The role of Hepatocyte Nuclear Factor 4α (HNF4α) in the metabolic regulation of its target genes

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by

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Dedication

This thesis is dedicated to James, Kay and Richard, and in memory of my father.
Acknowledgement

Firstly, I would like to acknowledge the support and encouragement of my two supervisors, Dr. Alfred Thumser and Dr. Nick Plant over the course of my studies. I am grateful to both supervisors for giving me the opportunity to return to laboratory-based research, despite a gap of several years from the benchside, and for devising an interesting project, with relevance to several scientific areas. As my principal supervisor, Dr. Alfred Thumser has guided my development over the last four years and I would like to thank him for giving me the freedom to learn and develop as scientist. I am indebted to him for bringing his practical knowledge and analytical skills to planning of experiments and interpretation of results. I would like to thank Dr. Nick Plant for his critical insights and contributions to progress meetings, as well as help with learning new techniques.

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Abstract:

The nuclear receptor Hepatocyte Nuclear Factor 4α (HNF4α; NR2A1) regulates the transcription of many genes involved in glucose and lipid metabolism. Genetic linkage analyses have implicated HNF4α in the disease processes leading to Type 2 Diabetes Mellitus and dyslipidaemia. The aim of this study was to investigate the regulation of target genes in the metabolic pathways of glycolysis, lipogenesis and gluconeogenesis by HNF4α.

Initially, the expression of HNF4α and its splice variants was investigated in three human hepatoma cell lines, HuH7, HepG2 and Hep3B, with the latter two cell lines shown to express the same range of HNF4α splice variants as human adult liver. The regulation of specific HNF4α target genes, L-PK, PEPCK and SREBP-1c, was subsequently investigated in HepG2 cells using a reporter gene approach. HNF4α was found to induce expression of reporter genes containing L-PK, PEPCK and SREBP-1c proximal promoter sequences. Insulin (1 μM), but not high glucose (25 mM), was found to stimulate HNF4α-driven expression of the SREBP-1c reporter gene, while co-expression of HNF4α with the nuclear receptor co-activators, PGC-1α or p300, led to a reduction in SREBP-1c reporter gene expression.

The changes in expression of various HNF4α target genes in response to physiological mediators of the fasting-fed cycle were characterised in HepG2 cells using a real-time quantitative PCR approach. The role of HNF4α, p300 and PGC-1α was further investigated by plasmid overexpression. HNF4α and PGC-1α were found to positively regulate PEPCK expression under cell culture conditions simulating fasting (cAMP), whilst overexpression of HNF4α, PGC1α and p300 reduced L-PK mRNA expression under fed conditions.

In conclusion, the results indicate that HNF4α is a transcriptional activator of both glucagon-stimulated gluconeogenic gene expression and insulin-stimulated glycolytic and lipogenic gene expression. It is hypothesised HNF4α forms separate multi-protein complexes to differentially regulate metabolic pathways under different metabolic states.
Abbreviations:

\( \lambda \) wavelength

8-Br-cAMP 8-bromoadenosine 3',5'-cyclic monophosphate

aa amino acid

ABCA1 ATP-binding cassette protein-A1

ACC acetyl coA carboxylase

ADP adenosine diphosphate

AF activation function

AICAR 5-aminoimidazole-4-carboxamide 1-\( \beta \)-D-ribofuranoside

AMP adenosine monophosphate

AMPK AMP-activated protein kinase

ANOVA analysis of variance

apo apolipoprotein

apoAI apolipoprotein AI

apoB apolipoprotein B

apoCIII apolipoprotein CIII

AR androgen receptor

ATP adenosine triphosphate

AU absorbance units

bHLH-ZIP basic helix-loop-helix leucine zipper

CoA coenzyme A

CBP CRE-binding protein binding protein

cDNA complementary DNA

C/EBP CAAT/enhancer-binding protein

CETP cholesteryl ester transfer protein

CHD coronary Heart Disease

ChORE carbohydrate response element

ChREBP carbohydrate response element binding protein

CMV cytomegalovirus

CoA coenzyme A

COUP-TF chicken ovalbumin upstream promoter-transcription factor

CRC chromatin-remodelling complex
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<tr>
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<td>Ct</td>
<td>threshold cycle</td>
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<td>CVD</td>
<td>cardiovascular disease</td>
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<td>DBD</td>
<td>DNA-binding domain</td>
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<td>DEPC</td>
<td>diethyl pyrocarbonate</td>
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<td>DMEM</td>
<td>dulbecco’s modified Eagle medium</td>
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<td>de novo lipogenesis</td>
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<td>GSIS</td>
<td>glucose-stimulated insulin secretion</td>
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<td>IBMX</td>
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<td>IDL</td>
<td>intermediate density lipoprotein</td>
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<td>IGT</td>
<td>impaired glucose tolerance</td>
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<td>kb</td>
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<td>mitogen-activated protein kinase</td>
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<td>medium chain acyl-coA dehydrogenase</td>
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<td>maturity-onset diabetes of the young</td>
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<td>MTP</td>
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<td>non-alcoholic fatty liver disease</td>
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<td>nuclear localisation signal</td>
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<td>N.S.</td>
<td>not significant</td>
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<td>PKB</td>
<td>protein kinase B / Akt</td>
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<td>PKC</td>
<td>protein kinase C</td>
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<td>peroxisome proliferator-activated receptor</td>
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<td>progesterone receptor</td>
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<td>PSG</td>
<td>penicillin-streptomycin-L-glutamine</td>
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<td>rous sarcoma virus</td>
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<td>RXR</td>
<td>retinoid X receptor</td>
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<td>SCAP</td>
<td>SREBP cleavage activating protein</td>
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<td>SCM</td>
<td>standard culture medium</td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>SEAP</td>
<td>secreted alkaline phosphatase</td>
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<td>SEM</td>
<td>standard error of the mean</td>
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<td>SFM</td>
<td>serum-free medium</td>
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<td>SMRT</td>
<td>silencing mediator for retinoid and thyroid receptors</td>
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<td>SNP</td>
<td>single nucleotide polymorphism</td>
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<td>SOB</td>
<td>super optimal broth</td>
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<td>Sp1</td>
<td>specificity protein 1</td>
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<td>SRC</td>
<td>steroid receptor co-activator</td>
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<td>ss</td>
<td>single-stranded</td>
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<td>STM</td>
<td>standard Transfection Medium</td>
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<td>T1DM</td>
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<td>T2DM</td>
<td>type 2 diabetes mellitus</td>
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<td>T₃</td>
<td>3,3',5-triiodo-L-thyronine</td>
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<td>TAF</td>
<td>TBP-associated factor</td>
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<td>Taq</td>
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<td>transformation buffer</td>
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<td>TBE</td>
<td>Tris-borate-EDTA</td>
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<td>TF</td>
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<td>T&lt;sub&gt;m&lt;/sub&gt;</td>
<td>melting temperature</td>
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<td>TR</td>
<td>thyroid receptor</td>
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<td>TZD</td>
<td>thiazolidinedione</td>
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<td>VCM</td>
<td>vehicle control medium</td>
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<td>VLDL</td>
<td>very low density lipoprotein</td>
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Chapter 1

Introduction
Chapter 1:

The increase in Type 2 Diabetes Mellitus (T2DM) over the last century presents a global public health problem, with the incidence of T2DM predicted to double by 2025 (Zimmet et al., 2001). It is hypothesised that a better understanding of the intracellular pathways in the liver, pancreas, muscle and adipose tissue, which lead to the state of insulin resistance and relative insulin deficiency, will facilitate the development of new therapeutic targets to prevent and treat this disease (Moller, 2001). The transcriptional control of metabolic pathways is one mechanism by which homeostasis of plasma glucose and lipid levels is maintained and elucidation of the molecular mechanisms underlying these processes is vital to understanding their dysregulation in metabolic disease (Desvergne et al., 2006).

Nuclear receptors (NRs) are ligand-activated transcription factors involved in integrating a wide range of metabolic and hormonal signals, and specific NRs have already been shown to have an important role in T2DM. Activation of the NR, peroxisome proliferator-activated receptor γ (PPARγ), by the anti-diabetic thiazolidinedione (TZD) class of drugs increases insulin sensitivity by inducing adipocyte differentiation and increasing triacylglycerol storage, resulting in decreased plasma non-esterified fatty acid (NEFA) levels (Kersten et al., 2000). Like the PPAR gene family, hepatocyte nuclear factor 4α (HNF4α) binds fatty acid metabolites, making HNF4α a potential target for therapeutic intervention (Section 1.7), however the regulation of HNF4α target genes under different metabolic conditions is less well defined. In view of the genetic evidence that HNF4α has a role in T2DM and related disorders (Section 1.6), further investigation of the role HNF4α in regulating genes in key metabolic pathways is called for.

Metabolic disorders are characterised by altered levels of hormones and dysregulation of metabolic pathways by hormonal stimuli. Therefore the metabolic stimuli (Section 1.1) and metabolic pathways (Section 1.3) relevant to disorders such as T2DM, dyslipidaemia and the metabolic syndrome (Section 1.2) will be described in the following sections.
1.1 Metabolic stimuli

1.1.1 Insulin and glucagon

Insulin and glucagon are peptide hormones produced by the \( \beta \)-cells and \( \alpha \)-cells in the Islets of Langerhans, which constitute 2% by mass of the pancreas (Pocock and Richards, 2006). The two hormones have counter-regulatory functions on glucose and lipid homeostasis, with insulin having an anabolic action on metabolism and glucagon a catabolic role (Table 1.1). Insulin secretion occurs in two phases: the first secretory phase lasting a few minutes followed by a more sustained release of insulin (Holt and Hanley, 2007). The primary stimulus for insulin secretion is increased plasma glucose concentration, however amino acids and hormones such as secretin, glucagon and glucagon-like peptide also stimulate insulin secretion (Pocock and Richards, 2006). Glucagon secretion is stimulated by hypoglycaemia, amino acids and the gastrointestinal hormone, cholecystokinin, and inhibited by insulin (Pocock and Richards, 2006). Plasma glucagon concentrations vary less than insulin concentrations, hence the ratio of insulin to glucagon is important in determining the overall effect on cell metabolism (Pocock and Richards, 2006).

<table>
<thead>
<tr>
<th>Target organ</th>
<th>Insulin</th>
<th>Glucagon</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>Suppresses gluconeogenesis and stimulates glycolysis</td>
<td>Stimulates gluconeogenesis</td>
</tr>
<tr>
<td>Liver and skeletal muscle</td>
<td>Stimulates glycogen synthesis</td>
<td></td>
</tr>
<tr>
<td>Liver and skeletal muscle</td>
<td>Suppresses glycogen synthesis</td>
<td></td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>Stimulates glucose uptake</td>
<td></td>
</tr>
<tr>
<td>Adipose tissue</td>
<td>Stimulates lipid uptake</td>
<td>Stimulates lipolysis</td>
</tr>
<tr>
<td>Liver and adipose tissues</td>
<td>Suppresses lipogenesis</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>Suppresses fatty acid oxidation</td>
<td>Stimulates fatty acid oxidation</td>
</tr>
<tr>
<td>Muscle</td>
<td>Suppresses protein breakdown</td>
<td></td>
</tr>
</tbody>
</table>


1.1.2 Glucocorticoids

Glucocorticoids are steroid hormones produced by the adrenal gland, with cortisol being the dominant glucocorticoid in humans and smaller amounts of corticosterone and cortisone being produced (Pocock and Richards, 2006). The hypothalamic-pituitary-adrenal (HPA) axis regulates glucocorticoid secretion, with production of corticotropin releasing hormone (CRH) by the hypothalamus stimulating release of adrenocorticotropic hormone (ACTH) from the anterior pituitary which in turn stimulates glucocorticoid synthesis (Pocock and Richards, 2006). Plasma cortisol levels increase during periods of fasting due to amplified
secretory bursts from the adrenal gland (Beer et al., 1989; Bergendahl et al., 1996). Re-
programming of the HPA axis resulting in raised cortisol levels have been proposed to be one
mechanism by which low birth weight is associated with metabolic syndrome (Section 1.2.3)
(Phillips et al., 1998). Glucocorticoids are a ligand for the glucocorticoid receptor (GR), a
member of the NR superfamily (Section 1.4.5).

1.1.3 Signalling pathways

The opposing actions of glucagon and insulin on intracellular processes are mediated by
different phosphorylation cascades downstream of the respective hormone receptors. The
glucagon receptor is coupled to a stimulatory G-proteins (Gs) which activate adenylyl
cyclase, leading to rises in intracellular cAMP concentration and activation of protein kinase
A (Figure 1.1A) (Frayn, 2003).

Binding of insulin to the insulin receptor results in receptor autophosphorylation of tyrosine
residues, association of insulin receptor substrates (IRS) and activation of
phosphatidylinositol 3-kinase (PI3K). PI3K generates phosphatidylinositol (3', 4', 5')-
triphosphate, leading to activation of phosphoinositide-dependent kinase (PDK), which in
turn activates protein kinase B (PKB) (Figure 1.1B) (Frayn, 2003). Mitogen-activated kinase
pathways (MAPK) are also modulated by insulin signalling, with the Ras-Raf-MEK1-ERK
cascade being activated by insulin, whereas the p38 MAPK cascade is antagonized by insulin
(Keeton et al., 2002). The PI3K cascade mediates many of the effects of insulin on the
expression of genes involved in metabolism, whilst the MAPK cascade activates genes
involved in cell proliferation and the cell cycle (Saltiel and Kahn, 2001).

As well as hormones signalling whole-body metabolite requirements, intracellular energy
status is signalled by AMP-activated protein kinase (AMPK), which is activated by an
increase the AMP/ATP ratio (Figure 1.1C) (Hardie, 2004). AMPK suppresses energy-
expending processes such as gluconeogenesis and lipogenesis (Sections 1.3.1 and 1.3.3) and
stimulates the production of ATP by catabolic pathways such as fatty acid oxidation (Viollet
et al., 2006). The oral hypoglycaemic drug, metformin, reduces hepatic glucose production
through activation of AMPK (Zhou et al., 2001).
Figure 1.1: Key signalling pathways in the regulation of metabolism: (A) Glucagon (B) Insulin and (C) AMPK. (A) Glucagon receptor is coupled to Gs protein which activates adenylyl cyclase, raising intracellular cAMP and activating PKA. Diagram adapted from Frayn (2003). (B) Insulin receptor autophosphorylation leads to binding and activation of IRS and activation of the PI3K and MAPK cascades. Diagram adapted from Saltiel and Kahn (2001). (C) AMPK is raised AMP concentration and inhibited by ATP. Adenylate kinase converts ADP to AMP. Diagram from Hardie (2004).
1.2 Metabolic disorders

1.2.1 Diabetes mellitus

Diabetes mellitus is characterised by elevated fasting blood glucose (hyperglycaemia) due to an absolute or relative deficiency in insulin secretion by pancreatic β-cells. Prolonged hyperglycaemia results in symptoms of polyuria and polydipsia, as the kidneys attempt to restore plasma glucose homeostasis by excreting excess plasma glucose. A diagnosis of diabetes mellitus is reached after measurement of a fasting plasma glucose concentrations exceeding 7.0 mmol/l or non-fasting plasma glucose concentration greater than 11.1 mmol/l on two separate occasions (American Diabetes Association, 2002). In addition, diabetes mellitus can be diagnosed by means of an oral glucose tolerance test (OGTT), whereby a glucose load is given orally and blood glucose measurements taken at intervals afterwards. A plasma glucose concentration exceeding 11.1 mol/l at the 2 hour timepoint in the OGTT constitutes a diagnosis of diabetes mellitus (American Diabetes Association, 2002). The term ‘impaired glucose tolerance’ (IGT) refers to an intermediate stage in the development of diabetes mellitus where insulin secretion is unable to fully compensate for an increase in blood glucose, with a definition of a 2 hour plasma glucose concentration of ≥ 7.8 mmol/l and < 11.1 mmol/l during an OGTT (American Diabetes Association, 2002).

The prognosis for patients with diabetes mellitus is a reduction in life expectancy by about one third, with the main cause of premature mortality being cardiovascular disease (CVD) (Holt and Hanley, 2007). The increased risk of suffering from CVD or a stroke are the macrovascular complications of the disease (Holt and Hanley, 2007). Microvascular complications of diabetes mellitus include retinopathy, nephropathy and neuropathy, which can result in loss of vision, kidney failure and limb amputation (Holt and Hanley, 2007).

The most common forms of diabetes mellitus, types 1 and 2 diabetes mellitus (T1DM and T2DM respectively) differ markedly in their aetiology, metabolic alterations and treatment (Table 1.2). T1DM develops in childhood and is caused by β-cell destruction by an autoimmune process in the majority of cases, whereas T2DM develops in middle/old age and is caused by insulin resistance accompanied by the inability of the pancreas to compensate by producing increased amounts of insulin. Insulin resistance is the reduced ability of the liver, skeletal muscle and adipose tissue to respond to insulin and maintain glucose homeostasis (Reaven, 1988). Obesity is the most common cause of insulin resistance (Bogardus, 1985).
<table>
<thead>
<tr>
<th>Table 1.2: Comparison of Types 1 and 2 Diabetes Mellitus.</th>
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<tbody>
<tr>
<td><strong>Defect</strong></td>
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<tr>
<td><strong>Age of onset (years)</strong></td>
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<tr>
<td><strong>Typical bodily physique</strong></td>
</tr>
<tr>
<td><strong>Prevalence (whole population)</strong></td>
</tr>
<tr>
<td><strong>Inheritance</strong></td>
</tr>
<tr>
<td><strong>Ketoacidosis</strong></td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
</tr>
</tbody>
</table>

Table adapted from Frayn (2003).

A feedback loop of hyperglycaemia and increased insulin secretion ultimately leads to pancreatic β-cell malfunction in T2DM (Kahn, 2003). First phase insulin secretion is lost in T2DM, with second phase secretion also being diminished as the disease progresses (Holt and Hanley, 2007).

Type 1 diabetics are normally treated with insulin, whereas Type 2 diabetics are often treated initially with diet and exercise guidelines and an oral hypoglycaemic agent. These orally ingested drugs include sulfonylureas, metformin and thiazolidinediones, which act by stimulating insulin secretion (sulfonylureas) and increasing insulin sensitivity in liver (metformin) and adipose tissue (thiazolidinediones) (Holt and Hanley, 2007).

Changes in diet and an increasingly sedentary lifestyle mean that an epidemic of diabetes mellitus (with the majority of cases being T2DM) is being predicted for many westernised countries, as well as > 50% increases in diabetes mellitus in developing countries such as China and India (Zimmet et al., 2001). Global prevalence of diabetes mellitus is predicted to rise from 2.8% to 4.4% between 2000 and 2030 (Wild et al., 2004). However, factors such as increasing levels of obesity and decreased mortality may mean that current predictions underestimate the increase in T2DM (Lipscombe and Hux, 2007). Increased prevalence of T2DM will place an increased public health burden on affected countries for treatment of diabetes and its complications (Zimmet et al., 2001).

### 1.2.2 Dyslipidaemia

Dyslipidaemias are disorders of lipid metabolism, whereby serum cholesterol or triglyceride concentrations, or concentrations of both, are above (hyperlipidaemias) or below (hypolipidaemias) the normal ranges (Durrington, 2003). A major contributing factor to the increased risk of developing CHD in diabetics is the dyslipidaemia associated with the
disease (Holt and Hanley, 2007). In fact, it has been suggested that T2DM develops as a result of alterations in lipid metabolism rather than glucose metabolism (McGarry, 1992). In the initial stages of the disease (i.e. before pancreatic β-cell insufficiency), increased plasma non-esterified fatty acids (NEFA) are linked to decreased fatty acid oxidation in muscle, hyperinsulinaemia and increased hepatic glucose production, thereby promoting insulin resistance (McGarry, 2002). Diabetic dyslipidaemia is characterised by hypertriglyceridaemia, low levels of high density lipoprotein (HDL) cholesterol and the presence of small dense low density lipoprotein (LDL) particles (discussed further in Section 1.3.4) (Durrington, 2003).

1.2.3 The Metabolic Syndrome

The considerable overlap in the aetiologies of T2DM, dyslipidaemia and CVD led to the proposal that the development of underlying insulin resistance precedes the development of overt disease (Reaven, 1988). The term ‘Metabolic Syndrome’ was therefore coined to refer to the clustering of insulin resistance, obesity, dyslipidaemia and hypertension as risk factors for developing CVD and T2DM (Reaven, 1988). The International Diabetes Federation definition for metabolic syndrome specifies the presence of central adiposity plus two of the following four factors: raised plasma triglyceride levels, reduced HDL cholesterol, raised blood pressure or raised fasting plasma glucose (Alberti et al., 2006). The development of non-alcoholic fatty liver disease (NAFLD), a condition marked by intrahepatic triglyceride accumulation, has been described as a feature of the metabolic syndrome (Marchesini et al., 2001), warranting further investigation of the alterations in hepatic metabolism under conditions of insulin resistance or hyperinsulinaemia (Desvergne et al., 2006).

1.3 Metabolic pathways

The function of metabolic pathways of gluconeogenesis, glycolysis, lipogenesis and lipoprotein metabolism and their dysregulation in metabolic disorders will be discussed in detail below.

1.3.1 Gluconeogenesis

Gluconeogenesis is the synthesis of glucose from non-carbohydrate precursors, i.e. lactate, amino acids and glycerol (Figure 1.2). Hepatic gluconeogenesis is stimulated by a decrease in the ratio of insulin to glucagon and contributes to glucose homeostasis in the post-absorptive state (e.g. an overnight fast), alongside the breakdown of liver glycogen stores. In
the case of starvation (> 24 hours fasting), liver glycogen stores are depleted and hepatic glucose output is derived entirely from gluconeogenesis (Frayn, 2003).

The flux through the gluconeogenic pathway is determined by the enzyme activities of phosphoenolpyruvate carboxykinase (PEPCK), fructose 1,6-bisphosphatase and glucose 6-phosphatase (G-6-Pase) (Figure 1.2) (Salway, 2004). PEPCK (EC 4.1.1.32) catalyses the first rate-determining step, converting oxaloacetate and GTP to phosphoenolpyruvate and GDP with the release of CO$_2$. Oxaloacetate formed by pyruvate carboxylase in the mitochondrion is converted to malate and transported to the cytosol, followed by the reconversion of malate to oxaloacetate by malate dehydrogenase (Figure 1.2). This has the net effect of removing carbons from the tricarboxylic acid (TCA) cycle, a process known as cataplerosis (Hakimi et al., 2005). PEPCK activity is not modified by allosteric regulation, therefore its activity is controlled primarily at the level of its gene transcription (Hanson and Reshef, 1997) (discussed further in Section 1.9.1). The second rate-determining step, catalysed by fructose 1,6-bisphosphatase (EC 3.1.3.11), is the hydrolysis of fructose 1,6-bisphosphate to form fructose 6-phosphate with the release of inorganic phosphate (Figure 1.2). Fructose 1,6-bisphosphatase activity is subject to allosteric inhibition by fructose 2,6-bisphosphate, the concentration of which is reduced in the fasting state as the glucagon to insulin ratio increases (Salway, 2004). The final step of the gluconeogenic pathway, the hydrolysis of glucose 6-phosphate to form glucose and inorganic phosphate, is catalysed by G-6-Pase (EC 3.1.3.9). The catalytic site of this enzyme is located in the lumen of the endoplasmic reticulum, with its substrate being supplied by a specific translocase (van Schaftingen and Gerin, 2002). G-6-Pase is regulated in the short term by the concentration of glucose 6-phosphate and G-6-Pase activity is regulated in the long term at the level of its gene expression (van Schaftingen and Gerin, 2002).

In T2DM, insulin fails to suppress endogenous glucose production (Firth et al., 1986; Mitrakou et al., 1990), with increased gluconeogenic rate responsible for the increase in hepatic glucose output (DeFronzo et al., 1989; Consoli et al., 1989; Magnusson et al., 1992). Hepatic insulin resistance results in lack of inhibition of PEPCK and G-6-Pase expression by insulin, as demonstrated in a liver insulin receptor knockout mouse model (Michael et al., 2000). Overexpression of PEPCK in transgenic mice also resulted in T2DM (Valera et al., 1994).
Figure 1.2: The gluconeogenic pathway. The formation of glucose from pyruvate is shown with metabolic intermediates. The key regulatory steps (wide arrows) and the enzymes which catalyse them are indicated in bold. Intracellular compartments are indicated in italics, with the TCA cycle indicated by a circle in the mitochondrion. Abbreviations: ER, endoplasmic reticulum.

1.3.2 Glycolysis

The glycolytic pathway is the first pathway in the catabolism of glucose by the cell (Figure 1.3). It differs from gluconeogenesis (Figure 1.2) in three different control points in the pathways: namely, the phosphorylation of glucose by glucokinase (EC 2.7.1.2), the phosphorylation of fructose-6-phosphate by phosphofructokinase (EC 2.7.1.56) and the conversion of phosphoenolpyruvate to pyruvate catalysed by liver-type pyruvate kinase (L-
Figure 1.3: Glycolytic and lipogenic pathways. Glucose is transported into the hepatocyte by glucose transporter GLUT2, followed by its phosphorylation by glucokinase, the first step of the glycolytic pathway (left-hand side). Key control points in the glycolytic pathway are indicated by wide arrows, namely glucokinase, phosphofructokinase and the final step of the pathway, L-PK. Fatty acids generated by de novo lipogenesis or recycled by the liver are converted to triglycerides and exported in the form of very low density lipoprotein (VLDL). Abbreviations: GPAT, glycerol 3-phosphate acyl transferase. Diagram adapted from Dentin et al. (2005).

PK) (EC 2.7.1.40), thus ensuring that the two pathways do not become a futile cycle. In the liver, the glycolytic pathway in the first stage of de novo lipogenesis in the synthesis of fatty acids from dietary carbohydrates (Section 1.3.3).

L-PK catalyses the transfer of a phosphoryl group from phosphoenolpyruvate to ADP, generating pyruvate and ATP (Figure 1.9). The reaction of this final step of the glycolytic pathway is irreversible and thus controls flux of pyruvate into the TCA cycle. L-PK activity is subject to allosteric regulation, with ATP and alanine (a product of downstream pathways) inhibiting its activity, and fructose 1,6-bisphosphate (a product of upstream
phosphofructokinase) stimulating its activity (Salway, 2004). L-PK is also phosphorylated by PKA downstream of glucagon action, resulting inhibition of its activity in the fasting state, whilst insulin stimulates protein phosphatase 2A, which dephosphorylates and activates L-PK. In the long term, L-PK protein levels are regulated by changes to L-PK mRNA levels (Section 1.9.2). Increased L-PK mRNA expression has been demonstrated in several rodent models of T2DM and obesity (Sugiyama et al., 1989; Hotta et al., 1996; Pérez et al., 1998; Shimomura et al., 2000).

1.3.3 Lipogenesis

*De novo* lipogenesis (DNL) is the synthesis of fatty acids from non-lipid precursors (*e.g.* from glucose) (Figure 1.3). Whereas both the liver and adipose tissue express the enzymes of fatty acid synthesis, it is the liver that is the primary site of lipogenesis in humans (Ferre and Foufelle, 2007). In the metabolism of glucose or fructose, pyruvate generated by the glycolytic pathway is transported into the mitochondrion, converted to acetyl CoA by pyruvate dehydrogenase and enters the tricarboxylic acid (TCA) cycle. Citrate, an intermediate in the TCA cycle, is transported into the cytosol. Citrate is then converted to acetyl CoA by ATP citrate lyase, the first step of the lipogenic pathway (Figure 1.3). The carboxylation of acetyl CoA to form malonyl CoA by acetyl CoA carboxylase (ACC) (EC 6.4.1.2) is the first committed step in the lipogenic pathway. ACC is subject to allosteric control, with citrate inducing polymerization of enzyme protomers and activating the enzyme (Brownsey et al., 2006). Malonyl CoA and free CoA (products of the reaction catalysed by ACC), as well as fatty acyl CoA (products of downstream reactions in the lipogenic pathway) are inhibitors of ACC (Brownsey et al., 2006). ACC activity is also regulated by phosphorylation, with insulin activating the enzyme and AMPK inhibiting its activity (Brownsey et al., 2006). ACC gene expression regulates protein levels of ACC in the longer term, with SREBP-1c (Section 1.4.3) being a key regulator of its transcription (Shimano et al., 1999).

The sequential addition of two-carbon units to malonyl CoA to form palmitic acid is catalysed by fatty acid synthase (FAS) (EC 2.3.1.85), the product of a single gene with seven different enzyme activities. FAS activity is not subject to allosteric regulation or post-translational modification, with the rate of FAS gene transcription being the primary means of controlling its activity (Sul and Wang, 1998) (discussed further in Section 1.9.4). Saturated fatty acids (*e.g.* palmitate) formed by FAS are converted to triglycerides for export.
or storage by the esterification of glycerol 3-phosphate, catalysed by glycerol 3-phosphate acyltransferase (GPAT) (EC 2.3.1.15).

Although hepatic lipogenesis contributes less than 5-7% of all fatty acids present in VLDL in the fed state for healthy individuals on a western (high fat) diet, the contribution from DNL can increase to 30% in the fed state following the feeding of high carbohydrate diets (Hellerstein et al., 1996). Increased DNL has been shown to be present in metabolic states associated with the metabolic syndrome (Section 1.2.3): First, DNL contributes to carbohydrate-induced hypertriacylglycerolaemia, in which the liver overproduces VLDL following chronic consumption of diets rich in carbohydrates (Parks and Hellerstein, 2000). Second, hepatic lipogenesis is increased in human obesity (Diraison et al., 2002). Increased lipogenesis is also evident in non-alcoholic fatty liver disease (NAFLD) (Section 1.2.3) (Diraison et al., 2003; Donnelly et al., 2005; Fabbrini et al., 2008). Changes in lipogenic gene expression are likely to contribute to the increased DNL observed in humans, as induction of lipogenic enzyme expression is seen in animal models of obesity and fructose-feeding (Lopez-Casillas et al., 1991; Fiebig et al., 1998).

1.3.4 Lipoprotein metabolism

Lipids are transported in the circulation as complex particles known as lipoproteins, composed of free cholesterol, phospholipid, cholesteryl ester, triacylglycerol and proteins. The protein components of lipoproteins, i.e. apolipoproteins, are produced by both the liver and intestine (apo referring to protein component before the formation of a lipid complex), with different lipoproteins containing different constituent apolipoproteins (Figure 1.4).

Dietary fatty acids and cholesterol absorbed in the intestine are packaged into chylomicrons, whilst endogenous lipids and those derived from hepatic DNL (Section 1.2.3) are exported from the liver as VLDL. Both chylomicrons and VLDL contain apoB (in the form apoB-48 and apoB-100, respectively) and both are referred to as triacylglycerol-rich lipoproteins (TRLs) due to the high percentage of esterified fatty acids (90% in the case of chylomicrons, 65% for VLDL) (Frayn, 2003). Following lipolysis of chylomicrons and VLDL by lipoprotein lipase (LPL) in the peripheral tissues, the size of TRLs is reduced to intermediate density lipoproteins (IDL). Further hydrolysis of fatty acids results in the formation of low density lipoproteins (LDL) with a high percentage of bound cholesterol (45%) (Frayn, 2003).
Small dense LDL is a form of LDL which has become depleted of most of its bound lipid and high levels carry an increased risk of developing of CHD (Griffin et al., 1994; Lamarche et al., 1997). Increased levels of small dense LDL arise due to overproduction or insufficient catabolism of very low density lipoprotein (VLDL) (Adiels et al., 2006). Small dense LDL is particularly susceptible to oxidation and is hence considered particularly atherogenic (Frayn, 2003). Oxidised LDL is taken up by macrophages by scavenger receptor-A (SR-A), leading to the formation of foam cells and the development of an atherosclerotic plaque in the vessel walls of major blood vessels (Figure 1.4) (Boyle, 2005).
The smallest of the lipoproteins, high density lipoproteins (HDL), are produced by the liver with constituent apoA1 and apoAII and perform the role of transporting excess cholesterol from the periphery to the liver (reverse cholesterol transport) for excretion as bile acids. Cholesterol is removed from cells in the periphery by the interaction of HDL particles with ATP-binding cassette protein-A1 (ABCA1) (Oram, 2003). Reverse cholesterol transport also involves the activities of plasma enzymes, lecithin-cholesterol acyl transferase (LCAT) and cholesteryl ester transfer protein (CETP), which facilitate the transfer of free cholesterol to HDL (LCAT) and the exchange of cholesteryl ester and triacylglycerol with TRLs (CETP) (Figure 1.4) (Rye et al., 1999). Since the action of HDL is antiatherogenic, low concentrations of HDL cholesterol (<1.0 mmol/l) are associated with obesity and the metabolic syndrome (Durrington, 2003).

ApoC-I, -II and -III are constituents of chylomicrons, VLDL and HDL, and are key modulators of the interaction of lipoproteins with a range of lipoprotein receptors involved in postprandial triglyceride metabolism (Jong et al., 1999). ApoCIII is of particular relevance to this project as this gene is a well-characterised target gene of HNF4α (Section 1.9.5) with an important role in postprandial triglyceride metabolism. ApoCIII has an inhibitory effect on the hydrolysis of TRLs by LPL (Jong et al., 1999). ApoCIII also has an inhibitory action on hepatic lipase (HL) and on the uptake of chylomicron and VLDL remnants by the liver via the lipolysis-stimulated receptor (LSR) (Kinnunen and Ehnholm, 1976; Windler and Havel, 1985; Mann et al., 1997).

In line with these modes of action, increased apoCIII plasma concentration is associated with hypertriglyceridaemia in human studies (Carlson and Ballantyne, 1976; Schonfeld et al., 1979; Malmendier et al., 1989). Transgenic mice overexpressing apoCIII exhibit hypertriglyceridaemia and reduced VLDL clearance (Ito et al., 1990; de Silva et al., 1994; Aalto-Setala et al., 1996), whilst apoCIII knockout mice are show reduced plasma triglyceride levels and increased clearance of postprandial TG-rich lipoproteins (Maeda et al., 1994). ApoCIII mRNA expression plays an important role in determining apoCIII levels (discussed further in Section 1.9.5).
1.4 Transcriptional regulation of gene expression

Since many of the key genes in metabolic pathways are regulated at the transcriptional level, the structure of genes and the role of transcription factors in their expression will be described below.

1.4.1 Gene transcription

The regulatory regions of genes consist of promoter and enhancer elements. Promoters are immediately upstream of the transcription start site and consist of core promoter elements such as the TATA box and binding sites for gene-specific transcription factors (Smale and Kadonaga, 2003; Kadonaga, 2004). Enhancer elements are sequences which can enhance gene transcription from a distance of up to several kilobases (kb) away from the transcriptional start site. Unlike promoter sequences, enhancer elements are not dependent on their 5' to 3' orientation with respect to the gene, and enhancers can be upstream or downstream of the transcribed gene, or within an intronic region (Latchman, 2005).

Eukaryotic genes are transcribed by three different RNA polymerases: RNA polymerase I is responsible for the transcription of the precursor of the 5.8S, 18S and 28S ribosomal RNA (rRNA) genes, RNA polymerase II responsible for the transcription of mRNA and RNA polymerase III transcribes the 5.8S rRNA, transfer-RNAs (tRNAs) and non-coding RNAs such as microRNAs (Latchman, 2005; Dieci et al., 2007). Transcription is initiated at an RNA polymerase II promoter by the sequential binding of general transcription factors (TFIIB-H) which form a multi-protein complex with RNA polymerase (the holoenzyme). Initially, TFIID binds the TATA element present at -30 bp to the transcriptional start site in the majority of promoters, together with TFIIA. TFIID consists of multiple proteins, one of which is TATA-binding protein (TBP), as well as TBP-associated factors (TAFs). TFIID recruits TFIIB which can recruit RNA polymerase II. Subsequently, TFIIF, TFII and TFIIH bind to the RNA polymerase II complex, leading to the activation of RNA polymerase II (Latchman, 2005).

1.4.2 Transcription factors

Transcription factors are DNA-binding proteins, composed of different functional domains which serve to bind DNA, facilitate dimerization with the same or different transcription factors (homo- or heterodimerization), and activate transcription (Table 1.3). DNA-binding
domains (DBDs) include the homeobox domain, consisting of a helix-turn-helix motif characterised by two α-helical regions separated by a β-sheet. This domain is present in many genes which are important in embryonic development (Latchman, 2005). The zinc finger DBD consists of a zinc atom coordinated by two cysteine and two histidine residues, in the case of the ubiquitous transcription factor, Sp1. Alternatively, a zinc atom is in a tetrahedral conformation with four cysteine residues, as observed in members of the NR family (Section 1.4.5) (Latchman, 2005). The forkhead box is another example of a DNA-binding domain which consists of a conserved 110 amino acid motif, also known as a winged helix, with three α-helices, three β-sheets and two loop structures (Burgering and Medema, 2003).

<table>
<thead>
<tr>
<th>Table 1.3: Transcription factor domains.</th>
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<tbody>
<tr>
<td><strong>Domain</strong></td>
</tr>
<tr>
<td>Homeobox</td>
</tr>
<tr>
<td>Cysteine-histidine zinc finger</td>
</tr>
<tr>
<td>Cysteine-cysteine zinc fingers</td>
</tr>
<tr>
<td>Forkhead box</td>
</tr>
<tr>
<td>Basic element</td>
</tr>
<tr>
<td>Leucine zipper</td>
</tr>
<tr>
<td>Helix-loop-helix</td>
</tr>
<tr>
<td>Acidic region</td>
</tr>
<tr>
<td>Glutamine-rich region</td>
</tr>
<tr>
<td>Proline-rich region</td>
</tr>
</tbody>
</table>

Abbreviations: ChREBP, carbohydrate response element binding protein; NF1, nuclear factor 1; Sp1, specificity protein 1; SREBP, sterol regulatory element-binding protein. Adapted from Latchman (2005).

The basic element DBD is often associated with the leucine zipper (ZIP) or helix-loop-helix (HLH) motifs which are required for the correct positioning of the the DBD (Latchman, 2005). In class III HLH transcription factors, such as SREBP (Section 1.4.3) and ChREBP (Section 1.4.4), the HLH motif is adjacent to a ZIP motif (Massari and Murre, 2000). The domains of transcription factors involved in transcriptional activation often include regions rich in acidic amino acids, resulting in a negatively-charged region. Alternatively, glutamine-rich and proline-rich regions are found in the transcriptional activation domains of Sp1 and the proto-oncogene, c-jun (Latchman, 2005).

**1.4.3 Sterol regulatory element-binding proteins (SREBPs)**

SREBPs are transcription factors of the bHLH-ZIP family with a central role in both fatty acid and cholesterol synthesis (Yokoyama et al., 1993), and were originally identified as the protein responsible for mediating the effect of sterol depletion on LDL receptor gene expression (Briggs et al., 1993; Wang et al., 1993). SREBPs are synthesised as membrane-
bound proteins and consist of a N-terminal transcription factor domain, a central region coding for two transmembrane domains and a regulatory C-terminal domain. The transmembrane domains anchor SREBPs in the endoplasmic reticulum membrane, in a complex with SREBP cleavage activating protein (SCAP) and Insig (Yang et al., 2002). Dissociation of SREBP-SCAP from Insig results in their translocation to the Golgi apparatus, followed by proteolytic processing of SREBP by site 1 and site 2 proteases (S1P and S2P), releasing the mature N-terminal SREBP transcription factor so that it can translocate to the nucleus and activate gene expression (Brown and Goldstein, 1997; Brown and Goldstein, 1999).

SREBP-1 and -2 are encoded by two genes in humans, located on chromosomes 17 and 22 (Hua et al., 1995). SREBP-1a and -1c are derived by alternate promoter usage, generating proteins with alternative first exons. Although SREBP-1a is the stronger transactivator of gene expression, the SREBP-1c isoform is nine-fold more abundant than SREBP-1a in human liver (Shimomura et al., 1997; Shinano et al., 1997). SREBP-1c is also known as adipocyte determination differentiation dependent factor-1 (ADD-1), due to its role in promoting adipose differentiation and the expression of FAS and lipoprotein lipase in adipocytes (Kim and Spiegelman, 1996). SREBP-2 primarily regulates the expression of genes involved in cholesterol biosynthesis, whilst SREBP-1c regulates genes of the lipogenic pathway (Figure 1.5) (Horton et al., 1998b; Horton et al., 2002).

SREBP-1 has been proposed to be a key regulatory gene in disorders of lipid metabolism and the metabolic syndrome (Muller-Wieland and Kotzka, 2002). In agreement with this hypothesis, up-regulation of hepatic SREBP-1c has been shown in mouse models of obesity, insulin resistance and T2DM (Shimomura et al., 1999a; Shimomura et al., 2000; Kakuma et al., 2000; Tobe et al., 2001). Genetic evidence in humans also points to a role for SREBP-1c in the development of metabolic disorders: Firstly, association of the SREBP-1c chromosomal locus with metabolic syndrome, obesity and T2DM has been revealed by genome wide scans (Kissebah et al., 2000; Wu et al., 2002; Demenais et al., 2003). Secondly, single nucleotide polymorphisms (SNPs) in the SREBP-1c gene are linked to insulin resistance and T2DM (Laudes et al., 2004; Harding et al., 2006). Whilst the activity of SREBP-1a and SREBP-2 are primarily regulated by sterol depletion at the level of their proteolytic processing, SREBP-1c is also regulated at the level of its gene expression (Sheng et al., 1995) (discussed further in Section 1.9.4).
Figure 1.5: SREBP target genes. SREBP-2 predominantly regulates the expression of enzymes in the cholesterol biosynthetic pathway, whereas SREBP-1c target genes catalyse the fatty acid biosynthetic pathway. Abbreviations used: CYP51, lanosterol 14α-demethylase; DHCR, 7-dehydrocholesterol reductase; FPP, farnesyl diphosphate; G6PD, glucose-6-phosphate dehydrogenase; GPAT, glycerol-3-phosphate acyltransferase; GPP, geranylgeranyl pyrophosphate synthase; HMG CoA, hydroxymethylglutaryl coenzyme A; PGDH, 6-phosphogluconate dehydrogenase. Diagram from Horton et al. (2002).

1.4.4 Carbohydrate response element-binding protein (ChREBP)

The transcription of numerous genes in the pathways of glycolysis and lipogenesis (Sections 1.3.2 and 1.3.3 respectively), including L-PK and FAS (Sections 1.9.2 and 1.9.4. respectively), are induced by feeding of a high carbohydrate diet via a carbohydrate response element (ChRE) in the gene promoters consisting of two E-box elements (Uyeda et al., 2002). These effects of excess carbohydrate were found to be dependent on the intracellular metabolism of glucose through the pentose phosphate pathway (Doiron et al., 1994; Doiron et al., 1996). The protein isolated as binding these elements in a glucose-responsive manner, designated ChREBP, is a member of the basic helix-loop-helix leucine zipper (bHLH-ZIP) family of transcription factors, which binds DNA as a heterodimer with Max-like factor (Mlx) (Yamashita et al., 2001; Stoeckman et al., 2004). The intracellular localisation of ChREBP is determined by its phosphorylation status, with phosphorylation by PKA bringing about its nuclear exclusion, and dephosphorylation by a xylulose 5-phosphate-activated protein phosphatase (Kawaguchi et al., 2001; Kabashima et al., 2003). ChREBP DNA-
binding is also negatively regulated by phosphorylation by PKA or AMPK (Kawaguchi et al., 2001; Kawaguchi et al., 2002).

1.4.5 Nuclear receptors

The NR superfamily comprises over 48 different members in humans, which have been classified into six groups based on evolutionary conservation (Table 1.4) (Laudet, 1997; Zhang et al., 2004). NR nomenclature is based on the subfamily and group, e.g. the thyroid receptor-α (TRα) is classified as sub-family 1, group A, member 1 with the symbol NR1A1 (Laudet et al., 1997). NRs play important roles in many physiological functions, with mutations and changes in the expression of NRs being implicated in the pathogenesis of many diseases, including PPARs in atherosclerosis and diabetes, the oestrogen receptor (ER) in breast cancer and the androgen receptor (AR) in prostate cancer (Kersten et al., 2000; Nilsson et al., 2004; Gottlieb et al., 2004).

<table>
<thead>
<tr>
<th>Nuclear receptor subfamily</th>
<th>Example members (NR symbol)</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TRα and β (NR1A1,2)</td>
<td>NRs binding metabolites (RARs, PPARs, FXR, PXR), vitamin D (VDR) and 3,5,3'-triiodo-L-thyronine (TR). RORs and rev-erbb/β are orphan NRs. CAR and PXR activity is also modulated by xenobiotics (Timsit and Negishi, 2007).</td>
</tr>
<tr>
<td>HNF4α, β and γ (NR2A1,2,3)</td>
<td>RXRα, RXRβ (NR2B1,2)</td>
<td>RXRs bind all-trans retinoic acid. COUP-TFs are orphan NRs. HNF4 binds fatty acids (discussed further in Section 1.4)</td>
</tr>
<tr>
<td>3</td>
<td>ERα, ERβ (NR3A1,2)</td>
<td>Steroid hormone receptors (with the exception of ERRα/β which are orphan NRs)</td>
</tr>
<tr>
<td>NURR1 (NR4A2)</td>
<td>Orphan receptors</td>
<td></td>
</tr>
<tr>
<td>LRH1 (NR5A2)</td>
<td>Orphan receptors</td>
<td></td>
</tr>
<tr>
<td>GCNF1 (NR6A1)</td>
<td>Orphan receptors</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>DAX1 (NR0B1)</td>
<td>Orphan NRs lacking DBD (DAX1, SHP) or LBD</td>
</tr>
</tbody>
</table>

NRs classified into subfamilies based on Laudet (1997). NRs defined as orphan NRs based on Benoit et al. (2006). Abbreviations: TR, thyroid receptor; RAR, retinoic acid receptor; PPAR, peroxisome proliferator-activated receptor; Rev-erb, reverse-Erb; ROR, RAR-related orphan receptor; FXR, farnesoid X receptor; VDR, vitamin D receptor; PXR, pregnane X receptor; CAR, constitutive androstane receptor; RXR, retinoid X receptor; COUP-TF, chicken ovalbumin upstream promoter-transcription factor; ER, estrogen receptor; ERR, ER-related receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; PR, progesterone receptor; AR, androgen receptor; NURR1, Nur-related factor; LRH1, liver receptor homolog-1; GCNF1, germ cell nuclear factor 1.
In the classical paradigm of steroid hormone receptor action, such as that of the GR, ligand binding results in a conformational change which activates the nuclear receptor and allows its translocation to the nucleus and binding to a hormone response element (HRE) in the target gene promoter (LaMarco and Vivanco, 1996).

NRs have a conserved domain structure (Figure 1.6) (Aranda and Pascual, 2001). The N-terminal A/B region varies in length in different NRs and contains a ligand-independent activation function-1 (AF-1) domain. The most conserved region of the NR protein is the DNA-binding domain (DBD) which contains two ‘zinc finger’ motifs (Section 1.4.2) (Kumar et al., 2004). NRs bind DNA as homodimers, heterodimers or monomers (Figure 1.7): steroid hormone receptors such as the AR, ER and GR bind palindromic repeats as homodimers, whilst NRs which form heterodimers with RXR such as PPAR and TR bind direct repeat (DR) sequences. There can be a variable number of bases inbetween the half-site sequences, with 1 bp spacing between two direct repeat sequences referred to as DR-1. Some orphan NRs (Section 1.4.7.) such as RORs, ERRs and NURR bind half-site sequences preceded by A/T as monomers (Benoit et al., 2006). The amino acid sequence of the zinc finger domain determines the binding affinity of the NR for different hormone response elements (HREs) (Figure 1.7), with the first zinc finger determining the sequence specificity and the second zinc finger determining the spacing between the half-sites of the HRE (Latchman, 2005).

Region D is referred to as the hinge region connecting the DBD and ligand-binding domain (LBD) and contains the nuclear localisation signal (NLS). The LBD (region E) is composed of twelve α-helices (named H1-H12) which are arranged in a globular structure, forming a ligand-binding pocket (LBP) (Aranda and Pascual, 2001). Other LBD functions include dimerization and transcriptional activation via a second AF domain (AF-2), which contains a conserved hydrophobic motif in H12 which interacts with a conserved leucine motif (LXXLL, L=leucine, X=any amino acid) present in co-activators. According to the ‘mouse trap’ model of nuclear receptor activation, conformational changes in the LBD upon ligand binding result in displacement of H12 and greater accessibility of AF-2 to coactivator binding (Moras and Gronemeyer, 1998). The F-domain is a variable region in nuclear receptors, however this region is known to be involved in the action of antagonists and transcriptional cofactors in the cases of the PR and ER (Xu et al., 1996; Skafar and Zhao, 2008).
1.4.6 Peroxisome proliferator-activated receptors (PPARs)

PPARs were identified as NRs with a role in proliferation of peroxisomes in rat liver in response to hepatocarcinogens (Isserman and Green, 1990), with three PPAR isoforms encoded by separate genes being characterised: PPARα (NR1C1), PPARδ (NR1C2, also known as PPARβ) and PPARγ (NR1C3) (Dreyer et al., 1992). PPARα is most highly expressed in the liver, heart, intestine and kidney, while PPARβ shows a ubiquitous pattern of
expression and PPARγ is most highly expressed in adipose tissue, with smaller amounts in heart, liver and skeletal muscle (Mukherjee et al., 1997). PPARs form heterodimers with RXR (NR2B) and bind DR-1 elements (Kliewer et al., 1992).

Endogenous PPARα ligands include long-chain unsaturated fatty acids (e.g. linoleic acid) and metabolites of arachidonic acid via the lipoxygenase pathway (e.g. leukotrienes), whilst the fibrate class of drugs (e.g. clofibrate, fenofibrate, Wy-14,643) are pharmacological activators (Desvergne and Wahli, 1999). PPARα is a key regulator of fatty acid catabolism, with target genes including enzymes of mitochondrial fatty acid oxidation and ketogenesis, e.g. medium chain acyl CoA dehydrogenase and HMG CoA synthase (Desvergne and Wahli, 1999). In the fasting state, PPARα is activated by fatty acids liberated from adipose tissue, leading to the stimulation of hepatic gluconeogenesis and fatty acid oxidation in the liver and cardiac muscle (Desvergne and Wahli, 1999; Barger and Kelly, 2000). The role of PPARα in the fasting state is demonstrated in PPARα-null mice which fail to utilise fatty acids as an energy source under fasting conditions and exhibit hypoglycaemia, hypoketonemia and the accumulation of intracellular lipid in the liver (Leone et al., 1999; Kersten et al., 1999).

