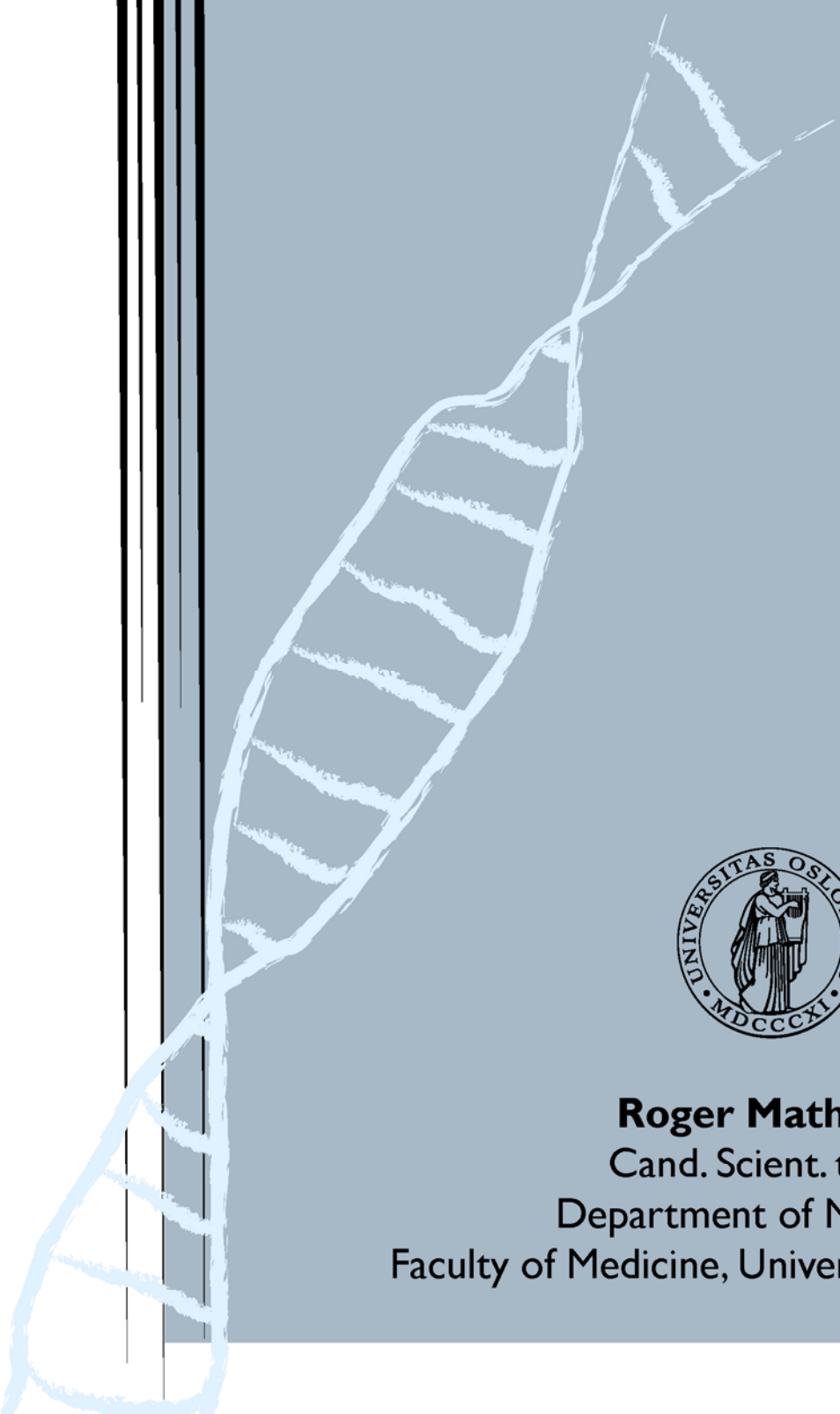
Identification of the lipid droplet-associated protein adipophilin as a novel liver X receptor target gene



Roger Mathisen
Cand. Scient. thesis
Department of Nutrition
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Acknowledgments

This work was conducted at Department of Nutrition, University of Oslo from August 2002 to May 2004, in the laboratory of Associate Professor Hilde Irene Nebb.

I would like to express my gratitude to supervisor Hilde Irene Nebb for including me in her group, for introducing me to the field of nutrigenomics, and for enthusiasm and support. I am also grateful to my co-supervisor Knut Tomas Dalen. Your detailed knowledge about molecular nutrition and enthusiastic interest in science has been a source for inspiration. I gladly express my gratitude for support, educational discussions and the challenges you have provided me with. Special thanks to Stine Ulven, Borghild Arntsen and all members of Nebb's research group for valuable advices and always being helpful and enthusiastic.

I would also like to acknowledge the following persons: Lise von Krogh for helping me with the front cover; Inger Ottestad and Helen Engelstad Kvalem for excellent collaboration during the last 5 years as students; My family and friends outside the world of nutrition for being supportive and patient with my lack of spare time during the last months; Ingvild Paur Knudsen, Tuva Dahl and Sverre Holm for support, fruitful discussions, never-ending coffee breaks and for being good company during the last years both in the lab and the reading room.

Finally, I would like to thank Tone Kristin Omsland for taking the time to read through my thesis, and for endless support and encouragement during the last 20 years.

Oslo, May 2004

Roger Mathisen

Abbreviations

ABC	ATP-binding cassette
ACC	Acetyl-CoA Carboxylase
ACO	Acyl-CoA Oxidase
ADP	Acetyl-Podocarpic Dimer
ADRP	Adipose differentiation-related protein
AF	Transactivation factor
aP-2	adipocyte protein-2
Apo	apolipoprotein
AR	Androgen Receptor
ARC	Activated Recruited Coactivator
BAT	Brown Adipose Tissue
bHLH-Zip	Basic Helix-Loop-Helix leucine Zipper family
BSA	Bovine Serum Albumin
cAMP	cyclic Adenosine 3',5' monophosphate
CAR	Constitutive Androstane Receptor
cEBP	CCAAT/enhancer binding protein
CETP	Cholesteryl Ester Transfer Protein
CIAP	Calf Intestinal Alkaline Phosphatase
CoA	Coenzyme A
CNS	Central Nervous System
CTD	C-Terminal Domain
Cyp7A1	Cholesterol 7-hydroxylase
DBD	DNA Binding Domain
DHA	Docosahexaenoic acid
DOKO	Double knock-out
DNA	Deoxyribonucleic acid
DPE	Downstream promoter element
DRn	Direct Repeat n
DRIP	Vitamin D Receptor Interacting Ptotein
EMSA	Electrophoretic mobility shift assay
ER	Estrogen Receptor
ERn	Everted Repeat n
FA	Fatty acid
FAS	Fatty Acid Synthase
FATP	Fatty Acid Transporter Protein
FCS	Fetal Calf Serum
FFA	Free Fatty Acids
FXR	Farnesoid X Receptor
GFP	Green Fluorescent Protein
GK	Glucokinase
GR	Glucocorticoid Receptor

HDL	High Density Lipoprotein
HRE	Hormone Responsive Element
HNF	Hepatic Nuclear Factor
HMG	3-hydroxy-3-methylglutaryl
IDL	Intermediate Density Lipoprotein
Inr	Initiator
IRn	Inverted Repeat n
IRS	Insulin Response Sequence
LBD	Ligand Binding Domain
LCAT	Lecithin cholesterol cyl transferase
LCPUFA	Long-chain polyunsaturated fatty acids
LD	Lipid droplet
LDAP	Lipid droplet-associated protein
LDL	Low Density Lipoprotein
LPDS	Lipoprotein deficient serum
L-PK	Liver-type Pyruvate Kinase
LPL	Lipoprotein lipase
LRH-1	Liver Receptor Homolog 1
LXR	Liver X Receptor
LXRE	LXR Responsive Element
MAPK	Mitogen Activated Protein Kinase
NHR	Nuclear Hormone Receptors
NR	Nuclear Receptor
GFP	Green fluorescent protein
pEGFP	Enhanced Green fluorescent protein plasmid
PEPCK	Phosphoenolpyruvate Carboxykinase
PGC-1	PPARgamma Coactivator-1
PIC	Preinitiation complex
PI-3	phosphatidylinositol 3-
PKA	Protein Kinase A
PLTP	Phospholipid transfer protein
PP	Peroxisomal Proliferator
PPAR	Peroxisomal Proliferator Activated Receptor
PPRE	PP responsive elements
PXR	Pregnane X Receptor
PUFA	Polyunsaturated fatty acids
RXR	Retinoic X Receptor
SCAP	SREBP cleavage activating protein
SCD	Stearoyl-CoA Desaturase
SF-1	Steroidogenic Factor-1
SGBS	Simpson-Golabi-Behmel Syndrome
SHP	Small Heterodimer Partner
SRB	Suppressor of RNA polymerase B
SREBP	Sterol Regulatory Element Binding Protein
SQS	Squalene Synthase

SXR	Steroid Xenobiotic Receptor
TAF	TBP Associated Factor
TAG	Triacylglycerol
TBP	TATA box Binding Protein
TF	Transcription Factor
TIP	Tail Interacting Protein
TNF-	Tumor Necrosis Factor-
TRAP	Thyroid hormone Receptor Associated Protein
TSS	Transcription Start Site
TTA	Tetradecyl Thioacetic Acid
TZD	Thiazolidinedione
USF	Upstream Stimulatory Factor
VLDL	Very Low Density Lipoprotein
WAT	White Adipose Tissue
22(R)-HC	22(R)-hydroxycholesterol

Summary

Liver X receptors are nuclear receptors that regulate cholesterol and fatty acid metabolism. Despite the critical role fatty acids play in energy metabolism, the information is scarce regarding molecular processes governing their deposition and recovery from lipid droplets (LDs). Adipophilin/Adipose differentiation-related protein (ADRP) is a prominent lipid droplet-associated protein (LDAP) and is postulated to be necessary for formation and cellular function of LD structures.

The present study was undertaken to reveal the gene regulation of adipophilin. This work shows that LXRα binds an identified LXR response element (LXRE) in the human adipophilin promoter. It also demonstrates adipophilin responsiveness upon LXR activation in transfection reporter assays.

Our data indicates that adipophilin is a novel and functional LXR target gene. This is the first report to demonstrate that a member of the lipid droplet-associated protein family is regulated by LXR and bring further evidence to LXRs interplay in cholesterol and lipid metabolism.

1. Introduction

1.1 Nutrition and gene regulation

The modern life style has led to an epidemic of obesity and comorbidities such as type 2 diabetes, hypertension, hyperlipidemia and cardiovascular disease (1). There is a growing realization that the effects of nutrition on health and disease cannot be understood without a profound understanding of how nutrients act at the molecular level. It is now evident that, as well as their function as fuel and co-factors, micro- and macronutrients can have important effects on gene and protein expression and, accordingly, on metabolism. The challenge is to identify nutrient-influenced molecular pathways and determine the down-stream effects of specific nutrients (2).

Transcription factors are the main agents through which nutrients influence gene expression. The nuclear hormone receptor superfamily of transcription factors, with 48 identified members in the human genome, is the most important group of nutrient sensors (3-5). Numerous receptors in this superfamily bind nutrients and their metabolites (Table 1.1). These include retinoic acid receptor (RAR), retinoid X receptor (RXR), peroxisome proliferator-activated receptors (PPARs), liver X receptor (LXR), vitamin D receptor (VDR), farnesoid X receptor (FXR), constitutively active receptor (CAR) and pregnane X receptor (PXR) (3-6).

Several physiological processes are regulated by nuclear receptors (NRs), and this superfamily mediate the transcription activity of endogenous and exogenous ligands (5;7-10). The above mentioned NRs heterodimerize with RXR to specific conserved nucleotide sequences, the so called hormone response elements (HREs) in the promoter regions of their target genes. In unliganded form, NRs are often associated with co-repressors either as complex on DNA or in cytosol. During ligand binding, NRs undergo a conformational change that results in the coordinated dissociation of co-repressors and the recruitment of co-activator proteins. This activation will

facilitate transcription. In metabolically active organs, such as the liver, intestine and adipose tissue, these transcription factors act as ligand sensors by changing the level of DNA transcription of specific genes in response to ligand changes (3). NRs have important roles in the regulation of numerous processes, including nutrient metabolism, embryonic development, cell proliferation and differentiation.

Table 1.1 Transcription-factor pathways mediating nutrient–gene interactions

Nutrient	Compound	Transcription factor
Macronutrients		
Fats	Fatty acids	PPARs, SREBPs, LXR, HNF4, ChREBP, RXR
	Cholesterol	SREBPs, LXRs, FXR, ROR
Carbohydrates	Glucose	USFs, SREBPs, ChREBP
Proteins	Amino acids	C/EBPs
Micronutrients		
Vitamins	Vitamin A	RAR, RXR
	Vitamin D	VDR
	Vitamin E	PXR
Minerals	Calcium	Calcineurin/NF-ATs
	Iron	IRP1, IRP2
	Zinc	MTF1
Other food components		
	Flavonoids	ER, NFκB, AP1
	Xenobiotics	CAR, PXR

Abbreviations used: AP1, activating protein1; CAR, constitutively active receptor; C/EBP, CAAT/enhancer binding protein; ChREBP, carbohydrate responsive element binding protein; ER, oestrogen receptor; FXR, farnesoid X receptor; HNF, hepatocyte nuclear factor; IRP, iron regulatory protein; LXR, liver X receptor; MTF1, metalresponsive transcription factors; NFκB, nuclear factor kB; NF-AT, nuclear factor of activated T cells; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; SREBP, sterol-responsive-element binding protein; USF, upstream stimulatory factor; VDR, vitamin D receptor.

1.2 The basal transcriptional machinery

Regulation of gene expression is complex and depends on binding of transcription factors to sequence specific sites in the promoter of their target genes. Usually several such response elements are located immediately 5' of the transcription start site (TSS) of the gene, but expression may also depend upon other response elements upstream and downstream from the transcription start. The complex regulation of genes involves several different sets of response elements (Figure 1.1). The core promoter is located upstream of TSS and often includes a TATA box and an initiator (Inr). The TATA box, the Inr and the downstream promoter element (DPE) bind the transcription factor II D (TFIID) Inr complex (11). Enhancers and silencers are located both in close proximity and distal to the core promoter while insulators usually are found far from TTS. Additional proximal sequence specific regulatory factors do not act as activators or repressors, but rather works as "docking sites" recruiting enhancer complexes to the core promoter (12). Silencers mediate repression of transcription, while insulators prevent enhancers associated with adjacent genes to inappropriately regulate other genes (13). Enhancers, silencers and insulators work over great distances in the promoter and increase or suppress activity of the core promoter. They can be located upstream, downstream or within the transcriptional unit, but do not have any promoter activity themselves (14).

A variety of A/T rich sequences can function as a TATA box, but the sequence 5'-TATATAAG-3' was identified as optimal for binding of the TATA box binding protein (TBP) (15). The initiator element is located at the TSS and contributes to the accurate initiation and strength of the promoter. The Inr is most probably involved in recognition and stabilisation of the TFIID complex. The spacing between the Inr and the TATA box strongly determines whether they act synergistically or independently. The interplay with the downstream promoter element is also important for stable TFIID binding. The downstream promoter element (DPE) acts together with Inr to recruit the TFIID complex (16), but DPE does not bind TBP itself. DPE does not

function independently of the Inr as the TATA box does and is most commonly found in TATA less promoters (17). The transcription factor II B (TFIIB) binding element is located immediately upstream of the TATA box in many promoters where it helps initiate transcription by recognizing the TFIIB factor (18).

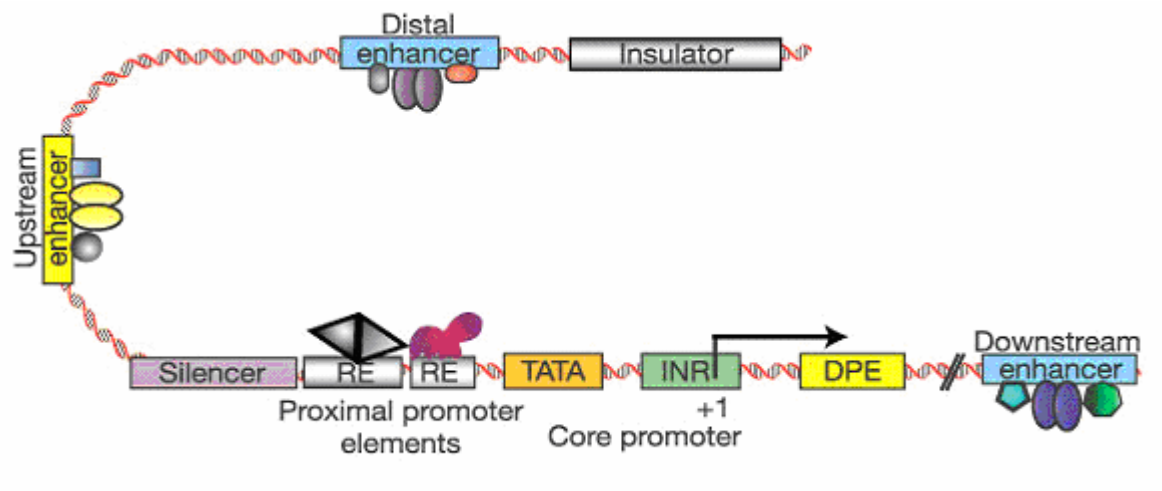


Figure 1.1 The transcriptional module responsible for controlling gene expression

A complex arrangement of multiple clustered enhancer modules interspersed with silencer and insulator elements which can be located 10–50 kb either upstream or downstream of a composite core promoter containing TATA box (TATA), response elements (RE), initiator sequences (INR), and downstream promoter elements (DPE) (14).

1.3 Nuclear receptors

The nuclear receptor superfamily describes an evolutionary conserved group of transcription factors (19). All NRs share a common structural organization (19;20). The N-terminal domain is the least conserved domain and contains a ligand-independent transactivation function (AF-1). Centrally is the DNA binding domain (DBD) responsible for sequence specific binding of the NRs to DNA and dimerization to other NRs. The DBD is highly conserved in NRs. The moderately conserved C-terminal domain contains a strong dimerization interface for other NRs, a ligand binding domain (LBD) and a ligand-dependent transactivation domain. The hinge domain between DBD and LBD allows a flexible three-dimensional structure. Two nuclear localization signal sequences are found in the hinge and C-terminal domain. Some NRs have an additional domain at the extreme C-terminal (F-domain). The function of this domain is poorly elucidated.

Most NRs bind to their cognate HRE as homo- or heterodimers (Figure 1.2). The response element sequence consists of hexamer half sites separated by variable length of nucleotides. A zinc-finger motif in each receptor monomer recognizes the nucleotide sequence on the target DNA. To recruit a receptor dimer, a functional HRE must contain two half sites arranged in a specific orientation and spacing. For instance, thyroid hormone receptors (T3Rs) preferentially bind to two AGGTCA half sites oriented as direct repeats with a four-base spacer (DR4); retinoic acid receptors (RARs) bind to the same AGGTCA half sites, but oriented as a DR5; estrogen receptors bind to AGGTCA half sites oriented as an inverted repeat with a three-base spacer (INV3); and androgen receptors (ARs) recognize an INV3 orientation containing AGAACA half sites (21).

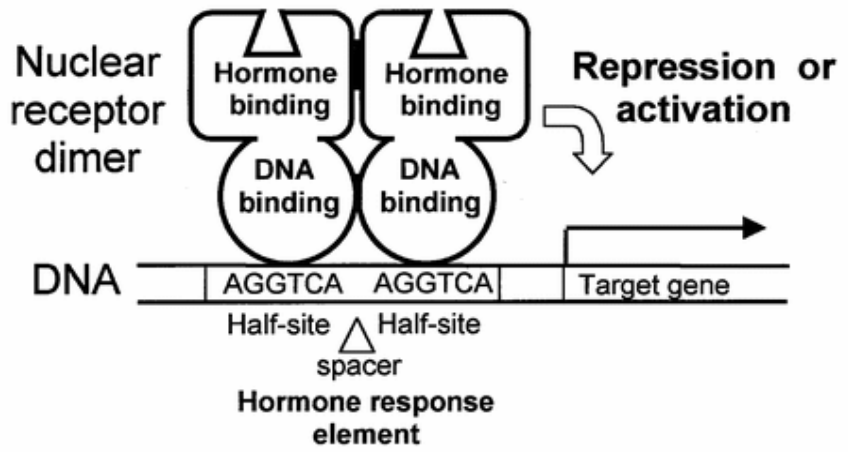


Figure 1.2 Nuclear receptor binding to DNA

A nuclear receptor dimer bound to a HRE upstream of a target gene. Each receptor is represented as two domains: DNA binding and hormone binding. The HRE shown here is composed of two AGGTCA half sites in a direct repeat separated by a spacer; different half-site sequences, spacings, and orientations select for the binding of different nuclear receptors. Adapted from (21).

NRs have been classified according to their binding and dimerization characteristics (Figure 1.3). The first class includes the classical steroid hormone receptors that bind as homodimers to inverted half sites separated by three nucleotides (IR3). The second class receptors bind to DR as heterodimers with RXR. The third class includes orphan NRs that bind as homodimers to DRs, while class four include orphan NRs binding as monomers to single response elements. Orphan NRs are receptors without any known physiological ligands. Recently, analysis based on the distribution of differentially conserved residues in the LBDs leads to the partition of the entire NR superfamily into two mutually exlusive classes and suggests the following proposals: Class I NR LBDs form RXR-like homodimers and Class II NRs form stable LBD heterodimers with RXR/USP or monomers (22) (Table 1.2).

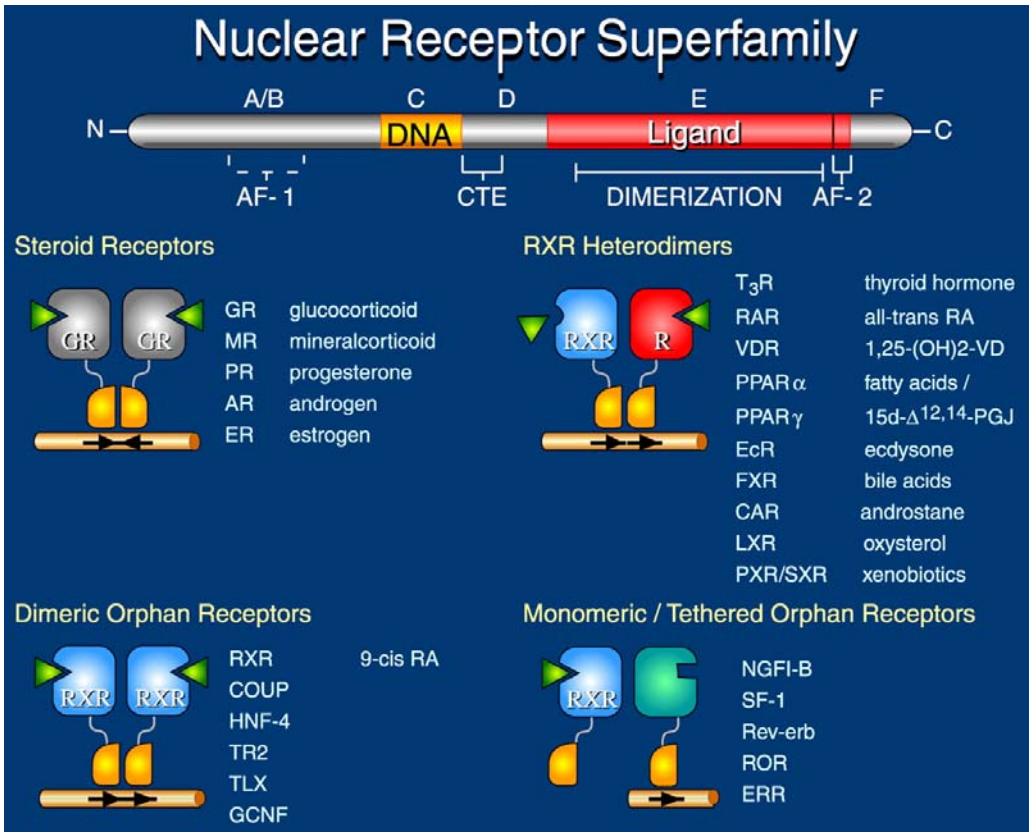


Figure 1.3 Structure/function organization of nuclear receptors

The six domains (A–F) of nuclear receptors comprise regions of conserved function and sequence. All of the nuclear receptors contain a central DBD (regionC), which is the most highly conserved domain and includes two zinc finger modules. A LBD (region E) and AF-II is contained in the C-terminal half of the receptor. Situated between the DBD and LBD is a variable length hinge domain (region D), and variable N-terminal region (A/B) contains AF-I activation function. Most receptors also contain a variable length C-terminal region F, the function of which is poorly understood. Many members of the nuclear receptor family form homo- or heterodimers, and amino acid sequences important for dimerization are contained within the DBD and LBD. Adapted from (19).

Class I	Class II
HNF4*	**TR****
DHR96	**RAR***
RXR*, **USP***	**PPAR***
TR2, TR4	RevErb, E75
DHR78	E78
TLL, PNR	**ROR**, HR3
COUP, SVP, EAR2	**EcR****
ER*	**LXR****, FXR
ERR*	**VDR****, **PXR****, CAR
GR*, **PR***, **AR**, MR	*NGFIB, Nurr1**, NOR1*
SF1, **LRH1**, FTZ-F1	**DHR38****
DHR39	
GCNF1	
Dax1, SHP	
All *C, elegans*	

Table 1.2 Class partition of nuclear receptors based on structure-based sequence analysis

Only a representative subset including all human receptors of the entire superfamily is shown here but the partition encompasses all NRs. Experimentally verified homodimers or heterodimers are indicated by (*) or (**), respectively. The known 3D LBD structures are in bold. Adapted from (22).

1.4 PPARs and LXRs

Peroxisome proliferator-activated receptors (PPARs) are important regulators of lipid and carbohydrate metabolism, and have become major targets for intervention in metabolic diseases (19). This subfamily of NRs comprises three distinct members encoded by separate genes: PPARα, PPARβ/δ and PPARγ. The PPARs bind to a PPRE (DR1) in the target gene promoter and regulates the gene expression (10;23).

PPARα is predominantly expressed in fat-burning tissues such as liver, kidney, heart, skeletal muscle and adipose tissue, but a lower level of expression is generally detected in all tissues examined (24). PPARα serves as the receptor for a structurally diverse class of compounds, including polyunsaturated fatty acids (PUFAs) and fibrates (25;26). PPARδ is ubiquitously expressed and its specific role remains unclear, even though it has been implicated in a wide range of physiological and pathophysiological prosesses such as embryonic implantation, wound healing, inflammation, cancer and osteoporosis, and recently in macrophage differentiation (27). Fatty acids such as palmitic acid and aracidonic acid, and the syntetic agonist GW501516 are known ligands for PPARδ (25;26).

PPARγ exists in two protein isoforms that are created by alternative promoter usage and alternative splicing at the 5' end of the gene (28). Whereas many tissues express a low level of PPARγ_1, PPARγ_2 is highly fat-selective and expressed at very high levels in adipose tissue. The differentiation and maintenance of adipose tissue is driven by the PPARγ. Natural high affinity ligands for PPARγ have not been identified, but endogenous PUFAs and 15-deoxy-Δ^{12-14}-prostaglandin J_2 (15-PGJ2) shows micromolar affinity for the receptor in line with their serum levels (29;30). Thiazolidinediones (TZDs), a new class of synthetic antidiabetic drugs, have been characterized as high affinity ligands for PPARγ (31).

Cholesterol and sterol homeostasis is another important regulatory pathway closely controlled by NR function. Liver X Receptors (LXRs) are major cholesterol sensors

in the body. The main functions of LXRs are to stimulate transition of cholesterol to bile acids in hepatocytes, inhibit cholesterol uptake in enterocytes and promote efflux of cholesterol from macrophages and possibly adipocytes (reviewed in (4)). All these activities decrease the amount of cholesterol in the body (4;32;33).

LXRα (NR1H3 (Nuclear Receptor Nomenclature Committee, 1999) also described as RLD-1) was first isolated from rat liver and later human liver (34;35). Later LXRβ (NR1H2, also described as OR-1/UR/NER/RIP15) (36-39) was identified. The two isoforms are higly related and share 78 % amino acid sequence identity in both DNA and LBDs.

Similar to other members of the nuclear receptor family, LXRs contain a zinc finger DNA-binding domain and a ligand-binding domain that accommodates specific small lipophilic molecules. Both isoforms form a heterodimer with RXR, and the heterodimers bind to a DR4 (AGGTCA nnnn AGGTCA) LXR response element (LXRE) in the promoter of target genes (35;39). LXR/RXR is a so-called permissive heterodimer, in that it can be activated by ligands for either LXR or RXR.

