Transcriptional Regulation of the Human Hepatic Lipase Gene

Relation to Glucose and Lipid Metabolism

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Contents

CHAPTER 1

General Introduction

1.1 Introduction to Hepatic Lipase

Hepatic Lipase (HL; EC 3.1.1.3) is an extracellular glycoprotein with phospholipase A1 and triacylglycerol hydrolase activity [1]. The human HL protein is encoded by the LIPC gene on chromosome 15q21. Most of this protein is synthesized in the parenchymal cells of the liver and secreted into the space of Disse [2-4] where it binds to heparin sulfate proteoglycans. Some synthesis of HL was also observed in macrophages [5]. The HL protein is also present in the steroidogenic adrenal glands, ovaries and, in small amounts, in the testes [6-9]. By using heparin, HL protein is displaced from its binding site. Human HL protein is a homodimer, the monomer has a molecular weight of 65 kDa. In the metabolism of plasma lipoproteins HL plays an important role; it mediates the conversion of high density lipoprotein subfraction 2 (HDL_2) to high density lipoprotein subfraction 3 (HDL_3), the conversion of intermediate density lipoprotein (IDL) to low density lipoproteins (LDL), and the formation of small dense LDL (sdLDL) from large buoyant LDL [1,10]. HL has a role in postprandial lipid transport where it facilitates the clearance of remnant lipoproteins by the liver [1]. In adrenals and ovaries the HL enzyme facilitates the delivery of HDL cholesterol for steroidogenesis, at least in the rat [11,12]. HL expression is determined by genetic [13,14], hormonal and nutritional factors [15], and by body composition [16-18]. HL activity is associated with a risk for Coronary Artery Diseases (CAD) [1,2]. Whether high HL expression is anti- or pro-atherogenic depends on other genetic or metabolic factors, e.g. concomitant hypertriglyceridemia [1,19]. Humans with visceral obesity and insulin resistance [16] or type 2 diabetes [21,22] show increased levels of HL expression. How HL gene expression is altered in insulin-resistant conditions is unknown. Relative to the common LIPC C-allele (referring to the C/T polymorphism at the -514 position), carriers of the T-allele have reduced post-heparin HL activity, and the T-allele is associated with dyslipidemia and insulin resistance in healthy controls and in Familial Combined Hyperlipidemia (FCH) [23]. In cell culture experiments using human HepG2 hepatoma cells, HL expression was found to be increased by elevated levels of fatty acids [24,25] and glucose [26,27], conditions that prevail in insulin resistance. How these metabolic factors affect HL expression is largely unknown.

1.2 Hepatic Lipase and the metabolism of lipoproteins

HL is a lipolytic enzyme important in plasma lipoprotein metabolism [3]. HL plays a role in the exogenous and endogenous pathway, as well in the reverse cholesterol pathway.

1.2.1 The exogenous pathway

In the exogenous pathway the digestion products of dietary fat are transported from the digestive system to body cells. In the intestinal cells, triglycerides (also named as triacylglycerol, abbreviated as TG) are re-synthesized and combined with cholesterol esters (CE). In the Golgi, chylomicrons are assembled from neutral lipids, phospholipids and cholesterol, and the apolipoproteins (apo) B-48, apo A-I, apo A-II and apo A-IV. The chylomicrons are secreted into the lymph which drains into the systemic circulation via the thoracic duct. Once in the systemic circulation, the chylomicrons have a half-life of only a few minutes and as a consequence they are only found in the serum after a high-fat meal [28-31]. When they enter the circulation, apo A-IV and apo A-I are lost to, and apo C-I, apo C-II, apo C-III and apo E are acquired from the HDL. The apo C-II is necessary as co-factor for LPL. Lipoprotein lipase (LPL) is present in adipose tissue and muscle. LPL mediates the lipolysis of TGs from TG-rich lipoproteins, resulting in the release of fatty acids [32,33], which are taken up by the fat and muscle cells. Due to this process, most of the TGs are lost from the chylomicrons. The surface components (apo Cs, free cholesterol, phospholipids) have been released and taken up by the HDL. This results in smaller CE-enriched particles which are named the chylomicron-remnants. The apo E on the chylomicron remnant enhances the uptake of the particle into the liver. The remnants, containing dietary cholesterol and residual TGs, end up in the lysosomes. Here, the remnants are degraded [28].

In the liver, HL is present extracellularly in the space of Disse. This enzyme plays an important role in the uptake and clearance of the atherogenic chylomicron-remnants, which may be one of its anti-atherogenic potentials. Studies in which mice and rats were injected with anti-HL antibodies showed impaired chylomicron remnant-removal [34,35]. In transgenic mice over-expressing HL, TG rich plasma lipoproteins like the chylomicron remnants were lower than in wild-type (WT) mice [36]. Studies by confocal microscopy showed that HL co-localized with chylomicron remnant clusters in the Space of Disse in HL transgenic mice. HL may contribute to the sequestration of chylomicron remnants by bringing the remnants in close proximity to their receptors [37].

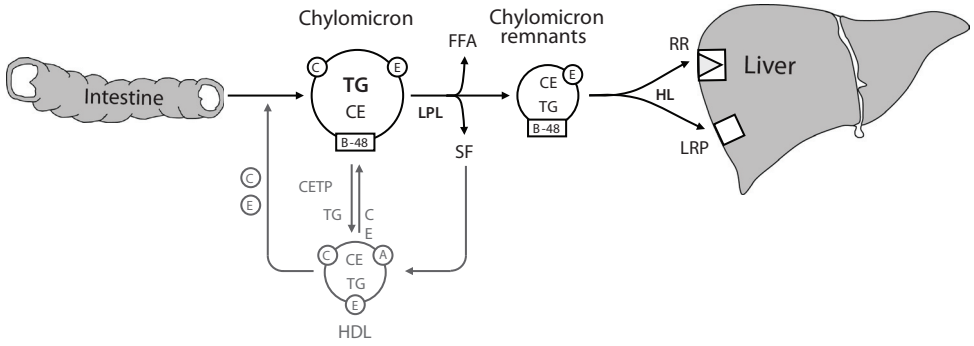

Figure 1. Pictorial scheme of the exogenous pathway of lipid transport.

The exogenous pathway and the role of HL herein, are described in the main text. A, apolipoproteins A-I, A-II or A-IV; B-48, apolipoprotein B-48; C, apolipoproteins C-I, C-II or C-III; CE, cholesterol-esters; CETP, cholesteryl ester transfer protein; E, apolipoprotein E; FFA, Free Fatty Acids; HDL, high density lipoproteins; HL, hepatic lipase; LPL, lipoprotein lipase; LRP, LDL receptor-related protein; RR, remnant receptor; SF, surface fragments; TG, triglycerides. The light grey part of the pictorial scheme shows exchange of material between de exogenous pathway with HDL and the reverse cholesterol transport pathway.

1.2.2 The endogenous pathway

Synthesis of VLDL takes place in the liver similar to the synthesis of chylomicrons in intestinal cells. TG is synthesized from "de novo" fatty acids, and from fatty acids mobilized from adipose tissue, or generated by intracellular digestion of internalized lipoprotein particles. CE is synthesized from cholesterol and fatty acids [38]. Apo B 100 is used in the assembly of VLDL from TG, CE and phospholipids. The nascent VLDL is secreted directly into the circulation where it acquires apo C-I, apo C-II, apo C-III and Apo E from HDL. The TG from the VLDL is converted by LPL to fatty acids, which are then taken up by the tissue cells. Surface fragments including the apo Cs – formed during the lipolysis of VLDL-TG – are taken up by HDL. The VLDL transforms into a smaller particle, named the intermediate density lipoprotein (IDL) [39].

The HL present in the liver hydrolyses the TG from the IDL. When still containing apo E, 80% of the IDL particles are taken up by the liver. When apo E is also lost from the remaining IDL, cholesterol-enriched LDL is formed. LDL delivers cholesterol and other LDL components (incl. fat-soluble vitamins) to peripheral cells that express LDL-receptors depending on their need for cholesterol. 60-90% of LDL uptake is LDL receptor-mediated; the remainder is removed from the plasma via scavenger receptors or via pinocytosis [39,40]. A large part of the LDL is taken up by the liver again. With higher HL activity, even more TG is hydrolysed from IDL particles resulting in small dense LDL (sdLDL). These sdLDL particles have less affinity for the LDL-receptor than normal-sized LDL [41]. Taken together, a high HL activity promotes the formation of sdLDL, which may contribute to the pro-atherogenic potential of HL.

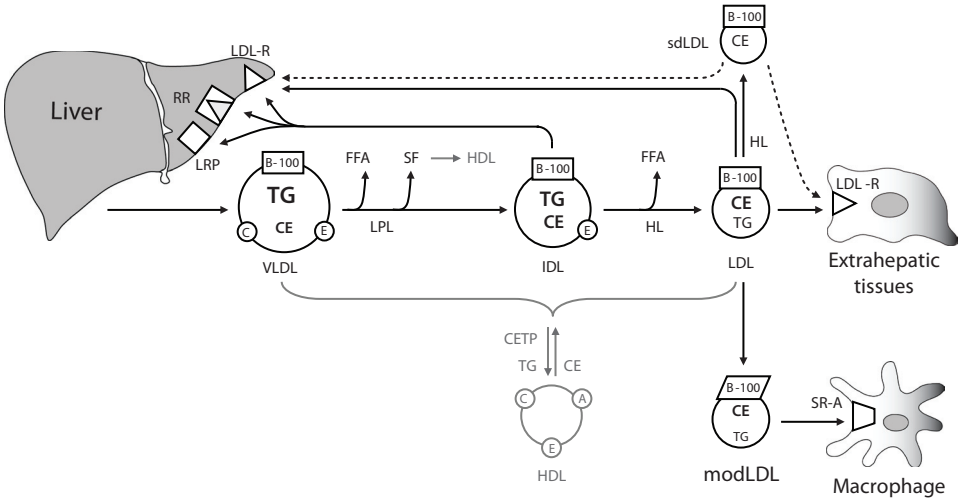

Figure 2. Pictorial scheme of the endogenous pathway of lipid transport.

The endogenous pathway and the role of HL herein are described in the main text. B-100, apolipoprotein B-100; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; LDL-R, LDL receptor; modLDL, modified LDL (e.g., by oxidation); sdLDL, small dense LDL; SR-A, Scavenger receptor class A; VLDL, very low density lipoprotein; other abbreviations are as for Figure 1. The light grey part pictorial scheme shows exchange of material between the endogenous pathway with HDL and the reverse cholesterol transport pathway. VLDL may also acquire apolipoproteins from HDL.

1.2.3 Reverse cholesterol transport

Nascent HDL originates from de novo synthesis in the liver and intestine, and from the surface fragments resulting from chylomicron lipolysis. They are composed of phospholipids, free cholesterol and apo A-I arranged as bilayers and are referred to as discoidal HDL. This nascent HDL as well as HDL_3 takes up free cholesterol from the peripheral cells, which is the beginning of the process of cholesterol transport from the periphery to the liver, named "reverse cholesterol transport" [39].

The efflux of cholesterol and phospholipids to apo A-I in HDL is mediated by the ATP-binding cassette, subfamily A, member 1 (ABCA1) receptor on peripheral cells [42]. In this process, cholesterol and phospholipids are initially integrated in the surface of the HDL. HDL also plays a role in the esterification of cholesterol in the circulation. Apo A-I and apo A-IV on HDL stimulate the enzyme lecithin:cholesterol acyltransferase (LCAT), which is bound to the HDL. This enzyme catalyses the conversion of free cholesterol into CE with a fatty acid derived from the lecithin [43], thereby converting discoidal HDL into a spherical HDL particle. Simultaneously, the HDL increases in size via small dense particles (HDL_3) to cholesterol rich particles (HDL_2). In addition, peripheral cells can deliver cholesterolesters to HDL_2 and HDL_3, through SR-BI and ATP-binding cassette, sub-family G, member 1 (ABCG1) receptors [44]. HDL_2 contain on average more CE molecules per particle than HDL_3. The liver then takes up the CE from HDL_2, either via a direct or indirect pathway. In the direct pathway, HDL delivers

cholesterol to the liver via endocytosis after binding to HDL receptors [45]. The whole HDL particle ends up in the lysosome and is completely degraded. Alternatively, by docking to SR-B1 both free and esterified cholesterol can be selectively removed from the HDL particles [46]. Here, the CE rich HDL particle is also endocytized. In the endosome it comes in contact with simultaneously internalized HL, and via its phospholipase A1 activity, the selective unloading of free and esterified cholesterol is promoted. After delivery of its cholesterol cargo the HDL particle recycles back to the surface of the liver cell [42]. Selective cholesterol uptake is also evident in steroidogenic organs, where HL and SR-B1 co-localize [12]. Thus HL mediates the remodelling of HDL_2 to HDL_3. As HDL_3 is a better acceptor of peripheral cholesterol and a better substrate for LCAT than HDL_2, this action of HL may further enhance reverse cholesterol transport [43].

Via the indirect pathway of reverse cholesterol transport, CE from HDL is delivered to the liver after Cholesteryl ester transfer protein (CETP)-mediated transfer to the apo B containing lipoproteins like VLDL in exchange for TGs. After conversion to IDL or LDL, HDL-derived CE is taken up mainly by the liver. The TG in HDL is hydrolysed by HL. CETP may also transfer CE from HDL to chylomicrons, which then end up in the liver as chylomicron remnants. Once taken up by the liver, the cholesterol esters are hydrolysed in the lysosome; the resulting free cholesterol is either esterified again or secreted into the bile. In humans, HDL cholesterol is transferred to the liver mainly through the indirect pathway [47].

Postheparin plasma HL activity correlates inversely with levels of HDL cholesterol in humans [48]. Individuals with a mutation of the HL gene that leads to reduced enzyme activity have moderately raised HDL cholesterol with enlarged, TG-rich HDL particles [49]. Like human patients, HL-deficient mice have increased plasma concentrations of HDL cholesterol and phospholipids compared with wild-type mice. This is even more pronounced when they are fed a high-fat and cholesterol-rich diet [50]. HDL cholesterol is reduced and HDL particles are smaller and denser in transgenic mice and rabbits that overexpress HL compared to wildtypes [51]. HL may lower HDL-cholesterol by facilitating cholesterol delivery to the liver, or by its role in remodelling of HDL towards smaller, denser HDL particles.

By promoting reverse cholesterol transport, HL may be anti-atherogenic. Besides its role in reverse cholesterol transport, HDL has also beneficial anti-inflammatory, anti-oxidative and anti-thrombotic properties, which contribute to its atheroprotective potential [52]. By lowering HDL, therefore, HL may be pro-atherogenic.

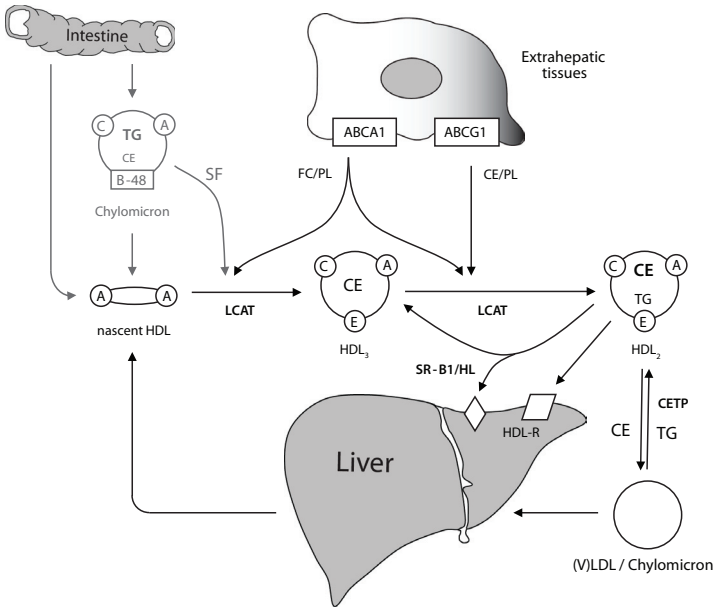

Figure 3. Pictorial scheme of the reverse cholesterol transport pathway.

Reverse cholesterol transport and the role of HL herein, is described in the main text. ABCA1, ATP-binding cassette, sub-family A, member 1; ABCG1, ATP-binding cassette, sub-family G, member 1; HDL-R, HDL receptor; LCAT, lecithin-cholesterol acyltransferase; SR-B1, scavenger receptor class B1; other symbols are as described for Figures 1 and 2.

1.3 Hepatic Lipase and Coronary Artery Disease

Coronary Artery Disease (CAD) contributes substantially to mortality in Western societies [53-55]. Atherosclerosis is a common cause of CAD. Multiple human and animal studies support the notion that HL has an impact on atherogenesis [1,14,56-59]. Although HL has been shown to modulate atherogenic risk, whether these studies support an atheroprotective or proatherogenic role depends on the model used. This has led to the suggestion that the role of HL in atherogenesis may depend on the presence of additional factors [1,49]. Connelly and Hegele suggested that HL deficiency, in the presence of a second genetic or environmental factor affecting lipoprotein levels, increases atherogenic risk [49]. Indeed, subjects with low HL activity suffer from increased CAD when simultaneously having genetically determined low CETP activity, despite higher HDL cholesterol levels [60]. In the Regression growth evaluation statin study (REGRESS), involving normocholesterolemic men with symptomatic CAD, increased progression of CAD is observed in homozygotes of both the LIPC T allele and CETP B2 allele, thus combining low HL with low CETP activity [61]. Jansen *et al.* [1] concluded in their review that high HL activity is anti-atherogenic in familial hypercholesterolemia (FH) and

pro-atherogenic in hypertriglyceridemia. In normolipidemia, HL seems to have little effect on CAD risk. In FH, the indirect pathway of reverse cholesterol is impaired, and a low HL activity will also reduce the direct pathway. In hypertriglyceridemic conditions there is an increase in CE transfer from HDL to apoB containing lipoproteins, which results in more TG-enriched HDL [62]. High HL activity will then lead to more sdLDL, and HL-mediated hydrolysis of HDL-TG will accelerate delipidation and subsequent clearance of apoA-I [1].

1.3.1 Role in HDL metabolism

In vitro and *in vivo* studies have shown that HL activity is inversely correlated with HDL cholesterol levels. HL may lower HDL cholesterol levels by facilitating the uptake and subsequent degradation of HDL particles by the liver [63-66]. In addition, HL facilitates the selective delivery of cholesterol (esters) during retro-endocytosis of HDL, leading to smaller HDL subclasses (e.g. HDL_3 and/or preβ HDL), which have increased activity of cholesterol efflux from cells [67]. HL thus stimulates the reverse cholesterol transport by promoting HDL cholesterol (ester) uptake in the liver as well by affecting HDL functionality. Involvement of HL in the reverse cholesterol transport may influence CAD risk [68-70]. The increased CAD risk, seen when low HL activity is present together with low CETP activity [60], can be explained by the role HL and CETP have in the reverse cholesterol transport. Whereas HL is mainly involved in the direct route, CETP is mainly involved in the indirect route [69]. When either of one of the pathways fails, the other route may compensate, and CAD risk is increased only when both pathways fail. HL also mediates the hydrolysis of HDL-TG resulting from CETP action, and thus also has a role in the indirect route by restoring CE acceptor particles [69,70]. Thus, low HDL may further deteriorate the indirect route of reverse cholesterol transport that is already compromised by the low CETP activity.

1.3.2 Role in IDL/LDL metabolism

HL is a determinant of LDL subclass distribution, which in turn attributes to atherogenic risk [20,71-73]. Human as well animal studies have shown that HL affects the metabolism of apo B-100 containing lipoproteins. Patients with HL deficiency present with hypercholesterolemia or hypertriglyceridemia and accumulate β-VLDL, chylomicrons remnants, IDLs, TG-rich LDLs, and HDLs [14,74-79]. Demant and coworkers [80] showed that the conversion of small VLDL into IDL particles was retarded by 50% and the conversion of IDL to LDL was inhibited by 90% of normal. This is consistent with the development of a type III-like lipoprotein profile described in HL deficient subjects [49]. In the EARS-II study, healthy young sons of CAD patients who carry the LIPC T allele have increased levels of atherogenic ApoC-III:B particles in addition to increased fasting lipids and HDL [81]. IDL-like lipoproteins have also accumulated in children with congenital hypothyroidism, a condition that associates with low HL activity levels as well [82]. IDL is an independent determinant of CAD risk [83]. Therefore, a low HL activity might lead to increased atherosclerosis due to the accumulation of IDL. Small-dense LDL is also considered to be highly atherogenic, but here accumulation may result from high instead of low HL

activity. Through lowering the number of sdLDL particles, HL activity has been proposed as a drug target in the treatment of CAD [57].

1.4 Hepatic Lipase and glucose metabolism and diabetes mellitus

1.4.1 Insulin and regulation of glucose metabolism

Glucose metabolism is regulated tightly to ensure sufficient glucose supply at all times to glucose-dependent organs, and simultaneously prevent the deleterious effects of too high glucose levels. Postprandially, glucose enters the blood from the intestine and is taken up by the organs. During fasting, the organs depend primarily on glucose produced by the liver. Cellular uptake of glucose from the circulation and hepatic glucose production are tightly regulated by a variety of mechanisms [84].

The most important hormone for glucose metabolism is insulin, which is synthesized and secreted by the β-cells of the islets of Langerhans [85]. Insulin stimulates glucose uptake from the blood and metabolism of glucose in most cells of the body. The islets also contain α-cells that, in case of low levels of glucose, secrete glucagon [86]. After secretion, insulin and glucagon first reach the liver via the portal vein. A condition of high insulin and/or low glucagon in the liver favors glucose uptake by the hepatocytes, whereas low insulin and/or high glucagon signals glucose output [87]. Hepatic glucose output is also induced by other hormones, notably cortisol and catecholamines [88,89]. Insulin, cortisol and catecholamines also affect glucose and energy metabolism in peripheral tissues. In cardiac and skeletal muscle and adipose tissue, insulin stimulates cellular uptake of glucose from the blood, whereas the other hormones mediate the mobilization of substrates necessary for glucose production by the liver. In addition, insulin has anabolic effects on peripheral tissues.

Due to the interplay of these hormones, blood glucose levels are maintained within narrow boundaries despite large fluctuations in glucose supply from the diet. When fasting blood glucose is above normal, or blood glucose increases above a certain threshold level and/or stays above normal for extended periods of time after a glucose load, the person is called glucose intolerant. This may be due to lack of insulin or to insulin resistance. Insulin resistance may be due to decreased sensitivity of tissues to insulin, and hence, more insulin is required to keep blood glucose low. Sensitivity to insulin may be reduced as a result of impaired intracellular signalling beyond the insulin receptor, or of the opposing effects of counter-regulatory hormones [90].

Fatty acids (FFAs) also seem to play an important role in determining insulin sensitivity [91]. There is competition between glucose and FFA as substrates for mitochondrial oxidation through the Randle cycle [92]. Increased FFA supply thereby reduces glucose uptake into the cell, and consequently more insulin is required. In particular, high availability of FFAs to the liver either from the diet or mobilized from adipose tissue by intracellular lipolysis, will reduce glucose disposal by the liver and increase demand for insulin [91].

1.4.2 Integration of lipid and glucose metabolism

In the fed state, plasma levels of insulin are high and glucagon and adrenaline are low. Under this condition, glucose derived from the diet is taken up by the liver and in part used for fatty acid de novo synthesis. These fatty acids are then converted to TG and exported from the liver in the form of VLDL, and hence end up in fat stores in adipose tissue and skeletal muscle. Insulin also stimulates storage of chylomicron-derived FFAs in adipose tissue and skeletal muscle. Insulin induces lipoprotein lipase activity in adipose tissue thereby increasing local lipolysis of chylomicron-TG and VLDL-TG. Via translocation of GLUT4 and subsequent uptake of glucose, insulin also increases intracellular supply of glycerol-3-phosphate necessary to esterify the delivered fatty acids into TG. In addition, insulin suppresses intracellular lipolysis by inhibiting hormone-sensitive lipase.

In the fasting state, plasma levels of insulin are low, and glucagon and adrenaline relatively high. In this condition, mobilization of FFAs from adipose tissue is activated by the loss of inhibition of intracellular lipolysis. The mobilized FFAs, especially from visceral fat depots, are mainly delivered to the liver. In the liver, FFAs are oxidized, thereby enabling the liver cells to synthesize glucose from non-carbohydrate substrates (gluconeogenesis). The FFAs not used for oxidation in the liver cells are again esterified to TG and exported from the liver in the form of VLDL. The VLDL-TG is then delivered to muscle via local lipoprotein lipase-mediated lipolysis.

1.4.3 Pathophysiology of Type 2 Diabetes Mellitus

Diabetes mellitus is a chronic disease, which is characterized by hyperglycaemia, and it results from defects in insulin secretion, insulin action or both [93]. Type 2 diabetes mellitus (T2DM) denotes all forms of diabetes with relative insulin deficiency, which can be caused by insulin resistance or secretory defects [93]. According to the European Health Report 2002 of the World Health Organization (WHO), between 85 and 95% of patients with diabetes suffer from T2DM [93,94]. Due to increasing obesity and lack of exercise T2DM is becoming more and more prevalent. The disease usually affects adults, but it increasingly occurs among teenagers as well. Genetic factors that influence β-cell development and function influence the susceptibility to T2DM and probably contribute stronger to young onset and relatively lean cases [95].

Insulin resistance in the muscle, adipose tissue or liver, occurs when the cells do not respond normally to the insulin. The β-cells initially compensate by secreting more insulin, which results in hyperinsulinemia and may further decrease insulin responsiveness. When the β-cells cannot increase insulin secretion any further or the β-cells become damaged, the blood sugar level increases strongly giving rise to full-blown T2DM phenotype. Basal hepatic glucose production is increased and peripheral glucose utilization is reduced [91].

T2DM is also characterized by hyperlipidemia, which includes hypercholesterolemia and hypertriglyceridemia [96]. Higher levels of TGs (mainly VLDL) and sdLDL particles and lower levels of HDL cholesterol are commonly found [97], giving rise to an atherogenic lipid profile. Increased FFA supply to the liver from the adipose tissue together with an increased hepatic VLDL secretion into the blood, and decreased TG clearance from the blood could give rise to the hyperlipidemia. Studies in animals and humans with diabetes showed that increased VLDL secretion might be caused directly by the decreased sensitivity to inhibition by insulin [98-100], and indirectly by increased flux of FFA to the liver [101]. Several but not all studies show lower TG clearance [102-105]. A decreased clearance of TG from the blood in T2DM patients has been linked to the impaired lipolysis of VLDL-TG by LPL. Insulin resistance would lead to lower levels of LPL at least in adipose tissue. Finally, skeletal muscle in T2DM patients have a reduced ability to oxidize the fatty acids [106,107]. According to Bandsma, the combination of increased intracellular lipolysis in adipose tissue with a lower uptake of FFA by the skeletal muscle could result in a re-direction of FFA from adipose tissue and skeletal muscle to the liver [91].

1.4.4 Involvement of Hepatic Lipase

Human studies indicate that HL activity is positively correlated with insulin levels [15]. HL activity increases with fasting insulin levels in normocholesterolemic coronary artery disease patients without diabetes [14], and plasma HL activity positively correlates with increased plasma insulin levels in response to an oral glucose load [108,109]. HL activity is increased during conditions with high plasma insulin levels, such as in T2DM [22,110,111] and obesity-related hyperinsulinaemia [112]. However, a direct stimulating effect of insulin on HL expression has not been unequivocally identified [113]. Instead, acute hyperinsulinemia actually reduces HL expression [114]. HL activity is also associated with parameters of insulin resistance in males without diabetes [14,23] and in familial combined hyperlipidemia (FCHL) [23]. In an animal model, HL expression is increased upon induction of insulin resistance; this is partially reversed by treatment with an insulin sensitizer [115]. Taken together, rather than a direct stimulating effect of insulin, some aspect of insulin resistance, e.g., hyperglycemia, appears to induce the increase in HL expression. Indeed, in HepG2 hepatoma cells, HL expression was found to be increased by elevated levels of glucose [26,27].

Omental fat mass, a parameter of visceral obesity with increased risk for development of T2DM, is also strongly correlated with HL expression, the more fat mass the higher HL activity [16]. As visceral obesity suggests increased fatty acid flow from the viscera to the liver, increased FFA supply to the liver may increases HL expression. Indeed, in HepG2 cells HL expression is increased by fatty acids [24,25]. HL expression may be elevated in insulin resistant states not only as a consequence of the hyperglycemia but also of the increased fatty acid delivery to the liver. How HL is upregulated in conditions of insulin resistance, hyperglycemia, and/or hyperlipidemia is unknown.

1.5 Regulation of Hepatic Lipase gene expression

1.5.1 Genomic organisation of the Hepatic Lipase gene

The HL gene (LIPC) resides on the long arm of chromosome 15 region q21 in humans [116-118]. The gene has a size of more than 100 kb and consists of 8 introns and 9 exons. The mRNA is 1.7 kb long. The size of the exons is between 118 and 234 bp [117]. Exon 1 encodes the signal peptide, and exon 4 a region that binds to the lipoprotein substrate. Exon 5 encodes a highly evolutionary conserved region representing the catalytic domain. Exons 6 and 9 encode sequences rich in basic amino acids thought to be important in anchoring the enzyme to the extracellular surface by interacting with glycosaminoglycans [117].

Two transcription start sites have been identified, a major one at -43 and a minor one at -77 from the translation initiation site [116,117]. The region from -1600 to +129 relative to the major transcription start site is considered to harbour most of the regulatory sequences of the gene [116,119]. This region contains four highly linked polymorphisms at -250G/A, -514C/T, -710C/T and -763A/G [120,121], which are collectively denoted as the LIPC C- and T-allele according to the nucleotide at -514. The T-allele is present in 17-24% of Caucasians, 40-50% of Japanese and Afro-Americans, and 55% of Native-Americans [13]. Carriers of the T-allele have a gene-dose dependent lowering of post-heparin HL activity by 15 to 30% [13,14,122]. In transfected HepG2 liver cells, promoter activity of the LIPC T-allele was also less than the LIPC C-allele [123,124].

The promoter region of the HL gene has putative TATA box-like sequences at around position -30 and position -65, and two CCAAT elements at around positions -470 and -1290. The TATA box-like sequence at -65 is homologous to a sequence known to bind hepatocyte-specific factors [116], and is now considered to be a HNF-1 binding site. There are multiple other regulatory elements in the promoter of the HL gene. Potential binding sites for thyroid hormone (TRE), glucocorticoids (GRE), estrogens (ERE), sterol (SRE) and cAMP (CRE) are present in the upstream regulatory region of the human HL gene [116]. The human HL gene also contains E-boxes, which may be involved in transcriptional regulation by glucose and/or insulin [123]. One of the polymorphism in the LIPC gene, the -514 C/T, resides in an E-box and is shown to affect the *in vitro* binding of Upstream Stimulatory Factor (USF) [124]. Hadzopoulou-Cladaras and Cardot found a strong negative regulatory region between positions +28 and +129 [125]. Additional negative regulatory regions were reported for the regions from -1576 to -1342 and from -623 to -407.

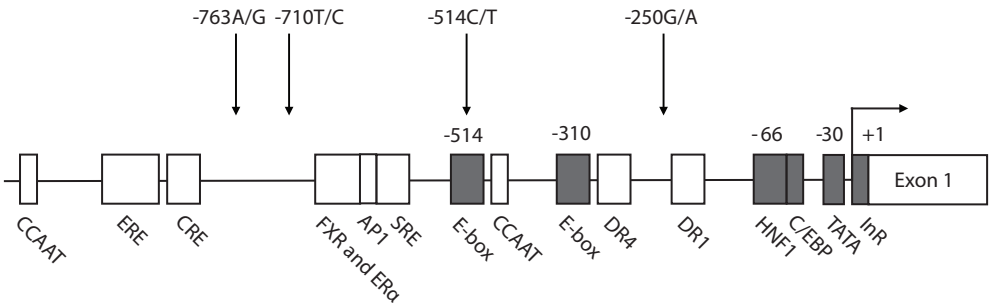

Figure 4. Potential regulatory elements within the proximal promoter region of the human HL gene.

The picture shows a schematic overview of the -1600 to +129 region. The grey boxes show the regulatory elements which have been studied in this thesis.

1.5.2 Transcription of the Hepatic Lipase gene

The HL gene is regulated by various hormones and nutritional states, both at the transcriptional and posttranscriptional level. *In vivo* and cell culture experiments in rats and humans have shown that thyroxin [126], glucocorticoids [127], insulin [127,128] and androgens [129] cause an increased secretion or post-heparin activity of HL. HL activity is decreased by estrogens [129,130], and activity decreases in diabetic [131] and hypothyroid [132] conditions. The enzyme activity is lower in premenopausal women than in men, but increases after menopause, possibly as a result of higher androgen and lower estrogen levels. In clinical studies of post menopausal woman, estrogen replacement therapy leads to a decrease of HL activity [130]. The up- or down regulation of the HL activity is however limited to about two fold [133].

For some of the above-mentioned hormones and metabolic conditions, changes in HL mRNA levels have been found. In addition, HL mRNA content in HepG2 cell is reported to be increased by heparin [134], mevinolin [135], and ligands of the farnesoid receptor FXR [136]. In rats, HL mRNA was decreased in the situation of a cholesterol rich diet [137]. In contrast to the moderate regulation by hormones and nutrition, the almost complete restriction of HL gene expression to differentiated liver cells is highly conspicuous [138,139]. Several groups have pointed to the HNF1 and HNF4α binding sites in the proximal promoter of the HL gene to explain this liver-specificity [119,125,140,141].

Only a few transcription factors have been identified to affect transcription of the HL gene or activity of the proximal promoter. Upstream stimulatory factor (USF), estrogen receptor α (ERα), and the farnesoid X receptor (FXR) have been confirmed functionally by transient transfection assays, whereas HNF1α and activator protein-1 (AP1) are only predicted based on sequence, binding assays, and localization within DNase I footprints from hepatic nuclear extracts. USF reportedly upregulates HL expression via the -510 to -515 region [124,142]. HNF1α is supposed to act by binding to the region from -75 to -43 [125]. ERα, FXR and AP1 negatively regulate HL promoter activity. The exact binding sites for FXR and ERα have not

been determined, but are located between -698 and -541 [136], and between -1,557 and -1,175 [142], respectively. AP1 is thought to bind at -564 to -558 [140].

1.5.3 Upstream Stimulatory Factors

Upstream Stimulatory Factors (USFs) belong to the basic helix-loop-helix leucine zipper (bHLH-Zip) family of transcription factors characterized by a highly conserved carboxy-terminal domain responsible for their dimerization and DNA binding. USFs were originally described for HeLa cells as proteins able to transactivate the adenovirus major late promoter by binding to the sequence CANNTG, referred to as an E-box motif [143,144]. Purification of USFs revealed two polypeptides of 43 (USF1) and 44 kDa (USF2), which were subsequently shown to be encoded by two distinct genes in human, rat, and mouse [145-147]. USF1 and USF2 bind to DNA as homo- as well as heterodimers, and share very similar DNA binding properties [148,149].

Many reports have implicated USF in the regulation of genes involved in glucose and lipid metabolism. In the liver, USF is largely involved in lipid and lipoprotein metabolism by regulating expression of genes for fatty acid synthase (FAS) [150], apolipoprotein (apo) A-II [151], apoCIII [152], apoAV [153], apoE [154], and HL [124]. Furthermore, USF1 is involved in the regulation of genes involved in glucose sensing, such as those for insulin [155], insulin growth factor-binding protein 1 (IGF-BP1) [156], the glucagon receptor [157], the islet-specific glucose-6-phosphatase catalytic subunit-related protein [158] and glucokinase [159]. The *USF1* gene on chromosome 1q21 has been linked to familial combined hyperlipidaemia (FCHL), particularly in males with hypertriglyceridemia [160,161]. Additional studies [162] suggest that the USF1, besides its involvement in the lipid and glucose metabolism, may be linked to type 2 diabetes. The USF1 gene is also linked to cardiovascular disease and all-cause mortality among females [163]. No such associations have been reported for the *USF2* gene. In Usf knockout mice, normal responsiveness required either Usf1/Usf2 heterodimers or Usf2 homodimers, whereas Usf1 homodimers gave rise to delayed glucose responsiveness [164]. Taken together, USF appear to play an important role in the transcriptional regulation of genes involved in insulin, glucose, fatty acids, and cholesterol metabolism.

1.5.4 Sterol Regulatory Element Binding Proteins

Like USFs, sterol regulatory element binding protein (SREBP)-1c and -2 belong to the family of bHLH-Zip transcription factors. Cholesterol, FFAs and insulin may affect gene expression through the sterol regulatory element binding protein (SREBP)-1c and -2 [165,166]. SREBPs are synthesized as precursors that are bound to the endoplasmic reticulum membrane and nuclear envelope [167,168]. When cells are full of cholesterol, precursor SREBPs are retained at the ER, thus preventing maturation. When cells are low in cholesterol, precursor SREBP is released and allowed to be transported by membrane flow to the Golgi. In the Golgi, part of the SREBP is cleaved off by site-specific proteases, and the soluble fragment (designated mature or nuclear SREBP or nSREBP) then diffuses into the nucleus. This nSREBP is composed of a transcriptional active N-terminal part and a bHLH-Zip domain for binding at specific DNA

elements. nSREBP activate transcription of genes with a SRE or E-box like enhancer element. SREBP responsive genes encode enzymes involved in cholesterol synthesis, like 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase, HMG-CoA reductase, farnesyl-diphosphate synthase, and squalene synthase, and the LDL receptor. Furthermore, SREBP stimulate the transcription of genes involved in fatty acid synthesis, such as acetyl-CoA carboxylase, fatty-acid synthase, glycerol-3-phosphate acyltransferase, and stearoyl-CoA desaturase [169]. SREBP can also repress transcription, for example of the LDL receptor-related protein (LRP1) and MTP gene [170,171]. Overexpression of SREBP-1 generally affects fatty-acid and triglyceride synthesis, whereas SREBP-2 overexpression promotes the cholesterol over triglyceride pathways [172].

1.6 Aim and scope of thesis

The regulation of hepatic lipase expression is of scientific interest because of its role in plasma lipoprotein metabolism and its potential role in atherogenesis. As argued above, whether HL is pro- or atherogenic may depend on the genetic background and hormonal, metabolic and/or nutritional states. Despite the potential impact of high or low HL expression, relative little is known about its transcriptional regulation. The aim of this thesis is therefore to determine the mechanism of transcriptional regulation of the HL gene under different metabolic conditions, and the involvement of relevant transcription factors.

Chapter 2 describes the search for highly conserved DNA elements within 30 kb of the 5'-flanking region of the HL gene from different species. Because of their high conservation, these elements may be important for the transcriptional regulation of HL, in particular for the strict liver-specificity.

Chapter 3 deals with the suppressing effect of diabetogenic hormones adrenaline and glucagon on HL expression in liver cells. We studied this by incubating HepG2 cells with a membrane-permeant cAMP homologue. With 5'-deletion analysis and reporter assays we pin-pointed the cAMP responsive element in the proximal HL promoter to a binding site for liver-enriched transcription factor C/EBPβ.

In **Chapters 4 and 5**, we studied the effect of glucose on HL gene expression, and the role of the transcription factor USF. In chapter 4, we hypothesized that high glucose activates the HL promoter through USF. To determine this we studied 1) whether glucose affects nuclear expression of USF proteins in hepatoma cells; and 2) whether this could explain the upregulation of HL expression seen in the high-glucose states. In chapter 5, the role of USF in activation of the HL promoter in HepG2 cells is studied in further detail. USF may affect HL promoter activity through a non-canonical E-box at -510/-516 that harbours the common -514C/T promoter polymorphism, a canonical E-box at -307/-312, as well as through the TATAA-Inr region.

Chapters 6 and 7 describe the opposite effect of the oleate and statins on HL expression in HepG2 cells. The increase in secretion of hepatic lipase as a result of supplementation with

oleate is abolished by atorvastatin. We hypothesized that oleate affects HL transcription through the transcription factors USF and SREBP. In chapter 6 the interaction of oleate and atorvastatin with the sterol-regulatory-element binding proteins (SREBP) at the proximal HL promoter region is studied in HepG2 cells. In chapter 7 the role of USF in the regulation of HL expression by oleate is studied in more detail.

Chapter 8 focuses on the suppressing effect of different polyunsaturated fatty acids on the regulation of the SREBP activity in HepG2 cells. We tested the hypothesis that supplementation of cells with the unsaturated fatty acids results in the redistribution of non-esterified cholesterol from the plasmamembrane to the endoplasmic reticulum and thus inhibition of SREBP maturation. We studied the effect of changing the fatty acid composition of cellular membranes on the SREBP activity for several unsaturated fatty acids, and the effect on intracellular distribution of non-esterified cholesterol in HepG2 cells.

Finally, the main findings of these studies are discussed in **Chapter 9**.

References

1. Jansen H, Verhoeven AJM, Sijbrands EJG. Hepatic lipase: a pro- or anti-atherogenic protein? *J Lipid Res.* 2002; **43:** 1352–1362.
2. Jansen H, Kalkman C, Zonneveld AJ, Hulsmann WC. Secretion of triacylglycerol hydrolase activity by isolated parenchymal rat liver cells. *FEBS Lett*. 1979; **98:** 299.
3. Sanan DA, Fan J, Bensadoun A, Taylor JM. Hepatic lipase is abundant on both hepatocyte and endothelial cell surfaces in the liver. J Lipid Res. 1997; **38:** 1002.
4. Breeveld B, Schoonderwoerd K, Verhoeven AJ, Willemsen R, Jansen H. Hepatic lipase is localized at the parenchymal cell microvilli in rat liver. *Biochem J*. 1997; **321:** 425.
5. González-Navarro H, Nong Z, Freeman L, Bensadoun A, Peterson K, Santamarina-Fojo. Identification of mouse and human macrophages as a site of synthesis of hepatic lipase. *J Lipid Res*. 2002; **43(5):** 671–675.
6. Jansen H, De Greef WJ. Heparin-releasable lipase activity of rat adrenals, ovaries and testes. *Biochem J*. 1981; **196**: 739–745.
7. Persoon NL, Sips HJ, Hülsmann WC, Jansen H. Monoclonal antibodies against salt-resistant rat liver lipase. Cross-reactivity with lipases from rat adrenals and ovaries. *Biochim Biophys Acta.* 1986; **875:** 286–292.
8. Doolittle MH, Wong H, Davies RC, Schotz MC. Synthesis of hepatic lipase in liver and extrahepatic tissues. *J Lipid Res*. 1987; **28:** 1326–1334.
9. Verhoeven AJM, Jansen H. Hepatic lipase mRNA is expressed in rat and human steroidogenic organs. *Biochim Biophys Acta* 1994; **1211:** 121–124.
10. Zambon A, Austin MA, Brown BG, Hokanson JE, Brunzell JD. Effect of hepatic lipase on LDL in normal men and those with coronary artery disease. *Arterioscler Thromb.* 1993; **13:** 147–153.
11. Jansen H, de Greef WJ. L-type lipase activity in ovaries of superovulated rats. Relation to cholesterol homeostasis. *Mol Cell Endocrinol*. 1988; **57(1-2):** 7–15.
12. Vieira-van Bruggen D, Kalkman I, van Gent T, van Tol A, Jansen H. Induction of adrenal scavenger receptor BI and increased high density lipoprotein-cholesteryl ether uptake by in vivo inhibition of hepatic lipase. *J Biol Chem*. 1998; **273(48):** 32038–32041.
13. Isaacs A, Sayed-Tabatabaei FA, Njajou OT, Witteman JC, van Duijn CM. The -514 C->The hepatic lipase promoter region polymorphism and plasma lipids: a meta-analysis. *J Clin Endocrinol Metab*. 2004; **89:** 3858–3863.
14. Jansen H, Verhoeven AJ, Weeks L, Kastelein JJ, Halley DJ, van den Ouweland A, Jukema JW, Seidell JC, Birkenhäger JC. Common C-to-T substitution at position -480 of the hepatic lipase promoter associated with a lowered lipase activity in coronary artery disease patients. *Arterioscler Thromb Vasc Biol*. 1997; **17(11):** 2837–2842.
15. Perret B, Mabile L, Martinez L, Terce F, Barbaras R, Collet X. Hepatic lipase: structure/function relationship, synthesis, and regulation. *J Lipid Res*. 2002; **43:** 1163–1169.
16. Carr MC, Hokanson JE, Deeb SS, Purnell JQ, Mitchell ES, Brunzell JD. A hepatic lipase gene promoter polymorphism attenuates the increase in hepatic lipase activity with increasing intra-abdominal fat in women. *Arterioscler Thromb Vasc Biol*. 1999; **19:** 2701–2707.
17. Despres JP, Ferland M, Moorjani S, Nadeau A, Tremblay A, Lupien PJ, Theriault G, Bouchard C. Role of hepatic-triglyceride lipase activity in the association between intra-abdominal fat and plasma HDL cholesterol in obese women. *Arteriosclerosis*. 1989; **9:** 485–492.
18. Nie L, Wang J, Clark LT, Tang A, Vega GL, Grundy SM, Cohen JC. Body mass index and hepatic lipase gene (LIPC) polymorphism jointly influence postheparin plasma hepatic lipase activity. *J Lipid Res*. 1998; **39:** 1127–1130.
19. Zambon A, Hokanson JE, Brown BG, Brunzell JD. Evidence for a new pathophysiological mechanism for coronary artery disease regression: hepatic lipase-mediated changes in LDL density. *Circulation*. 1999; **99(15):** 1959–1964.
20. Deeb SS, Zambon A, Carr MC, Ayyobi AF, Brunzell JD. Hepatic lipase and dyslipidemia: interactions among genetic variants, obesity, gender, and diet. *J Lipid Res*. 2003; **44:** 1279–1286.

21. Tan KC, Shiu SW, Chu BY. Effects of gender, hepatic lipase gene polymorphism and type 2 diabetes mellitus on hepatic lipase activity in Chinese. *Atherosclerosis*. 2001; **157:** 233–239.
22. Berk-Planken II, Hoogerbrugge N, Stolk RP, Bootsma AH, Jansen H. Atorvastatin dose-dependently decreases hepatic lipase activity in type 2 diabetes: effect of sex and the LIPC promoter variant. *Diabetes Care*. 2003; **26**: 427–432.
23. Pihlajamäki J, Karjalainen L, Karhapää P, Vauhkonen I, Taskinen MR, Deeb SS, Laakso M. G-250A substitution in promoter of hepatic lipase gene is associated with dyslipidemia and insulin resistance in healthy control subjects and in members of families with familial combined hyperlipidemia. *Arterioscler Thromb Vasc Biol*. 2002; **20:** 1789–1795.
24. Botma GJ, van Deursen D, Vieira D, van Hoek M, Jansen H, Verhoeven AJM. Sterol-regulatory-element binding protein inhibits upstream stimulator factor-stimulated hepatic lipase gene expression. *Atherosclerosis*. 2005; **179:** 61–67.
25. Nimmo L, McColl AJ, Rosankiewicz JZ, Richmond W, Elkeles RS. Regulation of hepatic lipase expression in HepG2 cells. *Biochem Soc Trans*. 1997; **25(4):** S689.
26. Tu AY, Albers JJ. Glucose regulates the transcription of human genes relevant to HDL metabolism. *Diabetes*. 2001; **50:** 1851–1856.
27. van Deursen D, Jansen H, Verhoeven AJ. Glucose increases hepatic lipase expression in HepG2 liver cells through upregulation of upstream stimulatory factors 1 and 2. *Diabetologia*. 2008; **51(11):** 2078–2087.
28. Aufenanger J. Chapter II – Biochemistry of lipoproteins. In: *Lipid metabolism and Atherosclerosis*. Stuttgart (Germany): Schattauer verlag GmbH; 1982.
29. Patsch JR, Gotto AM, Jr, Olivecrona T, Eisenberg S. Formation of high density lipoprotein 2-like particles during lipolysis of very low density lipoproteins in vitro. *Proc Natl acad Sci USA*. 1978; **75:** 4519.
30. Redgrave TG, Small DM. Quantitation of the transfer of surface phospholipid of chylomicrons to the high density lipoprotein fraction during the catabolism of chylomicrons in the rat. *J Clin Invest*. 1979; **64(1):** 162-171.
31. Tall AR, Green PH, Glickman RM, Riley JW. Metabolic fate of chylomicron phospholipids and apoproteins in the rat. *J Clin Invest*. 1977; **64:** 977.
32. Olivecrona T, Liu G, Hultin M, Bengtsson-Olivecrona G: Regulation of lipoprotein lipase. *Biochem Soc Trans*. 1993; **21:** 509–513.
33. Goldberg IJ: Lipoprotein lipase and lipolysis: central roles in lipoprotein metabolism and atherogenesis. *J Lipid Res*. 1996; **37:** 693–707.
34. Sultan F, Lagrange D, Jansen H, Griglio S. Inhibition of hepatic lipase activity impairs chylomicron remnant-removal in rats. *Biochim Biophys Acta*. 1990; **1042(1):** 150–152.
35. de Faria E, Fong LG, Komaromy M, Cooper AD. Relative roles of the LDL receptor, the LDL receptor-like protein, and hepatic lipase in chylomicron remnant removal by the liver. *J Lipid Res*. 1996; **37(1):** 197–209.
36. Dichek HL, Brecht W, Fan J, Ji ZS, McCormick SPA, Akeefe H, Conzo L, Sanan DA, Weisgraber KH, Young SG, Taylor JM, Mahley RW. Overexpression of hepatic lipase in transgenic mice decreases apolipoprotein B-containing and high density lipoproteins. *J. Biol. Chem.* 1998; **273:** 1896–1903.
37. Yu KC, Cooper AD. Postprandial lipoproteins and atherosclerosis. *Front Biosci*. 2001; **6:** D332–354.
38. Spector AA, Haynes WG. LDL cholesteryl oleate: a biomarker for atherosclerosis? *Arterioscler Thromb Vasc Biol.* 2007; **27(6):** 1228–1230.
39. Aufenanger J. Lipoproteins. In: *Principles of Laboratory Medicine*. Zawta B (Editor.). Mannheim (Germany): Boehringer Mannheim GmbH; 1995.
40. Dietschy JM, Turley SD, Spady DK. Role of liver in the maintenance of cholesterol and low density lipoprotein homeostasis in different animal species, including humans. J Lipid Res. 1993; **34:** 1637–1659.
41. Chait A, Brazg RL, Tribble DL, Krauss RM. Susceptibility of small, dense, low-density lipoproteins to oxidative modification in subjects with the atherogenic lipoprotein phenotype, pattern B. *Am J Med*. 1993; 94(4): 350–356.
42. Schmitz G, Langmann T. Structure, function and regulation of the ABC1 gene product. *Curr Opin Lipidol*. 2001; **12(2):** 129–183.

43. Ohashi R, Mu H, Wang X, Yao Q, Chen C. Reverse cholesterol transport and cholesterol efflux in atherosclerosis. *Q J Med*. 2005; **98:** 845–856.
44. Wang D, Lan D, Chen W, Matsuura F, Tall AR. PNAS 2004, 101, 9774-9
45. Fidge NH. High density lipoprotein receptors, binding proteins, and ligands. *J Lipid Res*. 1999; **40(2):** 214–216.
46. Acton SL, Kozarsky KF, Rigotti A. The HDL receptor SR-BI: a new therapeutic target for atherosclerosis? *Mol Med Today*. 1999; **5(12):** 518–524.
47. Jonas A. Lecithin cholesterol acyltransferase. *Biochim Biophys Acta.* 2000; **1529(1-3):** 245–256.
48. Blades B, Vega GL, Grundy SM. Activities of lipoprotein lipase and hepatic triglyceride lipase in postheparin plasma of patients with low concentrations of HDL cholesterol. *Arterioscler Thromb*. 1993; **13:** 1227–1235.
49. Connelly PW, Hegele RA. Hepatic lipase deficiency. *Crit Rev Clin Lab. Sci*. 1998; **35:** 547–572.
50. Homanics GE, de Silva HV, Osada J, Zhang SH, Wong H, Borensztajn J, Maeda N. Mild dyslipidemia in mice following targeted inactivation of the hepatic lipase gene. *J Biol Chem*. 1995; **270:** 2974–2980.
51. Santamarina-Fojo S, Gonzalez-Navarro H, Freeman L, Wagner E, Nong Z. Hepatic lipase, lipoprotein metabolism, and atherogenesis. *Arterioscler Thromb Vasc Biol*. 2004; **24:** 1750–1754.
52. Tomlinson B. Raising high-density lipoprotein: for better or worse? *Heart*. 2010; **96(17):** 1339–1340.
53. Anderson KM, Castelli WP, Levy D. Cholesterol and mortality: 30 years of follow-up from the Framingham Study. *J Am Med Assoc*. 1987; **257:** 2176–2180.
54. Schaefer EJ, Lamon-Favo S, Cohn SD, Schaefer MM, Ordovas JM, Castelli WP, Wilson PWF. Effects of age, gender, and menopausal status on plasma low-density lipoprotein cholesterol and apolipoprotein B levels in the Framingham Offspring Study. *J Lipid Res*. 1994; **35:** 779–792.
55. Kreger BE, Odell PM, D'Agostino RD, Wilson P. Long-term intra-individual cholesterol variability: natural course and adverse impact on morbidity and mortality: the Framingham Study. *Am Heart J*. 1994; **127:** 1607–1614.
56. Santamarina-Fojo S, Haudenschild C, Amar M. The role of hepatic lipasein lipoprotein metabolism and atherosclerosis. *Curr Opin Lipidol.* 1998; **9:** 211–219.
57. Zambon AA, Deeb SSD, Pauletto PB, Crepaldi GA, Brunzell JD. Hepatic lipase: a marker for cardiovascular disease risk and response to therapy. *Curr Opin Lipidol.* 2003; **14:** 179–189.
58. Zambon A, Deeb SS, Hokanson JE, Brown BG, Brunzell JD. Common variants in the promoter of the hepatic lipase gene are associated with lower levels of hepatic lipase activity, buoyant LDL, and higher HDL2 cholesterol. *Arterioscler Thromb Vasc Biol.* 1998; **18:** 1723–1729.
59. Beisiegel U. New aspects on the role of plasma lipases in lipoprotein catabolism and atherosclerosis. *Atherosclerosis.* 1996; **124:** 1–8.
60. Hirano K, Yamashita S, Kuga Y, Sakai N, Nozaki S, Kihara S, Arai T, Yanagi K, Takami S, Menju M, Ishigama M, Yoshida Y, Kameda-Takemura K, Hayashi K, Matsuzawa Y. Atherosclerotic disease in marked hyperalphalipoproteinemia. Combined reduction of cholesteryl ester transfer protein and hepatic triglyceride lipase. *Arterioscler Thromb Vasc Biol.* 1995; **15:** 1849–1856.
61. van Acker BA, Botma GJ, Zwinderman AH, Kuivenhoven JA, Dallinga-Thie GM, Sijbrands EJ, Boer JM, Seidell JC, Jukema JW, Kastelein JJ, Jansen H, Verhoeven AJ; REGRESS Study Group, *Atherosclerosis*. 2008; **200(1):** 161–167.
62. Ginsberg HN. Diabetic dyslipidemia: basic mechanisms underlying the common hypertriglyceridemia and low HDL cholesterol levels. *Diabetes.* 1996; **45(Suppl. 3):** S27–30.
63. Johnson WJ, Bamberger MJ, Latta RA, Rapp PE, Phillips MC, Rothblat GH. The bidirectional flux of cholesterol between cells and lipoproteins. Effects of phospholipid depletion of high density lipoprotein. *J Biol Chem*. 1986; **261:** 5766–5776.
64. Lambert G, Amar MJ, Martin P, Fruchart-Najib J, Foger B, Shamburek RD, Brewer Jr HB, Santamarina-Fojo S. Hepatic lipase deficiency decreases the selective uptake of HDL cholesteryl esters in vivo. *J. Lipid Res.* 2000; **41:** 667–672.
65. Lambert G, Chase MB, Dugi K, Bensadoun A, Brewer Jr HB, Santamarina-Fojo S. Hepatic lipase promotes the selective uptake of high density lipoprotein-cholesteryl esters via the scavenger receptor B1. *J Lipid Res.* 1999; **40:** 1294–1303.

66. Collet X, Tall AR, Serajuddin H, Guendouzi K, Royer L, Oliveira H, Barbaras R, Jiang XC, Francone OL. Remodeling of HDL by CETP in vivo and by CETP and hepatic lipase in vitro results in enhanced uptake of HDL CE by cells expressing scavenger receptor B-I. *J Lipid Res.* 1999; **40:** 1185-1193.

67. Fielding CJ, Fielding PE. Cellular cholesterol efflux. *Biochim Biophys Acta.* 2001; **1533:** 175-189.

68. von Eckardstein A, Nofer JR, Assmann G. Acceleration of reverse cholesterol transport. *Curr Opin Cardiol.* 2000; **15:** 348–354.

69. von Eckardstein, A, Nofer JR, Assmann G. High density lipoproteins and arteriosclerosis. Role of cholesterol efflux and reverse cholesterol transport. *Arterioscler Thromb Vasc Biol.* 2001; **21:** 13–27.

70. Hill SA, McQueen MJ. Reverse cholesterol transport– a review of the process and its clinical implications. *Clin Biochem.* 1997; **30:** 517–525.

71. Couture P, Otvos JD, Cupples LA, Lahoz C, Wilson PW, Schaefer EJ, Ordovas JM. Association of the C-514T polymorphism in the hepatic lipase gene with variations in lipoprotein subclass profiles: The Framingham Offspring Study. *Arterioscler Thromb Vasc Biol.* 2000; **20:** 815–822.

72. Cohen JC, Vega GL, Grundy SM. Hepatic lipase: new insights from genetic and metabolic studies. *Curr Opin Lipidol.* 1999; **10:** 259–267.

73. Campos H, Dreon DM, Krauss RM. Associations of hepatic and lipoprotein lipase activities with changes in dietary composition and low-density lipoprotein subclasses. *J Lipid Res.* 1995; **36:** 462–472.

74. Breckenridge WC, Little JA, Alaupovic P, Wang CS, Kuksis A, Kakis G, Lindgren F, Gardiner G. Lipoprotein abnormalities associated with a familial deficiency of hepatic lipase. *Atherosclerosis.* 1982; **45:** 161–179.

75. Hegele RA, Little JA, Vezina C, Maguire GF, Tu L, Wolever TS, Jenkins DJA, Connelly PW. Hepatic lipase deficiency: Clinical, biochemical, and molecular genetic characteristics. *Arterioscler Thromb.* 1993; **13:** 720–728.

76. Carlson LA, Holmquist L, Nilsson-Ehle P. Deficiency of hepatic lipase activity in post-heparin plasma in familial hyper-[alpha]-triglyceridemia. *Acta Med Scand.* 1986; **219:** 435–447.

77. Brand K, Dugi KA, Brunzell JD, Nevin DN, Brewer HBJ, Santamarina-Fojo S. A novel A-G mutation in intron I of the hepatic lipase gene leads to alternative splicing resulting in enzyme deficiency. *J Lipid Res.* 1996; **37:** 1213–1223.

78. Knudsen P, Antikainen M, Ehnholm S, Uusi-Oukari M, Tenkanen H, Lahdenpera S, Kahri J, Tilly-Kiesi M, Bensadoun A, Taskinen MR, Ehnholm C. A compound heterozygote for hepatic lipase gene mutations Leu334–_Phe and Thr383–_Met: correlation between hepatic lipase activity and phenotypic expression. *J Lipid Res.* 1996; **37:** 825–834.

79. Brunzell JD, Deeb SS. Familial lipoprotein lipase deficiency, apoC-II deficiency, and hepatic lipase deficiency. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, Kinzler KW, Vogelstein B, eds. *The Metabolic and Molecular Bases of Inherited Disease*. New York, NY: McGraw-Hill; 2001: 2789–2816.

80. Demant T, Carlson LA, Holmquist L, Karpe F, Nilsson-Ehle P, Packard CJ, Shepherd J. Lipoprotein metabolism in hepatic lipase deficiency: studies on the turnover of apolipoprotein B and on the effect of hepatic lipase on high density lipoprotein. *J. Lipid Res*. 1988; **29:** 1603–1611.

81. Jansen H, Chu G, Ehnholm C, Dallongeville J, Nicaud V, Talmud PJ. The T allele of the hepatic lipase promoter variant C-480T is associated with increased fasting lipids and HDL and increased preprandial and postprandial LpCIII:B: European Atherosclerosis Research Study (EARS) II. *Arterioscler. Thromb. Vasc. Biol.* 1999; **19:** 303–308.

82. Asami T, Ciomartan T, Uchiyama M. Thyroxine inversely regulates serum intermediate density lipoprotein levels in children with congenital hypothyroidism. *Pediatr. Int.* 1999; **41:** 266–269.

83. Kraus RM. Relationship of intermediate and low-density lipoprotein subspecies to risk of coronary artery disease. *Am. Heart J.* 1987; **113:** 578-582.

84. Baynes J, Dominiczak M. Glucose homeostasis and fuel metabolism. In: *Medical Biochemistr*. Mosby Ltd., 2 ed., 2004; 243–266.

85. Henquin JC, Dufrane D, Nenquin M. Nutrient control of insulin secretion in isolated normal human islets. *Diabetes*. 2006; **5(12):** 470–477.

86. Cherrington AD. Banting Lecture 1997. Control of glucose uptake and release by the liver in vivo. *Diabetes*. 1999; **48:** 1198–1214.

87. Rizza RA, Mandarino LJ, and Gerich JE. Dose-response characteristics for effects of insulin on production and utilization of glucose in man. *Am J Physiol*. 1981; **240:** E630–E639.

88. Sacca L, Vigorito C, Cicala M, Corso G, and Sherwin RS. Role of gluconeogenesis in epinephrine-stimulated hepatic glucose production in humans. *Am J Physiol*. 1983; **245:** E294–E302.

89. Hansen I, Firth R, Haymond M, Cryer P, and Rizza R. The role of autoregulation of the hepatic glucose production in man. Response to a physiologic decrement in plasma glucose. *Diabetes*. 1986; **35:** 186–191.

90. Saltiel AR, Kahn CR. Insulin signalling and the regulation of glucose and lipid metabolism. *Nature*. 2001; **414(6865):** 799-806.

91. Bandsma RH, Smit GP, Kuipers F. Disturbed lipid metabolism in glycogen storage disease type 1. *Eur J Pediatr*. 2002; **161:** S65–69.

92. Bevilacqua S, Buzzigoli G, Bonadonna R, Brandi LS, Oleggini M, Boni C, Geloni M, Ferrannini E. Operation of Randle's cycle in patients with NIDDM. *Diabetes*. 1990; **39(3):** 383–389.

