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Biochemische Endokrinologie
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# Molecular Mechanisms of Action of Thyroid Hormone: The Liver Fatty Acid Binding Protein as a Model

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Für meine Eltern

Il faut imaginer Sisyphe heureux.

Le Mythe de Sisyphe, *Albert Camus*

One must imagine Sisyphus happy.

The Myth of Sisyphus, *Albert Camus*

Wir müssen uns Sisyphos als einen glücklichen Menschen vorstellen.

Der Mythos von Sisyphos, *Albert Camus*

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**Abstract**

The thyroid hormone (T3) has a profound influence on normal development and metabolic balance. Hyperthyroid individuals have a markedly elevated basal energy expenditure, which includes an increased hepatic fatty acid (FA) turnover. Conversely, a decrease of T3 has the reverse effect. At least two genomic mechanisms of molecular action have been postulated, (i) a direct one and (ii) an indirect one, working via intermediary factors. Unlike direct T3 effects, which can be adequately explained by T3 response elements (TREs) in promoter regions of T3 responsive genes, mechanisms of indirect effects are still elusive. A characteristic of indirect T3 effects is a latency period of 12 to 48 hours before an elevation of the target gene mRNA concentration can be observed in response to T3 in vivo.

The aim of this thesis was to investigate mechanisms by which indirect effects of T3 are mediated. Microarray analysis, conducted earlier in our laboratory, revealed a latency period in the case of liver fatty acid binding protein (LFABP) gene expression in rats. LFABP, an important hepatic FA transport protein, was therefore chosen as a model gene.

Northern hybridisation confirmed the microarray results. Sequence analysis of the human and rat LFABP promoters revealed a putative TRE in the human promoter, but not in the rat sequence. Transient transfection experiments did not show an effect of T3 and thyroid hormone receptor on either rat or human LFABP promoter regions, thus a T3 effect via a TRE does not appear to be likely. Putative peroxisome proliferator response elements (PPREs) were found by sequence analysis of the promoters, and confirmed through electrophoretic mobility shift assays. Transient transfections showed a clear response of the rat LFABP promoter region to clofibric acid, a peroxisome proliferator, and peroxisome proliferator activated receptor alpha (PPARα), thus verifying an active PPRE in the rat LFABP promoter. Rat PPARα itself was not positively regulated by T3 in vivo.

Given the fact that rat PPARγ co-activator 1 (PGC-1) shows a direct response to T3 in vivo and is known to interact with PPARα, the following working hypothesis was created: T3 increases PGC-1 gene expression. The synthesis of the protein is time consuming, which explains the latency period. PGC-1 then interacts with PPARα, which is bound to the PPRE in the LFABP promoter region, and increases LFABP gene activity. This mechanism offers an attractive explanation for the latency period of indirect T3 effects.

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# 1. Introduction

## 1.1. Liver Fatty Acid Binding Protein

### 1.1.1. Characterisation of Liver Fatty Acid Binding Protein

In the early 1970s, mammalian fatty acid binding proteins (FABPs) were discovered as abundant proteins in the cytosol of intestinal mucosa, liver, and other tissues, which bind long chain fatty acids (LCFA) [24]. Today it is known that FABPs and related proteins encompass a so called mammalian cytosolic 14-15 kDa FABP gene family [33]. The different FABPs are distributed in a tissue specific manner, with liver FABP (LFABP) being found in the liver and the small intestine.

LFABP is a 14.2 kDa protein, comprising 3-5% of the cytoplasmatic liver proteins of physiologically normal rats [25]. Not only LCFA but also other hydrophobic ligands, including acyl coenzyme A, heme, and bile acids bind to LFABP. It plays an important role in hepatocellular fatty acid (FA) transport and homeostasis. Approximately 60% of intracellular LCFAs are bound to LFABP, thus maintaining the LCFA concentration in this aqueous environment. It is also thought to participate in LCFA transport to, and from, cellular compartments, including the nucleus [15, 33], figure1.

The putative transport function to the nucleus appears to be ligand-dependent. Once LFABP has entered the nucleus with its ligand, it interacts with nuclear receptors (e.g. peroxisome proliferator activated receptor alpha, PPARα) which also bind to hydrophobic ligands. Thereby playing a putative indirect role in gene expression [15, 42].

### 1.1.2. Regulation of LFABP Gene Expression

LFABP concentration is altered in vivo and in vitro by a number of factors on the mRNA, as well as on the protein level.

Clofibric acid, thyroid hormone, growth hormone, fatty acids and 9-cis-retinoic acid were reported to increase LFABP mRNA and protein concentrations [3, 4, 23, 27]. However, a lack of insulin (diabetic rats) or thyroid hormone (hypothyroid rats) lower these concentrations [23]. Euthyroid rats have a hepatocellular LFABP protein concentration that is slightly higher than the protein concentration found in hypothyroid individuals, but clearly below protein levels of rats in hyperthyroid

**Physiological Role of LFABP**

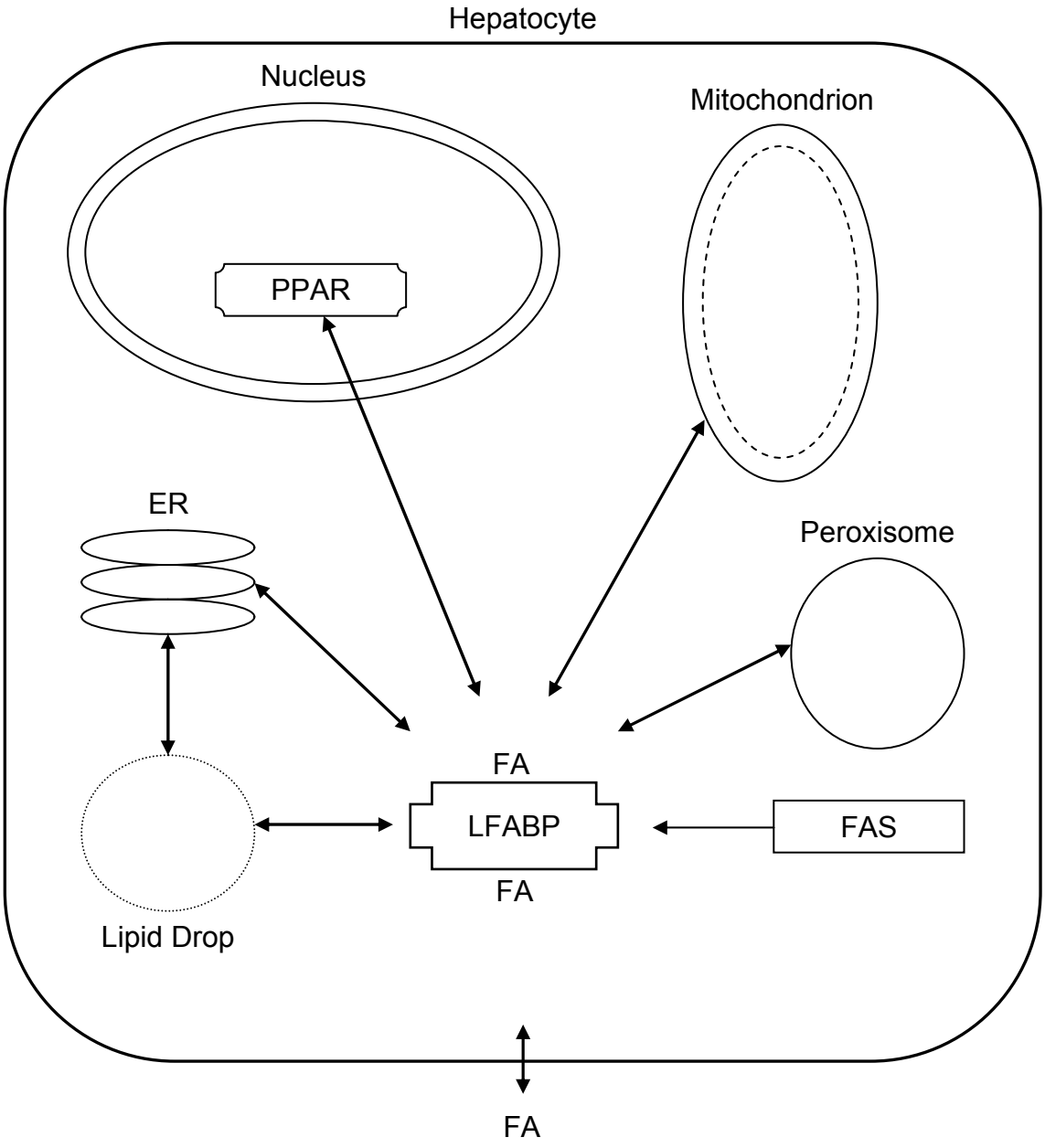



***Fig.1:*** *Physiological role of LFABP in hepatocytes.* Fatty acids enter the cell or are synthesised in the cell. They bind to LFABP at a molar ratio of 2:1 (FA:LFABP). Acyl coenzyme A, heme, bile acids, and other hydrophobic substances are also ligands. LFABP is thought to facilitate transport to and from cellular compartments, including the nucleus where it can interact with PPARs. Approximately 60% of all long chain FAs in hepatocytes are bound to LFABP, which hereby maintains the concentration in the cytosol. ER = endoplasmic reticulum, FA = fatty acid, FAS = fatty acid synthetase, LFABP = liver fatty acid binding protein, PPAR = peroxisome proliferator activated receptor.

states [23]. Females show higher concentrations of LFABP mRNA and protein than male individuals [2, 3]. The effect of glucocorticoids is controversial. One study reports that livers of adrenalectomised rats have an increased LFABP protein concentration, and that substitution of glucocorticoids reverses this effect [23]. However, a second study revealed no change of LFABP protein after adrenalectomy, but a decrease when glucocorticoids were substituted [8].

At the beginning of the work on this thesis the following was known about the mouse and rat LFABP promoters: two binding elements of hepatocyte nuclear factor 1α (HNF1α) in the mouse LFABP promoter have been shown to be active [1], and a putative peroxisome proliferator response element (PPRE) was reported to be in the rat LFABP promoter [27].

### 1.1.3. Pathophysiological Role of LFABP in Diabetes Mellitus

An association between diabetes mellitus and changes in FA metabolism is well established. In diabetic states, glucose utilisation is decreased and FAs are used as an alternative energy source. It has been proposed that elevated serum FA levels and a consecutive influx of FAs to the liver, combined with changes in hepatic FA metabolism result in hepatic steatosis [16].

The diabetes mellitus form formerly known as maturity onset diabetes of the young 3 (MODY3) in humans, is caused by mutations in the HNF1α gene. HNF1α deficient mice also exhibit a diabetic phenotype with elevated serum FAs, and were used to investigate the effects of HNF1α and LFABP on hepatic FA metabolism. The livers of these mice show severe hepatocyte lipid accumulation and a lowered LFABP mRNA expression. Due to the assumed central role of LFABP in hepatic FA homeostasis, it was suggested that LFABP is indeed an important factor for the development of this phenotype in HNF1α null mice, and possibly also in MODY3 patients [11].

## 1.2. Thyroid Hormone and It's Molecular Mechanism of Action

### 1.2.1. Synthesis and Physiological Significance of Thyroid Hormone

Peptidergic neurones in the hypothalamus synthesise the thyrotropin releasing hormone (TRH), which stimulates the synthesis and release of thyrotropin (TSH) by pituitary thyrotropic cells. TSH is known to increase the release and production of thyroid hormones by the thyroid gland. In turn, thyroid hormones mediate a

negative feedback regulation of TRH and TSH secretion. This regulation is known as the hypothalamic-pituitary-thyroid-complex [41].

The precursor of the thyroid hormones thyroxine (T4) and triiodothyronine (T3) is synthesised in the follicular cells of the thyroid gland. Follicles are sacs which consist of a wall of cuboidal cells (= follicular cells) and an interior filled with the proteinaceous colloid. Large amounts of precursors of thyroid hormones are stored in the colloid. In the follicular cells the precursors can be converted to T3 and T4 and released to the blood. Here the 5'deiodinase monodeiodinases T4 to T3, which is considered to be the active hormone. More than 70 % of the circulating T3 is formed at the periphery [32, 41].

The Thyroid hormone has a profound influence on normal development and metabolic balance. Severe deficiency of T3 in infancy causes retardation of mental development and growth. Metabolic changes caused by the alteration of T3 concentration are extensive. The most obvious effect of an elevation of T3 levels is an increase in basal energy expenditure, that includes an increased hepatic FA turnover. Conversely, a decrease of T3 concentration lowers basal energy expenditure [20, 32].

### 1.2.2. Molecular Mechanism of Thyroid Hormone Action

Thyroid hormone action is mediated by nuclear receptors (TR). In the early 1970s TRs were found through the use of radioactively labelled ligands, but they had not been cloned and characterised until 1988. Initially the receptors were identified as homologues of the viral v-erb-A oncogene [29, 36] and classified as members of the steroid and thyroid hormone receptor family [7]. Two genes, designated TRα and TRβ, encode TR. Multiple isoforms are generated by alternative splicing.

TR exerts its function directly through thyroid hormone response elements (TRE), that often contain the consensus sequence AGGTCA. TR binds TREs constitutively, independent of ligand occupancy. Usually TR forms heterodimers with the retinoid x receptor (RXR). Unliganded TR:RXR heterodimers repress the transcription by a group of co-repressor proteins that interact directly with the DNA bound receptors. When TR binds T3, the receptor changes its conformation and recruits co-activator proteins (e.g. PPARγ co-activator 1, PGC-1) that enhance transcriptional activity.

Activation and repression is likely to be explained by a change of chromatin structure. The co-activators are associated with the formation of euchromatin (open chromatin) that is thought to increase the transcription rate. Conversely, the co-repressors enhance the arrangement of heterochromatin (condensed chromatin), which has a negative effect on transcription activity [44].

This is not the only mechanism of genomic T3 action. Several genes lack TREs but the mRNA or protein concentration is influenced by altered levels of T3 [18, 26, 31]. Different gene induction kinetics, which cannot be explained by the mechanism described above have also been reported. An “early” induction kinetic is characterised by an increase of mRNA levels approximately 6 hours after stimulation. A “late” kinetic shows a rise of mRNA levels after about 12 to 48 hours. An “early/late” kinetic combines the features of the first two kinetics (some authors only distinguish between the first two kinetics, “early/late” effects are then considered to be “late” effects). The “early” kinetic can be explained by a TRE in a gene promoter, but the molecular mechanisms of “late” kinetics remain elusive.

Cytochrome c is an example of a gene, that lacks a TRE, but is regulated by T3. The induction kinetic of mRNA levels after T3 administration shows characteristics of a “late” pattern [30]. The nuclear respiratory factor 1 (NRF1) binds a recognition site in the cytochrome c promoter and activates the transcription of the gene. In turn NRF1 mRNA level is increased after stimulation with T3, thus providing an explanation for the findings [18, 26, 31]. NRF1 is a very interesting candidate with which to explain indirect gene induction by T3. However, a number of genes showing “late” induction kinetics were identified that lack binding sites for NRF1 [38, 40]. So it is likely that other intermediary factors are also important.

A microarray analysis conducted in our laboratory revealed that LFABP is also a gene that displays characteristics of a "late" kinetic in response to T3 in male Wistar rats [38].

