Regulation of gene expression by thyroid hormone and thyroid hormone dependent cofactors

Analyses in selected human and rat (*Rattus norvegicus,* Berkenhout 1769) cell lines

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Regulation of gene expression by thyroid hormone and thyroid hormone dependent cofactors

Analyses in selected human and rat (*Rattus norvegicus,* Berkenhout 1769) cell lines

Doctoral Thesis

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by

Anne Wulf

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Sehr geehrte Damen und Herren,

hiermit bestätige ich, dass die von Frau Anne Wulf mit dem Titel "Regulation of gene expression by thyroid hormone and thyroid hormone dependent cofactors - analyses in human and rat (Rattus norvegicus) cell lines" vorgelegte Doktorarbeit in korrektem Englisch geschrieben ist.

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1 Introduction

1.1 Physiological basis of thyroid hormone action

Thyroid hormones produced by the thyroid gland influence important biological functions such as growth, development, and metabolism and play a role in virtually all vertebrate tissues. They control diverse metabolic activities, leading to an increase in basal oxygen consumption. One consequence of this activity is the rise of body heat production. Thyroid hormones stimulate almost all aspects of carbohydrate metabolism, including enhancement of insulin-dependent entry of glucose into cells and increased gluconeogenesis and glycogenolysis to generate free glucose. Increased thyroid hormone levels further affect both fat synthesis (lipogenesis) and lipolysis. Thyroid hormones are also associated with cell proliferation and apoptosis (reviewed in Yen, 2001).

Hormone synthesis and secretion in the thyroid gland is highly regulated by a negative feedback system that further involves the hypothalamus and the pituitary. There are two principal thyroid hormones produced by the thyroid gland, thyroxine (commonly known as T4) and triiodothyronine (T3). The majority of hormone secreted by the thyroid gland is T4, but the primarily active form is T3. T4, which circulates in the blood, is converted into T3 by three types of deiodinases. Since this conversion is located intracellularly, iodothyronine is transported across the cell membrane via at least three specific transporters (Friesema et al., 2005).

T3 is particularly important in the early human development, as lack of it leads to severe mental retardation and impairment of growth, a disease called cretinism (reviewed in Oppenheimer et al., 1997; Koibuchi et al., 2000). Developing countries still fight cretinism as endemic health problem when environmental iodine is low. In adulthood, T3 is crucial in keeping the metabolic balance in the body. Disorders of the T3 balance rank among the most common endocrine human diseases.

In wildlife, T3 is especially important for amphibian metamorphosis, which is characterised by a non-functional thyroid gland in embryos and a converted, functional thyroid gland during larval development (Berry et al., 1998). Endocrine disruptors -chemicals that mimic or interfere with endogenous hormones- have therefore become an area of increasingly ecological concern, since the most pronounced impact has been reported for the thyroid hormone system. It has been shown that endocrine disruptors cause potential reproductive and developmental defects (Opitz et al., 2006).

Thus, the study of thyroid hormone action has important medical and biological implications.

1.2 Molecular basis of thyroid hormone action

T3 exerts its function by non-genomic and genomic effects. The non-genomic effects have a smaller magnitude and are mainly related to alterations of intracellular Ca^{2+} concentrations or activities of particular kinases (reviewed in Zhang & Lazar, 2000). Non-genomic effects are not impaired by RNA inhibition and occur within minutes.

In comparison, T3-mediated modulation on the transcription level has greater impacts and is typically detectable after several hours. Genomic effects are induced by binding of T3 to high-affinity thyroid hormone receptors (TRs). TRs belong to the large superfamily of nuclear receptors that also includes the receptors for steroid, vitamin D, retinoic acid as well as "orphan" receptors, whose ligands if any have not been identified (Mangelsdorf et al., 1995).

All members of the family share a similar modular structure, containing six regions of domains designated A through F (Fig. 1). The variable N-terminal domain (A/B), which in some cases encodes an activation function-1 (AF-1), is followed by a DNA binding domain (DBD) (C). This highly conserved domain folds into two zinc finger motifs. The ligand binding domain (LBD) includes the linker region (D) together with the C-terminal part of the receptor (E/F). A ligand-dependent transcriptional activation domain, denoted as activation function-2 (AF-2), is located in the F region. Besides ligand binding, the LBD of nuclear receptors is required for homo- and heterodimerization, nuclear localization and interaction with cofactors.

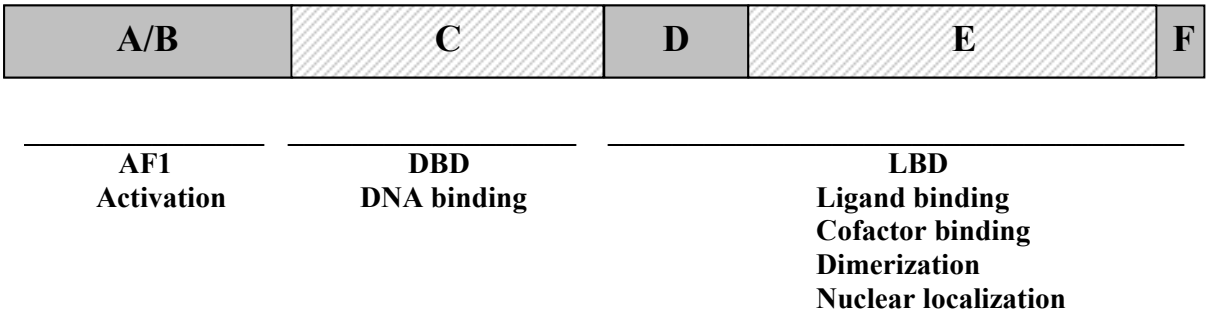


Fig. 1. General organization of major TR domains

Nuclear receptors like TR are composed of several functional domains A-F.

TRs are encoded by two distinct but closely related genes TRα and TRβ. In humans, alternative 5' exon splicing and separate promoters generate at least four different isoforms (TRα1, α2, β1 and β2), which are known to be expressed at the protein level (Lazar, 1993; Yen, 2001). These TRs are highly similar within their DBDs, hinge regions and LBDs, but have unique N-terminal regions. Overall, they share a 90% similarity at the amino acid level. TRs are expressed in specific temporal and tissue-specific manners. The onset of TRα1 expression starts soon in embryonic development and lasts through adulthood. TRα1 is ubiquitously expressed in tissues and cell types (Langlois et al., 1997). In comparison, TRβ expression appears later in development and parallels the occurrence of circulatory T3 and T4. Whereas isoform TRβ1 is found in different tissues, TRβ2 is expressed specifically only in the adult pituitary and hypothalamus (Hodin et al., 1989; Sakurei et al., 1989). Different phenotypes are displayed by knockout mice deficient in either TRα or TRβ, suggesting distinct functional roles for these isoforms (Flamant & Samarut, 2003).
Thyroid receptors mediate both positive and negative regulation of gene expression, depending upon the target gene promoter.

1.3 T3-mediated positive gene regulation

In case of positively regulated genes, the mechanism of transcriptional control is relatively well understood. These genes contain positive thyroid hormone response elements (pTREs), where TR binds preferentially as heterodimer together with the retinoid X receptor (RXR) (Miyamoto et al., 1994). The typical pTRE is referred to as DR+4, a direct repeat of the consensus sequence "AGGTCA", separated by a space of four random nucleotides (Brent et al., 1989). However, the P-box, a sequence within the first zinc finger responsible for TRE recognition, identifies the two half-sites also in other configurational arrangements, for example as palindromes or inverted repeats (Umesono & Evans, 1989; Ribeiro et al., 1994).

Binding of TR to DNA in the absence of T3 leads to transcriptional repression. Unliganded TR recruits corepressors like nuclear receptor corepressor (NCoR) and silencing mediator for retinoid and thyroid receptors (SMRT) (reviewed in Hu & Lazar, 2000). Both corepressors (CoRs) share an analog composition, containing C-terminal nuclear receptor interacting domains and a number of N-terminal repressing domains. Since knockout of NCoR is lethal, SMRT cannot compensate for a loss of NCoR, possibly due to a crucial role of NCoR in TR-mediated erythrocyte development (Jepsen et al., 2000). CoRs assemble in large complexes that include histone deacetylases and Sin3 among other proteins (Alland et al., 1997). The

recruitment of deacetylases leads to changes in histone acetylation and consequently induces chromatin compaction and transcriptional repression (Heinzel et al., 1997; McKenna & O'Malley, 2002).

Binding of T3 to TR changes the receptor conformation and the receptor dissociates from the CoR complex. In exchange, TR recruits an array of CoAs that include steroid coctivator 1 (SRC-1) and related factors (reviewed in Leo & Chen, 2000). A signature LXXLL motif within CoAs (where L stands for leucine, and X stands for any amino acid) was shown to mediate association with ligand-activated receptors (Heery et al. 1997). CoAs posses intrinsic histone acetyltransferase (HAT) activity and recruit additional HAT enzymes that relax the chromatin structure (Ito and Roeder, 2001; Rachez & Freedman, 2001). This chromatin remodelling is thought to allow efficient access of the basal transcription machinery to DNA, resulting in transcriptional activation.

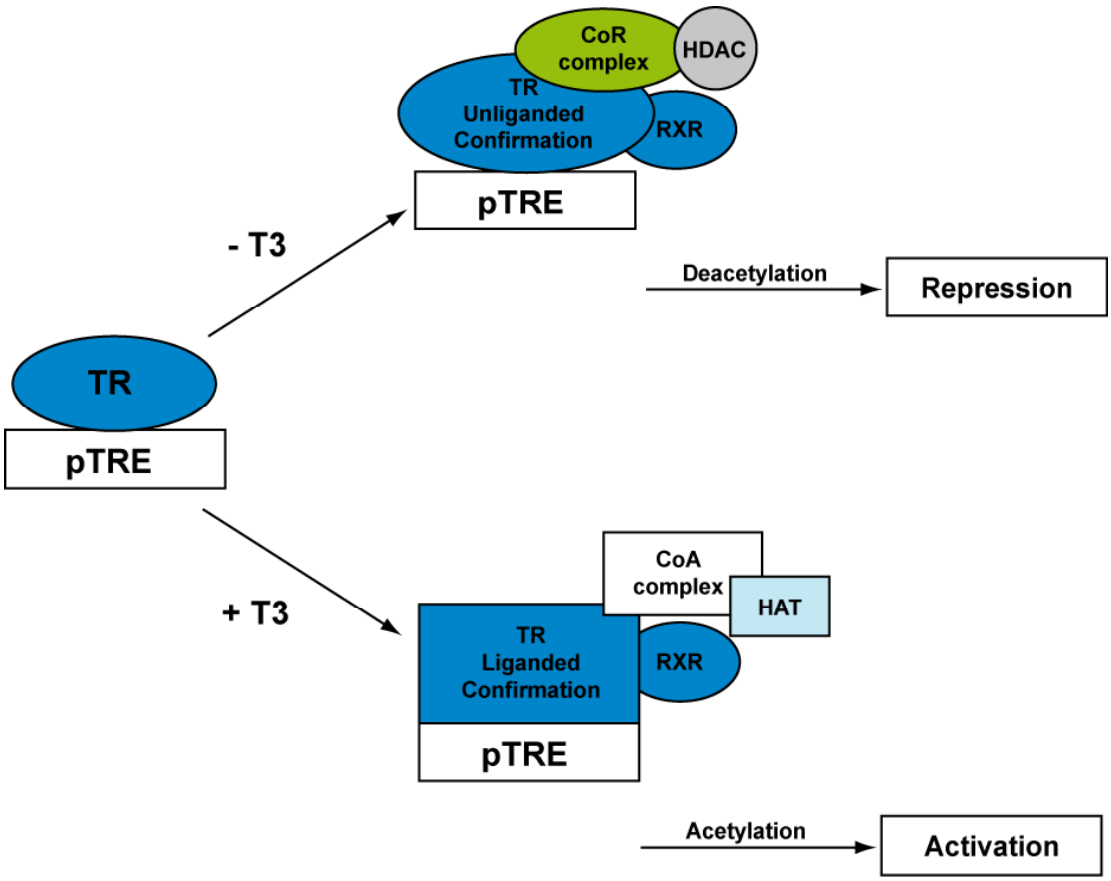


Fig. 2. Model for T3-dependent positive gene regulation

In the absence of T3, TR/RXR bind to a corepressor (CoR) complex that has histone deacetylase (HDAC) activity. In the presence of T3, TR/RXR recruit a coactivator (CoA) complex that has histone acetylase (HAT) activity. Chromatin modfication by HDAC or HAT enzymatic activity results in a closed or open chromatin structure that leads to transcriptional repression or activation.

The LBD of TR serves as the primary site of receptor interaction with both CoRs and CoAs. Coactivator recruitment depends primarily on a highly conserved motif, referred to as AF-2, in the extreme C-terminus of the LBD (Jeyakumar et al., 1997). AF-2 is located in helix 12 and creates a surface together with residues in helices 3, 5, and 6, which interacts with CoAs upon ligand binding. In the absence of ligand, the terminal helix 12 is thought to be reoriented, preventing the binding of CoAs whereas this reorientation favours interaction with CoRs (Marimuthu et al., 2002; Nagy & Schwabe, 2004).

1.3.1 Different T3-mediated gene expression patterns

The positive regulation of promoters that contain pTREs has been relatively well studied and has been described for a number of genes (Petty et al., 1990; Sap et al., 1990). However, an increasing amount of genes were identified as T3 target genes that apparently lack TRE sequences. Although it is possible that these genes could contain pTREs in so far unexplored or atypical regions, this possibility remains rather unlikely given the number of investigated genes. Interestingly, the T3-mediated expression pattern of genes lacking pTREs varies noticeably in its time course. In hypothyroid rats, the mRNA levels of numerous target genes rise within 6 h upon T3 administration ("early expression") and a number of pTREs have been characterized within these "early" regulated target genes like mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) or Spot14 (Weitzel et al., 2001a; Zilz et al., 1990).
In contrast, mRNA synthesis of a second subset of genes stays rather unaffected within the first 12-24 h, but shows marked up-regulation after that lag period ("late expression"). Of note, the identification of pTRE sequences in "late" expressed genes has failed until now (Weitzel et al., 2003). Noteworthy, these two different T3-mediated regulation patterns have also been identified during *Xenopus* metamorphosis and in a rat pituitary cell line, indicating a conserved alternative principle of T3 action (Denver et al., 1997; Miller et al., 2001).
Consequently, several lines of evidence indicate that there is a possible second mechanism for positive regulation besides the direct binding of TR to pTREs. The data suggest that "early" regulation (<6 h) is mediated via TREs whereas "late" expression (>12 h) occurs via an intermediate factor. The time needed for the synthesis of this intermediate factor could account for the observed lag period that precedes the up-regulation of "late" induced genes. Therefore, a candidate intermediate factor should be endogenously regulated by T3 probably via TRE, and this regulation should occur "early" within 6 h upon T3 administration.
On one side, the indirect mechanism of T3 action could be exerted by other transcription factors. In several T3 target genes, which are late induced in the presence of T3, binding sites

for factors like SP1, NRF-1 or NRF-2 have been detected. Furthermore, these recognition sites have been shown to be critical for promoter activities (Zaid et al., 1999; Virbasius & Scarpulla, 1994*)*. While the ubiquitously expressed factor SP1 itself is not regulated by T3, expression of NRF-1 and NRF-2 in response to T3 has been proven (Weitzel et al., 2001b; Rodríguez-Peña et al., 2002). However, only NRF-1 shows T3-mediated regulation within 6 h as requested for an intermediate factor. NRF-1 binding sites were characterized in late-induced genes like cytochrome c and mitochondrial transcription factor Tfam (Evans & Scarpulla, 1989; Virbasius & Scarpulla, 1994). Knockout experiments of NRF-1 in mice resulted in a major reduction of mitochondria with correlated embryonic lethality (Huo & Scarpulla, 2001). Consistently, T3 strongly influences mitochondrial biogenesis and activity, further indicating a possible involvement of NRF-1 in T3-mediated gene regulation.