93. European Commision. *Public Health* (http://ec.europa.eu/health/ph_information/dissemination/diseases/diabetes_en.htm, accessed 6 May 2010).

94. The European health report 2002. WHO regional publication, European series. 2002; No. 97.

95. Voight BF, Scott LJ, Steinthorsdottir V, et al. Twelve type 2 diabetes susceptibility loci identified through large-scale association analysis. Nat Genet. 2010. **42**; 579-89.

96. Yoshino G, Hirano T, and Kazumi T. Dyslipidemia in diabetes mellitus. *Diabetes Res Clin Pract*. 1996; **33:** 1–14.

97. Reaven GM, Chen YD, Jeppesen J, Maheux P, and Krauss RM. Insulin resistance and hyperinsulinemia in individuals with small, dense low density lipoprotein particles. *J Clin Invest*. 1993; **92:** 141–146.

98. Lewis GF, Uffelman KD, Szeto LW, and Steiner G. Effects of acute hyperinsulinemia on VLDL triglyceride and VLDL apoB production in normal weight and obese individuals. *Diabetes* 1993; **42:** 833–842.

99. Bourgeois CS, Wiggins D, Hems R, and Gibbons GF. VLDL output by hepatocytes from obese Zucker rats is resistant to the inhibitory effect of insulin. *Am J Physiol* 1995; **269:** E208–E215.

100. Sparks JD and Sparks CE. Obese Zucker (fa/fa) rats are resistant to insulin's inhibitory effect on hepatic apo B secretion. *Biochem Biophys Res Commun* 1994; **205:** 417–422.

101. Lewis GF, Carpentier A, Adeli K, and Giacca A. Disordered fat storage and mobilization in the pathogenesis of insulin resistance and type 2 diabetes. *Endocr Rev*. 2002 ; **23:** 201–229.

102. Howard BV, Reitman JS, Vasquez B, and Zech L. Very-low-density lipoprotein triglyceride metabolism in non- insulin-dependent diabetes mellitus. Relationship to plasma insulin and free fatty acids. *Diabetes*. 1983; **32:** 271–276.

103. Kissebah AH, Alfarsi S, Evans DJ, and Adams PW. Integrated regulation of very low density lipoprotein triglyceride and apolipoprotein-B kinetics in non-insulindependent diabetes mellitus. *Diabetes*. 1982; **31:** 217–225.

104. Blades B and Garg A. Mechanisms of increase in plasma triacylglycerol concentrations as a result of high carbohydrate intakes in patients with non- insulindependent diabetes mellitus. *Am J Clin Nutr*. 1995; **62:** 996–1002.

105. Yost TJ, Froyd KK, Jensen DR, and Eckel RH. Change in skeletal muscle lipoprotein lipase activity in response to insulin/glucose in non-insulin-dependent diabetes mellitus. *Metabolism*. 1995; **44:** 786–790.

106. Blaak EE, Wagenmakers AJ, Glatz JF, Wolffenbuttel BH, Kemerink GJ, Langenberg CJ, Heidendal GA, and Saris WH. Plasma FFA utilization and fatty acid-binding protein content are diminished in type 2 diabetic muscle. *Am J Physiol Endocrinol Metab*. 2000; **279:** E146–E154.

107. Blaak EE, Aggel-Leijssen DP, Wagenmakers AJ, Saris WH, and van Baak MA. Impaired oxidation of plasma-derived fatty acids in type 2 diabetic subjects during moderate-intensity exercise. *Diabetes*. 2000; **49:** 2102–2107.

108. Katzel LI, Coon PJ, Busby MJ, Gottlieb SO, Krauss RM, Goldberg AP. Reduced HDL2 cholesterol subspecies and elevated postheparin hepatic lipase activity in older men with abdominal obesity and asymptomatic myocardial ischemia. *Arterioscler. Thromb*. 1992; **12:** 814–823.

109. Pollare T, Vessby B, Lithell H. Lipoprotein lipase activity in skeletal muscle is related to insulin sensitivity. *Arterioscler Thromb*. 1991; **11:** 1192–1203.

110. Tan KCB, Shiu SWM, Chu BYM. Roles of hepatic lipase and cholesteryl ester transfer protein in determining low density lipoprotein subfraction distribution in Chinese patients with non-insulin-dependent diabetes mellitus. *Atherosclerosis*. 1999; **145:** 273–278.

111. Baynes C, Henderson AD, Anyaoku V, Richmond W, Hughes CL, Johnston DG, Elkeles RS. The role of insulin insensitivity and hepatic lipase in the dyslipidaemia of type 2 diabetes. *Diabet Med*. 1991; **8:** 560–566.

112. Cominacini L, Garbin U, Davoli A, Campagnola M, De Santis A, Pasini C, Pastorino AM, Bosello O. High-density lipoprotein cholesterol concentrations and postheparin hepatic and lipoprotein lipases in obesity: relationships with plasma insulin levels. *Ann Nutr Metab*. 1993; **37:** 175–184.

113. Rashid S, Watanabe T, Sakaue T, Lewis GF. Mechanisms of HDL lowering in insulin resistant, hypertriglyceridemic states: the combined effect of HDL triglyceride enrichment and elevated hepatic lipase activity. *Clin Biochem*. 2003; **36:** 421–429.

114. Baynes C, Henderson AD, Richmond W, Johnston DG, Elkeles RS. The response of hepatic lipase and serum lipoproteins to acute hyperinsulinaemia in type 2 diabetes. *Eur J Clin Invest*. 1992; **22:** 341-346.

115. Lewis GF, Murdoch S, Uffelman K, Naples M, Szeto L, Albers A, Adeli K, Brunzell JD. Hepatic lipase mRNA, protein, and plasma enzyme activity is increased in the insulin-resistant, fructose-fed Syrian golden hamster and is partially normalized by the insulin sensitizer rosiglitazone. *Diabetes*. 2004; **53:** 2893–2900.

116. Ameis D., Stahnke G., Kobayashi J., McLean J., Lee G., Buscher M., Schotz M.C. und Will H. (1990). Isolation and characterization of the human hepatic lipase gene. *J Biol Chem*. 1990; **265:** 6552–6555.

117. Cai S.J., Wong D.M., Chen S.H. und Chan L. Structure of the human hepatic triglyceride lipase gene. *Biochemistry*. 1989; **28:** 8966–8971.

118. Warden C.H., Davis R.C., Yoon M.Y., Hui D.Y., Svenson K., Xia Y.R., Diep A., He K.Y. und Lusis A.J. Chromosomal localization of lipolytic enzymes in the mouse: pancreatic lipase, colipase, hormonesensitive lipase, hepatic lipase, and carboxyl ester lipase. *J Lipid Res*. 1993; **34:** 1451–1455.

119. Chang S.F., Scharf J.G. und Will H. Structural and functional analysis of the promoter of the hepatic lipase gene. *Eur J Biochem*. 1997; **247:** 148–159.

120. Guerra R., Wang J., Grundy S.M. und Cohen JC. A hepatic lipase (LIPC) allele associated with high plasma concentrations of high density lipoprotein cholesterol. *Proc Natl Acad Sci USA*. 1997; **94:** 4532–4537.

121. Murtomaki S., Tahvanainen E., Antikainen M., Tiret L., Nicaud V., Jansen H. und Ehnholm C. Hepatic lipase gene polymorphisms influence plasma HDL levels. Results from Finnish EARS participants. European Atherosclerosis Research Study. *Arterioscler Thromb Vasc Biol*. 1997; **17:** 1879–1884.

122. Hakonson, JE, Cheng S, Snell-Bergeon JK, Fijal BA, Grow MA, Hung C, Erlich HA, Erlich J, Eckel RH, Rewers M. A common promoter polymorphism in the hepatic lipase gene (LIPC-480C>T) is associated with an increase in coronary calcification in type 1 diabetes. *Diabetes*. 2002; **51:** 1208–1213.

123. Deeb SS, Peng R. The C-515T polymorphism in the human hepatic lipase gene promoter diminishes its activity. *J Lipid Res*. 2000; **41(1):** 155–158.

124. Botma GJ, Verhoeven AJ, Jansen H. Hepatic lipase promoter activity is reduced by the C-480T and G-216A substitutions present in the common LIPC gene variant, and is increased by Upstream Stimulatory Factor. *Atherosclerosis*. 2001; **154(3):** 625–632.

125. Hadzopoulou-Cladaras M, Cardot P. Identification of a cis-acting negative element which modulates human hepatic triglyceride lipase gene expression. *Biochemistry*. 1993; **32:** 9657–9667.

126. Kihara S, Wölle J, Ehnholm C, Chean L, Oka K. Regulation of hepatic triglyceride lipase by thyroid hormone in Hep G2 cells. *J Lipid Res*. 1993; **34:** 961–970.

127. Jansen H, Hülsmann WC. On hepatic and extrahepatic postheparin serum lipase activities and the influence of experimental hypercortisolism and diabetes on these activities. *Biochim Biophys Acta*. 1975; **398:** 337–346.

128. Parkes JG, Chan P, Goldberg DM. Secretion of triglyceride lipase from rat hepatocytes in culture: modulation by insulin and phenobarbital. *Biochem Cell Biol*. 1986; **64:** 1147–1152.

129. Colvin PL, Auerbach BJ, Case LD, Hazzard WR, Applebaum-Bowden D. A dose-response relationship between sex hormone-induced change in hepatic triglyceride lipase and high-density lipoprotein cholesterol in postmenopausal women. *Metabolism*. 1991; **40:** 1052–1056.

130. Applebaum-Bowden D, McLean P, Steinmetz A, Fontana D, Matthys C, Warnick GR, Cheung M, Albers JJ, Hazzard WR. Lipoprotein, apolipoprotein, and lipolytic enzyme changes following estrogen administration in postmenopausal women. *J Lipid Res*. 1989; **30:** 1895–1906.

131. Hülsmann WC, Oerlemans MC, Geelhoed-Mieras MM. Effect of hypothyroidism, diabetes and polyunsaturared fatty acids on heparin- releasable rat liver lipase. *Biochem Biophys Res Comm*. 1977; **79:** 784–788.

132. Nozaki S, Kubo M, Sudo H, Matsuzawa Y, Tarui S. The role of hepatic triglyceride lipase in the metabolism of intermediate-density lipoprotein – postheparin lipolytic activities determined by a sensitive, nonradioisotopic method in hyperlipidemic patients and normals. *Metabolism*. 1986; **35:** 53–58.

133. Perret B, Mabile L, Martinez L, Terce F, Barbaras R, Collet X. Hepatic Lipase: structure/function relationship, synthesis, and regulation. *J Lipid Res*. 2002; **43:** 1163–1169.

134. Busch SJ, Martin GA, Barnhart RL, Jackson RL. Heparin induces the expression of hepatic triglyceride lipase in a human hepatoma (Hep G2) cell line. *J Biol Chem*. 1989; **264:** 9527–9532.

135. Busch SJ, Barnhart RL, Martin GA, Flanagan MA, Jackson RL(1990): Differential regulation of hepatic triglyceride lipase and 3-hydroxy-3-methylglutaryl-CoA reductase gene expression in a human hepatoma cell line, Hep G2. *J Biol Chem*. 1990; **265:** 22474–22479.

136. Sirvent A, Verhoeven AJ, Jansen H, Kosykh V, Darteil R, Hum DW, Fruchart J-C, Staels B. Farnesoid X receptor represses hepatic lipase gene expression. *J. Lipid Res*. 2004; **45:** 2110–2115.

137. Benhizia F, Lagrange D, Malewiak M-I, Griglio S. In vivo regulation of hepatic lipase activity and mRNA levels by diets which modify cholesterol influx to the liver. *Biochim Biophys Acta*. 1994; **1211:** 181–188.

138. Doolittle MH, Wong H, Davis RC, Schotz MC: Synthesis of hepatic lipase in liver and extrahepatic tissues. *J Lipid Res*. 1987; **28:** 1326-1334.

139. Semenkovich CF, Chen SH, Wims M, Luo CC, Li WH, Chan L: Lipoprotein lipase and hepatic lipase mRNA tissue specific expression, developmental regulation, and evolution. *J Lipid Res*. 1989; **30:** 423–431.

140. Oka K, Ishimura-Oka K, Chu MJ, Chan L. Transcription of the hepatic lipase gene is modulated by multiple negative elements in Hep G2 cells. *Gene*. 1996; **180:** 69–80.

141. Rufibach LE, Duncan SA, Battle M, Deeb SS: Transcriptional regulation of the human hepatic lipase (LIPC) gene promoter. *J Lipid Res*. 2006; **47:** 1463–1477.

142. Jones DR, Schmidt RJ, Pickard RT, Foxworthy PS, Eacho PI. Estrogen receptor-mediated repression of human hepatic lipase gene transcription. *J Lipid Res*. 2002; **43(9):** 383–391.

143. Sawadogo M, Van Dyke MW, Gregor PD, Roeder RG. Multiple forms of the human gene-specific transcription factor USF. I. Complete purification and identification of USF from HeLa cell nuclei. *J Biol Chem*. 1988; **263(24):** 11985–11993.

144. Sawadogo M. Multiple forms of the human gene-specific transcription factor USF. II. DNA binding properties and transcriptional activity of the purified HeLa USF. *J Biol Chem*. 1988; **263(24):** 11994–12001.

145. Henrion AA, Vaulont S, Raymondjean M, Kahn A. Mouse USF1 gene cloning: comparative organization within the c-myc gene family. *Mamm. Genome.* 1996; **7:** 803–809.

146. Aperlo C, Boulukos KE, Pognonec, P. The basic region/helix-loop-helix/leucine repeat transcription factor USF interferes with Ras transformation. *Eur. J. Biochem*. 1996; **241:** 249–253.

147. Lin Q, Luo X, Sawadogo M. Archaic structure of the gene encoding transcription factor USF. *J. Biol. Chem*. 1994; **269:** 23894–23903.

148. Viollet B, Lefrançois-Martinez AM, Henrion A, Kahn A, Raymondjean M, Martinez A. Immunochemical characterization and transacting properties of upstream stimulatory factor isoforms. *J Biol Chem*. 1996; **271(3):** 1405–1415.

149. Sirito M, Walker S, Lin Q, Kozlowski MT, Klein WH, Sawadogo M. Members of the USF family of helix-loop-helix proteins bind DNA as homo- as well as heterodimers. *Gene Expr*. 1992; **2(3):** 231–240.

150. Casado M, Vallet VS, Kahn A, Vaulont S. Essential role in vivo of upstream stimulatory factors for a normal dietary response of the fatty acid synthase gene in the liver. *J Biol Chem*. 1999; **274(4):** 2009–2013.

151. Ribeiro A, Pastier D, Kardassis D, Chambaz J, Cardot P. Cooperative binding of upstream stimulatory factor and hepatic nuclear factor 4 drives the transcription of the human apolipoprotein A-II gene. *J Biol Chem*. 1999; **274(3):** 1216–1225.

152. Pastier D, Lacorte JM, Chambaz J, Cardot P, Ribeiro A. Two initiator-like elements are required for the combined activation of the human apolipoprotein C-III promoter by upstream stimulatory factor and hepatic nuclear factor-4. *J Biol Chem*. 2002; **277(17):** 15199–15206.

153. Nowak M, Helleboid-Chapman A, Jakel H, Martin G, Duran-Sandoval D, Staels B, Rubin EM, Pennacchio LA, Taskinen MR, Fruchart-Najib J, Fruchart JC. Insulin-mediated down-regulation of apolipoprotein A5 gene expression through the phosphatidylinositol 3-kinase pathway: role of upstream stimulatory factor. *Mol Cell Biol*. 2005; **25(4):** 1537–1548.

154. Salero E, Giménez C, Zafra F. Identification of a non-canonical E-box motif as a regulatory element in the proximal promoter region of the apolipoprotein E gene. *Biochem J*. 2003; **370(Pt 3):** 979–986.

155. Read ML, Clark AR, Docherty K. The helix-loop-helix transcription factor USF (upstream stimulating factor) binds to a regulatory sequence of the human insulin gene enhancer. *Biochem J*. 1993; **295 (Pt 1):** 233–237.

156. Matsukawa T, Inoue Y, Oishi Y, Kato H, Noguchi T. Up-regulation of upstream stimulatory factors by protein malnutrition and its possible role in regulation of the IGF-binding protein-1 gene. *Endocrinology*. 2001; **142(11):** 4643–4651.

157. Portois L, Tastenoy M, Viollet B, Svoboda M. Functional analysis of the glucose response element of the rat glucagon receptor gene in insulin-producing INS-1 cells. *Biochim Biophys Acta*. 2002; **1574(2):** 175–186.

158. Martin CC, Oeser JK, Svitek CA, Hunter SI, Hutton JC, O'Brien RM. Identification and characterization of a human cDNA and gene encoding a ubiquitously expressed glucose-6-phosphatase catalytic subunit-related protein. *J Mol Endocrinol*. 2002; **29(2):** 205–222.

159. Iynedjian PB. Identification of upstream stimulatory factor as transcriptional activator of the liver promoter of the glucokinase gene. *Biochem J*. 1998; **333 (Pt 3):** 705–712.

160. Pajukanta P, Lilja HE, Sinsheimer JS, Cantor RM, Lusis AJ, Gentile M, Duan XJ, Soro-Paavonen A, Naukkarinen J, Saarela J, Laakso M, Ehnholm C, Taskinen MR, Peltonen L. Familial combined hyperlipidemia is associated with upstream transcription factor 1 (USF1). *Nat Genet*. 2004; **36(4):** 371–376.

161. Coon H, Xin Y, Hopkins PN, Cawthon RM, Hasstedt SJ, Hunt SC. Upstream stimulatory factor 1 associated with familial combined hyperlipidemia, LDL cholesterol, and triglycerides. *Hum Genet*. 2005; **117(5):** 444–451.

162. Elbein SC, Hoffman MD, Teng K, Leppert MF, Hasstedt SJ. A genome-wide search for type 2 diabetes susceptibility genes in Utah Caucasians. *Diabetes*. 1999; **48(5):** 1175–1182.

163. Komulainen K, Alanne M, Auro K, Kilpikari R, Pajukanta P, Saarela J, Ellonen P, Salminen K, Kulathinal S, Kuulasmaa K, Silander K, Salomaa V, Perola M, Peltonen L. Risk alleles of USF1 gene predict cardiovascular disease of women in two prospective studies. *PLoS Genet*. 2006; **2(5):** e69.

164. Vallet VS, Casado M, Henrion AA, Bucchini D, Raymondjean M, Kahn A, Vaulont S. Differential roles of upstream stimulatory factors 1 and 2 in the transcriptional response of liver genes to glucose. *J Biol Chem*. 1998; **273:** 20175-20179.

165. Worgall TS, Sturley SL, Seo T, Osborne TF, Deckelbaum RJ. Polyunsaturated fatty acids decrease expression of promoters with sterol regulatory elements by decreasing levels of mature sterol regulatory element-binding protein. *J Biol Chem*. 1998; **273(40):** 25537–25540.

166. Worgall TS, Johnson RA, Seo T, Gierens H, Deckelbaum RJ. Unsaturated fatty acid-mediated decreases in sterol regulatory element-mediated gene transcription are linked to cellular sphingolipid metabolism. *J Biol Chem*. 2002 8; **277(6):** 3878–3885.

167. Sato R, Yang J, Wang X, Evans MJ, Ho YK, Goldstein JL, Brown MS. Assignment of the membrane attachment, DNA binding, and transcriptional activation domains of sterol regulatory element-binding protein-1 (SREBP-1). *J Biol Chem*. 1994; **269(25):** 17267–17273.

168. Wang X, Sato R, Brown MS, Hua X, Goldstein JL. SREBP-1, a membrane-bound transcription factor released by sterol-regulated proteolysis. *Cell*. 1994; **77(1):** 53–62.

169. Sato R, Miyamoto W, Inoue J, Terada T, Imanaka T, Maeda M. Sterol regulatory element-binding protein negatively regulates microsomal triglyceride transfer protein gene transcription. *J Biol Chem*. 1999; **274(35):** 24714–24720.

170. Llorente-Cortés V, Royo T, Otero-Viñas M, Berrozpe M, Badimon L. Sterol regulatory element binding proteins downregulate LDL receptor-related protein (LRP1) expression and LRP1-mediated aggregated LDL uptake by human macrophages. *Cardiovasc Res*. 2007; **74(3):** 526–536.

171. Moon YS, Latasa MJ, Griffin MJ, Sul HS. Suppression of fatty acid synthase promoter by polyunsaturated fatty acids. *J Lipid Res*. 2002; **43(5):** 691–698.

172. Horton JD, Shimomura I, Brown MS, Hammer RE, Goldstein JL, Shimano H. Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2. *J Clin Invest*. 1998; **101(11):** 2331–2339.

CHAPTER 2

Comparative genomics and experimental promoter analysis reveal functional liver-specific elements in mammalian hepatic lipase genes

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2.1 Abstract

Background: Mammalian hepatic lipase (HL) genes are transcribed almost exclusively in hepatocytes. The basis for this liver-restricted expression is not completely understood. We hypothesized that the responsible cis-acting elements are conserved among mammalian HL genes. To identify these elements, we made a genomic comparison of 30 kb of 5'-flanking region of the rat, mouse, rhesus monkey, and human HL genes. The *in silico* data were verified by promoter-reporter assays in transfected hepatoma HepG2 and non-hepatoma HeLa cells using serial 5'-deletions of the rat HL (-2287/+9) and human HL (-685/+13) promoter region.
Results: Highly conserved elements were present at the proximal promoter region, and at 14 and 22 kb upstream of the transcriptional start site. Both of these upstream elements increased transcriptional activity of the human HL (-685/+13) promoter region 2-3 fold. Within the proximal HL promoter region, conserved clusters of transcription factor binding sites (TFBS) were identified at -240/-200 (module A), -80/-40 (module B), and -25/+5 (module C) by the rVista software. In HepG2 cells, modules B and C, but not module A, were important for basal transcription. Module B contains putative binding sites for hepatocyte nuclear factors HNF1α. In the presence of module B, transcription from the minimal HL promoter was increased 1.5-2 fold in HepG2 cells, but inhibited 2-4 fold in HeLa cells.
Conclusion: Our data demonstrate that searching for conserved non-coding sequences by comparative genomics is a valuable tool in identifying candidate enhancer elements. With this approach, we found two putative enhancer elements in the far upstream region of the HL gene. In addition, we obtained evidence that the -80/-40 region of the HL gene is responsible for enhanced HL promoter activity in hepatoma cells, and for silencing HL promoter activity in non-liver cells.

2.2 Background

Understanding transcriptional regulation of gene expression is a major challenge in molecular biology. In eukaryotes, regulation of gene expression is achieved through the complex interaction of transcription factors, which bind to specific DNA sequence motifs. These motifs are predominantly located in the upstream region of genes. Over the last decades, numerous transcription factors have been identified, each with its own specific DNA binding sequence (TFBS). Transcription factors that are potentially involved in the regulation of a particular gene are usually identified by the presence of the specific DNA binding motif in the upstream regulatory region. These binding motifs are compiled in libraries such as the Transfac database [1], and programs such as MatInspector enable pattern recognition with the entries in this database [2]. Unfortunately, most transcription factors bind to short, degenerate sequences, which occur very frequently in the eukaryotic genome. Only a very small fraction of all predicted binding sites is biologically relevant [3]. Recently, new strategies for the *ab initio* identification of functionally significant *cis*-acting regulatory sequences have been developed, based on the assumption that regulatory elements are conserved among multiple species [4-8], and that multiple TFBS tend to specifically cluster together [9,10]. The rVista computational tool for identification of functional regulatory elements combines the comparative sequence analysis of orthologous genes with the analysis of clustering of predicted TFBS [11,12]. In this study, we tested the validity of this approach to identify functional TFBS for the mammalian hepatic lipase genes, by comparing the *in silico* data with experimental promoter-reporter assays.

Hepatic lipases (HL) are synthesized and secreted almost exclusively by hepatocytes [13-15]. Although synthesis of HL has been shown to occur in mouse adrenals [16], and in mouse and human macrophages [17], this is negligible compared to expression in liver. The HL activity present in adrenals and ovaries [18] originates predominantly from liver, and is transported through the circulation to these organs [19,20]. In liver, the enzyme is bound to cell surface proteoglycans within the sinusoids, from where it can be released by heparin. Hepatic lipase plays an important role in plasma lipoprotein metabolism and intracellular lipid homeostasis [21], by mediating cholesterol influx into liver cells from high-density lipoproteins (HDL), and clearance of remnant lipoproteins from the circulation by the liver. HL is an important determinant of plasma HDL cholesterol levels, and is implicated in the protection against development of premature atherosclerosis by HDL [21]. HL gene expression in humans and rodents is regulated by various hormones and nutritional states mainly at the transcriptional level, but up- or downregulation is limited to about two-fold [15]. In contrast to this moderate regulation by hormones and nutrition, the almost complete restriction of HL gene expression to differentiated liver cells is highly conspicuous [13,14]. Several groups have pointed to the HNF1 and HNF4a binding sites in the proximal promoter of the HL gene to explain this liver-specificity in humans [22-25]. Since the liver-restricted expression is a common feature of most, if not all, mammalian HL genes, we hypothesize that the regulatory elements responsible for liver-specific expression are conserved among mammals. We therefore searched the upstream

regulatory region of the rat, mouse, rhesus monkey and human genes for the presence of conserved clusters of TFBS motifs, and combined the *in silico* data with experimental promoter-reporter assays in cultured cells of hepatic versus non-hepatic origin. This unbiased approach led to the identification of two putative enhancer elements in the far upstream region, and of highly conserved sequence modules within the proximal promoter of the HL genes.

2.3 Results

2.3.1 Interspecies comparison of genomic HL sequences

Of the mammalian HL genes, genome sequence including part of the 5'-flanking region is available for human, chimpanzee, rhesus monkey, rat, mouse and hedgehog (Ensembl ***e!***42:Dec 2006) [26]. Pairwise alignment of the HL coding sequences shows the expected, high degree of sequence identity (Table 1). This high homology also extends into the 5'-UTR and upstream-regulatory region, with sequence identity ranging from 53 to 98 % over the proximal 0.9-1.4 kb. Multiple sequence alignment of 30-kb of the 5'-flanking region available for five HL genes (all except for the hedgehog, which known sequence is too short) was performed by the mVista web-tool (Figure 1). The chimp and macaque sequences are highly homologous to the human sequence, as 95 % and 64 % of the 30-kb region showed at least 95 % sequence identity over a 100-bp window, respectively. Similarly, 5 % of the rat sequence showed this high degree of sequence identity with the orthologous mouse sequence. Because of the near-identity of the chimp to the human sequence, we only included the latter sequence in subsequent *in silico* analysis. The global genomic sequence comparison showed a particularly high conservation among the four genes immediately upstream of the transcriptional start site (P=7*10^{-7}). Three additional islands of highly significant homology (P<10^{-5}) were identified further upstream. Conservation of a 475 bp element at -14 kb was even more significant (P=4*10^{-11}) than the proximal HL promoter region (Figure 1). The element at -22 kb (P=2*10^{-6}) contained a 173bp sequence that was completely identical among the three primate sequences.

To test the potential enhancer function of two of the most conserved elements in the far upstream regulatory region, promoter-reporter assays were performed with human HL promoter constructs in transiently transfected HepG2 hepatoma cells. The -14 kb element (679 bp) and the -22 kb element (387bp) were inserted in the hHL-685Luc plasmid. As a control, we also tested the activity of the non-conserved -10kb sequence (502 bp). The transcriptional activity of the human HL -685/+13 promoter region was increased 3- and 2-fold with the -14 kb and -22 kb elements inserted in the sense orientation, respectively (Figure 2A). In contrast, the -10 kb sequence slightly but significantly reduced HL promoter activity (n=3; P<0.05). Qualitatively similar effects were obtained when the elements were inserted in the anti-sense orientation (Figure 2B). Hence, the conserved sequences at -14 kb and -22 kb have moderate enhancer activity of the proximal HL promoter region.

Table 1. Pairwise sequence comparison of the coding and the 5′-flanking sequence of mammalian HL genes.

	Upstream regulatory sequence							
cDNA sequence	81		53	53	61	57	56	hedgehog[a]
	79	76		86	64	55	59	mouse
	79	76	92		63	57	57	rat
	85	82	79	79		94	94	rhesus[a]
	85	82	80	80	96		98	chimp
	85	82	79	79	96	99		human
	rabbit[b]	hedgehog	mouse	rat	rhesus	chimp	human	

Sequence identity (%) was determined by pairwise alignment using the DNAMAN software package (optimal alignment; gap open penalty 10.0, gap extension penalty 5.0). cDNA sequence was from the translation start ATG up till the stopcodon; the upstream regulatory region was from -1400 up till the translation start ATG.

[a]: Because of a gap in upstream regulatory region of the public sequence of the rhesus monkey and hedgehog HL gene (Ensembl *e!*42: Dec 2006), alignments with the rhesus and hedgehog sequence was done with the sequence from -900 and -1000 up till the start ATG, respectively.

[b]: the rabbit HL cDNA sequence was taken from [38].

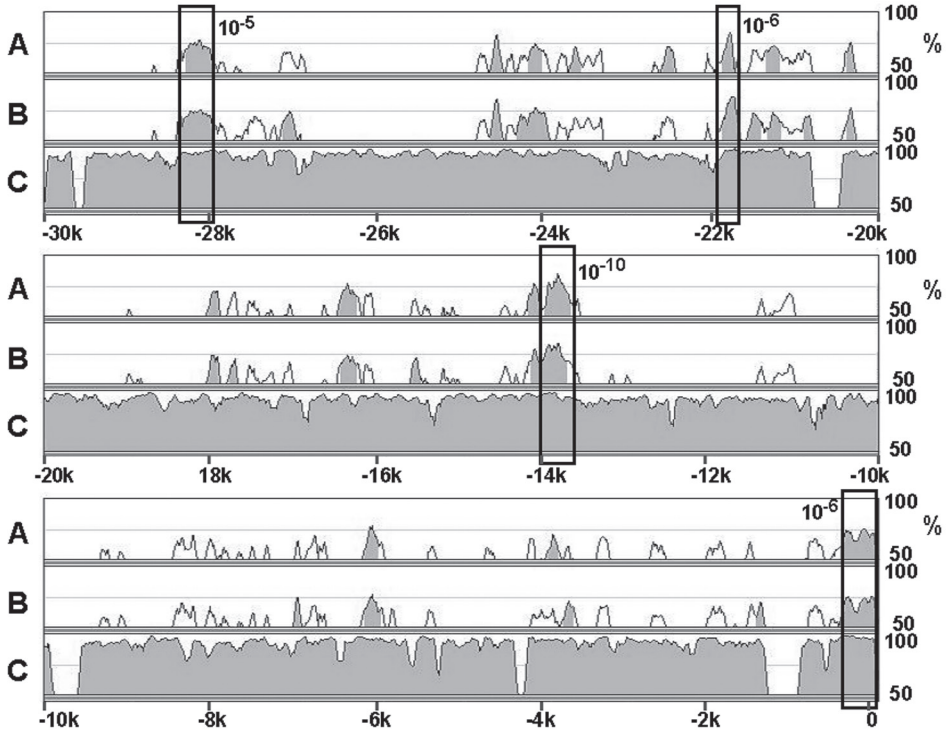

Figure 1. Alignment of the 5′-flanking region of four mammalian HL genes.

From the rat, mouse, macaque and human HL genes, exon-1 and 30 kb of upstream sequence was aligned by the MLAGAN algorithm of the mVista program. The sequences of rat (**A**), mouse (**B**) and macaque (**C**) are aligned to the human HL sequence (x-axis); numbering is relative to the transcriptional start site. Conserved regions (>70% homology over 100 bp window) are shaded. The boxes indicate conserved regions among the four sequences, as determined by RankVista ($P \leq 10^{-5}$), with the P-values given above.

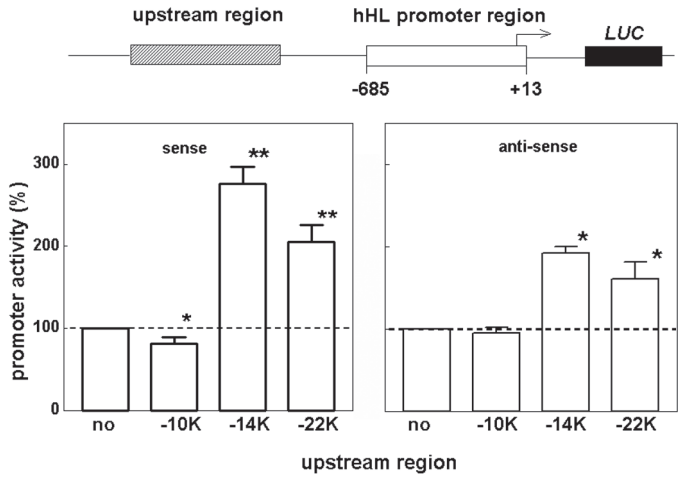

Figure 2. Possible enhancer activity of the conserved sequences in the far upstream HL regulatory region.

HepG2 cells were transiently transfected with the indicated promoter-reporter constructs. At 48 h post-transfection, transcriptional activity was determined as the firefly over renilla luciferase activity. Data are expressed as percentage of the ratio measured in the hHL-685luc - transfected cells. Data are means ±SD from 3-4 independent experiments, each performed in quadruplicate. *and **: $P<0.05$; and $P<0.01$, respectively

2.3.2 Comparative genomics of the proximal 2 kb HL upstream regulatory region

Submission of 2-kb upstream sequence of the rat HL gene to the MatInspector software program (core similarity >0.75; matrix similarity >0.70) returned over 2000 potential TFBS, randomly distributed over the entire sequence. A similar number of sites was predicted for the orthologous mouse, human and rhesus macaque sequences. When we searched for clustered TFBS motifs that are conserved between the rat and human sequence, using the web-tool rVista, three separate modules were identified within the proximal promoter region (Figure 3). Module A (-240 to -200 in the human sequence relative to the transcriptional start site), for which AP1, AP2, CAAT, COUP, C/EBP, HNF4α, PPAR and USF binding sites are predicted, corresponds to the DR1 site recently identified by Rufibach *et al.* [25]. Module B (-80 to -40) potentially contains AP2, CAAT, C/EBP, HNF1, HNF4, PPAR and Sp1 sites, and corresponds to the previously characterized HNF1 site [22-24]. Module C (-25 to + 5), which may bind AP2, C/EPB, HNF4, PPAR and USF factors, contains the transcription start site preceded by a conserved pyrimidine-rich motif, and therefore likely represents the Inr involved in binding of the transcription initiation complex. These three modules were also found to be conserved among the human and mouse HL gene. The human-mouse comparison revealed an additional, conserved module (-295 to -265), with potential binding sites for AP2, C/EBP, HNF1, HNF4, PPAR and Sp1, and which partly overlaps the DR4-site recently described by Rufibach *et al.* [25]. Similar results were obtained in pairwise comparisons between orthologous sequences of macaque and rat, and of macaque and mouse. Despite the high homology in the intervening sequence between

modules A and B, the rVista program did not recognize conserved clusters of TFBS among the human, macaque, rat and mouse. Irrespective of which transcription factors actually bind to these sites, the results of the interspecies sequence comparison by rVista suggest that the three highly conserved sequence modules in the proximal HL promoter region are involved in common features of transcriptional regulation. This is further supported by the fact that these three modules correspond to distinct DNA footprints of the human HL sequence in rat liver [22] and human HepG2 cells [23].

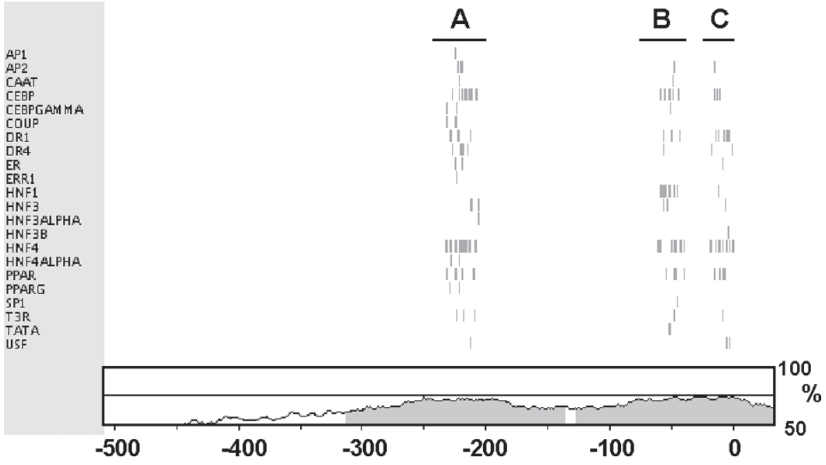

Figure 3. Identification of functional regulatory sequences in the proximal promoter region of the HL gene by rVista.

Of the proximal promoter regions of the rat and human HL genes, 600 bp were submitted to the rVista sequence analysis software, and searched for conserved clusters of TFBS for a selection of 50 transcription factors known to be expressed in mammalian liver. A vertical line indicates the position of the conserved TFBS relative to the human sequence (x-axis, numbering is relative to the transcriptional start site). Homology between the rat and human sequence is given as described for figure 1. Three clusters of conserved TFBS are identified, and designated A (-240/-200), B (-80/-40) and C (-25/+5). In human-mouse comparison, these clusters are also evident with an additional cluster at -295/-265.

2.3.3 Functional characterization of the rat HL promoter region

To corroborate the *in silico* results, promoter-reporter assays were performed with promoter fragments of the rat HL gene in transiently transfected HepG2 cells. Plasmids were constructed with progressively 5′-deleted promoter fragments spanning the -2287/+9 region of the rat HL gene in front of the CAT reporter gene. Compared to the SV40 promoter, the rHL-2287 construct showed low CAT expression (Figure 4). Upon deleting the 5′-end of the HL promoter fragments to position -1048, CAT expression became even lower, and was no longer significantly different from promoter-less pCAT-Basic, suggesting that there is weak enhancer activity between nucleotides -1697 and -2287. Further deletion to position -754 slightly increased promoter activity to levels significantly above background. Shortening the insert from -754 to -446

resulted in a 5-fold increase in promoter activity, suggesting the presence of a strong negative regulatory element in this region of the rat HL gene. CAT expression was not significantly affected by deleting the insert from -446 to -211. The presence of the weak enhancer element between -2287 and -1697, and the negative element between -754 and -446 corresponds to positive and negative elements in the human HL upstream regulatory region observed by Oka *et al*. [23]. We assume, therefore, that both these elements are present in homologous parts of the rat and human gene. Indeed, the global alignment of the four species by mVista detected homology at these parts of the gene (Figure 1), but homology did not exceed the 70 % over 100 bp mark used as threshold in this analysis. Apparently, potentially important elements may be missed due to the high stringency of the conservation rule in the mVista program.

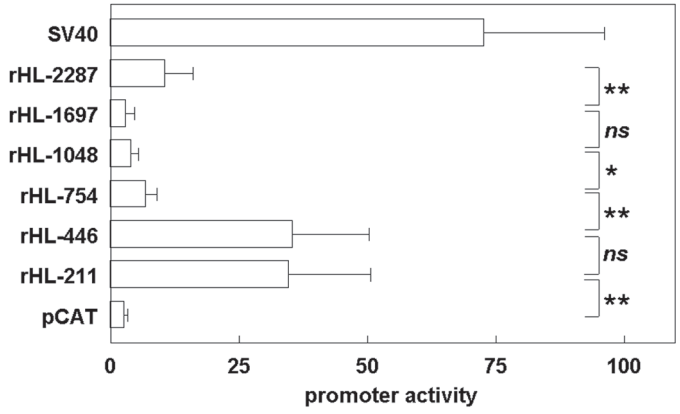

Figure 4. Effect of serial 5'-deletions of the rat HL upstream region on transcriptional activity in HepG2 cells.

HepG2 cells were transiently transfected with the indicated promoter-reporter constructs. At 48 h post-transfection, cells were lysed and expression of CAT and b-galactosidase protein was determined. Data are expressed as the ratio of CAT over b-galactosidase expression. Data are means ±SD from 4-7 independent experiments, each performed in triplicate. *: $P<0.05$; **: $P<0.01$; n.s.: not significant.

To test the importance of the conserved sequence modules within the -220 to +9 region, further 5'-deletions in the rat HL promoter region were made (Figure 5). Transcriptional activity of the rHL-127 construct, in which module A has been removed, was not significantly different from that of the rHL-446 or rHL-221 constructs. Similarly, removal of the highly conserved intervening sequence between modules A and B (rHL-86 and rHL-75) had no significant effect on CAT expression. In contrast, additional removal of most of module B in rHL-39 reduced transcriptional activity by approximately 60 %. With rHL-23, in which the remainder of module B as well as the putative TATA-box has been deleted, CAT expression decreased further. Despite absence of the TATA-box, CAT expression of the rHL-23 construct was significantly higher than of promoter-less pCAT-Basic, which may be due to residual promoter activity of module C.

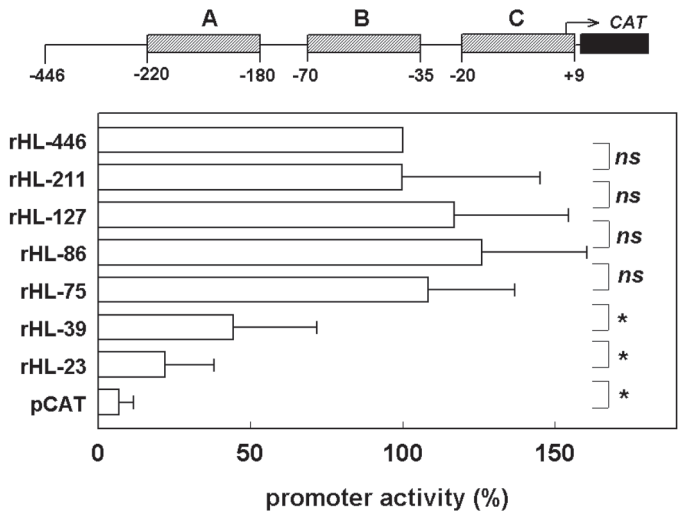

Figure 5. Effect of serial 5'-deletions of the rat HL promoter region on transcriptional activity in HepG2 cells.

Experiments were performed as described in the legends to figure 4. Data were expressed as percentage of the ratio measured in the rHL-446 CAT - transfected cells, and are means ±SD from 3-5 independent experiments, each performed in triplicate. *: $P<0.05$; n.s.: not significant.

2.3.4 Comparison with the proximal human HL promoter region

Similar promoter-reporter assays were performed with the -685/+13 region of the orthologous human HL gene, except that the luciferase gene was used as reporter (Figure 6). Luciferase activity of the hHL-306 construct was similar to hHL-685, whereas activity of the hHL-79 construct was slightly, but not significantly, higher. This is in line with the rat promoter data, which show little effect of module A, and of the intervening sequence between modules A and B, on basal transcriptional activity in HepG2 cells. The luciferase activity of the hHL-36 construct, in which entire module B has been removed, was only 25% of the hHL-79 construct. The transcriptional activity of hHL-36, which contains a *bona fide* TATA box and entire module C, was 7-fold higher than background. This confirms that modules B and C are crucial for basal transcriptional activity in HepG2 cells, with module B being most important.

2.3.5 Role of module B in liver cell-specific HL transcription

To test whether modules A and B are involved in liver-specific expression of the HL gene, we compared transcriptional activity of different rat HL promoter fragments in HepG2 cells with non-hepatic HeLa cells (Figure 7). Promoter activity in each cell line was expressed as percentage of that of the rHL-39 construct, because this fragment represents the minimal promoter with the TATA-box and transcription start site. In the hepatoma cells, the activity of the rHL-75 construct was 1.5-2 fold higher than the minimal promoter construct. In HeLa

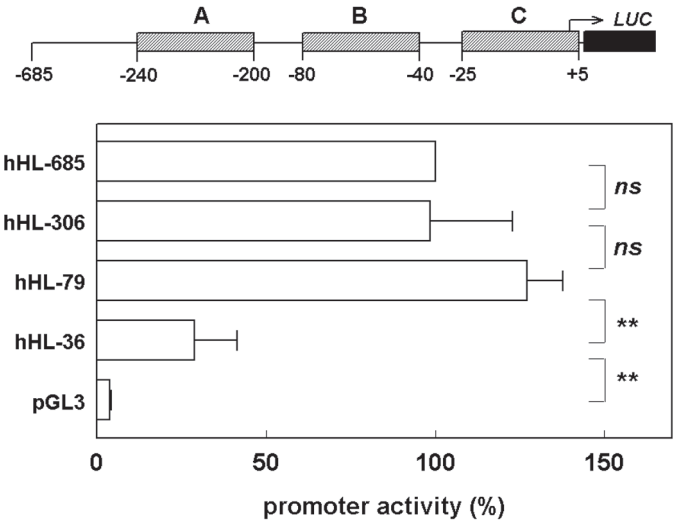

Figure 6. Effect of serial 5′-deletions of the human HL promoter region on transcriptional activity in HepG2 cells.

HepG2 cells were transiently transfected with the indicated promoter-reporter constructs. At 48 h post-transfection, transcriptional activity was determined as the firefly over renilla luciferase activity. Data are expressed as percentage of the ratio measured in the hHL-685luc – transfected cells. Data are means ±SD from 4 independent experiments, each performed in quadruplicate. *: $P<0.05$; n.s.: not significant.

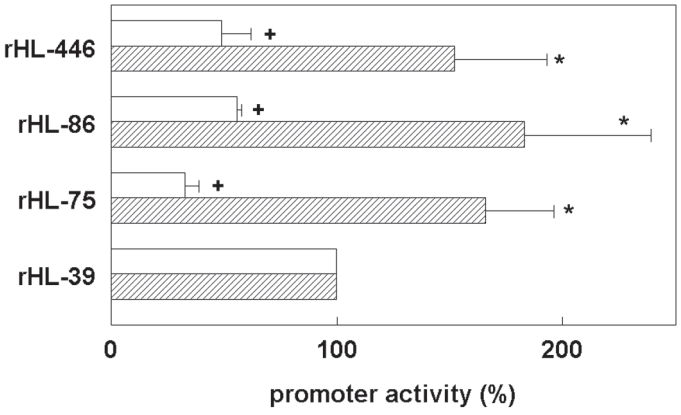

Figure 7. Transcriptional activity of the rat proximal HL promoter region in HepG2 and HeLa cells.

HepG2 (hatched bars) and HeLa cells (open bars) were transiently transfected with the indicated promoter-reporter constructs. At 48 h post-transfection, transcriptional activity was determined as the ratio of CAT over b-galactosidase expression level. Data are expressed as percentage of the ratio measured in the rHL-39 CAT – transfected cells. Data are means ±SD from 3 independent experiments, each performed in triplicate. *,+: $P<0.05$ compared to 100% in HepG2 and HeLa cells, respectively.

cells, contrastingly, transcriptional activity of rHL-75 was 2-4 fold lower than the minimal promoter construct in HeLa cells. Consequently, there was a marked, 3-5-fold difference in relative promoter activity between these two cell lines. Similar results were obtained with the longer rat HL constructs that all contained module B. The data were minimally affected by the simultaneous presence of module A (Figure 7). Qualitatively similar results were obtained with human HL promoter fragments (data not shown). We conclude therefore, that module B plays a pivotal role in liver-restricted expression of the HL gene, by moderately activating transcription in liver cells, and simultaneously suppressing activity in non-hepatic cells.

2.4 Discussion

Global alignment of the 5'-flanking region of mammalian HL genes revealed three highly conserved elements ($P \leq 10^{-5}$) that lie far upstream of the HL promoter (Figure 1). Two of these elements, at -14kb and -22kb, show moderate enhancer activity in HepG2 cells. What discriminates the conserved -14kb and -22kb elements from the non-functional, non-conserved -10kb sequence is unclear, as all three sequences contain a similar repertoire of TFBS for liver-expressed transcription factors (data not shown). Further studies are required to clarify the mechanism responsible for the enhancer activity of the two highly conserved elements in the HL gene. The finding of two hitherto unknown enhancers supports the hypothesis that conserved non-coding sequences may identify functional regulatory elements. Experimentally, we also found a positive and a negative regulatory sequence between -2.2 and -0.4 kb of the rat HL gene that coincided with homology peaks, but were not recognized by the Rankvista analysis of the sequence comparison. Rubin's group recently demonstrated strong *in vivo* enhancer activity for almost half of the elements that are ultra-conserved among human/mouse/rat [8,27]. Our study further illustrates the power of the approach, and suggests that gene regulatory functions may also reside in somewhat less conserved elements among mammalian genomes.

We also tested whether global genome comparisons can also aid in identification of functional regulatory elements within highly conserved sequences, using the proximal HL promoter region as a model. Within this proximal promoter region, three modules are identified with conserved clusters of TFBS motifs. These modules A, B and C correspond with the previously identified regulatory elements DR1 [25], HNF1 [22-24] and Inr [22-24], respectively. However, we missed an additional module (-295 to –265) that has recently been identified as a functional DR4 site [25]. The cluster of TFBS within this module appeared to be conserved among human and mouse, but not among human and rat. Despite the relatively high homology between the mouse and rat over the proximal 5'-flanking region of the HL gene (Table 1), the outcome of the genomic sequence analysis differed whether the rat or the mouse sequence was used. Hence, although searching genomic sequences for conserved

clusters of TFBS is a valuable tool in predicting functionally important regulatory elements, this approach is sub optimal.

For two of the modules that are conserved among the four species, a significant contribution to basal transcription was confirmed by promoter-assays in HepG2 cells. For module C (-25/+5), this is not surprising since it contains the transcriptional start site itself, as well as a pyrimidine-rich stretch that may serve as an initiator region (Inr). Module B (-80 to -40) overlaps with a protected region in DNAse footprinting in rat liver [22] as well as in HepG2 cells [23], and contains a HNF1 binding site that has been implicated in liver-specific expression of the human HL gene by other groups [22-24]. Experimentally, we could not confirm a major role for module A (-240 to -200) in determining basal transcription activity in HepG2 cells. This is surprising since it corresponds to a functional DR1 site [25], and perfectly matches with a protected region in DNAse footprinting in rat liver and human HepG2 nuclear extracts [22, 23], suggesting that this part of the HL promoter is occupied by transcription factors under basal conditions. Similarly, we could not confirm the role of the DR4 module (-295 to -265) conserved among human and mouse, in basal transcriptional activity in HepG2 cells. We propose, therefore, that this part of the HL promoter region is involved in modulation of gene transcription under different hormonal or nutritional conditions.

We show here that the conserved module B (-80 to -40) plays a dual role in mediating liver-restricted transcription of the HL gene. On the one hand, the module mediates moderate stimulation of minimal promoter activity in liver-derived HepG2 cells, and on the other hand, it mediates inhibition of minimal promoter activity in the non-hepatic HeLa cells. Of the potential TFBS identified in module B, the liver-enriched HNF1 is a likely candidate for effecting the liver-specific activation of the HL promoter. Other groups have already suggested an important role for the HNF1 binding site [22-24], and *in vitro* HNF1 binding to this sequence has been demonstrated by gelshift assays [24]. Furthermore, HNF1α knockout mice have 3.4 fold lower HL mRNA levels than control mice [28]. In primary hepatocytes, HL secretion increases with HNF1α gene dosage [28]. However, HL mRNA and HL secretion are not completely lost by HNF1α knockout, indicating that HNF1α is not the only transcription factor determining HL expression in liver. HL secretion was only observed with hepatoma cell lines that express HNF1α or HNF1β mRNA [24], but not all cell lines with detectable HNF1α or -β expression do also secrete HL. In fact, HL secretion correlated with expression of HNF4 rather than with HNF1 mRNA [24]. The HNF4a gene itself is a target of HNF1α [29]. Since potential HNF4a binding sites were detected in the conserved module A (as well as in the -295/-265 module), the liver-specific stimulation of HL promoter activity may well be mediated by HNF4α. In fact, HNF4α is bound to the promoter regions of almost half of the actively transcribed genes in human liver [29] and therefore contributes to a large fraction of liver-specific gene expression. Sequence modules that contain both HNF1 and HNF4 binding sites are among the strongest predictors of liver-specific transcription [10]. Rufibach *et al.* [25] proposed that HNF1α and HNF4α independently and additively activate HL promoter activity. Which transcription factor(s) mediate inhibition of minimal promoter activity in cells of non-hepatic origin, remain(s) unknown at present.

2.5 Conclusions

In summary, we have shown here that a global multispecies comparison of non-coding sequences, followed by a search for conserved clusters of TFBS, has predicted the most important sequences involved in basal transcription of the HL gene. This *in silico* analysis does not identify all regulatory sequences in a particular gene, but enables the intelligent design of experiments towards identification of functional cis-regulatory elements and transactivating factors in gene regulation. This study illustrates the power of comparative genomics in the identification of TFBS that are functional in gene expression.

2.6 Methods

2.6.1 Database analysis

The annotated data of the mammalian genome sequence projects were accessed through the Ensembl genome server (***e!***42: Dec 2006) [30]. The exon-1 and 5'-upstream regulatory sequence of the hepatic lipase gene was available only for human (ENSG00000166-035), rat (ENSRNOG00000015747), mouse (ENSMUSG00000032207), hedgehog (ENSETEG00000015177), chimpanzee (ENSPTRG00000007115) and rhesus macaque (ENSMMUG00000009566). Multiple sequence alignment was performed with DNAMAN software package version 3.2 (Lynnon BioSoft, Quebec, Canada). Global sequence alignments were performed with the publicly available web-based tool mVista [12,31] using the MLAGAN algorithm. A search for potential TFBS in the upstream regulatory region of a particular HL gene was performed online at Genomatix using the MatInspector program [2,32]. Clusters of TFBS that are conserved among the rat, mouse, human and macaque HL promoter regions were identified by the publicly available web-tool rVista [11,12,31].

2.6.2 Isolation of exon-1 and the 5'-flanking region of the rat HL gene

A rat genomic library in l DASH II (Stratagene, La Jolla, CA, USA) was used for isolation of the HL promoter region, using a HL cDNA probe corresponding to exons-1 and -2. The probe was generated by RT-PCR on 1 µg rat liver RNA using the oligonucleotides (5'-GGT AAG ACG AGA GAC ATG G-3', nt 1-19; numbering according to [33]) and (5'-CCC GTG GAT GAT CAT GAC AA-3', nt 285-266) as forward and reverse primers, respectively. The RT-PCR product was isolated by agarose gel electrophoresis, and radiolabeled using [α^{32}-P]dCTP and the Megaprime kit from Amersham (Amersham, UK). Filters containing 10^6 plaques were hybridized overnight at 42°C with 50 ng of the labeled cDNA probe in hybridization buffer (50 % (v/v) formamide, 0.5% (w/v) SDS, 0.1 mg/ml denaturated herring sperm DNA and 2 x PIPES buffer; [34]). After washing in 0.2 x sodium chloride/sodium citrate/0.5% SDS at 65°C for 5 min, the filters were exposed to autoradiography film. Two positive clones were identified, which were plaque-purified three times. One of these clones was selected for further analysis. Phage DNA was isolated and

digested with *EcoR*I. A 6 kb fragment [35] was subcloned into pBluescript KS$^-$ (pBsE6) and its identity with the 5'-regulatory region of the rat HL gene was verified by sequence analysis.

Construction of reporter plasmids

The clone in pBluescript containing the 6 kb *EcoR*I fragment of the rat HL gene (pBsE6) was used to generate promoter-reporter constructs in pCAT-Basic (Promega, Madison, WI, USA). By digestion with *Pst*I and *Xba*I, a 1.85-kb *Pst*I/*Pst*I, a 0.32-kb *Pst*I/*Xba*I and a 0.15-kb *Xba*I/*Xba*I fragment was isolated. First, the 0.32-kb *Pst*I/*Xba*I (-446/-127; numbering according to [35]) fragment was cloned into pCAT-Basic. From this construct, the rHL-446 CAT plasmid was generated by insertion of the 0.15-kb *Xba*I (-127/+9) fragment. Subsequently, rHL-2287 CAT was generated by insertion of the 1.85-kb *Pst*I (-2287/-446) fragment into rHL-446. The rHL-127 CAT construct was made by subcloning the 0.15-kb *Xba*I (-127/+9) fragment into pCAT-Basic.

From the rHL-2287 CAT vector, the 5'-truncated rHL-1697, rHL-1041 and rHL-747 constructs were generated by PCR using *Hind*III-restriction site-containing oligonucleotides 3F, 4F and 5F as upstream primer, respectively, and the CAT-gene specific oligonucleotide CATrev2 as downstream-primer (Table 2). After digestion of the PCR products with *Hind*III and *Pst*I, the DNA fragments were purified by electrophoresis through agarose gel, and subsequently ligated into the rHL-446 CAT plasmid that had been digested with the same restriction enzymes. Similarly, the rHL-211 construct was generated from the rHL-446 CAT by PCR using oligonucleotides 9F and CATrev2 as upstream and downstream primer, respectively, followed by ligation into the *Hind*III and *Xba*I sites of rHL-446 CAT. Finally, the rHL-75, rHL-39 and rHL-23 constructs were generated from pBsE6 using 7F, 6F and 11F as upstream, and T3Primer as downstream primer, respectively, followed by digestion and ligation into the *Hind*III and *Pst*I sites of pCAT-Basic; subsequently, the resulting plasmids were digested with *Xba*I followed by self-ligation.

Human HL promoter constructs were made in the pGL3-Basic luciferase reporter plasmid (Promega, Madison, WI, USA), starting from the hHL(-685/+13)-CAT plasmid described previously [36]. An upstream *Sac*I restriction site was introduced by PCR using the HHL-685Sac primer (Table 2) and the downstream HHL+13Xba primer. After digestion with *Sac*I and *Xba*I, the gel-purified DNA products were ligated into the *Sac*I and *Nhe*I sites of pGL3, thus generating the hHL-685Luc plasmid. Similarly, hHL-306Luc, hHL-79Luc and hHL-36Luc plasmids were generated by using HHL-306Nhe, HHL-79Kpn, and HHL-36Kpn as upstream primers, respectively.

To test the enhancer activity of conserved upstream sequences, the -14kb, -22kb and -10kb elements were inserted into the enhancer site of the hHL-685Luc plasmid using the *BamHI* and *SalI* restriction sites. Human genomic DNA was isolated from a buffy coat, and the -14kb, -22kb and –10kb elements were PCR amplified using specific primers (Table 2). The PCR products were cloned into the pGEM T-easy vector (ProMega, Madison, WI, USA). After digestion of the plasmids with *BamHI* and *SalI*, the inserts were cloned into hHL-685Luc in either sense or anti-sense orientation.

All clones were verified by DNA sequencing using the Thermo-sequenase dye terminator kit (Amersham, UK) and the ABI 377 sequencer (Applied Biosystems, Foster City, CA, USA).

Table 2. Oligonucleotides used to generate serial 5'-deletions of the rat and human HL promoter region.

Name	species and orientation[a]		position[b]	5'→3' sequence[c]
3F	r	F	-1697/-1675	cggaagc TTA GCA GAC AGC GAT TGG C
4F	r	F	-1048/-1030	cggaag CTT GCC TCC TCC TGA GTG C
5F	r	F	-754/-736	cggaagc TTG TCC AGG GCG TCC ATA C
9F	r	F	-211/-196	cggaagctt AGC TTG GCT CAA AAG G
8F	r	F	-86/-71	cggaagctt GTG TTC AAA TAC TGG G
7F	r	F	-75/-58	cggaagct TGG GTA ACA TGT TTT AGG
6F	r	F	-39/-22	cggaag CTT CCA CAA CTA AAT ACC
11F	r	F	-23/-8	cggaagctt CCA AGA AGC ATT CTG G
HHL-685Sac	h	F	-685/-667	ccgagctc TGG TCG CCT TTT CCC TAC C
HHL-306Nhe	h	F	-306/-291	gcatgctagc GAA GCC ACC TAC CCC G
HHL-79Kpn	h	F	-79/-55	ggggtacc TAA CAT GTT GAG AGG
HHL-36Kpn	h	F	-36/-20	ggggtac CAA AGT ATC TAA TAG GC
HHL+13Xba	h	R	+13/-6	gctctaga CTT GGT AAT TTC TGA AGC C
HHL-10Kfw	h	F	-10457/-10437	gtcgac GAA GGA TCA GGT GAG GGA TGG
HHL-10Krev	h	R	-9956/-9975	ggatcc GCT TCA AGG GCA ATG AAA GC
HHL-14Kfw	h	F	-14202/-14183	gtcgac GAC TTG GGG ATA TCC ACA TC
HHL-14Krev	h	R	-13524/-13549	ggatc CTG GTA AAA GGA CAT GAA CAA TAT GG
HHL-22Kfw	h	F	-21929/-21909	gtcgac GAT GGG AAA TGG ACC TAC AGC
HHL-22Krev	h	R	-21543/-21562	ggatcc GAT GAG GAC TGA TTC TCA GC
CATREV2[d]	-	R		gca act gac tga aat gcc tc
T3primer[e]	-	R		att aac cct cac taa ag

[a]: r and h: rat and human, respectively; F and R: forward and reverse orientation.
[b]: numbering of the rat and human sequence according to [35] and Ensembl *e!*42 [26], respectively.
[c]: rat and human HL specific parts of the primer sequences are given in capitals.
[d]: oligonucleotide specific for pCAT-Basic.
[e]: oligonucleotide specific for pBluescript.

2.6.3 Promoter reporter assays

HepG2 hepatoma cells and HeLa cells were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (ICN, Costa Mesa, CA, USA) supplemented with 10% (v/v) fetal calf serum (Gibco, Breda, Netherlands) and penicillin/streptomycin. Transfection of HepG2 cells with CAT-reporter constructs was performed by the calcium-phosphate co-precipitation method. At 24 h before transfection, the cells were plated in 6-wells plates at 20-30 % confluence. At 3 h before transfection, the medium was refreshed. Cells were co-transfected with 2.5 µg/well of the CAT reporter test plasmid and 0.2 µg/well of control RSV β-galactosidase expression plasmid

(Promega) [36]. Parallel transfections with SV40-CAT-Control and empty pCAT-Basic plasmids were used as controls. Fourty-eight hours post-transfection, cell lysates were prepared. CAT and β-galactosidase were determined by ELISA (Roche). Promoter activity was expressed as pg CAT/ng β-galactosidase to correct for differences in cell number and transfection efficiency.

Transfections of HepG2 and HeLa cells with the luciferase–reporter constructs were performed in 24-wells plates with Lipofectamine Plus (Invitrogen, Groningen, Netherlands) using 0.4 µg of the luciferase-reporter construct and 20 ng of pRL-CMV (Promega) per well [37]. Cell extracts were prepared at 48 h post-transfection. The luciferase activity in the cell extracts was determined with the FireLight kit (Perkin-Elmer, Boston MA, USA) and the Packard Top Count NXT luminometer. Data were normalized for the Renilla activity measured in the same sample.