## 1.3. Peroxisome Proliferator Activated Receptors and their Molecular Mechanism of Action

### 1.3.1. Characterisation of Peroxisome Proliferator Activated Receptors

The first peroxisome proliferator activated receptor (PPAR) was cloned from mouse cDNA and identified as a member of the steroid hormone receptor superfamily [11]. Shortly after characterisation of this first PPAR, a group of three

isotypes was discovered in Xenopus laevis. The receptors were termed xPPARα, xPPARβ, and xPPARγ, respectively [6]. Corresponding isotypes were identified in other vertebrates. In accordance with the Xenopus nomenclature, the mammalian PPARα, PPARβ and PPARγ could be distinguished. Xenopus PPARβ and mammalian PPARβ are less clearly homologous than the other two isotypes, and hence the mammalian PPARβ is also named PPARδ. Further isotypes are created by alternative splicing and multiple PPAR isotype promoters [5].

Like TRs, PPARs bind to response elements in the DNA and regulate the expression of target genes by similar mechanisms as described for TR (see section 1.2.2.). By comparing sequences of a number of PPREs, the following consensus sequence was found: 5' AGGNCAAAGGTCA 3' [5].

### 1.3.2. Effects Mediated by Peroxisome Proliferator Activated Receptors

The first PPAR ligands discovered were so called peroxisome proliferators, e.g. clofibric acid [11]. Soon a variety of other ligands were discovered, including polyunsaturated fatty acids (PUFAs), thereby providing an explanation for the effects of PUFAs on metabolism, for the first time. Some of these ligands bind to all three isotypes (e.g. PUFAs), others bind with high affinities to individual isotypes (e.g. fibrates and thiazoledindiones interact with PPARα and PPARγ, respectively) [5].

PPARs are expressed in a number of tissues and organs, with PPARα being the dominant isotype in the liver, a main organ of metabolism. Here PPARα plays an important role in energy homeostasis by regulating some key enzymes of fatty acid transport (e.g. fatty acid translocase, and fatty acid transporter protein) and β-oxidation (e.g. acyl-CoA oxidase). This is only one example of PPAR mediated effects, others include adipogenesis, adaptation to stress, altering circulating lipoproteins and cholesterol metabolism, control of inflammatory responses, and carcinogenesis. It is not only the insight into these variegated mechanisms, but also the approaches to the treatment of patients which the investigation of this enormous variety of effects may provide [5].

Some PPAR ligands have already found their way into the treatment of metabolic disorders. The PPARα specific agonist clofibric acid and it's derivatives are used to treat hyperlipoproteinaemia because they decrease very low density lipoprotein (VLDL) and triglyceride serum concentrations. Thiazoledindiones (also termed glitazones) are PPARγ agonists that have been introduced recently for

treatment of diabetes mellitus type 2 (DM2). Even though their exact mechanism of action is not yet completely understood, they are known to lower insulin resistance, an important pathophysiological aspect of DM2 [13].

## 2.1. Background and Objective

Many efforts have been made to gain insights into the mechanisms, by which thyroid hormone action is mediated. Some of these mechanisms have been much studied, however, they do not explain all observations. Since the discovery of thyroid hormone receptors, direct T3 effects via TREs are well understood. But mechanisms of “late” effects are still elusive. As described in the introduction, it has been proposed that intermediate factors may play important roles.

The aim of this thesis was to increase our understanding of indirect mechanisms of thyroid hormone action. Microarray experiments, conducted earlier in our laboratory, revealed a “late” induction kinetic of rat liver LFABP mRNA in response to T3 in vivo [38]. This gene, which plays an important role in hepatic FA transport and homeostasis, was therefore chosen as a model.

The first step of this thesis was to control the in vivo microarray results and try to reproduce them in permanent liver cell lines. Then, computer aided analysis of the cloned rat and human promoter sequences were carried out. The promoters were then analysed further by transient transfection assays and electrophoretic mobility shift assays. A possible influence of T3 on expression of rat PPARα mRNA in vivo was also studied. Finally, possible indirect mechanisms of T3 action were discussed.

## 3. Results

### 3.1 Effect of Thyroid Hormone on LFABP mRNA Concentration

#### 3.1.1. Thyroid Hormone Influence on Rat LFABP Gene Activity in vivo

Prior to investigating this subject further, the results obtained from the microarrays had to be confirmed by northern hybridisation. As described above, microarrays showed a late induction pattern of rat LFABP mRNA concentration in response to T3, in the livers of male Wistar rats. In order to validate the results, the total RNA extracted from the livers of three hypothyroid male Wistar rats was pooled. The same was done with the total liver RNA from three male Wistar rats 6, 24, or 48 hours after injection of T3, respectively.

Figure 2 shows the results: the first signal represents LFABP mRNA in livers of hypothyroid rats. A moderate but statistically significant increase of rat LFABP (rLFABP) mRNA after 6 hours (1.6 fold) and 24 hours (1.5 fold), compared to the hypothyroid state, can be observed. After 48 hours, a clear 3.7 fold rise is detected. These findings clearly confirm the previous microarray results.

**T3 Induced Change of Rat LFABP mRNA Concentration in vivo**

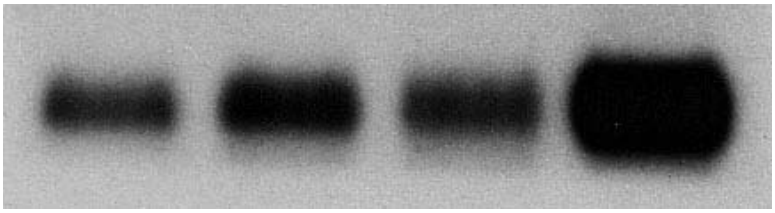



***Fig. 2:*** *T3 mediated change of rat LFABP mRNA in livers of male Wistar rats.* Total liver RNA from three individual hypothyroid rats was pooled 0, 6, 24, 48 hours after injection of T3, respectively (0h = hypothyroid animals). Relative to hypothyroid state, a 1.6 fold (6 hours), 1.5 fold (24 hours) and 3.7 fold (48 hours) rise of mRNA concentration is detectable, respectively. All changes are statistically significant. rLFABP mRNA concentration was normalised to ubiquitin signal.

#### 3.1.2. LFABP mRNA in HepG2 and Fao Cells

To investigate the T3 effect on LFABP mRNA in permanent cell lines, an analogous experiment was conducted. Fao cells, highly differentiated rat

hepatoma cells, and HepG2 cells, derived from human hepatoma cells, were used. Half of the cells were transfected with chicken thyroid hormone receptor alpha (cTR$\alpha$) and human retinoid x receptor beta (hRXR$\beta$), to ensure the presence of functional receptors. Cells were either not stimulated (0 hours), or stimulated with T3, 6 hours, 16 hours, 24 hours, and 48 hours prior to extraction of total RNA.

LFABP mRNA was not detectable in both cell lines by northern hybridisation. Whereas mRNA of adenine nucleotide translocase 2 (ANT2) was present, as shown in figure 3. ANT2 was chosen as control, to monitor the efficiency of the northern hybridisation technique.

**ANT2 mRNA in Fao and HepG2 Cells**

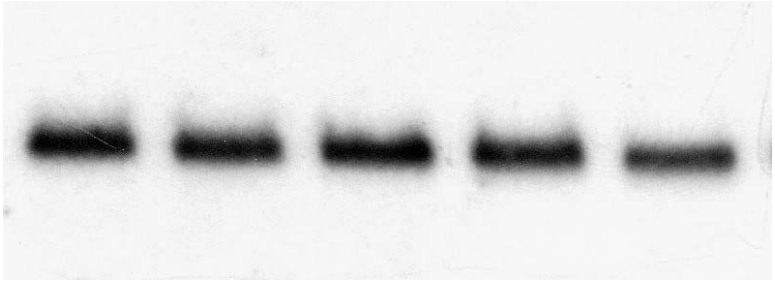



***Fig. 3a:*** *ANT2 mRNA in Fao cells.* Total RNA was extracted 0, 6, 16, 24, and 48 hours after stimulation with T3, respectively. ANT2 was used as positive control to monitor the efficiency of northern hybridisation. See section 3.1.2. for details.

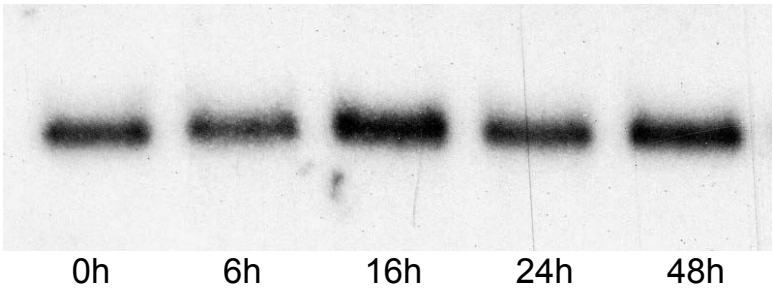



***Fig. 3b:*** *ANT2 mRNA in HepG2 cells.* Total RNA was extracted 0, 6, 16, 24, and 48 hours after stimulation with T3, respectively. ANT2 was used as positive control to monitor the efficiency of northern hybridisation. See section 3.1.2. for details.

**Sequence of Rat LFABP Promoter**

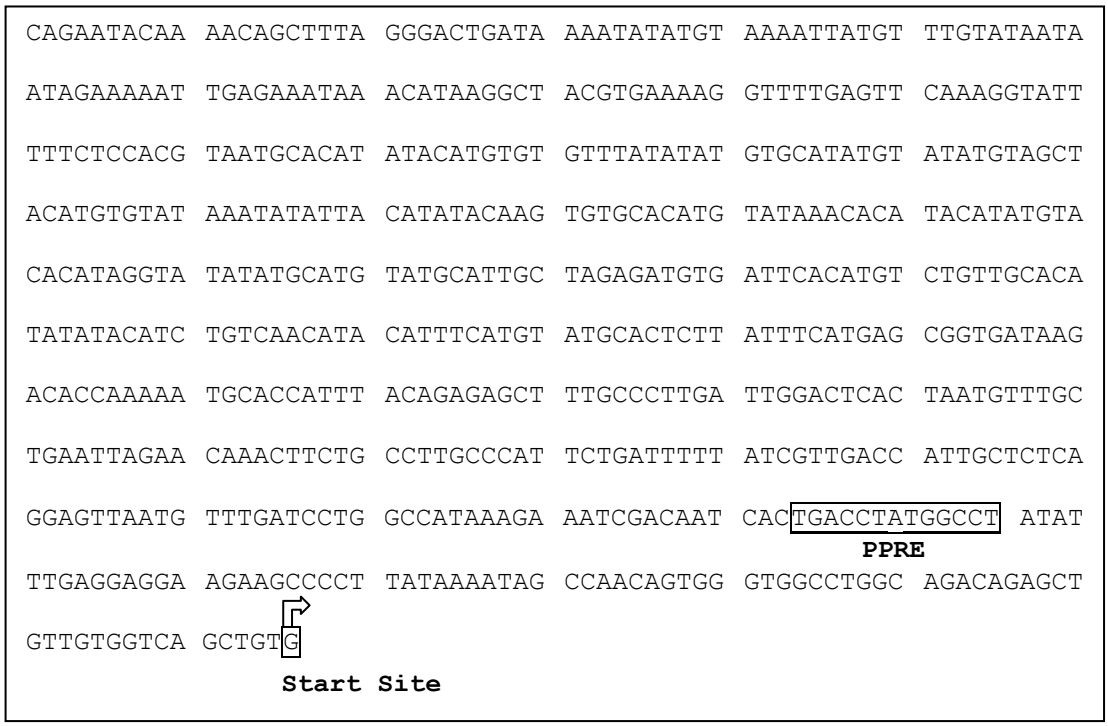



***Fig. 4a:*** *Sequence of rat LFABP promoter*. Positions –615 to +1, relative to transcription start site. A peroxisome proliferator response element (PPRE) was found at positions –92 to –80.

## 3.2. Analysis of Rat and Human LFABP Promoters in Response to Thyroid Hormone and Clofibric Acid in vitro

### 3.2.1. Computer Aided Analysis of Rat and Human LFABP Promoter Regions

Promoter regions of genes are essential for the regulation of gene expression and analysing them was therefore inevitable for this work. The first step was a computer aided analysis of the cloned promoter regions. Positions –615 to +155, relative to transcription start side, of the rLFABP promoter (accession number M13501, positions 7 through 155), and positions -1623 to +132 of the hLFABP promoter region (accession number AC092836, positions 62924 through 61170) were searched for putative binding sites of transcription factors.

A peroxisome proliferator response element (PPRE) previously found in the LFABP promoter of Fao cells is also in the natural rat LFABP promoter sequence (5'-TGACCTATGGCCT-3' at positions –92 to –80) [27]. Putative binding sites for the thyroid hormone receptor (TRE), NRF-1, or a cAMP response element (CRE) were not found (figure 4a).

Analysis of the hLFABP promoter revealed two putative PPREs (5'-TGACCTCTGGCCG-3' at positions –14 to –2 and 5'-GGACCTGTGGCCT-3' at positions –1241 to –1229) , and a putative TRE (5'-AGATCATAAGGTTA-3', positions –484 to –471). At positions –980 to –987, a putative CRE was found (5'-TGACGCCA-3'). There were, however, no NRF-1 response elements in the sequence of the promoter fragment (figure 4b).

### 3.2.2. Effect of Thyroid Hormone on Rat LFABP Promoter Region in vitro

The second step of this analysis entailed a transient transfection assay, using the cloned promoters.

HepG2 cells were transfected with pGL3-615rLFABP (rat LFABP promoter in pGL3 basic reporter gene vector) and adequate receptors. Figure 5 displays the results obtained from cells that were harvested 48 hours after T3 stimulation (n=12). The first two columns show the relative luciferase activity of cells transfected with pGL3-615rLFABP alone. Only a 1.27 fold increase of reporter gene activity is observed when T3 was added. The cTRα was co-transfected to ensure the presence of functional TR. Similarly in columns 4 and 5, a 1.48 fold increase of the signal is obvious, but statistically not significant. Finally a heterodimerisation partner of TR, hRXRα, was also co-transfected along with cTRα and pGL3-615rLFABP. Again, no significant alterations were observed.

Luciferase activities presented here are normalised to β-galactosidase activities. An alternative normalisation method is to express luciferase activity relative to the protein concentration of the cells. Then too, similar results were obtained (data not shown).

An experiment was conducted in which HepG2 cells were transfected with pGL3-615rLFABP and receptors harvested 96 hours after stimulation with T3 (n=3). In contrast to all other transfections, basal pGL3 basic activity was not determined. This experiment was intended to ascertain whether 48 hours of incubation after T3 administration are long enough. However this was only one series of transfection, giving three readings for each condition, and is not up to the standard of proper scientific results. Again, T3 does not affect the reporter gene activity, independently of the normalisation method. Luciferase activities in figure 6 are expressed relative to β-galactosidase activities.

In all, these experiments do not show an effect of T3 on the rat LFABP promoter.

## Sequence of Human LFABP Promoter

***Fig.4b:*** *Sequence of human LFABP promoter.* Positions –1623 to +1, relative to transcription start site. Two putative peroxisome proliferator response elements (PPRE) are at positions -14 to -2 and -1241 to -1229, respectively. Positions -484 to -471 encompass a putative thyroid hormone response element (TRE). A possible cAMP response element (CRE) is at positions -980 to -987.

