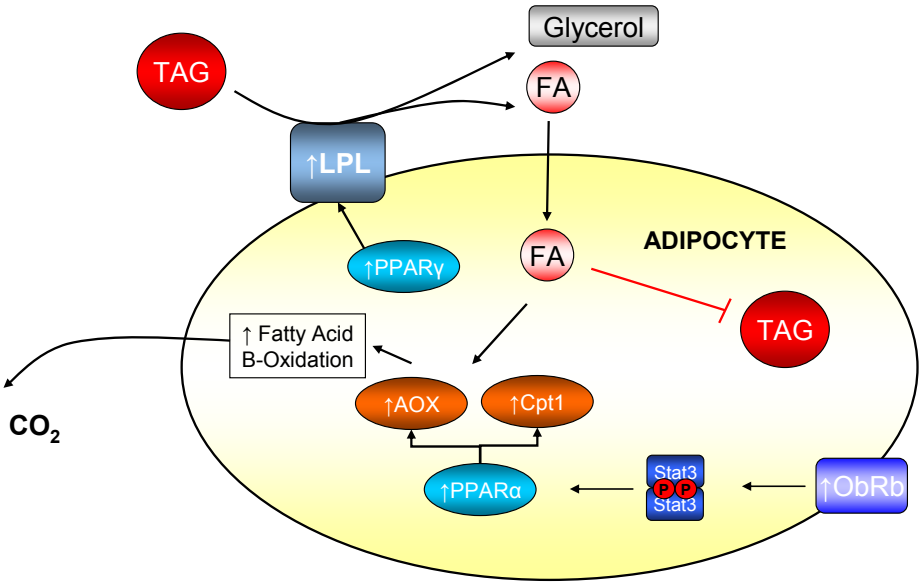


Figure 7.1 Proposed effect of neonatal leptin treatment on adipocytes according to altered gene expression profiles. It is proposed that the increase in expression of PPARs and their target genes facilitate an increase in fatty acid β-oxidation within adipocytes.

7.3 Future work

Several issues remain unresolved in this study. Firstly, no genes were shown to be altered in response to maternal undernutrition in IUGR offspring. Therefore, to confirm the hypothesis that

undernutrition induces an increased capacity to store fat, future work should measure the expression of genes of fatty acid synthesis and storage in both IUGR offspring and those treated with leptin. Work in this report also indicated that leptin activates PPARα via a non-canonical Stat3 mechanism, however, Stat3/Sp1 protein/DNA binding needs to be demonstrated upon activation by leptin. This could be verified by EMSA to show that Stat3/Sp1 can bind the unique region of the PPARα promoter in leptin treated cells, and secondly by performing a transient ChIP using Sp1/Stat3 antibodies to show binding at promoter upon leptin activation.

Clearly, the mechanism which induces the persistent alterations in PPARα gene expression still needs to be clarified. For example, if altered leptin receptor signaling is responsible, leptin receptor needs to be analysed for epigenetic alterations. This should include both DNA methylation and histone modifications associated with its promoter. In addition, further analysis of the PPARα promoter needs to be performed to determine if there are any alterations in DNA methylation downstream of the region analysed in this report. Further studies are also needed to determine if there are any differences in histone modifications present at the PPARα P2 promoter in leptin treated offspring. As yet histone modifications in the PPARα promoter region of the different offspring groups are unknown. Histone status could be investigated using ChIP assays to look at differences in histone modification between leptin treated and saline treated offspring. In particular, methylation of H3 K27 should be measured at the unique region of the P2 promoter, to determine if PcG proteins are involved in repression.

To support the mechanisms proposed in this discussion, further work needs to be undertaken. To explore the hypothesis concerning leptin's actions on the hypothalamus, further animal studies are needed to identify alterations in the programmed neonate. This work should identify if neonates are hypoleptinemic, and if the leptin surge is blunted or premature in these IUGR offspring and if this is due to adipose specific alterations in leptin expression. Furthermore any alterations in the leptin surge should be followed up with an analysis of hypothalamic projections and leptin specific hypothalamic signaling, in particular gene expression and methylation of appetite control genes, to determine leptin sensitivity. Furthermore, all these factors need to be measured in leptin treated offspring also. To support the proposed mechanisms acting at the level of the adipocytes, specific gene expression and epigenetic data should be measured in key genes at the time of neonatal leptin treatment to understand the changes in epigenetic regulation as they are set up. This should therefore include investigating the epigenetic and gene expression status of genes before and after neonatal leptin treatment. In particular changes associated with repression at the PPARα P2 promoter need to be investigated.