2.6.4 Statistics

Experimental data are expressed as mean ± SD. Differences were tested for statistical significance by paired Student's *t*-test.

List of abbreviations: CAT, chloramphenicol acetyltransferase; HDL, high-density lipoprotein; HL, hepatic lipase; HNF, hepatocyte nuclear factor; kb, kilo basepairs; TFBS, transcription factor binding sites.

Authors' contributions: DvD and GJB carried out the biochemical assays. HJ and AJMV conceived of the study and participated in its design and coordination. AJMV carried out the comparative genomic analysis, and drafted the manuscript. All authors read and approved the final manuscript.

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References

1. Wingender E, Chen X, Fricke E, Geffers R, Hehl R, Liebich I, Krull M, Matys V, Michael H, Ohnhauser R *et al*: The TRANSFAC system on gene expression regulation. *Nucleic Acids Res* 2001, **29**(1):281-283.
2. Quandt K, Frech K, Karas H, Wingender E, Werner T: MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res* 1995, **23**(23):4878-4884.
3. Fickett JW, Wasserman WW: Discovery and modeling of transcriptional regulatory regions. *Curr Opin Biotechnol* 2000, **11**(1):19-24.
4. Zhang Z, Gerstein M: Of mice and men: phylogenetic footprinting aids the discovery of regulatory elements. *J Biol* 2003, **2**(2):11.
5. Hardison RC: Conserved noncoding sequences are reliable guides to regulatory elements. *Trends Genet* 2000, **16**(9):369-372.
6. Levy S, Hannenhalli S, Workman C: Enrichment of regulatory signals in conserved non-coding genomic sequence. *Bioinformatics* 2001, **17**(10):871-877.
7. Cora D, Herrmann C, Dieterich C, Di Cunto F, Provero P, Caselle M: Ab initio identification of putative human transcription factor binding sites by comparative genomics. *BMC Bioinformatics* 2005, **6**(1):110.
8. Pennacchio LA, Ahituv N, Moses AM, Prabhakar S, Nobrega MA, Shoukry M, Minovitsky S, Dubchak I, Holt A, Lewis KD *et al*: In vivo enhancer analysis of human conserved non-coding sequences. *Nature* 2006, **444**(7118):499-502.
9. Wagner A: Genes regulated cooperatively by one or more transcription factors and their identification in whole eukaryotic genomes. *Bioinformatics* 1999, **15**(10):776-784.
10. Krivan W, Wasserman WW: A predictive model for regulatory sequences directing liver-specific transcription. *Genome Res* 2001, **11**(9):1559-1566.
11. Loots GG, Ovcharenko I, Pachter L, Dubchak I, Rubin EM: rVista for comparative sequence-based discovery of functional transcription factor binding sites. *Genome Res* 2002, **12**(5):832-839.
12. Frazer KA, Pachter L, Poliakov A, Rubin EM, Dubchak I: VISTA: computational tools for comparative genomics. *Nucleic Acids Res* 2004, **32**(Web Server issue):W273-279.
13. Doolittle MH, Wong H, Davis RC, Schotz MC: Synthesis of hepatic lipase in liver and extrahepatic tissues. *J Lipid Res* 1987, **28**(11):1326-1334.
14. Semenkovich CF, Chen SH, Wims M, Luo CC, Li WH, Chan L: Lipoprotein lipase and hepatic lipase mRNA tissue specific expression, developmental regulation, and evolution. *J Lipid Res* 1989, **30**(3):423-431.
15. Perret B, Mabile L, Martinez L, Terce F, Barbaras R, Collet X: Hepatic lipase: structure/function relationship, synthesis, and regulation. *J Lipid Res* 2002, **43**(8):1163-1169.
16. Schultz CJ, Blanchette-Mackie EJ, Scow RO: Adrenal and liver in normal and cld/cld mice synthesize and secrete hepatic lipase, but the lipase is inactive in cld/cld mice. *J Lipid Res* 2000, **41**(2):214-225.
17. Gonzalez-Navarro H, Nong Z, Freeman L, Bensadoun A, Peterson K, Santamarina-Fojo S: Identification of mouse and human macrophages as a site of synthesis of hepatic lipase. *J Lipid Res* 2002, **43**(5):671-675.
18. Jansen H, De Greef WJ: Heparin-releasable lipase activity of rat adrenals, ovaries and testes. *Biochem J* 1981, **196**(3):739-745.
19. Hixenbaugh EA, Sullivan TR, Jr., Strauss JF, 3rd, Laposata EA, Komaromy M, Paavola LG: Hepatic lipase in the rat ovary. Ovaries cannot synthesize hepatic lipase but accumulate it from the circulation. *J Biol Chem* 1989, **264**(7):4222-4230.
20. Galan X, Peinado-Onsurbe J, Julve J, Ricart-Jane D, Robert MQ, Llobera M, Ramirez I: Inactive hepatic lipase in rat plasma. *J Lipid Res* 2003, **44**(12):2250-2256.
21. Jansen H, Verhoeven AJ, Sijbrands EJ: Hepatic lipase: a pro- or anti-atherogenic protein? *J Lipid Res* 2002, **43**(9):1352-1362.
22. Hadzopoulou-Cladaras M, Cardot P: Identification of a cis-acting negative DNA element which modulates human hepatic triglyceride lipase gene expression. *Biochemistry* 1993, **32**(37):9657-9667.
23. Oka K, Ishimura-Oka K, Chu MJ, Chan L: Transcription of the human hepatic lipase gene is modulated by multiple negative elements in HepG2 cells. *Gene* 1996, **180**(1-2):69-80.

24. Chang SF, Scharf JG, Will H: Structural and functional analysis of the promoter of the hepatic lipase gene. *Eur J Biochem* 1997, **247**(1):148-159.
25. Rufibach LE, Duncan SA, Battle M, Deeb SS: Transcriptional regulation of the human hepatic lipase (LIPC) gene promoter. *J Lipid Res* 2006, **47**(7):1463-1477.
26. Hubbard TJ, Aken BL, Beal K, Ballester B, Caccamo M, Chen Y, Clarke L, Coates G, Cunningham F, Cutts T *et al*: Ensembl 2007. *Nucleic Acids Res* 2007, **35**(Database issue):D610-617.
27. Prabhakar S, Poulin F, Shoukry M, Afzal V, Rubin EM, Couronne O, Pennacchio LA: Close sequence comparisons are sufficient to identify human cis-regulatory elements. *Genome Res* 2006, **16**(7):855-863.
28. Shih DQ, Bussen M, Sehayek E, Ananthanarayanan M, Shneider BL, Suchy FJ, Shefer S, Bollileni JS, Gonzalez FJ, Breslow JL *et al*: Hepatocyte nuclear factor-1alpha is an essential regulator of bile acid and plasma cholesterol metabolism. *Nat Genet* 2001, **27**(4):375-382.
29. Odom DT, Zizlsperger N, Gordon DB, Bell GW, Rinaldi NJ, Murray HL, Volkert TL, Schreiber J, Rolfe PA, Gifford DK *et al*: Control of pancreas and liver gene expression by HNF transcription factors. *Science* 2004, **303**(5662):1378-1381.
30. **Ensembl** [www.ensembl.org]
31. **Vista** [http://genome.lbl.gov/vista]
32. **Genomatix Software GmbH** [www.genomatix.de]
33. Komaromy MC, Schotz MC: Cloning of rat hepatic lipase cDNA: evidence for a lipase gene family. *Proc Natl Acad Sci U S A* 1987, **84**(6):1526-1530.
34. Sambrook J FE, Maniatis T: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor: Cold Spring Harbor Press; 1989.
35. Sensel MG, Legrand-Lorans A, Wang ME, Bensadoun A: Isolation and characterization of clones for the rat hepatic lipase gene upstream regulatory region. *Biochim Biophys Acta* 1990, **1048**(2-3):297-302.
36. Botma GJ, Verhoeven AJ, Jansen H: Hepatic lipase promoter activity is reduced by the C-480T and G-216A substitutions present in the common LIPC gene variant, and is increased by Upstream Stimulatory Factor. *Atherosclerosis* 2001, **154**(3):625-632.
37. Botma GJ, van Deursen D, Vieira D, van Hoek M, Jansen H, Verhoeven AJ: Sterol-regulatory-element binding protein inhibits upstream stimulatory factor-stimulated hepatic lipase gene expression. *Atherosclerosis* 2005, **179**(1):61-67.
38. Warren RJ, Ebert DL, Mitchell A, Barter PJ: Rabbit hepatic lipase cDNA sequence: low activity is associated with low messenger RNA levels. *J Lipid Res* 1991, **32**(8):1333-1339.

CHAPTER 3

Down-regulation of hepatic lipase expression by elevation of cAMP in human hepatoma but not adrenocortical cells

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3.1 Abstract

Expression of hepatic lipase (HL) in the liver is reduced during prolonged fasting. This effect is mainly mediated via catecholamines, which signal through elevation of Ca^{2+}_i as well as cAMP. We have studied the effect of cAMP on HL expression in cell culture. Overnight incubation of HepG2 cells with 10-300 mM 8-bromo-cyclic AMP resulted in a dose-dependent, up to 50% reduction in secretion of HL, but had no effect on secretion of α_1-antitrypsin, or overall protein synthesis. HL mRNA levels were decreased 1.5 fold, as determined by semi-quantitative and real-time RT-PCR. In HepG2 cells transiently transfected with human HL (-685/+13) or rat HL (-446/+9) promoter-reporter constructs, cAMP induced a similar dose-dependent suppression of HL promoter activity. cAMP responsiveness in HepG2 cells was mediated by a conserved 10-bp response element at -45/-36, that represents a potential binding site for CCAAT/enhancer-binding protein beta (C/EBPβ). cAMP reduced expression of the 45kDa C/EBPβ protein and binding of C/EBPβ to the proximal promoter region of the human HL gene by 50%, as determined by immunoblotting and chromatin immunoprecipitation assay, respectively. In human H295R adrenocortical cells, cAMP failed to suppress HL promoter activity, and only slightly reduced C/EBPβ expression. We conclude that the fall in HL expression during prolonged fasting may be mediated through elevation of cAMP and lowering of C/EBPβ expression.

Abbreviations: Br-cAMP: 8-bromo-cyclic AMP; CAT: chloramphenicol acyltransferase; DMEM: Dulbecco's modified Eagle's medium; HDL and LDL: high- and low-density lipoproteins; HL: hepatic lipase; PBS: phosphate-buffered saline; PMSF: phenylmethylsulfonylfluoride

3.2 Introduction

Hepatic lipase (HL; EC 3.1.1.3) is a lipolytic enzyme that is synthesized and secreted almost exclusively by liver parenchymal cells (reviewed in (Jansen *et al*., 2002; Perret *et al*., 2002). The protein is bound extracellularly to the liver at heparin-sensitive sites, where it plays an important role in the metabolism of plasma lipoproteins. HL mediates the conversion of cholesterol-enriched HDL_2 to cholesterol-poor HDL_3 and the formation of small dense LDL from large buoyant LDL, and HL plays a role in postprandial lipid transport by facilitating the clearance of remnant lipoproteins by the liver (Jansen *et al*., 2002). Hepatic lipase activity is also expressed in adrenals and ovaries, where the enzyme may play a role in delivery of HDL cholesterol for steroidogenesis (Jansen and de Greef, 1988; Vieira-van Bruggen *et al*., 1998). The amount of HL in human post-heparin plasma is influenced by genetic (Isaacs *et al*., 2004; Jansen *et al*., 1997), hormonal and nutritional factors (Perret *et al*., 2002), as well as by body composition (Carr *et al*., 1999; Despres *et al*., 1989; Nie *et al*., 1998).

Several studies in humans suggest that HL activity varies in parallel with insulin levels (Perret *et al*., 2002). HL activity increases with fasting plasma insulin levels in non-diabetic, normocholesterolemic coronary artery disease patients (Jansen *et al*., 1997), and plasma HL activity is positively correlated with increased plasma insulin levels in response to an oral glucose load (Katzel *et al*., 1992; Pollare *et al*., 1991). HL activity is elevated under conditions with high plasma insulin, such as in type 2 diabetes (Baynes *et al*., 1991) and obesity-related hyperinsulinaemia (Cominacini *et al*., 1993). However, in glycemic clamp studies with normal and type 2 diabetic men insulin administration caused a decrease in hepatic lipase activity (Baynes *et al*., 1992). *In vitro* studies have not shown clear upregulation of HL expression by insulin (Jensen *et al*., 1989). Hence, there is currently no evidence for a direct upregulation of human HL expression by insulin.

In rats, post-heparin plasma HL activity is strongly depressed during fasting (Peinado-Onsurbe *et al*., 1991, 2000). This effect has been attributed to the increased catecholamine levels during fasting (Peinado-Onsurbe *et al*., 1991). Indeed, *in vitro* studies with rat hepatocytes show an acute inhibition of HL secretion by α_{1B}-adrenergic agonists at the post-transcriptional level (Neve *et al*., 1998; Peinado-Onsurbe *et al*., 1991, 2000), mainly through the mobilization of intracellular Ca^{2+} (Neve *et al*., 1997, 1998). However, α_{1B}-adrenergic agonists also increase the generation of cAMP in liver cells (Morgan *et al*., 1983; Nomura *et al*., 1993), and cAMP elevation in rat hepatocytes has been shown to inhibit secretion of HL activity (Klin *et al*., 1996). In addition, overnight but not short-term treatment of freshly isolated rat hepatocytes with glucagon, which predominantly signals through elevation of cAMP, has been shown to decrease HL secretion (Jensen *et al*., 1989; Peinado-Onsurbe *et al*., 1991; Schoonderwoerd *et al*., 1984). *In vivo*, glucagon levels in portal blood vary oppositely to insulin levels, and catecholamines increase glucagon secretion into portal blood. We hypothesize therefore, that hepatic lipase secretion is increased in parallel with insulin, due to the opposite changes in catecholamines and glucagon, and through signaling via intracellular cAMP. In this study, we examined the

mechanism by which elevation of cAMP reduces human hepatic lipase expression using HepG2 hepatoma cells as model system. HepG2 cells express only few α_{1B}- or β-receptors (Kost *et al*., 1992; Sanae *et al*., 1992). In addition, most studies suggest that glucagon receptors are markedly downregulated in hepatoma cells (Hornbuckle *et al*., 2004; Mirel *et al*., 1978). We therefore used membrane-permeant cAMP to study the effect of receptors that signal through this second messenger.

3.3 Materials and methods

3.3.1 Materials

8-Bromo-cAMP (Br-cAMP) and 8-bromo-cGMP (Br-cGMP) were purchased from Sigma (St. Louis, IL, USA), and dibutyryl-cAMP (db-cAMP), cycloheximide, Trasylol and restriction enzymes were from Roche (Germany). PMSF was from Merck (Darmstadt, Germany). Media, fetal bovine serum and Tran^{35}S label (1100 Ci/mmol) were from ICN (Costa Mesa, CA, USA). Glycerol trioleate (50-80 mCi/mmol), [α-^{32}P]dCTP, the Megaprime DNA labelling kit and Hybond-filters, and horseradish peroxidase-conjugated donkey anti-rabbit IgG and Hyper ECL film were all from Amersham Pharmacia Biotec (Amersham, U.K.). Heparin was obtained from Leo Pharmaceuticals (Weesp, Holland). Rabbit antibodies against human α_1-antitrypsin and Zysorbin (formaldehyde-fixed *Staphylococcus aureus* membranes) were from Dakopatts (Glostrup, Denmark) and Zymed Laboratories (San Francisco, CA, USA), respectively. Rabbit antibodies against C/EBPβ (C19) and B23 (nucleophosmin, a nucleolar marker protein) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Super signal West Pico Chemiluminescent Substrate was from Pierce, Rockford, IL., USA. Oligonucleotides were custom-made by Eurogentec (Seraing, Belgium). Other chemicals were from Sigma.

3.3.2 HepG2 cell culture and hepatic lipase secretion

Human hepatoma HepG2 cells were grown as monolayer cultures in DMEM supplemented with 10 % (by vol) fetal bovine serum at 37°C in a humidified air/CO_2 (19:1) atmosphere. The cells were split 1:10 into new flasks once a week. Medium was refreshed once a week. For secretion experiments, cells were seeded into six-well plates. At confluence, the medium was replaced by 1 ml of fresh medium containing 25 U/ml heparin. Incubations were started with the addition of Br-cAMP and continued for 16 h, unless otherwise stated. Then, the medium was collected on ice for analysis of secreted HL activity.

HL activity was determined by a triacylglycerol hydrolase assay at pH 8.5 in 0.6 M NaCl with a gum acacia-stabilized glycerol [^{14}C]trioleate emulsion as substrate (Verhoeven and Jansen, 1990). Activities are expressed as m-units (nmol of free fatty acids released per min). Of total lipase activity in the media, more than 95% was sensitive to inhibition by polyclonal anti-human HL IgG's (Verhoeven and Jansen, 1990).

3.3.3 α_1-Antitrypsin and total protein synthesis

Synthesis and secretion of α_1-antitrypsin was measured by the incorporation of [^{35}S]methionine into immunoprecipitated protein (Verhoeven *et al*., 1999). HepG2 cells were incubated overnight in normal medium containing 80 µCi/ml of Tran^{35}S-label with or without Br-cAMP. Then, the culture plates were put on ice, and the medium was collected into vials containing unlabelled methionine (1 mM final concentration) and a cocktail of protease inhibitors (1 mM EDTA, 10 units/ml Trasylol, 0.1 mM benzamidine, 2 µg/ml leupeptin, 2 µg/ml antipain, 2 µg/ml chymostatin and 2 µg/ml pepstatin; all final concentrations). The medium was incubated for 15 min (on ice) with Zysorbin in the presence of 0.2 mg/ml human serum albumin and centrifuged (10 min 10,000g) to remove material that bound non-specifically to the Protein A. The supernatants were then incubated for 1 h (4°C) with rabbit anti-human α_1-antitrypsin (1:100), followed by overnight incubation with Zysorbin. After centrifugation, the pellets were washed twice in phosphate-buffered saline (PBS) containing 1% (v/v) Triton X-100, 0.25% sodium deoxycholate and 1 mM PMSF, and twice in PBS. The pellets were resuspended in Laemmli's sample buffer, and after boiling for 5 min, the precipitated proteins were separated by SDS/PAGE (7.5% gel). The ^{35}S-labelled proteins were visualized by exposure of the dried gels to a phosphor screen (GS-393 Molecular Imaging System; Bio-Rad, Hercules, CA, USA).

Incorporation of [^{35}S]methionine into trichloroacetic acid-precipitable material was taken as a measure of total protein synthesis. HepG2 cells were incubated with Tran^{35}S-label, and cell-free medium was prepared, as described above. The cells in the wells were washed twice in PBS, then lysed in PBS containing 1% Triton X-100, 1% sodium deoxycholate, 1 mM methionine, 10 mM Hepes (pH 7.4) and the protease inhibitor cocktail described above. After 30 min on ice, the lysate was collected from the wells, and the lysate was cleared by centrifugation for 10 min at 10,000g. Aliquots (5 µl) of the cell-free medium and the cleared lysate were spotted in triplicate onto Whatmann 3MM filters, and the radioactivity on the filters was quantified by phosphor imaging, without or with treatment with boiling trichloroacetic acid (Verhoeven and Jansen, 1990).

3.3.4 Quantification of HL mRNA.

Total RNA was isolated from the HepG2 cells with phenol/guanidinium isothiocyanate (Chomczynski and Sacchi, 1987). RNA was quantified by spectrophotometry at 260 nm. The amount of HL mRNA was determined by semi-quantitative and by real-time RT-PCR. For the former method, a single-tube RT-PCR was performed using 5'-GTG GGC ATC AAA CAG CCC-3' as forward and 5'-CAG ACA TTG GCC CAC ACT G-3' as reverse primer, and known amounts of *in vitro* generated HL cRNA lacking an internal 80 bp-fragment, as described previously (Verhoeven *et al*., 1994). Real-time quantitative PCR analysis was performed using the MyIQ single color real-time PCR Detection system (Bio-Rad). First, 4 µg total RNA was reverse transcribed using random hexamer primers and M-MLV Reverse Transcriptase (Promega, Leiden, Netherlands) in a final volume of 50 ml. cDNA (2 ml) was added in a 25 ml final volume containing 0.2 mM dNTPs, 0.5 U Platinum *Taq* DNA polymerase (Invitrogen), 3 mM $MgCl_2$,

0.4 mM of forward and reverse primers, and 0.15xSYBR Green I (Sigma). The PCR program consisted of a denaturing step (3 min 95°C), followed by 40 cycles of 15 sec at 95°C and 45 sec at 60°C. The relative abundance of HL mRNA was determined by the DC_T method using the efficiency of amplification derived from the log-linear part of the PCR. HL mRNA levels were normalized to acidic ribosomal phosphoprotein PO (36B4) mRNA. Primers used were: HL forward: 5'-ATC GCC GTC CGC AAC ACC-3' (nt 394-411, numbering according to the coding sequence (Datta *et al*., 1988)); HL reverse: 5'-ACC CAG CTG TAC CCA ATT AGG-3' (nt 510-489); 36B4 forward: 5'-CCT TCT TGG CTG ATC CAT CTG C-3' (nt 877-898, numbering according to the cDNA sequence (Rich and Steitz, 1987)); 36B4 reverse: 5'-CCG ACT CCT CCG ACT CTT CC-3'(nt 980-999).

3.3.5 Human and rat HL promoter-reporter constructs

Human and rat HL promoter fragments were generated and subcloned into the pGL3-basic luciferase or the pCAT-Basic reporter vector (Promega) as detailed elsewhere (van Deursen *et al*., 2007). All human and rat HL promoter fragments extended to +13 and +9 at their 3'-end, respectively. From the human HL-685 luciferase plasmid, a promoter construct lacking the internal -45/-36 sequence was generated by the PCR overlay technique (Higuchi *et al*., 1988), using 5'-TTA TTA AAT GGG CAG T-A AAG TAT CTA ATA GGC-3' and 5'-ATT AGA TAC TTT –AC TGC CCA TTA ATA ATT A-3' as forward and reverse mutagenic primers, respectively, in combination with two plasmid specific primers. All inserts in pGL3-Basic and pCAT-Basic were verified by cycle sequencing using the Thermo Sequenase dye terminator kit (Amersham) and the ABI 377 sequencer. Plasmid DNAs were isolated with the Wizard Midiprep System (Promega).

3.3.6 Transfection assays in HepG2 cells

Transfection of HepG2 cells with CAT-reporter constructs was performed by the calcium-phosphate co-precipitation method. At 24 h before transfection, the cells were plated in 6-wells plates at 30% confluence. At 3 h before transfection, the medium was refreshed. Cells were co-transfected with 2.5 µg/well of the CAT reporter test plasmid and 0.2 µg/well of control RSV β-galactosidase expression plasmid (Promega) (Botma *et al*., 2001). Parallel transfections with SV40-CAT-Control and empty pCAT-Basic plasmids were used as positive and negative controls, respectively. At 3 h and 24 h after transfection, the medium was refreshed and test agents were added to the fresh medium. Fourty-eight hours post-transfection, cell lysates were prepared. CAT and β-galactosidase were determined by ELISA (Roche). Promoter activity was expressed as pg CAT/ng β-galactosidase to correct for differences in cell number and transfection efficiency.

Transfections of HepG2 cells with the luciferase-reporter constructs were performed in 24-wells plates with Lipofectamine Plus (Invitrogen, Groningen, Netherlands) using 0.4 µg of the luciferase-reporter construct and 20 ng of pRL-SV40 (Promega) per well (Botma *et al*., 2005). At 3 h and 24 h after transfection, the medium was refreshed and test agents were added to the fresh medium. Cell extracts were prepared at 48 h post-transfection. The luciferase activity in

the cell extracts was determined with the FireLight kit (Perkin-Elmer, Boston MA, USA) and the Packard Top Count NXT luminometer. Data were normalized for the Renilla activity measured in the same sample.

3.3.7 Transfection assays with NCI-H295R cells

The human adrenocortical cell line NCI-H295R was obtained from Dr. B. Staels, Lille, France. Cells were cultured as described previously (Botma *et al*., 2007). At 24 h before transfection, cells were plated in 6-well plates at 50% confluence. Transfections were performed with Lipofectamine-Plus using 1.0 µg CAT reporter and 0.25 µg/well RSV β-galactosidase expression plasmid per well. The cells were incubated with Br-cAMP, and promoter activity was determined as described above for the HepG2 cells.

3.3.8 Immunoblotting

Near-confluence cultures of HepG2 and NCI-H295R cells were incubated for 24 h with or without Br-cAMP. Nuclear extracts were prepared according to Schreiber *et al*. (1989). The amount of protein in each extract was determined with the DC protein assay (Bio-Rad), using bovine serum albumin as standard. Extracts (50 mg) were electrophoresed on denaturing 10% SDS-polyacrylamide gels (Laemmli, 1970), and the separated proteins were transferred to a nitrocellulose membrane (Protean, Schleicher & Schuell, Düsseldorf, Germany). The membrane was blocked overnight with 4% milk powder/0.05% Tween-20 in Tris-buffered saline, and then incubated for 2 h with either anti-C/EBPβ (1:1000) or anti-B23 (1:10000), respectively, followed by a 1h incubation with secondary antibody at 1:8000, all in blocking buffer. The secondary antibody was visualized by enhanced chemiluminescence. The images were quantified by densitometry using the GS-800 Calibrated Densitometer from Bio-Rad.

3.3.9 Chromatin immunoprecipitation assays

These assays were performed according to the instructions of Upstate Biotechnology (Lake Placid NY, USA) with some modifications, using a single 10-cm culture dish of HepG2 cells for each immunoprecipitation reaction. At near-confluence, cross-links were formed with 1% formaldehyde. Thereafter, cell lysates were prepared and sonicated until DNA was sheared into <1000 bp fragments. An aliquot of sheared chromatin was used as input control, the remainder was diluted 10-fold in immunoprecipitation buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl; pH 8.0). After preclearing for 30 min at 4°C with 60 ml protein A-Sepharose beads preabsorbed with salmon sperm DNA (Upstate), the chromatin solution was incubated overnight at 4°C with 4 ml anti-C/EBPβ antibodies. As negative control, a chromatin fraction was incubated in parallel without antibody. Then, 60 ml protein A-Sepharose beads were added. After 1 h incubation at 4°C, the beads were collected by centrifugation. After extensive washing, bound material was eluted with 1% SDS; 0.1 M $NaHCO_3$. Cross-links were reversed by incubation for 4 h at 65°C in the presence of 0.2 M NaCl, followed by incubation for 30 min at 65°C with 2 mg/ml RNase A in 10 mM EDTA; 40 mM Tris (pH 6.8). The DNA was isolated

with a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and subjected to real-time quantitative PCR analysis, as described above. Amplification was done with the proximal HL promoter specific primers 5'-GGC AGT CTT CCC TAA CAA AGT ATC-3' (nt -51/-28) and 5'-TGT CCA TTT CTC CGT TTC ACC-3' (nt +30/+50) and the distal HL gene specific primers 5'-CTT GGG ATT TGC TTG CTT TAT C-3' (nt –6071/-6050) and 5'-ATT TGA TGA CCT GAG AAT GAC C-3' (nt -5965/-5986). Quantitation was done by the ΔC_T method using the 1% input-reaction as a reference.

3.3.10 Statistics

Experimental data are expressed as mean ± SD. Differences were tested for statistical significance by paired Student's *t*-test.

3.4 Results

3.4.1 Effect of cAMP on HL secretion and HL mRNA

Overnight incubation of HepG2 cells with db-cAMP and Br-cAMP resulted in a dose-dependent decrease in secretion of hepatic lipase activity (Figure 1A). With Br-cAMP, maximal inhibition of about 50% was observed at a final concentration of 0.3 mM, and a half-maximal effect was reached at less than 0.03 mM. Db-cAMP was less effective, with a similar maximal inhibition observed at about 3 mM, and a half-maximal effect at about 0.3 mM. Over the same concentration range, 8-bromo-cGMP did not affect hepatic lipase secretion. The inhibition of hepatic lipase secretion induced by Br-cAMP was not paralleled by a significant reduction of α_1-antitrypsin secretion (Figure 1B), or by an inhibition of overall protein synthesis or secretion (Figure 1C). This suggests that the inhibitory effect of cAMP was rather specific for hepatic lipase.

With 0.3 mM Br-cAMP, secretion of hepatic lipase initially proceeded unaffected. Only after 4 h of incubation with the cAMP homolog, hepatic lipase activity in the extracellular medium became significantly lower than in parallel controls (Figure 2A). Between 8 and 24 h, secretion of hepatic lipase activity continued but at a much lower rate than in control incubations. Simultaneously, intracellular HL activity was reduced from 0.68 ± 0.08 to 0.54 ± 0.04 mU/well (mean ± SD, n=3; $p<0.05$). For comparison, we also incubated cells with cycloheximide, which instantaneously and completely inhibited overall protein synthesis (Verhoeven *et al*., 1999). Here, inhibition of hepatic lipase secretion became apparent between 4 and 8 h, and was complete thereafter (Figure 2A). This delayed inhibition is interpreted to indicate maturation and secretion of hepatic lipase that was already present in the cells at the start of the incubation with cycloheximide (Verhoeven *et al*., 1999). These data suggest therefore, that synthesis and secretion of hepatic lipase continues in the presence of Br-cAMP, but at a much reduced rate. By semi-quantitative RT-PCR, HL mRNA expression was 1.5- to 2-fold less in cells incubated overnight with 0.3 mM Br-cAMP than in parallel controls (Figure

2B). By real-time PCR, HL mRNA in Br-cAMP treated cells was 1.38 ± 0.13 fold less than in control cells (mean ±SD, n=3; p<0.05). Hence, the inhibition of HL secretion induced by elevation of cAMP is at least partly explained by a reduction of HL mRNA expression.

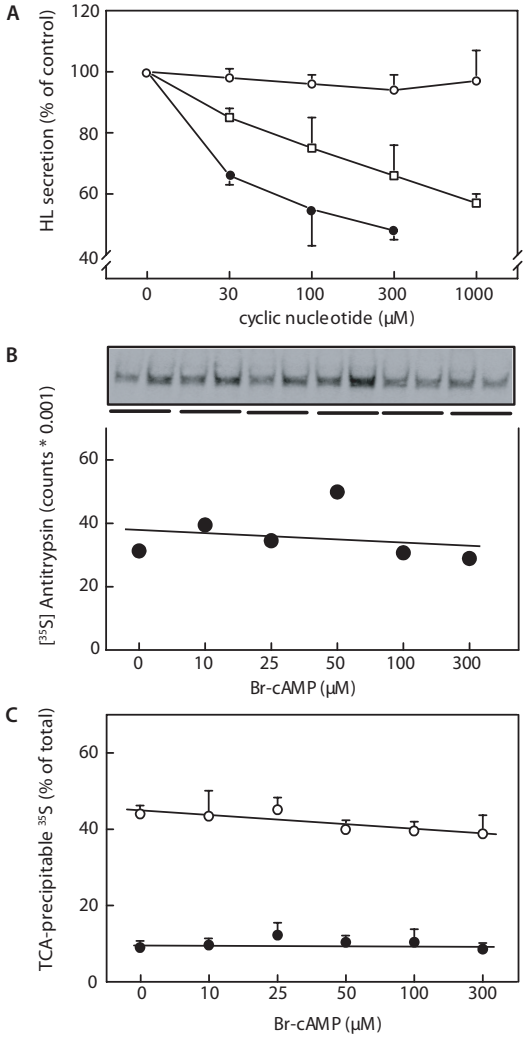

Figure 1. Effect of cAMP homologs on protein synthesis and secretion of HL and α_1-antitrypsin by HepG2 cells.

In **A**, HepG2 cells were incubated overnight with medium containing heparin and the indicated final concentration of Br-cAMP (●), Br-cGMP (O) or dibutyryl-cyclic AMP (□). At the end of the incubation, HL activity was determined in the extracellular medium. Data are expressed as % of the HL activity in the extracellular medium of parallel control incubations (100% = 0.79 ± 0.05 mU/well; mean ± SD, n=3), and are mean ± SD for three independent experiments. In **B** and **C**, HepG2 cells were incubated with the indicated concentration of Br-cAMP in the presence of 80 mCi/ml Tran^{35}S-label. At the end of the incubation, cell-free media and cell lysates were prepared. In **B**, α_1-antitrypsin was immunoprecipitated from the cell-free media, and the ^{35}S-label was quantified after SDS-PAGE by phosphorimaging. In C, incorporation of ^{35}S-label in total protein in cell free media (●) and cell lysates (O) was determined by TCA precipitation, and expressed as percentage of total ^{35}S radioactivity in each fraction. Data are mean ± SD; n =3.

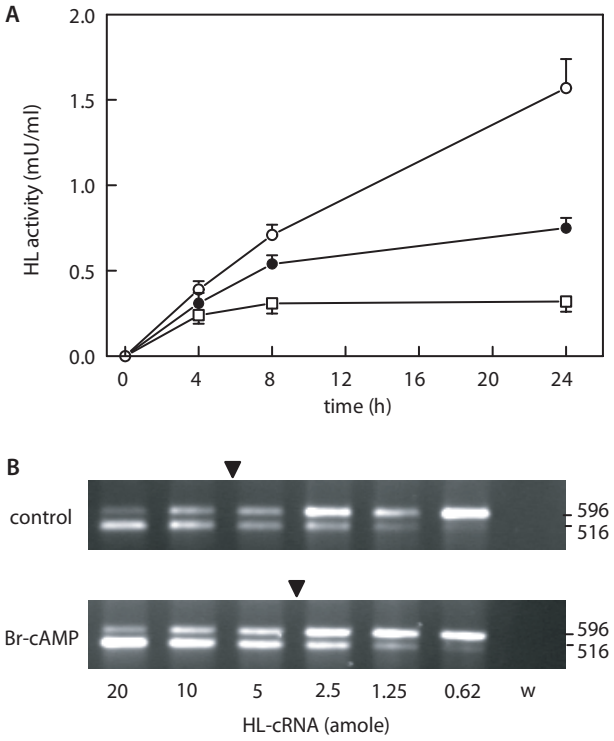

Figure 2. Effect of Br-cAMP on HL secretion and HL mRNA in HepG2 cells.

In **A**, HepG2 cells were incubated for the indicated time with heparin without further additions (O), or with 0.3 mM Br-cAMP (●) or 10 mg/ml cycloheximide (□). Hepatic lipase activity was determined in the extracellular medium. Data are mean ± SD for three independent experiments. In **B**, cells were incubated for 24 h without or with 0.3 mM Br-cAMP (upper and lower lane, respectively), and then total cell RNA was prepared. HL mRNA expression was determined by semiquantitative RT-PCR, in which 0.5 mg total cell RNA was mixed with known amounts of a HL cRNA (given in attomoles) that lack an internal 80nt fragment (Verhoeven *et al*., 1994). After RT-PCR, the amplimers of HL mRNA and the HL-cRNA (596 and 516 bp, respectively) were separated by agarose gel electrophoresis. When signal intensities of both amplimers within a single RT-PCR mixture are identical, the amount of HL mRNA is identical to the amount of added HL-cRNA (indicated by the arrows). Data are representative of two similar experiments.

3.4.2 Effect of cAMP on HL promoter activity in HepG2 cells.

To test whether reduced HL mRNA expression is the result of reduced transcription, we performed promoter-reporter assays in transiently transfected HepG2 cells. We used the proximal -685/+13 and -446/+9 promoter region of the human and rat HL gene, cloned in front of a luciferase and CAT reporter gene, respectively. As shown in Figure 3A, the activity of both HL promoter-reporter constructs was 60-75 % lower in cAMP-treated cells than in parallel controls. In contrast, the activity of reporter constructs containing the viral SV40 control promoter (Figure 3A) or the CMV control promoter (data not shown), were not reduced but rather increased by cAMP treatment. With the human HL promoter construct, maximal

inhibitory effect was observed with 0.1 mM Br-cAMP, and half-maximal effect was reached at about 0.01 mM (Figure 3B). Hence, HL promoter activity is similarly, or even slightly more, sensitive to inhibition by Br-cAMP as secretion of HL activity. We conclude therefore, that the inhibitory effect of cAMP elevation on HL secretion is largely explained by reduced transcription of the HL gene.

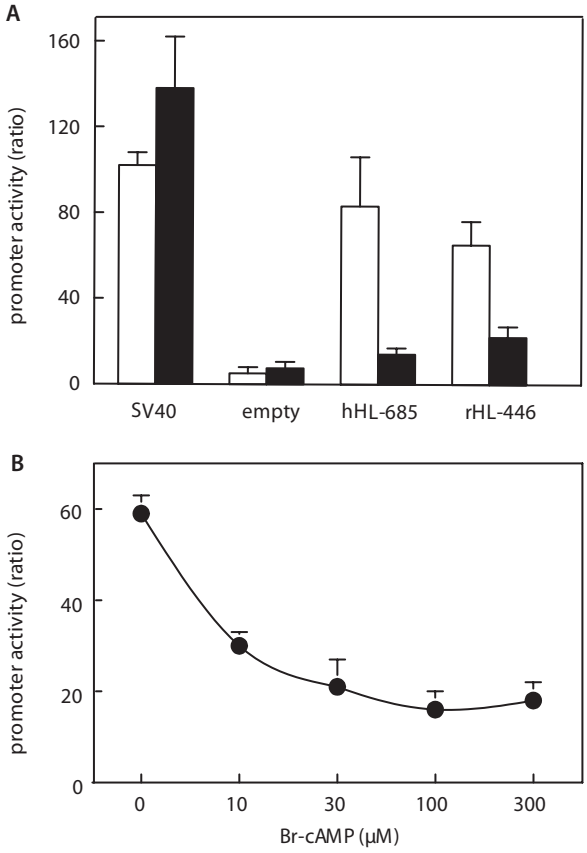

Figure 3. Inhibition of HL promoter activity by Br-cAMP in transfected HepG2 cells.

In **A**, HepG2 cells were transfected with the indicated promoter-CAT constructs, and then incubated without or with 0.3 mM Br-cAMP for 44 h (open and hatched bars, respectively). Then, cell lysates were prepared and analysed for CAT and b-galactosidase levels. In **B**, HepG2 cells were transfected with the hHL-685 reporter construct, and then incubated for 44 h with the indicated concentration of Br-cAMP. Promoter activities were expressed as the ratio between CAT and b-galactosidase level measured in the lysate, except for the data with the SV40-promoter constructs, which are expressed as the ratio divided by 10. Data are mean ± SD for three independent experiments, each performed in quadruplicate.

3.4.3 Identification of a cAMP-responsive element in the HL promoter.

In the best-studied cAMP responsive liver gene PEP-CK, the cAMP effect is mediated through CREB-, AP1- and C/EBP-like transcription factors (Roesler, 2000). Recently, HNF4α has been

shown to mediate cAMP responsiveness to the CYP7A1 gene (Song and Chiang, 2006). With the Match program (Kel *et al*., 2003), a number of consensus binding sites for these cAMP-sensitive transcription factors were found in both the proximal human (Figure 4A) and rat HL promoter region (Figure 4C). To test through which of these elements cAMP may affect HL promoter activity, serial 5'-deletion constructs were generated, and tested for their ability to respond to cAMP. Statistically significant inhibition of human HL promoter activity by 0.3 mM Br-cAMP was observed with the 5'-deleted fragments up till -79 (Figure 4B). This ruled out a major role for the putative CREB, AP1 and HNF4 consensus binding sites in this part of the human HL promoter. Further deletion to -39 removed the HNF1 and a putative C/EBPβ binding site, and completely abolished the Br-cAMP-induced inhibition (Figure 4B). Instead, HL promoter activity was slightly increased by Br-cAMP, similar to the empty control vector. For the rat HL promoter, statistically significant inhibition of promoter activity by Br-cAMP was observed with serial 5'-deletions from -446 to -75 (Figure 4D). The rat HL promoter contains a HNF1 site and an adjacent C/EBPβ binding site at a similar position as the human HL promoter. Further deletion of the rat HL promoter to -39, thereby removing the HNF1 site but not the adjacent C/EBPβ binding site, did not affect responsiveness to Br-cAMP. However, Br-cAMP no longer inhibited transcriptional activity when the rat HL promoter was shortened to -23, which removed this C/EBPβ binding site and the TATA box.

Comparison of the activity of 5'-deletion constructs of the human and rat HL promoter pinpointed cAMP responsiveness to a 10bp sequence consisting of the potential C/EBPβ binding site (Figure 5A). Deletion of this -45/-36 sequence from the human HL -685 promoter-reporter construct completely abolished responsiveness to Br-cAMP (Figure 5B). To test whether C/EBPβ binds to this site in HepG2 cells, we performed chromatin immunoprecipitation assays using anti-C/EBPβ antibodies. When compared to the distal HL promoter fragments, and to the no-antibody controls (mock), the proximal promoter region of the HL gene (-51/+30) encompassing the putative C/EBPβ binding site was specifically amplified from immunoprecipitated DNA (Figure 5C). Moreover, the amount of proximal HL promoter that was immunoprecipitated from the chromatin of Br-cAMP-treated cells was reduced to approximately 50% of parallel controls ($p<0.05$; $n=3$). These data suggest an important role for the potential C/EBPβ site immediately 3' of the HNF1 site in effecting cAMP responsiveness to the human and rat HL promoter.

Next, we tested the effect of cAMP on expression of C/EBPβ protein in nuclear extracts of HepG2 cells, using the nucleolar protein nucleophosmin (B23) as loading control. C/EBPβ ran as a 45 kDa protein, with a minor band at 20 kDa, corresponding to LAP and LIP isoforms, respectively (Figure 6). The amount of the 45kDa C/EBPβ protein was reduced by approximately 50 % after 24 h incubation of the cells with 0.3 mM Br-cAMP. This coincided with the increased abundance of a 32 kDa immunoreactive protein. The expression of the20 kDa isoform of C/EBPβ was not affected by cAMP. Taken together, the cAMP induced reduction in HL gene expression may be mediated by the decreased nuclear expression of the 45 kDa C/EBPβ isoform.

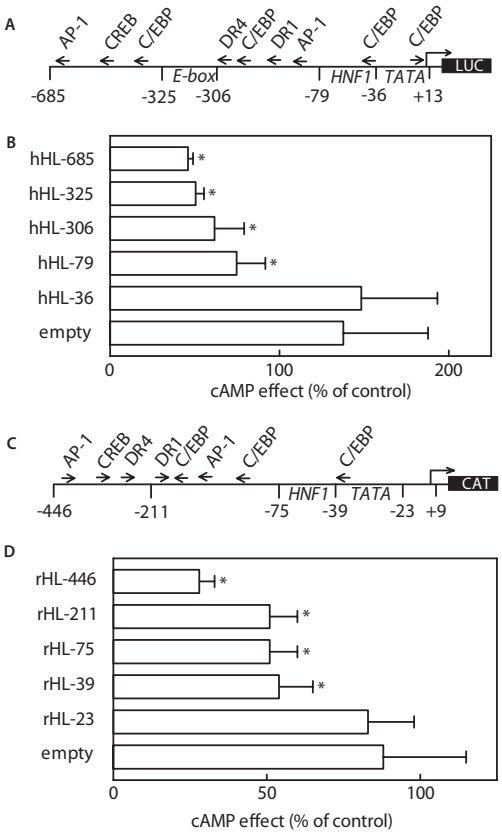

Figure 4. Identification of the cAMP-responsive elements in the human and rat HL promoter region by serial 5′-deletions.

A en **C** give a schematic representation of the human and rat HL promoter constructs, respectively. The orientation and approximate binding sites for putative cAMP-responsive transcription factors identified by the Match program (core similarity >0.85; matrix similarity >0.90) (Kel *et al*., 2003) are indicated by the arrows. In addition, the DR1 and DR4 sites involved in HNF4α binding are also indicated (Rufibach *et al*., 2006). Based on the locations of these binding sites, 5′-deletions were generated and tested for promoter activity in the absence and presence of 0.3 mM Br-cAMP. **B** and **D** show the cAMP effect on the 5′-deletion constructs made from the human and rat HL promoter region, respectively. Promoter activity in the presence of Br-cAMP was expressed as percentage of promoter activity in its absence. Data are mean ±SD for 3-5 independent experiments. *: statistically significant effect of Br-cAMP ($p<0.05$)

3.4.4 Effect of cAMP on HL promoter activity in adrenocortical cells

The human HL gene is also transcribed in human adrenocortical cells (Botma *et al*., 2007). In contrast to the HepG2 cells, treatment of H295R adrenocortical cell line with 0.3 mM Br-cAMP was shown to increase HL promoter activity (Botma *et al*., 2007). Upon transient transfection into the H295R cells, the hHL-685 and hHL-325 constructs both showed significantly higher promoter activity when incubated with than without Br-cAMP ($p<0.05$; $n=3$). Qualitatively similar results were obtained for the rat HL-446 reporter construct (Figure 7). In nuclear extracts of the H295R cells, both 45 and 20 kDa C/EBPβ isoforms were expressed (Figure 6).

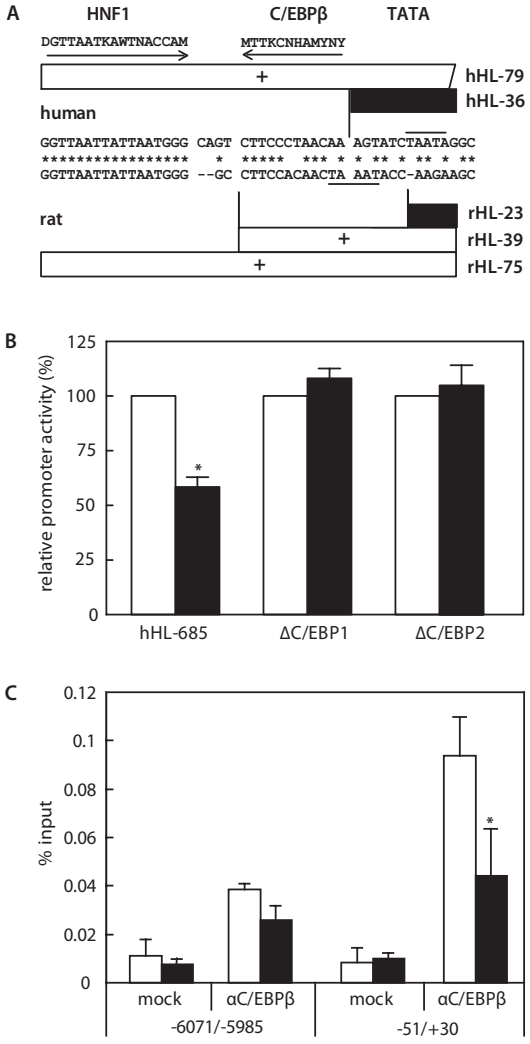

Figure 5. cAMP responsiveness of the HL gene resides in a potential C/EBPb consensus-binding site.

In **A**, the proximal promoter region of the human and rat HL gene (-66/-20 and -58/-15 region, respectively) was aligned, and conserved nucleotides were indicated by the asterisks. The TATA box is given by a horizontal line. The HNF1a and C/EBPβ consensus binding site is taken from the Transfac database (Matys *et al*., 2003), and given in the IUPAC 15-letter code on top of the aligned sequence. The upstream part and the exact 5'-end of the various human and rat promoter fragments used in reporter assays is aligned to the sequence, and drawn schematically by the horizontal bars. Open bars indicate constructs whose promoter activity is inhibited by cAMP (+), whereas closed bars indicate the constructs that are no longer responsive to cAMP (-). In **B**, cAMP responsiveness of human HL (-685/+13)-reporter plasmid and two independent clones with an internally deleted C/EBPβ binding site (-45/-36) was determined as described in the legends to Figure 4B. Data are expressed as percentage of luciferase activity in control cells, and are means ± D for three independent experiments each performed in quadruplicate. In **C**, binding of C/EBPβ to a distal (left panel) and proximal (right panel) part of the upstream region of the HL gene in control cells (open bars) and 0.3 mM Br-cAMP-treated HepG2 cells (closed bars) was determined by chromatin immunoprecipitation assays using anti- C/EBPβ antibodies (aC/EBPβ) or no antibodies (mock). Data are expressed as % of input DNA, and are means ± SD for three independent experiments. *: statistically significant effect of Br-cAMP ($p<0.05$)

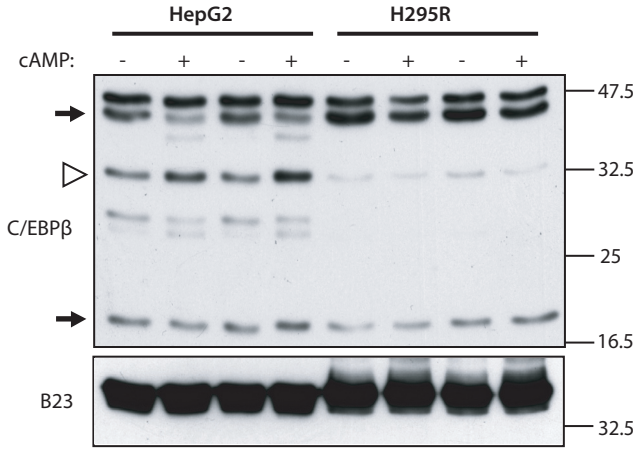

Figure 6. Effect of Br-cAMP on C/EBPβ expression in HepG2 and H295R cells.

Cells were incubated for 24 h without (-) or with 0.3 mM Br-cAMP (+), and then nuclear extracts were prepared. Of each fraction, 50 µg protein was immunoblotted with anti- C/EBPβ and anti-B23 antibodies. The position of the molecular size markers (in kDa) is indicated. The positions of the C/EBPβ proteins corresponding to LAP (45 kDa) and LIP (20 kDa) are indicated by the arrows, and of a immuno-related protein (32 kDa) by the arrowhead. The band at 47 kDa is a crossreacting band (SantaCruz).

After correction for B23 in the loading control, the abundance of the 45 kDa isoform in the H295R cells was twice as high as in HepG2 cells, whereas the abundance of the 20 kDa isoform was slightly lower than in HepG2 cells. Incubation of the H295R cells with Br-cAMP only slightly reduced the abundance of the 45 kDa isoform to 90 % of control, whereas the abundance of the 20 kDa isoform and the immuno-reactive 32 kDa protein remained unaffected.

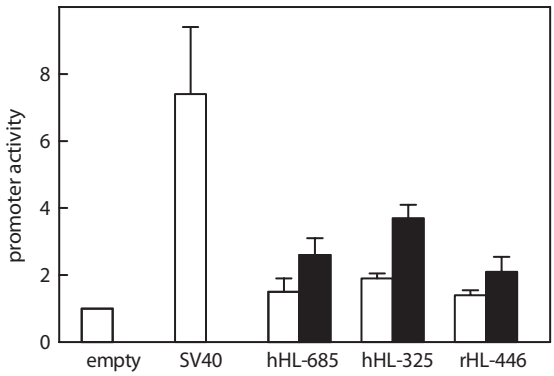

Figure 7. Effect of Br-cAMP on HL promoter activity in transfected H295R cells.

Experiments were performed as described in the legends to Figure 3. Promoter activities are expressed relative to the CAT/β-galactosidase ratio observed with the empty promoter construct, and are mean ± SD for three independent experiments, each performed in triplicate

3.5 Discussion

Here we show that the synthesis and secretion of HL by human hepatoma HepG2 cells is strongly inhibited by prolonged incubation with membrane-permeant cAMP homologs, which mimics fasting conditions. To the best of our knowledge, HL expression in the fasted versus the well-fed state has not been compared in human individuals. In rats, expression of HL has been shown to be lower in the fasting than in the well-fed state (Peinado-Onsurbe *et al*., 1991, 2000; Stam *et al*., 1984), an effect that is shown to be mediated by adrenaline through α_{1B} adrenergic receptors (Neve *et al*., 1998). In freshly isolated rat hepatocytes, α_{1B} agonists acutely reduce hepatic lipase secretion (Neve *et al*., 1997; Peinado-Onsurbe *et al*., 1991; Schoonderwoerd *et al*., 1984) by inhibiting the intracellular maturation of newly synthesized hepatic lipase protein, an effect that is mediated by the increase in intracellular Ca^{2+}-concentration (Neve *et al*., 1998). This acute, post-translational effect of adrenaline on HL secretion is paralleled by a similar fall in albumin secretion, and is therefore not considered to be specific for HL (Galan *et al*., 2002). α_{1B} adrenergic agonists also elevate intracellular cAMP, which may result in an acute increase in HL secretion (Morita *et al*., 1994). However, we show here that elevation of cAMP results in reduction of hepatic lipase secretion. In contrast to the acute effects of elevated Ca^{2+}_i, the effect of elevated cAMP occurs at the level of transcription. This appears to be specific for HL, as secretion of a_1-antitrypsin is not affected, and transcriptional activity of some viral promoters is increased rather than reduced by cAMP. A similar reduction in rat hepatic lipase expression has been described for glucagon (Jensen *et al*., 1989) or parathyroid hormone (Klin *et al*., 1996), which both act through elevation of cAMP. We cannot exclude the possibility that these effects are the result of cross-talk between cAMP and Ca^{2+} signaling pathways. However, the finding that cAMP responsiveness is mediated through the well-known cAMP responsive transcription factor C/EBPβ (reviewed in Wilson and Roesler, 2002), suggests that the effects are mediated through cAMP itself.

Our study pinpoints cAMP responsiveness of the human and rat HL promoter to a potential C/EBPβ binding site. This C/EBPβ site is contiguous with the functionally important HNF1 binding site, and is not only conserved among human and rat, but also in the mouse, rabbit and rhesus monkey HL genes (van Deursen *et al*., 2007). C/EBPβ is highly expressed in liver, intestine, adipose and lung tissue (Lekstrom-Himes and Xanthopoulos, 1998), as well as in adrenocortical cells (this paper). We show here that cAMP-treatment reduces the nuclear expression of active C/EBPβ protein and simultaneously lowers C/EBPβ binding to the proximal HL promoter region in HepG2 cells. This may therefore, explain the reduced HL gene expression in the hepatoma cells. Besides active 45kDa C/EBPβ protein (termed LAP), the C/EBPβ gene also translates into a truncated 20kDa protein (termed LIP) that lacks the activation domain of LAP, and acts as a dominant-negative repressor. As in liver tissue (Carmona *et al*., 2005; Manchado *et al*., 1994), LIP expression in HepG2 cells is rather low compared to LAP. Although 20kDa LIP expression is not markedly affected by cAMP, its expression relative to

45-kDa LAP is increased. The resulting increase in repressor activity of LIP may further attribute to the observed reduction in HL gene expression.

Our results indicate that C/EBPβ is a positive transactivator of the HL gene, and that the inhibition of HL gene expression by cAMP is mediated by decreased nuclear expression of C/EBPβ. Surprisingly, cAMP failed to suppress HL promoter activity in human adrenocortical H295R cells. We show here that C/EBPβ expression in these cells is hardly affected by cAMP elevation, which may be related to the lack of CREB activity in these cells (Groussin *et al*., 2000). The mechanism by which cAMP reduces C/EBPβ expression in HepG2 cells remains unknown. One possibility is that phosphorylation of C/EBPβ by CREB may result in increased degradation of C/EBPβ protein. Alternatively, CREB may phosphorylate and activate another transactivator protein, thereby displacing C/EBPβ from the proximal promoter region of the HL gene.

If the *in vitro* observations also hold *in vivo*, several mechanisms appear to exist that ensure that HL expression is reduced during prolonged fasting. One wonders therefore, why it is physiologically important to lower HL expression during fasting. HL is involved in the clearance of chylomicron remnants by the liver, and indirectly in the removal of surface fragments that are generated by lipolysis of chylomicrons and VLDL (reviewed in Jansen *et al*., 2002). Therefore, it makes sense that HL expression is higher in the prandial fase than in the fasting state. In the prandial fase, adrenaline and glucagon levels fall, which will result in a fall in intra-hepatocyte cAMP levels and thus, HL expression will rise. Although there may be an additional benefit to lower HL expression during prolonged fasting, we propose that the cAMP effects observed are a reflection of the increased expression of HL during the prandial state mediated, among others, by the lowering of intra-hepatic cAMP concentration.

References

1. Baynes, C., Henderson, A.D., Anyaoku, V., Richmond, W., Hughes, C.L., Johnston, D.G., Elkeles, R.S., 1991. The role of insulin insensitivity and hepatic lipase in the dyslipidaemia of type 2 diabetes. *Diabet. Med.* **8**, 560-566.
2. Baynes, C., Henderson, A.D., Richmond, W., Johnston, D.G., Elkeles, R.S., 1992. The response of hepatic lipase and serum lipoproteins to acute hyperinsulinaemia in type 2 diabetes. *Eur. J. Clin. Invest.* **22**, 341-346.
3. Botma, G.J., Heuveling, M., Lamers, J.M., Jansen, H., Verhoeven, A.J., 2007. Cloning, expression, and promoter analysis of hepatic lipase derived from human hyperplastic adrenals: evidence for alternative mRNA splicing. *Cell. Biochem. Biophys.* **47**, 149-158.
4. Botma, G.J., van Deursen, D., Vieira, D., van Hoek, M., Jansen, H., Verhoeven, A.J., 2005. Sterol-regulatory-element binding protein inhibits upstream stimulatory factor-stimulated hepatic lipase gene expression. *Atherosclerosis* **179**, 61-67.
5. Botma, G.J., Verhoeven, A.J., Jansen, H., 2001. Hepatic lipase promoter activity is reduced by the C-480T and G-216A substitutions present in the common LIPC gene variant, and is increased by Upstream Stimulatory Factor. *Atherosclerosis* **154**, 625-632.
6. Carmona, M.C., Hondares, E., Rodriguez de la Concepcion, M.L., Rodriguez-Sureda, V., Peinado-Onsurbe, J., Poli, V., Iglesias, R., Villarroya, F., Giralt, M., 2005. Defective thermoregulation, impaired lipid metabolism, but preserved adrenergic induction of gene expression in brown fat of mice lacking C/EBPbeta. *Biochem. J.* **389**, 47-56.
7. Carr, M.C., Hokanson, J.E., Deeb, S.S., Purnell, J.Q., Mitchell, E.S., Brunzell, J.D., 1999. A hepatic lipase gene promoter polymorphism attenuates the increase in hepatic lipase activity with increasing intra-abdominal fat in women. *Arterioscler. Thromb. Vasc. Biol.* **19**, 2701-2707.
8. Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156-159.
9. Cominacini, L., Garbin, U., Davoli, A., Campagnola, M., De Santis, A., Pasini, C., Pastorino, A.M., Bosello, O., 1993. High-density lipoprotein cholesterol concentrations and postheparin hepatic and lipoprotein lipases in obesity: relationships with plasma insulin levels. *Ann. Nutr. Metab.* **37**, 175-184.
10. Datta, S., Luo, C.C., Li, W.H., VanTuinen, P., Ledbetter, D.H., Brown, M.A., Chen, S.H., Liu, S.W., Chan, L., 1988. Human hepatic lipase. Cloned cDNA sequence, restriction fragment length polymorphisms, chromosomal localization, and evolutionary relationships with lipoprotein lipase and pancreatic lipase. *J. Biol. Chem.* **263**, 1107-1110.
11. Despres, J.P., Ferland, M., Moorjani, S., Nadeau, A., Tremblay, A., Lupien, P.J., Theriault, G., Bouchard, C., 1989. Role of hepatic-triglyceride lipase activity in the association between intra-abdominal fat and plasma HDL cholesterol in obese women. *Arteriosclerosis* **9**, 485-492.
12. Galan, X., Peinado-Onsurbe, J., Robert, M.Q., Soley, M., Llobera, M., Ramirez, I., 2002. Acute regulation of hepatic lipase secretion by rat hepatocytes. *Biochem. Cell Biol.* **80**, 467-474.
13. Groussin, L., Massias, J.F., Bertagna, X., Bertherat, J., 2000. Loss of expression of the ubiquitous transcription factor cAMP response element-binding protein (CREB) and compensatory overexpression of the activator CREMtau in the human adrenocortical cancer cell line H295R. *J. Clin. Endocrinol. Metab.* **85**, 345-354.
14. Higuchi, R., Krummel, B., Saiki, R.K., 1988. A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucleic Acids Res.* **16**, 7351-7367.
15. Hornbuckle, L.A., Everett, C.A., Martin, C.C., Gustavson, S.S., Svitek, C.A., Oeser, J.K., Neal, D.W., Cherrington, A.D., O'Brien, R.M., 2004. Selective stimulation of G-6-Pase catalytic subunit but not G-6-P transporter gene expression by glucagon in vivo and cAMP in situ. *Am. J. Physiol. Endocrinol. Metab.* **286**, E795-808.
16. Isaacs, A., Sayed-Tabatabaei, F.A., Njajou, O.T., Witteman, J.C., van Duijn, C.M., 2004. The -514 C->T hepatic lipase promoter region polymorphism and plasma lipids: a meta-analysis. *J. Clin. Endocrinol. Metab.* **89**, 3858-3863.
17. Jansen, H., de Greef, W.J., 1988. L-type lipase activity in ovaries of superovulated rats. Relation to cholesterol homeostasis. *Mol. Cell. Endocrinol.* **57**, 7-15.
18. Jansen, H., Verhoeven, A.J., Sijbrands, E.J., 2002. Hepatic lipase: a pro- or anti-atherogenic protein? *J. Lipid Res.* **43**, 1352-1362.

19. Jansen, H., Verhoeven, A.J., Weeks, L., Kastelein, J.J., Halley, D.J., van den Ouweland, A., Jukema, J.W., Seidell, J.C., Birkenhager, J.C., 1997. Common C-to-T substitution at position -480 of the hepatic lipase promoter associated with a lowered lipase activity in coronary artery disease patients. *Arterioscler. Thromb. Vasc. Biol.* **17**, 2837-2842.

20. Jensen, E., Hansson, P., Floren, C.H., Nilsson, A., Nilsson-Ehle, P., 1989. Cell-density-dependent release of hepatic lipase from cultured rat hepatocytes. *Horm. Metab. Res.* **21**, 4-7.

21. Katzel, L.I., Coon, P.J., Busby, M.J., Gottlieb, S.O., Krauss, R.M., Goldberg, A.P., 1992. Reduced HDL2 cholesterol subspecies and elevated postheparin hepatic lipase activity in older men with abdominal obesity and asymptomatic myocardial ischemia. *Arterioscler. Thromb.* **12**, 814-823.