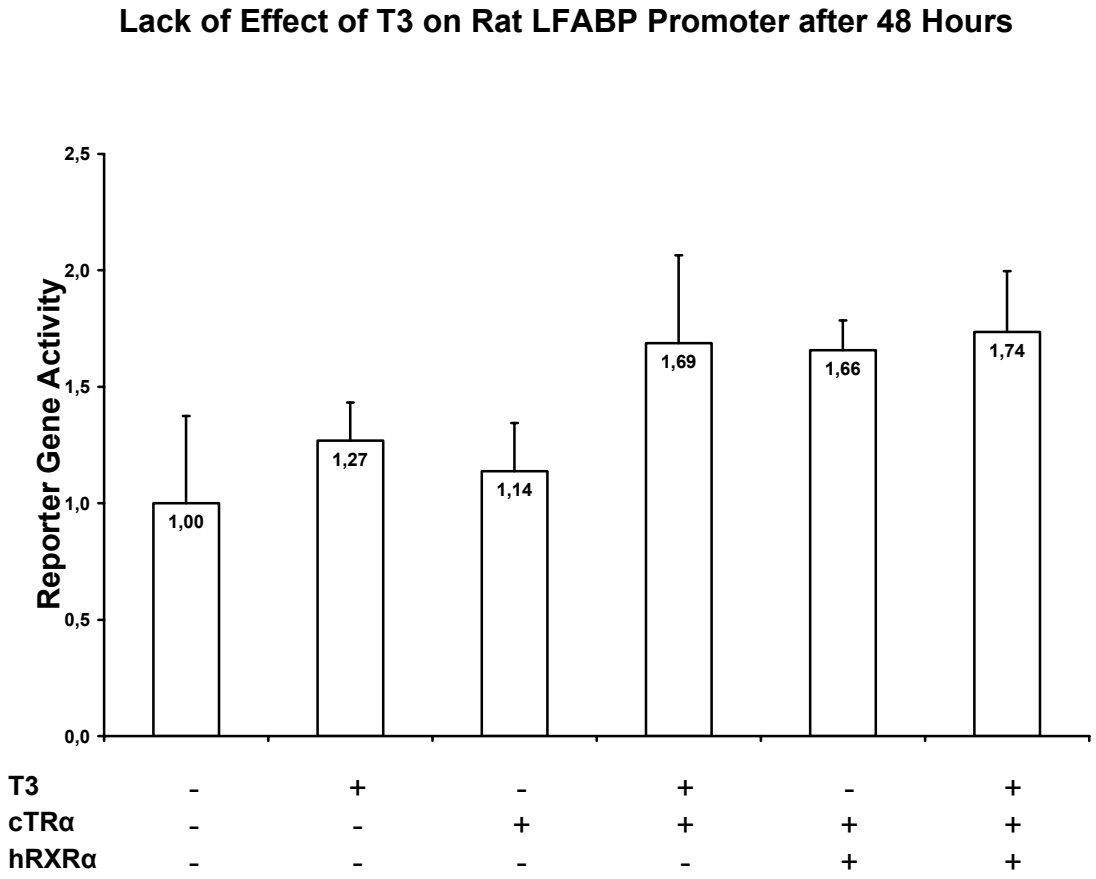



***Fig. 5:*** *Lack of effect of T3 on rat LFABP promoter in pGL3 basic vector (pGL3-615rLFABP).* Changes of reporter gene activity are statistically not significant. See section 3.2.2. for further details. Transfected HepG2 cells were harvested 48 hours after addition of T3. Luciferase activities were normalised to β-galactosidase signals and are expressed relative to basal promoter activity. Results are means ± S.E.M. T3 = thyroid hormone, cTRα = chicken thyroid hormone receptor α, hRXRα = human retinoid-x-receptor α.

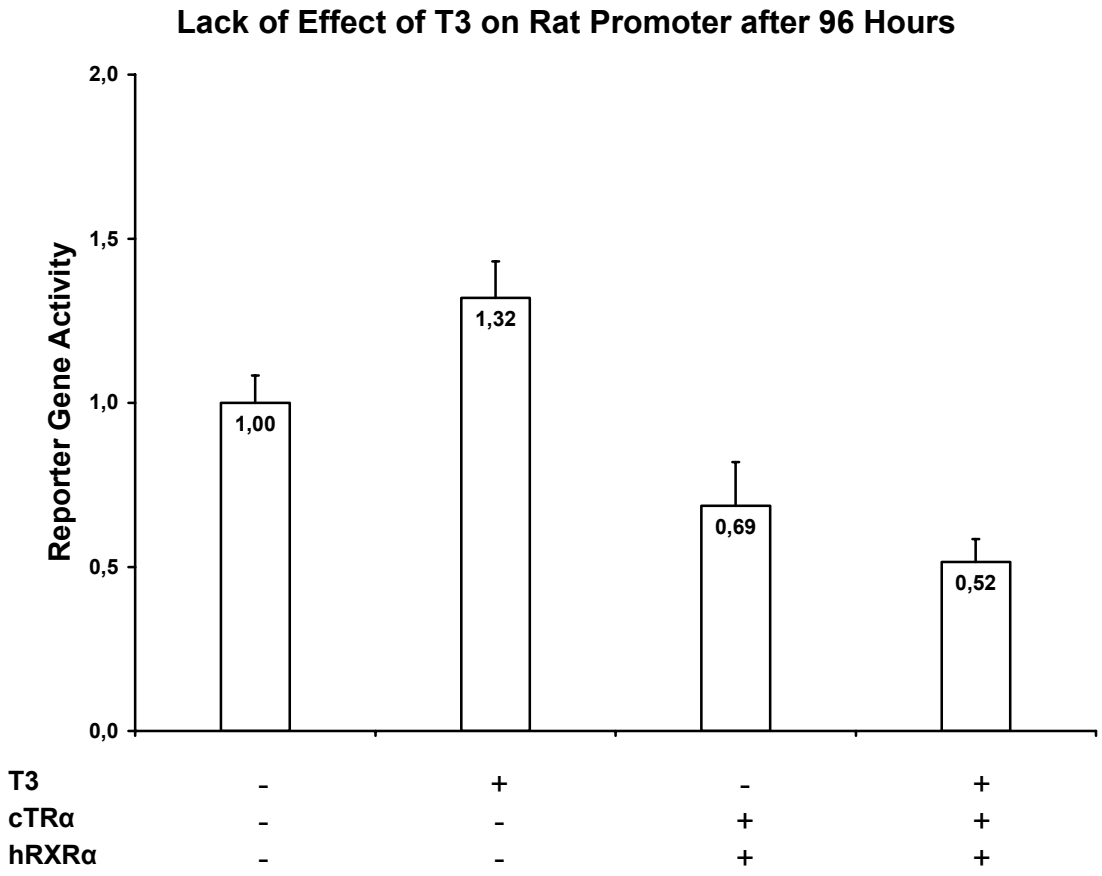



***Fig 6:*** *Lack of effect of T3 on rat LFABP promoter in pGL3 basic vector (pGL3-615rLFABP).* Changes of reporter gene activity are statistically not significant. See section 3.2.2. for further details. Transfected HepG2 cells were harvested 96 hours after addition of T3. Luciferase activities were normalised to β-galactosidase signals and are expressed relative to basal promoter activity. Results are means ±S.E.M. T3 = thyroid hormone, cTRα = chicken thyroid hormone receptor α, hRXRα = human retinoid-x-receptor α.

### 3.2.3. Effect of Thyroid Hormone on Human LFABP Promoter Region in vitro

As the human LFABP promoter contains a putative TRE, it was of special interest to study the T3 effect on this region. A series of transient transfection assays were conducted, analogous to those described in section 3.2.2..

First HepG2 cells were harvested 24 hours after transfection (n=12). This period of time was chosen, because promoters which contain active TREs usually show positive results within 24 hours. The findings are displayed in figure 7. Columns 1 and 2 represent relative reporter gene activities when no receptors were co-transfected. No significant change can be seen in response to T3. The last two columns chart the results when cTRα and hRXRα were co-transfected, along with pGL3-1623hLFABP (human LFABP promoter in pGL3 basic reporter gene vector). Again, T3 does not alter the promoter activity. The two middle columns,

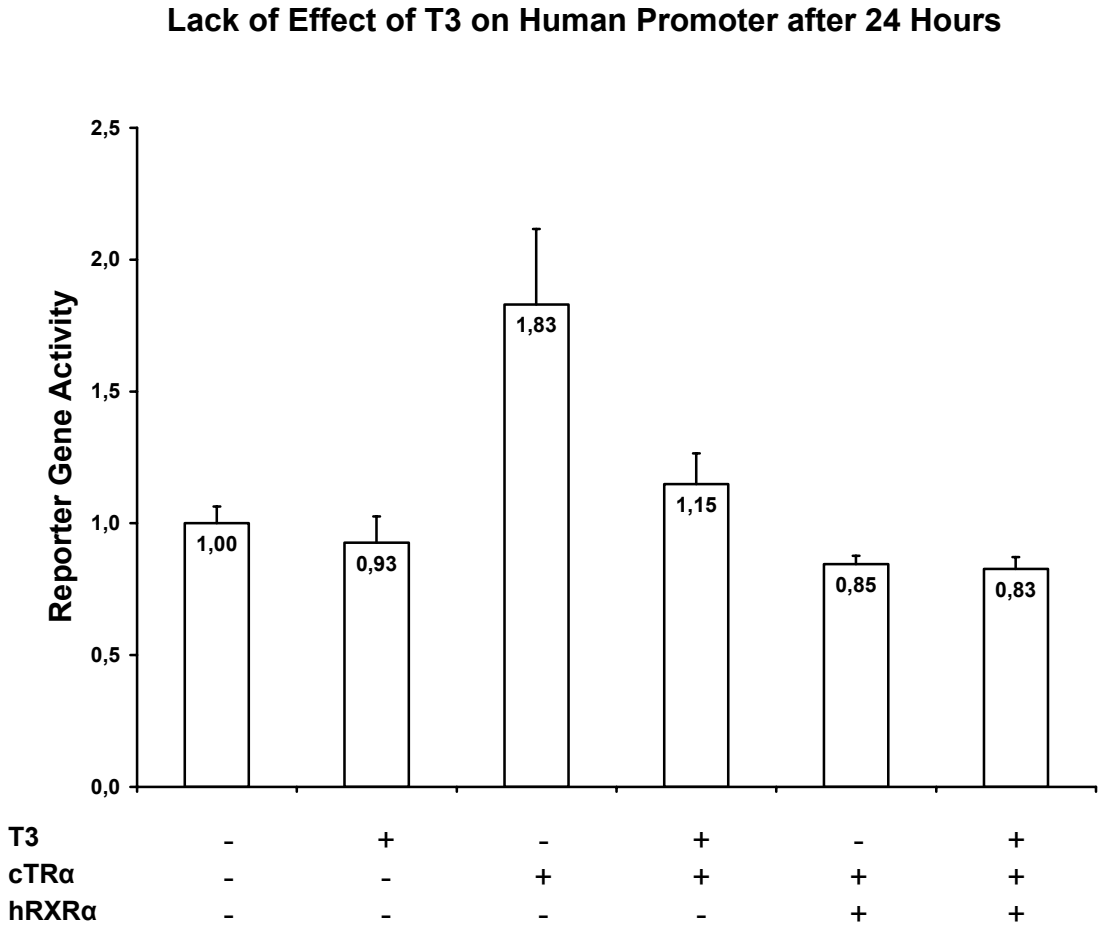



***Fig. 7:*** *Lack of effect of T3 on human LFABP promoter in pGL3 basic vector (pGL3-1623hLFABP).* Changes of reporter gene activity (-T3 compared to +T3) are statistically not significant. See section 3.2.3. for further details. Transfected HepG2 cells were harvested 24 hours after addition of T3. Luciferase activities were normalised to β-galactosidase signals and are expressed relative to basal promoter activity. Results are means ±S.E.M. T3 = thyroid hormone, cTRα = chicken thyroid hormone receptor α, hRXRα = human retinoid-x-receptor α.

representing reporter gene activity when cTRα was co-transfected, seem to show a T3 effect, Albeit an unexpected one: reporter gene activity in cells stimulated with T3 is lower than in cells that were not stimulated. However this is firstly not statistically significant, and secondly it must be stressed that the S.E.M. was high. These results are normalised to β-galactosidase activities, expressing luciferase-activity relative to total protein concentration reveal similar findings (data not shown).

Another series of experiments was conducted in an analogous manner, the only difference being the time of harvesting after stimulation, 48 instead of 24 hours (n=12). This was done to ensure a sufficient period of time for synthesising the reporter gene product. The results can be seen in figure 8, and are similar to those

obtained after 24 hours. Again, columns number 1, 2, 5, and 6 do not show a T3 effect on the promoter, whereas the middle columns show an increase of luciferase activities, which is lowered when T3 was added. Here also, the relatively high S.E.M. must be pointed out.

These experiments show that neither the putative TRE in the human LFABP promoter region is active, nor is there any other direct effect of T3 on the promoter.

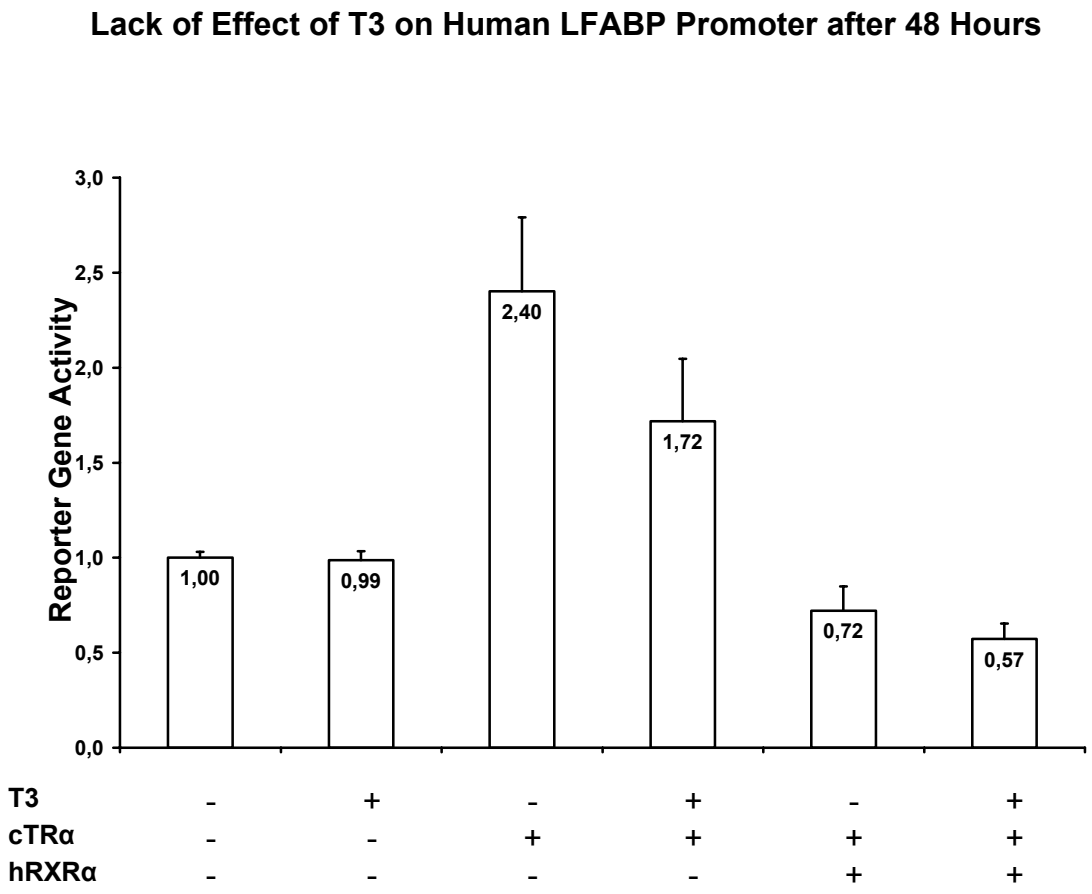



***Fig. 8:*** *Lack of effect on human LFABP promoter in pGL3 basic vector (pGL3-1623hLFABP).* Changes of reporter gene activity (-T3 compared to +T3) are statistically not significant See section 3.2.3. for further details. Transfected HepG2 cells were harvested 48 hours after addition of T3. Luciferase activities were normalised to β-galactosidase signals and are expressed relative to basal promoter activity. Results are means ±S.E.M. T3 = thyroid hormone, cTRα = chicken thyroid hormone receptor α, hRXRα = human retinoid-x-receptor α.

