Promoter Variants and Variant Promoters of the Hepatic Lipase Gene

Promoter verschillen en verschillende promoters van het lever lipase gen

Proefschrift

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Voor Delfina en Julia

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Chapter 1.0

Introduction

1.1 Role of Hepatic lipase in lipoprotein metabolism

1.1.1 Hepatic lipase

Hepatic lipase (HL; E.C. 3.1.1.34) is a glycoprotein with triglyceridase, phospholipase A1 and some esterase activity, but no cholesterylesterase activity. During the assembly of the HL protein the rat HL protein acquires two and the human four oligosaccharide chains. Most HL is synthesised in liver parenchymal cells and secreted into the space of Disse were it binds to heparan sulfate proteoglycans. HL activity is also present in the steroidogenic organs as adrenal glands, ovaries and testis. The HL protein is synthesised in the liver and transported via the bloodstream to these steroidegenic organs where it accumulates. With the use of heparin, HL can be displaced from its binding site in the same manner as lipoprotein lipase (LPL). The human and rat mature HL have an apparent molecular weight of 65 and 58 kDa, respectively.

HL is an important enzyme in lipoprotein metabolism, and is involved in the exogenous as well as endogenous lipid transport pathway, and in "reverse cholesterol transport". In the exogenous lipid transport pathway, lipoproteins are formed by, and transported from, the intestine to, finally, the liver while in the endogenous lipid transport pathway lipoproteins are formed by, and transported from, the liver to the peripheral tissues. In the reverse cholesterol transport pathway, high-density lipoproteins (HDL) transport cholesterol from the peripheral tissues back to the liver, where cholesterol can be re-used or be excreted from the body via the bile. HL is considered a key enzyme in reverse cholesterol transport, which will be discussed in more detail in § 1.1.2. In figure 1 the central role of HL in lipoprotein metabolism is pictured.

In the exogenous lipid transport pathway, triglycerides and cholesterol are absorbed in the small intestine and incorporated into chylomicrons, which contain the apolipoproteins apoB48, apoAI and apoAIV. After secretion into the lymphatic system, chylomicrons enter the bloodstream via the thoracic duct. In the circulation, ApoAI and apoAIV are lost and apoCI, apoCII, apoCIII and apoE are acquired from HDL. ApoCII is an essential cofactor for LPL, enabling the LPL present in muscle and adipose tissue to hydrolyse the triglycerides. The chylomicrons become smaller in size and enriched in cholesteryl esters by the removal of triglycerides. Finally, chylomicron remnants are formed, which are considered to be highly atherogenic [54, 114]. Via the low density lipoprotein receptor (LDL-R) or the LDL-R-related-protein (LRP) the liver clears the chylomicron remnants from the circulation [118]. HL has been shown to be internalized by liver cells together with chylomicron remnants [89]. In rats, immunoinhibition of HL with anti-HL antibodies leads to impaired clearance of chylomicron remnants [94]. It has been suggested that heparan sulphate proteoglycans (HSPG) and/or HL present on the surface of hepatocytes first bind the remnants and then bring the remnants in close proximity of one or both of the above receptors for hepatic uptake [118]. Catalytic activity of HL does not appear to be necessary for this ligand function [30]. When the clearance is impaired, the chylomicron remnants circulate longer, resulting in longer exposure to the atherogenic remnants. This is observed for instance in patients with hypertriglyceridemia, familial combined hyperlipidemia and non-insulin-dependent-diabetes-mellitus (type 2 diabetes) [118].

In the endogenous lipid transport pathway, hepatocytes form TG and CE from dietary, endogenous and de novo synthesized fatty acids and cholesterol, and assemble them with apoB100 into very low density lipoproteins (VLDL). VLDL is secreted directly into the circulation. Here, the VLDL particle acquires apoCI, apoCII, apoCIII and apoE from HDL. In muscle and adipose tissue the triglycerides of VLDL are hydrolysed by LPL, using apoCII as co-factor. When the VLDL particle looses triglycerides, it becomes a smaller particle called a VLDL remnant or IDL (intermediate density lipoprotein). IDL is regarded as a major determinant of the risk for atherosclerosis [62]. The liver, via the LDL-R and apoE receptor, can take up IDL, or its triglycerides can be hydrolysed by HL; in the latter process the IDL looses its apoE thus effectively becoming an LDL particle. In the process from VLDL to LDL, the particle is enriched in cholesteryl esters. Peripheral tissues and the liver can take up the LDL particle via the LDL-R. With a higher HL activity, the LDL particle becomes more depleted of TG and hence, more small-dense LDL (sdLDL) particles are formed. These sdLDL are smaller, have less affinity for the LDL-R, and are more prone to oxydative modification, and hence are far more atherogenic than normal LDL.

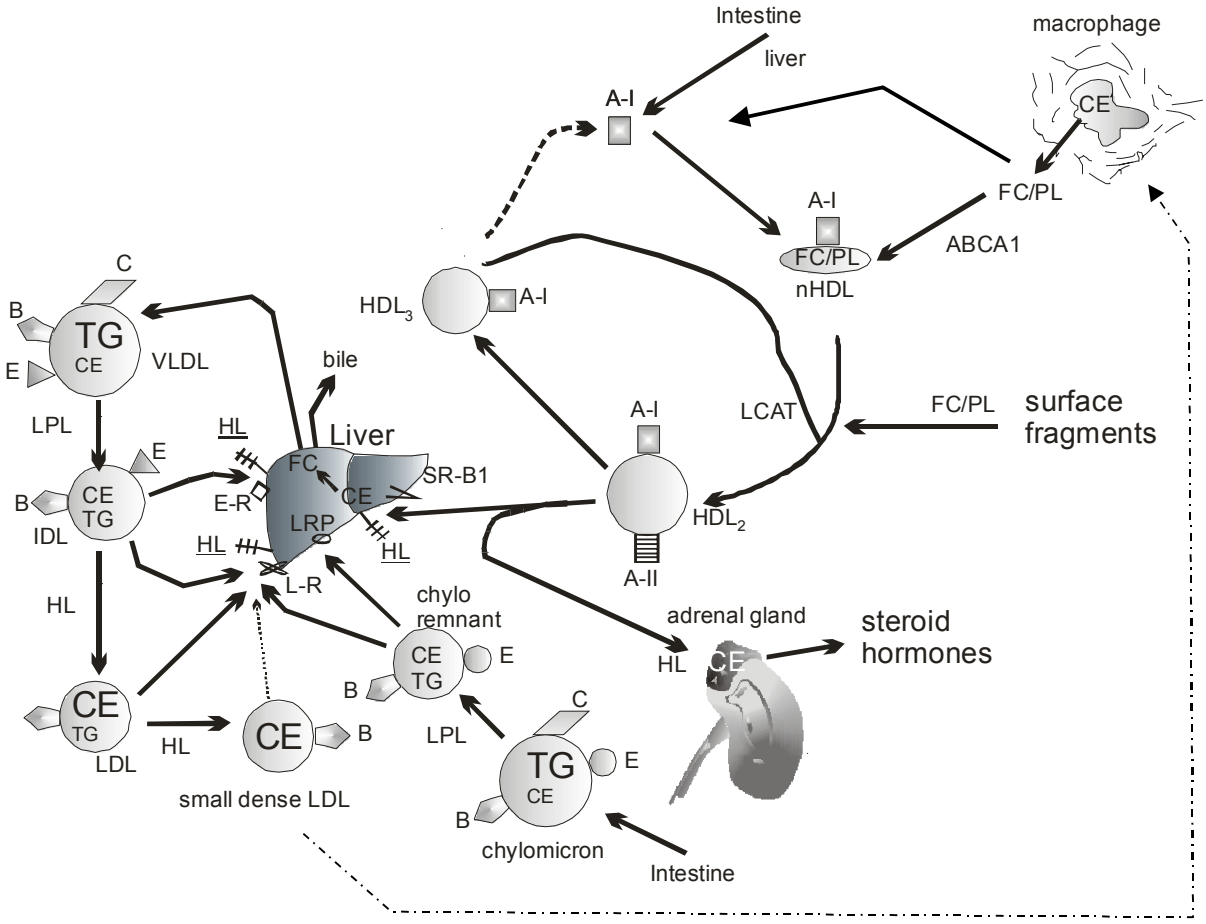

Figure 1: **Central role of hepatic lipase in lipoprotein metabolism**
The metabolic pathways are described in the text.
A-I, apolipoprotein A-I; A-II, apolipoprotein A-II; ABCA1, ATP-binding cassette transporter A1; B, apolipoprotein B; C: apolipoprotein C2; CE, cholesteryl-esters; chylo remnant, chylomicron remnant; E, apolipoprotein E; E-R, apoE receptor; FC, free cholesterol; HL, hepatic lipase catalytic active; HL, hepatic lipase as ligand; L-R, LDR receptor; LPL, lipoprotein lipase; nHDL, nascent HDL; PL, phospholipid; SR-B1, scavenger receptor B1; LCAT, lecithin–cholesterol acyltransferase; VLDL, very low density lipoprotein.

1.1.2 Hepatic lipase and reverse cholesterol transport

The reverse cholesterol transport pathway is the process in which tissue cholesterol returns to the liver via HDL. Cholesterol from the plasma membrane of arterial wall cells is loaded onto lipid-poor nascent HDL particles to form HDL_3, or onto HDL_3 to form HDL_2 particles. The efflux of cholesterol and phospholipids to apoA-I is mediated by the ATP-binding cassette, subfamily A, member 1 (ABCA1) [82]. In this process the free cholesterol and phosholipids are integrated in the surface of the HDL particle. Free cholesterol is esterified with a fatty acid derived from the phospholipid by the enzyme lecithin:cholesterol acyltransferase (LCAT) loosely bound on the HDL. Upon esterification, the cholesterol migrates from the phospholipid coat to the hydrophobic core, and the preß-HDL matures into a spherical α-HDL. Similarly, the HDL_3 particle is converted to a larger, CE-enriched HDL_2 particle.

The liver takes up cholesteryl esters from HDL_2 through one of several mechanisms. First, the liver can internalize the whole HDL particle via endocytosis, after binding at putative HDL receptors [27]. In this process, the whole particle ends up in the lysosome and is completely degraded. Perhaps, the ligand function of HL may play a role in this process, as outlined above for the clearance of chylomicron remnants. Secondly, cholesteryl esters can be removed from HDL_2 by the process of 'selective cholesterol uptake', which is mediated by SR-BI and HL. SR-BI [1] promotes the selective removal of both free and esterified cholesterol from HDL particles. In this process, the HDL_2 particle is endocytized but then recycled back to the surface of the liver cell (retro-endocytosis) [82]. In the endosomes close contact of HDL with HL may lead to selectively unloading of free and esterified cholesterol. This process probably requires the phospholipase A_1 activity of HL, as pre-treatment of HDL with HL or other phospholipases enhances its cholesterol delivery capacity to liver cells [4, 67]. Selective cholesterol uptake is also evident in steroidogenic organs, in which HL and SR-BI co-localize. In rat adrenals, HL cooperates together with SR-BI in the selective uptake of HDL (see § 1.1.3) [106]. Finally, cholesteryl esters of HDL_2 can be transported to the liver by an indirect route involving LDL and the LDL-R. Cholesteryl ester transfer protein (CETP) promotes the transfer of cholesteryl esters from HDL to the apoB containing lipoproteins VLDL and LDL in exchange for triglycerides. The liver can then take up the LDL via the LDL-R. The triglycerides in HDL are subsequently hydrolysed by HL. The combined action of CETP and HL results in the remodelling of HDL2 into HDL3, which again can act as acceptors of ABC-AI mediated cholesterol efflux from peripheral cells. Taken together, HL proofs to be a crucial factor in reverse cholesterol transport, both via the direct and the indirect route.

1.1.3 Hepatic lipase and steroidogenesis

The steroidogenic organs ovaries, testis and adrenal glands synthesise the steroid hormones estrogens and progestagens, androgens, and gluco-and mineralocorticoids, respectively. Since these hormones are derived from cholesterol, the supply of cholesterol is crucial for the maintenance of steroidogenesis. Like in most other cells, the steroidogenic cells are able to synthesize cholesterol *de novo*. However, if the rate of cholesterol synthesis is slower than the rate of hormone synthesis, the required cholesterol can be taken up from the circulation, either from LDL or from HDL. In humans, the major source of cholesterol for steroidogenesis may be LDL. In the rat, the major source has been shown to be HDL [33] . HDL

cholesterol is predominantly taken up by the steroidogenic cells by the selective cholesterol uptake mechanism mediated by SR-BI and HL, similar to the liver. In rat, human, hamster, and bovine adrenal cortex an enzyme activity (liver-type lipase) highly similar to HL has been detected [19, 46, 47], as well as in rat and mouse ovaries [39, 47, 109] and human granulosa cells [75]. In adrenals, HL activity is localized in the zona fasciculata [74]. In ovaries, HL has been detected in the corpora lutea, while very little activity was detected in pre-ovulatory follicles [49, 74]. The HL activity measured in these organs varies in parallel with the generation of steroid hormones [28, 48, 109], suggesting that HL is important for the cholesterol supply during steroidogenesis.

The cooperation between HL and SR-BI has been studied with immuno-inhibition experiments. In vivo inhibition of rat HL activity using HL anti-bodies resulted in a 5.2 and 1.6 fold increase of rat adrenal SR-BI mRNA and protein, respectively, within four hours [106]. The uptake of HDL cholesteryl esters by the adrenals increased by 68% [106]. Possibly, to compensate for the impaired uptake of free cholesterol due to inhibition of HL, an excess of SR-BI protein is synthesised, resulting in the increased uptake of HDL cholesteryl esters. Apparently, HL is not needed for adrenal SR-BI mediated selective uptake of HDL, but HL influences the uptake by SR-BI. The exact way of cooperation between HL and SR-BI is not known yet. Possibly, remodelling of HDL2 by HL may facilitate its binding to SR-BI, or may facilitate selective cholesterol uptake from HDL2. When HL is inhibited, more SR-BI is needed to fulfil the demand for cholesterol in the adrenal gland.

Despite the presence of HL activity in these steroidogenic organs, no full length HL mRNA has been found. Instead, in rat adrenals and ovaries a truncated HL mRNA is synthesised, which lacks the first two exons of the HL gene [104, 105]. Pulse-labeling experiments in rat adrenocortical cells showed synthesis of a HL immunoreactive protein of 40-45 kDa, that remained mainly intracellularly [104]. For rat ovaries it has been shown that during superovulation the truncated HL mRNA was induced in parallel with synthesis of a similarly sized 47 kDa HL immunoreactive protein [105]. The function of this intracellular HL-like protein is still unknown.

1.1.4 Hepatic lipase deficiency

As mentioned above, HL has multiple roles in lipoprotein metabolism. Predictably, HL deficiency will affect the exogenous and endogenous lipid transport pathway, and the reverse cholesterol transport pathway, each with a potentially different outcome for atherosclerotic risk. As discussed in § 1.1.1, HL promotes the clearance of the atherogenic chylomicron and VLDL remnants. HL deficiency impairs the clearance of both atherogenic lipoprotein particles and could therefore increase atherosclerotic risk. On the other hand, HL stimulates the conversion of VLDL remnants to LDL, especially to sdLDL, which is also highly atherogenic, so HL deficiency would predictably be pro-atherogenic. HL converts HDL_2 into the smaller and denser HDL_3. During this conversion, cholesterol is taken up by the liver, and plasma HDL cholesterol becomes lower. Epidemiologically, high HDL cholesterol protects against the development of atherosclerosis. In case of HL deficiency plasma HDL cholesterol would be elevated, and this condition would be considered anti-atherogenic. However, if the cholesterol flow from the periphery is impaired because of HL deficiency, the accumulation

ofHDL_2 would not protect against atherosclerosis. Jansen has described very clearly all arguments for the pro- and anti-atherogenic potential of hepatic lipase [54]. This is summarized in table 1.

In HL knock out mice, chylomicron remnant removal is suppressed [40], but only after a high fat load. Fertility of these mice was reduced, in line with reduced cholesterol supply for steroid hormone production in ovaria [109]. In HL knock out mice crossed with apo E deficiency, plasma cholesterol was increased, but the susceptibility to atherosclerosis was decreased [69]. Rabbits naturally express very low HL activity [17], and readily develop atherosclerotic plaques on a high cholesterol diet [58]. Overexpression of HL in transgenic rabbits attenuates the rise in plasma lipids induced by cholesterol feeding, but increased plaque size [100]. In contrast, overexpression of HL in transgenic mice reduces aortic cholesterol deposition [12]. Hence, in animal models HL can be either pro- or anti-atherogenic, depending on the genetic background of the animals.

HL deficiency in the human population is very rare [18]. Possibly it is rare because the phenoltypical manifestation of HL mutations is not recognized. Coronary artery disease has been reported in subjects with HL deficiency, but the numbers are too low to decide whether human HL deficiency affects atherosclerotic risk [18]. Among others, four mutations in the coding sequence [35, 59] and one in an intron [23] of the HL gene that affect HL expression have been described. Knudsen described a Finnish family with HL deficiency due to a compound heterozygosity for L334F and T383M [59]. In vitro studies showed that each mutation reduced specific enzyme activity of the HL protein to 30% and 80% of wild type, respectively [24, 59]. Plasma lipoproteins of the affected patients showed a slight increase in large, buoyant LDL and an increase in HDL_2 [59]. Subjects who are compound heterozygotes for S267F and T383M were found to have type III hyperlipidemia and premature atherosclerosis [36]. The S267M mutation led to a catalytically inactive HL protein [24]. Heterozygotes for either the R186H or L334F mutation presented with moderately increased plasma triglycerides, and increased triglycerides in LDL, HDL_2 and HDL_3 [60]. This indicates that heterozygote carriers of these HL mutations have decreased HL activity and an atherogenic lipid profile. Several polymorphisms in the coding sequence that lead to aminoacid substitutions have been described (V73M, N193S, F234S), but these polymorphisms do not markedly affect specific enzyme activity [35, 98].

Mutations in the coding part of the HL gene are relatively rare, particularly when compared with the closely related lipoprotein lipase (LPL) gene (see § 1.2.1). Recently a common polymorphism in the HL promoter region has been discovered [32, 51]. This polymorphism consists of four SNPs that are in almost complete linkage disequilibrium [32, 41]. Therefore, two different alleles can be distinguished, which are designated as the *LIPC* C- and T-allele, according to the –514SNP. The frequency of the T-allele varies between 17-24 % in Caucasians to 40-50 % in Japanese and Afro-Americans, and even to 55 % in some American Indian populations [45]. Several studies have shown that the T-allele is associated with a lowered HL activity [8, 51, 96, 103] and elevated HDL cholesterol [32, 51, 52, 96, 103].
In vitro, promoter activity of the LIPC T-allele was less than the LIPC C-allele [21] (chapter 6). In some but not all studies, carriers of the T-allele have a higher incidence for coronary artery disease despite the higher HDL cholesterol [23, 51, 91].

Table 1: **Arguments for pro- and anti-atherogenic potential of hepatic lipase.**

Impact on Human Lipoprotein Metabolism	
Pro-atherogenic	- Decreases LDL size
Anti-atherogenic	- Stimulates HDL cholesterol (ester) uptake (reverse cholesterol transport)
	- Stimulates post-prandial lipid clearing
	- Stimulates IDL clearing
	- Stimulates preβ HDL, HDL3 formation
Animal Studies	
Pro-atherogenic	- Deficiency attenuates atherosclerosis in apoE k.o. mice
	- Overexpression augments plaque size in rabbits
Anti-atherogenic	- Over-expression in mice decreases aortic cholesterol deposition
	- Inhibition of activity in apoA-II overexpressing mice increases aortic cholesterol deposition
	- Animals low in HL show diet-induced hyperlipidemia
Associations with Human Lipoprotein Profile	
Pro-atherogenic	- Activity inversely correlated with HDL (2)
	- Activity inversely correlated with LDL size
Anti-atherogenic	- Activity inversely correlated with post-prandial lipids
	- Activity inversely correlated with LpCIII:B
Association with Human Atherosclerosis Promoting Diseases and Other Conditions	
Pro-atherogenic	- Activity high in males compared to females
	- Activity positively associated with insulin-resistance and high in type 2 diabetes
	- Activity positively correlated with omental fat mass, fasting insulin
	- Activity high in FH
Anti-atherogenic	- Activity low in hypothyroidism
Association with Human CAD	
Pro-atherogenic	- Decrease in activity during hypolipidemic drug treatment associated with Increased LDL size and decreased CAD
Anti-atherogenic	- Activity low in CAD patients (if accompanied by low CETP)
	- Activity inversely associated with calcification in homozygote FH
	- Activity predictor of CAD regression after dietary intervention
	- Deficiency associated with increased CAD risk

The table summarizes arguments in favour of a pro- or anti-atherogenic potential of HL based on different experimental approaches. (From Jansen et al. J Lipid Res. 2002 43:1352-62 [54])

Connelly and Hegele proposed that moderate HL deficiency increases atherogenic risk, especially in the presence of a second genetic or environmental factor affecting lipoprotein levels [18]. This may also hold for carriers of the *LIPC* T-allele. Support for this hypothesis comes from a Japanese study, which showed that subjects with high HDL cholesterol levels exhibited increased coronary artery disease, but only when both HL and cholesteryl ester transfer protein (CETP) activity were low [38]. Although the LIPC genotype was not determined in this study, the low HL activity in these subjects is likely due to the HL promoter polymorphism because of the high T-allele frequency among the Japanese [45]. Since multiple gene products play a role in lipoprotein metabolism it may be worthwhile to investigate the interaction of the HL promoter polymorphism with other genes, such as CETP Taq1B [85, 112],

EL 2,237 3'UTR or Thr111Ile [80], LPL HindIII [80] or S447X [43, 85], apoE ε2, ε3 or ε4 [5, 85 112], microsomal triglyceride transfer protein (MTP) -493 G/T [112], insuline gene VNTR-Hph*I* [111], PPARγ P12A [93], and apoCIII -482C > T [5, 53] in determining the risk for cardiovascular disease.

1.1.5 Hepatic lipase and type 2 diabetes

Type 2 diabetes, previously called non-insulin-dependent diabetes mellitus (NIDDM), manifests normally after the age of 40, especially in obese, but its prevalence in younger subjects and even in children is rapidly increasing [9, 77]. The clinical picture of type 2 diabetes is diverse. According to the World Health Organization, type 2 diabetes may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with or without insulin resistance [113]. The disease is thought to gradually progress from glucose intolerance with mild insulin resistance, via a hyperglycemic and hyperinsulinemic diabetic state, to the severe insulin deficient state similar to type 1 diabetes. Patients in the early stage of disease progression are able to produce insulin. The target tissues respond less than normal to the insulin, and the pancreatic β-cells compensate this relative insulin insufficiency with increased release of insulin. This in turn further deteriorates the responsiveness of target tissues to insulin, thus entering a vicious cycle of increased insulin secretion and reduced insulin responsiveness. Finally the islets of Langerhans are worn out and are no longer able to produce insulin. The primary cause of insulin resistance may lie either in muscle, in adipose tissue or in the liver. Finally, all these organs become resistant to insulin. Insulin resistance of muscle results in decreased postprandial glucose disposal. Insulin resistance of adipose tissue results in increased lipolysis, giving rise to increased fatty acid delivery to the liver and muscle, which in turn will affect glucose handling by these tissues. Insulin resistance of the liver manifests as increased gluconeogenesis.

The underlying mechanism of the insulin resistance has not been unravelled yet. Reduced control of *de novo* glucose synthesis is a likely candidate. The liver and the kidney are the only organs capable of *de novo* glucose synthesis and secretion [25]. Normally, in fasted conditions, the basal *de novo* synthesis of glucose in the liver maintains normal blood glucose levels. In type 2 diabetes this glucose synthesis in the fasted state is normal to slightly elevated [42]. Normally, in fed conditions (hyperinsulinemic or hyperglycaemic), the basal level of *de novo* synthesis of glucose in the liver is suppressed, and fatty acids are stored as triglycerides in adipose tissue. In type 2 diabetes, the basal level of *de novo* synthesis of glucose in the liver is not, or less, suppressed [22]. This results in elevated plasma glucose and/or increased insulin levels. Recently, the group of Peter Voshol showed compelling evidence that triglyceride production in mice liver increases glucose output [108]. The increased triglyceride levels in liver may result from increased delivery of fatty acids from insulin resistant adipose tissue. Alternatively, the liver lipogenic pathway may be less affected by insulin resistance than glucose *de novo* synthesis. Either way, the increased TG production will lead to increased secretion of VLDL, and the type 2 diabetic patients become hypertriglyceridemic.

Although the genetic background of type 2 diabetes is not known yet, the basic clinical picture is well defined. Besides hyperglycemia, type 2 diabetic subjects also experience

obesity, hypertension, and dyslipidemia. The dyslipidemia is characterised by high triglycerides (mainly as VLDL), low HDL cholesterol and an elevated number of small, dense LDL particles. LPL and HL are the two most important plasma lipolytic enzymes involved in plasma lipoprotein metabolism. In the postprandial state, insulin stimulates LPL expression in adipose tissue [26], which increases lipolysis of the triglycerides in chylomicrons and VLDL. In a small study with type 2 diabetic patients and diabetic dyslipidemia more that 50% of the subjects were shown to have a polymorphism in the LPL gene with a decreased LPL activity [120]. It has been suggested that in type 2 diabetes LPL gene variants are a predictor for diabetic dyslipidemia [120].

HL hydrolyzes the triglycerides in IDL, and maybe also in the chylomicron remnants. Similar to LPL, HL activity also appears to be up-regulated by insulin [15, 81] (see § 1.2.3). In the hyperinsulinemic stage of type 2 diabetes, HL activity is higher than in normoglycemic healthy controls [8, 99]. An increased HL activity will increase TG lipolysis in IDL leading to predominantly small, dense LDL, as well as to lower HDL cholesterol levels, typical of the diabetic dyslipidemia. The common LIPC T allele of the HL gene is associated with a decreased HL activity, increased triglycerides and decreased HDL cholesterol compared with the C allele. Interestingly, HL activity of carriers of the T allele did not increase in parallel with fasting plasma insulin, whereas HL activity in homozygotes for the C allele did [51] (see 1.2.3.3). Hence, the common –514C→T substitution in the promoter region of the HL gene may represent a case of gene specific insulin resistance, similar to what has been described for the apoCIII –482 C→T and –455 T→C polymorphisms which fall within a previously identified insulin response element in the apoCIII gene [64].

The frequency of LIPC-T allele carriers was shown to be higher among insulin resistant subjects, such as type 2 diabetes and familial combined hyperlipidemia, than in normoglycemic controls [63]. The –514C→T substition was also associated with higher liver fat content and increased whole-body insulin resistance [93]. Carriers of the –514T allele exhibited decreased insulin sensitivity, when on a saturated but not unsaturated fat diet [29]. Moreover, the HL promoter polymorphism may also affect the progression of type 2 diabetes. In two studies the HL promoter polymorphism was shown to predict the development of type 2 diabetes. In the STOP-NIDDM trial [119], subjects with impaired glucose tolerance (IGT) were monitored for a period of approximately three years. Subjects homozygote for the LIPC T allele had a 2.3 fold higher risk for conversion to type 2 diabetes compared with subjects homozygote for the LIPC C allele [119]. In The Finnish Diabetes Prevention Study (FDPS), however, the effect of the LIPC genotype is just the other way around as the LIPC C allele appeared to protect from type 2 diabetes [102]. In this study, subjects with impaired glucose tolerance (IGT) were also monitored for a period of three years. The conversion rate from IGT to type 2 diabetes was 17.8% among subjects with two LIPC C alleles opposed to 10.7% among carriers of the LIPC T allele [102]. The LIPC T allele appears to protect the carrier against the conversion of IGT to type 2 diabetes. In other studies insulin resistance, type 2 diabetes and or glucose intolerance is mostly associated with the LIPC T allele [51, 53, 93, 117]. Overall, most studies appear to indicate that the LIPC T-allele is associated with an increased risk for insulin resistance. In the two similar studies on the conversion from IGT to type 2 diabetes, the effect of the HL promoter polymorphism is opposite. One possible explanation for these opposing results may be the low numbers of LIPC T homozygotes involved in these studies. To test the

role of the HL promoter polymorphism, such studies should best be performed among populations with a high T-allele frequency, such as the Japanese, and not with Caucasians with the lowest reported T-allele frequency, despite the higher incidence of type 2 diabetes. Such studies are urgently needed to establish the role of the HL promoter polymorphism in the conversion of IGT to type 2 diabetes.

Although the genetic and environmental background of type 2 diabetes is very divers, it is likely that the HL genotype is one of the genetic factors contributing to the development of type 2 diabetes. The combined effect of all these factors will result in a particular clinical picture. A complicating factor is the interaction of the LIPC promoter polymorphism with dietary fat intake [72, 97]. Homozygote LIPC T subjects on a low fat diet were shown to have larger HDL particles and lower triglycerides levels, but homozygote LIPC T subjects on a high fat diet were prone to a more atherogenic lipid profile, a lower HDL cholesterol and a strong increase of triglycerides.

1.2 Genetics of hepatic lipase

1.2.1 Lipase gene family

Hepatic lipase is a member of the lipase gene family, based on amino acid composition and gene organisation. The first identified members of this family were pancreatic lipase (PL), lipoprotein lipase (LPL) and hepatic lipase. Later on more lipases were added to the family: PL related proteins 1 and 2, phosphatidylserine phospholipase A1, Drosophila yolk proteins and endothelial lipase (EL) [6, 7, 37, 57, 61, 79]. These water-soluble lipases all hydrolyse ester bonds of water-insoluble substrates as triglycerides and or phospholipids. Especially the HL, LPL and EL-genes are closely related, both on the basis of their coding sequences, as on their exon-intron organization. HL has 9 exons and 8 introns whereas LPL and EL have 10 exons and 9 introns. The length of the first intron of the HL gene is extremely long compared to the other genes of the family. The 10th exon of LPL forms a large untranslated region. Despite this close kinship, their substrate specificity markedly differs, as LPL exhibits triglyceridase activity, and EL phospholipase A1 activity. HL is intermediate between LPL and EL by combining both catalytic activities [79].

The lipase genes have probably evolved from a primordial ancestor gene by gene duplication. The new genes then evolved separately from each other. Kirchgessner suggested that in time introns have been lost or gained, and introns have been shifted due to intron-sliding [57]. As mentioned above, known mutations in the coding part of the HL gene are rare compared to the LPL gene, for which more than 100 different mutations have been described to date [68]. One explanation for this striking difference may be that the HL gene has evolved from the LPL gene rather recently by a gene duplication event, and not enough time has elapsed since then to acquire multiple mutations. The PL gene is more distantly related, since it has 13 exons and 12 introns. Hence, the PL and LPL genes may have separated far earlier in evolution than the HL and LPL genes. In table 2 the exon and intron lengths of the HL, EL, LPL and PL genes are listed.

Table 2: **Exon and intron length of members of the lipase gene family**.

	exon 1	intron 1	exon 2	intron 2	exon 3	intron 3	exon 4	intron 4	exon 5	intron 5	exon 6	intron 6	exon 7
Human HL	145	106212	185	3267	183	566	118	3090	234	2354	243	12291	118
Rat HL	178	100526	191	2605	183	1477	118	1953	234	2906	243	10505	118
Mouse HL	333	111318	191	5508	183	1002	118	2085	234	3617	243	8855	118
Human EL	349	2911	182	1943	180	1815	112	5820	222	5824	243	704	121
Human LPL	88	8651	161	3428	180	1361	112	698	234	1487	243	3176	121
Human PL	34	134	49	592	155	490	126	2188	135	1349	109	1505	120

	intron 7	exon 8	intron 8	exon 9	intron 9	exon 10	intron 10	exon 11	intron 11	exon 12	intron 12	exon 13
Human HL	2523	219	4992	158								
Rat HL	1499	219	3723	234								
Mouse HL	1405	219	3664	283								
Human EL	1073	219	2971	105	3650	2408						
Human LPL	1520	183	1031	105	3090	812						
Human PL	99	120	1880	119	906	130	2828	109	1247	168	3499	123

Coding regions of the lipase genes show a notably high homology. The catalytic domain consists of the amino acid residues serine (residue 147), aspartate (residue 176), and histidine (residue 263), in exon 3, 4 and 5 of the HL gene, respectively (calculated using the sequence according to Cai [14]), is present in all lipases, and is in fact characteristic for esterases. This catalytic triad is brought together in the three-dimensional structure of the protein, which results in the activation of the serine residue [10, 115]. The active site serine residue is always part of the conserved pentapeptide motif Gly-X- Ser-X-Gly [116]. The hydrophobic nature [70] of this sequence enables catalytic activity with water-insoluble substrates such as phospholipids and triglycerides in lipoproteins or fat droplets. A loop or lid structure covers the active site and prevents access of the substrate to the catalytic site. Upon binding of the enzyme to the surface of a lipoprotein or a lipid droplet, the enzyme undergoes a conformational change and the lid is moved away from the catalytic site. Recently, Lohse suggested that the family of acid lipases, including gastric lipase, lysosomal acid lipase and lingual lipase form another branch of the lipase family [65]. The overall structural homology with the other lipases is lost but the Gly-X- Ser-X-Gly motif and the catalytic triad are preserved [66].

1.2.2 Genomic organisation of hepatic lipase

The human HL gene (LIPC) is located on chromosome 15 region q21 and spans over 125 kb (calculated from the known human DNA sequence using Ensembl [44]). Previously, using DNA cloning techniques the HL gene size was predicted to be roughly 60 kb [20, 92]. Using the web-based genome browser Ensembl version 38 [44], the exact location was

determined at q21.3. The HL gene consists of 9 exons separated by 8 introns. The total length of the exons and introns is calculated by Ensembl to be 136,898 basepairs (bp) for the human HL gene and 124,572 bp for the rat HL gene. The 5' upsteam regulatory and 3'-flanking regions are not included in this calculation, therefore the gene is even longer. The major and minor transcription start sites have been identified independently by Cai [14], Ameis [2] and Chang [16]. In a growing number of papers, the A at 43 nt upstream of the translation start codon identified by Ameis is taken as the major start site. Cai and Chang have identified the transcription start site at 165 and 42 nt upstream of the translation start codon, respectively. Recently, we have shown the presence of an alternative promoter within intron-2 of the HL gene (chapter 3). Intron 1 is strikingly long compared to intron 1 of LPL and EL, and introns of other genes. It is not known whether the long size of this intron has implications for gene transcription and/or splicing efficiency.

1.2.3 Regulation of hepatic lipase expression

1.2.3.1 Gene expression

Gene expression begins with the transcription of the coding part of a gene into mRNA, followed by translation and posttranslational modifications into a mature protein. Regulation of gene expression can occur at all these levels, but regulation at the level of transcription initiation is considered to be most important and most effective. For the accurate and efficient transcription of a gene, a promoter of minimally 100 bps long is required. A eukaryotic promoter consists of several conserved DNA elements to which the general transcription factors (GTFs) and RNA polymerase II (PolII) bind. Most promoters have a short DNA sequence consisting of Ts and As located approximately 30 bps upstream of the transcriptional start site, and which is called the ''TATA''-box. Other elements important for binding of polII are the initiator recognition site (Inr) at approx. –2 to +5, the downstream promoter element (DPE) at approx. +28 to + 32, and the TFIIB-recognition element (BRE) at approximately –38 to -32 relative to the transcriptional start site (fig. 2). Together, these elements constitute the core promoter. All promoters contain at least one of these elements, but none is absolutely essential for proper promoter function.

Transcription initiation begins at the site of the promoter with the assembly of a large protein complex termed the preinitiation complex (PIC). This complex of almost 60 proteins consists of PolII and multiple general transcription factors (fig. 2). The proteins that form the PIC are highly conserved between yeast and man. The assembly of the PIC starts with the binding of TFIID to the TATA box via its TATA-binding subunit (TBP). Subsequently, other GTFs are recruited to the complex, followed by binding of PolII. Eleven to fifteen bp's surrounding the transcription start site change in confirmation and are melted [110]. The template strand of the promoter is positioned within the active site of PolII. The first step of the actual transcription starts with the synthesis of the first phosphodiester bond of the RNA strand. Upon phosphorylation of the C-terminal domain (CTD) of polII by TFIIH, the PIC falls apart and polII then starts moving along the template DNA strand while elongation the RNA strand. Thus, a basal transcription rate is achieved.

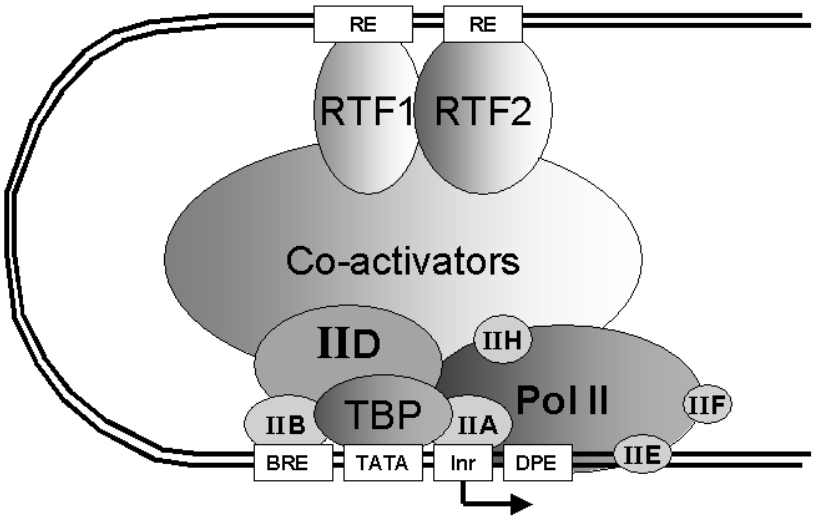

Figure 2: **Transcription initiation by RNA polymerase II at the promoter of a eukaryotic gene.**
After TBP binding to the promoter, the general transcription factors II A through II H are sequentially recruited to the promoter together with RNA polymerase II (Pol II), thus forming a preinitiation complex. The efficiency of the formation of the preinitiation complex, and the activation of RNA polymerase II, largely depends on the binding of specific regulatory transcription factors (RTFs) to their respective responsive elements (REs) elsewhere in the gene. Once bound to the DNA, these RTFs are brought in close proximity of the promoter by bending of the DNA, and the RTFs interact with the preinitiation complex, either directly or via co-activator proteins. As a result, the RNA polymerase II is released from the promoter and starts moving along the DNA while synthesizing the RNA. The protein complex at the promoter disassembles. Another RNA polymerase II is recruitd to the promoter, and the synthesis of an additional RNA molecule is initiated, as described above (Voet D & Voet JG. 2004, Biochemistry, 3rd Ed, John Wiley & Sons, Inc., Chichester, UK (pp. 1446-1482) [107].

Transcription is strongly accelerated, or slowed down, by binding of specific activator proteins to enhancer and silencer elements, respectively, present within the same gene. These elements are short DNA sequences usually no more than 6-10 bps long, which bind a specific activator protein. These elements influence transcription independently of their orientation and can be located within a wide range of upstream and downstream gene-flanking region, and even within introns. The activator proteins bound to these elements can directly interact with the PIC on the promoter. Alternatively, co-activator proteins interact with the DNA bound transcription factors and the PIC. The DNA-bound activator proteins are brought in close proximity of the PIC on the promoter by looping of the DNA. Interaction of the DNA bound transcription factor, either directly or indirectly, with the proteins of the PIC affects the formation of the PIC at the promoter, and/or affects the phosphorylation state of the CTD of polII. Each gene contains several enhancer and silencer elements, which all may have input on the PIC at the promoter. As a result, the combined binding of specific transcription factors to enhancer and silencer elements within the gene strongly affects the frequency of transcription initiation, and hence the number of RNA molecules formed.

1.2.3.2 5' Regulatory region of hepatic lipase

In the human HL gene two putative TATA boxes have been identified by sequence comparison [2, 14] at approximately –27 and -63 bp upstream of the transcription start site. It has not been investigated which of these TATA boxes is the actual TATA box. It is likely that the TAATA sequence at around position -27 is used for initiation of transcription, since most TATA boxes are located approximately 30 bp upstream of the transcription start site. The second putative TATA box is located within a site that binds hepatocyte-enriched nuclear factor 1 (HNFI) [16, 34]. Several studies have shown that the proximal promoter region contains multiple enhancer and negative regulatory elements [16, 34, 71]. In comparison with strong ''promoters'' like the Rous Sarcoma Virus (RSV) and the Simian Virus 40 (SV40) promoter, the HL promoter constructs used for investigation exhibit a relatively low promoter activity [16, 34, 71] (chapter 6). The region +29/+123 is a strong negative regulatory element [34], and has therefore been excluded from all subsequent promoter studies. Additional silencing elements have been identified in more distal parts of the 5'-flanking region of the HL gene [16, 34, 71] (chapter 2), However, even if all these silencers are deleted, the HL promoter activity remains low compared to the viral promoters [16]. Using DNase I footprint analysis in rat liver or human HepG2 nuclear extracts, Hadzopoulou-Cladaras and Oka independently observed multiple protected regions within the proximal promoter region, which is suggestive for potential transcription factor binding sites [34, 71] (fig. 3).

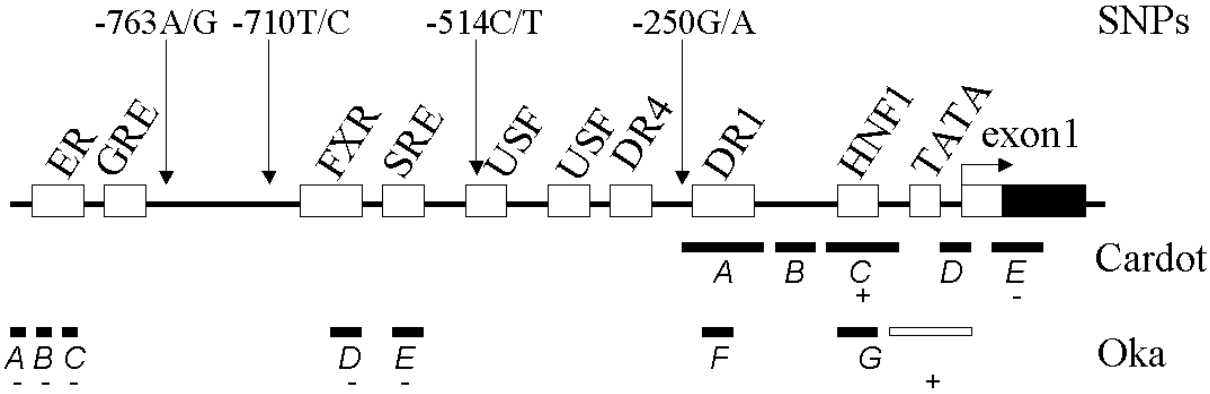

Figure 3: **Potential regulatory elements in the proximal promoter region of the human HL gene.**
A schematic of the –1600 to + 200 region of the human HL gene is given with the approximate locations of putative transcription factor binding sites, whose function has been detected or proposed in the literature (see text for details). The location of the four common SNPs in the HL promoter region is also shown. The numbered, thick underlined sequences indicate the DNAse footprints that have been identified by Hadzopoulou-Cladaras & Cardot (1993) [34], and by Oka et al. (1996) [71], in nuclear extracts of rat liver and HepG2 cells, respectively. The + and – sign indicate whether the footprint is shown to be in a positive or negative regulatory region of the HL gene. The open bar indicates a positive element identified by Oka et al. [71].