1.5 LXRs in cholesterol and lipid homeostasis

The LXRβ isoform is ubiquitously expressed in adults (38), whereas the expression of LXRα is predominantly restricted to tissues known to play important roles in lipid metabolism, such as liver, skeletal muscle, adipose tissue, kidney and small intestine, but a lower expression level is also seen in spleen, pituitary and adrenal gland (34;35;40). The major breakthrough in understanding the biological functions of LXRs was the identification of their ligands.

The first LXR activators were identified by screening organic tissue extracts and natural compound libraries. Oxidized forms of cholesterol (oxysterols), which are intermediary substrates in the rate-limiting steps of steroid hormone biosynthesis, bile acid synthesis, and in the conversion of lanosterol to cholesterol, have been identified as the potential physiological ligands for LXRs (Figure 1.4) (41). The most potent LXR activators are 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol and 24(S),25-epoxycholesterol (42). Acetyl-Podocarpic Dimer (APD) (43), T0901317 (44), and GW3965 (45) were later identified as synthetic compounds with ability to activate LXRs. The two latter ones have been widely used in studies to characterize functions of the LXRs. PUFAs are found to be competitive inhibitors of LXR ligands, antagonizing LXR activity by inhibiting binding of the RXR/LXR heterodimer to the LXRE (46;47). Several genes encoding proteins involved in important regulatory steps of body cholesterol transport have been shown to be regulated by LXRs (Reviewed in (48)). LXRs have also been shown to affect major genes encoding proteins that control triglyceride metabolism (48). Thus, LXRs seems to provide peripheral tissues with fatty acids while bringing cholesterol back to the liver.

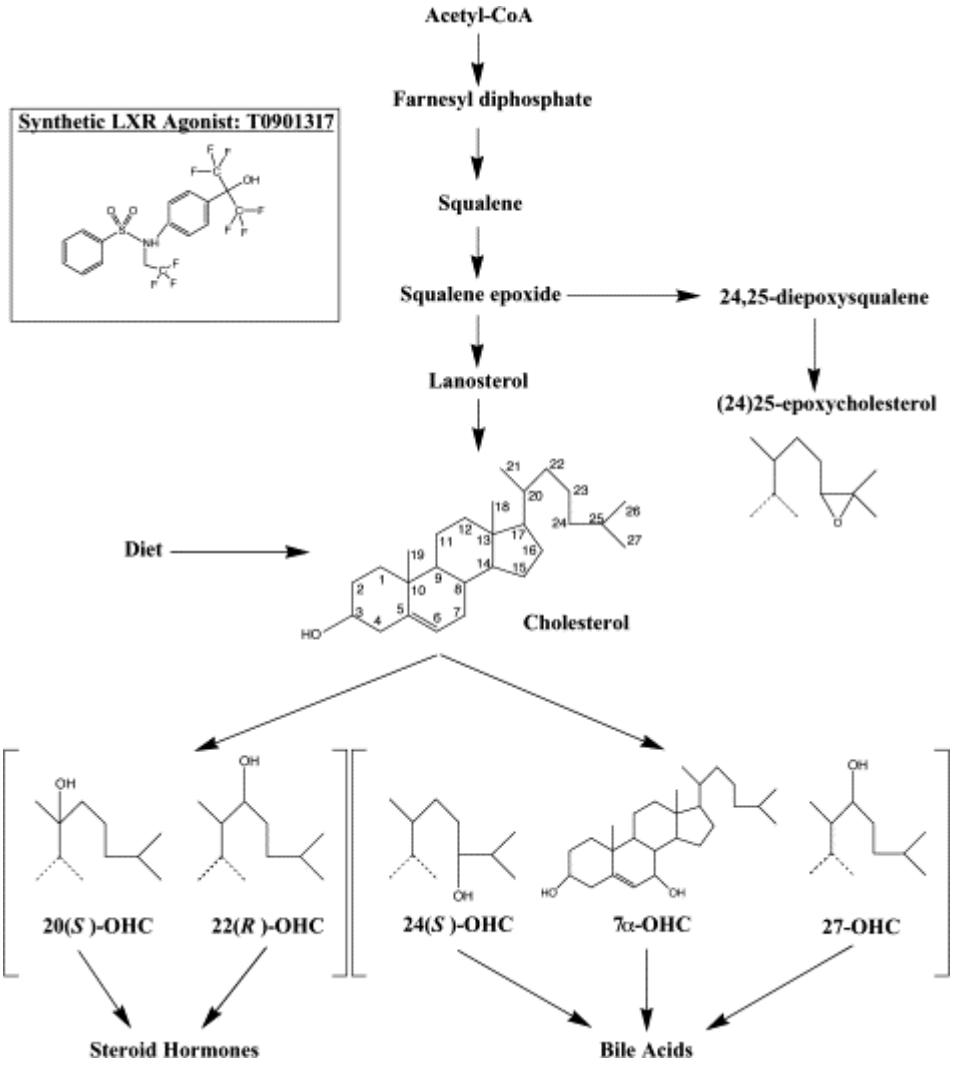


Figure 1.4 Endogenous synthesis of oxysterol ligands for LXR

The synthesis of (24)25-epoxycholesterol, cholesterol and oxidized derivatives of cholesterol and their conversion to bile acids or steroid hormones are shown. The endogenous LXR agonists are bracketed. Many intermediates have been omitted for simplicity. The structure of the synthetic LXR agonist, T0901317, is shown for comparison.

LXRs appear to control all the major steps in the reverse cholesterol transport pathway (Figure 1.5 and Table 1.4). In this process cholesterol is transported from extrahepatic tissues into the liver to be excreted as cholesterol or bile acids into the bile, and ultimately into the gut. Of particular importance in this pathway is that LXRs induce expression of ATP-binding cassettes A1 (ABCA1) (49) and G1 (ABCG1) (50), which are involved in transport of cholesterol and phospholipids from cells to extracellular cholesterol acceptors, notably the lipid-poor apolipoproteins apoAI and apoE. LXRs also induce expression of apoE in macrophages and adipocytes (51), and might enhance reverse cholesterol transport in a tissue-specific manner.

The lipid transfer proteins regulated by LXRs include the phospholipid transfer protein (PLTP) (52) and cholesterol ester transfer protein (CETP) (53). PLTP is involved in the generation of efficient acceptors of cellular cholesterol (preβ-HDL (high density lipoprotein)). In this process PLTP transfers excess lipoprotein surface phospholipids (surface remnants) to lipid-poor apolipoprotein A-I (apo A-I). The remnants are formed when lipoprotein lipase (LPL) hydrolyzes triglyceride-rich lipoproteins such as very low density lipoprotein (VLDL) (54). In addition, PLTP generates preβ-HDL through remodelling of circulating HDL particles. CETP, in turn, transports cholesteryl esters from HDL particles to the apolipoprotein B-100-containing lipoprotein particles (VLDL, intermediate density lipoprotein or IDL, and low density lipoprotein or LDL) (55). This leads to hepatic clearance of the cholesteryl esters when the IDL and LDL particles are taken up by the liver. Finally, in the mouse, but not in humans, LXRs also increase hepatic transcription of 7-α-hydroxylase (56), which is involved in bile acid synthesis and thus drives secretion of the cholesterol taken up by the liver into the bile.

Recent studies have revealed that LXRs are involved in the regulation of triglyceride metabolism and storage (44;57;58). LXRs stimulate fatty acid synthesis in the liver, and the increased quantities of fatty acids in the liver cells then become available for the synthesis of triglycerides, which are subsequently secreted into the circulation as

major components of the triglyceride-rich lipoproteins (VLDLs). The genes involved in fatty acid synthesis and activated by the LXRs include the gene encoding the sterol regulatory element-binding protein 1c (SREBP-1c) and the fatty acid synthase (FAS) (59). Moreover, LXRs also control the synthesis of lipoprotein lipase (LPL) (60), an enzyme located on the luminal surface of vascular endothelial cells hydrolyzing triglycerides and liberating fatty acids into adipose tissue for storage and into skeletal muscle for energy expenditure. Thus, LXRs are involved in fatty acid metabolism by promoting both their hepatic synthesis and their peripheral uptake.

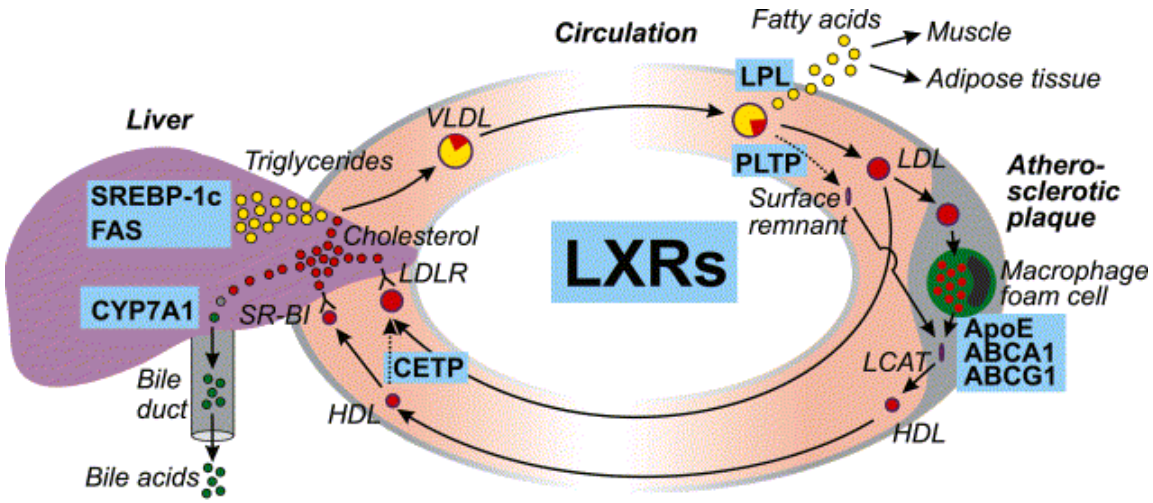


Figure 1.5 LXRs in the regulation of lipid homeostasis of the body

LXRs stimulate hepatic fatty acid synthesis by upregulating the SREBP-1c and FAS. The FAs formed are incorporated into VLDL particles in the form of TG. The TGs of VLDL are hydrolyzed into FFA by LPL in the capillary bed. The resulting LDL particles, the major cholesterol-containing lipoproteins in the circulation, accumulate in the arterial wall, notably in macrophages, causing the formation of foam cells. Efflux of unesterified cholesterol from the macrophages to apoAI- or E-containing small phospholipid disks, surface remnants, is facilitated by ABCA1 and ABCG1. The surface remnants are derived from the surface material of the VLDL particles upon action of LPL and PLTP. Once enriched with unesterified cholesterol, the surface remnants mature into spherical HDL particles as the unesterified cholesterol is esterified by LCAT. The cholesteryl esters of the HDL particles can be selectively taken up by the scavenger receptor BI (SR-BI) in the liver or, alternatively, be transported to larger apoB-100-containing lipoprotein particles (LDL) by CETP. The LDL particles are taken up by the hepatic LDL receptors (LDLR). Cholesterol in the liver can be used for synthesis of VLDL particles, or can be secreted into the bile, either as such or after being converted into bile acids (BA). In this conversion CYP7A1 is rate-limiting. Adapted from (61;62).

Table 1.4 LXR target genes in metabolism

Target gene	Function	Direction/Ref
Lipid cholesterol metabolism		
Cyp7α*	Rate-limiting enzyme in the conversion of cholesterol to bile acids	↑ LXRE (41)
CETP	Mediates transfer of cholesterol esters from HDL to triglyceride-rich lipoproteins	↑ LXRE (53)
ABCA1	Mediates the active efflux of cholesterol from cells to apolipoproteins	↑ LXRE (49;50;63;64)
ABCG1	Mediates the active efflux of cholesterol and phospholipids from cells to apolipoproteins	↑ (63;65)
ABCG4	Cellular transmembrane transport of endogenous lipid substrates	↑ (66)
ABCG5/8	Important role in entero-hepatic sterol transport	(67)
SREBP1c	Transcription factor that regulates expression of lipogenic enzymes	↑ LXRE (58;68;69)
SCD-1/2**	Rate-limiting enzyme in the cellular synthesis of MUFA from saturated fatty acids, an important step in producing TG.	↑ (44)
FAS	Catalyzes the formation of long-chain fatty acids from acetyl-CoA	↑ LXRE (44;59)
ApoE	Facilitates cholesterol efflux outside the enterohepatic axis	↑ LXRE (51;70)
ApoC	Cofactor for LPL in hydrolysis of triglyceride	↑ LXRE (70)
LXRα	Autoregulation	↑ LXRE (71-74)
LPL	Hydrolyzes triglycerides in circulating large lipoproteins	↑ LXRE (60)
PLTP	Transfer phospholipids from triglyceride-rich lipoproteins to HDL	↑ (52)
SR-B1	HDL receptor involved in reverse cholesterol transport	↑ LXRE (75)
Angpt13	A family member of the secreted growth factor angiopoietins	↑ LXRE (76)
Carbohydrate metabolism		
PEPCK	Rate-limiting enzyme in gluconeogenesis	↓ (77-79)
PDK4	Glycolysis inhibitor	↑ (78)
GLUT4	Glucose transporter	↑ LXRE (80;81)
Inflammatory response		
TNF-α	Proinflammatory cytokine	↑/↓ LXRE (82;83)
Others		
11β-HSD-1	Catalyzes the conversion of inactive cortisone to active cortisol	↓ (79)

Only genes with an LXRE found in the promoter and regulated by exposure to an LXR agonist are included.↑ Upregulated; ↓ downregulated. *Not directly LXR regulated in human. **regulated by SREBP-1. Adapted from (84)

1.6 Lipid droplet-associated proteins

To survive periods of nutrient scarcity, animals have evolved the ability to store energy in lipid droplets (LDs) during periods of nutrient excess. LDs are present in the cytoplasm of most eukaryotic cells and consist of triacylglyceride and steryl ester-rich cores surrounded by phospholipid monolayers (3;85-87). Despite the critical role fatty acids play in energy metabolism, there is relatively little information on the molecular processes that govern their deposition and recovery from the lipid droplets.

LDs are thought to form by budding from endoplasmatic reticulum (ER) through an unusual and poorly characterized mechanism. First, neutral lipids synthesized in the ER membrane accumulate in the center of the bilayer and form disks. Next, the disks bulge into the cytoplasm as they enlarge and eventually bud from the ER as LDs, acquiring ER derived phospholipid monolayers in the process. Although no proteins are known to reside in the hydrophobic LD core, several proteins co-localize on the droplet surface. These include P_{200} (88), caveolin-2β (89), vimentin (85;90), mouse adipose differentiation-related protein (ADRP) / human adipophilin (hereafter referred to as adipophilin) (91;92), perilipin (93;94), S3-12 (95) and tail-interacting protein of 47 kDa (TIP47) (96;97). Recently, nearly 40 specifically associated proteins in droplets were identified, suggesting that a specific set of proteins are present in lipid droplets (98). Based on the protein profile, the droplet appears to contain the molecular machinery to synthesize, store, utilize, and degrade various lipids derived from enzymatic activity. In addition, this compartment may be directly involved in membrane traffic and possibly phospholipid recycling, suggesting that the lipid droplet is not a simple cell inclusion but a metabolically active organelle (98).

Perilipin, adipophilin and TIP47 exhibit high sequence identity within an N-terminal motif termed PAT1 and a more distal located PAT2 domain (96;99). A fourth protein, S3-12 has been described along with these PAT family members. S3-12 contains a repeated 33 amino acid motif also found in adipophilin (100), and it shares protein sequence identity to both adipophilin and TIP47 in the C-terminus, but not to

perilipin (99) (Figure 1.6). The precise functions of these lipid droplet-associated proteins (LDAPs) are not known. Perilipin is the best characterized member, and is located on the surface of larger TAG droplets in mature adipocytes (93) and on cholesterol ester droplets in steroidogenic cells (101).

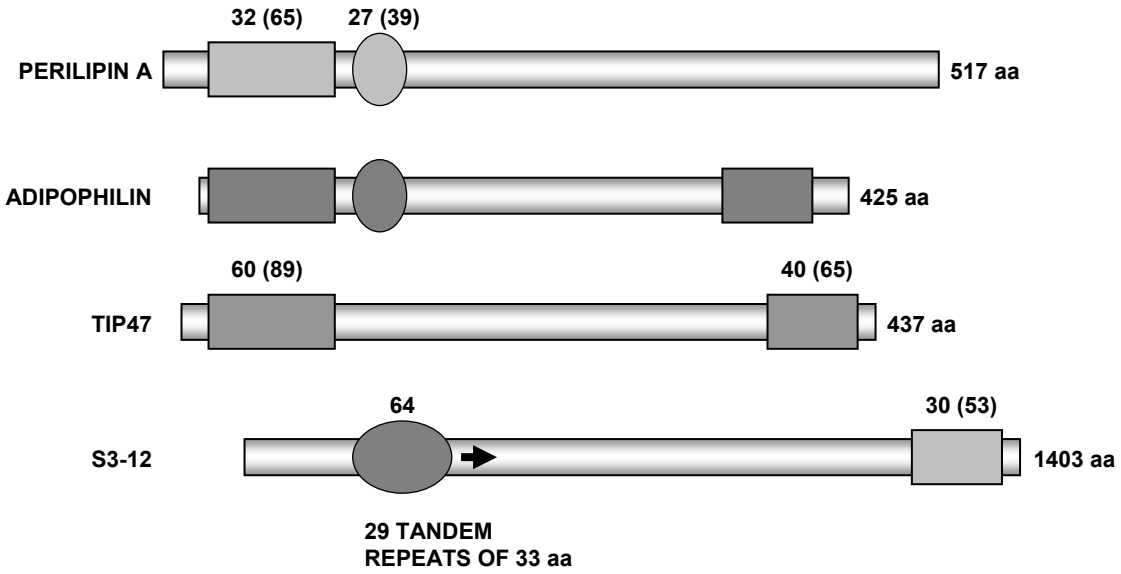


Figure 1.6 Sequence relationships of lipid droplet-associated proteins

The three indicated regions within adipophilin exhibit sequence similarities with the three other proteins shown. Values above each region indicate percent identity and in parantheses percent similarity to adipophilin. Relatedness to adipophilin is also indicated by shading, with darker shading denoting a greater degree of homology. The reported 33-amino acid repeat is not found in TIP-47 (102). Adapted from (85).

Perilipin is the major protein kinase A (PKA) substrate in adipocytes (103), and PKA activation induces phosphorylation of perilipin, ultimately resulting in increased lipolysis, whereas insulin stimulation acts contrarily by facilitating dephosphorylation of the perilipin protein core (104-106). The importance of perilipin in lipid metabolism has been demonstrated in vivo. Consistent with the role of perilipin as a barrier to lipolysis (Figure 1.7), perilipin knockout mice have reduced TAG in their

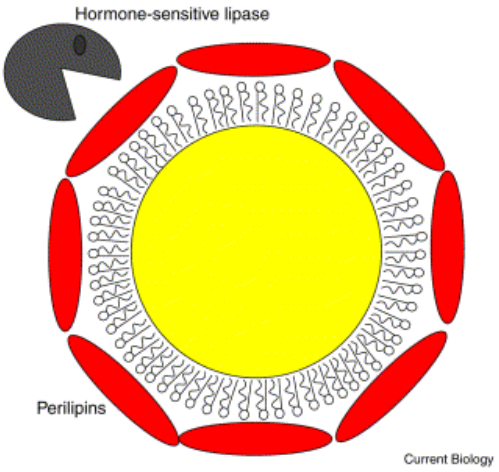


Figure 1.7 Role of perilipins in adipocyte lipid droplets

Lipid droplets contain a neutral lipid core surrounded by a phospholipid monolayer. Perilipins associate with lipid droplets and could plausibly coat the entire surface. Exogenous perilipin expression in 3T3-L1 pre-adipocytes retards triacylglycerol hydrolysis, possibly through sterically blocking access of hormone-sensitive lipase to the droplet surface. Adapted from (90).

adipose tissue (107), are resistant to both genetic and diet-induced obesity (107), show enhanced basal lipolytic rate (108) and fail to respond maximally to lipolytic stimuli (109). TIP47 is thought to act as a cargo selection device for trafficking of mannose-6-phophate receptors (MPRs) from late endosomes to Golgi (110) in addition to its association to lipid droplets (96;97). S3-12 was originally cloned as a surface/membrane-associated protein in adipocytes (100), but later observations suggests that S3-12 coats nascent LDs in adipocytes (95).

Adipophilin is a 50-kDa protein initially cloned from a mouse adipocyte cDNA library (92). Initial studies showed that adipophilin mRNA was expressed primarily in adipose tissue and was induced early during adipocyte differentiation (111;112). However, recent studies indicate that adipophilin is a prominent LDAP found in many mammalian cell types, including hepatocytes, trophoblasts, testicular Leydig and Sertoli cells, adrenal cortex cells, adipocytes, muscle cells, and mammary epithelial cells, either during development (113) or in the mature functioning cell (85;91;113). Adipophilin has been shown to stimulate long chain fatty acid uptake (114), milk lipid secretion (115) and induce formation of lipid droplets (116). Conversely its expression is increased at the transcriptional level in the presence of fatty acids (117). There is also proposed a role for adipophilin in the transfer of lipid between lipofibroblasts and alveolar epithelial cells (118).

Adipophilin may be involved in regulating deposition and release of lipids at LDs and its expression in LDs appears to be modulated by sterol carrier protein-2 (119). Furthermore, adipophilin binds to fatty acids (120) and cholesterol (121). By immunofluorescence microscopy using monoclonal antibody raised to an amino-terminal peptide of adipophilin, the labeling was found as a ring around lipid droplets (122). These properties suggest significance of adipophilin for LD functions, but how the protein attaches to the LD surface has not been demonstrated in detail.

Tauchi-Sato et al recently showed that the surface of LDs is a phospholipid monolayer of unique fatty acid composition (123). The result indicates that the LD surface is a kind of membrane, or a hemi-membrane, but how proteins are bound or integrated in it is not known in detail. In contrast to several other proteins localized to LDs, adipophilin does not have a long hydrophobic domain indicating that adipophilin has two independent domains related to its localization and lipid droplet biogenesis. In lipoproteins are α-helices known to be lipid binding elements (124). It has been suggested that the presence of 11-mer repeats (Figure 1.8) may play an important role in lipid binding of a number of proteins including adipophilin (125), but biological evidence of the importance of this repeat is still lacking.

The molecular mechanisms for regulation of adipophilin expression have not been well understood, but Dalen et al have recently shown that adipophilin, perilipin and S3-12 are PPARγ target genes in adipose tissue (102). A DR1 element in the mouse adipophilin promoter that recruits PPARβ/δ in macrophages has been described (126), but analyses in our laboratory have failed to demonstrate that the human version of this DR1 element is able to recruit RXRα/PPARγ and transactivate the human adipophilin promoter in adipocyte cells (unpublished data). Adipophilin has also altered expression opposit to S3-12 and perilipin in obese compared to lean rats, suggesting that adipophilin is differently and perhaps more complexly regulated. Recent observations in our laboratory have elucidated human adipophilin (112) mRNA expression prior to PPARγ (102), suggesting that adipophilin expression is not dependent on PPARγ as reported for S3-12 and perilipin.

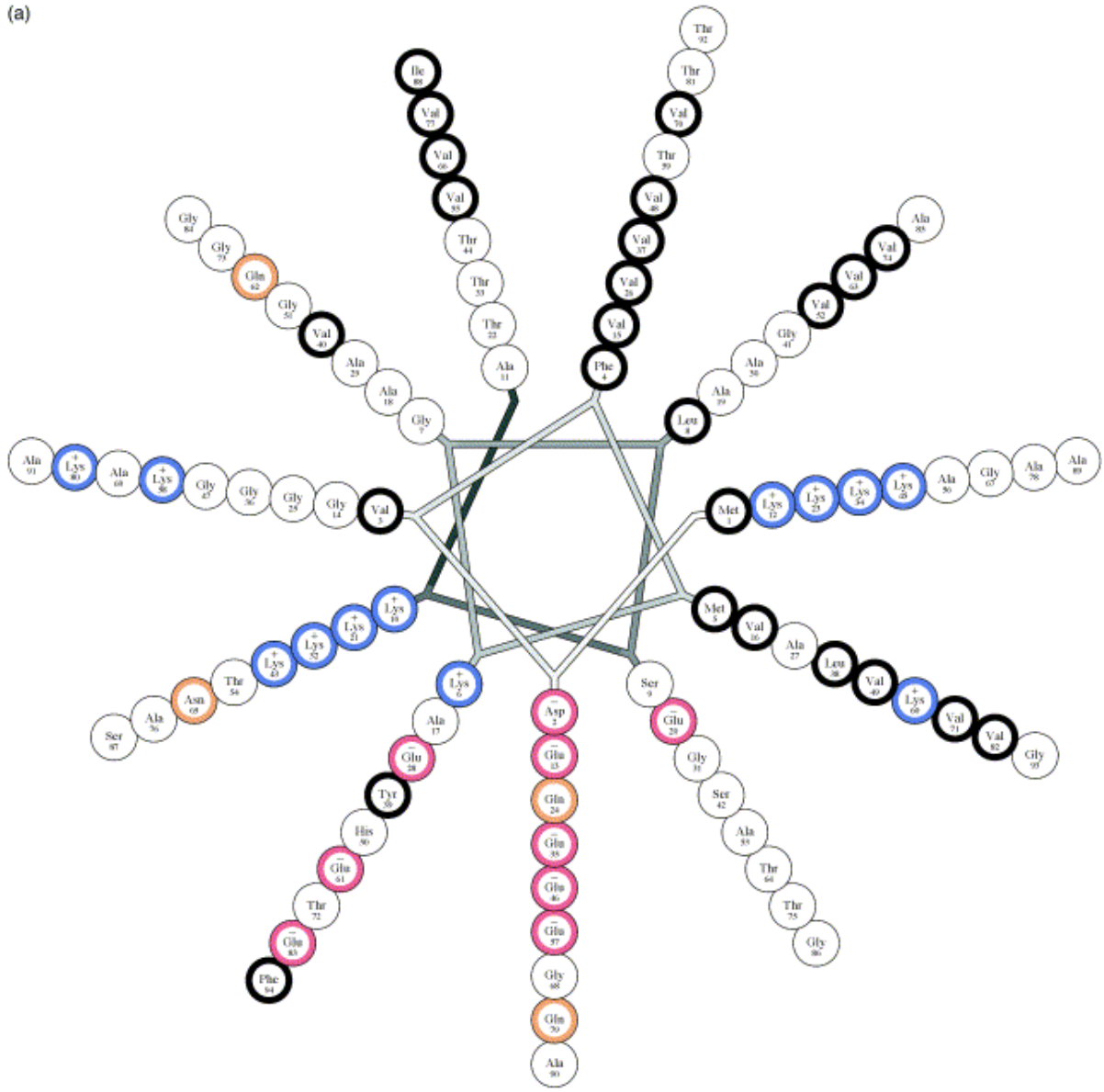


Figure 1.8 Helical pinwheel plot

The 11-mer helix contains hydrophobic amino acids shown on the upper side of this pinwheel plot and polar amino acids on the other side. This structure might give adipophilin both lipid binding and hydrophilic properties. Hydrophobic residues are in black, charged residues in red or blue, and polar residues in yellow. 11-mer repeat: HxxHxxxHxxx, where H are hydrophobic and x are non-hydrophobic residues. Adapted from (125).

2. Objectives

This work takes part in a project with the overall goal to identify novel PPARγ and LXRα/β target genes, and to further identify the biological functions and putative roles of these genes in common metabolic and cardiovascular diseases.

The objective of this thesis was to study gene regulation of the lipid droplet-associated protein adipophilin. Preliminary data suggests that the human adipophilin promoter contains a putative DR4 that may be a functional LXRE. It was therefore of great interest to investigate whether human adipophilin was a novel LXR target gene.