22. Kel, A.E., Gossling, E., Reuter, I., Cheremushkin, E., Kel-Margoulis, O.V., Wingender, E., 2003. MATCH: A tool for searching transcription factor binding sites in DNA sequences. *Nucleic Acids Res.* **31**, 3576-3579.

23. Klin, M., Smogorzewski, M., Ni, Z., Zhang, G., Massry, S.G., 1996. Abnormalities in hepatic lipase in chronic renal failure: role of excess parathyroid hormone. *J. Clin. Invest.* **97**, 2167-2173.

24. Kost, D.P., DeFrances, M.C., Lee, C.R., Michalopoulos, G.K., 1992. Patterns of alpha-1-adrenergic receptor expression in regenerating and neoplastic hepatic tissue. *Pathobiology* **60**, 303-308.

25. Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

26. Lekstrom-Himes, J., Xanthopoulos, K.G., 1998. Biological role of the CCAAT/enhancer-binding protein family of transcription factors. *J. Biol. Chem.* **273**, 28545-28548.

27. Manchado, C., Yubero, P., Vinas, O., Iglesias, R., Villarroya, F., Mampel, T., Giralt, M., 1994. CCAAT/enhancer-binding proteins alpha and beta in brown adipose tissue: evidence for a tissue-specific pattern of expression during development. *Biochem. J.* **302**, 695-700.

28. Matys, V., Fricke, E., Geffers, R., Gossling, E., Haubrock, M., Hehl, R., Hornischer, K., Karas, D., Kel, A.E., Kel-Margoulis, O.V., Kloos, D.U., Land, S., Lewicki-Potapov, B., Michael, H., Munch, R., Reuter, I., Rotert, S., Saxel, H., Scheer, M., Thiele, S., Wingender, E., 2003. TRANSFAC: transcriptional regulation, from patterns to profiles. *Nucleic Acids Res.* **31**, 374-378.

29. Mirel, R.D., Morris, H.P., DiAugustine, R.P., 1978. Membrane receptor function and the loss of glucagon-stimulated adenylate cyclase activity in hepatomas. *Endocrinology* **102**, 1237-1246.

30. Morgan, N.G., Waynick, L.E., Exton, J.H., 1983. Characterisation of the alpha 1-adrenergic control of hepatic cAMP in male rats. *Eur. J. Pharmacol.* **96**, 1-10.

31. Morita, T., Sakata, K., Kanagawa, A., Ueki, H., 1994. Stimulatory release of hepatic lipase activity from cultured rat hepatocytes by sodium orthovanadate: rapid increase in cyclic adenosine monophosphate content. *Biol. Pharm. Bull.* **17**, 577-580.

32. Neve, B.P., Verhoeven, A.J., Jansen, H., 1997. Acute effects of adrenaline on hepatic lipase secretion by rat hepatocytes. *Metabolism* **46**, 76-82.

33. Neve, B.P., Verhoeven, A.J., Kalkman, I., Jansen, H., 1998. Maturation and secretion of rat hepatic lipase is inhibited by alpha1B-adrenergic stimulation through changes in Ca2+ homoeostasis: thapsigargin and EGTA both mimic the effect of adrenaline. *Biochem. J.* **330**, 701-706.

34. Nie, L., Wang, J., Clark, L.T., Tang, A., Vega, G.L., Grundy, S.M., Cohen, J.C., 1998. Body mass index and hepatic lipase gene (LIPC) polymorphism jointly influence postheparin plasma hepatic lipase activity. *J. Lipid Res.* **39**, 1127-1130.

35. Nomura, T., Kondo, H., Hasegawa, S., Watanabe, T., Yokoyama, R., Ukai, K., Tachibana, M., Sumi-Ichinose, C., Nomura, H., Hagino, Y., 1993. Alpha 1B-adrenoceptor-mediated stimulation of Ca2+ mobilization and cAMP accumulation in isolated rat hepatocytes. *Eur. J. Pharmacol.* **246**, 113-120.

36. Peinado-Onsurbe, J., Julve, J., Galan, X., Llobera, M., Ramirez, I., 2000. Effect of fasting on hepatic lipase activity in the liver of developing rats. *Biol. Neonate* **77**, 131-138.

37. Peinado-Onsurbe, J., Soler, C., Galan, X., Poveda, B., Soley, M., Llobera, M., Ramirez, I., 1991. Involvement of catecholamines in the effect of fasting on hepatic endothelial lipase activity in the rat. *Endocrinology* **129**, 2599-2606.

38. Perret, B., Mabile, L., Martinez, L., Terce, F., Barbaras, R., Collet, X., 2002. Hepatic lipase: structure/function relationship, synthesis, and regulation. *J. Lipid Res.* **43**, 1163-1169.

39. Pollare, T., Vessby, B., Lithell, H., 1991. Lipoprotein lipase activity in skeletal muscle is related to insulin sensitivity. *Arterioscler. Thromb.* **11**, 1192-1203.
40. Rich, B.E., Steitz, J.A., 1987. Human acidic ribosomal phosphoproteins P0, P1, and P2: analysis of cDNA clones, in vitro synthesis, and assembly. *Mol. Cell Biol.* **7**, 4065-4074.
41. Roesler, W.J., 2000. What is a cAMP response unit? Mol. Cell. Endocrinol. 162, 1-7.
42. Rufibach, L.E., Duncan, S.A., Battle, M., Deeb, S.S., 2006. Transcriptional regulation of the human hepatic lipase (LIPC) gene promoter. *J. Lipid Res.* **47**, 1463-1477.
43. Sanae, F., Kohei, K., Nomura, M., Miyamoto, K., 1992. Studies on responsiveness of hepatoma cells to catecholamines. VI. Characteristics of adrenoceptors and adenylate cyclase response in rat ascites hepatoma cells and human hepatoma cells. *J. Pharmacobiodyn*. **15**, 303-309.
44. Schoonderwoerd, K., Hulsmann, W.C., Jansen, H., 1984. Regulation of liver lipase. II. Involvement of the alpha 1-receptor. *Biochim. Biophys. Acta* **795**, 481-486.
45. Schreiber, E., Matthias, P., Muller, M.M., Schaffner, W., 1989. Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res*. **17**, 6419.
46. Song, K.H., Chiang, J.Y., 2006. Glucagon and cAMP inhibit cholesterol 7alpha-hydroxylase (CYP7A1) gene expression in human hepatocytes: discordant regulation of bile acid synthesis and gluconeogenesis. *Hepatology* **43**, 117-125.
47. Stam, H., Schoonderwoerd, K., Breeman, W., Hulsmann, W.C., 1984. Effects of hormones, fasting and diabetes on triglyceride lipase activities in rat heart and liver. *Horm. Metab. Res.* **16**, 293-297.
48. van Deursen, D., Botma, G.J., Jansen, H., Verhoeven, A.J., 2007. Comparative genomics and experimental promoter analysis reveal functional liver-specific elements in mammalian hepatic lipase genes. *BMC Genomics* **8**, 99.
49. Verhoeven, A.J., Carling, D., Jansen, H., 1994. Hepatic lipase gene is transcribed in rat adrenals into a truncated mRNA. J. *Lipid Res*. **35**, 966-975.
50. Verhoeven, A.J., Jansen, H., 1990. Secretion of rat hepatic lipase is blocked by inhibition of oligosaccharide processing at the stage of glucosidase I. *J. Lipid Res.* **31**, 1883-1893.
51. Verhoeven, A.J., Neve, B.P., Jansen, H., 1999. Secretion and apparent activation of human hepatic lipase requires proper oligosaccharide processing in the endoplasmic reticulum. *Biochem. J.* **337**, 133-140.
52. Vieira-van Bruggen, D., Kalkman, I., van Gent, T., van Tol, A., Jansen, H., 1998. Induction of adrenal scavenger receptor BI and increased high density lipoprotein-cholesteryl ether uptake by in vivo inhibition of hepatic lipase. J. *Biol. Chem.* **273**, 32038-32041.
53. Wilson, H.L., Roesler, W.J., 2002. CCAAT/enhancer binding proteins: do they possess intrinsic cAMP-inducible activity? *Mol. Cell. Endocrinol.* **188**, 15-20

CHAPTER 4

Glucose increases hepatic lipase expression in HepG2 liver cells through upregulation of Upstream Stimulatory Factors 1 and 2

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4.1 Abstract

Aims/hypothesis: Elevated Hepatic Lipase (HL) expression is a key factor in the development of the atherogenic lipid profile in type 2 diabetes and insulin-resistance. Recently, genetic screens revealed a possible association of type 2 diabetes and familial combined hyperlipidemia with the Upstream Stimulatory Factor 1 (*USF1*) gene. Therefore, we investigated the role of USFs in the regulation of HL.

Methods: The expression of USF1, USF2 and HL was measured in HepG2 cells cultured in normal or high glucose medium (4.5 and 22.5 mmol/l, respectively), and in livers of streptozotocin-treated rats.

Results: Nuclear extracts of high-glucose cells contained 2.5±0.5-fold more USF1 protein and 1.4±0.2-fold more USF2 protein than normal-glucose cells (mean ±SD, n=3). This coincided with higher DNA binding of nuclear proteins to the USF consensus DNA binding site. Secretion of HL activity (2.9±0.5-fold), abundance of HL mRNA (1.5±0.2-fold), and HL(-685/+13) promoter activity (1.8±0.3-fold) increased in parallel. In chromatin immunoprecipitation assays, the proximal HL promoter region was immunoprecipated with anti-USF1 and anti-USF2 antibodies. Co-transfection with USF1 or USF2 cDNA stimulated HL promoter activity 6-16 fold. USF and glucose responsiveness were significantly reduced by removal of the -310E-box from the HL promoter. Silencing of the USF1 gene by RNA interference reduced glucose responsiveness of the HL(-685/+13) promoter region by 50 %. The hyperglycemia in streptozotocin-treated rats was associated with similar increases in nuclear USF expression in rat liver nuclei, but not with increased binding of USF to the rat HL promoter region.

Conclusions/interpretation: Glucose increases HL expression in HepG2 cells via elevation of USF1 and USF2. This mechanism may attribute to the development of the typical dyslipidemia of type 2 diabetes.

Abbreviations: FCHL, familial combined hyperlipidemia; HL, hepatic lipase; SREBP: sterol-regulatory-element binding protein; TG, triglyceride; USF, upstream stimulatory factor; qPCR: quantitative real-time PCR.

4.2 Introduction

Morbidity and mortality in patients with type 2 diabetes is largely dominated by the occurrence of atherosclerotic cardiovascular disease [1]. The insulin resistant state that precedes the development of type 2 diabetes is also associated with increased risk for cardiovascular disease [2-4]. The dyslipidemia that is typical for the insulin resistant states such as central obesity, the metabolic syndrome, type 2 diabetes and familial combined hyperlipidemia (FCHL) [5,6] is an important contributor to the development of coronary artery disease, and consists of hypertriglyceridemia, low HDL cholesterol and the preponderance of small, dense LDL particles [3,7]. Hepatic lipase (HL) plays a key role in lipoprotein metabolism and in the remodeling of HDL and LDL [8]. HL is a lipolytic enzyme that mediates in the formation of small dense LDL and the reduction in HDL cholesterol levels [8,9]. Elevated HL expression is associated with the dyslipidemia in the metabolic syndrome, and in type 2 diabetes [9,10], and the HL gene is associated with the lipoprotein abnormalities in familial combined hyperlipidemia [11].

HL expression is increased in type 2 diabetes [12,13]. Although insulin resistant states are commonly associated with hyperinsulinism, a direct stimulating effect of insulin on HL expression has not been unequivocally established [10]. Instead, acute hyperinsulinemia actually reduces HL expression [14]. HL activity correlates with parameters of insulin resistance in non-diabetic males [15,16] and in FCHL [16]. In an animal model, HL expression was increased upon induction of insulin resistance, which could be partially reversed by treatment with an insulin sensitizer [17]. It is likely therefore, that some aspect of insulin resistance induces the increase in HL expression. HL activity is strongly increased with omental fat mass, a parameter of visceral obesity with increased risk for development of type 2 diabetes [18]. This suggests that HL expression may increase with fatty acid supply to the liver. Indeed, *in vitro* HL expression is increased by fatty acids [19,20]. Recently, transcription of the HL gene in HepG2 cells was shown to be increased by glucose [21]. Hence, HL expression may be elevated in insulin resistant states as a consequence of the hyperglycemia and increased fatty acid delivery to the liver.

We recently found that transcription of the HL gene in HepG2 cells was stimulated by the Upstream Stimulatory Factor USF1 [22], a transcription factor that controls expression of several genes involved in glucose and lipid homeostasis [23]. USF1 binds as a homodimer, or as a heterodimer with highly homologous USF2, to E-box motifs in gene regulatory sequences. In the liver, expression of the genes for glucokinase [24], fatty acid synthase [25], apoA-II [26], apoA-V [27], apoC-III [28], and apoE [29] are all upregulated by USF. In liver as well as in other tissues, USF play an important role in the regulation of genes by insulin [24,25,27] or glucose [30-34]. Interestingly, the *USF1* gene on chromosome 1q21 has been linked with type 2 diabetes [35], FCHL [36,37] and cardiovascular disease and all-cause mortality among females [38]. Allelic variants of the USF1 gene may confer susceptibility to core features of the metabolic syndrome, such as glucose intolerance and dyslipidemia [36,39,40].

USF1 and USF2 are ubiquitously expressed. It is unclear how they convey glucose or insulin responsiveness to susceptible target genes. In the *USF1* gene a number of polymorphisms were found [36,38,40], some of which are associated with an unfavorable oral glucose and fat tolerance test [41], an increased adipocyte lipolysis [42], and a decreased expression of USF target genes in fat biopsies [40]. In non-hepatic cells, glucose has been shown to increase nuclear expression of either USF1 or USF2 [31-34]. We hypothesized therefore, that the expression of USF1 or USF2 itself is subject to regulation by glucose. In this study we tested (i) whether glucose affects nuclear expression of USF proteins in hepatoma cells, and (ii) whether this could explain the upregulation of hepatic lipase expression seen in the high-glucose states.

4.3 Materials and methods

4.3.1 Cell culture and hepatic lipase secretion

Monolayer cultures of HepG2 cells were routinely maintained at 37°C, 5% CO_2/95% air in DMEM/10% FCS, 50 IU penicillin and 50 mg/ml streptomycin, containing either high or low glucose (all from Gibco BRL, Breda, The Netherlands). Glucose was determined using glucose test strips and the AccuTrend sensor (Roche, Almere, Netherlands), and was 22.5 and 4.5 mM in fresh high and low glucose medium, respectively. Glucose in the cell-conditioned media was measured daily. The medium was refreshed every second day, or daily if medium glucose had dropped below 1 mM, which occurred occassionally and only when cell cultures were near-confluent. The cultures were split every 5 days. To avoid complications due to acute osmotic effects, cells were maintained at either high or low glucose medium for at least four passages before the start of the experiments.

For determination of hepatic lipase secretion, cells were grown in 6-wells plates and 2 ml medium/well. Upon confluence, the media were removed and the cells were incubated for 8 h in 1 ml/well of fresh medium containing 25 IU heparin (Leo Pharmaceuticals, Breda, The Netherlands). Hepatic lipase activity was assayed in the cell-free media as described before [43]. Enzyme activity was expressed as mU (nmoles of free fatty acids released per min from triolein substrate).

4.3.2 RNA analysis

Total RNA was isolated from a confluent T25 flask of HepG2 cells using the TRIzol reagent (Invitrogen, Leek, The Netherlands). RNA was quantified by spectrophotometry at 260 nm. The quality of the RNA preparations was judged from the pattern of ribosomal RNA after agarose gel electrophoresis. Four microgram of total RNA was reverse transcribed using random hexamer primers and M-MLV Reverse Transcriptase (Promega, Leiden, Netherlands) in a final volume of 50 ml. Real-time quantitative PCR analysis was performed using the MyiQ single color real-time PCR Detection system (Bio-Rad, Hercules CA, USA). cDNA (2 ml) was added in a 25 ml final volume containing 0.2 mM dNTPs, 0.5 U Platinum *Taq* DNA polymerase (Invitrogen)

3 mM $MgCl_2$, 0.4 mM of forward and reverse primers, and 0.15xSYBR Green I (Sigma, St. Louis IL, USA). The PCR program consisted of a denaturing step (3 min 95°C), followed by 40 cycles of 15 sec at 95°C and 45 sec at 60 °C. Primers were selected by the Beacon Designer software (Premier Biosoft, Palo Alto CA, USA): HL forward: 5'-ATC GCC GTC CGC AAC ACC-3' (nt 394-411, numbering according to the coding sequence [44]); HL reverse: 5'-ACC CAG CTG TAC CCA ATT AGG-3' (nt 510-489); 36B4 forward: 5'-CCT TCT TGG CTG ATC CAT CTG C-3' (nt 877-898, numbering according to the cDNA sequence [45]); 36B4 reverse: 5'-CCG ACT CCT CCG ACT CTT CC-3' (nt 980-999). The relative abundance of HL mRNA was determined by the ΔC_T method using the efficiency of amplification derived from the log-linear part of the PCR. HL mRNA levels were normalized to acidic ribosomal phosphoprotein PO (36B4) mRNA.

4.3.3 Determination of USF1 and USF2 expression

Nuclear extracts were prepared by the method of Schreiber *et al*. [46] from the cells of one confluent T25 culture flask; 4 h before cell lysis, culture media were refreshed. Protein concentrations ranged from 1-2 mg/ml, as determined by DC protein assay (Bio-Rad). Proteins (20 mg) were separated by SDS-PAGE on 10% gels, and blotted to nitrocellulose membrane (Hybond ECL; Amersham Biosciences, Amersham, UK). Molecular sizes were determined from the Dual Color Precision Plus Protein Standards (Bio-Rad) run in parallel. After blocking with 0.5% milk powder in 0.1% Tween-20/Tris-buffered saline (20 mM Tris pH 7.6 in 150 mM NaCl), USF1 and USF2 protein was detected by a 1:500 dilution of rabbit polyclonal anti-human USF1 and anti-human USF2 (both C20; Santa-Cruz Biotechnology, CA, USA), respectively. B23 protein (nucleophosmin) was detected by 1:10,000 dilution of mouse monoclonal anti-B23 (a kind gift from Dr. P.K. Chan). Bound rabbit and mouse antibodies were detected by a 1:5,000 dilution of the respective HRP-coupled secondary antibodies and visualised using ECL chemiluminescent substrates (Amersham). The images were quantified by densitometry using the GS-800 Calibrated Densitometer from BioRad.

Human USF1 and USF2 proteins were synthesized *in vitro* using the TnT reticulocyte lysate system (Promega) according to the manufacturer's instructions, using USF1 cDNA and USF2 cDNA in pcDNA3 (Invitrogen) as expression vectors, respectively. The USF1 expressing vector was obtained from Dr. B. Staels. The entire coding sequence of USF2 cDNA was generated by RT-PCR on total HepG2 RNA using 5'-gcg aat tCC ATG GAC ATG CTG GAC-3'as forward primer and 5'-gct cta gaG CGT GGT GGT GGC GG-3' as reverse primer. The primers contained an extra *EcoR*I and *Xba*I restriction site, respectively (sequences underlined). These restriction sites were used to insert the cDNA into the pcDNA3 vector. The insert was verified by DNA sequencing (BaseClear, Leiden, The Netherlands).

4.3.4 Electrophoretic mobility shift assays

Double-stranded ^{32}P-labelled oligonucleotides AdML containing the USF consensus-binding site were 5'-GGT GTA GGC CAC GTG ACC GGG TGT AAG CTT-3' and 5'-GGA AGC TTA CAC CCG GTC ACG TGG CCT ACA-3' [22]. Alternatively, double-stranded biotinylated oligonucleotides

corresponding to the -300/-320 region of the human HL gene were used. Oligonucleotides were incubated for 30 min at room temperature with 2.5 µg protein of HepG2 nuclear extracts, or with 1 µl of a reticulocyte lysate with *in vitro* synthesized USF1 or USF2 protein (see above) in a final volume of 20 µl, as described previously [22]. In supershift assays, the nuclear extract protein was pre-incubated for 30 min on ice with 1 µl of anti-USF1 (C-20), anti-USF2 (C-20) or anti-SREBP2 (N-19; all from Santa-Cruz). Protein–DNA complexes were separated in a 4% non-denaturing polyacrylamide gel in 0.5* TBE buffer (44 mM Tris, 44 mM boric acid, 0.1 mM EDTA, pH 8.0). For detection of the radiolabelled oligonucleotides, the gels were dried and exposed to autoradiography film. For detection of biotinylated oligonucleotides, the DNA in the gel was blotted onto nitrocellulose membranes, followed by immunoblotting and enhanced chemoluminescence using the LightShift Chemiluminescent EMSA Kit from Pierce (Rockford IL, USA).

4.3.5 Measurement of HL promoter activity

Transcriptional activity of the human HL (-685/+13) promoter fragment was determined by transient transfection of the HepG2 cells with the HL-685/+13 luciferase vector as described previously [20], except that an medium was refreshed also at 24 h post-transfection. Co-transfection with pRL-TK (Promega) was used as internal control, and luciferase activity data were normalized on the basis of the renilla activity. Each transfection assay was performed in quadruplicate.

From the HL(-685/+13) promoter construct in pGL3, the 5'-deleted HL(-305/+13) promoter construct was prepared by PCR using appropriate oligonucleotide primers. In the HL(-685/+13) construct, the -310Ebox CACGTG was scrambled by standard PCR-directed mutagenesis into GCTAGC (Em). Similarly, the -514E-box CACGGG (-514C) was changed into CATGGG (–514T). Mutations were verified by automated sequencing (BaseClear).

4.3.6 RNA Interference Analysis

RNA silencing of the USF1 and USF2 gene was performed by transient transfection with pSilencer 3.1-H1 hygro siRNA expression vector (Ambion, Austin TX, USA) that generate short-hairpin RNA molecules (shRNA). The sequences of the shRNA molecules directed against USF1 and USF2 partially or completely overlapped with the targeting sites used by McMurray & McCance [47], respectively. The sequences of the shRNA molecules were for USF1: 5'-GGT GGG ATT CTA TCC AAA GCT TCA AGA GAG CTT TGG ATA GAA TCC CAC CTT TTT T-3', and for USF2: 5' GGA GAT ACT ACG GCT GTG TCC AAG CTT GGA CAC AGC CGT AGT ATC TCC TTT TT-3'. As controls, parallel transfections were performed with empty pSilencer vector, or with pSilencer constructs targeted against two non-related genes (aquaporins AQP3 and AQP7, a kind gift of Dr.B. Tilly and C. Lim, Dept. Biochemistry, Erasmus MC Rotterdam).

4.3.7 Chromatin immunoprecipitation assays

A single 10-cm culture dish of HepG2 cells grown in high glucose medium was used for each immunoprecipitation, which was performed as described by Upstate Biotechnology (Lake Placid NY, USA) with some modifications. At near-confluence, formaldehyde was added to the medium at a final concentration of 1%, and the incubation was continued for 10 min at 37°C. Then the cells were harvested, and resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl; pH 8.0). After 10 min ice, the lysates were sonicated until DNA was sheared into 200-400 bp fragments, followed by centrifugation (10 min, 15,000g). Supernatants were diluted 10-fold in immunoprecipitation buffer (1 % Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl; pH 8.0) and precleared for 30 min at 4°C with 60 ml protein A-Sepharose beads that had been preabsorbed with salmon sperm DNA (Upstate Biotechnology). Thereafter, the chromatin solution was incubated overnight at 4°C with 8 µl anti-USF1 or anti-USF2 antibodies (Santa Cruz). For a negative control, a supernatant fraction was incubated in parallel without addition of antibody. After incubation with 60 µl protein A-Sepharose for 1 h at 4°C, the beads were collected by centrifugation, and washed successively with low-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl; 20 mM Tris-HCl; pH 8.0), high-salt buffer (same as the low salt buffer but with 500 mM NaCl), LiCl buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl; pH 8.0), and twice with Tris-EDTA; pH 8.0. Bound material was eluted with 1% SDS;0.1 M $NaHCO_3$, and cross-links were reversed by incubation for 4h at 65 °C in the presence of 0.2 M NaCl. The eluate was then brought to 10 mM EDTA and 40 mM Tris; pH 6.5, and proteins were digested with 40µg/ml proteinase K (Sigma) for 1 h at 45°C. The DNA was isolated with a QIAquick PCR purification kit (QIAGEN, Hilden, Germany) and subjected to real-time quantitative PCR analysis, as described above. Amplification was done with the proximal HL promoter specific primers 5'-CAC AAG CAT CAC CAA TTT CAC-3' (nt -480/-460) and 5'-GCT GGC TCA GGA AAG TGG-3' (nt -369/-352); as a negative control, distal HL gene specific primers 5'-CTT GGG ATT TGC TTG CTT TAT C-3' (nt -6071/-6050) and 5'-ATT TGA TGA CCT GAG AAT GAC C-3' (nt –5965/–5986) were used. Quantitation was done by the DC_T method using the 1% input-reaction as a reference. Alternatively, the PCR products were resolved by 3 % agarose-gel electrophoresis and visualized by ethidiumbromide staining.

4.3.8 Livers from diabetic rats

Female Wistar rats weighing 250 to 350 g were housed in a reversed light-cycle room and had free access to water and standard rodent chow. Animal experiments were carried out according to the regulations of the local Animal Care and Use Committee. Animals were made diabetic by a single intraperitoneal injection of 55 mg/kg of streptozotocin (STZ; Sigma) in 100 mM sodium citrate (pH 4.5) [49,50]. Control animals were injected with vehicle only. Seven weeks later, rats were sacrificed by an isoflurane overdose. Glucose was determined in full blood samples. Liver portions were quickly removed into liquid nitrogen, and stored at -80°C until further analysis. For immunoblotting assays, nuclear extracts were prepared from frozen 100 mg liver aliquots. Of each extract, 50 µg protein was tested for expression of USF1 and USF2 as described above,

and 1 µg for histone H3 using rabbit anti-H3 antibody (Abcam, Cambridge, UK; 1:2500). The images were quantified by densitometry as described above, and data were normalized for H3 staining.

For chromatin immunoprecipitation assays, 100 mg liver aliquots were powdered in liquid nitrogen, and immediately thawed in 1 ml of 1% formaldehyde in PBS. After 10 min incubation at 37°C, the homogenate was diluted once in 2 % SDS, 20 mM EDTA, 100 mM Tris-HCl (pH 8.0). Thereafter, DNA shearing, immunoprecipitation, DNA isolation and qPCR analysis was performed as described above. Amplified regions of the rat HL gene were –392/–282 (primers 5′-GGG GCT TTT ACC TCT CTT TGG G-3′ and 5′-TGA CCT CTG TAT TGT TGC CTG TG-3′) and –7328/–7214 (primers 5′-CAC AGC AAC ACG AGC CTC AG-3′ and 5′-TGC AGT GTA GAA TTT GTG GCA TAC C-3′). For determination of HL activity, frozen 100 mg liver aliquots were homogenized in 2 ml phosphate-buffered saline (PBS) containing 50 IU/ml heparin on ice using a Polytron homogenizer. HL activity was determined in the homogenate, and was completely inhibited by preincubation with excess anti-rat HL antiserum [43].

4.3.9 Statistics

All data are expressed as means ± SD. Statistical analyses were performed using Student's t-test. Statistical significance was defined as $p<0.05$.

4.4 Results

4.4.1 Glucose increases both HL expression and nuclear accumulation of USF1 and USF2

HepG2 cells that had been cultured to confluence at 22.5 mM glucose were incubated for an additional 8h in medium containing normal (4.5 mM), intermediate (9 mM) or high (22.5 mM) glucose. Secretion of HL activity increased with increasing glucose concentration (Figure 1A). In the high-glucose medium, HL secretion was 2.9±0.5-fold higher than in the normal-glucose medium (Figure 1A). After 8 h of incubation under these conditions (confluent wells and reduced medium volume), glucose was completely depleted from the normal-glucose medium. In the high-glucose medium, glucose was reduced to 13.0±1.9 mM (n=3). In medium with intermediate glucose concentration, extracellular glucose decreased from 9 to 1.6±0.3 mM (n=3). HL secretion was also intermediate between the normal- and high-glucose cells (Figure 1A). In further experiments, cells were incubated with higher volumes of medium per well, and the medium was refreshed daily to keep extracellular glucose above 1 mM throughout the incubation. Under these conditions, the abundance of HL mRNA, as determined by RT-real-time quantitative PCR, in high-glucose cells was 1.5±0.2-fold higher than in parallel normal-glucose cells (p=0.028, n=3). To test the effect of glucose on HL transcription, cells were transiently transfected with the HL-685/+13 luciferase promoter-reporter construct. Luciferase activity in the high-glucose cells was 1.8±0.3-fold higher than in the normal-glucose cells (p=0.036;

n=3). This suggests that glucose dose-dependently increased HL secretion, largely by affecting transcription of the HL gene.

Nuclear extracts were prepared from normal- and high-glucose HepG2 cells, and the amount of USF1 and USF2 protein was determined by immunoblotting. Nucleophosmin (B23), an abundant nuclear protein involved in ribosome biogenesis [48], was used as loading control. As shown in Figure 1B, the amount of USF1 was 2.5±0.5-fold (n=3, p=0.042) higher in the cells grown in high-glucose compared to normal-glucose medium. USF2 was slightly, but significantly, higher in the high-glucose cells (1.4±0.2-fold, n=3, p=0.010).

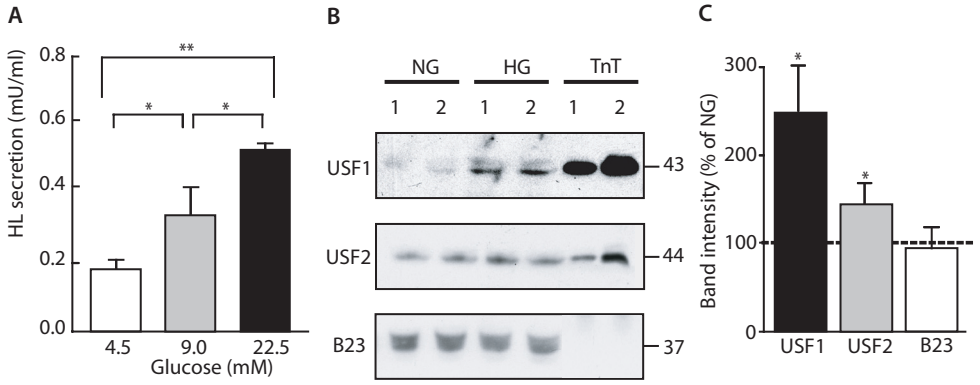

Figure 1. High glucose increases HL secretion and nuclear accumulation of USF proteins.

In panel A, HepG2 cells were incubated for 8 h in medium containing heparin and the indicated amounts of glucose. At the end of the incubations, hepatic lipase activity was determined in the cell-free media. Data are means ±S.D. for 3-5 independent experiments. In B, HepG2 cells were cultured with 4.5 (normal glucose [NG]) or 22.5 mM (high glucose [HG]) glucose. At confluence, nuclear extracts were prepared and the presence of USF1 and USF2 protein was detected by immunoblotting. B23 protein served as internal control. TnT 1 and 2 are 0.5 ml and 1.0 ml, respectively, of *in vitro* transcribed and translated USF, which serve as positive controls. The molecular sizes are indicated in kDa. In C, the data were quantified by densitometry, the two parallels were averaged, and the data were expressed as percentage of the NG controls. Data are means ± S.D. for three to four independent experiments. * and **: p<0.05 and p<0.01, respectively.

4.4.2 Glucose increases DNA binding activity of USF

Electrophoretic mobility shift assays were performed using HepG2 nuclear extracts and double-strand oligonucleotides containing a USF consensus-binding site. As shown in figure 2A, a similar band shift was produced upon incubation with nuclear extracts from the high- and normal-glucose cells. With the high-glucose cells, however, markedly more probe was shifted than with the normal-glucose cells. The shifted band co-migrated with in-vitro synthesized USF1 and USF2 proteins run in parallel. In supershift assays, the band was partially shifted to higher positions in the gel after preincubation of the nuclear extracts with antibodies against either USF1 or USF2 (Figure 2B), but not with antibodies against related SREBP2. A complete supershift was induced by preincubation with anti-USF1 and anti-USF2 combined. These data indicate that glucose increases both the amount and DNA binding activity of USF proteins in

HepG2 cell nuclei, and suggests that the USF proteins induced by glucose are active in DNA binding.

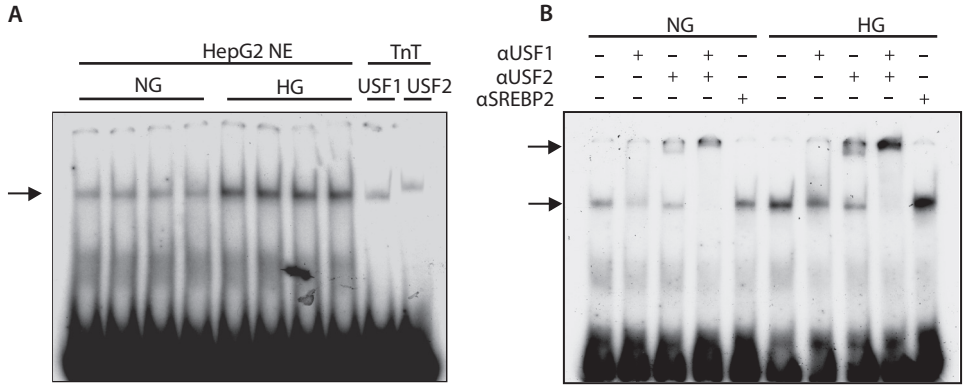

Figure 2. Glucose increases E-box DNA binding activity in HepG2 nuclei.

Gel shift (panel A) and supershift (panel B) assays were performed using ^{32}P-labeled, double-stranded oligonucleotides containing consensus USF binding sites, and nuclear extracts (2.5 µg protein) from HepG2 cells that were grown to confluence in 4.5 mM or 22.5 mM glucose (NG and HG, respectively). In **A**, nuclear extracts from four different incubations were run in parallel, and with TnT reticulolysate extracts that had been incubated with pcDNA3-USF1 or pcDNA3-USF2. In **B**, the nuclear extracts were pre-incubated with the antibody indicated prior to binding to the DNA. The arrows indicate the positions of the shifted and supershifted bands.

4.4.3 Glucose increases USF1 and USF2 binding to the HL promoter in chromatin

Chromatin was isolated from normal- and high-glucose cells, and USF1 and USF2 were immunoprecipitated. As shown by PCR, the proximal HL promoter region co-immunoprecipated with both anti-USF antibody preparations (Figure 3A). Significantly more PCR product was generated with DNA immunoprecipitated from high-glucose than from normal-glucose cells using primers specific for the proximal HL promoter region (-480/-352) (Figure 3B). In contrast, the amount of PCR product generated with distal HL gene specific primers (-6071/–5965) was negligible, and not affected by glucose conditioning. Hence, both USF1 and USF2 are bound to the proximal HL promoter region in the HepG2 nuclei *in situ*, and binding is higher in high-glucose than in normal-glucose cells.

4.4.4 Glucose-induced increase of HL promoter activity is reduced by removal of potential USF binding sites

We previously reported that co-transfection with the USF1 expression vector pCX-USF1 dose-dependently increased transcriptional activity of the HL -685/+13 promoter fragment [20]. Here we show that co-transfection of the HL -685/+13 luciferase construct with 10 and 24 ng of pcDNA3-USF1 increased luciferase activity approximately 6- and 16 fold, respectively (Figure 4B). In this HL promoter fragment, E-boxes that potentially bind USF are present around

positions -514 and -310 (Figure 4A). We have not been able to show binding of endogenous USFs to these sites in gel shift assays with HepG2 nuclear extracts, in agreement with the low binding affinity compared with the AdML consensus site [22]. However, purified USFs have been shown to bind to the -514 region of the HL promoter [22], and Fig. 4C demonstrates binding of USF1 and USF2 to the -310 region in nuclear extracts of HepG2 cells transiently transfected with the respective expression plasmids. Mutation of the -514 E-box from CACGGG (-514C) into the allelic variant CATGGG (-514T) reduced responsiveness of the HL -685/+13 promoter fragment to co-transfected USF1 and USF2, but this reduction did not reach statistical significance (Figure 4B, left panel). Scrambling of the -310 E-box sequence from CACGTG (-310E) to GCTAGC (-310Em) reduced responsiveness to USF1 and USF2 to approximately 50 % (p=0.006 and 0.001, respectively; n=4; Fig. 4 B, right panel). This suggests that USF1 and USF2 upregulate HL promoter activity in part through binding to the -310 E-box.

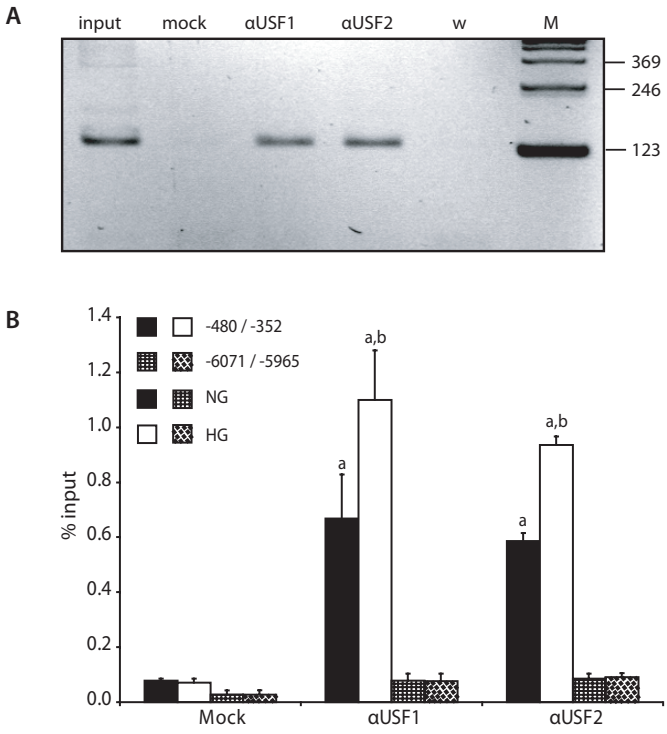

Figure 3. Chromatin immunoprecipitation assays with HepG2 cells.

Chromatin was immunoprecipitated from HepG2 cells with anti-USF1 or anti-USF2, and the DNA was analysed by PCR using HL specific primers. In **A**, part of the proximal HL promoter region (-480/-352) was amplified (30 cycles 30 s 95°C, 30 s 55°C, 45 s 72°C) using 4 out of 60 µl immunoprecipitate, and PCR products were analysed by agarose-gel electrophoresis and ethidiumbromide staining. As positive and negative controls, 1% input DNA and no-antibody immunoprecipation (mock) was included, respectively. The w is the PCR water control; M indicates the 123-bp DNA ladder from Roche. In **B**, DNA immunoprecipitated from normal-glucose (NG) and high-glucose (HG) cells was analysed by qPCR, amplifying either the proximal (-480/-352) or distal (-6071/-5965) upstream region of the HL gene. Data are mean ±S.D. for three independent experiments. [a] and [b]: statistically significant difference from mock and normal glucose, respectively.

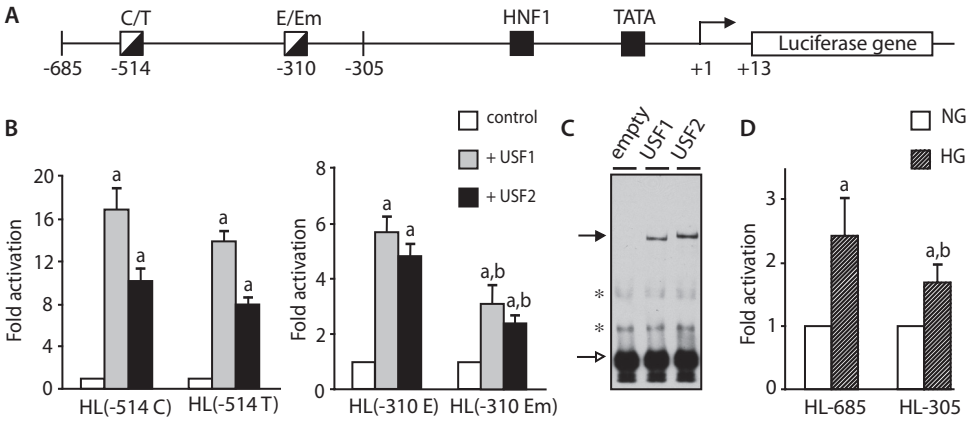

Figure 4. E-box motifs in the HL promoter fragment are required for full responsiveness to USF1 and glucose.

Panel **A** is a schematic representation of the HL -685/+13 promoter fragment, indicating the position of two potentially USF-binding E-box motifs. The role of these E-boxes was tested by either mutating these E-boxes (panel B), or by removal of both E-boxes by 5'-deletion (panel D). In **B**, the effect of the -514C→T mutation or the -310E→Em mutation was tested on responsiveness to stimulation with USF1 and USF2 in transient co-transfection assays. High-glucose HepG2 cells were co-transfected with 24 ng (left panel) or 10 ng (right panel) of USF1- (gray bars) and USF2- (dark bars) expression vectors. Luciferase activities were expressed relative to the activities measured in cells co-transfected with empty pcDNA3 vector. ([a] and [b]: statistically significant difference from control cells and from the wildtype construct, respectively.) In panel **D**, the glucose responsiveness of the HL-685/+13 promoter fragment is compared with the HL-305/+13 promoter fragment. HepG2 cells grown in 4.5 (open bars) and 22.5 mM glucose (hatched bars) were transiently transfected with both promoter fragments, and the luciferase activity was determined. Activities in the normal-glucose cells were set at 1.0, and the activities in the high-glucose cells were expressed as fold increase relative to normal-glucose cells. Data are means ± S.D. for 4-5 independent experiments. ([a] and [b]: statistically significant difference from normal-glucose cells and from the full-length construct, respectively). Panel **C** shows a gelshift of biotinylated -300/-320 region of the HL gene, and nuclear extracts of HepG2 cells that were transiently transfected with 100 ng of USF1 or USF2 expression vector, or with empty pcDNA3 vector (mock). The arrows indicate the position of the shifted bands; the asterisk indicates a non-specific band.

Compared to the HL-685/+13 promoter reporter construct, the HL-305/+13 construct lacks both the potential USF binding sites at -514 and -310. Both reporter constructs were tested in parallel for promoter activity. Whereas in this series of experiments the activity of HL-685/+13 construct was 2.4±0.6-fold higher in the high-glucose than in the normal-glucose cells, this figure was only 1.6±0.3-fold with the shorter construct (p=0.037; n=5; Figure 4D). Hence, the E-boxes in the HL promoter are also important for full glucose responsiveness.

4.4.5 Glucose-induced increase of HL promoter activity is reduced by silencing of the USF1 gene

Finally, the USF1 and USF2 gene was silenced by RNA interference. Transfection of HepG2 cells with pSilencer plasmids that code for shRNA directed against USF1 and USF2 markedly reduced the amount of USF1 and USF2 protein in nuclear extracts, respectively (Figure 5A). In normal-glucose cells, HL promoter activity was slightly but significantly reduced by co-transfection with shUSF1 and shUSF2 (Figure 5B). In the high-glucose cells, the HL promoter activity was

reduced to approximately 50 % of parallel controls, and the upregulation by high-glucose was almost completely prevented by co-transfection with shUSF1. Similar trends were observed with shUSF2, but the effects did not reach statistical significance. shRNA constructs directed against non-related AQP3 or AQP7 did not affect HL promoter activity in the high-glucose cells (Figure 5C).

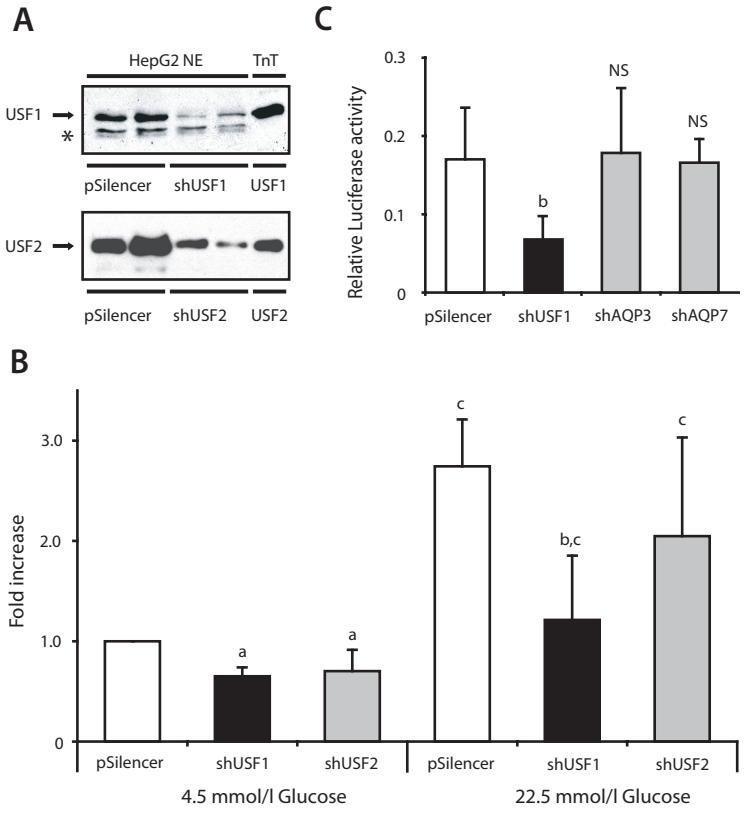

Figure 5. Silencing USF1 reduces glucose-induced up-regulation of HL promoter activity.

HepG2 cells were co-transfected with the HL-685/+13 luciferase reporter, and 100 ng of the indicated shRNA construct in pSilencer. Panel **A** shows the effect of shUSF1 on USF1 protein and of shUSF2 on USF2 protein in nuclear extracts (upper and lower panel, respectively). Of shUSF- and mock-transfected cells, 25 mg nuclear protein was separated by SDS-PAGE in parallel with *in vitro* synthesized USF protein, followed by immunoblotting with anti-USF1 and anti-USF2, respectively. The asterisk indicates a cross-reacting band. In panel **B**, HL promoter activity was determined in cells that were co-transfected with 100 ng shUSF1, shUSF2 or pSilencer. Luciferase data were expressed as fold stimulation relative to the activity with pSilencer measured in the normal glucose cells. Data are means ±S.D. for 5 independent experiments. In C, the effect of the shUSF1 construct was compared with shRNA constructs directed against two unrelated proteins (AQP3 and AQP7). Cells were incubated in high-glucose medium. Data are means ± S.D of 5 parallel incubations. [a] and [b]: statistical significant differences of sh versus pSilencer in normal and high glucose conditions, respectively; [c]: high versus normal glucose; n.s.: non-significant.

4.4.6 HL expression in livers of streptozotocin-treated rats

To test whether chronically elevated glucose affects USF and HL expression *in vivo*, we used livers of streptozotocin-treated rats. Whole blood glucose concentration in streptozotocin-treated rats was 23.9±1.2 mmol/L compared to5.9±0.5 mmol/L in control rats ($p<0.05$; n=3). In livers of the treated rats, nuclear expression of USF1 was 2.1-fold (19.0±2.8 versus 9.0±0.9 a.u., $p<0.05$; n=3) higher, and USF2 was 1.4-fold (72.7±11.5 versus 51.0±5.7 a.u., $p<0.05$; n=3) higher than in parallel controls (Figure 6A). In chromatin immunoprecipitation assays, the proximal region of the rat HL gene (-392/-282) were immunoprecipated with anti-USF1 and anti-USF2, but not a distal region of the HL gene (-7329/-7214), indicating that USF proteins are specifically bound to the proximal promoter region of the HL gene in rat liver (Figure 6B). In contrast to HepG2 cells, binding of USF proteins to the upstream region of the HL gene was not significantly affected by the high glucose levels, as similar amounts of DNA were immunoprecipitated from livers of streptozotocin-treated and control rats. HL activity in liver homogenates amounted to 4.3±0.4 mU/mg protein in streptozotocin-treated rats compared to 10.6±2.8 mU/mg protein in control animals ($p<0.05$; n=3).

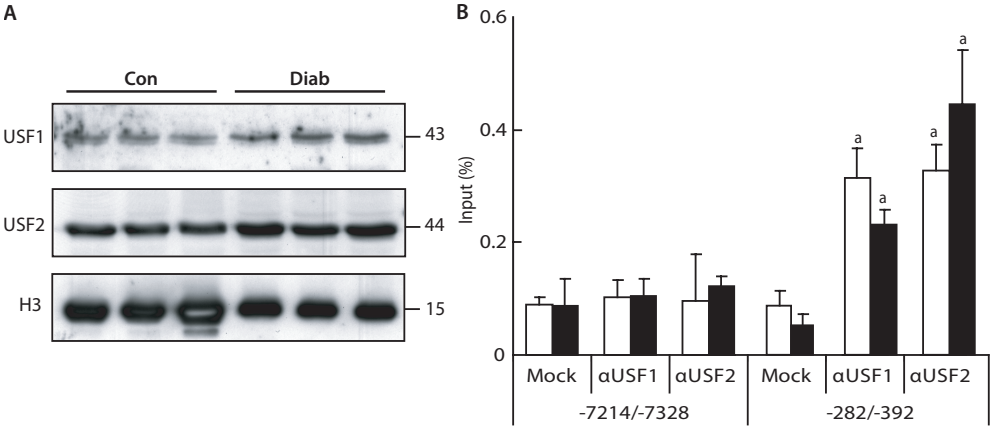

Figure 6. Effect of chronically elevated blood glucose on USF expression and chromatin immuno-precipitation in rat liver.

In **A**, expression of USF1 and USF2 was determined in liver nuclear extracts of three control rats (Con) and three streptozotocin-treated rats (Diab), using histon H3 as loading control. The molecular sizes are indicated in kDa. In **B**, chromatin immunoprecipitation assays were performed with chromatin isolated from livers of control and streptozotocin-treated rats (open and closed bars, respectively), and anti-USF1 and anti-USF2. Immunoprecipitated DNA was analysed by qPCR using HL specific primers targeted at the indicated upstream regions of the rat HL gene. Data are mean ±S.D. for three independent experiments. [a] and [b]: statistically significant difference from mock and from control liver, respectively.

4.5 Discussion

Our study shows that HepG2 cells express more USF1 and USF2 protein in their nuclei under high- than low-glucose conditions. As extracellular glucose concentrations in our experiments frequently reach very low levels, one may argue that USF proteins disappear from the nuclei in the low-glucose conditions. However, we favor the interpretation that high-glucose induces the nuclear accumulation of USF proteins, as increased expression of both USF proteins is also observed in livers of hyperglycemic, streptozotocin-treated rats. In HepG2 cells, this is parallelled by increased binding of USFs to the promoter region of the HL gene, and a parallel increase in HL expression. The human HL gene is a potential target for USFs, as USF proteins are bound to the proximal HL promoter region in HepG2 cells (Figure 3), the proximal HL promoter region contains functionally important E-boxes, and HL promoter activity is dose-dependently upregulated by over-expression of USF1 or USF2 (Figure 4) [20,22]. Silencing of the *USF1* gene by RNA interference strongly reduced the glucose-induced upregulation of HL promoter activity (Figure 5). Therefore, the USF proteins that accumulate in the nuclei of HepG2 cells are important in mediating the glucose-induced increase in HL expression. Glucose responsiveness of the HL promoter was not completely lost upon deletion of the E-boxes. Hence, the glucose-induced upregulation of HL promoter activity may be mediated by USFs via additional, non-E box elements. Co-transfection with pcDNA3-USF1 and -USF2 also increased transcription of proximal HL promoter constructs through binding of USFs to the transcription initiation site (manuscript in preparation). In rat liver chromatin, USF1 and USF2 protein apparently bind to the proximal HL promoter region at non-E box elements, as this region of the rat HL gene lacks canonical E-boxes. Alternatively, glucose may activate the HL promoter through additional mechanisms that are independent of USFs. Recently, ChREBP was identified as a major glucose responsive transcription factor in liver cells; its consensus DNA binding site, however, consists of two E-boxes separated by 5 bps [51]. By the MatInspector program (matrix similarity >0.82; [52]), such a potential binding site was found within 2 kb of the proximal USF1 and USF2 but not of the HL promoter region. Moreover, fatty acids decrease expression of glucose responsive gene through decrease of ChREBP activity [53], whereas fatty acids increase HL [19,20] and nuclear USF expression (van Deursen and Verhoeven, unpublished observations) in HepG2 cells. A major role for ChREBP in mediating glucose-responsiveness to the HL gene therefore appears unlikely.

In contrast to HepG2 cells, the accumulation of USF1 and USF2 in liver nuclei of streptozotocin-treated rats was not accompanied by increased binding of USFs to the proximal HL promoter region. In addition, HL expression was reduced in this *in vivo* model. Apparently, increased nuclear expression of USF proteins is not the only factor that regulates USF binding activity and HL expression. In the *in vivo* model, the liver cells are not only chronically exposed to hyperglycemia and low insulin, but also to many other variables not mimicked in our *in vitro* model, such as elevated fatty acid levels [50], glucocorticoids [54] and glucagon [55]. Our preliminary data show that addition of 1 mM BSA-bound oleate to the high-glucose medium

further increases USF1 expression, HL promoter activity and secretion of HL in HepG2 cells. In contrast, glucocorticoids and glucagon lower HL expression in rat hepatocytes [56-57], possibly through mechanisms that override the positive effect of increased nuclear expression of USFs. One possibility is that USFs become phosphorylated thereby affecting their DNA binding activity [23,27]. Whether increased nuclear expression of USF protein leads to increased USF binding to the proximal HL promoter, and subsequently to upregulation of HL expression, apparently depends on the species studied, or on the prevailing metabolic or hormonal status. Alternatively, the hyperglycemia-induced, USF-mediated upregulation of HL expression may be restricted to human hepatoma cells.

USF1 and USF2 are ubiquitously expressed. In various non-hepatoma cells, glucose has been shown to increase either nuclear USF1 [32] or USF2 [34] alone, or both [33]. In HepG2 cells, glucose increases predominantly nuclear USF1, and silencing of the *USF1* but not *USF2* gene abolished the increase in HL promoter activity. In mouse liver, glucose responsiveness is mainly signalled through the USF1-USF2 heterodimer, but the homodimers are also transcriptionally active [30]. Hence, upregulation of either USF1 or USF2 may affect expression of target genes. The relative importance of USF1 and USF 2 overexpression may depend on which of the two proteins is limiting in the cell type used. Taken together, our data suggest that the nuclear accumulation of USF1 and USF2 signals glucose responsiveness to the hepatic lipase gene, and likely also to other USF target genes. The mechanism that leads to accumulation of USF1 and USF2 by glucose is presently unknown. In adipocytes, glucose has to be metabolized in the glycolytic pathway beyond glucose-6-phosphate to the triose phosphates [31]. Further research is required to show whether this also holds for liver cells.

USF1 and USF2 are involved in the coordinate regulation of glucose and lipid metabolism [23,40], in which the liver plays a central role. The finding of increased nuclear expression of USF proteins may therefore bear relevance to the aetiology of insulin resistance and type 2 diabetes. The associated dyslipidemia (low HDL, high TG, increased small-dense LDL) may result from the increased transactivation by USFs not only of hepatic lipase, but also of other HDL- and TG- related genes such as apoA-II [26], apoA-V [27], apoC-III [28], apoE [29] or hormone-sensitive lipase [31]. Increased USF1 transactivation of its target genes has been suggested to explain the development of the metabolic syndrome [35,39], the dyslipidemia associated with FCHL [36,40], and the development of diabetic complications [32,34]. Metabolic syndrome, type 2 diabetes and FCHL have all been linked to the *USF1* gene [35-37]. A number of risk alleles of the *USF1* gene have been identified, which all represent variants of the non-coding sequence [36,38,41]. It is not clear how non-coding polymorphisms in the *USF1* gene may contribute to these metabolic disorders. The polymorphism in intron 7 of the *USF1* gene [36] has been shown to affect binding of nuclear proteins to this region [38], suggesting that this polymorphism affects transcriptional or post-transcriptional regulation of USF1 expression. Possibly, these polymorphisms interfere with the glucose-mediated nuclear accumulation of USF1 in the liver. Further studies are required to elucidate the mechanism by which glucose affects USF1

and USF2 expression, and how genetic variants of the *USF1* gene may alter responsiveness to glucose.