In silico comparison of the sequence of the proximal 1.4 kb promoter region of the human, rat, mouse and rhesus monkey HL genes revealed a homology between 57 and 93 %, which is close to the sequence homology among the exon sequences. In the upstream 25 kb region, four other islands with high sequence homology between the four orthologous HL genes were observed (chapter 2). This high degree of sequence conservation suggests that the proximal promoter region, and the high-homology ''spots'' further upstream may contain important regulatory elements.

1.2.3.3 Transcriptional regulation of hepatic lipase

Hepatic lipase is almost exclusively synthesised in differentiated hepatocytes [75]. HL is expressed at a low level in fetal liver, but expression levels dramatically increase postnatally [86]. Small amounts of HL mRNA have recently been detected by the sensitive RT-PCR assay, in mouse adrenals [84], and in mouse and human macrophages [31]. Therefore, the transcription of HL expression must be almost exclusively liver specific. The 5' regulatory region contains multiple consensus binding sites for liver-enriched HNF1 and HNF4. The HNF1 binding site located at nt -52/-66 is considered to be responsible for liver specific expression [16, 34, 71]. This sequence was shown to bind HNF1 in vitro [16]. Several experiments confirmed that this HNF1 site acts as an enhancer element, but there is no experimental proof that the HNF1 site at nt -52/-66 accounts for liver specific expression [16, 78]. It is possible that other not yet investigated HNF1 sites account for the liver specific expression. HNF1 is also expressed in other cell types, such as pancreatic β-cells or kidney cells [73], so additional cis- and trans-acting factors are required for the liver-specific expression of the HL gene. Another possibility is that in non-hepatic tissues HL expression is actively repressed by binding of transcription factors at one of the many silencer sites in the proximal HL promoter region.

Recently, Rufibach and co-workers showed that HNF4α binds to a DR1 and a DR4 direct repeat element located in the proximal HL promoter region, and stimulates HL expression [78]. Several transcription factors are known to bind to such DR elements, including retinoid X receptor alpha (RXRα), apolipoprotein A-I regulatory protein-1 (ARP-1, or COUP-TFII), retinoic acid receptor alpha (RARα), peroxisome proliferator-activated receptor (PPAR) and HNF4α. ARP-1 is shown to compete with HNF4α for binding to the two DR sites in the HL promoter, and to block HNF4α's ability to activate HL expression [78].

Sirvent and co-workers have shown that the farnesoid X receptor (FXR; NR1H4) represses HL transcriptional activity [90]. FXR is a member of the nuclear receptor super family that is highly expressed in liver, kidney, adrenals, and intestine [88], and is activated by bile acids. FXR repression was mediated via elements located in the proximal HL promoter region between nucleotides -698 and –541, but this region lacks a canonical FXR site, and the precise location of the FXR sensitive element has not been determined.

HL expression is regulated by several hormones and nutrients, mainly at the transcriptional level [75]. HL activity is lower in women compared to men suggesting regulation by sex hormones. Downregulation of HL expression by estrogens is observed in studies with rat liver and hepatoma cell lines [101]. Androgens, which activity is lower in women than in men, increases HL activity [56]. In post menopausal woman, androgen levels rise and estrogen levels fall, resulting in an increased HL activity. In clinical studies of post menopausal women, estrogen replacement therapy leads to a decrease of HL activity [3, 11]. The effect of the estrogen receptor-α (ERα) on the HL 5' regulatory region was tested in HepG2 cells using a HL promoter construct and a ERα expression vector. Incubation with 17β-estradiol (E_2), which activates ERα, decreased HL expression with 50% [55]. The region of estrogen responsiveness was localized to -1557/-1175 of the HL promoter [55]. An androgen responsive element has not been described for the HL promoter. Glucocorticosteroids like dexamethasone and triamcinolone decrease HL activity in the liver [50]. ACTH-induced hypercorticism in rats resulted in a decrease of HL activity in the liver [49, 83]. These data suggest that ACTH reduces HL expression through the induction of glucocorticoids. A putative glucocorticoid-response

element (GRE) is present at position –979 of the human HL gene [2] and –773 of the rat HL gene [87]. If the glucocorticoids regulate HL expression through this GRE, it must act as a silencer.

In type 1 diabetes, HL activity was found to be 50% [15] compared to normoglycemics, and HL activity increased after insulin administration [81]. In type 2 diabetes, high insulin levels were shown to be associated with high HL activity [95], but acute insulin injection did not increase, but instead reduced HL activity [13]. These observations suggest that HL expression is not regulated by insulin per sé, but rather by another parameter of insulin resistance. Nevertheless, the insulin responsiveness of the HL gene is considered by some investigators to be mediated by upstream stimulatory factor (USF) [121]. USFs are transcription factors involved in the regulation of glucose and lipid metabolism by insulin. In HepG2 cells, HL promoter activity is dramatically upregulated by co-transfection with USF cDNA (chapter 6). USFs bind to E-box sequences (CAnnTG) in promoter regions of susceptible genes. The proximal promoter region of the human HL gene contains a canonical E-box at position –310 (CACGTG) and a non-canonical E-box at position –514 (CACGGG). This latter E-box is disrupted by the –514C→T substition (CATGGG) in the common HL promoter polymorphism. Indeed, USF was shown to bind to HL specific oligonucleotides with the –514C nucleotide but not with the -514T nucleotide (chapter 6). Interestingly, post-heparin plasma HL activity increased with fasting insulin levels in homozygotes for the LIPC C-allele but not in carriers of the T-allele [51]. Moreover, the –514C→T polymorphism is associated with glucose intolerance in healthy subjects [53, 111], with insulin resistance in non-diabetic subjects [29, 76, 93] and familial combined hyperlipidemia [76], and predicts the conversion to type 2 diabetes for subjects with impaired glucose tolerance [102, 119].

1.3 Scope of this thesis

As reviewed in the foregoing, HL plays an important role in plasma lipoprotein metabolism and intracellular cholesterol and lipid homeostasis. Huge interest in HL originates from its potential role in atherogenesis. HL appears to have both pro- and anti-atherogenic properties [54], and whether HL contributes to, or prevents the early development of atherosclerosis is thought to depend on additional genetic and environmental factors [54]. HL also plays a role in the supply of HDL cholesterol for steroidogenesis in adrenals and ovaries. Despite the potential impact of HL expression, relatively little is known about its transcriptional regulation. In this thesis, several aspects of the transcriptional regulation of the hepatic lipase gene are addressed.

In chapter two, we identified 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 are potentially important for the transcriptional regulation of HL, in particular for the strict liver-specificity. The importance of elements within the highly conserved proximal promoter region was confirmed by 5'-deletion analysis and promoter-reporter assays.

In chapters three and four, the expression of HL in rat and human adrenal glands is studied, respectively. In the rat adrenals, the HL gene is transcribed into a variant HL

messenger RNA, which lacks exons 1 and 2. We found that this variant HL mRNA is synthesized from an alternative promoter present within intron-2 of the HL gene. This alternative HL promoter has low activity in both adrenals and liver of the rat. In human hyperplastic adrenals obtained from adrenalectomy of Cushing's disease patients, we found no evidence for the utilization of an alternative HL promoter. Instead, low expression of full-length HL mRNA was observed in the human adrenals.

In chapter five, we describe the presence of a common polymorphism in the proximal HL promoter region. This polymorphism consists of four highly linked single nucleotide substitutions, and is associated with reduced HL expression. First, we describe two strategies for the determination of the HL promoter genotype. Secondly, we describe the interaction of the polymorphism with HDL cholesterol and plasma triglyceride levels in a healthy human population. Thirdly, we found a strong interaction of the HL promoter polymorphism with the CETP genotype in determining the risk for coronary artery disease.

In chapter six, we show that the HL -514 C→T substitution reduces transcriptional activity of promoter-reporter constructs in transiently transfected human hepatoma HepG2 cells. This substitution occurs within a potential binding site for the transcription factor USF. Over-expression of USF-1 resulted in a strong upregulation of the HL promoter activity. The HL -514 C→T substitution indeed abolished USF-1 binding to this site in vitro, and reduced the upregulation by USF-1. Hereby, we offer a mechanism for the functionality of the common HL promoter polymorphism.

In chapter seven, we hypothesize that HL expression is regulated as an integral part of intracellular lipid homeostasis. We show for HepG2 cells, that HL expression is transcriptionally regulated by fatty acids and by pharmacological inhibition of cholesterol-synthesis. These effects are likely mediated via the transcription factor Sterol-regulatory-element binding protein (SREBP). We demonstrate that SREBP2 reduces HL promoter activity, by abolishing the stimulatory effect of USF-1.

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Chapter 2.0

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

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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 sequences were found in the proximal promoter region, and at 14 and 24 kb upstream of the transcriptional start site. With the rVista program, which searches for conserved clusters of transcription factor binding sites (TFBS), elements were identified at –220/–180 (module A), –70/–34 (module B), and –20/+9 (module C) of the proximal promoter region. 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 HNF1 and HNF4. 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 clusters of TFBS by comparative genomics is a valuable tool in designing experiments aimed at identification of functional cis-regulatory elements. With this approach, we obtained evidence that the –70/-34 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.

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. 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 realization that multiple TFBS tend to specifically cluster together [4, 5] in regions that are conserved among multiple species [6-9]. 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 [10, 11]. 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 [12-14]. Although synthesis of HL has been shown to occur in mouse adrenals [15], and in mouse and human macrophages [16], this is negligible compared to expression in liver. The HL activity present in adrenals and ovaries [17] originates predominantly from liver, and is transported through the circulation to these organs [18, 19]. 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 [20], 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 [20]. 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 [14]. 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 [12, 13]. Several groups have pointed to the HNF1 binding site in the proximal promoter of the HL gene to explain this liver-specificity in humans [21-23]. However, HL expression is not correlated with HNF1 levels in different hepatoma cell lines [23]. 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 highly conserved sequence modules in the proximal promoter of the HL genes. One of these modules contains putative binding sites for the liver-enriched transcription factors HNF1 and HNF4, and mediates the transcriptional activation in HepG2 cells but suppression in non-hepatic HeLa cells.

Results

Interspecies comparison of genomic HL sequences

Of the mammalian HL genes, genome sequence including exon-1 and the 5'-flanking region is available only for human, rat, mouse and rhesus monkey (Ensemble v.36 – December 2005). Hence, multiple sequence comparisons were restricted to these four species. Pair wise 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 of these genes, with sequence identity ranging from 57 to 93 % over the proximal 1.4 kb. Multiple sequence alignment of 30-kb of the 5'-flanking region of the four HL genes was performed by the mVista web-tool (fig. 1). Over the entire 30-kb region, the human sequence is 95 % homologous to the macaque sequence (regions with loss of homology are due to gaps in the known macaque sequence), whereas homology with the orthologous rat and mouse sequences was 10 and 11 %, respectively.

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

Sequence identity (%) was determined by pairwise alignment using the DNAMAN software package (optimal alignment; gap penalty 10.0, gap extend 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]: Since exon 7 (118 bp) of the HL gene has not yet been identified for the rhesus monkey genome (Ensemble v.36 – Dec2005), the exon 7 sequence was excluded in the alignment of the cDNA sequences with rhesus. Similarly, a 335-bp gap in the upstream regulatory region of the rhesus gene was excluded in alignments with the monkey sequence.

		upstream regulatory sequence			
cDNA sequence		86	61	57	rat
	92		61	59	mouse
	79	79		93	rhesus[a]
	79	79	95		human
	rat	mouse	rhesus[a]	human	

This global genomic sequence comparison showed a particularly high conservation among the four orthologous genes immediately upstream of the transcriptional start site (P=10^{-6}), where more than 70 % homology was observed over a 100 bp-window. Four additional islands of highly significant homology (P<0.001) were identified further upstream. Conservation of the 475 bp module at –14 kb was even more significant (P=10^{-7}) than for the proximal HL promoter region, despite the presence of a gap in the known macaque sequence within this region (fig. 1). Whether these highly conserved sequences in the genomic region far upstream of the HL promoter are involved in the regulation of HL expression, awaits further study. Here, we focus on the proximal 2 kb of the 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 were predicted for the orthologous mouse, human and rhesus macaque sequences. When we searched for clustered TFBS motifs that are conserved among the rat and human sequence, using the web-tool rVista, three separate modules were identified within the proximal promoter region (fig. 2). For module A (-220 to –180 in the rat sequence relative to the transcriptional start site) AP1, AP2, CAAT, COUP, DR4, HNF1, HNF4, PPAR and USF binding sites are predicted. Module B (–70 to –34) potentially contains AP2, CAAT, DR4,

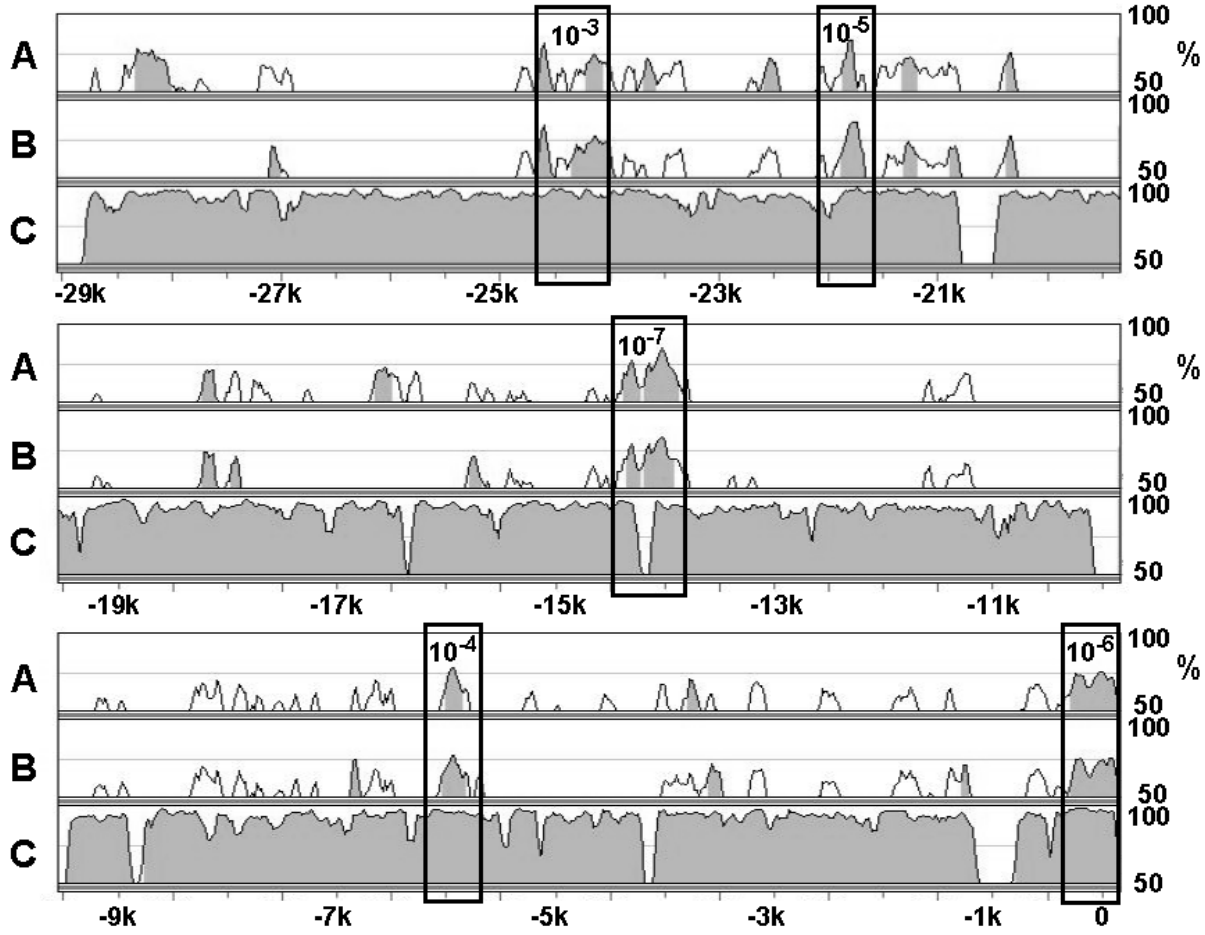

Figure 1: **Alignment of the 5'-flanking region of four mammalian HL genes.**
From the rat, mouse, macaque and human HL genes, exon-1 and 29 kb of upstream sequence was aligned by the MLAGAN algorithm of the mVista program. The sequences of rat (A), mouse (B) and macaque (C) are aligned to the human 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 ($P \leq 0.001$), with the P-values given above.

HNF1, HNF4, PPAR and Sp1 sites, whereas module C (-20 to + 9) may bind HNF4 and PPAR factors. Module C 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 (-275 to –240), with potential binding sites for AP2, C/EBPβ, CAAT, HNF3β, HNF4 and PPAR. 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 TFBS conserved 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 [21] and human HepG2 cells [22].

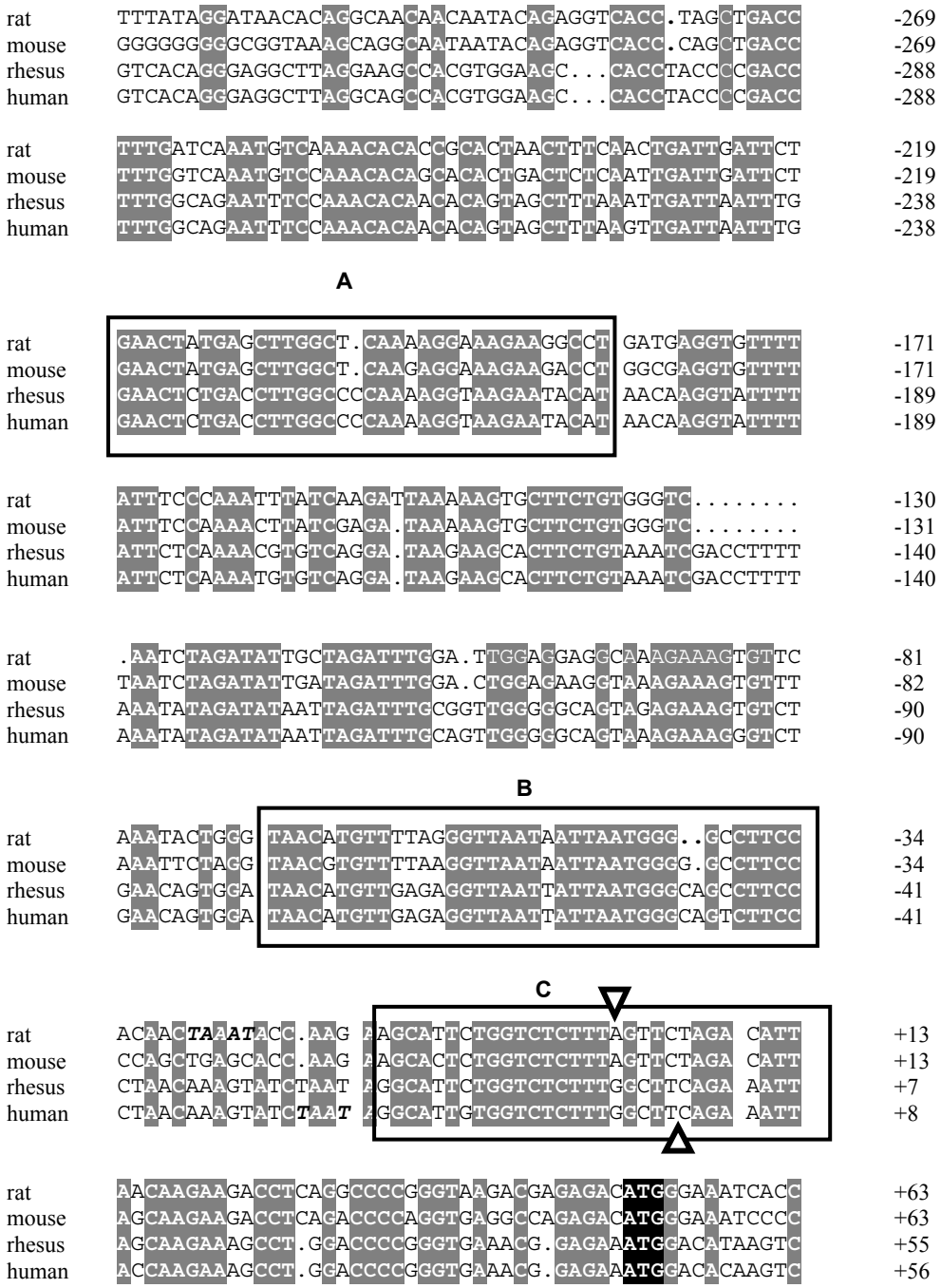

Figure 2: **Identification of functional regulatory sequences in the proximal promoter region of the HL gene by rVista.**

The proximal promoter regions of the rat, mouse, macaque and human HL genes, were aligned. Identical nucleotides are shown in grey. The translation start site ATG is indicated in black. The TATA box in the rat and human sequence is in italics. Numbering is according to the major transcription start site that has been identified for the rat [28] and human [23] HL gene (indicated by the open arrowheads). The start site for the mouse and macaque genes has not been experimentally identified; these start sites were tentatively assigned to the same position as for the rat and human HL genes, respectively. Note that the high sequence similarity between the rat (and mouse) sequence and the human (and macaque) sequence decreases upstream of position –285 and –300, respectively. Conserved and clustered TFBS were identified by pair-wise rat-human and mouse-human sequence comparisons, using the rVista utility. A, B and C denote the three conserved modules identified.

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 hepatoma 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 (fig. 3).

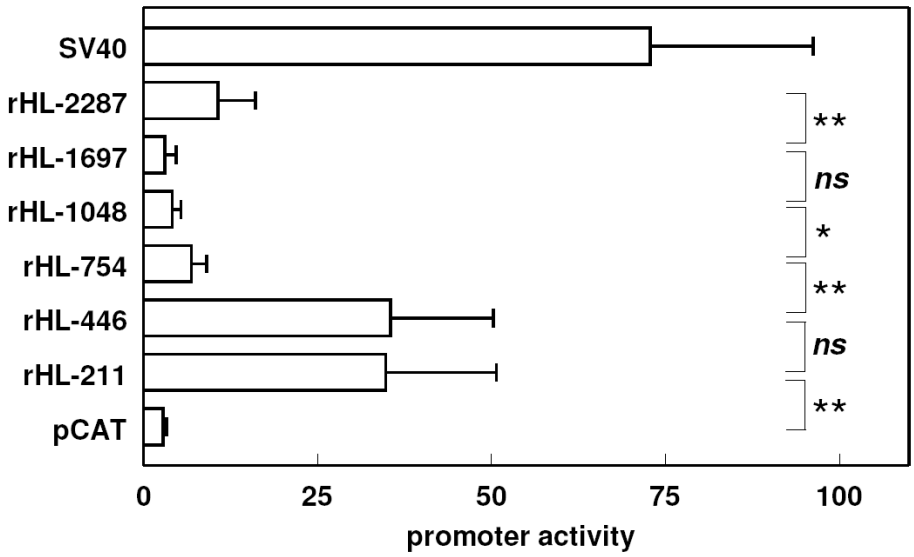

Figure 3: **Effect of serial 5'-deletions of the rat HL upstream region on transcriptional activity in HepG2 cells.**

HepG2 cells were transiently transfected with the indicated promoter-reporter constructs. At 48 h post-transfection, cells were lysed and expression of CAT and β-galactosidase protein was determined. Data are expressed as the ratio of CAT over β-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.

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 correspond to peaks of homology among the rat, mouse and human HL sequence in the mVista plot (fig. 1). However, homology did not exceed the 70 % over 100 bp mark used as threshold in this analysis.

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 (fig. 4).

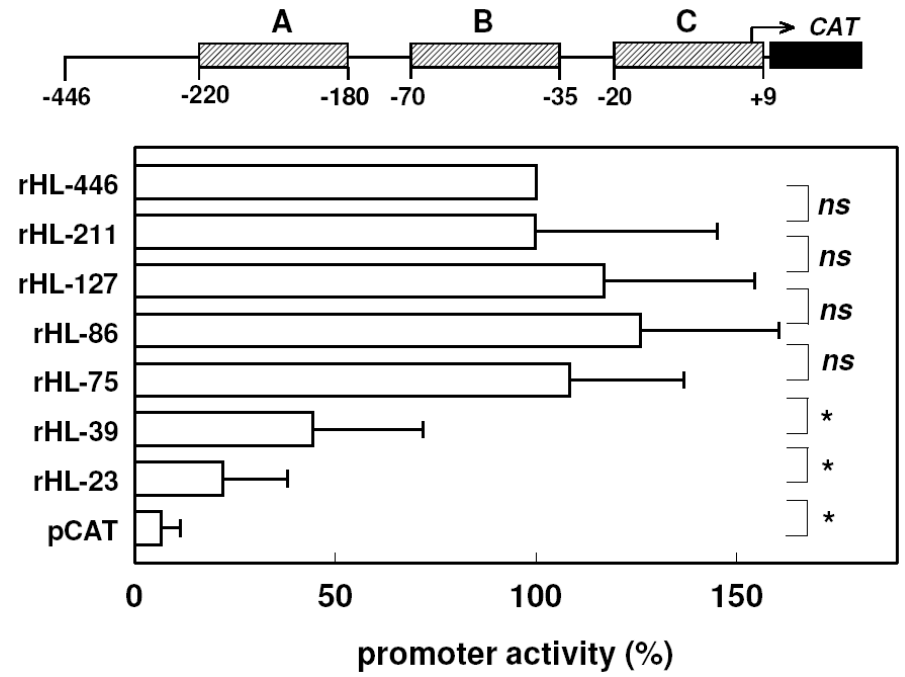

Figure 4: **Effect of serial 5'-deletions of the rat HL promoter region on transcriptional activity in HepG2 cells.**

Experiments were performed as described in the legends to figure 3. 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.

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.

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 (fig. 5). 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 the 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.

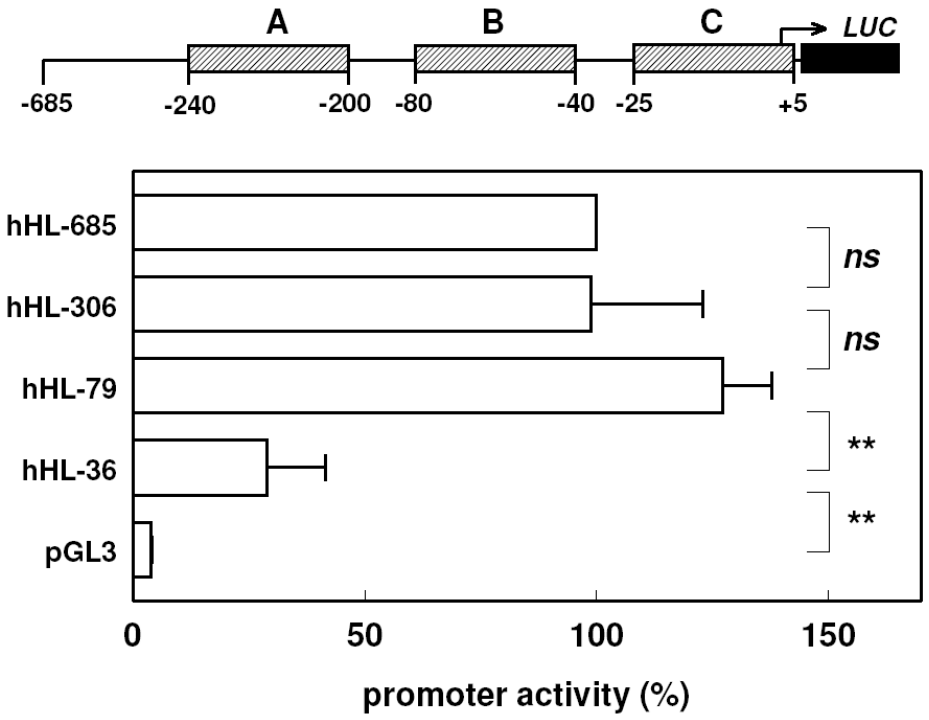

Figure 5: **Effect of serial 5'-deletions of the human HL promoter region on transcriptional activity in HepG2 cells.**
HepG2 cells were transiently transfected with the indicated promoter-reporter constructs. At 48 h post-transfection, transcriptional activity was determined as the firefly over renilla luciferase activity. Data are expressed as percentage of the ratio measured in the hHL-685 luc - transfected cells. Data are means ± SD from 4 independent experiments, each performed in quadruplicate. *: $P<0.05$; n.s.: not significant.

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 (fig. 6). 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 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 (fig. 6). 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.

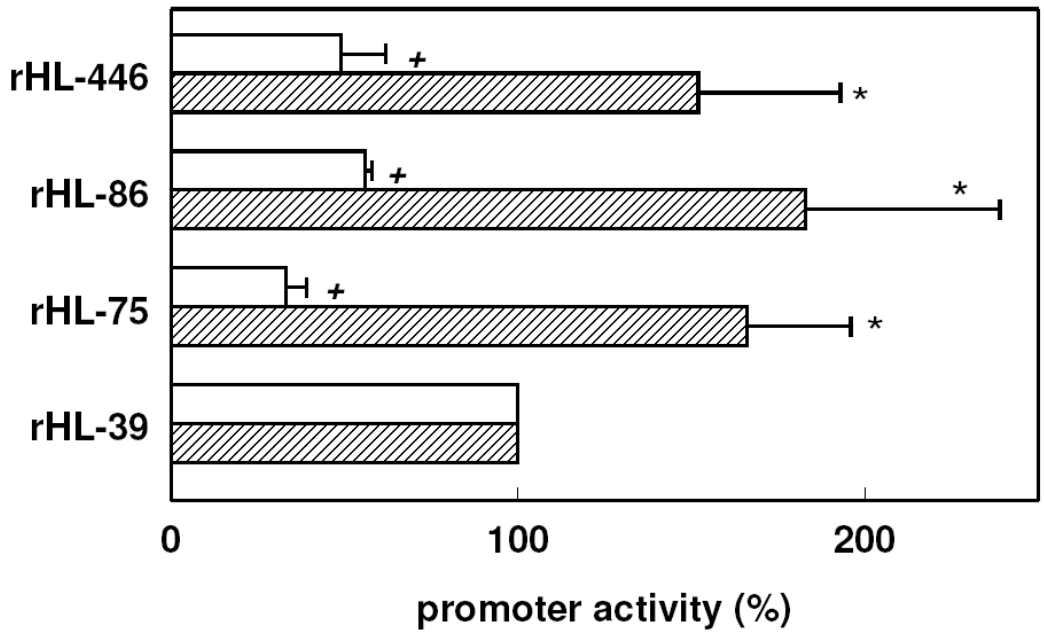

Figure 6: **Transcriptional activity of the rat proximal HL promoter region in HepG2 and HeLa cells.** HepG2 (hatched bars) and HeLa cells (open bars) were transiently transfected with the indicated promoter-reporter constructs. At 48 h post-transfection, transcriptional activity was determined as the ratio of CAT over β-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.

Discussion

Global alignment of the 5'-flanking region of four orthologous HL genes revealed three highly conserved modules ($P \leq 10^{-5}$), one of which represents the promixal promoter region (fig. 1). The two other sequences lie far upstream of the HL promoter. Whether these far upstream sequences are somehow involved in transcriptional regulation of the HL gene warrants further investigation. Within the proximal promoter region, three modules are identified with conserved clusters of TFBS motifs. Although overall homology between the mouse and rat is very high over the proximal 5'-flanking region of the HL gene (table 1), we included both the mouse and rat in our search for conserved sequences. By doing so, we could eliminate an additional module (-275 to –240), with potential binding sites for Ap2, C/EBPβ, CAAT, HNF3β, HNF4 and PPAR, that was less conserved between human and rat. Our experimental data indicated that this region was not important for basal transcription of the HL gene. 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 (-20/+9), 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 (-70 to –34) overlaps with a protected region in DNAse footprinting in rat liver [21] as well as in HepG2 cells [22], and contains a HNF1 binding site that has been implicated in liver-specific expression of the human HL gene by other groups [21-23]. Experimentally, we could not confirm a major role for module A (-220 to –180) in determining basal transcription activity in HepG2 cells. This is surprising since it perfectly matches with a protected region in DNAse footprinting in

rat liver and human HepG2 nuclear extracts [21, 22], suggesting that this part of the HL promoter is occupied by transcription factors under basal conditions. We propose, therefore, that this part of the HL promoter may be involved in modulation of gene transcription under different hormonal or nutritional conditions. Taken together, the method of comparative genomics to detect functional TFBS in conserved sequence modules has predicted the most important sequences involved in basal transcription of the HL gene.

Our experimental data identified two additional regulatory sequences in the 5'-flanking region of the rat HL gene that are not predicted by the global comparative genomics approach. A weak enhancing activity was observed between –1697 and –2287, and a strong negative regulatory element between –446 and –755 (fig. 3). Both elements were observed by Oka *et al.* [22] for the human HL upstream regulatory region at similar distances from the transcriptional start site. 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 (fig. 1), but these homologies remain below the threshold set for the detection of sequence conservation in the mVista program. Apparently, potentially important elements may be missed due to the high stringency of the conservation rule in the mVista program.

We show here that the conserved module B (-70 to –34) 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. A similar tissue-specific effect was observed for the –138 to -50 region of the human HL gene [22], which contains most of module B. 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 [21-23], and in vitro HNF1 binding to this sequence has been demonstrated by gelshift assays [23]. Furthermore, HNF1α knockout mice have 3.4 fold lower HL mRNA levels than control mice [24]. In primary hepatocytes, HL secretion increases with HNF1α gene dosage [24]. 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 [23], 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 [23]. The HNF4α gene itself is a target of HNF1α [25]. Since potential HNF4 binding sites were detected in the conserved modules B and C, the liver-specific stimulation of HL promoter activity may well be mediated by HNF4. In fact, HNF4α is bound to the promoter regions of almost half of the actively transcribed genes in human liver [25] 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 [5]. Which transcription factor(s) mediate inhibition of minimal promoter activity in cells of non-hepatic origin, remain(s) unknown. More detailed studies are required to unravel this novel mechanism for liver-restricted expression of the HL gene.

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, dramatically reduces the number of potentially functional cis-regulatory sites. This *in silico* analysis 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.

Materials and Methods

Database analysis

The annotated data of the mammalian genome sequence projects were accessed through the Ensemble genome server at http://www.ensembl.org/ (Ensemble v.36 - Dec2005). The exon-1 and 5'-upstream regulatory sequence of the hepatic lipase gene was available only for human (ENSG00000166035), rat (ENSRNOG00000015747), mouse (ENSMUSG00000032207) 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 [11] using the MLAGAN algorithm through the website http://genome.lbl.gov/vista/. A search for potential TFBS in the upstream regulatory region of a particular HL gene was performed at www.genomatix.de using the MatInspector software package [2]. 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 [10, 11] using the website mentioned above.

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

A rat genomic library in λ 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 Komaromy [26]) 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 ºC 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; [27]). 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 *Eco*RI. A 6 kb fragment [28] 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 Sensel [28]) 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. Finally, the 0.15-kb *Xba*I (-127/+9) fragment was subcloned into pCAT-Basic to generate rHL-127 CAT.

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.

Table 2 Oligonucleotides used to generate serial 5'-deletions of the rat and human HL promoter region.
[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 Sensel [28] and Chang [23], 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.

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
CATREV2[d]	-	R		gca act gac tga aat gcc tc
T3primer[e]	-	R		att aac cct cac taa ag

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 [29]. 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.

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).

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) [29]. Parallel transfections with SV40-CAT-Control and empty pCAT-Basic plasmids were used as controls. Forty-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 [30]. 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.

Statistics

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

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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Chapter 3.0

Transient induction of a variant hepatic lipase messenger RNA by corticotropic hormone in rat adrenals

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Abstract

Hepatic lipase (HL) is present not only in liver, but also in steroidogenic organs, where it is thought to mediate cellular uptake of plasma cholesterol. In rat adrenals and ovaries, the HL gene is transcribed into a variant messenger RNA (mRNA) that lacks exons 1 and 2. Treatment of male Wistar rats with corticotropin resulted in a transient 9-fold increase in the variant HL mRNA in the adrenals, which was paralleled by synthesis of 47- to 49-kilodalton HL-related proteins. In contrast, a delayed, but sustained, 6-fold increase in adrenal HL activity was observed. This difference in time course suggests that the HL activity does not reflect HL-like proteins expressed from the variant mRNA. By Northern blotting, the variant HL mRNA was 2.6 kilobase. By screening a rat genomic library, the 5′ end of the variant HL mRNA was located in intron 2 immediately upstream of exon 3. Primer extension analysis mapped the 5′ end at nucleotide 465 upstream of exon 3. In promoter-reporter assays, the intron 2 region (−233/+350 with respect to the putative start site) showed no apparent basal activity in HepG2 hepatoma and NCI-H295R adrenocortical cells. The putative promoter in intron 2 was up-regulated in NCI-H295R human adrenocortical cells by treatment with 8-bromo-cyclic adenosine monophosphate. We conclude that intron 2 of the rat HL gene has an alternative promoter with low activity in adrenals, ovaries, and liver. In rat adrenals, this promoter is transiently activated by corticotropin.

Introduction

Hepatic lipase (HL; EC 3.1.1.34) is extracellularly located in the liver of most vertebrates, where it plays an important role in lipoprotein metabolism. The lipase is involved in the conversion of intermediate-density lipoprotein into low-density lipoprotein and in the uptake of very low-density lipoprotein and chylomicron remnants by the liver [1-3]. The enzyme also plays a major role in the metabolism of high-density lipoprotein (HDL). Notably, HL is known to facilitate the uptake of HDL–cholesteryl esters by liver cells [4-6]. Adrenal cortex and ovaries of a number of species including rat contain an extracellularly bound lipase activity that is indistinguishable from HL [7-9]. This lipase activity is thought to originate from liver and to be transported to the steroidogenic organs via the bloodstream [9] in association with HDL [10]. The activity in these organs varies in parallel with steroid hormone output [11-13]. At least in the rat, these organs depend largely on HDL cholesterol (HDL-C) as a source for steroid hormone production. The tissue distribution of HL is similar to that of scavenger receptor class B1 (SR-B1), which mediates delivery of HDL-C to the sites of steroidogenesis [14]. Hepatic lipase may therefore facilitate the uptake of HDL-C via SR-B1. In line with this, SR-B1 expression in adrenal glands is up-regulated when HL activity is inhibited by injection with anti-HL antibodies [15].

Synthesis and secretion of HL protein have not been detected in nonhepatic tissues, except for recent reports on mouse adrenals [16] and mouse and human macrophages [17]. In rat adrenals and ovaries, the HL gene is not expressed into the full-length HL messenger RNA (mRNA). Instead, an alternative form of HL mRNA is found in these tissues [18, 19], in which the first 2 coding exons of the HL gene are replaced by an intron-like sequence of unknown

origin. We designate this novel sequence as exon 1A. This variant HL mRNA is predicted to translate into a protein that lacks the N-terminal part of liver HL, including the signal sequence and the lid that covers the catalytic pocket. This variant HL mRNA is also expressed in rat liver, but at a much lower level than full-length HL mRNA [18, 19]. In rat ovaries the amount of variant HL mRNA varied in parallel with cholesterol demand for steroidogenesis [19]. In rat adrenals and ovaries, the expression of the variant HL mRNA coincides with the de novo synthesis of 47- to 49-kilodalton (kd) proteins that are immunologically related to HL [18, 19]. For rat ovaries, we have shown that the immunorelated proteins are transiently induced by gonadotropic hormones in parallel with the induction of the variant HL mRNA [19]. These HL-related proteins remained mainly intracellular. Taken together, these observations suggest a possible role for these HL-related proteins in intracellular cholesterol handling in the steroidogenic organs.

In this study, we determined the temporal relationship between HL activity, HL gene transcription, and de novo synthesis of the 47- to 49-kd HL-related proteins in rat adrenals after stimulation with corticotropin. Because the first 2 exons are not used in the expression of the variant HL mRNA, we assumed that transcription occurs from another, hitherto unidentified, promoter. We decided to identify the alternative promoter and to characterize its 5′-flanking region.

Materials and methods

Rat adrenal glands

Male Wistar rats (200-250 g body weight) were fed ad libitum with a standard chow diet (Hope Farm, Wilnis, Netherlands). Animals were killed by decapitation under light diethyl ether narcosis. Adrenal glands were quickly excised, and the surrounding adipose tissue was trimmed off. Hypertrophic adrenals were obtained by treating the animals for the indicated time by daily subcutaneous injection with 50 μg of a synthetic corticotrophin analogue (Synacthen, Novartis, Basel, Switzerland). For each animal, one adrenal was rapidly frozen in liquid nitrogen and used for RNA isolation or measurement of HL activity; the second adrenal was placed in medium and immediately used in pulse-labeling experiments.

Hepatic lipase activity and immunoprecipitation assays

Three adrenals from 3 different animals were pooled and homogenized in 10 volumes of phosphate-buffered saline (pH 7.4) containing 5 U/mL of heparin and 1 mmol/L benzamidine (4°C) using a Polytron homogenizer (Kinematica, Lucerne, Switzerland). After centrifugation (2 minutes, 10 000*g*, 4°C), the postnuclear supernatant was assayed for HL activity 7, which is defined here as the triacylglycerol hydrolase activity that is sensitive to immunoinhibition with antirat HL immunoglobulin G's (IgGs) [19]. Enzyme activities were expressed as milliunits (nanomole of free fatty acids released per minute).

Three freshly dissected adrenal glands from 3 animals were pooled and minced using a razor blade. The slices were pulse-labeled with Tran^{35}S-label (ICN, Cosa Mesa, CA) for 2 hours, and HL-related proteins were immunoprecipitated with goat antirat HL IgGs immobilized onto Sepharose 4B beads (Pharmacia, Uppsala, Switzerland), as described

previously[18, 19]. The immunoprecipitated proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide gels followed by fluorography using Amplify (Amersham Biosciences, Amersham, UK).

Reverse transcriptase–polymerase chain reaction

Total RNA was isolated from 2 to 4 adrenals by the method of Chomczynski and Sacchi [20]. RNA concentrations were determined by spectrophotometry at 260 nm [21]. The quality of the isolated RNA was judged from the ratio of 28S over 18S ribosomal RNA upon electrophoresis in a 1% agarose/Tris-borate-EDTA (TBE) gel. Hepatic lipase mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were determined by reverse transcriptase–polymerase chain reaction (RT-PCR) starting from 1 μg total tissue RNA. After synthesis of random-primed complementary DNA (cDNA), the mixture was divided into parallel PCR incubations. Amplification was performed for 35 cycles with HL specific primer pairs or 20 cycles with GAPDH-specific primer pairs. Hepatic lipase specific forward primers were RHL-3 (5′-CGG GGG CTC CTT CCA GCC TGG-3′, nt 756-776; numbering according to the rat cDNA sequence [22]), RHL-12 (5′-TGG CTT GCT AGA AAC CTG G-3′, nt 297-315), or INT (5′-GCA TTG TCC TTG AGC CTG AG-3′, positions −112 to −93 upstream of exon 3 [18]), whereas RHL-2 (5′-CAG ACA TTG GCC CAC ACT-3′, nt 1307-1289) and RHL-9 (5′-GGC ATC ATC TGG AGA AAG GC-3′, nt 660-641) were used as reverse primers. The GAPDH-specific primers 5′-TCT TCT TGT GCA GTG CCA GC-3′ (nt 35-54) and 5′-CTC TCT TGC TCT CAG TAT CC-3′ (nt 1120-1101) span the entire coding sequence [23]. All RT-PCR experiments included no-template and no-RT controls.