However, a drawback in the model, which favours NRF-1 or other transcription factors as intermediate proteins, is the fact that a binding site common for all "late" induced genes could not be identified up to now.

Therefore, proteins that influence transcriptional activity without directly binding to DNA seem to be more likely candidates. Cofactors interact with transcription factors, providing a platform for the recruitment of regulatory protein complexes that exert powerful effects on gene transcription. Since they do not interact directly with target promoters, a common recognition motif is missing.

Members of the PPARγ coactivator 1 family are highly regulated in hypothyroid rats in response to T3. mRNA levels of PGC-1α and PERC/PGC-1β were up-regulated 13-fold and 5-fold respectively within the first 6 h after administration of T3 (Weitzel et al., 2003). Noteworthy, PGC-1α has been shown to be a potent coactivator of many nuclear receptors like TR and NRF-1 (Zhang et al., 2004; Wu et al., 1999). Consequently, PGC-1α is a possible candidate for mediating the "late" induction of T3 target genes.

Furthermore, the impact of PGC-1α expression resembles several T3 effects. First, overexpression of PGC-1α induces mitochondrial biogenesis and functions (Wu et al., 1999; Lehman et al., 2000). Second, PGC-1α is a key modulator of hepatic gluconeogenesis (Herzig et al., 2001). Finally, the cofactor plays an important role in linking nuclear receptors to the transcriptional program of adaptive thermogenesis (Puigserver et al., 1998; Tiraby et al., 2003). Taken together, the characteristics of PGC-1α suggest that the cofactor is involved in the molecular action of thyroid hormones. However, so far it is unknown, how PGC-1α excerts this function and how the cofactor itself is regulated in response to T3.

1.4 T3-mediated negative gene regulation

An intriguing aspect of the action of thyroid hormones is that within the same cell, some target genes are stimulated, whereas others are repressed. Negative regulation of gene expression is an essential part of the physiological action of thyroid hormones. A significant percentage of T3-regulated genes in liver were shown to be suppressed (Feng et al., 2000). Negative regulation is crucial in the hypothalamus-pituitary-thyroid axis (HPT axis). Hypothalamic thyrotropin-releasing hormone (TRH) and thyroid stimulating hormone gene (TSH) as part of the HPT axis are suppressed via feedback-mechanism, thereby controlling thyroid hormone homeostasis.

A clinically important feature associated with T3-mediated gene suppression has been described for certain patients with acute or chronic sickness. During disease, an extensive distortion in peripheral and central thyroid hormone metabolism takes place, which is described as “nonthyroid illness”. Among other things, the syndrome is characterized by decreased serum T3 and low TSH levels. In this case, TSH does not increase upon low T3 levels as part of the negative feedback regulation, but the mechanism involved in the down-regulation of TSH is not clarified (Boelen et al., 2006).

Another interesting aspect of T3-mediated negative gene regulation was observed in proliferating cell lines of diverse origin. In these cell lines, genes are negatively regulated, which are normally activated under T3 stimulation in liver (Ota & Mariash, 2003; own results). This paradoxical regulation is caused by an interaction between T3 and TR, but the factors involved are not identified up to now.

Contrary to positive regulation, transcriptional control of negatively regulated genes is poorly understood. Little is known about the underlying mechanism of the regulation seen *in vivo* and *in vitro*. Three major potential models have been proposed to explain T3-dependent gene suppression (reviewed in Lazar, 2003) (Fig. 2).

In a first concept, T3-mediated repression involves the direct binding of TR to so-called negative TREs (nTRE), which have been suggested in the promoters, first exons or 3’-untranslated regions of target genes (Villa et al., 2004; Perez-Juste et al., 2000; Zhang et al., 1992) (Fig. 2A). Of note, negative regulatory elements have been reported for the TRH and TSH promoters (Satoh et al., 1996; Breen et al., 1997). However, a consensus sequence for nTRE has not been determined. Direct binding of TR to nTREs must involve a reversal of the ligand-dependent switch between CoR and CoA recruitment. Due to assumed allosterical alteration at the nTRE site, TR now recruits CoAs in the absence of T3, whereas CoR binding

is favoured in the presence of the ligand. Studies with non-TRE binding TR mutants support the requirement of TR/DNA interaction for negative regulation (Shibusawa et al., 2003a). This model therefore suggest a mechanism which corresponds to binding of TR to pTREs, but with opposite readout.

In a second concept, T3-dependent negative regulation involves recruitment of TR to the promoter without direct DNA binding but via protein-protein interaction (Fig. 2B).

In this model, binding to another protein rather than to DNA might allosterically alter the conformation of TR, leading to the reversal of CoR/CoA recruitment and subsequently T3-mediated gene suppression. Alternatively, TR might block the activity of the interacting protein in a ligand-dependent way. The components of AP-1 complexes have been shown to interact with TR in response to T3 (Pfahl, 1993). Furthermore, it has been demonstrated that interaction of TR with CREB, GHF-1/Pit-1 or TBP induced T3-dependent negative regulation possibly without direct TR-DNA binding (Méndez-Pertuz et al., 2003; Sanchez-Pacheco et al., 1995; Kim et al., 2005).

In a third concept, TR and other transcription factors compete for common cofactors. This model involves a DNA binding independent but rather “squelching” mechanism (Fig. 2C). It has been proposed to explain negative regulation of genes, for which nTREs have not been identified. In the absence of T3, unliganded TR in solution sequesters CoRs from other transcription factors bound at the target promoter, consequently withdrawing histone deacetylases (HDACs). An increase in histone acetylation and recruitment of CoAs to the promoter causes gene activation. Upon binding of T3, TR recruits coactivators, while dissociated CoRs gain access to the promoter, leading to gene repression (Tagami et al., 1999).

Different studies have supported several aspects of the fundamental different models. However, the exact ligand-dependent negative regulation mechanism remains to be elucidated.

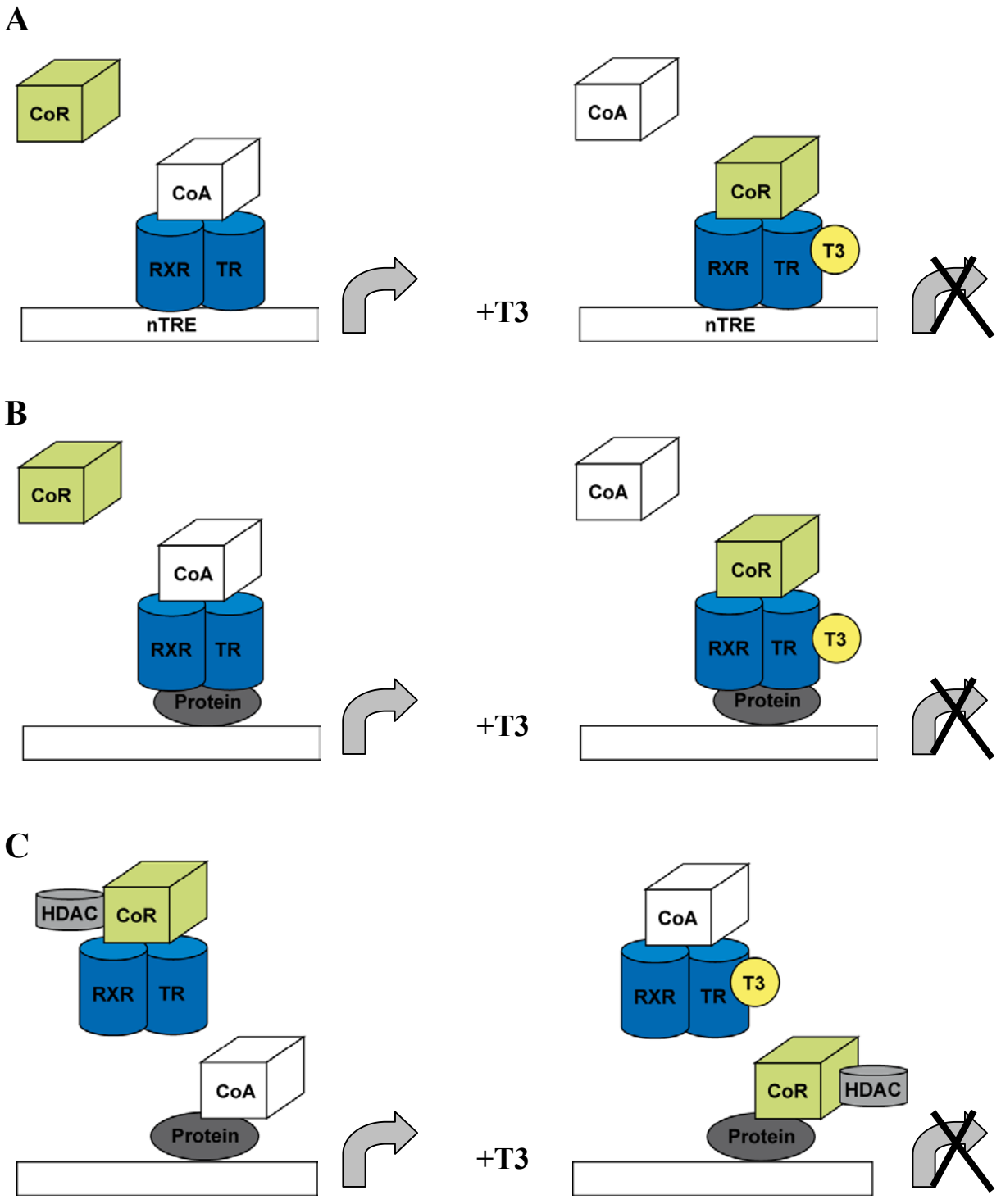


Fig. 3. Potential mechanisms for T3-dependent negative gene regulation

(A) TR binds to nTRE, which triggers a switch from coactivator to corepressor binding in response to T3. **(B)** Negative regulation involves recruitment of TR to the promoter without direct DNA binding but via protein-protein interaction. **(C)** TR and other transcription factors compete for common cofactors.

2 Background and Objective

Over the last years, we have greatly gained insights in the molecular mechanisms of thyroid hormone action in normal and disease states. The development of transgenic and knockout mouse models has shed light on the roles of TRs in the regulation of specific target genes and general development. The recent discovery of thyroid transporters has finally clarified how thyroid hormone is transported across the cell membrane.

T3 regulates the expression of a wide array of target genes and plays a critical role in several metabolic pathways. The mechanism of this transcriptional control is relatively well described for genes containing positive thyroid hormone receptor response elements (pTREs). However, several aspects of T3 action have been undetermined until now. The regulation of genes lacking TREs and which generally show a delayed induction kinetic in reponse to T3 has remained unclear. Several lines of evidence indicate that "late" expression (>12 h) in response to T3 occurs via an intermediate factor. Cofactor PGC-1α is involved in the control of different metabolic pathways, thereby ressembling the effects of T3 regulation. It is therefore a candidate protein for an intermediate factor of T3 action. However, up to now the molecular regulation of the PGC-1α promoter by T3 is unclear.

Besides positive regulation, molecular action of T3 involves suppression of gene expression.

In contrast to positive gene regulation, the mechanism through which TRs control the transcription of negatively regulated genes is less well understood and object of controversial discussion.

The aim of this thesis work was to investigate how thyroid hormones regulate gene expression besides the classical pathway via a pTRE in the target gene promoter.

For the investigation of "late" gene expression kinetics, a cell culture model was supposed to be established that mimics the observed "early" and "late" induced regulation patterns *in vivo*. In particular, it was tried to determine if these gene expression patterns are maintained independently of PGC-1α inactivation. A knockdown strategy via siRNA was chosen to investigate gene expression in the absence of PGC-1α. Furthermore, promoter analysis of PGC-1α should clarify how this cofactor is regulated in response to T3. Given the conflicting models for T3-mediated negative gene regulation, a second aim of this work was to analyze gene repression by using different luciferase assay systems. Particularly, the experiments should address the question of whether DNA binding of TR is dispensable for T3-mediated gene suppression.

3 Material and Methods

3.1 Material

3.1.1 Lab supplies

Consumable lab supplies were obtained from Eppendorf (Hamburg) and Greiner (Frickenhausen).

3.1.2 Chemicals and Enzymes

All chemicals and reagents used throughout this work were of analytical grade or of maximal purity available and were obtained (if not noted otherwise) from Merck (Darmstadt), Sigma (Deisenhofen), Serva (Heidelberg), BD (Franklin Lakes, NJ USA), Invitrogen (Carlsbad, CA USA), Biomol (Hamburg), GE Healthcare (Little Chalfont, UK), Roth (Karlsruhe) and BioRad (Munich). Enzymes were obtained from Roche Applied Biosciences (Mannheim), Sigma-Aldrich (St. Louis MO, USA), MBI Fermentas (St. Leon-Roth) and Invitrogen (Carlsbad, CA USA). Restriction enzymes and DNA-modifying enzymes were purchased from Roche Applied Biosciences (Mannheim), New England Biolabs (Ipswich MA, USA) or MBI Fermentas (St-Leon-Roth).

3.1.3 Media, solutions and buffers

Millipore distilled 2-fold deionised water was used for preparing media and buffers. Media were sterilized by autoclaving. Heat labile components were filtrated (0.22 μm pore size) (Schleicher and Schuell, Dassel).