The focus of the work presented in this thesis was aimed at:

- Examine whether human adipophilin show responsiveness upon LXR activation in transfection studies
- Test whether mutation of the putative DR4 will abolish induction by LXR activation in transfection studies
- Examine if LXRα bind to the DR4 in the adipophilin promoter

3. Materials

3.1 Bacteria

One Shot INVαF competent E. coli	Invitrogen

3.2 Cell lines

Human liver HepG2 cells	ATCC
Humane stellate LI90 cells	Japanese Collection of Research Bioresources (JCRB)
Monkey kidney COS-1 cells	ATCC
Simpson-Golabi-Behmel syndrome (SGBS) cells	Wabitsch, University of Ulm

3.3 Cell medium

Dulbecco's Modified Eagle's Medium (DMEM)	Sigma
Fetal Calf Serum	Sigma

3.4 Chemicals

γ-^{32}P[ATP]	Amersham
[α-^{32}P] dCTP	Amersham
2-mercaptoethanol	Sigma
Acetic acid	Sigma
Acrylamide (40 %)/Bic solution 19:1 (161-0144)	BioRad
Agarose	MedProbe
Ammonium per sulphate	BioRad
Ampicillin	Sigma
Apotransferrin	Sigma
Boric acid	Sigma
Bovine Serum Albumin	Sigma
Bromophenol blue	BioRad
$CaCl_2$	Merck
Chloroform	Sigma
Coenzyme A	Sigma
Hydrocortisone	Sigma
Xylene Cyanole FF	BioRad
DEPC	Sigma

Dexamethasone	Sigma
Dextrose	Sigma
DMSO (Dimethyl Sulfoxide)	Sigma
DTT (DL-Dithiothreitol)	Sigma
EDTA (ethylenediaminetetraacetic acid)	Sigma
Ethanol	Merck
Ethidium bromide	Fluka
Ficoll	Sigma
Formaldehyde	Sigma
Formamide	Sigma
Glucose	Sigma
Glycerol	Fluka
Glycin	Sigma
HCl	Chemi-teknikk AS
Hepes (Hydroxyethylpiperazineethanesulfonic acid)	Sigma
IBMX (Isobutylmethylxanthine)	Sigma
Insulin	Sigma
Isopropanol	Arcus
KCl	Merck
KH_2PO_4	Merck
LG100268	GSK
L-glutamine	BioWhittaker
Luciferin	Sigma
Methanol	Merck
$MgSO_4$ x $7H_2O$	Sigma
$MgSO_4$	Sigma
MOPS	Sigma
$Na_2HPO_4x2H_20$	Merck
NaAc (Sodium Acetate)	Merck
Na-citrate	Merck
NaCl	Sigma
NaH_2PO_4	Merck
NaOH	Chemi-teknikk AS
Igepol CA-630 (NP-40)	Sigma
ONPG (o-nitrophenyl-ß-D-galactopyranoside)	Sigma
PBS (Phosphate buffered saline)	BioWhittaker
Phenol	Sigma
PIPES (Piperazin ethansulfonsäure)q	AppliChem
Polyvinylpyrrolidone	Sigma
RNase AWAY	Molecular Bio Products
Rosiglitazone	Alexis
Salmon sperm DNA	Invitrogen
SDS	Sigma
Streptomycin	BioWhittaker
T0901317	Alexis
T3 (Triiodothyronine)	Sigma

TEMED (Tetramethylethylenediamine)	BioRad
Tris-base	Sigma
TRIZOL	Invitrogen
Trypsin	BioWhittaker

3.5 Enzymes and buffers

Buffer B 10X Buffer	Promega
CIAP	Promega
Kinase buffer	Promega
Klenow enzyme	Promega
Labelling buffer	Promega
Ligase 10xBuffer	Gibco BRC
Lysis buffer	Gibco BRC
MULTI-CORE™ 10X Buffer	Promega
Pvu II	Promega
T4 DNA Ligase	Promega
T4 Polynuceotide kinase	Promega

3.6 Equipment

Cell flasks and plates	Corning Incorporated
EMSA gel and electrophoresis equipment	BioRad
Falcon tubes	Falcon
Hypercasette Autoradiography Casettes	Eastman Kodak Company
Hyperfilm MP RPN 2115	Amersham Pharmacia Biotech
Nylon membrane /Amersham Hybond	Amersham
Omnifix	Braun
Parafilm	American National Can
Plastic / glad pack	Tybring-Gjedde
Watman paper / gel blotting paper	Schleicher & Schuell

3.7 Growth media for bacteria

Agar	Merck
Peptone	Merck
Yeast extracts	Merck

3.8 Instruments

AX105 DeltaRange (Weight)	Mettler Toledo

Biofuge fresco (Centrifuge)	Heraeus instruments
Cell counter model Z1	Coulter electronics
Electrophoresis power supply EPS 500/400	Pharmacia
Freezer, -86C	Forma Scientific
Gallenkamp Orbital Incubator	Bergmann
Gel dryer model 583	BioRad
Heλiosγ ThermoSpectronic	Nerliens
Hiclave HV-50 (Autoclave)	HMC/Dipl. Ing Houm
iEMS Reader MF	Labsystems
Incubator	Forma Scientific
Incubator	Thermaks
Kubota KS-5200C (Centrifuge)	Medinor
Labinco L21	Labinco BV, Netherlands
Labinco L46 (Vortex)	Houm
Leica DMIL/MPS 60 (Microscope)	Leica
Luminometer TD-20/20	Turner Design
MilliQ-syntesis	Millipore
Nicon Digital Camera DXM 1200	Nicon
Nicon eclipse TS 100	Nicon
Personal Densitometer SI	Mol Dynamics/Pharmacia
PhosphoImager SI	Mol Dynamics/Pharmacia
Photo-print IP-214-SD	Saveen
PhotoZoom (Microscope)	Cambridge Instruments
Pipeteboy Comfort	Integra Biosciences
PMH 92 LABpH meter	Nerliens/Radiometer
PowerPac 300	BioRad
Techne TE-10D tempunit	Tamro Lab AS
Thermed 5002 electronics	GFC
Transiluminator	LKB, Bromma
Ultraviolett crosslinker	Amersham Life Science

3.9 Kits

JETQUICK Plasmid Maxiprep Kit	Genomed
JETQUICK Plasmid Miniprep Spin Kit	Genomed
ProbeQuant G50 Micro Columns	Amersham Biosciences
QIAEX II Gel Extraction Kit	QIAGEN

3.10 Plasmids and oligonucleotides

pCMX	Mangelsdorf (Dallas, TX)
pCMX-hLXRα	Mangelsdorf (Dallas, TX)

pCMX-RXRα	Mangelsdorf (Dallas, TX)
pEGFP-N1	Clontech
pGL3-Basic	Promega
Primers	Sigma
pSV-β-Gal	Promega

3.11 Software and internet resources

Adobe Illustrator 10	Adobe
Automatic camera tamer software (ACT-1) V 2.11	Nikon
Genesis Labsystems version 2.12 / 1303	Labsystems
Microsoft Office and Microsoft XP	Microsoft
NEBcutter Version 2.0	New England BioLabs Inc.
PubMed,	National Library of Medicine
Reference manager 10	ISI ResearchSoft
SPSS 11	SPSS Inc.
Wisconsin Package Version 10.0	Genetics Computer Group

4. Methods

4.1 Essential DNA techniques

4.1.1 Spectrophotometric quantitation of nucleic acid

The nucleic acids in both DNA and RNA absorb light in the ultraviolet range (200-400 nm), with an absorption peak at 260 nm. Proteins have an absorption peak at 280 nm. Spectrophotometric readings should be taken at both wavelengths and Kalckar's formula (the OD_{260}/OD_{280} ratio) should be used to provide an estimate for the purity of the nucleic acid. Pure preparations of DNA or RNA have the OD_{260}/OD_{280} values of 1.8 and 2.0 respectively. The concentration of DNA or RNA can be estimated by Beer-Lamberts law: $A = \varepsilon \cdot c \cdot l$, were A is absorbance, ε is extinction coefficient, c is concentration of sample and l is length of light pathway (cm). An OD_{260} measurement of 1.0 with l equal to 1 centimetre corresponds to approximately 50 μg/ml double stranded DNA, 40 μg/ml single stranded DNA or RNA and 20 μg/ml single stranded oligonucleotides (127).

Protocol

Dilute the sample in dH_20 and measure the absorbance at 260 and 280 nm in a spectrophotometer. Use quartz cuvettes when measuring, since plastic absorbs light in the ultraviolet range. Use dH_20 for zeroing the spectrophotometer.

4.1.2 Separation of DNA by agarose gel electrophoresis

Agarose gel electrophoresis is a standard method used to separate and identify DNA fragments. Large molecules migrate more slowly because of greater friction drag, since they have greater difficulties migrating trough the pores in the gel than smaller molecules. The DNA is negatively charged at physiological pH and will migrate towards the anode in an electric field. The DNA molecules are visualised by ethidium

bromide, usually added to the gel before it sets. The ethidium bromide intercalates between the bases in a double stranded DNA, and the complex is fluorescent when exposed to ultraviolet light (127).

Protocol

Solutions

Sample buffer (10 x)

50 % glycerol; 50 mM EDTA; 0.25 % (w/v) bromophenol blue; 0.25 % cyanine

TBE buffer (stock solution 5 x)

54 g Tris-base; 27.5 g boric acid; 20 ml 0.5 M EDTA (pH 8.0); dH_20 to a total volume of 1 litre

Ethidium bromide (stock solution)

Dilute 10 mg ethidium bromide per ml

Experimental procedure

Seal the open ends of a plastic tray and place it horizontal on the table. Prepare electrophoresis buffer (0.5 x TBE). Add powder agarose in an aliquot of the buffer and melt it in a microwave oven. (Use 0.5 g agarose in 50 ml buffer to make 1% agarose gel). Cool the solution to 50-60°C and add ethidium bromide to 1 µg/ml final concentration in gel. Place the comb 0.5-1 mm above the plate in position close to the cathode and pour the agarose solution into the mold. Remove the comb carefully and mount the gel in the electrophoresis apparatus. Add TBE buffer to cover the gel and wash the wells with the same buffer. Mix DNA with 6 x sample buffer and load the mixture into the well. Attach the electric leads and apply voltage. Run the electrophoresis at 3-10 V/cm (50-100 V in small gel chambers) for 1 to 3 hours. Detect DNA fragments with an UV detector or in a FlourImager.

4.1.3 Preparation of cloning vectors

Restriction enzymes recognise specific, often palindromic, sequences in double stranded DNA and cleave these by hydrolysis of the phosphodiester bonds in DNA. Restriction enzymes typically recognise specific DNA sequences that are four, five or six nucleotides in length. The localisation of cleavage sites within the sequence differs from enzyme to enzyme. Some cleave both strands exactly in the middle of a sequence, creating fragments with blunt ends. Others cleave at similar locations some basepairs apart on opposite strands in the DNA, creating DNA fragments with single stranded termini.

Calf Intestinal Alkaline Phosphatase (CIAP) catalyzes the hydrolysis of 5´-phosphate groups from DNA, RNA, and ribo- and deoxyribonucleoside triphosphates. This enzyme is used to prevent recircularization and religation of linearized cloning vector DNA by removing phosphate groups from both 5´-termini and may also be used for the dephosphorylation of 5´ phosphorylated ends of DNA or RNA for subsequent labeling with [^{32}P] ATP and T4 Polynucleotide Kinase. CIAP is active on 5´ overhangs 5´ recessed and blunt ends (127).

Protocol

Solutions

Pvu II, Buffer B 10X Buffer, MULTI-CORE™ 10X Buffer, Bovine Serum Albumin, CIAP stop buffer, NaAc, Phenol (TE-saturated), Chloroform.

Experimental procedure

The following procedure is designed for a typical single stranded reaction, containing 0.2-0.5 µg DNA. For digestion of larger amounts of DNA, the reaction mixture should be scaled appropriately. It is important to optimise the temperature, incubation time, pH and salt concentration for optimal digestion of DNA. Use buffer, incubation time and temperature recommended by the manufacturer. Optimal for PvuII: storage at –20°C in 10mM Tris-HCl (pH 7.4), 50mM NaCl, 0.1mM EDTA, 1mM DTT, 0.5mg/ml BSA, 50% glycerol. Incubate in buffer B at 37°C. Cut the cloning vector

with chosen restriction enzyme(s) in appropriate buffer. Incubate for at least 2 hours at 37 °C or another temperature depending on enzyme(s) used. 1 µl vector (1 µg/µl); 1 µl restriction enzyme buffer; 0.1 µl BSA (1µg/µl); 0.1 µl Enzyme A (10 U/µl) (0.1 µl Enzyme B); 77 µl dH_2O. Test the cutting by applying 1 µl of the reaction mixture on an agarose gel. Calculate the amount of CIAP to be used in the dephosphorylation reaction based on the amount of vector used and the size of the cloning vector. Formula: [amount vector (µg)/size vector (kb)*3.03 pmol ends]*[volume of diluted CIAP solution*0.01 U CIAP/pmol ends].

Amount (ug)	Size (kb)	5'-ends (pmol)	dilution CIAP (X ul in 50 ul)
10	3	10,1	5,1
10	4	7,6	3,8
10	4,5	6,7	3,4
10	5	6,1	3,0
10	6	5,1	2,5

Mix the digested vector with the following:40 µl digestion mixture; 5 µl CIAP buffer; 4 µl H_2O; 1 µl CIAP (diluted) = 50 µll total. Incubate at 37 °C for 15 minutes. Then add an additional 1 µl og CIAP (diluted) and incubate for another 15 minutes. Add 300 µl CIAP stop buffer and mix well. Add 175 µl phenol and 175 µl chloroform and mix vigorously. Centrifuge at 13.000 rpm for 5 minutes. Transfer the aqueous phase to a new tube and add 350 µl chloroform. Centrifuge at 13.000 rpm for 5 minutes. Measure the amount of the aqueous phase (~330 µl). Add 0.10x 4M NaAc and 2.5x EtOH (330 µl digestion mixture; 33 µl NaAc; 907.5 µl EtOH) and precipitate the DNA at -80°C for at least 2 hours. Centrifuge at 15.000 rpm at 4 °C for 20 minutes. Discard the aqueous phase and wash the pellet with cold 70 % EtOH. Centrifuge at 15.000 rpm at 4 °C for 20 minutes and discard the aqueous phase. Air-dry the pellet for ~5 minutes and resolve the DNA pellet in ~30 µl H_2O. Estimate the concentration of the cloning vector by electrophoresis, and dilute the vector with H_2O.

4.1.4 Agarose gel extraction

QIAEX II Agarose Gel Extraction is design for the extraction of 40 to 50-kb fragments from 0.3-2 % standard or low-melt agarose gels in TAE or TBE buffers (128).

Protocol

Excise the DNA band from the agarose gel with a scalpel and transfer gel slice to a microfuge tube. Weigh the gel slice in a colourless tube. Add 300 µl Buffer QX1 and 200 µl H_2O to each 100 mg gel. Resuspend QIAEX II by vortexing for 30 seconds and add it to the sample according to the DNA amount; <2 µg DNA add 10 µl, 2-10 µg DNA add 30 µl. Incubate at 50°C for15 minutes to solubilize the agarose and bind the DNA. Mix by vortexing every 2 minutes to keep the buffer in suspension. Check if pH <7.5 and add NaAc if pH is above this level. Centrifuge the sample for 30 sek at maximum speed and carefully remove supernatant with a pipet. Wash the pellet with 500 µl of Buffer QX1, resuspend the pellet by vortexing and centrifuge the sample for 30 seconds. Remove all traces of supernatant with a pipet. Wash the pellet twice with 500 µl of Buffer PE like the Buffer QX1 wash. Air-dry the pellet for 30 minutes. Elute DNA by adding 20 µl H_20 and resuspend the pellet by vortexing. Incubate at 50°C for 5 minutes. Centrifuge for 30 seconds and carefully pipet the supernatant (contains purified DNA) to a clean tube.

4.1.5 Ligation of DNA

Ligation of DNA is catalysed by DNA ligase, an enzyme that joints to pieces of double stranded DNA. T4 DNA Ligase catalyzes the joining of two strands of DNA between the 5´-phosphate and the 3´-hydroxyl groups of adjacent nucleotides in either a cohesive-ended or blunt-ended configuration. The enzyme has also been shown to catalyze the joining of RNA to either a DNA or RNA strand in a duplex molecule but will not join single-stranded nucleic acids (129). The reaction is performed in eppendorf tubes with small volumes (10-15 µl) facilitating annealing of

the two DNA fragments with compatible termini. After annealing, the DNA ligase seals the single stand nick in the DNA (127).

Protocol

It is recommended to use a 1:3, 1:1, or 3:1 molar ratio of vector:insert DNA when cloning a fragment into a plasmid vector. These ratios will vary with other types of vectors, for example, cDNA and genomic cloning vectors. The following example illustrates the conversion of molar ratios to mass ratios for a 3.0 kb plasmid and a 0.5 kb insert DNA fragment: (ng of vector × kb size of insert)/ kb size of vector × (molar ratio of (insert/ vector)) = ng of insert. Assemble the following reaction in a sterile microcentrifuge tube: Vector DNA 100ng; Insert DNA 17ng; Ligase 10X Buffer 1µl; T4 DNA Ligase (Weiss units) 0.1–1U; Nuclease-Free Water to final volume of 10µl. Incubate the reaction at room temperature for 3 hours, 4°C overnight, or 15°C for 4–18 hours.

4.2 DNA cloning in plasmid vectors

Closed circular plasmid DNA is cleaved with one or more restriction enzymes and ligated in vitro to foreign DNA bearing compatible termini, and then the ligation products are transformed into an appropriate strain of E.coli.

4.2.1 Transformation of E. coli

Introduction of foreign DNA to bacteria is called transformation. In this process, bacteria take up free DNA from solution. Linear fragments must be incorporated in the bacteria genome for replication. Plasmids replicate by themselves by acting as extra circular genomes. Transformation cells have to be selected from other cells in the transformation mixture. To ensure this, plasmids contain markers, usually a gene encoding resistance to an antibiotic. Transformed cells are selected by growing the transformation mixture in medium containing the same antibiotic, where only transformed cells are able to live and multiply. During ligation, re-ligation of the plasmid can be a problem. To minimize the possibility for picking cells transformed with a re-ligated vector, the transformed cells are selected by the α-complementation system. The vector carries a short DNA segment containing the regulatory sequence and the coding information of the first 146 amino acid sequence in the β-galactosidase gene. Within this region is a polycloning site that does not interfere with the enzyme activity of the produced N-terminal sequence of the β-galactosidase enzyme. The genome in the E.coli strain codes for the carboxyl-terminal sequence of the β-galactosidase gene. Neither of these fragments are themselves active, but if they associate they form an active enzyme. Insertion of fragments in the polycloning site of the plasmid interferes with the production of the N-terminal fragment making it unable of this α-complementation. An active enzyme hydrolyses X-gal, making a blue product indicating that the colony represents bacteria with re-ligated plasmids without inserts (127).

Transformation using calcium chloride

E.coli cells are grown to log phase. Cells are concentrated by centrifugation and resuspended in a solution containing calcium chloride. Exposure to calcium ions renders the cells able to take up DNA. Plasmid DNA is mixed with cells and presumably adheres to them. The mixture of DNA and cells is then heat shocked, which allows the DNA to efficiently enter the cells. The cells are grown in non-selective medium to allow synthesis of plasmid-encoded antibiotic resistance proteins, and then plated on antibiotic-containing medium to allow identification of plasmid-containing colonies (130).

Protocol

Solutions

LB medium with ampicillin

To 1L of distilled water, add: 10 peptone; 5g Yeast extract; 10g NaCl.

Adjust the pH to 7.0 with NaOH. Autoclave to sterilize. Allow the autoclaved medium to cool to 55°C and add ampicillin (final concentration 100µg/ml). For LB plates, include 15g agar prior to autoclaving.

$CaCl_2$ solution (250 ml)

2.21 g $CaCl_2$; 0.756 g PIPES pH 7.0; 44.1 ml 85 % glycerol. Filter-sterilizing or autoclave.

SOC medium

Tryptone (pancreatic digest of casein), 2% (w/v); Yeast extract, 0.5% (w/v); 8.6 mM NaCl; 2.5 mM KCl; 20 mM $MgSO_4$; 20 mM Glucose

Experimental procedure

Prepare competent cells

Inoculate a single colony of E.coli cells into 50 ml LB medium. Grow overnight at 37°C with moderate shaking. Inoculate 4 ml of the culture into 400 ml LB medium in a sterile 2-liter flask. Grow at 37°C, shaking at 250 rpm, to an OD_{590} of 0.375 (~3 hours). Aliquot culture into eight 50-ml pre-chilled, sterile polypropylene tubes and

leave the tubes on ice for 10 minutes. Centrifuge cells 7 minutes at 1100 x g, 4°C. Allow centrifuge to decelerate without brake. Pour of supernatant and resuspend each pellet in 10 ml ice-cold $CaCl_2$ solution. Centrifuge cells 5 minutes at 1100 x g (2500 rpm), 4°C. Discard supernatant and resuspend each pellet in 10 ml ice-cold $CaCl_2$ solution. Keep resuspended cells on ice for 30 minutes. Centrifuge cells 5 minutes at 1100 x g, 4°C. Discard supernatant and resuspend each pellet in 2 ml ice-cold $CaCl_2$ solution. Dispense cells into pre-chilled, sterile polypropylene tubes. Freeze immediately at -70°C.

Transform competent cells

Aliquot 10 ng of DNA in a test tube and place on ice. Rapidly thaw competent cells by warming between hands and dispense 90 µl immediately into test tubes containing DNA. Gently swirl tubes to mix, then place on ice for 30 minutes. Heat shock cells by placing tubes into 42°C water bath for 1.5 minutes. Place immediately on ice and add 410 µl SOC medium. Mix gently and incubate for 1 hour at 37°C.

4.2.2 Mini preparation of plasmid DNA

Mini preparation is used for the preparation of small plasmid DNA. The DNA yield is up to 30 µg. The procedure employs a modified alkaline/SDS method to prepare the clear lysate. After neutralization, the lysate is applied directly onto a spin column and the plasmid DNA is bound to the absorption matrix. It is then washed to remove RNA, proteins and all other impurities. The purified plasmid DNA is eluted from the spin column in TE buffer or water (131).

Protocol

Solutions

G1: Cell suspension solution: 50 mM Tris/Hcl (pH 8.0); 10 mM EDTA; 100 µg RNase A

G2: Cell lysis solution: 200 mM NaOH; 1% SDS

G3: Neutralisation/Binding solution: Contains acetate and guanidine hydrochloride

G4: Wash solution (reconstituted): Contains ethanol, NaCl, EDTA and Tris/HCl

GX: Wash: Contains acetate, guanidine hydrochloride, EDTA and ethanol

Experimental procedure

Transfer one single bacterial colony into 2 ml LB-medium containing 50 μg/ml ampicillin and incubate overnight at 37°C with vigorous shaking. E. coli cells are pelleted by centrifugation. Remove all traces of medium carefully. Add 250 μl of solution G1 to the pellet and resuspend the cells by vortexing or with a pipette until the suspension is homogeneous. Add 250 μl of solution G2 and mix gently, but thoroughly, by inverting the tube several times. Incubate at room temperature for 5 minutes. Add 350 μl of solution G3 and mix gently but thoroughly, by inverting the tube until a homogeneous phase is obtained. Centrifuge the mixture at 13 000 rpm for 10 minutes at room temperature. Place a spin column into a 2 ml receiver tube. Load the supernatant from step 4 into the spin column. Centrifuge at maximum speed for 1 minute. Discard the flowthrough. Empty the receiver tube, and reinsert the spin column into the receiver tube. Add 500 μl of solution GX and centrifuge at maximum speed for 1 minute. Discard flowthrough and place the column back into the same receiver tube. Empty the receiver tube and reinsert the spin column into the receiver tube. Add 750 μl of reconstituted solution G4 and centrifuge at maximum speed for 1 minute. Discard flowthrough and place the spin column back into the same receiver tube. Centrifuge again at maximum speed for 1 minute. Place the spin column into a new 1.5 ml microfuge tube and add 50 μl of sterile water or buffer directly onto the center of the silica matrix of the spin column. Centrifuge at maximum speed for 2 minutes.

4.2.3 Maxi preparation of plasmid DNA

Maxi preparation of DNA is used for isolation of highly purified large amounts of plasmid DNA. Instead of phenol:chloroform extraction, the DNA is purified on an

anion exchanger in the first purification step. The DNA is then precipitated with isopropanol, washed, dried and dissolved in dH_20/TE-buffer (132).

Protocol

Solutions

E1: Cell resuspending solution: 50 mM Tris; 10mM EDTA; HCl ad pH 8.0

E2: Cell lysis solution: 200 mM NaOH; 1.0% SDS (w/v)

E3: Neutralization: 3.1 M potassium acetate; acetic acid ad pH 5.5

E4: Column equilibration solution: 600 mM NaCl; 100mM sodium acetate; 0.15% TritonX-100; acetic acid ad pH 5.0

E5: Column washing solution: 800 mM NaCl; 100mM sodium acetate; acetic acid ad pH 5.0

E6: DNA elution solution: 1250 mM NaCl; 100 mM Tris-HCl pH 8.5

Experimental procedure

Transfer one single plasmid containing bacterial colony into 500 ml LB-medium containing 50 μg/ml ampicillin. Grow bacteria overnight at 37 °C with vigorous shaking. Split the bacterial medium in 150-200 ml centrifugation tubes. Do not use more than 200 ml medium pr. Tube. Centrifuge at 6.000g for 5 minutes at 4°C, and remove the supernatant. Apply 30 ml solution E4, and let the column empty by gravity flow. Resuspend the bacterial pellet in 10 ml cold solution E1. Add 10 ml of solution E2, mix the solution gently, and incubate at room temperature for 5 minutes. Add 10 ml cold solution E3, mix the solution immediately, but gently, by shaking the tube. Centrifuge the solutions at 15.000g for 10 minutes at 20°C. Apply the supernatant from step 6 to the pre-equilibrated column and allow it to enter the resin by gravity flow. Wash the column with 60 ml solution E5, and elute with 15 ml solution E6. Precipitate the DNA with 0.7 volumes of isopropanol (10.5 ml) at room temperature, and centrifuge immediately at 15.000g for 30 minutes at 4°C. Remove

the supernatant carefully. Wash the plasmid pellet with 5 ml 70 % ethanol, and centrifuge again at 15.000g for 5-10 minutes at 4°C. Remove the supernatant, and put the centrifugation tube upside down for 5 minutes, then turn the tubes and let the pellet air dry in room temperature for approximately 15 minutes. Redissolve the plasmid pellet in approximately 400 µl, dH_2O depending on the size of the pellet, and transfer to a clean eppendorf tube. Wash the tube again with 100 µl dH_2O to increase the yield.

4.3 General techniques for culturing cell lines

To avoid contamination of micro organisms, cells in culture are handled using aseptic techniques. As an extra precaution, the cell culture medium is supplemented with antibiotics to prevent bacterial growth. The cells are cultured in an incubator at 37°C, with 5% CO_2 and humidified air to simulate an in vivo situation. When taken out of the incubator, the cells are immediately placed in sterile cell culture hood. Gloves are ethanolized, and all equipments in use should be sterile, and all bottles or equipment brought into the hood ethanolized.

4.3.1 Thawing of frozen cells

Cells are thawed by agitation in 37°C water bath (within 40-60 seconds). As soon as the ice is melted, the ampule is removed from the water bath. All of the operations from this point on are carried out under strict aseptic conditions. The cell suspension are transferred and diluted with the recommended culture medium in culture flask.

4.3.2 Cell counting

The Coulter counter estimates the number of cells in the suspension. This apparatus measure changes in current when a particle pas trough two electrodes in a current leading solution. These changes correspond with the volume of the particle. A particle size between 7-16 μm has been found to be equivalent to the size of the cells and was defined as the interval for particle counting.

4.3.3 Cell Cultures

Monkey kidney COS-1 cells (ATCC no. CRL 1650)

COS-1 is a fibroblast-like cell line established from CV-1 simian cells which were transformed by origin-defective mutant of SV40 which codes for wild type T antigen. The line contains T antigen, retains complete permissiveness for lytic growth of

SV40, supports the replication of ts A209 virus at 40C and supports the replication of pure populations of SV40 mutants with deletion on the early region. This line contains a single integrated copy of the complete early region of SV40 DNA, and is a suitable host for transfection, especially for vectors requiring expression of SV40T antigen (133). COS-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, penicillin (50 U/ml) and streptomycin (50 μg/ml). Cells were kept subconfluent prior to experiments.

Human liver Hep G2 cells (ATCC no HB-8065)

This human cell line was derived from tissue of a 15-year old male Caucasian. The cells are epithelial in morphology, have a modal chromosome number of 55 and are not tumorigenic in nude mice. The cells produce alpha-fetoprotein, albumin, alpha2-macroglobulin, alpha1-antitrypsin, transferring, alpha1-antichymotrypsin, haptoglobin, ceruloplasmin, plasminogen, complement (C3, C4), C3-activator, fibrinogen; alpha1-acid glycoprotein, alpha2-HS glycoprotein, beta-lipoprotein and retinol binding protein (134-136). Hep G2 cells were cultured as COS-1 cells.

Humane stellate LI90 cells (NIHS/JCRB 0160)

The LI-90 cells are human hepatic ito (fat storing) stellate-shaped mesenchymal tumor cells that exist in the space of Disse of the liver and contain many fat droplets in cytoplasm. Cells were derived from tissue of a 55-year old Japanese female with an epitethelioid hemangioendothelioma (137). LI 90 cells were cultured as COS-1 cells.