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References

1. Varughese GI, Tomson J, Lip GY (2005) Type 2 diabetes mellitus: a cardiovascular perspective. *Int J Clin Pract* **59**:798-816
2. Sobel BE, Schneider DJ (2005) Cardiovascular complications in diabetes mellitus. *Curr Opin Pharmacol* **5**:143-148
3. Reaven GM (2005) Compensatory hyperinsulinemia and the development of an atherogenic lipoprotein profile: the price paid to maintain glucose homeostasis in insulin-resistant individuals. *Endocrinol Metab Clin North Am* **34**:49-62
4. Nigro J, Osman N, Dart AM, Little PJ (2006) Insulin Resistance and atherosclerosis. *Endocr Rev* **27**:242-259
5. Bredie SJ, Tack CJ, Smits P, Stalenhoef AF (1997) Nonobese patients with familial combined hyperlipidemia are insulin resistant compared with their nonaffected relatives. *Arterioscler Thromb Vasc Biol* **17**:1465-1471
6. Brunzell JD, Hokanson JE (1999) Dyslipidemia of central obesity and insulin resistance. *Diabetes Care* **22**(Suppl 3):C10-13
7. Ginsberg HN (2006) Review: Efficacy and mechanisms of action of statins in the treatment of diabetic dyslipidemia. *J Clin Endocrinol Metab* **91**:383-392
8. Jansen H, Verhoeven AJM, Sijbrands EJG (2002) Hepatic lipase: a pro- or anti-atherogenic protein? *J Lipid Res* **43**:1352-1362
9. Deeb SS, Zambon A, Carr MC, Ayyobi AF, Brunzell JD (2003) Hepatic lipase and dyslipidemia: interactions among genetic variants, obesity, gender, and diet. *J Lipid Res* **44**:1279-1286
10. Rashid S, Watanabe T, Sakaue T, Lewis GF (2003) Mechanisms of HDL lowering in insulin resistant, hypertriglyceridemic states: the combined effect of HDL triglyceride enrichment and elevated hepatic lipase activity. *Clin Biochem* **36**:421-429
11. Allayee H, Dominguez KM, Aouizerat BE, Krauss RM, Rotter JI, Lu J, Cantor RM, de Bruin TWA, Lusis AJ (2000) Contribution of the hepatic lipase gene to the atherogenic lipoprotein phenotype in familial combined hyperlipidemia. *J. Lipid Res* **41**:245-252
12. Tan KCB, Shiu SWM, Chu BYM (1999) Roles of hepatic lipase and cholesteryl ester transfer protein in determining low density lipoprotein subfraction distribution in Chinese patients with non-insulin-dependent diabetes mellitus. *Atherosclerosis* **145**:273-278
13. Berk-Planken II, Hoogerbrugge N, Stolk RP, Bootsma AH, Jansen H (2003) Atorvastatin dose-dependently decreases hepatic lipase activity in type 2 diabetes: effect of sex and the LIPC promoter variant. *Diabetes Care* 26:427-432
14. Baynes C, Henderson AD, Richmond W, Johnston DG, Elkeles RS (1992) The response of hepatic lipase and serum lipoproteins to acute hyperinsulinaemia in type 2 diabetes. *Eur J Clin Invest* **22**:41-346
15. Jansen H, Verhoeven AJM, Weeks L, Kastelein JJ, Halley DJ, van den Ouweland A, Jukema JW, Seidell JC, Birkenhäger JC (1997) Common C-to-T substitution at position -480 of the hepatic lipase promoter associated with a lowered lipase activity in coronary artery disease patients. *Arterioscler Thromb Vasc Biol* **17**:2837-2842
16. Pihlajamäki J, Karjalainen L, Karhapää P, Vauhkonen I, Taskinen MR, Deeb SS, Laakso M (2000) G-250A substitution in promoter of hepatic lipase gene is associated with dyslipidemia and insulin resistance in healthy control subjects and in members of families with familial combined hyperlipidemia. *Arterioscler Thromb Vasc Biol* **20**:1789-1795
17. Lewis GF, Murdoch S, Uffelman K, Naples M, Szeto L, Albers A, Adeli K, Brunzell JD (2004) Hepatic lipase mRNA, protein, and plasma enzyme activity is increased in the insulin-resistant, fructose-fed Syrian golden hamster and is partially normalized by the insulin sensitizer rosiglitazone. *Diabetes* **53**:2893-2900
18. Carr MC, Hokanson JE, Deeb SS, Purnell JQ, Mitchell ES, Brunzell JD (1999) A hepatic lipase gene promoter polymorphism attenuates the increase in hepatic lipase activity with increasing intra-abdominal fat in women. *Arterioscler Thromb Vasc Biol* **19**:2701-2707
19. Nimmo L, McColl AJ, Rosankiewicz JZ, Richmond W, Elkeles RS (1997) Regulation of hepatic lipase expression in HepG2 cells. *Biochem Soc Trans* **25**:S689

20. Botma, GJ, van Deursen D, Vieira D, van Hoek M, Jansen H, Verhoeven AJM (2005) Sterol-regulatory-element binding protein inhibits upstream stimulator factor-stimulated hepatic lipase gene expression. *Atherosclerosis* **179**:61-67
21. Tu AY, AlbersJJ (2001) Glucose regulates the transcription of human genes relevant to HDL metabolism. *Diabetes* **50**:1851-1856
22. Botma GJ, Verhoeven AJM, Jansen H (2001) Hepatic lipase promoter activity is reduced by the C-480G and G-216A substitutions present in the common *LIPC* variant and is increased by upstream stimulatory factor. *Atherosclerosis* **154**:625-632
23. Corre S, Galibert MD (2005) Upstream stimulating factors: highly versatile stress-responsive transcription factors. *Pigment Cell Res* **18**:337-348
24. Iynedjian PB (1998) Identification of upstream stimulatory factor as transcriptional activator of the liver promoter of the glucokinase gene. *Biochem J* **333**:705-712
25. Wang D, Sul HS (1997) Upstream stimulatory factor binding to the E-box at –65 is required for insulin regulation of the fatty acid synthase promoter. *J Biol Chem* **272**:26367-26374
26. Ribeiro A, Pastier D, Kardassis D, Chambaz J, Cardot P (1999) Cooperative binding of upstream stimulatory factor and hepatic nuclear factor 4 drives the transcription of the human apolipoprotein A-II gene. *J Biol Chem* **274**:1216-1255
27. Nowak M, Helleboid-Chapman A, Jakel H, Martin G, Duran-Sandoval D, Staels B, Rubin EM, Pennacchio LA, Taskinen MR, Fruchart-Najib J, Fruchart JC (2005) Insulin-mediated down-regulation of apolipoprotein A5 gene expression through the phosphatidylinositol 3-kinase pathway: role of upstream stimulatory factor. *Mol Cell Biol* **25**:1537-1548
28. Pastier D, Lacorte JM, Chambaz J, Cardot P, Ribeiro A (2002) Two initiator-like elements are required for the combined activation of the human apolipoprotein *C-III* promoter by upstream stimulatory factor and hepatic nuclear factor-4. *J Biol Chem* **277**:15199-15206
29. Salero E, Gimenez C, Zafra F (2003) Identification of a non-canonical E-box motif as a regulatory element in the proximal promoter region of the apolipoprotein E gene. *Biochem J* **370**:979-986
30. Vallet VS, Casado M, Henrion AA, Bucchini D, Raymondjean M, Kahn A, Vaulont S (1998) Differential roles of upstream stimulatory factors 1 and 2 in the transcriptional response of liver genes to glucose. *J Biol Chem* **273**:20175-20179
31. Smih F, Rouet P, Lucas S, Mairal A, Sengenes C, Lafontan M, Vaulont S, Casado M, Langin D (2002) Transcriptional regulation of adipocyte hormone-sensitive lipase by glucose. *Diabetes* **51**:293-300
32. Bidder M, Shao JS, Charlton-Kachigian N, Loewy AP, Semenkovich CF, Towler DA (2002) Osteopontin transcription in aortic vascular smooth muscle cells is controlled by glucose-regulated upstream stimulatory factor and activator protein-1 activities. *J Biol Chem* **277**:44485-44496
33. Weigert C, Brodbeck K, Sawadogo M, Häring HU, Schleicher ED (2004) Upstream stimulatory Factor (USF) proteins induce human *TGF*-b*1* gene activation via the glucose-response element –1013/-1002 in mesangial cells. *J Biol Chem* **279**:15908-15915
34. Wang S, Skorczewiski J, Feng X, Mei L, Murphy-Ullrich JE (2004) Glucose up-regulates thrombospondin 1 gene transcription and transforming growth factor-b activity through antagonism of cGMP-dependent protein kinase repression via upstream stimulatory factor 2. *J. Biol. Chem.* **279**:34311-34322
35. Ng MC, Miyake K, So WY, Poon EW, Lam VK Li JK, Cox NJ, Bell GI, Chan JC (2005) The linkage and association of the gene encoding upstream stimulatory factor 1 with type 2 diabetes and metabolic syndrome in the Chinese population. *Diabetologia* **48**:2018-2024
36. Pajukanta P, Lilja HE, Sinsheimer JS, Cantor RM, Lusis AJ, Gentile M, Duan XJ, Soro-Paavonen A, Naukkarinen J, Saarela J, Laakso M, Ehnholm C, Taskinen MR, Peltonen L (2004) Familial combined hyperlipidemia is associated with upstream transcription factor 1 (*USF1*). *Nat Genet* **36**:371-376
37. Coon H, Xin Y, Hopkins PN, Cawthon RM, Hasstedt SJ, Hunt SC (2005) Upstream stimulatory factor 1 associated with familial combined hyperlipidemia, LDL cholesterol, and triglycerides. *Hum Genet* **117**:444-451
38. Komulainen K, Alanne M, Auro K, Kilpikari R, Pajukanta P, Saarela J, Ellonen P, Salminen K, Kulathinal S, Kuulasmaa K, Silander K, Salomaa V, Perola M, Peltonen L (2006) Risk Alleles of USF1 Gene Predict Cardiovascular Disease of Women in Two Prospective Studies. *PloS Genetics* **2**:e69

39. Shoulders CC, Naoumova RP (2004) USF1 implicated in the aetiology of familial combined hyperlipidaemia and the metabolic syndrome. *Trends Mol Med* **10**:362-365

40. Naukkarinen J, Gentile M, Soro-Paavonen A, Saarela J, Koistinen HA, Pajukanta P, Taskinen MR, Peltonen L (2005) USF1 and dyslipidemias: converging evidence for a functional intronic variant. *Hum Mol Genet* **14**:2595-2605

41. Putt W, Palmen J, Nicaud V, Tregouet DA, Tahri-Daizadeh N, Flavell DM, Humphries SE, Talmud PJ (2004) Variation in *USF1* shows haplotype effects, gene:gene and gene:environment associations with glucose and lipid parameters in the European Atherosclerosis Research Study II. *Human Mol Genet* **13**:1587-1597

42. Hoffstedt J, Ryden M, Wahrenberg H, van Harmelen V, Arner P (2005) Upstream transcription factor-1 gene polymorphism is associated with increased adipocyte lipolysis. *J Clin Endocrinol Metab* **90**:5356-5360

43. Verhoeven AJM, Neve BP, Jansen J (1999) Secretion and apparent activation of human hepatic lipase requires proper oligosaccharide processing in the endoplasmic reticulum. *Biochem. J.* **337**:133-140

44. Datta S, Luo CC, Li WH, VanTuinen P, Ledbetter DH, Brown MA, Chen SH, Liu SW, Chan L (1988) Human hepatic lipase. Cloned cDNA sequence, restriction fragment length polymorphisms, chromosomal localization, and evolutionary relationships with lipoprotein lipase and pancreatic lipase. *J Biol Chem* **263**:1107-1110

45. Rich BE, Steitz JA (1987) Human acidic ribosomal phosphoproteins P0, P1, and P2: analysis of cDNA clones, in vitro synthesis, and assembly. *Mol Cell Biol* **7**:4065-4074

46. Schreiber E, Matthias P, Müller MM, Schaffner W (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res* **17**:6419

47. McMurray HR, McCance DJ (2003) Human papillomavirus type 16 E6 activates TERT gene transcription through induction of c-Myc and release of USF-mediated repression. *J Virol* **77**:9852-9861

48. Hingorani K, Szebeni A, Olson MOJ (2000) Mapping the functional domains of nucleolar protein B23. *J Biol Chem* **275**:24451-24457

49. Van Linthout S, Riad A, Dhayat N, Spillmann F, Du J, Dhayat S, Westermann D, Hilfiker-Kleiner D, Noutsias M, Laufs U, Schultheiss HP, Tschöpe C (2007) Anti-inflammatory effects of atorvastatin improve left ventricular function in experimental diabetic cardiomyopathy. *Diabetologia* **50**:1977-1986

50. King LM, Sidell RJ, Wilding JR, Radda GK, Clarke K (2001) Free fatty acids, but not ketone bodies, protect diabetic rat hearts during low-flow ischemia. *Am J Physiol Heart Circ Physiol* **280**:H1173-1181

51. Ma L, Robinson LN, Towle HC (2006) ChREBP*Mlx is the principal mediator of glucose-induced gene expression in the liver. J *Biol Chem* **281**:28721-28730

52. Cartharius K, Frech K, Grote K, Klocke B, Haltmeier M, Klingenhoff A, Frisch M, Bayerlein M, Werner T (2005) MatInspector and beyond: promoter analysis based on transcription factor binding sites. *Bioinformatics* **21**:2933-42

53. Kawaguchi T, Osatomi K, Yamashita H, Kabashima T, Uyeda K (2002) Mechanism for fatty acid "sparing" effect on glucose-induced transcription: regulation of carbohydrate-responsive element-binding protein by AMP-activated protein kinase. *J Biol Chem* **277**:3829-3835

54. Odedra BR, Dalal SS, Millward DJ (1982) Muscle protein synthesis in the streptozotocin-diabetic rat. *Biochem J* **202**:363-368

55. Bhathena SJ, Voyles NR, Smith S, Recant L (1978) Decreased glucagon receptors in diabetic rat hepatocytes. *J Clin Invest* **61**:1488-1497

56. Jansen H, van Tol A, Auwerx J, Skretting G, Staels B (1992) Opposite regulation of hepatic lipase and lecithin: cholesterol acyltransferase by glucocorticoids in rats. *Biochim Biophys Acta* **1128**:181–185

57. Jensen E, Hansson P, Floren CH, Nilsson A, Nilsson-Ehle P (1989) Cell-density-dependent release of hepatic lipase from cultured rat hepatocytes. *Horm Metab Res* **21**:4-7

CHAPTER 5

Upstream Stimulatory Factors 1 and 2 activate the human hepatic lipase promoter via E-box dependent and independent mechanisms

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5.1 Abstract

We studied the transcriptional regulation of the HL gene by USF1 and USF2 in HepG2 cells. The transcriptional activity of the HL(-685/+13) promoter construct was increased up to 25-fold by co-transfection with USF1 and USF2. Silencing of USF1 by RNA interference reduced promoter activity by 30-40%. Chromatin immunoprecipitation assays showed binding of endogenous USF1 and USF2 to the proximal HL promoter region. In gel shift assays, USF1 and USF2 bound to E-boxes at -307/-312 and -510/-516, and to the TATA-Inr region. Although the -514C→T substitution abolished *in vitro* USF binding to the -510/-516 E-box, the increase in HL promoter activity by USF1 and USF2 was unaffected. Deletion and mutation analysis of the HL promoter region, and insertion of multiple E-box copies in front of a heterologous promoter, revealed that upregulation by USFs was mainly mediated through the -307/-312 E-box and the TATA-Inr region. We conclude that in HepG2 cells USF1 and USF2 regulate transcriptional activity of the HL gene through their binding to the E-box at -307/-312 and the TATA-Inr region.

5.2 Introduction

Hepatic lipase (HL; E.C. 3.1.1.3) is an extracellular protein with phospholipase A_1 and triacylglycerol hydrolase activity that is almost exclusively synthesized and secreted by the liver (reviewed by [1,2]). HL plays an important role in plasma lipoprotein metabolism. The enzyme facilitates the delivery of HDL cholesterol to the liver parenchymal cells, and is involved in the formation of small-dense LDL from TG-rich VLDL and IDL, and the clearance of chylomicron remnants by the liver [3]. In several human association studies and genetically modified animal models, a high expression of HL has been shown to protect against the development of atherosclerosis. In other studies, however, HL has been shown to be pro-atherogenic (reviewed in [3]). Whether high HL expression is pro- or anti-atherogenic may depend on other genetic or metabolic factors, such as concomitant hypertriglyceridemia [3,4]. HL expression is increased in central obesity with insulin resistance [5] and in type 2 diabetes [6,7]. The HL gene contributes to the atherogenic lipoprotein profile (high plasma triglycerides, low HDL cholesterol, reduced LDL particle size) in familial combined hyperlipidemia (FCH) [8]. A high HL expression is associated with low HDL cholesterol and reduced LDL particle size [9]. The common HL promoter polymorphism is associated with dyslipidemia and insulin resistance in healthy controls and in FCH [10]. How HL gene expression is altered in insulin resistant conditions is unknown. In cell culture experiments using human HepG2 hepatoma cells, HL expression was found to be increased by fatty acids [11,12] and glucose [13,14], conditions that prevail in insulin resistance. How these metabolic factors affect HL expression is largely unknown.

We recently showed that expression of the HL gene in HepG2 cells is strongly upregulated by Upstream Stimulatory Factor USF1 [12,15]. USF1 as well as USF2 have been implicated in the coordinate regulation of glucose and lipid metabolism [16,17]. USFs activate expression of responsive genes through binding as homo- or heterodimers at E-box elements in their proximal promoter regions. Besides hepatic lipase, USFs have been shown to regulate expression of several other lipid and lipoprotein genes in mammalian liver, such as fatty acid synthase (FAS) [18], apolipoprotein (apo)A2 [19], apoCIII [20], apoA5 [21] and apoE [22]. In USF1 and USF2 knockout mice, glucose homeostasis is severely impaired [18,23], and glucokinase [24], the glucagon receptor [25] and insulin-like growth factor-binding protein 1 [26] are among the liver genes that are directly regulated by USFs. The human *USF1* gene lies on chromosome 1q in a region that is strongly linked to type 2 diabetes, although the USF1 gene itself seems not to be a major contributor to type 2 diabetes susceptibility [27]. Recently, the *USF1* gene has been linked to the increased plasma cholesterol and triglycerides levels in familial combined hyperlipidaemia [28,29]. Hence, upregulation of the HL gene by USFs may be (patho)physiologically important.

In a previous study, we postulated that USF1 regulates HL gene expression through the non-canonical E-box at -510/-516 of the HL gene [15]. This region contains the common -514C/T promoter polymorphism. The -514C→T substitution within the proximal promoter

region of the human HL gene is associated with a 50% decrease in HL expression [30]. This polymorphism was shown to interfere with USF1 binding *in vitro* [15]. Despite the loss of USF binding capacity, however, promoter activity of the -514T and C alleles were similarly upregulated by USF1 over-expression [15]. Therefore, we decided to study the mechanism by which USFs upregulate HL gene transcription in further detail. Our results show that USF1 and USF2 increase transcriptional activity of the proximal HL promoter through binding to a canonical E-box at -307/-312, as well as through a non-E-box sequence in the TATA/Inr region.

5.3 Materials and methods

5.3.1 Cell Culture

Hepatoma HepG2 cells and COS-1 kidney cells (both from American Type Culture Collection, Rockville, MD, USA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO-BRL, Breda, Netherlands), supplemented with 10% foetal calf serum (FCS), 50 IU/ml penicillin and 50 µg/ml streptomycin. The cells were kept at 37°C in a humidified atmosphere of 5% CO_2 in air. Twenty-four hours before the start of the experiment, the cells were plated at 30% confluence in 6- or 24-well culture plates (Nunc, Roskilde, Denmark).

5.3.2 Plasmids

Recombinant DNA techniques were performed according to standard procedures [31]. All oligonucleotides were custom-made by Sigma (Cambridge, UK). Enzymes used were purchased from Roche (Basel, Switzerland). All inserts were verified by DNA sequencing (BaseClear, Leiden, Netherlands).

5.3.2.1 Luciferase reporter constructs based on the human HL(-685/+13) region

A series of 5'-deletion fragments were generated by PCR, starting from the HL(-685/+13)-luc parent plasmid (further named HL-685) described previously [12,32]. All inserts contained the same 3'-end (+13 relative to the transcription start site identified by [33]). Unless otherwise stated, these constructs correspond to the common LIPC C-allele with a C at position -514 and a G at position -250. When indicated, a HL-685 construct corresponding to the T-allele (-514T; -250A) was used [12]. In the HL-685 reporter construct, the E-box at -312/-307 (the -310E-box) was mutated into an *Nhe*I site in two parallel PCR reactions. In the first reaction, the 3'-half of the HL-685/-13 region was amplified using 5'-GCA TGC TAG CGA AGC CAC CTA CCC CG-3' as mutagenic primer in combination with a vector-specific primer. After digestion of the product with *Nhe*I and *Xba*I, the insert was cloned into the *Nhe*I site of pGL3-basic, thus generating HL-305. In the second reaction, the 5'-half was amplified using using 5'-GCA TGC TAG CGC TGC CTA AGC CTC CC-3' as mutagenic primer in combination with a vector-specific primer. After digestion with *Sac*I and *Nhe*I, the fragment was inserted into the corresponding sites of HL-305

construct, thus generating the HL–685Em construct. Similarly, the E-box at -510/-516 in the HL-685 construct was mutated into a *Nhe*I site, thus generating the HL-514Em construct.

5.3.2.2 TK-luc plasmids containing three copies of human HL E-boxes

Three copies of wild type or mutated HL -514 or -310 E-boxes, including 12 bp flanking sequence on either side, were inserted in antisense orientation in front of the heterologous TK promoter in the TKpGL3BKO reporter vector (a TKpGL3 vector in which the endogenous *Bam*HI site had been removed; a kind gift from Dr. B. Staels, Lille, France) using double stranded oligonucleotides with *Bgl*II and *Bam*HI overhangs. The following oligonucleotide pairs were used (wildtype and mutated E-boxes are underligned; HL specific sequences are indicated in capital letters): -514C: 5'-gat ccA GCT CCT TTT GAC A**C**G GGG GTG AAG GGT TTA-3' and 5'-gat ctA AAC CCT TCA CCC CC**G** TGT CAA AAG GAG CTG-3'; -514T: 5'-gat ccA GCT CCT TTT GAC A**T**G GGG GTG AAG GGT TTA-3' and 5'-gat ctA AAC CCT TCA CCC CC**A** TGT CAA AAG GAG CTG-3'; -310E: 5'-gat ccG GCT TAG GCA GCC ACG TGG AAG CCA CCT ACA-3' and 5'-gat ctG TAG GTG GCT TCC ACG TGG CTG CCT AAG CCG-3'; -310Em: 5'-gat ccG GCT TAG GCA GCG CTA GCG AAG CCA CCT ACA-3' and 5'-gat ctG TAG GTG GCT TCG CTA GCG CTG CCT AAG CCG-3'. The oligonucleotides were phosphorylated in a polynucleotide kinase reaction and then annealed pair-wise by incubation at 95°C for 5 min, followed by 15 min at 65°C, 15 min at 37°C and 15 min at room temperature. Concatemers were generated by ligation with T4 DNA ligase in the presence of *Bam*HI and *Bgl*II for 1 hour at 37°C, followed by ligation into the *Bgl*II site of TKpGL3BKO in the presence of *Bam*HI. The copy number and orientation of the E-boxes in the resulting plasmids were determined by restriction analysis with *Hind*III and *Xho*I.

5.3.2.3 HL promoter constructs in pLuc-MCS

In the HL(-13/+13) construct, the Inr region of the human HL gene was inserted into the *Sac*I site of pLuc-MCS (Stratagene, La Jolla, CA, USA) between the TATA box and the reporter gene, using the double-stranded oligonucleotides 5'-gTC TCT TTG GCT TCA GAA ATT ACC AAG aag ct-3' and 5'-tCT TGG TAA TTT CTG AAG CCA AAG AGA cag ct-3'. In HL(-36/-13), the TATA box of the pLuc-MCS vector was substituted by the TATA-box of the human HL gene using the *Bgl*II and *Sac*I sites and the double-stranded oligonucleotides 5'-gat caA AGT ATC ATC TAA TAG GCA TTG Tga gct-3' and 5'-cAC AAT GCC TAT TAG ATT AGA TAC TTt-3'. Similarly, the -36/+13 region of the human HL promoter was inserted into the *Bgl*II/*Sac*I sites of pLuc-MCS, thus generating the HL(-36/+13) reporter construct. Finally, HL(-685/-49) reporter constructs were made in pLuc-MCS using the *Bgl*II site and PCR with appropriately designed primers bearing an extra *Bgl*II site.

5.3.2.4 Other plasmids

The expression vector pcDNA3.1 and control vector pRL-SV40 were obtained from Invitrogen (Breda, Netherlands) and Promega (Leiden, Netherlands), respectively. The expression vector pUSF1, which contains human USF1 cDNA in pcDNA3.1, was kindly provided by Dr. B. Staels

(Lille, France). To generate an expression vector for human USF2, we amplified USF2 cDNA from human liver RNA by RT-PCR, using the oligonucleotides 5′-gcg aat tcC ATG GAC ATG CTG GAC-3′ and 5′-gct cta gaG CGT GGT GGT GGC GG-3′ as forward and reverse primers, respectively. After digestion with *Eco*RI and *Xba*I, the PCR product was inserted into the corresponding sites of pcDNA3.1. From pUSF1 and pUSF2, Δb-mutants were generated by deleting the 42bp fragment that codes for the basic region (aa 203-216) that constitutes the DNA binding domain [34]. Δb-USF1 was made with the Transformer Site-Directed Mutagenesis Kit (Clontech Laboratories, Mountain View, CA, USA), according to manufacturers instructions, using the mutagenic primer 5′-GGA CGA CTC GGG ATG AGC G*A GAC AAG ATC AAC AAC TGG-3′ (* indicates the position of the 42 residues to be removed). Δb-USF2 was generated by the PCR overlay technique [35] using the USF2-specific primers described above, and both the forward and reverse primers of the sequence 5′-GAA CAC CCC GAG ATG AG* GAC AAG ATC AAC AAC TGG-3′. After digestion with *Eco*RI and *Xba*I, the PCR product was inserted into pcDNA3.1.

5.3.3 Promoter reporter assays

Promoter reporter assays were performed in transiently transfected HepG2 cells, as described previously [32]. Luciferase reporter constructs (0.4 µg/well) were co-transfected with 15 ng/well pRL-SV40 and the indicated amounts of USF expression vectors, or pcDNA3.1. After 48 hours, cell extracts were made (Roche, Basel, Switzerland), and firefly and renilla luciferase activities were determined with the Fire Light kit and the Packard Top Count NXT Luminometer (Perkin-Elmer, Boston MA, USA). In 30 independent experiments with HL-685, luciferase and renilla counts were 85,000±79,000 cps and 478,000±394,000 cps, respectively. Promoter activities were expressed as the ratio between firefly and renilla counts (0.14±0.12; n=30 for HL-685).

5.3.4 Over-expression of USF1 and USF2

HepG2 cells and COS-1 cells were cultured in 6-wells plates and transfected with the indicated amounts of pUSF1 or pUSF2 expression vectors using Lipofectamine Plus (Invitrogen, Breda, Netherlands). At 48 h post-transfection, nuclear extracts were prepared from the HepG2 cells according to Schreiber *et al.* [36]. Whole-cell extracts were prepared from the COS-1 cells in 20 mM Hepes-KOH (pH 7.6), 400 mM KCl, 1 mM EDTA, 10 mM DTT, 1 mM PMSF and 10 % glycerol by three cycles of freeze-thawing. The amount of protein in each extract was determined with the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, USA), using bovine serum albumin as standard. Extracts (20 µg) were electrophoresed on denaturing 10% SDS/polyacrylamide gels [37], and the separated proteins were transferred to a nitrocellulose membrane (Protean, Schleicher & Schuell, Düsseldorf, Germany). The membrane was incubated for 1h with rabbit polyclonal IgG antibodies (1:4000) against human USF1 or USF2 (SC-229X and SC-862X; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 0.5% milk powder/0.05% Tween-20 in Tris-buffered saline (TBS), followed by 1h incubation with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amerham Biosciences, UK), diluted 1:5000 in TBS. Subsequently, the secondary antibody was visualised by enhanced chemiluminescence (Super Signal West

Pico Chemiluminescent Substrate, Pierce, Rockford, IL, USA) and exposure to Hyper ECL film (Amerham Biosciences, UK).

USF1 and USF2 proteins were also synthesized *in vitro* using the TnT T7 Quick Coupled Transcription/Translation System (Promega) with pUSF1 or pUSF2 as DNA template according to the manufacturer's instructions.

5.3.5 RNA Interference Analysis

RNA silencing of the USF1 and USF2 gene was performed by transient transfection with pSilencer 3.1-H1 hygro siRNA expression vector (Ambion, Austin TX, USA) that generate short-hairpin RNA molecules (shRNA). The sequences of the shRNA molecules directed against USF1 and USF2 partially or completely overlapped with the targeting sites used by McMurray & McCance [38], respectively. The sequences of the shRNA molecules were 5'-GGT GGG ATT CTA TCC AAA GCT TCA AGA GAG CTT TGG ATA GAA TCC CAC CTT TTT T-3' and 5' GGA GAT ACT ACG GCT GTG TCC AAG CTT GGA CAC AGC CGT AGT ATC TCC TTT TT-3' for USF1 and USF2, respectively. As controls, parallel transfections were performed with empty pSilencer vector, and with shAQP7 directed against non-related AQP7 gene (a kind gift from B. Tilly, Dept. Biochemistry, ErasmusMC, Rotterdam).

5.3.6 Quantification of HL mRNA

Total RNA was isolated from the HepG2 cells, and HL mRNA was determined by reverse-transcription followed by real-time quantitative PCR analysis (RT-qPCR) using acidic ribosomal phosphoprotein PO (36B4) mRNA as reference, as described previously [14].

5.3.7 Electrophoretic mobility shift assays

Gel shift assays were performed with the Lightshift Chemiluminescent EMSA kit from Pierce. The -310E and -310Em oligonucleotides correspond to the -324/-294 region of the HL promoter. The AdML and Ad-Inr probes contain the USF-consensus binding site (5'-GTG TAG GCC ACG TGA CCG GGT GTA AGC TTC-3', [39]) and the Inr region (5'-GCG TTC GTC CTC ACT CTC TTC CGC ATC GTG-3'; [40]) of the adenovirus major late promoter, respectively. When indicated, competitor oligonucleotides or antibodies (1µl) were added to the reaction mixture 10 min before the probe. Reaction mixtures were separated on non-denaturating 6% polyacrylamide gels. The DNA in the gel was transferred to Biodyne B nylon membranes (Pierce), the DNA was UV-crosslinked to the membrane, and the biotin-label was visualized with the Chemiluminescent Nucleic Acid Detection kit (Pierce).

5.3.8 Chromatin immunoprecipitation assays

These assays were performed as described previously [14] using anti-USF1 or anti-USF2 antibodies. As negative control (mock), a chromatin fraction was incubated in parallel without antibody. Different parts of the HL gene were amplified by PCR (30 s 95°C, 30 s 55°C, 45 s 72°C; 30 cycles) using 4 out of 60 µl immunoprecipitate and appropriate sets of HL specific

primers. The PCR products were resolved by 3% agarose-gel electrophoresis and visualized by ethidiumbromide staining. The images were quantified by densitometry using the GS-800 Calibrated Densitometer from Bio-Rad.

5.3.9 USF1 knockout mice

Livers from homozygote and heterozygote USF1-knockout mice [23] and wildtype litter mates were collected in liquid N_2. For each genotype, three 4-month-old animals (two females, one male) were used. Total RNA was isolated with TRI reagent (Molecular Research Center Inc., Cincinnati, Ohio, USA). HL mRNA in each liver sample was determined in triplicate by RT-qPCR using β-actin mRNA as reference. HL-specific primers used were 5'-GAC TGG ATC TCC CTG GCA TA-3' and 5'-AGG TGA ACT TTG CTC CGA GA-3'. β-actin-specific primers were 5'-AGG CCC AGA GCA AGA GAG G-3' and 5'-TAC ATG GCT GGG GTG TTG AA-3'.

5.3.10 Statistics

Data are given as mean ±SD. Statistical analyses were performed with Stata/SE 8.2 for Windows (StataCorp LP, Texas, USA) using Student's *t*-test. Statistical significance was defined as $p<0.05$.

5.4 Results

5.4.1 USF1 and USF2 upregulate HL promoter activity

pUSF1 and pUSF2 both upregulated the transcriptional activity of the HL-685 promoter construct dose-dependently (Figure 1). Maximal activation was 25-fold with pUSF1, and 12-fold with pUSF2. Both dose-response curves were biphasic, with a first phase up to 10 ng plasmid, where a 6-fold and 3-fold increase in HL promoter activity was obtained, respectively. Transfection with pUSF1 and pUSF2 also induced a dose-dependent increase in USF1 and USF2 protein level, respectively. Silencing of the USF1 and USF2 gene markedly reduced the amount of USF1 and USF2 protein in nuclear extracts, respectively (Figure 2A). Promoter activity of the HL–685 luc construct was significantly reduced by co-transfection with shUSF1 or shUSF2, but not with non-related shAQP7 (Figure 2B).

In cultures of HepG2 cells transfected with 100 ng pUSF1, HL mRNA level was 1.25±0.05-fold higher than in mock-transfected cells ($p<0.01$; $n=3$). Compared to pSilencer, HL mRNA level in shUSF1- and siAQP 7 transfected cell cultures was reduced to 0.71±0.11-fold ($p<0.05$; $n=3$) and 1.10±0.29-fold (n.s.), respectively. Assuming a transfection efficiency of 30 % (van Deursen, unpublished), this would indicate a doubling of HL mRNA level, and an almost complete loss of HL mRNA in cells transfected with USF1 and shUSF1, respectively.

Since transfection of HepG2 cells with 10 ng pUSF1 or pUSF2 gave moderate over-expression of USF1 and USF2 proteins, respectively, this dose of expression plasmid was chosen for further transfection studies.

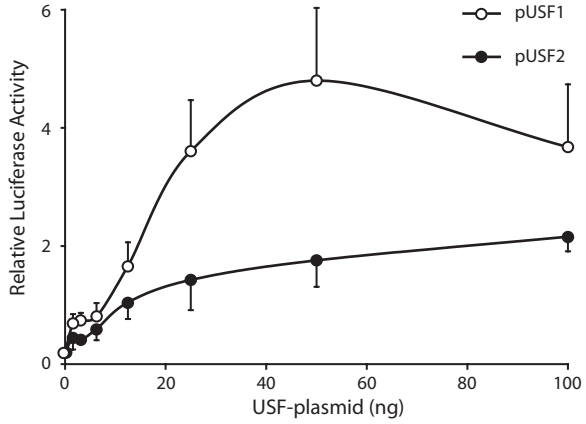

Figure 1. USF1 and USF2 strongly up-regulate HL promoter activity.

HepG2 cells were co-transfected with HL–685 and the indicated amounts of pUSF1 or pUSF2 (open and closed symbols, respectively), and HL promoter activity was determined. Data are mean ±SD for three independent experiments.

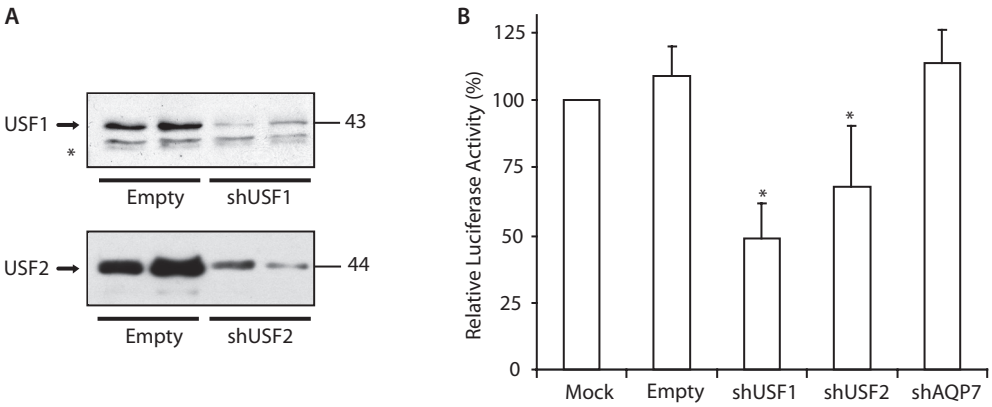

Figure 2. Silencing of the USF1- and USF2-gene reduces HL promoter activity.

HepG2 cells were co-transfected with 10 ng HL–685 and 100 ng of the indicated shRNA construct. Panel **A** shows the effect of shUSF1 on USF1 protein and of shUSF2 on USF2 protein (upper and lower panel, respectively) in HepG2 nuclear extracts (NE; 25 mg). The asterisk indicates a non-specific band. In panel **B**, HL promoter activity was determined in cells that were co-transfected without (mock), or with 100 ng of the indicated sh construct. Luciferase data were expressed as percentage of the activity with mock transfection. Data are mean ± SD for 5 independent experiments. * indicates statistical significant difference of sh construct versus mock.

5.4.2 Role of DNA binding domain of USF1 and USF2

When transfected with Δb-USF1 or Δb-USF2 plasmids, HepG2 cells express mutant USF proteins that were slightly smaller than wildtype USF1 or USF2, respectively (Figure 3A). In gel shift assays, the mutant proteins did not bind to the -310 E-box of the human HL promoter (Figure 3B), nor to the AdML probe containing a USF consensus binding site (not shown). The mutant USF plasmids showed no effect on the luciferase activity of the pHL-685 construct (Fig. 3C). When co-transfected, the effect of pUSF1 as well as pUSF2 was almost completely abolished with Δb-USF1 (figure 3C) as well as by Δb-USF2 (not shown). These observations are in line with the hypothesis that USF1 and USF2 form heterodimers [41]. Taken together, these data indicate that activation of the HL-685 promoter construct by USF1 and USF2 requires their binding to DNA.

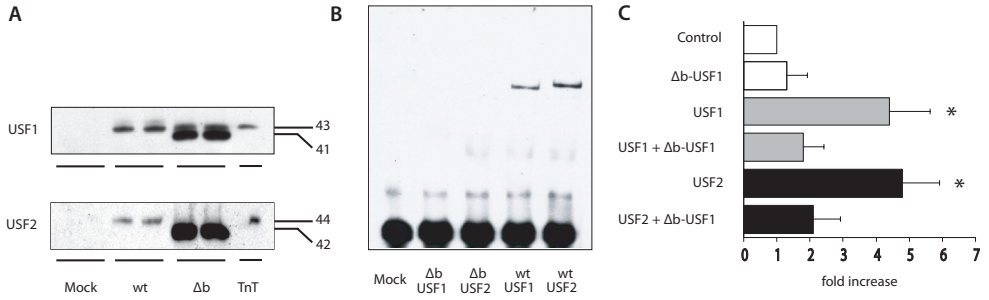

Figure 3. Up-regulation of HL promoter activity by USF requires DNA binding.

In **A**, cells were transfected without (mock), or with 10 ng wildtype (wt) or 100 ng Δb-mutant (Δb) of pUSF1 (upper panel) or pUSF2 (lower panel), and nuclear extracts (20 µg) were analysed by Western blotting. TnT extracts (0.5 µl) with *in vitro* made USF1 or USF2 protein were run in parallel (TnT). The molecular sizes are indicated in kDa. Note that USF1 and Δb USF1 protein appear as doublets, which probably reflect differentially phosphorylated forms [47]. In **B**, gel shift assays were performed with the AdML probe and 4 µg of nuclear extracts, prepared from the HepG2 cells transfected with 100 ng of the indicated expression plasmid. In **C**, HepG2 cells were co-transfected with HL-685 and pUSF1 (10 ng), pUSF2 (10 ng) and Δb-USF1 (100 ng), and the effect on HL promoter activity was determined. Data are expressed as fold increase with HL promoter activity in control cells set at 1.0. Data are mean ±SD for 4 independent experiments; *: $p<0.05$ *vs* control.

5.4.3 Gel shift assays using USF and E-boxes from the HL promoter

Screening of the proximal HL promoter region for potential USF binding sites identified a non-canonical E-box at -510/-516 (-514 E-box; ..CACGGG..) and a canonical E-box at -307/-312 (-310E-box; ..CACGTG..). The former E-box was no longer recognized in the proximal HL promoter region corresponding to the -514T allele (..CATGGG..). We have previously shown that USF1 binds to -514C but not -514T oligonucleotides [15]. Similar results were obtained with USF2 (data not shown). To test the binding of USF1 and USF2 to the -310 E-box, we used nuclear extracts of COS cells over-expressing USF1 or USF2. Both nuclear extracts produced a mobility shift with the -310E probe similar to the AdML probe (Figure 4). Unlabeled -310E

but not -310Em oligonucleotides, competed with the labeled -310 E-probe for USF1 as well as USF2 binding. In shift assays with nuclear extracts of COS cells that were co-transfected with pUSF1 and pUSF2, an almost complete supershift was observed with either anti-USF1 or anti-USF2 alone. These data suggest that USFs bind at the -310 E-box as homo- and heterodimers.

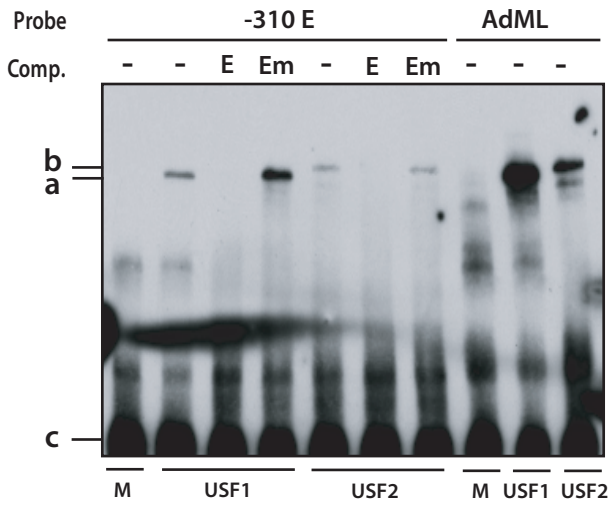

Figure 4. **Gel shift assays using USF and the -310 E-box of the HL promoter region.**

Gel shifts were performed with the indicated probes, without (–) or with a 50-fold excess of the indicated competitors (comp.). Whole cell extracts (20 µg) of COS cells transfected with pUSF1 or pUSF2 were used. Extracts from mock-transfected COS cells served as negative control (M). The positions of the USF1 shifted band, the USF2 shifted band, and unbound oligonucleotides, are indicated as a, b and c, respectively.

5.4.4 5'-deletions of the HL (-685/+13) promoter

To identify which part of the HL promoter region is responsible for the upregulation by USF, we generated 5' deletions of the HL-685 promoter construct. As shown in figure 5, the HL-325 construct was still upregulated 5-fold by pUSF1 and 2.5-fold by pUSF2 over-expression. With the HL-305 construct, the upregulating effect of pUSF1, and to a lesser extent pUSF2, was significantly reduced. This suggests that the -310 E-box is important for the upregulation of the HL promoter by USF1 as well as USF2. However, the HL-79 and HL-36 constructs were also significantly upregulated by USF1 and USF2. The transcriptional activity of the HL-36 construct, which contains the TATA-box and Inr, was only 41±17 % (n=3) of the full-length construct, but this activity was still increased 3-fold upon co-transfection with pUSF1 and 2-fold with pUSF2 (Figure 5). Apparently, some responsiveness to USF1 and USF2 also resides in the -36/+13 region of the human HL promoter.

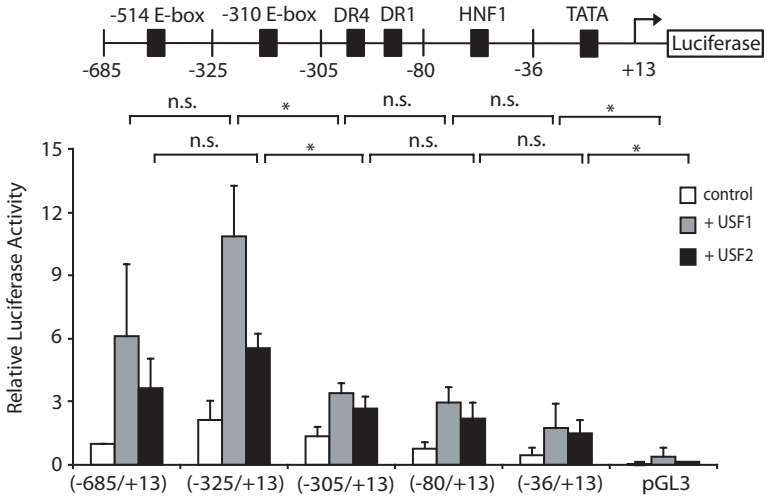

Figure 5. Effect of 5'-deletions on upregulation of the HL-685 by USF.

A series of 5'-deletions of the HL-685 construct in pGL3-basic was made. The upper panel schematically shows the deletions relative to the locations of potential regulatory elements. HepG2 cells were co-transfected with these promoter-reporter constructs without (open bars), or with 10 ng pUSF1 (grey bars) or 10 ng pUSF2 (black bars). The activity of HL–685 construct in control HepG2 cells was taken as 1.0 (n.s.: non-significant; *: $p<0.05$). Data are mean±sd for 3-6 independent experiments.

5.4.5 Role of the E-boxes in the up-regulation of the HL promoter by USF.

Three copies of the region containing either the -514 or the -310 E-box were cloned upstream of a heterologous TK promoter. Similar constructs were made with mutated -514 E-boxes (-514C→T) or scrambled –310 E-boxes (Em). In control HepG2 cells, activity of the TK promoter was increased 1.95±0.15-fold ($p<0.01$; n=3) by insertion of the -310 E-box, but not the -310 Em box or the -514 E-boxes. The activity of the $TK(-514T)_3$ construct was similar to the corresponding $TK(-514C)_3$ construct (106±60%; n=6; p=0.80 by paired *t*-test), whereas the activity of the $TK(–310Em)_3$ construct was reduced to 56±11% (n=4; p=0.004 by paired *t*-test) relative to the $TK(-310E)_3$ construct. As shown in figure 6A, co-transfection with pUSF1 or pUSF2 failed to increase luciferase expression of $TK(-514C)_3$, $TK(-514T)_3$ and $TK(-310Em)_3$. In contrast, the activity of $TK(-310E)_3$ was increased 6-fold by USF1 and USF2 over-expression.

Next, we mutated the E-boxes in the HL(-685/+13) construct. The -514C→T substitution slightly but not significantly reduced the transactivation by USF1 [14] and USF2 (not shown). Similarly, scrambling of the -514E box did not significantly reduce transactivation by USF1 (from 7.1±0.8 to 5.7±1.5-fold; p=0.15; n=4). Scrambling of the -310 E-box significantly reduced the responsiveness to USF1 and USF2 from 5-6 fold to 2.5-3-fold (p=0.004 and 0.0002, respectively; n=4; Figure 6B). Double mutants showed similar responsiveness to pUSF1 and pUSF2 over-expression as the HL(-310Em) construct (not shown).

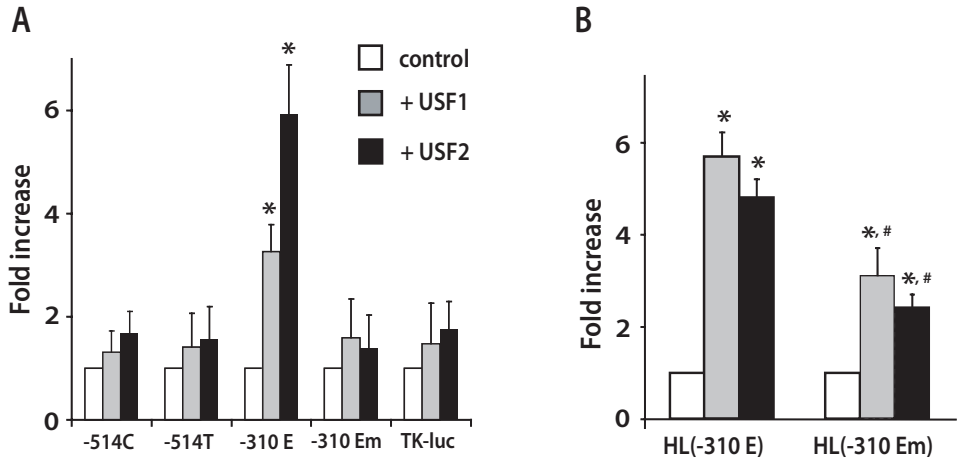

Figure 6. Effect of the E-boxes on USF responsiveness.

In **A**, three copies of the wild type (-514C or -310E) or mutant (-514T or -310Em) boxes were inserted in front of the heterologous TK promoter. In **B**, the E-boxes within the HL–685 construct have been mutated. HepG2 cells were co-transfected with these constructs without, or with 10 ng pUSF1 or pUSF2 (open, gray and black bars, respectively), and transcriptional activity was determined. Data are expressed relative to transcriptional activity of each construct in control cells. Data are mean ±SD for three independent experiments. * and #: statistically significant difference from control cells and from the wildtype construct, respectively.

Finally, the -685/-49 region of the HL gene was cloned in front of a heterologous TATA-Inr region of the pLuc-MCS vector. Co-transfection with 10 ng pUSF2 resulted in a 3.6±1.6-fold increase in luc activity (n=8). This activity was reduced to 82±7% thereof ($p<0.05$ by paired *t*-test, n=4) when the -514 E-box was mutated (-514C>T). When the –310 E box was scrambled, activity in USF2 over-expressing cells was reduced to 36±19 % ($p<0.01$; n=7), and to 34±10% in the double mutants ($p<0.005$, n=6), similar to the empty vector (29±10 %; n=3). Qualitatively similar results were obtained with pUSF1.

5.4.6 Activation of the HL promoter by USF via the TATA-Inr region

USFs have also been reported to activate gene expression via Inr-like sequences in the AdML promoter (Ad-Inr) and other genes. As shown in figure 7A, purified USF1 produced a band shift with the Ad-Inr probe. In competition experiments, the gel shift was completely abolished by unlabeled double-stranded oligonucleotides of the HL(-36/+13) region, and markedly reduced with HL(-36/-13) and HL(-13/+13) oligonucleotides containing the HL TATA box and Inr, respectively. These data suggest that USF1 can bind to the HL(-36/+13) region of the HL promoter.

To investigate the effect of USF over-expression, both the HL-TATA and Inr region were swapped with similar regions from the pLuc-MCS vector. Substitution of the vectors TATA-box

for the HL(-36/+13) region resulted in a construct that was 3- and 6-fold up-regulated by co-transfection with pUSF1 and pUSF2, respectively (Figure 7B). Substitution for the HL(-36/–13) region containing only the TATA box did not significantly affect responsiveness to pUSF1 and pUSF2. Combining the -13/+13 region containing the HL Inr with the heterologous TATA box from pLuc-MCS almost completely abolished responsiveness to pUSF1 over-expression, but transcriptional activity was still upregulated 2.5 fold by over-expression of USF2. Taken together, part of the effect of USF on HL promoter activity may be mediated through binding at the TATA-Inr region.

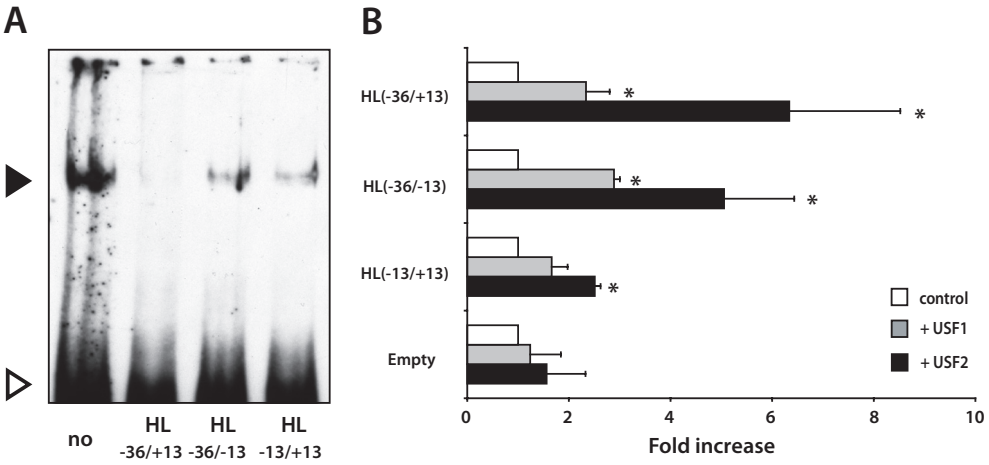

Figure 7. E-box independent upregulation by USF through the TATA/Inr-region.

In **A**, gel shifts were performed with 100 ng of purified USF1 protein (ProteinOne, Bethesda, MD, USA) and biotinylated Ad-Inr, with or without competitors in 100-fold excess. The closed and open arrowheads point to the position of the bound and unbound probes, respectively. In **B**, the HL-TATA and Inr region were swapped with similar regions from the pLuc-MCS vector (Stratagene), and the effect on luciferase activity was determined. The effect of USF1 and USF2 over-expression on luciferase activity is shown (gray and black bars, respectively). Transcriptional activity of each construct in control cells was taken as 1.0 (open bars). Data are mean±sd from one experiment performed in quadruplicate (*: $p<0.05$, from control), which is representative of two similar experiments.

5.4.7 Chromatin immunoprecipitation assays

From the DNA immunoprecipitated with anti-USF1 and anti-USF2, the region between -674 to + 233 of the HL gene was readily amplified (Figure 8). No PCR product was obtained for the HL gene further upstream or downstream, and hence the proximal HL promoter region has been specifically immunoprecipitated. These data indicate that in HepG2 cells endogenous USF1 and USF2 are bound to the proximal HL promoter region, encompassing the E-boxes and the TATA/Inr region. The strongest signals were obtained for the -480/-369 region, which is midway between the two E-boxes. In comparison, the signals for the -674/-571 region were

rather low. This observation suggests, but does not proof, that endogenous USFs are bound to the -310 E-box rather than the -514 E-box.

5.4.8 Liver HL mRNA expression in USF1 knockout mice.

To test whether USFs are involved in the *in vivo* regulation of HL expression, we determined HL mRNA levels in livers of adult USF1 knockout mice by RT-qPCR. After normalization for β-actin, HL mRNA levels were 1.17±0.15, 1.20±0.22 and 1.04±0.30 (arbitrary units, n=3) in USF1-/-, +/- and +/+ mice, respectively. Hence, in mice basal HL expression appears not to be affected by endogenous USF1.

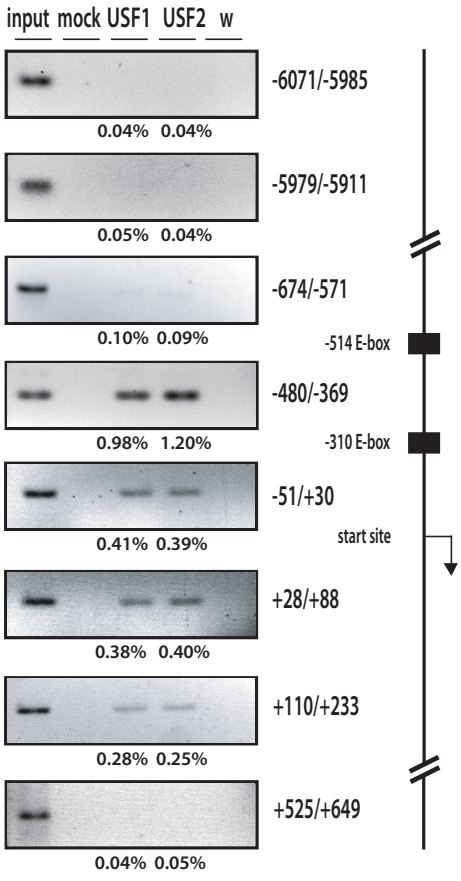

Figure 8. Chromatin immunoprecipitation with anti-USF1 and anti-USF2.

Chromatin was isolated from HepG2 cells, and the indicated regions of the HL gene were amplified by PCR from DNA immunoprecipitated with anti-USF1 or anti-USF2. As positive and negative controls, 1% input DNA and no-antibody precipitation (mock) were included, respectively; w depicts the PCR water control. Signal intensities were expressed as percentage of the input, and are given underneath the PCR bands.

5.5 Discussion

Interest in this topic originated from our previous observation that the common -514C/T polymorphism which results in a 50% loss of *in vivo* HL expression [30] and a similar reduction of *in vitro* HL promoter activity [15,42], affects a potential USF binding site, but despite this, both alleles were similarly up-regulated by over-expression of USF1 [15]. In this study we confirm that this E-box has little effect on transcriptional activity in HepG2 cells. Instead, we report here that USF1 and USF2 also bind to a canonical E-box at -312/-307 as well as to the TATA-Inr region of the HL promoter *in vitro*. Our ChIP analysis suggests that both USF proteins are also bound to this region in the chromatin context of HepG2 cells. In addition, these two sites convey sensitivity of the HL promoter as well as a heterologous TK promoter to USF1 and USF2 over-expression. Mutated USF proteins that lack DNA binding ability are ineffective, suggesting that USFs mediate their effect through binding at the -310 E-box and the TATA-Inr region. The -514E box is not conserved among mammals in the genomic database, whereas the -310E-box and flanking region is conserved among primates only (Ensembl, accessed July 2008). In line with this, knockout of the USF1 gene in the mouse did not affect basal HL expression in liver. Hence, regulation of HL expression by USFs may be restricted to primates. In rat liver, however, ChIP assays suggest that USF1 and USF2 proteins are bound to the proximal HL promoter region [14]. Alternatively, USFs may affect HL expression only under non-basal conditions, such as high extracellular glucose.

USFs are ubiquitous transcription factors that are involved in, among other, the coordinate regulation of lipid and glucose metabolism. How USFs are regulated by glucose, lipid or insulin status is not clear. In rat liver, USF1 is decreased by fasting, and increased by refeeding [17]. In cultured cells, high extracellular glucose levels enhance expression of USF1 and/or USF2 protein [43-46]. USF1 is also subject to regulation by various kinases [16,47], which all increase its DNA binding and transactivation activity, although the opposite effect has been reported for phosphorylation by the PI3-Kinase signaling pathway [21]. In HepG2 cells, insulin increases USF1 phosphorylation, which reduces expression of the ApoA5 gene [21]. There is little evidence that HL gene is directly regulated by insulin. Rather, HL expression is associated with insulin resistant states characterized by elevated plasma glucose, fatty acids and triglycerides [5-7]. In HepG2 cells, HL expression is upregulated by high glucose [13] and by fatty acids [11], conditions that are associated with increased levels of USF1 and USF2 proteins (van Deursen *et al*., submitted). Similar to the HL gene [8,10], the USF1 gene has been linked to familial combined hyperlipidaemia (FCHL) and metabolic syndrome traits [28,48]. The associated dyslipidemia (low HDL, high TG, increased small-dense LDL) may result from the increased transactivation by USFs not only of hepatic lipase, but also of other HDL- and TG related genes such as apoA2 [19], apoC-III [20], apoE [22,49] or hormone-sensitive lipase [46]. Increased USF1 transactivation of its target genes has been suggested to explain the development of the metabolic syndrome [48], the dyslipidemia associated with FCHL [28], and the development of diabetic complications [43,45]. Taken together, these observations suggest

that the association of the HL gene with insulin resistance and/or FCHL may be a consequence of the USF mediated regulation of HL expression.

Since USFs appear to affect HL gene expression only marginally through the -514E-box, the question arises what causes the reduction in HL expression due to the common LIPC promoter polymorphism. This polymorphism consists of four highly linked SNPs, of which the functionality has been attributed to the -514C→T and -250G→A substitutions [15,42]. Transcription factors other than USF may bind to the -514E-box. Several bHLH-Zip transcription factors bind to E-boxes, such as c-Myc, Max, Mad andTFE3. However, the gel shift observed with the AdML probe and HepG2 nuclear extract was completely abolished by the combination of anti-USF1 and anti-USF2, leaving little room for other E-box binding factors (data not shown). Therefore, it seems unlikely that a major effect on HL expression is mediated through E-box binding transcription factor other than USF through the -310 E-box, and by inference, through the polymorphic -514E-box. Alternatively, the functionality of the LIPC promoter polymorphism may reside in the -250G→A substitution, either alone or in combination with the -514C→T substitution. A search through the Transfac database did not reveal a candidate whose binding is affected by the -250G→A substitution. This SNP is adjacent to the DR1 site -238/-226 that recently has been shown important for transcriptional activation by HNF4 [50], and may possibly interfere with transactivation of HNF4.

USFs appear to regulate the expression of a number of genes involved in plasma lipoprotein metabolism. HL functions in the hydrolysis of triglycerides and phospholipids in HDL and IDL. An elevated HL expression by USF, hence in conditions of high glucose and fatty acids levels, may be important for the delivery of phospholipid to the liver cell necessary to maintain high VLDL secretion rates by the liver [3]. ApoE is a ligand for TG-rich lipoproteins, ApoA5 is an activator and apoCIII an inhibitor of lipoprotein lipase, and apoA2 is an activator of hepatic lipase. Whereas apoA5 expression is reduced by USF [21], expression of apoCIII [20], apoA2 [19,20] and apoE [22] are all upregulated by USF. This suggests that HL may be regulated coordinately with the apolipoproteins. For the apoCIII and apoA2 promoters, transcriptional activation through binding of USFs requires the simultaneous binding of HNF4 to an adjacent binding site [19,20]. Interestingly, HL gene expression is not only up-regulated by USF but also by HNF4 [50]. The -310E-box to which USFs bind is adjacent to the DR4 site (-302/-287) involved in the transactivation by HNF4. It is tempting to speculate that similar to the apoCIII and apoA2 genes, regulation of the HL gene is due to cooperative binding of USF and HNF4. Further studies are required to test this hypothesis.

Acknowledgments

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References

1. B. Perret, L. Mabile, L. Martinez, F. Terce, R. Barbaras, X. Collet, Hepatic lipase: structure/function relationship, synthesis, and regulation, *J Lipid Res* **43** (2002) 1163-1169.
2. S. Santamarina-Fojo, H. Gonzalez-Navarro, L. Freeman, E. Wagner, Z. Nong, Hepatic lipase, lipoprotein metabolism, and atherogenesis, *Arterioscler Thromb Vasc Biol* **24** (2004) 1750-1754.
3. H. Jansen, A.J. Verhoeven, E.J. Sijbrands, Hepatic lipase: a pro- or anti-atherogenic protein?, *J Lipid Res* **43** (2002) 1352-1362.
4. A. Zambon, J.E. Hokanson, B.G. Brown, J.D. Brunzell, Evidence for a new pathophysiological mechanism for coronary artery disease regression: hepatic lipase-mediated changes in LDL density, *Circulation* **99** (1999) 1959-1964.
5. M.C. Carr, J.E. Hokanson, S.S. Deeb, J.Q. Purnell, E.S. Mitchell, J.D. Brunzell, A hepatic lipase gene promoter polymorphism attenuates the increase in hepatic lipase activity with increasing intra-abdominal fat in women, *Arterioscler Thromb Vasc Biol* **19** (1999) 2701-2707.
6. K.C. Tan, S.W. Shiu, B.Y. Chu, Effects of gender, hepatic lipase gene polymorphism and type 2 diabetes mellitus on hepatic lipase activity in Chinese, *Atherosclerosis* **157** (2001) 233-239.
7. P. Berk, II, N. Hoogerbrugge, R.P. Stolk, A.H. Bootsma, H. Jansen, Atorvastatin dose-dependently decreases hepatic lipase activity in type 2 diabetes: effect of sex and the LIPC promoter variant, *Diabetes Care* **26** (2003) 427-432.
8. H. Allayee, K.M. Dominguez, B.E. Aouizerat, R.M. Krauss, J.I. Rotter, J. Lu, R.M. Cantor, T.W. de Bruin, A.J. Lusis, Contribution of the hepatic lipase gene to the atherogenic lipoprotein phenotype in familial combined hyperlipidemia, *J Lipid Res* **41** (2000) 245-252.
9. S.S. Deeb, A. Zambon, M.C. Carr, A.F. Ayyobi, J.D. Brunzell, Hepatic lipase and dyslipidemia: interactions among genetic variants, obesity, gender, and diet, *J Lipid Res* **44** (2003) 1279-1286.
10. J. Pihlajamaki, L. Karjalainen, P. Karhapaa, I. Vauhkonen, M.R. Taskinen, S.S. Deeb, M. Laakso, G-250A substitution in promoter of hepatic lipase gene is associated with dyslipidemia and insulin resistance in healthy control subjects and in members of families with familial combined hyperlipidemia, Arterioscler Thromb Vasc Biol 20 (2000) 1789-1795.
11. L. Nimmo, A.J. McColl, J.Z. Rosankiewicz, W. Richmond, R.S. Elkeles, Regulation of hepatic lipase expression in HepG2 cells, *Biochem Soc Trans* **25** (1997) S689.
12. G.J. Botma, D. van Deursen, D. Vieira, M. van Hoek, H. Jansen, A.J. Verhoeven, Sterol-regulatory-element binding protein inhibits upstream stimulatory factor-stimulated hepatic lipase gene expression, *Atherosclerosis* **179** (2005) 61-67.
13. A.Y. Tu, J.J. Albers, Glucose regulates the transcription of human genes relevant to HDL metabolism: responsive elements for peroxisome proliferator-activated receptor are involved in the regulation of phospholipid transfer protein, *Diabetes* **50** (2001) 1851-1856.
14. D. van Deursen, H. Jansen, A.J. Verhoeven, Glucose increases hepatic lipase expression in HepG2 liver cells through upregulation of upstream stimulatory factors 1 and 2, *Diabetologia* **51** (2008) 2078-2087.
15. G.J. Botma, A.J. Verhoeven, H. Jansen, Hepatic lipase promoter activity is reduced by the C-480T and G-216A substitutions present in the common LIPC gene variant, and is increased by Upstream Stimulatory Factor, *Atherosclerosis* **154** (2001) 625-632.
16. S. Corre, M.D. Galibert, Upstream stimulating factors: highly versatile stress-responsive transcription factors, *Pigment Cell Res* **18** (2005) 337-348.
17. D. Wang, H.S. Sul, Upstream stimulatory factors bind to insulin response sequence of the fatty acid synthase promoter. USF1 is regulated, *J Biol Chem* **270** (1995) 28716-28722.
18. M. Casado, V.S. Vallet, A. Kahn, S. Vaulont, Essential role in vivo of upstream stimulatory factors for a normal dietary response of the fatty acid synthase gene in the liver, *J Biol Chem* **274** (1999) 2009-2013.
19. A. Ribeiro, D. Pastier, D. Kardassis, J. Chambaz, P. Cardot, Cooperative binding of upstream stimulatory factor and hepatic nuclear factor 4 drives the transcription of the human apolipoprotein A-II gene, *J Biol Chem* **274** (1999) 1216-1225.

20. D. Pastier, J.M. Lacorte, J. Chambaz, P. Cardot, A. Ribeiro, Two initiator-like elements are required for the combined activation of the human apolipoprotein C-III promoter by upstream stimulatory factor and hepatic nuclear factor-4, *J Biol Chem* **277** (2002) 15199-15206.

21. M. Nowak, A. Helleboid-Chapman, H. Jakel, G. Martin, D. Duran-Sandoval, B. Staels, E.M. Rubin, L.A. Pennacchio, M.R. Taskinen, J. Fruchart-Najib, J.C. Fruchart, Insulin-mediated down-regulation of apolipoprotein A5 gene expression through the phosphatidylinositol 3-kinase pathway: role of upstream stimulatory factor, *Mol Cell Biol* **25** (2005) 1537-1548.

22. E. Salero, C. Gimenez, F. Zafra, Identification of a non-canonical E-box motif as a regulatory element in the proximal promoter region of the apolipoprotein E gene, *Biochem J* **370** (2003) 979-986.

23. V.S. Vallet, M. Casado, A.A. Henrion, D. Bucchini, M. Raymondjean, A. Kahn, S. Vaulont, Differential roles of upstream stimulatory factors 1 and 2 in the transcriptional response of liver genes to glucose, *J Biol Chem* **273** (1998) 20175-20179.

24. P.B. Iynedjian, Identification of upstream stimulatory factor as transcriptional activator of the liver promoter of the glucokinase gene, *Biochem J* **333** (Pt 3) (1998) 705-712.

25. L. Portois, M. Tastenoy, B. Viollet, M. Svoboda, Functional analysis of the glucose response element of the rat glucagon receptor gene in insulin-producing INS-1 cells, *Biochim Biophys Acta* **1574** (2002) 175-186.