Table 1. Media

Substance	Composition
LB media	0,5% (w/v) Yeast, 1% (w/v) Tryptone, 1% (w/v) NaCl, pH 7.2
LB agar	LB media, 1.5% (w/v) Agar
Cell culture media	Dulbecco's modified Eagle's medium D-MEM plus Glutamax, supplemented with 10% foetal calf serum and penicillin (50 U/ml)/ streptomycin (50 μg/ml) (all Gibco-Invitrogen, Carlsbad CA, USA)

Table 2. Solutions and buffers

Substance	Composition
10x TAE	0.4 M TRIS, 1 mM Acetic Acid, 0.5 M EDTA, pH 8.0 (using NaOH)
10x TE	100 mM TRIS, 10 mM EDTA
10x TBE	890 mM TRIS, 890 mM Boric Acid, 20 mM EDTA
10x PBS	137 mM NaCl, 27 mM KCl, 43 mM Na_2HPO_4*7 H_2O, 14 mM KH_2PO_4
10x HBS	8.18% (w/v) NaCl, 0.2% Na_2HPO_4, 5.94% (w/v) HEPES, pH 7.2
10x TBS	500 mM TRIS, 1.38 NaCl, 27 mM KCl, pH 8.0
1x TBST	1x TBS, 0.05% TWEEN 20
20x SSC	0.3 M Na_3Citrat, 3 M NaCl, pH 7.0
20x SSPE	0.3 M Na_3Citrat, 0.2 M NaH_3PO_4*H_2O, 20 mM EDTA, pH 7.4
100 mM KPO_4	77 mM K_2HPO_4, 23 mM KH_2PO_4, pH 7.8
$CaCl_2$ solution	solved to a concentration of 2 mM, sterile filtered, aliquoted and stored at -20 °C
DEPC-treated H_2O	H_2O + 0.1% Diethylpyrocarbonate
Cell lysis solution	Galacto-Light Plus Lysis Solution (Applied Biosystems, Foster City CA, USA), 0.5 mM DTT
Luciferase substrate buffer	25 mM Glycyl-Glycin, 15 mM $MgSO_4$*7x H_2O, 4 mM EGTA, 1 mM DTT, 15 mM KPO_4, 2 mM ATP, pH 7.8, 2 mM ATP (add before use)
Luciferin	Luciferin (Roche Applied Biosciences, Mannheim) was solved to a concentration of 100 mM in H_2O, aliquoted and stored at -80°C.
Luciferin solution	25 mM Glycyl-Glycin, 10 mM DTT, 0.2 mM Luciferin
RF1	100 mM RbCl, 50 mM $MnCl_2$*4x H_2O, 30 mM CH_3COOK, 10 mM CaCl*2x H_2O, 15% Glycerin, pH 5.8
RF2	10 mM MOPS (pH 6,8), 10 mM RbCl, 75 mM $CaCl_2$*2x H_2O, 15% Glycerin, pH 6.8
Buffer A	10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA (pH 8.0), 10 mM EGTA (pH 8.0), 0.1 mM DTT, 0.05 mM AEBSF

Continuation Table 2. Solutions and buffers

Substance	Composition
Buffer B	20 mM HEPES (pH 7.9), 0.1 mM NaCl, 0.1 mM EDTA (pH 8.0), 10 mM EGTA (pH 8.0), 0.1 mM DTT, 0.1 mM AEBSF
Protein lysis buffer	50 mM HEPES (pH 7.9), 10% (v/v) Glycerin, 150 mM NaCl, 10 mM Na_2HPO4, 1% (v/v) Triton X-100, 2 mM EDTA (pH 7.5), 1 mM AEBSF
2x SDS sample buffer	2% (v/v) SDS, 62.5 mM TRIS-HCl, 10% (v/v) Glycerin, 2% Mercapto-ethanol, 0.01% (w/v) Bromphenole blue, pH 6.8
Resolving gel buffer	125 mM TRIS-HCl, 0.1% SDS, pH 6.8
Stacking gel buffer	375 mM TRIS-HCl, 0.1% SDS, pH 8.8
PAGE buffer	25 mM TRIS, 192 mM Glycin, 0.1% (v/v) SDS
Transfer buffer	195 mM Glycine, 25 mM TRIS, 0,01% SDS, 20% Methanol
Björrum-Schäfer-Nielsen buffer	48 mM TRIS, 39 mM Glycin, 20% (v/v) Methanol
Coomassie	0.1% (w/v) Coomassie blue R-250, 16% (v/v) Acetic acid
Ponceau	0.5% (w/v), Ponceau S, 1% (v/v) Acetic acid
Blocking buffer I	1x TBS, 5% (w/v) Milk powder (Roth, Karlsruhe)
Blocking buffer II	1x TBS, 2.5% (w/v) Milk powder (Roth, Karlsruhe)
10x Nick buffer	500 mM TRIS-HCl (pH 7.5), 100 mM $MgCl_2$, 1 mM DTT, 500 µg/ml BSA
10x Band-Shift-Buffer	100 mM TRIS-HCl (pH 7.5), 500 mM NaCl, 1 mM EDTA, 10 mM DTT, 5 mM $MgCl_2$, 1 mg/ml BSA, 50% (v/v) Glycerin
6x DNA/RNA loading buffer	50% (v/v) Glycerin, 1 mM EDTA, 0.4% (w/v) Bromphenol blue, 0.4% (w/v) Xylene cyanol
10x RNA gel running buffer	200 mM MOPS, 50 mM NaAcetat, 5 mM EDTA, pH 7.0
Northern blot solution 1	65% (v/v), Formamide, 21% (v/v) Formaldehyde, 14% (v/v) 10x RNA gel running buffer

Continuation Table 2. Solutions and buffers

Substance	Composition
50x Denhardt's	1% (w/w) Ficoll, 1% (w/w) Polyvinylpyrrolidone, 1% (w/w) BSA
Prehybrydisation solution	50% (v/v) Formamide, 5x SSPE, 5x Denhardt's, 0.1% SDS, 200 mg tRNA Baker's Yeast
Ampicillin	Final concentration: 50 μg/ml
Kanamycin	Final concentration: 50 μg/ml

3.1.4 Cell lines

Table 3. Permanent cell lines used in this work

Name	Origin	Source
HepG2	Human hepatocyte carcinoma cells	ATCC: HB-8065
GH3	Rat pituitary tumour cells	ATCC: CCL-82.1
GC	Rat pituitary tumour cells	Gift of D Gourdji, Lyon, France

3.1.5 Plasmids and Oligonucleotides

Oligonucleotides used as primers for PCR, DNA sequencing and probe generation were purchased from MWG Biotech (Ebersberg). Oligonucleotides for siRNA were obtained from Qiagen (Hilden).

Table 4. Vectors used in this work

Vector	Relevant characteristics	Source
pGL3-basic	Promoterless, Firefly luciferase reporter gene	Promega, Mannheim
pRL-MA	Tk promoter, Renilla luciferase reporter gene	Promega, Mannheim
pSG5	Expression vector	Stratagene, La Jolla CA, USA
VP16-pAASV	Expression vector, VP16 activation domain	Gift of T Tagami, Kyoto, Japan
pcDNA3-GAL4 DBD	Expression Vector, GAL4 DNA binding domain	Gift of W Strätling, Hamburg
5xUAS-tk-luc	5 copies of the upstream activator sequence, Tk promoter, Firefly luciferase reporter gene	Gift of W Strätling, Hamburg
pBLCAT3	Promoterless, Chloramphenicol acetyl transferase reporter gene	Gift of A Martin-Requero, Madrid, Spain
pCMV6-XL4	Expression vector	Omnigene, Camebridge MA, USA
pCMX	Expression vector	Gao and Goldfarb, 1995

Table 5. Promoter constructs used in this work

Promoter	Vector	Source	Cloning primer (5'-3')
Rat mGPDH (-316/+109)	pGL3-basic	JM Weitzel	-
Rat mGPDH (-316/+109)	pRL-MA	this work	PF5_NdeI (ggaattccatatgcagctggcgagg) PF5_Spe1 (gactagtatcggtcgtcacacgcc)
hTSHα (802/+22)	pGL3-basic	this work	WL390 (tctaagccagttccttacgg) WL391 (cttatgagttctcagtaactgc)
hTSHα (802/+22)	pBLCAT3	this work	WL459 (attaaagctttctaagccagttccttacgg) WL458 (attatctagacttatgagttctcagtaactgc)
hPGC-1α (4kb) (-4483/+78)	pGL3-basic	Gift of J Rhee, Boston, USA	-
hPGC-1α (2kb) (-2533/+78)	pGL3-basic	Gift of J Rhee, Boston, USA	-
hPGC-1α (TRE) (-4025/-4010)	pRL-MA	this work	AW15_2 (ggaattccatatgtgcactttgcttagctactagtc) AW16_2 (gactagtagctaagcaaagtgcacatatggaattcc)
5xUAS-tk-luc	pGL3-basic	Gift of W Strätling, Hamburg	-
DR+4-tk-luc	pGL3-basic	Gift of PJ Hofmann, Berlin	-

Table 6. Vector constructs used in this work

Gene	Vector	Source	Cloning primer (5'-3')
cTRα (full size)	pSG5	JM Weitzel, Hamburg	-
cTRα (full size)	pcDNA3-GAL4 DBD	this work	WL344/3 (cgacgggtaccatggaacagaagcccagcac) WL345 (cgacgggatcccctacacctcctggtcctcg)
cTRα (C1) (aa 50-408)	pSG5	this work	WL455 (attagaattctgcgtggtgtgcggggacaa) AW4 (cgacgatcgatcctacacctcctggtcctcg)
cTRα (C1) (aa 50-408)	pcDNA3-GAL4DBD	this work	WL429 (attaggtacctgcgtggtgtgcggggacaa) WL431 (atcaggatccctacacctcctggtcctcga)
cTRα (C2) (aa 112-408)	pSG5	this work	WL456 (attagaattctgcatctccgtgggcatggc) AW4 (cgacgatcgatcctacacctcctggtcctcg)
cTRα (C2) (aa 112-408)	pcDNA3-GAL4 DBD	this work	WL430 (attaggtacctgcatctccgtgggcatggc) WL431 (atcaggatccctacacctcctggtcctcga)
VP16-cTRα (full size)	pAASV	this work	AW1 (cgacggaattcaccatggaacagaagc) AW4 (cgacgatcgatcctacacctcctggtcctcg)
hRXRγ (full size)	pCMV6-XL4	JM Weitzel, Hamburg	-
mNCoR (full size)	pCMX	Gift of I Bach, Hamburg	-
mNCoR-IDC (aa 2278-2444)	pcDNA3-GAL4 DBD	this work	WL501 (gattaggtaccagttttgatgataaagttgaag) WL502 (gattatctagatcagtcgtcactatcagac)
mNCoR-F1 (aa 1564-2444)	pcDNA3-GAL4 DBD	this work	WL451 (cgacgggtaccttggatccagctatgccc) WL502 (gattatctagatcagtcgtcactatcagac)
hSRC-1e (full size)	pSG5	Gift of M Parker, London, UK	-
hSRC-1 (LXXLL) (aa 493-1007)	pcDNA3-GAL4 DBD	this work	WL470 (attaggtaccgttacttctggattggcaac) WL471 (attatctagaggctagggagattggcagt)
hSRC-1 (Qrich) (aa 1050-1185)	pcDNA3-GAL4 DBD	this work	WL494 (attaggtaccgcacctaaccagcttcgacttc) WL495 (attatctagattagctggtagaagcaggtggggtt)

Table 7. Primer for real-time PCR

Target gene	Primer	Sequence
Adenine nucleotide translocator 2 (ANT2)	ANT2_for	gtgggcaaagctggagc
	ANT2_rev	cagtgacagactgtgcaatc
Acyl coenzyme A oxidase (AOX)	WL382	cagggaactcatcttcgagg
	WL383	gctgagccagaactattgcg
Chloramphenicol acetyl transferase (CAT)	WL461	acctataaccagaccgttcagc
	WL462	aatcgtcgtggtattcactcc
Cytochrome c (Cyt c)	WL342	ggcaagcataagactgga
	WL343	tatcctctccccaggtga
Glucose-6-phosphatase (G-6-Pase)	WL523	catctacaatgccagcctcc
	WL524	accatctcttggccttctcc
Stimulatory G protein alpha-subunit (GSα)	GSα_for	taccatctctgtgatcctcttc
	GSα_rev	gtgagggtagcagtagtgac
Mitochondrial glycerol-3-phosphatase dehydrogenase (mGPDH)	WL313	gatctcaggctgagcaatgg
	WL314	tgagcttctctggaaggagg
Nuclear respiratory factor-1 (NRF-1)	WL340	gcagctgatgaggtaactgc
	WL341	agaacaatggcttgctgtcc
Peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α)	WL200	cgcagagatatgagaagcg
	WL201	aagcgtcacaggtgtaacgg
Peroxisome proliferator-activated receptor γ coactivator-1β (PGC-1β)	WL329	cttcccccaagagcactg
	WL330	gcacagggcacacagag
Ribosomal protein S27	WL394	ccaggataaggaaggaattcctcctg
	WL395	ccagcaccacattcatcagaa
Mitochondrial transctiption factor A (Tfam)	WL220	tgaagttgggcgaagtgatc
	WL221	atgcattcagtgggcagaag

Table 8. Oligonucleotides for EMSA

Name	Oligonucleotide
PGC-TRE wt	ctgctgcactttgcttagctgtag
PGC-TRE 5'mut	ctgc*gaattc*ttgcttagctgtag
PGC-TRE 3'mut	ctgctgcactttgc*gaattc*gtag
TRE-mGPDH	accgtggccccgcctgacccggtgact

Table 9. siRNA oligonucleotides directed against PGC-1α

Name	mRNA target sequence
si1	aacatatcacaggagctccaa
si2	aattgaagagcgccgtgtgat
si3	ggtggattgaagtggtgtaga

3.1.6 Antibodies

Table 10. Antibodies used in this work

Antibody	Source
Rabbit polyclonal anti-TRα1 antibody	#H0204, Santa Cruz Biotechnology, Santa Cruz CA, USA
Rabbit polyclonal anti-mGPDH antibody directed against amino acids 42-206	JM Weitzel, Hamburg
Rabbit polyclonal anti-PGC-1α antibody directed against amino acids 777-797	#516557, Calbiochem/Merck, Darmstadt
Rabbit polyclonal anti-PGC-1α antibody directed against the N-terminus (~300 amino acids)	Gift of Natasha Kralli, La Jolla CA, USA
Peroxidase conjugated Goat anti-Rabbit antibody	#111-035-045, Dianova, Hamburg
Rabbit polyclonal anti-Acetyl Histone H3 antibody	#07-360, Upstate/Millipore, Billerica MA, USA

3.2 DNA methods

3.2.1 Plasmid isolation

Qiagen Plasmid Mini, Midi and Maxi Kits (Qiagen, Hilden) were used for small and large-scale plasmid preparations according to the manufacturer's protocol.

3.2.2 Polymerase chain reaction

For analytical PCR, Taq DNA polymerase (Amersham Pharmacia Biotech/GE Healthcare, Little Chalfont, UK) was used. For amplification of long DNA fragments as well as generation of DNA mutations, PfuTurbo DNA Polymerase was used (Stratagene, La Jolla CA, USA). The standard reaction mixture (50 µl) consisted of template, 0.5 µM of each primer, 200 µM dATP, dGTP, dCTP and dTTP and 2.5 U of polymerase. PCR was performed in the T3 Thermocycler (Biometra, Göttingen). An initial denaturation step for 5 min at 95 °C was followed by 25 to 35 cycles of denaturation (30 s at 95 °C), primer annealing (30 s at annealing temperature) and primer elongation at 72 °C. The annealing temperature was in general 2 °C lower than the lowest predicted melting temperature T_M of the primers. The calculation of T_M was based on the nucleotide composition of the primers: T_M (°C) = 4x(G + C) + 2x(A + T). Elongation time was determined according to the size of the amplified DNA fragment. Routinely, an elongation time of 60 s was applied for the amplification of 1000 bp. 10 µl of the PCR mixture were routinely analyzed by agarose gel electrophoresis. Amplified DNA fragments from PCR reactions were purified using the QIAquick spin PCR purification kit (Qiagen, Hilden). Alternatively, purification was achieved through agarose gel electrophoresis followed by gel extraction.