Fibrates exert a hypolipidaemic effect via PPARα activation of genes involved in reverse cholesterol transport (apoAI, apoAII), as well as inhibition of apoCIII expression (through competition with HNF4α) (Staels et al., 1995; Peters et al., 1997; Staels et al., 1998).

PPARδ binds both saturated and unsaturated fatty acids including arachidonic acid and eicosapentaenoic acid, as well as arachidonic acid derivatives via the cyclooxygenase pathway), prostaglandins (Willson et al., 2000). PPARδ has been shown to have a role in promoting hepatic glucose disposal and muscle fatty acid oxidation, with PPARδ agonists increasing insulin sensitivity in animal models of diabetes (Lee et al., 2006). PPARδ suppresses the expression of inflammatory mediators involved in macrophage activation and the potential for PPARδ agonists in the modulation of atherosclerosis progression is currently the subject of research (Barish et al., 2006).

Endogenous PPARγ ligands also include prostaglandin derivatives, whilst the anti-diabetic drugs TZDs (e.g. troglitazone, pioglitazone and rosiglitazone) are selective agonists for PPARγ (Desvergne and Wahli, 1999). PPARγ is a key regulator of adipogenesis and stimulates the expression of SREBP-1c (Section 1.3.3) (Tontonoz et al., 1994; Kim and
Spiegelman, 1996). PPARγ expression is up-regulated by feeding a high-fat diet and in obesity, and decreased during fasting (Vidal-Puig et al., 1996; Vidal-Puig et al., 1997). In the fed state, PPARγ promotes the uptake and utilisation of fatty acids in adipose tissue by stimulating the expression of LPL, fatty acid transport proteins (FATP and CD36), adipocyte specific fatty acid binding protein (aP2), long chain acyl CoA synthase and PEPCK, which generates glycerol required for triglyceride synthesis (Walczak and Tontonoz, 2002; Chakravarty et al., 2005). PPARγ is also important in macrophage biology and atherosclerosis (Section 1.3.4), playing a role in both lipid uptake via CD36 and efflux via its target gene, LXRα (Walczak and Tontonoz, 2002).

1.4.7 Orphan nuclear receptors

Orphan NRs are those for which no ligand has been identified or the physiological relevance of the ligand is unclear (Benoit et al., 2006). Orphan NRs are present in all six sub-families of nuclear receptors, as well as orphan NRs lacking a DBD or LBD which form a separate sub-family (group 0) (Table 1.4). In some cases, orphan NRs lack a LBP due to bulky amino acids side chains filling this space (for example, NURR1) or display constitutive activity with an empty LBP (for example, ERRγ) (Greschik et al., 2002; Wang et al., 2003). Alternatively, ligands have been identified for orphan NRs which have a structural role in the activity of the NR. Fatty acids have been shown to play a role in the structural stability of HNF4 (Section 1.7.2), whilst the Drosophila homologues of RXR and Rev-erb, USP (NR2B4) and E75 (NR1D3), bind phospholipid or heme moieties respectively as ‘structural ligands’ (Benoit et al., 2006).

1.4.8 Chromatin structure and modification

The organisation of DNA into chromatin in the nucleus of eukaryotic cells is one reason for the complexity of transcriptional regulation in higher organisms. The DNA double-helix is coiled around histone proteins which form an octamer consisting of core histones H2A, H2B, H3, and H4. Histone H1 binds to the DNA linking the nucleosomes and is involved in the formation of higher order chromatin structure (Berg et al., 2006). Promoters and regulatory regions of actively transcribed genes differ from inactive chromosome regions in that the former are less tightly complexed with histones and more accessible to RNA Polymerase II and the basal transcription factors necessary for the initiation of transcription (Berg et al., 2006).
The reversible acetylation of lysine residues in the N- and C-terminal tails of histones H3 and H4 is an important mechanism of regulating the accessibility of the basal transcriptional machinery to DNA (Figure 1.8). Acetylation disrupts ionic interactions of DNA with the highly charged tails of histone molecules, leading to a more open nucleosomal structure (Spencer and Davie, 1999).

Figure 1.8: Influence of histone acetylation and DNA methylation on chromatin structure and transcription. In regions of actively transcribed DNA, DNA is unmethylated and histones are acetylated (pink circles). The latter weakens the interaction between the negatively charged DNA and positively charged histones and allowing binding of TFs to promoter sequences. Deacetylation of histones and methylation of DNA (green circles) results in transcriptional repression via the recruitment of methyl-CpG-binding proteins (MeCP2) and HDACs. Diagram from Schrem et al. (2002).
Some NR co-activators have intrinsic histone acetyltransferase (HAT) activity, for example, steroid receptor co-activator-1 (SRC-1), cAMP response element binding protein-binding protein (CBP) and p300 (further discussed in Section 1.8.4) (Spencer et al., 1997; Kalkhoven, 2004). The reverse process catalyzed by histone deacetylases (HDACs) leads to a more restricted nucleosomal structure. Co-repressors of NRs, for example, silencing mediator for retinoid and thyroid receptors (SMRT), recruit HDACs as a mechanism of gene silencing (Moehren et al., 2004). It should be noted that NR co-activators catalyze other covalent histone modifications, including methylation, phosphorylation and ubiquitination, all of which play roles in the structure of chromatin (Hermanson et al., 2002). Nuclear receptor co-activators also include ATP-dependent chromatin remodelling complexes (CRCs), which alter nucleosome positioning in a non-covalent manner. For example, a requirement for the ATPase BRG-1 (a constituent of the SWI/SNF class of CRCs) has been shown in GR-mediated transcriptional activation (Trotter and Archer, 2007).

DNA methylation of cytosine nucleotides also plays an important role in determining whether a gene is in an active or inactive state (Figure 1.8). Promoter regions often contain a high percentage of cytosine and guanine nucleotides (genomic regions known as CpG islands) (Gardiner-Garden and Frommer, 1987). Methylated CpG bases are recognised by methyl CpG binding proteins, which exert a repressive effect through interaction with HDAC-containing complexes (Figure 1.8) (Nan et al., 1998). DNA methylation can also have a direct repressive effect on gene transcription by preventing transcription factor binding to DNA (Bird and Wolffe, 1999).

As will be discussed in the following sections, recruitment of co-factors with histone-modifying capabilities is highly relevant to the transcriptional factor at the heart of this project, HNF4α. Different aspects of HNF4α biology including genetics, structure and modulation of transcription activity will be described in detail in the following four sections.

1.5 HNF4α: an overview

HNF4 was identified in rat liver cell extracts and was initially designated as an orphan member of the nuclear receptor family due to the lack of an endogenous ligand being identified (Sladek et al., 1990). Hepatocyte nuclear factors (HNFs) are a group of transcription factors from different transcription factor families, whose expression is enriched in the liver and is required for liver development and function (Schrem et al., 2002).
Three genes for HNF4 exist: HNF4α (NR2A1), the originally identified isoform and two further HNF4 genes, HNF4β and HNF4γ (Sladek et al., 1990; Holewa et al., 1997; Drewes et al., 1996). HNF4α is expressed in mammals, including humans, rat and mouse (Chartier et al., 1994; Sladek et al., 1990; Hata et al., 1995). The HNF4β was identified in *Xenopus laevis* (NR2A3) gene, however it is absent in mammalian genomes (Holewa et al., 1997). The HNF4γ gene is expressed in mammals and located on human chromosome 8 and on mouse chromosome 3 (Drewes et al., 1996; Taraviras et al., 2000).

HNF4α is expressed predominantly in the liver, the kidney, small intestine, colon and pancreas, with small amounts also present in the testis (Sladek et al., 1990; Miquerol et al., 1994; Drewes et al., 1996). HNF4γ is expressed in the pancreas, kidney, small intestine and testis, but is absent from the liver (Drewes et al., 1996). In comparison with other nuclear receptors such as PPARs which heterodimerize with RXRs, HNF4α undergoes exclusive homodimerization and is found to reside primarily in the nucleus (Jiang et al., 1995).

HNF4α dimers binds direct repeat-1 (DR-1) elements which consist of two half-sites of six bases separated by a single nucleotide (Sladek and Seidel, 2001).

### 1.5.1 HNF4α target genes

HNF4α regulates a wide variety of target genes involved in nutrient transport and the metabolism of lipids, sterols and glucose and amino acids (Figure 1.9). In addition, HNF4α regulates genes involved in many other aspects of liver function, including blood coagulation and immunity (Figure 1.9) (Sladek and Seidel, 2001). Of particular interest in the study of metabolic diseases is the role of HNF4α in the regulation of genes involved in metabolic pathways which are active under opposing conditions: For example, HNF4α target genes include key regulatory genes in both gluconeogenesis, e.g. PEPCK, and glycolysis, e.g. L-PK, (Hall et al., 1995; Diaz Guerra et al., 1993). In addition, HNF4α target genes include both apolipoprotein B (apoB), a constituent of LDL, and apolipoprotein A1 (apoA1), a constituent of HDL (Chan et al., 1993; Metzger et al., 1993).


1.6 Genetic evidence for the role of HNF4α in metabolic disorders

1.6.1 Maturity Onset Diabetes of the Young

Maturity-onset diabetes of the young (MODY) is defined as an early-onset form of Type 2 diabetes which is inherited in an autosomal dominant pattern (Hattersley, 1998). Patients characteristically develop symptoms of hyperglycaemia and show impaired insulin secretion before the age of 25, without the prior development of insulin resistance or obesity (Fajans et al., 2001). The early-onset and monogenic nature of MODY makes it ideal for genetic analysis, compared to late-onset Type 2 diabetes where multi-generation pedigrees are difficult to obtain (Hattersley, 1998). To date six genes have been identified as causing MODY types 1-6 which are typically diagnosed at different ages and vary in the severity of symptoms and complications (Table 1.5).
Table 1.5: Comparison of different types of MODY.

<table>
<thead>
<tr>
<th>MODY Type</th>
<th>Gene</th>
<th>Age of onset</th>
<th>Most common treatment</th>
<th>Frequency of complications</th>
</tr>
</thead>
<tbody>
<tr>
<td>MODY 1</td>
<td>HNF4α</td>
<td>Postpubertal</td>
<td>Oral hypoglycemic agent, insulin</td>
<td>Frequent</td>
</tr>
<tr>
<td>MODY 2</td>
<td>Glucokinase</td>
<td>Childhood</td>
<td>Diet and exercise</td>
<td>Rare</td>
</tr>
<tr>
<td>MODY 3</td>
<td>HNF1α</td>
<td>Postpubertal</td>
<td>Oral hypoglycemic agent, insulin</td>
<td>Frequent</td>
</tr>
<tr>
<td>MODY 4</td>
<td>IPF-1 (PDX-1)</td>
<td>Early adulthood</td>
<td>Oral hypoglycemic agent, insulin</td>
<td>Rare</td>
</tr>
<tr>
<td>MODY 5</td>
<td>HNF1β</td>
<td>Postpubertal</td>
<td>Insulin</td>
<td>Non-diabetic renal dysfunction present</td>
</tr>
<tr>
<td>MODY 6</td>
<td>NeuroD1 (BETA2)</td>
<td>Early adulthood to middle age</td>
<td>Insulin</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Table adapted from Hattersley (1998) and Fajans et al. (2001) with additional information on MODY5 and MODY6 (Lindner et al., 1999; Malecki et al., 1999)

With the exception of the MODY 2 gene which codes for glucokinase, the other five identified MODY genes code for transcription factors (Table 1.5). Whilst glucokinase mutations directly affect the metabolism of glucose to glucose-6-phosphate, MODY-linked transcription factors regulate the expression of several genes involved in glucose-stimulated insulin secretion (GSIS), including glycolysis, mitochondrial ATP generation, ion channels and insulin gene transcription (Figure 1.10) (Fajans et al., 2001).

Linkage studies localised the gene responsible for MODY 1 to the long arm of chromosome 20 (Bell et al., 1991), with the HNF4α gene subsequently being identified as the MODY 1 gene (Yamagata et al., 1996). As with other MODY types, MODY 1 is characterised by deficient pancreatic insulin secretion, rather than insulin resistance (Herman et al., 1994). Even in the prediabetic state, HNF4α mutation-carriers show an inability of pancreatic β-cells to increase insulin secretion when blood glucose concentrations rise above 7 mmol/l (Byrne et al., 1995). In contrast to normal individuals or other types of MODY, where insulin secretory response by β-cells is primed by prior infusion of glucose or arginine, HNF4α mutation-carriers lack this priming response (Byrne et al., 1995; Herman et al., 1997). The molecular basis for pancreatic dysfunction in MODY 1 is likely to reside in deficient transcription of HNF4α downstream genes involved in glucose-stimulated insulin secretion (Figure 1.10). HNF4α has been shown to regulate several genes in glucose transport and metabolism, subunits of the ATP-dependent K+ channel, as well as the expression of another
Figure 1.10: Role of MODY genes in pancreatic β-cell function. The MODY2 gene, glucokinase, catalyses the conversion of glucose to glucose-6-phosphate, thereby acting the pancreatic glucose sensor. The metabolism of glucose via the glycolytic pathway and Krebs cycle leads to the generation of ATP. Raised ATP levels trigger the closure of ATP-sensitive K^+ channels, resulting in membrane depolarisation and opening of voltage-dependent Ca^{2+} channels. Ca^{2+} influx and release from intracellular stores leads to the fusion of insulin-containing granules with the plasma membrane. MODY genes coding for transcription factors (HNF4α, HNF1α, IPF-1, HNF1β and NeuroD1) code for transcription factors which regulate the expression of insulin, and other genes involved in GSIS. Diagram from review by Fajans et al. (2001).

Interestingly, patients with MODY1 also show decreased plasma triglyceride and lipoprotein levels, suggesting that hepatic lipid metabolism is also affected by HNF4α haploinsufficiency.
(Lehto et al., 1999; Shih et al., 2000). Neonatal hypoglycaemia and macrosomia due to foetal hyperinsulinaemia have been also observed in heterozygous carriers of HNF4α mutations, suggesting that over-secretion of insulin may precede the decline in insulin secretion observed later in life for MODY patients, reminiscent of the development of T2DM (Section 1.2.1) (Pearson et al., 2007; Fajans and Bell, 2007; Kapoor et al., 2008).

1.6.2 Late-onset Type 2 Diabetes Mellitus

Unlike MODY, late-onset T2DM is a polygenic disorder, with nevertheless a substantial genetic component as demonstrated by studies with identical twins: if one identical twin has T2DM, the other twin has a 90% chance of developing the condition (Holt and Hanley, 2007). However, a genetic predisposition to develop T2DM can be modulated by environmental factors such as diet and physical activity (McCarthy and Menzel, 2001). It is hypothesised that genes which conferred a selective advantage during periods of starvation throughout human evolution have become detrimental in westernised society where calorie-rich food is in excess (Neel, 1962).

The HNF4α gene is located on chromosome 20q, a region which was associated with T2DM in several studies (Bowden et al., 1997; Ji et al., 1997). Single nucleotide polymorphisms (SNPs) in the HNF4α P2-promoter region were initially identified as being associated with T2DM in Finnish and Ashkenazi Jewish populations, with odds ratios of 1.33 and 1.45 respectively (i.e. there is a 33% or 45% greater likelihood of suffering from T2DM in carriers of the HNF4α SNP compared to non-carriers) (Silander et al., 2004; Love-Gregory et al., 2004). Several subsequent studies found a more modest association of HNF4α P2 SNPs with T2DM in Caucasian populations (Damcott et al., 2004; Hansen et al., 2005; Bonnycastle et al., 2006), Pima Indians (Muller et al., 2005), Americans of Mexican descent (Lehman et al., 2007) and Japanese (Hara et al., 2006). However, HNF4α P2-promoter SNPs were not associated with an increased risk of T2DM in other studies (Vaxillaire et al., 2005; Winckler et al., 2005; Yokoi et al., 2006). The association of P1 promoter and intragenic SNPs with T2DM differed between the studies of Silander et al. (2004) and Love-Gregory et al. (2004), whilst P1 promoter variants were shown to carry a more significant risk of T2DM than P2 promoter variants in the Amish (Damcott et al., 2004). In a longitudinal study investigating progression from impaired glucose tolerance to T2DM (Section 1.1.2), female carriers of a minor P2 promoter SNP allele had an odds ratio of 1.7 of developing T2DM compared to
no carriers, whilst no association was found in men (Andrulionyte et al., 2006). Differences in the individual SNPs carrying the greatest risk observed between studies could be due to interactions between genotype and environmental factors such as nutrition (Love-Gregory and Permutt, 2007).

1.6.3 Dyslipidaemia and the Metabolic Syndrome

Recent studies have shown association of HNF4α gene variants with types of dyslipidaemia and traits which fall under the classification of metabolic syndrome (Section 1.2.3). HNF4α SNPs have recently been associated with familial combined hyperlipidaemia (FHCL) where serum cholesterol and/or triglycerides are raised, in Finnish and Mexican cohorts (Weissglas-Volkov et al., 2006). Two of the individual SNPs identified in this study, located downstream of the P2 promoter (rs2144908) and in the P1 promoter region (rs2425640) were also associated with T2DM, suggesting genetic and phenotypic overlap between dyslipidaemia and T2DM (Silander et al., 2004; Love-Gregory et al., 2004; Damcott et al., 2004). The rs2425640 SNP and haplotypes composed of multiple SNPs were also associated with parameters of glucose tolerance and insulin sensitivity in Finnish FHCL families. In the same report, different HNF4α haplotype variants were associated with raised total cholesterol levels in Finnish low-HDL cholesterol families, providing evidence for association of HNF4α variants with a different trait of the metabolic syndrome (Weissglas-Volkov et al., 2006). Classification of Finnish FHCL and low-HDL cholesterol families for features of the metabolic syndrome also found association of distinct HNF4α haplotypes with the metabolic syndrome (Weissglas-Volkov et al., 2006).

1.6.4 HNF4α knockout models

Further evidence for the role of HNF4α in the pathogenesis of diabetes and dyslipidaemia comes from targeted disruption of the HNF4α gene in mouse models. HNF4α null animals die during gestation due to impaired gastrulation (Chen et al., 1994b). An alternative strategy to investigate HNF4α function was to disrupt the HNF4α gene in embryonic stem cells (Stoffel and Duncan, 1997). The latter were induced to form visceral endoderm, which displays properties of both the liver and endocrine pancreas (Stoffel and Duncan, 1997). HNF4α−/− stem cells showed decreased mRNA expression of several genes involved in glucose metabolism and GSIS including liver-pyruvate kinase (L-PK), aldolase B, GLUT2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Stoffel and Duncan, 1997).
A conditional knockout of the HNF4α gene in β-cells resulted in mice with fasting hypoglycaemia and hyperinsulinaemia (Gupta et al., 2005). These findings in relatively young animals (five-month old) are in agreement with recent results showing the same symptoms in human neonates with a HNF4α mutation (Pearson et al., 2007; Fajans and Bell, 2007; Kapoor et al., 2008) (Section 1.6.1). However a separate β-cell HNF4α knockout model did not replicate these findings (Miura et al., 2006). Both β-cell knockout mouse models showed impaired glucose tolerance due to defective insulin secretion, with both first and second phase insulin response being diminished (Section 1.1.1) (Gupta et al., 2005, Miura et al., 2006). Both studies pointed to K\textsubscript{ATP} channel dysfunction contributing to the impaired insulin secretion, with Gupta et al. (2005) finding reduced K\textsubscript{ATP} subunit (Kir6.2) mRNA expression responsible, whilst Miura et al. (2006) observed increased conductivity of the K\textsubscript{ATP} channels in knockout vs. control animals.

Conditional gene knockout of HNF4α in the liver resulted in mice with an ‘atheroprotective serum lipid profile’ of decreased serum triglyceride and cholesterol concentrations (Hayhurst et al., 2001). However, the conditional hepatic HNF4α null mice also exhibited hepatic accumulation of lipid and increased serum bile acids (Hayhurst et al., 2001). Genes involved in VLDL secretion (apolipoprotein B and microsomal triglyceride transfer protein) showed reduced expression in hepatic HNF4α knockout mice. LDL and HDL concentrations were lower in HNF4α knockout animals compared to controls, which were proposed to be due to increased scavenger receptor B1 expression, facilitating increased HDL uptake (Hayhurst et al., 2001). Genes involved in hepatic glucose metabolism were affected in another liver-specific knockout of the HNF4α gene, with decreased expression of glycogen synthase and gluconeogenic enzymes (PEPCK and glucose-6-phosphatase) (Parviz et al., 2003).

### 1.7 HNF4α structure and function

#### 1.7.1 HNF4α functional domains

The classical nuclear receptor domains A-F containing DNA-binding, dimerization, ligand-binding and activation function motifs has been mapped in the HNF4α protein sequence (Figure 1.11) (Hadzopoulou-Cladaras et al., 1997). AF-1 encompasses the first N-terminal 24 amino acids, whilst amino acids 128 to 366 are required for AF-2 (Hadzopoulou-Cladaras et al., 1997). The F-domain in HNF4α is relatively large (90 amino acids) compared to that
of other NRs and shares a proline-rich repressor region with similarity to the PR (Iyemere et al., 1998). The presence of the F-domain inhibits HNF4α-dependent transcriptional activity and plays a role in the interaction of HNF4α with the co-repressor, SMRT (Sladek et al., 1999; Ruse et al., 2002).

![Figure 1.11: HNF4α structural domains.](image)

The HNF4α F-domain inhibits transcriptional activity and hence was termed the negative regulatory domain (NRD). Diagram from Hadzopoulou-Cladaras et al. (1997).

### 1.7.2 Fatty acids and HNF4α

Despite initially being classified as an ‘orphan’ nuclear receptor, fatty acids have subsequently been shown to have a role in HNF4α ligand binding and activity. Fatty acyl coenzyme A (CoA) thioesters have been proposed to be the proximate ligand for HNF4α based on in vitro binding assays and cotransfection experiments (Hertz et al., 1998). These experiments also suggested that CoA thioesters of polyunsaturated fatty acids (PUFA) have an inhibitory effect of HNF4α activity whilst maximal transactivation is achieved in the presence of the saturated palmitoyl-CoA (Hertz et al., 1998). However, X-ray crystallographical studies found that the HNF4α LBD contained a non-esterified fatty acid (Wiseley et al., 2002). The bound fatty acid moiety was found to be a saturated or monounsaturated fatty acid of lengths between C16 to C18, however the bound fatty acid was not exchangeable with 14C-labelled palmitic acid, suggesting that fatty acids form a permanent cofactor in the HNF4α molecule as opposed to an exchangeable ligand (Wiseley et al., 2002; Dhe-Paganon et al., 2002).

Recently, it has been proposed that there is cross-talk between the regions of HNF4α involved in binding of free fatty acid and acyl CoA (Hertz et al., 2005). In line with X-crystallographic studies, the central region of HNF4α from amino acids 133-382 was found to be involved in the binding of free fatty acids, whilst the C-terminal region (amino acids 370 – 455) was found to be required for binding and hydrolysis of acyl CoAs (Hertz et al., 2005). However, in contrast to the findings of X-ray crystallographical studies, the products
of acyl CoA hydrolysis were able to exchange with the free fatty acid bound in the free fatty acid pocket and modulate HNF4α transcriptional activity (Hertz et al., 2005).

The suppression of HNF4α activity by polyunsaturated fatty acyl-CoA (Hertz et al., 1998) has been proposed be one mechanism by which PUFAs exert a hypolipidaemic effect on hepatic lipid metabolism (Davidson, 2006). For example, the inhibitory effect of PUFAs on the transcription of the glycolytic gene, L-PK, is mediated by the HNF4α binding site in the L-PK promoter (Section 1.9.2) and polyunsaturated fatty acyl CoA was found to inhibit HNF4α binding to the G-6-Pase promoter (Pan et al., 2000; Rajas et al., 2002). HNF4α has been proposed to be an alternative target for hypolipidaemic drugs, such as clofibrate (in the form of their CoA-conjugates), which are traditionally thought to work as PPARα agonists (Bar-Tana, 2004).

### 1.7.3 Splice variants of HNF4α

The HNF4α gene contains ten exons which undergo alternative splicing to yield nine theoretical splice variants, six of which (HNF4α1, HNF4α2, HNF4α3, HNF4α4, HNF4α7 and HNF4α8) have been identified in vivo (Figure 1.5) (Sladek and Seidel, 2001). Splice variants are also generated through alternative promoter usage, with usage of proximal promoter P1 to yield transcripts containing exon 1A (HNF4α1-6) or usage of the distant upstream promoter P2 to generate transcripts containing exon 1D (HNF4α7-9) (Figure 1.12).

HNF4α1 was the first splice variant to be isolated in rat liver nuclear extracts (Sladek et al., 1990), with a second isoform, HNF4α2 (previously referred to as HNF4B), being identified in rat, human and mouse liver (Hata et al., 1992; Chartier et al., 1994; Hata et al., 1995). HNF4α2 contains an extra 10 amino acid peptide in the C-terminal F domain, which was reported to be less inhibitory on co-activator binding than the HNF4α1 F-domain (Sladek et al., 1999). The third splice variant of HNF4α, HNF4α3 (previously referred to as HNF4C), is formed by use of an alternative stop codon in exon 8, resulting in a shorter F domain of only 40 amino acids (Kritis et al., 1996). The shorter F domain may be less inhibitory in its interaction with the AF-2 region than the longer F domain of HNF4α1 and HNF4α2 (Suaud et al., 1999). HNF4α4 was cloned from a human kidney cDNA library and contains an addition exon, 1B, coding for an additional 30 amino acids in the N-terminal A/B domain (Figure 1.12) (Drewes et al., 1996).
According to published sequences for HNF4α4 (or HNF4α5 based on the nomenclature of Sladek and Seidel (2001), Figure 1.12), the cloned splice variant contained the 10 amino acid peptide in the C-terminal region common with HNF4α2 (Drewes et al., 1996). Transient transfection experiments indicated that HNF4α4 had a much lower transactivation potential compared to HNF4α2 (Drewes et al., 1996). HNF4α4 has been found to be expressed in enterocyte cell lines, with high expression of the HNF4α4 variant being associated with a less differentiated status (Suaud et al., 1997).

The HNF4α7 and HNF4α8 splice variants are associated with decreased differentiation of hepatic cells: HNF4α7 was initially identified in an undifferentiated murine hepatocyte cell line and both the HNF4α7 and HNF4α8 variants are expressed in murine foetal-like hepatoma cells (Nakhei et al., 1998; Torres-Padilla et al., 2001). HNF4α7 and HNF4α8 originate from an alternative promoter (P2), situated ~46 kb upstream of the P1 promoter in the human genome, and contain an alternative first exon, exon 1D (Thomas et al., 2001).
HNF4\(\alpha\)1 (P1 promoter) and HNF4\(\alpha\)7 (P2 promoter) splice variants undergo a differential pattern of expression in mouse liver development with HNF4\(\alpha\)7 being expressed prenatally, whilst the expression of HNF4\(\alpha\)1 is increased shortly before birth and postnatally (Torres-Padilla et al., 2001). In line with these findings, the two promoters are regulated by different transcription factors, with the P2 promoter being subject to negative regulation by HNF4\(\alpha\)1 (Figure 1.13) (Briancon et al., 2004). Compared to their expression in the adult liver, P2 promoter splice variants (HNF4\(\alpha\)7, HNF4\(\alpha\)8 and HNF4\(\alpha\)9) are expressed relatively highly in the islets of Langerhans in the pancreas (Thomas et al., 2001; Boj et al., 2001; Briancon and Weiss, 2006).

As well as their different roles in development, HNF4\(\alpha\) P1- and P2-promoter variants (referred to as HNF4\(\alpha\)1 and HNF4\(\alpha\)7 for simplicity) possibly have different metabolic functions, as shown by knock-in mice, expressing only HNF4\(\alpha\)1 or HNF4\(\alpha\)7 (Briancon and Weiss, 2006). HNF4\(\alpha\)7-only mice display dyslipidaemia, with reduced serum cholesterol and triglycerides and mild hepatosteatosis, whilst HNF4\(\alpha\)1-only mice were found to have impaired glucose tolerance (Briancon and Weiss, 2006). Dyslipidaemia observed in the HNF4\(\alpha\)7-only mice may result from the inability of HNF4\(\alpha\) to stimulate expression of genes involved in VLDL secretion (apoB and MTP) and catabolism (apoCII) (Briancon and Weiss, 2006).

![Figure 1.13: Regulation of HNF4\(\alpha\) expression from its P1 and P2 promoters.](image)

The HNF4\(\alpha\) splice variants HNF4\(\alpha\)1-5 are transcribed by the P1 promoter whilst HNF4\(\alpha\)7-9 are transcribed by the P2 promoter. The alternative promoters are under the control of different liver-enriched transcription factors (HNFs). The glucocorticoid receptor (GR) binds the enhancer element (ENH) upstream of promoter P1. HNF4\(\alpha\)1/2 also negatively regulates transcription from the P2 promoter. Diagram from Briancon et al. (2004).
As this project focuses on the regulation of HNF4α transcriptional activity by metabolic stimuli such as insulin and glucagon (Section 1.1.1), the different regulatory mechanisms through which metabolic stimuli could influence HNF4α activity, such as post-translational modification, interactions with co-factors and changes in HNF4α mRNA expression, will be discussed in the following section.

1.8 Metabolic regulation of HNF4α activity

1.8.1 Regulation of HNF4α activity by post-translational modification

One mechanism by which metabolic stimuli influence HNF4α transcriptional activity is through post-translational modification of the HNF4α protein by phosphorylation or acetylation. There are several potential sites for serine (S) and threonine (T) phosphorylation within the HNF4α protein (Figure 1.14) (Jiang et al., 1997). HNF4α is phosphorylated by PKA within the DBD at S134, leading to reduced DNA-binding activity (Viollet et al., 1997). Phosphorylation of S304 by AMPK within the LBD inhibits HNF4α dimerization and brings about a reduction in HNF4α protein levels (Leclerc et al., 2001; Hong et al., 2003).

![Diagram showing phosphorylation sites on HNF4α](image)

**Figure 1.14: HNF4α serine/threonine phosphorylation sites.** Phosphorylation sites are shown as identified by phosphopeptide mapping (Jiang et al., 1997), from which this diagram is taken. Positions are given in the rat HNF4α protein sequence and putative phosphorylation sites indicated by the symbols indicated below. Arrows refer to studies confirming HNF4α being a target for intracellular protein kinases: PKC at S78 (Sun et al., 2007), PKA at S134 (Viollet et al., 1997), p38 MAPK at S158 (Guo et al., 2006) and by AMPK at S304 (Leclerc et al., 2001).
Recent reports demonstrate that S158 is a target of p38 MAPK, which has a positive effect on HNF4α DNA-binding and transcriptional activity, and protein stability (Guo et al., 2006; Xu et al., 2007). Finally, phosphorylation of HNF4α at S78, which is located between the two zinc fingers of the DBD, by protein kinase C (PKC) disrupts its DNA-binding activity, protein stability and nuclear localisation (Sun et al., 2007). In addition, HNF4α is subject to phosphorylation at tyrosine residues, which also play a role in its DNA-binding activity and subnuclear localisation, although the specific residues involved were not characterised (Ktistaki et al., 1995). HNF4α transcriptional activation and nuclear retention is promoted by acetylation of lysine residues within the NLS by the co-activator CBP (Soutoglou et al., 2000).

1.8.2 Regulation of HNF4α activity through interaction with co-factors

From a structural point of view, the interaction of HNF4α with co-factors is key to its adopting an active conformation. In the case of ligand-activated NRs, ligand binding triggers a conformation change and repositioning of the AF-2 domain in the twelfth α-helix (H12) of the LBD, whereas H12 of HNF4α remains in an open position even with a fatty acid in the ligand binding pocket (Figure 1.15) (Duda et al., 2004). The crystal structure of fatty acid-bound HNF4α with a co-activator peptide (SRC-1) showed H12 to be in a closed, active conformation, suggesting that co-factor binding could be a decisive event in HNF4α activation (Figure 1.15) (Duda et al., 2004).

Both HNF4α AF-1 and AF-2 domains have been shown to mediate the interaction of HNF4α with co-factors (Wang et al., 1998; Green et al., 1998). Variation of these regions in HNF4α splice variants could be one mechanism facilitating differential interactions with co-activators and hence differential target gene expression. Differences in the F domain of HNF4α1, HNF4α2 and HNF4α3 influenced their interactions with cofactors (Sladek et al., 1999; Suaud et al., 1999). Furthermore, HNF4α variants transcribed from the P1 promoter (HNF4α1-5) contain the activation module AF-1 in the A/B region whereas those transcribed from the P2 promoter (HNF4α7-9) do not. The weaker transactivation potential of HNF4α8 compared to HNF4α2 was attributed to its lack of interaction with co-factors which bind to the AF-1 region, such p300 (Eckhoute et al., 2003). The interaction of co-factors with HNF4α can be modulated by promoter context: For example, transactivation of the apoB

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promoter by HNF4α was found to be independent of the co-activator GRIP-1, whilst expression of an apoAI reporter plasmid (Section 1.11) showed synergism in the interaction of HNF4α, p300 and GRIP-1 (Torres-Padilla and Weiss, 2003).

The interaction of HNF4α with co-factors may be governed by metabolic stimuli, as co-activators are themselves phosphoproteins and targets of protein kinase cascades (Wu et al., 2005). Two HNF4α co-activators of importance in this project, PGC-1α and p300, will be described in greater detail below.

1.8.3 Peroxisome proliferator-activated receptor-γ coactivator-α (PGC-1α)

PGC-1α is a 798 amino acid protein that functions as a ‘bridging’ co-activator, acting as a molecular scaffold to facilitate interactions between a transcriptional activator and the RNA polymerase holoenzyme (Section 1.4.1) (Acevedo and Kraus, 2004). PGC-1α facilitates the
recruitment of further co-activators through different protein domains: Through its N-terminal activation domain, PGC-1α interacts with co-activators that display HAT activity such as p300 and SRC (Section 1.4.8), whilst the PGC-1α C-terminus interacts with thyroid hormone receptor–associated protein/vitamin D receptor–interacting protein complex (TRAP/DRIP, or Mediator), which interacts directly with RNA Polymerase (Figure 1.16A) (Puigserver, 1999; Wallberg et al., 2003). PGC-1α is also involved in splicing of pre-mRNAs via its C-terminus (Finck and Kelly, 2006).

PGC-1α was discovered as a cold-inducible co-activator of PPARγ in the process of thermogenesis in brown adipose tissue (Puigserver, 1998). In addition to brown adipose tissue, PGC-1α is expressed in other tissues with a high oxidative capacity such as heart, slow-twitch skeletal muscle and liver (Finck and Kelly, 2006). PGC-1α interacts with a wide range of NRs, transcription factors involved in mitochondrial biogenesis (nuclear respiratory factors (NRFs)) and muscle-specific gene expression (myocyte enhancing factor-2 (MEF2)) (Figure 1.16A) (Finck and Kelly, 2006). Expression of the PGC-1α gene is induced in the liver in response to fasting stimuli (glucagon and glucocorticoids), leading to activation of gluconeogenic gene expression (Yoon et al., 2001). The interaction of HNF4α and PGC-1α is crucial to the PGC-1α-dependent stimulation of PEPCK and G-6-Pase expression (Rhee et al., 2003). The PGC-1α LXXLL motif interacts with the HNF4α AF-2 domain (Yoon et al., 2001). PGC-1α is also known to interact with other transcription factors in the regulation of PEPCK including CREB and Foxo1 (Herzig et al., 2001; Puigserver et al., 2003). PGC-1α is activated by p38 MAPK phosphorylation downstream of cytokine stimulation in muscle cells (Puigserver et al., 2001).

### 1.8.4 p300

p300 is a transcriptional co-activator with intrinsic HAT activity. p300 shares regions of high homology with CBP and often the two co-activators are referred to interchangeably in the literature, however CBP contains a unique polyglutamine stretch towards the C-terminus which is linked to neurogenerative diseases (Kalkhoven, 2004). p300 is a large protein of over 2400 amino acids with regions which facilitate its interactions with other proteins (CH1, CH3, KIX domains) and with acetylated residues (bromodomain), and contain its HAT domain (Figure 1.16B) (Chan and La Thangue, 2001). p300 can interact with basal transcription factors, TBP and TFIIB, and RNA Polymerase II through its N- and C-termini,
thereby acting as a molecular scaffold to stabilize the basal transcription complex (Kalkhoven, 2004).

HNF4α interacts with p300 through the HNF4α AF-1 and AF-2 domains and impaired recruitment of p300 is a possible mechanism for the reduced activity of HNF4α mutations found in MODY (Eeckhoute et al., 2001; Eeckhoute et al., 2003). S1834 of p300 is a target for phosphorylation by PKB; a mechanism which plays a role in the stimulation of glucokinase gene expression by insulin (Roth et al., 2004). On the other hand, phosphorylation of S89 by AMPK blocks the interaction of p300 with nuclear receptors (Yang et al., 2001).
1.8.5 Regulation of HNF4α gene expression

Alterations in HNF4α gene expression levels may be one mechanism which leads to changes in expression of HNF4α target genes. HNF4α mRNA expression has been shown to be up-regulated in the fasting state in vivo (Yoon et al., 2001). Expression of the HNF4α gene in primary rat liver hepatocytes was also found to be stimulated by glucagon and dexamethasone, a synthetic glucocorticoid (Oyadomari et al., 2000). A glucocorticoid response element in an enhancer region ~6 kb upstream of the P1-promoter is responsible for induction by glucocorticoids (Figure 1.13) (Bailly et al., 2001). Oyadomari and coworkers (2000) also found that insulin antagonized the effects of glucagon and dexamethasone and reduced HNF4α mRNA levels. In line with this finding, in streptozotocin-induced diabetic rats that cannot produce insulin, levels of HNF4α mRNA are elevated and can be normalised by insulin treatment (Oyadomari et al., 2000).

PEPCK, L-PK, SREBP-1c, FAS, apoCIII and PPARα are HNF4α target genes in differentially regulated metabolic and transcriptional pathways (Section 1.3). As the expression of these HNF4α target genes were studied in this project, the factors influencing their mRNA expression will be discussed in the following section.

1.9 Transcriptional regulation of HNF4α target gene expression

1.9.1 PEPCK

PEPCK is a key regulatory gene in the gluconeogenic pathway (Section 1.3.1). The transcription of PEPCK is tightly regulated according the fasting-fed cycle: glucagon/cAMP and glucocorticoids up-regulate its transcription whilst insulin acts as a dominant inhibitory stimulus (Lamers et al., 1982; Grammer et al., 1983; Sasaki et al., 1984). The PEPCK promoter has been described as consisting of three regulatory regions (Figure 1.17) (Hanson, 2005). Region 1 contains a cAMP response element (CRE), which binds CRE-binding protein (CREB) amongst other members of the leucine zipper family of transcription factors (Chakravarty et al., 2005). Region 2 is also important in the up-regulation of PEPCK expression in response to glucagon/cAMP and contains a binding site for CAAT/enhancer-binding protein (C/EBP) (Liu et al., 1991). Region 3 is required for the induction of PEPCK expression by glucocorticoids and contains a proximal and distal glucocorticoid regulatory unit (GRU), each consisting of a glucocorticoid regulatory element and accessory factor
(AF)-binding sites, which bind transcription factors other than the GR and are necessary for the transcriptional response to glucocorticoids (Imai et al., 1990). The -90 bp CRE also interacts with the proximal GRU in the induction of PEPCK expression by glucocorticoids (Imai et al., 1993). There are two sterol regulatory elements (SREs) in the PEPCK promoter (at positions -322 to -313 and -590 to -581 bp), and SREBP-1c has been implicated in the insulin inhibition of PEPCK expression (Chakravarty et al., 2004).

HNF4α was initially identified as binding to the accessory factor site 1 (AF1) of the proximal GRU and has subsequently been shown to play a role in stimulation of PEPCK expression via the AF1 site in distal GRU (Hall et al., 1995; Cassuto et al., 2005). The interaction of HNF4α with the fasting-induced co-activator, PGC-1α (Section 1.8.3), is important in the fasting-induced expression of PEPCK (Yoon et al., 2001; Rhee et al., 2003).

**Figure 1.17: Regulatory elements of the PEPCK gene promoter.** Diagram showing regulatory elements in the PEPCK promoter and the transcription factors and co-factors which bind to them. Abbreviations used: ATF-3: activating transcription factor 3, CRE: cAMP regulatory element, CREB: CRE-binding protein, COUP-TF, Chicken Ovalbumin Upstream Promoter-Transcription factor, PPARγ2: Peroxisome Profilerator-Activated Receptor-γ2, SREBP-1: Sterol Regulatory Element Binding Protein-1, GRU: Glucocorticoid Regulatory Unit, T3R: Thyroid Hormone Receptor, Pol II, RNA Polymerase II, NFκB: Nuclear Factor κB. Diagram adapted from Hanson (2005) with notation of HNF4α binding to dAF1 element (Cassuto et al., 2005).
1.9.2 L-PK

L-PK is a key regulatory gene in the glycolytic pathway (Section 1.3.2). L-PK gene transcription is regulated in an opposite manner to that of PEPCK: L-PK mRNA expression is induced by glucose and insulin following feeding of carbohydrates, and repressed by glucagon (Raymondjean et al., 1991). The differential regulation of L-PK mRNA expression facilitates the utilisation of diets rich in carbohydrates, whilst in the fasted state, the gluconeogenic pathway is active and fatty acids are the primary source of energy production in the liver (Salway, 2004). Thyroid and glucocorticoid hormones play a permissive role in the hepatic expression of L-PK (Munnich et al., 1984; Vaulont et al., 1986). As the glycolytic pathway generates substrates for the TCA cycle and lipogenic pathways (Sections 1.2 and 1.3), n-3 PUFA and PPARα agonist Wy14,643 which have hypolipidaemic effects on hepatic lipogenesis (Section 1.7.3 and 1.7.4) also suppress L-PK mRNA expression (Jump et al., 1994; Pan et al., 2000). The anti-hyperglycaemic drug, metformin, stimulates L-PK mRNA expression, thereby both promoting hepatic glucose utilisation and suppressing hepatic gluconeogenesis (Fulgencio et al., 2001).

The -200 bp region of the L-PK proximal promoter contains key elements in the regulation of L-PK gene expression by glucose, insulin and glucagon (Thompson and Towle, 1991; Bergot et al., 1992) (Figure 1.18). This region contains binding sites (named L1 to L4) for four transcription factors (in order of proximity to the transcriptional start site): HNF1, Nuclear Factor-1 (NF-1), HNF4 and ChREBP (Vaulont et al., 1986; Yamashita et al., 2001). ChREBP (Section 1.4.4) is activated by intracellular glucose metabolism and stimulates L-PK mRNA expression by binding to the L4 element (also known as the carbohydrate response element, ChORE or ChRE) (Yamashita et al., 2001). HNF4α binds to the adjacent L3 element and cooperates with ChREBP in the induction of L-PK gene expression by glucose (Diaz Guerra et al., 1993; Liu et al., 1993). Both ChREBP and HNF4α mediate the negative effects of glucagon (and its second messenger, cAMP) on L-PK transcription (Gourdon et al., 1999; Kawaguchi et al., 2001). The inhibition of L-PK expression by n-3 PUFA is mediated via the L3 element, with n-3 PUFA being found to transiently suppress the binding of HNF4α to the L-PK promoter (Liimatta et al., 1994; Xu et al., 2006).
Figure 1.18: L-PK promoter elements and factors affecting L-PK gene transcription. L-PK promoter elements are identified as sequence motifs bound by ChREBP-Mlx (ChORE composed of two E boxes, HNF4α (DR-1 element), NF-1 and HNF1. Promoter elements are also labelled L1 to L4 in line with nomenclature used by Vaulont et al. (1986). Factors inducing L-PK expression are indicated in the upper part of the diagram, whilst factors repressing L-PK gene expression are indicated in the lower part of the diagram, with arrows reflecting the promoter elements through which these factors act. Diagram adapted from Xu et al. (2006).

1.9.3 SREBP-1c

Of the three isoforms of SREBP (Section 1.4.3), only SREBP-1c gene expression is subject to regulation by nutritional status, with decreased expression in the fasting state and increased transcription upon refeeding, an effect which is common to adipose tissue, liver and muscle (Kim et al., 1998; Horton et al., 1998a; Bizeau et al., 2003). The transcription of SREBP-1c in hepatocytes is induced by insulin and glucose, and inhibited by glucagon and PUFAs (Kim et al., 1999; Foretz et al., 1999b; Shimomura et al., 1999b; Hasty et al., 2000; Matsuzaka et al., 2004). The induction of SREBP-1c in the fed state stimulates the expression of downstream lipogenic genes such as ACC and FAS (Horton et al., 1998a; Foretz et al., 1999b). The human SREBP-1c promoter shares only 42% identity with that of the mouse, with novel binding sites being identified for HNF4α and PDX-1, the gene responsible for MODY4 (Section 1.6.1) (Tarling et al., 2004). Overexpression of HNF4α or PDX-1 stimulated expression from the human SREBP-1c promoter, raising the possibility that these
transcription factors are involved in the regulation of SREBP-1c gene expression in human tissues (Tarling et al., 2004).

1.9.4 FAS

The activity of FAS is regulated at the level of its gene expression, as FAS is not modulated allosterically or by covalent modification. Expression of the FAS gene is activated in the fed state, with insulin, glucocorticoids and glucose contributing to its induction (Paulauskis and Sul, 1989; Rufo et al., 1999). In common with L-PK and SREBP-1c, FAS gene transcription is suppressed by cAMP and PUFAs (Paulauskis and Sul, 1989; Blake and Clarke, 1990).

Insulin Response Elements at -65 and -332 bp bound by upstream stimulatory factors (USFs) are important in mediating the effect of insulin on FAS gene transcription (Moustaid et al., 1994; Wang and Sul, 1995; Moon et al., 2000). The binding of SREBP-1 to a SRE at -150 bp in the FAS promoter is also regulated by nutritional state (Latasa et al., 2000; Latasa et al., 2003). Transgenic mice overexpressing SREBP-1 showed increased FAS mRNA expression (Shimano et al., 1996; Shimano et al., 1997; Horton et al., 1998a), whereas SREBP-1 knockout animals show an impaired response of FAS to refeeding (Shimano et al., 1999). The SREBP-1c isoform has been identified as the major mediator of insulin action on FAS expression (Foretz et al., 1999a). In addition, ChREBP is a key mediator of the effect of glucose on FAS expression (Dentin et al., 2004; Ishii et al., 2004; Iizuka et al., 2004). A ChoRE has been identified in an enhancer region ~7 kb upstream of the FAS gene transcriptional start site (Rufo et al., 2001). As in the case of the L-PK gene (Section 1.9.2), the FAS ChoRE is adjacent to a HNF4α DR-1 site, with both factors cooperating in the carbohydrate induction of the FAS gene (Adamson et al., 2006).

1.9.5 apoCIII

In view of the physiological role of apoCIII in inhibiting LPL activity (Section 1.3.4), the expression of apoCIII mRNA is suppressed by insulin, thus facilitating the hydrolysis of chylomicrons in the fed state (Chen et al., 1994a). However in a state of insulin resistance, as occurs in T2DM, raised apoCIII plasma concentrations are observed in humans, with increased apoCIII mRNA expression also observed in the db/db diabetic mouse model (Gervaise et al., 2000; Altomonte et al., 2004). The transcription factor, forkhead box O1 (Foxo1), is a key mediator of the inhibitory action of insulin on apoCIII gene transcription,
acting via insulin response element (IRE) -470/-453 bp upstream of the transcriptional start site (Altomonte et al., 2004).

HNF4α is a transcriptional activator of apoCIII expression (Taylor et al., 1996), with HNF4α binding sites being present in the proximal promoter (-67/-87 bp) and two enhancer elements (-734/-716 bp and -2880/-2929 bp) (Sladek et al., 1990; Kardassis et al., 1997; Vergnes et al., 1997). Acute-phase response cytokines (e.g. tumor necrosis factor-α (TNFα) and interleukin 1 (IL-1)) decrease HNF4α activity at the apoCIII promoter (Nikolaidou-Neokosmidou et al., 2006). The latter is mediated by the nuclear factor-κB (NF-κB) pathway, a key pathway in immune and inflammatory processes, activation of which results in translocation of the transcription factor NF-κB from the cytoplasm to the nucleus (Perkins and Gilmore, 2006). The MAPK signalling pathway (Section 1.1.1) has also been shown to inhibit HNF4α-dependent transcriptional activation of apoCIII gene expression (Reddy et al., 1999).

1.9.6 PPARα

In line with its role in the fasting state (Section 1.4.5), PPARα mRNA expression is induced by glucocorticoids and inhibited by insulin (Steiniger et al., 1994; Lemberger et al., 1994). HNF4α is a positive regulator of PPARα transcription, as shown by decreased PPARα mRNA expression in HNF4α conditional knockout models (Hayhurst et al., 2001; Gupta et al., 2005, Miura et al., 2006), and a functional response element for HNF4α has been identified in the human PPARα promoter (Pineda Torra et al., 2002).

As described in the above section, HNF4α regulates a range of hepatic target genes whose expression is alternately controlled by metabolic stimuli. Genetic evidence (Section 1.6) also points to HNF4α playing a role in the development of T2DM and dyslipidaemia. Since hepatic HNF4α target genes such as PEPCK, L-PK and SREBP-1c contribute to overproduction of glucose and triglycerides by the liver in these metabolic disorders, HNF4α function in the liver was investigated in the course of this research. The choice of model and different techniques used to study the impact of HNF4α activity on hepatic gene expression are therefore discussed in the following two sections.
1.10 Models of liver metabolism

In vivo models present the most physiological setting in which to study liver metabolism, however investigation of human liver metabolism in vivo is prevented by the dangers associated with liver biopsy. Studies in animals provide an alternative approach, however this approach is costly and genetic manipulation is a more lengthy process compared to the use of in vitro models. In vitro models for the study of hepatic metabolism include primary hepatocytes and cultured cells derived from hepatomas. There are advantages and disadvantages to both primary and cultured cells: First, human hepatocytes have limited availability and display phenotypic instability (Castell et al., 2006). Differences between human and rat liver may make primary rat hepatocytes less than ideal as a model; for example, differences exist between human and rat liver in the expression levels of PPARα and the action of peroxisome proliferators (Palmer et al., 1998). Human hepatoma cell lines offer the advantage of being of human origin and ease of manipulation for repeated experiments and procedures such as transient transfection (Castell et al., 2006). Three commonly used human hepatoma cell lines are described in greater detail below.