26. T. Matsukawa, Y. Inoue, Y. Oishi, H. Kato, T. Noguchi, Up-regulation of upstream stimulatory factors by protein malnutrition and its possible role in regulation of the IGF-binding protein-1 gene, *Endocrinology* **142** (2001) 4643-4651.

27. E. Zeggini, C.M. Damcott, R.L. Hanson, M.A. Karim, N.W. Rayner, C.J. Groves, L.J. Baier, T.C. Hale, A.T. Hattersley, G.A. Hitman, S.E. Hunt, W.C. Knowler, B.D. Mitchell, M.C. Ng, J.R. O'Connell, T.I. Pollin, M. Vaxillaire, M. Walker, X. Wang, P. Whittaker, K. Xiang, W. Jia, J.C. Chan, P. Froguel, P. Deloukas, A.R. Shuldiner, S.C. Elbein, M.I. McCarthy, Variation within the gene encoding the upstream stimulatory factor 1 does not influence susceptibility to type 2 diabetes in samples from populations with replicated evidence of linkage to chromosome 1q, *Diabetes* **55** (2006) 2541-2548.

28. P. Pajukanta, H.E. Lilja, J.S. Sinsheimer, R.M. Cantor, A.J. Lusis, M. Gentile, X.J. Duan, A. Soro-Paavonen, J. Naukkarinen, J. Saarela, M. Laakso, C. Ehnholm, M.R. Taskinen, L. Peltonen, Familial combined hyperlipidemia is associated with upstream transcription factor 1 (USF1), *Nat Genet* **36** (2004) 371-376.

29. H. Coon, Y. Xin, P.N. Hopkins, R.M. Cawthon, S.J. Hasstedt, S.C. Hunt, Upstream stimulatory factor 1 associated with familial combined hyperlipidemia, LDL cholesterol, and triglycerides, *Hum Genet* **117** (2005) 444-451.

30. A. Isaacs, F.A. Sayed-Tabatabaei, O.T. Njajou, J.C. Witteman, C.M. van Duijn, The -514 C->T hepatic lipase promoter region polymorphism and plasma lipids: a meta-analysis, *J Clin Endocrinol Metab* **89** (2004) 3858-3863.

31. F.E. Sambrook J, Maniatis T, Molecular Cloning: a laboratory manual, 2nd Ed ed., Cold Spring Harbor Laboratory Press, New York, 1989.

32. D. van Deursen, G.J. Botma, H. Jansen, A.J. Verhoeven, Comparative genomics and experimental promoter analysis reveal functional liver-specific elements in mammalian hepatic lipase genes, *BMC Genomics* **8** (2007) 99.

33. D. Ameis, G. Stahnke, J. Kobayashi, J. McLean, G. Lee, M. Buscher, M.C. Schotz, H. Will, Isolation and characterization of the human hepatic lipase gene, *J Biol Chem* **265** (1990) 6552-6555.

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

35. R. Higuchi, B. Krummel, R.K. Saiki, A general method of in vitro preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions, *Nucleic Acids Res* **16** (1988) 7351-7367.

36. E. Schreiber, P. Matthias, M.M. Muller, W. Schaffner, Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells, *Nucleic Acids Res* **17** (1989) 6419.

37. U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature* **227** (1970) 680-685.

38. H.R. McMurray, D.J. McCance, Human papillomavirus type 16 E6 activates TERT gene transcription through induction of c-Myc and release of USF-mediated repression, *J Virol* **77** (2003) 9852-9861.

39. R.W. Carthew, L.A. Chodosh, P.A. Sharp, An RNA polymerase II transcription factor binds to an upstream element in the adenovirus major late promoter, *Cell* **43** (1985) 439-448.
40. H. Du, A.L. Roy, R.G. Roeder, Human transcription factor USF stimulates transcription through the initiator elements of the HIV-1 and the Ad-ML promoters, *Embo J* **12** (1993) 501-511.
41. A. Kiermaier, J.M. Gawn, L. Desbarats, R. Saffrich, W. Ansorge, P.J. Farrell, M. Eilers, G. Packham, DNA binding of USF is required for specific E-box dependent gene activation in vivo, *Oncogene* **18** (1999) 7200-7211.
42. S.S. Deeb, R. Peng, The C-514T polymorphism in the human hepatic lipase gene promoter diminishes its activity, *J Lipid Res* **41** (2000) 155-158.
43. M. Bidder, J.S. Shao, N. Charlton-Kachigian, A.P. Loewy, C.F. Semenkovich, D.A. Towler, Osteopontin transcription in aortic vascular smooth muscle cells is controlled by glucose-regulated upstream stimulatory factor and activator protein-1 activities, *J Biol Chem* **277** (2002) 44485-44496.
44. C. Weigert, K. Brodbeck, M. Sawadogo, H.U. Haring, E.D. Schleicher, Upstream stimulatory factor (USF) proteins induce human TGF-beta1 gene activation via the glucose-response element-1013/-1002 in mesangial cells: up-regulation of USF activity by the hexosamine biosynthetic pathway, *J Biol Chem* **279** (2004) 15908-15915.
45. S. Wang, J. Skorczewski, X. Feng, L. Mei, J.E. Murphy-Ullrich, Glucose up-regulates thrombospondin 1 gene transcription and transforming growth factor-beta activity through antagonism of cGMP-dependent protein kinase repression via upstream stimulatory factor 2, *J Biol Chem* **279** (2004) 34311-34322.
46. L. Shi, S. Liu, D. Nikolic, S. Wang, High glucose levels upregulate upstream stimulatory factor 2 gene transcription in mesangial cells, *J Cell Biochem* **103** (2008) 1952-1961.
47. E. Cheung, P. Mayr, F. Coda-Zabetta, P.G. Woodman, D.S. Boam, DNA-binding activity of the transcription factor upstream stimulatory factor 1 (USF-1) is regulated by cyclin-dependent phosphorylation, *Biochem J* **344** Pt 1 (1999) 145-152.
48. M.C. Ng, K. Miyake, W.Y. So, E.W. Poon, V.K. Lam, J.K. Li, N.J. Cox, G.I. Bell, J.C. Chan, The linkage and association of the gene encoding upstream stimulatory factor 1 with type 2 diabetes and metabolic syndrome in the Chinese population, *Diabetologia* **48** (2005) 2018-2024.
49. F. Smih, P. Rouet, S. Lucas, A. Mairal, C. Sengenes, M. Lafontan, S. Vaulont, M. Casado, D. Langin, Transcriptional regulation of adipocyte hormone-sensitive lipase by glucose, *Diabetes* **51** (2002) 293-300.
50. L.E. Rufibach, S.A. Duncan, M. Battle, S.S. Deeb, Transcriptional regulation of the human hepatic lipase (LIPC) gene promoter, *J Lipid Res* **47** (2006) 1463-1477.

CHAPTER 6

Sterol-regulatory-element binding protein inhibits upstream stimulatory factor-stimulated hepatic lipase gene expression

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6.1 Abstract

Hepatic lipase (HL) not only plays an important role in plasma lipoprotein transport, but may also affect intracellular lipid metabolism. We hypothesize, that HL expression is regulated as an integral part of intracellular lipid homeostasis. Addition of oleate (1 mM) to HepG2 cells increased HL secretion to 134±14% of control ($p<0.02$), and increased the transcriptional activity of a 698-bp HL promoter-reporter construct 2-fold. Atorvastatin (10 mM) abolished the oleate-stimulation. The transcriptional activity of an SREBP-sensitive HMG-CoA synthase promoter construct was reduced 50% by oleate, and increased 2-3 fold by atorvastatin. Co-transfection with an SREBP-2 expression vector reduced HL promoter activity and increased HMG-CoA synthase promoter activity. Upstream stimulatory factors (USF) are also implicated in maintenance of lipid homeostasis. Co-transfection with a USF-1 expression vector stimulated HL promoter activity 4-6 fold. The USF-stimulated HL promoter activity was not further enhanced by oleate, but almost completely prevented by atorvastatin or co-transfection with the SREBP-2 vector. Opposite regulation by USF-1 and SREBP-2 was also observed with a 318-bp HL promoter construct that lacks potential SRE-like and E-box binding motifs. We conclude that the opposite regulation of HL expression by fatty acids and statins is mediated via SREBP, possibly through interaction with USF.

6.2 Introduction

Hepatic lipase (HL) is considered to be a focal point for the development and treatment of coronary artery disease [1,2]. HL plays an important role in plasma lipoprotein metabolism [3,4]. Its activity in post-heparin plasma is a major determinant of HDL cholesterol level and LDL size [5]. High HL activity is associated with low HDL cholesterol level and may contribute to the formation of small-dense LDL. Consequently, increased HL activity leads to development of the atherogenic lipid profile, as e.g. found in type 2 diabetes [6,7]. In view of its role in the metabolism in several lipoproteins, pro- as well as anti-atherogenic properties are ascribed to high HL expression (reviewed in [4,8]). Taken together, HL is an important enzyme, and changes in its activity are clinically relevant. However, it is not clear why HL activity changes under different conditions. By interaction with plasma lipoproteins, HL promotes the cellular uptake of lipids, and in this way, may affect intracellular lipid homeostasis [8]. Vice versa, factors that influence hepatic lipid metabolism appear to modulate HL activity. Fatty acids stimulate HL secretion from HepG2 cells [9], which may explain the positive correlation between HL activity and omental fat mass observed in women [10]. Treatment of patients with statins results in a dose-dependent lowering of post-heparin plasma HL activity [5,7,11], particularly in patients with elevated plasma triglycerides [5,7]. We hypothesize that HL plays an important role in intracellular hepatic lipid homeostasis, and that its expression is regulated accordingly. Therefore, we studied whether HL expression is modulated by factors involved in fatty acid and cholesterol metabolism.

6.3 Materials and methods

6.3.1 Cell culture

Monolayer cultures of HepG2 cells were maintained at 37°C, 95% O_2, 5% CO_2 in Dulbecco's modified Eagle's medium (ICN, Costa Mesa, Ca, USA) supplemented with 10% FCS, 50 IU/ml penicillin and 50 µg/ml streptomycin (Gibco BRL, Breda, Netherlands). Twenty-four hours before the start of the experiments, HepG2 cells were plated at 30% confluence in 6- or 24-wells culture dishes.

6.3.2 Hepatic lipase secretion

To study the effect of non-esterified fatty acids (NEFA), bovine serum albumin bound oleate (molar ratio 1:6) was added to the medium to a final oleate concentration of 1 mmole/L. The control medium contained less than 0.05 mmole/L NEFA (NEFA C-kit, Wako Chemicals GmbH, Germany). The oleate-enriched as well control media were refreshed every 12 hours during a period of 48 hours. When indicated, atorvastatin was present at a final concentration of 10µmole/L in the control and oleate-enriched-media. During the last 12 h, heparin (25 IU/mL; Leo Pharmaceuticals, Breda, The Netherlands) was present in the medium. The hepatic lipase

activity was assayed as described before [12]. Enzyme activities were expressed as mUnits (nmoles of free fatty acids released per min). Atorvastatin was kindly provided by Pfizer, N.Y., USA.

6.3.3 Cellular cholesterol and triglyceride biosynthesis

To determine the effect of atorvastatin on cholesterol(-ester) biosynthesis in HepG2 cells, [2-^{14}C]acetic acid (Amersham, UK) was added in trace amounts to the extracellular medium at 48 h after atorvastatin, and the incubation was continued for an additional 2 h. Then, the medium was removed, the cells were washed twice with ice-cold PBS, and the lipids were extracted with hexane:isopropanol (3:2, by vol.). After evaporation of the extraction fluid, lipids were dissolved in heptane:isopropanol (1:4; by vol.), and unlabelled cholesterol and cholesterolesters were added as carriers. The lipids were separated by thin-layer chromatography using heptane:diethyl ether:acetic acid (60:40:1; by vol.). The lipids were made visible by iodine vapor, and the cholesterol and cholesterolester spots were scraped off and the radioactivity was determined by scintillation counting.

The triglyceride content of the cell extracts was determined enzymatically by the TG kit of Roche (Almere, The Netherlands).

6.3.4 Construction of plasmids

Two promoter-fragments of the human hepatic lipase gene (*LIPC*) were used. First, a 698-bp fragment of the human HL promoter (-685 to +13 relative to the transcriptional start site identified by [13]) was used as described before [14]. This HL promoter fragment corresponds to the "wild-type" sequence, having a C at position -514 and a G at -250 [14]. Secondly, a 318-bp fragment (-305 to+13) was generated from the former fragment by PCR. This fragment was chosen to eliminate both potential E-boxes at -514 and -310, and the potential SRE at -553, which have been identified in the HL promoter by the TransFac database [15]. As a reporter vector we used either pCAT-Basic or pGL3-Basic (Promega, Leiden, The Netherlands). The HL promoter fragments were subcloned into the reporter vectors using suitable restriction sites.

An SREBP-responsive luciferase reporter vector (pSRE-luc) was constructed by cloning part of the hamster HMG-CoA synthase promoter region into pGL3-Basic [16]. This fragment contained the generic TATA-box and three SRE-elements. From total RNA obtained from hamster liver, the -325 to -225 region, and the -30 to + 36 region of the HMG-CoA synthase gene were amplified by RT-PCR, and the fragments were ligated into the *Sac*I/*Nhe*I restriction sites, and in the *Xho*I/*Hind*III sites of pGL3-Basic, respectively.

The expression vector pSREBP2 containing the coding sequence of mature human SREBP-2 (amino acids 1-481) in pcDNA3 was kindly provided by B. Staels, Institute Pasteur, Lille, France. The expression vector pCX-USF (a kind gift from R.G. Roeder, Rockefeller University, New York, USA) encoded for the 43-kDa human USF-1 [17]. An RSV-β-galactosidase expression vector (Promega, Leiden, The Netherlands) was used as a control for transfection efficiency.

6.3.5 Promoter-reporter assays

Three hours before transfection, the medium was refreshed. Transfections of CAT –reporter constructs were performed in 6-well plates by calcium phosphate co-precipitation using 2.5 µg of the HL-promoter/CAT-construct and 0.2 µg of an RSV-β-galactosidase expression vector per well. Three hours after transfection, oleate and atorvastatin were added as indicated. In each experiment, parallel transfections with promoter-less pCAT-Basic were included as negative controls. At 48 h after transfection, cell extracts were prepared. The amounts of CAT and b-galactosidase protein were determined by ELISA (Roche, Almere, The Netherlands). Promoter activity was expressed as pg CAT/ng β-galactosidase to correct for differences in cell number and transfection efficiency.

Transfections of the luciferase–reporter constructs were performed in 24-wells plates with Lipofectamine Plus (Invitrogen, Groningen, The Netherlands) using 0.4 µg of the luciferase-reporter construct per well. The luciferase activity in the cell extracts was determined with the FireLight kit (Perkin-Elmer, Boston MA, USA) and the Packard Top Count NXT luminometer. Promoter activity was expressed as units of luciferase activity/µg cell protein to correct for differences in cell number. Since the RSV promoter of the RSV-β-galactosidase expression vector was highly upregulated by pSREBP2 cotransfection (data not shown), we corrected only for the amount of protein per cell-extract. In three independent experiments without pSREBP2 cotransfection, the β-galactosidase expression was similar among parallel incubations, indicating that intra-assay variation in transfection efficiency was relatively small.

6.3.6 Statistics

Unless otherwise indicated, data are expressed as means ±S.D. of three to four independent experiments. Data were analyzed using one-way-ANOVA to compare differences between groups followed by Student-Newman-Keuls.

6.4 Results

6.4.1 Effect of oleate and atorvastatin on secretion of HL

HepG2 cells secreted 0.4-1.5 mU lipase activity (0.3-1.0 mU/mg cell protein) into the extracellular medium during a 12-h incubation with heparin. When the medium was supplemented with 1 mM BSA-bound oleate, the cells rapidly removed the oleate from the medium, and converted it almost stoichiometrically into intracellularly stored triglycerides (data not shown). Repetitious medium changes were necessary to maintain an extracellular oleate concentration between 1 and 0.5 mM througout a 48 h incubation period. Under these conditions, the presence of BSA-bound oleate increased HL activity in the medium to 134±14% of parallel controls (n=5, $p<0.02$). Incubation of the cells for 48 h with 10 µM of the HMG-CoA reductase inhibitor atorvastatin slightly, but not significantly, reduced HL secretion to 90 ± 6% of controls (n=5; p=0.15). In parallel incubations, atorvastatin reduced [2-^{14}C]acetate incorporation into

cholesterol and cholesterolesters to 30±14% and 37±1 % (n=3; p<0.05) of untreated controls, respectively. In co-incubations with oleate, atorvastatin reduced HL secretion to 102±8% of untreated controls (n=5; p<0.01 with respect to oleate-treatment; n.s. with respect to untreated controls). HL mRNA levels, as determined by real-time RT-PCR, showed the same tendency as HL secretion, but the differences were not statistically significant (data not shown).

6.4.2 Effect of oleate and atorvastatin on HL promoter activity

HepG2 cells were transiently transfected with a CAT-reporter vector containing the -685 to +13 region of the human HL gene (HL698-CAT), and the cells were subsequently incubated for 48 h with or without BSA-bound oleate or atorvastatin (Figure 1). Oleate increased the HL promoter activity approximately two-fold. Atorvastatin alone slightly reduced HL promoter activity, but this did not reach statistical significance. Co-incubation of the cells with the statin almost completely abolished the oleate-induced HL promoter activity.

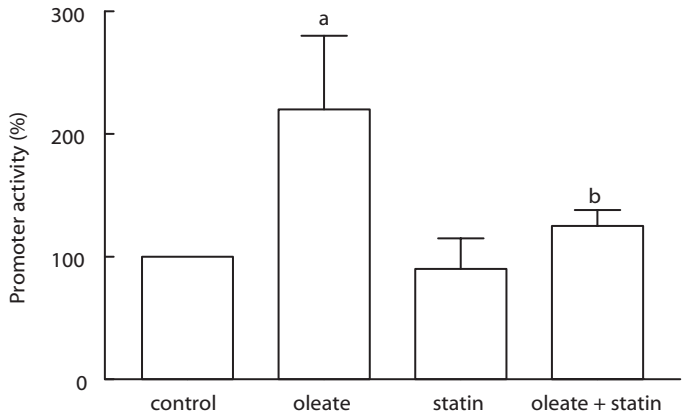

Figure 1. Effect of oleate and atorvastatin on HL promoter activity.

HepG2 cells were transiently transfected with HL698-CAT construct. Albumin-bound oleate (ratio 1:6) was added to the extracellular medium at a final concentration of 1 mM, and atorvastatin to a final concentration of 10 µM. The media were refreshed every 12 h. After 48 h, the cells were collected and the amount of CAT was determined. The promoter activity in the control incubation was taken as 100%. Data are mean ±S.D. for three independent experiments, each performed in triplicate. a en b indicate statistically significant (p<0.05) differences with the control and oleate incubations, respectively.

6.4.3 Interaction with Sterol-Regulatory-Element Binding Proteins (SREBP)

We next investigated whether the effects of oleate and atorvastatin could have been exerted via SREBP. To enable functional assay for mature SREBP in HepG2 cells, we generated an SREBP-sensitive luciferase reporter construct (pSRE-luc), on the basis of the HMG-CoA synthase promoter region containing three sterol regulatory elements [16]. This construct was similar

to that reported by Worgall *et al*. [16] except for use of the pGL3-Basic backbone. To validate our probe, we transfected HepG2 cells with pSRE-luc and subsequently incubated the cells for 48 h in medium supplemented with 20 % lipoprotein-deficient serum (LPDS) or FCS (Figure 2A). The luciferase-activity was ten-fold higher with 20 % LPDS than with 20 % FCS ($p<0.001$; n=4), in accordance with the cellular uptake of cholesterol from lipoproteins present in FCS and subsequent suppression of the maturation of SREBP [18]. Increasing the amount of FCS from 0 to 20% dose-dependently reduced luciferase activity. From this dose-response curve, we concluded that the SRE-luc probe is sensitive to changes in SREBP activity at 10% FCS, the condition that is used throughout this study.

As shown in figure 2B, incubation of the cells with oleate resulted in a 50 % reduction of the SREBP activity ($p<0.001$; n=6), whereas atorvastatin increased SREBP activity 2-4 fold ($p<0.05$; p=4). Apparently, the HL promoter activity is regulated by oleate and atorvastatin opposite to the concurrent changes in SREBP activity. Co-transfection of the cells with pSREBP2 encoding mature, constitutively active SREBP-2, increased pSRE-luc driven luciferase activity by more than 6 fold, and simultaneously reduced HL promoter activity to 25% of control ($p<0.01$; n=4; Figure 2B). These observations indicate that oleate decreases, and atorvastatin increases, SREBP-activity in HepG2 cells, and suggest that SREBP exert a negative effect on HL promoter activity.

6.4.4 Interaction with Upstream Stimulatory Factors (USF)

We previously reported that the HL promoter is strongly upregulated by USF [14]. Since SREBP and USF are both bHLH-ZIP-type transcription factors that recognize E-box sequences, we studied the possible involvement of USF in the regulation by oleate and atorvastatin. In line with our previous report, co-transfection of HepG2 cells with the pCX-USF expression vector increased the activity of the HL698-CAT construct to 390±50% (n=3, $p<0.001$). Unlike control cells, incubation of the USF-1 over-expressing cells with oleate had no additional stimulatory effect (Figure 3). Atorvastatin on the other hand almost completely abolished the USF-induced stimulation of HL promoter activity. In subsequent experiments we compared the effect of co-transfection with pCX-USF and pSREBP2 on the promoter activity of an HL698-luc construct and the pSRE-luc probe (Figure 4). Co-transfection with pCX-USF upregulated the HL promoter, but the endogenous SREBP activity was not significantly affected (Figure 4A). Co-transfection with pSREBP2 markedly increased the pSRE-luc driven luciferase activity, whereas the HL promoter activity was slightly but significantly reduced. When the cells were co-transfected with both pCX-USF and pSREBP2, the USF-mediated upregulation of the HL promoter was almost completely abolished. Suppression of the USF effect by co-transfection with pSREBP2 was dose-dependent (Figure 4B), and was already evident at 15 ng of the pSREBP2 plasmid per well, the lowest amount tested. On the other hand, the SREBP-2 mediated increase of the pSRE-luc activity was reduced by co-transfection with pCX-USF in some, but not all experiments (Figure 4A). These observations indicate that SREBP interfere with the USF-mediated upregulation of the HL promoter.

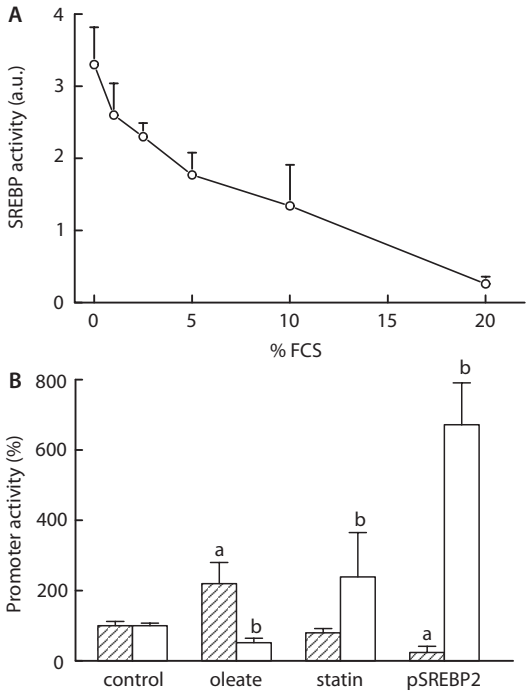

Figure 2. Determination of SREBP activity in HepG2 cells with an SREBP-sensitive luciferase probe.

HepG2 cells were transiently transfected with pSRE-luc, and then incubated for 48 h in the presence of the indicated additions. In **A**, the incubation medium contained 20 % serum (by vol.), either LPDS or increasing amounts of FCS in LPDS. Data are means ±SD for one experiment performed in quadruplicate. In **B**, the medium was kept at 10% FCS. Cells were transfected with either HL698-CAT (hatched bars) or pSRE-luc (open bars). Cells were then incubated for 48 h with albumin-bound oleate (final 1 mM) or atorvastatin (final 10 μM), In one set of incubations, the cells were cotransfected with 120 ng/well of pSREBP2. The promoter activities in the control incubations were set at 100 %. Data are means ±SD of three experiments each performed in triplicate. a en b indicate statistically significant ($p<0.05$) differences with the control HL promoter activity and pSRE-activity, respectively.

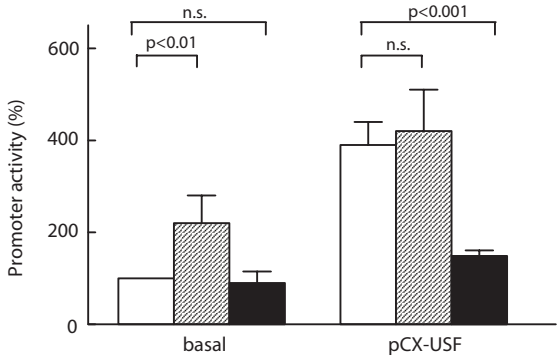

Figure 3. Effect of oleate and atorvastatin on HL promoter activity in USF-1 overexpressing cells.

Experiments were performed as indicated in the legend to figure 1, except that in part of the incubations, cells were co-transfected with 500 ng/well of pCX-USF. Open bars: no further additions; hatched bars: 1 mM albumin-bound oleate; filled bars: 10 μM atorvastatin. The promoter activity in the control incubation without USF-overexpression was taken as 100%. Data are mean ± S.D. for three independent experiments, each performed in triplicate. n.s.: not statistically significant.

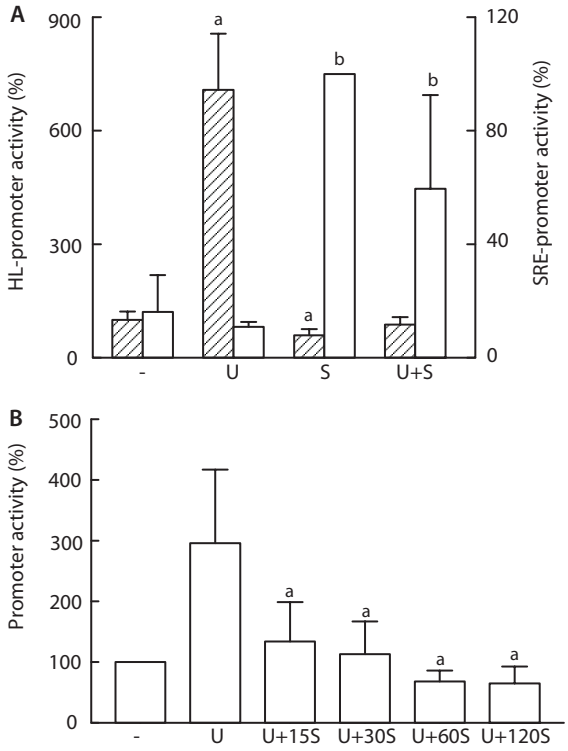

Figure 4. Effect of pCX-USF or pSREBP2 co-transfection on HL and SRE promoter activity.

In **A**, HepG2 cells were transfected either with HL698-luc (hatched bars) or pSRE-luc (open bars) with or without co-transfection with 100 ng/well pCX-USF (U) or pSREBP2 (S). After 48 h of incubation, cell extracts were analyzed for luciferase activity. a en b indicate statistically significant ($p<0.05$) differences with the control HL promoter activity and pSRE-activity, respectively. In **B**, cells were transfected with HL698-luc with or without 100 ng/well pCX-USF, and the indicated amounts (in ng/well) pSREBP2. The HL promoter activities were expressed as a percentage of that in the incubations without pCX-USF or pSREBP2. The SRE-promoter activities were expressed as a percentage of maximum activity (after co-transfection with pSREBP2). Data are means ± S.D for three independent experiments. a indicates statistically significant difference from the USF-activated promoter activity.

6.4.5 Role of potential SRE and E-box sequences in the HL promoter region

A search of the -685/+13 region of the human HL gene sequence through the Transfac database revealed the presence of a potential SRE at position -553, and potential E-boxes at -514 and -310. To test whether the opposite effects of SREBP and USF are mediated through one of these DNA elements, we subcloned the -305/+13 region into pGL3-Basic (HL318-luc). Removal of the 5'-half of the HL promoter region including the potential SRE and E-box elements had no significant effect on transcriptional activity when transfected into HepG2 cells (Figure 5). HL318-luc was downregulated by co-transfection with pSREBP2, similar to HL698-luc. The transcriptional activity of HL318-luc was also significantly upregulated by cotransfection with pCX-USF, albeit to a less extent than HL698-luc. Finally, the stimulatory

effect of USF-1 overexpression on the HL318-luc activity was also abolished by co-transfection with pSREBP2. Hence, the opposite effects of USF and SREBP on HL promoter activity appear to occur independent of the -685/-305 region of the HL gen, including the potential SRE at -553 and E-boxes at -514 and -310.

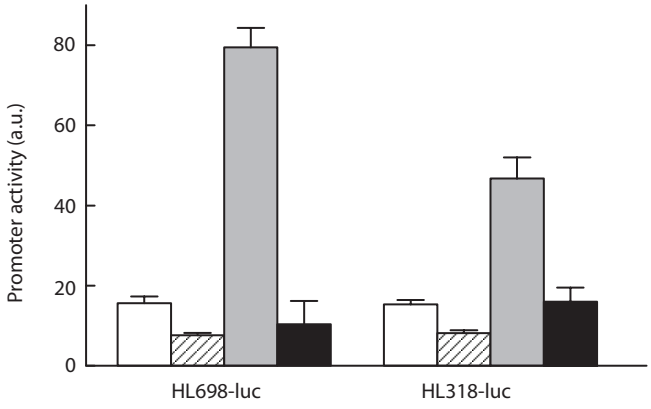

Figure 5. Opposite regulation of the HL promoter activity is independent of SRE and E-box sequences.

HepG2 cells were transfected either with HL698-luc (left panel) or HL318-luc (right panel) without (open bars) or with 120 ng/well pSREBP2 (hatched bars), 100 ng/well pCX-USF (gray bars), or both (filled bars), and 48 h later cell extracts were analyzed for luciferase activity. Data are means ±S.D for four parallel incubations.

6.5 Discussion

We show here, that oleate stimulates the secretion of HL from HepG2 cells and the activity of the proximal HL promoter. NEFA may influence gene expression in several ways, among others by stimulating PPAR-alpha activity [19]. PPAR-alpha consensus sequences have not been identified in the proximal HL promoter. Ciprofibrate, a potent PPARa agonist, did not affect the secretion of endogenous HL (not shown). This suggests that the effect of oleate on HL expression was not mediated via PPARα. Unsaturated fatty acids including oleate have been reported to affect gene expression also by lowering the mature, active form of SREBP-1c and -2 [16,20]. Our data with the SREBP-sensitive luciferase probe are consistent with this, but do not allow discrimination between either SREBP isoform. SREBP-1c and -2 are transcription factors that bind either to SRE or SRE-like sequences, or to E-boxes; binding of SREBP to these sequences generally stimulates gene expression [18,21]. The HL promoter fragment used contains a potential SRE at around position -553, and E-box sequences at around -514 and -310. However, atorvastatin and cotransfection with pSREBP-2, which stimulate SREBP activity as demonstrated by the increased activity of an SRE-reporter construct, did not stimulate HL

promoter activity under basal conditions. Rather, atorvastatin and pSREBP2 cotransfection reduced HL promoter activity. To our knowledge, a strong negative effect of SREBP-2 on gene transcription via an SRE-like element has only been reported for the microsomal triglyceride transfer protein MTP [22]. Removal of the potential SRE and E-boxes from the HL promoter did not prevent the downregulation by atorvastatin or pSREBP2 cotransfection. This rules out an important contribution of the potential SRE at -553 and E-boxes at -514 and -310 in the regulation of the HL promoter by SREBP's.

Previously, we have found that HL promoter activity is strongly stimulated by USF [14]. USF are ubiquitously expressed transcription factors involved in lipid and glucose homeostasis and insulin regulation. In cells that overexpress USF-1, atorvastatin or cotransfection with pSREBP2 completely abolished the USF mediated upregulation of the HL promoter. Thus, SREBP inhibited USF-stimulation of HL promoter activity. Assuming that, under basal conditions, USF-stimulated HL promoter activity is inhibited by endogenous SREBP, the stimulatory effect of oleate may be explained by the lowering of mature, active SREBP [16,20]. When USF-1 was overexpressed, oleate did not further stimulate HL promoter activity, possibly since the relatively high amount of USF-1 overcomes inhibition by endogenous SREBP under these conditions. When SREBP activity is further increased (atorvastatin, cotransfection with pSREBP2), the USF stimulation is abolished. We hypothesize that SREBP act negatively on HL expression by preventing, or inhibiting, the USF-mediated upregulation of the HL promoter.

The mechanism by which SREBP interfere with USF-action on the HL promoter is not clear yet. Both SREBP and USF may bind to so-called E-boxes, and competition for E-box binding may be proposed. The proximal HL promoter contains two E-box sequences, an imperfect one at position -514 and a canonical E-box at position -310. In the common HL gene variant, the -514C→T transition renders the -514 E-box ineffective in USF binding [14]. Post-heparin plasma HL activity in carriers of the -514T allele has been shown by us and several other groups to be lower than in -514C homozygotes [7,23]. In promoter-reporter assays, the HL-514T promoter shows 30-50% less activity than the HL-514C counterpart [14,24]. This suggests that E-boxes, at least the one at -514, are involved in the regulation of HL expression. However, removal of these E-boxes from the proximal HL promoter only partly reduced the upregulation by USF-1 overexpression, and left inhibition by SREBP-2 essentially unaffected (Figure 5). Apparently, upregulation of the proximal HL promoter by USF, and inhibition by SREBP, occur partly via non-E-box dependent mechanisms. USF and SREBP may oppositely regulate gene expression directly via binding to hitherto unidentified DNA sequences in the HL promoter, or via recruitment of other transcription factors [25]. Studies are underway in our laboratory to delineate the mechanism for regulation of the proximal HL promoter by USF and SREBP.

Our results link the regulation of HL expression directly to fatty acid and cholesterol homeostasis in the liver. They explain why HL expression is enhanced in conditions with a high fatty acid supply to the liver such as an increased omental fat mass [10] or type 2 diabetes [7]. Since fatty acids from visceral fat stores are the main precursors for VLDL-triglycerides, this

mechanism ensures that HL activity is synchronized with hepatic triglyceride secretion. This offers the molecular basis for the observed high HL activity in type 2 diabetes and the positive correlation between HL activity and plasma triglyceride levels. Atorvastatin abolished the stimulation of HL expression by oleate without having a significant effect on the basal activity. Extrapolating these data to humans predicts that statins affect HL more strongly if fatty acid supply to the liver is high. Indeed, Zambon and coworkers found a substantial HL-lowering effect of hypolipidemic treatment in subjects with hyperlipidemia [5]. We found a dose-dependent lowering of HL activity by atorvastatin in hypertriglyceridemic type 2 diabetes [7]. Our results also predict that HL expression will be low in conditions with high levels of mature SREBP. This is in line with the often found inverse correlation between expression of SREBP-stimulated genes (e.g. LDL receptor) and HL activity [8], e.g. during statin treatment. These data strongly support the hypothesis that HL is part of intracellular, hepatic lipid homeostasis and is regulated accordingly. The implication of this notion is that changes in plasma lipoproteins due to HL are the ultimate result of the participation of HL in lipid homeostasis.

Acknowledgements

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Abbreviations: CAT: chloramphenicol acetyltransferase; FCS: foetal calf serum; HL: hepatic lipase; LPDS: lipoprotein deficient serum; Luc: luciferase; NEFA: non-esterified fatty acids; PBS: phosphate-buffered saline; SRE: sterol-regulatory element; SREBP: SRE-binding protein; USF: Upstream Stimulatory Factor.

References

1. Zambon A., Brown BG, Deeb SS, Brunzell JD. Hepatic lipase as a focal point for the development and treatment of coronary artery disease. *J Investig Med* 2001;**49**:112-118.
2. Dugi KA, Brandauer K, Schmidt N, Nau B, Schneider JG, Mentz S, Keiper T, Schaefer JR, Meissner C, Kather H, Bahner ML, Fiehn W, Kreuzer J. Low hepatic lipase activity is a novel risk factor for coronary artery disease. *Circulation* 2001;**104**:3057-3062.
3. Cohen JC, Vega GL, Grundy SM. Hepatic lipase: new insights from genetic and metabolic studies. *Curr Opin Lipidol* 1999;**10**:259-267.
4. Santamarina-Fojo S, Haudenschild C, Amar, M. The role of hepatic lipase in lipoprotein metabolism and atherosclerosis. *Curr Opin Lipidol* 1998;**9**:211-219.
5. Zambon A, Hokanson JE, Brown BG, Brunzell JD. Evidence for a new pathophysiological mechanism for coronary artery disease regression: hepatic lipase-mediated changes in LDL density. *Circulation* 1999;**99**:1959-1964.
6. Tan KC, Shiu SW, Chu BY. Roles of hepatic lipase and cholesteryl ester transfer protein in determining low-density lipoprotein subfraction distribution in Chinese patients with non-insulin-dependent diabetes mellitus. *Atherosclerosis* 1999;**145**:273-278.
7. Berg-Planken IIL, Hoogerbrugge N, Stolk RP, Bootsma AH, Jansen H. Atorvastatin dose-dependently decreases hepatic lipase activity in type 2 diabetes: effect of sex and the LIPC promoter variant. *Diabetes Care* 2003;**26**:427-32.
8. Jansen H, Verhoeven AJM, Sijbrands EJG. Hepatic Lipase: a pro- or anti-atherogenic protein? *J Lipid Res* 2002;**45**:1352-1362.
9. Nimmo L, McColl AJ, Rosankiewicz JZ, Richmond W, Elkeles RS. Regulation of hepatic lipase expression in HepG2 cells. *Biochem Soc Trans* 1997;**25**:S689.
10. Carr MC, Hokanson JE, Zambon A, Deeb SS, Barrett PH, Purnell JQ, Brunzell JD. The contribution of intraabdominal fat to gender differences in hepatic lipase activity and low/high density lipoprotein heterogeneity. *J Clin Endocrinol Metab* 2001;**86**:2831-2837.
11. Hoogerbrugge N, Jansen H. Atorvastatin increases low-density lipoprotein size and enhances high- density lipoprotein cholesterol concentration in male, but not in female patients with familial hypercholesterolemia. *Atherosclerosis* 1999;**146**:167-174.
12. Neve BP, Verhoeven AJM, Jansen H. Acute effects of adrenaline on hepatic lipase secretion by rat hepatocytes. *Metabolism* 1997;**46**:76-82.
13. Ameis D, Stahnke G, Kobayashi J, McLean J, Lee G, Buscher M, Schotz MC, Will H. Isolation and characterization of the human hepatic lipase gene. *J Biol Chem* 1990;**265**:6552-6555.
14. Botma GJ, Verhoeven AJM, Jansen H. Hepatic lipase promoter activity is reduced by the C-480T and G-216A substitutions present in the common LIPC gene variant, and is increased by Upstream Stimulatory Factor. *Atherosclerosis* 2001;**154**:625-632.
15. Quandt K, Frech K, Karas H, Wingerden E, Werner T. MatInd and MatInspector - new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res* 1995;**23**:4878-4884.
16. Worgall TS, Sturley SL, Seo T, Osborne TF, Deckelbaum RJ. Polyunsaturated fatty acids decrease expression of promoters with sterol regulatory elements by decreasing levels of mature sterol regulatory element-binding protein. *J Biol Chem* 1998;**273**:25537-25540.
17. Du H, Roy AL, Roeder RG. Human transcription factor USF stimulates transcription through the initiator elements of the HIV-1 and the Ad-ML promoters. *EMBO J* 1993;**12**:501-511.
18. Edwards PA, Tabor D, Kast HR, Venkateswaran A. Regulation of gene expression by SREBP and SCAP. *Biochim Biophys Acta* 2000;**1529**:103-113.
19. Torra IP, Chinetti G, Duval C, Fruchart JC, Staels B. Peroxisome proliferator-activated receptors: from transcriptional control to clinical practice. *Curr Opin Lipidol* 2001;**12**:245-254.
20. Worgall TS, Johnson RA, Seo T, Gierens H, Deckelbaum RJ. Unsaturated fatty acid-mediated decreases in sterol regulatory element- mediated gene transcription are linked to cellular sphingolipid metabolism. *J Biol Chem* 2002;**277**:3878-3885.

21. Horton JD, Shah NA, Warrington JA, Anderson NN, Park SW, Brown MS, Goldstein JL. Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. *Proc Natl Acad Sci USA* 2003;**100**:12027-12032

22. Sato R, Miyamoto W, Inoue J, Terada T, Imanaka T, Maeda M. Sterol regulatory element-binding protein negatively regulates microsomal triglyceride transfer protein gene transcription. *J Biol Chem* 1999;**274**:24714-24720.

23. Jansen H, Verhoeven AJM, Weeks L, Kastelein JJ, Halley DJ, van den Ouweland A, Jukema JW, Seidell JC, Birkenhäger JC. Common C-to-T substitution at position -480 of the hepatic lipase promoter associated with a lowered lipase activity in coronary artery disease patients. *Arterioscler Thromb Vasc Biol* 1997;**17**:2837-2842.

24. Deeb SS, Peng R. The C-514T polymorphism in the human hepatic lipase gene promoter diminishes its activity. *J Lipid Res* 2000;**41**:155-158.

25. Santamarina-Fojo S, Remaley AT, Neufeld EB, Brewer HB Jr. Regulation and intracellular trafficking of the ABCA1 transporter. *J Lipid Res* 2001;**42**:1339-1345.

CHAPTER 7

Activation of Hepatic Lipase Expression by Oleic Acid: Possible Involvement of USF1

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7.1 Abstract

Polyunsaturated fatty acids affect gene expression mainly through PPARs and SREBPs, but how monounsaturated fatty acids affect gene expression is poorly understood. In HepG2 cells, oleate supplementation has been shown to increase secretion of hepatic lipase (HL). We hypothesized that oleate affects HL gene expression at the transcriptional level. To test this, we studied the effect of oleate on HL promoter activity using HepG2 cells and the proximal HL promoter region (700 bp). Oleate increased HL expression and promoter activity 1.3-2.1 fold and reduced SREBP activity by 50%. Downregulation of SREBP activity by incubation with cholesterol+25-hydroxycholesterol had no effect on HL promoter activity. Overexpression of SREBP2, but not SREBP1, reduced HL promoter activity, which was effected mainly through the USF1 binding site at -307/-312. Oleate increased the nuclear abundance of USF1 protein 2.7±0.6-fold, while USF1 levels were reduced by SREBP2 overexpression. We conclude that oleate increases HL gene expression via USF1. USF1 may be an additional fatty acid sensor in liver cells.

7.2 Introduction

Polyunsaturated fatty acids (PUFAs) affect gene expression through interaction with specific transcription factors, notably peroxisome proliferator-activated receptors (PPARs) and sterol regulatory element binding proteins (SREBPs) [1,2]. PUFAs are ligands for PPARs, and binding results in the formation of an active transcription factor [3]. SREBP1 and -2 are cholesterol-sensitive transcription factors that are involved predominantly in regulation of fatty acid synthesis and cholesterol homeostasis, respectively [4]. Immature SREBP proteins are present in the endoplasmic reticulum membrane; upon transport to the Golgi, the transcriptionally active N-terminal part of the protein (nSREBP) is released by proteolytic cleavage [5]. PUFAs are shown to modulate both the synthesis and maturation of the SREBPs [1,4,6]. In short-term feeding experiments, the mRNA profile of mouse liver almost completely overlapped between PUFAs and specific PPARα agonists, suggesting that PUFAs mainly act through PPARα [7]. PUFAs also reduce SREBP activity or nSREBP1 protein in rat and mouse liver *in vivo* [8-11], rat hepatocytes [10,11] and human HepG2 hepatoma cell lines [6,12].

Monounsaturated fatty acids (MUFAs), notably oleate, are the most abundant fatty acids in human plasma. Compared to PUFAs, the effect of MUFAs on liver gene expression is relatively small [7]. How gene expression is affected by MUFAs is poorly understood. It is assumed that similar to PUFAs, MUFAs signal through PPARs and SREBPs. However, short-term feeding of mice with triolein showed relatively limited overlap in mRNA profile with PUFAs or with PPARα agonists; of the 114 genes affected by triolein feeding, 65 (57%) were unique to triolein, whereas of the 519 genes affected by PUFAs, only 89 (17%) were unique for PUFAs [7]. In addition, oleate is much less effective than PUFAs in suppressing SREBP activity and nSREBP1 protein levels [6,12]. This suggests that MUFAs may affect gene expression through mechanisms other than PPARs and SREBPs.

We are interested in how the human hepatic lipase (HL) gene is upregulated by oleic acid. Hepatic lipase (EC 3.1.1.3) is an extracellular enzyme present on cell membranes in liver sinusoids, where it has an important role in plasma lipid and lipoprotein metabolism [13,14,15]. Post-heparin plasma HL activity is elevated in type 2 diabetes [16], increases with the HOMA-index, a measure of insulin resistance, in non-diabetic males [17], and increases with visceral fat mass [18,19]. Hence, HL activity appears to be high under conditions with increased supply of fatty acids to the liver. In rats, diets rich in either saturated fats [20] or fish oil [21] reduced post-heparin plasma HL activity, but the effect of selective MUFA enrichment has not been reported. HepG2 cells supplemented with oleate showed increased HL expression [22,23], which is due at least in part to increased transcription of the HL gene [23]. In human studies, treatment with PPARα agonists minimally elevated HL activity [24,25], whereas in rats fenofibrate strongly suppressed HL expression [26]. It seems unlikely therefore that the effect of oleate on HL expression is explained by activation of PPARα. Treatment with statins, which act predominantly through elevation of SREBP activity, consistently results in reduction of HL activity [16,27]. In HepG2 cells, atorvastatin as well as forced expression of nSREBP2 reduced

HL secretion and HL gene transcription [23]. However, feeding rats a cholesterol-enriched diet, which suppresses SREBP activity, was also reported to reduce HL expression [28]. Our previous studies using HepG2 cells suggested that SREBP2 interferes with the sensitivity of the HL gene to upregulation by Upstream Stimulatory Factors (USFs) [23]. USF1 and 2 are ubiquitous transcription factors involved in the regulation of many genes including the insulin-responsive and lipogenic enzymes expressed in liver [29,30]. Binding of USFs to their cognate site in the HL promoter region strongly increased its transcription [31,32]. Overexpression of nSREBP2 in HepG2 cells appeared to abolish this responsiveness to USFs [23]. Hence, the HL gene may be an indirect target of the SREBPs.

In the present paper, we tested the hypothesis that HL gene expression is affected by oleate at the level of transcription. As a model, we used the proximal promoter region of the HL gene upon transient transfection of HepG2 cells. Our results show that supplementation of HepG2 cells with oleate increases the nuclear abundance of USF1, which may at least in part explain the stimulatory effect of oleate on HL promoter activity [33].

7.3 Results and Discussion

7.3.1 Oleate increases HL expression and down-regulates SREBP activity

When HepG2 cells were supplemented with oleate (1 mM BSA-bound), and then incubated for 48 h, secretion of HL activity (Figure 1A) and luciferase activity of the HL-685 promoter construct (Figure 1B) were significantly increased. By this time however, oleate was no longer detectable in the extracellular medium [23]. When an extra addition of oleate was given after 24 h, secretion of HL activity and HL-685 luciferase activity further increased (Figures 1A and B), suggesting a dose-response relationship. HL mRNA increased in parallel to HL secretion and HL-685 luciferase activity (Figure 1C). Simultaneously, SRE-luc activity and HMG-CoA reductase (HMGR) mRNA, an SREBP2 target gene, were significantly suppressed by oleate supplementation (Figure 1C).

7.3.2 HL promoter activity is down-regulated by SREBP-2 but not by SREBP-1

To test whether the HL gene is a target of SREBP1 or SREBP2, we transfected HepG2 cells with pSREBP1 and pSREBP2, which encode the nuclear form of SREBP1 and SREBP2. The activity of HL–685 was dose-dependently down-regulated by pSREBP2 up till 50% (Figure 2A). HL promoter activity was not significantly affected by pSREBP1. Qualitatively similar results were obtained with the HL–325 construct (not shown). In parallel, both pSREBP2 and pSREBP1 increased SRE-luc activity 8-14 fold (Figure 2B). Hence, HL promoter activity is down-regulated by SREBP2, but not by SREBP1.

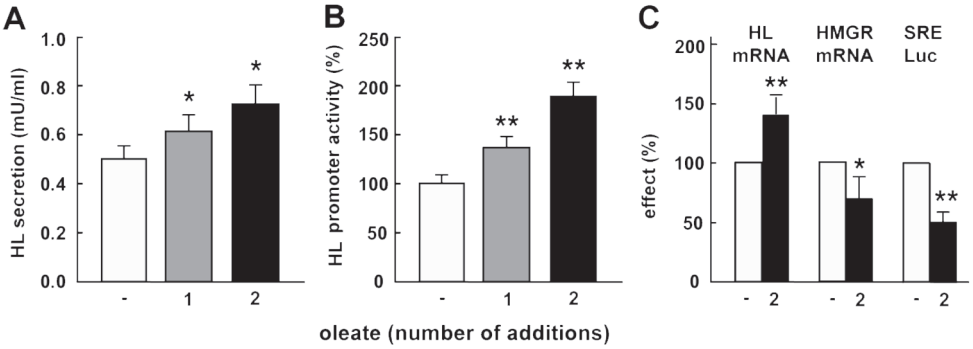

Figure 1. Oleate increases HL expression and down-regulates SREBP activity.

HepG2 cells were incubated for 48h without further additions (-), with BSA-bound oleate added once at the start of the incubation (1) or with oleate added both at the start and again after 24h (2). At the end of the incubation, secretion of HL activity (A), HL-685 luciferase activity (B) and HL mRNA (C) was determined. In parallel, HMG-CoA reductase (HMGR) mRNA and SRE-luciferase (SRE-Luc) was measured (C). (n=3-5; *: $P<0.05$ and **: $P<0.01$ vs. control).

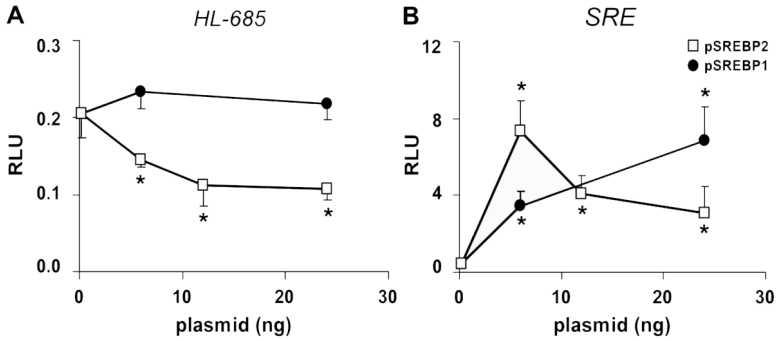

Figure 2. Effect of nSREBP2 and nSREBP1 on HL-685 promoter activity.

HepG2 cells were transfected with the plasmidsHL-685 **(A)** or SRE-luc **(B)**, and the indicated amounts of pSREBP1 or pSREBP2 (open and closed symbols, respectively). Relative luciferase activity (RLU) was determined after 48h (n=3; *: $P<0.05$ compared to control).

To determine the correlation between SREBP activity and down-regulation of the HL promoter, we determined luciferase activity at different time points after co-transfection. Suppression of HL-685 activity was only apparent at 48 and 72h with both 6 and 24 ng of pSREBP2 (Figure 3A). In fact, after 24h, HL promoter activity was slightly increased. In contrast, SRE luc activity was already strongly and maximally increased after 24h, and remained high thereafter (Figure 3B).

When HepG2 cells were incubated with cholesterol+25-hydroxycholesterol, SREBP activity was strongly suppressed to 8±3% of control (n=6, $P<0.001$). The activity of the HL-685 construct was not significantly affected (109±40%, n=7). Apparently, lowering of SREBP activity *per se* is not sufficient to increase HL promoter activity. Combined with the delayed

response of HL promoter activity to overexpression of nSREBP2, this suggests that the HL gene is not a direct target of SREBP2. This can also be deduced from the oligonucleotide microarray data from Horton *et al.* [34], who showed that HL mRNA was reduced in SREBP1 and SREBP2 transgenic mice but not increased in SREBP-cleavage activating protein (SCAP) knockout mice.

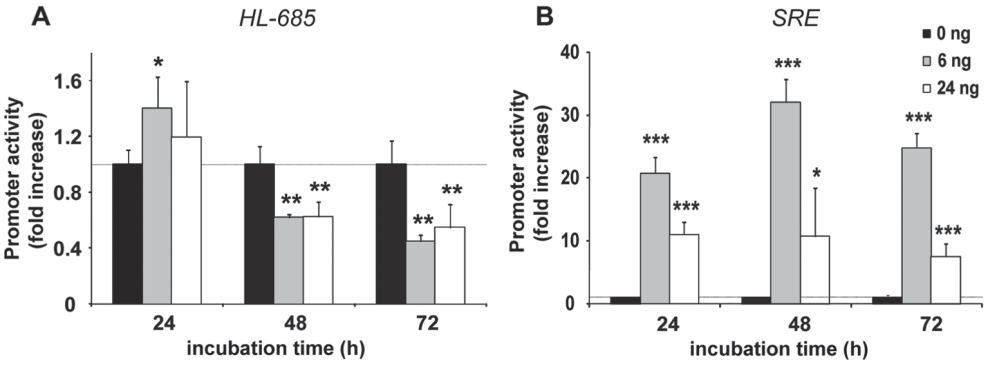

Figure 3. nSREBP2 overexpression suppresses HL-promoter activity time-dependently.

HepG2 cells were transfected with HL-685-luc **(A)** or SRE-luc **(B)**, either without or with 6 or 24 ng pSREBP2 (dark, grey and white bars, respectively). Luciferase activities were determined at the incubation times indicated, and expressed as fold increase relative to the no-pSREBP2 control; (n=4; *: $P<0.05$, **: $P<0.01$, and ***: $P<0.001$ vs. control).

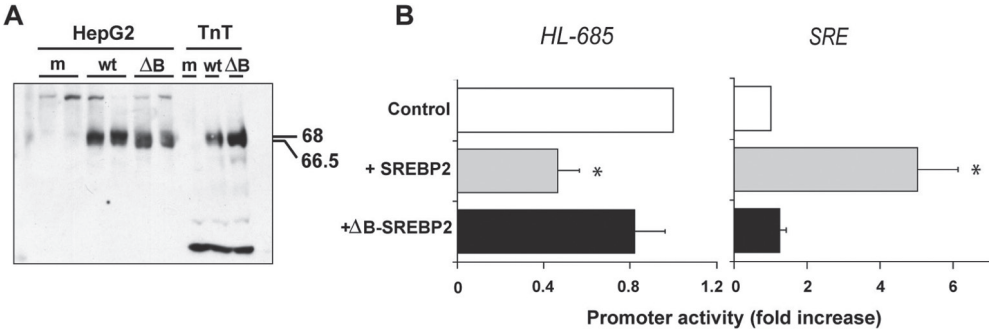

Figure 4. The DNA binding domain of nSREBP2 is required for suppression of HL-685.

(A) HepG2 cells were transfected in a 6-wells plate with 1 µg of empty pcDNA3 vector (m=mock), 1 µg wildtype (wt) or 1 µg ΔB-SREBP2. SREBP2 protein was detected in nuclear extracts by immunoblotting with RS004 anti-SREBP2. TnT extracts with *in vitro* made SREBP2 or ΔB–SREBP2 protein (all 0.5 µl) were run in parallel (TnT). Molecular sizes are indicated in kDa. **(B)** HepG2 cells were co-transfected in a 24-wells plate with 24 ng pSREBP2 or ΔB-SREBP2, and either HL-685- (left panel) or SRE-luc (right panel). Data are expressed as fold increase with respect to mock transfection (control); (n=4; *: $P<0.05$ vs. control).

7.3.3 Down-regulation of the HL promoter by SREBP2 requires an intact DNA binding domain

We then constructed the DB-SREBP2 expression plasmid, which encodes a mutant form of nSREBP2 lacking the basic, DNA binding domain [35,36]. Wildtype and ΔB-SREBP2 plasmids induced similar amounts of the protein product when expressed *in vitro* and upon transfection into HepG2 cells (Figure 4A). In contrast to the wildtype protein, the mutant protein was not detectable on immunoblots using the monoclonal anti-SREBP2 from IgG-1D2 hybridoma raised against aa 48-403 of human SREBP2, but both proteins were detectable by polyclonal RS004 anti-SREBP2. Co-transfection of HepG2 cells with up till 100 ng of the ΔB-SREBP2 plasmid did not significantly affect the activity of HL-685 and SRE-luc (Figure 4B). Hence, the DNA binding domain of SREBP2 is required for the down-regulation of HL promoter activity.

7.3.4 nSREBP2 exerts its effect predominantly via the –307/-312 region of the HL promoter

To identify which part of the HL promoter is responsible for the down-regulation by SREBP2, a series of 5'-deletions was generated from the HL-685 promoter fragment. As shown in figure 5, overexpression of SREBP2 caused a 40% reduction in activity of HL-325, similar to the parent construct. The HL–305 construct was only slightly, but significantly, down-regulated by SREBP2, whereas shorter constructs were no longer affected. Down-regulation of HL-305 was only apparent at 72h; at earlier time points, HL-305 activity was not significantly affected by overexpression of SREBP2 (83±16% of control, n=5). We conclude therefore, that the down-regulation of the HL promoter by SREBP2 is predominantly effected through the -325/-305 region.

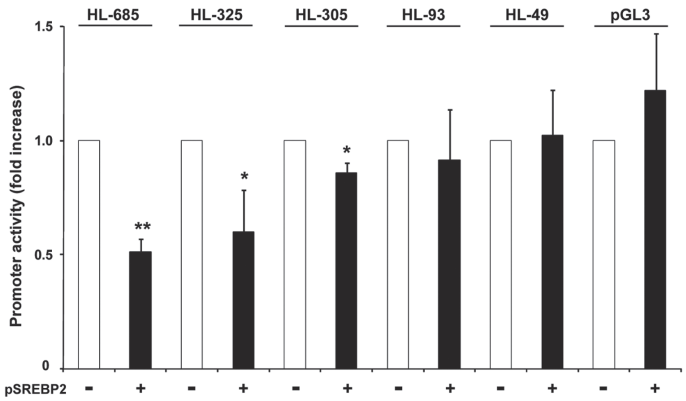

Figure 5. Effect of 5'-deletions on the down-regulation of HL-685 activity by nSREBP2.

HepG2 cells were co-transfected with the indicated HL constructs (or empty pGL3-basic) without (-) or with (+) 6 ng pSREBP2. Luciferase activity was determined after 72h. The activity without pSREBP2 was taken as 1.0 (n=3-4; *: $P<0.05$, ** $P<0.01$ vs. control).

We have previously demonstrated the presence of a functional E-box at -307/-312 of the HL promoter region, which mediates transactivation by upstream stimulatory factors (USF) 1 and 2 [31]. To test the importance of this E-box and endogenous USF proteins in mediating the effect of SREBP2, we made an HL-685 construct in which the E-box was mutated (HL–685Em). In addition, we inhibited expression of endogenous USF1 by RNA interference (siUSF1). The activity of HL-685 and HL-325 was suppressed by approximately 60% by siUSF1 and SREBP2 alone (Figure 6). The effects of siUSF1 and SREBP2 were not additive. In contrast, an unrelated siRNA did not significantly affect HL promoter activity. Removal or mutation of the -307/-312 E-box in the HL-305 and HL-685m constructs, respectively, resulted in the almost complete loss of sensitivity to siUSF1 as well as SREBP2 (Figure 6). These findings strongly indicate that nSREBP2 exerts its effect through USFs and the E-box at -307/-312.

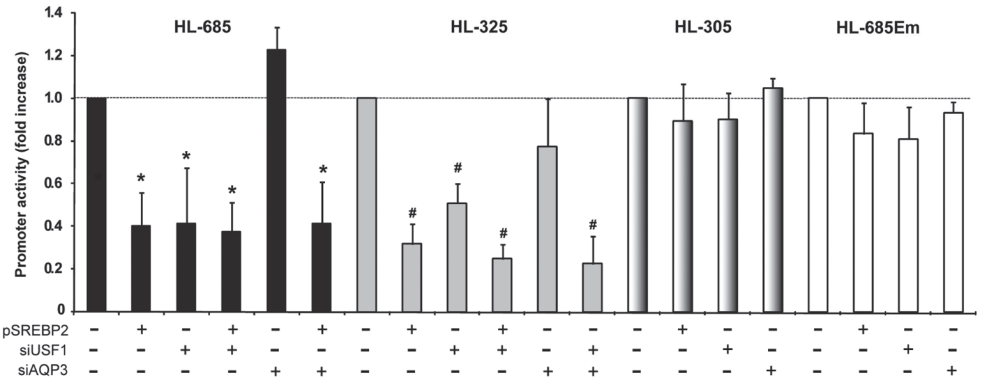

Figure 6. The effect of SREBP2 and USF1 is mediated by the -310 E-box of the HL gene.

HepG2 cells were transfected with the indicated HL-promoter constructs without (-) or with (+) 6 ng pSREBP2 and/or 100 ng siUSF1 or siAQP3. Luciferase activity was determined after 48h; data are expressed as fold increase relative to the transfection with the respective HL-promoter construct (n=3-11; *: $P<0.05$ vs. control of HL-685, #: $P<0.05$ vs. control of HL–325).

7.3.5 Nuclear USF1 is increased by oleate and reduced by nSREBP2 overexpression

Nuclear extracts were prepared from HepG2 cells after incubation for 48h without or with 1 mM of BSA-bound oleate. As shown in Figure 7A, the amount of USF1 was 2.7±0.6-fold higher in the oleate-treated cells (n=3, $P=0.04$), whereas USF2 expression was not significantly increased (1.2±0.2-fold, n=3). We have previously shown by chromatin immunoprecipitation assays that USF1 is bound to the proximal region of the human HL gene in HepG2 cells [31,32], that the degree of binding correlates with HL gene expression levels [31], and that co-transfection of HepG2 cells with USF1-expression plasmids results in strong upregulation of HL expression [23,31].

When cells were transfected with pSREBP2, the amount of USF1 protein was reduced to 45-55% of control, similar to the effect seen with siUSF1 (Figure 7B). The amount of endogenous USF1 protein was further reduced to 15-20% of untreated controls by the combined transfection with SREBP2 and siUSF1.