7.4 Implications

Accumulating evidence in human epidemiological cohorts and animal models suggest that the increased prevalence of obesity and its metabolic disturbances may be programmed in utero by a poor maternal nutrition. Furthermore, studies are emerging implicating altered epigenetic regulation of gene transcription in the underlying mechanisms. In this IUGR rat study, neonatal leptin treatment during a specific period of plasticity reversed the programmed phenotype, which may have involved alteration to epigenetic marks both centrally and peripherally. As the IUGR rat model exhibits similarities to the disturbances identified in human IUGR, the underlying mechanisms may be similar in humans, thus implicating leptin treatment during an equivalent period of developmental plasticity as a therapeutic intervention in individuals who have hypoleptinemia, in order to minimise the risk of chronic disease such as obesity and its metabolic disturbances in adult life.

Appendix 1

Clustal 2.0.12 multiple sequence alignment

Figure 1. Multiple sequence alignment of mouse, rat and human PPARα exon 3. Sequences of mouse, rat and human PPARα exon 3 were aligned using Clustal W2.0.12. Bases conserved between all 3 species are marked with *. Identity for rat and mouse is 92%, identity for rat and human is 77% and identity for mouse and human is 78%.

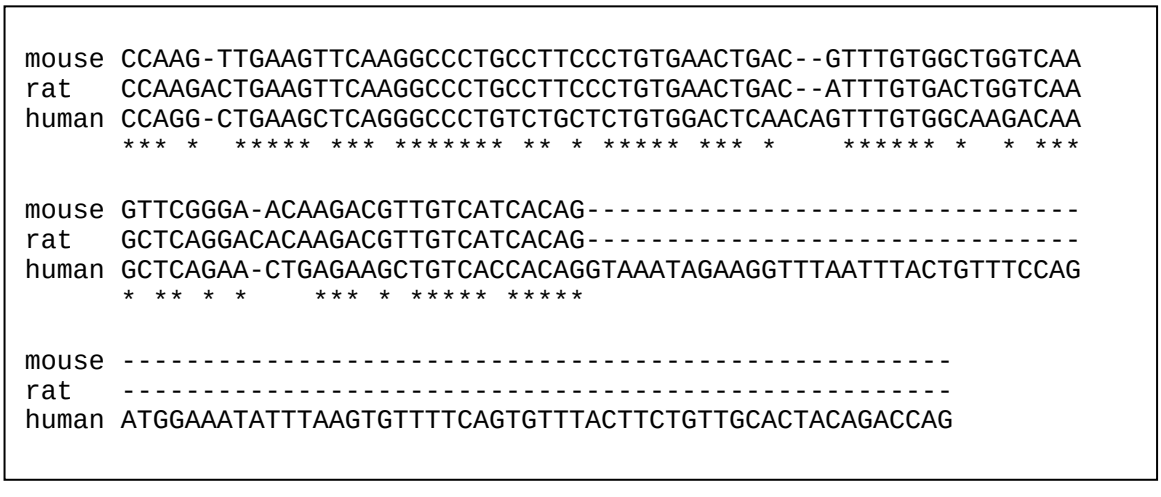


Figure 2. Multiple sequence alignment of mouse and rat PPARα exon 2 and human PPARα exon 1A. Sequences of mouse and rat PPARα exon 2 and human PPARα exon 1A were aligned using Clustal W2.0.12 [259]. Bases conserved between all 3 species are marked with *. Identity for rat and mouse is 94%, identity for rat and human is 75% and identity for mouse and human is 74%.

Figure 3. Multiple sequence alignment of mouse PPARα exon 1B, rat PPARα exon 1E and human PPARα exon X before 5'RACE. Sequences of mouse exon 1B, rat exon 1E (prior to the 5'RACE procedure) and human PPARα exon X were aligned using Clustal W2.0.12 [259]. Sequences conserved between all 3 species are marked with *. Identity for rat and mouse is 94%, identity for rat and human is 64% and identity for mouse and human is 52%.

Figure 4. Multiple sequence alignment of mouse PPARα exon 1B, rat PPARα exon 1E and human PPARα exon X after 5'RACE. Sequences of mouse exon 1B, rat exon 1E (after the 5'RACE procedure) and human PPARα exon X were aligned using Clustal W2.0.12 [259]. Sequences conserved between all 3 species are marked with *. Identity for rat and mouse is 94%, identity for rat and human is 50% and identity for mouse and human is 52%.