Hepatic lipase mRNA was semiquantified by competitive RT-PCR using an internally deleted complementary RNA as competitor [18].

Genomic library screening for exon 1A

Standard molecular biology techniques were used [21]. A rat genomic library in λ DASH II (Stratagene, La Jolla, CA) was screened with the oligonucleotide INT that recognizes the sequence in the variant HL mRNA upstream of exon 3 [18]. Briefly, a total of 10^6 plaques were screened with ^{32}P-end–labeled INT. After hybridization, the final washing condition was 5 minutes at room temperature in 0.15 mol/L NaCl/0.015 mol/L sodium citrate/0.1% (wt/vol) SDS (pH 7.0). Two plaques that were positive on duplicate filters were plaque-purified 3 times [21]. The DNA from these 2 clones was analyzed by restriction mapping using the endonucleases *Bgl*II, *Hin*dIII, *Pvu*II, *Sac*I, *Sma*I, and *Xho*I (Roche, Almere, Netherlands), either alone or in combination with *Eco*RI. The digestion products were separated on a 0.7% agarose gel, followed by denaturation with 0.5 mol/L NaOH, 1.5 mol/L NaCl, and overnight blotting to Hybond membranes. The membranes were hybridized with different ^{32}P-end–labeled HL-specific oligonucleotides: RHL-11 (5′-CTG TGG ACA AGG CGT GGG-3′, nt 78-95), RHL-13 (5′-TTG TCA TGA TCA TCC ACG GG-3′, nt 266-285), RHL-14 (5′-CAC CCA CTA TCT TCC AGA TCC-3′, nt 314-334), RHL- 8 (5′-TTA ATT GGG TAC AGC CTG GG-3′, nt 508-527), RHL-3, and INT, which recognize exons 1, 2, 3, 4, 5 and 1A, respectively. After stripping, the filters were rehybridized with a different probe. A 5.6-kilobase (kb) *Eco*RI-*Eco*RI fragment that was positive for INT was isolated from clone I and subcloned into pBluescript KS$^-$. From this construct, a 577-base pair (bp) *Hin*dIII-*Bgl*II

fragment was isolated and subcloned into pBluescript KS$^-$. Sequencing of both strands was done with the Thermo-sequenase dye terminator kit (Amersham Biosciences) and the ABI 377 sequencer (Applied Biosystems, Foster City, CA).

Genomic library screening for exon 1 and the 5′-regulatory region

The same genomic library was also used for isolation of the normal HL promoter region, using an HL cDNA probe corresponding to exons 1 and 2. The probe was generated by RT-PCR on 1 μg liver RNA using the oligonucleotides 5′-GGT AAG ACG AGA GAC ATG G-3′ (nt 1-19; numbering according to Komaromy [22]) and 5′-CCC GTG GAT GAT CAT GAC AA-3′ (nt 266-285) as forward and reverse primers, respectively. The RT-PCR product was isolated by agarose gel electrophoresis and ^{32}P-labeled using [α^{32}-P]dCTP and the Megaprime kit from Amersham; 10^6 plaques were screened with this probe; final wash step was for 5 minutes at 65°C in 0.03 mol/L NaCl/0.003 mol/L sodium citrate/0.5% SDS (pH 7.0). Two positive clones were identified, which were plaque-purified 3 times. One of these clones was selected for further analysis. Phage DNA was isolated and digested with *Eco*RI. A 7-kb fragment [24] was cloned into pBluescript KS$^-$ and its identity with the 5′-regulatory region of the rat HL gene was verified by sequence analysis.

Southern blot analysis of genomic DNA

Rat genomic DNA was isolated from the liver of a healthy 3-month-old male Wistar rat [21]. In parallel incubations, 10 μg of this genomic DNA was digested overnight with 10 U of the restriction enzymes indicated. After size separation by agarose gel electrophoresis, the DNA fragments were transferred to a Hybond membrane and denaturated in 1.5 mol/L NaCl/0.5 mol/L NaOH. The filter was screened with 20 μg of ^{32}P-labeled *Hin*dIII-*Bgl*II 577-bp DNA fragment. The filter was finally washed for 5 minutes at 60°C in 0.03 mol/L NaCl/0.003 mol/L sodium citrate/0.5% SDS (pH 7.0).

Northern blotting

A rat multiple-tissue Northern blot (Clontech, Palo Alto, CA), which contained 2 μg poly(A)-enriched RNA from several tissues, was probed with oligonucleotide AIB (antisense to INT). After stripping, the blot was reprobed successively with ^{32}P-labeled oligonucleotide RHL-14 (specific for exon 3) and a 1.1-kb human GAPDH cDNA probe, according to the manufacturer's instructions. After hybridization with the oligonucleotides, the blot was washed for 10 minutes at 60°C in 0.3 mol/L NaCl/0.03 mol/L sodium citrate/0.5% SDS (pH 7.0), and then exposed to autoradiography film. After hybridization with the cDNA probe, the blot was washed finally for 20 minutes with 0.015 mol/L NaCl/0.0015 mol/L sodium citrate/0.5% SDS (pH 7.0) at 65°C.

Primer extension analysis

Primer extension analysis was performed according to Sambrook [21] using total RNA isolated from the liver of a healthy 3-month-old Wistar rat. Three different primers, corresponding to sequences located in the variant HL mRNA upstream of exon 3, were used: AIB (antisense to INT), ext-1 (5′-GAT TTC TCA ATC TCG TGC AG-3′, nt −169 to −150; numbering relative to the exon 3 sequence), and ext-2 (5′-GTC ATT GTC TGA ATC TTT

CCC-3′, nt −342 to −322). Of the ^{32}P-end–labeled primers, 2×10^5 cpm was hybridized to 50 μg total RNA in the presence of annealing buffer (0.15 mol/L KCl, 1 mmol/L EDTA, 10 mmol/L Tris-HCl, pH 8.3). The primer-RNA mixture was treated for 2 minutes at 95°C, incubated for 5 minutes at 70°C followed by slow cooling to room temperature. After ethanol precipitation, the pellet was resuspended in 25 μL of reverse transcriptase cocktail (0.56 mmol/L of each deoxynucleotide triphosphates (dNTP), 50 μmol/L Tris [pH 8.3], 50 μmol/L KCl, 5 mmol/L dithiotreitol (DTT), 5 mmol/L $MgCl_2$, 20 U RNAsin (Promega, Madison, WI), 100 U MMLV-RT (Promega)), and primer extension was performed by incubation at 42°C for 90 minutes. After phenol-chloroform extraction and ethanol precipitation, the extended primer was resuspended in 5 μL of 10 mmol/L Tris-HCl/1 mmol/L EDTA buffer (pH 8.0). After addition of 3 μL formamide loading buffer (United States Biochemicals, Cleveland, OH) and denaturation at 70°C, the mixture was run on a 6% polyacrylamide/7 mol/L urea sequencing gel alongside a radioactive sequencing ladder. The latter was prepared from the 577-bp *Hin*dIII-*Bgl*II fragment using the same primer as in the extension reaction. DNA radioactive sequencing was performed using the Sequenase-2 kit from United States Biochemicals. After gel electrophoresis, the sequencing gel was exposed to autoradiography film.

Promoter activity

For in vitro studies, the 577-bp *Hin*dIII-*Bgl*II fragment was cloned into the pCAT-Basic reporter plasmid (Promega) to generate the intron 2 (−233/+350)–CAT construct. Similarly, the 5′-upstream regulatory region of the rat HL gene was cloned into pCAT-Basic. From the 7-kb *Eco*RI-*Eco*RI fragment containing the 5′-regulatory region of the HL gene, the *Pst*I-*Xba*I (−437 to +9) fragment was used (HL [−437/+9]–CAT).

HepG2 hepatoma cells were cultured at 37°C and 5% CO_2 in Dulbecco modified Eagle medium (ICN) supplemented with 10% (vol/vol) fetal calf serum (Gibco, Breda, Netherlands) and penicillin/streptomycin. At 24 hours before transfection, the cells were plated in 60-mm culture dishes at 20% to 30% confluence. At 3 hours before transfection, the medium was refreshed. Transfections were performed by the calcium-phosphate coprecipitation method using 10 μg of the CAT reporter test plasmid and 0.4 μg of the RSV β-galactosidase expression plasmid (Promega), as described previously [25]. Promoter activity was expressed as picogram of CAT/nanogram of β-galactosidase to correct for differences in cell number and transfection efficiency.

NCI-H295R adrenocortical cells were cultured in Dulbecco's modified Eagle medium/Ham F12 medium (Gibco), supplemented with 2% fetal calf serum, 15 mmol/L HEPES, 25 mmol/L $NaHCO_3$, 10 μg/mL of insulin-transferrin–sodium selenite (Roche), 10 nmol/L hydroxycortisone (Merck, Darmstadt, Germany), 10 nmol/L β-estradiol (Sigma, St Louis, MO), and penicillin/streptomycin. At 24 hours before transfection, the cells were plated in 6-well plates at 50% confluence. Transfections were performed with Lipofectamine-Plus (Invitrogen, Breda, Netherlands) according to the manufacturer's instructions, using 1.0 μg CAT reporter and 0.25 μg RSV β-galactosidase expression plasmid per well. When indicated, 8-bromo-cyclic adenosine monophosphate (8-Br-cAMP; Sigma) was added to the medium 4 hours after transfection at a final 300 μmol/L. Incubations were continued for 48 hours, and promoter activity was determined as described above.

Data analysis

The annotated data of the rat genome project were accessed through the internet at http://www.ensembl.org/Rattus_norvegicus/. Similarly, data on the human, mouse, and dog genome projects were accessed at http://www.ensembl.org/. Sequence alignments were performed with mVista [26] through http://genome.lbl.gov/vista/. A search for potential promoter sites and transcription factor binding sites was performed with the Genomatix PromoterInspector and MatInspector computer programs at http://www.genomatix.de/, respectively.

Experimental data are expressed as mean ± SD. Differences were tested for statistical significance by Student *t* test.

Results

Corticotropin-induced expression of the HL gene in rat adrenals

Male adult rats were treated for several days by daily injections with synthetic corticotropin. This resulted in a gradual, up to 4-fold increase in adrenal weight after 6 days. The HL activity in the adrenals increased from 10.2 ± 0.8 to 15.4 ± 0.6 mU per 2 adrenals at day 2 of treatment (n = 3; $P < .05$). The activity continued to increase to 64.4 ± 1.1 mU per 2 adrenals at day 6 (n = 3; $P < .05$) and remained high at least until 9 days of corticotropin treatment (fig.1). On the basis of adrenal weight, adrenal HL activity gradually increased from 107 ± 13 mU/g in control animals to 136 ± 28 mU/g after 4 days of corticotropin treatment (n = 3, NS) and 213 ± 50 mU/g after 6 days (n = 3, $P < .05$).

Reverse transcriptase–polymerase chain reaction on RNA isolated from control adrenals using the primers RHL-2 and RHL-3 yielded the expected 552-bp product, whose identity with part of HL cDNA was confirmed by restriction mapping. Upon treatment of the rats with corticotropin, the amount of RT-PCR product transiently increased, with highest levels observed at days 1 and 2 of treatment (fig. 1A). In contrast, the amount of PCR product generated with GAPDH-specific primers was hardly affected by corticotropin treatment. Quantification of HL mRNA by competitive RT-PCR using an HL complementary RNA with an internal 80-nt deletion as competitor showed that control adrenals contained approximately 0.4 amol of HL mRNA per microgram of total RNA (fig. 1B), in agreement with our previous report [18]. Upon corticotropin treatment, the amount of HL mRNA gradually increased to 3.5 amol/μg total RNA at day 2. Thereafter, the amount of HL mRNA decreased again to near-control levels after 6 to 9 days. Hence, the effect of in vivo corticotropin treatment on adrenal HL mRNA expression showed a strikingly different pattern compared with HL activity in rat adrenals.

Although part of the HL gene transcript was detected in rat adrenals, and shown to be transiently up-regulated by stimulation with corticotropin, we were unable to amplify the 5′ end of HL mRNA including exon 1 or 2, either from control [18] or from stimulated adrenals (fig. 1A). Using the oligonucleotides INT and RHL-9, which are specific for exons 1A and 5, respectively, the expected 481-bp PCR product was obtained with all adrenal RNA preparations.

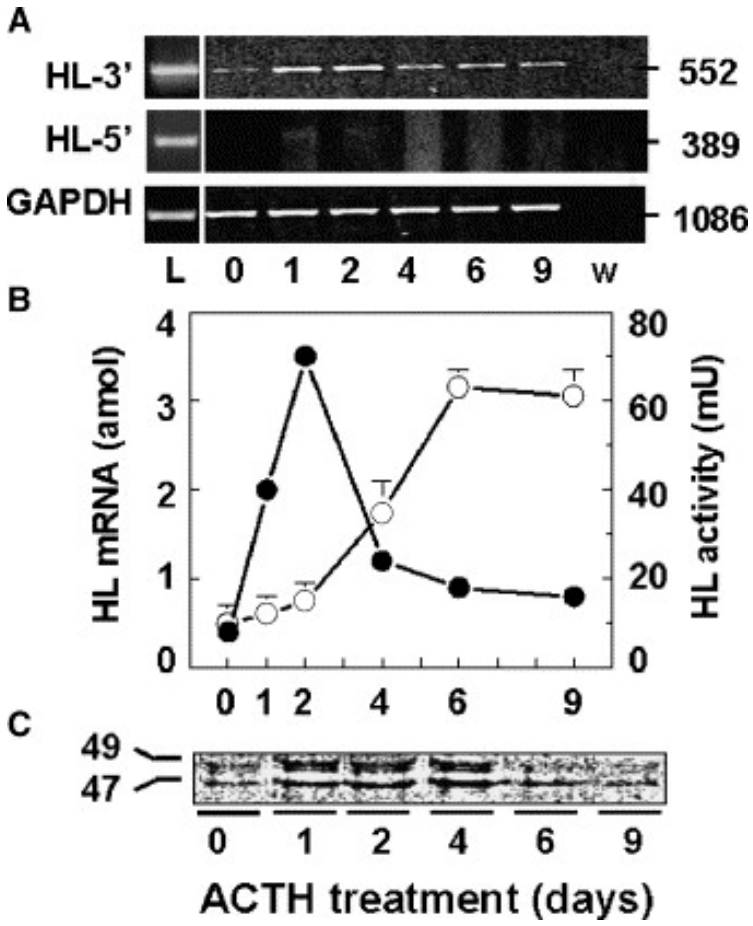

Figure 1: **Transient HL gene expression in rat adrenals induced by corticotropin treatment.**
Male rats were treated with corticotropin for the number of days indicated, and then the animals were killed and the adrenals dissected. Of each animal, one adrenal was used for RNA isolation, whereas the other was used for protein analysis. A, RT-PCR was performed on total RNA isolated from a pool of 3 adrenals using the primers RHL-12 and RHL-9, and RHL-2 and RHL-3, to detect the 5′ and 3′ part of HL mRNA, respectively. GAPDH mRNA was used as external standard. The numbers indicate the sizes (in bp) of the RT-PCR products. B, The time-dependent increase in the amount of adrenal HL mRNA (●; in amol/μg total RNA) is compared with adrenal HL activity (○; in mU per 2 adrenals; mean ± SD, n = 3). C, The freshly isolated adrenals of 3 animals were sliced and then pulse-labeled with [^{35}S]methionine for 2 hours. After cell lysis, HL-like proteins were immunoprecipitated from the lysates. The immunoprecipitated proteins were separated by SDS-PAGE and visualized by fluorography. The location of the 47- and 49-kd bands in the gel is indicated. The data are representative for 3 similar experiments. L and w indicate liver total RNA and water, as positive and negative controls, respectively.

The amount of PCR product generated by this primer pair was also transiently increased after 1 and 2 days of corticotropin treatment, in parallel with the PCR product generated with the primers RHL-2 and RHL-3 (data not shown). Therefore, the transient increase in the amount of HL gene transcript in rat adrenals is entirely due to up-regulation of the variant form in which exons 1 and 2 are replaced by exon 1A.

De novo synthesis of HL-related proteins

The de novo synthesis of HL-related proteins was studied by pulse-labeling with [^{35}S]methionine in whole-adrenal slices followed by immunoprecipitation with polyclonal anti-HL IgGs. With the adrenals from control rats, no immunoreactive proteins were found in the 55- to 60-kd range corresponding to full-length HL. Instead, 2 major ^{35}S-labeled protein

bands with apparent molecular weight in the 47- to 49-kd region were detected (fig. 1C). Upon in vivo stimulation of the adrenals with corticotropin, the ^{35}S incorporation into these bands increased several-fold. The incorporation of radioactivity was highest at days 2 and 4, and decreased again thereafter to near-control levels at days 6 and 9. Hence, the corticotropin-induced up-regulation of de novo synthesis of the 47- to 49-kd proteins occurred concomitantly with the up-regulation of the variant HL gene transcript in the adrenals.

Mapping of exon 1A to intron 2 of the rat HL gene

To determine the location of exon 1A in the rat genome, we screened a rat genomic library with oligonucleotide INT, which recognizes the known part of exon 1A. From the library, 2 positive clones (I and II) were identified and isolated. A restriction map of both clones was obtained after digestion with a number of restriction enzymes and hybridization with oligonucleotide probes specific for exons 2, 3, 4, and 1A (fig. 2). The map reveals that the clones contained overlapping sequences of the rat HL gene. Clone I contains exons 2 to 3 and clone II contains exons 2 to 4. Neither clone hybridized with an oligonucleotide specific for exon 1 or 5. From this analysis, we deduced that intron 2 spans approximately 2.5 kb, whereas intron 3 spans approximately 3 kb. Because exon 1 is not included in clone I, and exon 5 is not included in clone II, the length of introns 1 and 4 exceed 5 and 9 kb, respectively. INT hybridized with DNA fragments that contained the intron 2 sequence (fig. 2). The location of exon 1A within intron 2 was confirmed by PCR on genomic rat DNA using primer pairs that flank intron 2. The used upstream and downstream primers were 5′-TTG TCA TGA TCA TCC ACG GG-3′ and 5′-CAC CCA CTA TCT TCC AGA TCC-3′, respectively. The resulting PCR product was approximately 2.5 kb and hybridized with the exon 1A–specific oligonucleotide INT (data not shown). Sequencing of the 3′ half of the PCR product established identity with the intron 2 sequence obtained from the isolated rat genomic clones.

To test the possibility that multiple HL-like genes exist in the rat genome, we subjected rat genomic DNA to digestion with 14 different restriction enzymes. Upon Southern blotting, the membrane was hybridized with the 577-bp *Hin*dIII-*Bgl*II fragment that contained the intron 2 sequence just upstream of exon 3. With 9 of 14 enzymes, an unambiguous single hybridizing band was found (fig. 3). The size of the hybridizing bands obtained with *Bgl*II, *Eco*RI, *Hin*dIII, and *Sac*I were in accordance with the digestion maps of clones I and II isolated from the rat genomic library. No hybridizing bands larger than approximately 900 bp were obtained after *Sau*3AI digestion. The results with the 4 other enzymes used were not informative. These results do not indicate the presence of multiple HL-like genes in the rat genome. Therefore, it is unlikely that the alternative transcript observed in rat liver and steroidogenic organs is product of a gene distinct from the HL gene.

A 5.7-kb *Eco*RI fragment containing entire intron 2 was isolated from clone I and subcloned into pBluescript KS^-. Further digestion and sequencing analysis of this clone localized the exon 1A sequence in intron 2 immediately upstream of, and contiguous with, exon 3 (fig. 4). After finishing this part of our study, the rat genome including the entire HL gene sequence has become available publicly (LIPH gene, ENSRNOG00000015747). Alignment of our sequence with that of the LIPH gene confirmed identity with intron 2 of the rat HL gene.

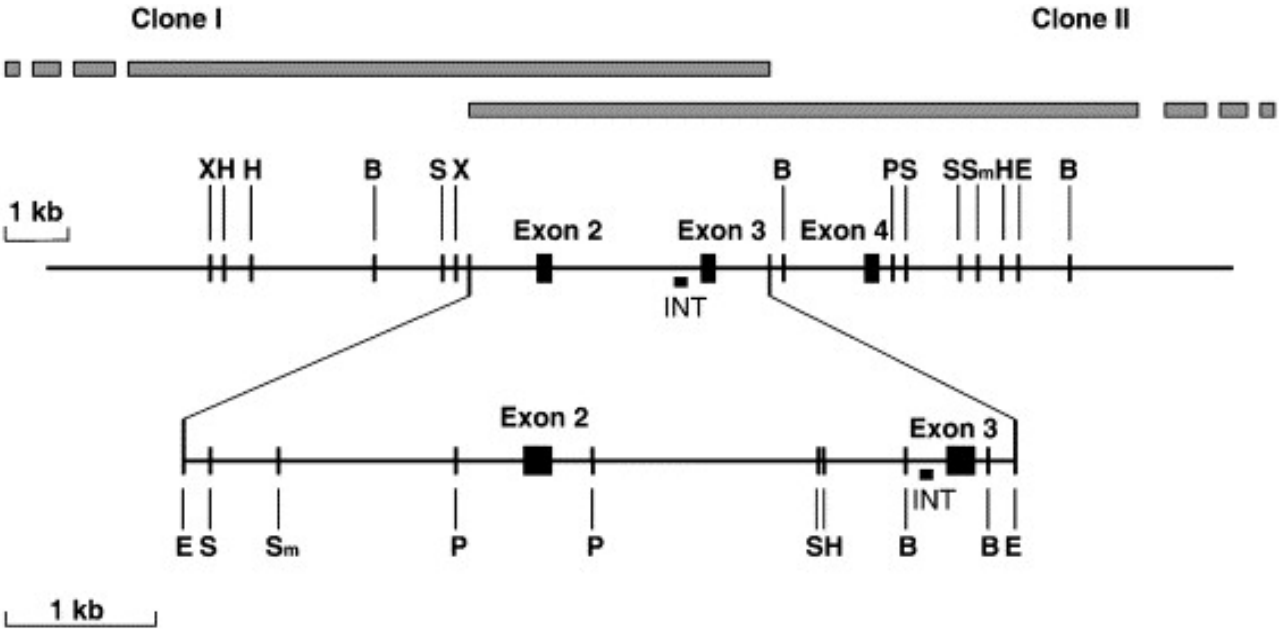

Figure 2: **Restriction map of rat genomic clones I and II.**
Clones I and II were isolated from the rat genomic library by hybridization to oligonucleotide INT. A partial restriction map was generated with the enzymes *Bgl*II (B), *Eco*RI (E), *Hin*dIII (H), *Pvu*II (P), *Sac*I (S), *Sma*I (Sm), and *Xho*I (X). The positions of exons 2, 3, and 4 are indicated, as well as the approximate position where the oligonucleotide INT hybridizes.

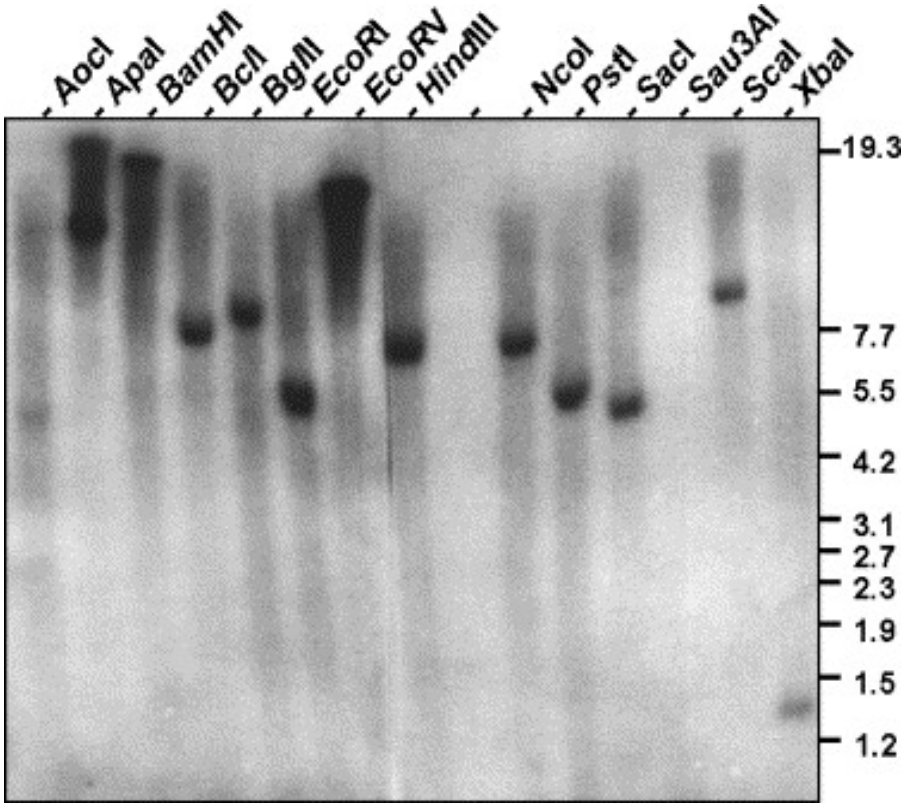

Figure 3: **Southern blot analysis of rat genomic DNA**.
Rat genomic DNA was digested with the endonucleases indicated. After separation by agarose gel electrophoresis, the DNA fragments were transferred to Hybond-N membrane. The membrane was hybridized with ^{32}P-labeled 577-bp *Hin*dIII-*Bgl*II fragment and exposed to autoradiography film. The migration of the molecular size markers (Roche) is indicated in kilobase.

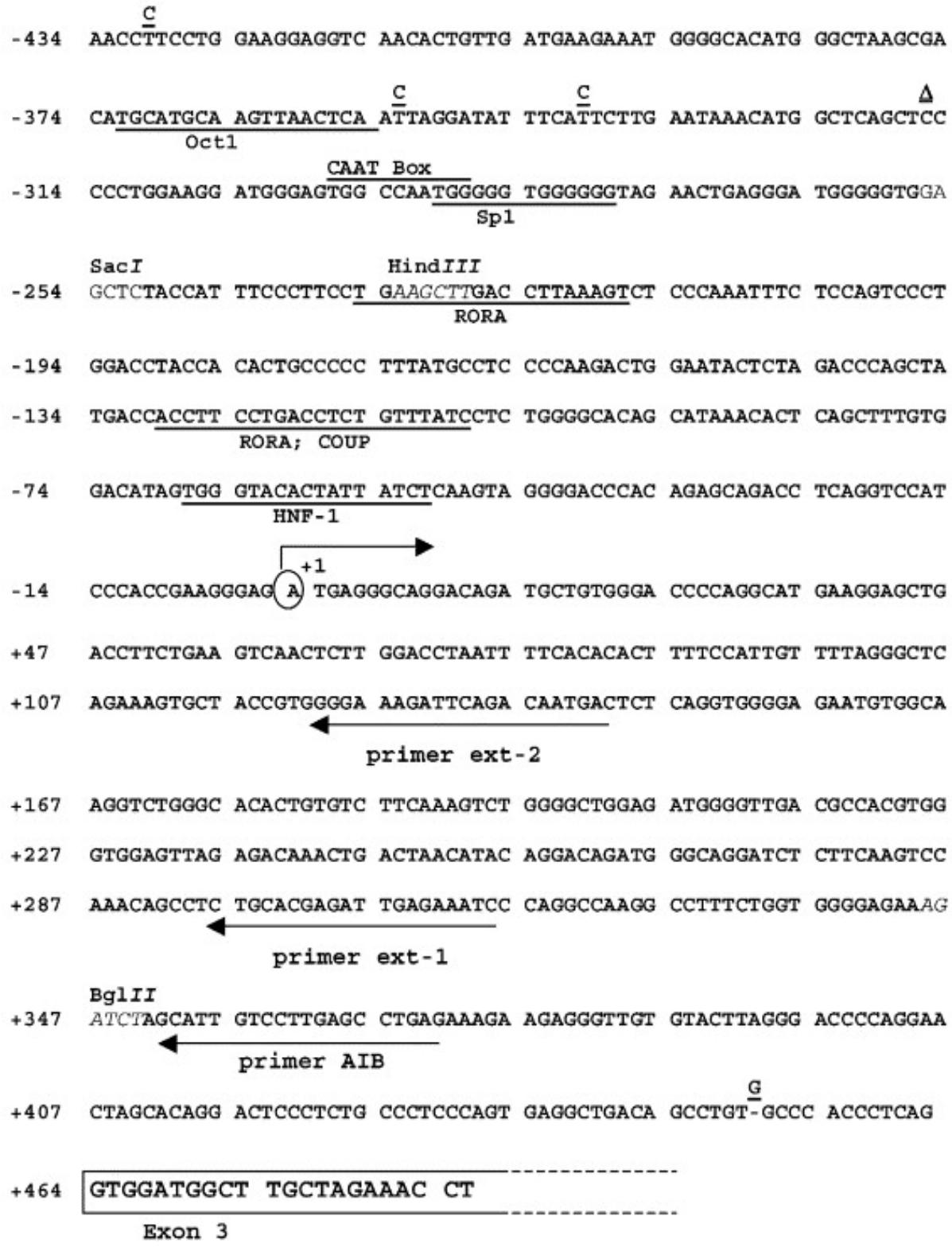

Figure 4: **Nucleotide sequence of the alternative promoter region in intron 2 of the rat HL gene.**
Numbering is according to the putative transcription start site (indicated by the arrow above the sequence); the first nucleotide of the transcript is denoted as +1. Exon 3 is boxed. Oligonucleotides used for primer extension are indicated by the arrows (5′→3′) underneath the sequence. The *Hin*dIII and *Bgl*II sites used for cloning into the reporter plasmid are indicated in italics. Putative transcription factor binding sites are underlined. Nucleotides that are at variance with data from the rat genome project (LIPH gene, ENSRNOG00000015747) are indicated. Δ indicates the presence of an extra nucleotide.

Expression of the variant HL mRNA in rat liver

Northern blot analysis of poly(A)-rich RNA isolated from different rat tissues is shown in figure 5. The exon 1A–specific oligonucleotide AIB (which is antisense to INT) hybridized with a single RNA band of approximately 2.6 kb. This signal was obtained with RNA from liver, but not with RNA from any other tissue on the blot. As expected [22, 27, 28], the exon 3–specific oligonucleotide RHL-14 hybridized with an RNA band of approximately 1.9 kb in liver, but not in any of the other tissues tested. The additional band of about 2.6 kb was not clearly visible. These data indicate that the variant HL mRNA is 2.6 kb long and is expressed in rat liver at a much lower level compared with full-length HL mRNA.

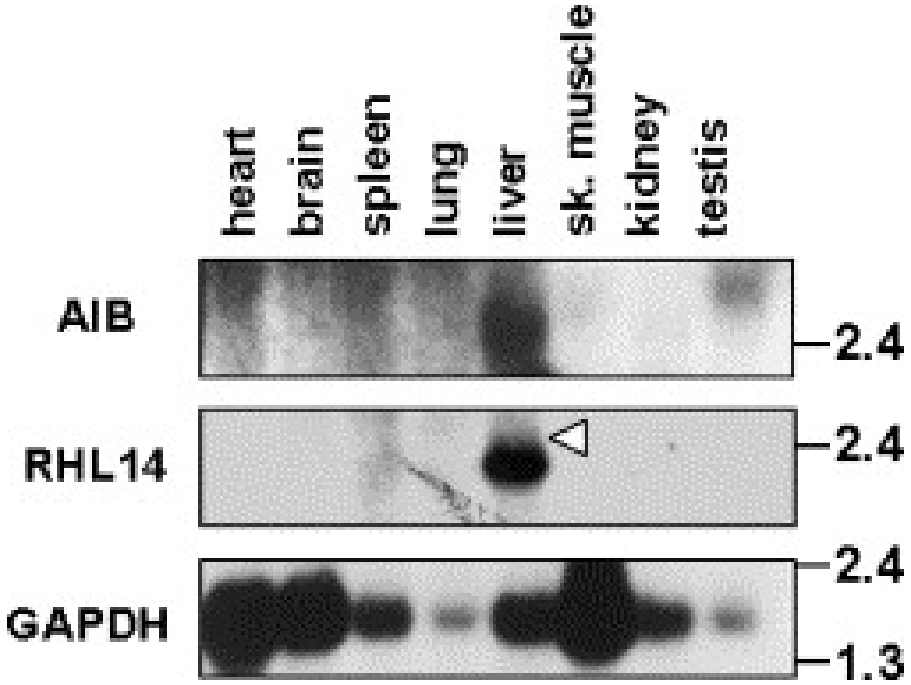

Figure 5: **Northern blot analysis.**
A rat multi-tissue Northern blot was probed successively with ^{32}P-labeled oligonucleotide AIB (specific for intron 2; specific activity, 0.5×10^9 dpm/μg; 5×10^6 dpm/mL), ^{32}P-labeled oligonucleotide RHL-14 (specific for exon 3; specific activity, 0.4×10^9 dpm/μg; 0.5×10^6 dpm/mL), and finally with a ^{32}P-labeled human GAPDH cDNA probe. The arrowhead in the middle panel points to a faint 2.6-kb shoulder band. Note that the differences in signal intensities with the GAPDH probe may reflect different expression levels rather than RNA loading. The migration of RNA markers (in kb) is indicated.

The 5′ end of the variant HL mRNA was localized by primer extension using rat liver RNA. The primers AIB and ext-1, which recognize a sequence immediately upstream of exon 3 and the sequence at −169 to 150 nt upstream of exon 3, respectively, gave distinct products larger than 350 and 250 nt (data not shown). With primer ext-2 (−342 to −322 nt), a single product of approximately 140 nt was obtained (fig. 6). Alignment of this product with the sequencing ladder of the 577-bp *Hin*dIII-*Bgl*II fragment pinpointed the 5′ end of the variant HL mRNA at the A residue, 465 nucleotides upstream of exon 3. These data suggest that the variant HL mRNA is transcribed from an alternative promoter within intron 2 upstream of this A-465 nucleotide.

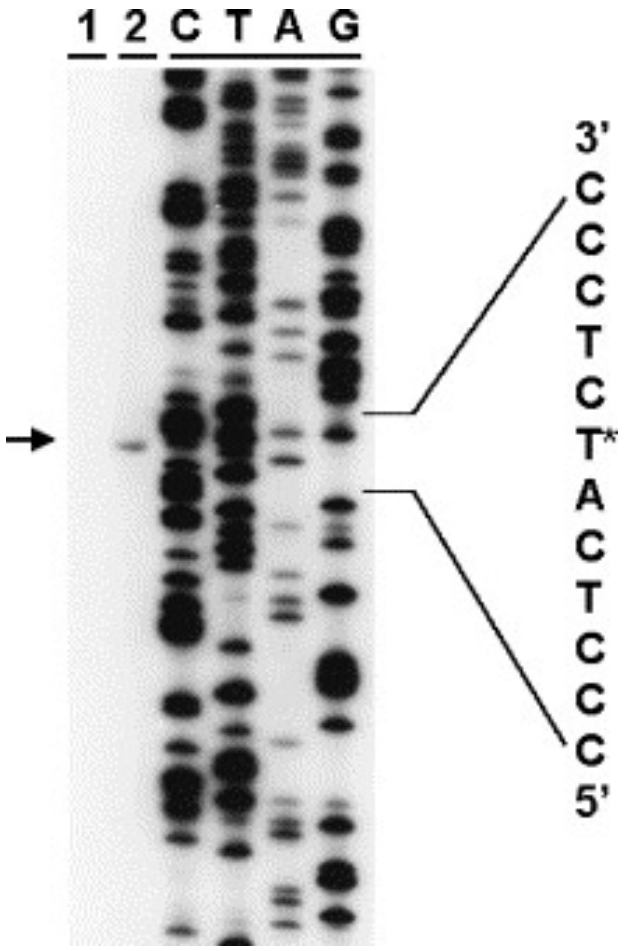

Figure 6: **Mapping of the variant HL mRNA 5′ end by primer extension analysis.**
The ^{32}P-labeled oligonucle-otide ext-2, complementary to intron 2 sequence (fig. 4), was hybridized to 50 μg of yeast RNA (lane 1) or rat liver RNA (lane 2). After extension of the oligonucleotide by reverse transcription, the DNA product was analyzed by polyacrylamide gel electrophoresis. The 5′ end of the transcript was determined by running in parallel DNA fragments of a sequencing reaction performed with ext-2 on the 577-bp *Hin*dIII-*Bgl*II fragment. The sequence represents the complementary strand of the intron 2 region. Asterisk indicates the transcription start site on the complementary strand.

Promoter activity

Screening the entire intron 2 sequence with the Genomatix PromoterInspector software did not identify any potential promoter region. However, this program also failed to find the authentic promoter in the rat and human HL gene when the appropriate genome sequences were submitted. Multiple alignment of the intron 2 sequences of the rat, mouse, human, and dog HL genes with mVista [26] showed little conservation except between rat and mouse (fig. 7). However, there was a remarkable homology of two 50-bp stretches among the 4 sequences, one upstream and one downstream of the putative transcription start at the A-465 nucleotide. The former sequence was also remotely related to the −50/−100 region of the authentic human HL promoter. Using MatInspector [29], a potential HNF-1 site was identified in this homologous sequence in rat intron 2 and the authentic human HL promoter at a similar position relative to the transcriptional start site. Several potential gene regulatory elements could be identified further upstream in the rat intron 2 sequence, such as RORA, COUP, oct-1, Sp1, and CCAAT-box sites. A clear TATA box, however, was not found.

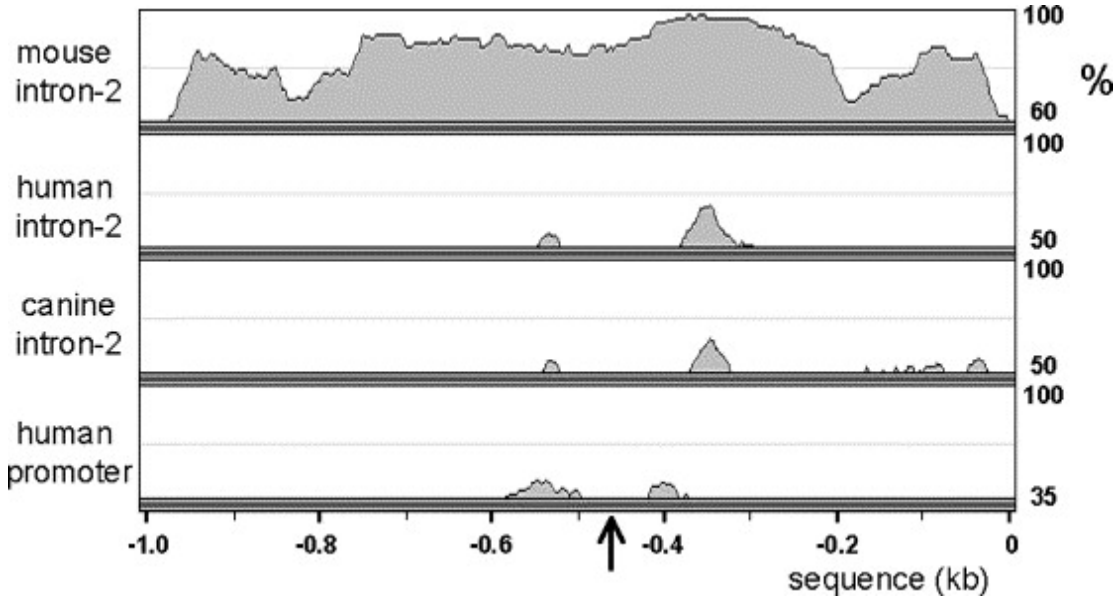

Figure 7: **Vista plot of the alignment of intron 2 from rat, mouse, human, and dog, and the human authentic HL promoter region.**
The −1000/+10 region of rat intron 2 (numbering relative to the start of exon 3) was aligned by the MLAGAN algorithm of the mVista program with corresponding regions of the mouse, human, and dog HL genes, and with the −500/+500 region of the human HL gene. The arrow indicates the position of the putative transcription start site in the rat intron 2 sequence.

The 577-bp *Hin*dIII-*Bgl*II fragment was cloned into the reporter vector pCAT-Basic to generate intron 2-CAT (−233/+350 with respect to the putative start site) to test the transcriptional activity of this intron 2 region in liver cells. As a reference the HL (−437/+9)–CAT plasmid was used, which contained the conventional rat HL promoter region. Upon transient transfection of the HepG2 human hepatoma cell line, the activity of the conventional HL promoter was 53% ± 9% of that of the SV40 promoter (n = 4). In this assay, the intron 2 region displayed only weak promoter activity (6.9% ± 1.9%), which was not significantly different from that of pCAT-Basic (fig. 8A). Similar results were obtained in transient transfection assays using NCI-H295R human adrenocortical cells, except that the activity of the conventional promoter (11.4% ± 3.5%) was slightly, but significantly ($P < .05$; n = 4) above the empty pCAT-Basic (7.1% ± 2.4%). Hence, the putative promoter of the variant HL appears to have low, or no, basal activity. Treatment of the adrenocortical cells with 8-Br-cAMP, which mimics the effect of corticotropin, slightly increased the activity of the conventional promoter (1.3 ± 0.1-fold; $P < .05$; n=4; fig. 8B), whereas the activity of the intron 2 region was stimulated 3.5 ± 1.0-fold ($P < .05$; n=4).

Possible translation product

The 3 possible reading frames of the alternative transcript that consists of exon 1A, followed by exons 3 to 9, were searched for possible translation products. By far the longest open reading frame starts at an AUG within exon 4 and would result in a 38-kd protein that is identical to the C-terminal part of HL (amino acids 134-472 [22]). This open reading frame extends 243 nt further upstream of this AUG and would correspond to an HL-related protein of maximally 47 kd. Despite repetitive double-strand sequencing reactions, no potential in-frame AUG codon was found in this region of the sequence.