### 3.2.4. Thyroid Hormone Concentration in Cell Culture Medium

The thyroid hormone is of course a critical variable in the experiments described above. To exclude the possibility that changes of T3 levels could be an explanation for the lack of clear T3 effects, T3 concentration was monitored in the cell culture medium. The analysis revealed concentrations above 100 nM under all experimental conditions.

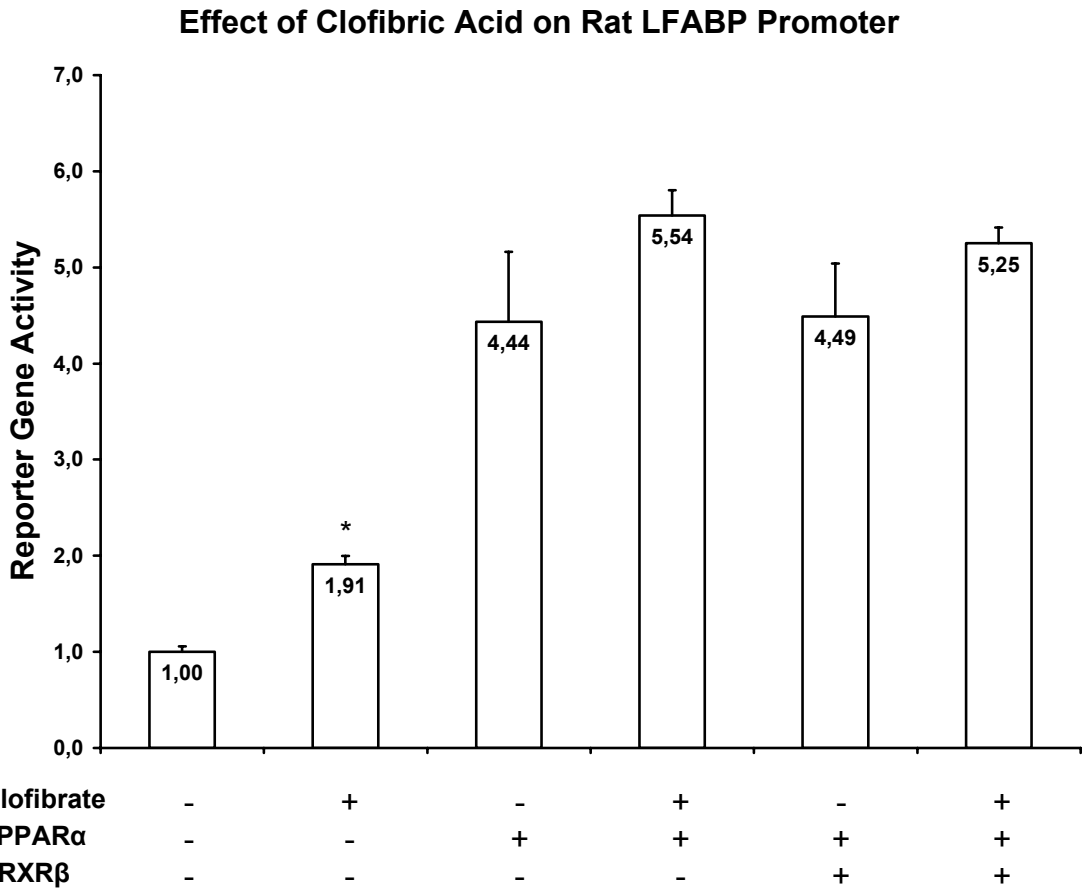



***Fig. 9:*** *Effect of clofibric acid on rat LFABP promoter in pGL3 basic vector (pGL3-615rLFABP)*. Stimulation of cells with clofibric acid results in a 1.91 fold increase of reporter gene activity (*p=0.025, columns 1 and 2). Co-transfection of receptors also causes a rise in gene activity, which is further increased by clofibric acid (1.25 fold columns 3 and 4, 1.16 fold columns 5 and 6, respectively). See section 3.2.5. for further details. Transfected HepG2 cells were harvested 24 hours after addition of clofibric acid. Luciferase activities were normalised to β-galactosidase signals and are expressed relative to basal promoter activity. Results are means ±S.E.M. Clofibrate = clofibric acid, xPPARα = Xenopus peroxisome proliferator activated receptor α, hRXRβ = human Retinoid-x-Receptor β.

### 3.2.5. Effect of Clofibric Acid on Rat LFABP Promoter Activity in Transient Transfections

LFABP and PPARs play important roles in fatty acid metabolism. It has been proven that the proteins interact in vitro [42], hence appearing to be co-operative. At the beginning of these experiments, the putative PPRE in the rLFABP promoter region was only confirmed in electrophoretic mobility shift assay (EMSA) experiments, i.e. it was not known if it is functional. A series of transient transfection assays, using pGL3-615rLFABP and appropriate nuclear receptors, was conducted. HepG2 cells were transfected, stimulated with clofibric acid, and harvested 24 hours after this stimulation (n=6).

Figure 9 displays the results. Stimulation of cells transfected with pGL3-615rLFABP causes a highly significant (p=0.025) 1.91 fold increase in reporter gene activity (columns 1 and 2). The co-transfection of Xenopus laevis PPARα (xPPARα) leads to a 4.44 fold increase. Further addition of clofibric acid results in a 5.54 fold rise of reporter gene signal, compared to basal promoter activity (middle columns). The co-transfection of hRXRβ, along with xPPARα and pGL3-615rLFABP, reveals similar findings (columns 5 and 6). A 4.49 fold increase when not stimulated and a 5.25. fold increment when stimulated, relative to basal promoter activity, can be observed.

These experiments indicate a clear response of the rLFABP promoter to xPPAR$\alpha$ and clofibric acid.

## 3.3. Interaction of Rat and Human Peroxisome Proliferator Response Elements with Nuclear Receptors

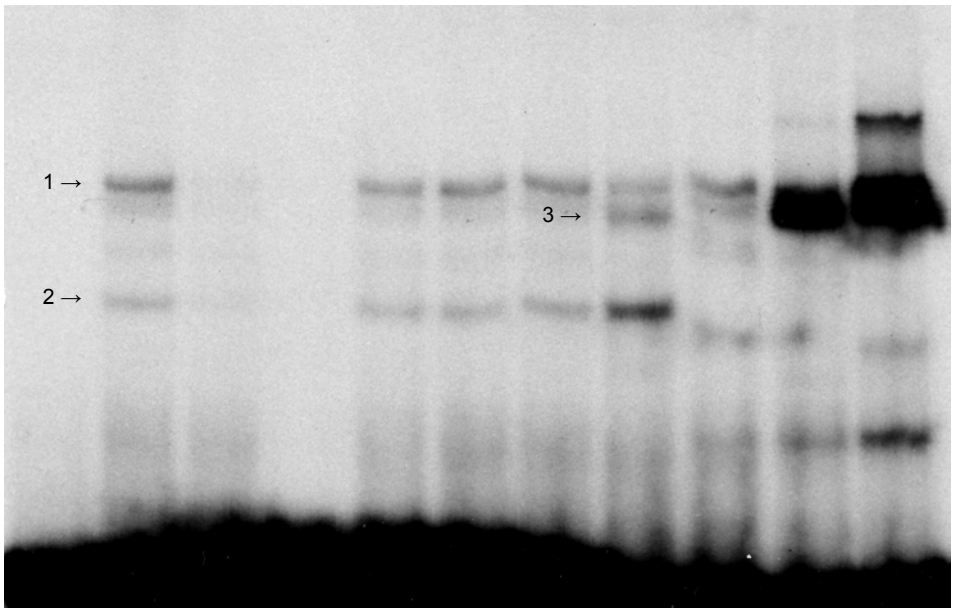
### 3.3.1. Analysis of the Peroxisome Proliferator Response Element in Rat LFABP Promoter by Electrophoretic Mobility Shift Assay

It has previously been demonstrated that mouse PPAR$\alpha$ and mouse PPAR$\beta$ together with mouse RXR$\beta$ bind to the PPRE contained in the rat LFABP promoter [27]. Still, it was not known whether these receptors bind to the putative PPRE in the human LFABP promoter. EMSA experiments were set up to compare the two PPREs. Oligonucleotides containing the PPRE found in rat LFABP promoter and the putative human PPRE were used in this assay.

Figure 10 shows the results of experiments in which the rat PPRE was investigated. Oligonucleotides containing the sequence of rat PPRE were used

(lane 1). With the addition of in vitro translated xPPARα and hRXRβ, two additional bands appeared on lane 2 (arrow 1 and arrow 2), but disappear when a specific competitor was added (lane 3), suggesting a specific binding reaction. This assumption is further supported by findings on lanes 4 through 6, where poly dIdC was added as non specific competitor at different concentrations (0.01g/l, 0.04g/l, and 0.001g/l, respectively) with the bands remaining visible.

**PPAR Interaction with Rat PPRE**




| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| xPPARα | - | + | + | + | + | + | + | - | - | - |
| hRXRβ | - | + | + | + | + | + | - | + | - | - |
| Sp.Com. | - | - | + | - | - | - | - | - | - | - |
| dIdC | - | - | - | 0.01 | 0.04 | 0.001 | - | - | - | - |
| pSPUTK | - | - | - | - | - | - | - | - | + | - |
| Lysate | - | - | - | - | - | - | - | - | - | + |

***Fig. 10:*** *EMSA analysis of rat PPRE sequence*. Labelled oligonucleotides containing the rat PPRE were used. Addition of in vitro translated xPPARα and hRXRβ cause formation of 2 bands (arrows 1 and 2), which disappear when a specific competitor was added (lane 3). Poly dIdC as non specific competitor has no effect on the bands (lanes 4 to 6). These findings suggest a specific protein/DNA interaction. xPPARα alone also induces both bands, whereas hRXRβ only causes formation of the upper band. Arrow 3 indicates a band that appears to be non-specific. See section 3.3.1. for further details. PPRE = peroxisome proliferator response element, xPPARα = Xenopus laevis peroxisome proliferator activated receptor α, hRXRβ = human retinoid x receptor β, Sp.Com. = specific competitor, dIdC = poly dIdC in g/l, pSPUTK = expression vector, Lysate = reticulocyte lysate.

The addition of in vitro translated xPPARα alone, without hRXRβ, to the probe, leads to the formation of the two bands as well (lane 7), although the lower one is more prominent than the upper one. hRXRβ was also added alone (lane 7), but this only leads to the formation of the upper band.

Lanes 9 and 10 are controls, where the empty in vitro translated expression vector pSPUTK and the reticulocyte lysate were used, respectively. A number of bands are visible, but one should be of particular importance. On each lane, a prominent band is detectable, which is at the same height as the band apparent on lane 7 (arrow 3).

Overall, this EMSA shows three bands of interest: two bands that comply with criteria of specific DNA-protein-interaction (arrows 1 and 2, figure 10), and an additional one (arrow 3, figure 10) which could be non-specific.

### 3.3.2. Analysis of the Peroxisome Proliferator Response Element in Human LFABP Promoter by Electrophoretic Mobility Shift Assay

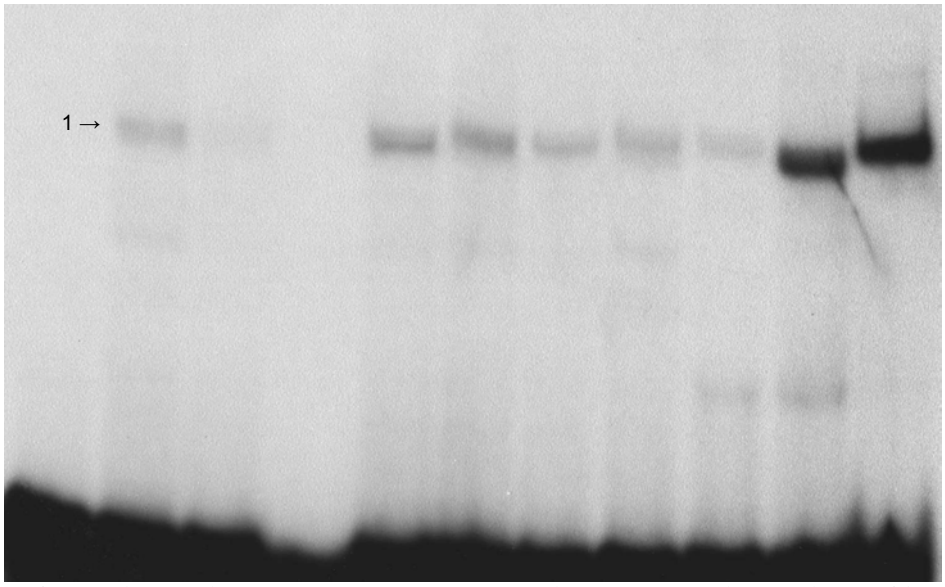
The PPRE in the human LFABP promoter region was analysed in experiments analogous to those described above. With one difference: the radioactively labelled oligonucleotides contained the PPRE found in the human LFABP promoter instead of it's rat counterpart (figure 11, lane 1). In vitro translated xPPARα and hRXRβ cause the formation of a band (lane 2, arrow 1), which disappears with the addition of 100 fold molar excess of unlabeled oligonucleotides. Bands at the same level are visible when poly dIdC was added as non specific competitor at different concentrations (0.01g/l, 0.04g/l, 0.001g/l, respectively). Thus meeting criteria for specific receptor-DNA-interaction.

On lanes 7 and 8, representing in vitro translated xPPARα and hRXRβ respectively, bands at the same height are detectable. Two bands, formed on the control lanes, deserve further mention. The first one on lane 9 is more prominent and slightly lower than the previous ones. On this lane in vitro translated vector pSPUTK was used. Lane 10 displays results, when reticulocyte lysate without added DNA was used. The very prominent band on the last lane is again at the same level as those that appear to be specific.