Simpson-Golabi-Behmel syndrome (SGBS) cells

SGBS cells are a human preadipocyte cell strain derived from stromal cell fraction of subcutaneous adipose tissue of a male infant with Simpsin-Golabi-Behmel syndrome, a rare X-linked disorder characterized by pre- and postnatal overgrowth (138). Although the biological mechanism behind the prolonged differentiation capacity is

still unknown, SGBS adipocytes are morphologically, biochemically and functionally identical to in vitro differentiated adipocytes from healthy subjects and are therefore an ideal human adipocyte *in vitro* model system. SGBS cells were cultured in DMEM/Nutrient Mix (0F) supplemented with 10% non- heat inactivated fetal calf serum (OF+10% FCS). Growth medium for all cells was supplemented with 2 mM L-glutamine, penicillin (100 U/ml), streptomycin (100 µg/ml) and vitmix (0.04 g biotin + 0.02 g D-pantotenate (or 0.04 g DL-pantotenate in 50 ml MQ water). Cells were not allowed to grow confluent prior to experiments.

4.3.4 Differentiation of SGBS cells

Protocol

Solutions

Adipogenic media 3FC

0F (- FCS)		10 ml
0,01 mg/ml	human apo-transferrin	10 µl of 10 µg/µl stock
$2x10^{-8}$M	insulin|	2 µl of 100 µM stock
10^{-7}M	cortisol	10 µl of 100 µM stock
0.2 nM	T3	10 µl of 200 nM stock

Quickdiff

3FC		10 ml
25nM	dexamethasone	10 µl of 25µM stock
500µM	IBMX	222 µl of 22.5 mM stock
2 µM	rosiglitazone	2 µl of 10 mM stock

Experimental procedure

Seed the cells in appropriate culture chamber/flask (25 cm^2 culturing flasks) before you start differentiation. Then let the cells grow confluent before you start the differentiation program. This increases the degree of differentiation substantially. Remove the medium. Add 5 ml Quick-diff medium, and incubate for 4 days. Change medium to freshly made 3FC (5 ml) after 4 days, and then incubate another 4 days. Then change medium each 3rd-4th day until experiment.

4.4 Promoter analysis

The information governing how transcription factors influence gene expression is laid down in the regulatory genomic sequences, not in the proteins themselves, and thus a tremendous amount of information can be mined from regulatory regions. Linking the results of functional analysis of gene regulation can allow rapid identification of a series of potential coregulated genes and thus facilitate target gene characterization and identification (139). Functional assays for promoter analysis makes it possible to assess a control region's ability to mimic accurately the expression pattern of the endogenous gene. Mutations can then be introduced into the control region to identify important regulatory elements and, ultimately, important transcription factors (140).

4.4.1 Computer analysis

The DNA sequences that comprise promoters do not provide much direct information about regulation. Promoter function is not coupled to fixed stretches of sequence homology, but rather to highly variable elements representing individual transcription factor binding sites that act as a binding site for their cognate protein. The sites are generally composed of 10 to 30 nucleotides; of these, usually only a small core of nucleotides, often separated by nonconserved sequences, establishes the criteria for a binding site. Because of this inherent variability, transcription factor binding sites cannot be efficiently described by their individual sequence. However, the flexibility of these sites can be defined by either an IUPAC consensus sequence or by weight matrices. IUPAC consensus sequences use ambiguous symbols (e.g., B=C, G or T; R=A or G) to describe the variability of nucleotide usage. Software that allows detection and characterization of individual binding sites is available from several sources, including Signal Scan (http://bimas.dcrt.nih.gov/molbio/signal), MATRIX SEARCH, Genetics Computer Group (gcg) Wisconsin Package Version 10.0 or MatInspector (http://www.gsf.de/biodv). A large collection of functional binding sites

derived from the literature can be found in the TRANSFAC database (http://transfac.gbf.de/TRANSFAC/) (139).

The computer analysis (80) was performed prior to my experiments and a limited protocol is added since this method was fundamental to further characterization of the human Adipophilin gene.

Protocol

Briefly, the full-length human adipophilin mRNA (accession: #BC005127) was used to identify the chromosomal localization, exon/inton boundary and the predicted transcription start (TS) site by BLAST against htgs sequences (annealed to: #BC005127). A short 5‘- regulatory sequence for the mouse adipophilin gene, was used as bait to identify a longer form of the mouse adipophilin promoter (accession: #L09734.1 annealed to #AL824707.5). The promoter sequences spanning 5.000 bp up- and downstream from TS was extracted and analyzed for presence of response elements by the use of consensus PPRE (RGGBSA A AGGTCA) and LXRE (DGGTYA HWHW MGKKCA) sequences generated by the gcg-program (Wisconsin Package Version 10.0, Genetics Computer Group (gcg), Madison, Wisc.) to localize potential PPRE elements.

4.4.2 Cloning and mutagenesis of the adipophilin promoter

Cloning and mutagenesis of the adipophilin promoter was performed prior to my experiments and a limited protocol is added since this method was fundamental to further examination of the human adipophilin gene.

Protocol

Briefly, the full-length human and murine adipophilin promoters were amplified with *Pfu* Turbo (Stratagene) from human (Clontech, #6550-1) and mouse (Clontech; #6650-1) genomic DNA with PCR settings as described previously (80).The following primers were used: 5'-human adipophilin (KpnI): 5'-

TAGGTACCAAAAACGTCTCCTTTGTCCTCTGGA-3', 3'- human adipophilin (KpnI): 5'-TAGGTACCTCTAACGCGTTTCCCTTTCGATAAT-3', 5'-mouse adipophilin (NheI): 5'-ATGCTAGCATCGCCTTGGGATCTAATCTTGGT-3' and 3'-mouse adipophilin (NheI): 5'-ATGCTAGCCTAACAGGAGAGCTGAGGGACGAG-3' Both promoter was first cloned into the pPCR-Script vector (Stratagene) prior to insertion into the luciferace reporter vector pGL3-Basic (Promega) to generate reporter vectors.

4.4.3 Transient transfection assay

Transient transfection assay is the most common functional assay to study transcription regulation. In this method plasmids containing the control region of interest are introduced by one of several transfection procedures into cells maintained in culture. Typically, the control region regulates transcription of a “reporter gene”, a gene whose mRNA or protein level can be measured easily and accurate. If the regulatory region of interest is a promoter, it is placed immediately upstream of the reporter, such that the inserted promoter will drive reporter gene transcription. If the control gene of interest is an enhancer or other control region that appears to function at a distance from the promoter, a well characterized promoter is usually placed upstream of the reporter gene, with the enhancer inserted upstream of the promoter or downstream of the reporter gene. At a specific time point following transfections of cultured cells with the resulting plasmid, the activity of the control region is assessed by measuring mRNA or protein synthesis from the reporter gene. This assay is considered to be transient because the plasmids remain episomal and rarely integrate into the host genome (140).

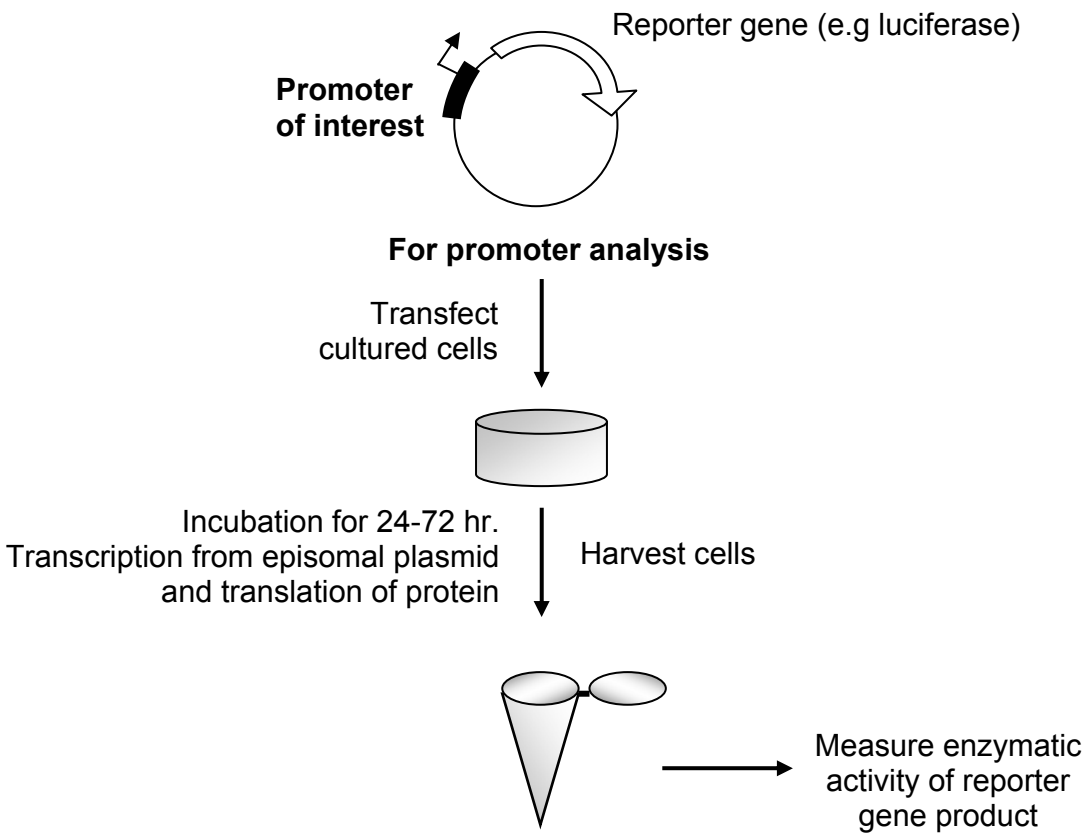


Figure 4.1 Transient transfection assay

The most commonly used reporter gene is the luciferase gene from the firefly *Photinus.* This gene encodes a 61-kD enzyme that oxidizes D-luciferin in the presence of ATP, oxygen and Mg^{2+}, yielding a fluorescent product that can be quantified by measuring the released light. In the conventional assay for luciferase, a flash of light is generated that decays rapidly after the enzyme and substrates are combined (141).

Figure 4.2 Bioluminescent reaction catalyzed by firefly luciferase

Protocol

Solutions

2 x Hepes-buffer-saline (2 x HBS)

1.6 g NaCl; 0.074 g KCl; 0.024 g $Na_2HPO_4x2H_20$; 0.2 g dextrose; 1 g hepes. Solve in ddH_2O to a total volume of 100 ml and adjust pH to 7.05 with NaOH. Sterilize by filtration.

$CaCl_2$ (250 mM)

Sterilize by filtration.

10 x PBS

1 tablet in 10 ml ddH_20 or 137 mM NaCl; 2.7 mM KCl; 4.3 mM Na_2HPO_4; 1.4 mM KH_2PO_4; The final pH should be 7.3. Sterilize by filtration.

2 x HBS /PBS

10 µl 10 x PBS / ml 2 x HBS

Luciferase assay reagent

470 µM luciferin; 530 µM ATP; 270 µM coenzyme A; 20 mM tricine; 3.74 mM $MgSO_4$ x $7H_2O$; 0.1mM EDTA; 33.3mM DTT; ad 100 ml H_2O.

Experimental procedure

Transient transfections of COS-1 and HepG2 cells were performed after modifications of the calcium phosphate precipitation method (142).

Ligands

Ligands were dissolved in DMSO before addition to the transfection medium at appropriate concentrations.

C*ells*

Split COS-1 or HepG2 cells 24 hours prior transfection in 6-well and 12-well plates respectively, with a cell density of 2 x 10^5 cells / subconfluent per well. Change to transfection medium prior transfection.

Plasmids

Each well receive 5 µg test plasmid, 3 µg pSV-β-Gal as internal control, 1 µg of pCMX, pCMX-RXRα or pCMX-hLXRα expression vectors and 2µg pGL3-basic to a total volume of 12 µg DNA in the experiments.

Transfection

Mix DNA in 2 x HBS and add calcium chloride in a phosphate buffer. Add this mix in droplets to the cells. The result is a formation of a DNA-calcium phosphate precipitate, which is layered on the cells. The precipitate is taken up by endocytosis. Incubate for 24 to 48 hours.

Harvesting and lysis

Equilibrate 1 x lysis buffer to room temperature before use. Carefully remove the growth medium from cells to be assayed. Rinse cells with PBS, being careful to not dislodge attached cells. Remove as much of the PBS rinse as possible. Add enough 1X lysis buffer to cover the cells (e.g., 250 µl per well of a 6-well plate). Rock culture dishes several times to ensure complete coverage of the cells with lysis buffer. Scrape attached cells from the dish. Transfer cells and all liquid to a microcentrifuge tube. Place the tube on ice. Vortex the microcentrifuge tube 10–15 seconds, then centrifuge at 12,000g for 15 seconds (at room temperature) or up to 2 minutes (4°C).

Luminometer

Dispense 100µl of the Luciferase Assay Reagent into luminometer tubes, one tube per sample. Program the luminometer to perform a 2-second measurement delay followed by a 10-second measurement read for luciferase activity. Sensitivity should be approximately 39.9. Add 10 µl of cell lysate to a luminometer tube containing the Luciferase Assay Reagent. Mix by pipetting 2–3 times or vortex briefly. Place the tube in the luminometer and initiate reading.

4.4.4 β-Galactosidase assay

β-galactosidase is a commonly used reporter molecule. The β-galactosidase enzyme assay system with reporter lysis buffer is a convenient method for assaying β-galactosidase activity in lysates prepared from cells transfected with β-galactosidase reporter vectors such as pSV-β-galactosidase control vector. The standard assay is performed by adding sample to the buffer that contains the substrate ONPG (o-nitrophenyl-β-D-galactopyranoside). Samples are incubated for 30 minutes, during which time the β-galactosidase hydrolyzes the colorless substrate to o-nitrophenol, which is yellow. The absorbance is then read at 420 nm with a spectrophotometer (143).

Protocol

Solutions

Z-buffer

60 mM Na_2HPO_4; 40 mM NaH_2PO_4; 10mM KCL; 1mM $MgSO_4$. Adjust to pH 7.0.
Add 50 mM 2-mercaptoethanol before use

o-nitrophenyl-ß-D-galactopyranoside (ONPG) reaction buffer

ONPG (4mg/ml) in 100 mM Phosphate buffer, pH 7.0

Experimental procedure

Mix 175 µl of 2-mercaptoethanol in 50 ml Z-buffer and add 1.5 ml to the eppendorf tube with 3 µl β-Galactosidase (0.1mU/µl). Make a standard curve by pipetting duplicates of 0, 10, 20, 30, 40, 50, 60 and 70 µl into a 96-well plate followed by 10 µl of each cell lysate from the transfection experiments in the resisting wells. Dilute with Z-buffer to a total volume of 200 µl in each well and add 40 µl *o*-nitrophenyl-ß-D-galactopyranoside (ONPG) (4 mg/ml). Place a cover on the plate. Incubate the plate at 37°C for 30 minutes or until a faint yellow colour has developed. Colour development continues for approximately 3 hours. Read the absorbance of the samples at 420 nm in a plate reader.

4.4.5 Green fluorescent protein transfection marker

Green fluorescent protein (GFP) is a 28-kD protein from the jellyfish *Aequorea victoria*. GFP contains an intrinsic peptide chromophore that emits green light following oxidation and excitation with ultraviolet or blue light. The pEGFP-N1 encodes a red-shifted variant of wild-type GFP, which has been optimized for brighter fluorescence and higher expression in mammalian cells (144;145). GFP are used for monitoring gene expression and can be used as a substitute for luciferase reporters, by measuring fluorescence in a flourometer. pEGFP-N1 can also be used simply to express EGFP in cell line of interest as a transfection marker (146).

Protocol

HepG2 cells cultured in 12-well plates were transfected with 2.5, 5.0 and 7.5 μg pEGFP-N1 using the calcium phosphate precipitation method (4.4.3). The DNA amount of 5.0 μg was based on experience with transfections in COS-1 cells (Cell amount reduced ~60 % in 12-well plates compared to 6-well plates). The amount of GFP positive cells were estimated visually after 48 and 72 hours based on experience with COS-1 cells.

4.5 DNA binding by regulatory transcription factors

4.5.1 Electrophoretic mobility shift assay (EMSA)

In an electrophoretic mobility shift assay (EMSA) a ^{32}P-labeled DNA fragment containing a specific DNA site is incubated with a cognate DNA-binding protein. The protein-DNA complexes are separated from free (unbound) DNA by electrophoresis through a nondenaturing polyacrylamide gel. The protein retards the mobility of the DNA fragments to which it binds. Thus, the free DNA will migrate faster than the DNA-protein complex. An image of the gel is used to reveal the positions of the free and bound ^{32}P-labeled DNA (147;148). EMSA was thus used to bind the LXR protein to its LXRE (LXRE) or probe identified upstream on the adipophilin gene (studied in transfections) (140;149).

Protocol

Solutions

Gel fixing solutions (1 litre)

700 ml dH_2O; 200 ml methanol; 100 ml acetic acid.

5x running buffer / EMSA buffer) (1litre)

TRIS-base 0.25 M 30.28 g; Glycin 1.9 M 142.63 g; EDTA 10 mM 3.72 g; dH_20 700 ml. pH=8.5, Add dH_20 to final volume of 1 litre.

2 x Binding buffer (for in vitro translated proteins)

Stock solution		in reaction	2 x buffer	50 ml
1 M	Tris-HCl (pH=8.0)	20 mM	40 mM	2 ml
1 M	KCl	80 mM	160 mM	8 ml
85 %	glycerol	8 %	16 %	8.8 ml
5 %	NP-40	0.1 %	0.2 %	2 ml
1 M	DDT	1 mM	2 mM	200 µl

Experimental procedure

Annealing of oligo

Dilute each oligo to 20 pmol/µl in 100 µl dH_20 (20 µl of each 100 pmol/µl oligo stock, 60 µl dH_2O). Boil the tube in water bath for 5 minutes, and let cool down at room temperature in the same solution until ~35°C (or until next day). Store annealed oligo at -20°C.

Radio labelling of oligo

Mix the following reagents and incubate in a water bath at 37°C for 90 minutes: 34 µl H_2O; 4 µl annealed probe (20 pmol/µl); 5 µ 10 x kinase buffer; 5 µ γ-^{32}P[ATP] (5 µCi/µl $\Rightarrow$ 25 µCi); 2 µl T4 Polynuceotide kinase (5 U/µl). Purify on ProbeQuant G50 Micro Columns (Amersham Biosciences), and dilute probe to 100 µl by adding 50 µl dH_2O. Store labelled probe at 4°C.

In vitro transcription and translation of proteins

Defreeze reticulocyte lysate (Master Mix) on ice, and take care not to expose the lysate to air more than necessary (as it reacts with CO_2). For same reason, do not store the lysate on dry ice. Mix the following reagents and incubate at 30°C for 60-90 minutes: 40 µl Reticulocyte lysate (Master Mix); 2.5 µl Plasmid (1 µg/µl); 1 µl Methionine; 6.5 µl DEPC. Freeze the in vitro translated proteins at -70°C until use. Might be stored for at least one year. Avoid repeated freezing/defreezing of lysate, and disperse the translated lysate in eppendorf tubes (~30 µl/each tube).

Preparation of native gel (2 gels)

Mix the following reagents (add TEMED as last component): 42 ml H_2O; 1.25 ml 5 x EMSA running buffer; 6.3 ml 40% acrylamide; 0.5 ml 10 % APS (ammonium per sulphate); 25 µl TEMED. 40% acrylamide = 40% Acrylamide/BIs solution 19:1 (BioRad, #161-0144). TEMED = N,N,N',N'-tetra-metylethylenediamine (BioRad, #161-0801). 10% APS is solved in dH_2O and stored at stocks of 1 ml at -20°C.

Let the gel polymerise for at least one hour before you use the gel. Do not let the gel dry in room temperature for to long, as this might make it difficult to remove the comb later on. Wrap it into a plastic bag containing 0.5xRunning buffer (~20 ml) to avoid drying.

Pre-running of gel

Dilute 10 x EMSA running buffer to 1 x EMSA running buffer concentration. Place the gel in position in the chamber, and wash the bottom of the gel (between the glass plates) to remove all air bobbles. Wash each well with buffer. Pre-run the gel 30 to 60 minutes at 100 V.

Binding reaction

Mix the samples as follows: 10 µl2 x binding buffer; 4 µl Protein (in vitro translated); 1 µl ssDNA; y µl competitor; 4-y µl dH_2O. Add 1 µl of radiolabeled probe (remember to dilute probe to the desired concentration). Incubate with radiolabeled probe for 20 minutes at room temperature.

Electrophoresis

Wash each well (just prior to addition of probe) and apply samples on gel. Wait 2 minutes and turn on the current. Run at 180 V for 3.5 hours. Wash the gel with water. Take special care of washing the bottom of the gel to remove radioactive free probe in the solution remaining between the glass plates. Remove the side dispensers and then remove one of the glass plates while letting the gel stick to the other one. Add Fix solution (just to cover the gel surface), and fix the gel for 5-10 minutes (reticulocyte lysate turns brown). Pour of the fix solution (against the bottom of the gel), and transfer the gel to a Watman paper (Press the Watman paper quickly and gently on top of the gel, and then remove the paper with the gel attached to it. Dry the gel at 70°C for 30-60 minutes. It is optimal to preheat the gel dryer to decrease the drying time required. Gels are then subjected to autoradiography at −70 °C (41).

4.6 RNA analysis

RNA analysis is used to measure the amount and size of RNAs transcribed from DNA and to estimate their abundance. Northern analysis is capable of obtaining these pieces of information simultaneously from a large number of RNA preparations, and is therefore fundamental to studies of gene expression in eukaryotic cells. The basic steps in this analysis are isolation and separation of RNA, transfer and fixation to a solid membrane, hybridization with radio-labelled probe against gene of interest, removal of unspecifically probe and finally detection, capture and analysis of an image of the specifically bound probe molecule (127).

4.6.1 Isolation of total RNA from cell cultures with TRIZOL

Protocol

Wash the cell layer once with 10-15 ml 1x PBS. Remove PBS and lyse the cells directly in the culture flask by adding 1.5 ml TRIZOL reagent to a 25 cm^2 culture flask. Wait for 2-3 minutes to allow the cells to lyse completely, and pass the cell lysate several times through the pipette and transfer to an RNase free tube. Freeze samples at -20°C, or at -70°C for longer storage. Incubate the homogenised samples for 5 minutes at 15-30°C to permit complete dissociation of nucleoprotein complex. Add 300 µl chloroform (0.2 ml/1 ml of TRIZOL reagent used for homogenisation). Shake vigorously by hand for 15 seconds, and then incubate the tube at 15-30°C for 2 to 3 minutes. Centrifuge the samples at no more than 12.000 g for 15 minutes at 2 to 8°C. After centrifugation, the mixture separates into a lower red, phenol-chloroform phase, an interphase, and a colourless upper aqueous phase. RNA remains exclusively in the upper aqueous phase. Transfer the aqueous phase to a fresh tube. Precipitate the RNA from the aqueous phase by mixing with isopropanol. Use 1.5 ml isopropanol (0.5 ml/1 ml of TRIZOL reagent used for homogenisation). Incubate the samples at 15 to 30°C for 10 minutes, and centrifuge by no more than 12.000 g for 30-40 minutes at 2 to 8°C. Remove the supernatant. Wash the RNA pellet with 75%

ethanol, adding at least 1.5 ml of ethanol (at least 1 ml of 75% ethanol/1 ml of TRIZOL reagent used for homogenisation). Mix gently and centrifuge at no more than 7500 g for 5 minutes at 2 to 8°C. Remove the supernatant, and air-dry the RNA pellet for 5 to 10 minutes. Dissolve in approximately 15 µl RNase free water (DEPC water), depending on the size of the pellet.

4.6.2 Northern analyses

Solutions

10x Running buffer (1 L)

0.4 M MOPS (pH 7.0) 83.72 g; 100 mM NaCl 8.2 g; 10 mM EDTA 3.72 g; Adjust pH with NaOH.

10x Loading dye (10 ml)

50% Glycerol 5.8 ml; 1 mM EDTA 20 µl - 0.5 M EDTA (pH 7.5 – 8.0); 0.4% Bromophenol blue; 0.04 g 0.4% Xylene cyanol 0.04 g.

20x SSPE (1 litre)

NaCl 175.3 g; NaH_2PO_4 x H_2O 27.6 g; (Na_2HPO_4 28.4 g); EDTA 7.4 g. Adjust pH to 7.4 with NaOH.

Hybridisation mix (0.5 L)

5x SSPE	125 ml	20x SSPE
10x Denhardt (without BSA)	1 g	Ficoll
	1 g	Polyvinylpyrrolidone
100 µg/ml salmon sperm DNA	5-6 ml	10 mg/ml freshly denatured ssDNA
50% formamide	250 ml	deionised formamide
2% SDS	10 g	SDS (or 200 mL 10% SDS)

Solution 1 (1 L): 100 ml 20x SSC (2x SSC) and 2.5 ml 20% SDS (0.05% SDS)

Solution 2 (1 L): 5 ml 20x SSC (0.1x SSC) and 5 ml 20% SDS (0.1% SDS)

Stripping solution 1 (1 L): 25 ml 20% SDS (0.5% SDS)

Stripping solution 2 (1 L: 100 ml 20x SSC (2x SSC)

Stock solutions
20x SSC (1 L): 175.3 g NaCl and 88.2 g Na-citrate. Adjust pH to 7.0 with a few drops of 10 M NaOH

20% SDS (1.0 L): 200 g SDS. Dissolve overnight without heating.

Experimental procedure

Preparation of gel
Make 400 ml of a 1% agarose gel by melting agarose, 288.6 ml H_2O (DEPC) and 40 ml 10x running buffer in a microwave. Then chill the solution to approximately 65°C before adding 71.4 ml formaldehyde. Formaldehyde should have a final concentration of 2.2 M. For each sample make a mix of the following: 4.5 µl RNA (up to 20 µg) and DEPC, 2.0 µl 10x buffer, 3.5 µl formaldehyde and 10.0 µl formamide (deionised). Heat the samples for 15 minutes at 65°C. Put on ice for 1 minute. Add 2 µl loading dye and centrifuge to get the sample in the bottom of the tube. Run the gel overnight. Start at 80 V, until samples have migrated into the gel, and then adjust the voltage down to approximately 40 V.

Blotting of gel
Rinse the gel in DEPC water. Put 10x SSPE in each of the buffer reservoirs in the gel chamber. Cut three layers of Whatman papers, soak them in 10x SSPE buffer and lay them one by one over the glass plate/support with ends reaching into 10x SSPE buffer in the chamber below. Remove air bubbles for each layer of Whatman paper. Cut a Nylon membrane (Amersham Hybond) and three pieces of Whatman papers with the same size as the gel. Soak the layers of Whatman papers in 10x SSPE and place the gel upside down on top of the Whatman papers. Make sure there are no air bubbles between the gel and the Whatman papers. Put Parafilm on top of the gel along each side of the gel to ensure that all blotting solution is transferred through the

gel only. Soak the nylon membrane in 2x SSPE and place it on top of the gel and Parafilms. Soak the Whatman papers in 2x SSPE and put them one by one on top of the stack. Put blotting paper on top of the stack. Put a glass plate on top and a weight (1 kg for a 20x20 cm gel). Blot the gel for at least 20 hours. Remove the blotting papers and the Whatman papers, remove the membrane and place it on a clean piece of Whatman paper and let it air-dry completely. Crosslink both sides of the membrane in the UV-crosslinker (Energy 700 at both sides).

Labelling of oligo

Pipette 30-50 ng of probe (X µl) into an eppendorf tube, add 5 µl primer, and dilute to total volume of 33 µl with dH_2O. Make a small hole in the top of the eppendorf tube and boil the mix for 5-10 min. After boiling, put the probe directly on ice to minimise renaturation of the probe. Centrifuge down the probe. Add 10 µl 5x labelling buffer, 5 µl [α-^{32}P] dCTP and 2 µl Klenow enzyme. Incubate at 37°C for 10 minutes to 1 hour. Separated the probe on a Sephadex G-50 column (ProbeQuant™ G-50 Micro Columns, 27-533-01) right after incubation. Reaction mix: 1 µl probe, 5 µl primer 28-1 µl H_20 | 10 µl buffer, 5 µl [α-^{32}P] dCTP, 2 µl Klenow enzyme. Mix the contents in the Sephadex G-50 spin column until a homogenous solution. Loosen the top of the column, remove the tip at the bottom, and put the column in an eppendorf tube. Centrifuge the column at 3000 rpm for 1 minute. Dry the tip of the column with paper to remove the remainder of the solution. Add the probe-mix in the middle of the dry column matrix. Centrifuge the column at 3000 rpm for 2 minutes. The probe passes through the column, while the nucleotides remain in the column. Count the incorporation by pipetting 2 x 1 µl of the flow-through in a scintillation counter. The count number should be about 1-5 $\cdot$ 10^6, (~10^8 total).