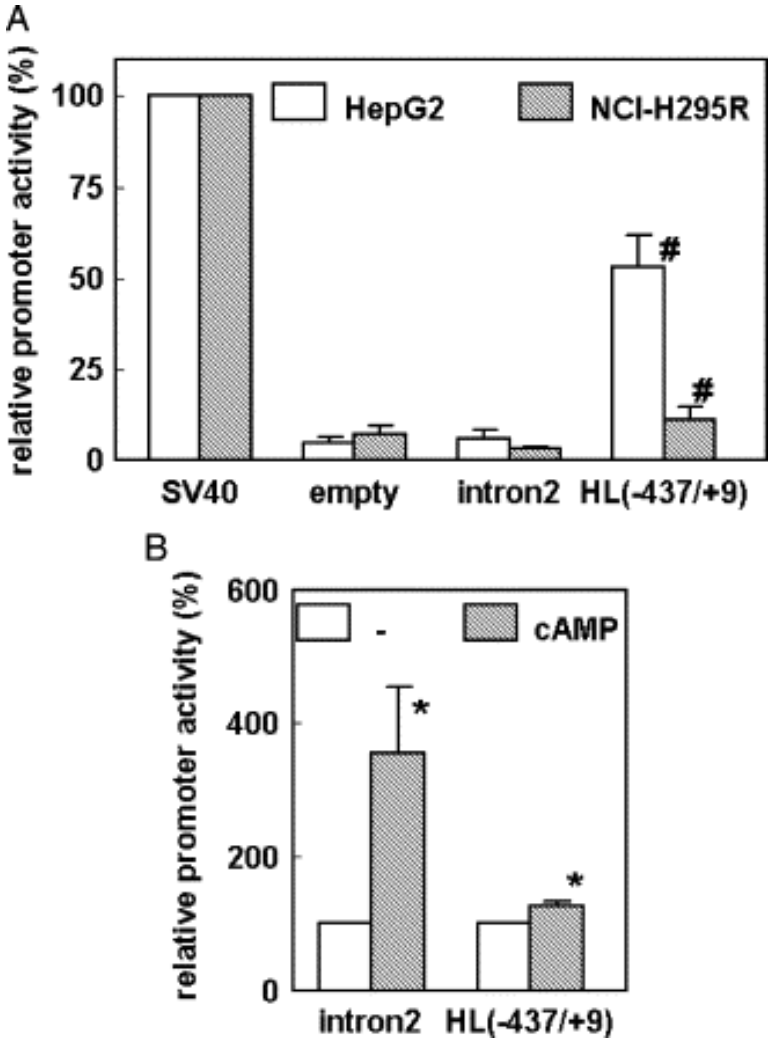

Figure 8: **Transcriptional activity of the intron 2 region in transiently transfected hepatoma and adrenocortical cells.**

A, Intron 2 and HL (−437/+9) CAT reporter plasmids were transfected into HepG2 (open bars) and NCI-H295R cells (hatched bars). As reference, parallel transfections with pCAT-Basic and pCAT-SV40 were performed. B, The plasmids were transfected into NCI-H295R cells, and then incubated for 44 hours without (open bars) or with 0.3 mol/L 8-Br-cAMP (hatched bars). At 48 hours posttransfection, CAT and *β*-galactosidase expression was determined. The results are calculated as CAT/*β*-galactosidase ratio, and given as percentage of the ratio obtained with pCAT-SV40 (panel A) or as a percentage of the ratio obtained without 8-Br-cAMP treatment (panel B). Data are means ± SD for 4 independent experiments. $^{\#}$ and $^{*}P < .05$ relative to empty plasmid and control medium, respectively.

Discussion

The data we present here suggest that the rat HL gene can be transcribed from an alternative promoter within intron 2. In rat liver, this alternate promoter is much less active than the conventional promoter. In adrenals and ovaries, transcription is exclusively from the alternative promoter. Its product is a variant HL mRNA of approximately 2.6 kb, which is identical to full-length HL mRNA except that exons 1 and 2 have been substituted by a long 5′

extension of exon 3. Rat liver contains approximately 0.4 amol of the variant HL mRNA compareed with 16 amol of the full-length HL mRNA per microgram of total RNA [18]. When expressed in human hepatoma HepG2 cells, the alternative promoter showed only a weak activity compared with the conventional rat HL promoter. This low promoter activity is in good agreement with the low expression levels of the variant HL mRNA compared with full-length HL mRNA in liver. The amount of the variant HL mRNA in whole rat adrenals and ovaries ranges from 0.4 to 4 amol/μg total RNA (this article) and 0.01 to 0.4 amol/μg total RNA [19], respectively, depending on the hormonal status of the animals. The expression of the variant HL mRNA in adrenals and ovaries varies in parallel with [^{35}S]methionine incorporation into 47- to 49-kd HL-immunorelated proteins (fig. 1C; [18, 19]). Sequence analysis of the variant HL transcript revealed that the longest open reading frame starts from an AUG within exon 4 (codon 134 [22]) and would translate into the C-terminal 38.5-kd part of the HL protein. The observed expression and induction of a 47- to 49-kd HL-immunorelated proteins parallel with the variant HL mRNA in both adrenals and ovaries would fit with this predicted translation product, except for its relatively large molecular mass on SDS-PAGE. Although the reading frame extends farther upstream, an in-frame AUG was not found in our sequence nor in the publicized rat genome data. Alternatively, the discrepancy in molecular size may be explained by extensive posttranslational modification. Because the variant HL proteins remain intracellular [18, 19], and the predicted sequence lacks a signal peptide essential for endoplasmic reticulum–Golgi targeting and contains only one N-glycosylation consensus site 22, it is unlikely that glycosylation makes up for the discrepancy between apparent and predicted molecular size. Rat full-length HL in circulation has recently been reported to increase in apparent molecular size from 55 to 59 kd over time while losing its catalytic activity [10]. The nature of these posttranslational modifications remains unknown.

Hepatic lipase has long been thought to be exclusively synthesized and secreted from liver parenchymal cells [27]. Recently, this view has changed by the demonstration of the synthesis of HL in human and mouse macrophages [17] and in the adrenals of newborn mice [16]. Nevertheless, it is still generally accepted that most, if not all, of the HL activity present in the adrenals and ovaries of rat, mouse, human, hamster, and cow originates from the liver [9, 27]. In rat adrenals and ovaries, no evidence for de novo synthesis and secretion of HL has been obtained [9, 27]. Instead, we have reported the presence of a variant HL mRNA in these organs. This variant HL mRNA in adrenals and ovaries appears to be unrelated to the HL activity found in these organs because induction of HL mRNA and synthesis of the 47- to 49-kd HL-related proteins, by corticotropic or gonadotropic hormones, respectively, is only transient and precedes the long-lasting expression of HL activity. Moreover, whereas the HL activity in adrenals is heparin-releasable, and present at extracellular sites [7], the 47- to 49-kd protein that cross-reacts with anti-HL IgGs remains mainly intracellular [18, 19]. The variant HL forms were only detected in in vitro pulse-labeling experiments with tissue slices from control and, particularly, corticotropin-activated adrenals. We [30], and others [10], have not been able to detect the presence of 47- to 49-kd HL-related proteins by immunoblotting in homogenates of adrenals from untreated rats. This suggests that expression of the variant HL forms in rat adrenals in vivo is very low, at least in untreated animals. As discussed previously, it is unlikely that the putative 47- to 49-kd protein product has the same catalytic activity as HL

because of the lack of the N-terminus. Synthesis of variant 47- to 49-kd HL proteins, therefore, may not be functionally important.

Expression of the variant HL mRNA was transiently increased in adrenal glands upon treatment of the animals with corticotropin. Expression was maximal at days 2 and 4 of treatment. After 6 to 9 days, the amount of HL mRNA has returned to near-normal levels as reported previously [18]. Similarly, both parameters were transiently induced in rat ovaries upon stimulation with the gonadotropins pregnant mare serum (PMS) and human chorionic gonadotropin [19]. The presence of a number of potential binding sites for regulatory transcrip-tion factors, such as Sp1, CCAAT, HNF1, RORA, COUP, and oct-1 sites, in the 5′-flanking region of the putative promoter in intron 2, opens the possibility for hormonal regulation of transcription from this promoter. Indeed, when expressed in human adrenocortical NCI-H295R cells, the alternative promoter was up-regulated more than 3-fold by treatment with cAMP.

The observation that both HL mRNA and HL-related proteins are transiently increased in rat adrenals and ovaries upon stimulation with corticotropin and gonadotropins [19], respectively, suggests that this response may be related to changes in cholesterol homeostasis of these steroid-producing cells in the early days of stimulation. This time frame overlaps with the reduction of intracellular cholesteryl ester content and parallels the increase in de novo cholesterol synthesis [31]. Therefore, the endogenous cholesterol levels may have become seriously reduced, and the need for HDL-C as exogenous source for steroidogenesis may have increased. Because the intracellular HL-related protein is probably catalytically inactive, the protein may have other functions in selective cholesterol uptake or intracellular cholesterol trafficking. Stimulation of adrenals as well as ovaries [19] results in a strong, sustained elevation of HL activity. This HL activity is not locally synthesized and therefore likely originates from circulation. Because HL in non-heparin plasma is virtually inactive [10], and the specific triglyceridase activity of HL isolated from adrenals is similar to liver HL[10], the increased adrenal HL activity is not simply due to increased entrapment of blood in the enlarged, hyperplastic glands. As HL in the circulation is mainly present on HDL [10], extracellular HL derived from liver may increase as a result of HDL trapping in the increased number of microvillar channels of the stimulated, hyperplastic adrenals [32], presumingly followed by unmasking of its catalytic activity. This increased HL activity may facilitate the influx of HDL-C in cooperation with SR-B1, which is induced in parallel with HL activity [33, 34].

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Chapter 4.0

Cloning, Expression and Promoter Analysis of Hepatic Lipase Derived from Human Hyperplastic Adrenals: Evidence for Alternative Splicing

Hepatic lipase mRNA in human adrenals

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Abstract

Human adrenals contain hepatic lipase (HL) activity, which is thought to facilitate the uptake of plasma cholesterol used in steroidogenesis. We show here that full-length HL mRNA is expressed in hyperplastic adrenals of Cushing's disease patients. In addition, a splice variant that lacks exon-3 was detected in the human adrenals and hepatoma (HepG2) cells, but not in liver. In CAT-reporter assays using human NCI-H295R adrenocortical cells, the HL(-685/+13) promoter region was transcriptionally active, and its activity was enhanced 2-fold by cAMP. In rat adrenals, the HL gene is exclusively transcribed from an alternative promoter within intron-2, resulting in a variant mRNA that lacks exons 1 and 2. By RT-PCR, we found no evidence for expression of such a variant mRNA in human adrenals, liver or HepG2 cells. The presence of both full-length mRNA and enzyme activity in human adrenals suggests that part of the HL activity is locally synthesized.

Introduction

Hepatic lipase (HL; E.C. 3.1.1.34) activity is present in the adrenal cortex of several mammals including man [1-4] as well as in rat ovaries [5-8]. The HL activity from rat, mouse and human adrenals is indistinguishable from HL in liver and post-heparin plasma [2, 5, 8-10]. In the liver, HL activity plays an important role in plasma lipoprotein metabolism [11]. Notably, the enzyme facilitates the cellular uptake of cholesterol carried in HDL [12, 13]. Several lines of evidence indicate that the HL activity in the steroidogenic organs is also involved in the uptake of cholesterol necessary for steroid production [6 ,8, 14, 15], probably by accelerating the SR-BI mediated uptake of HDL cholesterol [16, 17].

It is generally assumed that the HL activity found in steroidogenic organs is not locally synthesized, but originates from liver and is transported to these organs via the circulation [4,7,9]. This is mainly based on the inability to detect full-length HL mRNA [7,9,18-20] and de novo synthesis of HL protein [9,18,19,20] in extrahepatic tissues of the rat. HL protein is present in rat plasma associated with HDL [4], and rat ovaries were shown to accumulate hepatic lipase from the circulation [7]. However, expression of HL mRNA and synthesis of HL protein have now been demonstrated in adrenals of neonatal mice [10] and in human and mouse macrophages [21]. We recently showed, that the HL gene is transcribed in rat adrenals and ovaries, but exclusively from an alternative promoter within intron-2 [19,20,22]. This gave a variant HL mRNA product in which the first two coding exons of the HL gene are substituted by a sequence that corresponds to intron-2 immediately upstream of exon-3. In addition, local synthesis of a possible translation product of 45-47 kDa that cross-reacts with poly- and monoclonal anti-HL antibodies was observed. The variant transcript is also present in rat liver as a small fraction of total HL gene products [19,22]. These observations open the possibility that, besides liver-derived HL, rat steroidogenic organs contain a second HL gene product with a hitherto undefined function in steroidogenesis.

In human adrenocortical tissue, part of the HL mRNA could be detected by RT-PCR demonstrating that the HL gene is transcribed in adrenals not only in rat but also in man [3]. The question arises whether in human adrenals the HL gene is also transcribed into a variant

mRNA similar to rat. We therefore used the RT-PCR approach to study the possible expression of multiple HL gene transcripts in human adrenals as well as in human liver and the human hepatoma cell line HepG2. Our data demonstrate the presence of full-length HL gene transcripts in human adrenals.

Materials and methods

Patient material

Four hyperplastic adrenal glands were obtained from three Cushing's disease patients who underwent bilateral adrenalectomy. A human liver sample was obtained from a patient that underwent liver transplantation. The tissues were kept on ice, rinsed in ice-cold PBS, cut into small pieces and then dropped in liquid nitrogen within 1-2 h after surgery. Tissues have been stored at -80 ^{o}C until use.

Cell lines

HepG2 human hepatoma cells were cultured in T75 flasks (Nunc, Roskilde, Denmark) at 37 °C and 5% CO_2 in DMEM (ICN, Costa Mesa, CA, USA) supplemented with 10% (v/v) fetal calf serum (Gibco, Breda, Netherlands) and penicillin/streptomycin. NCI-H295R human adrenocortical cells were cultured in T25 flasks in DMEM/Ham F12 medium (Gibco), supplemented with 2 % fetal calf serum, 15 mM Hepes, 25 mM $NaHCO_3$, 10 μg/ml of insulin-transferrin-sodium selenite (Roche, Almere, Netherlands), 10 nM hydroxycortisone (Merck, Darmstadt, Germany), 10 nM β-estradiol (Sigma, St. Louis, USA) and penicillin/ streptomycin. Every 7 days, the cultures were split 1:10 and 1:3 into new flasks, respectively. Media were refreshed once a week.

RNA isolation

Total RNA was isolated from approximately 100 mg of adrenal or liver tissue by the method of Chomczynski & Sacchi [23]. Similarly, total RNA was isolated from HepG2 and NCI-H295R cells, each starting from two T25 flasks of confluent cell culture. Total RNA isolated from 100 mg of liver from a male Wistar rat was used as a reference. RNA concentrations were determined by spectrophotometry at 260 nm [24]. The quality of the RNA preparations was judged from the ratio of 28S over 18S ribosomal RNA after denaturation and electrophoresis in a 1 % agarose/TBE gel [24].

RT-PCR

First strand cDNA synthesis and subsequent amplification by PCR was performed in a single tube starting with 1 μg of total RNA, as described previously [19]. No-template controls as well as no-RT controls were included in each experiment. Oligonucleotides used for amplification of HL cDNA are specified in table 1. The oligonucleotides int2 and INT recognize intron-2 of the human and rat HL gene, respectively, immediately upstream of exon-3 [19,25]. For the amplification of human SR-B1 cDNA, the oligonucleotides 5'-cgg aat TCA GGG GTG TTT GAA GGC-3' (nt 637-654) and 5'-cgg gat CCT GAA TGG CCT CCT TAT CC-3' (nt 1234-1215) were used as forward and reverse primer, respectively (capital

letters correspond to the SR-B1 cDNA sequence, numbering according to Calvo and Vega [26]. All primer pairs used span at least one intron.

Post-PCR analysis

PCR products were separated by electrophoresis through a 1 % agarose gel, and visualized by ethidium bromide staining [24]. Digestion with restriction enzymes (Roche) was performed directly on the post-PCR mixtures. DNA molecular weight marker VI was from Roche. The intensities of the PCR bands were assessed by densitometry using the GS-800 Calibrated Densitometer (BioRad, Hercules, CA, USA).

Table 1: **Primers used in PCR analysis of human and rat HL gene expression.**

part of HL gene	name	orientation	species	Sequence (5' → 3')	nt	Ref.
exon 1	HL-4	F	h	ACC AAG AAA GCC TGG ACC C	8-26	25
exon 2	HL-13	F	h	TCT GGT GAT GAT AAT CCA CGG G	253-274	36
	RHL-13	F	r	TGT CAT GAT CAT CCA CGG G	267-285	37
exon 3	HL-12	F	h	CGG CGT GCT AGA AAA CTG G	286-304	36
	RHL-12	F	r	TGG CTT GCT AGA AAC CTG G	297-315	37
exon 4	HL-8	F	h+r	TAA TTG GGT ACA GCC TGG G	498-516	36
	HL-9	R	h+r	GGC ATC ATC TGG AGA AAG GC	660-641	37
exon 5	HL-1	F	h+r	GTG GGC ATC AAA CAG CCC	701-718	36
	HL-3	F	h+r	CGG GGG CTC CTT CCA GCC TGG	741-761	36
exon 8	HL-2	R	h+r	CAG ACA TTG GCC CAC ACT G	1292-1274	36
exon-9	HL-22	R	h	TGT GAG ATT CTT TAT GTA ATT TTG GC	1641-1616	25
intron 2	int	F	h	AAA CTA AGG CGA CCC TCC CT	-	25
	INT	F	r	GCA TTG TCC TTG AGC CTG AG	-	19

F and R: forward and reverse orientation, respectively; h and r: identical to human and rat sequence, respectively.

For Southern blotting, the DNA in the gel was transferred onto Hybond-N (Amersham Biosciences, Amersham, UK) and the membrane was probed either with a 32P-labeled oligonucleotide (HL-8) or full-length rat HL cDNA. The oligonucleotide was labeled with polynucleotide kinase (Roche) and [γ-32P]ATP (Amersham); the cDNA was labeled using the Megaprime kit and [α-32P]dCTP (Amersham). Standard hybridization and washing conditions were used [24]. The 32P-labeled bands on the blot were visualized by autoradiography.

For sequence analysis, the DNA band of interest was purified from the gel by the freeze-squeeze method [24], and the DNA was TA-cloned [27] into pBluescript KS$^-$ (Stratagene, La Jolla, CA, USA). Automated sequencing was performed on an ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA) using the Thermo-sequenase dye terminator kit (Amersham) in conjunction with the vector specific SK primer (Stratagene).

Promoter activity

The promoter-reporter plasmids used have been described previously. Of the human HL gene, the –685/+13 region and the –312/+13 region were inserted into the pCAT-Basic reporter plasmid (Promega, Madison, WI, USA), to generate HuHL698-CAT and HuHL325-CAT vectors, respectively [28]. Of the rat HL gene, the PstI-XbaI (–437 to +9) fragment was used to generate RHL446-CAT reporter vector [22]. In intron-2-CAT, the 577 bp HindIII-BglII fragment of the rat alternative promoter region located in intron-2 of the rat HL gene was inserted into pCAT-Basic [22].

At 24 h before transfection, the NCI-H295R cells were plated in 6-well plates at 50 % confluence. Transfections were performed with Lipofectamine-Plus (Invitrogen, Breda, Netherlands) according to the manufacturer's instructions, using 1.0 μg CAT reporter and 0.25 μg RSV β-galactosidase expression plasmid per well. Parallel transfections with empty pCAT-Basic plasmids were used as controls. When indicated, synthetic ACTH (Synacthen; Novartis, Basel, Switzerland) or 8-bromo-cyclic AMP (8-Br-cAMP; Sigma) was added to the medium 4 h after transfection at a final 0.4 mU/ml and 300 μM, respectively. Fourty-eight hours post-transfection, cell lysates were prepared. CAT and β-galactosidase expression 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.

HepG2 cells were transfected with the calcium-phosphate co-precipitation method using 10 μg of the CAT reporter test plasmid and 0.4 μg of the RSV β-galactosidase expression plasmid (Promega). At 24 h before transfection, the cells were plated in 60 mm culture dishes at 20-30 % confluence. At 3 h before transfection, the medium was refreshed. Incubations were continued for 48 h after transfection, and promoter activity was determined as described above.

Statistical analysis

Data are expressed as mean ± SD. Differences were tested for statistical significance by paired Student *t*-test.

Results

Human hyperplastic adrenals

With the RNA isolated from a human hyperplastic adrenal, a single major product of 596 bp was generated by RT-PCR using the oligonucleotides HL-1 and HL-2 which recognize both human and rat HL cDNA (fig. 1). This PCR product was observed with RNA prepared from all four adrenals studied. A similar PCR product was generated with RNA from human liver as well as rat liver (fig. 1). Digestion of the PCR product from human liver, human adrenals and rat liver with *Bcl*I resulted in the predicted product of 545bp. An additional fragment corresponding to 45 bp and 51 bp was generated with the human and rat preparations, respectively, in agreement with the restriction maps of human and rat HL cDNA. *Pst*I digestion of the rat liver PCR product gave the expected 400bp and 196bp fragments, whereas the human PCR products were not digested. With 1 μg of human or rat

adrenal RNA, the 596 bp PCR product became UV-detectable after 28-30 cycles of amplification, whereas with 1 µg of rat liver RNA this band was already observed after 20 cycles. This suggests that HL mRNA abundance in the human adrenals is similar to rat adrenals, which is 40-50-fold lower than in rat liver [19]. These observations extend our previous report [3] and demonstrate that at least part of the HL mRNA is expressed in human hyperplastic adrenals.

In rat adrenals, exons 1 and 2 of the HL gene are not expressed in the RNA. We tested whether this is also the case for the human adrenals. RT-PCR was performed using HL-2 as downstream primer in combination with different forward primers (fig. 2A). With HL-8 and HL-12, which are specific for exon-4 and exon-3, respectively, PCR products were obtained of the expected size, and which hybridized to full-length rat HL cDNA (fig. 2B).

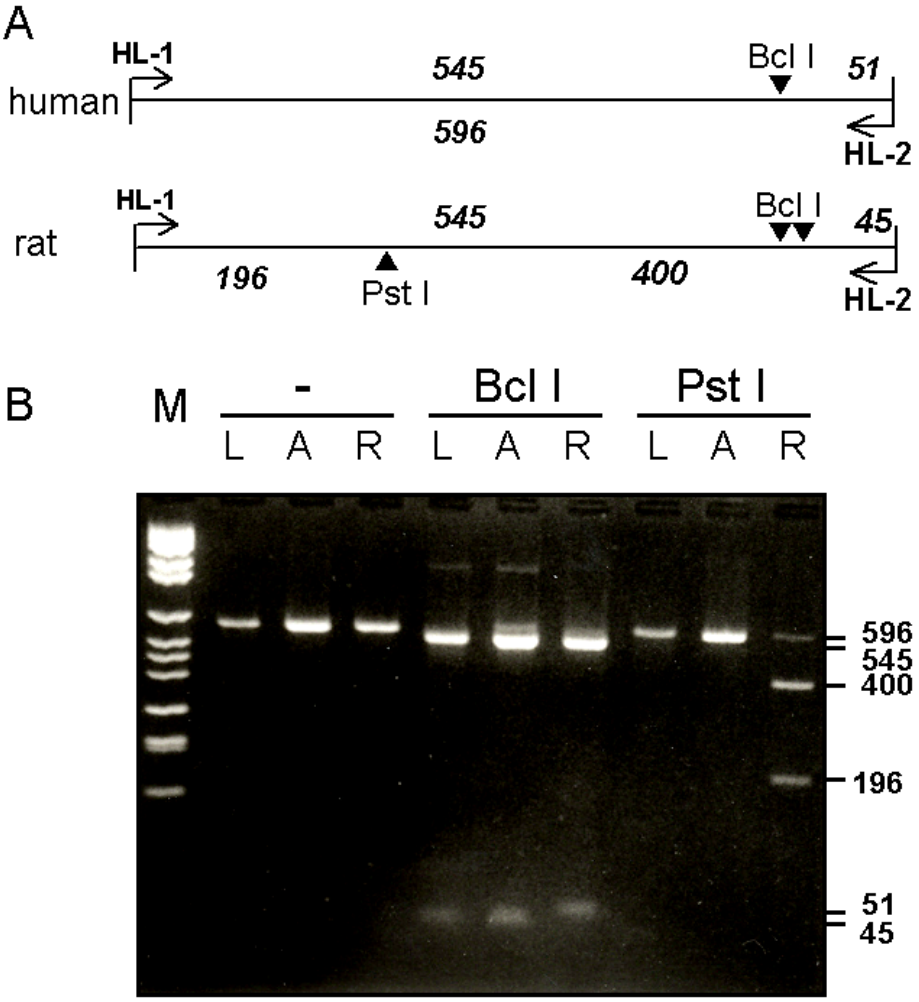

Figure 1: **Detection of HL mRNA in human adrenal glands by RT-PCR.**
RT-PCR was performed with 1 µg of total RNA prepared from human liver (L), from human hyperplastic adrenal (A) and from rat liver (R), using the oligonucleotides HL-1 and HL-2 as forward and reverse primer, respectively. After RT-PCR, the reaction mixtures were incubated without any restriction enzyme (-), or in the presence of *Bcl*I or *Pst*I. Panel A shows the *Bcl*I and *Pst*I restriction maps of the 596bp PCR product deduced from the human and rat HL cDNA sequences [36,37]. Panel B shows the RT-PCR and digestion products after separation by agarose gel electrophoresis. DNA size markers were run in the lane marked M. Expected PCR and digestion fragment sizes are indicated in bp. The no-template and no-RT controls were all negative. Data are representative for three similar experiments.

With exon-2 specific HL-13, a 1.0kb and a 0.85kb product was generated with RNA from human adrenals and HepG2 cells. In three independent experiments, the intensity of the 0.85kb band was 51 ± 8 % and 57 ± 14 % of that of the 1.0 kb band with adrenal and HepG2 RNA, respectively. RNA from human liver only generated the 1.0kb RT-PCR product. Both amplimers were sequenced after cloning into pBluescript. The 1.0-kb band appeared to be identical to the published human HL cDNA sequence. This finding indicates that exon-2 of the HL gene is represented in the RNA from human adrenals, which contrasts with rat adrenals. Additional RT-PCR's with other combinations of primers, including HL-4 and HL-22, demonstrated that in fact the entire coding part of the HL gene was expressed in the RNA of human hyperplastic adrenals (not shown). The sequence of the 0.85kb amplimer was also identical to human HL cDNA except that the entire third exon was missing (fig. 3); in this amplimer exon-2 was immediately followed by exon-4.

Finally, we tested whether the human HL gene is transcribed from an alternative promoter within intron-2, as described for the rat gene [19, 22]. RT-PCR was performed using HL-2 in combination with the oligonucleotide int, which recognizes intron-2 immediately upstream of exon-3, and which is used routinely in our laboratory in the amplification of exon-3 from genomic DNA. No products were observed when total RNA was used from either human adrenals, HepG2 cells or human liver (fig. 4).

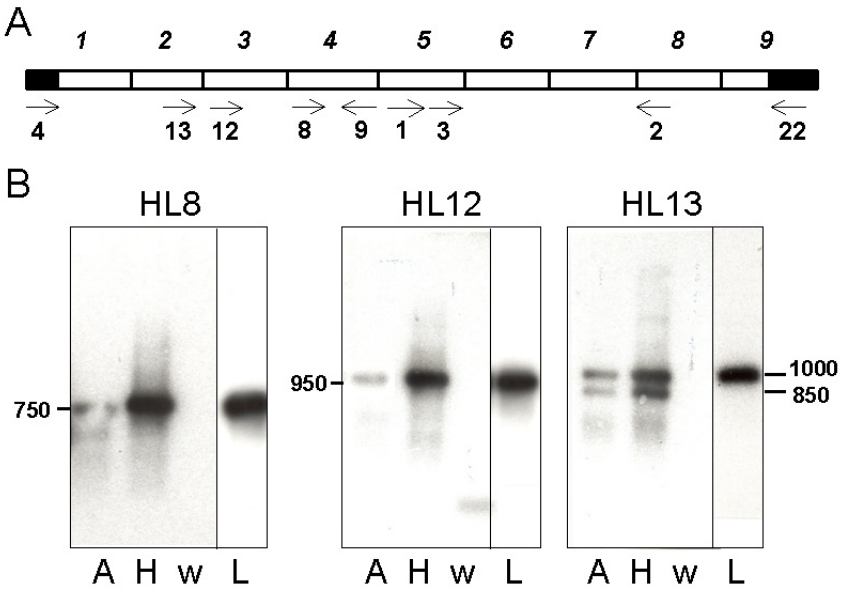

Figure 2: **Southern-blot analysis of HL mRNA from human hyperplastic adrenals after RT PCR.**
Panel A schematically shows the exon organization of human HL cDNA. The open bar indicates the coding sequence with the exon numbers given above. The closed bars represent the untranslated regions. The arrows define the position and 5'→3' orientation of all the oligonucleotides used in this study. Panel B shows the results of the RT-PCR analysis. RT-PCR was performed on total RNA from human adrenals (A), HepG2 cells (H), human liver (L), and on a parallel water control (w). After first-strand DNA synthesis, the incubation was split into three aliquots for amplification with the reverse primer HL-2 and the different forward primers indicated on top of each panel. After gel electrophoresis and blotting, the membranes were probed with ^{32}P-labeled rat HL cDNA. The data on human liver are from an overnight exposure to autoradiography film, whereas the other data are from a 48 h exposure. Approximate amplimer sizes are indicated in bp. Data are representative for three independent experiments.

In parallel reactions, PCR products of the expected size that hybridized with an internal probe were generated with HL-12 and HL-13 as forward primers. Analogous RT-PCR reactions on rat liver RNA gave good yields with all three forward primers tested, including the intron-2 primer. Hence, transcription from an alternative promoter in intron-2, as described for the rat HL gene, is not evident in human adrenals, human liver or HepG2 cells.

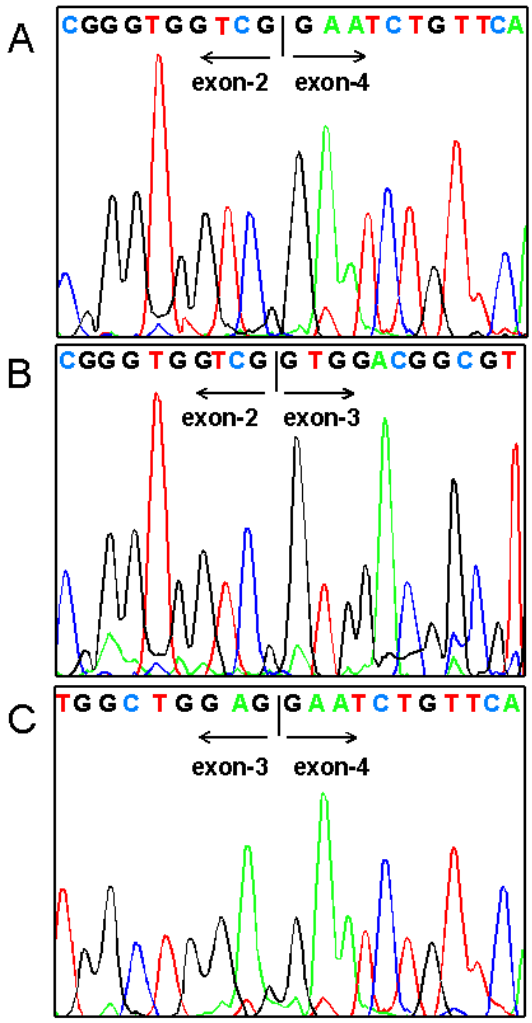

Figure 3: **Exon-3 is skipped in some of the HL gene transcripts in human adrenals.**
The 0.85kb RT-PCR product (panel A) and the 1.0kb RT-PCR product (panel B and C) obtained with the HL-13/HL-2 primer pair (cf. Fig. 2B) was gel-purified, TA-cloned into pBluescript and then analyzed by automated DNA sequencing. The figure shows part of the chromatogram that contained the exon-2/exon-4 boundary in the 0.85kb product (A), and the exon-2/exon-3 (B) and exon-3/exon-4 boundaries (C) in the 1.0kb product.

Human adrenocortical cell line

After 45 cycles of amplification, RT-PCR on RNA from NCI-H295R adrenocortical cells with the HL-2/HL-3 primer pair yielded the expected 552bp product (fig. 5). This PCR product was not visible in ethidiumbromide-stained gels after 35 cycles of amplification used routinely, suggesting that the abundance of HL mRNA is less than in HepG2 cells or hyperplastic adrenals. For comparison, we also used oligonucleotides specific for human SR-B1 in the RT-PCR. A product of the expected size (610bp) was readily obtained, confirming previous reports that SR-B1 is expressed in this cell line [29].

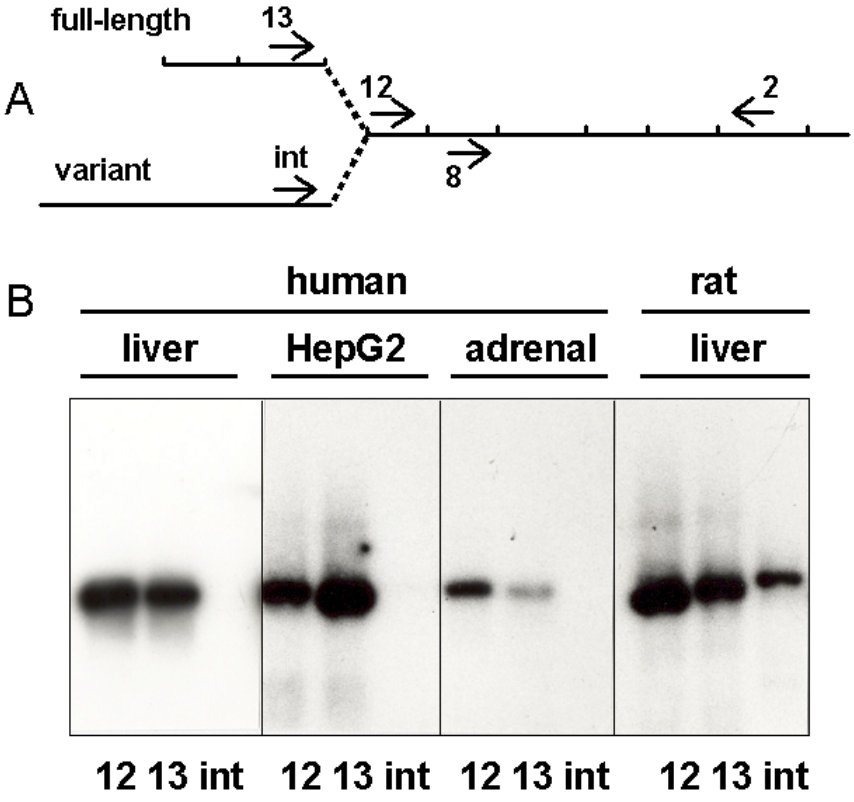

Figure 4**: Intron-2 sequence is not expressed in HL mRNA from human adrenals or liver.**
RT-PCR was performed on RNA from human liver, HepG2 cells and hyperplastic adrenals. In combination with HL-2, different forward primers were used that are specific either for exon-3 (HL-12), exon-2 (HL-13) or intron-2 (int). In parallel, a similar set of incubations was performed with RNA from rat liver using primers specific for the homologous regions in the rat HL gene. After separating the PCR-mixtures on an agarose gel, the DNA was blotted to Hybond-N and the membrane was probed with ^{32}P-labeled HL-8 oligonucleotide. Panel A schematically shows the position of the used oligonucleotides in human and rat HL cDNA. Panel B shows an autoradiogram of the resulting blot. Data are representative for two independent experiments.

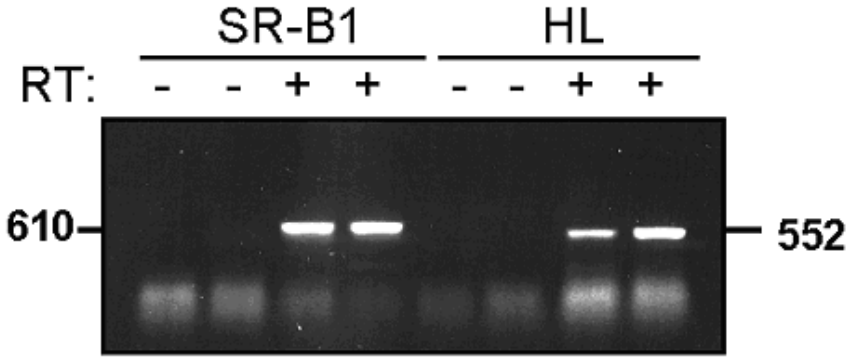

Figure 5: **HL mRNA is expressed in the NCI-H295R human adrenocortical cell line.**
RT-PCR was performed on RNA prepared from two independent human NCI-H295R cell cultures, without (-) or with (+) reverse-transcriptase in the RT-reaction. Amplification was for 45 cycles using either SR-B1 specific primers, or the HL specific primers HL-2 and HL-3, PCR products were separated by agarose gel electrophoresis. The expected amplimer sizes are indicated in bp. Data are representative for two independent experiments.

To determine the transcriptional activity of the human HL promoter region in NCI-H295R adrenocortical cells, cells were transiently transfected with reporter plasmids containing a 698 bp or a 325 bp fragment (fig. 6). Both HL promoter fragments showed almost 3-fold higher activity than the promoter-less pCAT-Basic. The activity of the 698 bp HL promoter fragment in the adrenocortical cells was comparable to that in the human hepatoma HepG2 cells. Shortening the HL promoter fragment from 698 bp to 325 bp left transcriptional activity in adrenocortical cells unaffected, whereas the activity in HepG2 cells was further increased. Similarly, the rat HL446 promoter fragment was active in both cell types, but CAT expression was much higher in the HepG2 than in the NCI-H295R cells. Compared to the conventional

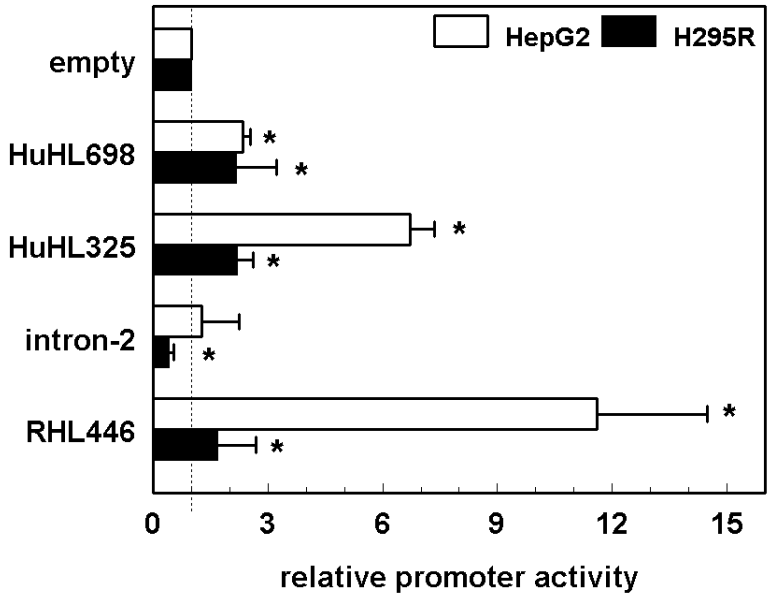

Figure 6: **HL promoter activity in transiently transfected HepG2 and NCI-H295R cells.**
The indicated promoter-CAT reporter plasmids were transfected into HepG2 and NCI-H295R cells (open en closed bars, respectively). At 48 h post-transfection, CAT and β-galactosidase expression was determined. The CAT data were normalized on the basis of β-galactosidase expression. Normalized CAT-data are expressed as fold increase with respect to the promoter-less pCAT-Basic. Data are means ± SD for three independent experiments. The asterisks indicate a statistically significant difference from pCAT-Basic ($P<0.05$).

human and rat HL promoter fragments, the alternative rat HL promoter in intron-2 showed negligible activity in this reporter assay (fig. 6). Treatment of transfected NCI-H295R cells with 0.4 mU/ml ACTH slightly but not-significantly increased transcriptional activity of the HL698 construct (1.3 ± 0.4-fold increase; n=3; n.s.). As shown in figure 7, incubation of the cells with membrane-permeant 8-Br-cAMP, which mimics the effect of the corticotropin, increased the activity of the HuHL698 promoter fragment 1.9 ± 0.2-fold (n=3; $P<0.05$), and that of the HuHL325 construct 1.7 ± 0.3-fold (n=3; $P<0.05$). Hence, the conventional HL promoter region is active in the human adrenocortical cells, and sensitive to regulation by cAMP.

Discussion

The presence of HL activity in the adrenal cortex of humans and other mammals, as well as in ovaries, has long been recognized. In the rat, several attempts to determine HL mRNA or HL *de novo* synthesis in these steroidogenic organs have been unsuccessful [7, 9, 18, 19]. This led to the hypothesis that this lipase actually is synthesized in the liver and subsequently

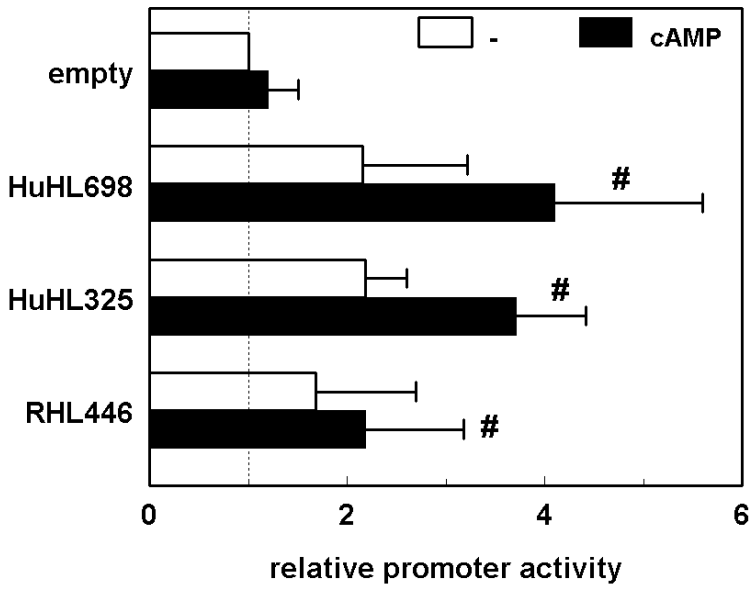

Figure 7: **Effect of 8-Br-cAMP on HL promoter activity in NCI-H295R cells.**
Experiments were performed as described in the legends to figure 6, except that cells were incubated in the presence (filled bars) or absence (open bars) of 300 µM 8-Br-cAMP. Data are means ± SD for three to four independent experiments. #: statistically significant effect of 8-Br-cAMP (P<0.05), as determined by paired Student t-test.

transported to the steroidogenic organs [4, 7, 9].Here, we show for the first time that full-length HL mRNA is expressed in the hyperplastic adrenals of patients with Cushing's disease. These adrenals are activated by increased pituitary secretion of ACTH, and contain elevated levels of HL activity [30, 31]. We corroborated these findings by promoter-reporter assays using the human adrenocortical NCI-H295R cell line. These assays demonstrated that the human HL promoter region is active in these cells, and that promoter activity in these cells is enhanced by 8-Br-cAMP, which mimics the effect of ACTH. An increased HL expression in these organs may be important to maintain the high steroid production, possibly by accelerating the SR-BI mediated uptake of HDL cholesterol [6, 14, 16, 17]. The presence of full-length HL mRNA in these activated adrenals strongly suggests that at least part of the adrenal HL activity originates from local synthesis.

The presence of full-length HL mRNA in the human adrenals contrasts with the situation in rat adrenals [19, 22] and ovaries [20], where only a variant HL gene transcript is found. This variant HL mRNA is also present in rat liver, but at a low abundance relative to full-length HL mRNA [19], and originates from the usage of an alternative promoter in intron-2 [22]. In the present study, no evidence was found for the expression of its counterpart in human

adrenals or in human liver. In this respect, the situation in the rat steroidogenic organs appears to be unique to rat.

In human adrenals as well as in HepG2 cells, a small fraction of the HL gene transcripts lacks exon-3. The open reading frame of this alternative splice product is maintained [25], which would result in a markedly shorter translation product. Since exon-3 is at the heart of the catalytic domain of HL, it is unlikely that this alternative translation product is properly folded, let alone, catalytically active [32]. The alternatively spliced RNA was not observed with the human liver preparation. Hence, alternative splicing of the primary HL gene transcript may reflect the altered phenotype of the hyperplastic adrenal and the HepG2 hepatoma cells rather than imply functional significance. Therefore, no attempts were made to demonstrate expression of the alternative translation product in either HepG2 cells or human adrenals.