The HuH-7 cell line originated from well-differentiated hepatocellular carcinoma tissue excised from a 57-year old Japanese male (Nakabayashi et al., 1982). The HuH7 cell line was found to secrete a range of liver-specific markers, including plasma proteins (including albumin, α1-antitrypsin, transferrin and LDL) and low activities of glucose-6-phosphatase and fructose-1,6-bisphosphatase. However HuH7 cells were not found to express liver-type pyruvate kinase or glucokinase, suggesting that whereas they have a liver-like phenotype, it is not complete (Nakabayashi et al., 1982).

The HepG2 cell line was established from a liver biopsy of a male Caucasian aged 15 years, with a well differentiated hepatocellular carcinoma (hepatoblastoma type) (Aden et al., 1979). HepG2 cells also secrete most of the major plasma proteins including albumin, α2-macroglobulin, α1-antitrypsin, transferrin and plasminogen, as well as LDL and HDL and the major apolipoproteins (Knowles et al., 1980; Thrift et al., 1986). HepG2 cells are able to metabolise chylomicrons, VLDL, LDL and HDL and are able to produce bile acids from cholesterol, making the cell line a useful model for the study of lipid metabolism (Javitt, 1990).
The Hep3B cell line was established from a liver biopsy of an African American male aged 8 years (Aden et al., 1979). Like the HepG2 cell line, Hep3B cells secrete a wide range of plasma proteins. Unlike the HuH7 and HepG2 cell lines, the Hep3B cell line expresses two polypeptides of the Hepatitis B Surface Antigen and contain an integrated copy of the Hepatitis B genome (Knowles et al., 1980).

1.11 Approaches to studying transcriptional regulation

Changes in gene expression can occur at the transcriptional, post-transcriptional, translational or post-translational levels. Whilst studying changes at the mRNA level does not take account of changes affecting protein expression or stability, transcriptional networks are undoubtedly important in the pathogenesis of T2DM: For example, five of the six genes responsible for MODY (Section 1.6.1) code for transcription factors. Since HNF4α is a transcription factor, it is appropriate to study changes in transcription of its target genes, as changes in the expression or activity of HNF4α will take effect at this level. Therefore two approaches for studying changes in mRNA expression and gene transcription are described below.

1.11.1 Reverse Transcriptase Polymerase Chain Reaction

Reverse transcriptase polymerase chain reaction (RT-PCR) enables the study of mRNA expression levels in cell extracts and tissue samples under different experimental conditions, with detection of low abundance transcripts possible. RT-PCR consists of two steps: the reverse transcription of RNA into complementary DNA (cDNA) and the amplification of the cDNA by PCR. RNA is reverse transcribed by an RNA-dependent DNA polymerase usually derived from a retrovirus such as Moloney Murine Leukaemia Virus (M-MuLV), which also possesses exoribonuclease activity and therefore degrades the RNA template following cDNA synthesis, resulting in single-stranded (ss) cDNA (Figure 1.19A). Primers for first-strand synthesis include poly-dT, which binds the RNA polyA 3'-tail, random hexamer and gene-specific oligonucleotides. The subsequent PCR reaction amplifies the gene of interest using gene-specific primers, which bind to both the sense (5'→3') and antisense (3'→5') DNA strands. During each cycle of PCR, double-stranded DNA is denatured to facilitate primer binding, which is followed with primer extension by thermostable Tag polymerase which originates from the bacterium Thermus aquaticus, in the presence of deoxyribonucleotide triphosphates (dNTPs). With the exception of the first cycle of PCR where single-stranded cDNA is converted to double-stranded DNA, each PCR cycle results
Figure 1.19: (A) Reverse Transcription (RT) reaction (B) Polymerase Chain Reaction (PCR). (A) RNA templates are reverse transcribed into cDNA and subsequently digested by the RNA-dependent DNA polymerase and ribonuclease (RNase) activities of the reverse transcriptase enzyme. (B) cDNA is converted into dsDNA and undergoes logarithmic amplification following each PCR cycle of dsDNA denaturation, primer annealing and primer extension by Taq DNA polymerase. Diagram based on Voet and Voet (1995).

in a doubling of the number of double-stranded DNA molecules, hence providing a logarithmic amplification of the targeted DNA region (Figure 1.19B).

The amplified DNA can be detected by gel electrophoresis (Section 2.2.5.5) or in real-time by using fluorescence-based probes (Section 2.2.8). The RT-PCR steps can take place in the
same tube (one-step RT-PCR) using gene-specific primers for both steps, or in separate reaction tubes (two-step RT-PCR), whereby cDNA is usually generated from all RNA templates present in the extract using poly-dT or random primers, followed by separate PCR reactions using gene-specific primers.

### 1.11.2 Reporter genes

Reporter genes consist of a plasmid with a reporter gene, expression of which can be controlled by the promoter or enhancer regions of interest. The promoter or enhancer region of interest is cloned and inserted into a plasmid containing a reporter protein. When the reporter plasmid is transfected into a cell line with the necessary transcription factors and cofactors to stimulate expression from the cloned promoter, RNA Polymerase II stimulates transcription of the reporter gene, followed by the production of the protein by the host cell translational machinery. Reporter proteins must be distinguishable from the endogenous proteins produced by the cell system being employed (Schenborn and Groskreutz, 1999). Detection methods include both enzyme-based assays such as luciferase, which is able to produce luminescence in the presence of its substrate, luciferin (Section 2.2.7.4), and the fluorescent properties of the reporter protein, such as green fluorescent protein (Schenborn and Groskreutz, 1999). Reporter gene constructs enable the dissection of gene promoters in terms of the DNA elements which mediate the effects of stimuli on expression of the gene of interest (e.g. cAMP, PUFAs) and the transcription factors which bind to the DNA elements.

### 1.12 Hypothesis, Aims and Objectives

HNF4α promotes the transcription of genes which are oppositely regulated by metabolic stimuli. Whilst some HNF4α target genes, e.g. PEPCK, are induced by glucagon, others, e.g. L-PK, are transcriptionally repressed by the same stimulus. Conversely, whilst PEPCK expression is inhibited by insulin, L-PK expression is activated by insulin. The current research therefore focussed on the question: How does HNF4α participate in both gene activation and repression under a same metabolic condition?

**Hypothesis:** Glucagon and insulin have opposite effects on HNF4α activity in terms of the regulation of gluconeogenic genes compared to those of glycolytic and lipogenic genes, i.e. glucagon stimulates HNF4α to promote gluconeogenesis, whilst insulin stimulates HNF4α-regulated glycolytic and lipogenic gene expression.
HNF4α has been shown to be a target for insulin signalling via its interaction with Foxo1, with the latter acting as a co-repressor of HNF4α activity (Hirota et al., 2003). Insulin relieves Foxo1-mediated repression of HNF4α by causing phosphorylation of Foxo1 and its exclusion from the nucleus (Hirota et al., 2003). These findings suggest that this or other interactions of HNF4α may play a role in its regulation target gene promoters. Since the transcription of HNF4α target genes, L-PK, SREBP-1c and FAS, is activated by insulin (Paulauskis and Sul, 1989; Raymond Jean et al., 1991; Shimomura et al., 1999b), it is hypothesized that insulin has a stimulatory effect on HNF4α activity at these gene promoters.

Contrasting effects of glucagon on HNF4α activity are reported in the literature. HNF4α is crucial to the action of PGC-1α in the activation of gluconeogenic gene expression in response to glucagon (Rhee et al., 2003). The latter report conflicts with the finding that glucagon has an inhibitory effect on HNF4α DNA-binding capacity (Viollet et al., 1997). It is therefore hypothesized that HNF4α activity is modulated by promoter context, such that glucagon activates HNF4α when bound to the promoters of gluconeogenic genes such as PEPCK.

To address the above hypothesis, the project aims were as follows:

**Aim 1:** To characterise the human hepatoma cell lines, HuH7, HepG2 and Hep3B, for the expression of HNF4α and its splice variants, and HNF4α target genes.

**Objectives:**
- To profile the expression of HNF4α splice variants in the HepG2, Hep3B and HuH7 cell lines using RT-PCR with splice-variant specific primers.
- To compare the expression profile of HNF4α splice variants in the human hepatoma cell lines with those of adult and foetal human liver.
- To study the expression of HNF4α and its hepatic target genes in a hepatoma cell culture system, using semi-quantitative RT-PCR under different conditions of glucose and insulin concentration.
Aim 2: To investigate the effects of feeding and fasting stimuli on HNF4α transcriptional activity by studying downstream effects on HNF4α target genes in the pathways of glycolysis, gluconeogenesis and lipogenesis.

Objectives:
- To investigate the effects of different metabolic stimuli on HNF4α reporter gene expression utilising reporter plasmids containing promoter sequences of key HNF4α target genes in the metabolic pathways of glycolysis, gluconeogenesis and lipogenesis together with HNF4α over-expression.
- To investigate the effect of insulin on the mRNA expression of lipogenic (SREBP-1c, SREBP-2, ACC, FAS) and glycolytic (L-PK) genes in HepG2 cells, and the role of HNF4α in these effects by HNF4α overexpression.
- To characterize the effects of a range of feeding and fasting stimuli on the mRNA expression of HNF4α target genes in oppositely regulated pathways in HepG2 cells, and to investigate the role of HNF4α in these effects by overexpression of dominant-negative HNF4α.

Aim 3: To investigate the mechanisms by which HNF4α differentially regulates its target genes under fasting vs. feeding conditions.

Objectives
- To ascertain the role of HNF4α co-activators, PGC-1α and p300, in the regulation of HNF4α-dependent reporter gene expression for genes from opposing metabolic pathways.
- To investigate the role of HNF4α and its co-activators, p300 and PGC-1α, in the regulation of HNF4α target genes by metabolic stimuli by overexpression of co-activators, individually and in combination with HNF4α overexpression.
Chapter 2
Materials and Methods
Chapter 2:

2.1 Materials

Dulbecco’s Modified Eagle Medium (DMEM) (1000 mg/l D-glucose, 110 mg/l sodium pyruvate), Dulbecco’s Modified Eagle Medium without phenol red (1000 mg/l D-glucose, 110 mg/l sodium pyruvate), Dulbecco’s Modified Eagle Medium without glucose and sodium pyruvate (DMEM G”), Medium 199 with Earle’s Salts, Foetal Bovine Serum (FBS), Non-essential amino acids (NEAA), Trypsin-EDTA (0.25% Trypsin 1 mM EDTA), Trypan Blue, Penicillin (10,000 units/ml) – Streptomycin (10 mg/ml) solution, Trizol Reagent and UltraPure™ DNase/RNase-free water were obtained from Invitrogen (Paisley, U.K.). Small (culture area 25 cm²), medium (culture area 80 cm²) and large cell culture flasks (culture area 175 cm²), multiwell tissue culture plates and cryovials were obtained from NuncIon (Roskilde, Denmark).

Sigma-Aldrich (Gillingham, U.K.) supplied 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), 8-bromoadenosine 3’,5’-cyclic monophosphate sodium salt (8-Br-cAMP), dexamethasone, diethyl pyrocarbonate (DEPC), D-Glucose solution, 3-isobutyl-1-methylxanthine (IBMX), human recombinant insulin solution, (10 mg/ml), L-glutamine (200 mM) - penicillin (10,000 U/ml) - streptomycin (10 mg/ml) solution, phosphate buffered saline tablets (PBS), sodium pyruvate solution and triido-L-thyronine. D-Glucose (anhydrous) was obtained from Fisher Scientific (Loughborough, U.K.).

Gel and PCR Clean-up System, Wizard Plus Miniprep DNA Purification System, agarose, restriction endonucleases, restriction endonucleases reaction buffers, 100 bp DNA markers, 1 kb DNA markers, RQ1 RNase-Free DNase and Bright-Glo® and Steady-Glo® Luciferase Assay Systems were supplied by Promega (Southampton, U.K.).

Amersham Biosciences (Little Chalfont, U.K.) Ready-to-Go RT-PCR Beads were used for RT-PCR. PCR Primers for semi-quantitative PCR were supplied as unmodified oligonucleotides by MWG Biotech (Ebersberg, Germany).

Fugene-6 Transfection Reagent was purchased from Roche Diagnostics (Indianapolis, IN, U.S.A.). Phospha-Light™ SEAP Reporter Gene Assay System, Applied Biosystems (Bedford, MA, U.S.A.) was used for quantification of SEAP activity.
Sterile white opaque 96-well microplates for luciferase assays, non-sterile white opaque 96-well opti-plates for SEAP assays and clear adhesive top-seals for microplates were obtained from Perkin Elmer (Zaventem, Belgium). Abgene (Epsom, U.K.) provided Thermo-Fast® 96 non-skirted, opaque white PCR plates and Absolute™ QPCR seals.

Qiagen (Hilden, Germany) supplied RNaseasy Mini Kit for RNA purification, QuantiTect Reverse Transcription Kit, QuantiTect SYBR Green PCR Kit and target gene-specific QuantiTect Primer Assays. Homo sapiens housekeeping gene detection kits for use with SYBR Green chemistry from Primer Design (Southampton, U.K.) were used for reference gene quantification by real-time PCR.

Cambrex (Rockland, ME, U.S.A.) low-melting point NuSieve GTG Agarose and 20 bp DNA ladder were used for gel analysis of real-time PCR products.

The RNA 6000 Nano Chip and RNA 6000 Nano Kit from Agilent Technologies (Waldbronn, Germany) were used for analysis of RNA integrity.

2.2 Methods

2.2.1 Cell culture

2.2.1.1 HuH7, HepG2 and Hep3B human hepatoma cell lines

Three human hepatoma cell lines were cultured in the course of the project:

- HuH7 (Cell Line No. JCRB0403, Japanese Collection of Research Bioresources - Cell Bank, Osaka, Japan)
- HepG2 (Cell Line No. 85011430, ECACC, Salisbury, U.K.)
- Hep3B (Cell Line No. 86062703 ECACC, Salisbury, U.K.)

The standard cell culture medium (SCM) for all three cell lines was Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1% non-essential amino acids (NEAA) and 1% L-glutamine (200 mM) - Penicillin (10,000 U/ml) - Streptomycin (10 mg/ml) solution (PSG). Cells were incubated in a humidified incubator at 37°C, 5% CO₂.

2.2.1.2 Passage of cells

Cells were grown in medium cell culture flasks (culture area 80 cm²) until approximately 80% confluent. Medium was removed by aspiration. Cells were washed twice with 10 ml sterile PBS before addition of 4 ml 0.25% Trypsin-1 mM EDTA (approximately 1 ml per 25 cm² culture area). The flask was gently rocked to ensure even coverage of the cells with the
protease and incubated at 37°C for 3 - 5 minutes until the cells had detached, as checked by microscopy. An equal volume of SCM : trypsin was added to the flask to neutralise trypsin activity, and the cells mixed by pipetting. Cells were passaged 1:4 to 1:6 by dividing the cell suspension between 4 - 6 new flasks (of equal culture area) and then incubated at 37°C, 5 % CO₂. The cell culture medium was refreshed every 2-3 days with 10 - 15 ml of SCM per flask.

2.2.1.3 Preparation of frozen stocks

Cells were grown in large cell culture flasks (culture area 175 cm²) with 25 ml standard cell culture medium until 90% confluent. Cells were passaged as described above with 7 ml Trypsin-EDTA per flask. After cells had detached, 7 ml SCM was added and 10 μl sample of the suspension removed for counting of cells using a haemocytometer (see Section 2.2.1.5). The cell suspension was centrifuged at 900 x g (2000 rpm, Sigma 6K10 centrifuge) for 5 minutes at room temperature. The cell pellet was resuspended in freezing medium containing 90% FBS, 10% DMSO (v/v) to a concentration ≈4 x 10⁶ cells/ml. Aliquots of the cell suspension were placed in an alcohol bath at -80°C to enable cooling at a rate of 1-3°C/min before being transferred to liquid nitrogen storage (ECACC Procedure for Freezing Cells).

2.2.1.4 Plating of frozen stocks

An ampoule containing 1 ml frozen cells in freezing medium was thawed rapidly in a 37°C water bath. The cell suspension was transferred to 10 ml SCM (37°C) and centrifuged at 900 x g (2000 rpm, Sigma 6K10 centrifuge) for 5 minutes at room temperature. The cell pellet was resuspended in 5 ml SCM and transferred to a small cell culture flask (culture area 25 cm²), and cultured under standard conditions.

2.2.1.5 Estimation of cell number using a haemocytometer

Cell number was estimated using a haemocytometer; the central region of haemocytometer contains two etched counting grids which can be viewed by light microscopy (Figure 2.1). A coverslip is attached to the haemocytometer by breathing on the coverslip and pressing it gently onto the central part of the haemocytometer. On the sides of the coverslip small rainbow-like rings can be seen - these are an indication of a proper fit, and are known as Newton's Rings.
For calculations of cell number, a 10 μl sample was removed from a suspension of cells and mixed with 90 μl Trypan Blue solution. Trypan Blue is excluded from live cells therefore only cells which appear white/yellow on the grid are counted, whilst cells which appeared blue are excluded. The Trypan Blue suspension was pipetted at the edge of the coverslip either side of each counting grid and allowed to fill the haemocytometer chamber by capillary action. The number of cells was counted in all 4 regions of the grid, each corresponding to 1 mm² (Figure 2.1), utilising both sides of the haemocytometer, and a mean no. of cells calculated. The concentration of cells in the original suspension was then calculated using Equation 2.1 (Freshney, 1994).

\[
\text{Concentration of cells in original suspension (}/ml) = \frac{\text{Mean cell count}}{\text{Volume of grid section (10}^{-4} \text{ ml})} \times \text{Dilution factor to original sample (10)}
\]

**Equation 2.1: Calculation of cell concentration.** The estimated concentration of cells in the original suspension is based on the mean cell count in a grid section of 1 mm² with depth 0.1 mm from a 10 μl sample diluted to a volume of 100 μl with Trypan Blue (dilution factor of 10).

### 2.2.1.6 Preparation of hormone stock solutions

Hormone stock solutions were prepared according to manufacturer's product information (Sigma):

- The solubility of 5-aminimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR, Formula Weight (FW) 258.23 g/mol) is > 10 mg/ml in water. 5 mg AICAR was
dissolved in 5 ml of serum-free DMEM (containing antibiotics and NEAA) to a concentration of 1 mg/ml.

- 8-bromoadenosine 3',5'-cyclic monophosphate sodium salt (8-Br-cAMP, FW 430.08 g/mol) is very soluble in aqueous solutions. 25 mg 8-Br-cAMP was dissolved in 10 ml serum-free DMEM (containing antibiotics and non-essential amino acids) to prepare a stock solution of 2.5 g/l.

- Dexamethasone (FW 392.5 g/mol) stock solution (1 mg/ml) was prepared by dissolving 25 mg dexamethasone in 25 ml filter-sterilised ethanol.

- Glucagon (FW 3482.75 g/mol) stock solution (0.1 mg/ml) was prepared by dissolving 1 mg glucagon in 10 ml 0.05 M acetic acid.

- 3-isobutyl-1-methylxanthine (IBMX, FW 222.24 g/mol) was dissolved in DM to a concentration of 100 g/l by warming at 37°C.

- 3,3',5-triiodo-L-thyronine (T₃) (FW 673.0 g/mol) stock solution was prepared by dissolving triiodothyronine in sterile 1 M NaOH to a concentration of 1 g/l.

Hormone stock solutions were mixed by pipetting and were frozen in aliquots -20°C.

Insulin (10 mg/ml) and glucose (450 g/l) were supplied as stock solutions. In the case of insulin, a final concentration of 100 nM was required, thus the supplied insulin solution (10 mg/ml) was diluted 1:100 in sterile water or experimental cell culture medium to a concentration of 0.1 mg/ml, before a second dilution was made to achieve the final concentration. Other stock solutions of hormones and protein kinase activators were diluted in cell culture medium to achieve the required final concentration and mixed by pipetting several times (Table 2.1). In the case of media containing dexamethasone, T₃ or IBMX, the appropriate volume of solvent (sterile ethanol, 1 M NaOH or DMSO respectively), was added to the standard medium to act as the vehicle control.
<table>
<thead>
<tr>
<th>Hormone / protein kinase activator</th>
<th>Concentration stock solution (g/l)</th>
<th>Molarity stock solution (mM)</th>
<th>Final concentration (μM)</th>
<th>Volume stock solution (μl)</th>
<th>Volume of cell culture medium (ml)</th>
<th>Final volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3</td>
<td>1</td>
<td>2.5</td>
<td>0.1</td>
<td>450</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>IBMX</td>
<td>1</td>
<td>2.5</td>
<td>0.1</td>
<td>450</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Insulin</td>
<td>1</td>
<td>2.5</td>
<td>0.1</td>
<td>450</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>8-Br-cAMP</td>
<td>1</td>
<td>2.5</td>
<td>0.1</td>
<td>450</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>1</td>
<td>2.5</td>
<td>0.1</td>
<td>450</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Glucagon</td>
<td>1</td>
<td>2.5</td>
<td>0.1</td>
<td>450</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Typical dilutions of hormone stock solutions in experimental cell culture medium. *Cell culture media contained 5.6 mM glucose therefore additional glucose solution was added to a calculated concentration of 19.4 mM.
2.2.2 Isolation of total RNA from cell culture samples

2.2.2.1 Trizol method

In experiments where gene expression was analysed by semi-quantitative RT-PCR, total RNA was purified using Trizol reagent (Invitrogen). Trizol reagent is a mono-phasic solution of phenol and guanidine isothiocyanate, which disrupts cell membranes and nucleoprotein complexes, whilst maintaining RNA integrity by inhibiting endogenous ribonucleases (RNases).

Cells were grown in six-well plates prior to RNA isolation. Cell culture media was removed and the cells washed twice with PBS (1 ml). Cells were lysed with 1 ml Trizol per 10 cm² culture area (1 ml per well) and lysate mixed thoroughly by pipetting. All stages were performed in a sterile laminar flow hood. RNase-free water was prepared by adding 0.1 % diethyl pyrocarbonate to deionised water and leaving overnight prior to autoclaving.

Total RNA was extracted according to Trizol product information (Invitrogen). Samples were incubated at room temperature for 5 minutes to permit the complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform was added per 1 ml Trizol and the tubes shaken vigorously by hand for 15 seconds. Samples were incubated at room temperature for 2 to 3 minutes and centrifuged for 15 minutes at 2 – 8°C at 12,000 × g (11,400 rpm, Eppendorf microcentrifuge model 5415D) in order to separate the total RNA in the aqueous phase from the organic phenol/chloroform phase. The upper aqueous phase was then transferred to a fresh microcentrifuge tube and RNA precipitated with 0.5 ml isopropanol per 1 ml Trizol reagent, facilitated by incubation at room temperature for 10 minutes. The samples were then centrifuged at 12,000 × g (11,400 rpm) for 10 minutes at 2 – 8°C.

Following centrifugation, the supernatant was removed and 1 ml of 75 % ethanol (in 0.1 % DEPC-treated water) was added per 1 ml of Trizol reagent to wash the RNA pellet and remove residual isopropanol. The sample was mixed by vortexing and centrifuged at 7,500 × g (8,900 rpm) for 4 minutes at 2 – 8°C. The supernatant was removed and the RNA pellet air-dried for 5 minutes. Traces of 75% ethanol were removed by blotting with tissue at the edge of the tube. RNA samples were normally dissolved in 50 μl RNase-free water. RNA samples were then kept on ice or frozen at -80°C.
2.2.2.2 RNA purification using microspin column chromatography

In experiments where gene expression was analysed by real-time quantitative PCR, total RNA was purified using RNeasy® Mini microspin columns (Qiagen), which contain a silica-based membrane that specifically binds RNA. Like Trizol reagent, this method uses a buffer containing the chaotropic agent guanidine isothiocyanate to lyse cells and disrupt the nucleic acid–protein interactions. However, this method of RNA purification is performed without phenol/chloroform, which has the advantage of avoiding carry-over of phenol that can affect the highly sensitive detection of gene transcript levels by real-time RT-PCR.

RNA was isolated from cultures of HepG2 cells seeded at $5 \times 10^5$ cells/well in 24-well plates according to the ‘Protocol: Purification of Total RNA from Animal Cells using Spin Technology’ (RNeasy Mini Handbook, 2006). Initial optimization experiments were performed to ensure that the total number of cells did not exceed the recommended maximum of $5 \times 10^6$ cells per 350 μl lysis buffer. In addition to presence of guanidine isothiocyanate in the lysis buffer (RLT), β-mercaptoethanol (10 μl per 1 ml) was added to inhibit RNase activity. At the endpoint of the experiment, cells were washed once with 1 ml PBS to remove traces of the cell culture medium and lysed with 350 μl Buffer RLT. The sample was mixed by pipetting and transferred to an RNase-free microcentrifuge tube. The lysate was homogenized by passing through a sterile 21-gauge needle attached to a 1 ml syringe at least 5 times. The homogenized lysate was normally frozen at -80°C and thawed at 37°C when required.

After thawing, one volume of 70% ethanol was mixed with the homogenized lysate for optimal binding of the RNA to the spin column matrix. The sample was transferred to an RNeasy spin column, centrifuged for 15 s at 8,000 × g (9,300 rpm, Eppendorf Microcentrifuge Model 5415D) and the flow-through discarded. The column was then washed three times with the supplied buffers RW1 (700 μl) and RPE (with the appropriate volume of ethanol added) (500 μl), each followed by centrifugation for 15 s (or 2 minutes for the final wash with buffer RPE) at 8,000 × g. The RNeasy column was then placed in a new collection tube and centrifuged at full-speed (14,000 × g) for 1 minute to allow complete evaporation of residual ethanol from the wash buffers. The RNA was eluted from the silica membrane by placing the column in 1.5 ml microcentrifuge tube and adding 50 μl RNase-
free water directly to the column membrane, followed by centrifugation for 1 minute at 8,000 x g. After elution, RNA samples were kept on ice or frozen at -80°C for long-term storage.

2.2.3 Measurement of nucleic acid concentration and quality

2.2.3.1 Measurement of nucleic concentration and purity by UV spectroscopy

Nucleic acid concentration was measured using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Montchanin, U.S.A.). Nuclease-free water was used to initialize and blank the spectrophotometer. Sample type was selected as DNA or RNA, as appropriate, and 1 μl of DNA or RNA sample was loaded.

![Figure 2.2: An example of Nanodrop ND-1000 Spectrophotometer output for the measurement of DNA concentration and purity. A spectroscopic scan is performed for wavelength range from 230 – 350 nm. The Nanodrop software displays absorbance at 260 and 280 nm (normalised to a 10 mm path length), concentration (ng/μl) and ratios of absorbances at 260/280 nm and 260/230 nm as measures of nucleic acid purity.](image]

A spectroscopic scan of wavelengths from 230 – 350 nm is made automatically and displayed together with calculations of nucleic acid concentration and purity (Figure 2.2). The measurement of concentration (ng/μl) is based on the absorbance at 260 nm, using a modified form of the Beer-Lambert equation with the wavelength-dependent extinction coefficients of 50 ng.cm/μl for dsDNA and 40 ng.cm/μl for RNA. The ratio of absorbances at 260/280 nm was used as the primary measure of nucleic acid purity. A ratio 260/280 > 1.8 indicated acceptably pure DNA solutions and a 260/280 ratio of ~2.0 as acceptably pure for RNA solutions. The ratio of absorbances at 260/230 nm provides a secondary indication of nucleic acid purity with a 260/230 ratio between 1.8 and 2.2. considered acceptable (Nanodrop ND-1000 Spectrophotometer V3.0.1 Users Manual (2004), Nanodrop Technologies). Increased
absorbance around either the 280 nm or 230 nm regions may suggest the presence of contaminating protein (280 nm) or phenol (230 nm).

2.2.3.2 Measurement of RNA integrity

RNA purity and integrity was also assessed in selected RNA samples, prior to their use in quantitative PCR analysis of gene expression, with a 2100 Bioanalyzer and RNA 6000 Nano Chip (Agilent Technologies, Waldbronn, Germany). This system consists of a microfluidic chip with 12 wells connected to a microcapillary gel system. An intercalating dye enables RNA to be detected by laser-induced fluorescence and migration time through the gel channel is indicative of the size of the RNA molecules (Mueller et al., 2000). The 2100 Bioanalyzer software displays an electropherogram of fluorescence against migration time alongside a densitometry simulation (gel view), together with measurements of RNA concentration (based on area under the trace), ratio of the 28S/18S ribosomal RNA (rRNA) and RNA Integrity Number (RIN). Traditionally, a ratio of the peak densities of 28S to 18S rRNA bands was considered the main determinant of RNA integrity, with a ratio of 2.0 indicating high quality RNA. However, other factors such as the presence of RNA degradation products or sheared genomic DNA also contribute to the performance of the RNA in downstream reactions (Imbeaud et al., 2005). The RIN method utilizes an algorithm which detects the presence of these factors in the electropherogram and assigns a RIN from 1 to 10, with 1 indicating a completely degraded sample and 10 indicating fully intact RNA (Schroeder et al., 2006).

The RNA 6000 Nano Chip was prepared according to manufacturer’s instructions (Agilent Technologies, 2006). A gel matrix was spin-filtered at 1500 x g for 10 minutes at room temperature (Eppendorf Microcentrifuge Model 5415D) and 65 μl aliquot used to prepare gel-dye mix: A dye-concentrate was equilibrated to room temperature and vortexed for 10 seconds before 1 μl of the dye concentrate added to 65 μl filtered gel. After vortexing, the gel-dye mix was centrifuged at 13,000 x g for 10 minutes at room temperature. The RNA 6000 Chip was primed with 9.0 μl gel-dye matrix, which was distributed throughout the chip with the aid of a syringe attached to the Agilent Chip Priming Station. A further 9.0 μl gel-dye mix was loaded into a second well of the chip. All twelve sample-wells of the chip, in addition to the well used for the RNA molecular size ladder, were loaded with 5.0 μl RNA 6000 Nano marker per well, used as an internal standard to correct for drift in migration time (Mueller et al., 2000). RNA samples were diluted with RNase-free water to the
concentration range 25 – 500 ng/µl. Aliquots (1.5 µl) of the RNA samples and RNA ladder were heated for 4 minutes at 65°C and then kept on ice; this process causes the denaturation of heterogeneous RNA secondary structures and the formation of the most energetically favourable secondary structure, hence reducing variability in the migration pattern of the different RNA molecules (Frequently Asked Questions: Lab-on-a-Chip RNA Assay, Agilent Technologies). 1.0 µl RNA ladder or 1.0 µl RNA sample was loaded into each of the sample wells. The chip contents were mixed by vortexing at 2400 rpm for 1 minute and the chip run on the 2100 Bioanalyzer within 5 minutes.

In total, the RNA integrity of 20 different RNA samples, purified using RNeasy® Mini spin columns (Section 2.2.2.2), were analyzed using the 2100 Bioanalyzer. In all cases, RIN values of ≥ 9.4 were calculated, indicative of high quality RNA.

![Figure 2.3: Typical Electropherogram for an RNA sample analysed using a 2100 Bioanalyzer (Agilent). Fluorescence (FU) is plotted against migration time (s) and peaks corresponding to the 18S and 28S rRNA bands are indicated. Alongside the graphical output, the 2100 Bioanalyzer software produces a densitometry simulation. RNA concentration is determined based on the area under the trace.](image-url)
2.2.4 Agarose gel electrophoresis of DNA

2.2.4.1 Standard agarose gel electrophoresis

Agarose gel electrophoresis facilitates the size separation of DNA (or RNA) molecules by virtue of their differing speed of migration through an agarose gel when an electrical voltage is applied across the gel. Agarose is an unbranched polysaccharide purified from seaweed, which dissolves upon boiling and sets to form a gel. Due to their overall negative charge, nucleic acids migrate towards the anode of a gel electrophoresis tank; DNA molecules of greater length will migrate more slowly through the pores of an agarose gel than smaller fragments. DNA conformation (e.g. supercoiled or linearised plasmid DNA) also influences the migration of DNA. The position of the migrated DNA is visualised by the presence of an intercalating agent, such as ethidium bromide which fluoresces under UV illumination (Sharp et al., 1973).

Tris-Borate-EDTA (TBE) was used as the buffer for preparing and running agarose gels. TBE was prepared as a 5x stock solution (0.45 M Tris, 0.45 M boric acid, 0.01 M EDTA, adjusted to pH 8.0 with HCl). Agarose gels were produced by dissolving agarose in 1x TBE buffer by microwave heating. The percentage of agarose in the gel was chosen according to the resolving power for DNA molecules of different lengths (Sambrook et al., 1989). For the analysis of PCR products (size range 150 – 600 bp), 2% (w/v) gels were prepared by dissolving 2 g agarose in 100 ml TBE. For the analysis of plasmid DNA, 0.9 % gels were prepared. Ethidium bromide was added to the agarose solution to a final concentration of 0.2 µg/ml in order visualise DNA under UV illumination. Once set, the agarose gel was placed in a gel tank containing 1x TBE. For the analysis of PCR products, 10 µl samples were loaded with 2 µl loading dye (Orange G) along with 100 bp DNA markers. Gels were run at 4-5 V/cm until the Orange G dye front had migrated three-quarters to full length of the gel. Gels were photographed using a Genesnap camera and software (Syngene, Frederick, Maryland, U.S.A.).

2.2.4.2 Low melting point agarose gel electrophoresis

In order to confirm the size of smaller amplicons (< 150 bp) from real-time PCR reactions, high concentration gels were prepared using low-melting point agarose. 3.5% NuSieve GTG Agarose (Cambrex Bioscience, Rockland, U.S.A.) gels were prepared according to the manufacturer's instructions. Agarose powder was added to chilled 1x TBE with stirring and
soaked in the buffer for 15 minutes at room temperature prior to heating in a microwave oven for 2 minutes at medium power, followed by bringing the solution to boiling point for 1 minute at full power. The solution was cooled to 50-60°C before pouring the gel. 10-15 μl real-time PCR product was mixed with 6x loading dye and loaded on the gel, alongside 20 bp DNA markers.

### 2.2.5 Measurement of gene expression by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR)

#### 2.2.5.1 Cell culture conditions

##### 2.2.5.1.1 Incubation of cell lines for the detection of HNF4α splice variants

HuH7, HepG2 and Hep3B cell lines were cultured under standard conditions (Section 2.2.1.1) until 90% confluence and then lysed with the appropriate volume of Trizol reagent (Section 2.2.2.1).

##### 2.2.5.1.2 Incubation of HuH7 cells with different concentrations of glucose

Medium containing 0.2 mM (low glucose), 5.5 mM (standard glucose) or 25.0 mM glucose (high glucose) was prepared using standard DMEM or DMEM G', containing 10% FBS, 1% PSG and 1% NEAA with the additional supplements (Table 2.2).

| Table 2.2: Preparation of low, standard and high glucose cell culture media. |
|-----------------|-----------------|-----------------|
| **Medium type** | **Basal medium** | **Supplements** |
| Low glucose (0.2 mM)* | DMEM G' | 1% sodium pyruvate |
| Standard glucose (5.5 mM) | DMEM | None |
| High glucose (25.0 mM) | DMEM | Anhydrous D-glucose |

High glucose (25.0 mM) medium was prepared by adding anhydrous D-glucose to standard cell culture medium and filter-sterilising. *Concentration of glucose due to glucose present in FBS used (10%).

HuH7 cells were grown under standard conditions (Section 2.2.1.1) in medium cell culture flasks (culture area 80 cm²) until 90% confluent and the medium refreshed one day prior to the start of the experiment. The cells were passaged as per standard method and the number of cells in the resulting suspension counted using a haemocytometer (Sections 2.2.1.2 and 2.2.1.5). The cell suspension was centrifuged at 900 × g (2000 rpm, Sigma 6K10 centrifuge) for 5 minutes, the supernatant removed and the cell pellet resuspended in either low, standard or high glucose medium. HuH7 cells were plated at a density of 1×10⁵ cells/well in six-well
plates with 5 ml medium per well (n = 3 for each media type and time point). RNA extracts in Trizol were prepared after 0, 24, 48, 72 and 96 h incubation (Section 2.2.2.1).

### 2.2.5.1.3 Incubation of HuH7 and HepG2 cells with glucose and insulin

Media containing different concentrations of glucose and insulin were prepared (Table 2.3). All media consisted of DMEM with 5% FBS, 1% NEAA acids and 1% PSG. D-Glucose solution and human insulin solution (10 mg/ml) were diluted (Section 2.2.1.6) to achieve the final concentrations shown in Table 2.3. HuH7 and HepG2 cells were passaged and seeded in six-well plates as previously (Section 2.2.5.1.2), with the cell pellet resuspended in one of the four types of media (Table 2.3). Cells were plated at a density of $1 \times 10^5$ cells/well in six-well plates and incubated for 48 hours at 37°C, 5% CO$_2$, after which cells were washed with 1 ml PBS per well and lysed with 1 ml Trizol per well.

<table>
<thead>
<tr>
<th>Medium No.</th>
<th>Basal medium</th>
<th>Glucose Concentration (mM)</th>
<th>Insulin concentration (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DMEM</td>
<td>5.5</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>DMEM</td>
<td>20.0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>DMEM</td>
<td>5.5</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>DMEM</td>
<td>20.0</td>
<td>100</td>
</tr>
</tbody>
</table>

### 2.2.5.2 Semi-quantitative one-step RT-PCR

For the semi-quantitative measurement of gene expression, one-step RT-PCR was performed using Ready-to-Go RT-PCR Beads contain M-MuLV Reverse Transcriptase, Taq DNA polymerase, 200 μM of each dNTP, RNAguard™ Ribonuclease Inhibitor, stabilisers and 10 mM Tris-HCl, 60 mM KCl, 1.5 mM MgCl$_2$ when reconstituted to a final volume of 50 μl (Amersham Biosciences, Little Chalfont, U.K.). Oligonucleotide primers were supplied by MWG Biotech (Ebersberg, Germany) in lyophilized form and resuspended in nuclease-free water to a concentration of 100 μM.

RT-PCR reactions were prepared on ice prior to initiation of the reverse transcription phase of the reaction. The appropriate amount of room temperature DEPC-treated water (as per Table 2.4) was added to the side of each tube to dissolve the RT-PCR bead and the tube tapped or pipetted gently in order to mix. RT-PCR tubes were incubated on ice for approximately 5 minutes until the bead was dissolved. 1 μl forward and 1 μl reverse primers
were added to each tube (Table 2.4) to a final concentration of 2 μM. Finally RNA was added to a final volume of 50 μl and the reaction contents mixed by pipetting. The tubes were transferred to a pre-warmed thermal cycler (Hybaid Model Omn-E). A program for one-step RT-PCR was run as outlined in Table 2.5, with an annealing temperature (T) of approximately 5°C less than the lowest melting temperature (Tm) of a primer pair, based on the Tm values supplied by MWG Biotech. After completion of the RT-PCR, tubes were placed on ice until use or stored at -20°C.

Table 2.4: Contents of each RT-PCR reaction (whole-bead).

<table>
<thead>
<tr>
<th>Material</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEPC-treated water</td>
<td>Z μl</td>
</tr>
<tr>
<td>Forward primer (100 pmol/μl)</td>
<td>1 μl</td>
</tr>
<tr>
<td>Reverse primer (100 pmol/μl)</td>
<td>1 μl</td>
</tr>
<tr>
<td>RNA template</td>
<td>Y μl</td>
</tr>
<tr>
<td>Total volume</td>
<td>50 μl</td>
</tr>
</tbody>
</table>

DEPC-treated water was added to a RT-PCR bead. Once the bead was dissolved, other contents were added.

Table 2.5: RT-PCR cycling conditions.

<table>
<thead>
<tr>
<th>RT-PCR Stage No.</th>
<th>Temperature (°C)</th>
<th>Duration (min)</th>
<th>No. of Cycles</th>
<th>Stage of RT-PCR reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>42</td>
<td>30</td>
<td>1</td>
<td>Reverse Transcription</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>5</td>
<td></td>
<td>Reverse Transcriptase denaturation</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>1</td>
<td>25</td>
<td>Denaturation</td>
</tr>
<tr>
<td></td>
<td>T</td>
<td>1</td>
<td>1</td>
<td>Annealing of primers</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>2</td>
<td></td>
<td>Extension</td>
</tr>
<tr>
<td>N/A</td>
<td>72</td>
<td>10</td>
<td>1</td>
<td>Final extension</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Holding Temperature</td>
</tr>
</tbody>
</table>

RT-PCR reactions consisted of cDNA synthesis with subsequent denaturation of the reverse transcriptase, followed by PCR cycling with an annealing temperature (T) of approximately 5°C less than the lowest calculated melting temperature (Tm) of the pair of primers. The annealing temperature (T) used for pairs of primers are specified in Table 2.6 and Table 2.7.
2.2.5.3 Detection of HNF4α splice variant mRNA expression in HuH7, HepG2 and Hep3B cell lines

Primers were used for RT-PCR which bound to sequences unique to each splice variant of HNF4α (Figure 2.4, Table 2.6). RT-PCR was performed using 400 – 2000 ng RNA per 50 μl reaction under standard cycling conditions (Table 2.4, Table 2.5) with annealing temperatures (T) as per Table 2.6. As a control for the amount of RNA present, RT-PCR with β-actin primers (Table 2.7) was also performed. 10 μl of the RT-PCR product were separated on a 2% agarose gel and photographed (Section 2.2.4).

Figure 2.4: Position of primers to HNF4α splice variants. The structure of the HNF4α gene is shown above together with the mRNA structure of the splice variants HNF4-α1, -α2, -α3, -α4, -α7 and -α8 below together the position of primer binding. The primer pair F1 and R1 amplify a fragment of the HNF4α transcript common to all splice variants. Splice variant specific primers bind to exons or exon boundaries (in the case of primer R9) unique to the specific splice variant. Diagram adapted from Sladek and Seidel (2001).

2.2.5.4 RT-PCR of HNF4α and target gene expression

For the analysis of the expression of HNF4α, apoCIII and FAS under conditions of variable glucose and insulin levels, RT-PCR beads (Table 2.4) were split into five aliquots after the addition of DEPC-treated water and primers (final concentration 2 μM) to make a final reaction volume of 10 μl after the addition of RNA. HNF4α, apoCIII and FAS mRNA expression was detected using gene-specific primers for HNF4α (all splice variants, Table
2.6) or target genes (Table 2.7), and normalised to β-actin expression. Approximately 100 ng RNA was used in each 10 μl reaction. RT-PCR products (10 μl) were electrophoresed on a 2% agarose gel and the relative amount of each product quantified, as described below (Section 2.2.5.5).

Table 2.6: PCR primers used for detection of HNF4α splice variants.

<table>
<thead>
<tr>
<th>HNF4α splice variant</th>
<th>Primer name</th>
<th>Primer sequence (5'→3')</th>
<th>T_m (°C)</th>
<th>T (°C)</th>
<th>Product size (bp)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNF4α (all) T</td>
<td>F1</td>
<td>CTGCTCGGAGCCACCAAGAGATCCATG</td>
<td>69.5</td>
<td>60</td>
<td>370</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>R1</td>
<td>ATCATCTGCCAGGTGATGCTCTGCA</td>
<td>64.6</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>HNF4α1</td>
<td>F1</td>
<td>As above</td>
<td>69.5</td>
<td>65</td>
<td>581</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>R9</td>
<td>GGTCTCAGGGGTTGACCACACTGCTGGTG</td>
<td>71.0</td>
<td>65</td>
<td>583</td>
<td>2</td>
</tr>
<tr>
<td>HNF4α2</td>
<td>F1</td>
<td>As above</td>
<td>69.5</td>
<td>65</td>
<td>583</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>R4C</td>
<td>CTGCTCCCTGGTGGGCCACCTCAC</td>
<td>74.3</td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>HNF4α3</td>
<td>F6</td>
<td>CTTGCGCAATGGATGCGACGAGTC</td>
<td>64.2</td>
<td>60</td>
<td>230</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>R6</td>
<td>AAACTGAGGGCAGAGAAGGGCTTGCA</td>
<td>68.2</td>
<td>65</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>HNF4α4</td>
<td>F7</td>
<td>CATGGACATGGCCGATACGAGTC</td>
<td>63.2</td>
<td>60</td>
<td>230</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>R7</td>
<td>CTGCC CCTGGTCGCGGCGACCTCAC</td>
<td>72.2</td>
<td>65</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>HNF4α7/8</td>
<td>F8</td>
<td>CTGGGCCATGCTGGAGGGTTTGAAGG</td>
<td>66.0</td>
<td>60</td>
<td>529</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>R3</td>
<td>CGAATGTCGCCGTTTGATC</td>
<td>56.0</td>
<td></td>
<td></td>
<td>3</td>
</tr>
</tbody>
</table>

F refers to forward primer, R refers to reverse primer. Primers F1 and R1 amplify a section of HNF4α common to all splice variants. References: (1) primers used by Suaud et al. (1997) with some modifications to match sequences as published by Chartier et al. (1994); (2) Primer R9 was designed to span the splice site between exons 9 and 10; (3) primers used by Eeckhoute et al. (2003).

Table 2.7: PCR primers used to detect HNF4α target gene expression.

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene</th>
<th>Primer sequence (5'→3')</th>
<th>Position in gene</th>
<th>Product size (bp)</th>
<th>T_m (°C)</th>
<th>T (°C)</th>
<th>Ref.</th>
<th>Genbank Accession Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>β-actin</td>
<td>ATCATGTTTGGACACCTCAA</td>
<td>404-424</td>
<td>318</td>
<td>51.2</td>
<td>57.3</td>
<td>1</td>
<td>X00351</td>
</tr>
<tr>
<td>R1</td>
<td></td>
<td>CAT CTC TTTGCTC GAA GTCCA</td>
<td>702-722</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F1</td>
<td>ApoCIII</td>
<td>CGG AGGATGGCTCA CTTCTCC</td>
<td>98-117</td>
<td>365</td>
<td>83.5</td>
<td>63.5</td>
<td>2</td>
<td>M28814</td>
</tr>
<tr>
<td>R1</td>
<td></td>
<td>CAGGGATAGGCTGGGGTGAAGG</td>
<td>444-463</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>FAS</td>
<td>GAAACTCGAGGACGTCTGC</td>
<td>6672-6689</td>
<td>156</td>
<td>58.0</td>
<td>58.2</td>
<td>3</td>
<td>NM_004104</td>
</tr>
<tr>
<td>R2</td>
<td></td>
<td>GACGAGTTGAGGGCCGAT</td>
<td>6811-6828</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

F refers to forward primer, R refers to reverse primer. The annealing temperature (T) used for PCR cycling was 46°C for β-actin, 57°C for apoCIII and FAS primers. References: (1) Eeckhoute et al. (2003); (2) Hsu et al. (2001); (3) Yang et al. (2003).
2.2.5.5 Measurement of gene expression by gel image digitization and quantification

Relative gene expression was measured using Scion Image software (Scion Corporation, Frederick, Maryland, U.S.A.) to digitize agarose gels and measure the relative intensity of the RT-PCR product of the gene of interest relative to that a housekeeping gene (β-actin).

2.2.5.5.1 Preparation and photography of agarose gels

RT-PCR products were run in lanes next to that of the housekeeping gene (β-actin) on a 2% agarose gel containing 0.2 μg/ml ethidium bromide, prepared as described in Section 2.2.4. Gels were photographed using a Genesnap camera and software (Syngene, Frederick, Maryland, U.S.A.). An unmodified digital image was saved in the TIFF file format.

2.2.5.5.2 Digitization and quantification of gel images

After loading Scion Image software, macros were activated under the Special > Load Macros menu and the option Gel plot 2 selected. The gel image was loaded under Menu, and the lanes containing the gene of interest and β-actin highlighted. The two lanes were plotted and the line tool was used to draw a base line from the left hand side of the peak in question to the right hand side in order to create a baseline of background emission due to ethidium bromide in the gel. A tangent was drawn to the main peak in the case of there being an extra peak or blurring before or after the main peak (Figure 2.5). The area between the peak and the baseline was selected and the number of square pixels was calculated for both the housekeeping and target gene peaks, and the process repeated three times. The mean ratio

\[
\frac{\text{Area under Target Gene peak (square pixels)}}{\text{Area under housekeeping gene peak (square pixels)}}
\]

was used to calculate the relative expression of the gene in question.
Figure 2.5: Quantification of gel bands using Scion Image software. (A) A gel photograph with bands corresponding to the RT-PCR products for the housekeeping and target genes is digitized in Scion Image and (B) the relative intensity of the area plotted. The background intensity is estimated by drawing a line at the bottom of the peak and then the area between the peak and the background measured in square pixels. In some cases, where there was blurring after the main gel peak, this was deducted by means of a tangent drawn from the main peak (example shown as diagonally striped area).

2.2.6 Plasmid DNA

2.2.6.1 Plasmid DNA information

2.2.6.1.1 Reporter gene information

L-PK reporter plasmids containing elements from the L-PK proximal promoter inserted into luciferase reporter plasmid pGL3-Basic (Promega) (Figure 2.6) were a gift from B. Viollet (INSERM, Paris, France).

The PEPCK reporter plasmid pPL32.Luc, containing the rat PEPCK proximal promoter (-467/+65) inserted into the luciferase plasmid pGL3 (Figure 2.6A) was a gift from D. K. Granner (Vanderbilt University Medical School, Tennessee, U.S.A.).
Figure 2.6: (A) Luciferase reporter plasmid pGL3 (B) L-PK promoter elements.

(A) pGL3-Basic vector (Promega). L-PK promoter constructs are ligated at the multiple cloning site upstream of the luc reporter gene. (B) L-PK promoter constructs containing oligomeric repeats of the ChREBP (L4) and/or HNF4α (L3) binding sites upstream of the 54 bp proximal promoter sequence.

The pGL4 843 SREBP-1c reporter plasmid contains the -780/+62 promoter region to the human SREBP-1c gene (GenBank accession no.: NT_010718), driving the expression of the firefly luciferase luc2 gene in the pGL4.10(luc2) reporter plasmid (Promega) (Figure 2.7A). This reporter plasmid was a gift from E. Tarling (University of Nottingham, U.K.).

pSEAP-3′Rep was a gift from K. Plant (FHMS, University of Surrey, U.K.) and contains the secretory alkaline phosphatase (SEAP) reporter gene under control of the cytomegalovirus (CMV) promoter (Figure 2.7B). It was generated by replacing the green fluorescent protein (GFP) coding region from pEGFP C2 (Clontech, Mountain View, CA, U.S.A.) with the SEAP coding region from pSEAP2 basic (Clontech) (K. Plant, personal communication). pSEAP-3′rep was co-transfected with luciferase reporter plasmids as a control for transfection efficiency.
2.2.6.1.2 Expression vector information

An expression vector containing the rat HNF4α1 cDNA sequence with the CMV promoter region (PCR3.HNF4) was obtained from B. Viollet (INSERM, Paris, France) (Viollet et al., 1997). An expression vector for haemagglutinin-tagged human HNF4α2 (pcDNA3.HA.hHNF4α2) was gifted by A. Fukamizu (University of Tsukuba, Japan) (Hirota et al., 2003).