3.2.3 Restriction digest

Various restriction enzymes were used for digestion of DNA. Restriction was carried out as suggested by the suppliers Roche Applied Biosciences (Mannheim) or New England Biolabs (Ipswich MA, USA). Reactions were incubated for 1 h or overnight at 37 °C. Restriction digests were controlled through gel electrophoresis.

3.2.4 Agarose gel electrophoresis

Agarose gels of 0.8- 1.5% with 0.001% (v/v) ethidium bromide were used to resolve DNA fragments of 8 kb to 0.2 kb respectively. Gels were prepared and run in 0.5x TAE buffer.

6x loading buffer was added to DNA and electrophoresis was performed at 70 V. A 100 base pair ladder or DRIgest III (both GE Healthcare, Little Chalfont, UK) was used as DNA size standards. DNA was visualized by illumination with UV light (IL-305-M UV transilluminator, Bachofer, Reutlingen) and documented with a Polaroid CU-5 camera (Bachofer, Reutlingen).

3.2.5 Extraction of DNA from gel

After separation by agarose gel electrophoresis, DNA fragments were extracted by using the QIAquick Gel Extraction Kit (Qiagen, Hilden), following the manufacturer's protocol.

3.2.6 DNA concentration

To determine the amount and quality of preparation, the DNA was measured at 260 nm with the BioPhotometer (Eppendorf, Hamburg). Since the DNA was usually intended to be used for transfection of mammalian cells, the ratio A_{260}/A_{280} needed to be >1.8.

3.2.7 DNA cloning

To avoid re-ligation, vectors were dephosphorylated with calf intestinal phosphatase (CIP) (Roche Applied Biosciences, Mannheim), following the manufacturer's instructions. The digested and dephosphorylated vector was purified with the QIAquick Gel Extraction Kit or QIAquick spin PCR purification kit (both Qiagen, Hilden). A molar ratio of roughly 1:3 was used between linearised plasmid and insert-DNA. T4 DNA ligase with appropriate buffer (USB, Cleveland OH, USA) was added to a final volume of 10-20 µl. The ligation reaction was incubated overnight at 15 °C and 2- 4 µl of ligation mixture were used for transformation purposes.

3.2.8 Preparation of competent cells

Escherichia coli NovaBlueComp cells (Novagene/Merck, Darmstadt) were prepared from a culture grown in 50 ml LB medium at 37 °C. At OD_{600nm} of 0.6, the culture was stored on ice for 15 min and centrifuged at 1000 rpm and 4 °C for 15 min. The pellet was resuspended in 17 ml of ice cold buffer RF1 and incubated on ice for 15 min. After centrifugation at 1000 rpm and 4 °C for 15 min, the pellet was resuspended gently in 4 ml of ice cold buffer RF2. Cells were incubated on ice for 10 min and aliquots of 0.2 ml were transferred into cooled tubes and stored at -80 °C.

3.2.9 Transformation

Competent cells were thawed on ice, DNA was added, mixed gently, and incubated on ice for 30 min. Cells were then subjected to a heat shock at 42 °C for 90 s and immediately transferred back to ice for 2 min. 500 µl of LB media were added and the transformed cells were incubated at 37 °C and shaking at 180 rpm for 1 h. Cells were plated on LB plates with corresponding antibiotics and incubated overnight at 37 °C.

3.2.10 DNA mutagenesis

DNA mutagenesis was performed by using the Quick-site change directed Mutagenesis Kit (Stratagene, Hamburg) according to the manufacturer's protocol.

3.2.11 DNA sequence analysis

250 to 500 ng of DNA were added to 2 µl of BigDye reaction mix, 8 µl of HT buffer (Applied Biosystems, Foster City CA, USA) and 12.5 pmol of primer in a volume of 20 µl. PCR was performed in the T3 Thermocycler (Biometra, Göttingen). An initial cycle was run at 95 °C (1 min), 96 °C (30 s), 50 °C (15 s) and 60 °C (4 min). In cycle 2 to 35, the denaturation step at 96 °C for 30 s was followed by primer annealing at 50 °C for 15 s and elongation at 60 °C for 4 min. PCR products were precipitated with 80 µl of 0.3 M sodium acetate and 250 µl of ethanol (100%). Centrifugation was performed at 14 000 rpm and 4 °C for 30 min. The DNA pellet was washed in ethanol (75%), spin and air-dried. DNA sequence analysis was performed at the DNA sequencing facility at the Institute of Cellular Biochemistry and Clinical Neurobiology, Hamburg.

3.3 RNA methods

3.3.1 RNA extraction

Media was removed from tissue culture plates and cells were washed twice with 1x PBS, removing as much PBS as possible. RNA was isolated using Trizol (Invitrogen, Carlsbad CA, USA) according to the manufacturer's protocol. Following Trizol isolation, a DNAse step was performed to remove residual DNA from RNA samples. Therefore, 1 µl of RNAse-free DNAse (Roche Applied Biosciences, Mannheim), 10 µl of DNAse buffer and 1 µl of RNAsin Ribonuclease Inhibitor (Promega, Mannheim) were added to 5 µl of RNA sample and

adjusted to 22 µl with DEPC-treated H_2O. The sample was incubated at 37 °C for 20 min. DNAse was inactivated at 75 °C for 5 min. The overall quality of RNA preparation was assessed by electrophoresis on denaturing agarose gel. Concentration of RNA was determined spectrophotometrically at 260 nm. The ratio A_{260}/A_{280} was used to assess the purity of the sample. RNA samples were stored at -80 °C until use.

3.3.2 Generation of cDNA probes

RNA was reverse transcribed with SuperScript TM II RNase H-Reverse Transcriptase (Invitrogen, Carlsbad CA, USA). Briefly, 1 µl of oligo dT (0.4 mg/ml), 1 µl of oligo N6 (0.4 mg/ml) and 1 µl of Ribonuclease inhibitor RNAsin (Promega, Mannheim) were added to 1 µg of total RNA and adjusted to a volume of 19 µl with DEPC-treated H_2O. The sample was incubated at 70 °C for 10 min and placed promptly on ice. Subsequently, 5 µl of 5x buffer, 3 µl of 0.1 M DTT, 1.5 µl of dNTP mix (10 mM each) and 1 µl of Reverse Transcriptase (10 U/ml) were added. Reactions were incubated at 42 °C for 1 h followed by 10 min at 65 °C to stop the reaction.

3.3.3 Real-time PCR

Quantitative PCR was performed at the LightCycler system, Version 3.5 with the quantitative SYBR green PCR kit and minor modifications to the manufacturer's protocol (Roche Applied Biosciences, Mannheim). The 15 µl of reaction mixture volume contained 1.5 µl of Master SYBR Green solution, 3 mM $MgCl_2$, 1 µl of each primer (25 µM) and 1.5 µl of cDNA. A negative control without DNA was included in each run. Amplification conditions were as follows: one cycle at 95° C for 10 min, followed by 45 cycles of denaturing for 10 s at 95° C, annealing at 57-61 °C (depending on the primer pair) for 5 s and extension at 72 °C for 15 s. The accumulation of product was monitored by SYBR Green fluorescence at the completion of each cycle. After amplification, melting curve analysis of the PCR product was used to differentiate between specific and non-specific amplification products. Melting curves were obtained by heating the PCR product from 65° C to 95 °C and by cooling at 40 °C for 30 s under continuous fluorescence monitoring. Fluorescence curve analysis was accomplished with the LightCycler software. The crossing point for each reaction was determined using the Second Derivative Maximum algorithm. Expression of target mRNAs was normalised by adjusting to the mRNA expression of housekeeping genes GSα or S27. The 2-ddCT method (Livak et al., 2001) was used to analyse the relative changes of target gene expression.

3.3.4 Northern blot hybridisation

10 µg of RNA was dissolved in 10 µl of DEPC-treated H_2O and 30 µl of solution 1 and was denatured at 65 °C for 15 min. Electrophoresis was performed in a 1% agarose gel containing 0.37% formaldehyde, 1x RNA gel running buffer and 0.001% ethidium bromide. Electrophoresis was performed at 120 V and RNA was subsequently fractionated by exposition to UV light for 10 min (312 nm; IL-305-M UV Transilluminator, Bachofer, Reutlingen). Capillary transfer to a nylon membrane (BiodyneA Transfer membrane, Pall, East Hills NY, USA) was performed with 1x RNA gel running buffer overnight. RNA was immobilised by UV cross-linking with the UV Stratalinker at 254 nm/ 1200 mJ (Stratagene, La Jolla CA, USA). The membrane was incubated in pre-hybridsation solution for 3 h at 43 °C. Rediprime II random prime labelling system (Amersham Pharmacia Biotech/GE Healthcare, Little Chalfont, UK) was used for generation of radioactively labeled cDNA probes according to the manufacturer's protocol. Probes were purified using Nick columns containing Sephadex G-50 DNA Grade (Amersham Pharmacia Biotech/GE Healthcare, Little Chalfont, UK).

After addition of labeled cDNA probe, incubation continued overnight. The membrane was washed once with 1x SSC + 0.1% SDS for 15 min at 42 °C, three times with 1x SSC + 0.1% SDS for 15 min at 65 °C and three times with 0.1x SSC + 0.1% SDS for 15 min at 65 °C. The blot was exposed to Super RX autoradiographic film (Fujifilm, Japan) for the appropriate time period and the film was visualized using the TLC Scanner II (Camag, Muttenz, Switzerland).

3.3.5 Determination of mRNA half-life times

GC cells were incubated overnight under standard conditions in D-MEM supplemented with 10% foetal calf serum and penicillin (50 U/ml)/ streptomycin (50 µg/ml) (all Gibco-Invitrogen, Carlsbad CA, USA). Subsequently, media was changed to serum-depleted medium 48 h prior to addition of 5 µg/ml of actinomycin D (Sigma, Taufkirchen). RNA was harvested (3.3.1) at the time of addition (0 h) and at various following time points depending on the gene of interest. Expression of mRNA was further investigated via Northern blot analysis (3.3.4) or real-time PCR (3.3.3).

3.4 Protein methods

3.4.1 Protein concentration

Concentration of proteins was determined by using the Bradford Assay from BioRad (Munich). Protein dilutions were measured with the BioPhotometer (Eppendorf, Hamburg) at 700 nm. The spectrophotometer reading of the protein dilutions, in the range of the BSA standard, was used to calculate the protein concentration.

3.4.2 Extraction of nuclear proteins

GC cells were washed with ice cold PBS, harvested and diluted in 40 ml of PBS. Centrifugation was performed for 10 min at 2500 rpm and 4 °C. Pellets were resuspended in 1.5 ml of ice cold Buffer A and incubated for 15 min on ice with occasional shaking. 100 µl of Buffer A with 10% TritonX-100 were added drop by drop and the sample was incubated on ice for additional 5 min. Centrifugation was performed at 1500 rpm and 4 °C for 5 min. The pellet was washed in Buffer A and transferred to a new tube. 100 µl of Buffer B were added and samples were incubated on a shaker for 30 min at 4 °C. Samples were centrifuged at 14 000 rpm and 4 °C for 10 min. Supernatant was transferred and the concentration of proteins was determined by Bradford analysis.

3.4.3 In vitro translation

For in vitro translation, the TnT Coupled Reticulocyte Lysate System (Promega, Mannheim) was used. Reaction was performed using 25 µl of TnT rabbit reticolocyte lysate, 2 µl of TnT reaction buffer, 1 µl of TnT RNA polymerase (SPG or T7, depending on the promoter of the expression vector), 1 µl of amino acid mixture (1 nM each), 40 U of RNAsin ribonuclease inhibitor, 1 µl of transcend biotin-lysyl-tRNA and 1 µg of DNA template in a final volume of 50 µl. The translation reaction was incubated at 30 °C for 90 min.

3.4.4 SDS PAGE and Western blot

SDS-PAGE was carried out essentially as described by Laemmli (1970) using the Minigel equipment from Biometra (Göttingen). In general, 8% or 10% resolving gels and 3% stacking gels were used. Cells were harvested in protein lysis buffer at 4 °C for 30 min under continous shaking. Subsequently, protein concentration was determined. 50 µg of protein were mixed

with 6× SDS loading buffer and denatured at 95 °C for 5 min. The "BenchMark TM Pre-stained Protein Ladder" (Invitrogen, Carlsbad CA, USA) or the "PageRuler Prestained protein Ladder" (MBI Fermentas, St. Leon-Roth) was used as protein size standard. Electrophoresis was performed at 80 V for approximately 1 h and additionally at 100 V for 2 h at room temperature in PAGE buffer. The gel was blotted to a nitrocellulose membrane (Protan BA85, Schleicher & Schuell, Dassel), using either a tank blot (Mini-Transblot-System, BioRad, Munich) or a semi-dry system (Biometra, Göttingen).

For the tank blot system, all components of the transfer sandwich had been soaked before in Björrum-Schäfer-Nielsen buffer and were assembled in the following way starting from the cathode: 2 pieces of soaking pads, 1 piece of filter paper, gel, membrane, filter paper and soaking pad. Transfer was run in Björrum-Schäfer-Nielsen buffer at a constant current of 100 V at 4 °C for 60 min.

For the semi-dry system, the gel, nitrocellulose membrane and 6 pieces of filter paper were saturated in transfer buffer. The transfer sandwich was stacked starting with three pieces of filter paper on the cathode, followed by the gel, the nitrocellulose membrane and three additional pieces of filter paper. The proteins were electrotransfered from the gel on the membrane at room temperature at 0.8 mA/ cm^2 for 90 min.

Coomassie and Ponceau staining of gel and membrane respectively were routinely performed in order to check the successful protein transfer. Therefore, gel and membrane were washed once in demineralized water and the gel was stained in Coomassie solution for 20 min whereas the membrane was stained in Ponceau solution for 10 min.

The membrane was destained in 1x PBS, followed by an incubation in blocking buffer I (5% milk powder in TBS) at 4 °C overnight to saturate non-specific protein binding sites. The membrane was incubated with a 1:1000 fold dilution of primary antibody in blocking buffer II (2.5% milk powder in TBST) at room temperature for 1 h. To remove unspecifically bound antibody, the blot was then washed three times in TBST for 10 min and subsequently incubated with the secondary antibody (1:10 000 diluted in blocking buffer II) at room temperature for 1 h. After incubation, the membrane was washed the following way: twice shortly in TBS, three times in TBST for 10 min, twice in TBST for 15 min, twice in TBST for 30 min and once in TBS for 30 min. Blots were revealed by enhanced chemiluminescence (ECL; Amersham Pharmacia Biotech/GE Healthcare, Little Chalfont, UK) according to the manufacturer's protocol. The blot was exposed to Super RX autoradiographic film (Fujifilm, Japan) for the appropriate time period and the film was visualized using the Curix 60 film developer (AGFA-Gevaert AB, Leverkusen).

3.5 Bacteria and cell culture

3.5.1 Culture of bacteria

Plasmids containing genomic DNA fragments were maintained in *E. coli* NovaBlueComp cells (Novagene/Merck, Darmstadt). *E. coli* cultures were routinely cultured at 37 °C on LB agar or in LB broth containing appropriate antibiotics in a final concentration of 50 µg/ml. All strains were kept as glycerol stock cultures both at -80 °C for long-term storage or on agar plates at 4 °C for short-term storage.