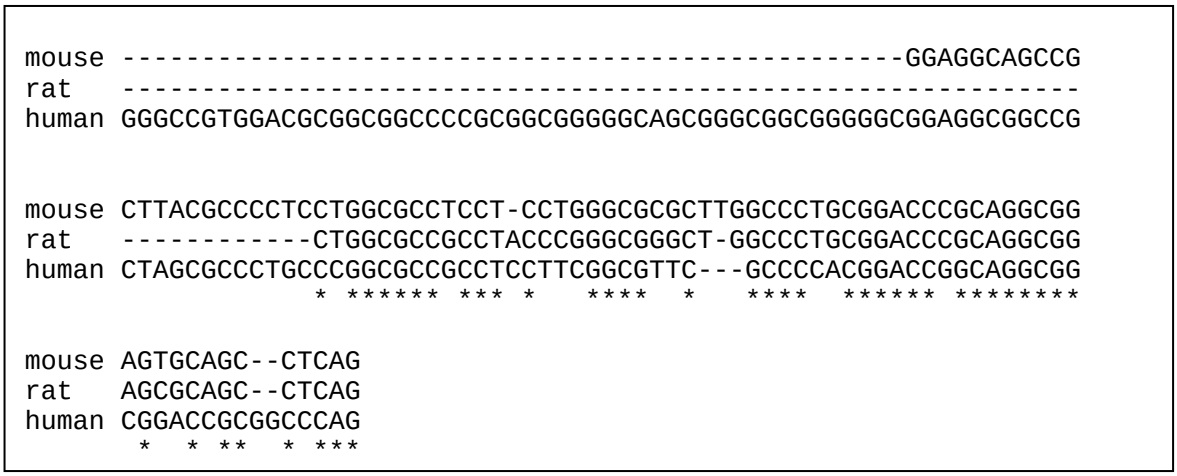


Figure 5. Multiple sequence alignment of mouse PPARα exon 1A, rat PPARα exon 1C and human PPARα exon A after 5'RACE. Sequences of mouse exon 1A, rat exon 1C (after the 5'RACE procedure) and human exon A were aligned using Clustal W2.0.12 [259]. Sequences conserved between all 3 species are marked with *. Identity for rat and mouse is 91%, identity for rat and human is 65% and identity for mouse and human is 71%.