Another series of experiments was conducted, in which an anti-human-PPARα-antibody was used in an attempt to create a supershift. This could have confirmed a specific protein-DNA interaction. Unfortunately this attempt did not lead to

utilisable results (not shown here). However it needs to be emphasized, that only in vitro translated Xenopus laevis PPARα and anti-human-PPARα-antibodies were available.

**PPAR Interaction with Human PPRE**




| Lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
|---|---|---|---|---|---|---|---|---|---|---|
| xPPARα | - | + | + | + | + | + | + | - | - | - |
| hRXRβ | - | + | + | + | + | + | - | + | - | - |
| Sp.Com. | - | - | + | - | - | - | - | - | - | - |
| dIdC | - | - | - | 0.01 | 0.04 | 0.001 | - | - | - | - |
| pSPUTK | - | - | - | - | - | - | - | - | + | - |
| Lysate | - | - | - | - | - | - | - | - | - | + |

***Fig. 11:*** *EMSA analysis of human PPRE sequence*. Labelled oligonucleotides containing the human PPRE were used. Addition of in vitro translated xPPARα and hRXRβ cause formation of a band (arrow 1), which disappears when a specific competitor is added (lane 3). Poly dIdC as non specific competitor has no effect on this band (lanes 4 to 6). These findings suggest a specific protein/DNA interaction. xPPARα and hRXRβ alone also induce the band, respectively (lanes 7 and 8). See section 3.3.2. for further details. PPRE = peroxisome proliferator response element, xPPARα = Xenopus laevis peroxisome proliferator activated receptor α, hRXRβ = human retinoid x receptor β, Sp.Com. = specific competitor, dIdC = poly dIdC in g/l, pSPUTK = expression vector, Lysate = reticulocyte lysate.

### 3.4.1. Effect of Thyroid Hormone on PPARα mRNA Concentration in vivo

TRs and PPARs are both important nuclear transcription factors in controlling FA metabolism and show complex patterns of gene regulation which are to some

extent co-operative. But do they also influence their gene expression mutually? A series of experiments was set up to investigate the effect of T3 on PPARα in livers of male Wistar rats. mRNA was prepared as described in 3.1.1. and analysed by quantitative RT-PCR. Ubiquitin was used as a “housekeeping” gene, to which the PPARα signal was normalised (n=10).

Figure 12 shows the changes of PPARα mRNA concentration after T3 injection (6h, 24h, 48h) relative to hypothyroid rats (0h). 6 hours after administration of thyroid hormone, a 35% decrease of mRNA concentration is detectable. But 18 and 42 hours later the level is at it's initial value again, exhibiting only a marginal T3 effect.

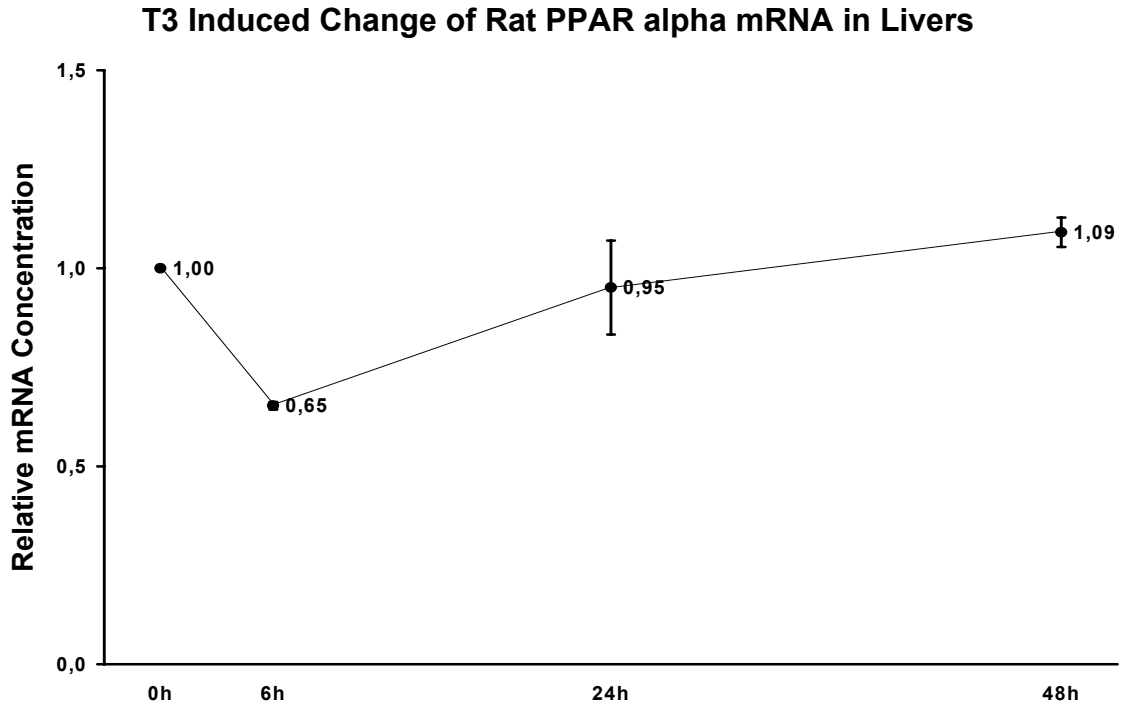



***Fig. 12:*** *Effect of T3 on rat PPARα mRNA in vivo*. Total liver RNA from three hypothyroid individual rats were pooled 0, 6, 24,and 48 hours after injection of T3, respectively (0h = hypothyroid animals). An initial decrease of concentration by 35% is detectable after 6 hours. In contrast, signals after 24 and 48 hours do not show significant differences in regard to the initial concentration. See section 3.4.1. for further details. Rat PPARα mRNA concentrations were normalised to ubiquitin signal and expressed relative to concentrations found in livers of hypothyroid rats. Results are means ± S.E.M. PPARα = peroxisome proliferator activated receptor α.

# 4. Discussion

## 4.1. Thyroid Hormone Influence on LFABP mRNA Concentration within 48 Hours in vivo

Microarrays are relatively new and powerful tools for the analysis of nucleic acids. Despite their huge potential and many advantages, they also have weak points that need to be kept in mind. One of them is reliability. Halgren et al. [10] reported that only 62.2% of 1189 cDNA clone sequences, which are used for many microarrays, were unobjectionable. So confirming relevant results by other methods is essential. Here northern hybridisation was chosen as the second technique. At the beginning of the work on this subject, it was known that rLFABP protein concentration in rat livers is elevated after administration of T3. This effect was described after an incubation period of 17-19 days [23]. This work, however, focuses on the change of mRNA concentrations during the first 48 hours because it could provide an indication of a possible underlying mechanism, which has not been investigated before.

The clear 3.7 fold increase of the northern hybridisation signal 48 hours after T3 administration to the rats is demonstrative, whereas the rises after 6 and 24 hours (1.6 and 1.5 fold, respectively) are moderate. This time course shows the characteristics of a late induction kinetic, thereby suggesting an indirect T3 effect.

## 4.2. Analysis of Human and Rat LFABP Promoters

### 4.2.1. Direct Effect of Thyroid Hormone on Rat and Human LFABP Promoters

The first step of this analysis of possible underlying mechanisms, was to investigate a direct T3 effect via a TRE in the rat and human LFABP promoters. Neither rat nor human promoter fragments showed a clear response to T3 in transient transfections. This means that the TRE-like sequence found in the human is not a functional TRE. These findings also support the assumption that T3 influences rLFABP gene expression indirectly.

These experiments were carried out under conditions analogous to those performed with the well characterised TRE-containing promoter B of the rat mitochondrial glycerol-3-phosphate dehydrogenase gene [37], thus showing that this cell system is suitable for detecting direct T3 effects. T3 concentrations in cell culture medium were also determined, so as to excludeT3 changes as possible sources of error. Another possible explanation of these negative results could be

**Model of Negative Gene Regulation by Thyroid Hormone**

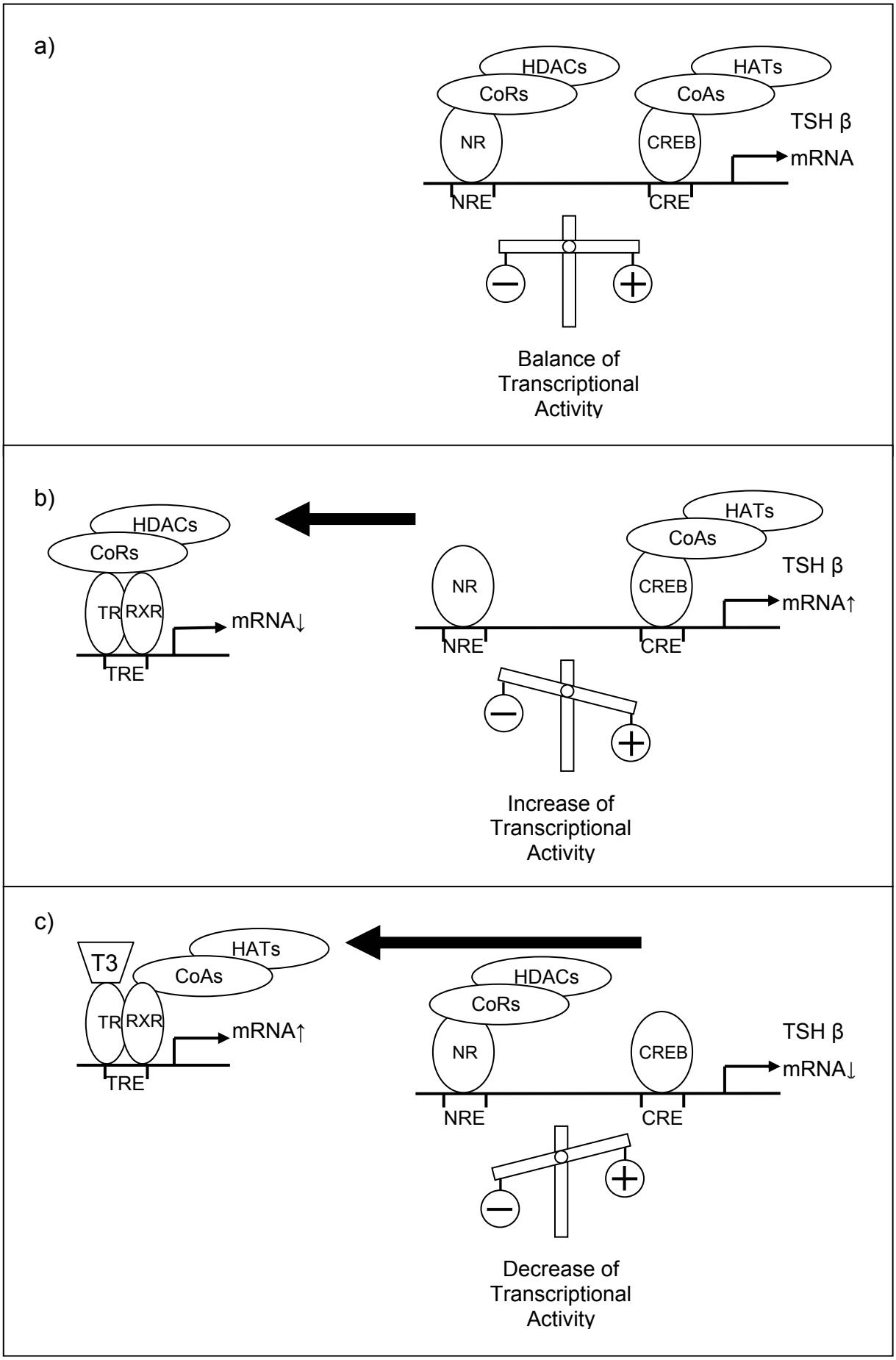


***Fig. 13 a-c*****:** *Model of negative regulation of THSβ gene by thyroid hormone.*
(a) The transcriptional activity of the TSHβ gene is determined by elements that have negative (NRE), as well as positive (CRE) effects on the gene expression. These effects are mediated by co-repressors (CoR) / histone deacetylases (HDAC) and co-activators (CoA) / histone acetyl-transferases (HAT), respectively. (b) When TR, together with RXR, binds to a TRE in the promoter of a target gene, CoRs and HDACs are sequestered from the TSHβ promoter. This shifts the transcriptional balance of TSHβ gene expression towards positively regulating factors. (c) Binding of T3 to TR results in a recruitment of CoAs and HATs, that are competed away from the TSHβ promoter. This causes a decrease of TSHβ gene expression.
See section 4.2.1. for further details. Modified from Tagami et al [34].
CoA = co-activator, CoR = co-repressor, CRE = cAMP response element, CREB = cAMP response element binding protein, HAT = histone acetyltransferase, HDAC = histone deacetylase, NR = nuclear receptor, NRE = negative response element, RXR = retinoid x receptor, T3 = thyroid hormone, TR = thyroid hormone receptors, TRE= thyroid hormone response element, TSHβ = thyrotropin β.

the length of the promoters, a TRE simply might not be in it. Seeing as the majority of active TREs are located several hundred base pairs upstream of transcription start sides [43], this alternative appears to be unlikely, even though it can't be excluded.

Although these findings do not support an activation of rat and human LFABP promoters, figures 7 and 8 show a T3 effect that needs to be discussed. Co-transfection with cTRα, along with pGL3-1623hLFABP, causes an increase of luciferase activity (columns 3). Interestingly, T3 stimulation of such cells reduces luciferase activity (columns 4).

Tagami et al. [34] proposed a model of negative regulation of the TSHβ gene by T3, which is helpful in explaining this unexpected observation. The promoter of the TSHβ gene contains a cAMP response element (CRE), which plays an important role in the regulation of this gene. Binding of cAMP response element binding protein (CREB) to the CRE causes recruitment of co-activators (CoAs) and histone acetyltransferases (HATs). This eventually leads to gene activation. On the other hand, there are also transcription factors bound to the promoter that recruit co-repressors (CoRs) and histone deacetylases (HDACs). These reduce the gene expression. Therefore gene expression is determined by a balance of HATs and HDACs (figure 13a).

Unliganded TR binds CoRs and HDACs, which causes a repression of genes containing TREs. HDACs are sequestered from transcription factors bound to the TSHβ promoter, which results in an overbalance of HATs and an increased TSHβ activity (figure 13b). In the presence of T3, TR binds CoAs and HATs, hereby enhancing transcription of genes containing TREs. The CoAs and HATs are competed away from the CREB on the TSHβ promoter, resulting in an overbalance of HDACs and a decrease of TSHβ expression (figure 13c).

The results of transient transfections could be explained by the following hypothesis: co-transfection with cTRα causes an over-expression of this TR. CoRs and HDACs, that would usually be bound to transcription factors on pGL3-1623hLFABP, are competed away from it. This results in an relative excess of CoAs and HATs on the promoter fragment and eventually in an elevated reporter gene activity (columns 3). On the other hand, addition of T3 doesn't cause a drop of luciferase activity below basal activity (columns 4). Possibly, liganded TR doesn't withdraw CoRs and HDACs from pGL3-1623hLFABP but from promoters other than this one.

Another question has to be addressed: why doesn't co-transfection with cTRα and hRXRα, without T3 stimulation, results in an elevated reporter gene activity? Perhaps an unliganded cTRα:hRXRα heterodimer binds other CoRs and HDACs than an unliganded cTRα homodimer.