In conclusion, we have shown here that human hyperplastic adrenals express full-length HL mRNA. In the human, adrenal HL may therefore not only originate from liver [4, 7], but may also be locally synthesized. The presence of HL mRNA and secretion of HL activity has been reported for human pre-ovulatory granulosa cells [33], suggesting that HL is locally synthesized in ovarian cells as well. Within the adrenal glands, HL activity is predominantly localized in the steroidogenic cortex [1, 2], and is required for optimal selective uptake of HDL cholesterol by adrenocortical cells [16]. In HL-null mice, the ACTH-induced increase in plasma corticosterone levels was attenuated, suggesting that adrenal HL is important for steroidogenesis [34]. In Cushing disease patients, adrenal HL activity is several-fold higher than in controls [30, 31], and the daily cortisol production is increased in parallel [30]. Taken together, these observations strongly suggest that adrenal HL has an important role in cholesterol supply for steroid production [34]. HL may facilitate the selective uptake of free cholesterol [12, 35], or of cholesteryl esters via the HDL/SR-B1 [17] and LDL-receptor pathways [34].

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Chapter 5.0

Human HL promoter polymorphism

Chapter 5.1

Methods for determination of the hepatic lipase promoter genotype

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Abstract

In the hepatic lipase promoter region four single nucleotide polymorphisms are present at positions –763 (A→G), -710 (T→C), -514 (C→T) and –250 (G→A), which are in almost complete linkage disequilibrium. Therefore, two different alleles can be distinguished, which are designated as the *LIPC* C- and T-allele according to the –514 polymorphism. Among different populations, the frequency of the T-allele varies between 17 and 55 %. The T-allele is associated with a lowered HL activity and elevated HDL cholesterol. Despite the higher HDL cholesterol, carriers of the T-allele may have a higher risk for coronary artery disease. In this study two different methods for determining the hepatic lipase promoter genotype are presented: the allele-specific oligonucleotide (ASO) hybridization and restriction endonuclease assay with Nla*III* or Dra*I*.

Introduction

The human *LIPC* gene spans approximately 160 kb of chromosome 15q21.3, and encodes for hepatic lipase [4, 14]. In the 5' upstream regulatory region of the *LIPC* gene four single nucleotide polymorphisms (SNPs) are present at positions –763 (A→G), -710 (T→C), -514 (C→T) and –250 (G→A) [7, 8]. These four SNPs affect potential restriction sites for Sph*I* (GCATG↓C), Ava*II* (G↓G(A/T)CC), Nla*III* (CATG↓), and Dra*I* (TTT↓AAA), respectively, which can be exploited for their analysis. The –514C/T polymorphism, which was previously denoted as the –480C/T polymorphism [10], is most widely used for LIPC genotyping [9], because this polymorphism was first identified [10], and has been shown to be functionally important in combination with the –250G/A polymorphism [2, 6].

Several methods have been described to determine the presence of a SNP. These methods can be subdivided into two categories [3, 5]. The first group of methods is designed to scan for new polymorphisms within a gene, and includes single-strand conformational polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), heteroduplex analysis (HET), ribonuclease cleavage (RNase), chemical cleavage analysis (CCM) and direct sequencing of the gene. Although these assays are highly informative, they are time - consuming and incompatible with high-throughput screening. The second group of methods is designed for screening for known SNPs, such as oligonucleotide ligation assay (OLA), primer-guided nucleotide incorporation assay, restriction site analysis, artificial introduction of restriction sites (AIRS), allele-specific oligonucleotide hybridization (ASO), and (automated) variations of these procedures. For screening for a known SNP in a large population, the ASO hybridization assay is an elegant method. In this assay, DNA that has been amplified by PCR is hybridised with either one of two allele-specific oligonucleotides (ASO). These ASOs are usually 15 to 25 nucleotides in length, with the polymorphic site located approximately in the middle. Large number of DNAs can be analysed simultaneously. However, the hybridisation signals are sometimes difficult to interpret, especially the heterozygotes.

The assay based on restriction site analysis is most widely used. The assay is straight forward provided that the SNP affects a potential restriction site. The analysis of digestion

products, however, is labor-intensive, and this type of analysis is not easily automated. In our laboratory, we use both the ASO assay as well as the restriction endonuclease assay for the determination of the *LIPC* genotype. The ASO assay is used to detect the –514C/T polymorphism, and digestion with *Nla*III and *Dra*I is used to detect the –514C/T and –250G/A polymorphism, respectively. In this chapter, these methods are described in detail.

Methods

Amplification of the proximal hepatic lipase promoter region.

Genomic DNA was prepared from buffy coats using a standard isolation method. With the polymerase chain reaction (PCR) a 551 bp fragment of the hepatic lipase promoter (nt -578/- 27 according to the sequence of Ameis et al. [1]; fig. 1) was amplified. Amplification was performed using approximately 0.5 µg of genomic DNA in a standard PCR reaction mixture (Eurogentec, Seraing, Belgium), with HLpr2S (5'-GGATCACCTCTCAATGGGTC-3') and HL25 (5'-GATACTTTGTTAGGGAAGACTGCC-3') as upstream and downstream primer, respectively. After an initial denaturation step (2 min at 95 °C), the DNA was amplified in 34 cycles of 30 sec at 95°C, 30 sec at 45°C and 90 sec at 72°C followed by a final extension step at 72 °C for 7 min.

Allele-specific oligonucleotide hybridisation assay.

Twenty-five microliters of the PCR mixture was denaturated by dilution with 75µl alkaline solution (0.4 M NaOH, 10 mM EDTA pH 8, slightly stained with xylene cyanol) and heating at 94°C for 10 min, followed by rapid cooling in an ice bath. A dot-blot apparatus (Bio-Rad, Richmond, USA) was assembled with two sheets of Whatman 3M filtration paper (Whatman, USA) and one sheet of Hybond N^+ membrane (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) on top. After wetting the membrane with 3x SSC (1x SSC contains 150 mM sodium chloride and 15 mM sodium citrate; pH 7), 50 µl of each sample was transferred to a well. Known homozygote and heterozygote controls were transferred in parallel. A vacuum was applied by applying slight suction until the liquid was evacuated from the wells. After disassembling the apparatus, the membrane was soaked in 3x SSC for 5 minutes, followed by cross-linking of the DNA to the membrane by exposure to 50 mJ UV radiation, and briefly baking the membrane at 65 to 80°C. This blotting procedure was performed in duplicate, to create two identical membranes.

The duplicate membranes were placed in separate hybridization bottles and incubated for 30 min at 37°C in 10 ml of prehybridization buffer (50 mM NaH_2PO_4, 0.75 M NaCl, 5 mM EDTA, 1% SDS, 0.05 mg/ml salmon sperm DNA, pH 7). For the *LIPC* -514C/T polymorphism, the C and T-allele specific oligos BHLp2SA: 5'-CACCCCC*G*TGTCAAA-3' and BHLp2SB: 5'-CACCCCC*A*TGTCAAA-3' were designed, respectively (fig. 1). For each ASO, 2 µl (1µM) was incubated with polynucleotide kinase (Eurogentec, Seraing, Belgium) and ^{32}P-γ-ATP (370 MBq/ml, Amersham) for 1h at 37^{o}C. The labelled oligonucleo-tides were separated from the free label using a Sephadex G50 gelfiltration column. After the prehybridization, the labelled ASOs were added to either one of the duplicate membranes in the prehybridization buffer, and hybridization was continued for one hour at 37°C. Thereafter,

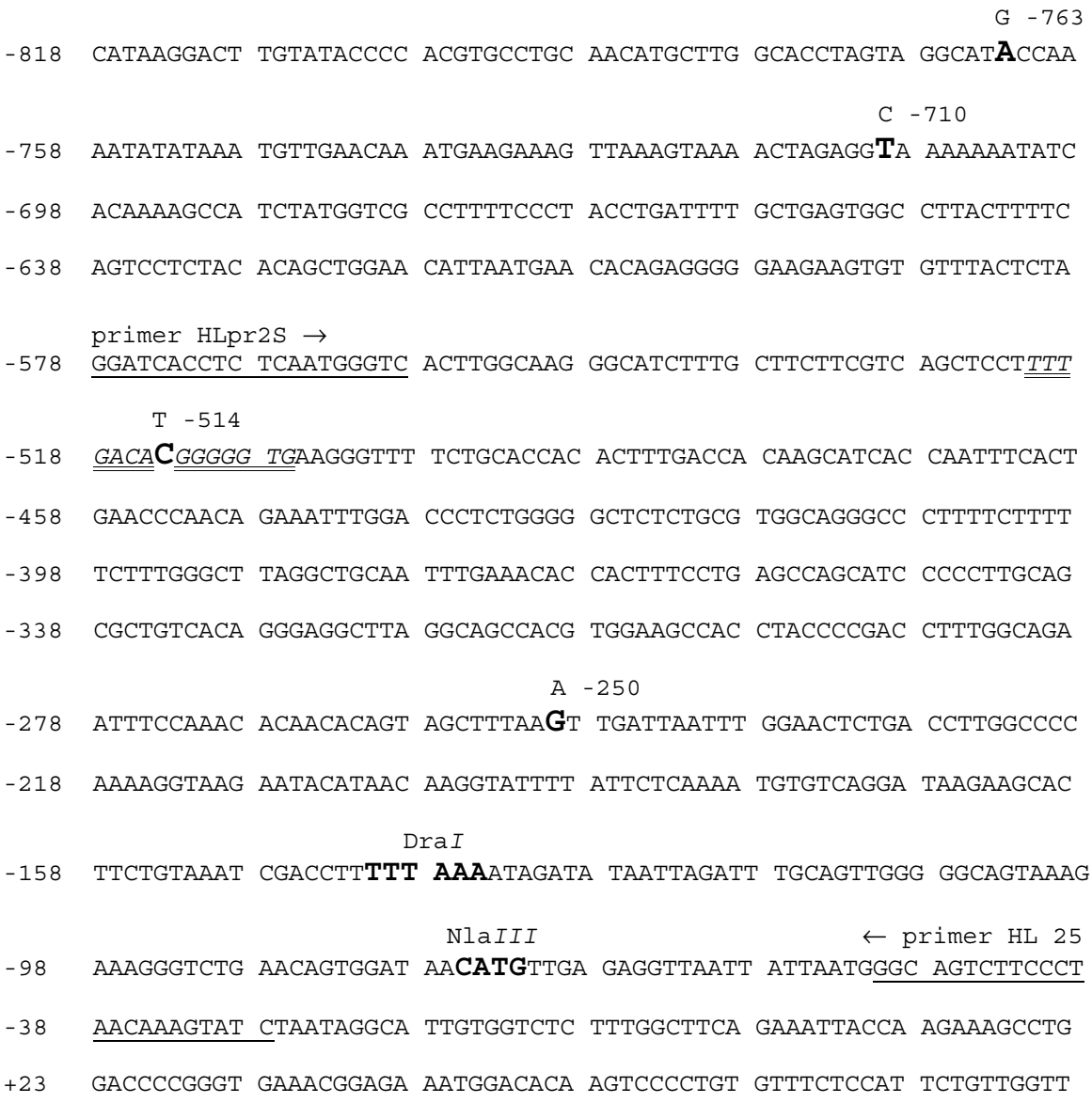

Figure 1: **The 5' upstream regulatory region of the HL gene, including part of exon 1, corresponding to the C-allele.**
The four single bold nucleotides represent the four SNPs. On top of them, the nucleotides of the T-allele are given. The two clusters of bold nucleotides represent the invariant Dra*I* and NlA*III* restriction sites. The underlined nucleotides represent the primers used for PCR. The double underlined nucleotides surrounding the –514 nucleotide represent the ASO primer.

the hybridisation buffers were removed and the membranes were washed for 5 minutes with, subsequently 3x SSC/0.1% SDS, 1x SSC/0.1% SDS, and 0.3x SSC/0.1% SDS. The air-dried membranes were covered with plastic foil, and then exposed to an autoradiographic film for 16 h at -80 °C. The films were developed in an automated Kodak developer.

With one labelled ASO probe, multiple membranes with hundreds of PCR samples can be simultaneously genotyped (fig. 2). Theoretically just one ASO probe would have been sufficient. When for instance only the -514C ASO probe is used, the dark spots are indicative for the -514CC homozygotes, and the grey spots having approximately half the intensity for the heterozygotes; the absence of a signal would indicate the -514TT homozygote. However, signals may also be absent for other reasons such as PCR failure or pipetting errors. Using the

duplicate membrane and the ASO specific for the second allele, the signals should be complementary, and reliability of genotyping is strongly enhanced.

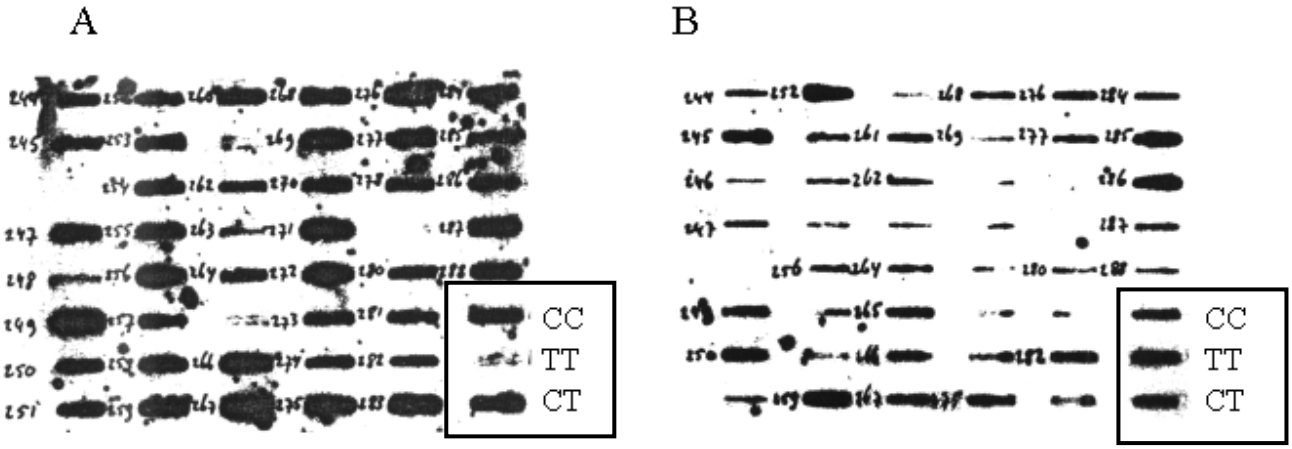

Figure 2: **Radiographs of duplicate dot-blotted PCR product on membranes after hybridization with (A) C-allele specific probe, (B) T-allele specific probe.**
On each membrane at the bottom on the right site, known controls have been blotted.

Restriction endonuclease assay.

PCR products with a length of 551 bp were generated as described above for the ASO assay. The PCR mixtures were incubated overnight at 37 ^{o}C, either with 10 U *Nla*III or with 10 U *Dra*I (both from New England Biolabs, Ipswich, MA, USA), for detection of the –514C/T and the –250G/A polymorphism, respectively. The digestion products were separated by electrophoresis through a 3% agarose gel, and visualized by ethidium bromide staining. The PCR primers have been designed such that the 551 bp PCR product included an invariant NlaIII and DraI restriction site, which serve as internal controls for restriction enzyme activity (fig. 1). In case of a –514CC homozygote, digestion with Nla*III* results in DNA fragments of 45 and 506 bp, whereas in case of a –514TT homozygote, the 506 bp is split into a 66 and 440 bp (fig. 3). A –514C/T heterozygote is diagnosticized by the presence of both the 440 and 506 bp digestion products. Digesting of the PCR product with Dra*I* will result in DNA fragments of 111 and 440 bp in case of a –250GG homozygote (fig. 4). In case of a –250AA homozygote, the 440 bp is split into a 114 and 326 bp product, and a –250GA heterozygote is detected by the presence of both the 326 and 440 bp digestion product. In each agarose gel, a sample of undigested PCR product was electrophoresed in parallel for reference and internal digestion control.

Results and discussion

We genotyped large numbers of DNAs for the LIPC –514C/T polymorphism with both the ASO hybridisation assay and the NlaIII digestion assay. The radiographs of the dot-blots incubated with the –514C ASO probe show much stronger signals compared with those incubated with the –514T ASO probe, despite similar specific radioactivities of both probes (fig. 2). This may have been caused by differences in annealing temperature of the ASO with its complementary sequence, as a result of the shift from a CG to a TA basepair. Interpretation

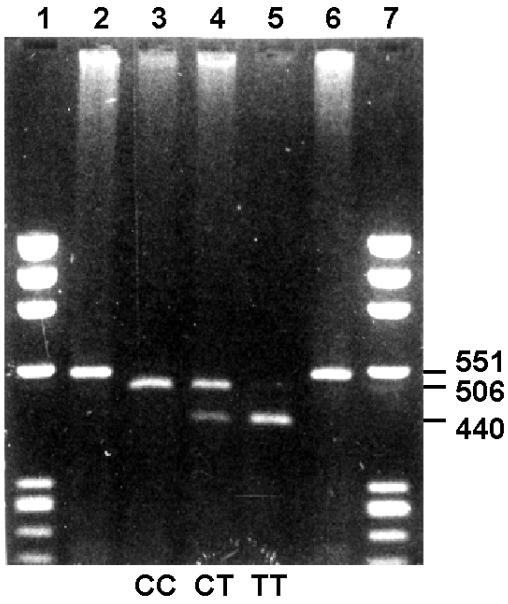

Figure 3: **The –514C/T SNP.**
After PCR and digestion with Nla*III* the DNA fragments were separated in a 3% agarose gel. Lanes 1and 7: DNA marker; lanes 2 and 6: undigested product; lane 3: homozygote CC; lane 4: heterozygote; lane 5: homozygote TT.

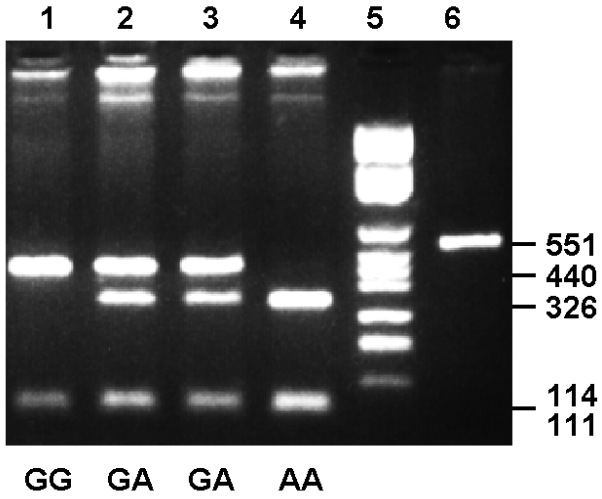

Figure 4**: The –250G/A SNP.**
After PCR and digestion with Dra*I* the DNA fragments were separated in a 3% agarose gel. Lane 1: homozygote GG; lane 2: heterozygote; lane 3: heterozygote; lane 4: homozygote AA; lane 5: marker; lane 6: undigested product.

of the results is also complicated by the observation that the –514T specific oligonucleotide hybridized to some extent to the –514C sequence, under the conditions used. Combined, it is not possible in all cases to genotype the –514SNP with absolute certainty. In cases where the ASO signals were not conclusive, we retested the DNA by PCR followed by *Nla*III digestion. This assay is robust and straightforward, but more expensive than the ASO. When large populations have to be screened the ASO method is preferred, but its success rate crucially depends on establishing the optimal hybridisation and washing conditions.

We also compared the performance of the –514C/T and –250G/A genotyping assays by digestion with *Nla*III and *Dra*I, respectively, in probands and family members of familial combined hyperlipidemia (FCH) patients (table I). Both assays use the presence of an invari-

Table I: **The LIPC –514C/T and –250G/A polymorphisms are highly linked in familial combined hyperlipidemia probands and family members.**

		Dra I (-250)			
		GG	**GA**	**AA**	**n**
Nla III (-514)	**CC**	81	4*	0	85
	CT	0	54	1*	55
	TT	0	0	12	12
n		81	58	13	152

*: from 2 different families

ant restriction site in the PCR product as an internal control for activity of the restriction enzyme. Due to this internal control, both assays give conclusive results. With *Nla*III, however, the size difference between non-digested and single-cut PCR product, and between single and double-cut PCR product, is relatively small, and correspondingly long separation times are required for clear distinction between the genotypes (fig. 3). In 147 out of 152 subjects, the –514C coincided with the –250G, and the –514T with the –250A. In only 5 subjects belonging to 2 different families, recombinants of these SNPs were observed (table I). This high degree of association reportedly extends to include the –763A/G and –710T/C polymorphisms in the proximal promoter region of the LIPC gene [7, 8]. Guerra reported for 25 subjects with hyperalphalipoproteinemia the presence of only two of the sixteen possible combinations of these four SNPs. Van't Hooft confirmed the presence of these two haplotypes for 183 of 186 healthy Swedish subjects. The haplotype –763A/-710T/-514C/-250G is now designated the *LIPC* -514C allele, whereas the haplotype –763G/-710C/-514T/-250A is designated the *LIPC* -514T allele. Apparently, these SNPs must have arisen simultaneously, and since then they must have only rarely recombined. This phenomenon is in agreement with the proposed expansion of the human population after going through a bottleneck, which may have occurred during the last 40.000-150.000 years [12, 13]. This bottleneck has either been attributed to a speciation event that led to modern man, or to a severe reduction in population size followed by a rapid expansion. The physical distance between the four SNPs is too short, and the number of generations since this bottleneck period too small, to allow for a significant number of single, let alone, double recombinations. Our data on the FCH families are in agreement with the presence of –514C/-250A recombinants in two nuclear families. Interestingly, this haplotype was introduced into these families via the spouses. From the study by Van't Hooft et al. [8], one can conclude that the –514C/T and –250G/A sites have recombined in 1-1.5 % of the European population. If one assumes an average crossover rate of 1.5 cM/Mb for chromosome 15 [11], one can calculate that it takes approximately 2500 to 3750 meioses, or 50.000 to 75.000 years, for the recombination to spread over 1 to 1.5 % of

the population, respectively. These figures are in good agreement with the hypothesis that the apparent bottleneck coincided with the immigration of modern man into Europa out of Africa [12, 13]. Alternatively, one cannot entirely exclude the possibility of evolutionary advantage of the two alleles over the other possible haplotypes.

Although rare recombinations of the four SNPs in the hepatic lipase promoter region have been detected by Van't Hooft [8] and by us, for screening purposes determination of either one of these SNPs suffices to haplotype the whole promoter region of the LIPC gene at least in Caucasian populations. Because of the exceptionally high degree of association between the four SNPs, determination of either one of the four SNPs will do the job. The –763 (A→G), –710 (T→C), –514 (C→T) and –250 (G→A) SNPs can be determined with restriction enzyme analysis using the enzymes Sph*I* (GCATG↓C), Ava*II* (G↓G(A/T)CC), Nla*III* (CATG↓), and Dra*I* (TTT↓AAA), respectively. For three SNPs the restriction site is introduced with the base substitution (*LIPC* T-allele), except for the SNP at position –710, where the Ava*II* restriction site is lost with the T→C substitution. We prefer to use the digestion with DraI. First, because the combination of –514/-250 SNPs have been shown to be functionally significant [2, 6]. And secondly, because in our hands interpretation of the DraI digestion profiles is superior to that of the NlaIII digestion profiles (fig. 4 versus 3).

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Chapter 5.2

The effect of the LIPC –514C/T polymorphism on plasma HDL levels is strongly affected by plasma triglycerides

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Abstract

The common LIPC –514C→T substitution is associated with reduced hepatic lipase activity and increased HDL cholesterol levels. In this study we tested whether the T-allele frequency differs in subjects with different HDL cholesterol levels. From a large population of healthy men, subpopulations were selected with the 10 % lowest, 10 % highest, and with median HDL cholesterol concentrations. The three groups were matched for environmental factors known to influence HDL levels. In the low HDL group 37 out of 92 subjects had plasma triglyceride levels > 2.3 mmol/L, compared to only 5 out of 57, and 0 out of 60 subjects in the median and high HDL group, respectively. In the normotriglyceridemic subjects, the T-allele frequency was 0.14, 0.17 and 0.28 in the low, median and high HDL group, respectively (χ^2= 8.444, df=2, p=0.015). Surprisingly, the allele frequency of the hypertriglyceridemic subjects within the low HDL group (n=37) is 0.27, similar to the normotriglyceridemic subjects of the high HDL group (n=60). We conclude that the effect of the LIPC –514C/T polymorphism on plasma HDL cholesterol levels is strongly affected by plasma triglyceride levels.

Introduction

Epidemiologically, a high concentration of plasma high density lipoprotein (HDL) cholesterol is associated with a reduced incidence of atherosclerotic cardiovascular diseases. The protective action of HDL has been attributed to its anti-oxydative and anti-inflammatory properties [4] as well as to its role in reverse cholesterol transport. HDL is a key factor in reverse cholesterol transport, in which peripheral cholesterol is taken up by HDL and subsequently delivered to the liver, from where the cholesterol may be excreted via the bile. A number of environmental factors influence the plasma HDL cholesterol concentration, such as alcohol consumption, diet, smoking and exercise [2]. In addition, a number of genetic factors regulate or modulate the HDL concentration, including lipoprotein lipase (LPL), cholesteryl ester transfer protein (CETP), hepatic lipase (HL), ATP-binding cassette transporter A1 (ABCA1), lecithin cholesteryl acyl transferase (LCAT) and several apolipoproteins [17].

HL is synthesized and secreted by liver parenchymal cells, and is present in liver sinusoids bound to proteoglycans. Here, it affects plasma lipoprotein metabolism. HL activity may lower HDL cholesterol concentration, by facilitating the uptake of HDL cholesterol by the liver [3, 15]. In addition, HL activity mediates the conversion of IDL to LDL [25] and facilitates the clearance of chylomicron remnants by the liver [24]. Besides, HL has been shown to affect plasma lipoprotein metabolism also independent of its catalytic activity [20]. A common polymerphism in the promoter of the HL gene is the most important genetic determinant of HL expression. In the HL promoter region four single nucleotide polymorphisms (SNPs) are present at positions –763 (A→G), -710 (T→C), -514 (C→T) and –250 (G→A) [9, 10]. These four SNPs are in almost complete linkage disequilibrium [9, 10]. Therefore, two different alleles can be distinguished, which are designated as the *LIPC* C- and T-allele according to the –514SNP. Among different populations, the frequency of the T-allele varies between 17 and 55 % [11]. Several studies have associated this T-allele with a lowered HL activity [5, 11, 19, 22] and

elevated HDL cholesterol [11-13, 19, 22]. In vitro, promoter activity of the LIPC T-allele was lower than the LIPC C-allele [6, 7]. In some but not all studies, carriers of the T-allele have a higher risk for coronary artery disease despite the higher HDL cholesterol [8, 12, 21].

In this study the association of the HL promoter polymorphism with HDL cholesterol levels is investigated in a population of healthy normocholesterolemic men. From this population, subjects were chosen that had the 10 % lowest or highest HDL cholesterol levels, or median HDL cholesterol levels.

Methods

Study population

Male subjects were selected from the Monitoring Project on Cardiovascular Disease Risk Factors study [23]. This study was a cross sectional investigation of the prevalence of risk factors for chronic diseases in a randomly selected sample of the Dutch population aged 20-59 years in three towns in the Netherlands (Amsterdam, Doetinchem, and Maastricht). Invitations to participate in the study were sent to a random sample of the population by municipal health services. A total of 17,138 male subjects were enrolled between 1987 and 1991 for questionnaires and physical examination. The questionnaires provided information about demographic variables, presence and (family) history of cardiovascular (-related) diseases, like hypertension, hypercholesterolemia and diabetes, and the presence and history of a number of other diseases. The physical examination included measurements of height, weight, waist-hip ratio, blood pressure, and lung function. Blood (non-fasting) samples were taken for determination of glucose, total and HDL cholesterol.

From this population subjects were selected and divided into three groups, on the basis of their plasma HDL cholesterol. In the low HDL group, subjects were selected from the 0-10th percentile of HDL cholesterol. In the high HDL group, subjects were selected from the 90th-100 percentile, whereas in the third group, subjects were selected from the 45-55th percentile of plasma HDL cholesterol. Subsequently, the three groups were matched with respect to age, blood pressure, exercise, alcohol intake and total plasma cholesterol. From the low, median and high HDL groups, sufficient DNA for determining the HL promoter polymorphism was available from 92, 57 and 60 subjects, respectively. The selection criteria and the matching procedure of the MORGEN study have been published before [18], and are summarized in table 1.

Hepatic lipase promoter genotyping

With polymerase chain reaction (PCR) a 551 bp fragment of the hepatic lipase gene (nt -578/- 27 according to the sequence of Ameis et al. [1]) was amplified. Amplification was performed using genomic DNA in a standard PCR reaction mixture (Eurogentec, Seraing, Belgium), with HLpr2S (5'-GGATCACCTCTCAATGGGTC-3') and HL25 (5'-GATACTTT GTTAGGGAAGACTGCC-3') as upstream and downstream primer, respectively. After an initial denaturation step (2 min at 95°C), the DNA was amplified in 34 cycles of 30 sec at 95°C, 30 sec at 45°C and 90 sec at 72°C followed by a final extension step at 72 °C for 7 min.

Table 1. Selection and matching of subjects investigated in the present study.

Total male subjects: 17,138

Exclusion criteria

- Age younger than 20 or older than 55
- Current smokers
- Subjects who drink more than one alcoholic beverage per day
- Subjects with BMI greater than 27 kg/m^2
- the presence or history of diabetes mellitus, cerebrovascular events, cancer and kidney stones
- treatment for hypertension or receiving anti-coagulants
- subjects with serum total cholesterol lower than 3.82 mmol/L (< 10th percentile) and higher than 6.50 mmol/L
- use of medications known to affect plasma lipids (e.g. diuretics, anabolic steroids, β-blockers, anti-epileptics and barbiturate derivates)

Total subjects included: 2,050

Group definition

three groups on the basis of plasma HDL cholesterol concentration:

- low HDL group: with HDL cholesterol ≤ 0.87 mmol/L (10th percentile, n=213);
- median HDL group: with HDL cholesterol between 1.09 and 1.15 mmol/L (45-55th percentile, n=250), and
- high HDL group: with HDL cholesterol ≥ 1.44 mmol/L (90th percentile, n=216).

Total subjects selected: 679

Matching procedure:

- age
- systolic and diastolic blood pressure
- percentage of inactivity in spare time
- number of alcoholic beverages per day
- plasma total cholesterol
- BMI; in order to make the groups comparable in average BMI it was necessary to make stratified samples by BMI (cut-off point 24.96 kg/m^2 which was the median BMI in the low HDL group). In each of the groups, 140 men were selected (34 above and 106 below a BMI of 24. 96 kg/m^2

Total subjects after matching: 420

HL genotyping

Number of subjects from which sufficient DNA was available

- low HDL group: 92 subjects
- median HDL group: 57 subjects
- high HDL group: 60 subjects

Subjects used in this study: 209

The four SNPs present in the proximal part of the HL promoter are in almost complete linkage disequilibrium [9, 10]. Therefore, any of the SNPs can be used to identify the presence of the LIPC C- and LIPC T-allele. Here, we chose for the –250 G/A polymorphism, which in the case of the T-allele generates a Dra*I* restriction site on top of the invariant Dra*I* site present in the same amplimer. The invariant Dra*I* site serves as an internal control for Dra*I* digestion. After PCR, the PCR mixtures were incubated overnight at 37^{o}C with 10 U Dra*I* (New

England Biolabs, Ipswich, MA, USA). The digestion products were separated by electrophoresis through a 3% agarose gel, and visualized by ethidium bromide staining.

Statistical analysis.

Data are presented as means ± S.D. Differences in allele frequency among all groups were tested for statistical significance by the χ^2 test. Differences between groups were tested for significance by Student t-test.

Results

HL promoter genotype and HDL cholesterol levels

The frequency of the T-allele in the three subpopulations is given in figure 1. The T-allele frequency is similar in the low and median HDL groups (0.190 and 0.184, respectively). In the high HDL group, the T-allele frequency is 0.283. The difference in allele frequency among the three study groups was not statistically significant (χ^2= 4.624, df=2, p=0. 10).
Within the high HDL group (fig. 2), subjects who are homozygote for the T-allele have a significantly higher HDL cholesterol level (1.83 ± 0.22 mmol/L; n = 5) than heterozygote subjects (1.57 ± 0.12 mmol/L, n = 24; p=0.01), and also a significantly higher HDL cholesterol level than homozygotes for the C-allele (1.59 ± 0.14 mmol/L, n = 31; p=0.003). Within the low and the median HDL group no significant differences were observed between the three HL genotypes.

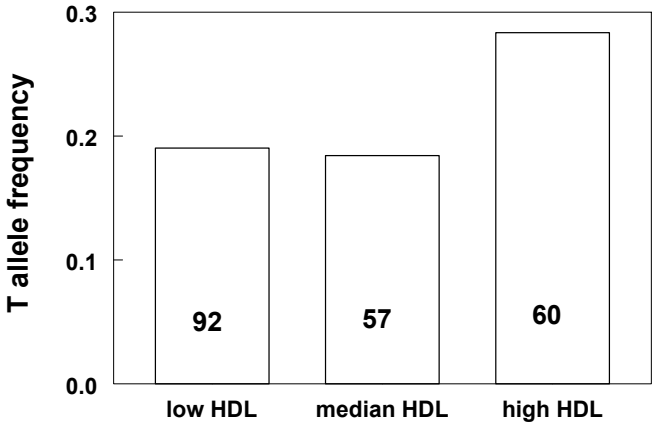

Figure 1: LIPC T allele frequencies in the three investigated HDL populations. Numbers indicate total number of subjects in each group.

HL promoter genotype and plasma triglyceride levels

The subjects had been selected and assigned to the three HDL groups on the basis of their plasma HDL cholesterol, and the groups were subsequently matched for total cholesterol. Table 2 shows that the plasma triglyceride concentration is inversely correlated with plasma HDL cholesterol between our study groups. In the low HDL group, 37 out of 92

Table 2 Lipid characteristics per HDL group.

	n	HDL cholesterol	Total cholesterol	Triglycerides
Low HDL group	92	0.79 ± 0.07	5.02 ± 0.72	2.41 ± 1.32
Median HDL group	57	1.12 ± 0.02[a]	5.09 ± 0.72	1.40 ± 0.55[a]
High HDL group	60	1.60 ± 0.16[a,b]	5.05 ± 0.62	1.01 ± 0.44[a,b]

Data (mmol/L) are mean ± SD. [a] and [b]: significantly different from low and median HDL group, respectively ($p<0.0001$).

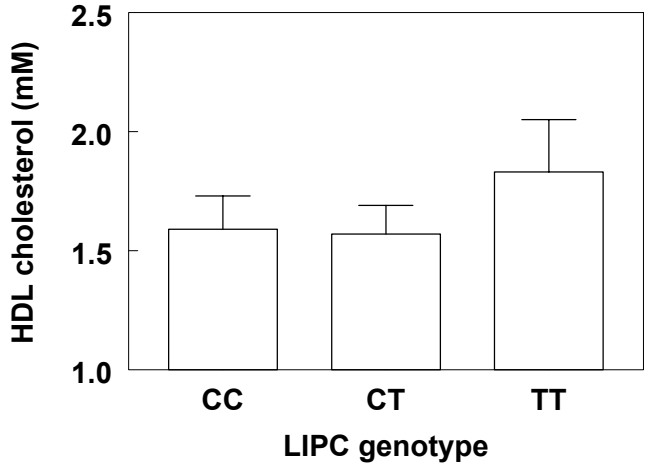

Figure 2: Relationship between HDL cholesterol and HL genotype in the high HDL group. HDL concentration in TT homozygotes differs significantly from heterozygotes,and from CC homozygotes (p=0.01 and p=0.003, respectively).

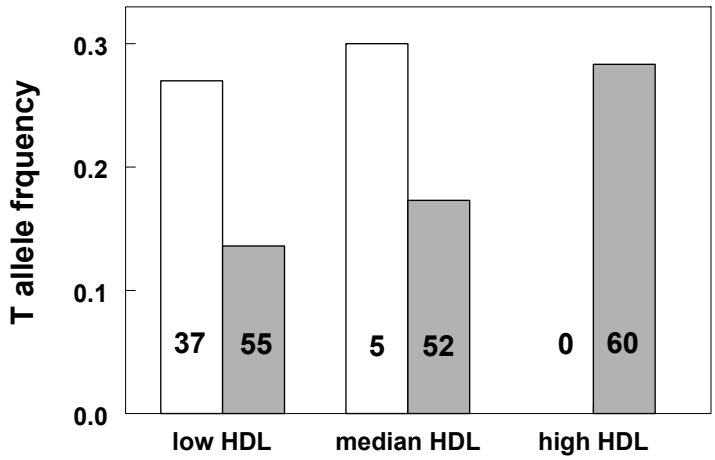

Figure 3: *LIPC* T allele frequencies in three HDL groups depend on triglyceride levels. Subjects within each group were subdivided in hypertriglyceridemic (plasma TG > 2.3 mmol/L) and normotriglyceridemics (filled and open bars, respectively). Numbers indicate total number of subjects in each (sub)group.

subjects (40 %) can be considered hypertriglyceridemic (plasma triglyceride > 2.3 mmol/L). In the median group this holds for only 5 out of 57 subjects (9 %), whereas in the high HDL group, none of the 60 subjects were hypertriglyceridemic. When the hypertriglyceridemic subjects were excluded, the mean plasma triglyceride levels in the low and medium HDL

groups were 1.61 ± 0.41 mmol/L (n = 55) and 1.30 ± 0.44 mmol/L (n=52), respectively. In these normotriglyceridemic subjects, the difference in plasma triglyceride levels between the low, median and high HDL groups still was highly significant ($p < 0.001$ for each comparison), and the trend towards higher plasma triglyceride levels with lower HDL levels remained. The *LIPC*-allele frequency was then recalculated for the three HDL groups, considering only the normotriglyceridemic subjects (fig. 3). The frequency of the T-allele was 0.14, 0.17 and 0.28 for the low, median and high HDL group, respectively. Statistically the difference between the T-allele frequency in the three HDL groups is significant (χ^2= 8.444, df=2, p=0.015). Within the low HDL group, the T-allele frequency of the normo- and hypertriglyceridemic subjects was 0.14 (n=55) and 0.27 (n=37), respectively (χ^2= 5.150, df=1, p= 0.023). Within the median HDL group, the allele frequencies were 0.17 (n=52) and 0.30 (n=5), respectively (χ^2= 0.978, df=1, p= 0.32). Hence, the T-allele frequency among hypertriglyceridemic subjects with low or median HDL levels appears to be similar to that in normotriglyceridemic subjects with high HDL levels, and approximately twice that in normotriglyceridemic subjects with low or median HDL levels.

Discussion

In a recent meta-analysis of 25 publications, Isaacs et al [11] present strong evidence for the argument that the *LIPC* T-allele is associated with a gene-dose dependent elevation of HDL cholesterol. In line with this, we show here for the high HDL group, that subjects who are homozygote for the T-allele have a significantly higher HDL cholesterol than heterozygotes and non-carriers (fig. 2). More importantly, the present study shows that this argument also holds the other way around: the frequency of the *LIPC* T-allele was considerably higher in the high HDL group than in both the low and median HDL group. This effect became highly significant when hypertriglyceridemic subjects were excluded from the analysis. Among the three selected HDL groups, which had been matched for total cholesterol, there is a strong positive correlation between HDL concentration and the frequency of the T-allele. The association of the HL promoter polymorphism with HDL cholesterol levels can be explained by its effect on HL activity. As summarized in the meta-analysis of Isaacs et al [11], the T-allele lowers post-heparin HL activity gene-dose dependently. The lower HL activity is most likely due to reduced HL transcription, since we and others have shown in vitro that transcriptional activity of the T-allele is approximately 40 % lower than the C-allele [6, 7]. HL plays a key role in reverse cholesterol transport, by facilitating uptake of HDL cholesterol by the liver. When HL activity is low, HDL cholesterol uptake by the liver will decrease and HDL cholesterol level will increase.

Unexpectedly, we found that the T-allele frequency is strongly increased in the hypertriglyceridemic subjects within the low HDL group. The T-allele frequency in these subjects is almost twice as high as the normotriglyceridic subjects within the low HDL group, and similar to the high HDL group. The T-allele frequency in the high triglyceride/low HDL cholesterol subjects is even higher than in a comparable healthy Caucasian population (0.27 versus 0.19 [12]).

Plasma triglyceride concentration was not used as a parameter in the matching procedure in the Morgen study. As shown in table 2, the average triglyceride concentration inversely correlated with average HDL cholesterol among the three HDL groups. In our study 42 out of 209 selected subjects presented with a plasma triglyceride > 2.3 mmol/L, and should thus be considered hypertriglyceridemic. By far the most hypertriglyceridemic subjects belong to the low HDL group, whereas none is present in the high HDL group. It should be stressed, that plasma triglyceride levels were measured in non-fasting blood. It is unlikely, however, that the elevated triglyceride levels are due to the non-fasting state per sé, because of the unequal distribution of hypertriglyceridemics among the three HDL groups, and because of the markedly increased T-allele frequency of the hypertriglyceridemics compared to the normotriglyceridemics in the low HDL group. Rather, other possible explanations should be considered. First, the low HL activity associated with the T-allele may result in an elevation of plasma triglycerides. Indeed, HL has been implicated in the hydrolysis of triglycerides in IDL, LDL and HDL, and in the clearance of chylomicron remnants [14]. Reduced lipolysis may be associated with reduced delivery of surface fragments to HDL, and hence to a lowering of HDL cholesterol. The meta-analysis of Isaacs et al [11], failed to show a significant effect of the T-allele on plasma triglyceride levels, but the publications used for this meta-analysis mostly involved normotriglyceridemic subjects. In the hypertriglyceridemic subjects from the low HDL group, the T-allele frequency is almost twice that in the normotriglyceridemic subjects from this group. An increased T-allele frequency is generally associated with lower HL activity and hence with high HDL [11], as is also shown in this study. It is not obvious why this HDL raising effect is reversed by the reduced lipolysis in part of the low-HDL subjects.

A second explanation for the enrichment of hypertriglyceridemia in the low HDL group may be that the elevation of plasma triglycerides increase CETP activity and hence transfer of cholesterol from HDL to triglyceride-rich lipoproteins [16]. Moreover, Kuivenhoven demonstrated for the same three HDL groups from the Morgen study that the CETP concentration in subjects of the low HDL group was significantly higher than in the median and high HDL groups [18]. This was explained by enrichment of CETP alleles that associate with increased CETP expression, and hence in lower HDL levels, in the low HDL group [18]. It is quite feasible, that the HDL raising effect of the *LIPC* T-allele is balanced, and even reversed, by the HDL lowering effect of increased CETP concentration and elevated acceptor lipoprotein levels in the hypertriglyceridemic subjects.

Taken together, we have shown here that the elevated HDL concentration is associated with an increased frequency of the *LIPC* T-allele in normotriglyceridemic, but not in hypertriglyceridemic subjects. This arm of the Morgen study was not designed to study the effect of genetic factors, such as the HL promoter polymorphism, on plasma triglyceride levels. Our data warrant further studies to delineate the effect of the HL promoter polymorphism in subjects selected on the basis of plasma triglyceride levels.