Appendix 2

PPARα alternative 5'UTR predicted secondary structures

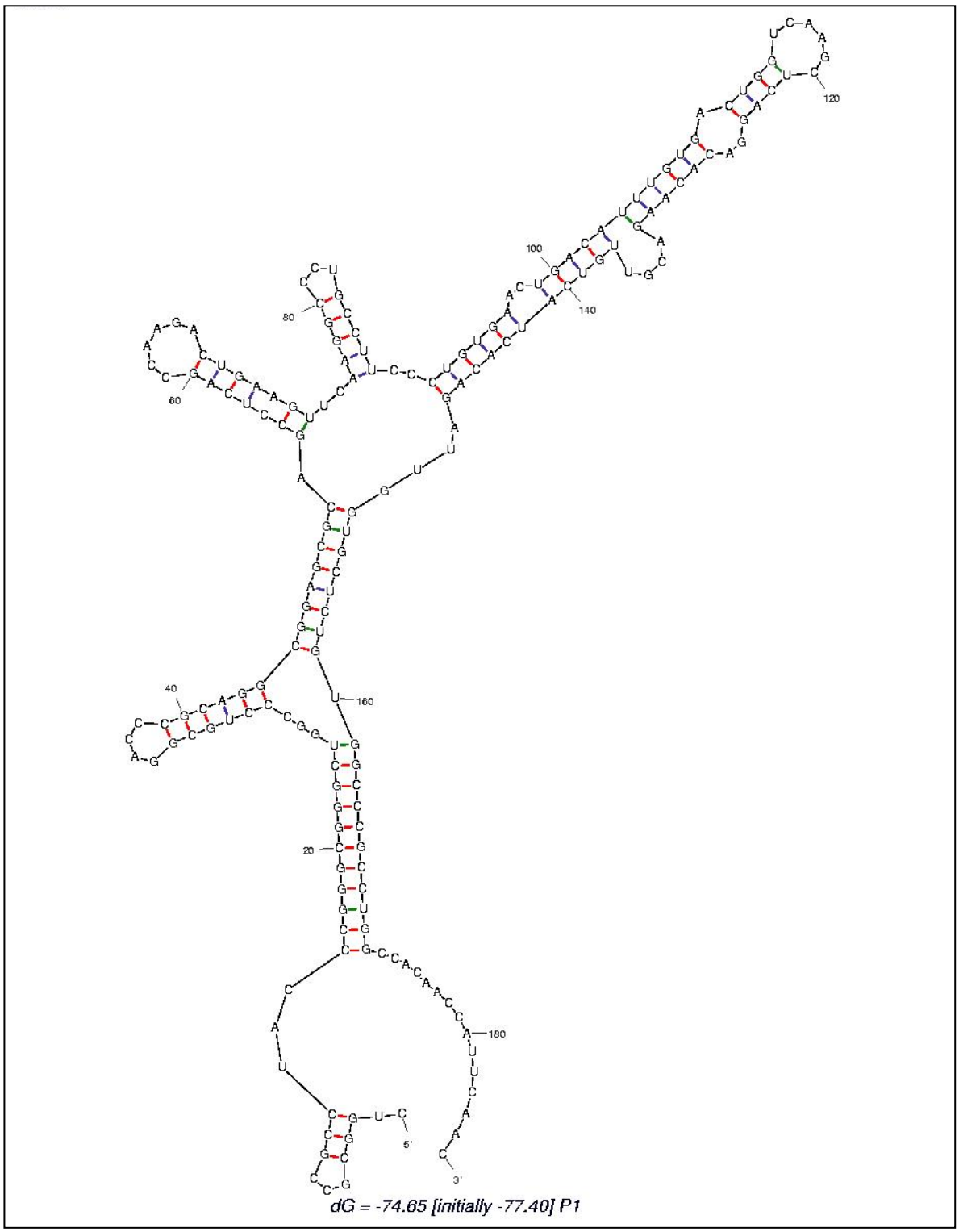


Figure 1. PPARα P1 5'UTR predicted secondary structure. The most energetically favourable conformation of the PPARα P1 5'UTR is shown as analysed by Zucker RNA mfold 2.3 software [260]. The minimum free energy was calculated as -74.65Kcal/mol.