### 4.2.2. The Peroxisome Proliferator Response Elements in Rat and Human LFABP Promoters

The second putative responsive element of interest in the rat promoter, the PPRE, has also been analysed. At the beginning of this work it was known that the rLFABP promoter contains a sequence to which mouse PPAR:RXR heterodimers bind in EMSA studies [27]. But it was not known if the consensus sequence is relevant for expression control of the gene.

Transient transfections do show a response to clofibric acid, a well known PPARα ligand. Interestingly, the results displayed in figure 9 also show that co-transfection of receptors alone, without addition of clofibric acid, increase reporter gene activity. Similar findings have been observed in the cases of other active PPREs [12], and are likely to be explained by the presence of endogenous ligands. This assumption is supported by another observation: transfections conducted with cell culture medium containing normal foetal calf serum (FCS) do

not show a further increase in reporter gene activity when clofibric acid is added to the co-transfected receptors (data not shown). The findings presented here are results from experiments in which delipidated FCS was used, and columns 4 and 6 of figure 9 do indeed show a further rise in luciferase activity. Even though this effect is statistically not significant, it suggests that lipophilic substances like FAs could act as endogenous ligands.

While these experiments were conducted, Poirier et al. published their analysis of the PPRE in rat LFABP promoter [28]. It shows findings that support those presented here. They include results obtained from transient transfection assays in which a promoter/reporter-gene construct containing a mutated PPRE was used: no increase of reporter gene activity is detectable when PPAR with ligands are added. This demonstrates the importance of this PPRE in mediating the effect.

The PPRE in rat LFABP promoter and the corresponding PPRE-like sequence in human promoter were further analysed by electrophoretic gel mobility shift assay experiments.

As displayed in figure 10, two bands (arrows 1 and 2) meet the criteria for specific protein/DNA interaction. It can be suggested that the upper band is caused by an interaction of receptor dimeres, whereas the lower is due to a binding of momoneres to the probes. According to this interpretation, xPPARα can bind as monomer and as a dimmer to the probe (two bands on lane 7), in contrast to hRXRβ, which only interacts as a homodimer with DNA (upper band on lane 8). Another band of interest is indicated by arrow 3, it is clearly visible on lane 7 and on both control lanes but not on any other lane. This suggests a non-specific interaction. A "super shift", with a specific PPARα antibody, could support the findings. Unfortunately, only Xenopus laevis PPARα for in vitro translation and antibodies directed against the first 98 amino acids of human PPARα were available. But PPARα from the two species differ highly in the sequence of the first 98 amino acids, i.e. the antibodies were not useful for these EMSA experiments.

These findings are in contrast to results published previously [27]. It was reported that only PPAR:RXR heterodimers interact with this PPRE, but not homodimers or monomers. Poirier et al. used in vitro translated mouse receptors, whereas here Xenopus laevis PPARα and human RXRβ were used. Other discrepancies in experimental conditions include buffers and the presence of salmon sperm DNA. This might explain the different findings.

EMSA experiments with oligonucleotides containing the sequence of the human PPRE also revealed bands which meet criteria for specific protein/DNA interaction (figure 11, arrow 1). Again, “super-shift” experiments would have been helpful to further underline the specificity of these protein/DNA interactions. But the use of the antibody directed against the first 98 amino acids of human PPARα didn't reveal useful results. This is most likely due to the fact that Xenopus laevis PPARα was used for in vitro translation.

In summary, the EMSA experiments demonstrate a specific protein/DNA interaction of xPPARα (together with hRXRβ) with the human and rat PPRE. Furthermore, transient transfections show that PPARα regulates rat LFABP gene via the PPRE.

### 4.3. PPARα mRNA Concentration after Thyroid Hormone Administration in vivo

The Thyroid hormone is known to play an important role in lipid metabolism by modifying the expression of a number of genes, which include LFABP, liver carboxlyesterase 10, and apolipoprotein A I [38]. But might T3 also influence PPARα gene activity, and thereby mediate some of it's indirect effects? mRNA concentrations of nuclear receptors are usually relatively low, so quantitative RT-PCR was needed for this investigation.

The results of these experiments (figure 12) do not support this idea. The initial 35% decrease of PPARα mRNA is relatively moderate and concentrations at the following points of time (24 and 48 hours) differ even less from the initial level. This initial drop and a subsequent elevation of mRNA concentration can be observed in a number of genes regulated by T3. Miller et al. reported that 24 of 358 responsive genes in GC cells, a permanent rat pituitary cell line, show an analogous pattern [17]. A new report from our laboratory reveals similar findings. 4 of 62 genes regulated by T3 in livers of male Wistar rats have a similar temporal expression kinetic [39]. The underlying mechanisms are still not understood, however.

In all, it appears to be unlikely that indirect T3 effects are mediated via a change of rPPARα gene expression.

### 4.4. Possible Indirect Mechanisms of Thyroid Hormone Action

Several mechanisms of indirect T3 action could provide an explanation for the findings presented here.

Firstly, extracellular or systemic endocrinological alterations in response to T3 might explain delayed effects. It is known, for example, that sympathetic activity is altered and serum gastrin levels are elevated, to name only two of an immense number of alterations [20-22]. Nevertheless, these extracellular effects eventually have to cause intracellular changes. Analysis of LFABP gene expression pattern in primary hepatocytes or permanent cell lines, which are derived from hepatocytes, could have been helpful, because extracellular effects would have been excluded. Despite several attempts, it was not possible to prepare valid primary cells. The use of HepG2 and Fao cells was also not suitable, because detection of LFABP mRNA by northern hybridisation failed.

Secondly, it could be due to a metabolic effect of T3. It is well known that serum FAs are raised in hyperthyroid states [21]. Also fatty acid synthesis rate in hyperthyroid rats is 8.5 times higher than in hypothyroid animals. FA synthesis rises after a lag time of 12-16 hours in hypothyroid rats to which T3 has been administered [9]. These FA could interact with hepatocellular PPARs and the active PPRE in the LFABP promoter, causing an increased LFABP gene expression rate. The latency period of 24-48 hours between injection of T3 and elevation of LFABP mRNA could be explained by the delay of FA synthesis in response to T3. However, not only FA synthesis is increased by T3 but also FA oxidation. Unfortunately no technique is available that can be used to determine intracellular FFA concentration precisely. But it is possible to measure the concentration of intracellular activated FAs, which is decreased in livers of starved hyperthyroid rats [19]. Assuming a correlation between intrahepatocellular activated FAs and free FAs, this hypothesis appears unlikely. It can be summarised, that due to experimental limitations, it is difficult to investigate this and other metabolic observations.

Thirdly, the indirect T3 mechanism of action could be mediated by intermediary gene regulating proteins, as explained in the introduction. These proteins have to meet two important criteria: (i) they have to be under direct control of T3, possibly via an active TRE. This means the change of their mRNA concentration after T3 administration has to show the characteristics of an early kinetic. Of course, the synthesis of the active protein would be time consuming, explaining a latency period before the protein can fulfil it's function. (ii) The intermediary proteins have to influence the transcription activity of target genes, i.e. transcription factors and other gene activity regulating factors are of interest. NRF-1 and PGC-1 are two

possible candidates, because it has been shown that their gene expression displays characteristics of an early kinetic in response to T3 [38].

Given these requirements, the following working hypothesis was created (figure 14): T3 enters the hepatocyte and binds to it's nuclear receptor. TR, together with it's heterodimerisation partner RXR, binds to a TRE in PGC-1 promoter and increases the transcription rate of the gene (this putative TRE is currently under investigation). After the synthesis of the protein, PGC-1 interacts with transcription factors that are already bound to the promoter, e.g. PPARα, thereby enhancing transcriptional activity of the LFABP gene. Of course PGC-1 could interact with other transcription factors on the promoter too, creating an accumulated effect.

It is not likely that the other attractive candidate, NRF-1, plays a role in regulating LFABP gene expression, due to the absence of a consensus sequence in both promoters. Nonetheless, it cannot be completely excluded. Firstly because of the limited length of the promoters, as already discussed above. Secondly, it needs to be kept in mind, that the computer aided search for putative consensus sequences doesn't provide definite results, quite often functional responsive elements are missed.

### 4.5. Conclusion

In summary, this data reveals a complex LFABP gene regulation in response to T3. Given the elevated hepatic turnover of FAs in hyperthyroid states, it is plausible that gene expression of LFABP as important FA transport protein is up-regulated. It is unlikely that this regulation is mediated via a direct T3/TR interaction with LFABP promoter, but by an intermediate protein. Unlike NRF-1, PGC-1 is a very likely candidate, which can interact with transcription factors like PPARα. At present it is difficult to show any effect PGC-1 might have in transient transfections with promoter/reporter gene constructs when natural promoter fragments are used. So far, effects have only been detected when PGC-1 has been co-transfected with PPARα and a reporter gene construct that contains three copies of a known PPRE upstream of the reporter gene [35]. So it is not surprising that the transient transfection experiments, conducted in our laboratory, didn't reveal an effect of PGC-1 on rat LFABP promoter fragments. At this point there is no suitable cell system available to investigate T3 effects mediated by PGC-1.

Further investigation with appropriate knock-out models or application of RNAi technique could shed light on this matter from a different perspective. Such research does indeed promise to be interesting.

Despite momentary experimental difficulties, there is a very interesting observation that supports the idea of a co-operative effect of T3 and PGC-1: PGC-1 effects are analogous to those of T3 regarding mitochondrial biogenesis, metabolic rate, and adaptive thermogenesis [14].

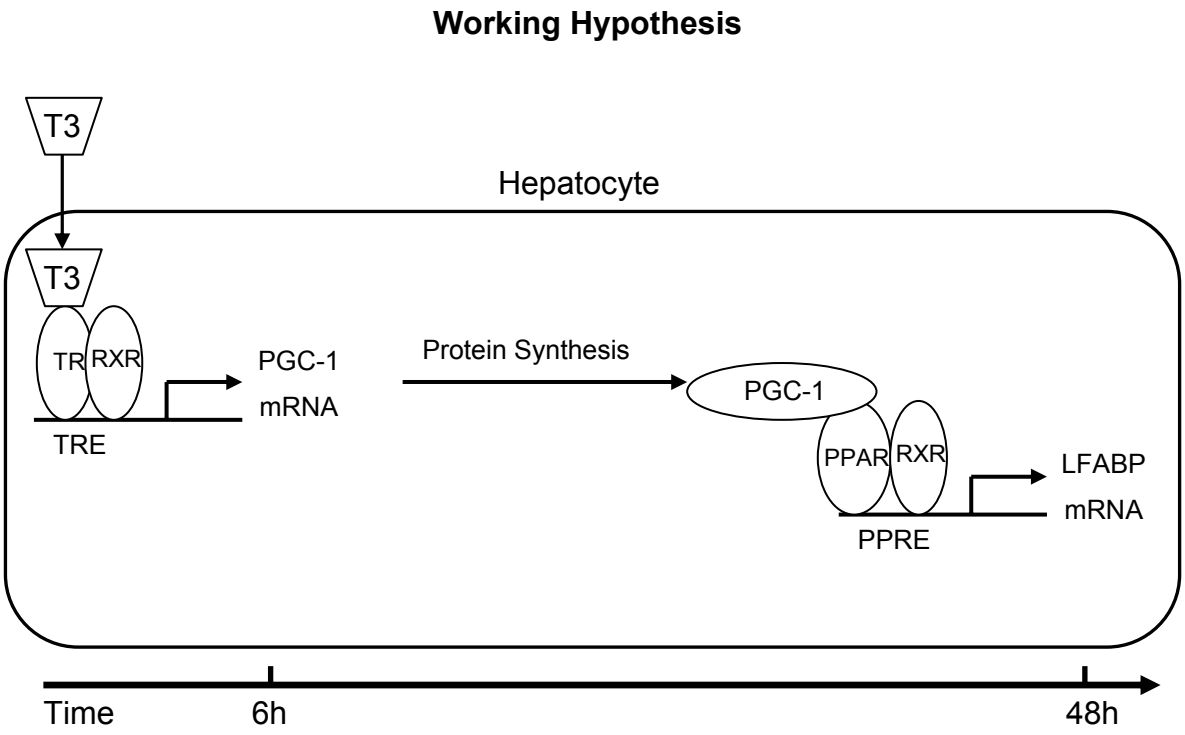



***Fig. 14:*** *Working hypothesis of indirect mechanism of T3 action.* T3 enters the cell, forms a complex with TR:RXR heterodimers, and binds to a putative TRE in the PGC-1 promoter. This results in an increased PGC-1 gene activity and eventually in an elevated PGC-1 protein concentration. Protein synthesis is time consuming and causes a latency period. After that PGC-1 associates with transcription factors bound to LFABP promoter. This results in a rise of LFABP mRNA concentration. For further details see section 4.4. LFABP = liver fatty acid binding protein, PPAR = peroxisome proliferator activated receptor, PGC-1 = peroxisome proliferator activated receptor gamma co-activator 1, PPRE = peroxisome proliferator response element, RXR = retinoid x receptor, T3 = thyroid hormone, TR = thyroid hormone receptor, TRE = thyroid hormone response element.

# 5. Material and Methods

## 5.1. Cloning and Subcloning of Promoters

### 5.1.1. DNA Sequences

DNA sequences were obtained from the National Centre for Biotechnology Information (NCBI, www.ncbi.nlm.nih.gov) of the USA. It offers access to public DNA databases and software tools for analysing genome data.

The promoter sequences of interest were then submitted to MatInspector (transfac.gbf.de). This internet based DNA sequence analysis tool is provided by the Gesellschaft für Biotechnologische Forschung (GBF). MatInspector searches for potential binding sites of nuclear receptors in a submitted DNA sequence.

To verify putative binding sites, the program GeneWorks (IntelliGenetics, version 2.45) was used. This program also identifies recognition sites of restriction endonucleases.

### 5.1.2. Cloning and Subcloning of Human and Rat LFABP Promoters

The human LFABP promoter was cloned from genomic HepG2 DNA, which had previously been isolated in our laboratory. The oligonucleotides AI 3 (TGCTGGAGGGAAAGGTCTGC) and AI 4 (GCTCTGCAGTTGGTACTTGC) were used for PCR (program HLFABP). The resulting DNA fragment, encompassing positions +132 to –1623 relative to transcription start side, was inserted into the pGEM T easy vector. The correct promoter sequence was verified by DNA sequencing (primers T7 (TAATACGACTVACTATAGGG) and SP6 (GAT TTAGGTGACACTATAG), program SEQU). The vector was then digested with Not I and Nde I and the resulting cohesive ends were blunted. After the pGL3 basic vector had been cut with Sma I, the promoter fragment was ligated upstream of the reporter gene, giving pGL3-1623hLFABP. Correct fragment orientation was confirmed by DNA sequencing (primers pGL3 for (CTAGCAAAATAGGCTGTCCC) and pGL3 rev (CTTTATGTTTTTGGCGTCTTCCA), program SEQU).