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Chapter 5.3

High HDL cholesterol associated with combined cholesteryl ester transfer protein and hepatic lipase gene variants does not protect against coronary artery disease.

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Abstract

Cholesteryl ester transfer protein (CETP) and hepatic lipase (HL) are two HDL modifying proteins that have both pro- and anti-atherogenic properties. We hypothesized that both proteins interact at the level of HDL cholesterol and atherosclerotic risk. To test this, we compared the distribution of the common HL promoter (*LIPC*-514C/T) and *CETP* Taq1B B1/B2 polymorphisms in male coronary artery disease patients (CAD; n=792) and non symptommatic controls (n=539). Cases and controls had similar allele frequencies, but the distribution of the combined genotypes differed (p=0.034). In CAD patients, 7.9 % of the *CETP*-B2B2 homozygotes had the *LIPC*-TT genotype, contrasting with only 1.2 % in controls (p=0.032). The HL lowering LIPC-T allele and the CETP lowering B2 allele synergistically increased HDL cholesterol from 0.86±0.19 mmol/L in the B1B1-CC (n=183) to 1.21 ± 0.25 mmol/L in the B2B2-CC patients (n=10). Compared to the B1B1-CC subjects, carriers of either one LIPC-T allele or one CETP-B2 allele had a lower CAD risk (OR 0.70, CI 95% 0.51-0.94; p=0.017). In contrast, the B2B2-TT subjects tended to have a 3-fold higher risk (OR 3.13, CI 95 % 0.88-11.2; p=0.079). In a two-year follow up, the loss of coronary lumen diameter in these patients was higher than in all other patients combined (0.34±0.70 versus 0.10±0.29 mm, p=0.044). We conclude that combined homozygosity for the CETP and HL lowering gene variants present with accelerated coronary atherosclerosis, despite the relatively high HDL cholesterol. Determination of the *CETP* and *LIPC* genotype is warranted to ascertain coronary risk and to design the optimal drug therapy in individual patients.

Introduction

Epidemiological studies have demonstrated an inverse relationship between the concentration of high density lipoprotein (HDL) cholesterol in plasma and the risk of coronary artery disease (CAD) [1, 2]. HDL exerts multiple antiatherogenic actions, due to its antiinflammatory, antioxidative, antithrombotic and direct vascular effects [3, 4], besides its role in the reverse cholesterol transport (RCT) pathway [5, 6]. The variation of plasma HDL cholesterol levels is determined by multiple environmental and genetic influences [7, 8]. Cholesteryl ester transfer protein (*CETP*) and hepatic lipase (*LIPC*) are two genes with common alleles that have been identified to determine HDL cholesterol levels [9, 10]. Both CETP and hepatic lipase (HL) proteins play crucial roles in the RCT pathway. After initial uptake of free cholesterol by HDL and subsequent esterification, the HDL cholesteryl esters can then be delivered to the liver by different pathways. One is an indirect route whereby cholesteryl ester is transferred by CETP from HDL to very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL), before being taken up by the liver [5, 6]. The other is a direct route in which HL and SR-B1 are involved [5, 6]. The total capacity of the RCT pathway to deliver cholesterol to the liver is the sum of these processes. A low CETP activity as well as a low HL activity has been associated with elevated HDL cholesterol levels [9, 10]. A high HDL level may reflect a high cholesterol uptake capacity and thus an efficient RCT. However, RCT may be compromised if a high HDL cholesterol results from impaired clearance.

Although low CETP and low HL are associated with elevated HDL levels, their protective role in atherosclerosis is disputed [11, 12]. Rare HL deficiency is associated with increased risk for CAD. HL activity is associated with the common *LIPC* promoter polymorphism. Carriers of the T-allele exhibit lower HL activity and elevated HDL [9, 13, 14], and were reported to have a slightly increased risk for CAD (reviewed by Jansen et al.[12]). However, the C-allele is associated with an atherogenic lipoprotein pattern characterized by the presence of small dense LDL and low HDL cholesterol, and lowering of HL activity by hypolipidemic treatment resulted in a reduced coronary stenosis in CAD patients, suggesting that high HL activity is proatherogenic[15]. The C-allele has recently been shown to be associated with increased atherosclerosis in the carotid arteries and prevalence of cerebrovascular events [16]. CETP concentration is associated with the common Taq1B polymorphism in intron-1 of the *CETP* gene, which is thought to result from its strong linkage disequilibrium with the C-629A polymorphism[17]. Carriers of the B2 allele exhibit lower plasma CETP levels and higher HDL cholesterol concentrations. However, in some but not all studies (reviewed by De Grooth et al.[11]), the B2 allele is associated with a reduced risk for coronary heart disease. CETP deficiency in Japanese-American men was associated with an increased prevalence of CAD despite modestly increased HDL levels [18]. In Japanese subjects with marked hyperalphalipoproteinemia, atherosclerotic disease was observed in 6% of the patients [19]. Interestingly, this subgroup of patients was characterized by a combined, severe reduction in both CETP and HL activities. Thus, strong evidence is provided that CETP deficiency, in particular when it occurs in combination with HL deficiency, can be proatherogenic despite modestly high HDL cholesterol. Nevertheless, inhibitors of CETP are currently investigated as potential atheroprotective drugs by virtue of their HDL raising effect [20].

From the literature the picture emerges that both CETP and HL can be either pro- or anti-atherogenic, depending on other metabolic or genetic factors [8, 11, 12, 15]. One possibility is that the effect of CETP on HDL cholesterol levels and atherosclerotic risk is affected by HL, and vice versa. To test this, we compared the prevalence, and clinical outcome, of the combined *LIPC* promoter and *CETP* TaqIB polymorphism in two groups of subjects: *(1)* a cohort of male patients with angiographically documented coronary atherosclerosis (the Regression Growth Evaluation Statin Study, REGRESS) and *(2)* a group of nonsymptomatic population controls.

Methods

Study population

Coronary artery disease (CAD) patients were participants of the Regression Growth Evaluation Statin Study (REGRESS). REGRESS, described in detail elsewhere, was designed as a randomized, placebo-controlled, multicenter study to assess the effect of 2 years of pravastatin treatment on the progression and regression of coronary atherosclerosis [21]. Included were 885 Caucasian men with angiographically documented CAD (>50% stenosis of at least 1 major vessel). All patients were <70 years of age, had total cholesterol levels between 4 and 8 mmol/L, and had triglyceride levels <4 mmol/L. The patients were randomized to receive pravastatin 40 mg once daily or matching placebo. REGRESS was

conducted under the auspices of the Interuniversity Cardiology Institute of The Netherlands. The study was approved by the Institutional Review Boards of each of the participating centers. Written informed consent was obtained from each patient. To assess the frequency of combined variants in *CETP* and *LIPC* in non-CAD subjects, normolipidemic healthy males with Dutch nationality were randomly selected from the population-based Monitoring Project on Cardiovascular Disease Risk Factors [22]. None of the control subjects had a history of major illness (i.e. cerebrovascular disease, myocardial infarction, coronary bypass or other heart surgery, diabetes mellitus or cancer), were taking any medication for hypertension or hypercholesterolemia, were using anticoagulants or were consuming alcohol in excess of three measures per day. Total cholesterol levels were required to be between 4 and 8 mmol/L, whereas the body mass index was required to be <33 kg/m^2. The control group was matched for age to the CAD group.

Genotyping

DNA was isolated from buffy coats according to standard procedures. The polymerase-chain-reaction (PCR)-based method of screening for the TaqIB polymorphism in intron 1 of the *CETP* gene gives rise to two alleles designated B1 and B2 [10]. Genotyping of the *LIPC* –514C>T promoter variant by allele specific oligohybridisation has been described before.[13] In cases of doubt, samples were reanalysed by *Nla*III digestion [13]. In addition, we determined the *LIPC* –250G>A variant by *Dra*I digestion [9]. Both SNPs were completely linked. Throughout, the –514C/-250G and –514T/-250A haplotypes are designated as the *LIPC*-C and *LIPC*-T allele, respectively.

Quantitative Coronary Angiography in CAD patients

In CAD patients, computer-assisted quantitative coronary angiography was carried out at the start and the end of the study, as described in detail elsewhere [21]. Primary end points were (a) the change in average minimal obstruction diameter (MOD) per patient and (b) the change in average mean segment diameter (MSD) per patient. Change in MOD mainly reflects focal progression-regression of atherosclerosis, and change in MSD mainly reflects diffuse progression-regression of atherosclerosis.

Other methods

All standard lipid laboratory tests, measured in CAD patients, were carried out at the Lipid Reference Laboratory, as published previously [21]. HL activities and CETP concentrations were determined as described in detail before [10, 13].

Data analysis

Cases and controls were classified into genotypic groups according to the presence or absence of the Taq1B restriction site in the *CETP* gene (B1 or B2 allele, respectively) and according to the *LIPC* variant. Homozygosity for the common alleles *CETP*-B1 and *LIPC*-C was coded as "*B1B1*" and "CC", respectively; homozygosity for the rare alleles *CETP*-B2 and *LIPC*-T was coded as "*B2B2*" and "*TT*", respectively; heterozygosity was coded as "*B1B2*" and "*CT*". Association of the *CETP* and *LIPC* gene variants was analyzed by Fisher's exact test. A population differentiation test was used to analyse whether the combined genotypes

were distributed equally between cases and controls. Genotypic effects on biochemical parameters were tested by ANOVA. A two-tailed p value of <0.05 was considered to indicate a statistically significant difference.

Results

Frequency of the CETP and LIPC variants

The *LIPC* and *CETP*-TaqIB genotypes were determined in 792 participants of REGRESS and in 539 nonsymptomatic controls (table 1). There was no significant difference in the frequency of the *CETP*-B2 allele (0.406 versus 0.419) or the *LIPC*-T allele (0.217 versus 0.221) between CAD patients and controls. In both groups the gene variants were in Hardy-Weinberg equilibrium. As published before, the B2 allele associated with lower CETP concentrations in plasma ($p<0.001$) [10]. The *LIPC*-T allele lowered HL activity in the REGRESS population ($p<0.01$) [13].

Table 1: **Variants in the cholesteryl ester transfer protein (*CETP*) gene and the hepatic lipase (*LIPC*) gene in CAD patients and controls**

	CETP Taq1B genotype					
	CAD patients			Controls		
LIPC genotype	B1B1	B1B2	B2B2	B1B1	B1B2	B2B2
CC	183	228	75	97	169	59
CT	79	149	41	65	101	24
TT	13	14	10	9	14	1

Data are number of individuals.

Association between CETP and LIPC genotypes

In the cases but not in the controls, the observed distribution of the combined *CETP* and *LIPC* gene variants was significantly different from the expected distribution had the variants occurred independently (table 1: cases, p=0.040; controls, p=0.23). The extent of this *CETP*- x *LIPC*-gene interaction differed significantly between cases and controls (p=0.019; derived from the interaction test between case/control group and the *LIPC* genotype in multinomial logistic regression of *CETP* genotype). As shown in figure 1, the proportion of B1B1 subjects carrying the *LIPC*-T allele was significantly lower in CAD patients than in controls (p=0.026). In the B2B2 subjects, the proportion of T allele carriers tended to be higher in the CAD patients than in the controls (p=0.11). In CAD patients, 7.9 % of the *CETP*-B2B2 homozygotes had the *LIPC*-TT genotype, contrasting with only 1.2 % in controls (χ^2=4.62, df=1, p=0.032). Since the *CETP* and *LIPC* gene are located on different chromosomes, the shortage of B1B1-TT subjects, and the enrichment in B2B2-TT subjects in the diseased cohort likely results from a CAD selection bias.

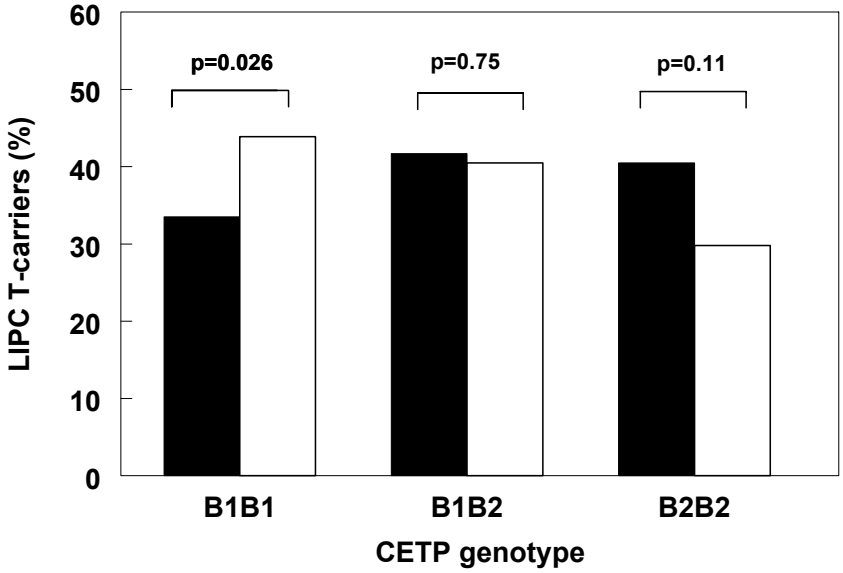
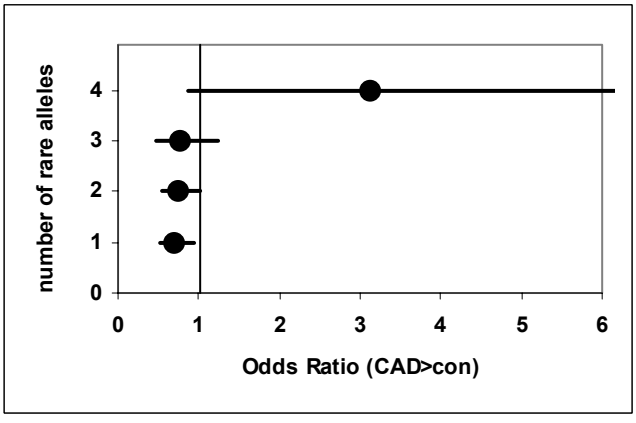


Figure 1: **Proportion of CAD patients and controls carrying the *LIPC*-T allele specified for each *CETP* genotype**. CAD patients: closed bars; nonsymptomatic controls: open bars.

Combined effect of CETP and LIPC genotype on CAD risk

The *CETP*-B2 allele and the *LIPC*-T allele each are associated with increased plasma HDL cholesterol. To test the interaction between both genes in determining the risk for CAD, we categorized the genotypes according to the number of these rare alleles. The distribution of genotypes in the CAD population was again significantly different from controls (χ^2=10.41, df=4, p=0.034). Compared to the double homozygotes for the common *CETP* and *LIPC* allele, the number of subjects with one rare allele was significantly lower in the CAD than in the control group (OR 0.70, CI 95% 0.51-0.94; p=0.017; fig. 2). This effect was independent of which rare allele was selected (one B2 allele: OR 0.64, CI 95 % 0.43-0.97, p=0.035; one T-allele: OR 0.75, CI 95 % 0.52-0.98, p=0.038). The number of subjects with two rare alleles tended to be lower in the CAD than in the control group (OR 0.74; CI 95 % 0.54-1.02; p=0.065), which was mainly attributable to the B2B2-CC subjects (OR 0.67; CI 95 % 0.44-1.03, p=0.065). In contrast, the B2B2-TT subjects having four rare alleles tended to be enriched in the CAD group (OR 3.13, CI 95 % 0.88-11.2; p=0.079), although this did not reach statistical significance in this test due to the low number. Taken together, these data suggest that subjects with one or two rare alleles have lower CAD risk, and that B2B2-TT subjects tend to have 3-fold higher CAD risk, than B1B1-CC subjects.

Figure 2: **Comparison of the distribution of *LIPC* and *CETP* genotypes in cases versus controls.** Cases and controls were categorized according to the number of the rare *LIPC*-T plus *CETP*-B2 alleles. Data represent odds ratios and 95 % confidence intervals for the comparison.



Effect of CETP and LIPC variants on plasma lipids in CAD patients

Against a background of the *LIPC*-CC homozygotes, the B2 allele increased the concentration of HDL cholesterol from 0.86±0.19 mmol/L to 0.95±0.24 mmol/L ($p<0.001$). Similarly, against a background of the *CETP*-B1B1 homozygotes, the T allele increased HDL cholesterol concentration from 0.86±0.19 mmol/L to 0.93±0.24 mmol/L ($p<0.001$). The effect of both variants was synergistic. This resulted in the highest HDL cholesterol concentrations in double homozygotes for the T and B2 alleles (fig. 3). This level was almost 40% higher than in the B1B1-CC patients ($p<0.001$), and almost 30% higher ($p<0.001$) than the average HDL cholesterol concentration in the single and double heterozygotes. When categorized according to the number of rare *CETP* and *LIPC* alleles, the concentration of HDL cholesterol was significantly increased in patients with three or four rare alleles compared to the B1B1-CC patients (fig. 4). In contrast to HDL cholesterol, total cholesterol and LDL cholesterol did not vary with the number of rare alleles, whereas plasma triglyceride levels slightly, but non-significantly, decreased with the number of rare alleles.

Compared to the other genotypes combined, the B2B2-TT patients had the highest plasma HDL cholesterol levels (table 2). Total cholesterol, LDL cholesterol and triglyceride concentrations were not different in patients with the B2B2-TT genotype compared to the other genotypes.

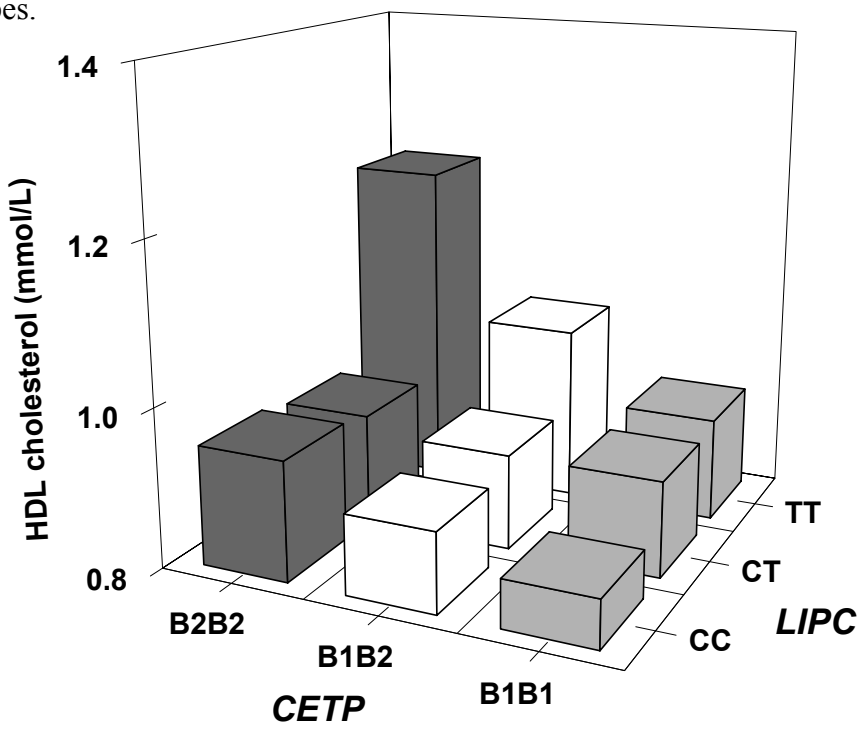


Figure 3: **HDL cholesterol concentration in 792 CAD patients, categorized according to their *CETP* and *LIPC* genotype**.

Effect of CETP and LIPC variants on MSD and MOD in CAD patients

At baseline, the angiographic parameters MSD and MOD did not differ between the various combined genotypes (table 3). During the two-year follow-up, the luminal narrowing was more severe in patients with the B2B2-TT genotype than in patients with the other genotypes combined. As shown in table 3, MOD loss was significantly higher in B2B2-TT cases, indicating increased progression of atherosclerosis in these patients compared to the

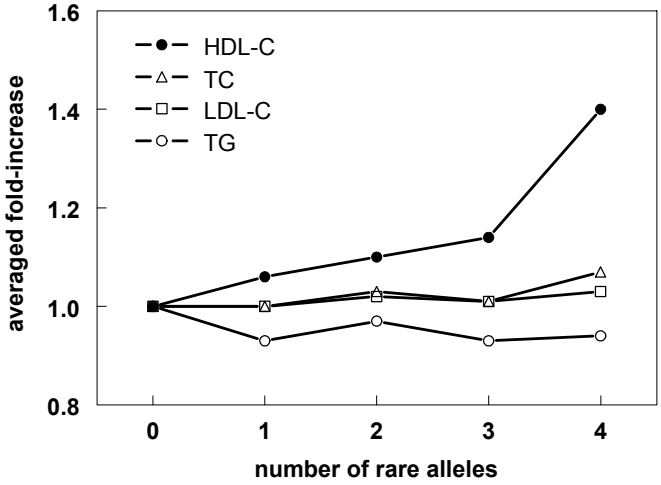


Figure 4: **Plasma lipids in 792 CAD patients, categorized according to the number of the rare *LIPC*-T plus *CETP*-B2 alleles.**
Results are expressed as averaged fold-increase compared to the B1B1-CC cases. HDL-C = high density lipoprotein cholesterol; TC = total cholesterol; LDL-C = low density lipoprotein cholesterol; TG = triglycerides; P-values: P<0.001 (HDL-C), P=0.09 (TC), P=0.60 (LDL-C), P=0.34 (TG).

other genotypes combined. The loss in MSD during follow-up was also higher in the B2B2-TT cases, but this difference did not reach statistical significance. Although patients receiving pravastatin and placebo treatment were combined in this follow-up analysis, the number of subjects with either treatment was equally distributed among the groups (B2B2-TT: n=5 versus n=5; other genotype combinations: n=400 versus n=382) and therefore could not have influenced the results.

Table 2: **Effect of combined homozygosity for the *CETP*-B2 allele and the *LIPC*-T allele on plasma lipids in 792 CAD patients.**

	CETP-LIPC Genotype		P-value
	B2B2-TT (n=10)	Other (n=782)	
HDL cholesterol (mmol/L)	1.21 ± 0.25	0.92 ± 0.21	<0.001
Total cholesterol (mmol/L)	6.42 ± 1.08	6.03 ± 0.86	0.16
LDL cholesterol (mmol/L)	4.43 ± 0.85	4.31 ± 0.79	0.64
Triglycerides (mmol/L)	1.76 ± 0.95	1.79 ± 0.76	0.89

Table 3: **Effect of combined homozygosity for the *CETP*-B2 allele and the *LIPC*-T allele on progression of atherosclerosis in 792 CAD patients.**

	CETP-LIPC Genotype		P-value
	B2B2-TT (n=10)	Other (n=782)	
MSD (mm)			
- baseline	2.81±0.46	2.73±0.37	0.62
- loss during 2-year follow-up	0.15±0.14	0.09±0.20	0.44
MOD (mm)			
- baseline	1.81±0.37	1.76±0.36	0.70
- loss during 2-year follow-up	0.34±0.70	0.10±0.29	0.044

MSD = mean segment diameter, MOD = minimal obstruction diameter

Discussion

This study was undertaken to test whether CETP and HL interact at the level of plasma HDL cholesterol and CAD risk, by comparing the distribution of the common SNPs in the *CETP* and *LIPC* gene among CAD patients and nonsymptomatic controls. The frequency of the *CETP* and *LIPC* genotypes were similar in cases and controls, indicating that these alleles per se do not significantly affect the risk of CAD. The combined *CETP* and *LIPC* genotypes, however, were differently distributed between CAD patients and controls. In the cohort of CAD patients, the rare *CETP*-B2 and *LIPC*-T alleles each increase HDL cholesterol by approximately 10 %, but in combination HDL cholesterol is increased by 40 %. Despite this high HDL cholesterol in the B2B2-TT double homozygotes, they are enriched in the CAD group, indicating that compared to the B1B1-CC homozygotes, they had a 3-fold higher CAD risk. This finding is consistent with the proatherogenic role of combined CETP and HL deficiency observed in the Japanese population [19]. Our data also clearly show that subjects with only one rare allele, either *CETP*-B2 or the *LIPC*-T, have a markedly reduced CAD risk compared to the B1B1-CC homozygotes. Based on the present study, we conclude that CETP and HL lowering gene variants mutually affect each other's atherogenic potential. Subjects with either one rare *LIPC* or *CETP* allele (approximately 12 % and 31 % of the Caucasian population, respectively) have a lower CAD risk, but B2B2-TT subjects (approximately 0.5 % of the Caucasian population) tend to have 3-fold higher CAD risk than B1B1-CC subjects (approximately 25 % of the Caucasian population), despite having the highest plasma HDL cholesterol.

Among the *CETP*/*LIPC* genotypes, the B1B1-CC subjects have the highest plasma CETP concentration [10, 17] and the highest HL activity [13, 14]. A high CETP and HL activity may be antiatherogenic by virtue of their ability to increase the rate of RCT [5, 6, 20]. However, both CETP and HL decrease the concentration of HDL cholesterol [7, 9, 10, 13, 14], indicating that they may also be proatherogenic. Moreover, CETP and HL are involved in the generation of atherogenic small-dense LDL [20, 23, 24]. Our finding that the CAD risk is markedly reduced in subjects with one rare *CETP* or *LIPC* allele, which results in lower CETP or HL activity, respectively, is in accordance with a predominantly pro-atherogenic role for CETP and HL. In the context of reverse cholesterol transport, this may indicate that when CETP or HL is decreased as a result of genetic variation, the return of cholesterol to the liver may be maintained provided the activity of HL and CETP, respectively, is not reduced. Average HDL and LDL cholesterol levels were not significantly different between groups of patients with 0 or 1 of the rare alleles, so the reduced CAD risk may haven been due to qualitative changes in HDL and LDL, such as reduced presence of small dense LDL or altered HDL_2/HDL_3 ratio [25].

The most striking result of this study is the 3-fold higher CAD risk for the B2B2-TT double homozygotes, despite their high HDL cholesterol levels. In addition, these CAD patients showed more progression of focal atherosclerosis, as reflected in MOD loss, than patients with other genotype combinations. Apparently, the antiatherogenic potential of the elevated HDL levels, and the presumed reduction in atherogenic small dense LDL, in these subjects are insufficient to protect against atherogenesis. We propose that the elevated HDL levels in these subjects are the result of a less efficient delivery of HDL cholesterol to the liver. In these double homozygous subjects, both CETP concentration and HL activity are

lowered, thereby reducing both the direct and the indirect pathway of RCT. Among nonsymptomatic controls homozygous for the *CETP*-B2 allele, the *LIPC*-C allele is enriched. In this situation the direct pathway of RCT compensates for the reduced return of cholesterol via the indirect route. Our findings are in agreement with the results of Hirano and colleagues, showing atherosclerotic cardiovascular disease in subjects with a concomitant reduction in CETP and HL, and not in subjects with a sole CETP or HL deficiency [19]. It is generally accepted that impairment of the reverse cholesterol transport system affects the potential of HDL as an antiatherogenic vehicle. Support for this hypothesis comes from transgenic mice models, demonstrating that experimental atherosclerosis can be reduced by stimulating hepatic cholesterol delivery [26]. In the present study, impaired reverse cholesterol transport may explain why subjects with the combined genotype B2B2-TT had highest HDL levels and why this combination of genotypes was observed more frequently than expected in CAD patients.

In epidemiological studies increased HDL cholesterol concentrations are protective against the development of atherosclerotic disease. In the individual patient this rule seems not always applicable. The functionality of the HDL particles may be an important determinant of its anti-atherogenic effect [6]. As outlined by von Eckardstein and colleagues [4, 27, 28], the underlying reasons for the increased HDL are important in determining whether the increase is protective, or in fact an indicator of proatherogenic risk. Our data also suggests that therapeutic interventions of coronary artery disease aimed at raising HDL cholesterol levels by CETP inhibition [20, 29] may not be effective, or may even be unfavorable, for patients who are homozygote for the *LIPC*-T allele. This warns against the use of CETP inhibitors for approximately 4 % of patients of Caucasian descent, but to a much larger part of the populations with a much higher T-allele frequency such as the Afro-Americans [30] and Japanese [31]. The development and application of rapid and simple molecular biology techniques to identify and characterize genetic variants and their significance enables the evaluation of the significance of increased HDL for the individual patient. In addition, genotyping for *LIPC* and *CETP* may help select candidates for treatment with HDL raising therapies, such as CETP inhibitors.

Acknowledgments

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Chapter 6.0

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

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Abstract

The common −216G→A and −480C→T substitutions in the promoter region of the human hepatic lipase (*LIPC*) gene show high allelic association, and are correlated with decreased hepatic lipase activity and increased high-density lipoprotein cholesterol levels. To test the functionality of these substitutions, CAT-reporter assays were performed in HepG2 cells. *LIPC* (−650/+48) but not (−650/+61) promoter constructs showed transcriptional activity. *LIPC* (−650/+48) constructs with both −216A and −480T exhibited significantly lower promoter activity (−45%) than the wild-type form. Activities of −289/+48 constructs were not significantly affected by the −216G→A substitution. The −480C/T site lies within a binding region for Upstream Stimulatory Factor (USF). Gel-shift assays showed that the binding affinity of USF protein for HL specific oligonucleotides was decreased four-fold by the −480C→T substitution. However, promoter activity of the −650/+48 constructs was not significantly affected by the −480C→T substitution alone. Co-transfection of HepG2 cells with USF[43] cDNA yielded a similar dose-dependent increase in activity of all −650/+48 constructs; the absolute difference in promoter activity increased but the relative difference between the variant promoter forms was maintained. Our studies demonstrate that the common *LIPC* promoter variation is functional, which explains the association of the −480T allele with a lower hepatic lipase activity in man.

Introduction

Human hepatic lipase (HL; triacylglycerol lipase, EC 3.1.1.3) is an extracellular liver enzyme with phospholipase A_1 and triacylglycerol hydrolase activity that plays an important role in plasma lipid transport [1]. HL is involved in the remodeling of high-density lipoproteins (HDL), thereby facilitating cholesterol transport from peripheral tissues to the liver [2-5]. HL also accelerates liver uptake of chylomicron remnants, either by changing the exposure of apoE on these particles and increasing their binding to apoE-recognizing receptors [6], or by acting as a ligand protein for chylomicron remnants [7, 8]. Studies in humans and genetically modified animals have shown that HL deficiency is generally associated with elevated HDL cholesterol levels, increased triglyceride levels in HDL and low density lipoprotein (LDL), presence of β-VLDL, and impaired metabolism of post-prandial triglyceride-rich lipoproteins [9-13], which are all considered to be risk factors for premature atherosclerosis. Indeed, coronary artery disease has been reported for subjects with HL deficiency [12, 13].

Mutations in the coding part of the human hepatic lipase gene (*LIPC*) that lead to a decreased expression of HL activity in post-heparin plasma are rare. We recently identified a common C→T substitution in the promoter region of the *LIPC* gene at nucleotide −480 [14, 15], which has also been described as the −514C→T by Guerra et al.[16]. This base substitution is associated with a 15–50% lowering of post-heparin HL activity [15, 17-19] and increased levels of HDL cholesterol and apoAI [15, 16, 19-21] or HDL and LDL triglycerides [18]. Moreover, it is associated with increased levels of remnant particles in plasma [21]. Interestingly, post-heparin plasma HL activity increased with fasting insulin levels in −480CC homozygotes but not in carriers of the −480T, suggesting that the base substitution may interfere with insulin

responsiveness [15]. The −480C→T substitution is in strong linkage disequilibrium with three other base substitutions in the 5′-regulatory region of the *LIPC* gene, one more proximal and two more distal; they are therefore collectively designated as the −480C and T alleles [16]. The −480T allele frequency varies from 0.2 to 0.5 among Caucasian and African American populations [15, 17, 20], respectively.

Although an association was found between post-heparin plasma HL activity and the *LIPC* promoter variants, individuals of the same *LIPC* genotype show a broad range of HL activities that largely overlaps with the other genotypes [15, 18, 19, 22]. This indicates that the differences in post-heparin plasma HL activity may be due to other factors that modulate expression of the *LIPC* gene, or to additional base variants in the gene. However, the functionality of these base substitutions has not been established. In the present study, we tested whether the difference in HL expression is causally related to the base substitutions in the *LIPC* promoter by studying its effect on in vitro promoter activity.

Materials and methods

Generation of wild-type and mutant promoter-CAT constructs

For in vitro promoter studies, the 5′-flanking region of the *LIPC* gene was cloned into reporter plasmids in front of the chloramphenicol *O*-acetyltransferase (CAT) gene. The −1068 to +61 fragment of the wild-type human HL gene in the reporter plasmid pGCAT-A was kindly donated by Dr H. Will (University of Hamburg, Germany) [23]. Since the wild-type fragment in pGCAT-A showed very low promoter activity when transfected into human hepatoma cells [23], the HL promoter fragment was subcloned into another reporter plasmid, pCAT-basic (Promega, Leiden, The Netherlands). An upstream *Pst*I restriction site was introduced by PCR at position −650 using the primer 5′-AAC TGC AGT GGT CGC CTT TTC CCT ACC-3′, whereas a downstream *Xba*I restriction site was introduced either at position +61 using primer 5′-GCT CTA GAC CCG GGG TCC AGG CTT TCT TGG-3′ or at position +48 using primer 5′-GCT CTA GAC TTG GTA ATT TCT GAA GCC-3′ (restriction sites underlined). After digestion with *Pst*I and *Xba*I, the PCR products were cloned into pCAT-basic upstream of the CAT reporter gene. Similarly, a −289/+48 promoter fragment was cloned into pCAT-basic after the introduction of a *Hin*dIII site at position −289 and an *Xba*I site at position +48 of the HL gene by PCR using the upstream primer 5′-CGGAAGCTT AGG CAG CCA CGT G-3′ and the *Xba*I-primer described above, respectively.

For site-directed mutagenesis, the 1.1 kb wild-type promoter fragment was subcloned into pBluescript KS$^-$ (Stratagene, La Jolla, CA, USA) using *Hin*dIII and *Sst*I. The −216A and −480T mutants were created independently using the Transformer™ site-directed mutagenesis kit (Clontech, Palo Alto, CA, USA). The mutagenic primers used were 5′ GCT CCT TTT GAC ATG GGG GTG AAG GG-3′ and 5′-CAC AGT AGC TTT AAA TTG ATT AAT TTG G-3′, respectively; the selection primer 5′-CCC TCG AGG TGC ACG GTA TCG-3′ was chosen to disrupt the unique *Sal*I site in pBluescript (mutations underlined). Subsequently, the mutant HL promoter fragments were subcloned into pCAT-basic as described above. Different combinations of the −480C/T and −216G/A variants were generated by appropriate exchange of 424 bp *Apa*I-*Xba*I fragments using an internal *Apa*I site.

All inserts in pCAT-basic were verified by cycle sequencing using the Thermo Sequenase dye terminator sequencing kit (Amersham Pharmacia Biotech, UK) and the ABI 377 sequencer. Plasmid DNA's used for transfections were isolated with the Wizard Midiprep System (Promega, Leiden, The Netherlands). All oligonucleotides were custom-made by Eurogentec (Seraing, Belgium). Restriction enzymes were from Boehringer Mannheim (Germany).

HepG2 transfections assays

Twenty-four hours before transfection, HepG2 cells were plated in 60 mm culture dishes at 20% confluency in Dulbecco's modified Eagle's medium (ICN, Costa Mesa, CA, USA) supplemented with 10% fetal calf serum (Gibco BRL, Breda, The Netherlands). Three hours before transfection, the medium was refreshed. DNA transfections were performed by calcium phosphate co-precipitation using 10 μg of the CAT-construct and 0.4 μg of a RSV-β-galactosidase expression vector per dish. In co-expression studies, the indicated amounts of the recombinant plasmid pCX-USF (a kind gift from Dr R.G. Roeder, Rockefeller University, New York, USA) were included, which contained cDNA encoding the 43-kDa human Upstream Stimulatory Factor (USF) isoform under the control of the CMV-promoter [24]. In each experiment, parallel transfections with promoter-less pCAT-basic and SV40 promoter-driven pCAT promoter-vector (Promega) were included as negative and positive controls, respectively. At 48–72 h after transfection, cell extracts were prepared and the amount of CAT and β-galactosidase antigen was determined by ELISA using kits from Boehringer Mannheim. Promoter activity was expressed as pg CAT/ng β-galactosidase to correct for differences in cell number and transfection efficiency. All transfection experiments were repeated at least once with independent plasmid DNA preparations.

Electrophoretic mobility shift assays

Oligonucleotides used were 5′-GTC AGC TCC TTT TGA CA(C/T) GGG GGT GAA GGG -3′ and 5′-CTT TT C CCT TCA CCC CC(A/G) TGT CAA AAG GAG C -3′, which contained either the polymorphic −480C or T of the *LIPC* promoter at a central position; the oligonucleotides 5′-GG T GTA GGC CAC GTG ACC GGG TGT AAG CTT -3′ and 5′-GG A AGC TTA CAC CCG GTC ACG TGG CCT ACA -3′ represent the USF binding site in the adenovirus major late promoter (AdML) (overlapping sequences underlined) [25]. Of each pair, one oligonucleotide was end-labeled using [γ-^{32}P]ATP (Amersham, UK) and polynucleotide kinase (Boehringer Mannheim). The labeled oligonucleotides were annealed with excess unlabeled complementary oligonucleotide by slow cooling from 95°C to room temperature. Double-stranded competitor oligonucleotides were prepared in parallel in the absence of labeled nucleotides. Of the labeled oligonucleotides, 4.5 fmol (10,000–25,000 dpm) were incubated for 30 min at room temperature with different amounts of purified calf brain USF protein [26] (a kind gift from Dr M. Timmers, University of Utrecht, The Netherlands) in a final volume of 10 μl of 10 mM Hepes/KOH buffer (pH 7.6) containing 60 mM KCl, 1 mM EDTA, 1 mM DTT, 4% Ficoll, 100 μg/ml poly(dI/dC) and 0.25 mg/ml of BSA. In some experiments, 1 μl of anti-human USF[43] (C-20; Santa-Cruz Biotechnology, CA, USA) was added just prior to the labeled oligonucleotide. Thereafter, protein–DNA complexes were separated in a 4%

(29:1) polyacrylamide gel in 0.5× TBE electrophoresis buffer (44 mM Tris, 44 mM boric acid, 0.1 mM EDTA, pH 8.0), and the dried gel was exposed to an autoradiographic film.

Statistics

Differences were tested statistically by one-way ANOVA followed by the Student–Newman Keuls-test, and were considered significant at $P<0.05$.

Results

Effect of the HL promoter variants on transcriptional activity

To test for the functionality of the −216 and −480 base substitutions, we initially prepared promoter-CAT constructs of the human HL promoter region from −650 to +61. This 3′-end corresponds to the *Sma*I-restriction site used by others for subcloning into reporter plasmids [22, 27, 28]. Upon transient transfection of human hepatoma HepG2 cells, these constructs expressed low promoter activity just slightly above the promoter-less reporter vector (fig. 1). Since a further lowering of promoter activity is hard to assess, we sought to design constructs with a higher starting activity in vitro. A 3′-deletion construct containing the −650 to +48 region showed a markedly increased promoter activity with levels three- to five-fold higher than pCAT-basic. A further increase in promoter activity to levels well above that of the SV40-promoter was obtained by an additional 5′-deletion from −650 to −298. We therefore, decided to use the *LIPC* (−650/+48)- and *LIPC* (−298/+48)-containing CAT-constructs for further experiments.

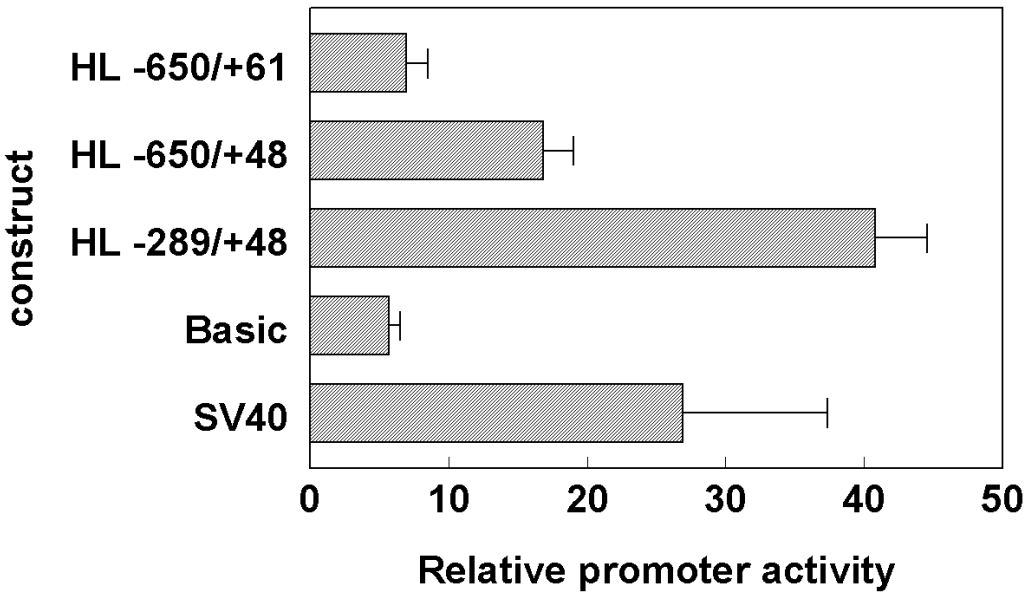

Figure 1: **Effect of 3′- and 5′-deletions on HL promoter activity in HepG2 cells.**
Expression studies were performed in HepG2 cells using CAT-constructs harboring the indicated regions of the wild-type human HL gene. Both negative (pCAT-basic) and positive (SV40-CAT) controls were included. Promoter activity was expressed as the amount of CAT expression relative to β-galactosidase expression. Data represent mean±S.D. for three independent experiments, each performed in triplicate.

The −216G→A mutation was introduced into the *LIPC* (−298/+48)-containing CAT-constructs by site-directed mutagenesis, and the effect on promoter activity was tested by transient transfection of HepG2 cells. Constructs with either −216G or A showed similar promoter activities (n.s., n=8) (fig. 2A). When the −216G→A and −480C→T mutations were introduced collectively into *LIPC* (−650/+48)-containing constructs, thus representing the two common alleles, transcriptional activity was reduced to almost half of that of the wild-type construct (56 ± 13%, n=7, P<0.05) (fig. 2B). As these two base substitutions are almost completely linked, we conclude that the common promoter polymorphism in the *LIPC* gene is functional.