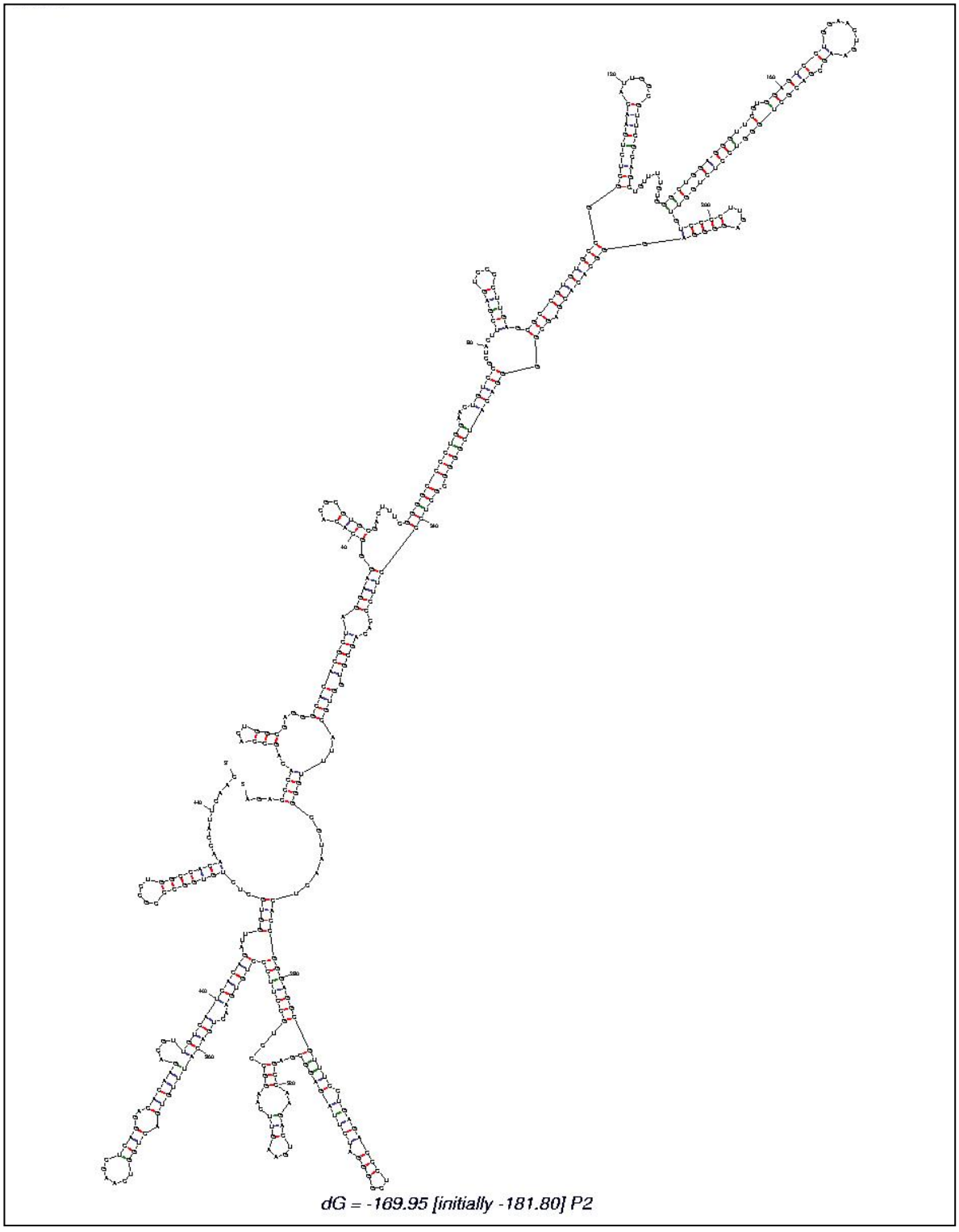


Figure 2. PPARα P2 5'UTR predicted secondary structure. The most energetically favourable conformation of the PPARα P2 5'UTR is shown as analysed by Zucker RNA mfold 2.3 software [260]. The minimum free energy was calculated as -169.95 Kcal/mol.