Prehybridisation

To make freshly denatured ssDNA, heat DNA at 100°C for 10 minutes, then put on ice for two minutes. Add ssDNA as the last component, and freeze the hybridisation mix at 20°C until use. Wet the membrane with hybridisation mix. Put the membrane

into a hybridisation tube, and add 10 ml hybridisation mix. Remove all air bubbles, and pre-hybridise the membrane in a tube at 42°C for 3 hours.

Hybridisation and whasing

Denature the probe by heating at 100°C for 10 min, and then put the probe on ice. Pipette the probe into 10-15 ml fresh hybridisation mix in a 15 ml Falcon tube. Remove the hybridisation solution, and replace with the hybridisation solution containing the probe. Remove all air-bubbles, and incubate at 42°C until next day. Wash the membrane once with 20 ml solution 1 at room temperature for 1-2 min. Then wash twice in 100-200 ml solution 1 at room temperature with moderate shaking for 15 minutes. Preheat a water bath to 50°C, and put a container containing solution 2 in the water bath. Wash the membrane 2 x 15 min at 50°C in 100-200 ml solution 2.

Exposure

Wrap the filter in plastic (Saran), and remove all air-bubbles, and most of the liquid. Expose the membrane for the acquired time.

Stripping

Heat a 0.5% SDS solution until boiling. Remove the solution from the hot plate and incubate the blot in the solution for 10 minutes. Transfer the blot to 2xSSC solution for 5 minutes at room-temperature. The blot is then ready for prehybridisation or storage at 4°C in plastic (Saran).

4.7 Statistical analysis

The results from transfection studies are presented as means ± SD, and are representative for three individual experiments performed in triplicates. The relatively small sample size in the transfection experiments complicates testing of normal distribution conformation of the data. Since assumptions of a parametric test cannot be met, a comparison using a non-parametric method which does not require any assumptions concerning the distributions of the data was carried out. The non-parametric alternative to the independent sample t test comparison for two samples, the Mann Whitney U test, was then conducted for significance testing. Statistically significance was set at the 5 % level ($P<0.05$).

5. Results

5.1 Characterization of the human adipophilin promoter

Preliminary data in Hilde Nebb's research group suggested that the human adipophilin gene had a DR4 element in the promoter region that might function as a LXRE. This information formed the basis of further examination of the gene regulation of adipophilin, using cell cultures, promoter analysis and DNA binding studies presented in this thesis.

5.1.1 Identification of a putative LXRE

Analysis of the proximal promoter of the human adipophilin gene using the Genetic Computing Group package, identified a DR4 element (-129 GGGTGA cact CGGGCT -114) (Figure 5.1 C). This type of DR4 response element has been shown to function as a high affinity binding site for heterodimers between RXR and LXR. To date, verified LXREs identified in LXR target genes correspond to a DR4, and the examined element shows high identity with the consensus sequence for LXREs (Table 5.1). Similar computer analysis techniques have been used to screen for PPARγ and LXR response elements in the GLUT4, S3-12 and perilipin promoters (80;102).

The high score candidate LXRE was found in the human promoter, but not in mouse (Figure 5.1A). Alignment of the human and mouse promoter suggest that the identified LXRE has undergone changes in the course of evolution resulting in species variation in the gene regulation of adipophilin (Figure 5.1, A and C). This supports a recent observation indicating that the adipophilin gene transcript is also differently transcribed during differentiation of human and mouse fibroblasts into adipocytes ((102) and Dalen, Personal communication).

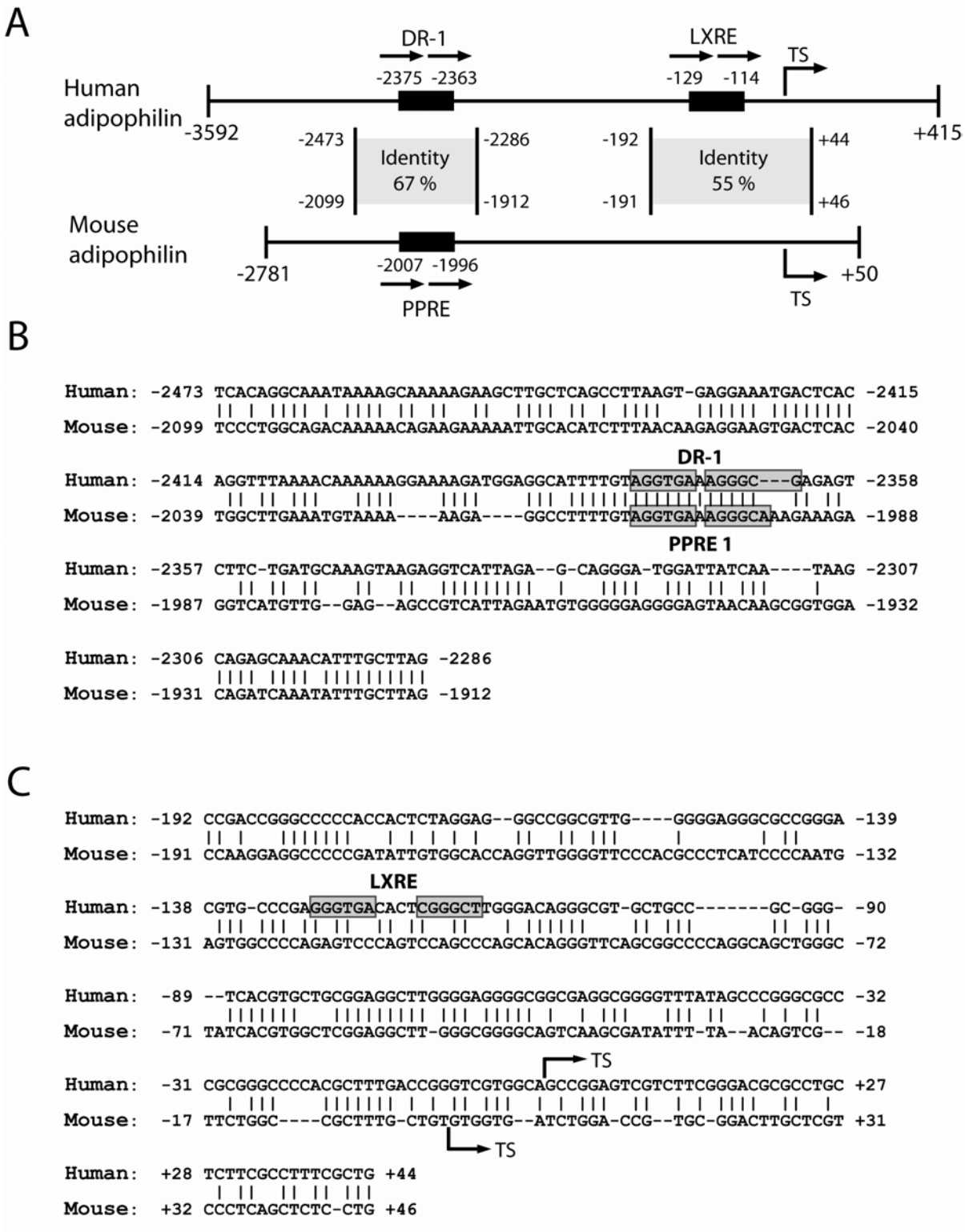


Figure 5.1: Characterization of the human adipophilin promoter

A) A schematic presentation of the cloned human adipophilin promoter (nucleotides -3592 to +415) and the mouse adipophilin promoter (nucleotides -2781 to +50). The transcription start site is marked by +1. The nucleotide position of the black boxes points out the localisation of the PPRE and LXRE. **B and C)** Sequence alignment and identity between the nucleotides surrounding the human and mouse adipophilin PPRE and LXRE. The two half sites in the conserved PPRE and LXRE are encircled by boxes, and conserved nucleotides are indicated by vertical lines.

Table 5.1 Functional LXR response elements identified in LXR target genes

Target gene	LXRE	Tissue	Ref
	→ →		
Cyp7α (mice)	**TGGTCA ctca AGTTCA**	Liver	(41)
CETP	**GGGTCA ttgt CGGGCA**	Liver	(53)
ABCA1	**AGGTTA ctat CGGTCA**	Macrophages, intestine, CNS	(49;67;150)
ABCG1 (LXRE1)	**TGGTCA ctca AGTTCA**	Macrophages, CNS	(65;150)
(LXRE2)	**AGTTTA taat AGTTCA**	Macrophages, CNS	(65)
SREBP1c	**GGGTTA ctgg CGGTCA**	Liver, adipose tissue, intestine, CNS	(58;150)
FAS	**GGGTTA ctgc CGGTCA**	Macrophages, liver	(59)
ACC	**GGGTTA cctc GGGTCA**	Not reported	(151)
ApoE	**GGGTCA ctgg CGGTCA**	Macrophages, adipose tissue	(51;70)
ApoCI/apoCII/apoCIV	**GGGTCA ctgg CGGTCA**	Macrophages	(70)
LXRα (human)	**AGGTTA ctgc TGGTCA**	Macrophages, adipose tissue	(71;72;74)
LXRα (mice)	**AGGTTA ctgc TGGCCA**	Adipose tissue	(73)
LPL	**TGGTCA ccac CGGTCA**	Macrophages, liver	(60)
PLTP	**AGGTTA ctag AGTTCA**	Macrophages, liver	(152)
SR-BI	**TGGACT tcat GGATCA**	Liver	(75)
Angpt13 (human)	**AGGTTA catt CGTGCA**	Not reported	(76)
GLUT4 (human)	**GGGTTA cttt GGGGCA**	Adipose tissue	(80;81)
TNFα	**GGGCTA tgga AGTCGA**	Macrophages	(153)
Akr1b7 (mice)	**AGGTCA tcca AGATGA**	Intestine	(154)
Consensus	**GGGTTA n4 CGGTCA** **T T C A TG** **A T**		
Adipophilin (human)	**GGGTGA cact CGGGCT**		

The table shows nucleotide sequences of identified functional LXREs. Nucleotide sequences are written from left to right. The consensus sequence for LXREs is idicated on the bottom.

5.2 Electrophoretic mobility shift assay

5.2.1 LXRα binds the human LXRE in the adipophilin promoter

To determine if the potential LXRE found in the human adipophilin promoter (Figure 5.1c) is able to bind the RXR/LXR heterodimer, an electrophoretic mobility shift assay (EMSA) with double stranded oligonucleotides containing the identified human adipophilin LXRE was performed (Figure 5.2A, oligonucleotide sequence).

No protein-DNA complex was observed without the presence of both *in vitro* translated RXRα and LXRα proteins as shown in Figure 5.2B (lane 1-4), but a specific protein-DNA complex with the LXRE was observed in presence of RXRα and LXRα proteins (lane 5). In the presence of increasing concentrations of wild-type competitor, the RXRα/LXRα complex was diminished (lanes 6-8). In contrast, the abundance of the RXRα/LXRα complex was unaffected by the mutant competitor (lanes 9-11). This result suggests a specific protein-DNA complex binding, since the binding was diminished by excess unlabeled wild type but not mutated oligonucleotides.

A

```
Adipophilin-LXRE-2s ACGTGCCCGAGGGTGACACTCGGGCTTGGGACAGGG
Adipophilin-LXRE-2a TGCACGGGCTCCCACTGTGAGCCCGAACCCTGTCCC

Adipophilin-LXRE-2s-mut ACGTGCCCGAGccTGACACTCccGCTTGGGACAGGG
Adipophilin-LXRE-2a-mut TGCACGGGCTCggACTGTGAGggCGAGCCCTGTCCC
```

B

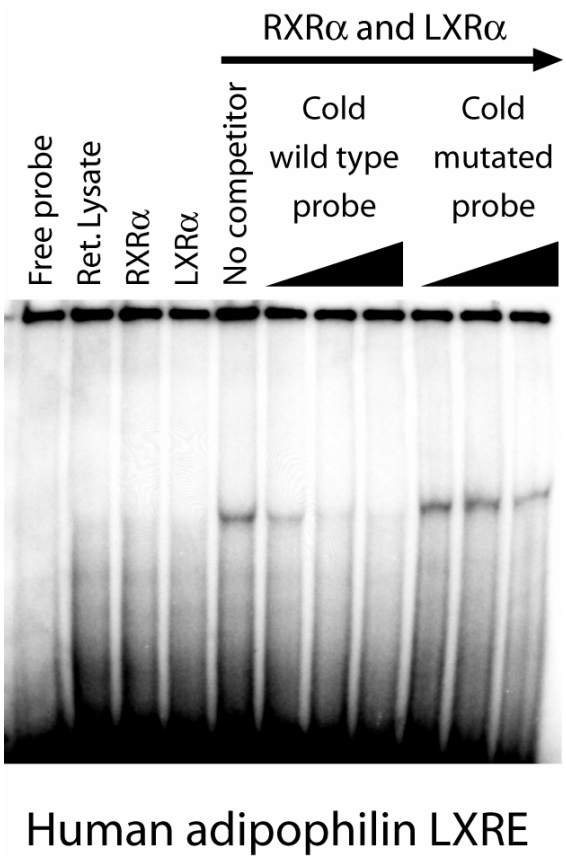


Figure 5.2 LXRE in the human adipophilin promoter recruits the RXRα/LXRα heterodimer

A) The nucleotide sequence for the oligonucleotides used. Each half-site in the DR4 element is indicated in underlined and boldface type, and the base substitution in the mutated oligonucleotides is given in the mutated oligonucleotides. **B)** Direct and specific binding of the RXRα/LXRα heterodimer to the LXRE in the human adipophilin promoter. The EMSA was performed with annealed double stranded ^{32}P-labeled human adipophilin LXRE nucleotides and incubated in the presence of *in vitro* translated RXRα and /or LXRα proteins as indicated. Cold competitor was added in excess; 4.8, 14.4 and 48 times compared to labeled probe, respectively (lanes 6-8).

5.3 Regulation of the adipophilin promoter in COS-1 cells

5.3.1 The adipophilin promoter is responsive to LXR activation

To determine the validity of the particular LXRE found in the human adipophilin promoter, the ability of a LXR agonist to activate the human adipophilin promoter was examined. The promoter was cloned (sequence spanning from -3592 to +415) and inserted into the pGL3-basic luciferase-reporter vector.

COS-1 cells were cultured in 6-well plates and transiently transfected with the full-length human adipophilin promoter. Co-transfection with RXRα and LXRα expression vectors and treatment for 72 hours with the LXR ligand T0901317 (0.1 μM), gave a 2.5-fold increase in reporter activity (Figure A1 / Appendix). This induction was minimal when compared to the cells receiving both receptor expression vectors and no ligand.

In an attempt to elevate the reporter activity measured in the full-length construct, investigation of factors that might influence the basal activity and thus the relative reporter response was conducted. Examination of CCAAT/enhancer binding protein α (cEBPα) indicated that it was not able to activate adipophilin expression (Figure A2 / Appendix). Since this luciferase-reporter also included a PPAR response element (located -2375 to 2363) that might influence the result, generation of a deletion construct lacking the PPAR response element was conducted (Figure 5.3). PvuII was used as restriction enzyme (Figure 5.4) and a deletion construct was generated as described in methods.

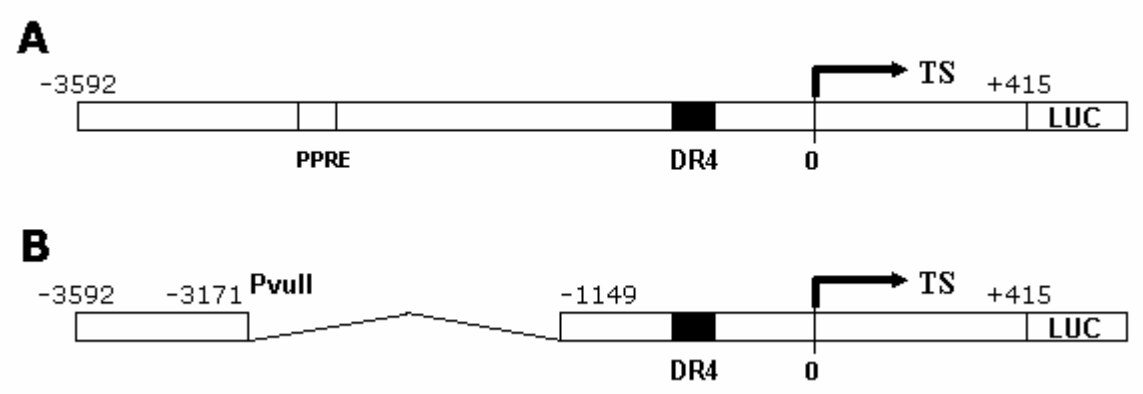


Figure 5.3 Human adipophilin full-length and deletion construct

A schematic presentation of the full-length and deletion construct made for the human adipophilin promoter. The arrow points out the transcription start site. **A)** Full-length construct. **B)** Deletion construct.

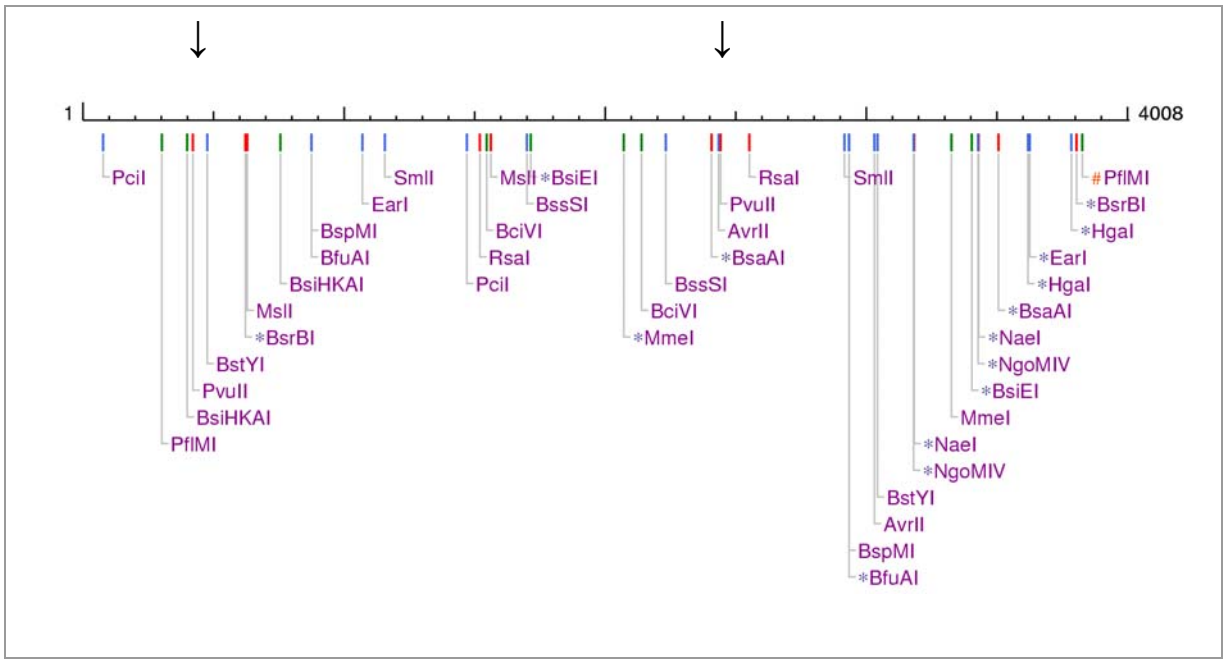


Figure 5.4 Digestion of the human adipophilin construct with restriction nuclease PvuII

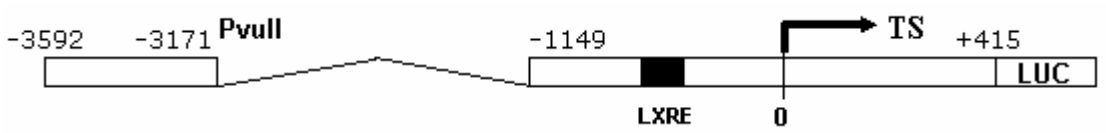
Presentation of PvuII cleavage sites (arrows). PvuII is a restriction endonuclease that cleaves DNA in a sequence specific manner to produce blunt ended products. The enzyme binds to a 12 base-pair site containing the consensus sequence CAGCTG. Cleavage occurs between the G and C bases.

5.3.2 Deletion construct shows elevated reporter activity

To examine whether the generated deletion construct lacking a fragment of 2022 nucleotides, including the PPAR response element, could elevate the reporter activity, COS-1 cells were transiently transfected with this construct (Figure 5.3B). Co-transfection with RXRα and LXRα expression vectors and treatment for 72 hours with the RXR ligand LG100268 (0.01 μM) and the LXR ligand T0901317 (0.1 μM), showed a statistically significant induction in the reporter activity ($P<0.05$).

Interestingly, the addition of both RXR and LXR expression vectors increased the reporter activity ($P<0.05$), indicating presence of endogenous ligands for the RXRα/LXRα heterodimer in COS-1 cells (Figure 5.5). A maximal 4.3-fold increase in reporter activity was observed following the addition of both receptor expression vectors and ligands. This induction was also statistically significant when compared to the cells receiving both receptor expression vectors and no ligand, indicating an increased responsiveness upon LXR activation. This result suggests that generation of a deletion construct and use of this construct in the transfection assay optimized LXR activation.

A



B

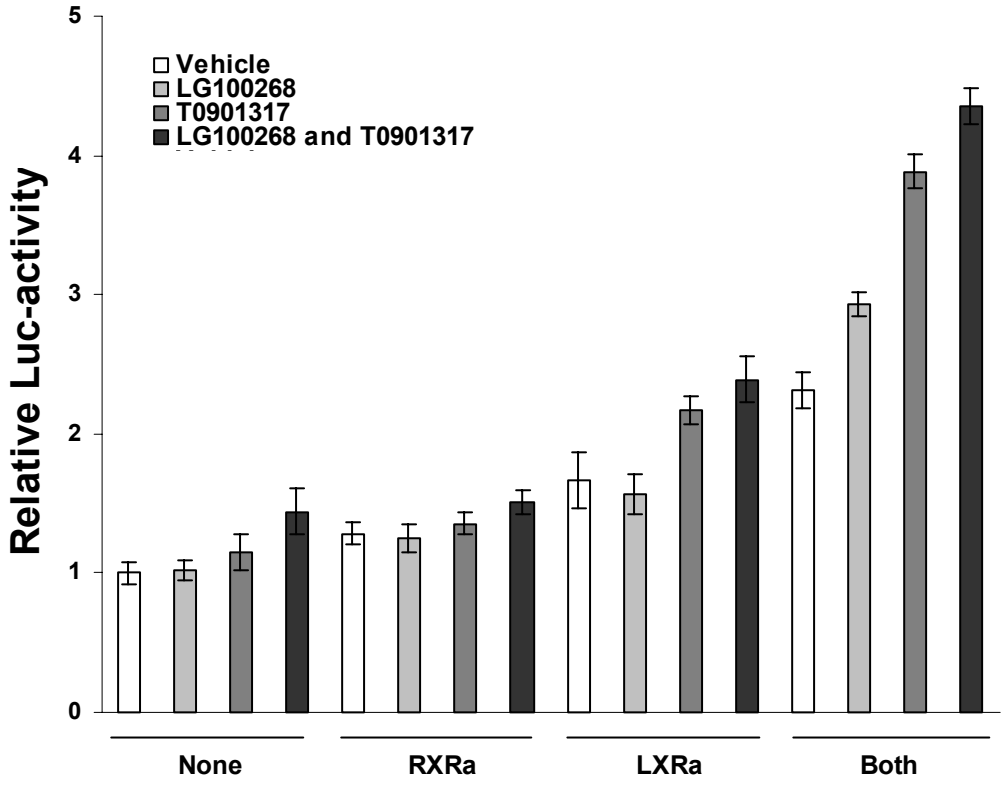


Figure 5.5 Transfection with a human adipophilin reporter in COS-1 cells shows that adipophilin is a LXR target gene.

A) A schematic presentation of the deletion construct made for the human adipophilin promoter. The arrow points out the transcription start site.

B) Transient transfection with the wild type deletion construct of human adipophilin luciferase reporter into COS-1 cells. The cells were co-transfected with β-galactosidase expression vector as an internal control and pCMX, pCMX-RXRα and/or pCMX-LXRα expression vectors as indicated. The medium was supplemented with vehicle (DMSO; white), RXRα ligand (LG100268; light grey), LXRα ligand (T0901317; dark grey) or both ligands (black). The result is representative for three individual experiments performed in triplicates. Results are given as mean $\pm$ SD. (None, vehicle = 1).

5.3.3 Mutation of LXRE abolish induction by LXRα agonist

Earlier studies have shown that transcriptional regulation of LXR target genes are mediated through a DR4 element (Table 5.1). To test if the DR4 element identified by computer analysis was indeed able to mediate transcriptional activation, LXRE mutation constructs were generated (Figure 5.6) and tested in transfection assays. Targeted mutations were introduced into the two half sites of the LXRE in the reporter construct (Figure 5.2A). Three different clones of the LXRE mutation construct were made to investigate potential errors generated in the PCR based mutation procedure.

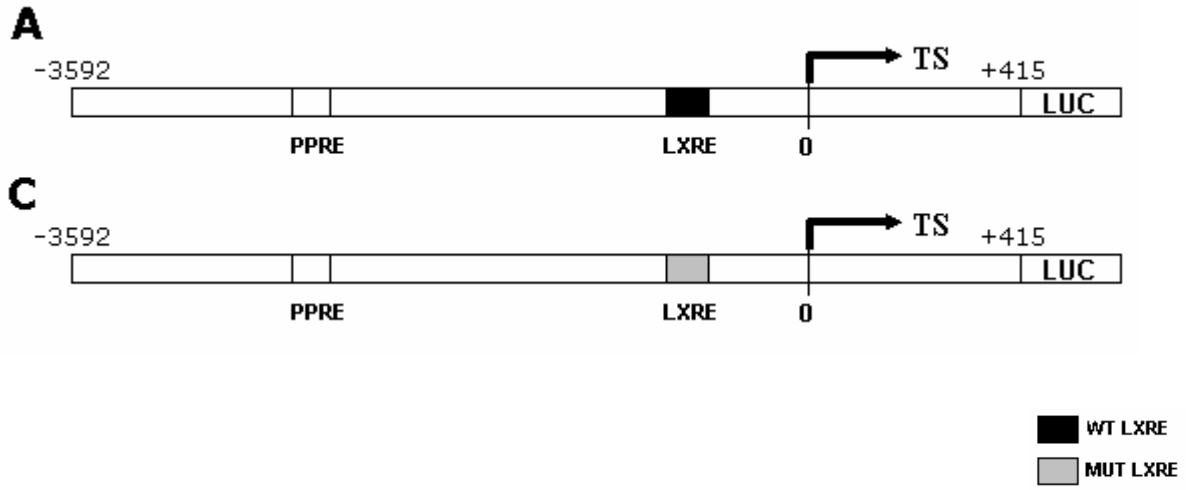


Figure 5.6 Human adipophilin promoter-luciferase reporters

A schematic presentation of the full-length and LXRE mutated construct made for the human adipophilin promoter. The arrow points out the transcription start site. **A)** Full-length construct. **C)** LXRE mutated deletion construct.

Transient transfections of the full-length luciferase reporter into COS-1 cells in combination with LXRα and RXRα expression vectors and stimulation with the LXR ligand T0901317 (0.1 μM), induced reporter activity (Figure 5.7). A maximal 2.5 fold increase in reporter activity was observed following addition of both receptor vectors and the ligand ($P<0.05$). Furthermore, three clones of the full-length construct containing mutated versions of the LXRE as described above, were also transfected into the COS-1 cells in the presence of LXRα and RXRα expression vectors and

T0901317 (0.1 μM). The ability of the RXRα /LXRα heterodimer complex to induce transcription of the adipophilin promoter was then significantly reduced. These data suggest that the LXR activated regulation of the human adipophilin promoter reporter is dependent on the LXRE characterized in Figure 5.1c.