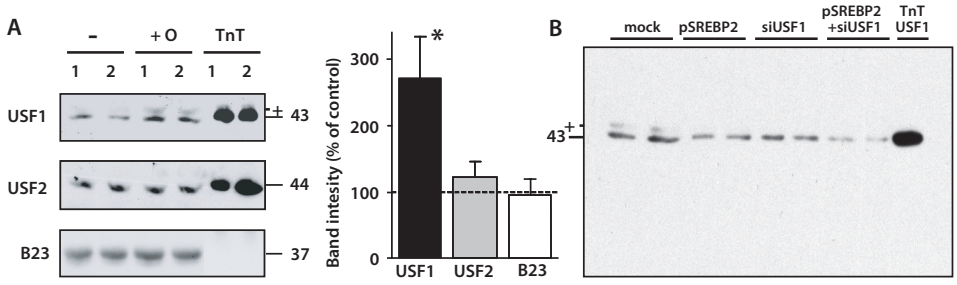

Figure 7. Effect of oleate and nSREBP2 on nuclear USF1 protein abundance.

(A) HepG2 cells were incubated for 48h in the absence (-) or presence (+O) of oleate. Thereafter, nuclear extracts were prepared and used for immunoblotting. B23 protein served as internal control. TnT 1 and 2 are 0.5 and 1.0 ml, respectively, of *in vitro* made USF protein (left panel). The data were quantified by densitometry and expressed as percentage of control (right panel; n=3-4; *: $P<0.05$ vs. control). **(B)** HepG2 cells were transfected without (mock) or with 100 ng pSREBP2, siUSF1, or both. Nuclear extracts were immunoblotted with anti-USF1. The molecular sizes are indicated in kDa. The bands marked with + may represent a modified form of USF1.

7.4 Discussion

Here we have demonstrated for the first time that the monounsaturated fatty acid oleate affects gene expression in liver cells by increasing the nuclear abundance of USF1. The USFs are ubiquitous transcription factors with a broad spectrum of target genes [29], in particular lipogenic and other insulin-responsive genes [30]. USF1 and USF2 are constitutively expressed, and their activity is modulated by reversible phosphorylation and acetylation [29,30,37,38]. In the regulation of the fatty acid synthase gene in liver cells, posttranslationally modified USF1 has recently been shown to be the sensor of nutritional status during fasting and refeeding [38]. Nevertheless, signalling through USF1 also occurs by regulating the nuclear abundance of USF1 [32,39,40]. Others have shown that upregulation of USF1 in HepG2 cells results in the elevated expression of several genes including apolipoproteins, angiotensinogen, and Cyp1A2. Upregulation of liver USF1 by oleate, and subsequent activation of lipogenic genes, would represent a feed-forward cycle leading to increased triglyceride and VLDL synthesis. Indeed, the USF1 gene has been implicated in the elevated plasma triglyceride trait of familial combined hyperlipidemia [30]. Here we showed that oleate increases HL expression in HepG2 cells. A high oleate supply to the liver results in increased production of VLDL. Increased HL

activity has been proposed to facilitate VLDL production by supplying the liver with sufficient phospholipid precursors [13]. An increase in nSREBP2 activity reflects a low intracellular cholesterol status, and loss of cholesterol via VLDL may be reduced by the nSREBP2-mediated suppression of HL activity.

It is not clear how oleate increases nuclear USF1 protein. PUFAs have been shown to affect gene expression predominantly through PPARs and SREBPs. It is not likely that the USF1 gene is upregulated by PPARa, since USF1 target genes are predominantly lipogenic and PPARa targets are predominantly involved in lipid oxidation. Oleate reduces SREBP activity in HepG2 cells, and overexpression of nSREBP2 reduces expression of USF1 protein and its target gene, HL. Suppression of HL expression by SREBP2 is not only seen upon forced overexpression of nSREBP2, but also upon treatment with a statin [16,23,27]. We may argue therefore that the oleate-mediated lowering of SREBP2 increases USF1 protein and hence hepatic lipase expression, though this was not actually shown. Although oleate is a relatively poor suppressor of nSREBP1, the USF1-gene may also be a target of SREBP1. However, the HL gene is not suppressed by overexpression of nSREBP1 as would be predicted when SREBP1 would affect USF1. In addition, HL promoter activity is not significantly upregulated by treating HepG2 cells with PUFAs [41], which predominantly lower nSREBP1. On the other hand, when SREBP activity in the HepG2 cells was strongly suppressed by incubation with cholesterol+25-hydroxycholesterol, HL promoter activity was not significantly upregulated, suggesting that lowering of SREBP1 and SREBP2 is not sufficient to increase nuclear USF1. In addition, USF1 has not been identified as a direct SREBP target gene in the comparative mRNA screen performed by Horton *et al.* [34]. We propose therefore that treatment of HepG2 cells with oleate upregulates USF1 expression and hence HL expression independently of SREBPs. Further studies are required to elucidate how oleate affects USF1 expression.

In general, SREBP1 and SREBP2 activate transcription of their target genes, and the HL gene would have been one of a few genes that are suppressed by SREBP2. From their comparative mRNA screen, Horton *et al.* [34] dismissed the HL gene being a direct SREBP target. The delay between nSREBP2 expression and HL suppression is in line with this notion. Our data show that SREBP2 overexpression affects HL promoter activity mainly through the functional USF binding E-box at -307/-312. USF1 has been shown to bind to SREBP1 [38], but this only occurs when both transcription factors are simultaneously bound to DNA in close proximity. Therefore, the possibility that USF1 is prevented from binding to the proximal HL promoter by complex formation with SREBPs in solution appears unlikely. SREBPs bind to E-boxes as well as SREs, but in contrast to SREs, binding to E-boxes does not result in transactivation [42]. SREBP2 overexpression may also reduce HL expression by competing with USF1 for binding at the -310 E-box. Hence, other mechanisms may be involved in the inhibition of HL expression by nSREBP2, in addition to the lowering of USF1 expression.

7.5 Methods

7.5.1 Cell culture

HepG2 cells (ATCC, Rockville, MD, USA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO-BRL, Breda, Netherlands), supplemented with 10% fetal calf serum (FCS), 50 IU/ml penicillin and 50 µg/ml streptomycin. The cells were kept at 37°C in a humidified atmosphere of 5% CO_2 in air. Twenty-four hours before the start of the experiment, the cells were plated at 30% confluence in 6- or 24-well culture plates (Nunc, Roskilde, Denmark).

Bovine serum albumin-bound oleate (molar ratio 1:6) was added to the medium to a final oleate concentration of 1 mM, which is the upper limit of free fatty acid concentrations found in human plasma [43]. The control medium contained less than 0.05 mM non-esterified fatty acids (NEFA C-kit, Wako Chemicals, Germany). In some experiments, the cells were incubated for 48h with 20 mg/mL cholesterol plus 2 mg/mL of 25-hydroxycholesterol (both from Sigma, St. Louis, USA); additions were made from 1000-fold stocks in ethanol. Unless indicated otherwise, the media including additions were refreshed after 24h. For determination of hepatic lipase secretion, cells were grown in 6-wells plates and 2 ml medium/well. After 48 h incubation, the media were removed and the cells were incubated for an additional 12h in 1 ml/well of fresh medium containing 25 IU heparin (Leo Pharmaceuticals, Breda, The Netherlands). Hepatic lipase activity was assayed in the cell-free media as described before [44]. Enzyme activity was expressed as mU (nmoles of free fatty acids released per min from triolein substrate).

Total cellular RNA was isolated using the Trizol reagent (Invitrogen, Breda, Netherlands), and the amount of HL mRNA and HMG-CoA reductase (HMGR) mRNA was determined by reverse transcription followed by real-time PCR (RT-qPCR) using MyIQ from BioRad, as described previously [38]. Primers used for HMGR mRNA were 5'-GAA GCT GTC ATT CCA GCC A-3' and 5'-GAA CTA CCA ACA TTC TGT GC-3'.

7.5.2 Plasmids

Recombinant DNA techniques were performed according to standard procedures [45]. Oligonucleotides were custom-made by Sigma (Cambridge, UK). Enzymes used were purchased from Roche (Basel, Switzerland). All inserts were verified by DNA sequencing (BaseClear, Leiden, Netherlands).

A series of reporter plasmids was used containing different 5'-deletions of the parent human HL(-685/+13)-luc plasmid (further named HL-685), as described previously [23,31]. All inserts contained the same 3'-end (+13 relative to the transcription start site). In HL-685Em the E-box at -307/-312 (the -310 E-box) was mutated into a NheI site [31].

pSRE-luc containing the generic TATA-box and three SRE-elements of the hamster HMG-CoA synthase in pGL3-Basic was used as a SREBP-responsive reporter construct [23]. pSREBP1 and pSREBP2, containing the coding sequence of human nSREBP1 and nSREBP2 in pcDNA3.1, were kindly provided by B. Staels (Institut Pasteur, Lille, France). From pSREBP2, the ΔB-SREBP2 mutant was generated by deleting the 39bp fragment that codes for the basic DNA

binding site (aa 331-343) [35,36]. The mutant was made by the PCR overlay technique [46] using SREBP2-specific forward and reverse primers of the sequence 5'-C CCC AAA GAA GGA GAA*TCC TCC ATC AAT GAC-3' (* denotes the position of the 39bp to be removed) in combination with vector specific primers. After digestion with BamHI and XbaI, the PCR product was inserted into pcDNA3.1 (Invitrogen, Breda, Netherlands).
RNA silencing of the USF1 gene was achieved with a shUSF1 plasmid as described previously [31,32]; shAQP3 plasmids against non-related AQP3 gene (a kind gift from B. Tilly, Biochemistry, Rotterdam) served as negative control. pRL-GAPDH, which contains the human glyceraldehyde 3-phosphate dehydrogenase gene promoter in pRL-null, was kindly provided by A.A.F. de Vries (LUMC, Leiden, Netherlands).

7.5.3 Promoter-reporter assays

Promoter reporter assays were performed in transiently transfected HepG2 cells, as described previously [23]. Cells were co-transfected with luciferase reporter constructs (0.4 µg/well), pRL-GAPDH (20 ng/well) and the indicated amounts of pSREBP1, pSREBP2 or shUSF1, complemented with pcDNA3.1. After 3h, the media were refreshed and BSA-bound oleate or cholesterol+25-hydroxycholesterol was added when indicated. Media were refreshed again at 24h. After 48h, cell extracts were prepared with lysis buffer (Roche, Basel, Switzerland), and firefly and renilla luciferase activities were determined with the FireLight kit (Perkin-Elmer, Boston MA, USA) and the Packard Top Count NXT luminometer. Luciferase activities were expressed as the ratio between firefly and renilla counts.

7.5.4 Protein expression

Nuclear extracts were prepared at 48h post-transfection [32], and protein concentrations were determined by DC protein assay (Bio-Rad). Alternatively, USF and SREBP2 proteins were expressed *in vitro* using the TnT T7 Quick Coupled Transcription/Translation system (Promega) with the appropriate expression vectors as DNA template. Nuclear extracts (20 mg) and TnT expression mixtures (0.5-1 ml) were separated by SDS-PAGE on 10% gels, and transferred to nitrocellulose membrane (Hybond ECL; Amersham Biosciences, Amersham, UK). After blocking overnight with 5% milk powder in TBS (20 mM Tris pH 7.6 in 150 mM NaCl), the membranes were incubated with either rabbit polyclonal anti-human SREBP2 (RS004, kindly provided by R. Sato, University of Tokyo, Japan [47]; 1:500 dilution in 5% milkpowder/0.05% Tween-20 in TBS), mouse monoclonal anti-SREBP2 (IgG-1D2 hybridoma supernatant (ATCC, Rockville, MD, USA [48]; 1:50 dilution in 0.5% milk powder/0.05% Tween-20 in TBS), or a 1:4000 dilution of rabbit polyclonal anti-human USF1 or USF2 (SC-229X and SC-862X, respectively; Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:4000 dilution in 0.5% milk powder/0.05% Tween-20 in TBS). Subsequently, the blots were incubated for 1h with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amerham Biosciences, UK) or goat anti-mouse IgG (Promega), diluted 1:2500 in 0.5% milkpowder/0.05% Tween-20 in TBS. The secondary antibody was visualized by enhanced chemiluminescence (Super Signal West Pico Chemiluminescent Substrate, Pierce,

Rockford, IL, USA) and exposure to Hyper ECL film (Amersham Biosciences, UK). The images were quantified by densitometry using the GS-800 Calibrated Densitometer from BioRad.

7.5.5 Statistics

Data are expressed as means±sd. Statistical significances were determined by Student's *t*-test.

7.6 Conclusions

We have shown here that oleate increases the nuclear abundance of USF1 in human hepatoma cells. Together with previous studies, this suggests that oleate affects expression of the HL gene through USF1. USF1 may be elevated by oleate secondary to suppression of nuclear SREBP2 activity, but our data suggests that oleate affects USF1 independently of SREBP2. Hence, USFs may represent an additional class of transcription factors, besides SREBPs and PPARS, through which fatty acids affect mammalian gene expression. USF1 has been shown to be a sensor of nutritional status during fasting and refeeding in the liver [38]. Our findings suggest that USF1 may function as a sensor of fatty acid supply to the liver.

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References and Notes

1. Jump, D.B. Dietary polyunsaturated fatty acids and regulation of gene transcription. *Curr. Opin. Lipidol.* **2002,** *13*, 155–164.
2. Bordoni, A.; Di Nunzio, M.; Danesi, F.; Biagi, P.L. Polyunsaturated fatty acids: from diet to binding to PPARs and other nuclear receptors. *Genes Nutr.* **2006**, *1*, 95–106.
3. Sonoda, J.; Pei, L.; Evans, R.M. Nuclear receptors: decoding metabolic disease. FEBS Lett. **2008**, *582*, 2-9.
4. Horton, J.D.; Shimomura, I. Sterol regulatory element-binding proteins: activators of cholesterol and fatty acid biosynthesis. *Curr. Opin. Lipidol.* **1999**, *10*, 143-150.
5. Ikonen, E. Cellular cholesterol trafficking and compartmentalization. *Nature Rev. Mol. Cell Biol.* **2008**, *9*, 125-138.
6. Worgall, T.S.; Sturley, S.L.; Seo, T.; Osborne, T.F.; Deckelbaum, R.J. Polyunsaturated fatty acids decrease expression of promoters with sterol regulatory elements by decreasing levels of mature sterol regulatory element-binding protein. *J. Biol. Chem.* **1998**, *273*, 25537–25540.
7. Sanderson, L.M.; de Groot, P.J.; Hooiveld, G.J.; Koppen, A.; Kalkhoven, E.; Müller, M.; Kersten, S. Effect of synthetic dietary triglycerides: a novel research paradigm for nutrigenomics. PLoS ONE **2008**, *3*, e1681.
8. Xu, J.; Cho, H.; O'Malley, S.; Park, J.H.; Clarke, S.D. Dietary polyunsaturated fats regulate rat liver sterol regulatory element binding proteins-1 and -2 in three distinct stages and by different mechanisms. *J. Nutr.* **2002**, *132*, 3333-3339.
9. Sekiya, M.; Yahagi, N.; Matsuzaka, T.; Najima, Y.; Nakakuki, M.; Nagai, R.; Ishibashi, S.; Osuga, J.; Yamada, N.; Shimano, H. Polyunsaturated fatty acids ameliorate hepatic steatosis in obese mice by SREBP-1 suppression. *Hepatology* **2003**, *38*, 1529-1539.
10. Le Jossic-Corcos, C.; Gonthier, C.; Zaghini, I.; Logette, E.; Shechter, I.; Bournot, P. Hepatic farnesyl diphosphate synthase expression is suppressed by polyunsaturated fatty acids. *Biochem. J.* **2005**, *385*, 787-794.
11. Botolin. D.; Wang, Y.; Christian, B.; Jump, D.B. Docosahexaneoic acid (22:6,n-3) regulates rat hepatocyte SREBP-1 nuclear abundance by Erk- and 26S proteasome-dependent pathways. *J. Lipid Res.* **2006**, *47*, 181-192.
12. Di Nunzio, M.; van Deursen, D.; Verhoeven, A.J.M.; Bordoni, A. n-3 and n-6 PUFAs suppress SREBP activity and increase flow of free cholesterol in HepG2 cells. *Br. J. Nutr*. **2009**, *103*, 161-7.
13. Jansen, H.; Verhoeven, A.J.M.; Sijbrands, E.J.G. Hepatic lipase: a pro- or anti-atherogenic protein? *J. Lipid Res.* **2002**, *43*, 1352-1362.
14. Perret, B.; Mabile, L.; Martinez, L.; Tercé, F.; Barbaras, R.; Collet, X. Hepatic lipase: structure/function relationship, synthesis, and regulation. *J. Lipid Res.* **2002**, *43*, 1163-1169.
15. Santamarina-Fojo, S.; González-Navarro, H.; Freeman, L.; Wagner, E.; Nong, Z. Hepatic lipase, lipoprotein metabolism, and atherogenesis. Arterioscler. Thromb. Vasc. Biol. **2004**, 24, 1750-1754.
16. Berk-Planken, I.I.; Hoogerbrugge, N.; Stolk, R.P.; Bootsma, A.H.; Jansen, H.; DALI Study Group. Atorvastatin dose-dependently decreases hepatic lipase activity in type 2 diabetes: effect of sex and the LIPC promoter variant. *Diabetes Care* **2003**, *26*, 427-432.
17. Jansen, H.; Verhoeven, A.J.; Weeks, L.; Kastelein, J.J.; Halley, D.J.; van den Ouweland, A.; Jukema, J.W.; Seidell, J.C.; Birkenhäger, J.C. Common C-to-T substitution at position -480 of the hepatic lipase promoter associated with a lowered lipase activity in coronary artery disease patients. *Arterioscler. Thromb. Vasc. Biol.* **1997**, *17*, 2837-2842.
18. Carr, M.C.; Hokanson, J.E.; Deeb, S.S.; Purnell, J.Q.; Mitchell, E.S.; Brunzell, J.D. A hepatic lipase gene promoter polymorphism attenuates the increase in hepatic lipase activity with increasing intra-abdominal fat in women. *Arterioscler. Thromb. Vasc. Biol.* **1999**, *19*, 2701-2707.
19. Carr, M.C.; Hokanson, J.E.; Zambon, A.; Deeb, S.S.; Barrett, P.H.; Purnell, J.Q.; Brunzell, J.D. The contribution of intraabdominal fat to gender differences in hepatic lipase activity and low/high density lipoprotein heterogeneity. *J. Clin. Endocrinol. Metab.* **2001,** *86*, 2831-2837.
20. Summerfield, J.A.; Applebaum-Bowden, D.; Hazzard, W.R. Effects of diet and age on lipoprotein lipase and hepatic triglyceride lipase activities in the rat. *Proc. Soc. Exp. Biol. Med.* **1984**, *175*, 158–163.

21. Benhizia, F.; Hainault, I.; Serougne, C.; Lagrange, D.; Hajduch, E.; Guichard, C.; Malewiak, M.I.; Quignard-Boulange, A.; Lavau, M.; Griglio, S. Effects of a fish oil-lard diet on rat plasma lipoproteins, liver FAS, and lipolytic enzymes. *Am. J. Physiol.* **1994**, *267*, E975–E982.
22. Nimmo, L.; McColl, A.J.; Rosankiewicz, J.Z.; Richmond, W.; Elkeles, R.S. Regulation of hepatic lipase expression in HepG2 cells. *Biochem. Soc. Trans.* **1997**, *25*, S689.
23. Botma, G.J.; van Deursen, D.; Vieira, D.; van Hoek, M.; Jansen, H.; Verhoeven, A.J. Sterol-regulatory-element binding protein inhibits upstream stimulatory factor-stimulated hepatic lipase gene expression. *Atherosclerosis* **2005**, *179*, 61–67.
24. Heller, F.; Harvengt, C. Effects of clofibrate, bezafibrate, fenofibrate and probucol on plasma lipolytic enzymes in normolipaemic subjects. *Eur. J. Clin. Pharmacol.* **1983**, *25*, 57-63.
25. Desager, J.P.; Horsmans, Y.; Vandenplas, C.; Harvengt, C. Pharmacodynamic activity of lipoprotein lipase and hepatic lipase, and pharmacokinetic parameters measured in normolipidaemic subjects receiving ciprofibrate (100 or 200 mg/day) or micronised fenofibrate (200 mg/day) therapy for 23 days. *Atherosclerosis* **1996**, *124*, S65-73.
26. Staels, B.; Peinado-Onsurbe, J.; Auwerx, J. Down-regulation of hepatic lipase gene expression and activity by fenofibrate. *Biochim. Biophys. Acta* **1992**, *1123*, 227-230.
27. Hoogerbrugge, N.; Jansen, H. Atorvastatin increases low-density lipoprotein size and enhances high-density lipoprotein cholesterol concentration in male, but not in female patients with familial hypercholesterolemia. Atherosclerosis **1999**, *146*, 167-174.
28. Benhizia, F.; Lagrange, D.; Malewiak, M.I.; Griglio, S. In vivo regulation of hepatic lipase activity and mRNA levels by diets which modify cholesterol influx to the liver. *Biochim. Biophys. Acta* **1994**, *1211*, 181–188
29. Corre, S.; Galibert, M.D. Upstream stimulating factors: highly versatile stress-responsive transcription factors. *Pigment Cell Res.* **2005**, *18*, 337-348.
30. Lee, J.C.; Lusis, A.J.; Pajukanta, P. Familial combined hyperlipidemia: upstream transcription factor 1 and beyond. *Curr. Opin. Lipidol.* **2006**, *17*, 101-109.
31. van Deursen, D.; van Leeuwen, M.; Vaulont, S.; Jansen, H.; Verhoeven, A.J. Upstream Stimulatory Factors 1 and 2 activate the human hepatic lipase promoter via E-box dependent and independent mechanisms. *Biochim. Biophys. Acta* **2009**, *1791*, 229-237.
32. van Deursen, D.; Jansen, H.; Verhoeven, A.J. Glucose increases hepatic lipase expression in HepG2 liver cells through upregulation of upstream stimulatory factors 1 and 2. *Diabetologia* **2008**, *51*, 2078-2087.
33. Part of this paper has been presented at the FoodOmics meeting in Cesena, Italy, in May 2009.
34. Horton, J.D.; Shah, N.A.; Warrington, J.A.; Anderson, N.N.; Park, S.W.; Brown, M.S.; Goldstein, J.L. Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 12027-12032.
35. Yokoyama, C.; Wang, X.; Briggs, M.R.; Admon, A.; Wu, J.; Hua, X.; Goldstein, J.L.; Brown, M.S. SREBP-1, a basic-helix-loop-helix-leucine zipper protein that controls transcription of the low density lipoprotein receptor gene. *Cell* **1993**, *75*, 187-197.
36. Sato, R.; Yang, J.; Wang, X.; Evans, M.J.; Ho, Y.K.; Goldstein, J.L.; Brown, M.S. Assignment of the membrane attachment, DNA binding, and transcriptional activation domains of sterol regulatory element-binding protein-1 (SREBP-1). *J. Biol. Chem.* **1994**, *269*, 17267-17273.
37. Nowak, M.; Helleboid-Chapman, A.; Jakel, H.; Martin, G.; Duran-Sandoval, D.; Staels, B.; Rubin, E.M.; Pennacchio, L.A.; Taskinen, M.R.; Fruchart-Najib, J.; Fruchart, J.C. Insulin-mediated down-regulation of apolipoprotein A5 gene expression through the phosphatidylinositol 3-kinase pathway: role of upstream stimulatory factor. *Mol. Cell Biol.* **2005**, *25*, 1537-1548.
38. Wong, R.H.; Chang, I.; Hudak, C.S.; Hyun, S.; Kwan, H.Y.; Sul, H.S. A role of DNA-PK for the metabolic gene regulation in response to insulin. *Cell* **2009**, *136*, 1056-1072.
39. Zhang, L.; Handel, M.V.; Schartner, J.M.; Hagar, A.; Allen, G.; Curet, M.; Badie, B. Regulation of IL-10 expression by upstream stimulating factor (USF-1) in glioma-associated microglia. *J. Neuroimmunol.* **2007**, *184*, 188-197.
40. Viswanathan, P.; Wood, M.A.; Walker, W.H. FSH transiently blocks FSH receptor transcription by increasing ID2 and decreasing Upstream Stimulatory Factor (USF) expression in rat Sertoli cells. *Endocrinology* **2009**, *150*, 3783-3791.

41. van Deursen, D.; di Nunzio, M. Unpublished observations.

42. Athanikar, J.N.; Osborne, T.F. Specificity in cholesterol regulation of gene expression by coevolution of sterol regulatory DNA element and its binding protein. *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 4935-4940.

43. Leichman, J.G.; Aguilar, D.; King, T.M.; Vlada, A.; Reyes, M.; Taegtmeyer, H. Association of plasma free fatty acids and left ventricular diastolic function in patients with clinically severe obesity. Am. J. Clin. Nutr. **2006**, *84*, 336-341.

44. Verhoeven, A.J.; Neve, B.P.; Jansen, H. Secretion and apparent activation of human hepatic lipase requires proper oligosaccharide processing in the endoplasmic reticulum. *Biochem. J.* **1999**, *337*, 133-140.

45. Sambrook, J.; Fritsch, E.F.; Maniatis, T. *Molecular Cloning, a Laboratory Manual*, 2nd Ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, N.Y., 1989.

46. Higuchi, R.; Krummel, B.; Saiki, R. A general method of *in vitro* preparation and specific mutagenesis of DNA fragments: study of protein and DNA interactions. *Nucl. Acids Res.* **1988**, *16*, 7351-7367.

47. Sato, R.; Miyamoto, W.; Inoue, J.; Terada, T.; Imanaka, T.; Maeda, M. Sterol regulatory element-binding protein negatively regulates microsomal triglyceride transfer protein gene transcription. *J. Biol. Chem.* **1999**, *274*, 24714-24720.

48. Yabe, D.; Brown, M.S.; Goldstein, J.L. Insig-2, a second endoplasmic reticulum protein that binds SCAP and blocks export of sterol regulatory element-binding proteins. *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 12753-12758.

CHAPTER 8

n-3 and n-6 polyunsaturated fatty acids suppress sterol regulatory element binding protein activity and increase flow of non-esterified cholesterol in HepG2 cells

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8.1 Abstract

The plasma lipid lowering effect of PUFA, one of their main beneficial effects, is considered to be related to the regulation of lipid biosynthesis through transcription factors including sterol regulatory element binding proteins (SREBP). In this study, we compared the effect of different PUFA on SREBP activity in HepG2 cells, using an SRE-luciferase reporter construct as probe. Supplementation with different fatty acids reduced SREBP activity in the order 20:5n-3=18:2n-6=20:4n-6 >> 18:3n-3=22:6n-3=22:5n-6 >> 18:1n-9. The suppression of SREBP activity greatly depended on the degree of incorporation of the supplemented PUFA into cellular lipids, and correlated positively with the unsaturation index ($r= 0.831$; $p<0.01$) of total cell lipids. Supplemented PUFA were also metabolized to longer and more unsaturated species. These processing activities were higher for n-3 than n-6 PUFA ($p<0.01$). We studied the effect of PUFA on the intracellular distribution of non-esterified cholesterol, using filipin staining and fluorescence microscopy with or without the cholesterol traffic blocker U18666A. The data show that the incorporation of PUFA increases non-esterified cholesterol flow from the plasma membrane to intracellular membranes. We conclude that suppression of SREBP activity by PUFA depends on the degree of incorporation into cellular lipids, and is associated with increased flow of non-esterified cholesterol between the plasma membrane and intracellular membranes.

8.2 Introduction

It is well documented that dietary intake of PUFA, and particularly a correct *n*-6:*n*-3 ratio, contributes to the prevention of many chronic diseases [1]. In order to explain how PUFA can influence so many biological processes, different mechanisms of action have been hypothesized, i.e. the modification of membrane fluidity and functionality through changes in membrane lipid composition [2], the alteration of eicosanoid signalling and the modulation of gene expression [3-5]. Their main beneficial effect, i.e. plasma lipid lowering, is nowadays considered to be related to the regulation of lipid biosynthesis through the transcription factors NF-κB [6], retinoid X receptor [7], PPAR [8], and sterol regulatory element binding proteins (SREBP) [9].

PUFA are thought to interact indirectly with SREBP [10,11], but the mechanism of this interaction is still unclear. It is unlikely that PUFA affect SREBP through liver X receptor (LXR), a major activator of SREBP-1c transcription, as fish oil fed rats showed suppression of hepatic SREBP-1c target genes, but not of LXR target genes such as cytochrome P450 family 7 subfamily A polypeptide 1 (CYP7A1), ATP-binding cassette (ABC)-G5, or ABC-G8 transporters [12]. In addition, in hepatocytes the treatment with EPA (20:5*n*-3) inhibited SREBP-1c controlled genes both in the absence and the presence of a synthetic LXR agonist [13]. Alternatively, PUFA may act by decreasing SREBP mRNA stability [14,15]. A recent study showed that DHA (22:6*n*-3) reduces the abundance of the nuclear form of SREBP-1 (nSREBP-1) in rat hepatocytes through 26S-proteasome- and Erk-dependent pathways [16].

Another possibility is that the SREBP suppression is linked to the incorporation of PUFA into cell membranes. Cholesterol is abundant in mammalian plasma membranes, accounting for as much as 50% (mol/mol) of total lipid. It globally modulates the molecular organization of the membrane, and its distinct affinity for different lipids drives the formation of membrane subdomains [17]. When PUFA are incorporated to a greater extent into membrane phospholipids, their poor affinity for cholesterol drives the formation of PUFA-enriched and cholesterol-depleted subdomains [18]. Since maturation of SREBP is regulated by the non-esterified cholesterol (NEC) content of the endoplasmic reticulum membranes [19], it is conceivable that PUFA inhibit SREBP processing by causing redistribution of NEC from the PM to the endoplasmic reticulum.

To test this hypothesis, we supplemented HepG2 cells with different concentrations of various PUFA, and we compared the degree of their incorporation into cell lipids with the activity of SREBP, measured by using an sterol regulatory element-luciferase (SRE-luc) construct as a probe. Furthermore, the distribution of NEC over the PM and intracellular membranes was assessed by fluorescence microscopy. We chose HepG2 cells as model system since the liver plays a central role in the regulation of cholesterol homeostasis.

8.3 Methods and materials

8.3.1 Materials

Dulbecco's modified Eagle's medium and Dulbecco's PBS were purchased from Lonza (Breda, The Netherlands), and Reporter Gene assay lysis buffer from Roche (Almere, The Netherlands). Fetal calf serum and Lipofectamine Plus were from Invitrogen (Groningen, The Netherlands), and U18666A and fatty acids from Sigma (St Louis, MO, USA). All other chemicals and solvents were of the highest analytical grade. The SRE luciferase-reporter construct SRE-luc was generated by insertion of part of the hamster hydroxyl-3-methylglutaryl-CoA synthase promoter region into pGL3-Basic [20]. pGAP-RL (a kind gift from Dr. AAF de Vries, Leiden, the Netherlands) contained part of the human glyceraldehyde 3-phosphate dehydrogenase (GAP) promoter, and was generated by insertion of the 0.5-kb HindIII-XhoI fragment of pGAP489CAT [21] into pRL-null.

8.3.2 Methods

8.3.2.1 HepG2 cells tissue culture

HepG2 human hepatoma cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, penicillin (100 IU/ml) and streptomycin (100 µg/ml) at 37°C, 95% air, 5% CO_2. Once per week, cells were split 1:10 into a new 75cm^2 flask; medium was refreshed once per week.

8.3.2.2 Fatty acid supplementation

Fatty acids were dissolved at different concentrations (6, 60 and 120 mM w/v) in 100% isopropanol, and bound to bovine serum albumin (BSA). Fatty acid-BSA complexes were prepared fresh each time at a final BSA concentration of 0.5% (in serum free Dulbecco's modified Eagle's medium), and cells were incubated for 21 h with fatty acids. Final isopropanol concentration in the media was kept below 1% (v/v). Control cells received corresponding amounts of BSA and isopropanol.

8.3.2.3 Determination of HepG2 fatty acid composition

Cells were seeded in six-well plates. After 24 h, at 75-80% confluence, cells were supplemented with fatty acid-BSA complexes at 60 µM-fatty acid concentration. After 21 h, cells were washed four times with ice-cold Dulbecco's PBS, scraped off and collected by centrifugation for 3 min at 1,000g and 4°C. Total cellular lipids were extracted according to Folch *et al*. [22], and methyl esterified according to Stoffel *et al*. [23]. The fatty acid composition (as methyl esters) was determined by GC (GC 8000, Fisons, Milan, Italy) using a capillary column (SP 2340, 0.2 µm film thickness) at a programmed temperature gradient (160–210°C, 8°C/min) as previously reported [24].

8.3.2.4 Luciferase assay

Cells were seeded in twenty-four-well plates. After 24 h, at 75-80% confluence, cells were transfected with SRE-luc (0.4 µg/well) and pGAP-RL (60 ng/well) using Lipofectamine Plus as described previously [20]. After 3 h, the medium was refreshed and supplemented with fatty acids at 6, 60 and 120 µM (w/v) concentrations. After 21 h, cells were washed three times with ice-cold Dulbecco's PBS and lysed. Luciferase activity was determined in the cell extracts with the Dual-Glo luciferase assay kit (Promega, Leiden, The Netherlands) using a Packard Top Count NXT luminometer (Meriden, CT, USA). Data were normalised for Renilla activity measured in the same sample to account for differences in cell viability and transfection efficiency. At 120µM-PUFA, Renilla expression levels were similar for the different fatty acids used, and amounted to 5721 (SD 471) counts (n 4) v. 6115 (SD 1986) counts (n 4) without fatty acid supplementation ($p=0.713$; paired t-test).

8.3.2.5 Filipin staining

HepG2 cells were seeded on coverslips. After 24 h, cells at about 20% confluence were supplemented with 60µM-fatty acid-BSA complex. In some experiments U18666A (2µg/ml), a blocker of intracellular cholesterol trafficking [25], was also added. Cells were washed after 21 h incubation, and fixated using 3% paraformaldehyde in Dulbecco's PBS for 60 min. Thereafter, the cells were washed three times, treated with 50 mM-glycine in Dulbecco's PBS for 30 min to quench paraformaldehyde, and stained with filipin (40 µg/ml) for a further 30 min. Coverslips were mounted on microscope slides and the epifluorescence was examined using an inverted Olympus IX50 microscope. Images were acquired and analysed using AnalySiS imaging software (Soft Imaging Systems, Münster, Germany) as previously described [26].

8.3.3 Statistical analysis

Data are presented as mean values and standard deviations of at least three independent experiments. Differences were tested for statistical significance by unpaired Student's t-test ($p<0.05$).

8.4 Results

The incorporation of supplemented PUFA into cell lipids was determined by GC (Table 1). All PUFA were readily incorporated at the expense of MUFA, while the molar content of SFA was not significantly affected. When incubated with linoleic acid (LA;18:2*n*-6), α-linolenic acid (ALA; 18:3*n*-3), arachidonic acid (ARA; 20:4*n*-6) or EPA, the corresponding elongated and more unsaturated fatty acids accumulated as well, suggesting that these PUFA are further metabolised upon cellular uptake. Metabolic conversion of the *n*-3 fatty acids was more efficient than the *n*-6 fatty acids. In fact in LA-supplemented cells the products:precursor ratio was 0.29 (SD 0.04), compared to 0.70 (SD 0.13) in ALA-supplemented cells ($p<0.01$). Supplementation

Table 1. Fatty acid composition (mol %) of HepG2 cells in control condition and after PUFA supplementation[1]. (Mean values and standard deviations for three independent experiments)

	Fatty acid supplemented															
			n-9		n-6						n-3					
	None		18:1 (OA)		18:2 (LA)		20:4 (ARA)		22:5 (DPA)		18:3 (ALA)		20:5 (EPA)		22:6 (DHA)	
Cellular lipids	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
14:0	4.32	0.56	3.86	1.02	4.23	0.5	4.74	0.93	4.42	0.39	4.27	0.57	4.32	0.21	3.94	0.65
16:0	31.34	0.96	28.43	2.76	30.15	1.94	32.14	1.32	31.63	0.68	31.26	2.79	32.26	0.91	30.99	1.41
16:1 n-7	10.26	0.32	8.44**	0.35	7.89**	0.77	8.28**	0.32	9.77	0.99	9.17	0.82	9.29	0.72	9.99	1.52
18:0	8.37	0.73	8.74	2.01	9.09	2.58	8.04	0.5	8.88	2.44	9	1.94	7.69	1.12	9.37	2.87
18:1 n-7/n-9	40.36	0.39	44.89	2.90	32.34**	2.58	32.44***	0.69	37.66*	1.11	31.52**	3.09	33.96***	0.91	38.02*	1.20
18:2 n-6	1.24	0.12	1.34	0.21	10.93***	1.16	1.09	0.03	1.30	0.16	1.32	0.23	1.25	0.08	1.33	0.02
18:3 n-3	1.21	0.15	1.50	0.19	0.98	0.18	0.94	0.13	1.12	0.13	7.13**	1.63	0.97	0.23	1.16	0.16
20:4 n-6	1.6	0.19	1.62	0.5	3.19**	0.27	10.57**	1.38	1.69	0.22	1.4	0.41	1.58	0.36	1.6	0.34
20:5 n-3	0.14	0.03	0.13	0.07	0.05**	0.01	0.04*	0.03	0.11	0.02	2.96***	0.25	4.79***	0.37	0.19	0.07
22:5 n-6	0	0	0	0	0	0	0.71***	0.34	2.37***	0.22	0	0	0	0	0	0
22:5 n-3	0.16	0.03	0.18	0.08	0.18	0.05	0.20	0.05	0.13	0.02	0.75*	0.25	1.95***	0.23	0.16	0.02
22:6 n-3	0.99	0.17	1.06	0.41	0.97	0.22	0.82	0.24	0.94	0.06	1.22	0.35	1.93**	0.17	3.26**	0.46

OA, oleic acid; LA, linoleic acid; ARA, arachidonic acid; DPA, docosapentaenoic acid; ALA, α-linolenic acid.
Mean value was significant different from that of control cells: * P<0.05; ** P<0.01; *** P<0.001.
[1]: Cells were incubated for 21 h with 60μM-fatty acids.

with oleic acid (OA; 18:1*n*-9) did not result in significant changes in relative content of OA, nor of any of the other fatty acids.

The effect of *n*-6 and *n*-3 PUFA on SRE-luc activity in HepG2 cells is shown in figure 1A and figure 1B, respectively. PUFA were supplemented at a very low concentration (6 µM) and at two physiological plasma concentrations (60 and 120 µM). Data were compared with the effects of corresponding concentrations OA. OA did not cause any reduction of SRE-luc activity at 6 µM, and only a mild reduction at 60 µM. At 6 µM, LA and ARA reduced SRE-luc activity by 50 % (Figure 1A). Maximal inhibition of 75% was observed at 60 and 120 mM. Docosapentaenoic acid (22:5*n*-6) had no effect at the low concentration, while its inhibitory effect was similar to LA and ARA at 120 µM. Among *n*-3 PUFA (Figure 1B), EPA appeared as effective as LA and ARA at all concentrations used. In contrast, the inhibitory effect of ALA and DHA was apparent only at 60 and 120 µM.

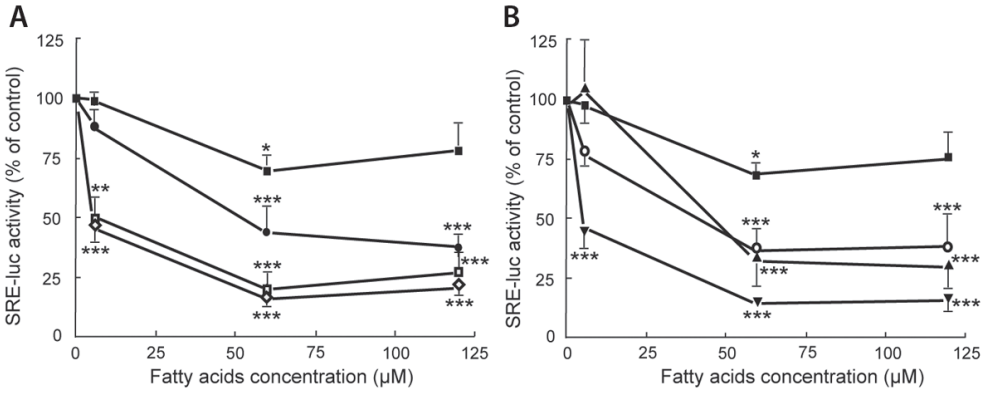

Figure 1. Effect of n-6 and n-3 PUFA supplementation on sterol regulatory element-luciferase (SRE-luc) activity.

HepG2 cells were incubated for 21 h with different concentrations of *n-6* (A) and *n-3* (B) fatty acids, and the effect on SRE-luc activity was determined. Fatty acids used were linoleic acid (□), arachidonic acid (◊), docosapentaenoic acid (●), α-linolenic acid (O), EPA (▼) and DHA (▲). The effects of PUFA were compared with similar concentrations of oleic acid (■). Data are expressed as percentage of control from three separate experiments each performed in quadruplicate. Values are means, with standard deviations represented by vertical bars. Mean value was significantly different from that of control cells: * P<0.05; ** P<0.01; *** P<0.001.

We then compared the degree of cellular lipid modification with the inhibition of SRE-luc activity obtained in 60µM-PUFA-supplemented cells. Interestingly, the more a supplemented fatty acid or its metabolites were incorporated into total cellular lipids, the stronger was its effect on SRE-luc activity. An inverse correlation was present between SRE-luc activity and the total PUFA conten of cellular membranes (r -0.79; p=0.02) (Figure 2A), and between SRE-luc activity and the unsaturation index of total cellular lipids (r -0.83; p<0.01) (Figure 2B). It is

therefore evident that the enrichment of PUFA in HepG2 cells is correlated to the degree of suppression of SREBP activity.

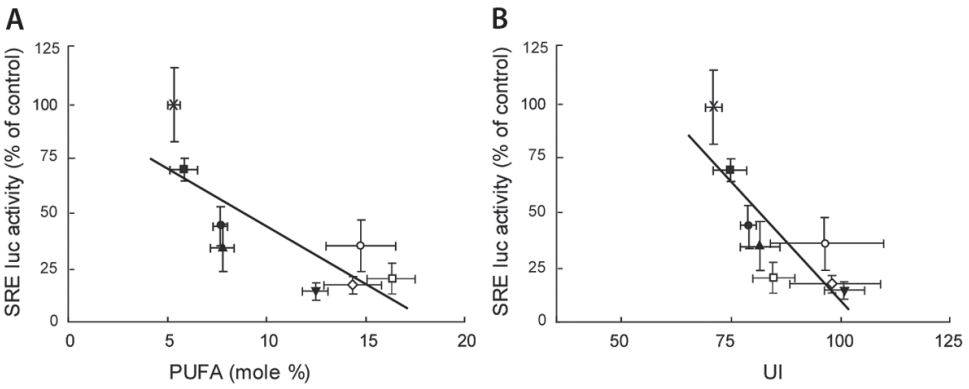

Figure 2. Correlations between regulatory element-luciferase (SRE-luc) activity and cell membrane fatty acid composition.

Cells were incubated for 21 h without (*) or with 60µM-fatty acids. Fatty acids used were oleic (■), linoleic (□), arachidonic (◊), docosapentaenoic (●), α-linolenic (O), eicosapentaenoic (▼) and docosahexaenoic (▲) acids. Total lipid fatty acid composition was obtained by GC, as reported in table 1. Unsaturation index (UI) was obtained by multiplying the relative molar content of each fatty acid by its number of double bonds, then adding up all obtained values. Values on PUFA content (A) or UI (B) and SRE-luc activity are means, with standard deviations represented by horizontal and vertical bars, respectively. SRE-luc activity negatively correlated with PUFA content (r -0.79; p=0.02) and UI (r -0.83; p<0.01).

To evaluate the intracellular distribution of NEC in HepG2 cells, filipin staining followed by fluorescence microscopy was performed in control and 60 µM-fatty acid-BSA-supplemented cells. In control cells, NEC appeared to be mainly localized at the cell periphery, in proximity to the PM, with a low intracellular staining intensity (Figure 3A). The relative distribution of cholesterol was not affected by any of the supplemented PUFA, as illustrated for EPA in figure 3B. Cholesterol is continuously transported from the PM to intracellular membrane, and vice versa. To estimate the effect of PUFA on the flow of cholesterol from the PM to intracellular membranes, we used U18666A, an inhibitor of cholesterol export from endo- and lysosomes [25]. In the presence of U18666A, filipin staining at the cell periphery was markedly reduced (Figure 3C). Simultaneously, intracellular staining appeared as small clusters of bright spots, indicating that NEC is confined to intracellular organelles. The NEC that accumulated in the intracellular organelles apparently reflects the amount of NEC that has been transported from the PM during incubation with U18666A. When cells were co-incubated with U18666A and EPA (Figure 3D), intracellular staining increased further and the cell periphery also stained strongly (cf. Figures 3C and D). Compared to U18666A alone, the number and size of the intracellular staining spots were markedly increased. The increased intracellular accumulation of NEC in EPA-supplemented cells indicates increased flow of cholesterol from the PM to endo- and lysosomes.

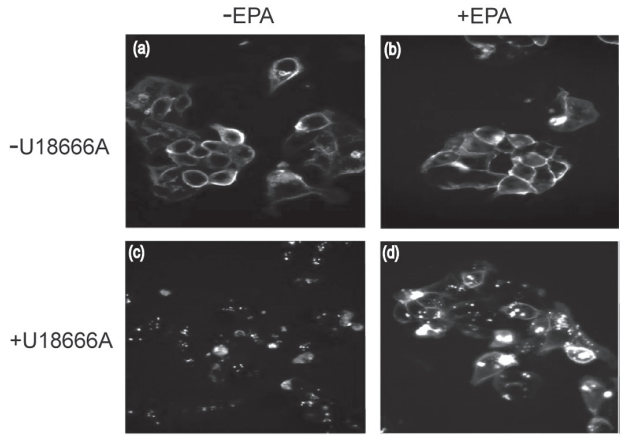

Figure 3. Effect of EPA and U18666A on non-esterified cholesterol distribution in HepG2 cells.

Cells were incubated for 21 h without (A,C) or with 60µM-bovine serum albumin-bound EPA (B,D), and without (A,B) or with U18666A (2µg/ml) (C,D), and then stained for non-esterified cholesterol by filipin. Data are representative of three independent experiments.

8.5 Discussion

It is 40 years since Keys & Parlin [27] developed predictive equations to quantify the effects of fatty acids and dietary cholesterol on plasma cholesterol concentrations, and dietary PUFA were reported as important regulators of cholesterol metabolism. It is widely accepted that PUFA modulate the expression of genes involved in lipid metabolism, and SREBP have emerged as key mediators of this regulation. Nevertheless, the exact mechanism(s) by which PUFA interact with SREBP is still unclear, as well as the effectiveness of different *n*-6 and *n*-3 PUFA. To clarify this, we supplemented HepG2 cells with both *n*-6 and *n*-3 PUFA, while the monounsaturated OA was used for comparison.

The degree of incorporation of supplemented PUFA and their metabolites appeared to be an important determinant of their inhibitory effect on SREBP activity. In fact, the more they were incorporated into total cellular lipids, the lower was SREBP activity. This is further illustrated by the inverse correlation between SREBP activity and the unsaturation index of cellular lipids. It is therefore evident that suppression of SREBP activity by PUFA in HepG2 cells depends on the degree of their enrichment in cellular lipids. Supplementation with 60-120µM-PUFA reduced SREBP activity in the order EPA = LA = ARA >> ALA =DHA = docosapentaenoic acid. In a recent review Jump *et al.* [28] indicated DHA as the most active PUFA in modulating hepatic gene transcription and the most potent suppressor of SREBP-1 nuclear abundance. Actually, in rat primary hepatocytes, DHA was more effective than EPA but ineffective below 100µM in reducing nSREBP-1 protein [16]. Worgall *et al.* [29] found that LA, ARA, ALA and

docosapentaenoic acid were similarly effective in HepG2 cells at 300μM; EPA and DHA were not included in this study. In rat hepatoma cells, ARA, EPA and DHA (150μM) were more effective in suppressing nSREBP-1 protein than LA and ALA (300μM) [30]. These differences in efficacy may be due to the different experimental models or the different PUFA:albumin ratios used. In addition, the reporter assay used in the present study does not discriminate between SREBP isoforms, and some PUFA may also suppress nSREBP-2. Supplemented PUFA were not only incorporated into cell lipids as such, but they were also converted to longer and more unsaturated species by the HepG2 cells. This is in contrast to Yu-Poth *et al.* [31] but in agreement with El-Badry *et al.* [32]. In accordance with this last paper, we found these processing activities to be higher for *n*-3 than *n*-6 PUFA, as indicated by the higher product:precursor ratios for 18:3*n*-3 than 18:2*n*-6.

Our fluorescence microscopy studies are consistent with an increased flow of NEC from the PM to intracellular membranes in PUFA-supplemented HepG2 cells. It is conceivable that incorporation of PUFA displaces cholesterol from the PM, thus increasing NEC flow to intracellular membranes. In the PM cholesterol is associated with sphingomyelin [29]. In HL-60 cells and human neutrophils, the increase in PUFA concentration stimulates neutral sphingomyelinase activity [33,34]. Treatment of cultured cells with sphingomyelinase, thereby degrading the major raft sphingolipids, leads to a rapid increase in intracellular cholesterol content and subsequent inhibition of SREBP maturation [35]. In control cells, NEC appeared to be mainly localised at the cell periphery, in proximity to the PM, with a low intracellular localisation. The similar staining pattern observed in PUFA-supplemented cells argues against a major re-distribution of NEC from the PM to intracellular membranes. However, cholesterol in the PM is continuously turning over and it is estimated that the entire PM cholesterol pool cycles to the ER and back with a half-time of 40 min [36]. Therefore, suppression of SREBP activity may result from subtle increases in intracellular NEC not detectable by filipin staining. However, in the presence of U18666A, which inhibits NEC basal movements, the increased accumulation of NEC in intracellular organelles strongly suggests increased trafficking of cholesterol between the PM and intracellular compartments. Hence, it may be the NEC dynamics in the intracellular membranes rather than the NEC concentration that affects the post-translational maturation of SREBP.

Taken together, our data suggest that both *n*-3 and *n*-6 PUFA suppress SREBP activity in HepG2 cells via the increased flow of NEC from the PM to intracellular membranes, in proportion to their accumulation in cellular lipids. Besides, the regulation of SREBP activity by PUFA may also involve other mechanisms, such as sphingomyelin hydrolysis and consequent ceramide production [37], PPAR activation [38], and accelerated degradation of nSREBP by a 26S proteasome-dependent pathway [16], mechanisms that may or may not be secondary to PUFA incorporation into cellular membranes. Lowering of liver SREBP-1 protein levels by PUFA-enriched oil feeding has been consistently reported for rats and mice [16,39,40], suggesting that our finding may hold also *in vivo*. Although additional studies are needed to elucidate how PUFA control SREBP activity, and thereby lipid metabolism, our data may contribute to the

further development of PUFA as nutritional therapeutic agents for management of cholesterol and lipid homeostasis.

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M. di N. and D. v. D. performed the analysis. A.J.M.V. and A. B. designed and supervised the study, and wrote this paper.

All authors state that there is no conflict of interest associated with this study.

References

1. Simopoulos AP (2008) The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. *Exp Biol Med* **233**, 674–688.
2. Rajamoorthi K, Petrache HI, McIntosh TJ *et al*. (2005) Packing and viscoelasticity of polyunsaturated ω-3 and ω-6 lipid bilayers as seen by ^{2}H NMR and X-ray diffraction. *J Am Chem Soc* **127**, 1576–1588.
3. Worgall TS & Deckelbaum RJ (1999) Fatty acids: links between genes involved in fatty acid and cholesterol metabolism. *Curr Opin Clin Nutr Metab Care* **2**, 127–133.
4. Benatti P, Peluso G, Nicolai R *et al.* (2004) Polyunsaturated fatty acids: biochemical, nutritional and epigenetic properties. *J Am Coll Nutr* **23**, 281–302.
5. Sampath H & Ntambi JM (2005) Polyunsaturated fatty acid regulation of genes of lipidmetabolism. *Annu Rev Nutr* **25**, 317–340.
6. Zhao Y, Joshi-Barve S, Barve S *et al*. (2004) Eicosapentaenoic acid prevents LPS-induced TNF-α expression by preventing NF-kB activation. *J Am Coll Nutr* **23**, 71-78.
7. Lengqvist J, Mata De Urquiza A, Bergman AC *et al*. (2004) Polyunsaturated fatty acids including docosahexaenoic and arachidonic acid bind to the retinoid X receptor alpha ligand-binding domain. *Mol Cell Proteomics* **3**, 692–703.
8. Edwards IJ & O'Flaherty JT (2008) Omega-3 fatty acids and PPARγ in cancer. *PPAR Res* **2008**, 358052.
9. König B, Spielmann J, Haase K *et al*. (2008) Effects of fish oil and conjugated linoleic acids on expression of target genes of PPAR alpha and sterol regulatory element-binding proteins in the liver of laying hens. *Br J Nutr* **100**, 355–363.
10. Nakamura MT, Cheon Y, Li Y *et al*. (2004) Mechanisms of regulation of gene expression by fatty acids. *Lipids* **39**, 1077–1083.
11. Bordoni A, Di Nunzio M, Danesi F *et al*. (2006) Polyunsaturated fatty acids: from diet to binding to ppars and other nuclear receptors. *Genes Nutr* **1**, 95–106.
12. Pawar A, Botolin D, Mangelsdorf DJ *et al*. (2003) The role of liver X receptor-alpha in the fatty acid regulation of hepatic gene expression. *J Biol Chem* **278**, 40736–40743.
13. Nakatani T, Katsumata A, Miura S *et al*. (2005) Effects of fish oil feeding and fasting on LXRalpha/RXRalpha binding to LXRE in the SREBP-1c promoter in mouse liver. *Biochim Biophys Acta* **1736**, 77–86.
14. Hannah VC, Ou J, Luong A *et al*. (2001) Unsaturated fatty acids down-regulate srebp isoforms 1a and 1c by two mechanisms in HEK-293 cells. *J Biol Chem* **276**, 4365–4372.
15. Jump DB (2002) Dietary polyunsaturated fatty acids and regulation of gene transcription. *Curr Opin Lipidol* **13**, 155–164.
16. Botolin D, Wang Y, Christian B *et al*. (2006) Docosahexaneoic acid (22:6,n-3) regulates rat hepatocyte SREBP-1 nuclear abundance by Erk- and 26S proteasome-dependent pathways. *J Lipid Res* **47**, 181–192.
17. Silvius JR (2003) Role of cholesterol in lipid raft formation: lessons from lipid model systems. *Biochim Biophys Acta* **1610**, 174–183.
18. Stillwell W, Shaikh SR, Zerouga M *et al*. (2005) Docosahexaenoic acid affects cell signaling by altering lipid rafts. *Reprod Nutr Dev* **45**, 559–579.
19. Weber LW, Boll M, Stampfl A (2004) Maintaining cholesterol homeostasis: sterol regulatory element-binding proteins. *World J Gastroenterol* **10**, 3081–3087.
20. Botma GJ, van Deursen D, Vieira D *et al*. (2005) Sterol-regulatory-element binding protein inhibits upstream stimulatory factor-stimulated hepatic lipase gene expression. *Atherosclerosis*. **179**, 61–67.
21. Aki T, Yanagisawa S, Akanuma H (1997) Identification and characterization of positive regulatory elements in the human glyceraldehyde 3-phosphate dehydrogenase gene promoter. *J Biochem* **122**, 271–278.
22. Folch J, Lees M, Sloane Stanley GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* **226**, 497–509.
23. Stoffel W, Chu F, Ahrens Jr JH (1959) Analysis of long chain fatty acids by gas liquid chromatography. *Anal Chem* **31**, 307–311.

24. Bordoni A, Angeloni C, Leoncini E *et al*. (2005) Hypoxia/reoxygenation alters essential fatty acids metabolism in cultured rat cardiomyocytes: protection by antioxidants. *Nutr Metab Cardiovasc Dis* **15**, 166–173.
25. Härmälä AS, Pörn MI, Mattjus P *et al*. (1994) Cholesterol transport from plasma membranes to intracellular membranes is inhibited by 3 beta-[2-(diethylamino)ethoxy]androst-5-en-17-one. *Biochim Biophys Acta* **1211**, 317–325.
26. Lim CH, Bijvelds MJ, Nigg A *et al*. (2007) Cholesterol depletion and genistein as tools to promote F508delCFTR retention at the plasma membrane. *Cell Physiol Biochem* **20**, 473–482.
27. Keys A & Parlin RW (1966) Serum cholesterol response to changes in dietary lipids. *Am J Clin Nutr* **19**, 175–181.
28. Jump DB, Botolin D, Wang Y, *et al.* (2008) Docosahexaenoic acid (DHA) and hepatic gene transcription. *Chem Phys Lipids* **153**, 3-13.
29. Worgall TS, Sturley SL, Seo T *et al*. (1998) Polyunsaturated fatty acids decrease expression of promoters with sterol regulatory elements by decreasing levels of mature sterol regulatory element-binding protein. *J Biol Chem* **273**, 25537–25540.
30. Le Jossic-Corcos C, Gonthier C, Zaghini I, *et al.* (2005) Hepatic farnesyl diphosphate synthase expression is suppressed by polyunsaturated fatty acids. *Biochem J* **385,** 787-794.
31. Yu-Poth S, Yin D, Kris-Etherton PM *et al*. (2005) Long-chain polyunsaturated fatty acids upregulate LDL receptor protein expression in fibroblasts and HepG2 cells. *J Nutr* **135**, 2541–2545.
32. El-Badry AM, Graf R, Clavien PA (2007) Omega 3 - Omega 6: What is right for the liver? *J Hepatol* **47**, 718–725.
33. Jayadev S, Linardic CM, Hannun YA (1994) Identification of arachidonic acid as a mediator of sphingomyelin hydrolysis in response to tumor necrosis factor alpha. *J Biol Chem* **269**, 5757–5763.
34. Robinson BS, Hii CS, Poulos A *et al*. (1997) Activation of neutral sphingomyelinase in human neutrophils by polyunsaturated fatty acids. *Immunology* **91**, 274–280.
35. Simons K & Ikonen E (2000) How cells handle cholesterol. *Science* **290**, 1721–1726.
36. Liscum L & Munn NJ (1999) Intracellular cholesterol transport. *Biochim Biophys Acta* **1438**:19–37.
37. Worgall TS, Johnson RA, Seo T *et al*. (2002) Unsaturated fatty acid-mediated decreases in sterol regulatory element-mediated gene transcription are linked to cellular sphingolipid metabolism. *J Biol Chem* **277**, 3878–3885.
38. König B, Koch A, Spielmann J *et al*. (2007) Activation of PPARα lowers synthesis and concentration of cholesterol by reduction of nuclear SREBP-2. *Biochem Pharmacol* **73**, 574-585.
39. Sekiya M, Yahagi N, Matsuzaka T, *et al.* (2003) Polyunsaturated fatty acids ameliorate hepatic steatosis in obese mice by SREBP-1 suppression. *Hepatology* **38**, 1529-1539.
40. Xu J, Cho H, O'Malley S, et al. (2002) Dietary polyunsaturated fats regulate rat liver sterol regulatory element binding proteins-1 and -2 in three distinct stages and by different mechanisms. *J Nutr* **132**, 3333-3339.

CHAPTER 9

General Discussion

9.1 Metabolic regulation of Hepatic Lipase in the liver

In rats, HL expression follows a diurnal rhythm with high activity in the fed state and low activity in the fasted state [1-3]. Several lines of evidence suggest that HL expression may fluctuate with feeding and fasting in humans as well. Postheparin plasma HL activity is increased in parallel with plasma insulin levels in response to an oral glucose load [4-6], and HL activity increases with fasting plasma insulin levels in nondiabetic, normocholesterolemic CAD patients [7]. HL activity is elevated under conditions with high plasma insulin, such as type 2 diabetes [8] and obesity related hyperinsulinaemia [9]. However, a direct stimulating effect of insulin on HL expression has not been unequivocally identified [10], indicating that HL expression is affected by another parameter varying concomitantly with insulin. In rats, the diurnal rhythm is mainly caused by catecholamines [1,11], of which plasma levels show an inverse relationship to insulin levels. In HepG2 cells, adrenaline inhibits HL expression through α1B receptors which signal through elevation of Ca_i^{2+} as well as cAMP [12]. The increase in intracellular Ca^{2+} concentration inhibits the intracellular maturation of newly synthesized proteins, including hepatic lipase [12]. In chapter 3 we showed that elevation of intracellular cAMP by incubating cells with membrane-permeable Br-cAMP inhibits HL expression at the level of transcription. This not only mimics the effect of adrenaline on liver cells, but also of glucagon, of which the concentration in the portal vein also varies opposite to insulin.

In chapter 4 we have shown that HL expression in HepG2 cells is upregulated by incubating the cells with high glucose concentrations. *In vivo*, plasma glucose concentration also varies to some extent with plasma insulin levels, suggesting that the effect of feeding-fasting on HL expression may also be mediated through extracellular glucose. One may question however whether incubating HepG2 cells with high glucose mimics the fed state. In the fed state, intracellular glucose in liver cells is kept low by insulin despite high extracellular glucose, thus maintaining a high glucose gradient over the cell membrane necessary for glucose uptake. In contrast, intracellular glucose has to be kept above extracellular glucose concentration in the fasted state to enable glucose output. Incubating HepG2 cells with high extracellular glucose may translate into high intracellular glucose, thus representing the fasted rather than fed state. In addition, we showed in chapters 6 and 7 that exposure of HepG2 to high oleate, thus mimicking increased FFA supply to the liver in the fasted state, increases rather than decreases HL expression. This observation is opposite to what one would expect if *in vivo* HL expression was relatively low during fasting and starvation. During feeding, the hormones signal increased HL expression, and the relatively low FFA levels decrease HL expression, whereas the high extracellular glucose but low intracellular glucose concentration may either increase or decrease HL expression depending on the perspective. The opposite situation exists in the fasting state (Table 1). We hypothesize that as a result of these partly opposing effects will result in only moderate changes in HL expression during the feeding-fasting cycle.

HL expression is increased in conditions of insulin resistance, such as obesity, T2DM and FCHL. These conditions are not only characterized by elevated insulin levels, but also by increased FFA delivery to the liver and high plasma glucose (and probably also high intrahepatocyte glucose). In addition, glucagon and perhaps also catecholamine levels are low. All these effectors lead to increased HL expression, which may explain high HL levels in insulin-resistant states (Table 1).