Vectors for wild-type human HNF4α1 (pDGT26.1) and dominant negative-HNF4α2 (DN-HNF4α) (pDGT23.1) were gifts from T. Leff (Wayne State University School of Medicine, Detroit, U.S.A.). The DN-HNF4α differs from wild-type in that it contains a point mutation at position 316, resulting in the replacement of a cysteine residue with arginine in the DNA binding domain, rendering one of the zinc finger motifs non-functional. DN-HNF4α retains its dimerization ability and forms defective heterodimers with wild-type HNF4α, interfering with transcriptional activity (Taylor et al., 1996).

An expression vector containing the human coding sequence of the co-activator p300 (pVR1012 p300) was donated by G. Nabel (NIH Vaccine Center, Maryland, U.S.A.) (Figure 2.8). J. K. Kemper (University of Illinois, U.S.A.) supplied an expression vector coding for
mouse PGC-1α (pcDNA3.PGC1) (Bhalla et al., 2004). Basic expression plasmids lacking inserted DNA, pSG5 (Stratagene) or pcDNA3.1 (Invitrogen), were used as controls for the amount of DNA in cases where cells were transfected with variable amounts of expression vectors for HNF4α, p300 or PGC-1α (Figure 2.9).

![Vector map of p300 expression vector (pVR1012 p300). Vector map provided by G. Nabel (NIH Vaccine Center, Maryland, U.S.A.).](image1)

![Vector maps of basic expression plasmids (A) pSG5 and (B) pcDNA3.1. (pSG5 Vector Instruction Manual, 2000; pcDNA3.1 Vector Instruction Manual, 2001.).](image2)
2.2.6.2 Preparation of competent *Escherichia coli* DH5α

*Escherichia coli* (E. coli) strain DH5α were made chemically competent based on the calcium-chloride method of Inoue *et al.* (1990). Luria-Bertani (LB) medium was prepared with 1% (w/v) bactotryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl. Super Optimal Broth (SOB) was prepared with 2% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂ and 10 mM MgSO₄, and adjusted to pH 6.7-7.0 with HCl. Bacterial growth media were sterilised by autoclaving. Transformation Buffer (TB) was prepared to contain 10 mM Pipes, 55 mM MnCl₂, 15 mM CaCl₂, 250 mM KCl, adjusted to pH 6.7 with KOH, and filter-sterilised.

A starter culture of *E. coli* DH5α in LB was inoculated 1:1000 into 100 ml SOB medium and grown at 20°C until the culture had reached OD₆₀₀ = 0.6 (≈20 hours). The bacterial culture was centrifuged at 1000 x g (2100 rpm, Sigma 6K10 centrifuge) for 10 minutes at 4°C. The cell pellet was resuspended in 32 ml ice-cold TB solution by gentle pipetting and incubated on ice for 10 minutes. The cell suspension was centrifuged 1000 x g as above for 10 minutes at 4°C and the cell pellet resuspended in 8 ml of ice-cold TB. DMSO was added to a final concentration of 7% (v/v). Aliquots of 100 µl were prepared on ice and frozen at -80°C.

2.2.6.3 Transformation of bacteria with plasmid DNA

Competent *E. coli* DH5α (Section 2.2.6.2) were thawed on ice and 1 – 5 µl plasmid DNA was added to 50 µl of competent bacteria, and incubated on ice for 30 minutes. The bacteria were heat-shocked for 45 seconds at 42°C and returned to ice for 5 minutes. 250 µl LB medium was added to each tube followed by incubation for ~1 hour at 37°C with shaking (this is an optional stage in the case of plasmids coding for ampicillin resistance, but required for kanamycin resistance plasmids). Bacteria were plated on LB-agar plates containing 1.5% agar and the selective antibiotic (50 µg/ml ampicillin or 50 µg/ml kanamycin) and incubated overnight at 37°C.

2.2.6.4 Preparation of plasmid DNA

Endotoxin-free, transfection grade DNA was prepared using Qiagen Plasmid Midi Kits, which utilise alkaline lysis of the bacterial culture followed by binding of plasmid DNA to an anion-exchange resin. Endotoxins such as lipopolysaccharide are present in the outer membrane of gram-negative bacteria, such as *E. coli*, which can lead to decreased transfection.
efficiency in human cell lines. Therefore an additional endotoxin removal step was included in the plasmid purification, after which only endotoxin-free buffers were used.

Plasmid DNA for transfection purposes was prepared according to the Qiagen Supplementary Protocol: ‘Isolation of endotoxin-free plasmid DNA using the QIAGEN® Plasmid Midi Kit’ (2001). A starter culture containing 2 – 5 ml LB containing the selective antibiotic (50 μg/ml ampicillin or 50 μg/ml kanamycin) was inoculated from a single colony of E.coli transformed with the plasmid of interest and incubated for ~8 hours at 37°C with shaking. The starter culture was diluted 1/1000 into 50 ml selective LB and grown for a further 12 – 16 hours at 37°C with shaking. The bacteria were harvested by centrifugation at 6000 × g (6500 rpm, Beckman J2-21 centrifuge) for 15 minutes at 4°C and the cell pellet resuspended in 4 ml supplied Buffer P1 by vortexing. Bacteria were lysed by addition of 4 ml Buffer P2, which contains sodium hydroxide and sodium dodecyl sulphate (SDS), thus causing disruption of cellular membranes and denaturation of DNA. The lysis reaction was not allowed to proceed for more than 5 minutes in order to prevent permanent denaturation of the plasmid DNA. After 5 minutes, 4 ml chilled Buffer P3 was added and mixed gently by inversion. The presence of acetic acid in Buffer P3 allows the renaturation of plasmid, however genomic DNA and cellular debris are precipitated in the presence of high salt. The lysate is then poured into a QIAfilter Cartridge, incubated for 10 minutes and filtered into a endotoxin-free tube. 1 ml endotoxin removal Buffer ER was added to the filtered lysate, mixed by inversion 10 times and incubated on ice for 10 minutes.

After endotoxin removal, the lysate was applied to a Qiagen-tip 100 chromatography column, previously equilibrated with Buffer QBT. RNA and low molecular weight impurities were then removed from the column with two washes with a medium salt Buffer QC (10 ml) and the column allowed to empty by gravity flow. DNA was eluted with a high salt buffer QN (5 ml) into a fresh endotoxin-free tube. The eluted DNA was precipitated with 3.5 ml room-temperature isopropanol and centrifuged at 15,000 × g for 30 minutes (4°C), in order to remove residual high salt buffer. The supernatant was removed and the DNA pellet washed with 2 ml of 70% ethanol and centrifuged at 15,000 × g for 10 minutes (4°C). After the supernatant was removed, the DNA pellet was air-dried for 5-10 minutes and redissolved in 100 μl endotoxin-free TE buffer. The concentration and purity of the prepared DNA was
measured using a Nanodrop Spectrophotometer (Section 2.2.3.). The DNA was diluted to a concentration of 100 ng/µl in endotoxin-free TE buffer prior to transfection experiments.

2.2.6.5 Restriction endonuclease digestion of plasmid DNA

The plasmids were subjected to restriction endonuclease (RE) digestion with a RE whose cutting site was present in its multiple cloning site (MCS) (and, if known, at other positions in the plasmid) in order to linearize the DNA and confirm the size of the prepared DNA (Table 2.8). Reagents were added to a sterile microcentrifuge tube (Table 2.9), mixed by pipetting and incubated at 37°C for 1-2 hours. 3 µl of gel loading buffer was then added and the digested DNA run on a 0.9% agarose gel (Section 2.2.4), alongside 1 kb DNA markers and samples of the uncut plasmids.

Table 2.8: List of restriction endonucleases used for diagnostic digestion of plasmid DNA.

<table>
<thead>
<tr>
<th>Plasmid name</th>
<th>Restriction Enzyme(s)</th>
<th>Product size(s) (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSG5</td>
<td><em>Bam HI, Eco RI</em></td>
<td>4100</td>
</tr>
<tr>
<td>pcDNA3.1</td>
<td><em>Xba I</em></td>
<td>5428</td>
</tr>
<tr>
<td>pcDNA3.1</td>
<td><em>Nco I</em></td>
<td>735, 1351, 3342</td>
</tr>
<tr>
<td>(L3)3-64.PK</td>
<td><em>Hind III</em></td>
<td>~5000</td>
</tr>
<tr>
<td>(L4L3)4-54PK</td>
<td><em>Hind III</em></td>
<td>~5000</td>
</tr>
<tr>
<td>pPL32 (PEPCK)</td>
<td><em>Bam HI</em></td>
<td>5318</td>
</tr>
<tr>
<td>pGL4 843 SREBP-1c</td>
<td><em>Kpn I (Acc 65 I)</em></td>
<td>5040</td>
</tr>
<tr>
<td>pGL4 843 SREBP-1c</td>
<td><em>Xho I</em></td>
<td>5040</td>
</tr>
<tr>
<td>pSEAPI-3’rep</td>
<td><em>Bam HI</em></td>
<td>1432, 4150</td>
</tr>
<tr>
<td>pDGT26.1 (human HNF4α1)</td>
<td><em>Bam HI</em></td>
<td>~8000</td>
</tr>
<tr>
<td>pDGT26.1 (human HNF4α1)</td>
<td><em>Hind III</em></td>
<td>~8000</td>
</tr>
<tr>
<td>pDGT23.1 (DN-HNF4α)</td>
<td><em>Xho I</em></td>
<td>~6500, ~1200</td>
</tr>
<tr>
<td>pDGT23.1 (DN-HNF4α)</td>
<td><em>Hind III</em></td>
<td>~6000, ~1700</td>
</tr>
<tr>
<td>pcDNA3.PGC1</td>
<td><em>Bam HI</em></td>
<td>~5500, ~2500</td>
</tr>
<tr>
<td>pcDNA3.PGC1</td>
<td><em>Hind III</em></td>
<td>~5500, ~2500</td>
</tr>
<tr>
<td>pVR1012 p300</td>
<td><em>Xho I</em></td>
<td>12100</td>
</tr>
<tr>
<td>pVR1012 p300</td>
<td><em>Hind III</em></td>
<td>9436 bp, 2748 bp</td>
</tr>
</tbody>
</table>
Table 2.9: Restriction digestion of plasmid DNA.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile water</td>
<td>3.0</td>
</tr>
<tr>
<td>Buffer (10x)</td>
<td>1.5</td>
</tr>
<tr>
<td>Plasmid DNA</td>
<td>10.0</td>
</tr>
<tr>
<td>Restriction Endonuclease (10 units/µl)</td>
<td>0.5 (5 U)</td>
</tr>
<tr>
<td>Final volume</td>
<td>15.0</td>
</tr>
</tbody>
</table>

Restriction digests were performed with 5 units of the restriction enzyme and a minimum of 250 ng of plasmid DNA and the appropriate buffer in a reaction volume of 15 µl.

2.2.7 Study of HNF4α reporter gene activity in HepG2 cells

2.2.7.1 Culture of HepG2 cells for reporter gene experiments

The standard transfection medium (STM) used for transfection experiments with L-PK reporter genes consisted of 199 medium with 3% FBS and 1% penicillin-streptomycin solution. 199 medium was chosen as this type of medium was used by Gourdon and coworkers in their original study of L-PK reporter genes in primary hepatocytes (Gourdon et al., 1999). The proportion of FBS in the cell culture medium was restricted to 3% to limit the concentration of hormones in the final medium. In addition, the same batch of FBS was used throughout the transfection experiments in order to avoid variation in the concentration of hormones or growth factors in the control medium.

Following optimisation of factors contributing to the efficiency of the luciferase assay (Section 2.2.7.4), the basal medium was changed to DMEM without phenol red (containing 3% FBS and 1% penicillin-streptomycin solution, as described above), as the presence of 20 mg/l phenol red in 199 medium can reduce relative luminescence output by 50% (Technical Manual: Bright-Glo Luciferase Assay System (2006), Promega). As phenol red in cell culture media serves as a pH indicator, opened bottles of DMEM without phenol red were discarded one month after opening to prevent changes in pH. STM containing DMEM without phenol red was used in experiments with the PEPCK and SREBP-1c reporter plasmids.

The typical protocol for culture of HepG2 cells for transfection was as follows (Bombail, 2003, with some modifications). HepG2 cells were grown to 90% confluence in SCM. On Day 1, the cells were trypsinized as per Section 2.2.1.2. The cell suspension was then
centrifuged at 230 × g (1000 rpm, Sigma 6K.10 centrifuge) for 5 minutes at room temperature and the cell pellet resuspended in 5 ml STM, and the number of cells/ml counted using a haemocytometer (Section 2.2.1.5). The cell suspension was diluted to a concentration of 5 × 10^5 cells/ml. The cells were seeded in a 96-well plate at a concentration of 5 × 10^4 cells/well, with 100 μl medium per well. The 96-well plate was incubated in a humidified container within the incubator (to avoid changes in cell culture medium volume caused by evaporation) for 24 hours under standard conditions (37°C, 5% CO₂).

On Day 2, 10 μl transfection mix (prepared as described below, Section 2.2.7.2) was added to each well of the 96-well plate. After 16 hours incubation (Day 3), the transfection medium was removed and replaced with STM supplemented with the metabolites/hormones under investigation. The cells were incubated for a further 24 hours before the luciferase and SEAP assays were performed (Day 4).

2.2.7.2 Preparation of transfection complexes
HepG2 cells were transfected with relevant plasmids, using Fugene-6 reagent (Roche Molecular Biochemicals). Fugene-6 is a lipid-based transfection agent which forms a complex with plasmid DNA and enables fusion with the cytoplasmic membrane and endosomal uptake into the cell (Fugene-6 Transfection Reagent Instruction Manual, 2000). Fugene-6 has been shown to have 6-fold higher transfection efficiency in HepG2 cells in comparison to a calcium-phosphate-based method (Kurachi et al., 1998).

For each assay, cells were transfected with 100 ng/well of the luciferase reporter plasmid, 25 ng/well SEAP-3' rep reporter plasmid as a control for transfection efficiency, along with expression vector(s) for HNF4α, p300 or PGC-1α (dependent on experiment). The basic expression vector pSG5 was used as a control for the amount of DNA so that all culture wells were transfected with the same quantity of DNA. Fugene-6 transfection reagent was used in a 3:1 ratio of volume of reagent (μl) to DNA (μg). The transfection mix was prepared as per manufacturer’s instructions (Fugene-6 Transfection Reagent Instruction Manual, 2000) by adding the required volume of Fugene-6 transfection reagent to additive-free basal medium (199 or DMEM without phenol red) to a volume of 100 μl, mixing by tapping the tube and incubated for 5 minutes at room temperature. The Fugene-6 transfection complex was then added to the plasmid DNA, mixed by tapping, and incubated for 30 minutes at room
temperature. The volume of transfection mixture was then made up to the appropriate
volume with additive-free basal medium and 10 μl of the transfection was added directly to
each well of the 96-well plate.

2.2.7.3 Secretory alkaline phosphatase (SEAP) assay
Prior to the luciferase assay, media samples (normally 20 μl) were removed from culture
wells (96-well plate) and the medium replaced with the same volume of STM. Media
samples were assayed for SEAP activity according to the manufacturer’s instructions
(modified from the Phospha-Light System Technical Manual (2000)). 6.25 μl of the media
was transferred to a white optiplate, 19 μl of 1× dilution buffer added per optiplate well and a
thermal seal applied to the plate to prevent evaporation. The plate was centrifuged at 1000
rpm for 1 minute to mix (193 x g, Eppendorf Centrifuge 5810) and then incubated at 65°C
for 30 minutes to destroy endogenous alkaline phosphatase activity. The optiplate was
removed from the oven and placed on ice for 2 minutes. 25 μl of assay buffer was added per
well and the optiplate incubated for 5 minutes at room temperature. 25 μl of reaction buffer
was added per well and the optiplate incubated for a further 15-20 minutes at room
temperature followed by measurement of luminescence. Luminescence was measured using
a Packard Luminicount II plate reader with a read length of 1.0 s, with the detector gain being
set automatically based on the well with the highest luminescence.

2.2.7.4 Luciferase assay

![Reaction catalysed by firefly luciferase enzyme](image)

Figure 2.10: Reaction catalysed by firefly luciferase enzyme. Firefly luciferase
catalyses the oxygenation of beetle luciferin in the presence of ATP, Mg²⁺ and O₂ to form
oxyluciferin, AMP and CO₂ with the concomitant emission of light which is measured

The luciferase gene was originally cloned from the bioluminescent firefly Photinus pyralis
(de Wet et al., 1987) and catalyses the oxygenation of luciferin with the concomitant
production of light (Figure 2.10). Unlike SEAP, luciferase is not secreted into the cell culture
medium, therefore cell lysis is required in order for the reaction to proceed. Luciferase
reporter gene activity in HepG2 cells was assayed with the homogenous Steady-Glo or
Bright-Glo Luciferase Systems (Promega) which contain both cell lysis reagents and luciferase assay substrates. The Steady-Glo reagent was used in initial experiments using L-PK reporter genes, however the Bright-Glo reagent was used for the majority of reporter gene experiments as it achieves higher sensitivity compared to the Steady-Glo reagent (Bright-Glo Luciferase Assay System Technical Manual, 2006).

To perform the luciferase assay, the cell culture plate was equilibrated to room temperature and 100 µl of luciferase reagent added to each well (containing 100 µl of medium). The cells and luciferase reagent were mixed using the mix function of the luminometer. In the case of the Steady-Glo reagent which has a longer signal half-life (~ 5 hours) compared to the Bright-Glo reagent (signal half life ~ 25 minutes), the plate was incubated for at least 5 minutes at room temperature to allow cell lysis to occur. For the Bright-Glo reagent, cell lysis was achieved after an incubation time of 2 minutes. After cell lysis, luminescence was measured using a Packard Luminount II plate reader, with a read length of 6.0 s and detector gain being set automatically depending on the well with the highest luminescence.

2.2.7.5 Calculation of induction

**Equations 2.2: Calculation of (A) Corrected luciferase gene reading and (B) Induction.** (A) Luciferase output from each well of a 96-well plate was corrected for transfection efficiency by dividing by the SEAP reading. (B) The effect of a hormone or metabolite on reporter gene expression was calculated by dividing the mean corrected luciferase reading for the condition with the hormone / metabolite by that for STM or vehicle control medium (VCM).

\[
\text{A} \quad \text{Corrected luciferase reading} = \frac{\text{Luciferase output}}{\text{SEAP output}}
\]

\[
\text{B} \quad \text{Induction} = \frac{\text{Mean corrected luciferase reading (Condition: + Hormone)}}{\text{Mean corrected luciferase reading (Condition: STM or VCM)}}
\]

Luciferase reporter gene expression was corrected for transfection efficiency by normalising each luciferase assay reading by the SEAP assay reading (Equations 2.2A). Fold-induction in reporter gene expression was calculated as the ratio of reporter gene expression in the
presence of the hormone or metabolite under investigation to that under the control condition (Equations 2.2B).

2.2.8 Study of HNF4α target gene expression using real-time PCR

2.2.8.1 Principles of real-time PCR

Real-time PCR provides a highly accurate and sensitive method for the quantification of gene transcripts, as the progress of the PCR reaction can be monitored in real-time and the amount of PCR product which has accumulated can be measured during the log-linear phase of the reaction (Figure 2.11A).

Figure 2.11: Principles of (A) Real-time methods for detection of PCR reaction (B) SYBR-Green biochemistry. (A) Real-time PCR cyclers detect the amount of PCR product which has accumulated after each PCR cycle and enable the linear phase of the PCR reaction to be accurately defined. (B) SYBR-Green fluorescent dye intercalates into double-stranded DNA and can be detected in this state by its specific excitation / emission spectra. (Qiagen, Critical Factors for Successful Real-time PCR, 2004).

The progress of the PCR reaction can be monitored using sequence-specific probes labelled with a fluorophore or a fluorescent dye that intercalates with double-stranded DNA. The latter approach was used in this project and the fluorescent dye, SYBR-Green I, used to monitor the accumulation of PCR product. The intercalation of SYBR-Green I dye with double-stranded DNA is proportional to the amount of double-stranded DNA and this interaction can be detected by virtue of the specific excitation / emission spectra of the SYBR-Green:DNA complex (excitation maximum $\lambda = 494$ nm, emission maximum $\lambda = 521$ nm).
SYBR-Green enables a range of gene targets to be detected using standard oligonucleotide primers, however as SYBR-Green also binds to primer-dimers and non-specific PCR products, melting curve analysis needs to be performed for each PCR run, to the fluorescence reporter signal corresponds to only the PCR product of interest (Section 2.2.8.7.2). In addition, real-time PCR products were analysed by agarose gel electrophoresis (Section 2.2.4.2), to validate that real-time PCR products of the correct size were present.

2.2.8.2 Culture of HepG2 cells for analysis of gene expression (Q-PCR)

A typical timeline for the analysis of gene expression in HepG2 cells by real-time quantitative PCR (Q-PCR) is outlined in Figure 2.12.

![Timeline](image)

Figure 2.12: Experimental timeline for the incubation of HepG2 cells for the analysis of gene expression by Q-PCR.

HepG2 cells were grown in SCM until 90% confluent and trypsinized (Section 2.2.1.2), followed by centrifugation at 230 x g (1000 rpm, Sigma 6K10 centrifuge). The cell pellet was resuspended in 5 ml SCM and a 10 μl aliquot removed for counting (Section 2.2.1.5). The cell suspension was then diluted to a concentration of 5x10^5 cells/ml and seeded in 24-well plates in a volume of 1 ml cell suspension/well. Following 24 hours incubation under standard conditions (37°C, 5% CO₂), cells were transfected with plasmid DNA (Day 2), in the case of experiments involving the over-expression of HNF4α, p300 or PGC-1α (Section 2.2.8.3), and incubated for a further 16 hours. On Day 3, the culture medium was removed and the cells washed twice with 1 ml PBS and the medium replaced with serum-free medium (SFM) (DMEM with 1% NEAA and 1% PSG). Following 24 hours serum-starvation, the medium was removed and the cells washed once with 1 ml PBS. The medium was replaced with SFM containing the hormones, metabolites or kinase activators under investigation (Section 2.2.1.6.). Following an experimental incubation (typically 6 hours), the medium was removed and cells washed once with 1 ml PBS prior to the addition of lysis buffer and RNA isolation (Section 2.2.2.2).
2.2.8.3 Overexpression of HNF4α, p300 and PGC-1α

Based on the results of transcription factor overexpression in reporter gene experiments, HepG2 cells were transfected with Fugene-6:DNA complexes using a 3:1 ratio of Fugene-6 transfection reagent (μl) to DNA (μg) (Section 2.2.7.2). The following small changes were applied in order to take into account the larger cell culture area of 24-well plates (1.9 cm²) compared to 96-well plates (0.4 cm²): Fugene-6 was initially diluted in additive-free DMEM to a volume ten times that of the Fugene-6 reagent (rather than 100 μl as previously, Section 2.2.7.2). The Fugene-6 complex was then divided between tubes containing the different plasmid DNAs being used in the experiment, plus sufficient additive free DMEM such that the volume of DNA-Fugene 6 transfection mix added to each well of the 24-well plate was 50 μl. A typical calculation including 10% excesses is shown in Table 2.10. Cells were transfected with between 125 - 500 ng/well DNA per expression plasmid. Control culture wells were transfected with an equal amount of the basic expression vector, pcDNA3.1 (Invitrogen), which lacks an inserted coding sequence (Section 2.2.6.1.2).

Table 2.10: Example calculation: Fugene-6:DNA transfection mix.

<table>
<thead>
<tr>
<th>Transfection No.:</th>
<th>1</th>
<th>2</th>
<th>Total</th>
<th>Transfection Mix*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Content</strong></td>
<td><strong>Control</strong></td>
<td><strong>Expression</strong></td>
<td><strong>Mix</strong></td>
<td></td>
</tr>
<tr>
<td>No. of wells (n)</td>
<td>10</td>
<td>10</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>No. of wells including excess (n+10%)</td>
<td>11</td>
<td>11</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>DNA (μg per well)</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fugene (μl):DNA (μg) ratio</td>
<td>3</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control plasmid (pcDNA3.1) (100 ng/μl) (μl)</td>
<td>55.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expression plasmid (100 ng/μl) (μl)</td>
<td>55.0</td>
<td>55.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Absolute volume Fugene-6 (μl)</td>
<td>16.5</td>
<td>16.5</td>
<td>33.0</td>
<td>36.3</td>
</tr>
<tr>
<td>Final volume Fugene-6 mix (μl)</td>
<td>165</td>
<td>165</td>
<td>330</td>
<td>363</td>
</tr>
<tr>
<td>Final vol. (μl)</td>
<td>550</td>
<td>550</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume additive-free DMEM (μl)</td>
<td>330</td>
<td>330</td>
<td>660</td>
<td>327</td>
</tr>
</tbody>
</table>

A transfection mix (right-hand column) of Fugene-6 was prepared in additive-free DMEM (*including 10% excess). Appropriate volumes of the transfection mix were added to tubes containing control plasmid, pcDNA3.1, or expression plasmid, plus additional DMEM to allow 50 μl Fugene-DNA complex to be added to each well of a 24-well plate.

2.2.8.4 RNA purification and DNase treatment of RNA

RNA was purified from HepG2 cells using Qiagen RNeasy Mini columns (Section 2.2.2.2). RNA concentration and purity were measured using a Nanodrop Spectrophotometer and, in the case of selected samples, RNA integrity was assessed using an Agilent 2100 Bioanalyzer.
(Section 2.2.3). The purified RNA was diluted to a final concentration of 100 ng/µl with RNase-free water.

DNase treatment of RNA was performed on all samples to ensure the removal plasmid DNA (in the case of over-expression experiments) and residual genomic DNA. The amount of RNA used in the DNase treatment and subsequent cDNA synthesis stages was optimised to ensure that the presence of plasmid DNA was not detected by Q-PCR in the RT- (RT minus) control. 500 ng RNA was DNase-treated using RQ1 DNase-free RNase (Promega) according to the manufacturer's protocol (Table 2.11). Reagents were pulse-centrifuged to mix and incubated at 37°C for 30 minutes. The DNase enzyme was inactivated by the addition of 1 µl DNase stop solution and a further incubation at 65°C for 10 minutes. 2 µl DNase-treated RNA (~100 ng) was used for cDNA synthesis.

<table>
<thead>
<tr>
<th>Table 2.11: Reaction composition of DNase treatment of RNA.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Component</strong></td>
</tr>
<tr>
<td>RNA (100 ng/µl)</td>
</tr>
<tr>
<td>RNase-free water</td>
</tr>
<tr>
<td>Reaction Buffer (10x)</td>
</tr>
<tr>
<td>RQ1 RNase-free DNase (1 unit/µl)</td>
</tr>
<tr>
<td>Volume (DNase reaction)</td>
</tr>
<tr>
<td>500 ng RNA was treated using in a reaction volume of 10 µl, containing 1 µl reaction buffer and 1 µl DNase.</td>
</tr>
</tbody>
</table>

2.2.8.5 cDNA synthesis

In contrast to semi-quantitative analysis of gene expression, where the RT reaction was performed using gene-specific primers as part of a one-step RT-PCR reaction (Section 2.2.5.2), cDNA was synthesized from RNA using an oligonucleotide primer mix consisting of oligo-dT and random nonamers, supplied as part of the QuantiTect Reverse Transcription kit (Qiagen). This mix of primers for first-strand synthesis is designed to amplify all regions of the RNA template. The QuantiTect Reverse Transcription kit also included a genomic DNA (gDNA) treatment stage, providing a second DNase treatment in addition to that described above (Section 2.2.8.4), which ensured that all plasmid and genomic DNA was removed.
cDNA was prepared according to the manufacturer's protocol for the QuantiTect Reverse Transcription kit (Qiagen), using 100 ng DNase-treated RNA (QuantiTect Reverse Transcription Handbook, 2005). All reagents were thawed at room temperature, briefly centrifuged to pool contents and kept on ice. All reactions were prepared on ice. For the first gDNA elimination stage (Table 2.12A), a mastermix of gDNA Wipeout Buffer and RNase-free water was prepared for the number of reactions (n) with an excess of 10% and divided between reaction tubes (12 µl per tube). Template RNA (2 µl DNase-treated RNA, ~100 ng) was then added to make a reaction volume of 14 µl, pulse-centrifuged to mix and incubated at 42°C for 2 minutes, and then returned to ice.

Table 2.12A: Reaction composition of gDNA elimination reaction.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNase-treated RNA solution (~100 ng)</td>
<td>2</td>
</tr>
<tr>
<td>gDNA Wipeout Buffer (7x)</td>
<td>2</td>
</tr>
<tr>
<td>RNase-free water</td>
<td>10</td>
</tr>
<tr>
<td>Total volume (gDNA elimination step)</td>
<td>14</td>
</tr>
</tbody>
</table>

Table 2.12B: Reaction composition of RT reaction.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Template RNA (gDNA elimination reaction)</td>
<td>14</td>
</tr>
<tr>
<td>Quantiscript Reverse Transcriptase</td>
<td>1</td>
</tr>
<tr>
<td>Quantiscript RT Buffer (5x)</td>
<td>4</td>
</tr>
<tr>
<td>RT Primer Mix</td>
<td>1</td>
</tr>
<tr>
<td>Total volume (RT step)</td>
<td>20</td>
</tr>
</tbody>
</table>

100 ng DNase-treated RNA underwent an additional gDNA treatment stage (A) prior to cDNA synthesis (B) using QuantiTect Reverse Transcription kit (Qiagen).

An RT mastermix consisting of Reverse Transcriptase, RT Buffer and RT Primer Mix (Table 2.12B) was prepared and 6 µl added to each tube containing the gDNA treatment reaction. An RT reaction was performed with reverse transcriptase replaced by 1 µl RNase-free water, in order to serve as a control for the presence of contaminating plasmid DNA or gDNA. The RT reaction tubes were incubated for 15 minutes at 42°C, followed by 3 minutes incubation at 95°C in order to denature the reverse transcriptase. Each 20 µl RT reaction was diluted to a volume of 100 µl with PCR-grade water to a concentration equivalent to 1 ng/µl RNA, mixed by tapping the tube and pulse-centrifugation and then stored at -20°C until required.
2.2.8.6 Quantitative real-time PCR using SYBR-Green I

2.2.8.6.1 Real-time PCR primer sets

PCR primers for detection of housekeeping genes (Primer Design) and target genes (Qiagen QuantiTect Primer Assays) were supplied as lyophilised oligonucleotides consisting of both forward and reverse primers, with the exception of SREBP-1c primers (Table 2.13). PCR primer sets were resuspended according to the manufacturer's instructions in nuclease-free water (housekeeping gene primer sets) or molecular biology grade TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) (QuantiTect Primer Assays) and vortexed.

SREBP-1c primers were supplied as HPLC-purified forward and reverse primers (MWG Biotech). The separate SREBP-1c forward and reverse primers were initially reconstituted in nuclease-free water to a concentration of 100 pmol/µl concentration and a primer mix of forward and reverse primers prepared in nuclease-free water (concentration of each primer: 8 pmol/µl). Reconstituted primer mixes were stored at -20°C in aliquots.

2.2.8.6.2 Preparation of real-time PCR reactions

Real-time PCR reactions were performed using QuantiTect SYBR Green PCR kit (Qiagen), which contains HotStar (heat-activated) Taq polymerase, PCR buffer, SYBR-Green I dye and ROX dye, the latter acting as an internal reference for normalisation of the SYBR-Green signal. Mastermixes were prepared using the manufacturers' recommended volumes of each reconstituted primer mix, SYBR Green Mix and PCR-grade water and 20 µl mastermix added to each well of a 96-well PCR plate (Table 2.14). 5 µl of each cDNA sample was then added per well. In addition, a 'no template control' (NTC) containing 5 µl PCR-grade water instead of cDNA was included as an indicator of contamination and a 5 µl RT- sample was included to ensure the efficacy of the DNase treatment of the RNA prior to cDNA synthesis (i.e. an indicator of genomic or plasmid DNA contamination). A thermal seal was applied to the top of the PCR plate (to avoid evaporation during the thermal cycling process) and the plate centrifuged for 1 minute at 1000 rpm (193 x g, Eppendorf Centrifuge 5810) in order to mix the primer-PCR mastermix and cDNA sample.
<table>
<thead>
<tr>
<th>Gene (symbol*)</th>
<th>Genbank Accession No.</th>
<th>Source (Reference)</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Housekeeping gene primers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 S rRNA (18S)</td>
<td>M10098</td>
<td>Primer Design</td>
<td>Not supplied</td>
</tr>
<tr>
<td>β-2-microglobulin (B2M)</td>
<td>NM_004048</td>
<td>Primer Design</td>
<td>Not supplied</td>
</tr>
<tr>
<td>Cytochrome c-1 (CYC1)</td>
<td>NM_001916</td>
<td>Primer Design</td>
<td>Not supplied</td>
</tr>
<tr>
<td>Eukaryotic translation initiation factor 4A (EiF4A2)</td>
<td>NM_001967</td>
<td>Primer Design</td>
<td>Not supplied</td>
</tr>
<tr>
<td>Topoisomerase 1 (TOP1)</td>
<td>NM_003286</td>
<td>Primer Design</td>
<td>Not supplied</td>
</tr>
<tr>
<td>Ubiquitin C (UBC)</td>
<td>NM_021009</td>
<td>Primer Design</td>
<td>137</td>
</tr>
<tr>
<td><strong>Target gene primers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetyl CoA carboxylase (ACACA)</td>
<td>NM_000664 NM_198835</td>
<td>Qiagen (QT00033761)</td>
<td>116</td>
</tr>
<tr>
<td>Apolipoprotein CIII (ApoCIII)</td>
<td>NM_000040</td>
<td>Qiagen (QT00012537)</td>
<td>78</td>
</tr>
<tr>
<td>Fatty acid synthase (FASN)</td>
<td>NM_004104</td>
<td>Qiagen (QT00014588)</td>
<td>71</td>
</tr>
<tr>
<td>Hepatocyte Nuclear Factor (HNF4A)</td>
<td>NM_128849 NM_000457</td>
<td>Qiagen (QT00019411)</td>
<td>90</td>
</tr>
<tr>
<td>Liver-type pyruvate kinase (PKLR)</td>
<td>NM_00298 NM_181871</td>
<td>Qiagen (QT00018156)</td>
<td>169</td>
</tr>
<tr>
<td>Phosphoenolpyruvate carboxykinase (cytosolic) (PCK1)</td>
<td>NM_002591</td>
<td>Qiagen (QT00011971)</td>
<td>97</td>
</tr>
<tr>
<td>Peroxisome proliferator-activated receptor-α (PPARA)</td>
<td>NM_001001928</td>
<td>Qiagen (QT00017451)</td>
<td>109</td>
</tr>
<tr>
<td>E1A binding protein p300 (EP300)</td>
<td>NM_001429</td>
<td>Qiagen (QT00094500)</td>
<td>72</td>
</tr>
<tr>
<td><em>Mus musculus</em> Peroxisome proliferator-activated receptor-γ coactivator 1-α (PPARGC1a)</td>
<td>NM_008904</td>
<td>Qiagen (QT00156303)</td>
<td>63</td>
</tr>
<tr>
<td><em>Homo sapiens</em> Peroxisome proliferator-activated receptor-γ coactivator 1-α (PPARGC1a)</td>
<td>NM_013261</td>
<td>Qiagen (QT00095578)</td>
<td>134</td>
</tr>
<tr>
<td>Sterol regulatory element binding protein-1c (SREBP-1c)</td>
<td>NM_004176</td>
<td>(Oberkofler et al., 2004)</td>
<td>80</td>
</tr>
<tr>
<td>Sterol regulatory element binding protein-2 (SREBF2)</td>
<td>NM_004599</td>
<td>Qiagen (QT00052052)</td>
<td>145</td>
</tr>
</tbody>
</table>

Housekeeping gene primer sets were supplied by Primer Design. The majority of target gene primer sets used were obtained as QuantiTect Primer Assays (Qiagen). All primer sets are designed to detect the human transcript unless stated otherwise below. *The gene symbol used by the manufacturer is given in parenthesis.
Table 2.14A: Composition of real-time PCR reactions using housekeeping gene or SREBP-1c primers.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume / reaction (µl)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reconstituted primer mix</td>
<td>1.25</td>
<td>400 nM</td>
</tr>
<tr>
<td>QuantiTect SYBR Green Mix (2x)</td>
<td>12.5</td>
<td>1 x</td>
</tr>
<tr>
<td>PCR-grade water</td>
<td>6.25</td>
<td></td>
</tr>
<tr>
<td>cDNA (1 ng/µl RNA equivalent)</td>
<td>5.0</td>
<td>0.2 ng/µl RNA equivalent</td>
</tr>
<tr>
<td>Final volume</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.14B: Composition of real-time PCR reactions using QuantiTect Primer Assays.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume / reaction (µl)</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>QuantiTect Primer Assay (10x)</td>
<td>2.5</td>
<td>1 x</td>
</tr>
<tr>
<td>QuantiTect SYBR Green Mix (2x)</td>
<td>12.5</td>
<td>1 x</td>
</tr>
<tr>
<td>PCR-grade water</td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>cDNA (1 ng/µl RNA equivalent)</td>
<td>5.0</td>
<td>0.2 ng/µl RNA equivalent</td>
</tr>
<tr>
<td>Final volume</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

2.2.8.6.3 Real-time PCR cycling conditions

Real-time PCR reactions were performed using an ABI PRISM 7000 Sequence Detection System cycler and associated software (Applied Biosystems, Foster City, U.S.A.). The cycling conditions were based on parameters outlined for the use of QuantiTect SYBR Green PCR mix and Primer Assays (Qiagen) with this real-time PCR cycler (Table 2.15) (QuantiTect SYBR Green PCR Handbook (2005), Qiagen; QuantiTect Primer Assay Handbook (2006), Qiagen). Following PCR cycling, a dissociation protocol with a starting temperature of 55°C was programmed (Section 2.2.8.7.2).

Table 2.15: Real-time PCR cycler conditions.

<table>
<thead>
<tr>
<th>Step</th>
<th>Time</th>
<th>Temperature</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial activation step</td>
<td>15 min</td>
<td>95°C</td>
<td>Activation of HotStar Taq DNA polymerase</td>
</tr>
<tr>
<td>3-step cycling (45 cycles):</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Denaturation</td>
<td>15 s</td>
<td>94°C</td>
<td>Denaturation double-strand DNA</td>
</tr>
<tr>
<td>Annealing</td>
<td>30 s</td>
<td>55°C</td>
<td>Primer binding</td>
</tr>
<tr>
<td>Extension</td>
<td>30 s</td>
<td>72°C</td>
<td>Synthesis of second strand DNA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Fluorescence data acquisition</td>
</tr>
</tbody>
</table>

PCR reactions were performed with the following cycling steps using an ABI PRISM 7000 real-time PCR cycler. Taq DNA polymerase was activated by 15 minutes activation at 95°C followed by 45 cycles consisting of denaturation, annealing and extension steps.
2.2.8.7 Analysis of real-time PCR data

2.2.8.7.1 Acquisition of real-time PCR data

The reporter signal generated by SYBR Green fluorescence was automatically corrected for the signal from the passive reference dye (ROX) to generate the normalised reporter signal (delta Rn) which reflects the progress of the PCR reaction. An amplification plot of delta Rn after each PCR cycle was produced using the ABI PRISM 7000 software (Figure 2.13). A baseline level, corresponding to background fluorescence separate from the progress of the PCR reaction, was set between 6 and 15 cycles. The threshold level, corresponding to the level of reporter signal at which relative abundance of transcripts was quantified, was adjusted manually to a region of the plot within the log-linear region of the reaction curves for all wells of the PCR plate. The threshold cycle (Ct) number at which the fluorescence reporter signal for each PCR reaction exceeded the threshold was then calculated and exported in the form of a Results Export File from the ABI PRISM 7000 program.

![Delta Rn vs Cycle Graph](image)

**Figure 2.13: Annotated amplification plot of delta Rn against cycle number.** Typical amplification plot of normalised fluorescence intensity (delta Rn) against cycle number produced for each PCR reaction (coloured lines) by ABI PRISM 7000 software. The baseline level corresponding to background fluorescence was set between 6 and 15 cycles and the threshold level (thick black line) set manually to a level corresponding to the exponential phase of the PCR reaction throughout all wells of the PCR plate. The threshold cycle (Ct) corresponds to the fractional cycle number at which the fluorescence reporter signal for a particular well crosses the threshold.
2.2.8.7.2 Interpretation of melting curve analysis

Following PCR cycling, a melting curve analysis of each sample was performed by measuring the fluorescence in each well over a temperature gradient (55°C - 85°C). The resulting dissociation plot of the first derivative of fluorescence reporter signal against temperature indicates the gradient of the melting curve at each temperature, i.e. a steep decrease in fluorescence reporter signal indicates increased denaturation of double-stranded DNA at that temperature. A single peak observed in the dissociation curve (Figure 2.14) corresponds to single PCR product (Nolan, 2004). The absence of multiple PCR products was also confirmed by gel electrophoresis (Section 2.2.4.2).

**Figure 2.14: Annotated dissociation curve analysis.** Typical dissociation curve of first derivative (of the fluorescence reporter signal) against temperature. Peaks attributable to primer-dimer and PCR product melting are indicated.

2.2.8.7.3 Calculation of PCR efficiency

A PCR efficiency of 100% assumes that there is a doubling of the number of PCR products following each cycle of PCR. However, PCR efficiency can vary with different primer sets due to different GC content of primers, efficiency of primer annealing or other experimental factors, leading to a PCR efficiency of less than 100%. It was necessary to calculate PCR efficiency for each primer set in order to quantify gene expression data without the use of a standard curve (Section 2.2.8.7.4). This was achieved by performing PCR reactions with several dilutions of a single cDNA sample (in PCR-grade water) for each gene-specific
primer set. The resulting C_t values were plotted against the logarithm of the amount of RNA (ng) used in the cDNA synthesis reaction and a trendline fitted to the data points (Figure 2.15). The slope (S) of the trendline was used to calculate PCR efficiency (E) using Equations 2.3A. The average PCR efficiency was calculated based on the results of slope values generated from two PCR experiments using dilutions of two different cDNA samples.

**Figure 2.15: Plot of C_t against log RNA (ng) for the calculation of PCR efficiency (E).** PCR efficiency (E) was determined from the slope of trendline relating C_t values to log_{10}[RNA].

### 2.2.8.7.4 Relative quantification of gene expression

Gene expression data for target genes was normalised to the expression of an endogenous reference gene (housekeeping gene) based on the ΔC_t method (Pfaffl, 2001). The relative expression of a particular gene within a biological repeat experiment was compared by subtracting the C_t value of the sample from the minimum C_t value (i.e. sample with the highest expression of the gene in question) in order to derive the ΔC_t value for each sample (Equations 2.3B). The ΔC_t value was then used to derive the relative expression level of a gene based on Equations 2.3C, which takes into account the PCR efficiency of the gene-specific primer set. For example, a ΔC_t value of -1.0 would equate to a 2-fold reduction in the abundance of the transcript in the sample in question compared to the sample with the highest expression (in the case of a PCR efficiency of 100%). The relative gene expression
quantities were then normalised to the expression of an endogenous reference gene, β-2-microglobulin (B2M), by a ratio-based method (Equations 2.3D). This is a similar approach as the ΔΔCt method (Livak and Schmittgen, 2001), however in this case it is possible to take into account different PCR amplification efficiencies (Pfaffl, 2001).

**Equations 2.3: Formulae used in analysis of real-time PCR data.** (A) PCR efficiency \( E \) was calculated from the slope \( S \) of the plot of \( C_t \) vs \( \log_{10}(RNA(ng)) \). (B) ΔCt for each sample is calculated by subtracting the \( C_t \) value for the sample from the minimum \( C_t \) values for all samples for a particular gene. (C) Calculation of the quantity of each sample (relative to the sample with the highest expression of the gene) using the values for PCR efficiency \( E \) and ΔCt for each sample. (D) The relative quantity of each target gene was normalised by dividing by the quantity of a housekeeping gene.

\[
\text{(A) } \quad \text{PCR Efficiency (E) } = 10^{-\frac{1}{S}} - 1 \\
\text{(B) } \quad \Delta C_t = \text{minimum } C_t \text{ (all values) } - C_t \text{ (sample)} \\
\text{(C) } \quad \text{Quantity} = (1 + E)^{\Delta C_t} \\
\text{(D) } \quad \text{Normalised gene expression level} = \frac{\text{Quantity: target gene}}{\text{Quantity: housekeeping gene}}
\]

### 2.2.8.8 Selection of most stable housekeeping gene (GeNorm method)

The choice of housekeeping gene to use as an endogenous reference for relative quantification of gene expression was made using the geNorm method and associated Visual Basic Application for Microsoft Excel (http://medgen.ugent.be/~jvdesomp/genorm/), which calculates housekeeping gene stability and the optimum number of housekeeping genes to use for normalisation (Vandesompele et al., 2002). The method uses the assumption that for two stably expressed housekeeping genes, the ratio between the two genes should be the same under each set of experimental conditions. The method outlined by Vandesompele and colleagues calculates the average pairwise variation in the expression of each housekeeping gene compared with other housekeeping genes within a set to define the average internal control gene stability measure \( M \). Stepwise omission of the housekeeping gene with the highest (i.e. least stable) \( M \) value results in the two housekeeping genes with the most stable expression under the tested experimental conditions (Figure 2.16). β-2-microglobulin (B2M) was selected as the most stable HKG based on being within the two most stable HKGs in two
geNorm analysis using samples from separate biological experiments investigating the effect of HNF4α over-expression on SREBP-1c gene expression in HepG2 cells (Chapter 5).

![Graph showing average expression stability values of remaining control genes](image)

**Figure 2.16:** Plot of average expression stability for a set of housekeeping genes. The analysis of average expression stability ($M$) of remaining housekeeping genes following stepwise exclusion of the least stable housekeeping gene was generated using the geNorm application for Microsoft Excel (Vandesompele *et al.*, 2002).

### 2.2.9 Sequencing of DNA

#### 2.2.9.1 Purification of HNF4α RT-PCR products

Briefly, 25 μl of RT-PCR or PCR product was separated on a 1.5% agarose gel for approximately 1 hour. The gel was visualized using a UV lamp and the band corresponding to the PCR product excised using a sterile razor blade and transferred to a sterile microcentrifuge tube with sterile forceps. The gel slice was then treated according to the manufacturer’s instructions for gel purification using the Wizard® SV Gel and PCR Clean-up System (Wizard SV Gel and PCR Clean-Up System Technical Bulletin, 2004). The weight of the gel slice was established using an empty microcentrifuge tube as a blank. The gel slice was dissolved in 10 μl Gel Binding Solution per 10 mg gel slice at 50-65°C for 10 minutes with regular vortexing. The dissolved gel slice was added to a SV Minicolumn, incubated for
1 minute at room temperature and centrifuged at 16,000 × g for 1 minute (14,000 rpm, Eppendorf Microcentrifuge Model 5415D). The column was then washed with 700 μl Membrane Wash Solution (diluted with 95% ethanol) for 1 minute at 16,000 × g, followed by a second wash with 500 μl Membrane Wash Solution for 5 minutes at 16,000 × g. The column was then placed in an empty collection tube and centrifuged for a further 1 minute to allow complete evaporation of ethanol. The minicolumn was then placed in a fresh 1.5 ml microcentrifuge tube and the DNA eluted with 50 μl nuclease-free water. DNA concentration and purity were determined using a Nanodrop ND-1000 Spectrophotometer (Section 2.2.3.1).

### 2.2.9.2 DNA sequencing and analysis

HNF4α1, HNF4α2 and HNF4α3 RT-PCR products were sequenced by the Functional Genomics Centre (FHMS, University of Surrey, U.K.) using a CEQ 2000XL DNA Analysis System (Beckman Coulter, Fullerton, U.S.A.). The sequencing of the HNF4α7/8 RT-PCR product was performed externally by Cogenics (Lark Technologies, Hope End, Takeley, U.K.).

<table>
<thead>
<tr>
<th>HNF4α splice variant</th>
<th>Primer name</th>
<th>Sequence (5'→3')</th>
<th>Tm (°C)</th>
<th>Position*</th>
</tr>
</thead>
<tbody>
<tr>
<td>a1/a2</td>
<td>seqprimerF1</td>
<td>TCCAGTTTCATCAAGCTCTTC</td>
<td>55.5</td>
<td>1153-1172</td>
</tr>
<tr>
<td>a1/a2</td>
<td>seqprimerF2</td>
<td>AGCTTGGAGGACTACATCAA</td>
<td>54.9</td>
<td>1100-1120</td>
</tr>
<tr>
<td>a7/a8</td>
<td>a7primerR1</td>
<td>TGTCTCATAAGCTTGACCTT</td>
<td>54.9</td>
<td>354-373</td>
</tr>
</tbody>
</table>

Sequencing primers were designed with a Tm of ~55°C and a GC content of ~50% using Primer3 primer design software. *Position of the primers in the Genbank nucleotide sequences for HNF4α1 (NM_178849) and HNF4α7/8 (AY680696).

In the case of the HNF4α3 RT-PCR product, primer R6 (Table 2.6) was used for primer extension. In the case of the HNF4-α1, HNF4α2 and HNF4α7/8 RT-PCR products, internal oligonucleotides were designed to achieve sequencing of the region to unique to the particular splice variant using Primer3 primer design software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Table 2.16). For example, the positions of the primers for sequencing the HNF4α1 and HNF4α2 splice variants were chosen so that the sequencing product would include the 3' end of the RT-PCR product which differs between the HNF4α1 and -α2 splice variants (Figure 2.4). The sequencing results were compared to the published
sequences for human HNF4α transcript variants using ClustalW sequence analysis program (http://www.ebi.ac.uk/clustalw/).

2.2.10 Statistical analysis

2.2.10.1 Descriptive statistics

In order to express differences in gene expression or reporter gene expression under different incubation conditions, the mean expression levels were calculated. The standard error of the mean (SEM) for each condition was calculated by dividing the standard deviation by the square root of number of data points per condition (n) (Watt, 1993).