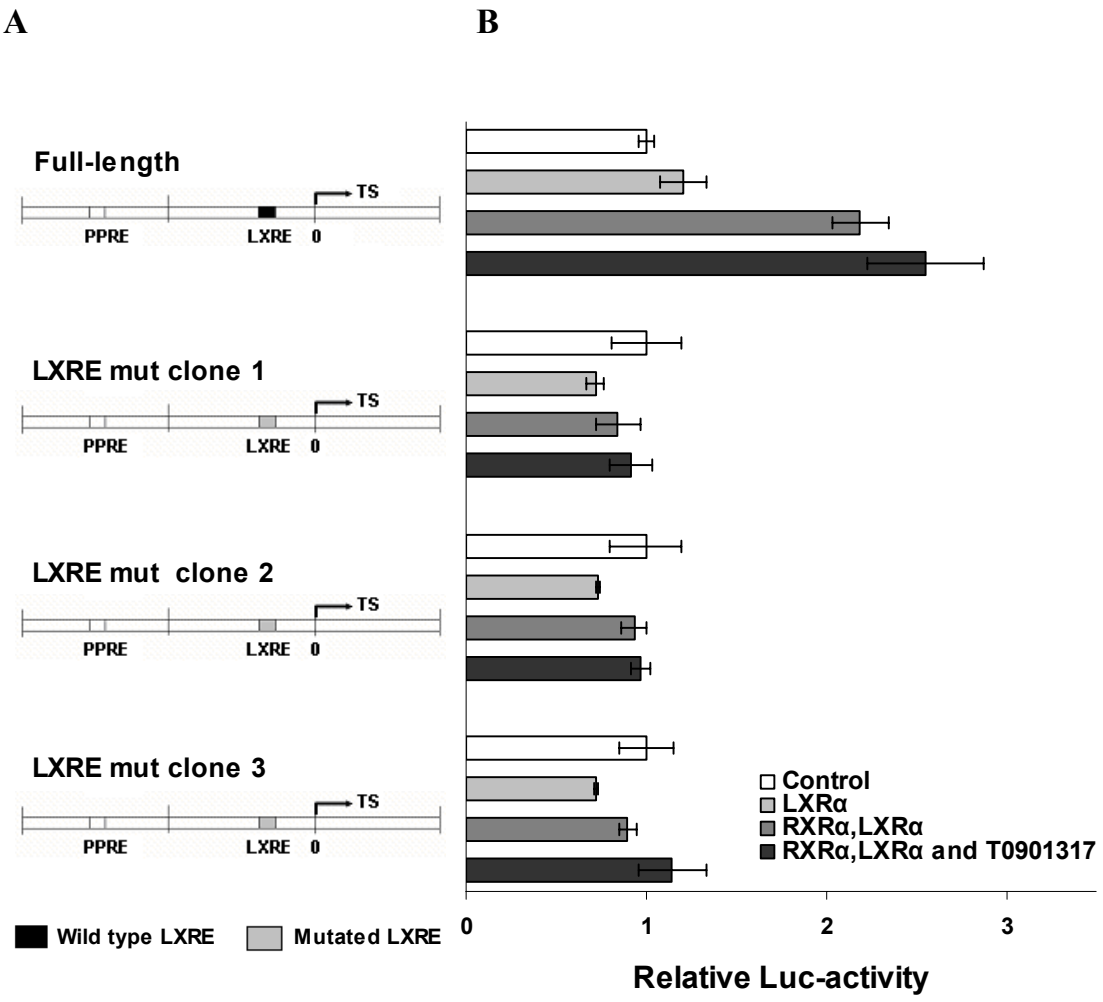


Figure 5.7 Mutation of the identified LXRE in the adipophilin gene abolished reporter activity after LXRα agonist treatment

A) A schematic presentation of the deletion construct and three different clones of the LXRE mutation construct made for the human adipophilin promoter. **B)** Transient transfection of full-length and clone 1-3 of the LXRE mutation construct of adipophilin reporters in COS-1 cells. Cells were co-transfected with no expression plasmid (white), LXRα expression plasmid (light grey), LXRα and RXRα expression plasmids (dark grey and black) stimulated with vehicle (white, light grey and dark grey) or T0901317 (0.1μM; black). Results are given as mean ± SD. (Control = 1).

Clone 1 from the experiments presented in Figure 5.7 was chosen for further studies. A deletion construct of clone 1 was generated using the PVU II restriction enzyme (Figure 5.8).

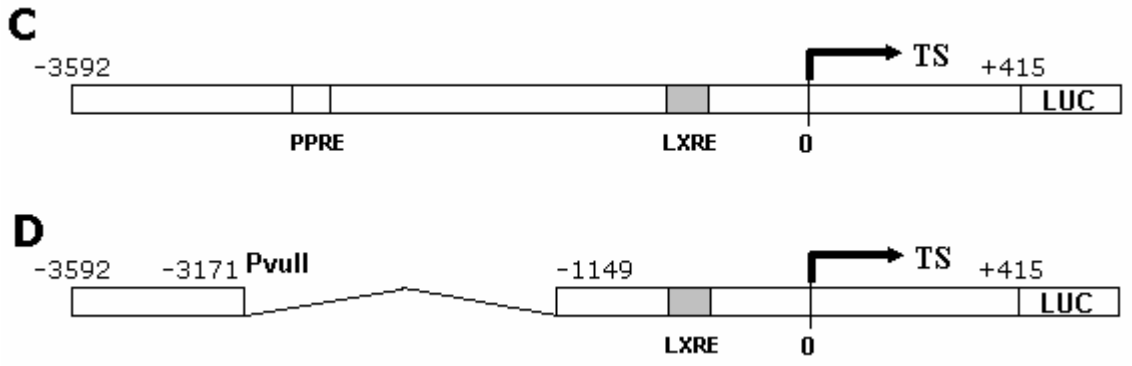


Figure 5.8 Human adipophilin LXRE mutated deletion reporter

A schematic presentation of the LXRE mutation construct and its corresponding deletion construct made for the human adipophilin promoter. The arrow points out the transcription start site. **C)** LXRE mutation construct. **D)** LXRE mutaded delition construct.

Clone 1 of the LXRE mutation constructs in Figure 5.7, its corresponding LXRE-mutated deletion construct, the full-length construct and the deletion construct lacking the PPAR response element were transfected into COS-1 cells in the presence of LXRα and RXRα expression vectors and the LXR ligand T0901317 (0.1 μM) (Figure 5.9).

Addition of both receptor expression vectors and the LXR ligand increased reporter activity only in the full-length and deletion construct as expected (Figure 5.9). This increase was statistically significant for both constructs ($P<0.05$). The LXRE mutated deletion construct completely abolished the induction by ligands.

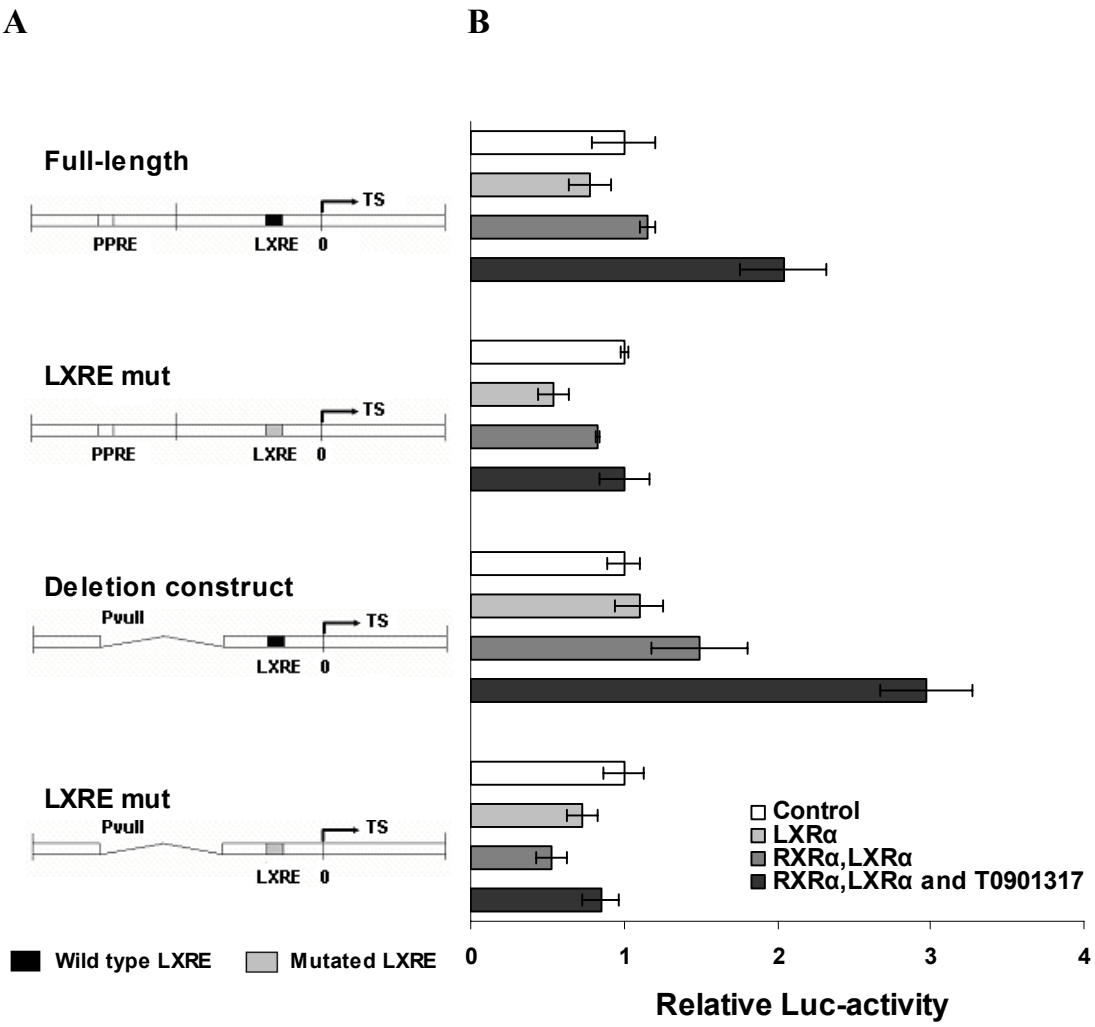


Figure 5.9 Transfections with deletion constructs and LXRE-mutated adipophilin promoters in COS 1-cells

A) A schematic presentation of the deletion and the LXRE mutation construct made for the human adipophilin promoter.

B) Transient transfection of full-length, LXRE-mutated full-length, deletion construct and LXRE-mutated deletion construct of adipophilin reporters in COS-1 cells. Cells were co-transfected with no expression plasmid (white), LXRα expression plasmid (light grey), LXRα and RXRα expression plasmids (dark grey and black) stimulated with vehicle (white, light grey and dark grey) or T0901317 (0.1μM; black). Results are given as mean ± SD. (None, Control = 1).

5.4 Regulation of the adipophilin promoter in HepG2 cells

5.4.1 The adipophilin promoter is a target for regulation by LXR

To determine the functionality and responsiveness of the identified LXRE in a more physiologically relevant cell strain than COS-1 cells, the impact of a LXR agonist alone or in combination with a RXR agonist was investigated in the human hepatic HepG2 cell line. Transfection optimizing analysis using GFP was first performed, and is described in methods and results shown in Figure 5.10 and 5.11.

HepG2 cells were cultured in 12-well plates and transiently transfected with the deletion construct in combination with RXRα and LXRα expression vectors and incubated for 72 hours with the RXR ligand LG100268 (0.01 μM) and the LXR ligand T0901317 (0.1 μM).

The addition of RXRα and LXRα expression vectors gave a statistically significant elevation in reporter activity, indicating a high level of endogenous agonists also in HepG2 cells (Figure 5.12). A maximum 3.2-fold increase in reporter activity was measured after treatment with both ligands ($P<0.05$). These results are consistent with results from transfection studies in COS-1 cells presented in Figure 5.5.

Interestingly, an additional transfection experiment using lipoprotein deficient serum (LPDS) gave an 8-fold increase in reporter activity ($P<0.05$) (A3 / Appendix). This experiment was carried out to investigate if there were any endogenous agonists in the previously used serum, but the differences between addition of both RXR and LXR expression vectors with or without ligands were not increased compared to previous experiments (~2-fold increase). This result might however suggest the presence of antagonists or inhibitors in the original serum (FCS).

Taken together, the results from EMSA and transfection studies indicate that the adipophilin promoter is a target for regulation by LXR.

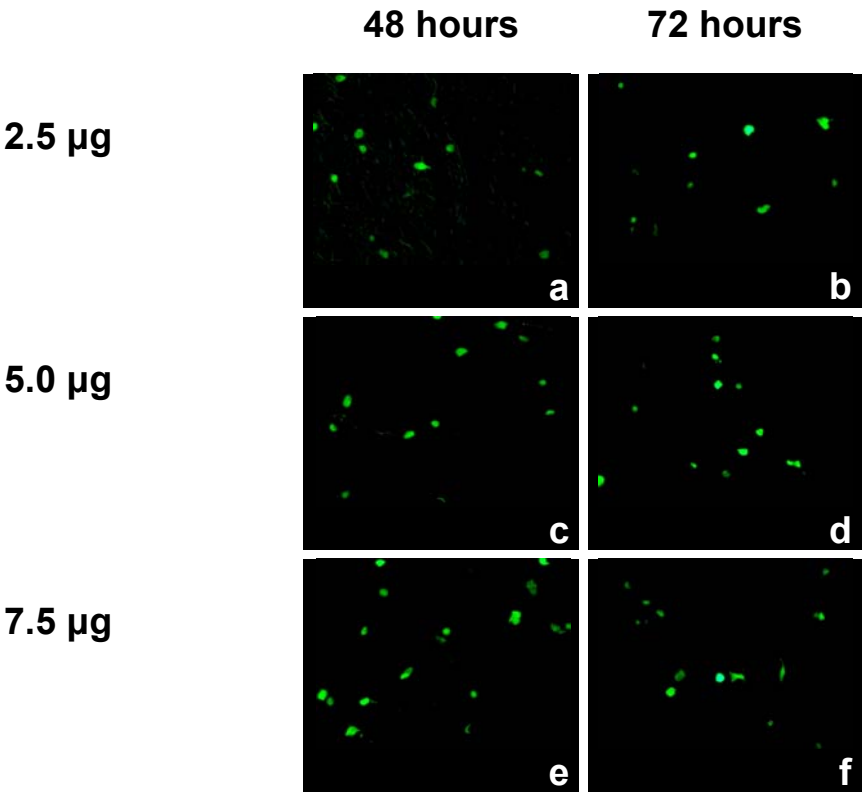
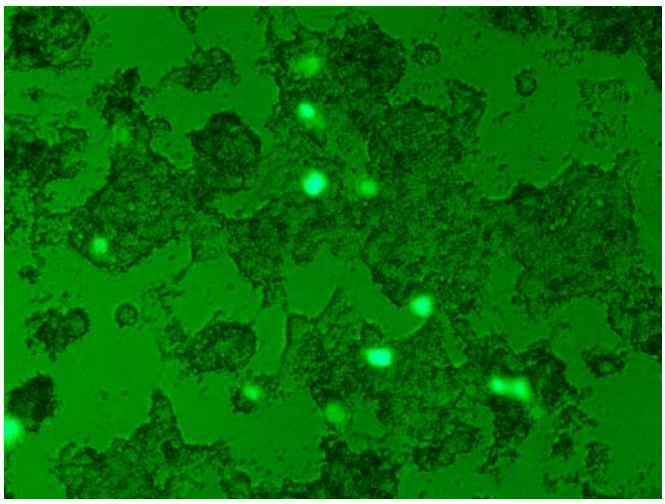


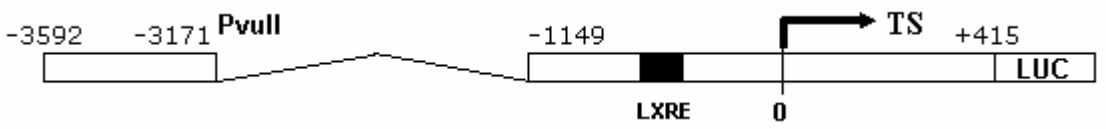
Figure 5.10 Determination of transient transfection efficiency in HepG2-cells

The plasmid EGFP with 2.5 (a and b), 5.0 (c and d) or 7.5 µg (e and f) DNA was transiently transfected into HepG2 cells using the calcium phosphate precipitation method. Differences in green fluorescence protein expression were determined visually after 48 and 72 hours. Each picture is representantive of three photos.

Figure 5.11
Amount of green florescence protein positive cells

The picture shows the amount of green fluorescence protein positive HepG2 cells after transfection with 5 µg DNA for 72 hours (Figure 5.10 d). Conditions similar to Figure 5.10 d / Figure 5.11 were chosen for further studies in HepG2 cells.





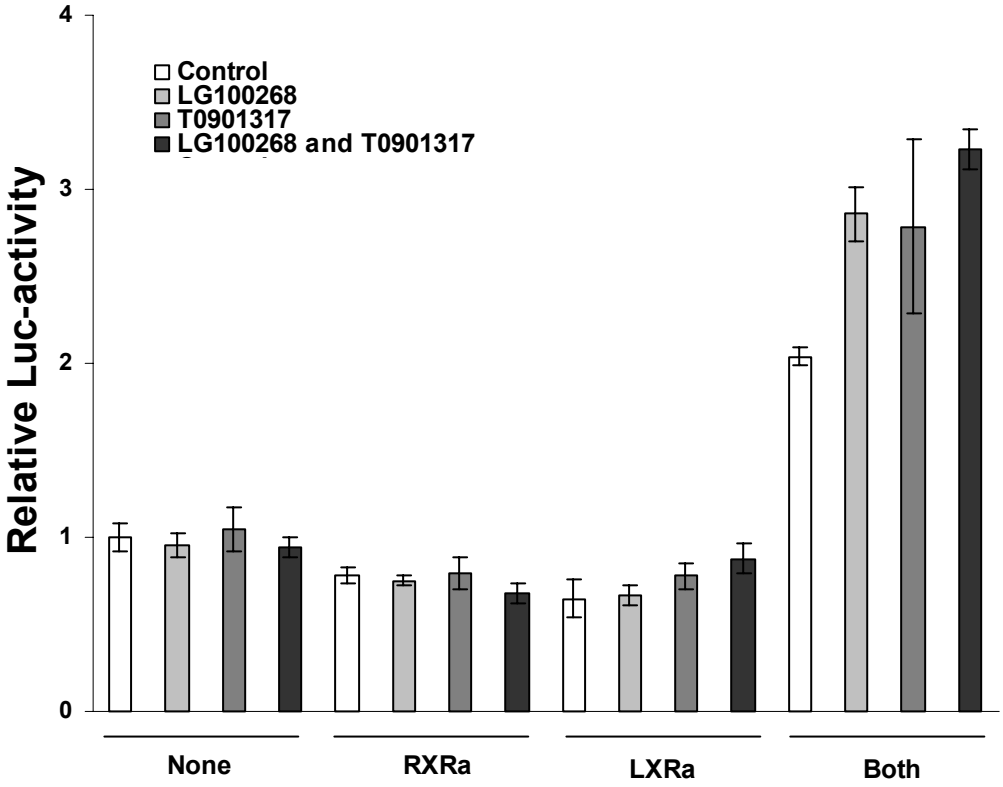


Figure 5.12 Transfection with a human adipophilin reporter construct in HepG2 cells shows LXR responsiveness

B) Transient transfection with the deletion construct of human adipophilin luciferase reporter into HepG2 cells. The cells were co-transfected with β-galactosidase expression vector as an internal control and RXRα and/or LXRα expression vectors as indicated. The medium was supplemented with vehicle (DMSO; white), RXRα ligand (LG100268; light grey), LXRα ligand (T0901317; dark grey) or both ligands (black). The result is representative for three individual experiments performed in triplicates. Results are given as mean ± SD. (None, Control = 1).

6. Discussion

6.1 Methodology

Analysis of reporter gene activity driven by various adipophilin constructs and binding studies *in vitro* have been central methods in this thesis. These are common methods frequently used in molecular biology research and have their advantages and limitations.

Analysis of the transcriptional potential of the adipophilin promoter was examined by cloning the promoter in front of the reporter gene and introducing this construct into COS-1 and HepG2 cells. Transcriptional regulatory elements might be located over great distances in the genome and can be found far upstream, far downstream as well as within gene introns. The reporter gene approach to identify response elements in promoters usually involves cloning of promoter fragments of 5 kb or less in front of the reporter gene. The responses or lack of responses, found in such systems do not necessary reflect the “true” responses since the promoter fragment is taken out of its endogenous environment in the genome. Thus, cloning parts of the promoter obviously does not include all the regulatory elements involved in the regulatory processes rendering the researcher with only limited information of the overall transcriptional mechanisms involved. However, this approach does provide basic information of the adipophilin promoter and its respective response elements being investigated.

The choice of transfection method may also influence the results in the promoter analysis. In this work transfections were performed after the calcium phosphate precipitation method. This method may not be the most optimal accessible, but it is widely used and can be performed quite easily with reagent-grade chemicals at a reasonable cost.

Cell lines express different levels of transcription factors and an ideal cell strain for transfection studies would be cells not expressing the expression vectors of interest. Experience with COS-1 cells in our laboratory indicates that these cells do not, or only to a modest extent, express LXRα. To make sure that COS-1 and HepG2 cells had adequate levels of transcription factors to give responses to the RXR and LXR agonists in the experiments, cells were co-transfected with RXR, LXR or both expression vectors. Transfection studies indicated that these cell lines did not have high levels of RXR and LXRα transcription factors prior to transfection, since an increased response was observed after co-transfection with expression vectors and no agonist treatment. This elevated activity also unravelled presence of endogenous ligands. To determine if compounds in the fetal calf serum (FCS) in the culture medium could be the source of LXR agonists, transfections with LPDS was performed (Figure A3 / Appendix). Differences in reporter activity between addition of both RXR and LXR expression vectors with or without ligands were not increased compared to previous experiments (~2-fold increase), but their maximal 5.5-fold and 8-fold increase compared to the control is an interesting result ($P<0.05$). A possible explanation is that the LPDS has less antagonists or inhibitors, resulting in an elevated transcriptional activity. Further transfection studies examining LXR target genes should therefore consider these results when it's desirable to optimize the reporter activity. Another potential candidate for the observed high basic activity was c/EBPα, a key transcription factor that acts as master regulator of many cellular responses and which are able to induce PPARγ expression (155;156). Transfection experiments with c/EBPα suggest that this transcription factor do not regulate adipophilin (Figure A2 / Appendix). Transfection studies with deletion constructs of the adipophilin promoter did neither reduce basic activity, but an elevated reporter activity was observed and might be explained by removal of regulatory elements such as the PPRE.

The optimal ligand concentrations for the COS-1 and HepG2 cell lines were also investigated. Several concentrations of T0901317 were examined in the transfection studies. Doses from 0.0001 to 5.0 μM were tested and a T0901317 concentration of

0.1 μM gave maximal increase in luciferase responses (results not shown). Higher doses might be toxic to the cells as the β-galactosidase activity rapidly decreased. For LG100268 a concentration of 0.01 μM was chosen to achieve maximal luciferase response in COS-1 and HepG2 cells. The ligand titration data were used as a tool to achieve maximal reporter responses in the transfection studies presented in the result chapter.

In these experiments the recently developed synthetic ligand T0901317, utilized to study the effects of LXR activation *in vivo*, was used. Docking studies of several endogenous agonists have suggested a common anchoring essentially identical to T0901317 (157). 22(R)-hydroxycholesterol, 24(S)-hydroxycholesterol and 24(S),25-epoxycholesterol are naturally-occurring agonists for LXR that bind at physiological concentrations and likely serve as endogenous modulators of LXR activities (41;42;158). A limitation in LXR research in general is that a large amount of information on the effects of LXR activation have relied on only a few LXR ligands on a wide variety of parameters in cultured mammalian cells. Unfortunately, other oxysterols may be of considerably more physiological importance. Moreover, the results of studies with these agonists have been frequently generalized to other oxysterols without experimentation. The limited availability of natural and stabile oxysterols might also be a major factor responsible for the very restricted number of studies of their in vivo effects.

COS-1 cells were chosen for the initial transfection studies since this cell line is an especially well suited host for transfections. The choice of cell lines is an important factor when promoter regions and response elements are characterized. Immortal cell lines commonly have an increased growth rate and a low differentiation level, which can make them different in terms of cell signalling responses and gene expression levels compared to normal cells. The use of primary cells would provide a more natural model system, but such cells are more difficult to obtain and keep in culture. However, observed effects in immortal cell lines could be confirmed in primary cells to further verify results obtained from cell lines.

SGBS cells were originally the preferred cell line to reproduce results from transfection studies. SGBS adipocytes are morphologically, biochemically and functionally identical to in vitro differentiated adipocytes and are therefore a suited human adipocyte *in vitro* model system. However, several components in the differentiation medium could interfere with the test parameters. Insulin, triiodothyronine and cortisol are important hormones in regulation of lipogenesis and might alter expression of genes including LXRα and adipophilin. These cells do neither fulfill the criteria for a cell line, since they are not immortalized and lose their capacity to proliferate and finally die after 70 generations. Figure A5 / Appendix shows successful differentiation of SGBS cells from fibroblasts like cells to cells with adipocyte like morphology with characteristic lipid droplets in cytoplasm. Work with SGBS cells revealed that more experience and optimizing of the protocol are necessary before reliable results can be obtained regarding studies on LXR, and to examine whether adipophilin is regulated by LXR in this cell type. Due to troubleshooting with SGBS cells, HepG2 cells were used to determine the functionality and responsiveness of the identified LXRE in a more physiologically relevant cell strain than COS-1 cells. The human stellate fat storing cell line LI90 replaced SGBS cells in RNA analysis, but investigations in this cell line did not leed to reliable results regarding regulation of adipophilin mRNA by LXR activation *in vitro.* Furher studies are needed to obtain these RNA data, however this is beyond the scope of this thesis.

EMSA was used in this work because it is by far the most frequently used assay, largely because it is the most straightforward and has proven to be the most sensitive and successful for detecting and characterizing specific protein-DNA interactions at an early stage of an analysis. Several methods are in use for the in vitro detection of and characterization of protein-DNA interactions, EMSA, DNase I footprint, exonuclease III footprinting, Southwestern blotting, Chromatin immunoprecipitation (ChIP), various chemical protection and interference assays and UV crosslinking. Procedures and experiences regarding these alternatives are not implemented in our lab and EMSA was chosen to give most reliable results.

The change in reporter activity observed in this work is in accordance with results reported on other LXR target genes (References in Table 5.1). However, Mann Whitney U test was conducted for significance testing of the data from transfection studies. It might be questionable if use of statistical analysis is necessary when the sample size is small and the differences between groups are as high as 300 to 400 %. Today many manuscripts submitted to journals only focus their conclusion on genes that show a change in activity of, say, more than a 2-fold (159), but several journals are now toughening up their criteria for accepting papers and some have published guidelines for stressing the importance of appropriate statistical analysis (159;160).

6.2 General discussion

In light of the potential importance of lipid droplet associated proteins in lipid metabolism, the transcriptional regulation of adipophilin was examined. This work shows that LXRα binds the LXRE in the human adipophilin promoter demonstrated in electrophoretic mobility shift assay. Furthermore, treatment of COS-1 and HepG2 cells with an agent that selectively activates LXRs increases human adipophilin expression in transfection studies. A DR4, a potential LXRE, was previously identified with computer analysis of the human adipophilin promoter. This promoter is active in COS-1 and HepG2 cells and its reporter activity is induced by treatment of agents that activate RXR or LXR. Mutation of the identified DR4 completely abolished the inducible response of the adipophilin promoter to a selective LXR agonist. Our data indicates that the DR4 element found in the adipophilin promoter is a functional LXRE and suggests that adipophilin is a target gene of LXR.

Previous observations have indicated (117;161;162) and demonstated (102) that the expression of adipophilin and two other members of the LDAP family, S3-12 and perilipin, are regulated by PPARs (102;117;162), which are important transcription factors in adipogenesis. Dalen et al further suggest that the anti-diabetic effects of PPARγ activation might to a certain extent be mediated by altered expression of LDAPs (102). The data from transfection studies presented in this thesis are the first to show that a member of the LDAP family is regulated by LXRα. Recent studies have identified LXRs as important regulators of cholesterol and lipid metabolism. It has been reported that LXR ligands increase expression of ABCA1 and induce apoA-I-dependent cholesterol efflux (64). LXR ligands have also been shown to induce expression of genes involved in lipogenesis and elevate plasma triglyceride levels (44;58). Recently, triglyceride and cholesterol metabolism have been linked identifying LXRα as a PPARγ target gene in macrophages (71) and adipose tissue (57). Nebb and colleagues have also shown that LXRs are necessary for SREBP-1c induction by insulin (163), but it is unclear whether LXRs are just required for

keeping a sufficient SREBP basal level. Interestingly, SREBP-1c is an important transcription factor in regulation of lipogenesis, and SREBP-1c target genes are inhibited in LXRα/β ”double knock-out” (DOKO) mice compared to control mice (8). An important observation in our laboratory, supporting the putative interplay between lipid and cholesterol metabolism, is that LXR increase lipid droplet size in adipocytes and induce expression of SREBP. Furthermore, LXRα/β DOKO mice show gradually decreased white and brown adipose tissue with age compared with control mice (57). This indicates that LXR have a function in remodelling of fat in adipose tissue.

Although, the function of adipophilin is not fully elucidated, there is some evidence for a role in lipid droplet formation and fatty acid uptake (114). Interestingly, long chain fatty acids stimulate adipophilin gene transcription (117). In addition, deletion of the gene encoding perilipin in mice suggests that adipophilin or other LDAPs might help regulate lipid uptake in addition to forming lipid storage droplets (108). Taken together, our findings of elevated adipophilin expression upon LXR activation and the potential role of adipophilin in lipid droplet formation might give further evidence to the link between lipid and cholesterol metabolism.