The rat LFABP promoter had been cloned in our laboratory previously, and had been inserted into the pGEM T easy vector. The immediate promoter region, encompassing positions –615 to +155, was excised with restriction endonucleases Sac I and Sac II. The cohesive Sac II end was converted into a blunt end. The DNA fragment was then ligated upstream of the luciferase gene contained in the

pGL3 basic vector, which had been digested with Sac I and Sma I, giving pGL3-615rLFABP. Correct ligation was confirmed by PCR (primers pGL3 for, pGL3 rev, WL 124 (CAGAATACAAAACAGCTTTAGG), and WL 125 (TGCTGACAGAAG GGATAGCC), program PCRCHECK).

### 5.1.3. Polymerase Chain Reaction

A PCR sample was prepared with the following components: template DNA (either plasmids or whole cells), two primers (1mM final concentration each), PCR Buffer (1x final concentration, Amersham Pharmacia Biotec), dNTPs (0.2mM final concentration), and Taq DNA polymerase (2.5U, Amersham Pharmacia Biotec). The reaction was carried out in an automated thermal cycler (T3 Thermocycler, Biometra). The different PCR programs are listed below.

### 5.1.4. Agarose Gel Electrophoresis

1% or 1.5% agarose gels containing 0.25μg/ml ethidium bromide were prepared. 5μl of DNA solution were added to 1μl of 6x loading buffer. To perform electrophoresis, the voltage was set to 10V/cm. BioRad Mini Sub Cell GT electrophoresis tanks and the BioRad Power Pac 300 power supply were used. 0.5x TBE was used as electrophoresis buffer. After electrophoresis, the gels were placed on the IL-305-M UV transilluminator (Bachofer) and photographed with a Polaroid CU-5 camera.

### 5.1.5. Extraction of DNA from Agarose Gels

The QIAquick Gel Extraction Kit (Qiagen) was used to extract DNA from agarose gels.

The DNA fragment was excised from agarose gel. To the volume of an agarose slice (100mg equal 100μl), three times the volume of buffer QG was added. The gel slice was dissolved at 50°C, and one gel slice volume of isopropanol was added to the sample. The sample was then applied to a QIAquick column and centrifuged for 1 minute at 10,000g. 0.75ml of wash buffer PE were added to the column and again centrifuged for 1 minute at 10,000g. To remove residual ethanol, the column was centrifuged for an additional 1 minute. DNA was eluted from the silica-gel membrane with 50μl of water.

### 5.1.6. Determination of DNA Concentration

The spectrophotometer (Pharmacia LKB Ultrospec III) was zeroed by reading the absorption of a cuvette filled with water at the particular wavelength. A DNA sample was diluted in an adequate volume of water. $A_{260}$ and $A_{280}$ were read and the concentration and purity of the sample was calculated.

### 5.1.7. Restriction Endonucleases

OPA restriction buffer (at a final concentration of 1x) and 5 through 15,000 units of restriction endonuclease were added to a DNA sample. This reaction mixture was incubated at 37°C for 2 hours. If cohesive ends were generated by the endonuclease but blunt ends were needed, 4 units of Klenow fragment and 5nmol of dNTPs were added and incubated at 37°C for 15 minutes. Inactivation of the Klenow fragment was achieved by heating the sample to 65°C for 20 minutes.

To prevent re-closure of a cleaved vector on itself, 1 unit of calf intestinal alkaline phosphatase (Roche) was added and incubated for 20 minutes at 37°C.

### 5.1.8. Ligation of DNA Fragments into Vectors

The pGEM-T easy Vector Kit (Promega) and the pGL3 basic Vector (Promega) were used for cloning PCR products and generating luciferase reporter gene vector constructs, respectively.

50ng of pGEM-T easy vector or pGL3 basic vector, insert DNA (at a insert:vector molar ratio of 3:1), ligation buffer (at a final concentration of 1x), and 3U of T4 DNA Ligase were incubated overnight at 4°C. To calculate the appropriate amount of insert DNA for the reaction, the following equation was used: ((ng of vector * kb size of insert)/(kb size of vector)) * (3/1) = ng of insert.

### 5.1.9. Transformation of Bacteria

Plasmids were introduced into competent E. coli cells (Nova Blue strain, Novagen).

0.5μl of a ligation reaction or plasmid DNA was added to a 20μl aliquot of competent cells and incubated on ice for 5 minutes. The sample was heated to 42°C for 30 seconds and then incubated on ice for another 2 minutes. 80 μl of SOC medium were added and the sample was plated on LB medium. The plate was incubated overnight at 37°C.

### 5.1.10. Growing of Bacteria

LB medium with ampicillin at a concentration of 25 μg/ml, was used to grow E. coli cells in liquid cultures as well as on solid plates. The cells were inoculated onto or into the medium and incubated overnight at 37°C.

### 5.1.11. Plasmid Preparation

The Qiagen Plasmid Midi and Maxi Kits were used for plasmid purification.

An overnight bacterial culture was grown in LB medium at 37°C with vigorous shaking (midi kit: 100 ml medium or maxi kit: 500 ml medium, respectively). The bacterial cells were harvested by centrifugation at 6000g for 15 minutes at 4°C (RC-5B Refrigerated Superspeed Centrifuge, Sorvall). The bacterial pellet was re-suspended in 4ml (midi kit) or 10ml (maxi kit) buffer P1. 4ml or 10ml of buffer P2 were added and incubated at room temperature for 5 minutes to lyse the cells. For neutralisation, 4ml or 10ml of buffer P3 were added and incubated on ice for 20 minutes. The sample was centrifuged at 20,000g for 30 minutes at 4°C and the supernatant was re-centrifuged at 20,000g for 15 minutes at 4°C. After equilibration of a Qiagen tip 100 or Qiagen tip 500 with 4ml or 10ml of buffer QBT, the supernatant was applied to the Qiagen tip. The resin was washed twice with 10ml or 30ml of buffer QC. The plasmid DNA was then eluted with 5ml or 15ml of buffer QF. Precipitation of plasmid DNA was achieved by adding 3.5ml or 10.5ml of isopropanol and centrifuging at 15,000g for 30 minutes at 4°C. The pellet was washed with 2ml or 5ml of 70% ethanol, centrifuged at 15,000g for 10 minutes, and air-dried. The DNA pellet was re-dissolved in a suitable volume of water.

### 5.1.12. Sequencing of DNA

Sequencing of DNA was performed at the DNA Sequencing Unit of the Institut für Zellbiochemie und Klinische Neurobiologie. The BigDye terminator sequencing kit (Perkin-Elmer Applied Biosystems) was used.

700 to 1200ng of DNA, 8μl of HT 2.5x Buffer, 2μl of BigDye reaction mixture, 12.5pmol of primer, and water to a final volume of 20μl were assembled. The program SEQU was set up and performed in an automated thermal cycler (T3 Thermocycler, Biometra). After PCR, 80μl of sodium acetate (0.3M) and 300μl of ethanol (100% vol.) were added to the sample, and it was incubated at room-temperature for 10 minutes. The samples were then centrifuged at 14,000g and

4°C for 30 minutes. The supernatant was decanted and 200µl of ethanol (75% vol.) were added. The sample was first centrifuged at 14,000g and 4°C for 10 minutes and then air dried.

### 5.2. Cell Culture

#### 5.2.1. Growing of Permanent Cell Lines

The cells were cultured with DMEM (Gibco Invitrogen Corporation) supplemented with 10% (v/v) delipidated foetal calf serum (Biochrom AG) in an incubator (B 50 60 EC-CO2, Heraeus Christ) at 37°C under a humidified atmosphere (5% $CO_2$).To separate cells of a culture of adherent cells, the medium was removed and the cells were washed with 1x PBS. To dissociate cells, a suitable volume of trypsin/EDTA-solution (0.05%/0.02%, w/v in PBS, Biochrom AG) was added and incubated in the incubator for 5 minutes. An appropriate volume of medium was added and pipetted up and down do separate the cells. Approximately 2/3 of the medium were removed and new medium was added.

For transfection, the dissociated cells were counted and seeded at a density of $10^5$ cells per 35 mm culture dish. 24 hours later, the cells were transfected, and after another 24 hours stimulated with hormones.

#### 5.2.2. Transient Transfection of Permanent Cell Lines

FuGENE 6 Transfection Reagent (Roche) was used for the transient transfection of eukaryotic cells.

Per 35mm culture dish, 3µl of FuGENE 6 were added to 100µl of serum-free DMEM medium and incubated for 5 minutes at room temperature. 1500ng of reporter gene construct, 100ng of pCH110 plasmid (cytomegalovirus β-galactosidase vector, to monitor transfection efficiency), and 500ng of receptor expression vectors were combined. The total DNA amount was kept constant by the addition of salmon-sperm-DNA. The FuGENE 6/ DMEM medium was pipetted to the DNA and incubated for 15 minutes at room temperature. The transfection sample was then added to a culture dish with cells. The medium was changed after 24 hours, with or without stimulation. To stimulate cells with T3, 150nmol of it were added per 35mm culture dish. For stimulation with clofibric acid, 300µmol of it, dissolved in ethanol, were added to one dish. Ethanol was added to those cells not stimulated with clofibric acid, in order to create comparable conditions.

### 5.2.3. Luciferase Activity Assay

A 35 mm culture dish with transfected cells was washed twice with ice-cold 1x PBS and the cells were lysed and solubilised in Lysis Solution (Galacto-Light, Applied Biosystems). The cells were centrifuged for 6 minutes at 4°C and 14,000g. The supernatant was used for the assay. 20μl of cell lysate and 100μl of luciferase-assay-buffer were mixed and placed in the luminometer chamber (Lumat LB 9501, Berthold). 100μl of luciferin-solution were injected into the sample and light output was measured for 10 seconds at room temperature. Two samples from each cell lysate were analysed.

### 5.2.4. β-Galactosidase Activity Assay

β-Galactosidase activity was measured with Galacto-Light chemiluminescent reporter gene assay (Applied Biosystems)

Cells were lysed as described in 5.2.3., 10μl of the cell lysate were added to 100μl of Reaction Buffer and incubated for 60 minutes at room temperature. Then the sample was placed in the luminometer and 100μl of Accelerator II were injected. The signal was read for 5 seconds.

### 5.2.5. Determination of Protein Concentration

Protein concentration was determined with the Bio-Rad Protein Assay (Bio-Rad)

2μl of protein solution, of unknown concentration or of known standard BSA concentration, were diluted in 98μl of PBS. 1ml of Bio-Rad Protein Assay solution was added. The sample was incubated for 20 to 60 minutes at room temperature and the extinction was measured in a spectrophotometer (Pharmacia LKB Ultrospec III).

### 5.2.6. Statistical Evaluation

The results obtained from luciferase and β-galactosidase activity assays were evaluated with the program Excel 97 (Microsoft).

Luciferase activity assay results were normalised to β-galactosidase activity or protein concentration to determine transfection efficiency. Activities of promoter/ reporter-gene constructs were normalised to pGL3 basic signals. The results are means ±S.E.M.

### 5.2.7. Preparation of Foetal Calf Serum

1g of charcoal , 0.1g of dextran T70 (Sigma) and 200ml of sterile water were stirred and incubated over night at 4°C. 1000 units of sulfatase (Sigma) were added to 500ml of FCS and incubated for 2 hours at 37°C. The charcoal solution was centrifuged for 10 minutes at 4000rpm (FABOFUGE, Heraeus Christ) and the supernatant was discarded. Prepared FCS and charcoal/dextran were combined and incubated for 30 minutes at 56°C to inactivate the FCS. After the sample had been placed on ice for 5 minutes, it was centrifuged for 15 minutes at 4°C and 4000rpm. The supernatant was removed and sterilised by filtration.

### 5.2.8. Determination of Thyroid Hormone Concentration in Cell Culture Medium

Determination of Thyroid Hormone Concentration was performed at the Hormonlabor of the Abteilung für Klinische Chemie, UKE.

## 5.3. Electrophoretic Mobility Shift Assay

### 5.3.1. Electrophoretic Mobility Shift Assay

The labelled DNA probe, band shift buffer (at a final concentration of 1x), the unlabeled DNA probe as specific competitor (100x molar excess relative to the labelled probe), poly(dI-dC) as non-specific competitor (at varying concentrations), and in vitro translated receptors were assembled for DNA-protein binding reaction and incubated at room-temperature for 30 minutes. A non-denaturing 5% polyacrylamide gel was prepared, 0.5x TBE was used as electrophoresis buffer. The gel was pre-run for 30 minutes at 200 V (electrophoresis tank: Sequencing Electrophoresis System Model S1, BRL Bethesda Research Laboratories Life Technologies; power supply: Desatronic 500/400, DESAGA Heidelberg). The binding reactions were inserted into the wells of the pre-run gel and the gel was run for 2 hours at 200V. To analyse the gel, it was transferred onto a sheet of 3MM Whatman paper and dried under vacuum and heat (Model 483 Slab Dryer, BioRad). Autoradiography was performed by placing the membrane onto a X-ray film (Super RX, Fuji Film) for 1 to 7 days at -80°C.

### 5.3.2. In vitro Translation

TnT Coupled Reticulocyte Lysate Systems (Promega) were used for in vitro translation.

The following reaction components were assembled: 25μl of TnT rabbit reticulocyte lysate, 2μl of TnT reaction buffer, 1μl of TnT RNA polymerase (SP6 or T7, depending on the promoter in the vector for in vitro translation), 1μl of amino acid mixture (1nM), 40U of RNasin ribonuclease inhibitor, 1μl of transcend biotin-lysyl-tRNA, 1μg of DNA template, and water to a final volume of 50μl. The translation reaction was incubated at 30°C for 90 minutes.

### 5.3.3. Preparation of DNA probes for Electrophoretic Mobility Shift Assay

Two oligonucleotides (200pmol of each) and NaCl (at a final concentration of 180mM) were assembled in a microcentrifuge tube. The tube was placed in hot (95°C) water and was allowed to cool to room-temperature. 5pmol of the annealed oligonucleotides, nick buffer (final concentration: 1x), dGTP, dATP, dTTP (10nmol of each), 5μl of $^{32}$P dCTP (50μCi), and 5U of Klenow fragment were incubated for 20 minutes at 37°C. The reaction was stopped by adding 2μl of EDTA. The DNA probes were purified as described in section 5.4.4. To determine specific activity of the DNA probe, 10fmol of it were diluted in 2ml of Ultima Gold LSC-cocktail (Packard) and read for Cerenkov counts in a Wallac 1409 scintillation counter (Wallac).

### 5.3.4. Sequences of Electrophoretic Mobility Shift Assay Probes

The oligonucleotides AI 11 (GGAATCGACAATCACTGACCTATGGCCTATAT TTGAGG) and AI 12 (GGCCTCCTCAAATATAGGCCATAGGTCAGTGATTG TCG) were annealed to generate an EMSA probe which contains the PPRE sequence of rLFABP promoter (positions -92 to -80). Oligonucleotides AI 13 (GGAATCAACAGCTGCTGACCTCTGGCCGCTATTCGAAG) and AI 14 (GTTCC CTTCGAATAGCGGCCAGAGGTCAGCAGCTGTTG) contained the analogous human sequence (positions -14 to -2).

## 5.4. Analysis of RNA

### 5.4.1. RNA Extraction

RNAClean Solution (Hybaid-AGS) was used to extract total RNA from cells and tissues.