Relative gene or reporter gene expression levels were expressed as a percentage of the control condition by division of each datapoint, already normalised to housekeeping gene expression for PCR analysis (Equations 2.3D), or SEAP expression for reporter gene analysis (Equations 2.2A), by the mean normalised value for the control condition (Equation 2.4). In this way, the mean relative expression level of the control condition is set as 100%, with all values being expressed as a percentage of the gene or reporter gene expression in the control condition.

Equation 2.4: Calculation of relative gene expression or reporter expression.

\[
\text{Relative gene expression level (\%)} = \frac{\text{Normalised gene expression level}}{\text{Mean normalised gene expression level (control condition)}}
\]

2.2.10.2 Comparison of group means

The statistical significance of differences between means of different experimental groups was assessed with SPSS for Windows (Version 13.0). The differences between two conditions was analysed by paired t-test, by pairing of the data points for each condition within a biological repeat. The statistical significance of differences between more than two different groups was calculated using a one-way analysis of variance (ANOVA). The dependent variable was selected as the relative gene expression value (for RT-PCR data) or corrected luciferase reading (for reporter gene data) and the independent variable was the incubation and/or transfection condition. One-way ANOVA compares the variance between
the different groups (due to the independent variable) with the variance within each group (due to chance) (Pallant, 2005). The F-statistic and its associated significance value \((p)\) were calculated. If the ANOVA showed overall statistical significance between groups \((p < 0.05)\), Tukey’s post-hoc test was used to analyse differences between two groups.

The data was also subject to Levene’s test for homogeneity of variance which tests the assumption that the variance in data is the same in each of the groups. If the result of this was a significance value \((p)\) of greater than 0.05, then this assumption is correct. However, if \(p < 0.05\), then the variance of the groups is not the same, and in this case, the Welch and Brown-Forsythe tests were used as robust tests of equality of means instead of one-way ANOVA (Pallant, 2005). The Games-Howell post-hoc test was used to identify significant differences between groups in this case, as this test does not assume equality of variances.

For data involving two independent variables, both of which were discrete (such as transfection condition and incubation condition), two-way ANOVA was performed in GraphPad Prism for Windows (Version 5). In the case of timecourse experiments, the effects of incubation condition and period of incubation were analysed by a repeated measures ANOVA with SPSS for Windows (Version 13.0).

2.2.10.3 Bivariate correlations
Correlations between pairs of continuous variables were performed by a two-tailed Pearson correlation test, with degree of correlation between the variables and its statistical significance being assessed from the resulting correlation coefficient \((R)\) and \(p\)-value. Correlation analysis was performed using SPSS for Windows (Version 13.0) or linear regression analysis in GraphPad Prism for Windows (Version 5).
Chapter 3

Characterisation of human hepatoma cell lines for the expression of HNF4α and its target genes
Chapter 3:

3.1 Introduction

HNF4α splice variants have been shown to have different properties in terms of their recruitment of co-factors and of their transactivation of different gene regulatory elements (Sladek et al., 1999; Torres-Padilla et al., 2002; Eeckhoute et al., 2003; Torres-Padilla and Weiss, 2003). Therefore, the expression of HNF4α target genes in human hepatoma cell lines could be influenced by the profile of the HNF4α splice variants which are expressed. The HNF4 isoform expression profile of colonic enterocyte cell lines has been associated with their phenotype: the more differentiated phenotype shown by Caco-2 cells is associated with a wider range of expressed HNF4 isoforms (HNF4α2, HNF4α4 and HNF4γ), whereas less differentiated HT-29 G- cells predominantly express the HNF4α4 splice variant (Saud et al., 1997). In hepatocytes, changes the expression and phosphorylation state of HNF4α1 and HNF4α2 are associated with changes in differentiation state (Runge et al., 1999).

**Aim:** To characterise the human hepatoma cell lines, HuH7, HepG2 and Hep3B, for the expression of HNF4α and its splice variants, and HNF4α target genes.

**Objective 1:** To profile the expression of HNF4α splice variants in the HepG2, Hep3B and HuH7 cell lines using RT-PCR with splice-variant specific primers, and compare the expression profile of HNF4α splice variants with those of adult and foetal human liver.

HepG2 cells have been previously reported to express HNF4α1 and HNF4α2 (Chartier et al., 1994), HNF4α3 (Kritis et al., 1996) plus splice variants derived from the P2 promoter (HNF4α7/8) (Thomas et al., 2001). However, the Hep3B and HuH7 cell lines have not been characterised for the profile of HNF4α splice variants expressed, which is important to determine how they compare to both the HepG2 cell line and in vivo situation. Comparison of HNF4α splice variant expression in the three human hepatoma cell lines with human liver samples will provide an indication of which cell line is the most ideal model for studying HNF4α function.
Objective 2: To study the expression of HNF4α and its hepatic target genes in a hepatoma cell culture system, using semi-quantitative RT-PCR under different conditions of glucose and insulin concentration.

Splice variants of HNF4α function differently at different target gene promoters (Torres-Padilla and Weiss, 2003), therefore cell lines with different expression patterns of HNF4α splice variants could show different patterns of HNF4α target gene expression. In addition, the differential interactions of HNF4α variants with co-factors (Eeckhoute et al., 2003; Eeckhoute et al., 2006; Pascussi et al., 2007) could entail differential modulation of target gene expression in response to metabolic stimuli such as glucose concentration and insulin, as HNF4α co-factors such as SRC-1 and p300/CBP are known to be targets of different signal transduction pathways (Ait-Si-Ali et al., 1999; Yang et al., 2001; Wu et al., 2005).

3.2 Expression of HNF4α and its splice variants in human hepatoma cell lines and human liver

3.2.1 Rationale

Three human hepatoma cell lines, HuH7, HepG2 and Hep3B, were investigated for the expression of total HNF4α and its splice variants HNF4α1, HNF4α2, HNF4α3, HNF4α4 (splice variants transcribed from promoter P1) and HNF4α7/8 (referring to all P2 promoter splice variants) (Introduction, Figure 1.12). In order to compare the profile of expressed splice variants in human liver cell lines with those expressed in the human liver, RT-PCR reactions for HNF4α and its splice variants were also performed on total RNA from human foetal and adult male and female liver samples. An RT-PCR reaction using primers for β-actin was also included, as a control for amount and quality of the mRNA in the sample.

3.2.2 Results

As previously reported, HepG2 cells were shown to express a wide range of HNF4α splice variants, including HNF4α1, HNF4α2, HNF4α3 and HNF4α7/8, however HNF4α4 was not detected (Figure 3.1). Sequencing of gel-purified HNF4α1 and HNF4α2 RT-PCR products confirmed that the C-terminal 30 bp insert unique to HNF4α2 was present in this RT-PCR product (Appendix 1, Box 8.2), whilst the 30 bp insert was found to be absent in the HNF4α1
RT-PCR product (Appendix 1, Box 8.1). An RT-PCR product of the predicted size for HNF4α 7/8 was also detected (Figure 3.1) and sequencing revealed the presence of exon 1D, the alternate first exon present in HNF4α 7/8, compared to exon 1A present in HNF4α 1-6 (Appendix 1, Box 8.4).

**Figure 3.1: Expression of HNF4α splice variants in the HepG2 cell line.** RT-PCR was performed with 2 µg total RNA from HepG2 cells cultured under standard conditions (Section 2.2.1) with primers to β-actin and HNF4α (all splice variants) and to the individual HNF4α splice variants (Section 2.2.5). Figure is representative of RT-PCR performed with two different samples of HepG2 RNA.

**Figure 3.2: Expression of HNF4α splice variants in the Hep3B cell line.** RT-PCR was performed with 400 ng total RNA from Hep3B cells cultured under standard conditions (Section 2.2.1) with primers to β-actin, HNF4α (all splice variants) and to the individual HNF4α splice variants (Section 2.2.5).
The Hep3B cell line also expressed HNF4α and showed the same profile of expressed HNF4α splice variants as the HepG2 cell line with HNF4α1, HNF4α2, HNF4α3 and HNF4α7/8 being detected by RT-PCR (Figure 3.2).

HNF4α was found to be expressed in HuH7 cells (Figure 3.3A); importantly, only the HNF4α3 splice variant was detected by RT-PCR using primers specific to different HNF4α splice variants (Figure 3.3B). This result suggests that HuH7 cells do not express HNF4α1, HNF4α2, HNF4α4 or HNF4α7/8 at a level detectable by RT-PCR. Sequencing of the HNF4α3 RT-PCR product showed sequence identity with the published HNF4α3 sequence (Appendix 1, Box 8.3).

HNF4α was expressed in all human liver samples (Figure 3.4). Bands corresponding to HNF4α1, HNF4α2 and HNF4α3 and a faint band for the HNF4α7/8 RT-PCR product were present following gel electrophoresis of the RT-PCR products of the adult male and female liver RNA samples (Figure 3.4A-B). RT-PCR products for the HNF4α1, HNF4α2 and HNF4α3 splice variants could also be identified in the human foetal liver RNA sample, however the HNF4α7/8 variant was not detectable (Figure 3.4C).

![Figure 3.3: Expression of (A) HNF4α and (B) HNF4α splice variants in the HuH7 cell line.](image)

**Figure 3.3: Expression of (A) HNF4α and (B) HNF4α splice variants in the HuH7 cell line.** HuH7 cells were cultured under standard conditions (Section 2.2.1) and total RNA isolated. RT-PCR was performed with 2 μg total RNA from HuH7 cells with primers to β-actin and (A) HNF4α (all splice variants) or (B) primers to specific HNF4α splice variants (Section 2.2.5). Gels are representative of RT-PCR performed with two different samples of HuH7 RNA.
3.3 Regulation of HNF4α and downstream gene expression in 
HuH7 and HepG2 cells by glucose and insulin concentration

3.3.1 Rationale

The liver is exposed to a wide range of blood glucose and insulin concentrations compared to peripheral tissues due to the transport of digested carbohydrate from the small intestine and insulin secreted by the pancreas direct to the liver via the hepatic portal vein (Frayn, 2003). The expression of HNF4α has been shown to be regulated by insulin in vivo (Oyadomari et al., 2000) and HNF4α regulates its own expression from both the P1 and P2 promoters (Bailly et al., 2001; Briancon et al., 2004; Magenheim et al., 2005). Given the involvement of HNF4α in the regulation of its target genes by high glucose concentration (Diaz Guerra et al., 1993; Adamson et al., 2006), the role of both glucose concentration and insulin in the expression of the HNF4α gene was investigated in HuH7 and HepG2 cells.

ApoCIII and FAS are HNF4α target genes (Sladek et al., 1990; Kardassis et al., 1997; Vergnes et al., 1997; Adamson et al., 2006) which are oppositely regulated by feeding stimuli. The expression of apoCIII is inhibited by insulin (Chen et al., 1994a), whilst FAS expression is stimulated by high glucose concentration and insulin (Paulauskis and Sul, 1989; Prip-Buus et al., 1995). Therefore the regulation of apoCIII and FAS mRNA expression by
insulin and glucose concentration was studied in human hepatoma cells. As HuH7 and HepG2 cells show different HNF4α splice variant expression patterns (Figures 3.1 and 3.2), it was hypothesised this could lead to differences in the regulation of apoCIII and FAS by insulin and glucose concentration, therefore these aspects were compared between the two cell lines.

3.3.2 Results

The effect of glucose on the overall expression of HNF4α was investigated by incubating HuH7 cells with glucose-containing media of different concentrations over a period of 96 hours and measuring the expression of HNF4α relative to the housekeeping gene β-actin by semi-quantitative RT-PCR (Figure 3.5). The effects of incubation time and glucose concentration on HNF4α expression were analysed by two-way ANOVA. Levels of HNF4α showed a tendency to increase over time under all three conditions, although this came short of significance ($p = 0.06$). Glucose concentration had a significant effect on the expression of HNF4α ($p < 0.05$). Although post-hoc tests did not identify significant differences between pairs of incubation conditions, HNF4α expression appeared to be lower under conditions of low glucose concentration (Figure 3.5A).

The expression of HNF4α and its target genes, apoCIII and FAS, in HuH7 cells and HepG2 cells was measured following an incubation period of 48 hours with medium of standard (5.5 mM) or high (20 mM) glucose concentration in the presence or absence of 100 nM insulin (Figure 3.6A and B), and the effects of glucose concentration and insulin on gene expression analysed by two-way ANOVA. The expression of HNF4α, apoCIII and FAS was not significantly modulated by glucose concentration or insulin in HuH7 cells (Figure 3.6A). On the other hand, glucose concentration had a significant effect on HNF4α expression in HepG2 cells ($p < 0.05$) (Figure 3.6B). Post-hoc tests did not show significant differences between two individual incubation conditions; however the difference in HNF4α expression between 5.5 mM and 20 mM glucose was greatest in the presence of insulin, with HNF4α expression 115% greater in the higher glucose concentration (Figure 3.6B). Due to high variability of data within each incubation condition, no significant differences between incubation conditions were found in apoCIII and FAS expression in HepG2 cells (Figure 3.6B). A non-significant 75% decrease in apoCIII mRNA levels was observed in HepG2
Figure 3.5: Expression of HNF4α under conditions of (A) low (B) standard and (C) high glucose concentration over a period of 96 hours in HuH7 cells. HuH7 cells were incubated in media containing low (0.2 mM), standard (5.5 mM) or high (25.0 mM) glucose concentration for 0, 24, 48, 72 and 96 hours after which RNA was isolated. RT-PCR was performed with 100 ng RNA with primers to β-actin and HNF4α and the RT-PCR products quantified by gel digitization. Expression of HNF4α was normalised to the expression of a housekeeping gene, β-actin, and expressed as a percentage. Values shown as mean ± SEM (n = 3) or as individual data points in the case, n = 1 or n = 2.
Figure 3.6: Effect of glucose concentration and insulin on the expression of HNF4α, apoCIII and FAS in (A) HuH7 cells and (B) HepG2 cells. Cells were incubated for 48 hours in media containing 5.5 mM or 20 mM glucose ± 100 nM insulin. RNA was isolated and RT-PCR performed with 100 ng RNA with primers to HNF4α, apoCIII and FAS. The RT-PCR products were quantified by gel digitization and the expression of each gene normalized to β-actin levels. Values shown as mean ± SEM (n = 3). Two-way ANOVA analysis of the effects of glucose concentration and insulin on the expression of each gene showed glucose concentration significantly altered HNF4α expression in HepG2 cells (p < 0.05).
cells incubated with 100 nM insulin (under 5.5 mM glucose conditions) compared insulin-
free medium (Figure 3.6B). Mean FAS mRNA levels were 50% higher in HepG2 cells under
conditions of 20 mM glucose in the presence of insulin compared to standard 5.5 mM glucose
insulin-free conditions although the large variance of data within each incubation condition
meant this effect was not statistically significant (Figure 3.6B).

As a large degree of variability was noted in the results of the above gene expression analyses
(Figures 3.5 and 3.6), the accuracy of the one-step RT-PCR method used in the above
experiments was investigated. The effects of cycle number and RNA concentration on the
accumulation of RT-PCR product DNA were investigated for a highly expressed gene (β-
actin) and a less abundant gene (apoCIII). Firstly, the linearity of the accumulated PCR
product as measured by gel digitization (Section 2.2.5.5) with respect to cycle number was
investigated (Figure 3.7). Accumulation of RT-PCR product was linear with respect to cycle
number within the range 10 to 25 cycles for β-actin (Figure 3.7A) and within the range 15 to
30 cycles for apoCIII (Figure 3.7B).

Secondly, the relationship between the abundance of mRNA transcripts and resulting levels
of RT-PCR products was investigated by quantifying RT-PCR product DNA for a range of
dilutions of a single RNA sample (Figure 3.8). A range of PCR cycles were also investigated
to assess whether this factor had any bearing on the relationship between these two factors.
Accumulation of β-actin RT-PCR product was linear with respect to RNA concentration
within the range 2.5 to 20 ng/μl when a PCR cycle number of 25 was used (Figure 3.8A).
However, when a similar RT-PCR experiment was performed for apoCIII detection, a linear
relationship between RNA concentration and RT-PCR product levels was not observed over
any part of the concentration range, or for any of the PCR cycle numbers employed (Figure
3.8B).
Figure 3.7: Relationship between number of PCR cycles and accumulation of RT-PCR products for (A) β-actin and (B) apoCIII. RT-PCR was performed with 100 ng RNA with primers for β-actin and apoCIII for the indicated number of PCR cycles. Levels of RT-PCR products were quantified by gel electrophoresis and digitisation. Correlation between the number of PCR cycles and accumulation of RT-PCR product was analysed by Pearson's correlation with $R^2$ and $p$-values as indicated.
Figure 3.8: Relationship between RNA concentration and accumulation of RT-PCR products for (A) β-actin and (B) apoCIII. RT-PCR was performed using different dilutions of a single RNA sample to give different RNA concentrations. Levels of RT-PCR products were quantified by gel electrophoresis and digitisation. Correlation between the RNA concentration and accumulation of RT-PCR product was analysed by Pearson’s correlation with $R^2$ and $p$-values as indicated.
3.4 Discussion

Three human hepatoma cell lines, HepG2, Hep3B and HuH7, were characterised for the expression of HNF4α and the expression of selected HNF4α target genes. Firstly, the expression of HNF4α at the mRNA level was confirmed in all three cell lines and the profile of expressed HNF4α splice variants was studied in each of the cell lines.

HepG2 cells were found to express HNF4α (Figure 3.1), in agreement with published results which suggest HNF4α mRNA expression is high in HepG2 cells and even exceeds that of cultured hepatocytes (Castell et al., 2006). HNF4α expression is also evident at the protein level in HepG2 cells (Naiki et al., 2004). Endogenous HNF4α activity in HepG2 cells is also suggested by the utilisation of the cell line to characterize the binding sites and interactions of HNF4α in reporter gene studies; for example, for the human sterol 27-hydroxylase (Garuti et al., 2002) and apoAI genes (Harnish et al., 1996).

HepG2 cells were found to express promoter P1-derived splice variants, HNF4α1, HNF4α2, HNF4α3, as well as low levels of transcripts from the P2-promoter (denoted HNF4α7/8) (Figure 3.1). HepG2 cells are documented to express HNF4α1, HNF4α2, HNF4α3 and HNF4α7/8 (Chartier et al., 1994; Kritis et al., 1996; Thomas et al., 2001; Castell et al., 2006), which validates this RT-PCR assay for the detection of HNF4α splice variants in the HuH7 and Hep3B cell lines, which had not been previously been investigated for this aspect.

Like HepG2 cells, Hep3B cells were found to express HNF4α and the HNF4α1, HNF4α2, HNF4α3 and HNF4α7/8 splice variants were detected (Figure 3.2). HuH7 cells were found to express HNF4α, however only the HNF4α3 splice variant was detected with the splice variant-specific primers (Figure 3.3). HNF4α expression has been detected at the protein level in HuH7 cells, although HNF4α mRNA levels were barely detectable by northern blotting (Naiki et al., 2002; Naiki et al., 2004). Although HNF4α3 was found to be an equally effective transactivator as HNF4α1 or HNF4α2 in one report (Kritis et al., 1996), another report suggested that HNF4α3 is a weaker transactivator at certain promoters (Saud et al., 1999), which could be a reason for the lack of expression of the HNF4α target gene, L-PK, in HuH7 cells (Nakabayashi et al., 1982).
HNF4α splice variants HNF4α1, HNF4α2 and HNF4α3 were detected in adult human liver (Figure 3.4), in agreement with published works (Chartier et al., 1994; Kritits et al., 1996). HNF4α4 was not detected by RT-PCR under the conditions used in any of the human hepatoma cell lines or liver samples (Figures 3.1 to 3.4), whereas the HNF4α4-specific exon IB was previously detected in human liver (Furuta et al., 1997). It is possible that HNF4α4 is more highly expressed in HNF4α-expressing tissues other than the liver, as this splice variant was cloned from a kidney cDNA library and also detected in the enterocytic Caco2 cell line.

The HNF4α7/8 splice variant was detected in human adult liver samples (Figure 3.4A and 3.4B), suggesting that P2-promoter transcripts are present in adult human liver, as are found in adult mouse liver (Nakhei et al., 1998). The low levels of RT-PCR product observed (Figure 3.4A and 3.4B) suggest that HNF4α transcripts derived from the P2 promoter are less abundant in adult liver than P1-promoter derived transcripts (HNF4α1-5) (Torres-Padilla et al., 2001). However, HNF4α7/8 was not detected in human foetal liver RNA (Figure 3.4C), whilst the expression of HNF4α1, HNF4α2 and HNF4α3 appeared to be at similar levels to those in adult liver samples (Figure 3.4), in disagreement with findings that HNF4α7/8 expression is higher during embryogenesis (Torres-Padilla et al., 2001). It is possible that the expression of HNF4α7/8 is different in human foetal development compared to that of mouse (Torres-Padilla et al., 2001), or that the foetal sample used here is relatively late in foetal development when P1 promoter-transcripts have already been up-regulated relative to those originating from the P2 promoter.

The impact of the different HNF4α splice variant expression profiles in HuH7 and HepG2 cells was investigated by studying the expression of HNF4α and its target genes, apoCIII and FAS, under different metabolic conditions. A timecourse study of HNF4α expression revealed differences in HNF4α expression under conditions of variable glucose concentration, as well as a tendency for HNF4α expression to increase with length of culture period (Figure 3.5). Increased confluence in HuH7 cells may be associated with increased HNF4α expression, as another hepatoma cell line, HepG2 cells, have been shown to adopt a more differentiated phenotype with increased confluence, exhibiting higher expression of HNF4α target genes, L-PK and aldolase B (Kelly and Darlington, 1989). A more than 60% reduction in HNF4α expression was observed under low glucose concentration (0.2 mM).
compared to standard conditions (5.5 mM glucose) at the 48, 72 and 96 hour time points (Figure 3.5). This effect may be due to an indirect effect of low glucose concentration on HNF4α mRNA expression. A possible mechanism may be via activation of AMPK brought about by decreased glycolytic flux and ATP concentration. HNF4α activity is suppressed by AMPK and negative feedback on HNF4α mRNA expression may be exerted via HNF-1, which forms a regulatory circuit with HNF4α (Hatzis and Talianidis, 2001; Leclerc et al., 2001; Hong et al., 2003).

In a separate experiment, HNF4α expression in HuH7 cells was also not modulated by standard (5.5 mM) vs. high (20 mM) glucose concentration or the presence of insulin (Figure 3.6A). On the other hand, a non-significant two-fold increase in HNF4α expression in HepG2 cells under conditions mimicking the fed state (20 mM glucose + 100 nM insulin) compared to standard conditions (5.5 mM glucose) was observed (Figure 3.6B). The latter result does not agree with other results which point to HNF4α expression being up-regulated under fasting conditions (Yoon et al., 2001). However, insulin was shown to antagonise the stimulatory effect of glucagon on HNF4α expression in the livers of streptozotocin-induced diabetic rats and in rat hepatocytes (Oyadomari et al., 2000), while HuH7 and HepG2 cells were incubated with insulin in the absence of glucagon (Figure 3.6), which may be a reason for insulin not having an inhibitory effect on HNF4α expression.

Different trends in the regulation of HNF4α target genes by glucose concentration and insulin were observed in HuH7 and HepG2 cells, although a high degree of variability meant that the trends were not significant at the 5% level. Whilst apoCIII expression in HuH7 cells was not modulated by glucose concentration or insulin, a 75% decrease in apoCIII levels in HepG2 cells incubated with 100 nM insulin compared to standard conditions, indicating that insulin had an inhibitory effect on the transcription of this HNF4α target gene in HepG2 cells, (Figure 3.6). ApoCIII has been shown to be down-regulated by insulin at the transcriptional level in vivo (Chen et al., 1994a). In HepG2 cells, mean FAS mRNA levels were also 50% higher under conditions of high glucose concentration in the presence of insulin compared to standard conditions (Figure 3.6B), agreeing with results obtained in vivo and in primary rat hepatocytes, showing that insulin and high glucose concentration induce expression of the FAS gene (Paulauskis and Sul, 1989; Prip-Buus et al., 1995). Together, the results of studying HNF4α target gene expression in HuH7 and HepG2 cells suggest that the apoCIII
and FAS genes are modulated to a greater extent by metabolic stimuli in the latter cell line. The differences in expression of HNF4α splice variants in the two cell lines may contribute to the differences observed in HNF4α target gene expression. Overexpression of HNF4α2 in pancreatic INS-1 cells brought about changes in a great number of downstream genes than overexpression of HNF4α8 (Erdmann et al., 2007), therefore the targets of HNF4α3, the dominant splice variant in HuH7 cells, may be more limited than those of the additional HNF4α splice variants expressed in HepG2 cells.

The high degree of variability in the RT-PCR results (Figure 3.5 and 3.6) suggests that the one-step RT-PCR method employed was not sufficiently quantitative for the purpose of measuring gene expression changes in HNF4α and its target gene mRNA levels under different incubation conditions. The linearity of the RT-PCR reaction with respect to cycle number and RNA concentration was further investigated (Figures 3.7 and 3.8 respectively). It was confirmed that the PCR reaction was in the linear phase of the reaction for both the housekeeping and target genes at a PCR cycle number of 25 (Figure 3.7), which was used for the gene expression analyses (Figures 3.5 and 3.6). Therefore it was concluded that PCR cycle number was not responsible for the variability in the results.

Investigation of the relationship between RNA concentration and PCR product (Figure 3.8), revealed that β-actin RT-PCR product abundance reflected starting transcript levels at the RNA concentration of 10 ng/μl used in the previous gene expression analyses (Figures 3.5 and 3.6), however the level of apoCIII RT-PCR product did not correlate with starting amounts of transcript (Figure 3.8B). As the PCR reaction was within its linear phase for both genes investigated (Figure 3.7), these experiments suggest that the RT stage of the RT-PCR reaction could be responsible for the problems in using this method to quantify transcripts of lower abundance such as apoCIII (Figure 3.8B) and that variation in the efficiency of the RT reaction could be one reason for the high variability of results. More reproducible results may have been obtained by using a two-step RT-PCR method, where the primers for cDNA synthesis are the same for all targets, as opposed to the use of gene-specific primers for cDNA synthesis in one-step RT-PCR (Pfaffl, 2001).

Another factor which may have contributed to the lack of significance in the results obtained was the choice of housekeeping gene (β-actin). Although β-actin has been shown to be a
stable housekeeping gene in some studies investigating the action of insulin on gene expression (Sutherland et al., 1995; Twisk et al., 1995), other reports indicate that its transcription is up-regulated by insulin (Buchou et al., 1991; Messina, 1992).

In conclusion, the profile of expressed HNF4α splice variants was established for the cell lines, HuH7, HepG2 and Hep3B. The HepG2 and Hep3B cell lines were shown to be closest to adult human liver in their expression of all HNF4α splice variants except HNF4α4, suggesting that these two cell lines are a closer model to human liver for the study of HNF4α action. The results of studying the expression of apoCIII and FAS in HuH7 and HepG2 cells also suggest that these genes are regulated in a more physiological manner in HepG2 cells than in HuH7 cells. As the Hep3B cell line contains an integrated hepatitis B viral genome, the expression of which is influenced by insulin and glucocorticoids (Chou et al., 1992; Chen et al., 1997), it is considered that the HepG2 cell line is the best model for studying HNF4α-mediated gene expression. As a semi-quantitative RT-PCR approach produced a high level of variability in the results, reporter gene assays and real-time PCR analysis were considered be potentially more accurate methods for subsequent studies of HNF4α-mediated gene expression.
Chapter 4

Regulation of HNF4α-dependent reporter gene expression by metabolic stimuli
Chapter 4:

4.1 Introduction

Reporter gene analysis enables the study of a gene promoter by delineating the roles of upstream DNA promoter elements and transcription factors which bind to them (Alam and Cook, 1990). Previously reporter gene constructs have been used to elucidate the role of HNF4α in the regulation of downstream genes in response to metabolic stimuli including hormones and metabolites, such as fatty acids and glucose (Gourdon et al., 1999; De Fabiani et al., 2000; Massillon et al., 2003; Roth et al., 2004; Adamson et al., 2006; Song and Chiang, 2006; Hamiiman et al., 2006).

The regulation of HNF4α transcriptional activity by metabolic signals is likely to be modulated by the promoter context and the other transcription factors and co-factors. The L-PK, PEPCK and SREBP-1c genes are important examples of oppositely-regulated HNF4α target genes in that each is a key regulatory gene in controlling the rates of glycolysis, gluconeogenesis and lipogenesis in the liver (Section 1.3). L-PK and SREBP-1c are induced in response to the feeding signals of insulin and glucose, whilst PEPCK gene expression is activated by the fasting signals of glucagon and glucocorticoids (Section 1.9) (Girard et al., 1997; Hanson and Reshef, 1997; Kim et al., 1998). Therefore reporter genes containing cognate HNF4α binding sites within proximal promoter elements from the L-PK, PEPCK and SREBP-1c genes were utilised in this study to investigate the influence of hormones and metabolites on HNF4α-dependent transcriptional regulation. The influence of hormones and metabolites on HNF4α transcriptional activity should be reflected by increases or decreases in the level of luciferase reporter gene expression (Alam and Cook, 1990).

Aim:
- To investigate the effects of feeding and fasting stimuli on HNF4α transcriptional activity in activating or inhibiting expression of reporter constructs of HNF4α target genes involved in the pathways of glycolysis (L-PK), gluconeogenesis (PEPCK) and lipogenesis (SREBP-1c).
Objective 1: To investigate the effects of glucose concentration and hormones (insulin, triiodo-L-thyronine and dexamethasone) on L-PK reporter gene expression in HepG2 cells.

Objective 2: To study the effect of metabolic stimuli (glucose concentrations, insulin, glucagon and dexamethasone) on HNF4α activity at the L-PK promoter using different reporter gene constructs together with HNF4α overexpression.

Objective 3: To study the regulation of PEPCK reporter gene expression by HNF4α, the effects of key hormones (insulin, dexamethasone) and the nuclear receptor co-activator PGC-1α.

Objective 4: To study the regulation of SREBP-1c reporter gene expression by HNF4α and the influence of metabolic stimuli (insulin and glucose concentration) and HNF4α co-activators (PGC-1α and p300).

4.2 Regulation of L-PK reporter gene expression by glucose concentration and hormones in HepG2 cells

4.2.1 Rationale

L-PK is a key regulatory gene in the glycolytic pathway, which is induced by the feeding stimuli of dietary carbohydrate and insulin, and repressed during fasting by glucagon and its downstream effector cAMP (Vaulont et al., 1986; Decaux et al., 1989). The -183/+11 proximal promoter region of the L-PK gene, which was previously found to confer responsiveness to dietary and hormonal factors in transgenic mice, consists of four transcription factor binding sites, named L1 – L4 (in order of proximity to the L-PK transcriptional start site) (Vaulont et al., 1989). The L1 site binds hepatocyte nuclear factor 1 (HNF1) and I.2 binds the ubiquitous nuclear factor 1 (NF1). HNF4α binds to the L3 site in the L-PK promoter which is contiguous to the ChREBP-binding site (L4) (Vaulont et al., 1989; Diaz Guerra et al., 1993; Yamashita et al., 2001).

The initial objective in using L-PK reporter constructs to study HNF4α-mediated gene expression was to reproduce the effect of induction by high glucose concentrations, as demonstrated by the original investigators in primary rat hepatocytes (Gourdon et al., 1999),
in the human hepatoma cell line HepG2, and confirm that the promoter functioned physiologically in response to high glucose concentration (25 mM). In addition to studying the effect of glucose concentration on L-PK reporter gene expression, the influence of hormones previously suggested to be important in permitting glucose-induced gene expression, namely insulin or the combination of insulin, triiodo-L-thyronine and the synthetic glucocorticoid, dexamethasone, was also investigated (Munnich et al., 1984; Doiron et al., 1994).

Transfections were performed in HepG2 cells with the L-PK reporter constructs (L4L3)4-54PK, which contains four oligomeric repeats of adjacent ChREBP- and HNF4α-binding sites and shows maximal induction by glucose, and (L3)3-54PK, which contains three oligomeric repeats of the HNF4α-binding site L3 ((Gourdon et al., 1999), reproduced in Materials and Methods, Figure 2.7).

4.2.2 Results

The (L4L3)4-54PK reporter construct was induced more than five-fold by an increase from 5.5 mM to 25 mM glucose under all conditions used (Figure 4.1). The expression of (L4L3)4-54PK reporter gene did not differ between 25 mM glucose medium and medium containing 25 mM glucose and 100 nM insulin. The level of reporter gene expression after incubation with 25 mM glucose HSM was lower than that following incubation with 25 mM glucose medium with the addition of only insulin. Thus, insulin, triiodo-L-thyronine and dexamethasone do not potentiate the induction of (L4L3)4-54PK reporter gene by 25 mM glucose. The presence or absence of added hormones did not modify the expression of the (L4L3)4-54PK reporter plasmid under conditions of low glucose concentration (5.5 mM).
**Figure 4.1: Effect of glucose concentration and hormones on L-PK reporter gene expression in HepG2 cells.** HepG2 cells were transfected with the luciferase reporter genes (L4L3)4-54PK and (L3)3-54PK along with pSEAPI-3'rep under control of the CMV promoter as a control for transfection efficiency. 16 h post-transfection, the media was changed and the cells incubated in media containing either 5.5 mM or 25 mM glucose ± 100 nM insulin, or hormone-supplemented medium (HSM) containing 100 nM insulin, 1 μM triiodo-L-thyronine and 1 μM dexamethasone for 24 hours. Luciferase readings were corrected for transfection efficiency based on SEAP expression levels. Values are shown as mean ± SEM (n = 4). Reporter gene expression between incubation conditions and reporter plasmids was compared by two-way ANOVA, followed by Bonferroni post-hoc tests. Results of selected post-hoc tests (where only one factor changed between the two groups analysed) are displayed: * p < 0.05; **/*** p < 0.001. A full summary of the post-hoc analysis is shown in Appendix Section 8.2.

The reporter expression of the (L3)3-54PK reporter plasmid was significantly lower than that of the (L4L3)4-54PK plasmid under all conditions containing 25 mM glucose, whilst the level of expression between the two reporter plasmids did not differ under 5.5 mM conditions (Figure 4.1). The expression of the (L3)3-54PK reporter gene was not inducible by high glucose concentration and was not altered by incubation with insulin or a combination of insulin, dexamethasone and triiodo-L-thyronine (HSM) under conditions of either low (5.5 mM) or high (25 mM) glucose concentration.
4.3 Regulation of L-PK reporter gene expression by HNF4α and metabolic stimuli

4.3.1 Rationale

The role of HNF4α in the modulation of L-PK reporter gene activity by hormones and metabolites was investigated using the (L4L3)4-54PK and (L3)3-54PK reporter plasmids in combination with overexpression of HNF4α.

4.3.2 Results

The role of HNF4α in the response of L-PK reporter genes containing both ChREBP and HNF4α binding sites ((L4L3)4-54PK) or only HNF4α binding sites ((L3)3-54PK) to 25 mM glucose and insulin was further investigated by human HNF4α2 overexpression (Figure 4.2). Incubation with insulin or 25 mM glucose and insulin resulted in higher reporter gene expression compared to standard medium (5.5 mM glucose). Incubation with 25 mM glucose medium without insulin did produce an induction of (L4L3)4-54PK reporter gene expression over expression levels at 5.5 mM glucose. Increased glucose concentration had an inhibitory effect on the expression of the (L3)3-54PK reporter plasmid when cells were incubated in the presence of insulin, while different glucose concentrations did not modulate expression of the reporter gene in the absence of insulin.

The effects of fasting hormones (glucagon and dexamethasone) compared to the feeding hormone (insulin) on the activity of HNF4α were investigated by transfecting HepG2 cells with the (L3)3-54PK reporter plasmid in the presence or absence of a human HNF4α2 expression vector (Figure 4.3). The reporter gene expression of the (L3)3-54PK plasmid was increased over 35-fold by co-transfection with a human HNF4α2 expression vector under all incubation conditions (Figure 4.3A). In the case of HNF4α2 overexpression, incubation of HepG2 cells with dexamethasone or glucagon, alone or in combination, did not alter (L3)3-54PK reporter gene expression, whilst incubation with insulin produced a significant 38% decrease in reporter gene expression relative to the control medium (Figure 4.3A). The combination of glucagon and insulin in the medium produced a significant 59% decrease relative to the control medium (Figure 4.3A). In the absence of the human HNF4α2 expression vector, no significant differences between the incubation conditions were observed (Figure 4.3B).
Figure 4.2: Effect of human HNF4α2 overexpression on the regulation on (L4L3)4-54PK and (L3)3-54PK reporter gene expression by glucose concentration and insulin. HepG2 cells were transfected with the luciferase reporter plasmids (L4L3)4-54PK or (L3)3-54PK and an expression vector for human HNF4α2 (3.3 ng/well). pSEAPI-3’rep was co-transfected as a control for transfection efficiency. 16 hours post-transfection, the media was changed and the cells incubated in media containing 5.5 mM or 25 mM glucose ± 100 nM insulin. Luciferase readings were corrected for transfection efficiency based on SEAP expression levels. Values are shown as mean ± SEM (n = 4). The effects of incubation condition and reporter plasmid were analysed by two-way ANOVA with Bonferroni post-hoc tests: * p < 0.05.
Figure 4.3: Effect of hormones on L-PK reporter plasmid (L3)3-54PK ± human HNF4α2 overexpression. HepG2 cells were transfected with the luciferase reporter gene (L3)3-54PK ± human HNF4α2 vector (3.3 ng/well). pSEAPI-3’rep was co-transfected as a control for transfection efficiency. 16 hours post-transfection, the media was changed and the cells incubated in 5.5 mM glucose media containing 30 nM glucagon, 1 μM dexamethasone or 100 nM insulin alone or in combination. Values are shown as mean ± SEM (n = 4) relative to the control incubation condition + human HNF4α2 overexpression. The effects of HNF4α overexpression and incubation condition were analysed by two-way ANOVA with Bonferroni post-hoc tests: * p <0.05, ** p <0.01, *** p <0.001.

Inset (B): (L3)3-54PK – HNF4α overexpression.
4.4 Regulation of PEPCK reporter gene expression by HNF4α, PGC-1α and hormones

4.4.1 Rationale

HepG2 cells were transfected with a luciferase reporter gene containing the PEPCK proximal promoter region (-467/+65 relative to the transcription start site), which includes the proximal GRU and cAMP regulatory element (CRE) (Quinn et al., 1988). The PEPCK reporter gene was transfected with an expression vector coding for rat HNF4α1, in order to confirm activation of PEPCK reporter expression by HNF4α and assess the contribution of HNF4α to the regulation of PEPCK gene expression by fasting and feeding hormones (dexamethasone and insulin respectively). In order to investigate the role of PGC-1α in co-activation of HNF4α, the effect of co-transfection with PGC-1α and HNF4α expression vectors on PEPCK reporter plasmid expression was also investigated.

The HNF4α expression vector was changed from one coding for human HNF4α2 (pcDNA3.HA.hHNF4α2), used in experiments with L-PK reporter plasmids (Section 4.3), to a rat HNF4α expression vector (PCR3.HNF4), as it was noted that the latter activated SREBP-1c reporter gene expression more effectively than the human HNF4α2 vector (data not shown).

4.4.2 Results

Overexpression of rat HNF4α1 produced a 50-fold increase in PEPCK reporter gene expression over basal levels, confirming HNF4α as a positive regulator of PEPCK expression (Figure 4.4). Incubation of the transfected cells with 1 µM insulin for 24 hours did not alter the level of HNF4α-stimulated PEPCK reporter gene expression, whilst incubation with 1 µM dexamethasone produced a 40% decrease in expression. Co-transfection with the co-activator PGC-1α halved the HNF4α-stimulated induction of PEPCK reporter gene expression, suggesting that PGC-1α represses HNF4α under control incubation conditions (i.e. the absence of external metabolic stimuli).
4.5 Regulation of SREBP-1c reporter gene expression by HNF4α, metabolic stimuli and co-activators

4.5.1 Rationale

The regulation of SREBP-1c gene expression by HNF4α was studied in HepG2 cells using a reporter plasmid containing the human SREBP-1c proximal promoter region (-780/+62 relative to the transcription start site). This region contains a putative binding site for HNF4α (Tarling et al., 2004). HNF4α overexpression was utilised to confirm HNF4α as a positive regulator of SREBP-1c expression and to assess the factors important in HNF4α-dependent activation of SREBP-1c expression. As feeding stimuli are important inducers of SREBP-1c expression in vivo (Kim et al., 1998), the influence of insulin and glucose concentration in the cell culture medium were investigated. The interaction of HNF4α with co-factors in
transcriptional regulation of SREBP-1c expression was studied by co-expression of PGC-1α or p300.

4.5.2 Results

4.5.2.1 Regulation of SREBP-1c reporter gene expression by HNF4α, insulin, glucose and PGC-1α

The modulation of SREBP-1c reporter gene expression by HNF4α, insulin, glucose and PGC-1α was studied in HepG2 cells (Figure 4.5). Two different expression vectors were used for HNF4α overexpression: one expressing the rat HNF4α1 coding sequence (Figure 4.5A) and another expressing the human HNF4α1 coding sequence (Figure 4.5B), enabling the comparison of species differences between rat and human HNF4α in human hepatoma cells.

Basal SREBP-1c reporter gene expression in the absence of exogenously expressed HNF4α was relatively low and a stimulatory effect of insulin could not be detected. Overexpression of rat HNF4α1 resulted in a mean 42-fold increase in reporter gene expression (Figure 4.5A) and overexpression of human HNF4α1 led to a mean 18-fold increase in reporter gene expression (Figure 4.5B). Incubation with 1 μM insulin increased the level of HNF4α-driven reporter gene expression a further 41% in the case of the rat HNF4α1 vector and 76% in the case of the human HNF4α1 vector under 5.5 mM glucose conditions (Figure 4.5A and B respectively). Incubation under high glucose (25 mM) conditions did not alter the level of HNF4α-stimulated reporter gene expression relative to standard glucose conditions (5.5 mM). The induction of HNF4α-driven reporter gene expression by insulin was similar under 25 mM glucose conditions as under standard glucose levels (5.5 mM), with a 60% increase observed for both rat and human HNF4α vectors (Figure 4.5A and B).

Overexpression of both HNF4α and PGC-1α produced a decrease in SREBP-1c reporter gene expression compared to HNF4α alone, with a significant reduction of 60% being observed for the rat HNF4α1 vector and a non-specific decrease of 44% for the human HNF4α1 vector (Figure 4.5A and B).
Figure 4.5: Regulation of SREBP-1c reporter gene expression by (A) rat or (B) human HNF4α1, insulin, glucose concentration and PGC-1α. HepG2 cells were transfected with a luciferase reporter gene containing the SREBP-1c proximal promoter (-780/+62), pSEAPI-3’rep as an internal transfection control, together with expression vectors for (A) rat or (B) human HNF4α1 (25 ng/well), PGC-1α (25 ng/well) or control pSG5 as indicated. After transfection, cells were incubated with STM (5.5 mM glucose) with the addition of 1 μM insulin and/or 25 mM glucose as indicated, for a further 24 hours. Corrected luciferase levels from a minimum of three independent experiments, performed in quadruplicate, were normalised to expression in the condition: + (rat or human) HNF4α overexpression and expressed as mean values ± SEM. Differences between conditions were analysed by one-way ANOVA with Tukey’s post-hoc tests. Results of selected post-hoc tests (where only 1 factor changed between the two groups analysed) are displayed: * p < 0.05; *** p < 0.001. The complete results of the post-hoc tests are shown in Appendix Section 8.2.
SREBP-1c reporter expression following incubation with insulin-containing medium was also suppressed by co-transfection of PGC-1α, with a reduction of 63% relative to HNF4α overexpression alone for both rat and human HNF4α vectors (Figure 4.5A and B).

4.5.2.2 Effect of HNF4α levels on SREBP-1c reporter gene expression

In order to confirm that HNF4α positively regulates SREBP-1c reporter gene expression in a concentration-dependent manner, HepG2 cells were transfected with the SREBP-1c reporter gene and a range of HNF4α vector concentrations (Figure 4.6). The concentration of rat HNF4α1 expression vector showed a good correlation with the level of SREBP-1c reporter

![Figure 4.6: Concentration-dependent regulation of SREBP-1c reporter gene expression by HNF4α.](image)

HepG2 cells were transfected with a SREBP-1c promoter (-780/+62) reporter gene, pSEAPI-3'rep as an internal transfection control, together with variable amounts of rat or human HNF4α1 vector as indicated. After transfection, cells were incubated with the standard transfection medium containing 1 μM insulin. Corrected luciferase readings are expressed relative to the condition: +25 ng/well rat HNF4α1 vector and as mean values ± SEM (n = 4). Correlation analysis is indicated by dashed lines and the Pearson correlation coefficient ($R^2$) and associated significance levels ($p$) are indicated.
gene expression within the range 0–100 ng/well expression vector ($R^2 = 0.98, p < 0.01$) (Figure 4.6). The concentration of the human HNF4α expression vector also positively correlated with the expression of the SREBP-1c reporter gene in the range 0–50 ng/well expression vector ($R^2 = 0.83, p < 0.01$), however reporter gene expression appeared to approach a plateau at a concentration of greater than 50 ng/well.

### 4.5.2.3 Effect of HNF4α co-activators PGC-1α and p300 on SREBP-1c reporter gene expression

In initial experiments investigating HNF4α-dependent regulation of SREBP-1c reporter plasmid expression (Section 4.5.2.1), PGC-1α overexpression was shown to reduce SREBP-1c reporter gene transcription when co-transfected with an expression plasmid for rat HNF4α1 under both standard incubation conditions and in the presence of 1 μM insulin (Figure 4.5A) and with an expression plasmid for human HNF4α1 under in the presence of 1 μM insulin (Figure 4.5B). To investigate whether this effect occurred in a concentration-dependent manner, SREBP-1c reporter expression was studied over a range of PGC-1α concentrations with a constant amount of either rat or human HNF4α1 expression vector (Figure 4.7A and B). Incubation conditions containing 1 μM insulin were chosen as these produced maximum HNF4α-activated reporter gene expression in initial experiments (Figure 4.5).

SREBP-1c reporter gene expression showed inverse correlation with increasing levels of PGC-1α. When rat HNF4α1 was overexpressed, a dose-dependent decrease was observed in the range 1–30 ng/well PGC-1α expression vector, however this fell short of statistical significance ($R^2 = 0.24, p = 0.05$) (Figure 4.7A). When human HNF4α1 was overexpressed, SREBP-1c reporter gene expression showed inverse correlation with PGC-1α expression vector levels in the range 10–100 ng/well expression vector (Figure 4.7B).

The effect of p300 on HNF4α-activated SREBP-1c reporter gene expression was tested over a range of p300 vector concentrations (Figure 4.8). When rat HNF4α1 was overexpressed, p300 overexpression produced inverse correlation with SREBP-1c reporter gene levels across the tested range (0–30 ng/well expression vector). In the case of human HNF4α1 overexpression, an inverse correlation of SREBP-1c reporter gene levels and p300 expression...
vector concentration was observed between 0 and 20 ng/well, with reporter gene expression approaching a plateau level at 30 ng/well expression vector.

Figure 4.7: Effect of PGC-1α concentration on HNF4α-stimulated SREBP-1c reporter gene expression. HepG2 cells were transfected with a SREBP-1c reporter plasmid and (A) rat or (B) human HNF4α1 expression vectors (25 ng/well) and a range of concentrations of PGC-1α expression vector as indicated. After transfection, cells were incubated in STM containing 1 μM insulin for 24 hours. Corrected luciferase readings are expressed relative to the condition: 0 ng/well PGC1-α for each HNF4α expression vector. Mean values ± SEM (n = 4) are displayed alongside correlation analyses (dashed lines) with the Pearson correlation coefficient (R²) and associated significance levels (p).
Figure 4.8: Effect of p300 concentration on HNF4α-stimulated SREBP-1c reporter gene expression. HepG2 cells were transfected with a SREBP-1c reporter gene and (A) rat or (B) human HNF4α1 expression vectors (25 ng/well) and a range of concentrations of p300 expression vector as indicated. After transfection, cells were incubated in STM containing 1 μM insulin for 24 hours. Corrected luciferase readings are expressed relative to the condition: 0 ng/well p300 for each HNF4α expression vector. Mean values ± SEM (n = 4) are displayed alongside correlation analyses (dashed lines) with the Pearson correlation coefficient ($R^2$) and associated significance levels ($p$).
4.5.2.4 Effect of insulin concentration on SREBP-1c reporter gene expression

In order to investigate the effect of insulin concentration on SREBP-1c reporter expression and the role of HNF4α in the effect of insulin, HepG2 cells were transfected with the SREBP-1c reporter plasmid with control, rat HNF4α1 or human HNF4α1 expression vectors (Figure 4.9A, B and C respectively), and then incubated with a range of insulin concentrations for 24 hours.

The insulin concentration of FBS measured using an ELISA assay was found to be 9.7 pM. Therefore, the insulin concentration of standard transfection medium (STM) due to the presence of 3% FBS was calculated to be 0.3 pM, corresponding to the lowest point of the range displayed as log[Insulin(M)] = -12.5 (Figure 4.9). It was important to establish the insulin concentration of FBS as insulin has been shown to have effects on gene expression at the levels found in FBS (Hasty et al., 2000).

SREBP-1c reporter gene expression in the absence of HNF4α overexpression (Figure 4.9A) resulted in approximately 100-fold lower reporter gene expression relative to the level of reporter gene expression in the presence of rat HNF4α1 overexpression (Figure 4.9B), therefore the inset to Figure 4.9A shows SREBP-1c reporter gene expression on a larger scale (0-2.2%, as opposed to 0-220%). In the absence of HNF4α overexpression, no correlation of SREBP-1c reporter gene expression with insulin concentration in the medium was observed (Figure 4.9A inset).

SREBP-1c reporter gene expression showed a positive correlation with insulin concentration in the presence of both rat and human HNF4α1 overexpression, with greatest correlation between 0.3 pM and 100 nM insulin (Figure 4.9B and C). Maximum SREBP-1c reporter gene expression was observed at 100 nM insulin, with 20-40% higher expression observed compared to 1 μM insulin, the concentration tested in initial experiments with the SREBP-1c reporter plasmid (Figure 4.5).
Figure 4.9: Insulin-dependence of SREBP-1c reporter gene expression in the presence of (A) control (B) rat or (C) human HNF4α1 expression vectors. HepG2 cells were transfected with a SREBP-1c promoter reporter plasmid with the indicated control (pSG5) (A) or HNF4α expression vectors as previously (B,C) (Figure 4.5). After transfection, cells were incubated in STM containing a range of insulin concentrations. Corrected luciferase readings are expressed relative to the condition: + rat HNF4α1 (STM without added insulin). Mean values are plotted ± SEM (n = 4). Correlation analyses are indicated by dashed lines with the Pearson correlation coefficient ($R^2$) and associated significance levels ($p$).
4.6 Discussion

In this chapter, the overall aim of studying the impact of fasting and feeding stimuli on HNF4α activity was investigated using reporter gene constructs containing sequences from the L-PK, PEPCK and SREBP-1c, representing key genes in the metabolic control of glycolysis, gluconeogenesis and lipogenesis.