The results from this thesis suggest that some of the reported up-regulation of adipophilin by PPARγ activation may be mediated by PPARγ induced expression of LXRα, at least in human. Bildirici et al recently reported elevated human adipophilin expression upon PPARγ activation in human trophoblasts (161). A putative and highly theoretically mechanism is that PPARγ ligands give a PPARγ induction of target genes including LXRα which then induces adipophilin expression (Figure 6.1). Our results might then bring new knowledge regarding a more direct gene regulation of adipophilin in human upon ligand activation of LXR. Nebb’s group have recently reported this cross-talk between PPARγ and LXR in adipose tissue, but physiological evidence is still scarce and need further investigation.

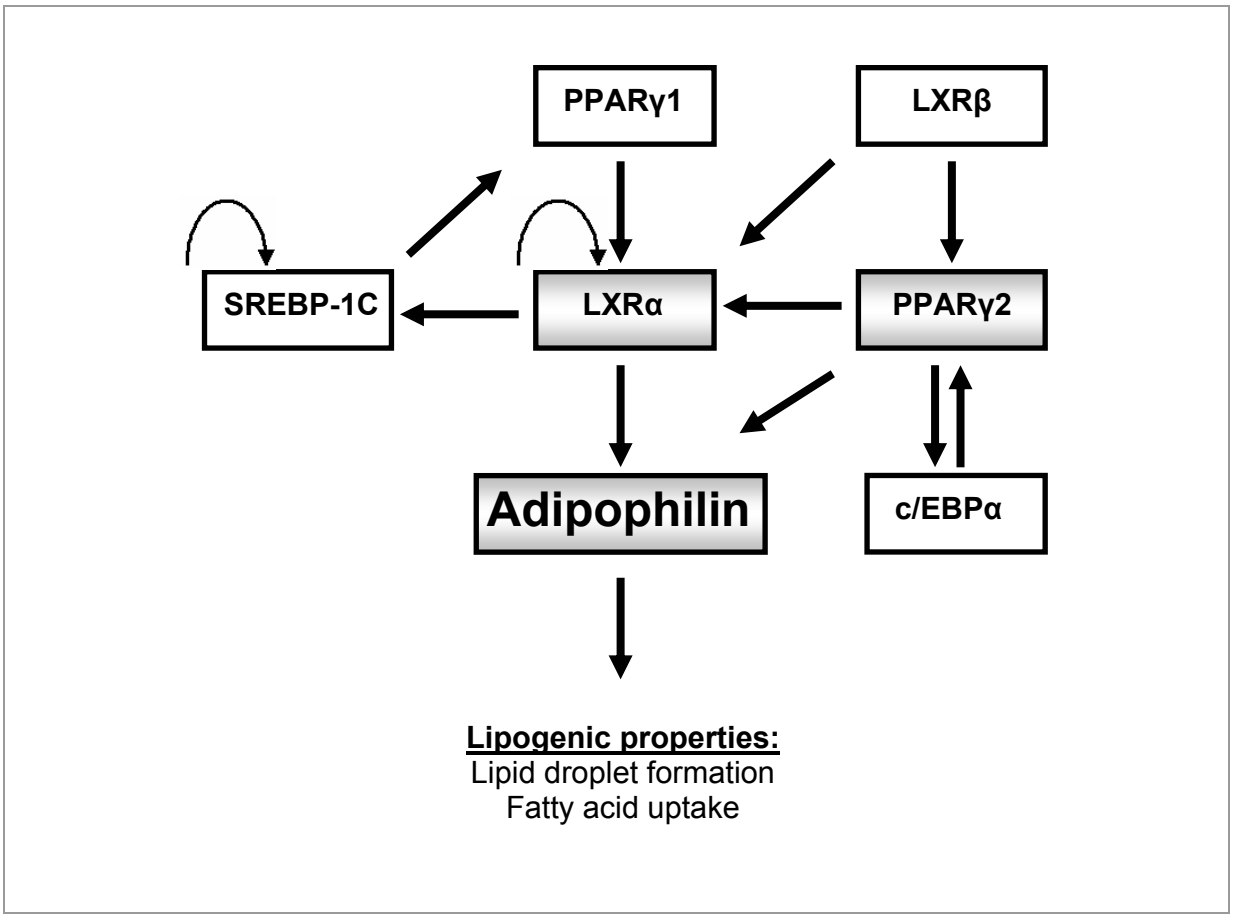


Figure 6.1 Transcription factors that might alter adipophilin expression

PPARγ2 is shown to be regulated by c/EBPα (155) and LXRβ, and has been reported to induce adipophilin expression (102). LXRα expression is induced by PPARγ (57;71;164) and LXRβ (71;72;74), and is shown to be autoregulated (71) and regulate SREBP-1c (58). SREBP-1c is also autoregulated (165) and regulate PPARγ1 (166). Our finding of LXRα responsive adipophilin expression suggests a direct regulation of the human adipophilin gene not dependent on PPARγ, and elucidates a potential complex interplay between transcription factors resulting in altered adipophilin expression.

Previous studies have shown that adipophilin mRNA is induced early during adipocyte differentiation (111;112) and is expressed prior to PPARγ (102). This observation indicates that adipophilin expression, unlike S3-12 and perilipin, is not dependent on PPARγ. In 3T3-L1 cells adipophilin protein levels increase during differentiation together with perilipin and S3-12. These findings suggest that adipophilin may play a certain role in the early stage of adipose differentiation. LXRα is induced later in adipose differentiation, supporting observations of this nuclear receptor as a PPARγ target gene (57). Our findings of a functional LXRE in the

human promoter indicate that adipophilin may also have a certain role later in adipose differentiation, since adipophilin induction matches the timing of LXRα induction (80;102). This might at least be plausible at the transcription level, but it is uncertain if translational modifications promote degeneration of mRNA or protein levels of adipophilin.

Heid et al have reported a very high sequence homology and immunological cross-reactivity over a range of species, including human, bovine, pig, dog, rat, and mouse, indicating that adipophilin has been strongly conserved during evolution. The human and mouse proteins share extensive homology with overall 84 % identical amino acids (91). However, alignment of the human and mouse promoter suggest that the adipophilin promoter has undergone changes in the course of evolution resulting in species variation in LXR responsiveness and thus the gene regulation of adipophilin. Caution should therefore be exercised when interpreting and extrapolating LXR findings from animal studies into human.

Elevated expression of LDAPs is proposed to increase the body's capacity to trap circulating fatty acids in white adipose tissue, resulting in elevated insulin sensitivity (102). Investigations of the gene regulation of LDAPs are therefore important to confirm if altered regulation of these genes are involved in diabetes type 2, obesity and its comorbidities. Because NRs bind small molecules which can be easily modified by drug design, and regulate a group of diverse and crucial biological functions such as metabolism, homeostasis, development and disease, they have become promising pharmacological targets. The ability of LXR ligands to promote cellular cholesterol efflux makes them potentially attractive agents for the modulation of human lipid metabolism. Their lipogenic activity, however, is a major limitation (59;167). Although the ability of LXR ligands to raise HDL levels is promising, the transient hypertriglyceridemia induced by the current available agonists is an undesired side effect. Clearly, a detailed understanding of the mechanism whereby LXR ligands raise triglyceride levels will be required before LXR can be optimized as an intervention target. Interestingly, recent evidence suggests that adipophilin

could play a regulatory role in lipid accumulation. For example, adipophilin facilitates uptake and transport of LCPUFAs in a dose dependent manner (114), and fatty acids are robust stimulus of adipophilin gene transcription (117). Development of specific LXR agonists not affecting the liver, only LXR target genes with fatty acid trapping properties could be an interesting challenge in pharmacological research. However, the knowledge of adipophilin and other LDAPs is still in its infancy and barely beyond the descriptive stage. Further investigations of their functions and gene regulations are important to unravel their potential role in lipid and cholesterol metabolism and as potential intervention targets in common metabolic diseases.

6.3 Conclusion

This work shows that LXRα bind to a DR4 response element in the human adipophilin promoter. Furthermore, treatments of COS-1 and HepG2 cells with an agent that selectively activate LXR increase human adipophilin expression in transfection studies. An identified LXRE, the DR4 element, is active in COS-1 and HepG2 cells and is induced by treatment with agonists that activate RXR or LXR. Mutation of the identified LXRE completely abolished the inducible response of the adipophilin promoter to selective LXR agonist.

Our data indicates that adipophilin is a novel and functional LXR target gene. This is the first report to demonstrate that a member of the LDAP family is regulated by LXR and bring further evidence to the cross-talk approach between cholesterol and lipid metabolism.

7. References

(1) Report of a Joint FAO/WHO Expert Consultation. Diet, nutrition and the prevention of chronic diseases. 2003. WHO Technical Report Series, No. 916.

(2) Muller M, Kersten S. Nutrigenomics: goals and strategies. Nat Rev Genet 2003; 4(4):315-322.

(3) Francis GA, Fayard E, Picard F, Auwerx J. Nuclear receptors and the control of metabolism. Annu Rev Physiol 2003; 65:261-311.

(4) Lu TT, Repa JJ, Mangelsdorf DJ. Orphan nuclear receptors as eLiXiRs and FiXeRs of sterol metabolism. J Biol Chem 2001; 276(41):37735-37738.

(5) Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K et al. The nuclear receptor superfamily: the second decade. Cell 1995; 83(6):835-839.

(6) Chawla A, Repa JJ, Evans RM, Mangelsdorf DJ. Nuclear receptors and lipid physiology: opening the X-files. Science 2001; 294(5548):1866-1870.

(7) Beato M, Herrlich P, Schutz G. Steroid hormone receptors: many actors in search of a plot. Cell 1995; 83(6):851-857.

(8) Evans RM. The steroid and thyroid hormone receptor superfamily. Science 1988; 240(4854):889-895.

(9) Kastner P, Mark M, Chambon P. Nonsteroid nuclear receptors: what are genetic studies telling us about their role in real life? Cell 1995; 83(6):859-869.

(10) Mangelsdorf DJ, Evans RM. The RXR heterodimers and orphan receptors. Cell 1995; 83(6):841-850.

(11) Smale ST, Kadonaga JT. The RNA polymerase II core promoter. Annu Rev Biochem 2003; 72:449-479.

(12) Calhoun VC, Stathopoulos A, Levine M. Promoter-proximal tethering elements regulate enhancer-promoter specificity in the Drosophila Antennapedia complex. Proc Natl Acad Sci U S A 2002; 99(14):9243-9247.

(13) Burgess-Beusse B, Farrell C, Gaszner M, Litt M, Mutskov V, Recillas-Targa F et al. The insulation of genes from external enhancers and silencing chromatin. Proc Natl Acad Sci U S A 2002; 99 Suppl 4:16433-16437.

(14) Levine M, Tjian R. Transcription regulation and animal diversity. Nature 2003; 424(6945):147-151.

(15) Wong JM, Bateman E. TBP-DNA interactions in the minor groove discriminate between A:T and T:A base pairs. Nucleic Acids Res 1994; 22(10):1890-1896.

(16) Burke TW, Kadonaga JT. Drosophila TFIID binds to a conserved downstream basal promoter element that is present in many TATA-box-deficient promoters. Genes Dev 1996; 10(6):711-724.

(17) Kutach AK, Kadonaga JT. The downstream promoter element DPE appears to be as widely used as the TATA box in Drosophila core promoters. Mol Cell Biol 2000; 20(13):4754-4764.

(18) Nikolov DB, Chen H, Halay ED, Usheva AA, Hisatake K, Lee DK et al. Crystal structure of a TFIIB-TBP-TATA-element ternary complex. Nature 1995; 377(6545):119-128.

(19) Olefsky JM. Nuclear receptor minireview series. Journal of Biological Chemistry 2001; 276(40):36863-36864.

(20) Robinson-Rechavi M, Escriva GH, Laudet V. The nuclear receptor superfamily. J Cell Sci 2003; 116(Pt 4):585-586.

(21) Privalsky ML. The role of corepressors in transcriptional regulation by nuclear hormone receptors. Annu Rev Physiol 2004; 66:315-360.

(22) Brelivet Y, Kammerer S, Rochel N, Poch O, Moras D. Signature of the oligomeric behaviour of nuclear receptors at the sequence and structural level. EMBO Rep 2004; 5(4):423-429.

(23) Kliewer SA, Forman BM, Blumberg B, Ong ES, Borgmeyer U, Mangelsdorf DJ et al. Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. Proc Natl Acad Sci U S A 1994; 91(15):7355-7359.

(24) Kliewer SA, Forman BM, Blumberg B, Ong ES, Borgmeyer U, Mangelsdorf DJ et al. Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. Proc Natl Acad Sci U S A 1994; 91(15):7355-7359.

(25) Forman BM, Chen J, Evans RM. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. Proc Natl Acad Sci U S A 1997; 94(9):4312-4317.

(26) Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS et al. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors alpha and gamma. Proc Natl Acad Sci U S A 1997; 94(9):4318-4323.

(27) Vosper H, Patel L, Graham TL, Khoudoli GA, Hill A, Macphee CH et al. The peroxisome proliferator-activated receptor delta promotes lipid accumulation in human macrophages. J Biol Chem 2001; 276(47):44258-44265.

(28) Tontonoz P, Hu E, Graves RA, Budavari AI, Spiegelman BM. mPPAR gamma 2: tissue-specific regulator of an adipocyte enhancer. Genes Dev 1994; 8(10):1224-1234.

(29) Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM. 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. Cell 1995; 83(5):803-812.

(30) Xu HE, Lambert MH, Montana VG, Parks DJ, Blanchard SG, Brown PJ et al. Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. Mol Cell 1999; 3(3):397-403.

(31) Lehmann JM, Moore LB, Smith-Oliver TA, Wilkison WO, Willson TM, Kliewer SA. An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). J Biol Chem 1995; 270(22):12953-12956.

(32) Edwards PA, Kast HR, Anisfeld AM. BAREing it all: the adoption of LXR and FXR and their roles in lipid homeostasis. J Lipid Res 2002; 43(1):2-12.

(33) Peet DJ, Janowski BA, Mangelsdorf DJ. The LXRs: a new class of oxysterol receptors. Curr Opin Genet Dev 1998; 8(5):571-575.

(34) Apfel R, Benbrook D, Lernhardt E, Ortiz MA, Salbert G, Pfahl M. A novel orphan receptor specific for a subset of thyroid hormone-responsive elements and its interaction with the retinoid/thyroid hormone receptor subfamily. Mol Cell Biol 1994; 14(10):7025-7035.

(35) Willy PJ, Umesono K, Ong ES, Evans RM, Heyman RA, Mangelsdorf DJ. LXR, a nuclear receptor that defines a distinct retinoid response pathway. Genes Dev 1995; 9(9):1033-1045.

(36) Seol W, Choi HS, Moore DD. Isolation of proteins that interact specifically with the retinoid X receptor: two novel orphan receptors. Mol Endocrinol 1995; 9(1):72-85.

(37) Shinar DM, Endo N, Rutledge SJ, Vogel R, Rodan GA, Schmidt A. NER, a new member of the gene family encoding the human steroid hormone nuclear receptor. Gene 1994; 147(2):273-276.

(38) Song C, Kokontis JM, Hiipakka RA, Liao S. Ubiquitous receptor: a receptor that modulates gene activation by retinoic acid and thyroid hormone receptors. Proc Natl Acad Sci U S A 1994; 91(23):10809-10813.

(39) Teboul M, Enmark E, Li Q, Wikstrom AC, Pelto-Huikko M, Gustafsson JA. OR-1, a member of the nuclear receptor superfamily that interacts with the 9-cis-retinoic acid receptor. Proc Natl Acad Sci U S A 1995; 92(6):2096-2100.

(40) Auboeuf D, Rieusset J, Fajas L, Vallier P, Frering V, Riou JP et al. Tissue distribution and quantification of the expression of mRNAs of peroxisome proliferator-activated receptors and liver X receptor-alpha in humans: no alteration in adipose tissue of obese and NIDDM patients. Diabetes 1997; 46(8):1319-1327.

(41) Lehmann JM, Kliewer SA, Moore LB, Smith-Oliver TA, Oliver BB, Su JL et al. Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. J Biol Chem 1997; 272(6):3137-3140.

(42) Janowski BA, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. Nature 1996; 383(6602):728-731.

(43) Sparrow CP, Baffic J, Lam MH, Lund EG, Adams AD, Fu X et al. A potent synthetic LXR agonist is more effective than cholesterol loading at inducing ABCA1 mRNA and stimulating cholesterol efflux. J Biol Chem 2002; 277(12):10021-10027.

(44) Schultz JR, Tu H, Luk A, Repa JJ, Medina JC, Li L et al. Role of LXRs in control of lipogenesis. Genes Dev 2000; 14(22):2831-2838.

(45) Collins JL, Fivush AM, Watson MA, Galardi CM, Lewis MC, Moore LB et al. Identification of a nonsteroidal liver X receptor agonist through parallel array synthesis of tertiary amines. J Med Chem 2002; 45(10):1963-1966.

(46) Ou J, Tu H, Shan B, Luk A, DeBose-Boyd RA, Bashmakov Y et al. Unsaturated fatty acids inhibit transcription of the sterol regulatory element-binding protein-1c (SREBP-1c) gene by

antagonizing ligand-dependent activation of the LXR. Proc Natl Acad Sci U S A 2001; 98(11):6027-6032.

(47) Yoshikawa T, Shimano H, Yahagi N, Ide T, Amemiya-Kudo M, Matsuzaka T et al. Polyunsaturated fatty acids suppress sterol regulatory element-binding protein 1c promoter activity by inhibition of liver X receptor (LXR) binding to LXR response elements. J Biol Chem 2002; 277(3):1705-1711.

(48) Repa JJ, Mangelsdorf DJ. The liver X receptor gene team: potential new players in atherosclerosis. Nat Med 2002; 8(11):1243-1248.

(49) Costet P, Luo Y, Wang N, Tall AR. Sterol-dependent transactivation of the ABC1 promoter by the liver X receptor/retinoid X receptor. J Biol Chem 2000; 275(36):28240-28245.

(50) Venkateswaran A, Repa JJ, Lobaccaro JM, Bronson A, Mangelsdorf DJ, Edwards PA. Human white/murine ABC8 mRNA levels are highly induced in lipid-loaded macrophages. A transcriptional role for specific oxysterols. J Biol Chem 2000; 275(19):14700-14707.

(51) Laffitte BA, Repa JJ, Joseph SB, Wilpitz DC, Kast HR, Mangelsdorf DJ et al. LXRs control lipid-inducible expression of the apolipoprotein E gene in macrophages and adipocytes.

(52) Cao G, Beyer TP, Yang XP, Schmidt RJ, Zhang Y, Bensch WR et al. Phospholipid transfer protein is regulated by liver X receptors in vivo. J Biol Chem 2002; 277(42):39561-39565.

(53) Luo Y, Tall AR. Sterol upregulation of human CETP expression in vitro and in transgenic mice by an LXR element. J Clin Invest 2000; 105(4):513-520.

(54) Goldberg IJ. Lipoprotein lipase and lipolysis: central roles in lipoprotein metabolism and atherogenesis. J Lipid Res 1996; 37(4):693-707.

(55) Oliveira HC, Ma L, Milne R, Marcovina SM, Inazu A, Mabuchi H et al. Cholesteryl ester transfer protein activity enhances plasma cholesteryl ester formation. Studies in CETP transgenic mice and human genetic CETP deficiency. Arterioscler Thromb Vasc Biol 1997; 17(6):1045-1052.

(56) Peet DJ, Turley SD, Ma W, Janowski BA, Lobaccaro JM, Hammer RE et al. Cholesterol and bile acid metabolism are impaired in mice lacking the nuclear oxysterol receptor LXR alpha. Cell 1998; 93(5):693-704.

(57) Juvet LK, Andresen SM, Schuster GU, Dalen KT, Tobin KA, Hollung K et al. On the role of liver X receptors in lipid accumulation in adipocytes. Mol Endocrinol 2003; 17(2):172-182.

(58) Repa JJ, Liang G, Ou J, Bashmakov Y, Lobaccaro JM, Shimomura I et al. Regulation of mouse sterol regulatory element-binding protein-1c gene (SREBP-1c) by oxysterol receptors, LXRalpha and LXRbeta. Genes Dev 2000; 14(22):2819-2830.

(59) Joseph SB, Laffitte BA, Patel PH, Watson MA, Matsukuma KE, Walczak R et al. Direct and indirect mechanisms for regulation of fatty acid synthase gene expression by liver X receptors. J Biol Chem 2002; 277(13):11019-11025.

(60) Zhang Y, Repa JJ, Gauthier K, Mangelsdorf DJ. Regulation of lipoprotein lipase by the oxysterol receptors, LXRalpha and LXRbeta. J Biol Chem 2001; 276(46):43018-43024.

(61) Kovanen PT, Pentikainen MO. Pharmacological evidence for a role of liver X receptors in atheroprotection. FEBS Lett 2003; 536(1-3):3-5.

(62) Tontonoz P, Mangelsdorf DJ. Liver X receptor signaling pathways in cardiovascular disease. Mol Endocrinol 2003; 17(6):985-993.

(63) Repa JJ, Turley SD, Lobaccaro JA, Medina J, Li L, Lustig K et al. Regulation of absorption and ABC1-mediated efflux of cholesterol by RXR heterodimers. Science 2000; 289(5484):1524-1529.

(64) Schwartz K, Lawn RM, Wade DP. ABC1 gene expression and ApoA-I-mediated cholesterol efflux are regulated by LXR. Biochem Biophys Res Commun 2000; 274(3):794-802.

(65) Kennedy MA, Venkateswaran A, Tarr PT, Xenarios I, Kudoh J, Shimizu N et al. Characterization of the human ABCG1 gene: liver X receptor activates an internal promoter that produces a novel transcript encoding an alternative form of the protein. J Biol Chem 2001; 276(42):39438-39447.

(66) Engel T, Lorkowski S, Lueken A, Rust S, Schluter B, Berger G et al. The human ABCG4 gene is regulated by oxysterols and retinoids in monocyte-derived macrophages. Biochem Biophys Res Commun 2001; 288(2):483-488.

(67) Repa JJ, Berge KE, Pomajzl C, Richardson JA, Hobbs H, Mangelsdorf DJ. Regulation of ATP-binding cassette sterol transporters ABCG5 and ABCG8 by the liver X receptors alpha and beta. J Biol Chem 2002; 277(21):18793-18800.

(68) DeBose-Boyd RA, Ou J, Goldstein JL, Brown MS. Expression of sterol regulatory element-binding protein 1c (SREBP-1c) mRNA in rat hepatoma cells requires endogenous LXR ligands. Proc Natl Acad Sci U S A 2001; 98(4):1477-1482.

(69) Yoshikawa T, Shimano H, Amemiya-Kudo M, Yahagi N, Hasty AH, Matsuzaka T et al. Identification of liver X receptor-retinoid X receptor as an activator of the sterol regulatory element-binding protein 1c gene promoter. Mol Cell Biol 2001; 21(9):2991-3000.

(70) Mak PA, Laffitte BA, Desrumaux C, Joseph SB, Curtiss LK, Mangelsdorf DJ et al. Regulated expression of the apolipoprotein E/C-I/C-IV/C-II gene cluster in murine and human macrophages. A critical role for nuclear liver X receptors alpha and beta. J Biol Chem 2002; 277(35):31900-31908.

(71) Laffitte BA, Joseph SB, Walczak R, Pei L, Wilpitz DC, Collins JL et al. Autoregulation of the human liver X receptor alpha promoter. Mol Cell Biol 2001; 21(22):7558-7568.

(72) Li Y, Bolten C, Bhat BG, Woodring-Dietz J, Li S, Prayaga SK et al. Induction of human liver X receptor alpha gene expression via an autoregulatory loop mechanism. Mol Endocrinol 2002; 16(3):506-514.

(73) Ulven SM, Dalen KT, Gustafsson JA, Nebb HI. Tissue specific auto-regulation of the LXRα gene facilitates induction of apoE in mouse adipose tissue. J Lipid Res. In press.

(74) Whitney KD, Watson MA, Goodwin B, Galardi CM, Maglich JM, Wilson JG et al. Liver X receptor (LXR) regulation of the LXRalpha gene in human macrophages. J Biol Chem 2001; 276(47):43509-43515.

(75) Malerod L, Juvet LK, Hanssen-Bauer A, Eskild W, Berg T. Oxysterol-activated LXRalpha/RXR induces hSR-BI-promoter activity in hepatoma cells and preadipocytes. Biochem Biophys Res Commun 2002; 299(5):916-923.

(76) Kaplan R, Zhang T, Hernandez M, Gan FX, Wright SD, Waters MG et al. Regulation of the angiopoietin-like protein 3 gene by LXR. J Lipid Res 2003; 44(1):136-143.

(77) Cao G, Liang Y, Broderick CL, Oldham BA, Beyer TP, Schmidt RJ et al. Antidiabetic action of a liver x receptor agonist mediated by inhibition of hepatic gluconeogenesis. J Biol Chem 2003; 278(2):1131-1136.

(78) Stulnig TM, Steffensen KR, Gao H, Reimers M, Dahlman-Wright K, Schuster GU et al. Novel roles of liver X receptors exposed by gene expression profiling in liver and adipose tissue. Mol Pharmacol 2002; 62(6):1299-1305.

(79) Stulnig TM, Oppermann U, Steffensen KR, Schuster GU, Gustafsson JA. Liver X receptors downregulate 11beta-hydroxysteroid dehydrogenase type 1 expression and activity. Diabetes 2002; 51(8):2426-2433.

(80) Dalen KT, Ulven SM, Bamberg K, Gustafsson JA, Nebb HI. Expression of the insulin-responsive glucose transporter GLUT4 in adipocytes is dependent on liver X receptor alpha. J Biol Chem 2003; 278(48):48283-48291.

(81) Laffitte BA, Chao LC, Li J, Walczak R, Hummasti S, Joseph SB et al. Activation of liver X receptor improves glucose tolerance through coordinate regulation of glucose metabolism in liver and adipose tissue. Proc Natl Acad Sci U S A 2003; 100(9):5419-5424.

(82) Landis MS, Patel HV, Capone JP. Oxysterol activators of liver X receptor and 9-cis-retinoic acid promote sequential steps in the synthesis and secretion of tumor necrosis factor-alpha from human monocytes. J Biol Chem 2002; 277(7):4713-4721.

(83) Fowler AJ, Sheu MY, Schmuth M, Kao J, Fluhr JW, Rhein L et al. Liver X receptor activators display anti-inflammatory activity in irritant and allergic contact dermatitis models: liver-X-receptor-specific inhibition of inflammation and primary cytokine production. J Invest Dermatol 2003; 120(2):246-255.

(84) Steffensen KR, Gustafsson JA. Putative metabolic effects of the liver X receptor (LXR). Diabetes 2004; 53 Suppl 1:S36-S42.

(85) Londos C, Brasaemle DL, Schultz CJ, Segrest JP, Kimmel AR. Perilipins, ADRP, and other proteins that associate with intracellular neutral lipid droplets in animal cells. Semin Cell Dev Biol 1999; 10(1):51-58.

(86) Murphy DJ. The biogenesis and functions of lipid bodies in animals, plants and microorganisms. Prog Lipid Res 2001; 40(5):325-438.

(87) Zweytick D, Athenstaedt K, Daum G. Intracellular lipid particles of eukaryotic cells. Biochim Biophys Acta 2000; 1469(2):101-120.

(88) Wang SM, Fong TH, Hsu SY, Chien CL, Wu JC. Reorganization of a novel vimentin-associated protein in 3T3-L1 cells during adipose conversion. J Cell Biochem 1997; 67(1):84-91.

(89) Fujimoto T, Kogo H, Ishiguro K, Tauchi K, Nomura R. Caveolin-2 is targeted to lipid droplets, a new "membrane domain" in the cell. J Cell Biol 2001; 152(5):1079-1085.

(90) Brown DA. Lipid droplets: proteins floating on a pool of fat. Curr Biol 2001; 11(11):R446-R449.

(91) Heid HW, Moll R, Schwetlick I, Rackwitz HR, Keenan TW. Adipophilin is a specific marker of lipid accumulation in diverse cell types and diseases. Cell Tissue Res 1998; 294(2):309-321.

(92) Jiang HP, Serrero G. Isolation and characterization of a full-length cDNA coding for an adipose differentiation-related protein. Proc Natl Acad Sci U S A 1992; 89(17):7856-7860.

(93) Greenberg AS, Egan JJ, Wek SA, Garty NB, Blanchette-Mackie EJ, Londos C. Perilipin, a major hormonally regulated adipocyte-specific phosphoprotein associated with the periphery of lipid storage droplets. J Biol Chem 1991; 266(17):11341-11346.

(94) Londos C, Brasaemle DL, Gruia-Gray J, Servetnick DA, Schultz CJ, Levin DM et al. Perilipin: unique proteins associated with intracellular neutral lipid droplets in adipocytes and steroidogenic cells. Biochem Soc Trans 1995; 23(3):611-615.