3.5.2 Culture of permanent cell lines

HepG2 and GC cells were grown in Dulbecco's modified Eagle's medium D-MEM plus Glutamax supplemented with 10% foetal calf serum (FCS) and penicillin (50 U/ml)/ streptomycin (50 µg/ml) (all Gibco-Invitrogen, Carlsbad CA, USA). Cell lines were grown at 37 °C and 5% CO_2. Splitting of cells was performed at a confluence of 75-80% by washing twice with 1x PBS and incubation with 1 ml of Trypsin/EDTA (0.05%/ 0.02% (w/v) in PBS) (Biochrome, Berlin) for 5 min at 37 °C. Detached cells were diluted in 10 ml medium and pelleted by centrifugation (1000 rpm, 5 min, room temperature). Three million cells were usually seeded per 75 cm^2 culture flask (Greiner, Frickenhausen).

3.5.3 Cryoconservation of cells

For cryoconservation, cells were removed from flasks and pelleted in 10 ml of D-MEM (1000 rpm, 5 min, room temperature). Cells were resuspended in Cell culture freezing medium with DMSO (Gibco-Invitrogen, Carlsbad CA, USA) and stored in liquid nitrogen till usage. For defrosting, cells were incubated for 2-5 min at 37 °C and resuspended in 12 ml of D-MEM.

3.6 Transient transfection of permanent cell lines

3.6.1 Calcium-phosphate transfection

0.5x 10^6 cells per well were seeded in 6-well plates in D-MEM with 10 % FCS and incubated overnight. Subsequently, medium was aspirated and fresh medium was added. For one well, 2 µg of reporter gene construct and 0.4 µg of any other factor were transfected. DNA for 3 wells was mixed with 62.5 µl of $CaCl_2$ and the volume was brought up to 375 µl with H_2O. Using a pipette hooked up to a pipette aid, the DNA-$CaCl_2$ solution was bubbled and 375 µl

of 2x HBS were added in drops. 230 µl of the transfection aliquots were added to each well and the transfected cells were incubated overnight. Subsequently, cells were either harvested or stimulated with tri-iodothyronine (T3). In case of stimulation, media was changed to D-MEM containing 100 nM T3 (Sigma, Taufkirchen) and cells were incubated for additional 24 h.

3.6.2 Liposome-mediated transfection

For chromatin immunoprecipitation experiments, lipid-mediated transfection was performed with Metafectene according to the manufacturer's protocol (Biontex, Munich) with small changes. Briefly, 0.5x 10^6 HepG2 cells per well were seeded in 6-well plates in D-MEM with 10% FCS and incubated overnight. For one well, 1 µg of promoter construct and 0.1 µg of any other factor were transfected. The DNA and 4 µl of Metafectene were each separately diluted in 50 µl of D-MEM (free of serum and antibiotics) and incubated for 5 min at room temperature. The DNA/D-MEM solution was added to the Metafectene/D-MEM solution and incubated for 20 min at room temperature. The initial culture medium in each well was discarded and replaced by fresh medium. The transfection mix was then overlaid onto the cells. Transfected cells were used for T3 treatment 24 h post-transfection. Therefore, the medium was changed to serum-free D-MEM containing 100 nM T3 and the cells were further incubated for 24 h.

3.7 Luciferase activity assay

For Firefly luciferase detection, cells were rinsed once with ice cold 1x PBS and covered by adding 100 µl of cell lysis solution to each well. Cells were incubated for 15 min at 4 °C, scraped from each well into separate microcentrifuge tubes and placed on ice. After vortexing for 10 s, the tubes were spin in a microcentrifuge for 5 min at 13 000 rpm and 4 °C. 10 µl of supernatant were immediately added to 100 µl of luciferase substrate buffer. Luciferase activity was determined in a luminometer (Lumat LB9501, Berthold, Bad Wildbad). After injections of 100 µl of luciferin solution to the sample, detection was performed with 10 s integration time.

For Renilla luciferase detection, the Renilla Luciferase Assay System (Promega, Mannheim) was used. Cells were washed twice with ice cold 1x PBS and covered by adding 500 µl of 1x Renilla Luciferase Assay Lysis Buffer to each well. Cells were transferred to a microcentrifuge tube, votexed and centrifuged at 14 000 rpm and 4 °C for 3 min. Detection

was performed according to the manufacturer's protocol in the luminometer (Lumat LB9501, Berthold, Bad Wildbad).
Values were given as light units and represented total luciferase activity per 5.0 x 10^6 cells as mean of duplicates. Measured luciferase values were related to total protein concentration.

3.8 Transfection of siRNA oligonucleotides

To design siRNA, target sequences were identified from the open reading frame of the corresponding PGC-1α cDNA. Selection criterion was the sequence 5'-AA(N19)UU-3', where N is any nucleotide. Potential target sites had a G/C content of 40 to 60%. Candidate sequences were then evaluated against the GenBank database to disqualify any target sequence with significant homology to other genes. siRNA was obtained from Qiagen (Hilden, Germany) and was provided in HPP grade as duplex as 5'-(N19)TT for sense and 5'-(N19)TT for antisense orientation.
The experimental approach for siRNA induced silencing was basically performed as described (Elbashir et al., 2001). GC cells were plated at a density of 0.6x 10^6 per well (6-well plates) and incubated overnight in 2.5 ml of Dulbecco's modified Eagle's medium D-MEM plus Glutamax supplemented with 10% foetal calf serum and penicillin (50 U/ml)/ streptomycin (50 µg/ml) (all Gibco-Invitrogen, Carlsbad CA, USA). Subsequently, media was changed to serum-depleted medium 24 h prior to transfection. Three different reagents were used according to the manufacturer's protocol to determine the best method for siRNA transfection in GC cells: Trans IT-TKO (Mirus, Madison WI, USA), Lipofectamine 2000 and Oligofectamine (both Invitrogen, Carlsbad CA, USA). Since TKO had the best transfection efficiency, this agent was used for further studies.
For siRNA transfection, two premixes were prepared and had the following compositions: premix 1 contained 220 nM of double-stranded siRNA in 120 µl of serum-free D-MEM, premix 2 contained 6.6 µl of TKO in 120 µl of serum-free D-MEM. Given quantities were used for transfection of one well. Both vials were allowed to incubate for 5 min. The premixes were then combined and allowed to incubate at room temperature for additional 20 min. Control treatments were prepared identically with unspecific siRNA. Cell medium was aspirated and 1 ml of serum-free medium was added. 200 µl of the liposome complexes were added to each well and gently mixed by shaking the plate. Transfected cells were incubated for 4 h and subsequently 1 ml of serum-free medium was added. In case of T3 stimulation, T3 was added to a final concentration of 100 nM. After 15 h, medium was replaced by fresh

serum-free medium. To detect the knockdown of the specific mRNA, quantitative RT-PCR was performed with samples harvested at different time points.

3.9 Detection of PGC-1α by immunoprecipitation

To detect PGC-1α protein in siRNA treated cells, an immunoprecipitation assay was used.
50 µg of 50 % slurry protein A sepharose CL-4B (Amersham Biosciences/GE Healthcare, Little Chalfont, UK) was coupled to a rabbit anti-PGC-1α antibody, directed against a C-terminal peptide from amino acids 777 to 797 (#516557, Calbiochem/Merck, Darmstadt). The sepharose was washed three times with TTBS. 1 µg of nuclear protein extract was added and incubation was performed according to the manufacturer's protocol. The agarose pellets were washed three times with TTBS (1500 rpm, 10 s) and suspended finally in 30 µl of 2x SDS sample buffer. For analysis, samples were incubated at 95 °C for 5 min, excessive agarose was pelleted and the supernatant fraction was resolved by SDS-PAGE. PGC-1α was detected by subsequent Western blot analysis using a PGC-1α antibody directed against the N-terminal ~300 amino acids (a kind gift of Natasha Kralli, La Jolla CA, USA).

3.10 Electrophoretic mobility shift assay

Sense and antisense oligonucleotides (200 pmol each) were added to NaCl (final concentration 180 mM), placed in hot water at 95 °C and allowed to cool to room-temperature overnight. 5 pmol of annealed oligonucleotides were incubated with 1x Nick buffer, dGTP, dATP, dTTP (10 nmol each), 5 µl of [^{32}P] dCTP (50 µCi) and 5 U of Klenow fragment (MBI Fermentas, St. Leon-Roth). The sample was incubated at 37 °C for 20 min. Reaction was stopped by adding 2 µl of EDTA. After labeling, oligonucleotides were purified using Nick Columns containing Sephadex G-50 DNA Grade according to the manufacturer's protocol (Amersham Pharmacia Biotech/GE healthcare, Little Chalfont, UK). Oligonucleotides were eluted from the column with 500 µl of TE buffer (pH 8.0). To determine the activity of the DNA probe, 10 fmol were diluted in 2 ml of Ultima Gold LSC-cocktail (Sigma, Taufkirchen) and read for Cerenkov counts in a Wallac 1409 scintillation counter (Wallac/Perkin-Elmer, Wellesley MA, USA).
1 µl of *in vitro* translated TRα1 was incubated with 40 fmol of labeled oligonucleotide probe, the unlabeled probe as specific competitor (100x molar excess relative to the labeled probe), and poly(dA-dT) as non-specific competitor. Binding reactions were performed at room temperature in band shift buffer (at a final concentration of 1x) for 30 min. A non-denaturing

5% polyacrylamide gel was prepared and pre-run in 0.5x TBE at 200 V for 30 min (Electrophoresis System Model S1, BRL Life Technologies/Invitrogen, Carlsbad CA, USA; Desatronic 400/500 power supply, DESAGA, Heidelberg). Samples were loaded and the gel was run at 200 V for 2 h. After electrophoresis, the gel was transferred to a layer of Whatman paper (Whatman, Brentford, UK) and dried under vacuum and heat in the Slab Dryer (BioRad, Munich). The dried gel was exposed to an autoradiography film at -80 ºC (Super RX, Fujifilm, Japan) and the film was subsequently visualized using the Curix 60 film developer (AGFA-Gevaert AB, Leverkusen).

3.11 Chromatin Immunoprecipitation

0.5x 10^6 HepG2 cells per dish were seeded into 6-well plates. Transient transfection for T3-mediated positively and negatively regulated promoter constructs was performed with Metafectene (3.6.2). One day after transfection, medium was changed and cells were stimulated with T3 (100 nM final concentration) for 24 h. Subsequently, proteins were cross-linked to DNA by changing to serum-free medium and adding formaldehyde to a final concentration of 1%. Cultures were incubated at 37 °C for 5 min. Cross-linking was stopped by rinsing the adherent cells twice with ice cold 1x PBS containing 1 mM protease inhibitor AEBSF (Roche Applied Biosciences, Mannheim). Cells from three wells were pooled and collected by centrifugation at 2000 rpm for 5 min. At this point, cells were either stored at -80 °C or the subsequent protocol was followed. For further application the Chromatin Immunoprecipitation (ChIP) Assay was used (Upstate/Millipore, Billerica MA, USA), according to the manufacturer's protocol with minor modifications. Cell pellets were resuspended in 400 µl of lysis buffer containing protease inhibitor (1 mM AEBSF) and the suspension was incubated on ice for 10 min. In order to shear the chromatin into length of <1000 bp, cell lysates were sonicated for 30 s in the Sonorex ultrasonic bath (Bandelin, Berlin). The cell debris was collected by centrifuging at 13 000 rpm and 4 °C for 20 min. The supernatant was transferred into 12 ml vessels and diluted 10-fold in dilution buffer. 20 µl of supernatant were saved back as total input control and stored at -80 °C. To reduce non-specific background, the other fraction was pre-cleared with 80 µl of a salmon sperm DNA/protein A agarose slurry for 30 min at 4°C with agitation. Agarose was removed by centrifugation for 2 min at 2000 rpm and 4°C, and the supernatant was transferred to a fresh centrifuge tube. Antibody directed against acetylated histone H3 (Upstate/Millipore, Billerica MA, USA) was added in a 1:500 dilution. The cell lysate/antibody mixture was incubated at 4 °C with rocking overnight. On day two, 60 µl of salmon sperm DNA/protein A agarose

slurry were added to input control or samples with antibody for 1 h at 4 °C with agitation. The agarose was pelleted by centrifuging for 4 min at 14 000 rpm and 4 °C. Pellets were washed consecutively for 3-5 min according to the manufacturer's protocol. In order to elute the Protein/DNA complexes, 250 µl of elution buffer were added, and the samples were incubated for 15 min on a rotating platform at room temperature. The procedure was repeated and the two eluates were pooled. Formaldehyde crosslinks were reversed by adding 20 µl of 5 M NaCl to 500 µl of eluate and the probes were incubated for 4 h at 65 °C. Protein/DNA crosslinks were also reversed in the input controls by adding 8 µl of 5 M NaCl to 200 µl of sample and treating the controls as described above. DNA was purified by phenol/chloroform extraction according to standard procedures and resuspended in 50 µl H_2O. PCR was performed with radioactively labeled nucleotides, using the forward primer WL325 (5'-gagctcggggtctgta-3') or WL328 (5'-ctaagcgttacttcactgag-3') and the reverse primer WL339 (5'-ctttatgtttttggcgtcttc-3') located within the luciferase gene of pGL3-basic. Amplification was performed using 15-20 µl as template DNA. PCR products were separated on 5% polyacrylamide gels and visualized by autoradiography on XAR films (Kodak, Rochester NY, USA).

3.12 Bioinformatics tools

Sequences were obtained from the GenBank at the National Centre for Biotechnology Information (NCBI). Restriction enzyme sites on DNA stretches were detected using the customer site provided by New England Biolabs.

3.13 Statistical evaluation

The statistical significance between the analyzed groups was determined using the two-sample T-test. Differences with a p–value of less than 0.05 were considered significant. Each experiment consisted of at least 3 replicates per condition.

4 Results

4.1 Establishment of a cell culture model

Induction of gene expression in response to T3 occurs in two different time patterns *in vivo*. Whereas one set of genes is up-regulated within 6 h after administration of T3, a second set remains unaffected for at least 12 h and positive regulation takes place first after this lag period. In order to investigate the latter regulation mechanism, a screening for a suitable cell line was carried out. The ideal cell line for this aim should contain endogenous receptors and be susceptible to T3 induction. Secondly, it should mimic the two *in vivo* observed regulation patterns. Finally, the cell line should be easily maintained under conventional culture methods.

T3-mediated induction was studied in GH3 and GC cells, two subclones of the growth hormone secreting GH cells. GH is a rat pituitary tumor cell line that is stimulated by T3 and expresses functional TRs (Herwig & Strauss, 1997). In the present study, T3 stimulation of GH3 and GC cells induced cell growth, which could be observed microscopically. This fact was routinely used to assess effectiveness of T3 stimulation within one experiment. To further confirm the susceptibility of GH3 or GC cells to T3, protein expression of the mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) was examined. Ubiquitously expressed, early-induced Promoter B of mouse GPDH is stimulated in a receptor-dependent manner via a TRE (Dümmler et al., 1996; Weitzel et al., 2001a). Different culture protocols were tested and proteins were routinely isolated after 48 h. Up-regulation of mGPDH was investigated in Western blot experiments. Best T3-mediated induction of mGPDH was found for GC cells, when cells were seeded at a density of 6x 10^5/9.6 cm^2, subsequently put on serum-free medium the next day and were finally stimulated 24 h later (Fig. 4). To exclude secondary effects due to cell stress, trypan blue staining was performed to confirm viability of cells.