In respect to the initial objective of comparing the effects of high glucose, insulin and other hormones on L-PK reporter gene expression in HepG2 cells with results in primary hepatocytes, it was found that the expression of an L-PK reporter gene construct containing both ChREBP (L4) and HNF4α (L3) binding sites was inducible by high glucose concentration (Figure 4.1), in agreement with the behaviour of the reporter construct in primary hepatocytes (Gourdon et al., 1999). The level of induction differed between HepG2 cells and primary rat hepatocytes, with 25 mM glucose inducing a five-fold increase in (L4L3)4-54PK reporter gene expression in HepG2 cells (Figure 4.1) compared to a 51-fold increase in primary rat hepatocytes (Gourdon et al., 1999). The level of induction by high glucose in HepG2 cells could be lower due to a number of reasons. Firstly, species differences could mean that the rat L-PK promoter is less responsive to glucose in human liver cells than in rat hepatocytes. Secondly, hepatoma cell lines are likely to be less sensitive to glucose levels than primary hepatocytes (Meienhofer et al., 1987). The (L3)3-54PK reporter plasmid containing only HNF4α binding sites (L3) and the 54 bp proximal promoter region, was not inducible by 25 mM glucose-containing medium (Figure 4.1), which is also in line with previous results (Gourdon et al., 1999).

The (L4L3)4-54PK reporter plasmid was induced by 25 mM glucose in the absence of added hormones (Figure 4.1), suggesting that the concentration of insulin, triiodo-L-thyronine and glucocorticoid hormones in the serum component of the medium (3%) is sufficient. Alternatively, the three hormones are not required for the observed effect of high glucose in HepG2 cells. The combination of insulin, triiodo-L-thyronine and glucocorticoids was shown to be required for the induction of L-PK in the refed state in vivo (Munnich et al., 1984). Ex vivo studies of (L4L3)4-54PK reporter gene expression in primary rat hepatocytes were also conducted in the presence of 100 nM insulin, 1 μM triiodo-L-thyronine and 1 μM dexamethasone (Gourdon et al., 1999).
HNF4α positively regulated the expression of the (L3)3-54PK reporter gene in HepG2 cells, as demonstrated by the substantial increases in reporter gene expression activity observed upon HNF4α overexpression (Figure 4.2). HNF4α overexpression also increased the expression of the (L3)3-54PK reporter gene in primary rat hepatocytes (Gourdon et al., 1999), although the fold-change (less than two-fold) was not as marked as that in HepG2 cells (over 35-fold), suggesting lower endogenous HNF4α activity in HepG2 cells compared to primary liver cells.

Insulin did not have a consistent effect on the expression of the (L3)3-54PK reporter gene in presence of HNF4α overexpression: a reduction of (L3)3-54PK reporter gene expression was observed in one experiment (Figure 4.3), whilst insulin had no effect in a subsequent experiment (Figure 4.2). On the other hand, insulin increased the expression of the (L4L3)4-54PK reporter gene under conditions of both standard and high glucose concentrations (Figure 4.2). These results suggest that insulin activates HNF4α via its interaction with ChREBP at the L-PK promoter; indeed, the functional cooperation and contiguity of the L4 (ChREBP) and L3 (HNF4α) elements has been found to be important in mediating the transcriptional response of the L-PK gene to feeding stimuli (Bergot et al., 1992; Diaz Guerra et al., 1993; Liu and Towle, 1995).

High glucose did not stimulate the expression of the (L4L3)3-54PK reporter gene when HNF4α was overexpressed (Figure 4.2), in contrast to results in the absence of HNF4α overexpression (Figure 4.1). Possibly, ChREBP levels become limiting in the presence of HNF4α overexpression and prevent significant induction of the (L4L3)4-54PK reporter plasmid by high glucose concentration.

Incubation of HepG2 cells with glucagon, or glucagon and dexamethasone, did not change the expression of the (L3)3-54PK reporter gene compared to control conditions (Figure 4.3). In contrast, the activity of (L3)3-54PK reporter was strongly repressed by cAMP, a second messenger of glucagon, in primary rat hepatocytes (Gourdon et al., 1999). HNF4α DNA-binding activity was previously shown to be reduced in nuclear extracts from the livers of fasted rats or following treatment of nuclear extracts with glucagon (Viollet et al., 1997), however the lack of inhibition of (L3)3-54PK reporter gene expression by glucagon in
HepG2 cells suggests that glucagon does not modulate HNF4α activity in a similar manner in this cell line.

Dexamethasone did not alter (L3)3-54PK reporter plasmid activity (Figure 4.2), suggesting that this fasting stimulus does not alter HNF4α activity at the L-PK promoter, and that an interaction between HNF4α and GR is not relevant to the modulation of HNF4α activity in the context of the L-PK promoter. This result is in keeping with published results showing that glucocorticoids exert their effects of L-PK mRNA expression at a post-transcriptional, rather than at a transcriptional level (Vaulont et al., 1986).

HNF4α overexpression stimulated PEPCK reporter gene expression, confirming HNF4α as a positive regulator of PEPCK reporter gene expression, in agreement with published work (Yamamoto et al., 2004). Incubation with 1 μM insulin did not alter the level of HNF4α-stimulated PEPCK reporter gene expression (Figure 4.4). In vivo, insulin inhibits both basal and cAMP and/or dexamethasone stimulated PEPCK expression (O'Brien and Granner, 1996), however the inhibitory effect of insulin was not apparent on basal PEPCK reporter gene expression performed in HepG2 or rat hepatoma H4IIE cells, with insulin exerting an inhibitory effect on cAMP- and dexamethasone-stimulated PEPCK reporter gene expression (Xing and Quinn, 1993; Ruckteschel et al., 2000). This result indicates that insulin does not activate or repress HNF4α when bound to the PEPCK promoter. This result also provides confirmation that the stimulatory effect of insulin on HNF4α-driven L-PK and SREBP-1c reporter gene expression is promoter-specific (Figures 4.3 and 4.5).

Incubation of HepG2 cells with dexamethasone reduced HNF4α-dependent PEPCK reporter gene levels by 40% (Figure 4.4). This is in contrast to the stimulatory action of dexamethasone on PEPCK mRNA expression in rat liver in vivo and on PEPCK reporter gene expression in H4IIE cells (Lamers et al., 1982; Xing and Quinn, 1993). However, dexamethasone has previously been shown to have a negative action on PEPCK reporter gene expression in HepG2 cells: When PEPCK reporter plasmid activity was stimulated by overexpression of PKA, incubation with dexamethasone brought about a 40% reduction in expression levels (Xing and Quinn, 1993). Furthermore, it was demonstrated that dexamethasone induces proteasomal degradation of the GR in HepG2 cells, resulting in decreased PEPCK mRNA expression (Sengupta and Wasylyk, 2001). In this manner, the
reduction in GR protein levels brought about by dexamethasone in HepG2 cells could limit
HNF4α-dependent PEPCK reporter expression, as HNF4α is known to interact
synergistically with the GR in activating PEPCK gene expression (Hall et al., 1995; Wang et
al., 1999; Stafford et al., 2001).

PGC-1α has previously been shown to stimulate hepatic gluconeogenic gene expression and
its interaction with HNF4α is essential in mediating this effect (Yoon et al., 2001; Rhee et
al., 2003). In light of this, the reduction in PEPCK reporter gene expression by co-
transfection of HNF4α and PGC-1α compared to HNF4α alone is surprising (Figure 4.4),
however it possible that additional stimuli such as glucocorticoids and/or glucagon are
required to mediate the synergistic interaction of HNF4α and PGC-1α. PGC-1α is known to
be activated through phosphorylation by p38 MAPK downstream of glucagon signalling
(Puigserver et al., 2001; Fan et al., 2004; Cao et al., 2005), therefore potentially the
phosphorylation state of PGC-1α under the basal incubation conditions does not permit its
co-activation of HNF4α (Figure 4.4).

Overexpression of either rat or human HNF4α1 increased SREBP-1c reporter gene
expression in HepG2 cells (Figure 4.5), in agreement with experiments employing the same
reporter gene and HNF4α overexpression in rat hepatoma McA-RH7777 cells (Tarling et al.,
2004). The fold-change upon HNF4α overexpression differed somewhat between the studies
in HepG2 and McA-RH7777 cells, with HNF4α1 overexpression producing 42- and 18-fold
increases in reporter gene levels over basal expression levels for the rat and human HNF4α1
expression vectors respectively (Figure 4.5A and B), whilst HNF4α1 and HNF4α2
overexpression resulted in 0.5- and 3-fold increases respectively in McA-RH7777 cells
(Tarling et al., 2004). Differences in the experimental parameters in terms of cell numbers or
transfection efficiency of the two cell lines could explain the low basal SREBP-1c reporter
gene expression in HepG2 cells. Alternatively, higher endogenous HNF4α activity in McA-
RH7777 cells could be responsible for the smaller fold-changes observed upon HNF4α
overexpression compared to HepG2 cells. The large increases in SREBP-1c reporter gene
expression upon HNF4α overexpression suggest that HNF4α activity could be one of the
factors limiting SREBP-1c expression in HepG2 cells.
Overexpression of rat HNF4α1 produced greater induction of SREBP-1c reporter gene expression over basal levels (42-fold) compared to human HNF4α1 (18-fold) (Figure 4.5A and B). The observed disparity could stem from differences in the efficiency of the promoters driving their expression, or alternatively the rat and human proteins could be metabolised differently within human HepG2 cells, for example, in their rates of degradation. The relationship between HNF4α expression vector concentration and SREBP-1c reporter gene levels also differs between the two HNF4α vectors, with SREBP-1c reporter gene expression approaching a plateau in the range 50-100 ng/well in the case of the human HNF4α expression vector, whilst rat HNF4α expression vector concentration positively correlated with SREBP-1c reporter gene expression throughout the tested range (0 – 100 ng/well) (Figure 4.6). These results also suggest that human HNF4α1 protein is metabolised differently to rat HNF4α1 in HepG2 cells.

The influences of insulin and glucose concentration on HNF4α-dependent activation of SREBP-1c reporter gene expression were also investigated. When HNF4α was overexpressed, SREBP-1c reporter gene expression was enhanced a further 40-80% in response to incubation with 1 μM insulin compared to STM (Figure 4.5). Insulin had a concentration-dependent effect on the SREBP-1c reporter gene, with maximal expression at 100 nM insulin (Figure 4.9). A decrease in SREBP-1c reporter gene expression was observed at 1 μM compared to 100 nM insulin, suggests a blunting of the cellular response to insulin at the higher concentration (Figure 4.9). This could be due to increased down-regulation of the insulin receptor and downstream pathways at the higher concentration (Frittitia et al., 2000).

Conditions of high glucose concentration (25 mM), on the other hand, did not significantly alter HNF4α-dependent SREBP-1c expression in the presence or absence of insulin (Figure 4.5). This agrees with other studies using a reporter gene approach that showed insulin stimulated SREBP-1c reporter gene expression, whilst high glucose had no effect (Deng et al., 2002; Dif et al., 2006). Overall, the induction of HNF4α-dependent SREBP-1c reporter gene expression by insulin suggests that insulin increases HNF4α transcriptional activity when bound to this promoter. Insulin could promote the formation of a transcriptionally active complex via changes in phosphorylation or nuclear abundance of HNF4α and other transcription factors involved (Mournier and Posner, 2006).
Other transcription factors which bind to downstream sites in the SREBP-1c promoter (Figure 4.10) have been shown to contribute to the mechanism by which insulin up-regulates SREBP-1c expression. Reporter gene constructs containing the -571/+90 and -257/+90 bp regions from the human SREBP-1c promoter are also stimulated in response to insulin in HEK-293 cells (Dif et al., 2006). The -571/+90 region of the human SREBP-1c promoter contains two liver X receptor elements (LXREs) and two downstream sterol response elements (SREs), which are one of the consensus binding motifs for SREBPs. Both LXR and SREBPs have been proposed to play a role in the induction of SREBP-1c expression by insulin. In hepatocytes, LXR was shown to play a central role in the induction of mouse SREBP-1c reporter gene expression by insulin (Chen et al., 2004), whilst the combinatorial action of SREBP, Sp1, nuclear factor Y (NF-Y) and LXR were found to contribute to the effect of insulin on the rat SREBP-1c reporter gene (Cagen et al., 2005). These findings suggest that interactions of HNF4α with one or more the aforementioned transcription factors could be important in mediating the stimulatory effect of insulin.

The presence of a putative HNF4α binding site in the human SREBP-1c promoter suggests that HNF4α could have a role in the tissue-specific expression of SREBP-1c in the liver and other organs which express HNF4α, such the pancreas and small intestine. Compared to adipose tissue and muscle, where SREBP-1c mRNA levels return to the levels of control animals upon refeeding after a period of fasting, SREBP-1c gene expression in liver is induced ~5-fold above control values upon refeeding (Gosmain et al., 2005). The induction of SREBP-1c in the liver in the absorptive state could be related to its exposure to higher levels of insulin than peripheral tissues (Frayn, 2003) and suggests that specific mechanisms in hepatocytes, which transmit signals downstream of insulin, facilitate the expression of

**Figure 4.10:** *In silico* analysis of the mouse and human SREBP-1c promoter regions. Mouse and human SREBP-1c proximal promoter regions were analysed *in silico* for transcription factor binding sites (Tarling et al., 2004).
SREBP-1c, enabling utilisation of dietary carbohydrate in the synthesis of fatty acids.

HNF4α could serve to amplify the stimulatory effects of insulin on SREBP, LXR or other transcription factors involved to induce SREBP-1c gene expression in the liver. Interestingly, a binding site for PDX-1 is present upstream of the HNF4α binding site within the human SREBP-1c promoter region (Figure 4.10). PDX-1 is a key transcription factor in the glucose-sensitive transcription of the insulin gene (Melloul et al., 1993), therefore the proximity of the PDX-1 and HNF4α binding sites suggests that their interaction could be important in the nutritional regulation of the SREBP-1c gene in the pancreas.

HNF4α-dependent transactivation of SREBP-1c reporter expression was repressed by PGC-1α, when co-transfected in both equal amounts as HNF4α and across a range of concentrations, up to a four-fold ratio of PGC-1α:HNF4α expression vector DNA (Figure 4.5 and Figure 4.7). This suggests that PGC-1α does not co-activate HNF4α in promoting SREBP-1c gene expression and potentially represses HNF4α activity or disrupts HNF4α interactions with other proteins in the SREBP-1c promoter. PGC-1α overexpression reduces the induction of SREBP-1c reporter gene expression by insulin from 41% to 29% in the case of rat HNF4α and from 76% to 14% in the case of human HNF4α overexpression (Figure 4.5A and B respectively). This suggests that repressive effect of PGC-1α on HNF4α activity is greater in the presence of insulin. As PGC-1α has been shown to be activated by phosphorylation downstream of glucagon and cAMP (Cao et al., 2005), insulin signalling could potentially reduce PGC-1α phosphorylation and activation via stimulation of cAMP phosphodiesterases (PDEs) which reduce intracellular cAMP levels (Houslay and Kilgour, 1990). Dephosphorylated PGC-1α shows enhanced interaction with co-repressors, such as p160MRB (Knutti et al., 2001; Fan et al., 2004), therefore PGC-1α could potentially recruit co-repressors to the SREBP-1c promoter under fed conditions corresponding to raised insulin concentration.

Alternatively, PGC-1α-mediated disruption of HNF4α-dependent SREBP-1c expression could act via the adjacent LXRE in the SREBP-1c promoter. PGC-1α has been shown to co-activate LXR:RXR heterodimers in the activation of human SREBP-1c reporter gene expression by oxysterols and 9-cis retinoic acid in HepG2 cells (Oberkofler et al., 2004). Possibly LXR activation by PGC-1α prevents HNF4α binding to its cognate site in the
SREBP-1c promoter, in a similar manner to the LXR-mediated displacement of HNF4α at the apoAI promoter in response to LXR ligands (Huuskonen et al., 2006).

Overexpression of the co-activator p300 also led to a dose-dependent decrease in HNF4α-dependent SREBP-1c expression in the presence of insulin (Figure 4.8), likewise suggesting that p300 could be disrupting the interaction(s) of HNF4α with other transcription factors or co-factors. p300 and closely related co-activator CBP have been shown to potentiate HNF4α transcriptional activity and promote nuclear retention by acetylation of HNF4α (Yoshida et al., 1997; Dell and Hadzopoulou-Cladaras, 1999; Soutoglou et al., 2000; Eeckhoute et al., 2001). However the magnitude of enhancement of HNF4α activity by co-expression of p300 was found to be promoter-specific using reporter constructs of different apolipoprotein gene promoters (Torres-Padilla and Weiss, 2003). p300 has been shown to be a substrate for protein kinase B (PKB/Akt) downstream of insulin and, whilst phosphorylation of p300 has been linked to insulin-stimulated expression of glucokinase by a complex including HNF4α and hypoxia inducible factor-1 (HIF-1), phosphorylation of p300 on Ser-1834 also disrupts its interaction with CAAT/enhancer-binding protein-α (C/EBPα) (Guo et al., 2001; Roth et al., 2004). The latter scenario could occur in the interaction of p300 with HNF4α in the context of the SREBP-1c promoter, as the effect of p300 co-transfection was not tested in the absence of insulin.

In summary, the combination of raised glucose concentration and insulin stimulated L-PK reporter gene expression for constructs containing both HNF4α and ChREBP binding sites, but not those containing only HNF4α binding sites. HNF4α overexpression increased the expression of L-PK reporter genes, with an additive effect of insulin for the construct containing HNF4α and ChREBP binding sites, whilst none of the metabolic stimuli tested modulated L-PK reporter gene expression for the construct containing only HNF4α-binding sites. HNF4α overexpression also stimulated PEPCK reporter gene expression, with incubation with dexamethasone or co-transfection of PGC-1α leading to reduced reporter gene levels. Insulin did not modulate PEPCK reporter gene expression. Lastly, HNF4α-activated SREBP-1c expression was stimulated by incubation of HepG2 cells with insulin, but not high glucose concentration. PGC-1α and p300 repressed SREBP-1c reporter gene
expression when co-transfected with HNF4α, indicating that these co-factors compete with other HNF4α partners when bound to the SREBP-1c promoter.
Chapter 5

Regulation of lipogenic and glycolytic mRNA expression in HepG2 cells by HNF4α and insulin
Chapter 5:

5.1 Introduction

The expression of SREBP-1c and L-PK reporter plasmids was found to be positively regulated by HNF4α overexpression and incubation of HepG2 cells with insulin (Chapter 4). In order to investigate whether HNF4α target genes in their endogenous chromatin-bound form were regulated in the same manner by HNF4α overexpression and insulin, the mRNA expression of SREBP-1c, L-PK and other target genes was studied in HepG2 cells using a quantitative real-time (Q-PCR) approach. Overexpression of HNF4α has previously been shown to increase the expression of a range of HNF4α target genes in HepG2 cells and to activate functions associated with the hepatic phenotype such as breakdown of ammonia (Naiki et al., 2004).

Hypothesis

HNF4α, in the presence of insulin, up-regulates lipid synthesis by regulating the expression of genes involved in glycolysis and lipogenesis.

Aims:

- To demonstrate, using real-time Q-PCR, that HepG2 cells increase the expression of SREBP-1c and SREBP-1c target genes (ACC, FAS) when transfected with a human HNF4α expression plasmid.
- To investigate up-regulation of other HNF4α target genes in the glycolytic (L-PK) and lipogenic (FAS) pathways by insulin.

Objective 1: To identify stable housekeeping genes in HepG2 cells under conditions of HNF4α overexpression and incubation with insulin, for the purpose of relative quantification of target gene expression.

Objective 2: To determine the relative mRNA expression levels of lipogenic (SREBP-1c, SREBP-2, ACC, FAS) and glycolytic (L-PK) genes in HepG2 cells following HNF4α overexpression and incubation with insulin-containing vs. control medium (STM).
Objective 3: To assess the time- and concentration-dependence of insulin action on SREBP-1c mRNA expression in HepG2 cells.

5.2 Identification of stable housekeeping genes in HepG2 cells

5.2.1 Rationale

The choice of housekeeping gene (also known as reference gene) used for relative quantification of gene expression is an important factor in the accurate quantification of target gene expression under different experimental conditions (Thellin et al., 1999). Therefore the most stably expressed housekeeping genes under the experimental conditions to be used in investigating the impact of HNF4α overexpression on SREBP-1c mRNA expression (Objective 2) were analysed using the GeNorm approach (Vandesompele et al., 2002) (see Section 2.2.8.8). The relative stability of six different housekeeping genes was analysed: 18S rRNA, β-2-microglobulin (B2M), cytochrome c-1 (CYC1), eukaryotic translation initiation factor 4A (EIF4A2), topoisomerase 1 (TOP1) and ubiquitin C (UBC).

Both rat and human HNF4α1 expression vectors were utilised previously in analysing the regulation of SREBP-1c reporter plasmid expression by HNF4α (Section 4.5). The human HNF4α1 expression vector was chosen for further experiments to eliminate any possible species-specific effects of rat HNF4α1 in a human cell line (discussed in Section 4.6). As only a single HNF4α expression vector was used for this and subsequent experiments, overexpression of human HNF4α1 will be referred to as HNF4α overexpression hereafter.

5.2.2 Results

The stability of expression of six housekeeping genes following transfection with human HNF4α1 or control expression vectors and incubation in the presence or absence of insulin was assessed using the GeNorm method (Figure 5.1). Two independent experiments were performed in order to confirm that the ranking of housekeeping gene stability did not differ significantly due to variation between biological repeats (Figure 5.1A and B). 18S and EIF4A2 were ranked as the least stable genes in both experiments; in comparison, B2M was ranked in the two most stably expressed genes in both analyses and was therefore chosen as the housekeeping gene for relative quantification of target gene expression.
Figure 5.1: GeNorm analysis of most stable housekeeping genes in HepG2 cells. HepG2 cells were transfected with a human HNF4α1 expression vector (125 ng/well) or control DNA (pcDNA3). 16 hours after transfection, cells were incubated with STM ± 1 μM insulin for a further 24 hours, after which RNA was isolated and cDNA prepared. The expression of 18S, B2M, CYC1, EIF4A2, TOP1 and UBC mRNA in samples from two independent experiments (A and B) was calculated using ΔCt values (Equation 2.3). The relative stability of the expression of the six housekeeping genes was analysed using the Genorm application for Microsoft Excel (Section 2.2.8.8).
5.3 Effect of HNF4α overexpression and insulin on the regulation of lipogenic and glycolytic gene expression in HepG2 cells

5.3.1 Rationale

The regulation of SREBP-1c mRNA expression by HNF4α and insulin in HepG2 cells was investigated by transfecting HepG2 cells with a HNF4α expression vector, followed by incubating the cells in the presence or absence of insulin for 24 hours. The incubation time of 24 hours was initially chosen because a 24 hour incubation with insulin-containing medium was previously shown to increase HNF4α-dependent SREBP-1c reporter gene expression (Section 4.5). Likewise, the amount of HNF4α expression vector DNA (125 ng/well) was based on the amount of expression vector DNA used in experiments with the SREBP-1c reporter plasmid (25 ng/well) (Section 4.4), scaled up to reflect the larger well size of a 24-well plate compared to a 96-well plate.

It was reasoned that if SREBP-1c expression were up-regulated following HNF4α overexpression, the expression of SREBP-1c target genes, ACC and FAS, would also increase (Oberkofler et al., 2004). The impact of HNF4α overexpression on the glycolytic gene (L-PK), also a target gene of HNF4α, was also assessed. The mRNA expression of HNF4α was measured in order assess efficiency of HNF4α overexpression. As two different genes for SREBP exist in the human genome (Yokoyama et al., 1993; Hua et al., 1993), SREBP-2 mRNA levels were also quantified in order to assess effects on SREBP-target genes not attributable to changes in SREBP-1c expression.

In order to investigate whether HNF4α overexpression led to changes in downstream target gene expression following incubation times of greater than 24 hours, timecourse experiments were also performed. It was reasoned that changes in HNF4α mRNA levels upon HNF4α overexpression may take effect at the protein level at a later time point than 24 hours (corresponding to 40 hours post-transfection). In turn, changes in SREBP-1c mRNA levels may also take a longer time period to be revealed at the level of SREBP-1c downstream genes (ACC and FAS).
5.3.2 Results

Figures 5.2 to 5.7 show the effects of HNF4α overexpression vs. control transfection on HNF4α and target gene expression prior to (0 h time point) and after incubation times of 24, 48, 72, 96 or 144 hours with (a) control medium (STM) or (b) STM containing 1 μM insulin. For the control incubation condition and for certain time points (72 and 144 hours) in the insulin-containing incubation condition, only a single biological replicate was performed. Statistical analysis by repeated measures ANOVA was performed with data from time points where a biological replicate was available.

HNF4α mRNA levels were significantly higher in HNF4α transfection condition compared to control vector (pcDNA3) after 24 and 48 hours incubation with insulin, while a difference in HNF4α expression levels between transfection conditions was not evident at the 0 h time point (Figure 5.2B). In the HNF4α transfection condition, HNF4α mRNA levels rose over 200% after 24 and 48 hours incubation compared to levels at the 0 h time point ($p < 0.01$), with a drop in expression levels evident after 72 and 96 hours incubation (Figure 5.2B). On the other hand, a non-significant 50% decrease in endogenous HNF4α mRNA levels (control transfection) was observed after 48 hours incubation with insulin-containing medium (Figure 5.2B). Under control incubation conditions ($n = 1$), HNF4α mRNA levels were also higher following transfection with the HNF4α expression vector compared to control vector at the 24 and 48 hours time points (Figure 5.2A).

After 24 hours incubation with insulin, SREBP-1c mRNA expression showed a modest increase (50%) under control transfection conditions (Figure 5.3B), although a 35% increase in SREBP-1c was also observed following 24 hours control incubation (Figure 5.3A). A 64% rise in SREBP-1c expression was observed after 96 hours incubation with insulin under conditions of HNF4α overexpression (Figure 5.3B). Maximal SREBP-1c expression was observed after 144 hours incubation with insulin, with rises of 290% and 100% observed for the control and HNF4α transfection conditions respectively (Figure 5.3B).
Figure 5.2: Effect on HNF4α overexpression on HNF4α mRNA expression in HepG2 cells under (A) control and (B) 1 μM insulin incubation conditions. HepG2 were transfected with a HNF4α expression vector (125 ng/well) or control DNA (pcDNA3.1). 16 hours after transfection (0 h time point), cells were incubated with STM ± 1 μM insulin for 24 - 144 hours as indicated, after which RNA was isolated and cDNA prepared. The expression of HNF4α was quantified by Q-PCR and normalised to the expression of a housekeeping gene, B2M, and expressed relative to mean expression level of the control transfection at the 0 h time point. Results are expressed as individual datapoints with 0 h time point data shown on both plots for purpose of comparison. The effects of HNF4α overexpression and incubation time on gene expression were analysed by repeated measures ANOVA where n > 1, with the results of Bonferroni post-hoc tests as indicated in the Key.
Figure 5.3: Effect on HNF4α overexpression on SREBP-1c mRNA expression in HepG2 cells under (A) control and (B) 1 μM insulin incubation conditions. Experiment and data analysis performed as detailed in Figure 5.2. Results are expressed as individual datapoints with 0 h time point data shown on both plots for purpose of comparison. The effects of HNF4α overexpression and incubation time on gene expression were analysed by repeated measures ANOVA where n > 1, with the results of Bonferroni post-hoc tests as indicated in the Key.
In the absence of insulin, little variation was apparent in ACC expression after different incubation time points or between transfection conditions (Figure 5.4A). In cells transfected with the control expression vector, ACC expression decreased with time following incubation with insulin-containing medium, with expression after 48 hours incubation 36% lower than at the 0 h time point \((p < 0.05)\), and after 96 hours incubation 48% lower than levels at the 0 h time point \((p < 0.05)\) (Figure 5.4B). For cells transfected with a HNF4α expression vector and incubated with insulin, ACC expression rose 93% between the 24 and 96 hour time points \((p < 0.01)\) and 87% between the 48 and 96 hours time points \((p < 0.01)\) (Figure 5.4B). However, post-hoc tests did not identify direct differences in the expression of ACC between the control and HNF4α transfection conditions at any time point (Figure 5.4B). For both transfection conditions, maximal ACC expression was observed after 144 hours incubation with insulin (Figure 5.4B).

FAS expression did not show significant alterations following different incubation times or between transfection conditions (Figure 5.5). For the control transfection, FAS expression after incubation with insulin remained within a similar range (65% - 103%) to that under control incubation conditions (89% - 119%) (Figure 5.5). In the case of HNF4α overexpression, FAS expression was within the range 53% - 128% following incubation with insulin and in the range 32% - 106% under control incubation conditions (Figure 5.5).

Although statistical comparison was not possible due to data only being available from single experiments, L-PK showed a drop in expression at the 24 hour time point, with a greater than 70% decrease observed for the HNF4α overexpression condition under both the control and insulin incubation conditions, and for the control transfection followed by incubation with insulin (Figure 5.6). L-PK levels after 48 hours incubation with insulin were also 70% lower than those prior to the incubation (0 h time point) for both control and HNF4α transfection conditions (Figure 5.6B).

SREBP-2 expression did not appear to vary considerably with incubation time or between transfection conditions, although statistical comparison was again not possible due data only being available from a single experiment (Figure 5.7).
Figure 5.4: Effect on HNF4α overexpression on ACC mRNA expression in HepG2 cells under (A) control and (B) 1 μM insulin incubation conditions. Experiment and data analysis performed as detailed in Figure 5.2. Results are expressed as individual datapoints with 0 h time point data shown on both plots for purpose of comparison. The effects of HNF4α overexpression and incubation time on gene expression were analysed by repeated measures ANOVA where n > 1, with the results of Bonferroni post-hoc tests as indicated in the Key.
Figure 5.5: Effect on HNF4\(\alpha\) overexpression on FAS mRNA expression in HepG2 cells under (A) control and (B) 1 \(\mu\)M insulin incubation conditions. Experiment and data analysis performed as detailed in Figure 5.2. Results are expressed as individual datapoints with 0 h time point data shown on both plots for purpose of comparison. The effects of HNF4\(\alpha\) overexpression and incubation time on gene expression were analysed by repeated measures ANOVA where \(n > 1\), with no significant differences observed between conditions.
Figure 5.6: Effect on HNF4α overexpression on L-PK mRNA expression in HepG2 cells under (A) control and (B) 1 μM insulin incubation conditions. Experiment and data analysis performed as detailed in Figure 5.2. Results are expressed as individual datapoints with 0 h time point data shown on both plots for purpose of comparison. The effects of HNF4α overexpression and incubation time on gene expression were analysed by repeated measures ANOVA where n > 1, with no significant differences observed between conditions.
Figure 5.7: Effect on HNF4α overexpression on SREBP-2 mRNA expression in HepG2 cells under (A) control and (B) 1 μM insulin incubation conditions. Experiment and data analysis performed as detailed in Figure 5.2. Results are expressed as individual datapoints with 0 h time point data shown on both plots for purpose of comparison. The effects of HNF4α overexpression and incubation time on gene expression were analysed by repeated measures ANOVA where n > 1, with no significant differences observed between conditions.
5.4 Time- and concentration- dependence of insulin action on SREBP-1c mRNA expression in HepG2 cells

5.4.1 Rationale
The possibility that short-term induction of SREBP-1c by insulin occurred over a shorter timescale than incubation periods of 24 hours or greater (Figures 5.2 to 5.7) was investigated by comparing SREBP-1c mRNA levels after 6 hours vs. 24 hours incubation with insulin-containing medium. In order to ascertain whether endogenous SREBP-1c mRNA expression in HepG2 cells was responsive to insulin concentration in the cell culture medium, different concentrations of insulin were compared.

Previously, a decrease in endogenous HNF4α mRNA expression was observed after 24 and 48 hours incubation with insulin-containing medium (Figure 5.2A(ii)), therefore the time- and concentration-dependence of this effect was followed up by measuring HNF4α mRNA expression after 6 and 24 hours across a range of insulin concentrations.

It was also considered that hormones present in the 3% serum component of STM used in the previous experiment (Figures 5.2 to 5.7) could affect endogenous SREBP-1c expression in HepG2 cells. It was noted that HepG2 cells were serum-starved in published studies investigating insulin effects on gene expression in this cell line (Xu et al., 1999; Hafner et al., 2006; Infantino et al., 2007). Therefore, a 24 hour serum starvation period was introduced prior to incubation with insulin, and serum-free medium (SFM) was used as the control medium (see Experimental outline, Figure 2.12).

5.4.2 Results
The mRNA expression of HNF4α and SREBP-1c in HepG2 cells was measured following 6 or 24 hours incubation with SFM medium containing 0, 1, 10 or 100 nM insulin (Figure 5.8). Based on linear regression analysis, HNF4α mRNA expression differed between the two incubation periods ($p < 0.05$), however a concentration-dependent effect of insulin on HNF4α mRNA expression was not evident after either 6 or 24 hours incubation (Figure 5.8A). SREBP-1c expression did not correlate with insulin concentration within the range 0 to 100 nM after either 6 or 24 hours incubation, and no significant differences between the two incubation periods was noted (Figure 5.8B).
Figure 5.8: Insulin-dependence of (A) HNF4α and (B) SREBP-1c mRNA expression in HepG2 cells after 6 and 24 hours incubation. HepG2 cells were seeded in 24-well plates and incubated in standard cell medium (SCM) for 40 hours, serum-starved for 24 hours and incubated with SFM containing a range of insulin concentration for 6 (Δ) or 24 (▲) hours as indicated. Following RNA isolation and cDNA synthesis, the expression of (A) HNF4α and (B) SREBP-1c was quantified by Q-PCR and normalised to the expression of a housekeeping gene, B2M, and expressed relative to expression level of the control incubation (0 nM insulin) at the 6 h time point. Results are expressed as individual datapoints. The effect of insulin concentration on HNF4α or SREBP-1c expression were analysed by linear regression analysis, with R² and p-values for each incubation time as indicated. The slopes and intercepts of the analyses for the different incubation times was compared (double-headed arrow). (N.S., not significant).
5.5 Discussion

Previously, SREBP-1c reporter gene expression in HepG2 cells was shown to be positively regulated by HNF4α and insulin (Section 4.5), therefore the regulation of endogenous SREBP-1c mRNA expression in HepG2 cells by HNF4α and insulin was investigated. Insulin did not positively regulate SREBP-1c mRNA levels in HepG2 cells. Although SREBP-1c mRNA expression increased by 50% following 24 hours incubation with insulin (Figure 5.3B, pcDNA3 transfection), a 35% increase in SREBP-1c expression was also observed after the first 24 hours of the control incubation (Figure 5.3A, pcDNA3 transfection), suggesting that this rise in SREBP-1c expression was not insulin-dependent. In a subsequent experiment (Figure 5.8B), SREBP-1c mRNA levels did not show dose-dependency over a range of insulin concentrations (0 to 100 nM), which was previously shown to induce SREBP-1c expression in primary rat hepatocytes after both 6 and 24 hour incubation time points (Foretz et al., 1999b). Induction of SREBP-1c by 100 nM was also evident in primary rat hepatocytes after 6 or 24 hours incubation time (Foretz et al., 1999; Shimomura et al., 2000). This leads to the conclusion that SREBP-1c expression in HepG2 cells is not regulated by insulin as occurs in vivo (Shimomura et al., 1999b).

The lack of insulin responsiveness in SREBP-1c expression in HepG2 cells could be linked to changes in the expression of SREBP-1a and -1c splice variants in this cell line compared to their tissue of origin. Whilst human adult liver expresses six-fold higher SREBP-1c than SREBP-1a, HepG2 express SREBP-1a as the dominant variant, with SREBP-1c mRNA levels half those of SREBP-1a (Shimomura et al., 1997). SREBP-1a has been shown to be a stronger transactivator of gene transcription compared to the -1c isoform (Shimano et al., 1997) and cancer cells require increased SREBP-1 activity in order to meet the requirement for fatty acid synthesis associated with rapid growth (Pizer et al., 1997; Li et al., 2000). HepG2 cells also show a lack of insulin responsiveness in the regulation of PEPCK gene expression and have been shown to lack a factor which is present in the insulin-responsive H4IE cell line (Quinn and Yeagley, 2005). Therefore HepG2 cells may be deficient in a transcription factor or co-factor required for the response of SREBP-1c expression to insulin.

Furthermore, the lack of activation of SREBP-1c expression upon HNF4α overexpression (Figure 5.3), despite a three-fold increase in HNF4α expression levels (Figure 5.2), could be
due to inaccessibility of the SREBP-1c promoter to HNF4α binding due to altered chromatin in the transformed cell line compared to in vivo. While a number of HNF4α target genes such as PEPCK and apoCIII were activated upon HNF4α overexpression in the human hepatoma cell lines, HepG2 and HuH7, the expression of the transthyretin gene was unaltered, which is unexpected (Naiki et al., 2002; Naiki et al., 2004). However, when primary hepatocytes were transfected with an adenoviral HNF4α vector, transthyretin mRNA expression increased, leading the authors to suggest that epigenetic alterations between primary cells and cell lines limited transthyretin expression in the latter (Naiki et al., 2005). Certain genes, which failed to be activated by ectopic HNF4α expression in the dedifferentiated rat hepatoma H5 cell line, were found to re-expressed upon treatment with the demethylating agent, 5-azacytidine (Spåth and Weiss, 1997).

In conclusion, HNF4α overexpression did not activate SREBP-1c mRNA expression in HepG2 cells compared control transfection conditions, in contrast to the results of HNF4α overexpression on SREBP-1c reporter gene expression (Chapter 4). A stimulatory effect of insulin on SREBP-1c expression was also not observed, which in combination with published results, leads to the conclusion that further validation of human SREBP-1c as a target gene of HNF4α is not possible in the HepG2 cell line. Prolonged incubation with insulin led to changes in SREBP-1c, ACC and L-PK, which could be associated with the development of insulin resistance in HepG2 cells. FAS and L-PK were also not up-regulated by HNF4α and/or insulin (Figures 5.5 and 5.6), suggesting that further investigation of the incubation conditions and incubation time effecting the transcription of FAS and L-PK is required.
Chapter 6

Role of HNF4α in the differential regulation of its target genes in HepG2 cells
Chapter 6:

6.1 Introduction

HNF4α regulates the transcription of a wide range of target genes, occupying some 40% of all gene promoters in the liver (Odom et al., 2004). Its target genes form key regulatory points in opposingly regulated metabolic pathways (Sladek and Seidel, 2001), such as gluconeogenesis vs. glycolysis, and de novo lipogenesis vs. lipid transport. Furthermore, HNF4α regulates the transcription of other transcription factors, forming part of complex cross-regulatory networks (Kuo et al., 1992; Odom et al., 2004; Kyrmizi et al., 2006).

Building on results studying regulation of HNF4α-dependent reporter gene expression by metabolic stimuli (Chapter 4), the differential regulation of HNF4α target genes in their chromosomal context was studied in HepG2 cells at the mRNA level by a quantitative real-time RT-PCR (Q-PCR) approach.

Hypothesis:

HNF4α differentially regulates its target genes by forming alternate complexes with co-activators in response to fasting vs. feeding stimuli.

Aim:

To investigate the mechanisms by which HNF4α differentially regulates its target genes under fasting vs. feeding stimuli.

Objective 1: To characterize the effects of metabolic stimuli on HNF4α target genes in HepG2 cells.

Objective 2: To investigate the timecourse of changes in target gene expression in response to metabolic stimuli.

Objective 3: To characterise the effects of overexpression of DN-HNF4α on target gene expression in HepG2 cells under standard incubation conditions.
Objective 4: To determine the role of HNF4α in the regulation of its target genes by metabolic stimuli by overexpression of DN-HNF4α.

Objective 5: To investigate the role of HNF4α co-activators, p300 and PGC-1α, in the regulation of HNF4α target genes by metabolic stimuli by overexpression of individual co-activators in HepG2 cells.

Objective 6: To investigate the role of HNF4α-coactivator complexes in the regulation of HNF4α target genes by metabolic stimuli by comparing target gene expression when HNF4α and co-activators are overexpressed vs. control transfection conditions.

6.2 Characterisation of the effects of metabolic stimuli on HNF4α target gene expression in HepG2 cells

6.2.1 Rationale

The transcriptional regulation of a range of HNF4α target genes by metabolic stimulation were studied in HepG2 cells. Target genes under investigation were chosen to include key regulatory genes in the metabolic pathways of gluconeogenesis (PEPCK), glycolysis (L-PK), lipogenesis (FAS), lipoprotein metabolism (apoCIII) as well as transcription factors, SREBP-1c and PPARα. Since SREBP-1c mRNA expression in HepG2 cells was observed to be unresponsive to insulin and HNF4α overexpression in Chapter 5, SREBP-1c was included here in order to confirm that SREBP-1c gene transcription was not modulated by any other metabolic stimuli. HNF4α mRNA expression was also quantified in order to relate possible changes in target gene expression due to changes in HNF4α expression levels.

Metabolic stimuli examined were those contained within the fasting vs. feeding cycle, by incubating HepG2 cells with the hormones dexamethasone, glucagon or insulin, or cell-permeable activators of downstream pathways. 8-Br-cAMP acts downstream of glucagon and activates protein kinase (PKA) by direct activation in the case of 8-Br-cAMP (Meyer and Miller, 1974). IBMX also mimics the effect of glucagon by raising intracellular cAMP levels via inhibition of cAMP PDE activity (IBMX) (Houslay and Kilgour, 1990). In addition to the actions of hormones and their downstream mediators on HNF4α target gene expression, the influence of AMPK was also studied. AMPK is activated physiologically by
low intracellular energy status reflected in an increased ratio of AMP/ATP (Winder and Hardie, 1999). The influence of AMPK activation on target gene expression was studied by incubating HepG2 cells with the synthetic AMPK activator, AICAR.

6.2.2 Results

HNF4α mRNA levels were not significantly affected by the presence of hormones and protein kinase activators compared to control serum-free medium conditions (Figure 6.1A). The fasting hormones, dexamethasone and glucagon, did not alter L-PK or PEPCK mRNA expression; however, 8-Br-cAMP, which acts downstream of glucagon, produced a 220% increase in PEPCK expression and a non-significant 48% inhibition in L-PK expression (Figures 6.1B and 6.2A). The PKA activator, IBMX, also stimulated PEPCK expression (100% increase) and produced a non-significant 38% decrease in L-PK expression (Figures 6.1B and 6.2A). Incubation with dexamethasone, AICAR or insulin did not lead to any significant changes in PEPCK or L-PK mRNA expression (Figures 6.1B and 6.2A).

The expression of apoCIII and FAS was not significantly affected by any of the incubation conditions, although apoCIII expression showed a non-significant decrease of 42% in the presence of insulin, while FAS expression showed a non-significant increase of 88% in the presence of insulin, compared to the control incubation (Figure 6.2B and C).

The expression of the transcription factors, PPARα and SREBP-1c, were not affected by incubation with any of the hormones or protein kinase activators (Figure 6.3A and B).

6.3 Timecourse of effects of metabolic stimuli on gene expression

6.3.1 Rationale

In published work, maximal PEPCK mRNA levels were observed two hours after exposure to glucagon in primary rat hepatocytes (Koo et al., 2005). Therefore, the possibility that maximal induction of PEPCK by 8-Br-cAMP in HepG2 cells occurred at an earlier time point than the 6 hour incubation period used in the previous experiment (Figure 6.1B) was investigated by means of a timecourse experiment. Likewise, changes in L-PK expression were observed over the same timecourse, in order to ascertain the timecourse of the inhibitory effect of 8-Br-cAMP on L-PK expression (Figure 6.2A). In addition, a timecourse experiment of changes in FAS and apoCIII mRNA expression was observed in order to
Figure 6.1: Effects of metabolic stimuli on (A) HNF4α and (B) PEPCK mRNA expression in HepG2 cells. HepG2 cells were serum-starved for 24 hours and incubated in SFM with the addition of 1 μM dexamethasone (Dex.), 100 nM glucagon, 1 mM 8-Br-cAMP, 0.5 mM IBMX, 0.2 mM AICAR or 100 nM insulin as indicated for 6 hours, after which RNA was isolated and cDNA prepared. The relative expression level of each gene was quantified by real-time PCR, normalised to the expression of B2M and expressed relative to the control incubation condition (SFM). Data is expressed as mean values ± SEM from a minimum of three independent experiments. Differences between multiple incubation conditions were analysed by one-way ANOVA followed by Tukey’s post-hoc test (* p < 0.05; *** p < 0.001).
Figure 6.2: Effects of metabolic stimuli on (A) L-PK, (B) apoCIII and (C) FAS mRNA expression in HepG2 cells. Experiment and data analysis performed as detailed in Figure 6.1.
confirm that the previously used 6 hour time point (Figure 6.2B-C) was optimal for further experiments investigating the action of insulin on these genes.

6.3.2 Results

The expression of PEPCK and L-PK was measured following incubation lengths of between 0.5 and 6 hours and the fold induction between 8-Br-cAMP-containing and control medium calculated at each time point (Figure 6.4). Changes in PEPCK expression with respect to incubation time followed a sigmoidal pattern, with maximum PEPCK mRNA levels observed after 6 hours incubation with 8-Br-cAMP (Figure 6.4A). Changes in L-PK expression, on the other hand, did not show a consistent pattern with respect to incubation time, with inhibition
of L-PK expression by 8-Br-cAMP only observed at the 0.5 and 2 hour time points (Figure 6.4B).

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**Figure 6.4**: Timecourse of 8-Br-cAMP action on (A) PEPCK and (B) L-PK gene expression. HepG2 cells were serum-starved for 24 hours and lysed (0 h time point) or incubated with SFM ± 1 mM 8-Br-cAMP for 0.5-6 hours as indicated, after which RNA was isolated and cDNA prepared. The relative expression level of PEPCK and L-PK was quantified by real-time PCR, normalised to the expression of B2M. The fold difference in expression at each time point between 8-Br-cAMP and control medium are shown as individual datapoints (n = 1).
Figure 6.5: Timecourse of insulin action on (A) apoCIII and (B) FAS gene expression. HepG2 cells were serum-starved for 24 hours and lysed (0 h time point) or incubated with SFM ± 100 nM insulin for 1-24 hours as indicated, after which RNA was isolated and cDNA prepared. The relative expression level of apoCIII and FAS was quantified by real-time PCR, normalised to the expression of B2M. The fold difference in expression at each time point between insulin and control medium are shown as individual datapoints (n = 1).
The timescale of potential actions of insulin on apoCIII and FAS expression was also investigated (Figure 6.5). Insulin produced a gradual decrease in apoCIII expression over an incubation period of 24 hours, with apoCIII at 67% of their starting levels after 24 hours (Figure 6.5A). Like PEPCK expression (Figure 6.4A), the graph of FAS expression followed a sigmoidal shape with respect to incubation time with a sharp increase in FAS mRNA levels after 4 hours and a plateau being approached after 24 hours (Figure 6.5B).

Taken together with the results of previous experiments (Figures 6.1-6.2), it was concluded from the timecourse experiments that changes brought about by 8-Br-cAMP and insulin on target gene expression were reflected in mRNA expression at the 6 hour time point (Figures 6.4 and 6.5). Therefore, this incubation time was used for further experiments.

### 6.4 Characterisation of effects of dominant-negative HNF4α (DN-HNF4α) on target gene expression

#### 6.4.1 Rationale

An expression vector coding for human HNF4α2 with a dominant-negative mutation in its DNA-binding domain (DN-HNF4α) (Taylor et al., 1996) was utilised to investigate the role of HNF4α in the regulation of its target genes in HepG2 cells. The DN-HNF4α protein contains a functional dimerisation domain that has been shown to disrupt the binding of wild-type HNF4α to DNA by producing defective heterodimers, leading to a reduction in HNF4α-mediated reporter gene expression (Taylor et al., 1996; Gourdon et al., 1999; Jahan and Chiang, 2005). Initially, different amounts of DN-HNF4α expression vector DNA were compared with control expression vector (pcDNA3) in order to ascertain the optimum dose for subsequent experiments (Section 6.5).

#### 6.4.2 Results

HepG2 cells were transfected with varying amounts of a DN-HNF4α expression vector or control expression vector DNA (pcDNA3) and the expression of HNF4α target genes, PEPCK and L-PK (Figure 6.6), apoCIII and FAS (Figure 6.7), measured 46 hours after transfection (Experimental timeline, Figure 2.12). Cells were incubated in SFM for the final six hours of the experiment, in order to match the incubation time which was used when incubating HepG2 cells with metabolic stimuli (Section 6.2). A decrease in PEPCK mRNA
levels with increasing DN-HNF4α expression vector concentration was observed ($R^2 = 0.48$, $p < 0.01$), however no significant differences were established when comparing the linear regression analyses of the control and DN-HNF4α expression vectors (Figure 6.6A). The expression of the other HNF4α target genes investigated (L-PK, apoCIII and FAS) was not modulated by DN-HNF4α expression vector concentration and did not show any differences in expression pattern compared to cells transfected with equal amounts of control expression vector (Figures 6.6B and Figure 6.7).

This DN-HNF4α expression vector has not previously been used to investigate effects on endogenous cellular mRNA expression, whilst its mechanism of action has been extensively characterised using reporter gene studies (Taylor et al., 1996; Gourdon et al., 1999; Jahan and Chiang, 2005). Therefore, the ability of DN-HNF4α to disrupt HNF4α-stimulated reporter gene expression was confirmed using L-PK (L3)3-54PK and SREBP-1c reporter genes (Chapter 4) (Figure 6.8).