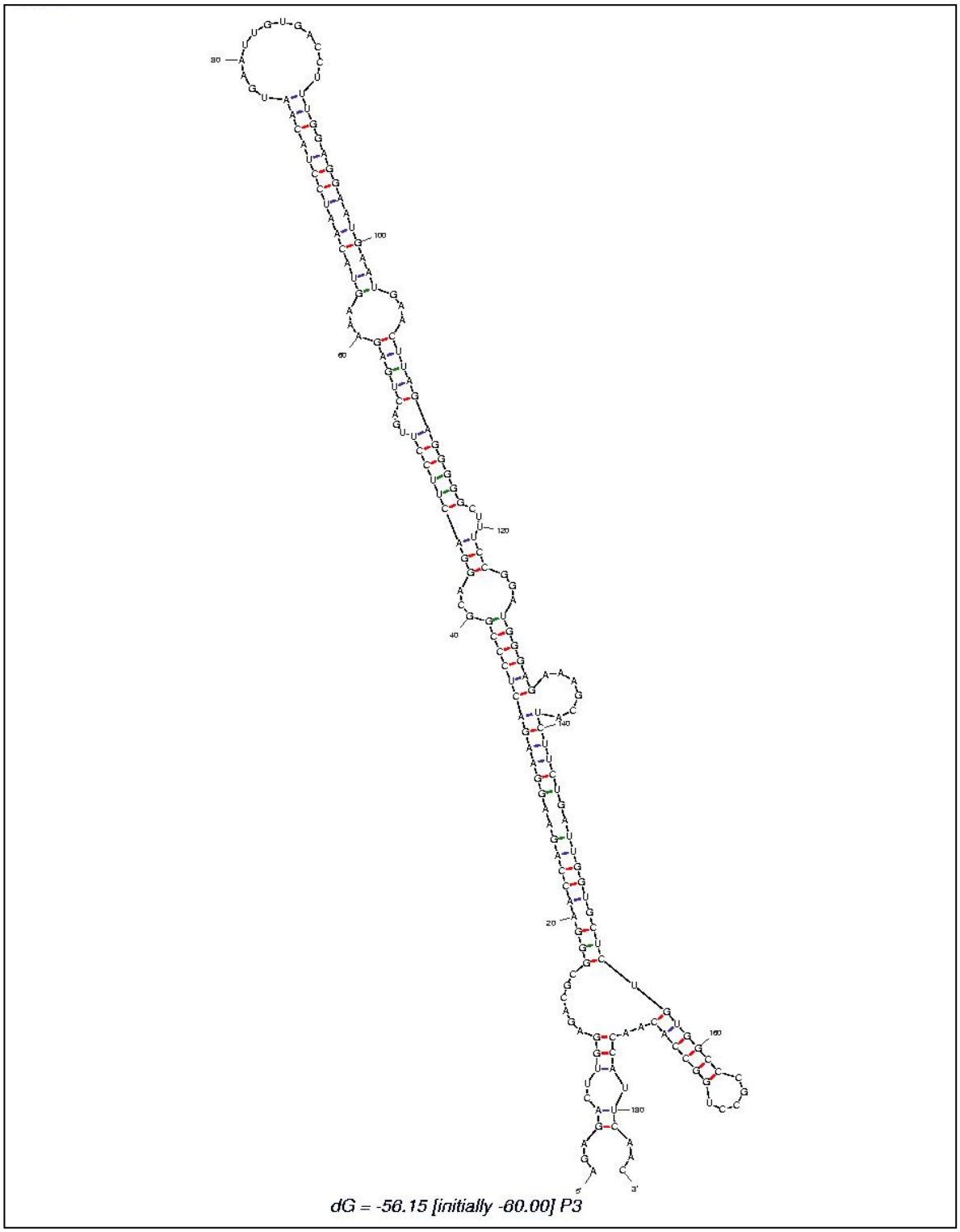


Figure 3. PPARα P3 5'UTR predicted secondary structure. The most energetically favourable conformation of the PPARα P3 5'UTR is shown as analysed by Zucker RNA mfold 2.3 software [260]. The minimum free energy was calculated as -56.15 Kcal/mol.