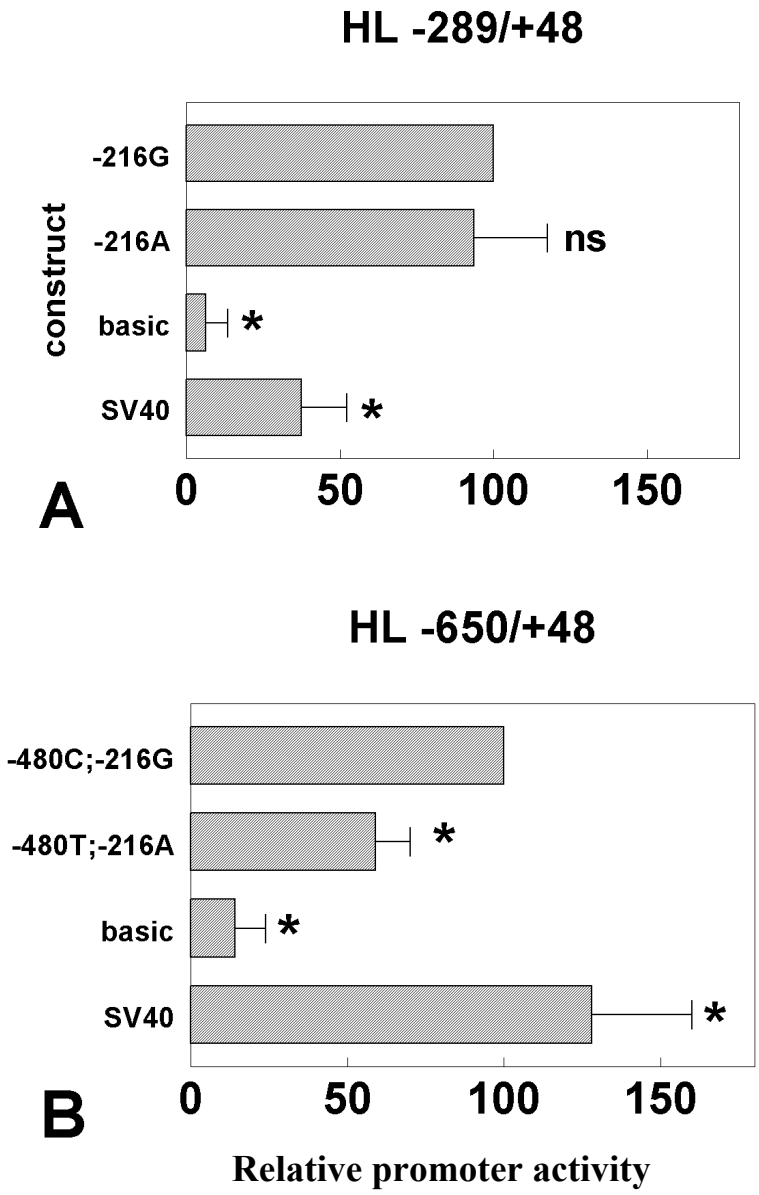

Figure 2: **Effect of base substitutions on HL promoter activity in HepG2 cells.**
Expression studies were performed in HepG2 cells using CAT-constructs harboring the −289/+48 region (panel A) or the −650/+48 region (panel B) of the human HL gene. Both negative (pCAT-basic) and positive (SV40-CAT) controls were included in each experiment. The CAT/β-galactosidase ratios obtained with the wild-type CAT constructs were set at 100%. Data represent mean±S.D. for five to eight independent experiments, each performed in triplicate. The asterisk indicates a statistically significant difference from the wild-type construct (P<0.05).

Differential binding of USF to HL −480C and −480T oligonucleotides

As the −216G→A substitution did not affect promoter activity of the short construct, we assumed that −480C→T substitution was responsible for the reduced promoter activity of the longer construct. A database search for homology with binding sites for known transcription factors [29] revealed that the −480C→T substitution disrupts a consensus binding site for USF, a potentially insulin-responsive transcription factor involved, among others, in the regulation of lipogenesis [25, 30]. To confirm the effect of the −480C→T substitution on USF binding, we performed electrophoretic mobility shift assays using double-stranded oligonucleotides harboring either the −480C or the −480T sequence, and USF protein purified from calf brain [26]. Binding conditions were optimized with oligonucleotides that contain a perfect consensus sequence for USF binding (AdML). An amount of USF protein was chosen that produced an approximately 50% shift of the AdML oligonucleotides. Under these conditions, a similar gel shift was observed with the −480C oligonucleotide, but the extent of binding was markedly less than with the AdML oligonucleotides (fig. 3A). Co-incubation with anti-USF[43] peptide antibodies completely prevented the gel shift and induced a supershift, thereby confirming the involvement of USF[43] protein (not shown). In contrast, the USF-dependent mobility shift was almost absent with the −480T oligonucleotide. In competition experiments using ^{32}P-labeled −480C oligonucleotide, unlabeled −480T oligonucleotide was able to completely abolish the gel-shift but this required levels that were four-fold higher than with unlabeled −480C oligonucleotide (fig. 3B). These observations demonstrate that USF binds to this region of the HL gene, and indicate that the affinity of binding to −480T is markedly lower than to −480C oligonucleotides. Hence, the −480C→T substitution interferes with binding of USF.

Effect of USF over-expression on HL promoter activity

To test the effect of USF on transcriptional activity, the *LIPC* (−650/+48) promoter-CAT constructs were transfected into HepG2 cells together with an expression vector containing human USF[43] cDNA. Promoter activities of both the wild-type and the double-mutant constructs gradually increased with co-transfection of increasing amounts of USF-encoding plasmids (fig. 4). The difference in promoter activity between both constructs was more pronounced in the USF-transfected than in the control cell cultures. In five independent experiments, the activity of the double mutant was 66±26% of that of the wild-type construct (mean±S.D., n=5, P<0.05) at the highest dose of USF-encoding plasmid used. Here, transcriptional activity of both the wild-type and double-mutant constructs was increased by a factor of 8.9±2.1 and 9.2±2.7 (mean±S.D., n=5, n.s.), respectively. Hence, despite the different binding affinities for USF, promoter activities were similarly sensitive to transactivation by USF.

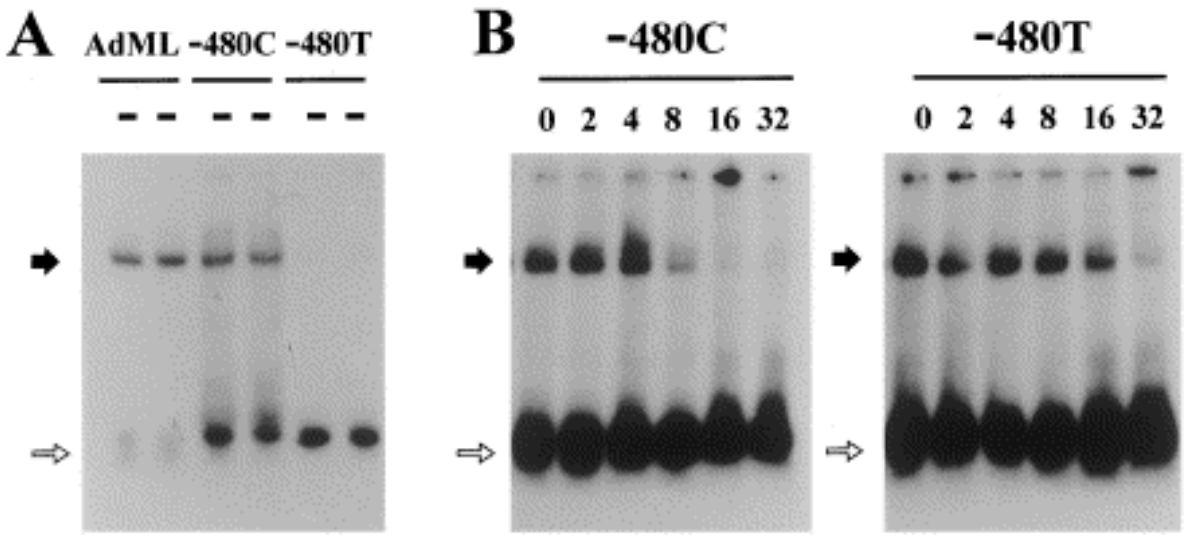

Figure 3: **Gel shifts of the −480C/T oligonucleotides by purified USF protein.**
Gel shift assays were performed with 30-bp oligonucleotides centered around the polymorphic −480 site of the human HL gene containing either C or T at this site, and with a 30-bp oligonucleotide containing the USF consensus binding site from the AdML. Of a preparation of USF protein highly purified from calf brain [26] (a gift from Dr M. Timmers), 0.1 µl was used throughout. In A, USF was incubated with the radiolabeled oligonucleotides indicated before electrophoretic separation and autoradiography. The specific radioactivity of the two HL-specific oligonucleotides was taken three-fold higher than of the AdML oligonucleotide to facilitate visualization. In B, radiolabeled wild-type HL −480C oligonucleotides were incubated with USF in the presence of increasing amounts of unlabeled competitor, either wild-type HL −480C (left panel) or mutant HL −480T (right panel). Numbers indicate the molar excess of unlabeled over labeled oligonucleotides. The positions of the bound and unbound oligonucleotides in the autoradiograms are indicated by filled and open arrowheads, respectively.

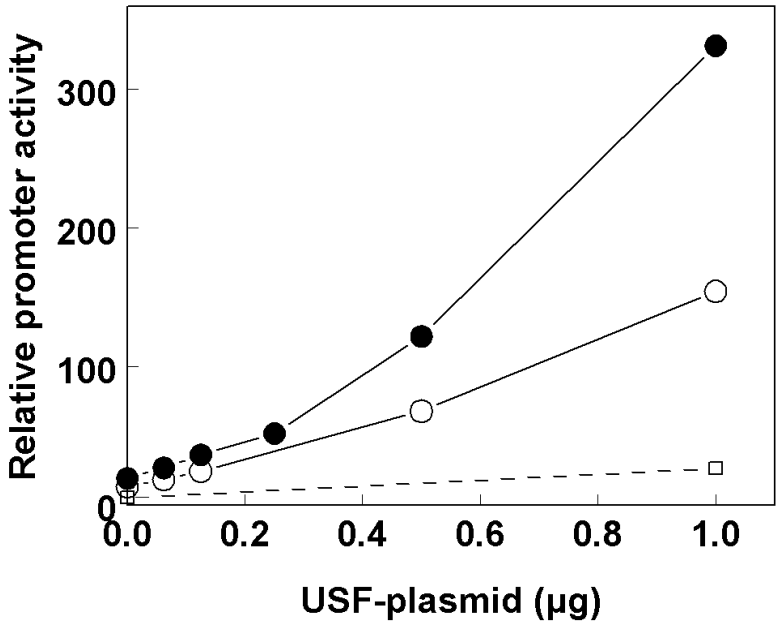

Figure 4: **Effect of co-expression of USF on HL promoter activity in HepG2 cells.**
HepG2 cells were co-transfected with HL promoter-CAT constructs and the indicated amounts of the human USF[43] expression vector pCX-USF. Reporter plasmids (10 µg) used were the HL(−650/+48) CAT-constructs harboring either the wild-type (−480C; −216G) or the double-mutant (−480T; −216A) promoter region (closed and open circles, respectively). The effect of co-transfection is also shown for the pCAT-basic (squares). Promoter activities are expressed relative to that of the SV40-promoter. Data are means of duplicate transfections, and are representative for two similar experiments.

We then tested the separate effects of the −480C→T and −216G→A mutations on promoter activity and USF transactivation of the *LIPC* (−650/+48) promoter-CAT constructs. Compared with the wild-type (−480C; −216G) promoter construct, the −480C→T substitution alone did not significantly affect expression of the CAT gene in control HepG2 cells or in the USF-transfected cells (fig. 5). The −216G→A mutation alone reduced transcriptional activity in control HepG2 cells (fig. 2B), but this did not reach statistical significance when the unprocessed data were tested by ANOVA (n=7). When the data in each experiment were first expressed as percentage of the parallel wild-type construct, however, the promoter activity of the −480C; −216A construct tested significantly lower than the wild-type construct and not statistically different from the double-mutant construct. In USF-transfected cells, the effect of the −216G→A substitution alone was no longer apparent (n=4). Collectively, these data suggest that the −216G→A substitution is more effective than the −480C→T substitution in the longer promoter constructs, though it does not affect promoter activity of the short constructs in the absence of the −480 region. The effect of the −216G→A substitution on promoter activity may be overcome by USF over-expression, provided that there is a high-affinity binding site for USF at position −480.

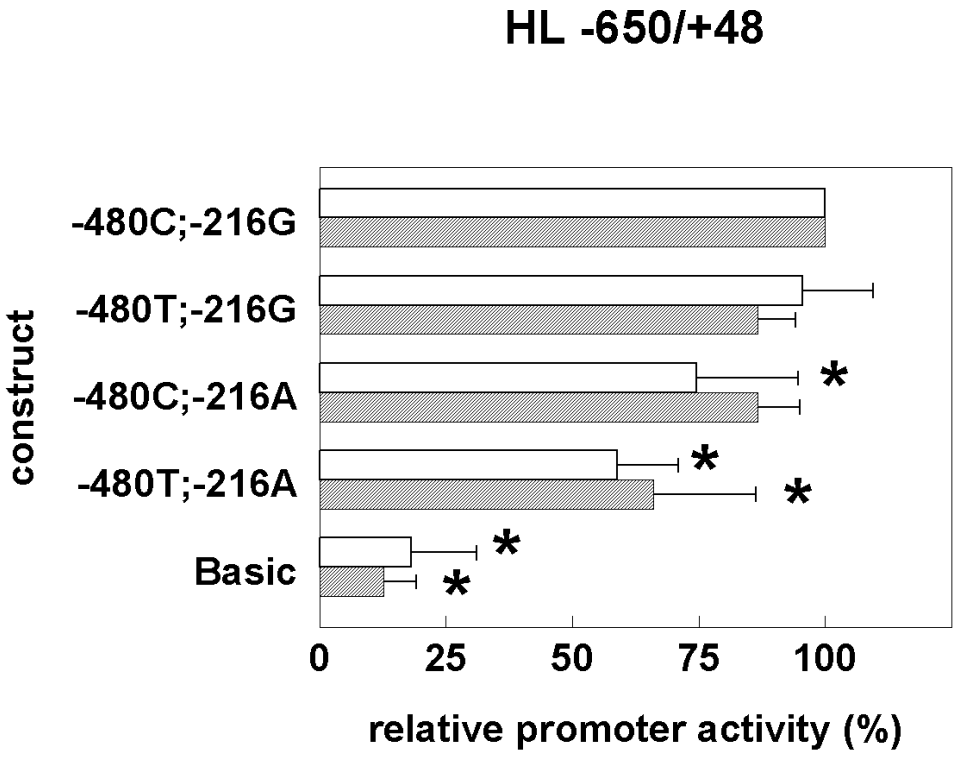

Figure 5: **Effect of base variations on HL promoter activity in HepG2 cells.**
Expression studies were performed in HepG2 cells using the indicated CAT-constructs harboring the −650/+48 region of the human HL, as described in the legends to figure 2. Cells were co-transfected without (open bars) or with 1.0 μg of pCX-USF (hatched bars). Promoter activities are expressed relative to that of the wild-type promoter CAT constructs. Data represent mean±S.D. for three to five independent experiments each performed in triplicate. The asterisk indicates a statistically significant difference from the wild-type construct (P<0.05).

Discussion

Several groups have shown that the −480T allele of the *LIPC* gene is associated with a 15–50% lower post-heparin plasma HL activity compared with the −480C allele [15, 17-19]. In these alleles, the −480C→T substitution is strongly linked to the −216G→A substitution, and to two other polymorphisms further upstream in the gene. We confirmed that in our previously reported population of normocholesterolemic CAD patients [15], the −480C→T substitution is almost completely associated with the *Dra*I and *Ava*II variants at −216 and −676, respectively (unpublished data, 1999). In reporter assays using the human hepatoma HepG2 cells, the combination of the −480C→T and −216G→A substitutions results in a reduction of *LIPC* promoter activity by 40–50%, which is in good agreement with the in vivo effect of this polymorphism on hepatic lipase expression. This observation demonstrates that the common polymorphism of the *LIPC* promoter is functional and can be explained solely by the combined effect of the −480C→T and −216G→A substitutions. However, an additional contribution of the two other base variants further upstream cannot be ruled out at present.

From the data presented here, the reduction in promoter activity cannot be unambiguously attributed to either one or the other base substitution alone. The −480C→T substitution alone did not significantly reduce promoter activity of the −650/+48 constructs either in control or in USF-over-expressing HepG2 cells. The −216G→A substitution alone did not affect the activity of the −289/+48 promoter fragment. One explanation for our data would be that each of the two base substitutions has a small effect on transcription, which when combined add up to statistically significant levels under the conditions tested. Alternatively, the effect of the −216G→A substitution may only become apparent in combination with the −480C→T substitution, or vice versa. Our data suggest that the −216G→A substitution alone may be effective in the −650/+48 constructs, i.e. in the presence of the −480 region. In addition, the effect of the −216G→A substitution on promoter activity may be overcome by USF over-expression, provided that there is a high-affinity binding site for USF at position −480. This suggests that the proteins that bind to these loci interact with each other.

Several regulatory elements have been reported in the 5′-regulatory region of the human *LIPC* gene, some of which include the −480C/T and the −216G/A positions [27, 28]. According to the Transfac DataBank [29], the −216G→A substitution affects the consensus binding site for c-Myb and CDP, which have no obvious relevance to lipid or lipoprotein metabolism. Interestingly, the −480C→T substitution disrupts one of the potential USF binding sites present in the promoter region of the *LIPC* gene. In vitro gel shift assays confirmed that this substitution reduced the binding of USF to this locus. The transcription factors USF[43] and USF[44] are expressed in various mammalian tissues including liver, and have recently been implicated in the regulation of lipogenic genes such as L-PK, Spot14 and fatty acid synthase [25, 30]. In rat livers, the expression of the USF[43] protein was markedly upregulated under conditions of high plasma glucose and insulin levels [25]. In vitro, *LIPC* promoter activity increased several-fold upon co-transfection of the HepG2 cells with USF[43] cDNA. Potentially, an increased liver expression of USF[43] in conditions with elevated plasma glucose and/or insulin, such as occur post-prandially and in hyperinsulinaemia, may lead to enhanced transcription of the hepatic lipase gene. Some evidence for the regulation of HL

expression in humans by glucose and/or insulin has been presented. Post-heparin plasma HL activity in men is positively correlated with plasma insulin levels in response to an oral glucose load [31, 32]. We recently reported that post-heparin plasma HL activity positively correlated with fasting plasma insulin levels in coronary artery disease patients homozygous for the −480C allele [15]. This correlation was not found in patients that were carriers of the −480T allele. In vitro, however, the −480C→T substitution alone did not significantly reduce promoter activity of the −650/+48 constructs either in control or in USF-overexpressing HepG2 cells, despite the reduced ability to bind USF protein. The relative increase in promoter activity by USF over-expression was independent of the presence of a C or a T at position −480. These observations suggest that the in vitro USF effect is mediated by transactivation through other elements, possibly through one of the additional USF consensus sites present in the *LIPC* (−650/+48) promoter fragment.

In summary, we show here for the first time that the common variation in the *LIPC* promoter is functional. Therefore, the reduced HL activity found in carriers of the −480T allele is likely due to reduced transcription. The molecular mechanism and the transcription factors involved remain to be established.

Acknowledgements

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Chapter 7.0

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

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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 transcripttionnal activity of a 698-bp HL promoter–reporter construct two-fold. Atorvastatin (10 μM) abolished the oleate stimulation. The transcriptional activity of a sterol-regulatory-element binding protein (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.

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 of several lipoproteins, pro- as well as anti-atherogenic properties are ascribed to high HL expression (reviewed in 4 and 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.

Materials and methods

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, 50IU/ml penicillin and 50 µg/ml streptomycin (Gibco BRL, Breda, The Netherlands). Twenty-four hours before the start of the experiments, HepG2 cells were plated at 30% confluence in 6- or 24-well culture dishes.

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 1mmol/L. The control medium contained less than 0.05 mmol/L NEFA (NEFA C-kit, Wako Chemicals GmbH, Germany). The oleate-enriched as well as control media were refreshed every 12 h during a period of 48 h. When indicated, atorvastatin was present at a final concentration of 10 µmole/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 mU (nmoles of free fatty acids released per min). Atorvastatin was kindly provided by Pfizer, NY, USA.

Cellular cholesterol and triglyceride biosynthesis

To determine the effect of atorvastatin on cholesterol(-ester) biosynthesis in HepG2 cells, [2-14C]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 volume). After evaporation of the extraction fluid, lipids were dissolved in heptane:isopropanol (1:4, by volume), 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 volume). 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).

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 Ameis et al.[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 SacI/NheI restriction sites, and in the XhoI/HindIII 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, NY, 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.

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 β-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-well 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 co-transfection (data not shown), we corrected only for the amount of protein per cell extract. In three independent experiments without pSREBP2 co-transfection, the β-galactosidase expression was similar among parallel incubations, indicating that intra-assay variation in transfection efficiency was relatively small.

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.

Results

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 throughout the 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 [2-14C]acetate incorporation into cholesterol and cholesterolesters to 30 ± 14% and 37 ± 11% (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).

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 (fig. 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.

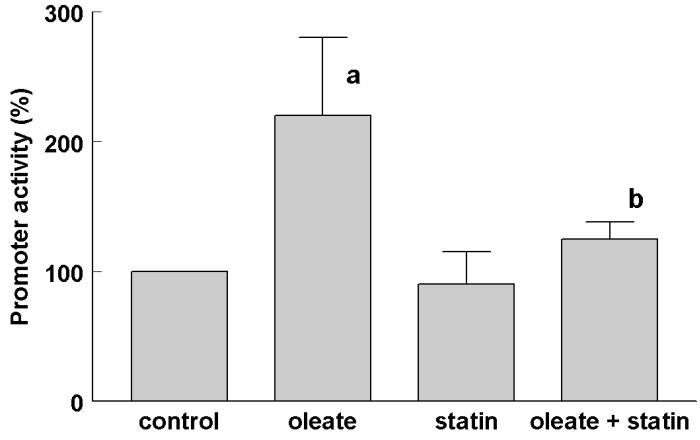

Figure 1: **Effect of oleate and atorvastatin on HL promoter activity.**
HepG2 cells were transiently transfected with HL698-CAT construct. Albumin-bound oleate (ratio 1:6) was added to the extracellular medium at a final concentration of 1 mM, and atorvastatin to a final concentration of 10 μM. The 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 means ± S.D. for three independent experiments, each performed in triplicate; a and b indicate statistically significant ($p < 0.05$) differences with the control and oleate incubations, respectively.

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 (fig. 2A). The luciferase activity was 10-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.

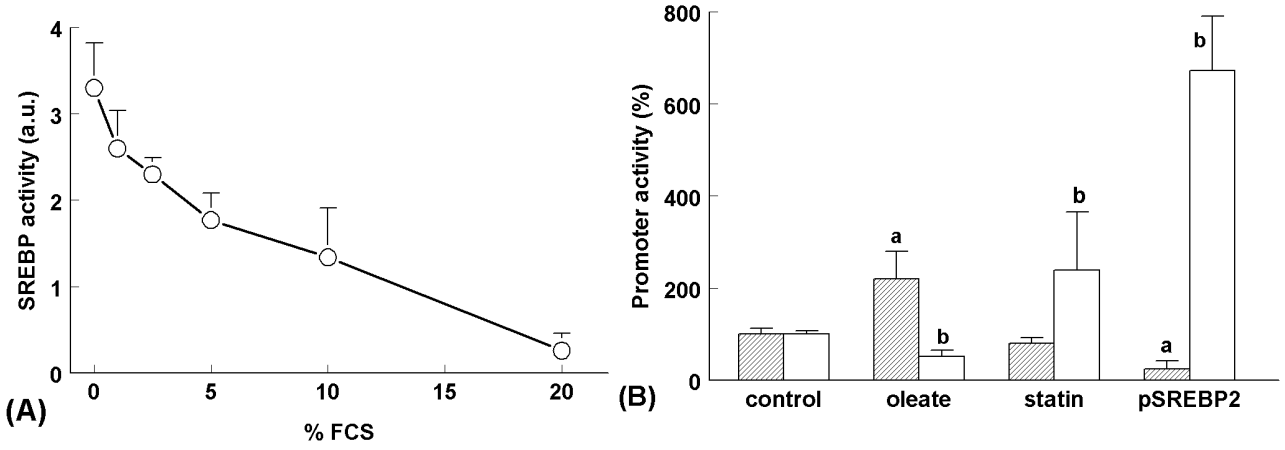

Figure 2: **Determination of SREBP activity in HepG2 cells with an SREBP-sensitive luciferase probe**. HepG2 cells were transiently transfected with pSRE-luc, and then incubated for 48 h in the presence of the indicated additions. (A) The incubation medium contained 20% serum (by volume), either LPDS or increasing amounts of FCS in LPDS. Data are means ± S.D. for one experiment performed in quadruplicate. (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 ± S.D. of three experiments each performed in triplicate; a and b indicate statistically significant ($p < 0.05$) differences with the control HL promoter activity and pSRE-activity, respectively.

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 ($p < 0.01$; $n = 4$; fig. 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.

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 (fig. 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 (fig. 4). Co-transfection with pCX-USF upregulated the HL promoter, but the endogenous SREBP activity was not significantly affected (fig. 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 (fig. 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 (fig. 4A). These observations indicate that SREBP interfere with the USF-mediated upregulation of the HL promoter.

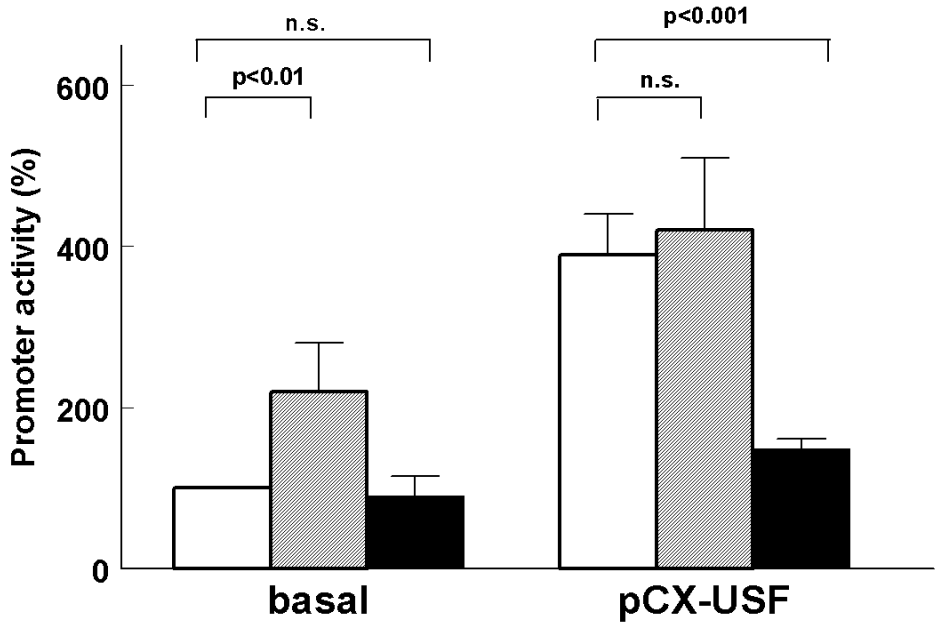

Figure 3: **Effect of oleate and atorvastatin on HL promoter activity in USF-1 overexpressing cells.**
Experiments were performed as indicated in the legend to figure 1 except that in part of the incubations, cells were co-transfected with 500 ng/well of pCX-USF. Open bars: no further additions; hatched 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 means ± S.D. for three independent experiments, each performed in triplicate; n.s.: not statistically significant.

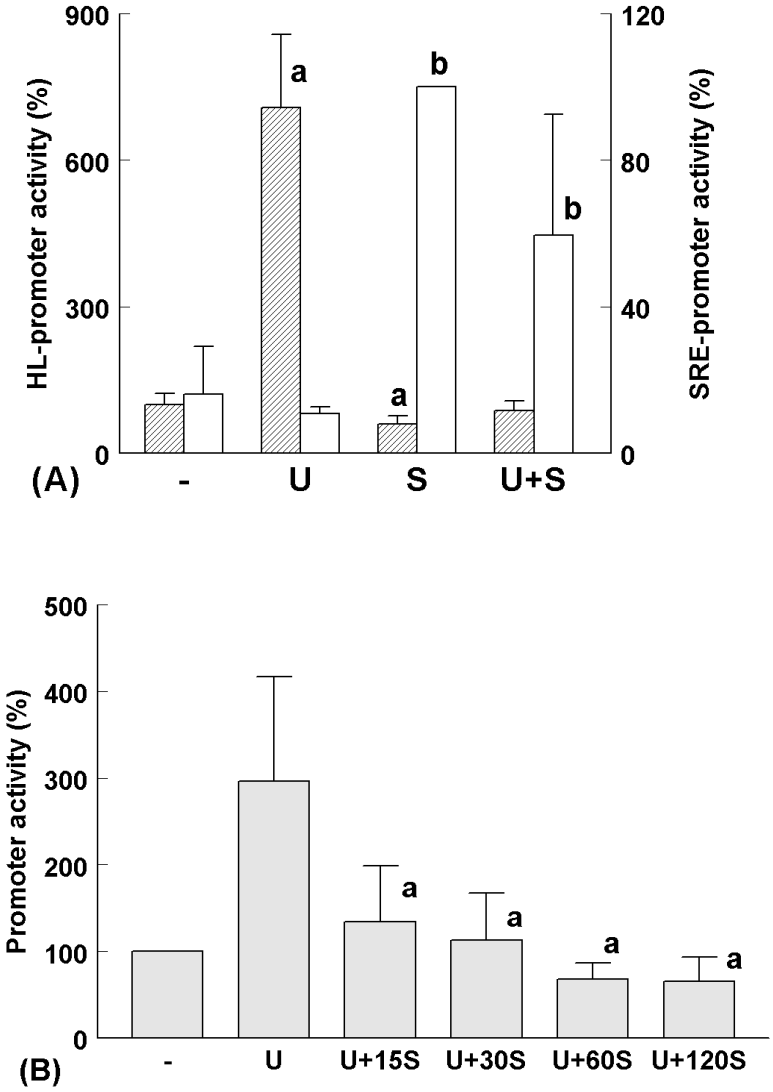

Figure 4: **Effect of pCX-USF or pSREBP2 co-transfection on HL and SRE promoter activity.**
(A) HepG2 cells were transfected either with HL698-luc (hatched bars) or pSRE-luc (open bars) with or without co-transfection with 100 ng/well pCX-USF (U) or pSREBP2 (S). After 48 h of incubation, cell extracts were analyzed for luciferase activity. a and b indicate statistically significant ($p < 0.05$) differences with the control HL promoter activity and pSRE-activity, respectively.(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 indepen-dent experiments; a indicates statistically significant difference from the USF-activated promoter activity.

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 (fig. 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

co-transfection 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 appears to occur independent of the −685/−305 region of the HL gene, including the potential SRE at −553 and E-boxes at −514 and −310.

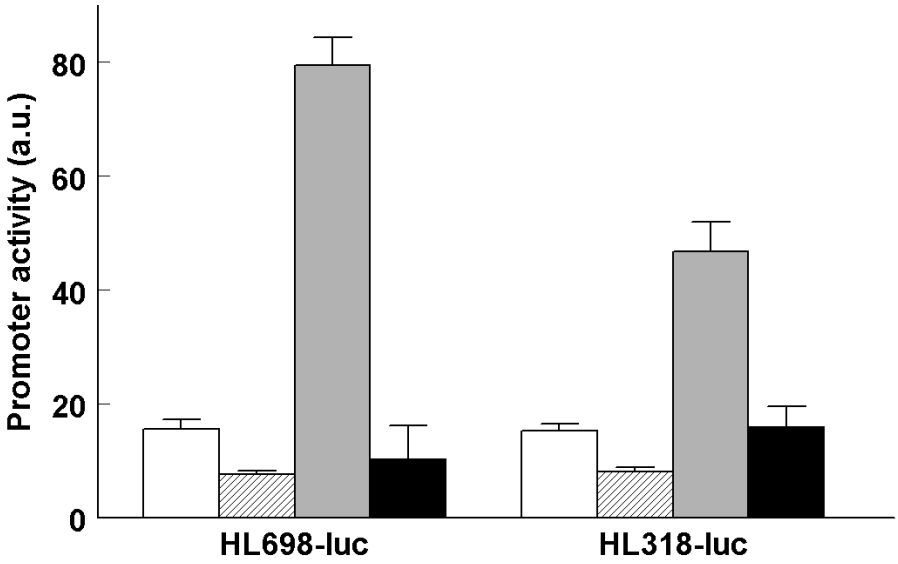

Figure 5: **Opposite regulation of the HL promoter activity is independent of SRE and E-box sequences.** HepG2 cells were transfected with either HL698-luc (left panel) or HL318-luc (right panel) without (open bars) or with 120 ng/well pSREBP2 (hatched bars), 100 ng/well pCX-USF (gray bars), or both (filled bars), and 48 h later cell extracts were analyzed for luciferase activity. Data are means ± S.D. for four parallel incubations.

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 PPARα activity [19]. PPARα consensus sequences have not been identified in the proximal HL promoter. Ciprofibrate, a potent PPARα 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 co-transfection 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 co-transfection 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 co-transfection. 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 co-transfection 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, co-transfection 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 (fig. 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 et al. 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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Chapter 8.0

General Discussion

HL promoter

The HL gene is almost exclusively transcribed and translated in liver cells. In chapter two we hypothesized that the responsible cis-acting elements for liver-restricted expression are conserved among mammalian HL genes. To identify these elements, a genomic comparison was made of the 5'-flanking region of the rat, mouse, rhesus monkey, and human HL genes. Although the Ensembl website contains genomic sequence of many more mammalians, only these four species have their first exon and hence upstream regulatory region identified. This is due to the extremely long length of intron 1 with over 100 kb sequence, which makes it very difficult to identify the transcription start site in silico. The proximal promoter region has elements that are highly conserved among these four species. This region contains putative binding sites for hepatocyte nuclear factors HNF1 and HNF4. These transcription factors enhance basal promoter activity of target genes in liver. Several groups have suggested that HNF1 is important for liver-specific HL expression. HNF1α knockout mice have 3.4 fold lower HL mRNA levels than control mice [37]. But it is not zero, so HNF1 may not be the master switch for liver-specific expression of the HL gene Besides in liver, HNF1 is also expressed in kidney, pancreas and the digestive tract [31], suggesting that HNF1 is not an exclusive liver-specific transcription factor. However, the notion that HNF1 is important for upregulation of liver-specific genes is generally accepted. It is possible that in other tissues the potential enhancing effect of HNF1 is opposed by another strong inhibitory transcription factor. Such an inhibitor may bind at the HL promoter and perhaps even at the HL HNF1 site (chapter two). Indeed, other groups have identified multiple negative elements in the proximal HL promoter region [9, 15, 30].

Another possibility is that HNF1 only activates those susceptible genes that are present in euchromatin, but not in heterochromatin, and that other factors determine in which part of the chromatin the gene is localized in a particular tissue. Genes expressed in specific tissues appear to be clustered together on the chromosome [27]. Genes within an active cluster are all expressed in that tissue. HL is located on chromosome 15 q21.3. The other genes in close proximity of the HL gene are not exclusively expressed in the liver, like ADAM10 (a disintergrin and metalloprotease). This protein is prominently expressed in all epithelial tissues but especially in the liver [16]. Another gene located near the HL gene is AQP9 (aquaporin 9), which is constitutively expressed at high levels in the liver but also in tanycytes, astrocytes and neurons of the brain [2]. Although these genes are not liver-specific, they are all expressed prominently in liver. Hence, the genomic region surrounding the HL gene may be present in the active part of the chromatin in liver. In heterochromatin, the chromosome is normally condensed and "closed" for transcription factors. In the euchromatin, modification or remodelling of the chromatin structure "opens" the chromatin so that transcription factors can reach the DNA. This process can be tissue specific for a particular DNA region. Possibly, the HL gene is located in a region of the chromatin that is in the "open" state in the liver, but not in other tissues, thus enabling HNF1 to induce HL transcription almost exclusively in the liver.

Alternatively, liver-restricted expression of HL may be mediated by other, not yet identified regulatory elements present in the far upstream or downstream region of the HL gene. Interestingly, the 30kb upstream region of the mammalian HL genes contains a number of conserved elements that may be important for liver-specific expression of HL. In future

research, the potential importance of these elements as enhancers of HL promoter activity should be tested, for example in reporter assays with transiently transfected hepatocytes and non-hepatocytes.

HL expression in steroidogenic tissues

As stated above, the HL gene is almost exclusively transcribed and translated in liver cells. The HL protein found in steroidogenic tissues closely resembles HL protein found in liver. However the molecular mass of the structural unit, as determined by radiation inactivation analysis [36] appears to differ for the rat: 57 kDa in liver and 51 kDa in adrenal glands and ovaries. At least for the rat, most of the "steroidogenic HL" is thought to originate from the liver and to be transported to the steroidogenic organs via the bloodstream. Therefore, it is likely that during transport to the steroidogenic organs the HL protein is modified.

With sensitive molecular-biological techniques HL mRNA was detected in rat adrenal glands and ovaries [40, 41]. In rat adrenals, the HL gene is transcribed into a variant HL mRNA, which lacks exons 1 and 2. In chapter three we present evidence that suggest that this variant HL mRNA is synthesized from an alternative promoter within intron-2 of the HL gene. The existence of alternative promoters has been documented now for a large number of genes, and is viewed as a means to increase the number of proteins expressed from the genome. The human genome contains up to 20,000–25,000 different protein-coding genes [19]. Compared with the total gene number in yeast (6,000) [12], fruit fly (14,000) [1] and the worm C. elegans (19,000) [8], the gene number in the mammalian genomes is only slightly higher despite the much higher complexity of these organisms. To explain this, Ewing and Green hypothesized that multifaceted use of genes could play a pivotal role in functional diversification of the genome without affecting total gene number [11]. This multifaceted use of a single gene can be provided by alternative splicing and promoters. Alternative splicing occurs in about half of all human genes, producing on average more than three different transcript variants per locus [26]. The various transcripts are translated into proteins with slightly different structure and function. Independently, Lopez and Black suggested that alternative splicing provides a mechanism for the fine-tuning of the function of a single gene [6, 28]. Just recently, the same hypothesis has also been made for alternative promoters. Based on the analysis of more than 14,000 genes, it was estimated that 52% of all genes have multiple promoters [23]. On average there are 3.1 alternative promoters per gene [23]. However, not all the products resulting from alternative splicing and promoters are functional. As argued below, the functionality of the rat HL alternative promoter is probably of little physiological importance. Future investigations are required to establish whether the alternative promoter in the rat HL gene (chapter three), adds to the protein repertoire of the mammalian genome, or just represents aberrant gene expression. On the basis of our present results, the latter explanation is most likely. This may also hold for the splice variant observed for the human HL gene (chapter four).

The amounts of HL mRNA detected in steroidogenic organs, even after induction, are very low. If the variant HL mRNA is translated into a functional protein, the amount probably is also very low. After induction, the increase of HL mRNA does not reflect the increase of HL activity, as HL mRNA has reached its maximum after two days and declines thereafter,

while HL activity is at its maximum only after six days [41] (chapter three). Moreover, in contrast with the HL protein synthesised in liver, the signal sequence is missing and the translation product would probably remain in the cytosol. As discussed in chapter three, it is unlikely that the possible translation product of the variant HL mRNA is catalytically active. The role of an inactive, intracellular HL protein in intracellular lipid metabolism remains unknown. In this light, and because of the low expression levels observed, it is unlikely that the truncated protein is physiologically important. In addition, the truncated message could not be detected by sensitive techniques in human hypertrophic adrenals (chapter four). If the variant expression product were of physiological importance, the alternative HL promoter would also be expected to operate in other species. The use of the alternative HL promoter in the rat liver and steroidogenic tissues may therefore just be another example of aberrant gene expression that becomes detectable with the highly sensitive RT-PCR assays. In the human adrenal glands, full length HL mRNA is detected instead. The amount of the full-length message has not been quantified, but probably this is also very low, since the HL mRNA could be detected only with sensitive techniques. Therefore, the physiological importance of locally synthesised HL can also be questioned. Recently HL expression has been shown for human macrophages [14]. We can also not exclude the possibility that the full-length message found by us, is produced in macrophages within the adrenal tissue.

Effect of HL promoter polymorphisms on lipid homeostasis

As summarised by Jansen et al. (2002), HL has both atherogenic and anti-atherogenic properties [22]. In other words, both a low and a high HL expression may be atherogenic, depending on the genetic and environmental background. This notion is also apparent from the distribution of HL genotypes among normocholesterolemic CAD patients (chapter five). In this study, both TT and CC homozygotes for the LIPC gene have an increased risk for CAD, depending on the CETP genotype. As shown in many publications now, the LIPC-C allele is associated with high HL activity, and the T allele is associated with lower HL activity [20].

As illustrated in figure 1A, HL plays an important role in reverse cholesterol transport. Through its phospholipase A_1 activity (and possibly its ligand function), HL facilitates the delivery of HDL cholesterol to the liver via the direct pathway. In addition, through its triglyceridase activity (and possibly its ligand function) HL also plays an important role in HDL remodelling in the indirect pathway. In the indirect pathway, CE is first transferred to (V)LDL in exchange for TG, after which the CE may either be taken up by the liver as IDL (or LDL) via LDL-receptors, or end up in CE-enriched, TG poor LDL (sdLDL). Remodeling of TG-enriched HDL to HDL_3 by HL ensures that reverse cholesterol transport is maintained. A high HL activity, especially in combination with high CETP activity, exemplified by the CC-B1B1 double homozygotes (chapter five), will lead to efficient reverse cholesterol transport, but also to low HDL cholesterol and elevated sdLDL (fig. 1B). Since these patients have an increased CAD risk, the increased formation of sdLDL combined with the low anti-oxydative and anti-inflammatory potential associated with low HDL apparently prevails over the enhanced reverse cholesterol transport. On the other hand, with a low HL activity in combination with a low CETP expression, as exemplified by the TT-B2B2 double

homozygotes (chapter five), both the direct and indirect pathway of reverse cholesterol pathway is impaired (fig. 1C). As a consequence, the CAD risk is increased despite the elevation of HDL cholesterol and the reduced formation of sdLDL.