In line with previous results (Chapter 4), the expression of (L3)3-54PK and SREBP-1c reporter genes was induced upon overexpression of wild-type human HNF4α2, with 14- and 33-fold increases over basal expression observed respectively (Figure 6.8). The repressive action of DN-HNF4α was investigated by co-transfection of a constant dose of wild-type HNF4α (25 ng/well) with increasing amounts of DN-HNF4α expression vector (Figure 6.8). (L3)3-54PK reporter gene expression was repressed by co-expression of DN-HNF4α, with a 30% inhibition of reporter gene expression observed with 25 ng/well DN-HNF4α compared to wild-type vector alone (Figure 6.8A). DN-HNF4α concentration and (L3)3-54PK reporter gene expression exhibited negative correlation within the range 0 – 25 ng/well DN-HNF4α expression vector ($R^2 = 0.78$, $p < 0.001$), with reporter gene expression reaching a plateau corresponding to ~25% inhibition at 50 and 100 ng/well DN-HNF4α (Figure 6.8A). SREBP-1c reporter gene expression also displayed negative correlation with DN-HNF4α concentration throughout the tested range (0 – 100 ng/well) ($R^2 = 0.55$, $p < 0.001$) (Figure 6.8B). The maximum inhibition of HNF4α-activated SREBP-1c reporter gene expression occurred with 100 ng/well DN-HNF4α, with a 25% reduction in reporter gene activity observed.
Figure 6.6: Effect of DN-HNF4α expression vector concentration on (A) PEPCK and (B) L-PK mRNA expression in HepG2 cells. HepG2 cells were transfected with 10, 125, 250 or 500 ng/well control (pcDNA3) or DN-HNF4α expression vector DNA. 16 hours after transfection, cells were serum-starved for 24 hours then incubated for a further 6 hours in SFM, after which RNA was isolated and cDNA prepared. The expression of PEPCK and L-PK was normalised to B2M and expressed relative to the lowest concentration of pcDNA3 (10 ng/well). Results are plotted as individual datapoints. The effect of expression vector concentration on gene expression was analysed by linear regression analysis, as indicated by dashed lines, with goodness of fit ($R^2$) and statistical significance ($p$) indicated on each plot. Differences between control and DN-HNF4α expression vectors were analysed by comparing the slopes and intercepts of the linear regression analyses, with no significant differences in PEPCK or L-PK mRNA expression being observed between control and expression vectors.
Figure 6.7: Effect of DN-HNF4α expression vector concentration on (A) apoCIII and (B) FAS mRNA expression in HepG2 cells. HepG2 cells were transfected with 10, 125, 250 or 500 ng/well control (pcDNA3) or DN-HNF4α expression vector DNA. 16 hours after transfection, cells were serum-starved for 24 hours then incubated for a further 6 hours in SFM, after which RNA was isolated and cDNA prepared. The expression of apoCIII and FAS was normalised to B2M and expressed relative to the lowest concentration of pcDNA3 (10 ng/well). Results are plotted as individual datapoints. The effect of expression vector concentration on gene expression was analysed by linear regression analysis, as indicated by dashed lines, with goodness of fit ($R^2$) and statistical significance ($p$) indicated on each plot. Differences between control and DN-HNF4α expression vectors were analysed by comparing the slopes and intercepts of the linear regression analyses, with no significant differences in apoCIII or FAS mRNA expression being observed between control and expression vectors.
Figure 6.8: Dose-dependence of DN-HNF4α effects on HNF4α-stimulated expression of (A) L-PK (L3)-54PK and (B) SREBP-1c reporter plasmids. HepG2 cells were seeded in STM in 96-well plates and transfected with luciferase reporter plasmids (L3)-54PK or SREBP-1c (-780/+62) (100 ng/well), with expression vector for human HNF4α1 (25 ng/well) and DN-HNF4α expression vector (0 - 100 ng/well) as indicated (symbol: ♦). Basal reporter gene expression in the absence of HNF4α overexpression was also measured (symbol: ◄). pSEAPI-3’rep was co-transfected as a control for transfection efficiency. Luciferase readings were corrected for transfection efficiency based on SEAP expression levels. Values are shown as mean ± SEM (n = 4), relative to reporter gene expression in the presence of wild-type HNF4α expression vector alone. The relationship between DN-HNF4α expression vector concentration and reporter gene expression was analysed by Pearson’s correlation, as indicated by a dashed line along with R² and p-values.
In addition to validation of the DN-HNF4α mechanism of action by reporter plasmid experiments, the wild-type and DN-HNF4α expression vectors were sequenced, confirming the presence of a point mutation (T → C) at position 316 in DN-HNF4α (Taylor et al., 1996) (Appendix Section 8.3, Box 8.5).

### 6.5 Effects of dominant-negative HNF4α on the regulation of gene expression by metabolic stimuli

#### 6.5.1 Rationale

The DN-HNF4α expression vector has previously been utilised to investigate the role of HNF4α in regulation of its target genes by metabolic stimuli: for example, in the stimulation of G-6-Pase expression by SCFAs and PKA (Massillon et al., 2003; Gautier-Stein et al., 2005), and in the stimulation of L-CPTI expression by cAMP (Louet et al., 2002). In the latter case, expression of DN-HNF4α prevented induction of L-CPTI reporter gene expression by cAMP, but did not change basal L-CPTI expression, indicating a role of HNF4α in the regulation of L-CPTI by cAMP (Louet et al., 2002).

As the expression of PEPCK and L-PK was found to be modulated by cAMP (Figures 6.1B and 6.2A), the role of HNF4α in these effects was investigated by overexpression of DN-HNF4α. In addition, apoCIII and FAS expression showed some evidence of regulation by insulin (although these effects were not significant) (Figure 6.2B-C), therefore the effect of the DN-HNF4α expression vector on apoCIII and FAS expression under conditions of insulin vs. control medium (SFM) was also measured. Since DN-HNF4α differs from wild-type HNF4α in the alteration of only a single base (Taylor et al., 1996), it is likely that RT-PCR analysis with primers to wild-type HNF4α would also detect DN-HNF4α transcripts and therefore HNF4α mRNA expression was measured in order to an indication of the expression level of DN-HNF4α in the experiment.

#### 6.5.2 Results

Overexpression of DN-HNF4α resulted in increases in HNF4α mRNA levels, however it is not possible to determine whether this increase reflects both endogenous (wild-type) and DN-HNF4α mRNA expression (Figure 6.9). Transfection with DN-HNF4α expression vector resulted in a 6.9-fold increase over the control transfection following incubation with
8-Br-cAMP (p < 0.01). Non-significant 2.3- and 3.4-fold increases over control HNF4α levels were also observed in DN-HNF4α-transfected cells incubated with control and insulin-containing medium respectively (Figure 6.9).

The role of HNF4α in the effects of 8-Br-cAMP on PEPCK and L-PK expression was investigated by comparing cells transfected with the DN-HNF4α vs. control expression vectors (Figure 6.10). Under control transfection conditions, PEPCK mRNA levels were 3.5-fold higher following incubation with 8-Br-cAMP. A mean 5.5-fold increase in PEPCK mRNA expression over control levels was observed in cells transfected with DN-HNF4α, however the difference between pcDNA vs. DN-HNF4α was not found to be significant in post-hoc tests (Figure 6.10A).

Figure 6.9: Effect of DN-HNF4α expression on the mRNA expression of HNF4α in HepG2 cells under different incubation conditions. HepG2 cells were transfected with 500 ng/well DN-HNF4α or control (pcDNA3) expression vector. 16 hours after transfection, cells were serum-starved for 24 hours, followed by a 6 hour incubation period with control medium (SFM) or medium containing 1 mM 8-Br-cAMP or 100 nM insulin as indicated. Total RNA was then isolated, cDNA prepared and gene expression measured by real-time PCR. The expression of HNF4α was normalised to B2M and expressed relative to the control transfection and incubation condition. Results are expressed as mean values ± SEM (n = 4). The effects of expression vector (pcDNA3 vs. DN-HNF4α) and incubation conditions on gene expression were analysed by two-way ANOVA with Bonferroni post-hoc tests (** p < 0.01).
Incubation of HepG2 cells with 8-Br-cAMP did not inhibit L-PK expression in control-transfected cells, whilst a 29% drop in L-PK expression was observed in DN-HNF4α-transfected cells ($p < 0.05$), although direct differences between pcDNA3 vs. DN-HNF4α were not revealed in post-hoc tests (Figure 6.10B).

In order to establish whether PEPCK or L-PK expression correlated with changes in HNF4α mRNA expression, linear regression analyses were performed relating HNF4α and target gene mRNA levels (Figure 6.11). This revealed that PEPCK expression positively correlated with HNF4α expression in the presence of 8-Br-cAMP ($R^2 = 0.82, p < 0.01$) (Figure 6.11A). A positive, though non-significant, correlation of L-PK with HNF4α mRNA levels under 8-Br-cAMP conditions was also observed ($R^2 = 0.41, p = 0.09$) (Figure 6.11B).

The role of HNF4α in the regulation of target genes whose expression is modified by insulin (apoCIII and FAS) was also investigated, however insulin did not significantly modulate the expression of either gene in cells transfected with either control or DN-HNF4α expression vectors (Figure 6.12). In addition, DN-HNF4α expression did not alter apoCIII or FAS expression compared to control vector (Figure 6.12). Linear regression analysis of the relationship between apoCIII or FAS and HNF4α mRNA levels also indicated that expression of DN-HNF4α did not significantly alter the expression of these target genes under either incubation condition (Appendix Section 8.4, Figure 8.1).
Figure 6.10: Effect of DN-HNF4α on the regulation of (A) PEPCK and (B) L-PK mRNA expression by cAMP. HepG2 cells were transfected with 500 ng/well DN-HNF4α or control (pcDNA3) expression vector. 16 hours after transfection, cells were serum-starved for 24 hours, followed by a 6 hour incubation period with control medium (SFM) or medium containing 1 mM 8-Br-cAMP as indicated. Total RNA was then isolated, cDNA prepared and gene expression measured by real-time PCR. The expression of PEPCK and L-PK was normalised to B2M and expressed relative to the control transfection and incubation condition. Results are expressed as mean values ± SEM (n = 4). The effects of expression vector (pcDNA3 vs. DN-HNF4α) and incubation conditions on gene expression were analysed by two-way ANOVA with Bonferroni post-hoc tests (* p < 0.05, *** p < 0.001).
**Figure 6.11:** Effect of DN-HNF4α on the regulation of (A) PEPCK and (B) L-PK mRNA expression by cAMP: Linear regression analysis. Analyses of correlation between (A) PEPCK or (B) L-PK and HNF4α mRNA expression levels were performed with data from control (closed triangles ▲) and 8-Br-cAMP (open triangles △) incubation conditions (from both pcDNA3- and DN-HNF4α-transfection conditions), from experiment as described in Figure 6.10. Results are presented as individual datapoints with correlation analyses indicated by a dashed line with adjacent $R^2$ and $p$-values. Differences between control and 8-Br-cAMP incubation conditions were analysed by comparing the slopes and intercepts of the linear regression analyses and a significant difference between the two lines is indicated by a double-arrow and adjacent $p$-value (N.S., not significant).
Figure 6.12: Effect of DN-HNF4α on the regulation of (A) apoCIII and (B) FAS mRNA expression by insulin. HepG2 cells were transfected with 500 ng/well DN-HNF4α or control (pcDNA3) expression vector. 16 hours after transfection, cells were serum-starved for 24 hours, followed by a 6 hour incubation period with control medium (SFM) or medium containing 100 nM insulin as indicated. Total RNA was then isolated, cDNA prepared and gene expression measured by real-time PCR. The expression of apoCIII and FAS was normalised to B2M and expressed relative to the control transfection and incubation condition. Results are expressed as mean values ± SEM (n = 4). The effects of expression vector (pcDNA3 vs. DN-HNF4α) and incubation conditions on gene expression were analysed by two-way ANOVA.
6.6 Effects of HNF4α co-activators p300 and PGC-1α on the regulation of PEPCK and L-PK expression

6.6.1 Rationale

The results of overexpression of DN-HNF4α suggested a role for HNF4α in the stimulation of PEPCK expression by cAMP (Figure 6.11A). The expression of L-PK in the presence of 8-Br-cAMP also appeared to be related to the expression of HNF4α (Figures 6.10B and 6.11B). Therefore, the role of HNF4α co-activators, p300 and PGC-1α, in these effects was further investigated by studying the impact of overexpressing each co-activator in HepG2 cells.

6.6.2 Results

HepG2 cells were transfected with expression vectors coding for p300, PGC-1α or control, and the expression of HNF4α, PEPCK and L-PK measured following a six hour incubation with 8-Br-cAMP-containing or control medium (Figure 6.13). HNF4α mRNA levels did not vary significantly between transfection or incubation conditions (Figure 6.13A). Incubation of HepG2 cells with 8-Br-cAMP resulted in induction of PEPCK expression in the control and PGC-1α transfection conditions respectively, with 2.7-fold and 2.9-fold increases in PEPCK mRNA levels observed respectively (Figure 6.13B). On other hand, the level of induction of PEPCK expression following incubation with 8-Br-cAMP in the p300 transfection condition was attenuated, with the 2.0-fold increase observed not reaching the level of significance (Figure 6.13B). In contrast, L-PK expression only showed inhibition by 8-Br-cAMP when p300 was overexpressed, but not in the control or PGC-1α transfection conditions (Figure 6.12C).

Although not found to be significant following a two-way ANOVA analysis, mean HNF4α levels were below the level of the control transfection and incubation condition (set as 100%), when PGC-1α was overexpressed (in both incubation conditions) and when p300 was overexpressed (in the presence of 8-Br-cAMP) (Figure 6.13A). Alterations in HNF4α expression were further investigated by studying the relationship between target gene and HNF4α expression levels under each incubation condition by correlation analysis (Figures 6.14 and 6.15).
Figure 6.13: Effects of p300 and PGC-1α overexpression on (A) HNF4α, (B) PEPCK and (C) L-PK mRNA expression in HepG2 cells. HepG2 cells were transfected with 500 ng/well control (pcDNA3), p300 or PGC-1α expression vector. 16 hours after transfection, cells were serum-starved for 24 hours, followed by a 6 hour incubation period with control medium (SFM) or medium containing 1 mM 8-Br-cAMP. Total RNA was isolated, cDNA prepared and gene expression measured by real-time PCR. Gene expression levels were normalised to B2M and expressed relative to the control transfection and incubation condition. Results are expressed as mean values ± SEM (n = 3). The effects of expression vector and incubation condition were analysed by two-way ANOVA, with the results of Bonferroni post-hoc tests as indicated (* p < 0.05, ** p < 0.01).
Under the control incubation condition, a highly significant relationship between PEPCK and HNF4α mRNA levels was evident ($R^2 = 0.84, p < 0.001$) (Figure 6.14). In the presence of 8-Br-cAMP, the correlation between PEPCK and HNF4α mRNA levels fell short of significance ($R^2 = 0.40, p = 0.07$) (Figure 6.14). Comparison of the intercepts of the linear regression analyses for the control and 8-Br-cAMP incubation conditions indicated that 8-Br-cAMP had a highly significant effect on PEPCK expression ($p < 0.0001$), when PEPCK mRNA levels were expressed as a function of HNF4α mRNA levels (Figure 6.14). This result suggests that HNF4α abundance could be a limiting factor in determining PEPCK expression.

Figure 6.14: Effects of p300 and PGC-1α overexpression: Relationship between HNF4α and PEPCK mRNA expression under control and 8-Br-cAMP incubation conditions. Analysis of correlation between HNF4α and PEPCK mRNA expression was performed with results presented in Figure 6.13 by combining data from all three transfection conditions (pcDNA3, p300 and PGC-1α). Results are presented as individual datapoints with the combined linear regression analysis for each incubation condition indicated by a dashed line with adjacent $R^2$ and $p$-value. Differences between control and 8-Br-cAMP incubation conditions were analysed by comparing the linear regression analyses and a significant difference between the intercepts of the two lines is indicated by a double-arrow and adjacent $p$-value.
L-PK expression positively correlated with HNF4α mRNA levels under both the control incubation condition ($R^2 = 0.47, p < 0.05$) and in the presence of 8-Br-cAMP ($R^2 = 0.91, p < 0.001$) (Figure 6.15). Comparison of the intercepts of the linear regression analyses for the control and 8-Br-cAMP incubation conditions indicated that 8-Br-cAMP also had a highly significant effect on L-PK expression ($p < 0.001$), when L-PK mRNA levels were expressed as a function of HNF4α mRNA levels (Figure 6.15). This result suggests that HNF4α abundance was modulated by the overexpression of PGC-1α or p300 and this had an impact on L-PK expression.

Figure 6.15: Effects of p300 and PGC-1α overexpression: Relationship between HNF4α and L-PK mRNA expression under control and 8-Br-cAMP incubation conditions. Analysis of correlation between HNF4α and L-PK mRNA expression was performed with results presented in Figure 6.13 by combining data from all three transfection conditions (pcDNA3, p300 and PGC-1α). Results are presented as individual datapoints with the combined linear regression analysis for each incubation condition indicated by a dashed line with adjacent $R^2$ and $p$-value. Differences between control and 8-Br-cAMP incubation conditions were analysed by comparing the linear regression analyses and a significant difference between the intercepts of the two lines is indicated by a double-arrow and adjacent $p$-value.
6.7 Role of HNF4α-co-activator complex in the differential regulation of PEPCK and L-PK expression

6.7.1 Rationale

Overexpression of either p300 or PGC-1α resulted in changes to PEPCK and L-PK expression which were related to endogenous HNF4α mRNA levels (Figures 6.14 and 6.15), suggesting that HNF4α quantity could be a limiting factor in the regulation downstream genes by either co-activator. Furthermore, nuclear receptors require the presence of co-activators with both bridging- and HAT-capabilities in order to activate transcription (Acevedo and Kraus, 2004). Activation of gene expression by PGC-1α involves the recruitment of HAT-type co-activators, p300 or CBP, owing to PGC-1α lacking HAT activity (Puigserver, 1999). Therefore, HNF4α and both co-activators (p300 and PGC-1α) were overexpressed in order to test the hypothesis that a complex of HNF4α, p300 and PGC-1α differentially regulates downstream genes, PEPCK and L-PK, under opposing metabolic conditions.

6.7.2 Results

HepG2 cells were transfected with expression vectors for HNF4α, p300 and PGC-1α or control DNA (pcDNA3) followed by incubation of the cells with 8-Br-cAMP and/or dexamethasone (fasting conditions), or insulin and/or high glucose concentration (25 mM) (feeding conditions). The mRNA levels of the overexpressed factors were measured (Figures 6.16 and 6.17), as well as those of target genes, PEPCK and L-PK (Figure 6.18 and Figure 6.23 respectively). For conditions containing dexamethasone, an additional incubation with vehicle control medium (0.04% ethanol) was performed in order to ensure dexamethasone-specific effects were attributable to the action of the glucocorticoid, not the vehicle (ethanol) (Appendix Section 8.4, Figures 8.2 and 8.3).

HNF4α mRNA expression was not modulated by incubation with any of the metabolic stimuli under control transfection conditions (Figure 6.15A). In the HNF4α-p300-PGC-1α transfection condition, HNF4α mRNA levels were between 35% and 146% greater than under the control transfection condition, however post-hoc tests only identified a significant difference in HNF4α expression between active vs. control transfection in the case of cells incubated with 8-Br-cAMP (p < 0.01) (Figure 6.16A).
In the control transfection condition, p300 expression was 72% greater when cells were incubated with 25 mM glucose medium compared to control medium ($p < 0.05$) (Figure 6.16B). A significant difference between 25 mM glucose- vs. dexamethasone-containing conditions was also identified ($p < 0.05$) (Figure 6.16B). p300 expression did not increase significantly upon transfection of HepG2 cells with a p300 expression vector under any of the incubation conditions, with the maximum percentage-change of 38% observed in the dexamethasone incubation condition not reaching the level of significance (Figure 6.16B).

As the PGC-1α expression vector used encoded mouse PGC-1α cDNA, it was possible to differentiate between the expression of endogenous PGC-1α gene in human HepG2 cells and expression vector-derived murine PGC-1α transcripts (Figure 6.17). Endogenous PGC-1α expression was significantly induced by 8-Br-cAMP and dexamethasone in both control ($p < 0.05$) and active ($p < 0.001$) transfection conditions (Figure 6.16A). Human PGC-1α was also greater in the presence of 8-Br-cAMP alone compared to the 25 mM glucose and insulin incubation condition under control transfection conditions ($p < 0.05$), but not in the active transfection condition (Figure 6.16A).

No differences in human PGC-1α expression between control and exogenous PGC-1α transfection conditions were observed, suggesting that the PCR reporter signal using human PGC-1α primers does not reflect expression-vector derived transcripts (Figure 6.17A). Mouse PGC-1α primers were also species-specific, with no signal being detectable in the majority of cases for samples from the control transfection condition (Figure 6.17B).

Expression of the PGC-1α expression vector was increased 3.9-fold by 8-Br-cAMP over control incubation conditions ($p < 0.001$) (Figure 6.17B). The combination of 8-Br-cAMP and dexamethasone almost doubled expression vector-derived PGC-1α transcript levels compared to 8-Br-cAMP alone ($p < 0.001$), suggesting the two compounds synergise to promote expression of the transfected DNA (Figure 6.17B).
Figure 6.16: Expression of (A) HNF4α and (B) p300 under different metabolic conditions. HepG2 cells were transfected with 125 ng HNF4α, p300 and PGC-1α expression vectors per well or control (375 ng/well pcDNA3). 16 hours after transfection, cells were serum-starved for 24 hours, followed by a 6 hour incubation period with control medium (SFM) or medium containing 1 mM 8-Br-cAMP, 1 μM dexamethasone, 100 nM insulin or 25 mM glucose as indicated. Total RNA was isolated, cDNA prepared and gene expression measured by real-time PCR. Gene expression levels were normalised to B2M and expressed relative to the control transfection and incubation condition. Results are expressed as mean values ± SEM (n = 4). The effects of transfection and incubation conditions were analysed by two-way ANOVA, with the results of Bonferroni post-hoc tests as indicated (* p <0.05, ** p <0.01).
Figure 6.17: Expression of (A) endogenous and (B) ectopically expressed PGC-1α under different metabolic conditions. HepG2 cells were transfected with control (pcDNA3) or HNF4α, p300 and PGC-1α expression vectors and incubated under different conditions as described in Figure 6.15. Gene expression levels of (A) endogenous human PGC-1α (hsPGC-1α) or (B) expression vector-derived mouse PGC-1α (mmPGC-1α) were normalised to B2M and expressed relative to (A) the control transfection and incubation condition or (B) the PGC-1α transfection and control incubation condition. Results are expressed as mean values ± SEM (n = 4). The effects of transfection and incubation conditions were analysed by two-way ANOVA, with the results of Bonferroni post-hoc tests as indicated (* p <0.05, *** p <0.001). The absence of brackets indicates the incubation condition was significantly different to all other incubation conditions.
The impact of overexpression of HNF4α, p300 and PGC-1α on downstream genes, PEPCK and L-PK was analysed. PEPCK expression was significantly induced by incubation of HepG2 cells with 8-Br-cAMP, with a mean 3.3-fold increase in expression observed under control transfection conditions ($p < 0.05$) (Figure 6.18). 8-Br-cAMP in the presence of dexamethasone caused a similar increase in PEPCK expression, with a mean 3.5-fold increase observed vs. the control incubation condition in the pcDNA3 transfection group ($p < 0.05$) (Figure 6.17). Overexpression of HNF4α, p300 and PGC-1α resulted in 2.0-fold higher PEPCK expression in the 8-Br-cAMP incubation condition compared to the control transfection condition ($p < 0.01$) (Figure 6.18).

![Figure 6.18: Role of HNF4α-p300-PGC-1α complex in the regulation of PEPCK mRNA expression by metabolic stimuli. HepG2 cells were transfected with control (pcDNA3) or HNF4α, p300 and PGC-1α expression vectors and incubated under different conditions as described in Figure 6.16. Total RNA was isolated, cDNA prepared and gene expression measured by real-time PCR. PEPCK expression levels were normalised to B2M and expressed relative to the control transfection and incubation condition. Results are expressed as mean values ± SEM ($n = 4$). The effects of expression vector and incubation condition were analysed by two-way ANOVA, with the results of Bonferroni post-hoc tests as indicated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The 8-Br-cAMP and 8-Br-cAMP + dex. incubation conditions were significantly different to all other incubation conditions.](image-url)
PEPCK expression following incubation with the combination of 8-Br-cAMP and
dexamethasone was 1.4-fold higher in the HNF4α-p300-PGC-1α transfection condition
compared to control, however this was not found to be significant in post-hoc tests (Figure
6.18).

As expression levels of the transfected HNF4α- and co-activator-expression vectors varied
between biological repeats (Figures 6.16 and 6.17), the influence of HNF4α, p300 and PGC-
1α expression levels on PEPCK expression was further analysed by linear regression analysis
(Figures 6.19 to 6.22). The correlation of PEPCK expression with HNF4α, p300 and both
endogenous (human PGC-1α) and transfected (mouse PGC-1α) was analysed. As PEPCK
expression was shown to be significantly stimulated by the incubation of HepG2 cells with 8-
Br-cAMP (alone and in combination with dexamethasone), correlation analyses of PEPCK
expression vs. expression of HNF4α, p300 and both
endogenous (human PGC-1α) and transfected (mouse PGC-1α) were presented here (Figures 6.19 to 6.21), alongside
correlation analyses of PEPCK expression under conditions mimicking the opposite
metabolic scenario of the fed state (25 mM glucose and insulin) (Figure 6.22). Correlation
analyses were also performed for the other incubation conditions investigated and are
presented in the Appendix (Figures 8.4-8.5, Section 8.4). The linear regression analysis for
each incubation condition was also compared with that of the control incubation condition in
terms of differences in intercept and slope of the two lines. A significant difference in the
intercepts was interpreted as evidence of the incubation condition modifying the expression
of the target gene (PEPCK), while a significant difference in the slopes of the lines suggested
that changes in the activity of the transcription factor or co-activator in question influenced
changes in expression of the target gene between the incubation conditions.

Differences in the intercepts of the linear regression analyses for the control and 8-Br-cAMP
conditions indicated that 8-Br-cAMP significantly increased the expression of PEPCK
(Figure 6.19A, B and D), in line with results of two-way ANOVA (Figure 6.18). When cells
were incubated with control medium, PEPCK expression correlated with HNF4α mRNA
levels (R^2 = 0.90, p < 0.0001, with the omission of one anomalous datapoint) (Figure 6.19A).
In the presence of 8-Br-cAMP, PEPCK mRNA levels positively correlated with HNF4α
mRNA levels when two anomalous values were omitted (R^2 = 0.72, p < 0.05) (Figure 6.19A).
p300 mRNA expression levels were not related to PEPCK expression levels under control or
8-Br-cAMP conditions (Figure 6.19B). Endogenous PGC-1α levels significantly correlated with PEPCK expression levels under control and 8-Br-cAMP incubation conditions (Figure 6.19C). A steeper gradient in the relationship between endogenous PGC-1α and PEPCK in the presence of 8-Br-cAMP compared to control medium suggests that PGC-1α has greater activity in the presence of 8-Br-cAMP. Exogenous PGC-1α transcript levels came close to a significant positive correlation with PEPCK expression under control ($R^2 = 0.47, p = 0.06$) and 8-Br-cAMP incubation conditions ($R^2 = 0.50, p = 0.05$) (Figure 6.19D).

Dexamethasone appeared to suppress PEPCK mRNA expression, based on linear regression analyses of HNF4α and endogenous PGC-1α vs. PEPCK expression levels (Figure 6.20A and C). A significant decrease in the slopes of the lines relating HNF4α or human PGC-1α mRNA levels with those of PEPCK suggested that dexamethasone suppresses the activity of both factors, resulting in decreased PEPCK mRNA expression (Figure 6.20A and C).

8-Br-cAMP and dexamethasone significantly stimulated PEPCK mRNA expression, based on differences in the intercepts of linear regression analysis for this incubation condition vs. control medium (Figure 6.21A, B and D). Unlike for 8-Br-cAMP alone (Figure 6.19A), a statistically significant linear relationship between HNF4α and PEPCK expression levels was not apparent in the presence of both 8-Br-cAMP and dexamethasone ($R^2 = 0.26, p = N.S.$) (Figure 6.21A). Under these incubation conditions, both endogenous PGC-1α and ectopically expressed PGC-1α mRNA levels positively correlated with PEPCK expression ($R^2 = 0.75, p < 0.01$) (Figure 6.21C) and ($R^2 = 0.69, p < 0.05$) (Figure 6.21D) respectively.

Linear regression analysis of PEPCK expression under high glucose concentration and insulin conditions suggested that this incubation condition diminishes PEPCK expression compared to control medium, with the intercept for the 25 mM glucose and insulin line being significantly lower than that for control medium (for the p300, human PGC-1α and mouse PGC-1α analyses (Figure 6.22B, C and D). When comparing the slopes of linear regression analyses for HNF4α between 25 mM glucose and insulin with control medium, a significant difference in the slopes of the lines was apparent ($p < 0.001$) (Figure 6.22A).
Figure 6.19: Correlation between expression of PEPCK and (A) HNF4α, (B) p300, (C) endogenous PGC-1α, and (D) exogenous PGC-1α mRNA levels under 1 mM 8-Br-cAMP incubation conditions. Linear regression analysis of the relationships between PEPCK and HNF4α/aco-activator mRNA expression levels was performed with data from control (closed triangles ▲) and 8-Br-cAMP (open triangles △) incubation conditions presented in Figures 6.16 to 6.18. Results are presented as individual datapoints with linear regression analyses indicated by a dashed line with adjacent $R^2$ and $p$-values. Anomalous datapoints (circled) omitted from analysis. Differences between control and 8-Br-cAMP incubation conditions were analysed by comparing the linear regression analyses and a significant difference between the intercepts or slopes of the two lines is indicated by a double-arrow and adjacent $p$-value or N.S. in the case of no significant differences. (If the difference is between the slopes, this is noted; no additional notation corresponds to a difference between the intercepts).
Figure 6.20: Correlations between expression of PEPCK and (A) HNF4α, (B) p300 (C) endogenous PGC-1α and (D) exogenous PGC-1α mRNA levels under incubation conditions of 1 μM dexamethasone. Linear regression analysis of the relationships between PEPCK and HNF4α / co-activator mRNA expression levels was performed with data from control (closed triangles ▲) and dexamethasone (Dex.) (open triangles △) incubation conditions as presented in Figures 6.16 to 6.18. Results are presented as individual datapoints with linear regression analyses indicated by a dashed line with adjacent $R^2$ and $p$-values. †Anomalous datapoints omitted from analysis (Figure 6.19). Differences between the incubation conditions were analysed by comparing the linear regression analyses and a significant difference between the intercepts or slopes of the two lines is indicated by a double-arrow and adjacent $p$-value or N.S. in the case of no significant differences. (If the difference relates to the slopes, this is noted; no additional notation corresponds to a difference between the intercepts).
Figure 6.21: Correlations between expression of PEPCK and (A) HNF4α, (B) p300 (C) endogenous PGC-1α and (D) exogenous PGC-1α mRNA levels under incubation conditions of 1 mM 8-Br-cAMP and 1 μM dexamethasone. Linear regression analysis of the relationships between PEPCK and HNF4α / co-activator mRNA expression levels was performed with data from control (closed triangles ▲) and 8-Br-cAMP +Dex. (open triangles △) incubation conditions as presented in Figures 6.16 to 6.18. Results are presented as individual datapoints with linear regression analyses indicated by a dashed line with adjacent $R^2$ and $p$-values. †Anomalous datapoints omitted from analysis (Figure 6.19). Differences between control and 8-Br-cAMP+Dex. incubation conditions were analysed by comparing the linear regression analyses and a significant difference between the intercepts or slopes of the two lines is indicated by a double-arrow and adjacent $p$-value or N.S. in the case of no significant differences. (If the difference is between the slopes, this is noted; no additional notation corresponds to a difference between the intercepts).
Figure 6.22: Correlations between expression of PEPCK and (A) HNF4α, (B) p300 (C) endogenous PGC-1α and (D) exogenous PGC-1α mRNA levels under incubation conditions of 25 mM glucose and 100 nM insulin. Linear regression analysis of the relationships between PEPCK and HNF4α / co-activator mRNA expression levels was performed with data from control (closed triangles ▲) and 25 mM glucose + Ins. (open triangles △) incubation conditions as presented in Figures 6.16 to 6.18. Results are presented as individual data points with linear regression analyses indicated by a dashed line with adjacent $R^2$ and $p$-values. †Anomalous data points omitted from analysis (Figure 6.19).

Differences between the incubation conditions were analysed by comparing the linear regression analyses and a significant difference between the intercepts or slopes of the two lines is indicated by a double-arrow and adjacent $p$-value or N.S. in the case of no significant differences. (If the difference relates to the slopes, this is noted; no additional notation corresponds to a difference between the intercepts).
Under ‘fed state’ conditions, PEPCK expression levels showed no relationship with those of HNF4α (R² = 0.00, p = N.S.) (Figure 6.22A), suggesting decreased HNF4α activity. A decreased slope in the relationship between HNF4α and PEPCK mRNA expression levels was also apparent for cells incubated with insulin or 25 mM glucose individually (Appendix, Figure 8.4A and 8.5A). A positive linear relationship between human PGC-1α and PEPCK expression levels was observed under for the 25 mM glucose and insulin incubation condition (R² = 0.53, p < 0.05) (Figure 6.22C), whilst exogenous PGC-1α expression did not correlate with PEPCK mRNA expression (Figure 6.22D). In presence of insulin only, human PGC-1α expression levels did not correlate with those of PEPCK and a significant decrease in the slope of the linear regression analysis compared to control also suggested that decreased PGC-1α activity had a negative impact on PEPCK mRNA expression (Appendix, Figure 8.4C). Likewise, exogenously expressed PGC-1α mRNA levels did not correlate with those of PEPCK in the presence of insulin or 25 mM glucose (Appendix, Figures 8.4D and 8.5D).

The expression of the oppositely regulated target gene, L-PK, was also analysed under the same range of conditions as PEPCK by two-way ANOVA (Figure 6.23). Whilst direct differences between the two transfection conditions were not noted in post-hoc tests, the influence of incubation condition on the expression of L-PK was different between the control and HNF4α-p300-PGC-1α transfection groups, with a significant interaction between transfection condition and incubation condition being shown by two-way ANOVA (p < 0.05). In the control transfection condition, the expression of L-PK was significantly lower (> 50%) when cells were incubated with 8-Br-cAMP compared to L-PK expression in the presence of dexamethasone, insulin, 25 mM glucose or 25 mM glucose with insulin (Figure 6.23). L-PK expression was also at least 40% lower in the presence of 8-Br-cAMP with dexamethasone compared to four other incubation conditions (dexamethasone, insulin, 25 mM glucose or 25 mM glucose with insulin) (Figure 6.23).

When HNF4α, p300 and PGC-1α were overexpressed, L-PK expression in the presence of 8-Br-cAMP was no longer decreased compared to any of the other incubation conditions. In addition, L-PK expression under the ‘fed-state’ conditions of 25 mM glucose or 25 mM glucose with insulin was decreased relative to the dexamethasone incubation condition (p < 0.05) (Figure 6.23). This suggests that overexpression of HNF4α in combination with p300 and PGC-1α reduces L-PK expression under conditions mimicking the fed-state (25 mM
glucose or 25 mM glucose with insulin), whilst attenuating the inhibitory action of 8-Br-cAMP on L-PK expression (Figure 6.23).

Figure 6.23: Role of HNF4α-p300-PGC-1α complex in the regulation of L-PK mRNA expression by metabolic stimuli. HepG2 cells were transfected with control (pcDNA3) or HNF4α, p300 and PGC-1α expression vectors and incubated under different conditions as described in Figure 6.15. Total RNA was isolated, cDNA prepared and gene expression measured by real-time PCR. L-PK expression levels were normalised to B2M and expressed relative to the control transfection and incubation condition. Results are expressed as mean values ± SEM (n = 4). The effects of expression vector and incubation condition were analysed by two-way ANOVA, with the results of Bonferroni post-hoc tests indicated with the least significant p-value (* p <0.05).

The relationship between the expression of HNF4α, p300 and PGC-1α and that of the L-PK gene was investigated by linear regression analysis, in order to gain insight into whether each transcription factor was linked to stimulation or repression of L-PK transcription under each set of incubation conditions. As differences in the expression of L-PK were observed between the two transfection groups under incubation conditions of 8-Br-cAMP, 25 mM glucose or 25 mM glucose with insulin (Figure 6.23), correlation analyses for these incubation conditions are presented here (Figures 6.24 to 6.26) with analysis of the remaining incubation conditions displayed in the Appendix (Section 8.4, Figures 8.6-8.8).
8-Br-cAMP induced a significant decrease in L-PK expression compared to control conditions, as judged by a lower intercept of linear regression analyses of HNF4α and exogenous PGC-1α vs. L-PK mRNA levels (Figure 6.24A and D). Both HNF4α and exogenous PGC-1α expression levels positively correlated with those of L-PK in the 8-Br-cAMP condition, \( R^2 = 0.93, p < 0.001 \) (with omission of an anomalous value) (Figure 6.24A) and \( R^2 = 0.62, p < 0.05 \) (Figure 6.24D) respectively. L-PK mRNA expression did not show concentration-dependence with p300 or endogenous PGC-1α expression levels (Figure 6.24B and C).

Based on linear regression analysis, incubation of HepG2 cells with dexamethasone had the opposite effect on L-PK compared to PEPCK mRNA expression, with higher intercepts in the relationships between HNF4α, p300 and mouse PGC-1α vs. L-PK mRNA levels in the dexamethasone condition compared to control (Appendix, Figure 8.6A, B and D). In contrast, 8-Br-cAMP combined with dexamethasone inhibited L-PK mRNA expression based on decreased intercepts of linear regression analyses for all four transcription (co-)factors vs. L-PK (Appendix, Figure 8.7A-D). Unlike in the presence of 8-Br-cAMP alone, L-PK mRNA expression did not significantly correlate with HNF4α or PGC-1α mRNA levels in the 8-Br-cAMP and dexamethasone incubation condition (Appendix, Figure 8.7A and C).

Under incubation conditions corresponding to the fed state (i.e. those with the addition of insulin, 25 mM glucose or both to the cell culture medium), no significant differences between these incubation conditions and the control condition were observed by linear regression analysis of HNF4α or co-activator vs. L-PK expression levels (Figures 6.25, 6.26 and Appendix, Figure 8.8), suggesting these stimuli did not induce L-PK mRNA expression in HepG2 cells. Changes in HNF4α mRNA expression did not appear to modulate L-PK expression under these conditions, nor did those of p300 or PGC-1α (Figures 6.25, 6.26 and Appendix, Figure 8.8). A negative trend in the direction of the linear regression plot for exogenous PGC-1α vs. L-PK mRNA expression was observable under conditions of 25 mM glucose and insulin, however this came short of significance \( (R^2 = 0.35, p = 0.12) \) (Figure 6.26D). Differences in the slopes between the analyses of PGC-1α vs. L-PK mRNA expression for the control and 25 mM glucose and insulin incubation conditions also fell short of significance \( (p = 0.08) \) (Figure 6.26D).
Figure 6.24: Correlation between expression of L-PK and (A) HNF4α, (B) p300 (C) endogenous PGC-1α and (D) exogenous PGC-1α mRNA levels under control and 1 mM 8-Br-cAMP incubation conditions. Analysis of correlation between L-PK and HNF4α / co-activator mRNA expression levels was performed with data from control (closed triangles ▲) and 8-Br-cAMP (open triangles △) incubation conditions, from experiment as described in Figures 6.16, 6.17 and 6.23. Results are presented as individual datapoints with correlation analyses indicated by a dashed line with adjacent R² and p-values. Anomalous datapoint (circle) omitted from correlation analysis of HNF4α vs. L-PK (8-Br-cAMP incubation). Differences between the incubation conditions were analysed by comparing the linear regression analyses and a significant difference between the intercepts or slopes of the two lines is indicated by a double-arrow and adjacent p-value or N.S. in the case of no significant differences. (If the difference relates to the slopes, this is noted; no additional notation corresponds to a difference between the intercepts).
Figure 6.25: Correlation between expression of L-PK and (A) HNF4α, (B) p300 (C) endogenous PGC-1α and (D) exogenous PGC-1α mRNA levels under incubation conditions of 25 mM glucose. Analysis of correlation between L-PK and HNF4α / co-activator mRNA expression levels was performed with data from control (closed triangles ▲) and 25 mM glucose (open triangles △) incubation conditions from experiment as described in Figures 6.16, 6.17 and 6.23. Results are presented as individual datapoints with correlation analyses indicated by a dashed line with adjacent R² and p-values. Differences between the incubation conditions were analysed by comparing the linear regression analyses and a significant difference between the intercepts or slopes of the two lines is indicated by a double-arrow and adjacent p-value or N.S. in the case of no significant differences. (If the difference relates to the slopes, this is noted; no additional notation corresponds to a difference between the intercepts).
Figure 6.26: Correlation between expression of L-PK and (A) HNF4α, (B) p300 (C) endogenous PGC-1α and (D) exogenous PGC-1α mRNA levels under incubation conditions of 25 mM glucose and insulin. Analysis of correlation between L-PK and HNF4α / co-activator mRNA expression levels was performed with data from control (closed triangles ▲) and 25 mM glucose and insulin (open triangles △) incubation conditions from experiment as described in Figures 6.16, 6.17 and 6.23. Results are presented as individual datapoints with correlation analyses indicated by a dashed line with adjacent $R^2$ and $p$-values. Differences between the incubation conditions were analysed by comparing the linear regression analyses and a significant difference between the intercepts or slopes of the two lines is indicated by a double-arrow and adjacent $p$-value or N.S. in the case of no significant differences. (If the difference relates to the slopes, this is noted; no additional notation corresponds to a difference between the intercepts).
6.8 Discussion

6.8.1 Effects of metabolic stimuli in HepG2 cells

In characterising the role of HNF4α in differentially regulated targets genes, the regulation of multiple target genes by a range of physiological mediators was initially characterised in HepG2 cells, prior to studying the impact of HNF4α and co-activators on their expression. Metabolic stimuli investigated included endocrine mediators of the fasting (glucagon, dexamethasone) and fed (insulin) cycle, as well as compounds stimulating intracellular signalling downstream of glucagon (8-Br-cAMP, IBMX). Postprandial increases in glucose concentration were investigated by raising the glucose concentration of medium from 5.5 mM to 25 mM. Metabolic regulation of target genes by the activation of intracellular energy-sensor, AMPK, was also investigated by incubating HepG2 cells with AICAR.

Glucagon did not stimulate the expression of PEPCK in HepG2 cells, however downstream effectors of glucagon, 8-Br-cAMP and IBMX, produced 220% and 100% increases in PEPCK mRNA levels respectively (Figure 6.1B). These findings suggest glucagon signalling in HepG2 cells is defective at the level of the glucagon receptor. The expression of G-6-Pase catalytic subunit which, like PEPCK, is stimulated by glucagon in vivo, was also refractory to glucagon in HepG2 cells (Hornbuckle et al., 2004). Transfection of HepG2 cells with a glucagon receptor expression vector restored glucagon sensitivity to G-6-Pase catalytic subunit reporter gene expression (Hornbuckle et al., 2004). In agreement with the results of 8-Br-cAMP on gluconeogenic gene expression in HepG2 cells presented here (Figure 6.1B), cAMP analogues also stimulated PEPCK reporter gene expression (Rucktaeschel et al., 2000), and G-6-Pase mRNA expression in this cell line (Hornbuckle et al., 2004). In a similar manner to their effects on PEPCK mRNA expression, L-PK mRNA expression was not altered by glucagon itself, whilst downstream activators, 8-Br-cAMP and IBMX, decreased L-PK mRNA levels, albeit non-significantly (Figure 6.2A).

Investigation of the timecourse of cAMP action on PEPCK expression suggested that PEPCK mRNA levels reached a maximum after 6 hours incubation with 8-Br-cAMP (Figure 6.4A), a similar timeframe to the induction of PEPCK gene expression observed in primary rat hepatocytes (maximal at 2 hours) (Koo et al., 2005) or human hepatocytes (maximal at 3 hours) (Song and Chiang, 2006). Measurement of L-PK mRNA levels over the same
timeframe (0 – 8 hours) did not reveal a consistent trend in the inhibitory action of 8-Br-cAMP on L-PK gene expression (Figure 6.4B). The extent of inhibition of L-PK expression also varied under the control transfection condition in later experiments with transfected cells, with 8-Br-cAMP not producing a significant down-regulation in L-PK mRNA levels vs. control medium (Figures 6.10B, 6.13C and 6.23). On the other hand, a robust induction of PEPCK by 8-Br-cAMP was observed in HepG2 cells transfected with control DNA (Figures 6.10A, 6.13B and 6.18). These results suggest that heterogeneity in the responsiveness of L-PK gene regulation by cAMP may exist in HepG2 cells despite limiting the passage number to within ten passages. Alterations in the regulation of L-PK by metabolic stimuli have also been observed in rat hepatoma cell lines (Meienhofer et al., 1987; Lefrancois-Martinez et al., 1994).

Dexamethasone did not stimulate PEPCK mRNA expression in HepG2 cells (Figure 6.1B), with linear regression analysis suggesting that dexamethasone had a repressive effect on PEPCK mRNA expression compared to control medium (Figure 6.20). Dexamethasone did not synergise with 8-Br-cAMP to increase PEPCK expression (Figure 6.18), in contrast to results in primary cells (Yoon et al., 2001). The absence of dexamethasone-stimulated PEPCK expression in HepG2 cells in the results presented here is in agreement with a previous report showing dexamethasone does not increase PEPCK reporter gene expression above basal levels and even reduces PKA-stimulated PEPCK expression (Xing and Quinn, 1993; Sengupta and Wasylyk, 2001). In contrast, the combination of dexamethasone and 8-Br-cAMP enhanced PGC-1α mRNA expression (Figure 6.17C) and dexamethasone alone also had slight stimulatory effect on L-PK mRNA expression (Appendix, Figure 8.4). In primary hepatocytes, the combination of 8-Br-cAMP and dexamethasone is also known to stimulate PGC-1α mRNA expression (Yoon et al., 2001). However, expression-vector derived PGC-1α mRNA levels were also increased by this treatment (Figure 6.17D), whilst expression-vector derived-HNF4α expression was not (Figure 6.16A). Possibly dexamethasone acts post-transcriptionally to stabilise PGC-1α and L-PK transcripts, an action of glucocorticoids previously reported for L-PK gene expression (Vaulont et al., 1986).

The apoCIII and FAS genes exhibited non-significant trends to be inhibited and stimulated by insulin respectively (Figure 6.2B-C and Figure 6.5). The degree of inhibition of apoCIII
mRNA expression brought about by insulin (> 40%, Figure 6.2B) is in agreement with the percentage inhibition of apoCIII reporter gene expression in HepG2 cells, which ranged from between 30% and 50% (Li et al., 1995; Dallinga-Thie et al., 2001). FAS mRNA expression was raised only 88% by incubation of HepG2 cells with insulin. In comparison to the > 19-fold increase in FAS mRNA levels brought about by insulin in vivo and in primary rat hepatocytes (Paulauskis and Sul, 1989; Stoeckman and Towle, 2002), the response in HepG2 cells appears blunted and inconsistent.

AICAR did not produce significant effects on the expression of HNF4α mRNA or that of its target genes (Figures 6.1 to 6.3), despite PEPCK, LPK, FAS and SREBP-1c being targets of AMPK (Foretz et al., 1998; Lochhead et al., 2000; Foretz et al., 2005). These findings could be attributable to the AICAR concentration used here (0.2 mM): whilst incubation of primary rat hepatocytes with 0.2 mM AICAR resulted in inhibition of HNF4α target gene expression, including that of L-PK and apoCIII, AICAR concentrations of 1 mM or greater were required to markedly down-regulate expression of the HNF4α target gene, apoAV, in HepG2 cells (Prieur et al., 2005).

PPARα gene expression was unaffected by any of the metabolic stimuli under investigation (Figure 6.3A), in contrast to results obtained in primary rat hepatocytes, which indicate that PPARα mRNA expression is stimulated by dexamethasone and inhibited by insulin (Steiniger et al., 1994). This could be due to differences in transcription factor binding sites in the human vs. rat PPARα promoters (Pineda Torra et al., 2002).

In line with results presented in Chapter 5, SREBP-1c mRNA expression in HepG2 cells was not enhanced by insulin in the cell culture medium (Figure 6.3B), nor by glucagon or cAMP, which have been shown to inhibit SREBP-1c gene expression in primary rat hepatocytes (Foretz et al., 1999b).

6.8.2 Differential gene regulation by HNF4α

HNF4α may differentially regulate its target genes under different metabolic states through changes in HNF4α abundance and through post-translational modifications affecting its interactions with target promoters and/or co-factors. The former was investigated by monitoring HNF4α mRNA levels in the presence of hormones and protein kinase activators.
HNF4α mRNA expression in HepG2 cells was not modulated by fasting stimuli (dexamethasone, glucagon, 8-Br-cAMP or IBMX) or insulin (Figures 6.1A, 6.9, 6.13A and 6.16A). In agreement with these results, HNF4α mRNA levels also remained stable in primary human hepatocytes when incubated with glucagon or 8-Br-cAMP (Song and Chiang, 2006), in contrast to results in rodent models which show that HNF4α mRNA expression is up-regulated under fasting conditions (Oyadomari et al., 2000; Yoon et al., 2001). No changes in HNF4α expression were brought about by incubation with dexamethasone or insulin (Figures 6.1A and 6.9). This contrasts with results in primary rat hepatocytes, where insulin was found to antagonise the stimulatory action of dexamethasone on HNF4α mRNA expression (Oyadomari et al., 2000). In summary, changes in endogenous HNF4α mRNA abundance do not play a role in the regulation of HNF4α target genes by metabolic stimuli in HepG2 cells.

Changes in the interactions of HNF4α with target promoters induced by metabolic stimuli could also regulate HNF4α target genes in a differential manner. This aspect was investigated by knockdown of HNF4α function in HepG2 cells. The HNF4α knockdown approach was performed by expressing a DN-HNF4α, which forms non-DNA binding heterodimers with wild-type HNF4α (Taylor et al., 1996). The impact of DN-HNF4α expression was studied on the expression of HNF4α target genes: PEPCK, L-PK, apoCIII and FAS (Figures 6.6 to 6.12). Overexpression of DN-HNF4α did not bring about any specific changes in the expression of any of the four target genes investigated under control incubation conditions (Figures 6.6 and 6.7), possibly due to insufficient expression of these genes under the conditions employed. In support of this hypothesis, HNF4α has been shown to bind to the apoCIII promoter in HepG2 cells, however recruitment of RNA polymerase II is deficient in this cell line (Martinez-Jimenez et al., 2006). On the other hand, expression of an alternative DN-HNF4α with an N-terminal truncation, lacking a DNA-binding domain, did decrease expression of apoCIII at the mRNA level in another study in HepG2 cells (Fraser et al., 1997).