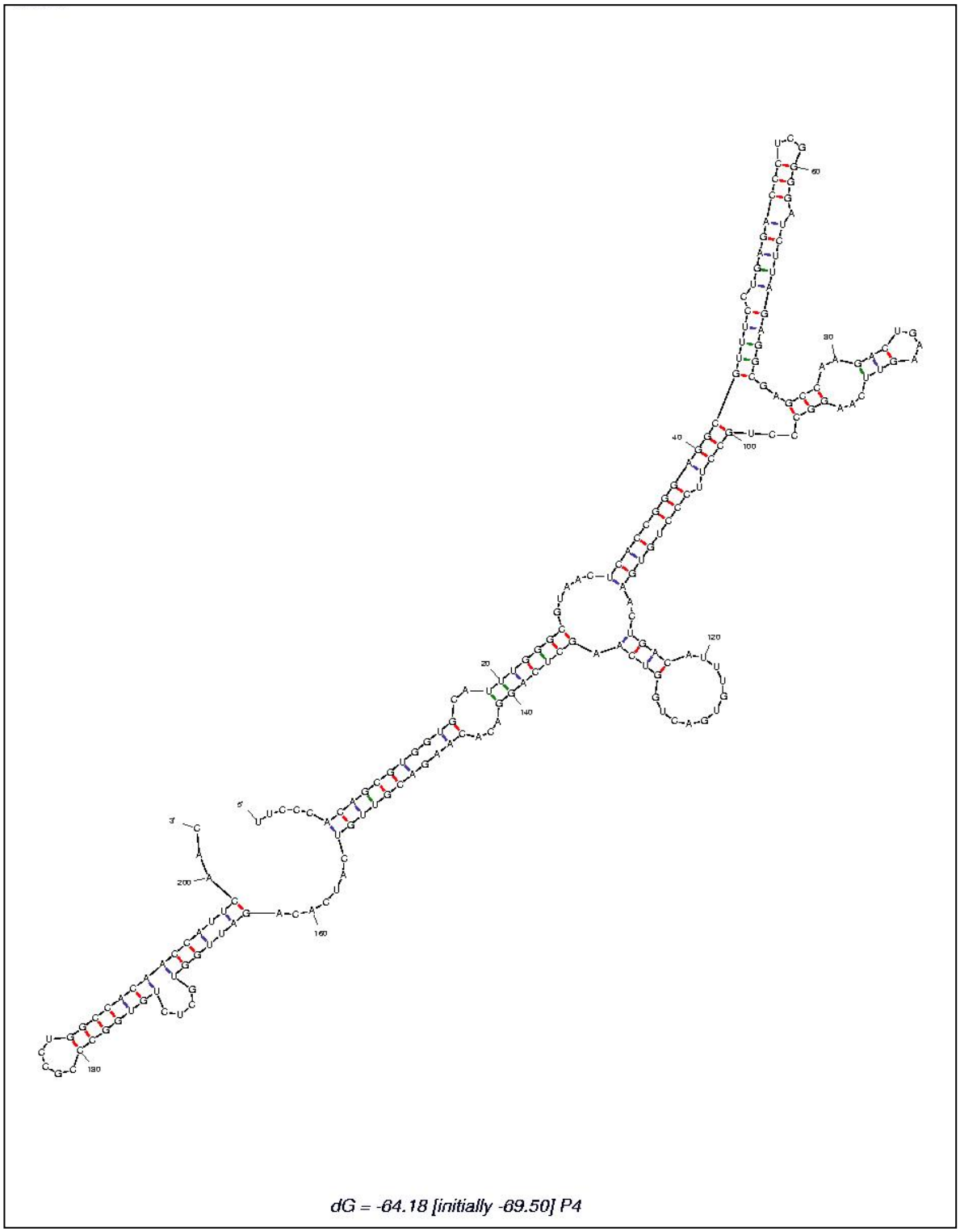


Figure 4. PPARα P4 5'UTR predicted secondary structure. The most energetically favourable conformation of the PPARα P4 5'UTR is shown as analysed by Zucker RNA mfold 2.3 software [260]. The minimum free energy was calculated as -64.18 Kcal/mol.

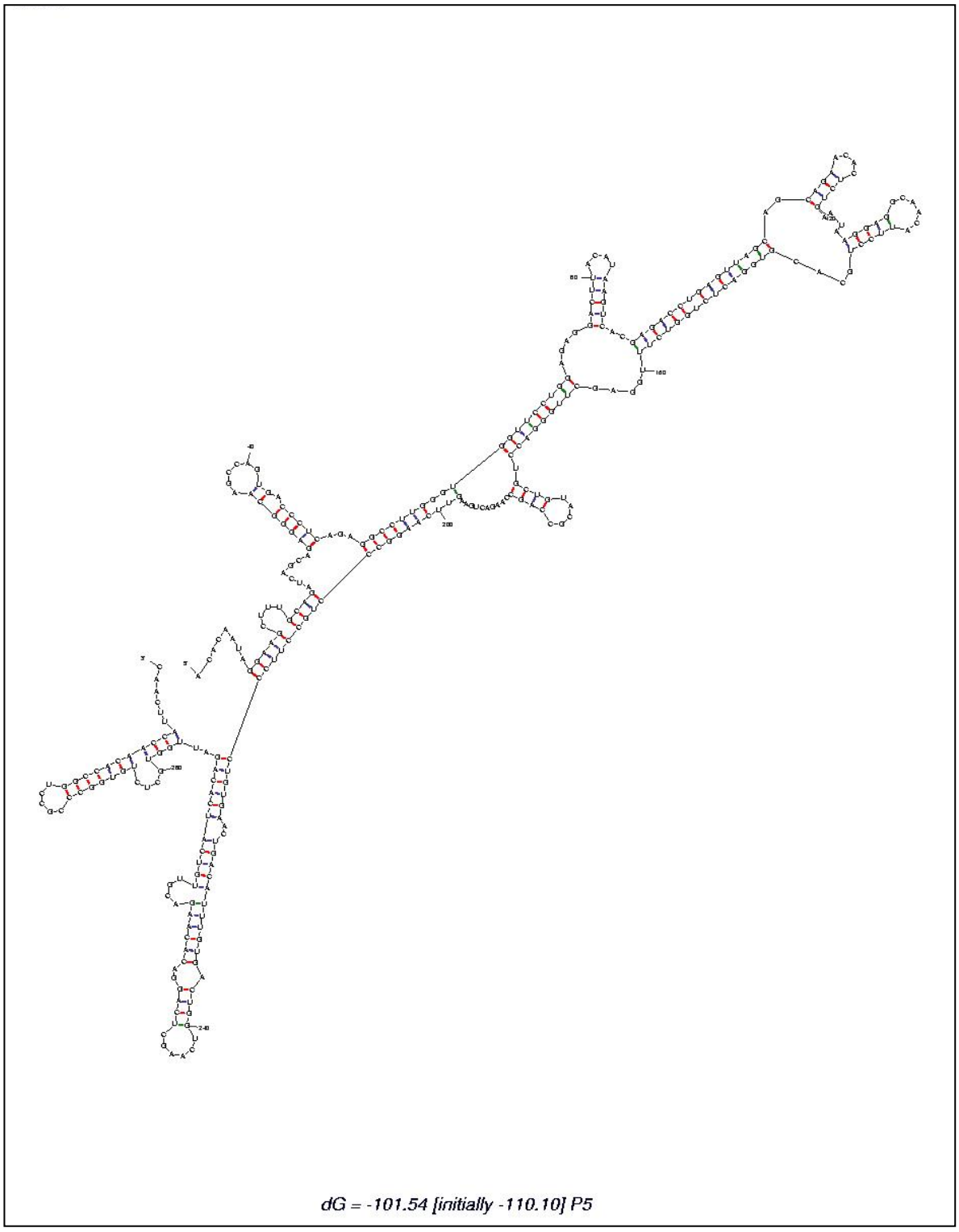


Figure 5. PPARα P5 5'UTR predicted secondary structure. The most energetically favourable conformation of the PPARα P5 5'UTR is shown as analysed by Zucker RNA mfold 2.3 software [260]. The minimum free energy was calculated as -101.54 Kcal/mol.