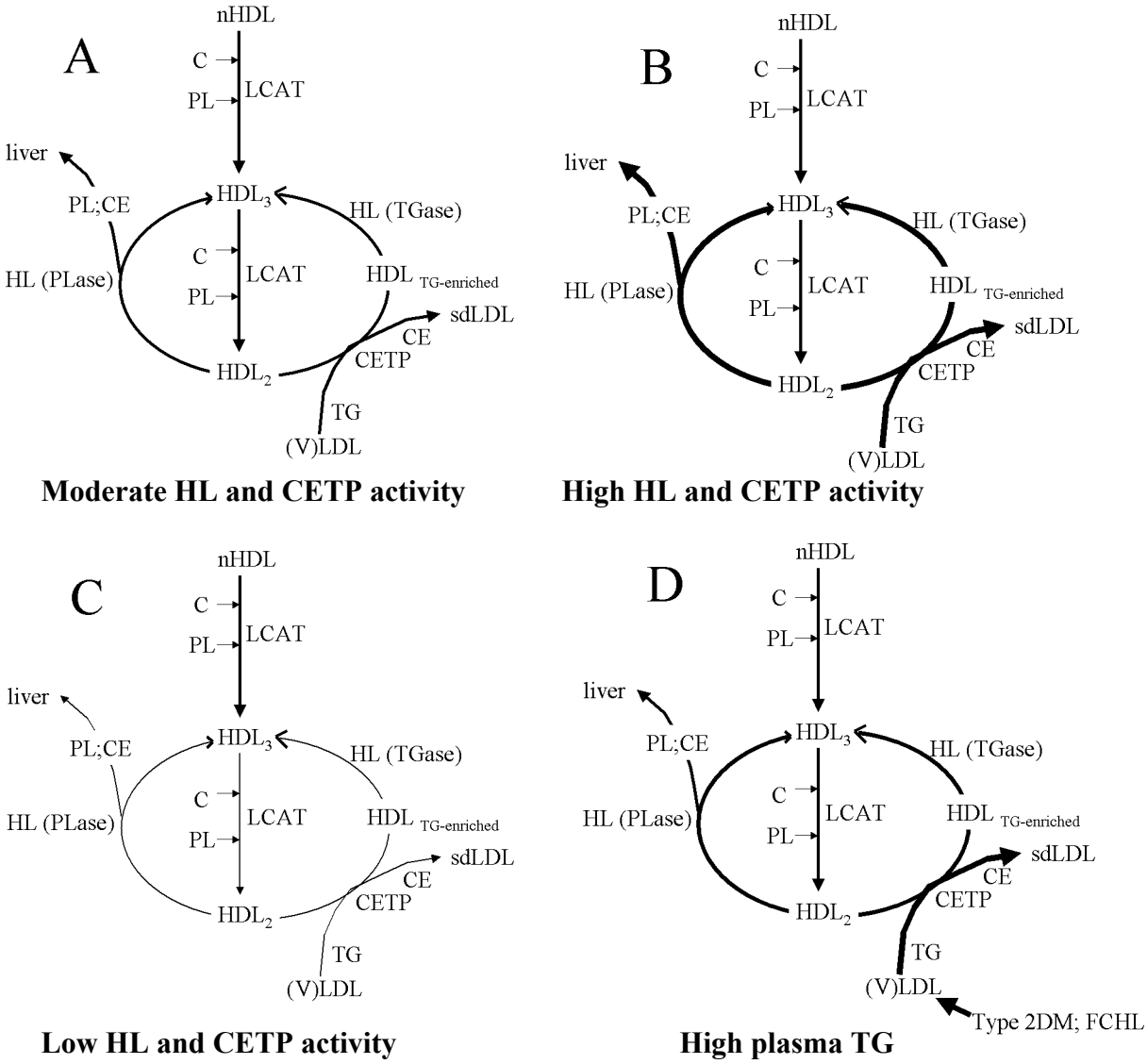

Figure 1: **HL and reverse cholesterol transport**.
Panel A: moderate HL and CETP activity; cholesterol derived from peripheral cells is efficiently delivered to the liver via the direct and indirect pathway, and HDL_2 is readily remodelled to HDL_3.
Panel B: high HL and high CETP activity; cholesterol delivery to the liver and remodelling of HDL_2 to HDL_3 is even more efficient, thus resulting in lower HDL and formation of more sdLDL.
Panel C: low HL and low CETP activity; cholesterol delivery to the liver and remodelling of HDL_2 to HDL_3 is impaired, and results in raised HDL cholesterol and less sdLDL.
Panel D: high plasma triglycerides; due to high (V)LDL levels CETP activity is high, which results in elevated HDL cholesterol and increased formation of sdLDL.
Thickness of arrows represents relative activity. Note that after CETP action, the cholesteryl esters in IDL (and LDL) can be delivered to the liver cell via the LDL-receptor; this part of the indirect pathway has been omitted for the sake of clarity. Abbreviations used: C, cholesterol; CE, cholesterol esters; DM, diabetes mellitus; FCHL, familial combined hyperlipidemia; HL, hepatic lipase; LCAT, lecithin-cholesterol acyltransferase; nHDL; nascent HDL; PL, phospholipids; PLase, phospholipase activity; sdLDL, small dense LDL; TGase, triglyceridase activity.

The increased CAD risk for the double LIPC-TT/CETP B2B2 homozygotes despite elevated HDL cholesterol illustrates that HDL levels in individual patients should be interpreted with caution. From epidemiological studies, the strong idea has emerged that high HDL cholesterol protects against development of atherosclerosis. In general this may be true, but not necessarily if high HDL results from impaired reverse cholesterol transport. When HL activity is low, the recycling of HDL_2 to HDL_3, which is able to take up cholesterol from the periphery, is impaired. Like HL, CETP has an important role in the indirect pathway of reverse cholesterol transport (fig. 1A); it promotes the flux of cholesteryl esters from HDL to the apoB containing particles, LDL and VLDL. The CETP Taq1B B2 allele is associated with a low CETP expression [24]. CETP lowering therapy has become attractive at this moment, as a low CETP activity is associated with increased HDL and reduced LDL cholesterol levels. Initial studies with the experimental CETP-lowering drugs JTT-705 and torcetrapib in humans indeed showed a substantial increase of HDL cholesterol and a minimal to up till 40% decrease of LDL cholesterol (reviewed by Barter [3]). However, whether this is associated with a corresponding decrease in CAD incidence remains to be demonstrated. The clinical benefit of CETP inhibition is thought to be primarily an increase of HDL cholesterol. CETP inhibition will only improve the risk profile of the patient if HDL cholesterol is adequately delivered to the liver. If CETP inhibition diminishes the cholesterol flow from HDL to VLDL and LDL in the indirect pathway of reverse cholesterol transport, the removal of cholesterol from HDL depends on the direct pathway, and hence on the action of HL. If HL expression is low, for instance in subjects with homozygote LIPC T allele, HDL cholesterol will accumulate, but this increase in HDL cholesterol will not protect against atherogenesis, especially if the CETP inhibition results only in a slight LDL cholesterol reduction [3]. Therefore, subjects who are considered for treatment with CETP lowering drugs should be examined for their HL promoter and CETP genotype.

In chapter five, we also determined the distribution of the LIPC-T allele in healthy males either with high, low or median HDL cholesterol. We found that the T-allele frequency among the three HDL groups strongly depended on the plasma TG levels. In normotriglyceridemic subjects, the T-allele frequency in the low HDL group was almost half that in the high HDL group, an observation that is in line with the general picture that the T-allele associates with lower HL activity and elevated HDL cholesterol [20]. Surprisingly, the T-allele frequency in the hypertriglyceridemic subjects, which are predominantly present in the low HDL group, is as high as in the high HDL group. This suggests that there are at least two different mechanisms responsible for the low HDL cholesterol in this group. In one model, the low HDL is associated with the C allele and hence may be an intrinsic property of HDL metabolism itself. In a second model, the low HDL is rather associated with the T-allele, and may be secondary to elevated plasma TG. A possible explanation for the latter model may be that increased levels of TG-rich lipoproteins offer increased substrate for CETP (fig. 1D). There will be more exchange of TG and CE between (V)LDL and HDL_2, resulting in lower HDL cholesterol. This may be exacerbated in the presence of low HL activity, such as in carriers of the T-allele, where subsequent remodelling of TG-enriched HDL to HDL_3 via HL is impaired (fig. 1D). This mechanism is particularly relevant to type 2 diabetes. These patients have an atherogenic lipoprotein profile (low HDL, high TG, increased sdLDL, impaired reverse cholesterol transport). Indeed, type 2 diabetes is a strong risk factor for atherosclerosis. It is

interesting to establish whether the atherosclerotic risk is much higher for type 2 diabetes patients with the TT genotype. If so, these patients may benefit most from early, aggressive lipid-lowering therapy [44].

Functionality of the SNPs in the HL promoter region

The common HL promoter polymorphism is functional, since the T-allele is associated with lower HL activity in post-heparin plasma, and with higher plasma HDL-cholesterol levels than the C-allele [20]. The HL promoter polymorphism consists of four SNPs, which are in strong linkage disequilibrium (chapter five). Van 't Hooft *et al.* performed reporter studies using the HL(-894/+49) promoter construct to test the impact of each of these SNPs on transcriptional activity [17]. They found that none of the SNPs changed basal HL promoter activity, and they concluded that the four SNPs must be in strong linkage disequilibrium with one or more yet unknown functional polymorphisms in the HL gene locus [17]. Although the presence of additional, linked and functional SNPs in the HL gene cannot be ruled out, we (chapter six) and Deeb & Peng [10] concluded from similar experiments that the SNPs at –514 and –250 are functional. One difference between these three studies is the HL promoter construct used. Whereas van't Hooft *et al.* [17] used the -894 to +49 region of the HL gene, Deeb & Peng [10] used the -639 to +29 region, and we have used the -685 to +13 region. Most researchers [9, 30] have made use of the SmaI site at +28 to create human HL promoter fragments. Hans Will noted that it is very difficult to perform HL promoter studies, because of the low HL transcriptional activity of these constructs (personal communication). In our initial studies on the human HL promoter we also used a HL promoter fragment with this convenient SmaI site on the 3' site (HL -685/+28). To our disappointment, this human HL promoter fragment showed very low promoter activity, not only when compared to a viral promoter, but also to a similar rat HL promoter (-437/+9) fragment (chapter six). The rat HL promoter also contains a SmaI site at a similar position as the human HL promoter, but in addition an XbaI site is located 28 bp upstream of this SmaI site, and this XbaI site was used by us to create the rat HL promoter fragment. We therefore decided to create a human HL promoter fragment that was 15 bp shorter on the 3' site (HL-685/+13). This resulted in a HL promoter construct with a much higher promoter activity (chapter six). The +28-to-+129 region of the human HL gene has been shown to have a strong negative effect on HL promoter activity [15]. In DNase footprint analysis with rat liver nuclear extracts, several protected elements have been identified in this region [15]. Although the +13-to-+28 region does not coincide with such a footprint, we propose this region still conveys considerable silencing activity. Unfortunately, the activity of HL promoter constructs described by van 't Hooft and co-workers were not compared with other reference promoters such as an SV40 or RSV promoter. Nevertheless, it is reasonable to assume that their promoter constructs show very low transcriptional activity. Perhaps, van 't Hooft et al. were unable to detect the effects of the four HL promoter SNPs because of this low transcriptional activity.

Influence of USF on HL promoter activity

In chapter six we have shown that the combination of the –514C→T and -250G→A substitutions results in a reduction of LIPC promoter activity by 40–50%, which is in good agreement with the in vivo effect of this polymorphism on HL expression. Similarly, Deeb & Peng [10] found that the –514C→T substitution was sufficient to lower in vitro transcriptional activity by about 30 %. In our hands, both the –514C→T and –250G→A are required for significantly lowering transcriptional activity. A search through the Transfac database failed to identify a candidate transcription factor, that binds to the –250G→A region. However, this site is close to a DNase I footprint [15, 30], and to a HNF4 half-site [15, 34]. Interestingly the –514C→T substitution disrupts a putative upstream stimulatory factor (USF) binding site (chapter six). Gel-shift assays showed that the binding affinity of USF protein was decreased four-fold by the –514C→T substitution. Taken together, our data indicate that the transcription factors that bind to, or close to, the –514 and –250 region, are required to maximally upregulate HL promoter activity.

One of our novel findings is that the human HL promoter is strongly upregulated by USF (chapter six). This notion comes from the identification of USF binding sites in the proximal HL promoter, and from the results of transient transfection assays on HepG2 cells with USF1 expressing plasmids. Further experiments are essential to definitely establish the role of USF in HL expression. First, in ChIP assays it must be demonstrated that USF is indeed bound to the proximal HL promoter in liver cells. Secondly, in the absence of USF, HL expression should be reduced, such as in USF knockout mice, or by silencing of USF by siRNA. Here, a complicating factor is the existence of two different USF genes, USF1 and USF2, which are active as homo- as well as heterodimers. Silencing or knockout of one gene may be compensated by the other. Thirdly, the element(s) in the HL promoter region responsible for the USF transactivating effect should be identified. USFs have been shown to act either through E-boxes or through Inr sites in promoters of susceptible genes. In the proximal human HL promoter region, two E-boxes are present. Besides the USF binding site at –514, which is abolished in the common HL promoter polymorphism, an invariant, canonical E-box is present at –310. In USF1 co-transfection experiments, we showed a similar dose-dependent increase in transcriptional activity of all HL promoter constructs, with or without a functional –514 E-box (Chapter six). The relative difference between the variant promoter forms measured under basal conditions was maintained in the USF1 over-expressing cells. Possibly, under conditions of USF1 over-expression the reduced transactivation due to the –514C→T substitution is overruled by the strong transactivation through either the –310 E-box or the Inr. Fourthly, the physiological context for this USF-mediated upregulation should be established. In rat liver, USF expression has been associated with the fasting/refeeding cycle, and hence, with plasma insulin levels [42]. In the fasting state whole-body metabolism is dominated by the mobilisation of energy stores, and maintaining blood glucose level by hepatic glucose production. In this state plasma insulin concentration is low. During refeeding, when plasma insulin is high, metabolism is geared towards energy storage and maintaining blood glucose level by accelerating glucose uptake by muscle, adipose and liver cells. In the liver, fatty acids are synthesised from glucose for export and storage as triglycerides. Wang & Sul [42] demonstrated that under these conditions, the expression of fatty

acid syntase (FAS), one of the rate-limiting enzymes of lipogenesis, is induced by insulin via elevation of USF. Post-heparin plasma HL activity is elevated in type 2 diabetes [5], a condition with high plasma insulin, so it is tempting to speculate that the USF-mediated activation of HL transcription plays a role under conditions with high insulin.

In non-diabetic subjects, post-heparin plasma HL activity is positively correlated with fasting plasma insulin levels [21], but only in the LIPC CC homozygotes and not in carriers of the T-allele. This observation also argues for the hypothesis that HL expression is upregulated by insulin via transactivation of USF and the –514 E-box. However, HL activity appears not to be directly upregulated by insulin [4]. Rather, the elevated HL activity in type 2 diabetes patients may be a reflection of the insulin-resistant state. Elevated fasting plasma insulin in non-diabetics is also an indicator of decreased insulin sensitivity. The interaction between the LIPC promoter polymorphism and insulin resistance has been confirmed now by other studies [13, 33, 38, 43]. Rather than insulin itself, HL transcription appears to be upregulated via USF by another parameter of insulin resistance. Among others, insulin resistance is associated with elevated plasma glucose and fatty acids. Recently, HL mRNA levels were shown to be increased by high glucose in HepG2 cells [39]. In the same in vitro model, oleate was shown to increase HL secretion (chapter seven) [29]. In obese women, post-heparin HL activity is positively correlated with omental fat mass, another parameter of insulin resistance, and taken to reflect increased flux of fatty acids to the liver [7]. Interestingly, in this latter study HL activity increases much stronger in LIPC CC homozygotes than in T-allele carriers. We propose therefore, that HL transcription is upregulated in the insulin-resistant state by either the elevated glucose or fatty acids, through USF transactivation, at least in part via the –514 E-box (fig. 2).

What may be the functional significance of increased HL expression under conditions of insulin resistance? Because HL plays an important role in plasma lipoprotein metabolism, notably in the reverse cholesterol transport pathway, and in the lipolysis of IDL, LDL and HDL triglycerides (fig. 1), it is likely that increased HL activity increases supply of cholesterol, phospholipids and fatty acids to the liver. It is hard to recognize the benefit of increased fatty acid supply under these conditions. Due to the insulin resistance of adipose tissue, ample fatty acids are already made available to the liver. The insulin-resistant state is characterized by elevated production and secretion of TG- and CE-containing VLDL, which may pose the need for additional cholesterol to the liver cell. Perhaps, the increased HL activity may facilitate the supply of the extra cholesterol from HDL. However, an intracellular demand for cholesterol is normally signalled by increased expression of nuclear SREBP (nSREBP), and we have shown that elevation of endogenous nSREBP is paralleled by a decrease rather than increase of HL secretion and HL promoter activity (chapter seven). Moreover, forced expression of nSREBP2 also reduced HL promoter activity (chapter seven). Hence, it seems unlikely that HL expression is increased to meet the cholesterol craving of the cell under this condition. The accelerated secretion of VLDL also urges the need for the hepatocyte to compensate for the concomitant loss of cellular phospholipids, notably of phosphatidylcholine (PC). From the building blocks needed for PC biosynthesis, glycerol and fatty acids are abundant in the hepatocytes under these conditions. Although intracellular levels of choline, or phosphorylcholine, are normally not limiting in the hepatocyte, it may become low under prolonged insulin resistance. In animal studies, both a choline deficiency

and an insufficiency to synthesise choline from ethanolamine, have been shown to reduce VLDL secretion [25]. Unfortunately, it is unknown whether HL expression is affected by this deficiency. Jansen et al [22] have previously presented arguments for existence of a physiological link between HL activity and VLDL secretion. Hence, HL activity may be increased in insulin resistance to satisfy the choline need for cellular phospholipid synthesis.

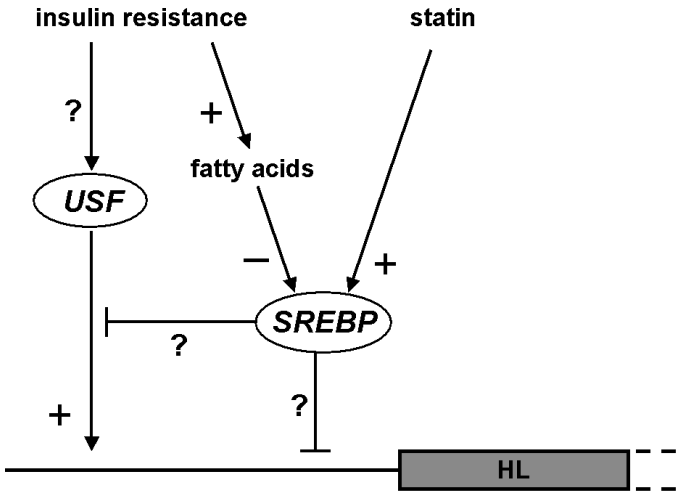

Figure 2: **Possible mechanism of stimulation of HL promoter activity.**
By reducing cholesterol denovo synthesis, statins increase mature SREBP, while fatty acids reduce mature SREBP. USF stimulates HL promoter activity. How insulin resistance influences HL promoter activity through USF, and how SREBP affects HL promoter activity, is not known yet.

Influence of SREBP on HL promoter activity

Another important finding is that the human HL promoter activity is reduced by nSREBP2 (chapter seven). Similarly, HL promoter activity in HepG2 cells was reduced when incubated with the HMG-CoA reductase inhibitor atorvastatin, especially in the presence of oleate or in USF1 over-expressing cells. This is not expected since nSREBP2 normally stimulates gene transcription and only a small number of genes have been shown to be down-regulated in nSREBP2 transgenic mice [18]. From the downregulated genes, only the MTP gene has been shown to be directly inhibited by nSREBP2 [18, 35]. Like nSREBP1, nSREBP2 affects gene expression by binding at sterol regulatory elements (SRE) or E-boxes in susceptible genes. In the proximal human HL promoter region, a potential SRE is present at around position –553, and E-boxes are present at –514 and –310. A HL promoter construct in which the potential SRE and the E-boxes had been removed was still upregulated by USF and downergulated by atorvastatin and nSREBP2. Therefore, nSREBP2 does not influence HL promoter activity through either the putative SRE or the E-boxes. For USF, transactivion via non-E-box-dependent mechanisms is not unprecedented, but how SREBP2 regulates HL promoter activity is not clear. One possibility is that SREBP2 interferes with USF transactivation. SREBP2 may compete with USF for binding to a site on the proximal HL promoter yet to be identified, thereby abolishing the transactivation by USF. Alternatively, USF and SREBP2 may bind to separate sites, thereby altering the interaction of bound USF with the basal transcription complex present at the transcriptional start site. Furthermore, SREBP2 may

affect USF expression, either transcriptionally or post-transcriptionally. Finally, SREBP2 may interfere with the formation of functional USF1 or USF2 homo- and heterodimers. Both USF and SREBP belong to the bZIP-HLH class of transcription factors. However, SREBPs appear not be able to heterodimerize with USF 32. Crucial to the understanding of the mode of action of SREBP2 is the identification of the responsive element in the proximal HL promoter region.

Figure 2 summarizes our current model of transcriptional regulation of the human HL gene. The HL gene is upregulated in the insulin-resistant state, either through the associated hyperglycemia or the increased flux of fatty acids to the liver. Glucose may increase transactivation of the HL gene by USF, and the fatty acids may reduce nSREBP activity, thereby reducing the inhibitory effect of SREBP on USF transactivation. USF may transactivate the HL gene via the E-boxes at –514 and –310, or through the Inr. Nuclear SREBP may interfere with USF transactivation, either by affecting USF expression, or by interfering with the stimulating effect of USF on HL promoter activity. The HMG-CoA reductase inhibitors, on the other hand, increase nSREBP activity, thereby reducing USF transactivation. This may explain the observation that atorvastatin reduces HL secretion and HL promoter activity in HepG2 cells especially when supplemented with oleate. A problem with this interpretation may be that statins affect cholesterol metabolism predominantly through SREBP2, whereas SREBP1 is mainly targeted by fatty acids [18]. Additional experiments are needed to show whether nSREBP1 affects HL promoter activity similar as nSREBP2, or that nSREBP2 is similarly affected by fatty acids as nSREBP1 in this model cell system.

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Summary

Hepatic lipase (HL) is an enzyme expressed predominantly in the liver, but HL activity is also present in adrenals and ovaries of most mammals (**chapter 1**). The enzyme has lipolytic activity towards phospholipids and triglycerides carried in plasma lipoproteins. HL plays an important role in the delivery of HDL cholesterol to the liver in the so-called "reverse cholesterol transport pathway". HL also mediates the formation of atherogenic small-dense LDL particles (sdLDL) from triglyceride-rich VLDL. Hence, HL activity has been implicated in the development of premature atherosclerosis, as well as in the protection against atherogenesis (reviewed in chapter 1). In addition, HL present in adrenals and ovaries mediates the uptake of HDL cholesterol, thereby supplying these tissues with cholesterol for steroidogenesis. Previous studies have shown that HL expression in the liver is regulated at the post-translational level, and more importantly, at the transcriptional level. Despite the potentially important physiological and pathological roles of HL, relatively little is known about the transcriptional regulation of HL gene expression in liver and steroidogenic organs. In this thesis, we describe studies on various aspects of the transcriptonal regulation of HL expression in human HepG2 cells, and in human and rat adrenals.

In **chapter 2**, we searched the HL gene sequence for potentially important regulatory sequences. We hypothesized that important regulatory elements would be conserved among HL genes from various mammals. In silico, the 5'-flanking region of the rat, mouse, rhesus monkey, and human HL genes were aligned, compared and analyzed. Highly conserved sequences were found at the proximal promoter region, and at 14 and 22 kb upstream of the transcriptional start site. In the proximal promoter region, three highly conserved clusters of transcription factor binding sites were identified. These clusters were analyzed using promoter-reporter assays. Two clusters were found to be important for basal transcription. The cluster at –70 to –34) contained the putative binding sites for hepatocyte nuclear factors HNF1 and HNF4. We show that this latter cluster is responsible for enhanced HL promoter activity in hepatoma cells, and for silencing of HL promoter activity in non-liver cells.

In rat adrenals and ovaries, the HL gene is transcribed into a variant mRNA in which exons 1 and 2 appear to be replaced by a ''new'' sequence. In **chapter 3** this novel sequence was identified. The variant rat HL mRNA was shown to be 2.6 kb. The 5′ end of the variant HL mRNA was located in intron 2 immediately upstream of exon 3. The transcription start site was mapped 465 bp upstream of exon 3. The 5' regulatory region of this alternative rat HL promoter showed no apparent basal activity in hepatoma cells, but in adrenocortical cells the promoter was upregulated upon incubation with cAMP, which mimicks the effect of corticotropins. Treatment of male rats with corticotropin resulted in a transient increase in the variant HL mRNA in the adrenals, which was paralleled by synthesis of 47- to 49-kilodalton HL-related proteins. In contrast, a delayed, but sustained, increase in adrenal HL activity was observed. This difference in time course suggests that the HL activity does not reflect HL-like proteins expressed from the variant mRNA.

In rat adrenals the HL gene is transcribed exclusively from the alternative promoter in intron-2. In **chapter 4** we show that the full-length HL mRNA is expressed in hyperplastic

adrenals of Cushing's disease patients. In addition, a splice variant that lacks exon-3 was detected in the human adrenals and hepatoma (HepG2) cells, but not in liver. The human HL promoter showed transcriptional activity in adrenocortical cells and its activity was enhanced 2-fold by treatment with cAMP. No evidence was found for the existence of an alternative promoter in the human HL gene similar to rat. The presence of both full-length mRNA and enzyme activity suggests that part of the HL activity is locally synthesized in human adrenals.

In the proximal hepatic lipase promoter region four single nucleotide polymorphisms are present at positions –763 (A→G), -710 (T→C), -514 (C→T) and –250 (G→A). These four polymorphisms are in almost complete linkage disequilibrium. Therefore, two different alleles can be distinguished, which are designated as the LIPC C- and T-allele according to the –514 polymorphism. In **chapter 5.1** two different methods for determining the hepatic lipase promoter genotype are presented: the allele-specific oligonucleotide (ASO) hybridization and restriction endonuclease assay with NlaIII or DraI.

The common LIPC –514C→T substitution is associated with reduced hepatic lipase activity and increased HDL cholesterol levels. In **chapter 5.2** we investigated whether the T-allele frequency differs in subjects with different HDL cholesterol levels. From a large population of healthy men, three subpopulations with either low, median, or high plasma HDL cholesterol concentrations were selected. The three groups were matched for environmental factors known to influence HDL levels. In normotriglyceridemic subjects, the T-allele frequency was 0.14, 0.17 and 0.28 in the low, median and high HDL group, respectively. Surprisingly, the allele frequency of hypertriglyceridemic subjects within the low HDL group was 0.27, similar to the high HDL subjects, who are all normotriglyceridemics. We conclude that the effect of the LIPC –514C/T polymorphism on plasma HDL cholesterol levels is strongly affected by plasma triglyceride levels.

Not only hepatic lipase, but also cholesteryl ester transfer protein (CETP) is a HDL-modifying protein that has both pro- and anti-atherogenic properties. In **chapter 5.3** we tested the hypothesis that both proteins interact at the level of HDL cholesterol concentration and atherosclerotic risk. The distribution of the common HL promoter -514C/T and CETP Taq1B B1/B2 polymorphisms in male coronary artery disease patients was compared with nonsymptomatic controls. Cases and controls had similar allele frequencies, but the distribution of the combined genotypes differed significantly. In coronary artery disease patients, 7.9 % of the CETP-polymorphism homozygotes had the LIPC-TT genotype, contrasting with only 1.2 % in controls. The HL lowering LIPC-T allele and the CETP lowering B2 allele synergistically raised HDL cholesterol concentration. Patients with the B2B2-CC genotype had higher HDL cholesterol levels than B1B1-CC patients. Compared to the B1B1-CC subjects, carriers of either one LIPC-T allele or one CETP-B2 allele had a lower coronary artery disease risk. In contrast, the B2B2-TT subjects tended to have a higher risk. In a two-year follow up, the loss of coronary artery lumen diameter in B2B2-TT patients was higher than in all other patients combined. We conclude that combined homozygosity for the CETP and HL lowering gene variants presents with accelerated coronary atherosclerosis, despite the relatively high HDL cholesterol level.

The functionality of the common −514C→T substitutions in the promoter region of the human hepatic lipase gene is investigated in **chapter 6**. *LIPC* (−685/+13) constructs with both −250G>A and −514C>T substitutions exhibited significantly lower promoter activity than their wild-type counterparts. Activities of −325/+13 constructs were not significantly affected by the −250G→A substitution. The −514C/T site lies within a binding region for Upstream Stimulatory Factor (USF). Gel-shift assays showed that the binding affinity of USF protein for HL specific oligonucleotides was decreased four-fold by the −514C→T substitution. However, promoter activity of the −685/+13 constructs was not significantly affected by the −514C→T substitution alone. Co-transfection of HepG2 cells with USF-1 cDNA yielded a similar dose-dependent increase in activity of all −685/+13 constructs. These studies demonstrate that the common *LIPC* promoter polymorphism is functional, and explain the association of the −514T allele with a 40-50% lower hepatic lipase activity in men.

In **chapter 7**, we tested the hypothesis that HL expression is regulated as an integral part of intracellular lipid homeostasis. Incubation of HepG2 cells with oleate resulted in a 2-fold increase in HL secretion and transcriptional activity of a HL(-685/+13) promoter–reporter construct. Co-incubation with atorvastatin abolished the oleate stimulation. The transcriptional activity of a sterol-regulatory-element binding protein (SREBP)-sensitive HMG-CoA synthase promoter construct was reduced by oleate, and increased 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 glucose and lipid homeostasis. Co-transfection with a USF-1 expression vector stimulated HL promoter activity several-fold. The USF-stimulated HL promoter activity was not further enhanced by oleate, but almost completely abolished by atorvastatin or co-transfection with the SREBP-2 expression vector. Opposite regulation by USF-1 and SREBP-2 was also observed with a 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.

Samenvatting

Het enzym hepatische lipase (HL) komt voornamelijk in de lever tot expressie, maar HL activiteit is ook aanwezig in de bijnieren en ovaria van de meeste zoogdieren (**hoofdstuk 1**). Het enzym vertoont lipolyse activiteit, met de fosfolipiden en triglyceriden in plasma lipoproteine deeltjes als substraat. HL bevordert ook de afgifte van HDL cholesterol aan de lever in het zogenaamde "omgekeerde cholesterol transport". HL bevordert daarnaast ook de omzetting van triglyceride-rijke VLDL deeltjes naar kleine LDL deeltjes (sdLDL) die bijzonder atherogeen zijn. Hepatische lipase wordt dan ook in verband gebracht zowel met de vroegtijdige ontwikkeling van atherosclerose, als met de bescherming daartegen (besproken in hoofdstuk 1). Het HL, dat aanwezig is in bijnieren en ovaria, bevordert daar de opname van HDL cholesterol die gebruikt wordt voor de synthese van steroidhormonen. Studies hebben uitgewezen dat de expressie van HL wordt gereguleerd op posttranslationeel niveau, maar vooral op het niveau van transcriptie. Over de transcriptionele regulatie van HL is nog relatief weinig bekend. Dit proefschrift beschrijft verschillende aspecten van de transcriptionele regulatie van HL in humane HepG2 levercellen, en in bijnieren van de mens en de rat.

In **hoofdstuk 2** hebben we gezocht naar potentieel regulatoire sequenties in het humane HL gen. De hypothese was dat belangrijke regulerende elementen geconserveerd zijn in HL genen van verschillende zoogdieren. De 5' flankerende gebieden van het HL gen van de rat, muis, rhesus aap en mens zijn met elkaar vergeleken. Sterk geconserveerde sequenties zijn gevonden in het proximale promoter gebied, en op 14 en 22 kb stroomopwaarts van de transcriptionele start plaats. In de proximale promoter zijn drie sterk geconserveerde clusters van bindingsplaatsen voor transcriptiefactoren aanwezig. Deze clusters werden geanalyseerd met behulp van promoter reporter assays. Twee clusters bleken belangrijk te zijn voor de basale transcriptie van het HL gen. Het cluster tussen –70 en –34 bevat potentiële bindingsplaatsen voor de leverspecifieke factor HNF1 en HNF4. Wij hebben laten zien dat dit cluster verantwoordelijk is voor HL promoter activiteit in levercellen, en voor de onderdrukking van HL promoter activiteit in niet-levercellen.

In rat bijnieren en ovaria wordt het HL gen getranscribeerd in een variante mRNA, waarin deexonen 1 en 2 zijn vervangen door een ''nieuwe'' sequentie. In **hoofdstuk 3** is deze nieuwe sequentie geïdentificeerd. Het variante rat HL mRNA bleek 2,6 kb groot te zijn. Het 5' eind van het variante HL mRNA is gelokaliseerd in intron 2 onmiddellijk stroomopwaarts van exon 3. De transcriptie startplaats bevindt zich 465 bp stroomopwaarts van exon 3. Het 5' regulerende gebied van deze alternatieve promoter vertoonde weinig activiteit in levercellen, maar in bijnierschors cellen werd de promoteractiviteit opgereguleerd door behandeling met cAMP, de second messenger voor het hormoon ACTH. Behandeling van mannetjes ratten met ACTH leidde tot een tijdelijke toename in de hoeveelheid van de variante HL mRNA in de bijnieren, hetgeen parallel plaatsvond met de synthese van een 47 – 49 kilodalton HL gerelateerd eiwit. De HL activiteit in de bijnieren nam daarentegen pas later toe, en bleef daarna verhoogd. Dit verschil in verloop met de tijd suggereert dat de gemeten HL activiteit in de bijnieren niet is toe te schrijven aan een translatieproduct van het variante HL mRNA.

In bijnieren van ratten wordt het HL gen uitsluitend getranscribeerd vanaf de alternatieve promoter in intron 2. In **hoofdstuk 4** beschrijven we, dat in hyperplastische bijnieren van patiënten met de ziekte van Cushing het volledige HL mRNA inclusief exon 1 en 2 tot expressie komt . Daarnaast is er in deze bijnieren, net als in HepG2 cellen, maar niet in de humane lever, een splice variant aanwezig die exon 3 mist. De humane HL promoter vertoonde transcriptionele activiteit in cellen van de bijnierschors, en deze activiteit werd verhoogd door behandeling met cAMP. Er zijn geen aanwijzigingen gevonden voor de aanwezigheid van een alternatieve promoter in intron 2 van het humane HL gen. De aanwezigheid van zowel volledig HL mRNA en enzymatische activiteit in de humane bijnieren suggereert dat een deel van de HL activiteit afkomstig is van locale synthese.

In het proximale lever lipase promoter gebied zijn vier basevarianten aanwezig op posities –763 (A→G), -710 (T→C), -514 (C→T) en –250 (G→A). Deze basevarianten zijn bijna compleet gekoppeld aan elkaar, zodat er sprake is van twee verschillende allelen. Deze allelen worden het LIPC C- en T-allel genoemd.. In **hoofdstuk 5.1** beschrijven we twee methoden voor de bepaling het lever lipase promoter genotype: de allel specifieke oligonucleotide (ASO) hybridisatie methode en de restrictie analyse met NlaIII of DraI.

De LIPC –514C→T substitutie is geassocieerd met een verlaagde HL activiteit en een verhoogd HDL cholesterol niveau. In **hoofdstuk 5.2** is onderzocht of de T allel frequentie verschilt in personen met een verschillende HDL cholesterol niveaus. Uit een grote populatie van gezonde mannen werden drie subpopulaties met een laag, een mediaan en een hoog plasma HDL cholesterol geselecteerd. In normo-triglyceridemische personen (TG < 2.3 mM) was de T-allel frequentie respectievelijk 0,14, 0,17 en 0,28 in de laag, mediaan en hoog HDL groep. Verassend genoeg was de allel frequentie van de hypertriglyceridemische personen (TG ≥ 2.3 mM) van de laag HDL groep vergelijkbaar met de allel frequentie van de hoog HDL groep die allen normo-triglyceridemisch zijn (0,27). We hebben geconcluderend dat het effect van het LIPC –514C/T polymorfisme op plasma HDL cholesterol sterk afhankelijk is van de plasma triglyceriden.

Net als HL is ook het cholesteryl ester transfer proteïne (CETP) een HDL modificerend eiwit met zowel pro- als anti- atherogene eigenschappen. In **hoofdstuk 5.3** hebben we de hypothese getest dat dat beide eiwitten een interactie hebben op het niveau van HDL cholesterol en risico op atherosclerose. Hierbij hebben we de verdeling van de algemeen voorkomende HL promoter (LIPC-514C/T) en CETP Taq1B B1/B2 polymorfismen vergeleken tussen mannelijke coronair vaatlijden patiënten enerzijds, en non-symptomatische controle personen anderzijds. De allelfrequenties waren vergelijkbaar tussen de patiënten en controle personen, maar de verdeling van de gecombineerde genotypen verschilde. In de patiënten waren 7,9 % van de CETP B2B2 homozygoten ook homozygoot voor het LIPC-TT genotype, terwijl dit in de controle groep slechts voor 1,2 % was. Het HL-verlagende LIPC-T allel en het CETP-verlagende B2 allel vertoonde een synergistische verhoging van het HDL cholesterol. Personen met het B2B2-CC genotype hebben in vergelijking met B1B1-CC patiënten een hoger HDL cholesterol. Dragers van één LIPC-T allel of één CETP B2 allel hebben een lager risico op coronair vaatlijden dan de B1B1-CC dubbel homozygoten. De

B2B2-TT personen daarentegen lijken een veel groter risico te hebben. Tijdens een follow-up van twee jaar was de coronaire lumen diameter in deze laatste groep patiënten sterker afgenomen dan bij alle andere groepen gecombineerd. De conclusie is dat dubbel-homozygoten voor de CETP- en HL-verlagende genvarianten een versnelde coronaire atherosclerose vertonen, ondanks hun relatief hoge HDL cholesterol.

De functionaliteit van de algemeen voorkomende −514C→T substitutie in het promoter gebied van het humane HL gen is beschreven in **hoofdstuk 6**. *HL*(−685/+13) constructen met beide −250G>A en −514C>T substituties hebben een significant lagere promoter activiteit dan het wildtype. De activiteit van −325/+13 constructen werd niet significant beïnvloed door de −250G>A substitutie. Het -514C/T polymorfisme is gelocaliseerd in een potentiële bindingsplaats voor Upstream Stimulatory Factor (USF). Gel-shift experimenten lieten zien dat de affiniteit van USF voor deze bindingsplaats een factor vier werd verminderd door de −514C→T substitutie. De promoter activiteit van de −685/+13 constructen werd niet significant verlaagd door de −514C→T substitutie alleen. Co-transfectie van HepG2 cellen met USF1 cDNA liet een dosisafhankelijke toename zien van de promoter activiteit van het −685/+13 construct zien, maar de procentuele stijging was vergelijkbaar voor het C- en T-allel. Deze studie heeft aangetoond dat het algemeen voorkomende *LIPC* promoter polymorfisme functioneel is, en de associatie van het −514T allel met een lagere HL expressie kan verklaren.

In **hoofdstuk 7** hebben we de hypothese getest dat de HL expressie wordt gereguleerd als een integraal onderdeel van de intracellulaire lipiden homeostase. Om dit aan te tonen werden HepG2 cellen geïncubeerd in aanwezigheid van oleaat. Hierdoor werd de HL secretie en de transcriptionele activiteit van een HL (-685/+13) promoter–reporter construct verhoogd. De cholesterolsynthese remmer atorvastatine deed deze stimulatie door oleaat teniet. De transcriptionele activiteit van een HMG-CoA synthase promoter construct werd verlaagd door oleate en verhoogd door atorvastatine, wat betekent dat het effect mogelijk loopt via Sterol-Regulerend-Element Bindend eiwit (SREBP). Co-transfectie met een SREBP-2 expressie vector verlaagde de HL promoter activiteit en verhoogde de HMG-CoA synthase promoter activiteit. Upstream Stimulatory Factors (USF) zijn net als SREBP's betrokken bij het in standhouden van de glucose- en lipid homeostasis. Co-transfectie met een USF-1 expressie vector stimuleerde de HL promoter activiteit. De USF-gestimuleerde HL promoter activiteit werd niet verder gestimuleerd door oleaat, maar werd bijna geheel tegengegaan door atorvastatine of co-transfectie met de SREBP-2 expressie vector. De tegengestelde regulatie door USF-1 en SREBP-2 werd ook waargenomen bij het HL (-305/+13) promoter construct, waarin potentiële SRE- en E-box elementen ontbreken. Deze experimenten laten zien dat de tegengestelde regulatie van de HL expressie door vetzuren en statinen wordt gemedieerd via SREBP, mogelijk door middel van competitie met USF.

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Curriculum Vitae

Gert Jan Botma werd samen met zijn tweelingbroer Henk Jan in Bussum geboren op 21 oktober 1966. Na het behalen van het HAVO diploma op de scholengemeenschap Godelinde te Naarden, werd het VWO diploma gehaald op het Willem de Zwijger college te Bussum. Na een jaar rechten te hebben gestudeerd werd in 1988 begonnen met de studie Biologie aan de Universiteit van Amsterdam. Na de propedeuse werd gekozen voor de studierichting Medische Biologie. Tijdens de eerste stage werd onderzoek gedaan op de afdeling van Haemostase, Thrombose and Atherosclerose van het AMC (Universiteit van Amsterdam) naar twee natuurlijk voorkomende mutaties in het lipoproteine lipase gen onder leiding van Dr. Taco Bruin en Dr. John J.P. Kastelein. De tweede wetenschappelijke stage werd uitgevoerd op de afdeling klinische chemie AMC (Universiteit van Amsterdam) onder leiding van Dr. Andre A. van den Berg en Dr. Abert van Gennip. Onderzocht werd de invloed van mycofenolzuur en deazauridine op het ribonucleotiden metabolisme. In 1994 werd de studie afgesloten en werd aangevangen met wederom een onderzoek op de afdeling van Haemostase, Thrombose and Atherosclerose van het AMC. Onder leiding van Drs. Jan Albert Kuivenhoven en Dr. John J.P. Kastelein werd onderzoek gedaan naar genetische determinanten van HDL concentraties en naar mutaties in het LCAT gen in relatie tot fish eye disease In 1995 werd gestart met een door de Nederlandse Hart Stichting gesubsidieerd AIO project onder leiding van Dr. Adrie J.M. Verhoeven en Prof. Dr. Hans Jansen op de afdeling Biochemie-COEUR (Cardiovasculair Onderzoeks-instituut Erasmus Universiteit Rotterdam), Faculteit Geneeskunde van de Erasmus Universiteit Rotterdam. Het project betrof: “The role of the 40 kD hepatic lipase protein in liver cholesterol homeostasis: regulation and function of the truncated HL gene product”. In 1999 werd het project ‘’Mechanism of action of atorvastatin: effects on the expression of hepatic lipase’’ gesponsord door Pfizer gestart. In 2001 werd de overstap naar het bedrijfsleven gedaan en wel in de positie van Drug Safety Officer bij Vigilex te Rotterdam. Vanaf 2003 is Gert Jan werkzaam bij Centocor te Leiden als Senior Pharmaco-vigilance Associate.

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Het grootste deel van de tijd bij Biochemie is door gebracht op Lab 7. Van Mirjam Heuveling heb ik de eerste kneepjes van de moleculaire biochemie geleerd. We hebben heel veel samen gewerkt en veel lol gehad, ik kijk er graag naar terug, Mirjam dank je wel. Ook met alle andere vaste medewerkers/gasten van Lab 7 was het een leuke tijd, Lab 7 stond voor jeugdige gezelligheid: Amad Javadi, Arthur Osterop, Karin Eizema, Marga van Setten, Han van Heugten, Astrid van de Tuijn en natuurlijk Adrie Verhoeven, de iets minder vaste gasten Jaap Deinum, Jeanette van Gool, Dick Dekkers en Karel Bezstarosti.
Soms had ik het gevoel dat er een stortvloed van stageres op mij afkwam, maar het bracht wel extra leven in de brouwerij: Lesley Siebert, Angelique Hardon, Jeroen Vermunt, Jeroen van den Bosch, Ruth Samuels, Galina Ladyjanskaja, Maya Kumar, Thaddeus Lancaster, Sjakila, Mohamed en Georgette, bedankt.
Zeker zijn er mensen vergeten maar de dank is voor iedereen.

Cecile Hanson en Rinus Machielse horen eigenlijk bij alle labs thuis, dus ook bij de onze. Bedankt voor jullie interesse in het werk maar ook voor de persoonlijke belangstelling.

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Se'irna