Overexpression of DN-HNF4α was associated with a non-significant increase in mean PEPCK mRNA expression from 350% to 550% relative to that of the control incubation and transfection condition (Figure 6.10A). This increase appears counterintuitive, when
compared to the downward trend in HNF4α-dependent reporter gene expression observed in the presence of DN-HNF4α, as shown here (Figure 6.8) and in published studies (Taylor et al., 1996; Gourdon et al., 1999; Jahan and Chiang, 2005). HNF4α mRNA expression was also higher in the presence of DN-HNF4α (Figure 6.9), although due to lack of specific primers, it was not possible to ascertain whether this increase corresponded to endogenous transcripts or DN-HNF4α mRNA. Further investigation of the effect of DN-HNF4α on PEPCK mRNA expression revealed that PEPCK mRNA levels positively correlated with those of HNF4α (Figure 6.11A). HNF4α gene expression has been shown to be subject to negative autoregulation and overexpression of another DN-HNF4α lacking DNA-binding activity resulted in increased endogenous HNF4α mRNA expression (Magenheim et al., 2005). Therefore, it is possible to speculate that the increase in HNF4α mRNA expression observed in the presence of DN-HNF4α (Figure 6.9) includes an increase in endogenous HNF4α transcripts, which could be responsible for the increased in PEPCK expression under 8-Br-cAMP conditions (Figure 6.11A). Increased endogenous HNF4α levels in the presence of DN-HNF4α may also explain why a four-fold excess of DN-HNF4α plasmid DNA over wild-type HNF4α expression vector only produced a 25% decrease in reporter gene expression (Figure 6.8).

Increased endogenous HNF4α mRNA expression in the presence of DN-HNF4α could also be responsible for the positive, though non-significant, trend in the relationship between HNF4α and L-PK mRNA levels in the presence of 8-Br-cAMP (Figure 6.11B). In the presence of DN-HNF4α but not control expression vector, 8-Br-cAMP also had a significant inhibitory effect on L-PK expression, suggesting that HNF4α has a role in mediating the negative effects of glucagon-cAMP on L-PK expression. HNF4α binding studies using rat liver extracts and reporter gene studies in rat hepatocytes also point to HNF4α being a key mediator of the negative effects of cAMP on L-PK expression (Viollet et al., 1997; Gourdon et al., 1999).

6.8.3 The role of co-activators in differential gene regulation by HNF4α

The mechanisms by which HNF4α differentially regulates transcription of PEPCK and L-PK were further investigated by studying the effects of co-activators, p300 and PGC-1α. When individual co-activators were overexpressed, induction of PEPCK expression by 8-Br-cAMP
was evident under the control and PGC-1α overexpression conditions, but not in the p300 overexpression condition (Figure 6.13B), suggesting that PGC-1α overexpression facilitates PEPCK induction by 8-Br-cAMP. PEPCK expression also showed positive correlation with HNF4α expression levels (Figure 6.14), suggesting that co-activator effects were dependent upon HNF4α levels, in agreement with studies showing the partnership of HNF4α and PGC-1α in PEPCK expression (Rhee et al., 2003). For the L-PK gene, inhibition of its expression was evident when p300 was overexpressed, but not in the control or PGC-1α conditions (Figure 6.13C). As with co-activator effects on PEPCK expression, HNF4α expression levels appeared to be a determining factor for L-PK expression (Figure 6.15).

The relationship between HNF4α and co-activators was further investigated by co-expression of HNF4α, p300 and PGC-1α under a range of metabolic conditions (Figures 6.16 to 6.26). Maximal mean PEPCK mRNA expression occurred in the presence of 8-Br-cAMP and overexpression of HNF4α, p300 and PGC-1α (Figure 6.18), which coincided with maximal HNF4α expression levels (Figure 6.16A).

HNF4α expression levels positively correlated with basal PEPCK expression (control medium) and over a limited range of HNF4α levels under incubation with 8-Br-cAMP (Figure 6.19A). However, the linearity of the relationship between the two variables did not extend to datapoints corresponding to maximum PEPCK or maximum HNF4α expression, suggesting that HNF4α levels are not the limiting factor in promoting PEPCK transcription, once full occupancy of the PEPCK promoter by HNF4α is achieved (Figure 6.19A). Further induction of PEPCK expression is then dependent on the interaction of HNF4α and PGC-1α, as illustrated by the linear relationship between exogenously expressed PGC-1α and PEPCK mRNA levels in the presence of 8-Br-cAMP, either alone or in combination with dexamethasone (Figures 6.19D and 6.21D). These results are in broad agreement with published work, which show that HNF4α expression is a requirement for the induction of PEPCK expression brought about by PGC-1α overexpression (Rhee et al., 2003). Increased degradation of the GR in the presence of dexamethasone (Sengupta and Wasylyk, 2001) could lead to reduced activity of HNF4α and PGC-1α in stimulating PEPCK expression, as evidenced by a lack of correlation between HNF4α and PGC-1α with PEPCK mRNA.
expression levels in the presence of dexamethasone (Figure 6.20), suggesting the GR could be a component of the complex with HNF4α and PGC-1α (Yoon et al., 2001).

Under the opposite metabolic conditions of high glucose concentration and insulin, PGC-1α expression levels also correlated with those of PEPCK, whereas PEPCK expression was independent of HNF4α expression levels, in contrast to the control incubation condition (Figure 6.22). This suggests that HNF4α and PGC-1α do not functionally interact under fed conditions at the PEPCK promoter. Indeed, decreased binding of HNF4α to the distal GRU upon incubation with insulin has been observed in vivo (Cassuto et al., 2005). Possibly, PGC-1α activates PEPCK expression via alternative protein-protein interactions under these conditions, such as with the GR (Knutti et al., 2000; Yoon et al., 2001).

For the oppositely-regulated HNF4α target gene, L-PK, the inhibitory effects of 8-Br-cAMP on L-PK expression were diminished when HNF4α was overexpressed together with p300 and PGC-1α (Figure 6.23). Further analysis revealed a positive correlation between HNF4α mRNA levels with L-PK mRNA levels in the presence of 8-Br-cAMP (Figure 6.24A), suggesting that HNF4α overexpression counteracts the inhibitory effect of cAMP on L-PK expression, but not under other metabolic conditions (Figures 6.23-6.25). This confirms that HNF4α is a critical factor in the response of L-PK to fasting stimuli, in line with published works (Bergot et al., 1992; Viollet et al., 1997; Gourdon et al., 1999). Exogenously-derived PGC-1α expression levels also positively correlated with L-PK expression under this metabolic condition (Figure 6.24D), suggesting that the HNF4α-PGC-1α interaction could also be active in permitting L-PK in the presence of 8-Br-cAMP. On the other hand, the decrease in L-PK expression in the presence of 25 mM glucose and insulin (Figure 6.23) coincided with a non-significant downward trend in the relationship between ectopically expressed PGC-1α and L-PK mRNA levels (Figure 6.26D). This result suggests that PGC-1α does not co-activate HNF4α under these conditions and may disrupt interactions between HNF4α and other factors bound to the L-PK promoter.

Unlike HNF4α and PGC-1α, p300 mRNA levels were not raised by p300 overexpression (Figure 6.16B) which may be one reason why p300 mRNA levels did not correlate with those of PEPCK or L-PK under any of the incubation conditions (Figures 6.19 – 6.22, Figures 6.24-
6.26 and Appendix, Figures 8.4-8.8). p300 mRNA levels may be tightly controlled at a post-transcriptional level, such that increased transcription of p300 did not impact on the p300 mRNA pool. In addition, p300 mRNA expression may already be at a level in HepG2 cells which does not limit the formation of an active complex at target promoters. Indeed, p300 was found to be expressed in HepG2 cells at levels comparable to those of primary human hepatocytes (Martinez-Jimenez et al., 2006). When p300 alone was overexpressed, L-PK expression was inhibited by incubation of cells with 8-Br-cAMP (Figure 6.13C), which suggests that p300 does not compete with the factors responsible for mediating this effect. This finding is in agreement with those of Gourdon et al. (1999), who found that p300 overexpression did not prevent the inhibition of L-PK reporter gene expression by cAMP.

In summary, HNF4α was found to play a role in cAMP-stimulated PEPCK expression based on the results of DN-HNF4α overexpression (Section 6.5). HNF4α was also a limiting factor in PEPCK expression when individual co-activators were overexpressed (Section 6.6). Overexpression of HNF4α together with p300 and PGC-1α raised cAMP-stimulated PEPCK expression, with PEPCK mRNA levels positively correlating with those of HNF4α and PGC-1α in the presence of 8-Br-cAMP, suggesting that HNF4α and PGC-1α interact to stimulate PEPCK expression under these conditions (Section 6.7). HNF4α mRNA expression was also found to be a limiting factor in L-PK expression in the presence of 8-Br-cAMP, based on DN-HNF4α and co-activator overexpression experiments (Sections 6.5 and 6.6). Overexpression of HNF4α, p300 and PGC-1α relieved the inhibitory action of 8-Br-cAMP on L-PK expression, whilst L-PK expression in the presence of 25 mM glucose was reduced (Section 6.7). Taken together, the results of studying PEPCK and L-PK expression under fasting vs. fed conditions suggest that HNF4α positively interacts with PGC-1α under fasting conditions, whilst a protein complex of HNF4α and PGC-1α does not activate transcription under fed conditions.
Chapter 7

Conclusion
Chapter 7:

7.1 Conclusion

The development of T2DM is associated with the dysregulation of metabolic pathways in response to endocrine stimuli which signal the nutritional status of the organism, namely insulin signalling the fed state and glucagon signalling the fasting state. In the state of insulin resistance associated with the development of T2DM, insulin fails to suppress hepatic gluconeogenesis. On the other hand, hepatic glycolysis and lipogenesis are stimulated by hyperinsulinaemia, which results from the compensatory response of the β-cell to insulin resistance in the liver, muscle and adipose tissue (Kahn, 2003). Since HNF4α is a candidate gene for T2DM, its regulation of target genes in the above-mentioned metabolic pathways may play a role in the pathogenesis of the disease (Gupta and Kaestner, 2004). Therefore, this project focussed on the role of HNF4α in the regulation of its hepatic target genes by metabolic stimuli and possible mechanisms which facilitate its involvement in differentially-controlled processes.

In vitro hepatic cellular models are a useful resource for the study of liver biology under disease states such as diet-induced obesity, or for prediction of in vivo toxicology of investigational new drugs (Gomez-Lechon et al., 2007; Castell et al., 2006). Therefore it was important to find a suitable model for studying HNF4α activity and the first aim of this study was to characterise the expression of HNF4α and its splice variants in three in vitro human liver models, the HuH7, HepG2 and Hep3B hepatoma cell lines. HepG2 and Hep3B cells were found to express a similar profile of HNF4α splice variants compared to human liver (Chapter 3), suggesting that these cell lines provide the best models for study of HNF4α activity. Since the HepG2 cell line displayed physiological trends in the regulation of apoCIII and FAS by insulin and high glucose concentration (Chapter 3), this cell line was thought to provide the best in vitro model for further exploration of the role of HNF4α in the metabolic regulation of its target genes (Chapters 4 to 6).

However, the regulation of HNF4α target genes in HepG2 cells differed in some aspects to results obtained in vivo and in primary hepatocyte model systems. For example, despite apoCIII being a well-established target gene of HNF4α (Section 1.5.4), apoCIII mRNA
expression in HepG2 cells was not modulated by overexpression of DN-HNF4α (Figure 6.7). In previous studies, many target genes of HNFs were found to be down-regulated in hepatocellular carcinomas (Xu et al., 2001a). ApoCIII was amongst the genes that were down-regulated in gene expression microarray profiling of different hepatocellular carcinoma samples, as were other HNF4α target genes, PEPCK, G-6-Pase, transthyretin, transferrin, GAPDH, and FAS (Xu et al., 2001a; Xu et al., 2001b). Overexpression of wild-type HNF4α was found to reactivate apoCIII expression in HepG2 cells, suggesting that apoCIII transcription responds to increased, rather than decreased, HNF4α activity (Naiki et al., 2004). These findings suggest that the HepG2 cell line displays lower HNF4α activity than primary hepatocytes, which can limit the expression of HNF4α target genes (Castell et al., 2006).

The metabolic flexibility of in vitro hepatocellular models in terms of responsiveness to hormonal stimulation, for example, in the up-regulation of glycogen synthesis in response to insulin is considered a key feature of differentiated liver function (Gomez-Lechon et al., 2001). In the current study, metabolic flexibility at the level of gene transcription in HepG2 cells was important for studying changes in HNF4α activity due to metabolic stimuli such as insulin, glucagon and glucocorticoids. Insulin was found to stimulate HNF4α-dependent SREBP-1c reporter gene expression (Chapter 4), however insulin did not activate the transcription of endogenous SREBP-1c gene transcription (Chapter 5). A trend for insulin to stimulate endogenous FAS gene expression was observed, however this was inconsistent (Figure 6.2). An intact insulin-PI3K-PKB signalling pathway has been utilised in HepG2 cells to study the effects of glucotoxicity on the development of insulin resistance, and intracellular effects of insulin such as stimulation of glycogen synthesis are observed in HepG2 cells (Nakajima et al., 2000), suggesting that insulin signalling cascades are active in HepG2 cells. Insulin is able to modulate the expression of some endogenous genes in HepG2 cells, such as the suppression of insulin-like growth factor-binding protein-1 and MTP gene expression by insulin (Cichy et al., 1998; Au et al., 2003), but not others, for example, PEPCK (Quinn and Yeagley, 2005). This suggests that the limited induction of SREBP-1c and its target gene, FAS, by insulin could be due to the expression of gene-specific factors being limiting in HepG2 cells (Quinn and Yeagley, 2005). The results of Chapter 5 in conjunction with published work suggest that SREBP-1c expression is down-regulated and
non-inducible in HepG2 cells, hence low SREBP-1c expression could limit the stimulation of FAS mRNA expression by insulin (Shimomura et al., 1997; Foretz et al., 1999a).

The induction of L-PK mRNA expression by high media glucose concentration was also limited in HepG2 cells (Figure 6.22). ChREBP is required for induction of L-PK expression by high glucose concentration (Yamashita et al., 2001), however the relative expression of this transcription factor in HepG2 cells compared to primary hepatocytes is unknown. Possibly low expression of ChREBP prevents an inductive effect of high glucose concentration on L-PK mRNA expression. Alternatively a repressor of L-PK expression in HepG2 cells could limit L-PK mRNA expression. An extinguisher of L-PK expression acting at an upstream region (between -1.2 and 2.1 kb) in the L-PK promoter was observed in reporter gene experiments with HepG2 cells (Raymondjean et al., 1991; Cognet et al., 1991). The latter conclusion would explain why induction of expression of a reporter plasmid containing elements of the L-PK proximal promoter (-196 bp) showed induction by high glucose concentration in HepG2 cells (Figure 4.1), whilst endogenous L-PK mRNA expression was not inducible by high glucose concentration (Figure 6.22). As discussed in Chapters 4 and 6, lack of dexamethasone effects on PEPCK expression in HepG2 cells are attributable to increased turnover of the GR in response to dexamethasone in this cell line (Xing and Quinn, 1993; O'Brien et al., 1995; Sengupta and Wasylyk, 2001). Overexpression of GR restores glucocorticoid-responsiveness to PEPCK reporter gene expression, confirming the expression of the GR as a key limiting factor in the effect of dexamethasone in HepG2 cells (Cassuto et al., 2005).

The second aim of the current study was to study the regulation of HNF4α transcriptional activity by fasting and feeding stimuli as reflected in downstream effects on target gene expression in order to evaluate the role of HNF4α in controlling the metabolic pathways of gluconeogenesis, glycolysis and lipogenesis.

As a model gene of the gluconeogenic pathway, PEPCK gene transcription is up-regulated in the fasting state by glucagon and glucocorticoids, hence the role of HNF4α in these effects was investigated. Although PEPCK reporter gene expression was not responsive to glucagon in HepG2 cells (Chapter 4), the downstream activator, 8-Br-cAMP, stimulated PEPCK mRNA expression in this cell line (Figures 6.1). Under conditions of DN-HNF4α, HNF4α
mRNA levels positively correlated with those in PEPCK in the presence of 8-Br-cAMP, suggesting that glucagon stimulates HNF4α activity and results in increased PEPCK transcription. Overexpression of HNF4α, PGC-1α and p300 also enhanced PEPCK mRNA expression in the presence of 8-Br-cAMP. From these results, it was concluded that cAMP (which acts downstream of glucagon) stimulates HNF4α activity and promotes the expression of PEPCK and potentially other gluconeogenic enzymes. This finding agrees published works, which point to HNF4α as a key player in up-regulation of PEPCK and G-6-Pase by glucagon and cAMP (Rhee et al., 2003; Gautier-Stein et al., 2005). In T2DM hepatic glucose output is increased, with one cause being a deficiency in insulin to inhibit PEPCK transcription (Postic et al., 2004). In this scenario, it is postulated that HNF4α would stimulate gluconeogenesis and contribute to increased hepatic glucose output. This would be exacerbated by the increased expression of PGC-1α observed in the diabetic state (Finck and Kelly, 2006).

The results of the current study also led to the conclusion that HNF4α plays a role in the inhibition of L-PK mRNA expression by glucagon, as demonstrated by the positive correlation between HNF4α and L-PK mRNA levels under 8-Br-cAMP incubation conditions (Figure 6.15). In addition, inhibition of L-PK mRNA expression by 8-Br-cAMP was overcome by HNF4α, p300 and PGC-1α overexpression (Figure 6.22), suggesting that the balance between HNF4α and other transcription factors is important in determining L-PK gene repression by cAMP. This finding is in agreement with studies in rat liver which demonstrate a role for HNF4α down-regulation of L-PK transcription in the fasting state (Viollet et al., 1997; Gourdon et al., 1999). It is hypothesised that increased HNF4α activity would result in increased flux through the glycolytic pathway, contributing to substrate availability for lipogenesis.

Investigation of the role of HNF4α in lipogenic gene expression focussed on the expression of the SREBP-1c and FAS genes, two key regulators of lipogenesis at the transcriptional and enzyme levels respectively (Sections 1.3.3 and 1.4.3). As the transcription of these target genes is stimulated by insulin (Section 1.8.3 and 1.8.4), the hypothesis that HNF4α transcriptional activity is enhanced by insulin was tested. The stimulation of HNF4α-dependent SREBP-1c reporter gene expression by incubation of HepG2 cells with insulin provides evidence for this hypothesis (Figures 4.5 and 4.9). On the other hand, results of
DN-HNF4α overexpression on FAS mRNA expression (Figure 6.12) were relatively inconclusive, in that insulin did not robustly induce FAS mRNA expression in HepG2 cells (Figure 6.2). Based on the results of SREBP-1c reporter gene studies, it was concluded that insulin stimulates HNF4α activity and promotes the expression of SREBP-1c. This finding suggests that HNF4α could contribute to the up-regulation of SREBP-1c expression observed in insulin resistant states (Shimomura et al., 2000) and thereby contribute to increased de novo lipogenesis observed in NAFLD (Donnelly et al., 2005).

The final aim of the current research was to investigate the mechanisms which enable HNF4α to differentially regulate target genes in different metabolic pathways. Further elucidation of these mechanisms may form a groundwork for the development of therapies targeted to reduce hepatic gluconeogenesis in T2DM or to inhibit hepatic lipogenesis in NAFLD. The findings of this project suggest that different protein complexes containing different HNF4α co-factors are involved in the activation of HNF4α target genes in the fasting state compared to the fed state. A hypothetical series of models was devised in order to explain experimental observations (Figure 7.1), which will be elaborated on below.

The co-activator PGC-1α is an important HNF4α partner in expression of gluconeogenic enzymes, apolipoproteins and bile acid synthesis enzymes (Rhee et al., 2003; Rhee et al., 2006; Bhalla et al., 2004). The findings from this project provide novel evidence that the interaction of HNF4α with PGC-1α are dependent on metabolic stimuli.

In studying regulation of PEPCK gene expression by HNF4α and PGC-1α, it was noted that incubation conditions altered the interaction between HNF4α and PGC-1α. Co-transfection of HepG2 cells with HNF4α and PGC-1α followed by incubation with standard culture medium repressed PEPCK reporter gene expression compared to HNF4α overexpression alone (Figure 4.4). On the other hand, HNF4α, PGC-1α and p300 overexpression in the presence of 8-Br-cAMP enhanced PEPCK mRNA expression (Figure 6.18). In combination, these results suggest that increased intracellular cAMP levels enhance the interaction of HNF4α and PGC-1α and that this interaction could be dependent on the phosphorylation state of either or both factors. In a dephosphorylated state, a negative regulatory domain of PGC-1α has been shown to interact with the co-repressor p160myb (Fan et al., 2004).

Phosphorylation of PGC-1α by p38 MAPK increases its transcriptional activity and relieves
its interactions with co-repressors (Puigserver et al., 2001; Fan et al., 2004). p38 MAPK activity has been shown to be stimulated by glucagon signalling in hepatocytes, with phosphorylation of PGC-1α by p38 MAPK playing a stimulatory role in gluconeogenesis (Cao et al., 2005).

HNF4α is also phosphorylated downstream of glucagon by PKA and also potentially by p38 MAPK (Viollet et al., 1997; Guo et al., 2006). HNF4α phosphorylation by PKA on S129 has been linked to decreased DNA-binding activity, whilst phosphorylation by p38 MAPK on S158 has been shown to enhance DNA-binding and trans-activation potential, and to stabilise HNF4α protein levels (Viollet et al., 1997; Guo et al., 2006; Xu et al., 2007). Therefore, it is postulated that a specific HNF4α phosphorylation pattern is brought about in the fasting state which facilitates the interaction of HNF4α with PGC-1α (Figure 7.1A).

A second stimulus in the fasting state is raised plasma glucocorticoid levels (Section 1.1.2). Since activation of PEPCK expression by glucocorticoids was not observed in HepG2 cells (as discussed in the first part of this section), it is hypothesised that HNF4α and PGC-1α form part of a multi-protein complex with the GR within the GRU of the PEPCK promoter (Figure 7.1A), based on other studies which show that glucocorticoids promote binding of HNF4α to the PEPCK promoter (Cassuto et al., 2005).

Under the opposite metabolic conditions corresponding to the fed state, results of Q-PCR analysis indicated that HNF4α and PGC-1α do not functionally interact, as a positive correlation with PEPCK mRNA expression levels was observed under these conditions for PGC-1α, but not for HNF4α (Figure 6.21). This result suggests that insulin and high glucose concentration alter the phosphorylation state of HNF4α and potentially disrupt its interactions with PGC-1α and the GR in the fed state.

Results of studying L-PK and SREBP-1c expression also led to the conclusion that PGC-1α is not the functional partner of HNF4α under fed state conditions. Co-transfection of HNF4α, p300 and PGC-1α tended to suppress L-PK mRNA expression under cell culture conditions mimicking the fed state (Figure 6.22). L-PK reporter gene expression was enhanced by high glucose concentration (Figure 4.1) and insulin enhanced reporter gene expression from L-PK promoter constructs containing HNF4α and ChREBP binding sites.
when HNF4α was overexpressed (Figure 4.2). These results suggest that HNF4α interacts with other protein partners in the fed state at the L-PK promoter. A candidate for the interaction with HNF4α at the L-PK promoter is ChREBP, although additional co-activator proteins may be involved (Figure 7.1B). In addition to the activation of ChREBP by dephosphorylation by glucose (Kawaguchi et al., 2001), the interaction of HNF4α with ChREBP is possibly further enhanced by an altered HNF4α phosphorylation status in the fed state. In the absence of glucagon signalling, HNF4α is likely to be dephosphorylated at S134 (Viollet et al., 1997).

The results of SREBP-1c reporter construct experiments suggest that insulin enhances HNF4α activity in the context of the SREBP-1c promoter. As noted for L-PK above, this enhancement of transcriptional activity could be through changes in HNF4α phosphorylation state brought about by insulin downstream signalling (Figure 7.1C). Co-transfection of HNF4α with PGC-1α or p300 repressed SREBP-1c reporter gene expression, (Figure 4.5 to 4.8), providing further evidence that HNF4α:co-activator interactions are dependent on promoter context and metabolic stimuli. Another transcription factor or co-activator may be a functional partner of HNF4α at the SREBP-1c promoter. A possible candidate for interaction with HNF4α at the SREBP-1c promoter is a member of the SREBP family, with an SRE motif present approximately 400 bp downstream of the putative HNF4α binding site in the human SREBP-1c promoter (Figure 4.10). HNF4α has been shown to interact in a non-DNA binding manner with SREBP-2 to activate sterol isomerase gene expression (Misawa et al., 2003). On the other hand, the interaction of SREBP-1 with HNF4α prevents PGC-1α recruitment to the PEPCK and CYP7A1 genes and inhibits their transcription in the fed state (Yamamoto et al., 2004; Li et al., 2006; Ponugoti et al., 2007). In the case of the SREBP-1c gene promoter, the converse scenario could occur: HNF4α and SREBP cooperate in activating SREBP-1c transcription, hence overexpressed PGC-1α competes with SREBP for binding of HNF4α, leading to decreased SREBP-1c gene expression (Figure 7.1C). As noted for PEPCK and L-PK, the phosphorylation state of HNF4α and its protein partners could determine whether a co-factor such as PGC-1α behaves as a co-activator or a co-repressor.
Figure 7.1: Model of HNF4α-dependent regulation of (A) PEPCK, (B) L-PK and (C) SREBP-1c gene expression in response to metabolic stimuli. (A) HNF4α interactions with PGC-1α and GR in stimulating PEPCK expression may be dependent on the phosphorylation state of HNF4α and PGC-1α. (B) HNF4α forms a nucleoprotein complex with ChREBP to activate L-PK expression in response to high glucose and insulin. (C) HNF4α activates SREBP-1c in response to insulin, potentially through an interaction with SREBP, which can be disrupted by PGC-1α and p300.
In summary, glucagon was found to activate HNF4α in promoting PEPCK expression through synergy of HNF4α with PGC-1α. Insulin activated HNF4α in promoting the expression of the L-PK and SREBP-1c genes, an effect repressed by PGC-1α or p300, suggesting that alternate protein complexes are central to the role of HNF4α in glycolytic and lipogenic gene expression. Overall, the current research provides new evidence that HNF4α activity is modulated by metabolic stimuli and suggests that the balance between insulin and glucagon in the circulation impacts on the subset of HNF4α target genes which are activated and their dysregulation in metabolic disease states.

7.2 Recommendations for future work

It is planned to publish the results of the current project in a peer-reviewed journal in due course. In addition, the current results could be built on using the following approaches:

- **Expansion of HNF4α target gene analysis**
  The proposed model (Figure 7.1) for the regulation of fasting vs. feeding-induced genes by HNF4α could be confirmed by extending the target genes analysed from the experiment where HNF4α, p300 and PGC-1α were overexpressed and effects on PEPCK and L-PK gene expression studied (Figures 6.16 - 6.25). Target genes which could be analysed by quantitative RT-PCR include additional genes of the gluconeogenic pathway (G-6-Pase), glycolytic pathway (GK, aldolase B) and lipogenic pathway (FAS, MTP).

- **HNF4α knock-down by small-interfering RNA**
  The role of HNF4α in metabolic regulation of its target genes could be further investigated by expression of small-interfering RNA (siRNA) against HNF4α, which knocks down gene function by inducing cleavage of mRNA coding for the siRNA-targeted gene. This approach has proved useful in demonstrating a role for HNF4α in the induction of FAS mRNA expression by carbohydrate in rat hepatocytes (Adamson et al., 2006). An siRNA approach would also lend itself to further investigation of the role of specific HNF4α splice variants in target gene expression.

- **Further validation of human SREBP-1c as a HNF4α target gene**
  Since further validation of human SREBP-1c as a HNF4α target gene was not possible due to limited SREBP-1c mRNA expression in HepG2 cells (Chapter 5), other approaches could be
employed. HNF4α binding to the putative HNF4α binding site in the human SREBP-1c promoter has yet to be demonstrated (Tarling et al., 2004). HNF4α binding to the SREBP-1 promoter could be investigated in vitro by electrophoretic mobility shift assays (EMSA) with oligonucleotides corresponding to the HNF4α element in the SREBP-1c promoter. Chromatin immunoprecipitation (ChIP) analysis with anti-HNF4α antibodies using primary human hepatocytes would demonstrate genomic binding of HNF4α to the SREBP-1c promoter.

**Role of HNF4α phosphorylation state in differential regulation**

The proposed role of HNF4α phosphorylation status in the regulation of differentially expressed target genes (Figure 7.1) could be tested by the utilisation of HNF4α phosphorylation site mutants. In this approach, the relevant serine (or threonine) residue is mutated to an alanine to generate a HNF4α which cannot be phosphorylated at that residue, or a phosphomimetic is generated by the mutation from serine to aspartate. Combined with inhibitors of the protein kinases of interest (e.g. p38 MAPK), this approach has enabled the identification of the HNF4α serine residue (S158) involved in the activation of target gene expression in response to inflammatory redox stimuli (Guo et al., 2006). A larger scale analysis of changes in the phosphorylation state of HNF4α, other transcription factors and co-factors in response to a particular metabolic stimuli could be undertaken using a phosphoproteomics approach (Ptacek and Snyder, 2006).

**HNF4α protein-protein interactions**

The potential interactions of HNF4α with protein partners such as PGC-1α, ChREBP and SREBP under different metabolic conditions (Figure 7.1) could be investigated by chromatin immunoprecipitation (ChIP) analysis, whereby transcription factors are crosslinked to DNA by formaldehyde treatment followed by immunoprecipitation with an antibody against the protein of interest and PCR with primers specific to elements of the target gene promoter (Das et al., 2004). Antibodies against the HNF4α protein partner of interest combined with PCR primers specific to the HNF4α binding site in the target gene promoter would indicate an interaction between HNF4α and a protein partner at the target gene promoter.
Metabolite profiling

In order to study the impact of changes in HNF4α activity on flux through metabolic pathways, a metabolomics approach could be employed combined with HNF4α knockdown or overexpression. The application of metabolomics methods to study metabolite profiles related to glucagon function in HepG2 cells has recently been presented (Thumser et al., 2007a) (see List of Abstracts).
Chapter 8

Appendix
**Chapter 8:**

### 8.1 Sequencing of HNF4α splice variant RT-PCR products

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CLUSTAL W (1.82) multiple sequence alignment

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**Box 8.1: ClustalW comparison of HNF4α1 sequence and the results of sequencing the HNF4α1 RT-PCR product.** HepG2 RNA was used in RT-PCR with primers specific to the HNF4α1 splice variant (Section 2.2.5). The HNF4α1 RT-PCR product was purified and sequenced using primer seqprimerF2 (Section 2.2.9). Results of the sequencing reaction (hnf4alseqF2) were compared with sequence based on human HNF4α1 sequence (Genbank Accession No.: NM_178849) (hnf4alprod) using ClustalW. Asterisks underneath the aligned sequences indicate that the sequences are identical. The sequencing results show 100% homology with the HNF4α1 sequence from base number 73 up to and including the final section which is unique to the HNF4α1 splice variant. The yellow/green boundary indicates the splice site between exons 9 and 10 of the HNF4α1 coding sequence which is unique to this splice variant.
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Alignment

CLUSTAL W (1.83) multiple sequence alignment

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</table>

Box 8.2: ClustalW comparison of HNF4α2 sequence and the results of sequencing the HNF4α2 RT-PCR product. HepG2 RNA was used in RT-PCR with primers specific to the HNF4α2 splice variant (Section 2.2.5). The HNF4α2 RT-PCR product was purified and sequenced using primer seqprimerF2 (Section 2.2.9). Results of the sequencing reaction (a2resultsF2CORR) were compared with sequence based on human HNF4α2 sequence (Genbank Accession No.: NM_000457) (HNF4a2RTPCR) using ClustalW. Asterisks underneath the aligned sequences indicate that the sequences are identical. The sequencing results show 100% homology with the HNF4α2 sequence from base number 33 (sequencing result) up to and including the final 26 bp section which is unique to the HNF4α2 splice variant (highlighted area).
Box 8.3: ClustalW comparison of HNF4α3 sequence and the results of sequencing the HNF4α3 RT-PCR product. HuH7 RNA was used in RT-PCR with primers specific to the HNF4α3 splice variant (Section 2.2.5). The HNF4α3 RT-PCR product was purified and sequenced (Section 2.2.9). Results of the sequencing reaction (seqresult) were compared with sequence based on human HNF4α3 sequence (Genbank Accession No.: NM_178850) (HNF4alphaSprod) using ClustalW. Asterisks underneath the sequences indicated sequences are identical. The sequencing results show 100% homology with the HNF4α3 sequence up to the last 30 bases where the sequencing reaction breaks down. Highlighted area (yellow) indicates the bases which are unique to the HNF4α3 splice variant. A similar ClustalW comparison with the HNF4α2 splice variant yielded a score of only 40%.
<table>
<thead>
<tr>
<th>SeqA Name</th>
<th>Len(nt)</th>
<th>SeqB Name</th>
<th>Len(nt)</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNF4a7</td>
<td>1329</td>
<td>seqresult</td>
<td>350</td>
<td>94</td>
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</tbody>
</table>

Box 8.4: ClustalW comparison of HNF4a7 sequence and the results of sequencing the HNF4a7/8 RT-PCR product. HepG2 RNA was used in RT-PCR with primers specific to the HNF4a7/8 splice variant (Section 2.2.5). The HNF4a7/8 RT-PCR product was purified and sequenced using antisense primer a7primerR1 (underlined bases) (Section 2.2.9). Results of the sequencing reaction (seqresult) were compared with sequence based on human HNF4a7 sequence (Genbank Accession No.: AY680696) (HNF4a7) using ClustalW. Asterisks underneath the sequences indicated sequences are identical. The sequencing results show 100% homology with the HNF4a7/8 sequence in exon 1D (highlighted area) which is unique to the HNF4a7/8 splice variant.
8.2 Results of statistical analysis (Chapter 4)

8.2.1 Key

<table>
<thead>
<tr>
<th>Symbol</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N.S.</td>
<td>≥ 0.05</td>
</tr>
<tr>
<td>*</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>**</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>***</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

8.2.2 Figure 4.1: Summary of two-way ANOVA and posthoc analysis

Effect of glucose concentration and hormones on L-PK reporter gene expression in HepG2 cells (Figure 4.1):

Results of two-way ANOVA

<table>
<thead>
<tr>
<th>Source of Variation</th>
<th>% of total variation</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interaction</td>
<td>24.55</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Incubation condition</td>
<td>27.09</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Reporter gene</td>
<td>35.86</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

Post-hoc analysis (Bonferroni):

<table>
<thead>
<tr>
<th>Incubation condition</th>
<th>5.5 mM glucose</th>
<th>25 mM glucose</th>
<th>5.5 mM glucose + Insulin</th>
<th>25 mM glucose + Insulin</th>
<th>5.5 mM glucose + HSM</th>
<th>25 mM glucose + HSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5 mM glucose</td>
<td>-</td>
<td>***</td>
<td>N.S.</td>
<td>***</td>
<td>N.S.</td>
<td>***</td>
</tr>
<tr>
<td>25 mM glucose</td>
<td>***</td>
<td>-</td>
<td>***</td>
<td>N.S.</td>
<td>***</td>
<td>N.S.</td>
</tr>
<tr>
<td>5.5 mM glucose + Insulin</td>
<td>N.S.</td>
<td>***</td>
<td>-</td>
<td>***</td>
<td>N.S.</td>
<td>***</td>
</tr>
<tr>
<td>25 mM glucose + Insulin</td>
<td>***</td>
<td>N.S.</td>
<td>***</td>
<td>-</td>
<td>***</td>
<td>*</td>
</tr>
<tr>
<td>5.5 mM glucose + HSM</td>
<td>N.S.</td>
<td>***</td>
<td>N.S.</td>
<td>***</td>
<td>-</td>
<td>***</td>
</tr>
<tr>
<td>25 mM glucose + HSM</td>
<td>***</td>
<td>N.S.</td>
<td>***</td>
<td>*</td>
<td>***</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 8.2: Post-hoc analysis - Incubation condition: Reporter gene: (L3)3-54PK

<table>
<thead>
<tr>
<th>Incubation condition</th>
<th>5.5 mM glucose</th>
<th>25 mM glucose</th>
<th>5.5 mM glucose + Insulin</th>
<th>25 mM glucose + Insulin</th>
<th>5.5 mM glucose + HSM</th>
<th>25 mM glucose + HSM</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5 mM glucose</td>
<td>–</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>25 mM glucose</td>
<td>N.S.</td>
<td>–</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>5.5 mM glucose + Insulin</td>
<td>N.S.</td>
<td>N.S.</td>
<td>–</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>25 mM glucose + Insulin</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>–</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>5.5 mM glucose + HSM</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>–</td>
<td>N.S.</td>
</tr>
<tr>
<td>25 mM glucose + HSM</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>–</td>
</tr>
</tbody>
</table>

Table 8.3: Post-hoc analysis (Reporter gene)

<table>
<thead>
<tr>
<th>Incubation condition</th>
<th>Reporter gene</th>
<th>(L4L3)4-54PK vs. (L3)3-54PK</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5 mM glucose</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>25 mM glucose</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>5.5 mM glucose + Insulin</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>25 mM glucose + Insulin</td>
<td>***</td>
<td></td>
</tr>
<tr>
<td>5.5 mM glucose + HSM</td>
<td>N.S.</td>
<td></td>
</tr>
<tr>
<td>25 mM glucose + HSM</td>
<td>***</td>
<td></td>
</tr>
</tbody>
</table>
8.2.3 Figure 4.5: Summary of one-way ANOVA and posthoc analysis

Regulation of SREBP-1c reporter gene expression by (A) rat and (B) human HNF4α, insulin, glucose concentration and PGC-1α (Figure 4.5):

Figure 4.5A

Result of one-way ANOVA: \( p < 0.001 \)

Post-hoc analysis using Tukey’s post-hoc tests:

<table>
<thead>
<tr>
<th>Incubation condition</th>
<th>5.5 mM glucose</th>
<th>5.5 mM glucose + insulin</th>
<th>5.5 mM glucose + HNF4α</th>
<th>25 mM glucose + insulin + HNF4α</th>
<th>25 mM glucose + PGC-1α + HNF4α</th>
<th>5.5 mM glucose + insulin + HNF4α + PGC-1α</th>
<th>5.5 mM glucose + PGC-1α + HNF4α</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5 mM glucose</td>
<td>-</td>
<td>N.S.</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>5.5 mM glucose + insulin</td>
<td>N.S.</td>
<td>-</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>5.5 mM glucose + HNF4α</td>
<td>***</td>
<td>***</td>
<td>-</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>25 mM glucose + HNF4α</td>
<td>***</td>
<td>***</td>
<td>N.S.</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>25 mM glucose + insulin + HNF4α</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>N.S.</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>5.5 mM glucose + HNF4α + PGC-1α</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>N.S.</td>
</tr>
<tr>
<td>5.5 mM glucose + insulin + HNF4α + PGC-1α</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

NS.
Figure 4.5B

Result of one-way ANOVA: \( p < 0.001 \)

Table 8.5: Results of post-hoc analysis: Figure 4.5B (Human HNF4α overexpression)

<table>
<thead>
<tr>
<th>Incubation condition</th>
<th>5.5 mM glucose</th>
<th>5.5 mM glucose + insulin</th>
<th>5.5 mM glucose + HNF4α</th>
<th>5.5 mM glucose + insulin + HNF4α</th>
<th>25 mM glucose</th>
<th>25 mM glucose + insulin</th>
<th>25 mM glucose + HNF4α</th>
<th>25 mM glucose + insulin + HNF4α + PGC-1α</th>
<th>5.5 mM glucose + HNF4α</th>
<th>5.5 mM glucose + insulin + HNF4α + PGC-1α</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.5 mM glucose</td>
<td>-</td>
<td>N.S.</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>N.S.</td>
<td>***</td>
</tr>
<tr>
<td>5.5 mM glucose + insulin</td>
<td>N.S.</td>
<td>-</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>*</td>
<td>***</td>
</tr>
<tr>
<td>5.5 mM glucose + HNF4α</td>
<td>***</td>
<td>***</td>
<td>-</td>
<td>***</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>5.5 mM glucose + insulin + HNF4α</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>-</td>
<td>***</td>
<td>N.S.</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>25 mM glucose + HNF4α</td>
<td>***</td>
<td>***</td>
<td>N.S.</td>
<td>***</td>
<td>***</td>
<td>-</td>
<td>*</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>25 mM glucose + insulin + HNF4α</td>
<td>***</td>
<td>***</td>
<td>N.S.</td>
<td>N.S.</td>
<td>*</td>
<td>-</td>
<td>***</td>
<td>***</td>
<td>***</td>
<td>***</td>
</tr>
<tr>
<td>5.5 mM glucose + HNF4α + PGC-1α</td>
<td>N.S.</td>
<td>*</td>
<td>N.S.</td>
<td>***</td>
<td>N.S.</td>
<td>***</td>
<td>N.S.</td>
<td>***</td>
<td>-</td>
<td>N.S.</td>
</tr>
<tr>
<td>5.5 mM glucose + insulin + HNF4α + PGC-1α</td>
<td>*</td>
<td>**</td>
<td>N.S.</td>
<td>***</td>
<td>N.S.</td>
<td>***</td>
<td>N.S.</td>
<td>***</td>
<td>N.S.</td>
<td>-</td>
</tr>
</tbody>
</table>
8.3 Results of sequencing DN-HNF4α expression vector

<table>
<thead>
<tr>
<th></th>
<th>WT-HNF4A</th>
<th>DN-HNF4A</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CTGACCCGCCCTACCCACCTCTCA</td>
<td>CTGACCCGCCCTACCCACCTCTCA</td>
</tr>
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<td>ATGTGCAGGTGTTGACGATGGGCAAT</td>
<td>ATGTGCAGGTGTTGACGATGGGCAAT</td>
</tr>
<tr>
<td></td>
<td>CTGGACCCAGCCTACACCACCCTGGAATTTGAGAATGTGCAGGTGTTGACGATGGGCAAT</td>
<td>CTGGACCCAGCCTACACCACCCTGGAATTTGAGAATGTGCAGGTGTTGACGATGGGCAAT</td>
</tr>
<tr>
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<tr>
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<td>GACACGTCCCCATCAGAAGGCACCAACCTCAACGCGCCCAACAGCCTGGGTGTCAGCGC</td>
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<td>CTGTGTGCCATCTGCGGGGACCGGGCCACGGGCAAACACTACGGTGCCTCGAGCTGTGAC</td>
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<td>GGCTGCAAGGGCTTCTTCCGGAGGAGCGTGCGGAAGAACCACATGTACTCCCTGCAGATTT</td>
</tr>
<tr>
<td></td>
<td>AGCCGGCCTCGGGGCTGGCATGAAGAAGGAAGCCGTCCAGAATGAGCGGGACGGGATCAGC</td>
<td>AGCCGGCCTCGGGGCTGGCATGAAGAAGGAAGCCGTCCAGAATGAGCGGGACGGGATCAGC</td>
</tr>
<tr>
<td></td>
<td>ACTCGAAGGTCAAGCTATGAGGACAGCAGCCTGCCCTCCATCAATGCGCTCCTGCAGGCG</td>
<td>ACTCGAAGGTCAAGCTATGAGGACAGCAGCCTGCCCTCCATCAATGCGCTCCTGCAGGCG</td>
</tr>
<tr>
<td></td>
<td>AAATGCTTCCGGGCTGGCATGAAGAAGGAAGCCGTCCAGAATGAGCGGGACGGGATCAGC</td>
<td>AAATGCTTCCGGGCTGGCATGAAGAAGGAAGCCGTCCAGAATGAGCGGGACGGGATCAGC</td>
</tr>
<tr>
<td></td>
<td>ACTCGAAGGTCAAGCTATGAGGACAGCAGCCTGCCCTCCATCAATGCGCTCCTGCAGGCG</td>
<td>ACTCGAAGGTCAAGCTATGAGGACAGCAGCCTGCCCTCCATCAATGCGCTCCTGCAGGCG</td>
</tr>
</tbody>
</table>

Box 8.5: ClustalW comparison of wild-type HNF4α sequence and the results of sequencing the DN-HNF4α expression vector (pDGT23.1). DN-HNF4α expression vector was sequenced with T7 promoter primer present in the basic vector (pcDNA3). Results of the sequencing reaction (DN-HNF4A) were compared with sequence of the wild-type human HNF4α2 sequence (WT-HNF4A) (Genbank Accession No.: NM_000457) using ClustalW sequence alignment program. It was confirmed that DN-HNF4α differs from wild-type in that it contains a T (green) → C (red) point mutation, as described by Taylor et al. (1996).
8.4 Further analysis (Chapter 6)

Figure 8.1: Effect of DN-HNF4α on the regulation of (A) apoCIII and (B) FAS mRNA expression by insulin: Linear regression analysis. Linear regression analysis of (A) apoCIII or (B) FAS vs. HNF4α mRNA expression levels were performed with data from control (closed triangles ▲) and insulin (open triangles △) incubation conditions (from both pcDNA3- and DN-HNF4α-transfection conditions), from experiment as described in Figure 6.12. Results are presented as individual datapoints with linear regression analyses indicated by a dashed line with adjacent $R^2$ and $p$-values. Differences between control and insulin incubation conditions were analysed by comparing the slopes and intercepts of the two linear regression analyses, as indicated by a double-arrow and adjacent $p$-value (N.S., not significant).
Figure 8.2: Comparison of (A) HNF4α, (B) p300 and (C) endogenous PGC-1α expression in HepG2 cells between dexamethasone-containing and control (EtOH)-medium. Experiment described in Figure 6.15. The effect of incubation with dexamethasone on gene expression was compared with control medium (0.04% EtOH) by two-way ANOVA with Bonferroni post-hoc tests (* p < 0.05, ** p < 0.01, *** p < 0.001).
Figure 8.3: Comparison of (A) exogenous PGC-1α, (B) PEPCK and (C) L-PK expression in HepG2 cells between dexamethasone-containing and control (EtOH)-medium. Experiment described in Figure 6.15. The effect of incubation with dexamethasone on gene expression was compared with control medium (SFM + 0.04% EtOH) by two-way ANOVA with Bonferroni post-hoc tests (* p < 0.05, ** p < 0.01, *** p < 0.001).
Figure 8.4: Correlation between expression of PEPCK and (A) HNF4a, (B) p300 (C) endogenous PGC-1α and (D) exogenous PGC-1α mRNA levels under 100 nM insulin incubation condition. Analysis of correlation between PEPCK and HNF4a / co-activator mRNA expression levels was performed with data from control (closed triangles ▲) and insulin (open triangles △) as presented in Figures 6.16 to 6.18. Results are presented as individual datapoints with correlation analyses indicated by a dashed line with adjacent R² and p-values. Differences between the incubation conditions were analysed by comparing the linear regression analyses and a significant difference between the intercepts or slopes of the two lines is indicated by a double-arrow and adjacent p-value or N.S. in the case of no significant differences. (If the difference relates to the slopes, this is noted; no additional notation corresponds to a difference between the intercepts).
Figure 8.5: Correlation between expression of PEPCK and (A) HNF4α, (B) p300 (C) endogenous PGC-1α and (D) exogenous PGC-1α mRNA levels under 25 mM glucose incubation condition. Analysis of correlation between PEPCK and HNF4α / co-activator mRNA expression levels was performed performed with data from control (closed triangles ▲) and 25 mM glucose (open triangles Δ) as presented in Figures 6.16 to 6.18. Results are presented as individual datapoints with correlation analyses indicated by a dashed line with adjacent $R^2$ and $p$-values. Differences between the incubation conditions were analysed by comparing the linear regression analyses and a significant difference between the intercepts or slopes of the two lines is indicated by a double-arrow and adjacent $p$-value or N.S. in the case of no significant differences. (If the difference relates to the slopes, this is noted; no additional notation corresponds to a difference between the intercepts).
Figure 8.6: Correlation between expression of L-PK and (A) HNF4α, (B) p300 (C) endogenous PGC-1α and (D) exogenous PGC-1α mRNA levels under 1 μM dexamethasone incubation condition. Analysis of correlation between L-PK and HNF4α / co-activator mRNA expression levels was performed with data from control (closed triangles △) and dexamethasone (open triangles △) as presented in Figures 6.16, 6.17 and 6.23. Results are presented as individual datapoints with correlation analyses indicated by a dashed line with adjacent R^2 and p-values. Differences between the incubation conditions were analysed by comparing the linear regression analyses and a significant difference between the intercepts or slopes of the two lines is indicated by a double-arrow and adjacent p-value or N.S. in the case of no significant differences. (If the difference relates to the slopes, this is noted; no additional notation corresponds to a difference between the intercepts).
Figure 8.7: Correlation between expression of L-PK and (A) HNF4α, (B) p300 (C) endogenous PGC-1α and (D) exogenous PGC-1α mRNA levels under 1 mM 8-Br-cAMP and 1 μM dexamethasone incubation condition. Analysis of correlation between L-PK and HNF4α / co-activator mRNA expression levels was performed with data from control (closed triangles ▲) and 8-Br-cAMP + dexamethasone (open triangles △) as presented in Figures 6.16, 6.17 and 6.23. Results are presented as individual datapoints with correlation analyses indicated by a dashed line with adjacent $R^2$ and $p$-values. Differences between the incubation conditions were analysed by comparing the linear regression analyses and a significant difference between the intercepts or slopes of the two lines is indicated by a double-arrow and adjacent $p$-value or N.S. in the case of no significant differences. (If the difference relates to the slopes, this is noted; no additional notation corresponds to a difference between the intercepts).
Figure 8.8: Correlation between expression of L-PK and (A) HNF4α, (B) p300 (C) endogenous PGC-1α and (D) exogenous PGC-1α mRNA levels under 100 nM insulin incubation condition. Analysis of correlation between L-PK and HNF4α / co-activator mRNA expression levels was performed with data from control (closed triangles △) and insulin (open triangles ▲) as presented in Figures 6.16, 6.17 and 6.23. Results are presented as individual datapoints with correlation analyses indicated by a dashed line with adjacent R² and p-values. Differences between the incubation conditions were analysed by comparing the linear regression analyses and a significant difference between the intercepts or slopes of the two lines is indicated by a double-arrow and adjacent p-value or N.S. in the case of no significant differences. (If the difference relates to the slopes, this is noted; no additional notation corresponds to a difference between the intercepts).
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