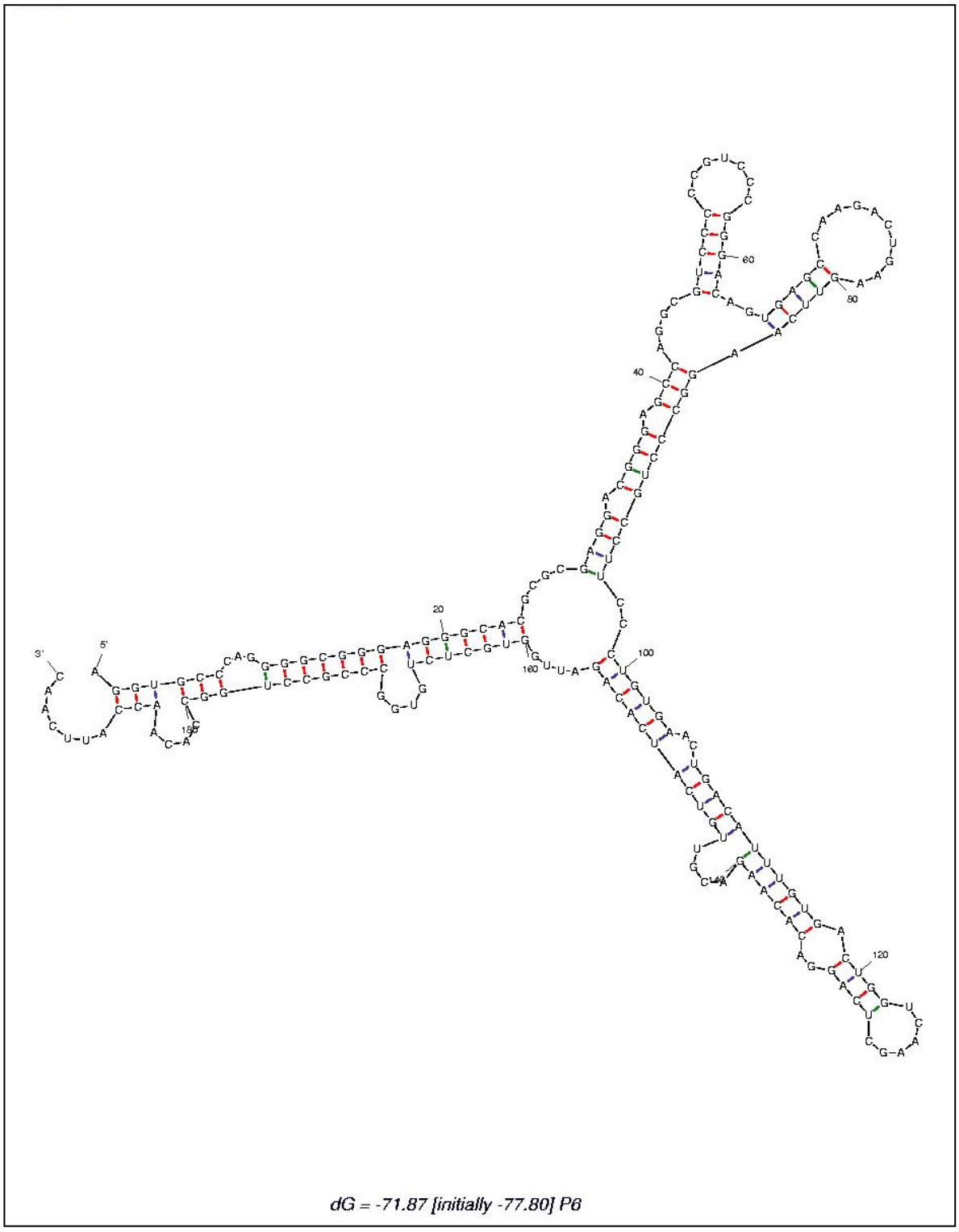


Figure 6. PPARα P6 5'UTR predicted secondary structure. The most energetically favourable conformation of the PPARα P6 5'UTR is shown as analysed by Zucker RNA mfold 2.3 software [260]. The minimum free energy was calculated as -71.87 Kcal/mol.

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