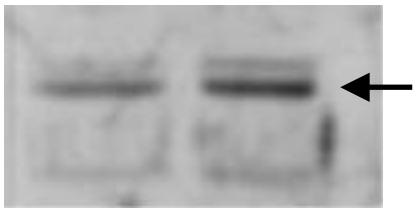


Fig. 4. Effects of T3 on mGPDH protein production

24 h after seeding, rat pituitary GC cells were incubated in serum free medium for one day before being treated with 100 nM T3 for 48 h. Protein were extracted and Western blot was performed. Equivalency of protein loading was monitored by Ponceau staining of the membrane after transfer.

To investigate whether GC cells show the same T3-mediated kinetics of gene expression as found *in vivo*, RNA was isolated from cells after T3 stimulation, transcribed into cDNA and quantified by real-time PCR. As examples for *in vivo* "early" induced genes, the induction of mGPDH, PGC-1α and PGC-1β, as well as the nuclear respiratory factor 1 (NRF-1) was investigated. All of the chosen genes show *in vivo* up-regulation within 6 h after induction with T3. In contrast, adenosine nucleotide translocator 2 (ANT2) and mitochondrial transcription factor A (Tfam) are susceptible to T3 stimulation *in vivo* after 15 to 24 h. Both genes do not contain described TREs. Gene expression patterns for selected genes are shown in Fig. 5. Gene regulation occurred for mGPDH within 6 h after administration of T3. Even though the expression level of PGC-1α was much smaller than seen in rat liver, the regulation was rapidly induced. By contrast, both ANT2 and Tfam were regulated after a lag period of 24 h. Furthermore, NRF-1 and PGC-1β responded with similar expression kinetics in rat liver and in GC cells (data not shown). In conclusion, GC cells feature the same T3-mediated kinetics of gene expression, which were shown *in vivo*.

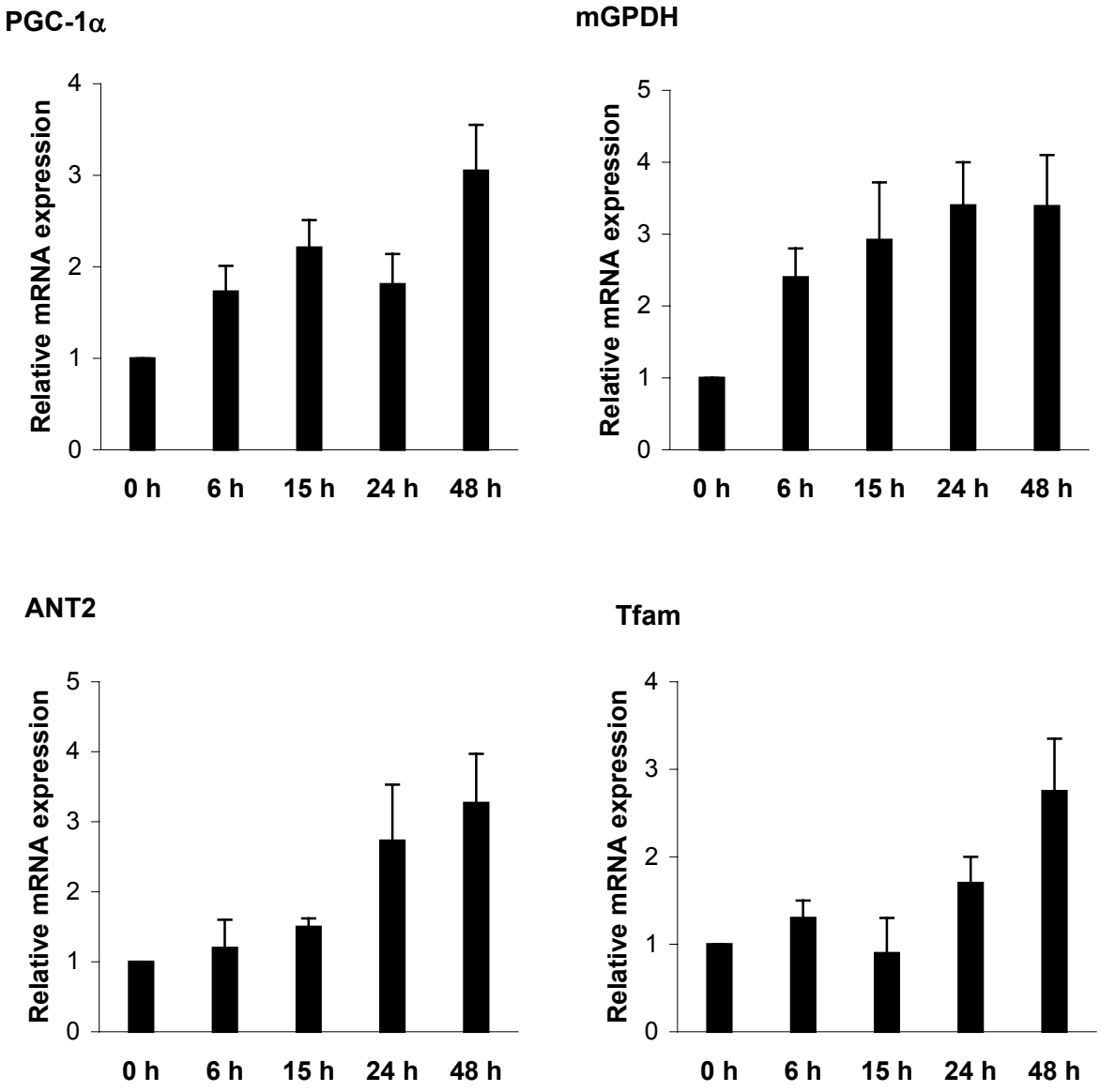


Fig. 5. Thyroid hormone-mediated gene expression pattern in GC cells

GC cells were treated with T3 for 6, 15, 24 and 48 h, following the established protocol. RNA was isolated and quantified by real-time PCR. Expression levels of PGC-1α, mGPDH, ANT2 and Tfam were normalized to the housekeeping gene GSα. Data are mean +/- SD of at least three independent experiments performed in duplicates and are expressed as fold activation relative to unstimulated expression values at the same time point.

4.2 RNA arrest studies of T3 target genes

In some cases, changes in the rate of gene expression are partly mediated by altered mRNA half-life. To investigate whether the T3-mediated up-regulation of the investigated genes was due to other influences than a *de novo* transcription rate, the half-life time of various genes was determined. Therefore, actinomycin D was used that binds to DNA duplexes, thereby interfering with the action of enzymes engaged in replication and transcription. Half-life time was investigated by either real-time PCR or Northern blot analysis. Fig. 6 and 7 indicate the half-life time of PGC-1α (~90 min) and ANT2 (~24 h). No differences were observed for the half-life time of the investigated genes in T3-induced cells compared to mock-induced cells. These results indicate clearly that increased mRNA levels in the presence of T3 were indeed due to *de novo* synthesis.

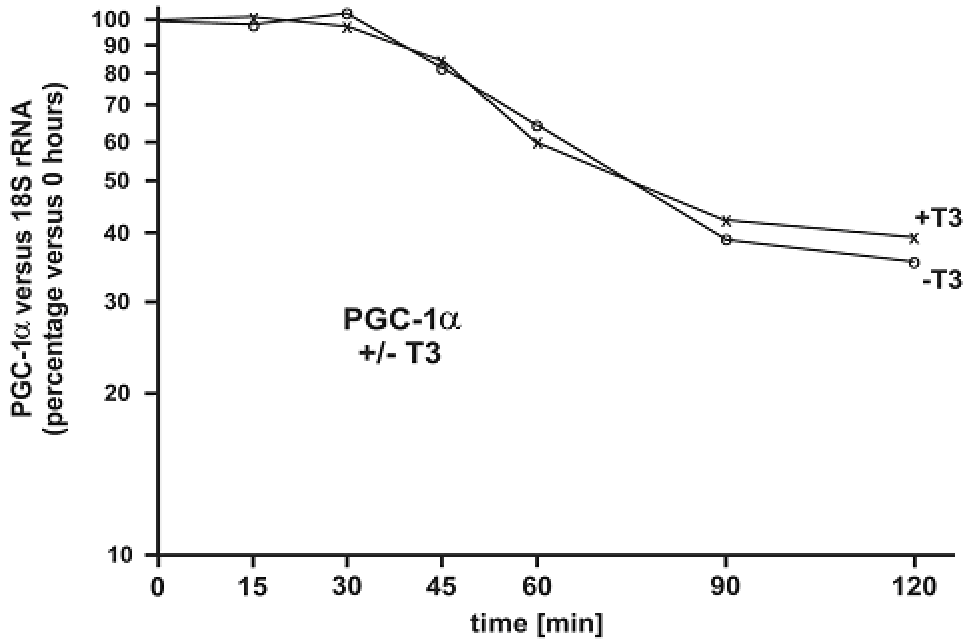


Fig. 6. Influence of T3 on mRNA half-life time of PGC-1α

24 h after seeding, GC cells were incubated with actinomycin D to block transcription in the absence and presence of T3 and cells were harvested at different time points. After isolation of RNA, expression of PGC-1α was quantified by real-time PCR. Level of mRNA was normalized to concentration of 18S rRNA with a known half-life time and expressed as percentage versus unblocked expression levels at 0 h.

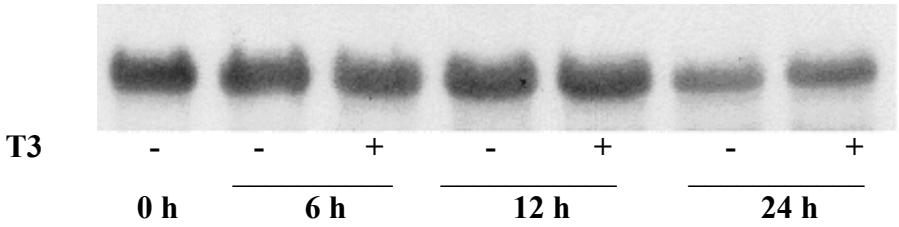


Fig. 7. Influence of T3 on mRNA half-life time of ANT2

24 h after seeding, GC cells were incubated with actinomycin D in the absence and presence of T3 and harvested after 0, 6, 12 and 24 h. After isolation, mRNA was analyzed by Northern blot hybridization. 10 µg of total RNA was used per lane and blotted nylon membranes were incubated with a radioactively labeled cDNA probe against ANT2.

4.3 Characterization of hPGC-1α 5'-flanking region

To further investigate the PGC-1α regulation in response to T3, a functional study of the 5'-flanking region of human PGC-1α promoter was conducted. The –4483/+78 bp promoter region was analyzed for the presence of thyroid response elements (TRE). Interaction between the thyroid hormone receptor TR and the promoter region was determined by a one-hybrid assay with full-length TR fused to the transactivation domain from viral protein 16 (VP16). Transient transfections were performed in HepG2 cells with two different PGC-1α promoter constructs in pGL3-basic reporter plasmid, containing either 5'-flanking region -4483/+78 bp (PGC-4kb) or -2533/+78 bp (PGC-2kb).

The results show, that luciferase activity was induced by 1.6-fold in the context of promoter construct PGC-4kb (Fig. 8). Only basal stimulation was observed when cells were transfected with construct PGC-2kb. These results indicate that a putative element responsible for T3 response is located within the proximal region of the promoter.

Potential transcription factor binding sites of PGC-1α were analyzed on the web site: *www.nubiscan.unibas.ch*. Bioinformatics analysis of the promoter using a consensus TRE sequence revealed the presence of several putative TREs. To test whether these motives indeed confer thyroid hormone responsiveness, the putative TRE oligonucleotides were individually placed upstream of a minimal tk promoter, driving expression of Renilla luciferase as reporter gene. One-hybrid assays were performed in HepG2 with VP16-TR. Only putative TRE, which is located at -4025/-4010 bp (PGC-TRE), conferred significant induction with VP16-TR on the neutral promoter (Fig. 8).

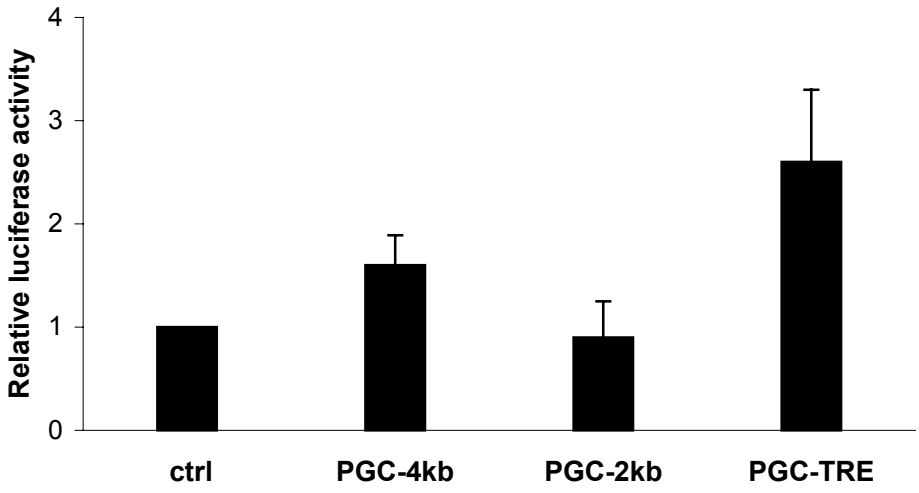


Fig. 8. Interaction between TR and the PGC-1α promoter as determined by one-hybrid assay

HepG2 cells were transiently cotransfected with 2 µg of either PGC-1α reporter gene construct (PGC-4kb, PGC-2kb, PGC-TRE) and 0.4 µg of control plasmid VP16-ctrl or VP16-TR. Cells were harvested after 24 h and luciferase activities were normalized to protein concentration of the cell extract. Data are mean +/- SD of at least three independent experiments performed in triplicates and are expressed as fold induction relative to VP16-empty activity for the respective reporter gene construct.

4.4 EMSA analysis of the putative PGC-TRE

In order to test whether TR can actually bind to the putative site PGC-TRE, gel mobility shift assays were performed. An oligonucleotide including the −4029 to −4006 bp region of the PGC-1α promoter sequence was radioactively labeled. For competition experiments, unlabeled oligonucleotides were generated, corresponding to the PGC-TRE element with mutations in either half-site (5'mut and 3'mut). Furthermore, a characterized TRE element from the mGPDH promoter was used (see Table 8 for sequences).