Culture medium was discarded from cells grown in monolayer cultures and the cells were washed twice with ice cold PBS. 1.5ml RNAClean solution was added per 63cm$^2$ culture dish. The cells were harvested and transferred into a microcentrifuge tube. 150μl of chloroform were added to the sample. After incubation for 5 min at 0°C, the sample was centrifuged for 15 minutes at 14,000g and 4°C. The aqueous phase was transferred to a fresh tube, an equal volume of isopropanol was added, and the samples were incubated for 15 minutes at -20°C. After incubation, the samples were centrifuged for 30 minutes at 14,000g and 4°C. The supernatant was removed and the RNA pellet was washed with 300μl of 70% ethanol. After centrifugation at 14,000g and 4°C for 30 minutes, the RNA pellet was air dried, and re-suspended in a suitable volume of $H_2O$/DEPC.

### 5.4.2. Northern Hybridisation

15μg RNA dissolved in 10μl $H_2O$ and 30μl solution 1 was denatured by heating to 65°C for 15 minutes. Electrophoresis was performed in a 1% agarose gel containing 0.37% formaldehyde, 1x RNA gel running buffer, and 0.001% ethidium bromide. The gel was run at 120V. In order to transfer the RNA from gel to membrane, the RNA was fractionated by exposition to UV light for 10 minutes (312nm; IL-305-M UV transilluminator, Bachofer). An overnight capillary transfer to a nylon membrane (BiodyneA Transfer Membrane, Pall) was performed by using 1x RNA gel running buffer as transfer buffer. Immobilisation of RNA was achieved by UV cross-linking in a UV light box (254nm, 1200mJ; UV Stratalinker, Stratagene). The membrane was placed in pre-hybridisation solution and incubated at 42°C for 3 hours. The labelled cDNA probe was added and incubation was continued overnight. The membrane was washed: once with 1xSSC+0.1%SDS for 15 minutes at 42°C, three times with 1xSSC+0.1% for 15 minutes at 65°C, and three times with 0.1xSSC+0.1%SDS for 15 minutes at 65°C. Autoradiography was performed by placing the membrane onto a X-ray film (Super RX, Fuji Film) for 4 to 7 days at -80°C. The films were scanned with a Camag TLC Scanner II Modell 1988.

### 5.4.3. Generation of cDNA Probes

Qiagen One Step RT-PCR (Reverse Transcriptase Polymerase Chain Reaction) Kit was applied to generate cDNA Probes for northern hybridisation.

A hLFABP specific cDNA probe was generated. For one reaction, the following components were assembled: 1.8μg of RNA from human HepG2 cells, 0.5μM of primers LX1 (GAGCCAGGAAAACTTTGAAG) and LX2 (CCAGTTTATTGTCACCT TCC), 1x Q-Solution, 1x Qiagen OneStep RT-PCR Buffer, 400μM of each dNTP, Qiagen OneStep RT-PCR Enzyme Mix, 10 units of RNasin ribonucleaseinhibitor, and water to a final volume of 50μl. The sample was placed in a thermal cycler (T3 Thermocycler, Biometra) and the program HLFABPSO was started. The sample was subjected to electrophoresis and the probe was extracted from the agarose gel.

A rLFABP probe for northern hybridisation had been generated previously in our laboratory. WL 88 (TGCCACCATGAACTTCTCC) and WL 89 (AGTCATGG TCTCCAGTTCGC) had been the primers and cDNA, derived from rat liver mRNA, had been used as template.

Full length cDNA of human ANT2 had been cloned earlier and was used as a probe in these experiments, since It makes detecting human and rat ANT2 mRNA possible.

### 5.4.4. Labelling of cDNA Probes

Rediprime II random prime labelling system (Amersham Pharmacia Biotech) was used to produce radioactive DNA probes.

Nick Columns containing Sepadex G-50 DNA Grade (Amersham Pharmacia Biotech) were applied for purification of radioactive DNA probes.

10ng cDNA probe was dissolved in 45μl TE buffer, denatured by heating to 100°C for 5 minutes, and then snap cooled by placing on ice for another 5 minutes. Next the denatured DNA was pipetted into the reaction tube (containing buffered solution of dATP, dGTP, dTTP, Klenow fragment, and random primers), 3μl [$^{32}$P] dCTP (Redivue $^{32}$P nucleotides, Amersham Pharmacia Biotech) were added to the reaction tube as well. The reaction solution was incubated at 37°C for 10 minutes.

To purify the DNA probes, a Nick Column was equilibrated with TE buffer. Then the sample containing the labelled DNA probe was added and the column was

washed with 450µl TE buffer. The purified DNA probes were eluted with 500µl TE buffer.

Prior to addition of DNA probe to pre-hybridisation solution, the DNA was denatured by heating to 100°C for 5 minutes and then cooled on ice for 5 minutes.

### 5.4.5. Quantitative Polymerase Chain Reaction

The Roche LightCycler and the LightCycler FastStart DNA Master SYBR Green I Kit were used for quantitative PCR.

1.5µl of cDNA template was added to 13.5µl of Mastermix and centrifuged at 2000rpm for approximately 10 seconds. The Mastermix consisted of Master SYBR Green solution (at a final concentration of 1x), $MgCl_2$ (3mM) forward primer and reverse primer (both 300nM). The cycler was run with program 57. Target gene readings were standardised to ubiquitin signal.

### 5.4.6. Preparation of Samples for Quantitative PCR

RNA from Wistar rats had been previously prepared in our laboratory. T3 was administered intraperitoneally to hypothyroid male Wistar rats and liver RNA was isolated after 0h, 6h, 12h, 24h, and 48h. At each point of time, RNA was extracted from three individual rats. RNAClean Solution (Hybaid-AGS) was used to isolate liver RNA.

To produce cDNA, 800ng of isolated RNA, 1µl of dN6 primer (1µg/µl, Amersham), 1µl of dT primer (1µg/µl, Amersham), and water to a final volume of 20µl were incubated for 10 minutes at 70°C. After placing the sample on ice, 6µl of 5x first strand buffer (Gibco), 3µl of dNTPs (10nM), and 1µl of Super Script RT II (Gibco) were added and incubated for 1 hour at 42°C.

### 5.4.7. Sequences of Oligonucleotides used for Quantitative PCR

LX 3 (TACATGACATGGAGACCTTG) and LX 4 (CATAGTGAAGTCAAACT TGG) were suitable for detecting rPPARα cDNA in quantitative PCR experiments. WL 102 (GGCGGTTTGTTCCTTCATC) and WL 103 (CGGGAATGCAGACTGA AGA) were used to determine relative concentration of ubiquitin.

### 5.4.8. Animal Treatment and Care

Total liver RNA from Wistar rats had been isolated earlier in our laboratory. Adult male Wistar rats (Charles River Wiga) were housed under controlled

conditions (22°C, constant humidity, 12h:12h dark-light cycle), food and water were provided ad lib. Rats were kept in the animal care facility of the Universitätsklinikum Hamburg-Eppendorf according to the UKE animal care guideline (Az. F 34/98). Hypothyroidism was induced by intraperitoneal injection of $Na^{131}I$ (Amersham Pharmacia, 250µCi per 100g body weight) 28 days before the experiments. Hyperthyroidism was provoked by intraperitoneal injection of T3 (Henning, 50µg per 100g body weight), which was repeated after 24 hours. Serum T3 and T4 were monitored. 0h, 6h, 24h, and 48h after T3 injection, the rats were decapitated, blood samples were taken, and the livers were prepared for RNA extraction.

### 5.5. Media, Solutions, PCR Programs, Plasmids

All chemicals were obtained from Sigma, unless otherwise specified. All oligonucleotides were synthesised by MWG.

#### 5.5.1. Media, Solutions and Buffers

| | |
|---|---|
| Accelerator II | Applied Biosystems |
| Band-Shift-Buffer: | 100mM TrisHCl; 500mM NaCl; 1mM EDTA; 10mM DTT; 5mM $MgCl_2$;1mg/ml BSA; 50% Glycerine. |
| BigDye: | Perkin-Elmer Applied Biosystems |
| Clofibric Acid: | 300µM, dissolved in Ethanol |
| Denhardt's, 50x: | Ficoll 1% (w/w); Polyvinylpyrrolidone 1% (w/w); Bovine Serum Albumin 1% (w/w) |
| DMEM: | Gibco |
| First Strand Buffer: | Gibco |
| Fugene6: | Roche |
| Gel Loading Buffer, 6x: | 50% Glycerol; 1mM EDTA; 0.4 Bromophenol blue; 0.4% Xylencyanol |
| $H_2O$/DEPC: | $H_2O$; 0.1% Diethylpyrocarbonate |
| HT 2.5x Buffer: | Perkin-Elmer Applied Biosystems |
| LB Agar: | LB Medium, 1.5% Bacto-Agar (Difco) |
| LB Medium: | 1% Bacto Tryptone (Difco); 0.5% Bacto Yeast Extract (Difco); 1% NaCl; pH 7.0 |
| Ligation Buffer: | 2x Rapid Ligation Buffer, Promega |

| | |
|---|---|
| LightCycler Solutions: | LightCycler FastStart Reaction Mix SYBR Green I (Roche) |
| Luciferase-Assay-Buffer: | 25mM Glycylglycine; 15mM $MgSO_4*7H_2O$; 4nM EGTA; 1mM DTT; 15mM $KPO_4$; 2mM ATP; pH 7.8 |
| Luciferin-Solution: | 25mM Glycylglycine; 10mM DTT; 0.2mM Luciferin |
| Lysis Solution: | Galacto-Light Plus Lysis Solution (Applied Biosystems); 0.5mM DTT |
| Nick-Buffer: | 500mM TrisHCl; 100mM $MgCl_2$; 1mM DTT; 500 µg/ml BSA |
| OPA Buffer: | Amersham |
| PBS: | 137mM NaCl; 2.7mM KCl; 4.3mM $Na_2HPO_4*7H_2O$; 1.4mM $KH_2\ PO_4$ |
| PCR Buffer, 10x: | Amersham |
| Pre-hybridisation Solution: | Formamide 50% (v/v); 5x SSPE; 5x Denhardt's; 0.1% SDS; 200µg/ml tRNA Baker's Yeast |
| Protein Assay: | Bio Rad |
| Qiagen Buffers: | Qiagen |
| Reaction Buffer: | Galacton Reaction Buffer Diluent; 1:100 Galacton-Plus (Applied Biosystems) |
| RNA Gel Running Buffer, 10x: | 200mM MOPS; 50mM Na Acetate; 5mM EDTA; pH 7.0 |
| RNA-Clean-Solution: | Hybaid-AGS |
| SOC Medium: | Novagen |
| Solution 1: | Formamide 65% (v/v); Formaldehyde 21% (v/v); 10x RNA Gel Running Buffer 14% (v/v) |
| SSC, 20x: | 3M NaCl; 0.3M $Na_3Citrate{\cdot}2H_2O$; pH 7.0 |
| SSPE, 20x: | 3M NaCl; 0.2M $NaH_3PO_4{\cdot}H_2O$; 20mM EDTA; pH 7.4 |
| TBE: | 890mM Tris base; 890mM Boric Acid; 20mM EDTA |
| TE: | 10mM Tris Cl; 1mM EDTA |
| TnT Reaction Buffer: | Promega |

### 5.5.2. PCR Programs

SEQU:

| | | |
|---|---|---|
| Cycle 1: | 95°C | 1 min |
| | 96°C | 30 sec |
| | 50°C | 15 sec |
| | 60°C | 4 min |
| Cycles 2-35: | 96°C | 30 sec |
| | 50°C | 15 sec |
| | 60°C | 4 min |

HLFABP:

| | | |
|---|---|---|
| Cycle 1: | 95°C | 5 min |
| | 53°C | 30 sec |
| | 72°C | 1 min 45 sec |
| Cycles 2-40: | 95°C | 30 sec |
| | 53°C | 30 sec |
| | 72°C | 1 min 45 sec |
| Cycle 41: | 95°C | 30 sec |
| | 53°C | 30 sec |
| | 72°C | 10 min |

PCRCHECK:

| | | |
|---|---|---|
| Cycle 1: | 95°C | 5 min |
| | 53°C | 30 sec |
| | 72°C | 30 sec |
| Cycles 2-41: | 95°C | 30 sec |
| | 53°C | 30 sec |
| | 72°C | 30 sec |
| Cycle 42: | 95°C | 30 sec |
| | 53°C | 30 sec |
| | 72°C | 10 min |

HLFABPSO:

| | | |
|---|---|---|
| Step 1: | 50°C | 30 min |
| Step 2: | 95°C | 15 min |
| Cycles 1-45: | 94°C | 1 min |
| | 50°C | 1 min |
| | 72°C | 1 min |
| Final Step: | 72°C | 10 min |

LightCycler program 57:

| | | | |
|---|---|---|---|
| Cycle 1: | 95°C | 10 min | 20°C/s |
| Cycles 2-46: | 95°C | 0 sec | 20°C/s |
| | 57°C | 5 min | 20°C/s |
| | 72°C | 20 min | 20°C/s |
| Cycle 47: | 95°C | 0 min | 20°C/s |
| | 65°C | 15 min | 20°C/s |
| | 95°C | 0 min | 0.1°C/s |

### 5.5.3. Receptors and Vectors

Xenopus laevis peroxisome proliferator activated receptor alpha (xPPARα) in pSPUTK (for EMSA) and pSG5 (for transfections), respectively (kind gifts from W. Wahli). Chicken thyroid hormone receptor alpha (cTRα) in pSG5. Human retinoid x receptor alpha (hRXRα) and Human retinoid x receptor beta (hRXRβ), both in pCMV6-XL4 (kind gifts from J. Köhrle).

## 6. References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## 9. Declaration

Ich versichere ausdrücklich, dass ich diese Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.

## 10. Abbreviations

| | |
|---|---|
| ANT2 | adenine nucleotide translocase 2 |
| c.. | chicken.. |
| CoA | co-activator |
| CoR | co-repressor |
| CRE | cAMP response element |
| CREB | cAMP response element binding protein |
| DM2 | diabetes mellitus type 2 |
| EMSA | electrophoretic mobility shift assay |
| FA | fatty acid |
| FABP | fatty acid binding protein |
| FCS | foetal calf serum |
| FFA | free fatty acid |
| h.. | human.. |
| HAT | histone acetyltransferase |
| HDAC | histone deacetylase |
| HNF1α | hepatocyte nuclear factor 1α |
| LCFA | long chain fatty acid |
| LFABP | liver FABP |
| MODY3 | maturity onset diabetes of the young type 3 |
| NR | nuclear receptor |
| NRE | negative response element |
| NRF1 | nuclear respiratory factor 1 |
| PCR | polymerase chain reaction |
| PGC-1 | PPARγ co-activator 1 |
| pGL3-1623hLFABP | positions –1623 to +132 of hLFABP promoter in pGL3 basic reporter gene vector |
| pGL3-615rLFABP | positions –615 to –155 of rLFABP promoter in pGL3 basic reporter gene vector |
| PPAR | peroxisome proliferator activated receptor |
| PPRE | peroxisome proliferator response element |
| PUFA | polyunsaturated fatty acid |
| RXR | retinoid x receptor |
| T3 | triiodothyronine (active thyroid hormone) |
| T4 | thyroxine |
| TR | thyroid hormone receptor |
| TRE | thyroid hormone response element |
| TRH | thyrotropin releasing hormone |
| TSH | thyrotropin |
| x.. | Xenopus laevis.. |