(95) Wolins NE, Skinner JR, Schoenfish MJ, Tzekov A, Bensch KG, Bickel PE. Adipocyte protein S3-12 coats nascent lipid droplets. J Biol Chem 2003; 278(39):37713-37721.

(96) Miura S, Gan JW, Brzostowski J, Parisi MJ, Schultz CJ, Londos C et al. Functional conservation for lipid storage droplet association among Perilipin, ADRP, and TIP47 (PAT)-related proteins in mammals, Drosophila, and Dictyostelium. J Biol Chem 2002; 277(35):32253-32257.

(97) Wolins NE, Rubin B, Brasaemle DL. TIP47 associates with lipid droplets. J Biol Chem 2001; 276(7):5101-5108.

(98) Liu P, Ying Y, Zhao Y, Mundy DI, Zhu M, Anderson RG. Chinese hamster ovary K2 cell lipid droplets appear to be metabolic organelles involved in membrane traffic. J Biol Chem 2004; 279(5):3787-3792.

(99) Lu X, Gruia-Gray J, Copeland NG, Gilbert DJ, Jenkins NA, Londos C et al. The murine perilipin gene: the lipid droplet-associated perilipins derive from tissue-specific, mRNA splice variants and define a gene family of ancient origin. Mamm Genome 2001; 12(9):741-749.

(100) Scherer PE, Bickel PE, Kotler M, Lodish HF. Cloning of cell-specific secreted and surface proteins by subtractive antibody screening. Nat Biotechnol 1998; 16(6):581-586.

(101) Servetnick DA, Brasaemle DL, Gruia-Gray J, Kimmel AR, Wolff J, Londos C. Perilipins are associated with cholesteryl ester droplets in steroidogenic adrenal cortical and Leydig cells. J Biol Chem 1995; 270(28):16970-16973.

(102) Dalen KT, Schoonjans K, Ulven SM, Weedon-Fekjaer MS, Bentzen TG, Koutnikova H et al. Adipose Tissue Expression of the Lipid Droplet-Associating Proteins S3-12 and Perilipin Is Controlled by Peroxisome Proliferator-Activated Receptor-γ. Diabetes. In press.

(103) Greenberg AS, Egan JJ, Wek SA, Moos MC, Jr., Londos C, Kimmel AR. Isolation of cDNAs for perilipins A and B: sequence and expression of lipid droplet-associated proteins of adipocytes.

(104) Clifford GM, Londos C, Kraemer FB, Vernon RG, Yeaman SJ. Translocation of hormone-sensitive lipase and perilipin upon lipolytic stimulation of rat adipocytes. J Biol Chem 2000; 275(7):5011-5015.

(105) Egan JJ, Greenberg AS, Chang MK, Londos C. Control of endogenous phosphorylation of the major cAMP-dependent protein kinase substrate in adipocytes by insulin and beta-adrenergic stimulation. J Biol Chem 1990; 265(31):18769-18775.

(106) Mooney RA, Bordwell KL. Counter-regulation by insulin and isoprenaline of a prominent fat-associated phosphoprotein doublet in rat adipocytes. Biochem J 1991; 274 (Pt 2):433-438.

(107) Martinez-Botas J, Anderson JB, Tessier D, Lapillonne A, Chang BH, Quast MJ et al. Absence of perilipin results in leanness and reverses obesity in Lepr(db/db) mice. Nat Genet 2000; 26(4):474-479.

(108) Tansey JT, Sztalryd C, Gruia-Gray J, Roush DL, Zee JV, Gavrilova O et al. Perilipin ablation results in a lean mouse with aberrant adipocyte lipolysis, enhanced leptin production, and resistance to diet-induced obesity. Proc Natl Acad Sci U S A 2001; 98(11):6494-6499.

(109) Sztalryd C, Xu G, Dorward H, Tansey JT, Contreras JA, Kimmel AR et al. Perilipin A is essential for the translocation of hormone-sensitive lipase during lipolytic activation. J Cell Biol 2003; 161(6):1093-1103.

(110) Diaz E, Pfeffer SR. TIP47: a cargo selection device for mannose 6-phosphate receptor trafficking. Cell 1998; 93(3):433-443.

(111) Eisinger DP, Serrero G. Structure of the gene encoding mouse adipose differentiation-related protein (ADRP). Genomics 1993; 16(3):638-644.

(112) Heid HW, Schnolzer M, Keenan TW. Adipocyte differentiation-related protein is secreted into milk as a constituent of milk lipid globule membrane. Biochem J 1996; 320 (Pt 3):1025-1030.

(113) Brasaemle DL, Barber T, Wolins NE, Serrero G, Blanchette-Mackie EJ, Londos C. Adipose differentiation-related protein is an ubiquitously expressed lipid storage droplet-associated protein. J Lipid Res 1997; 38(11):2249-2263.

(114) Gao J, Serrero G. Adipose differentiation related protein (ADRP) expressed in transfected COS-7 cells selectively stimulates long chain fatty acid uptake. J Biol Chem 1999; 274(24):16825-16830.

(115) Mather IH, Keenan TW. Origin and secretion of milk lipids. J Mammary Gland Biol Neoplasia 1998; 3(3):259-273.

(116) Imamura M, Inoguchi T, Ikuyama S, Taniguchi S, Kobayashi K, Nakashima N et al. ADRP stimulates lipid accumulation and lipid droplet formation in murine fibroblasts. Am J Physiol Endocrinol Metab 2002; 283(4):E775-E783.

(117) Gao J, Ye H, Serrero G. Stimulation of adipose differentiation related protein (ADRP) expression in adipocyte precursors by long-chain fatty acids. J Cell Physiol 2000; 182(2):297-302.

(118) Schultz CJ, Torres E, Londos C, Torday JS. Role of adipocyte differentiation-related protein in surfactant phospholipid synthesis by type II cells. Am J Physiol Lung Cell Mol Physiol 2002; 283(2):L288-L296.

(119) Atshaves BP, Storey SM, McIntosh AL, Petrescu AD, Lyuksyutova OI, Greenberg AS et al. Sterol carrier protein-2 expression modulates protein and lipid composition of lipid droplets. J Biol Chem 2001; 276(27):25324-25335.

(120) Serrero G, Frolov A, Schroeder F, Tanaka K, Gelhaar L. Adipose differentiation related protein: expression, purification of recombinant protein in Escherichia coli and characterization of its fatty acid binding properties. Biochim Biophys Acta 2000; 1488(3):245-254.

(121) Atshaves BP, Starodub O, McIntosh A, Petrescu A, Roths JB, Kier AB et al. Sterol carrier protein-2 alters high density lipoprotein-mediated cholesterol efflux. J Biol Chem 2000; 275(47):36852-36861.

(122) Nakamura N, Fujimoto T. Adipose differentiation-related protein has two independent domains for targeting to lipid droplets. Biochem Biophys Res Commun 2003; 306(2):333-338.

(123) Tauchi-Sato K, Ozeki S, Houjou T, Taguchi R, Fujimoto T. The surface of lipid droplets is a phospholipid monolayer with a unique Fatty Acid composition. J Biol Chem 2002; 277(46):44507-44512.

(124) Frank PG, Marcel YL. Apolipoprotein A-I: structure-function relationships. J Lipid Res 2000; 41(6):853-872.

(125) Bussell R, Jr., Eliezer D. A structural and functional role for 11-mer repeats in alpha-synuclein and other exchangeable lipid binding proteins. J Mol Biol 2003; 329(4):763-778.

(126) Chawla A, Lee CH, Barak Y, He W, Rosenfeld J, Liao D et al. PPARdelta is a very low-density lipoprotein sensor in macrophages. Proc Natl Acad Sci U S A 2003; 100(3):1268-1273.

(127) Sambrook J. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 1989.

(128) QIAGEN. QIAEX II Handbook for DNA Extraction from Agarose Gels. [20021]. 1999.

(129) Engler MJ, Richardson CC. In: *The Enzymes*. Boyer, P.D., ed., Academic Press, New York, NY., 1982.

(130) Seidman CE, Struhl K, Sheen J, Jessen T. Introduction of plasmid DNA into cells. Current Protocols in Molecular Biology. John Wiley & Sons Inc, 1997.

(131) Genomed. JETQUICK Plasmid Miniprep Spin Kit 250. Oktober 2000[400250]. 2000.

(132) Genomed. JETSTAR Plasmid Maxiprep Kit. [220020]. 1994.

(133) Gluzman Y. SV40-transformed simian cells support the replication of early SV40 mutants. Cell 1981; 23(1):175-182.

(134) Aden DP, Fogel A, Plotkin S, Damjanov I, Knowles BB. Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. Nature 1979; 282(5739):615-616.

(135) Knowles BB, Howe CC, Aden DP. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. Science 1980; 209(4455):497-499.

(136) Darlington GJ, Kelly JH, Buffone GJ. Growth and hepatospecific gene expression of human hepatoma cells in a defined medium. In Vitro Cell Dev Biol 1987; 23(5):349-354.

(137) Murakami K, Abe T, Miyazawa M, Yamaguchi M, Masuda T, Matsuura T et al. Establishment of a new human cell line, LI90, exhibiting characteristics of hepatic Ito (fat-storing) cells. Lab Invest 1995; 72(6):731-739.

(138) Wabitsch M, Brenner RE, Melzner I, Braun M, Moller P, Heinze E et al. Characterization of a human preadipocyte cell strain with high capacity for adipose differentiation. Int J Obes Relat Metab Disord 2001; 25(1):8-15.

(139) Werner T, Fessele S, Maier H, Nelson PJ. Computer modeling of promoter organization as a tool to study transcriptional coregulation. FASEB J 2003; 17(10):1228-1237.

(140) Carey M, Smale ST. Transcriptional Regulation in Eukaryotes. Consepts, Strategies, and Techniques. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2000.

(141) Promega. Luciferase Assay System. Technical Bulletin No. 281. 2002.

(142) Graham FL, van der Eb AJ. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology 1973; 52(2):456-467.

(143) Rosenthal N. Identification of regulatory elements of cloned genes with functional assays. Methods Enzymol 1987; 152:704-720.

(144) Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. Science 1994; 263(5148):802-805.

(145) Prasher DC, Eckenrode VK, Ward WW, Prendergast FG, Cormier MJ. Primary structure of the Aequorea victoria green-fluorescent protein. Gene 1992; 111(2):229-233.

(146) BD Biosciences Clontech. Product protocol: pEGFP-N1 N-terminal Protein Fusion Vector (Catalog #6085-1). 2004.

(147) Fried MG, Bromberg JL. Factors that affect the stability of protein-DNA complexes during gel electrophoresis. Electrophoresis 1997; 18(1):6-11.

(148) Kerr LD. Electrophoretic mobility shift assay. Methods Enzymol 1995; 254:619-632.

(149) Promega. Quick protocol for: TNT-T7 quick coupled transcription translation system. Technical Manual No.045. 2000.

(150) Whitney KD, Watson MA, Collins JL, Benson WG, Stone TM, Numerick MJ et al. Regulation of cholesterol homeostasis by the liver X receptors in the central nervous system. Mol Endocrinol 2002; 16(6):1378-1385.

(151) Zhang Y, Yin L, Hillgartner FB. Thyroid hormone stimulates acetyl-coA carboxylase-alpha transcription in hepatocytes by modulating the composition of nuclear receptor complexes bound to a thyroid hormone response element. J Biol Chem 2001; 276(2):974-983.

(152) Mak PA, Kast-Woelbern HR, Anisfeld AM, Edwards PA. Identification of PLTP as an LXR target gene and apoE as an FXR target gene reveals overlapping targets for the two nuclear receptors. J Lipid Res 2002; 43(12):2037-2041.

(153) Landis MS, Patel HV, Capone JP. Oxysterol activators of liver X receptor and 9-cis-retinoic acid promote sequential steps in the synthesis and secretion of tumor necrosis factor-alpha from human monocytes. J Biol Chem 2002; 277(7):4713-4721.

(154) Volle DH, Repa JJ, Mazur A, Cummins CL, Val P, Henry-Berger J et al. Regulation of the Aldo-Keto Reductase Gene akr1b7 by the Nuclear Oxysterol Receptor LXR{alpha} in the Mouse Intestine: Putative Role of LXRs in Lipid Detoxification Processes. Mol Endocrinol 2004.

(155) Ramji DP, Foka P. CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem J 2002; 365(Pt 3):561-575.

(156) Rosen ED, Walkey CJ, Puigserver P, Spiegelman BM. Transcriptional regulation of adipogenesis. Genes Dev 2000; 14(11):1293-1307.

(157) Svensson S, Ostberg T, Jacobsson M, Norstrom C, Stefansson K, Hallen D et al. Crystal structure of the heterodimeric complex of LXRalpha and RXRbeta ligand-binding domains in a fully agonistic conformation. EMBO J 2003; 22(18):4625-4633.

(158) Janowski BA, Grogan MJ, Jones SA, Wisely GB, Kliewer SA, Corey EJ et al. Structural requirements of ligands for the oxysterol liver X receptors LXRalpha and LXRbeta. Proc Natl Acad Sci U S A 1999; 96(1):266-271.

(159) Tilstone C. DNA microarrays: vital statistics. Nature 2003; 424(6949):610-612.

(160) Firestein GS, Pisetsky DS. DNA microarrays: boundless technology or bound by technology? Guidelines for studies using microarray technology. Arthritis Rheum 2002; 46(4):859-861.

(161) Bildirici I, Roh CR, Schaiff WT, Lewkowski BM, Nelson DM, Sadovsky Y. The lipid droplet-associated protein adipophilin is expressed in human trophoblasts and is regulated by peroxisomal proliferator-activated receptor-gamma/retinoid X receptor. J Clin Endocrinol Metab 2003; 88(12):6056-6062.

(162) Rosenbaum SE, Greenberg AS. The short- and long-term effects of tumor necrosis factor-alpha and BRL 49653 on peroxisome proliferator-activated receptor (PPAR)gamma2 gene expression and other adipocyte genes. Mol Endocrinol 1998; 12(8):1150-1160.

(163) Tobin KA, Ulven SM, Schuster GU, Steineger HH, Andresen SM, Gustafsson JA et al. Liver X receptors as insulin-mediating factors in fatty acid and cholesterol biosynthesis. J Biol Chem 2002; 277(12):10691-10697.

(164) Chawla A, Boisvert WA, Lee CH, Laffitte BA, Barak Y, Joseph SB et al. A PPAR gamma-LXR-ABCA1 pathway in macrophages is involved in cholesterol efflux and atherogenesis. Mol Cell 2001; 7(1):161-171.

(165) Amemiya-Kudo M, Shimano H, Yoshikawa T, Yahagi N, Hasty AH, Okazaki H et al. Promoter analysis of the mouse sterol regulatory element-binding protein-1c gene. J Biol Chem 2000; 275(40):31078-31085.

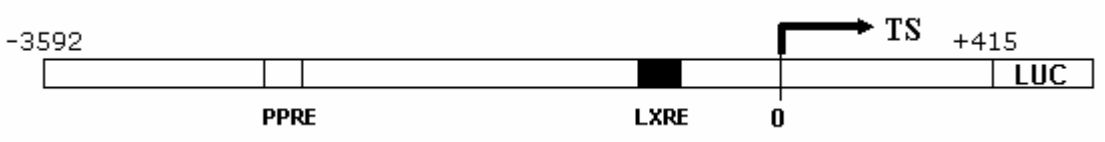
(166) Fajas L, Schoonjans K, Gelman L, Kim JB, Najib J, Martin G et al. Regulation of peroxisome proliferator-activated receptor gamma expression by adipocyte differentiation and determination factor 1/sterol regulatory element binding protein 1: implications for adipocyte differentiation and metabolism. Mol Cell Biol 1999; 19(8):5495-5503.

(167) Grefhorst A, Elzinga BM, Voshol PJ, Plosch T, Kok T, Bloks VW et al. Stimulation of lipogenesis by pharmacological activation of the liver X receptor leads to production of large, triglyceride-rich very low density lipoprotein particles. J Biol Chem 2002; 277(37):34182-34190.

(168) Haugen F, Zhaid N, Dalen KT, Hollung K, Nebb HI, Drevon C.A. Resistin expression in cultured adipocytes is reduced by arachidonic acid. J Lipid Res. In press.

Appendix

A



B

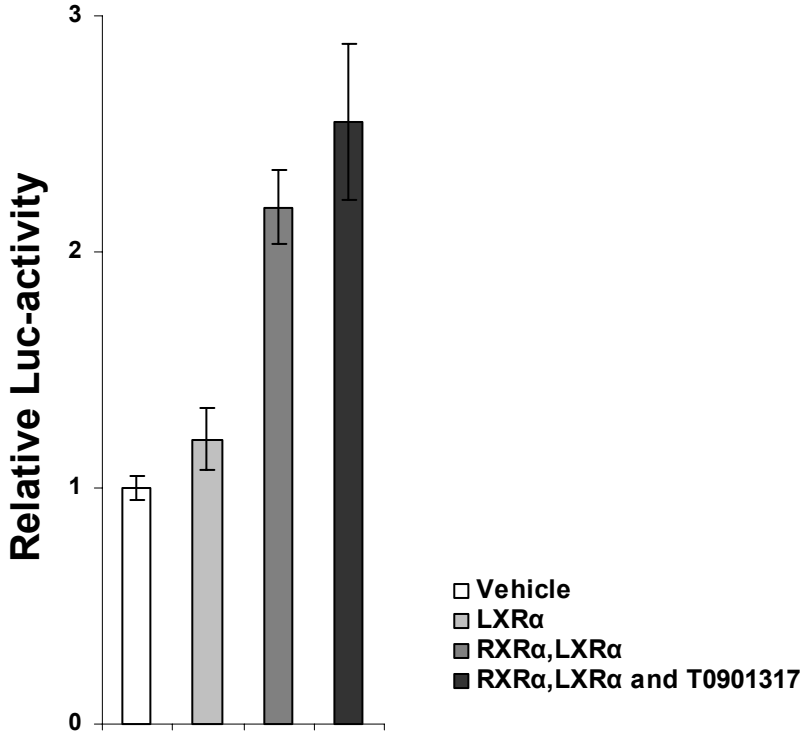


Figure A1 Transfection with human adipophilin promoters shows modest responsiveness to LXR activation

A) A schematic presentation of the human adipophilin promoter construct. **B)** Transient transfection with the full-length construct of human adipophilin luciferase reporter into COS-1 cells. Cells were co-transfected with no expression plasmid (white), LXRα expression plasmid (light grey), LXRα and RXRα expression plasmids (dark grey and black) stimulated with vehicle (white, light grey and dark grey) or T0901317 (0.1μM; black). The result is representative for three individual experiments performed in triplicates. Results are given as mean ± SD. (None, vehicle = 1).

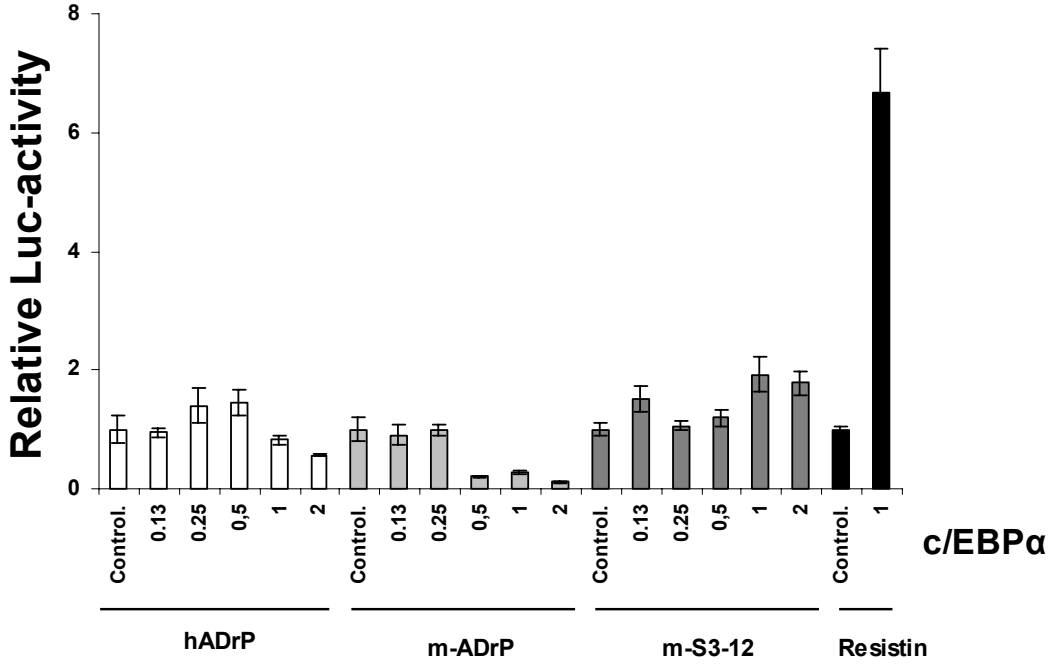


Figure A2 Transfection with c/EBPα

Transient transfection with reporter constructs of human adipophilin (hADrP), murin adipophilin (m-ADrP), murin S3-12 (m-S3-12) and resistin. Cells were co-transfected with c/EBPα (μM). Resistin (168) is known to be induced by c/EBPα. Results are given as ± SD. (Control = 1).

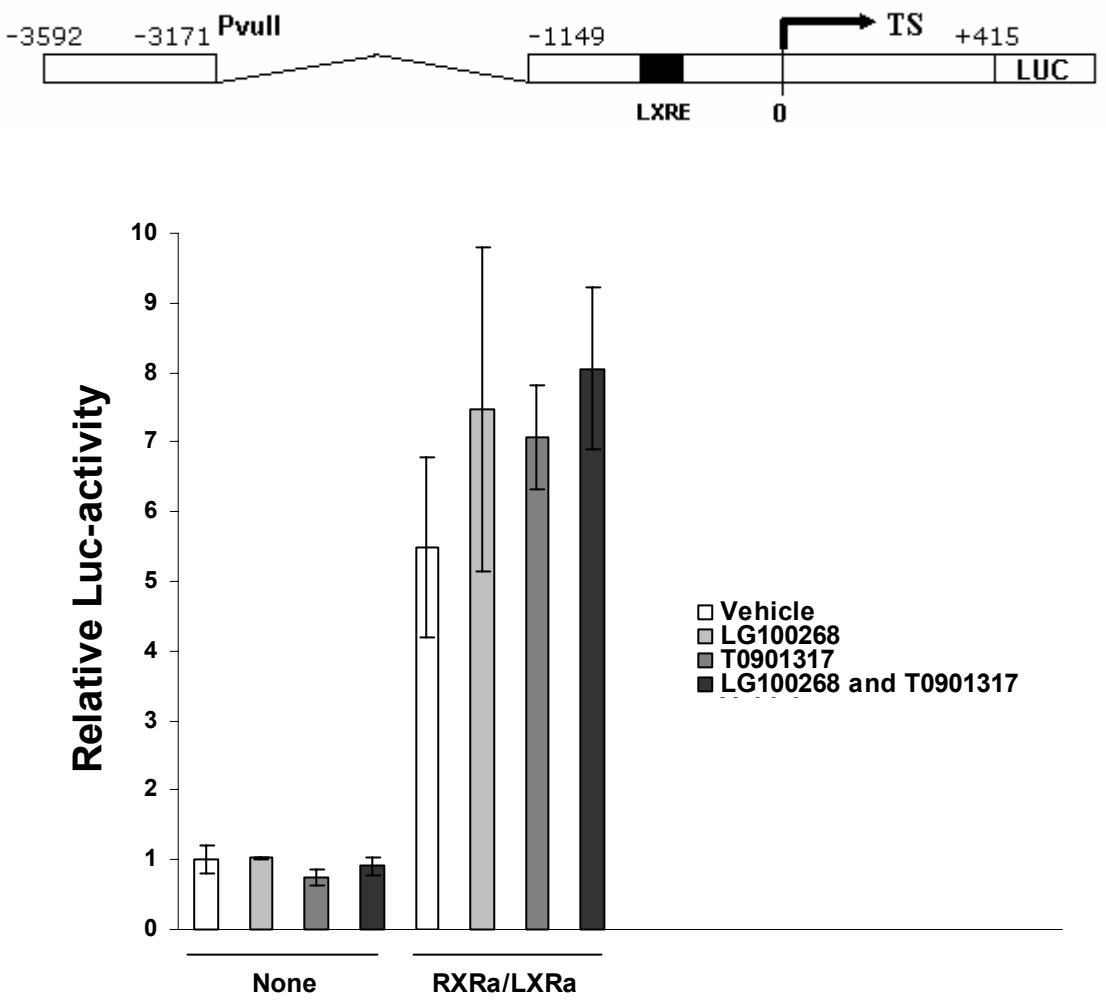


Figure A3 Transfection in HepG2 cells cultured in medium with LPDS

Transient transfection with the deletion construct of human adipophilin luciferase reporter into HepG2 cells cultured in medium with LPDS. The cells were co-transfected with RXRα and LXRα expression vectors as indicated. The medium was supplemented with vehicle (DMSO; white), RXRα ligand (LG100268; light grey), LXRα ligand (T0901317; dark grey) or both ligands (black). Results are given as mean ± SD. (None, vehicle = 1).

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TGCTGAACCCGCGCTTTCGACCCTTGCCTTTGTGCGTTGAGGCTTCTTTCCTGCCAACAGATTTAGGGCGATTG
CTATCGAATGTCCCTTGGCCTGCTTGGGTGGTTGTGGGAAAACAATATTTGTCGAAGGGACATTATCGAAAGGG
AAACGCGTTAGA

Figure A4 Human adipophilin promoter – cloned

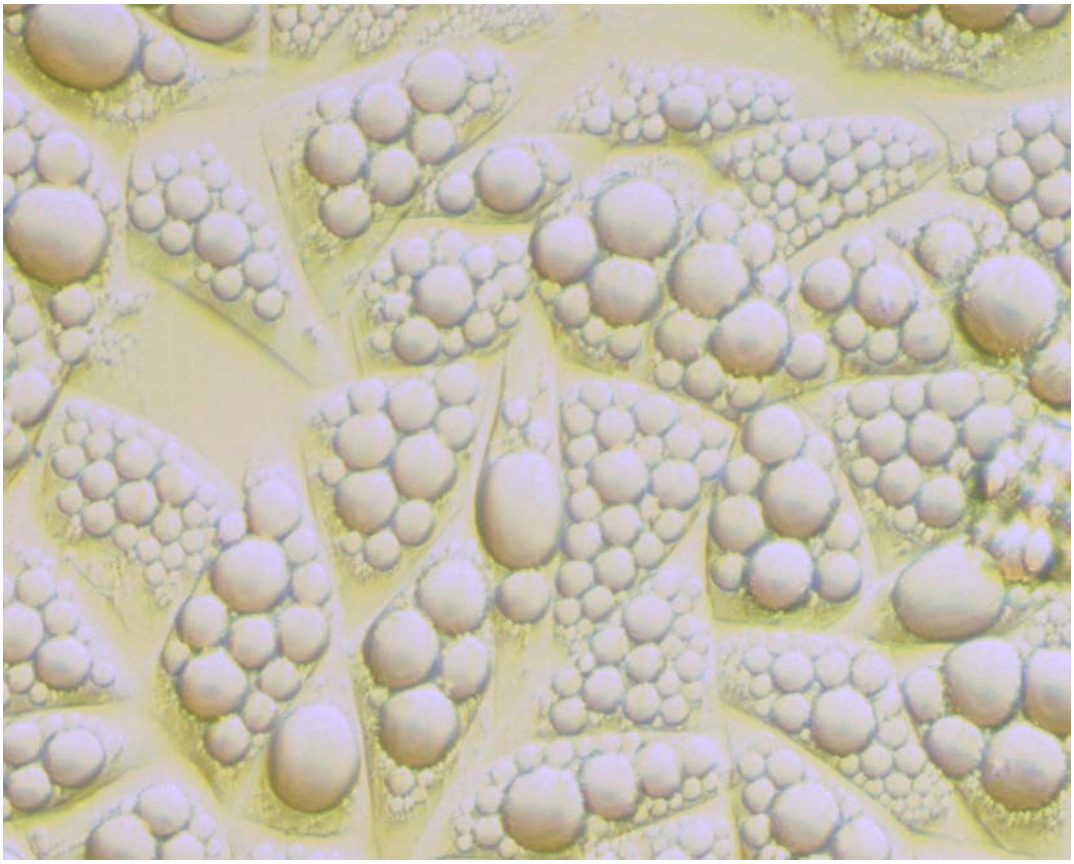

Figure A5 Successful differentiation of SGBS cells

SGBS cells were morphologically fibroblast-like and differentiated into mature adipocytes with characteristic lipid droplets. SGBS adipocytes are morphologically identical to in vitro differentiated adipocytes from healthy subjects and are a human adipocyte in vitro model system.