A specific band-shift was observed in the gel mobility shift assay when the radioactively-labeled oligonucleotide PGC-TRE site was incubated with *in vitro* translated TR (Fig. 9, arrow). To determine whether binding of the TR receptor was sequence specific, each of the unlabeled fragments was used as a competitor. Addition of 100-fold molar excess of wild type oligonucleotide abolished the DNA-TR complex, pointing to the specificity of the binding. In addition, no band shift was observed after competition with unlabeled TRE-mGPDH. When the assay was performed using the mutated oligonucleotides 5'mut or 3'mut at 100-fold excess, all retarded DNA–protein complexes were still formed. The binding between the

putative PGC-TRE and TR is therefore specific. Consequently, PGC-1α contains a TRE-like sequence at – 4 kb and is directly regulated by T3.

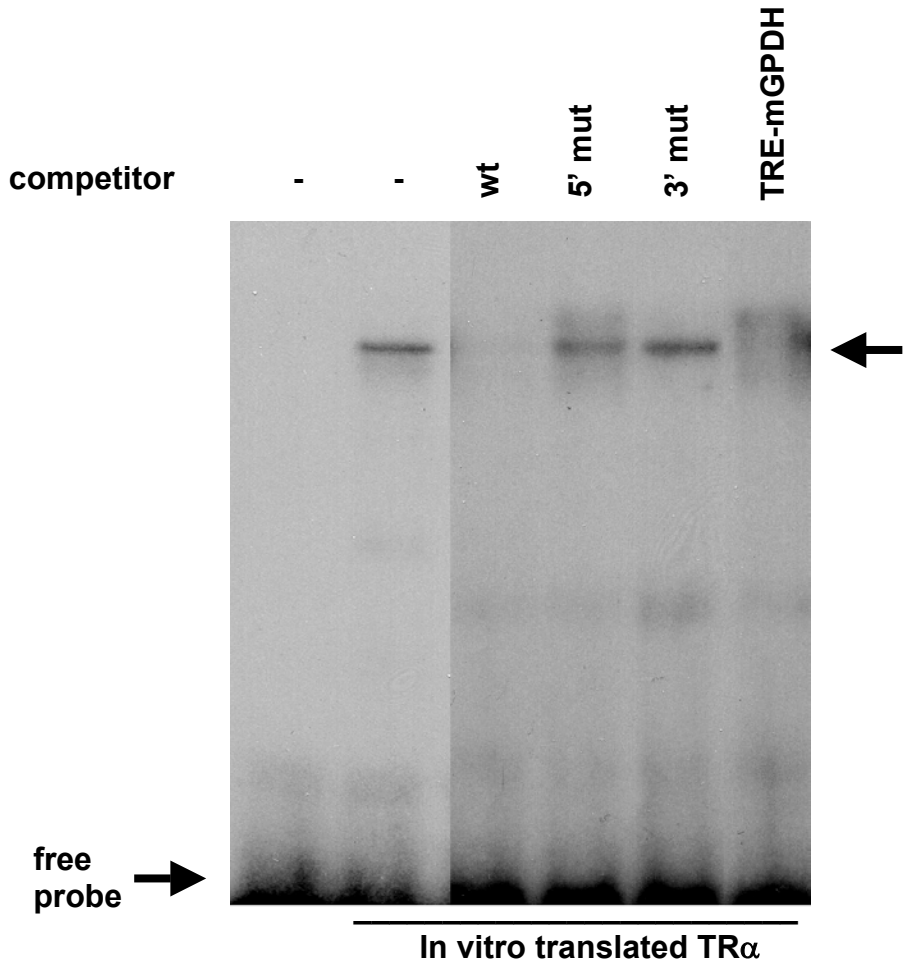


Fig. 9. Binding of TR to PGC-TRE in electrophoretic mobility shift assay

In vitro translated TR was incubated with the labeled PGC-TRE site (-4029/-4006 bp). For competition experiments, a 100-fold molar excess of either wild type PGC-TRE site, mutated PGC-TRE within either the 5' half-site (5'mut) or 3' half-site (3'mut), or the TRE from the mGPDH promoter (TRE-mGPDH) was added. Specific protein-DNA complexes are indicated by an arrow.

4.5 Knockdown of PGC-1α in GC cells

To determine the role of PGC-1α as possible intermediate factor of T3 action, a knockdown strategy via siRNA was chosen and T3-mediated gene expression patterns were investigated in the presence of reduced PGC-1α concentration.

4.5.1 Optimization of transfection efficiency

The key to successful transfection is the careful optimization of reaction conditions for each individual cell type. For transfection of siRNA in GC cells, three different cationic lipid agents were tested: TransIT-TKO (Mirus), Lipofectamine 2000 and Oligofectamine (both Gibco-Invitrogen). Transfection efficiency was analyzed in flow cytometry. 200 nM of siRNA was used per well. To distinguish living from dead cells, DNA dye 7-ADD was used. Signal threshold was set, so that no more than 1% of mock-treated cells were declared as false-positive. Uptake of siRNA differed depending on the specific cationic lipid transfection reagent used (Fig. 10). After transfection of cells with TKO, 99.4% siRNA-positive cells could be detected. After transfection using Oligofectamine or Lipofectamine, only 42.16% or 13.14% siRNA-positive cells were measurable respectively.

Since TKO showed by far the highest transfection efficiency, Oligofectamine and Lipofectamine were not considered anymore in the present study. To further optimize siRNA transfection, different TKO concentrations were tested (Fig. 11). When using more then 5 µl of TKO, saturation of siRNA positive cells was achieved.

Lipid transfection reagents are known to exhibit substantial toxic side effects, as reflected by cell viability. Therefore, toxicity of TKO was assessed by determining the percentage of living cells after transfection. The number of surviving cells decreased with rising concentration of TKO (Fig. 11).

As consequence, 6 µl of TKO were used for each transfection sample. This amount led to maximal transfection efficiency in relation to the cell surviving rate.

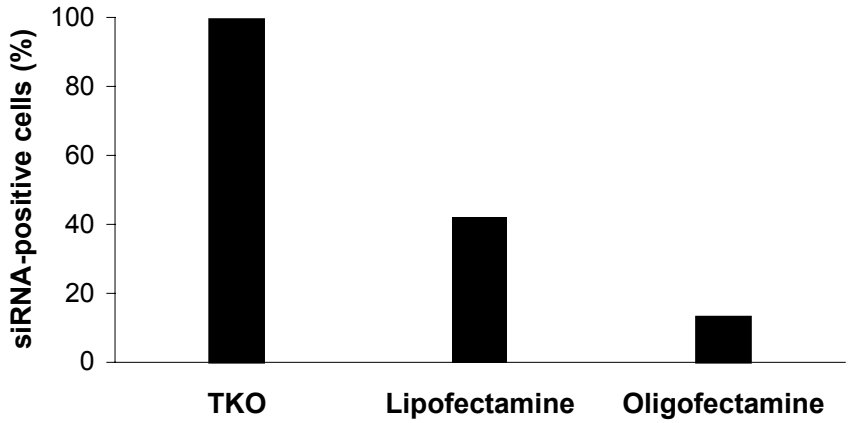


Fig. 10. Transfection efficiency of different cationic lipid agents

6x 10^5 GC cells/well were plated in 6-well plates in their appropriate medium. 24 h later, the medium containing 10% FCS was replaced with serum-free medium and the cells were incubated for additional 24 h. GC cells were transfected by using 8 µl of TKO, 8 µl of Lipofectamine or 6 µl of Oligofectamine and 200 nM of fluorescently labeled siRNA. Transfection efficiency was determined by FACS analysis and compared to mock-treated cells without siRNA.

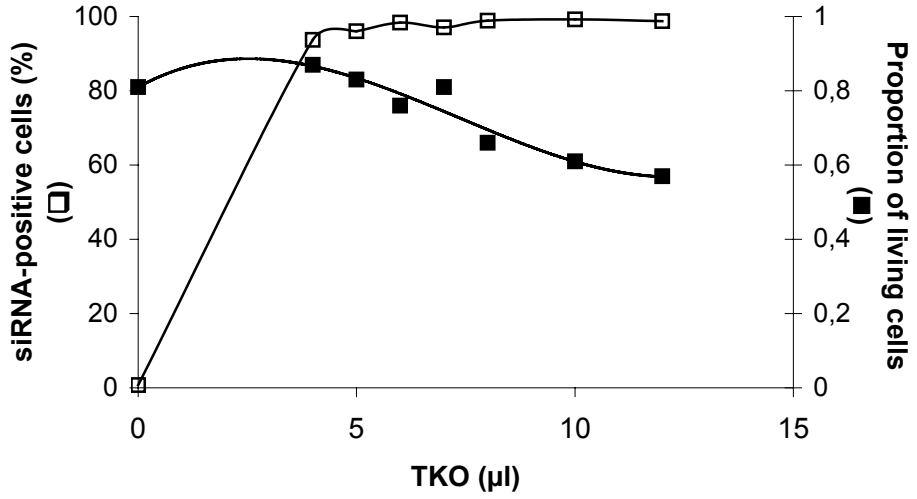


Fig. 11. Optimization of TKO concentration

Cells were incubated as described above and siRNA transfection was performed, by using 200 nM of flourescently labeled siRNA and rising concentrations of TKO. 24 h after transfection, cells were harvested. To distinguish living from dead cells, DNA dye 7-ADD was used. Transfection efficiency (❑) as well as determination of surviving cells (■) was assessed by FACS analysis.

4.5.2 siRNA directed against PGC-1α

Selection of suitable sequences was performed following the defined rules by Tuschl (siRNA user guide). The used mRNA sequence for PGC-1α was deposed at NCBI under accession number NM_031347. Three sequences were tested since not all regions of mRNA are equally accessible for RNA interference (see Table 9 for sequences). For control experiments, cells were mock-incubated with a non-specific siRNA oligonucleotide under identical experimental conditions. Only one siRNA directed against PGC-1α was efficient in inducing RNAi. The two other siRNAs did not lead to PGC-1α knockdown (Fig. 12). When GC cells were transfected with *si2***,** the level of PGC-1α mRNA was strongly decreased already after 4 h. Time course analysis showed that the level of PGC-1α remained low until the fifth day after transfection (Fig. 13A). Further increase of siRNA concentration did not exhibit a stronger down-regulation effect, possible due to saturation of the RNAi machinery (data not shown). The level of control mRNA (GSα) was not affected under the conditions employed (data not shown). To prove that mRNA knockdown correlates with protein knockdown, Western blot experiments were performed. As shown in Fig. 13B, the expression of PGC-1α protein was specifically reduced by the siRNA duplex, but it was unaffected by use of non-specific siRNA.

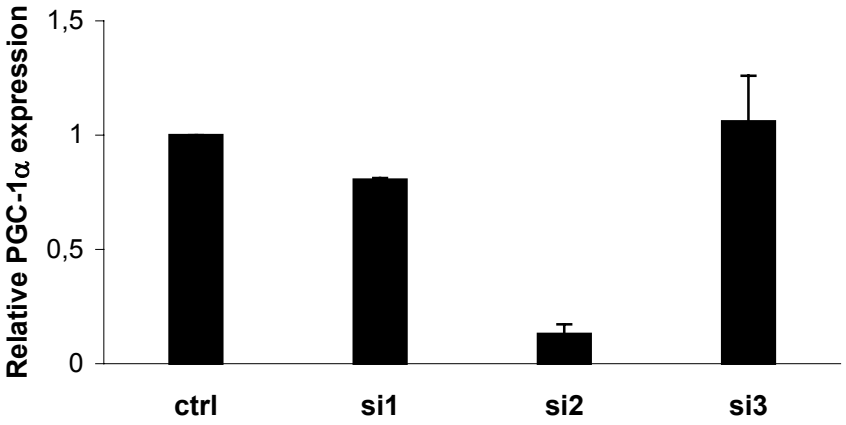


Fig. 12. Analysis of different siRNA oligonucleotides directed against PGC-1α

siRNA transfection was performed using 6 µl of TKO and 200 nM of either siRNA. 24 h later, RNA was isolated and quantified by real-time PCR. Expression level of PGC-1α was normalized to GSα. Data are mean +/- SD of at least at three independent experiments performed in duplicates and are expressed as relative expression compared to mock-treated cells.

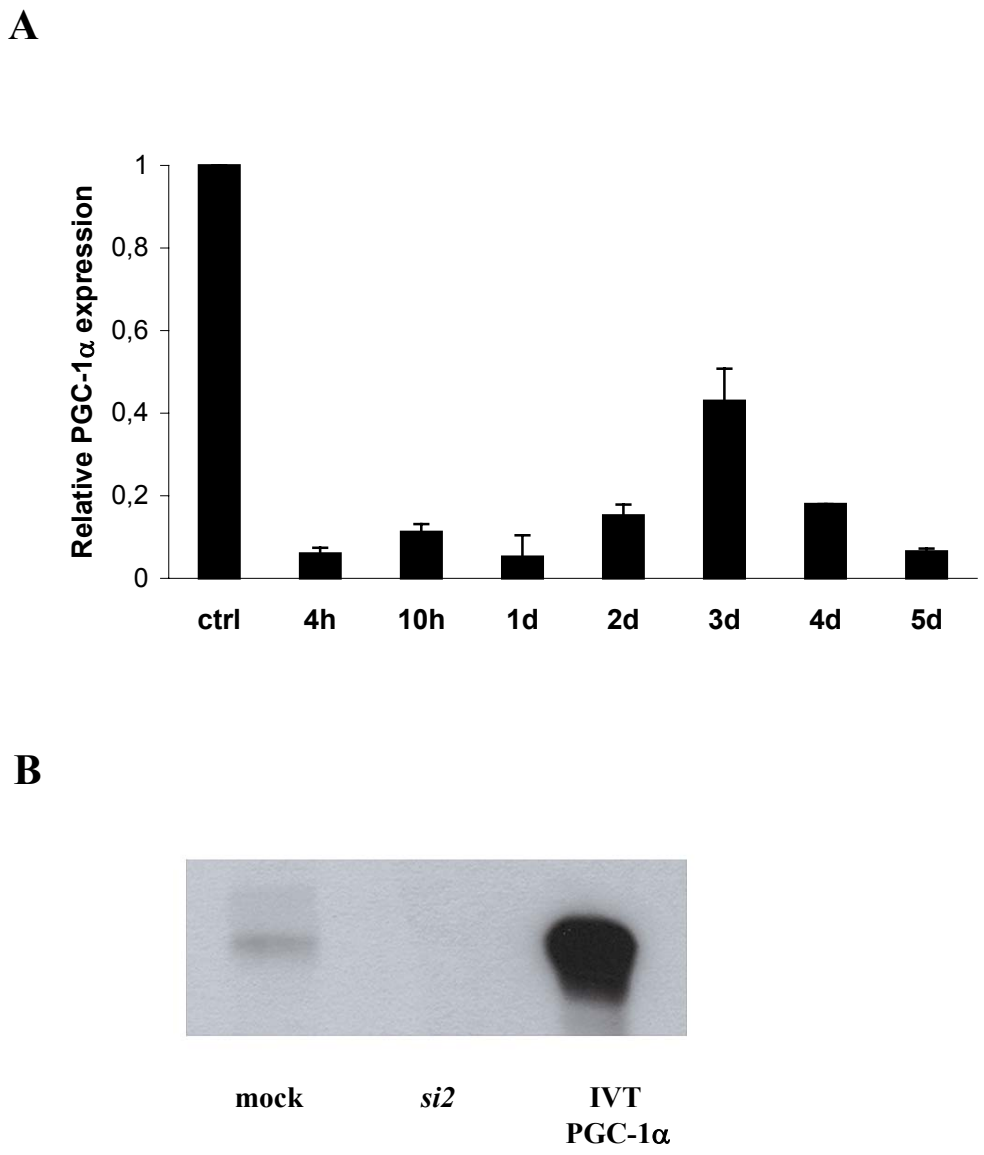


Fig. 13. Knockdown of PGC-1α on RNA and protein level in GC cells

(A) Cells were incubated as described above, and siRNA transfection was performed using 6 µl of TKO and 200 nM of *si2* or unspecific siRNA. Cells were harvested at different time points, RNA was isolated, transcribed and quantified by real-time PCR. Expression level of PGC-1α was normalized to GSα. Data are mean +/- SD of at least three independent experiments performed in duplicates and are expressed as relative expression compared to mock-treated cells at the same time point. **(B)** Nuclear extracts of cells transfected with *si2* and unspecific siRNA were immunoprecipitated with anti-PGC-1α antibody directed against the C-terminus. Western blot analysis was performed, using an antibody directed against the N-terminus of PGC-1α. *In vitro* translated (IVT) PGC-1α was used as positive control.