Table 1. HL expression according to nutritional status, obesity/insulin resistance and levels of metabolites and hormones.

fasting			feeding			obesity / insulin resistance		
Glucose ↑	→	HL ↑	Glucose ↓	→	HL ↓	Glucose ↑	→	HL ↑
FFA ↑	→	HL ↑	FFA ↓	→	HL ↓	FFA ↑	→	HL ↑
Epinephrine ↑ Glucagon ↑ cAMP ↑	→	HL ↓	Epinephrine ↓ Glucagon ↓ cAMP ↓	→	HL ↑	Epinephrine ↓ Glucagon ↓ cAMP ↓	→	HL ↑
Corticoids ↑	→	HL ↓	Corticoids ↓	→	HL ↑	Corticoids ↓	→	HL ↑
Insulin↓	→	HL ↓	Insulin ↑	→	HL ↑	Insulin ↑	→	HL ↑
HL ↓			HL ↑			HL ↑ ↑ ↑		

Why HL expression is relatively high in the fed state, and even higher in insulin-resistant conditions is unknown. In the fed state, a high HL expression is beneficial for the clearance of chylomicron remnants by the liver as well as for the removal by HDL of surface fragments that are generated in excess by lipolysis of the chylomicrons [reviewed in 13]. In insulin-resistant states, the liver has to deal with a high flux of fatty acids and VLDL synthesis and secretion. Increased HL expression may aid in the supply of building blocks necessary for VLDL production, such as phospholipids, choline or cholesterol [13]. Future experiments are needed to elucidate the relationship between insulin resistance and HL expression. Mice could be made insulin-resistant specifically in the liver, either by diet [14] or by genetic modification (e.g. apoC3 knockout [15] or liver-specific insulin receptor knockout (LIRKO) [16]) to determine the effect on HL expression. Alternatively, HL expression could be studied in mice or rats, in which fatty acid flux to the liver has been interrupted. The hepatic uptake of fatty acids is mainly regulated by the plasma fatty acids concentration, which is primarily determined by lipolysis in visceral adipose tissue [17]. In insulin-resistant states, insulin fails to suppress the activity of hormone-sensitive lipase, which results in enhanced lipolysis and flux of fatty acids to the plasma pool [18]. Inhibition could be achieved pharmacologically; e.g. by niacin or acipimox, agents that have been used to lower apoB100 lipoproteins and increase HDL-cholesterol. Acute administration of niacin or acipimox has been shown by numerous investigators to inhibit the lipolysis and reduce plasma FFAs [19,20]. Fatty acid supply to the liver could also be reduced by gene modification, eg in CD36 deficient mice that lack the fatty acid translocase

involved in fatty acid uptake [21], or in hepatocytes that have been treated with antibodies directed against FABPpm, thereby inhibiting fatty acid uptake [22,23]. In addition, the effect on HL expression could be determined upon inhibition of VLDL synthesis and secretion, such as in choline deficiency [24]. Niacin and acipimox also reduce VLDL synthesis and secretion by inhibiting intrahepatic TG synthesis [20]. Interestingly, the FATS study showed that niacin treatment in humans led to lower HL expression [25].

9.2 Regulatory elements in the HL gene, and their effect on transcription

Genomic DNA sequence elements that have functional value are likely maintained in evolution despite speciation. Elements that are important for regulation of transcription are probably highly conserved. Of the intergenic sequences that are conserved between species evolutionary as far apart as mouse and fugu fish, most but not all showed enhancing activity in mouse embryos [26,27]. To identify potential regulatory elements in the HL gene, we searched the upstream region for clusters of transcription factor binding sites in sequence elements that are conserved among mammalian HL genes. Indeed, we showed in chapter 2 that two of the three elements identified in the far upstream region displayed enhancer activity. However, the -10 kb sequence was not functional. For two of the three conserved elements within the proximal promoter region, functional activity was confirmed experimentally in chapters 2 and 3. The third element at -240 to -200 (designated as module A) contains a DR1 site which has been shown by Rufibach *et al*. [28] to be important in the HNF4α-dependent transactivation of the HL promoter. Why the conserved region at -10 kb does not show functionality is unclear. This is puzzling since this region contains a similar repertoire of transcription factor binding sites as the two other far upstream conserved elements. Nevertheless, these findings illustrate the power of this approach in identifying potentially important regulatory sequences. On the other hand, in chapters 4 till 7, the canonical E-box at around -310 is shown to be important for glucose-, fatty acid- and SREBP-mediated regulation of HL gene transcription via USF, although this E-box is not located within a highly conserved region of the gene. No E-box is present in similar locations of the rat and mouse genes. This may indicate that regulation of murine HL expression by USF is mediated through elements elsewhere in the gene, for example the Inr region. Alternatively, HL expression is regulated by USF in humans but not in rodents, as suggested by the finding of normal HL expression in livers of USF1-knock out mice (chapter 5). It would be interesting to test whether HL expression in rat and mouse is sensitive to regulation by glucose or fatty acids.

The conserved element in the proximal HL promoter at position -80 to -40 (designated module B) has an important role in the liver-specific transcription of the HL gene. It stimulated HL promoter activity in HepG2 cells, but inhibited the activity in HeLa cells. This element contains consensus binding sites for the liver-enriched transcription factors HNF1 and

C/EBPβ. Several researchers have presented data that suggest the important role of HNF1α in liver-specific expression of HL, as has been discussed in chapter 2. However, HNF1 alone does not explain the almost exclusive expression of HL in liver, as HNF1 is expressed in most other endoderm-derived cells as well [29]. C/EBPβ is also expressed in many tissues other than liver, partly overlapping with HNF1α [30]. An additional candidate could be HNF4, since HL expression in different hepatoma cell lines showed a high correlation with HNF4 mRNA levels [31]. HNF4α is bound to the promoter region of almost half of the genes active in human liver [32]. The DR1 site in the conserved module A (-240 to -200) potentially binds HNF4α, and its role in HL expression has been demonstrated by Rufibach *et al*. [28]. The presence of enhancers for HNF1 and HNF4 are among the strongest predictors of liver-specific transcription [33]. It is possible that liver-restricted expression is effected by the simultaneous binding of HNF1α, HNF4α and C/EBPα to the proximal HL promoter.

Another possibility is that the liver-restricted expression of HL is due to epigenetic control. Depending on the local chromatin structure the HL gene including the promoter region may or may not be accessible to regulatory factors such as HNF1α. HL expression is then only possible in cells in which the HL gene finds itself in a loosely packed, open chromatin structure. Even in cells expressing HNF1α, the HL gene would not be expressed if it were present within a tightly packed, closed chromatin structure. Whether chromatin is loosely or tightly packed depend on local cues within the DNA, such as DNA methylation at CpG islands, or the histone proteins in adjacent nucleosomes. DNA methylation usually marks silenced genes, whereas histone modifications can either mark active or inactive genes. Further studies are required to determine the involvement of epigenetics in tissue-specific expression of the HL gene, and to explain why some but not all highly conserved elements in the upstream region of the HL gene are functional. DNA methylation and histone modification status in the vicinity of the HL gene should be studied by ChIP assays [34]. In addition, Formaldehyde-Assisted Isolation of Regulatory Elements (FAIRE) assays should be performed on hepatic and non-hepatic tissues to determine whether the HL promoter region is free of nucleosomes [35] and thus accessible for regulatory proteins such as HNF1.

9.3 Transcriptional regulation of the HL promoter

9.3.1 Involvement of USF

We have shown in chapters 4 and 7, that the nuclear abundance of USF1, and to a less degree of USF2, is increased in HepG2 cells upon incubation with oleate or glucose at around concentrations that may be present in the portal vein in the fasted and the postprandial state, respectively. Furthermore, we have shown that USF upregulates HL gene expression, and evidence was presented that oleate and glucose increase HL expression in HepG2 cells via elevation of USF1. The *USF1* gene, notably a polymorphism in intron 7, has been linked to familial combined hyperlipidemia (FCH), a lipid disorder characterized by insulin resistance and

elevated serum cholesterol and/or triglycerides [36-38]. Komulainen *et al*. [39] showed that the polymorphism in intron 7 affects the binding of nuclear proteins to that region of USF1 gene, and thus may affect the transcriptional or posttranscriptional regulation of USF1 expression. Furthermore, the *USF1* gene is also associated with type 2 diabetes [40,41], metabolic syndrome [36,37,40,42], and both cardiovascular disease and all-cause mortality among women [32]. Since these are conditions in which the HL expression is increased, USF1 may be the link between insulin resistance, elevated expression of HL, and dyslipidemia. According to Lee *et al*., genetic and functional evidence is supportive of a role USF 1 in the elevated plasma triglyceride trait of familial combined hyperlipidemia [43]. The increase of USF1 by oleate and herewith activating lipogenic genes could represent a feed-forward cycle causing an increase of triglyceride and VLDL synthesis. The increased HL expression in conditions of insulin resistance and/or FCHL may therefore be a consequence of the USF mediated regulation of HL expression.

It is unknown how glucose or fatty acids can cause nuclear accumulation of USF1. In adipocytes, USF1 is involved in the upregulation of hormone-sensitive lipase (HSL) expression by glucose. For this effect, glucose must be metabolised in the glycolytic pathway [44], suggesting that a glycolytic intermediate between glucose-6-phosphate and triose phosphates increases USF activity. Additional research is needed to find out whether this is also the case for liver cells. USF activity has also been shown to be modulated by phosphorylation and acetylation [45-48]. Wong *et al*. recently showed that in response to feeding and fasting and insulin signalling, USF is posttranslationally modified by DNA-PK (phosphorylation) and HDAC9 (deacetylation), thereby regulating the fatty acid synthase gene in liver cells [48]. Future studies should be aimed at elucidating the mechanism by which USF activity and nuclear abundance is increased by glucose and fatty acids. Understanding the mechanism of USF activation by glucose and fatty acids may explain the observed linkage between the *USF1* gene, insulin resistance and dyslipidemia.

As shown in chapter 5, USF mediates its effect on HL expression mainly through binding at the -310Ebox and the Inr region. Compared to the -310 E-box, the non-canonical E-box harbouring the common -514C/T polymorphism had only little contribution to upregulation by USF1. This polymorphism has been shown to affect HL expression *in vivo*, and HL promoter activity and USF binding in gel-shift assays *in vitro* [49]. *In vitro* promoter activity of the -514T allele was already lower than the -514C allele in control HepG2 cells, and both alleles were similarly upregulated by over-expression of USF1, suggesting that the different HL expression may not be due to differential response to USF [49]. The -514C/T polymorphism is highly linked with three other polymorphisms within the proximal HL promoter region, so that the difference in promoter activity may either be due to one of the other polymorphisms, or to a factor different from USF. The -250G/A polymorphism lies just inside the highly conserved module A of the proximal HL promoter (chapter 2), and is adjacent to the DR1 site (-238/-226) that has been shown important for transcriptional activation by HNF4 [28]. We hypothesize therefore that the functional difference between the LIPC-C and -T allele results from the -250G/A polymorphism. Future studies must focus at this polymorphism to test this hypothesis.

As already mentioned, the -310 E-box and the Inr play important roles in USF-mediated transcription of the proximal promoter of the human HL gene. Several studies have demonstrated that USF can affect transcription via the Inr independent of an E-box [50-53]. The genomic region around the -310 E-box is conserved in primates only (Ensembl, accessed July 2009). In fact, a canonical E-box is not present in 2 kb of upstream region of the rat and mouse HL gene. HL expression in mice was not affected by knock-out of USF1. Hence, the USF-regulated HL expression may be restricted to primates. In rat liver, ChIP assays showed that USF1 and USF2 are bound to the proximal HL promoter region (see chapter 4), but the increased abundance of USF1 and USF2 in liver nuclei of streptozotocin-treated rats was not accompanied by increased USF binding. Nevertheless, liver HL activity was decreased in the streptozotocin-treated rats. This suggests that in the rat, USFs are mainly bound to the Inr, and that HL expression is not determined only by the amount of USF but also by its posttranslational modification, as discussed above.

9.3.2 Involvement of the transcription factor SREBP

In chapter 6 and 7, we presented experiments that suggest that HL expression by fatty acids and statins is mediated via SREBP, possibly through interaction with USF. SREBP2, but not SREBP1, overexpression inhibited HL promoter activity and reversed the positive regulating effect of USF, and sensitivity of the HL promoter to inhibition by SREBP2 was mediated mainly through the -310 E-box. In chapter 8, we showed that incubation of HepG2 cells with oleate led to reduced SREBP activity. Taken together, the oleate-mediated decrease in SREBP-activity may cause an increase in USF activity, and thereby activate HL expression. That the HL gene is not a direct target of SREBP is suggested by the observed delay between increased SREBP activity and suppression of HL promoter activity in HepG2 cells upon transfection with nSREBP plasmids (chapter 7). This can also be concluded from the microarray data of Horton *et al*. [54] in three mice strains with absent or suppressed SREBP activity: HL expression was significantly higher in SREBP1 and SREBP2 knockout compared to wildtype mice, but not affected in SCAP-transgenic mice. Lowering of SREBP activity, however, does not always lead to an increase of HL expression. In rats suppression of SREBP activity in liver by cholesterol-enriched diets was accompanied by a reduced HL expression [55]. In HepG2 cells PUFAs were more effective than oleate in suppressing SREBP activity (chapter 8), but at the same concentrations (60-120 μM) PUFAs failed to increase HL promoter activity [56]. Almost complete suppression of SREBP activity in HepG2 cells incubated with cholesterol+25-OHcholesterol also had no effect on HL promoter activity (chapter 7). It is clear that lowering SREBP activity is not sufficient to reduce HL expression. Possibly, other transcription factors are involved in mediating the effect of SREBP on HL expression. USF1 and USF2 are candidates. However, a study of Horton *et al*. [54] did not reveal the USF1 and USF2 genes as direct targets for SREBP either. Whether SREBP affects, directly or indirectly, the post-translational modification and hence, transactivation activity of USF1 and USF2, should be the focus of future research.

Other mechanisms may explain the observation that downregulation of HL promoter activity by nSREBP via the USF-binding site at around position -310. There could be competition between SREBP and USF for binding to the -310 E-box. SREBPs are able to bind not only to the SRE but also to an E-box, but transactivation by nSREBP only results from binding to SRE leads to transactivation [57]. Thus, SREBPs may compete with USF for binding to the -310 E-box, and hence inhibit USF-mediated transactivation. Alternatively SREBPs may bind to USF thereby preventing the latter from binding to the proximal HL promoter region. SREBP and USF have been shown to bind to each other forming hetero-dimer complexes [58]. However, this mechanism seems less likely, as heterodimer formation only occurred when both factors bind simultaneously to DNA at adjacent positions. It should be noted that most of our experiments were performed in HepG2 cells that were transfected with either SREBP or USF expressing plasmids, or both. Competition between over-expressed transcription factors may result from competition for coactivator proteins necessary for the transcription process [59]. ChIP assays to determine whether USF and SREBP proteins are bound to the proximal region of the HL gene under conditions with high versus low SREBP activity in HepG2 cells as well as *in vivo* in animal livers are a necessary first step in elucidating the mechanism.

9.3.3 Gene regulation by fatty acids

It is still poorly understood how fatty acids affect gene expression, but binding as a ligand to nuclear receptors, notably PPARs [60-62], and modulation of the synthesis and maturation of SREBPs [63-65] are the most likely modes of action. Short-term feeding of mice with specific triglycerides showed that the majority of genes regulated by unsaturated fatty acids in liver were also regulated by a specific PPARα agonist, and almost every single gene regulated by dietary unsaturated fatty acids remained unaltered in mice lacking PPARα [66]. Feeding mice with triolein was less effective in changing gene expression than feeding them oil enriched with PUFAs, notably DHA. In contrast, several studies have consistently reported that SREBP activity or nSREBP1 protein levels are decreased in rats and mice livers by PUFA-enriched oil feeding [67-70]. Moreover, oleic acid is much less effective than PUFAs in suppressing SREBP activity and nSREBP1 protein levels [64, chapter 8]. DHA was the most effective suppressor of SREBP activity (chapter 8), in agreement with the review of Jump *et al.* [63]. There were both quantitative and qualitative differences between the effect of oleate and PUFAs on gene expression in the liver. Whereas of the 519 genes affected by PUFAs, 83% were also upregulated by triolein or the PPARα agonist, of the 114 genes affected by triolein feeding, 57% were unique to triolein [66]. Taken together, these data suggest that unsaturated fatty acids, notably oleic acid, may affect regulation of subset of genes through yet other transcription factors. In chapter 7, we showed that the nuclear abundance of USF1 is upregulated upon incubation of HepG2 cells with oleate. We hypothesized that USF1 may be another fatty acid sensor in liver cells. It would be interesting to know whether PUFAs similarly upregulate USF1 in the nucleus, or whether this is unique to oleate. Future experiments should be aimed to elucidate the mechanism by which fatty acids affect the nuclear abundance and transactivating activity of USF.

Compared to PUFAs, the supplemented oleic acids are relatively poorly integrated into cellular lipids of the HepG2 cells (chapter 8). Probably, oleate is converted to triglycerides instead, which is subsequently secreted to the extracellular medium (chapter 6). Signaling of oleate supply to liver cells via USF may thus be physiologically relevant. A higher oleate supply to the liver or liver cells has been shown to cause an increase in VLDL production, and hence in the need for phospholipid precursors. By increasing HL expression, the uptake of HDL and remnants by the liver will be facilitated, thereby guaranteeing the supply of sufficient phospholipid precursors to maintain VLDL secretion [13].

In contrast to SREBP and USF, a possible role for PPARs in the regulation of the human HL gene by fatty acids has not been addressed in this thesis. PPAR consensus sequences have not been identified in the proximal HL promoter. In humans, treatment with PPARα agonists only marginally increased post-heparin plasma HL activity [71,72], whereas in rats fenofibrate strongly decreased HL expression [73]. Ciprofibrate, another potent PPARα agonist, did not affect the secretion of HL activity by HepG2 cells (chapter 6). This all suggests that the influence of oleic acid on HL gene expression is not likely mediated via PPARα.

9.4 Conclusion

The current thesis presents a number of studies into the transcriptional regulation of HL expression in human hepatoma cells, which have further expanded our knowledge on the central role of HL in lipoprotein metabolism, insulin resistance and intracellular glucose and lipid metabolism. The most important new finding of this thesis is that HL expression is upregulated by glucose as well as by the monounsaturated fatty acid oleate through the

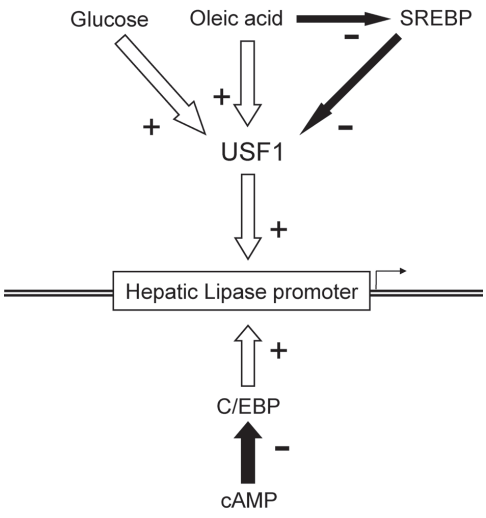

Figure 1. New findings in this thesis.

increased nuclear abundance of USFs, notably USF1. Previously it was thought that USFs affect HL expression through the polymorphic -514C/T E-box; here we showed the involvement rather of the -310 E-box and the Inr region of the HL promoter. Furthermore, USF1 also appears to be involved in the suppressing effect of the cholesterol-sensing SREBP2. HL expression varies with feeding and fasting which has been attributed to suppression by catecholamines; here we have established a role for cAMP and the transcription factor C/EBPβ (Figure 9.1). Taken together, these studies have shown that HL expression is regulated as integral part of glucose and lipid metabolism in the liver.

References

1. Peinado-Onsurbe J, Soler C, Galan X, Poveda B, Soley M, Llobera M, Ramirez I. Involvement of catecholamines in the effect of fasting on hepatic endothelial lipase activity in the rat. *Endocrinology*. 1991; **129:** 2599–2606.
2. Peinado-Onsurbe J, Julve J, Galan X, Llobera M, Ramirez I. Effect of fasting on hepatic lipase activity in the liver of developing rats. *Biol Neonate*. 2000; **77:** 131–138.
3. Stam H, Schoonderwoerd K, Breeman W, Hulsmann WC. Effects of hormones, fasting and diabetes on triglyceride lipase activities in rat heart and liver. *Horm Metab Res*. 1984; **16:** 293–297.
4. Perret B, Mabile L, Martinez L, Terce F, Barbaras R, Collet X. Hepatic lipase: structure/function relationship, synthesis, and regulation. *J Lipid Res*. 2002; **43:** 1163–1169.
5. Katzel LI, Coon PJ, Busby MJ, Gottlieb SO, Krauss RM, Goldberg AP. Reduced HDL2 cholesterol subspecies and elevated postheparin hepatic lipase activity in older men with abdominal obesity and asymptomatic myocardial ischemia. *Arterioscler Thromb*. 1992; **12:** 814–823.
6. Pollare T, Vessby B, Lithell H. Lipoprotein lipase activity in skeletal muscle is related to insulin sensitivity. *Arterioscler Thromb*. 1991; **11:** 1192–1203.
7. Jansen H, Verhoeven AJ, Weeks L, Kastelein JJ, Halley DJ, van den Ouweland A, Jukema JW, Seidell JC, Birkenhager JC. Common C to T substitution at position −480 of the hepatic lipase promoter associated with a lowered lipase activity in coronary artery disease patients. *Arterioscler Thromb Vasc Biol*. 1997; **17:** 2837–2842.
8. Baynes C, Henderson AD, Anyaoku V, Richmond W, Hughes CL, Johnston DG, Elkeles RS. The role of insulin insensitivity and hepatic lipase in the dyslipidaemia of type 2 diabetes. *Diab Med*. 1991; **8:** 560–566.
9. Cominacini L, Garbin U, Davoli A, Campagnola M, De Santis A, Pasini C, Pastorino AM, Bosello O. Highdensity lipoprotein cholesterol concentrations and postheparin hepatic and lipoprotein lipases in obesity: relationships with plasma insulin levels. *Ann Nutr Metab*. 1993; **37:** 175–184.
10. Rashid S, Watanabe T, Sakaue T, Lewis GF. Mechanisms of HDL lowering in insulin resistant, hypertriglyceridemic states: the combined effect of HDL triglyceride enrichment and elevated hepatic lipase activity. *Clin Biochem*. 2003; **36:** 421–429.
11. Neve BP, Verhoeven AJ, Jansen H. Acute effects of adrenaline on hepatic lipase secretion by rat hepatocytes. *Metabolism*. 1997; **46:** 76–82.
12. Neve BP, Verhoeven AJ, Kalkman I, Jansen H. Maturation and secretion of rat hepatic lipase is inhibited by alpha1B-adrenergic stimulation through changes in Ca^{2+} homoeostasis: thapsigargin and EGTA both mimic the effect of adrenaline. *Biochem J*. 1998; **330:** 701–706.
13. Jansen H, Verhoeven AJ, Sijbrands EJ. Hepatic lipase: a pro- or anti-atherogenic protein? *J Lipid Res*. 2002; **43:** 1352–1362.
14. Muurling M, Jong MC, Mensink RP, Hornstra G, Dahlmans VE, Pijl H, Voshol PJ, Havekes LM. A low-fat diet has a higher potential than energy restriction to improve high-fat diet-induced insulin resistance in mice. Metabolism. 2002; **51(6):** 695–701.
15. Duivenvoorden I, Teusink B, Rensen PC, Romijn JA, Havekes LM, Voshol PJ. Apolipoprotein C3 deficiency results in diet-induced obesity and aggravated insulin resistance in mice. *Diabetes*. 2005; **54(3):** 664–671.
16. Biddinger SB, Hernandez-Ono A, Rask-Madsen C, Haas JT, Aleman JO, Suzuki R, Scapa EF, Agarwal C, Carey MC, Stephanopoulos G, Cohen DE, King GL, Ginsberg HN, Kahn CR. Hepatic insulin resistance is sufficient to produce dyslipidemia and susceptibility to atherosclerosis. *Cell Metab*. 2008; **7:** 125–134.
17. Tamura S, Shimomura I. Contribution of adipose tissue and de novo lipogenesis to nonalcoholic fatty liver disease. *J Clin Invest*. 2005; **115:** 1139–1142.
18. Goldberg IJ, Ginsberg HN. Ins and outs modulating hepatic triglyceride and development of nonalcoholic fatty liver disease. *Gastroenterology*. 2006; **130:** 1343–1346.
19. Fuccella LM, Goldaniga G, Lovisolo P, Maggi E, Musatti L, Mandelli V, Sirtori CR 1980 Inhibition of lipolysis by nicotinic acid and by acipimox. *Clin Pharmacol Ther*. 1980; **28:** 790–795.
20. Kamanna VS, Kashyap ML. Mechanism of action of niacin. *Am J Cardiol*. 2008; **101(8A):** 20B–26B.

21. Goudriaan JR, Dahlmans VE, Teusink B, Ouwens DM, Febbraio M, Maassen JA, Romijn JA, Havekes LM, Voshol PJ. CD36 deficiency increases insulin sensitivity in muscle, but induces insulin resistance in the liver in mice. *J Lipid Res*. 2003; **44(12):** 2270–2277.
22. Stremmel W, Strohmeyer G, Berk PD. Hepatocellular uptake of oleate is energy dependent, sodium linked, and inhibited by an antibody to a hepatocyte plasma membrane fatty acid binding protein. *Proc Natl Acad Sci USA*. 1986; **83:** 3584–3588.
23. Stremmel W, Strohmeyer G, Borchard F, Kochwa S, Berk PD. Isolation and partial characterization of a fatty acid binding protein in rat liver plasma membranes. *Proc Natl Acad Sci USA*. 1985; **82:** 4–8.
24. Yao ZM, Vance DE. The active synthesis of phosphatidylcholine is required for very low density lipoprotein secretion from rat hepatocytes. *J Biol Chem*. 1988; **263(6):** 2998–3004.
25. Zambon AA, Deeb SSD, Pauletto PB, Crepaldi GA, Brunzell JD. Hepatic lipase: a marker for cardiovascular disease risk and response to therapy. *Curr Opin Lipidol*. 2003; **14:** 179–189.
26. Pennacchio LA, Ahituv N, Moses AM, Prabhakar S, Nobrega MA, Shoukry M, Minovitsky S, Dubchak I, Holt A, Lewis KD, Plajzer-Frick I, Akiyama J, De Val S, Afzal V, Black BL, Couronne O, Eisen MB, Visel A, Rubin EM. In vivo enhancer analysis of human conserved non-coding sequences. *Nature*. 2006; **444:** 499–502.
27. Ahituv N, Zhu Y, Visel A, Holt A, Afzal V, Pennacchio LA, Rubin EM. Deletion of ultraconserved elements yields viable mice. *PLoS Biol*. 2007; **5(9):** e234.
28. Rufibach LE, Duncan SA, Battle M, Deeb SS: Transcriptional regulation of the human hepatic lipase (LIPC) gene promoter. *J Lipid Res*. 2006; **47:** 1463–1477.
29. Ott MO, Rey-Campos J, Cereghini S, Yaniv M. vHNF1 is expressed in epithelial cells of distinct embryonic origin during development and precedes HNF1 expression. *Mech Dev*. 1991; **36(1-2):** 47–58.
30. Antonson P, Xanthopoulos KG. Molecular cloning, sequence, and expression patterns of the human gene encoding CCAAT/enhancer binding protein alpha (C/EBP alpha). *Biochem Biophys Res Commun*. 1995; **215(1):** 106–113.
31. Chang SF, Scharf JG, Will H. Structural and functional analysis of the promoter of the hepatic lipase gene. *Eur J Biochem*. 1997; **247:** 148–159.
32. Odom DT, M, Peltonen L. Risk alleles of USF1 gene predict cardiovascular disease of women in two prospective studies. *PloS Genetics*. 2006; **2:** e69.
33. Krivan W, Wasserman WW. A predictive model for regulatory sequences directing liver-specific transcription. *Genome Res*. 2001; **11:** 1559–1566.
34. Imai S, Kikuchi R, Kusuhara H, Yagi S, Shiota K, Sugiyama Y. Analysis of DNA methylation and histone modification profiles of liver-specific transporters. *Mol Pharmacol*. 2009; **75(3):** 568–576.
35. Giresi PG, Kim J, McDaniell RM, Iyer VR, Lieb JD. FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) isolates active regulatory elements from human chromatin. *Genome Res*. 2007; **17(6):** 877–885.
36. Pajukanta P, Lilja HE, Sinsheimer JS, Cantor RM, Lusis AJ, Gentile M, Duan XJ, Soro-Paavonen A, Naukkarinen J, Saarela J, Laakso M, Ehnholm C, Taskinen MR, Peltonen L. Familial combined hyperlipidemia is associated with upstream transcription factor 1 (USF1). *Nat Genet*. 2004; **36:** 371–376.
37. Shoulders CC, Naoumova RP. USF1 implicated in the aetiology of familial combined hyperlipidaemia and the metabolic syndrome. *Trends Mol Med*. 2004; **10:** 362–365.
38. Coon H, Xin Y, Hopkins PN, Cawthon RM, Hasstedt SJ, Hunt SC. Upstream stimulatory factor 1 associated with familial combined hyperlipidemia, LDL cholesterol, and triglycerides. *Hum Genet*. 2005; **117:** 444–451.
39. Komulainen K, Alanne M, Auro K, Kilpikari R, Pajukanta P, Saarela J, Ellonen P, Salminen K, Kulathinal S, Kuulasmaa K, Silander K, Salomaa V, Perola M, Peltonen L. Risk alleles of USF1 gene predict cardiovascular disease of women in two prospective studies. *PloS Genetics*. 2006; **2:** e69.
40. Ng MC, Miyake K, So WY, Poon EW, Lam VK, Li JK, Cox NJ, Bell GI, Chan JC. The linkage and association of the gene encoding upstream stimulatory factor 1 with type 2 diabetes and metabolic syndrome in the Chinese population. *Diabetologia*. 2005; **48(10):** 2018–2024.
41. Meex SJ, van Vliet-Ostaptchouk JV, van der Kallen CJ, van Greevenbroek MM, Schalkwijk CG, Feskens EJ, Blaak EE, Wijmenga C, Hofker MH, Stehouwer CD, de Bruin TW. Upstream transcription factor 1 (USF1) in risk of type 2 diabetes: association study in 2000 Dutch Caucasians. *Mol Genet Metab*. 2008; **94(3):** 352–355.

42. Naukkarinen J, Gentile M, Soro-Paavonen A, Saarela J, Koistinen HA, Pajukanta P, Taskinen MR, Peltonen L. USF1 and dyslipidemias: converging evidence for a functional intronic variant. *Hum Mol Genet*. 2005; **14(17):** 2595–2605.

43. Lee JC, Lusis AJ, Pajukanta P. Familial combined hyperlipidemia: upstream transcription factor 1 and beyond. *Curr. Opin. Lipidol*. 2006; **17:** 101–109.

44. Smih F, Rouet P, Lucas S, Mairal A, Sengenes C, Lafontan M, Vaulont S, Casado M, Langin D. Transcriptional regulation of adipocyte hormone-sensitive lipase by glucose. *Diabetes*. 2002; **51:** 293–300.

45. Corre S, Galibert MD. Upstream stimulating factors: highly versatile stress-responsive transcription factors. *Pigment Cell Res*. 2005; **18:** 337–348.

46. Cheung E, Mayr P, Coda-Zabetta F, Woodman PG, Boam DS. DNA-binding activity of the transcription factor upstream stimulatory factor 1 (USF-1) is regulated by cyclin-dependent phosphorylation. *Biochem J*. 1999; **344 (Pt 1):** 145–152.

47. Nowak M, Helleboid-Chapman A, Jakel H, Martin G, Duran-Sandoval D, Staels B, Rubin EM, Pennacchio LA, Taskinen MR, Fruchart-Najib J, Fruchart JC. Insulin-mediated down-regulation of apolipoprotein A5 gene expression through the phosphatidylinositol 3-kinase pathway: role of upstream stimulatory factor. *Mol Cell Biol*. 2005; **25:** 1537–1548.

48. Wong RH, Chang I, Hudak CS, Hyun S, Kwan HY, Sul HS. A role of DNA-PK for the metabolic gene regulation in response to insulin. *Cell*. 2009; **136:** 1056–1072.

49. Botma GJ, Verhoeven AJM, Jansen H. Hepatic lipase promoter activity is reduced by the C-480T and G-216A substitutions present in the common LIPC gene variant, and is increased by Upstream Stimulatory Factor. *Atherosclerosis*. 2001; **154:** 625–632.

50. Du H, Roy AL, Roeder RG. Human transcription factor USF stimulates transcription through the initiator elements of the HIV-1 and the Ad-ML promoters. *EMBO J*. 1993; **12(2):** 501–511.

51. Roy AL, Du H, Gregor PD, Novina CD, Martinez E, Roeder RG. Cloning of an inr- and E-box-binding protein, TFII-I, that interacts physically and functionally with USF1. *EMBO J*. 1997; **16(23):** 7091–7104.

52. Marmillot P, Scovell W. Enhancement of transcription factor, USF, binding to the adenovirus major late promoter: effect of dithiothreitol and high mobility group protein-1. *Biochim Biophys Acta*. 1998; **1395(2):** 228–236.

53. Coulson JM, Edgson JL, Marshall-Jones ZV, Mulgrew R, Quinn JP, Woll PJ. Upstream stimulatory factor activates the vasopressin promoter via multiple motifs, including a non-canonical E-box. *Biochem J*. 2003; **369(Pt 3):** 549–561.

54. Horton JD, Shah NA, Warrington JA, Anderson NN, Park SW, Brown MS, Goldstein JL. Combined analysis of oligonucleotide microarray data from transgenic and knockout mice identifies direct SREBP target genes. *Proc. Natl. Acad. Sci. USA*. 2003; **100:** 12027–12032.

55. Benhizia F, Lagrange D, Malewiak MI, Griglio S. In vivo regulation of hepatic lipase activity and mRNA levels by diets which modify cholesterol influx to the liver. *Biochim. Biophys. Acta*. 1994; **1211:** 181–188.

56. van Deursen & Di Nunzio, unpublished observations.

57. Athanikar JN; Osborne TF. Specificity in cholesterol regulation of gene expression by coevolution of sterol regulatory DNA element and its binding protein. *Proc. Natl. Acad. Sci. USA*. 1998; **95:** 4935–4940.

58. Wong RH, Chang I, Hudak CS, Hyun S, Kwan HY, Sul HS. A role of DNA-PK for the metabolic gene regulation in response to insulin. *Cell* 2009; **136:** 1056–1072.

59. Natesan S, Rivera VM, Molinari E, Gilman M. Transcriptional squelching re-examined. *Nature*. 1997; **390(6658):** 349–350.

60. Jump DB. Dietary polyunsaturated fatty acids and regulation of gene transcription. *Curr Opin Lipidol*. 2002; **13(2):** 155–164.

61. Bordoni A, Di Nunzio M, Danesi F, Biagi PL. Polyunsaturated fatty acids: From diet to binding to ppars and other nuclear receptors. *Genes Nutr*. 2006; **1(2):** 95–106.

62. Sonoda J, Pei L, Evans RM. Nuclear receptors: decoding metabolic disease. *FEBS Lett*. 2008; **582:** 2–9.

63. Jump DB, Botolin D, Wang Y, Xu J, Demeure O, Christian B. Docosahexaenoic acid (DHA) and hepatic gene transcription. *Chem Phys Lipids*. 2008; **153(1):** 3–13.

64. Worgall TS, Sturley SL, Seo T, Osborne TF, Deckelbaum RJ. Polyunsaturated fatty acids decrease expression of promoters with sterol regulatory elements by decreasing levels of mature sterol regulatory element-binding protein. *J Biol Chem*. 1998; **273(40):** 25537–25540.

65. Horton, J.D.; Shimomura, I. Sterol regulatory element-binding proteins: activators of cholesterol and fatty acid biosynthesis. *Curr. Opin. Lipidol*. 1999; 1**0:** 143–150.

66. Sanderson LM, de Groot PJ, Hooiveld GJ, Koppen A, Kalkhoven E, Müller M, Kersten S. Effect of synthetic dietary triglycerides: a novel research paradigm for nutrigenomics. *PLoS ONE*. 2008; **3:** e1681.

67. Le Jossic-Corcos C, Gonthier C, Zaghini I, Logette E, Shechter I, Bournot P. Hepatic farnesyl diphosphate synthase expression is suppressed by polyunsaturated fatty acids. *Biochem J*. 2005; **385(Pt 3):** 787–794.

68. Botolin D, Wang Y, Christian B, Jump DB. Docosahexaneoic acid (22:6,n-3) regulates rat hepatocyte SREBP-1 nuclear abundance by Erk- and 26S proteasome-dependent pathways. *J Lipid Res*. 2006; **47(1):** 181–192.

69. Xu J, Cho H, O'Malley S, Park JH, Clarke SD: Dietary polyunsaturated fats regulate rat liver sterol regulatory element binding proteins-1 and -2 in three distinct stages and by different mechanisms. *J Nutr*. 2002; **132:** 3333–3339.

70. Sekiya M, Yahagi N, Matsuzaka T, Najima Y, Nakakuki M, Nagai R, Ishibashi S, Osuga J, Yamada N, Shimano. Polyunsaturated fatty acids ameliorate hepatic steatosis in obese mice by SREBP-1 suppression. *Hepatology*. 2003; **38:** 1529–1539.

71. Heller F, Harvengt C. Effects of clofibrate, bezafibrate, fenofibrate and probucol on plasma lipolytic enzymes in normolipaemic subjects. *Eur. J. Clin. Pharmacol*. 1983; **25:** 57–63.

72. Desager JP, Horsmans Y, Vandenplas C, Harvengt C. Pharmacodynamic activity of lipoprotein lipase and hepatic lipase, and pharmacokinetic parameters measured in normolipidaemic subjects receiving ciprofibrate (100 or 200 mg/day) or micronised fenofibrate (200 mg/day) therapy for 23 days. *Atherosclerosis*. 1996; **124:** S65–73.

73. Staels B, Peinado-Onsurbe J, Auwerx J. Down-regulation of hepatic lipase gene expression and activity by fenofibrate. *Biochim. Biophys. Acta*. 1992; **1123:** 227–230.

Summary

In **chapter 1**, we gave an overview of the physiology of HL, its relationship to atherogenesis and diabetes, and outlined the regulation of its expression.

Mammalian hepatic lipase (HL) genes are transcribed almost exclusively in hepatocytes. In **chapter 2** we proposed that liver specific expression is due to cis-acting elements that are conserved among mammalian HL genes. To identify the potentially important upstream regulatory elements, we made a genomic comparison of 30 kb of 5'-flanking region of the rat, mouse, rhesus monkey and human HL genes. We found that the proximal promoter region and three 200-400 bp regions that lie far upstream of the mammalian HL promoter have strong conservation. Promoter-reporter assays in transiently transfected HepG2 cells confirmed that two distal elements at -22kb and -14kb upstream show increased activity towards the proximal promoter (-685/+13) region. In contrast, a conserved element at -10 kb appeared to be non-functional in this assay. Within the proximal promoter region, three elements were conserved, designated as A, B, and C. Module C (-25/+5) contains the transcriptional start site and a pyrimidine-rich sequence that may represent the initiator region (Inr). Module B (-80 to -40) contains a potential HNF1 binding site. This element stimulated the promoter activity in liver-derived HepG2 cells, and inhibited the promoter activity in the non-hepatic HeLa cells. The region A (-240 to -200) corresponds to a DR1 site, but its function in transcription could not be confirmed experimentally.

HL activity varies with feeding and fasting, suggesting that it is upregulated by insulin or suppressed by glucagon or epinephrine. There is little evidence that HL expression is directly regulated by insulin. In **chapter 3** we hypothesized that hepatic lipase expression is downregulated by the increase in cAMP induced by catecholamines and glucagon. We showed that the synthesis and secretion of HL by HepG2 cells is strongly inhibited by incubation with the membrane permeant cAMP homologue, 8-bromo-cAMP (Br-cAMP). This effect on HL expression appears to be on the level of transcription and appears to be specific for HL. The effect of Br-cAMP was mediated through the -45/-36 element of the proximal HL promoter, which contains a putative binding site for liver-enriched C/EBPβ. Br-cAMP treatment decreases the nuclear expression of liver-enriched C/EBPβ protein and chromatin-immunoprecipitation (ChIP) assays showed that Br-cAMP also lowers the binding of this protein to the proximal HL promoter region in HepG2 cells. In adrenocortical H295 cells however, Br-cAMP failed to reduce transcriptional activity of the proximal HL promoter and also hardly reduced C/EBPβ expression. This finding underscores the importance of C/EBPβ in the regulation of HL expression by cAMP.

The human HL promoter contains a number of E-boxes, which are potential binding sites for ubiquitously expressed USF. In **chapter 4**, we hypothesized that glucose regulates the HL promoter via USF. When maintained in a high glucose environment, increased protein levels of USF1 and USF2 were found in the nuclei of HepG2 cells, compared to cells kept under low-glucose conditions, in parallel with increased HL expression. Increased expression of both

USF proteins was also observed in livers of hyperglycaemic, streptozotocin-treated rats. In ChIP assays, we found that USF proteins are bound to the proximal HL promoter region, and that binding is increased under high glucose conditions. Overexpressing USF1 or USF2 caused a dose-dependent upregulation of HL promoter activity in HepG2 cells. Silencing of USF1 by RNA interference strongly reduced this up regulating activity. These findings suggest that high glucose conditions result in elevated HL expression due to increased nuclear activity of USF proteins.

In **chapter 5** we studied in more detail the mechanism of transcriptional regulation of HL by the transcription factors USF1 and USF2 and the involvement of E-boxes. The common HL promoter -514C-to-T polymorphism, which associates with reduced HL expression *in vivo*, disrupts a non-canonical E-box. By using transient transfection assays with tandem repeats of either the -514C or -514T elements, we showed that the -514 E-box has little involvement in the transcriptional regulation of HL by USF in HepG2 cells. Overexpression of USF1 and USF2 increased transcription of the HL promoter primarily through binding to a canonical E-box sequence at the -310 position. Mutations of the -514 and -310 E-box or scrambling this sequence within the HL promoter reduced the USF responsiveness of the HL promoter region. We found that USF1 and USF2 increase transcription of the HL promoter through additional binding of the USF proteins at the transcription initiator element (Inr) of the HL core promoter. These findings indicate that USFs mediate their effect predominantly through binding at the -310 E-box and the TATA-Inr region of the proximal HL promoter.

In **chapter 6** we hypothesized that HL is not only regulated by glucose but also by fatty acids as integral part of intracellular lipid homeostasis. The incubation of HepG2 cells with oleate resulted in an increased HL secretion to 1.5-fold of control and increased the transcriptional activity of a 698-bp HL promoter-reporter construct (HL-luc) two-fold. This oleate stimulation was abolished with 10 μM atorvastatin. Incubation with oleate reduced the activity of transiently transfected SREBP-sensitive HMG-CoA synthase promoter construct (SRE-luc) by 50%; while atorvastatin increased its activity 2-3-fold. Cotransfection with an mature SREBP-2 (nSREBP-2) expression vector increased the SRE-luc activity but reduced the HL-luc activity. When stimulating the HL promoter activity by overexpression of USF, oleate did not further enhance its activity. On the other hand, atorvastatin or co-transfection with the nSREBP-2 vector did completely abolish the USF enhancing effect on HL/luc. The observations suggest that the opposite regulation of HL expression by fatty acids and statins is mediated via SREBP and USF.

In **chapter 7** we further tested the mechanism by which oleic acid influences Hepatic Lipase expression on the transcriptional level. Oleic acid not only up-regulated HL promoter activity, but also down-regulated the SREBP activity in HepG2 cells, and forced overexpression of nSREBP2 but not nSREBP1 suppressed the HL promoter activity. However, in cells transfected with nSREBP2, we observed a delay between the increase in SRE-luc activity, and the decrease in HL(-685/+13)-luc activity. When we suppressed SREBP activity in the HepG2 cells by incubation with cholesterol+25-hydroxycholesterol, we observed no significant upregulation

of the HL promoter activity. Thus, the HL gene does not appear a direct target of SREBP. We further showed that treatment of HepG2 cells with oleate increased the nuclear abundance of USF1. In addition, nuclear USF1 was reduced by overexpression of nSREBP2. Furthermore, overexpressed nSREBP2 exerted a negative effect on HL promoter activity mainly through the -310 E-box previously implicated in USF binding. This suggests that USF may be a fatty acid sensor acting in competition with SREBP2 in liver cells.

Polyunsaturated fatty acids (PUFAs) and monounsaturated fatty acids (MUFAs) differentially affect cholesterol homeostasis. In **chapter 8**, we compared how different fatty acids affect SREBP activity, measured as SRE-luc activity, with the extent of their incorporation into cellular lipids in HepG2 cells. We showed that n-3 and n-6 PUFAs were much more effective than oleate in suppressing SREBP activity when incubated at 120 μM concentration. The amount of PUFA that was incorporated into total cellular lipids was inversely proportional to SREBP activity. Besides the incorporation into cellular lipids the PUFAs were also converted to longer and more unsaturated types of fatty acids by the HepG2 cells. These processing activities were higher for n-3 than n-6 PUFA. DHA (22:6 n-3) was the most effective suppressor of SREBP activity. Incorporation of PUFA into cellular lipids increased flow of non-esterified cholesterol from the plasma membrane to the intra-cellular membranes, as illustrated by filipin staining in cells in which the reverse transport had been blocked by U18666A. This increased content of cholesterol in intracellular membranes may result in the inhibition of SREBP maturation and activation. Suppression of SREBP activity by PUFA depends on the degree of incorporation into cellular lipids, and is associated with increased flow of non-esterified cholesterol between the plasma membrane and intracellular membranes.

Finally, the main findings and directions for future research are discussed in **chapter 9**.

Samenvatting

Hoofdstuk 1 geeft een overzicht van de fysiologie van hepatisch lipase (HL) en de relatie met atherogenese en insuline resistentie, en geeft een inleiding van de regulatie van HL gen expressie.

Het HL gen van zoogdieren komt bijna uitsluitend tot expressie in hepatocyten. In **hoofdstuk 2** hebben wij in 30 kb van het 5'-flankerende gebied van een aantal zoogdier HL genen gezocht naar overeenkomstige stukken DNA, in de veronderstelling dat de elementen die verantwoordelijk zijn voor de lever-specifieke expressie geconserveerd zijn. Wij vonden dat het proximale promotorgebied en drie distale 200-400 bp gebieden, sterk geconserveerd zijn. Promoter-reporter assays in getransfecteerde HepG2 cellen bevestigden dat de distale elementen op -22kb en -14kb de activiteit van de proximale HL promotor (-685/+13) verhoogden. Een geconserveerd element op -10 kb bleek niet functioneel te zijn in deze assay. Binnen het proximale promotorgebied waren drie elementen geconserveerd, aangeduid als module A, B, en C. Module C (-25/+5) bevat de transcriptionele startsite en een pyrimidinerijke sequentie mogelijk overeenkomend met de initiator regio (Inr). Module B (-80 tot -40) bevat een potentiële HNF1 bindingsplaats. Dit element bevorderde de HL promotoractiviteit in HepG2 cellen, en remde de promotoractiviteit in de HeLa cellen. Module A (-240 tot -200) bevat een DR1-type bindingsplaats, maar de functie in transcriptie kon niet experimenteel kon worden bevestigd.

De HL activiteit in postheparine plasma is hoger in gevoede dan in gevaste condities, tenminste in proefdieren. Dit suggereert dat HL expressie wordt verhoogd door insuline of onderdrukt door glucagon of adrenaline. Er zijn weinig aanwijzingen voor een direct effect van insuline op HL. In **hoofdstuk 3** hebben we onderzocht of HL expressie in HepG2 cellen wordt onderdrukt door cAMP, dat immers in hepatocyten wordt verhoogd door catecholamines en glucagon. De synthese en secretie van HL bleek inderdaad sterk te worden geremd door incubatie van de HepG2 cellen met de membraanpermeabele cAMP-homoloog, 8-bromo-cAMP (Br-cAMP). De activiteit van de HL (-685/+13) promotor werd ook onderdrukt door Br-cAMP, en dit effect kwam tot stand via het -45/-36 element, een potentiële bindingsplaats voor C/EBPβ. Incubatie met Br-cAMP verlaagde de hoeveelheid C/EBPβ eiwit in de celkernen, en de hoeveelheid C/EBPβ eiwit dat volgens chromatin-immunoprecipitatie (ChIP) assays is gebonden aan de proximale promotor van het HL gen in HepG2 cellen. In H295 bijnierschorscellen werd de transcriptionele activiteit van de HL proximale promotor en de nucleaire hoeveelheid C/EBPβ eiwit niet door Br-cAMP verminderd. Dit onderstreept het belang van C/EBPβ in de regulatie van HL expressie door cAMP.

De proximale promotor van het humane HL gen bevat een aantal E-boxen, potentiële bindingsplaatsen voor Upstream Stimulatory Factors USFs. In **hoofdstuk 4** hebben we onderzocht of HL expressie wordt gereguleerd door glucose via USF. HepG2 cellen synthetiseren en secreteren meer HL in een hoog-glucose medium (22.5 mM glucose) dan in

een laag glucose medium (5 mM). Ook was de hoeveelheid USF1 en USF2 eiwit in de celkernen verhoogd. Expressie van beide USF eiwitten was ook verhoogd in levers van hyperglycemische, streptozotocine-behandelde ratten. USF1 en USF2 bleken in ChIP assays gebonden te zijn aan de proximale promotor van het HL gen in HepG2 cellen, en deze binding was verhoogd in het hoog-glucose medium. Overexpressie van USF1 of USF2 gaf een dosis-afhankelijke verhoging van de activiteit van de proximale HL promotor, terwijl deze activiteit was verlaagd na onderdrukking van USF1 expressie met behulp van RNAi. Deze resultaten suggereren dat glucose de expressie van het HL gen verhoogd door de hoeveelheid USF1 en USF2 in de celkern te verhogen.

In **hoofdstuk 5** is onderzocht hoe USF1 en USF2 de transcriptie van het HL gen stimuleren, en de rol van de E-boxen daarbij. Eerst is gekeken naar de -514 E-box. Het veelvoorkomende HL promotor -514C-naar-T polymorfisme, dat is geassocieerd met een verminderde HL expressie *in vivo*, vermindert de USF binding aan deze E-box in gelshift assays *in vitro*. In promoter-reporter assays met getransfecteerde HepG2 cellen bleek echter geen significant verschil in transcriptionele activiteit tussen promotor constructen met meerdere kopieën van of de -514C of de -514T E-boxen. Dit suggereert dat de rol van de -514 E-box in de activatie van HL expressie door USF in HepG2 cellen gering is. Overexpressie van USF1 en USF2 verhoogden de proximale HL promotor activiteit vooral door binding aan de E-box op positie -310. Aangebrachte mutaties in de -310 E-box verminderde de gevoeligheid van de proximale HL promotor voor USF. In gel-shift assays bleek het USF1 eiwit ook te binden aan de TATA-Inr sequentie van de HL promotor, en in HepG2 cellen werd de activiteit van de minimale HL promotor constructen met alleen de TATA-Inr sequentie in HepG2 cellen door USF1 en USF2 gestimuleerd. Deze resultaten tonen aan dat USF de activiteit van de proximale HL promotor stimuleren voornamelijk via binding aan de -310 E-box en de TATA-Inr sequentie.

HepG2 cellen produceren ook meer HL na toevoeging van oliezuur aan het kweekmedium. In **hoofdstuk 6** laten we zien dat dit effect van 1 mM oliezuur wordt teniet gedaan wanneer de cellen tegelijkertijd worden geïncubeerd met 10 uM atorvastatine, een remmer van de cholesterolsynthese en daardoor een activator van de cholesterol- en vetzuur gevoelige SREBP transcriptiefactoren. Oliezuur verhoogde de activiteit van de HL (-685/+13) promotor, hetgeen onderdrukt werd door atorvastatine. De SREBP activiteit werd gemeten met een promotor-reporter assay waarbij HepG2 cellen worden getransfecteerd met een SRE-bevattend luciferase reporter plasmide (SRE-luc). In deze assays werd de SREBP activiteit verlaagd door oliezuur en verhoogd door atorvastatine. Overexpressie met constitutief actief SREBP2 (nSREBP-2) verhoogde de SRE-luc en verminderde de proximale HL promotor activiteit. Zowel atorvastatin als overexpressie van nSREBP-2 deed het stimulerende effect van USF1 op de HL promotor activiteit teniet, terwijl oliezuur de HL promotor activiteit niet verder stimuleerde in HepG2 cellen waarin USF1 tot overexpressie komt. Geconcludeerd werd dat HL expressie tegenovergesteld wordt gereguleerd door oliezuur en statines, mogelijk door een competitieve werking van SREBP en USF.

In **hoofdstuk 7** is de mogelijk competitieve werking van SREBP en USF in de regulatie van de proximale HL promotor verder onderzocht in HepG2 cellen. De HL promotor activiteit werd dosisafhankelijk onderdrukt door overexpressie van nSREBP2, maar niet door overexpressie van nSREBP1. De remming van de HL promotor activiteit door transfectie met nSREBP2 vector trad echter veel later op dan de stijging in SREBP activiteit. Oliezuur onderdrukte de SREBP activiteit, maar een bijna volledige remming van endogene SREBP activiteit door incubatie van de HepG2 cellen met cholesterol+25-hydroxycholesterol had geen effect op de HL promotor activiteit. Incubatie van HepG2 cellen met oliezuur verhoogde de hoeveelheid USF1 eiwit in celkernextracten, terwijl in celkernen aanwezige USF1 door overexpressie van nSREBP2 de hoeveelheid USF1 verminderde. De vertraagde remming van de HL promotor activiteit door overexpressie van nSREBP2 wordt hoofdzakelijk gemedieerd via de -310 E-box, dat ook belangrijk is voor de regulatie door USF. Geconcludeerd werd dat het HL gen geen directe target is van SREBP-2, maar dat SREBP2 competeert met de werking van USF. USF functioneert mogelijk als vetzuur sensor in levercellen.

In **hoofdstuk 8** hebben wij het effect van verschillende meervoudig onverzadigde vetzuren (PUFAs) en oliezuur op de SREBP activiteit in HepG2 cellen bestudeerd, en vergeleken met de mate van integratie in de cellulaire lipiden. De SREBP activiteit werd weer gemeten in cellen getransfecteerd met de SREBP-gevoelige luciferase reporter plasmide. Bij concentraties van 60-120 uM bleken de n-3 en n-6 PUFA's veel efficiënter dan oliezuur in het onderdrukken van de SREBP activiteit. DHA (22:6 n-3) was de meest effectieve onderdrukker van SREBP activiteit. De SREBP activiteit was omgekeerd evenredig met de hoeveelheid geincorporeerde PUFA's, en met de mate van onverzadigdheid van de cellulaire lipiden. De n-3 PUFA's werden ook meer omgezet in langere en meer onverzadigde types van vetzuren dan de n-6 PUFA's. Incubatie van HepG2 cellen met n-3 PUFA's verhoogde de flow van niet-veresterde cholesterol van het plasmamembraan naar de intracellulaire membranen. Dit bleek uit een verhoogde intracellulaire cholesterolophoping in cellen waarin de omgekeerde flow van cholesterol naar het plasmamembraan was geblokkeerd door U18666A. Deze toename in de hoeveelheid cholesterol in intracellulaire membranen is een mogelijk verklaring voor de remming van de SREBP maturatie en daarmee van de SREBP activiteit door PUFA's.

De algemene bevindingen en suggesties voor vervolgonderzoek zijn bediscussieerd in **hoofdstuk 9**.

List of Publications

- Di Nunzio M, van Deursen D, Verhoeven AJ, Bordoni A. n-3 and n-6 Polyunsaturated fatty acids suppress sterol regulatory element binding protein activity and increase flow of non-esterified cholesterol in HepG2 cells. *Br J Nutr*. 2010; 103(2): 161-167.

- van Deursen D, van Leeuwen M, Vaulont S, Jansen H, Verhoeven AJ. Upstream Stimulatory Factors 1 and 2 activate the human hepatic lipase promoter via E-box dependent and independent mechanisms. *Biochim Biophys Acta*. 2009; 1791(4): 229-237.

- van Deursen D, van Leeuwen M, Akdogan D, Adams H, Jansen H, Verhoeven AJ. Activation of hepatic lipase expression by oleic acid: possible involvement of USF1. *Nutrients*. 2009; 1(2): 133-147.

- van Deursen D, Jansen H, Verhoeven AJ. Glucose increases hepatic lipase expression in HepG2 liver cells through upregulation of upstream stimulatory factors 1 and 2. *Diabetologia*. 2008; 51(11): 2078-2087.

- van Deursen D, Botma GJ, Jansen H, Verhoeven AJ. Down-regulation of hepatic lipase expression by elevation of cAMP in human hepatoma but not adrenocortical cells. *Mol Cell Endocrinol*. 2008; 294(1-2): 37-44.

- van Deursen D, Botma GJ, Jansen H, Verhoeven AJ. Comparative genomics and experimental promoter analysis reveal functional liver-specific elements in mammalian hepatic lipase genes. *BMC Genomics*. 2007; 8: 99.

- van Deursen D, Van Leeuwen M, Jansen H, Verhoeven A. Hepatic Lipase is upregulated by Glucose and Fatty acids via induction of Upstream Stimulatory Factors. *Atherosclerosis Suppl*. 2006; 7(3): W3:4.

- Botma GJ, van Deursen D, Vieira D, van Hoek M, Jansen H, Verhoeven AJ. Sterol-regulatory-element binding protein inhibits upstream stimulatory factor-stimulated hepatic lipase gene expression. *Atherosclerosis*. 2005; 179(1): 61-67.

PhD Portfolio

Erasmus MC
Universitair Medisch Centrum Rotterdam

Name PhD student: Diederik van Deursen	PhD period: 2002-2007
Erasmus MC Department: Internal Medicine / Biochemistry	Promotor: Prof. Dr. E.J.G. Sijbrands
Research School: Cardiovascular Research School Erasmus University Rotterdam (COEUR)	Supervisor: Dr. A.J.M. Verhoeven

1. PhD training

	Year	ECTS
General academic skills		
- Biomedical English (old curriculum, incl. Cambridge exam)	2003	3.5
Research skills		
- Statistics (NIHES)	2005	6.0
- Animal Experimentation Course (article 9)	2004	4.5
- Biomedical Research Techniques (postgraduate school MolMed)	2004	1.5
In-depth courses (e.g. Research school, Medical Training)		
- COEUR courses (6 courses)	2003-2005	9
- NHF course Cardiac function and adaptation	2005	3
International conferences		
- Ernst-Klenk-Symposium (Cologne, Germany)	2003	0.9
- joint MGC-Cancer Research UK Graduate Student Conference (Lille, France & Oxford, UK)	2003, 2006*	1.8
- European Lipoprotein Club (Tutzing, Germany)	2004**	1.2
- Scandinavian Society for Atherosclerosis Research (Humlebæk, Denmark)	2005*	1.2
- Anglo-Danish-Dutch Diabetes Group (Beetsterzwaag, Netherlands)	2006**	1.2
- International Symposium on Atherosclerosis (Rome, Italy)	2006**	1.5
- International Symposium on High Density Lipoproteins (Parma, Italy)	2006*	0.6
Seminars and workshops		
- COEUR seminars	2003-2006	2.5
- Netherlands Lipoprotein Club (NLC) seminars	2003-2006	1.2
- Dutch Atherosclerosis Society (Ermelo)	2003, 2004	0.6
- NHF Day of Science (Leiden & Amsterdam)	2004*, 2005*	0.6
	* poster presentation	
	** oral presentation	

2. Teaching activities

	Year	ECTS
Supervision of trainees		
- Medical students (I. Davelaar, D. Akdogan)	2004, 2006	3
- Laboratory technician trainees (J. Koster, R. Voorzaat)	2004, 2007	4

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Diederik

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Curriculum Vitae

Diederik van Deursen is geboren op 19 april 1972 te Schiedam. Na het behalen van zijn MAVO-diploma aan de scholengemeenschap Buiten te Veste te Schiedam in 1988, werd het MLO diploma, richting Technische Microbiologie behaald in 1993 aan het Reynevelt College te Delft. Vervolgens werd in 1997 het HLO diploma, richting Biochemie behaald aan de Hogeschool Rotterdam & Omstreken te Delft. De MLO en HLO praktijkstages werden uitgevoerd bij de afdeling Stralengenetica & Chemische Mutagenese (Universiteit Leiden, o.l.v. drs. M.F. van Oosterwijk) en TNO Preventie en Gezondheid, afdeling Vaat- en Bindweefsel Onderzoek (Leiden, o.l.v. dr. P.E. Slagboom en drs. B.T. Heijmans). Van 1997 tot en met 1998 was hij werkzaam als farmaceutisch analist bij Chefaro te Rotterdam. In 1998 werd begonnen met de studie Biologie aan de Universiteit Leiden. Zijn eerste wetenschappelijke stage werd uitgevoerd op de afdeling Moleculaire Celbiologie van het Instituut Moleculaire Plantkunde (Leiden, o.l.v. dr. N.E.M. Quaedvlieg en dr. C.L. Díaz). Tijdens zijn tweede stage op het Department of Biological Sciences aan de Kent State Unversity in Ohio, USA, werd onder leiding van prof. dr. J.R.D. Stalvey onderzoek verricht naar de effecten van *in utero* blootstelling van ratten aan de dioxine TCDD en op de 3-beta-hydroxysteroid dehydrogenase (3β-HSD) expressie in de bijnieren van ratten. In 2001 werd het doctoraalexamen Biologie, specialisatie Moleculaire Biologie behaald. In 2002 begon Diederik zijn promotieonderzoek op de afdeling Biochemie van het Erasmus MC onder begeleiding van dr. A.J.M. Verhoeven en prof. dr. H. Jansen. De resultaten uit dit onderzoek zijn beschreven in dit proefschrift. In 2006 behaalde hij ook een research master in clinical research aan de Erasmus Universiteit Rotterdam. Tussen 2007 en 2010 werkte Diederik als project manager / study director bij het contract research laboratorium MicroSafe (Merck Millipore) te Leiden. Momenteel is hij werkzaam bij het farmaceutisch bedrijf HAL Allergy te Leiden op de afdeling Analytical Development.