To exclude a remaining activity of PGC-1α, T3-mediated up-regulation was investigated. As depicted in Fig. 14, PGC-1α mRNA was not induced in siRNA-treated cells in response to T3. Thus, knockdown of PGC-1α was successful.

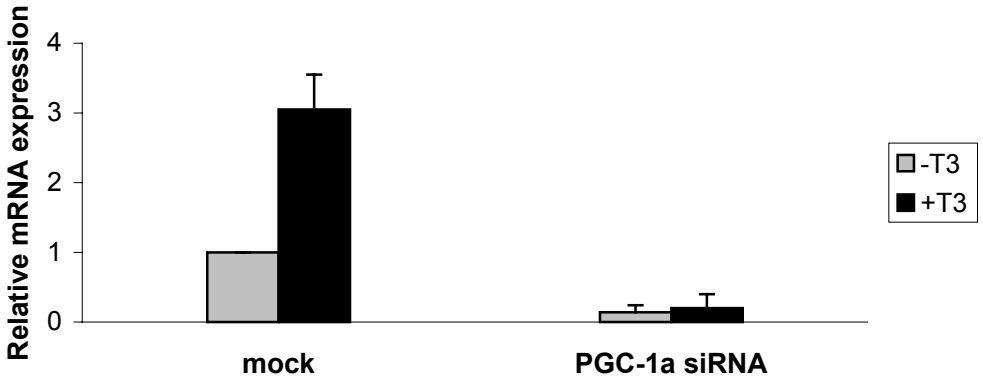


Fig. 14. T3-mediated PGC-1α regulation in mock- and siRNA-treated GC cells

4 h after knockdown of PGC-1α, cells were incubated with T3 and harvested after 24 h. RNA was isolated and expression level of PGC-1α in mock- and siRNA-treated cells in response to T3 was compared by real-time PCR. Data are mean +/- SD of at least at three independent experiments performed in duplicates.

4.5.3 Consequences of PGC-1α knockdown

Since PGC-1α is a known regulator of various enzymes, the effect of PGC-1α knockdown on the expression of several target genes was investigated. 24 h after transfection of *si2*, reduced mRNA expression of cytochrome c, glucose-6-phosphatase and acyl coenzyme A oxidase were observed. Therefore, it was proven that the successful knockdown of PGC-1α resulted in functional consequences (Fig. 15).

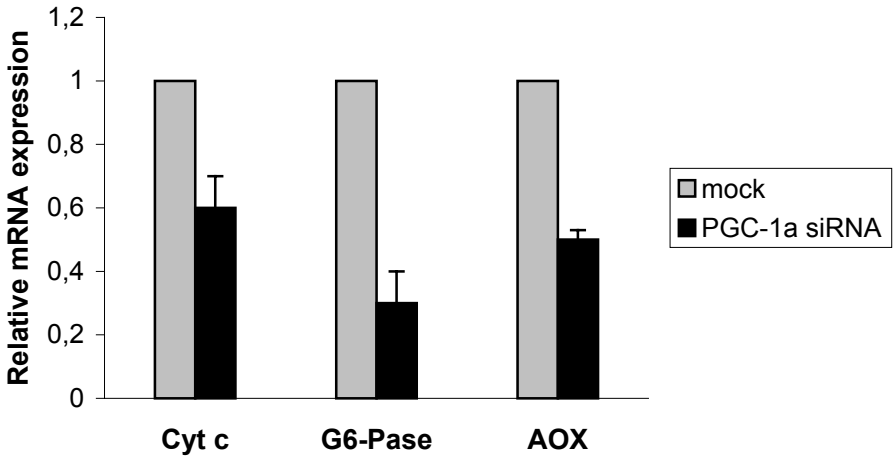


Fig. 15. Expression levels of PGC-1α target genes

Cells were incubated as described above, and siRNA transfection was performed using *si2* directed against PGC-1α or unspecific siRNA. RNA was isolated after 24 h and quantified by real-time PCR. Expression levels of cytochrome c (Cyt c), glucose-6-phosphatase (G6-Pase) and acyl coenzyme A oxidase (AOX) were normalized to GSα. Data are mean +/- SD of at least three independent experiments performed in duplicates.

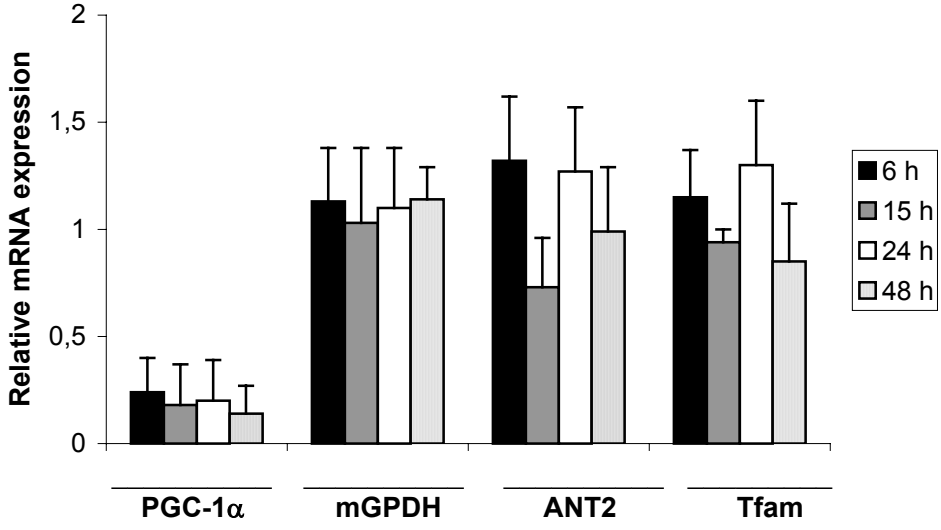


Fig. 16. Consequences of PGC-1α knockdown on gene expression in the absence of T3

Transfection of siRNA was performed using *si2* directed against PGC-1α or unspecific siRNA. RNA was isolated after 24 h and quantified by real-time PCR. Expression levels of mGPDH, ANT2 and Tfam were normalized to GSα. Data are mean +/- SD of at least at three independent experiments performed in duplicates and are expressed as relative expression compared to mock-treated cells at the same time point.

Whether knockdown of PGC-1α affects the mRNA levels of the genes of interest was investigated in the absence of T3. As shown in Fig. 16, down-regulation of the cofactor did not alter mRNA expression of mGPDH, ANT2 and Tfam compared to control. A further experiment targeted the question whether the siRNA approach would change T3-mediated expression kinetics. 4 h after knockdown of PGC-1α, cells were incubated with T3 and harvested at several time points. RNA levels of various genes in mock and siRNA-treated cells in response to T3 were compared by real-time PCR (Fig. 17).
Of note, no alteration of mRNA levels of either mGPDH, ANT2 or Tfam was identified. Since it is possible, that knockdown of one protein is compensated by the expression of another functionally related protein, it was investigated whether down-regulation of PGC-1α altered PGC-1β and NRF-1 expression. However, no compensatory up-regulation was observed (data not shown).

Taken together, a cell culture protocol was successfully established so that GC cells feature the same T3-mediated kinetics of gene expression as observed *in vivo*. It was consequently shown that increased mRNA levels of PGC-1α and other genes in the presence of T3 are due to *de novo* mRNA synthesis rather than due to other mechanisms. Expression of PGC-1α mRNA in response to T3 is regulated in the classical way via a TRE in the promoter which could be identified for the first time in this study at –4025/-4010 bp. The accomplished design and application of siRNA against PGC-1α indicates that T3-mediated “late” expression of genes is maintained independently of PGC-1α activation. However, PGC-1α is involved in the subsequent regulation of several T3 controlled metabolic pathways like fatty acid oxidation or gluconeogenesis, which could be succesfully shown in these siRNA experiments.

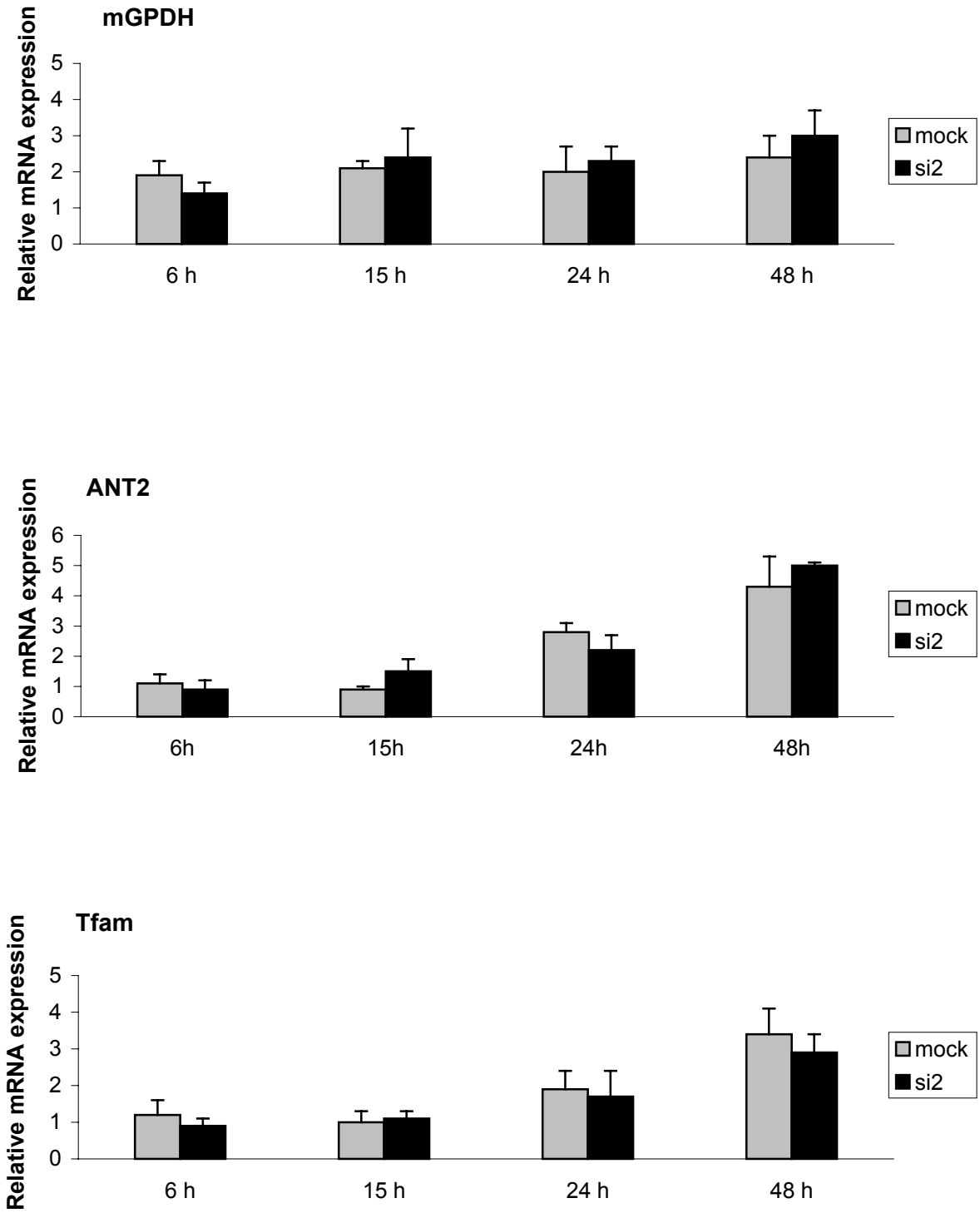


Fig. 17. Consequences of PGC-1α knockdown on T3-mediated gene expression patterns

siRNA transfection was performed using *si2* directed against PGC-1α or unspecific siRNA. RNA was isolated after 24 h and quantified by real-time PCR. Expression levels of mGPDH, ANT2 and Tfam were normalized to GSα. Data are mean +/- SD of at least at three independent experiments performed in duplicates and are expressed as relative expression compared to cells incubated in the absence of T3.

4.6 T3-mediated negative gene regulation

Gene suppression in response to T3 is another unsolved aspect of molecular T3 action. Several mechanisms have been proposed to explain the T3-mediated transcriptional control of negatively regulated genes. However, up to now the mechanism for ligand-dependent negative gene regulation remains unclear.

4.6.1 Comparison of T3 effect on different promoters

To study the principle of T3-mediated transcriptional control, positive and negative gene regulation was compared using HepG2 cells. Thyroid stimulating hormone (TSH) is a heterodimer, consisting of α and β subunit. The β-chain (TSHβ) is specific to TSH, while the α-chain (TSHα) is common to all glycoprotein hormones. TSH is part of the HPT axis, and transcription for both subunits is subject to feedback inhibition by T3. For ligand-dependent negative regulation, the TSHα subunit promoter was investigated. To study T3-mediated transcriptional activation, the mitochondrial glycerol-3-phosphate dehydrogenase (mGPDH) promoter was used, which contains a well-characterized pTRE. Luciferase activities of TSH (-802/+22 bp)-luc and mGPDH (-316/+109 bp)-luc reporter constructs were examined in transient cotransfection assays in the absence or presence of T3. When TSH-luc was cotransfected with TRα/RXR into HepG2 cells, luciferase expression was increased approximately 4-fold. After treatment with T3, TSHα activity was reduced to the basal activity without T3 (Fig. 18A).

Since the luciferase reporter gene itself was discussed to mediate the T3-dependent reporter gene suppression in different cell lines, chloramphenicol acetyl transferase (CAT) was used as reporter gene. The outcome of transfection experiments for the TSH-CAT construct in HepG2 cells is illustrated in Fig. 18B, confirming that this fragment is negatively responsive to T3. In comparison, mGPDH-Luc showed slight repression after cotransfection of TR/RXR, while supplement of T3 induced reporter gene activity about 5-fold. Similar results were obtained by using Renilla luciferase as reporter gene (Fig. 18C and D).

Observed promoter activities for TSH and mGPDH were not cell specific, since their T3-dependent regulation was confirmed by using different cell lines like human kidney HEK293 cells or rat pituitary GC cells (data not shown).

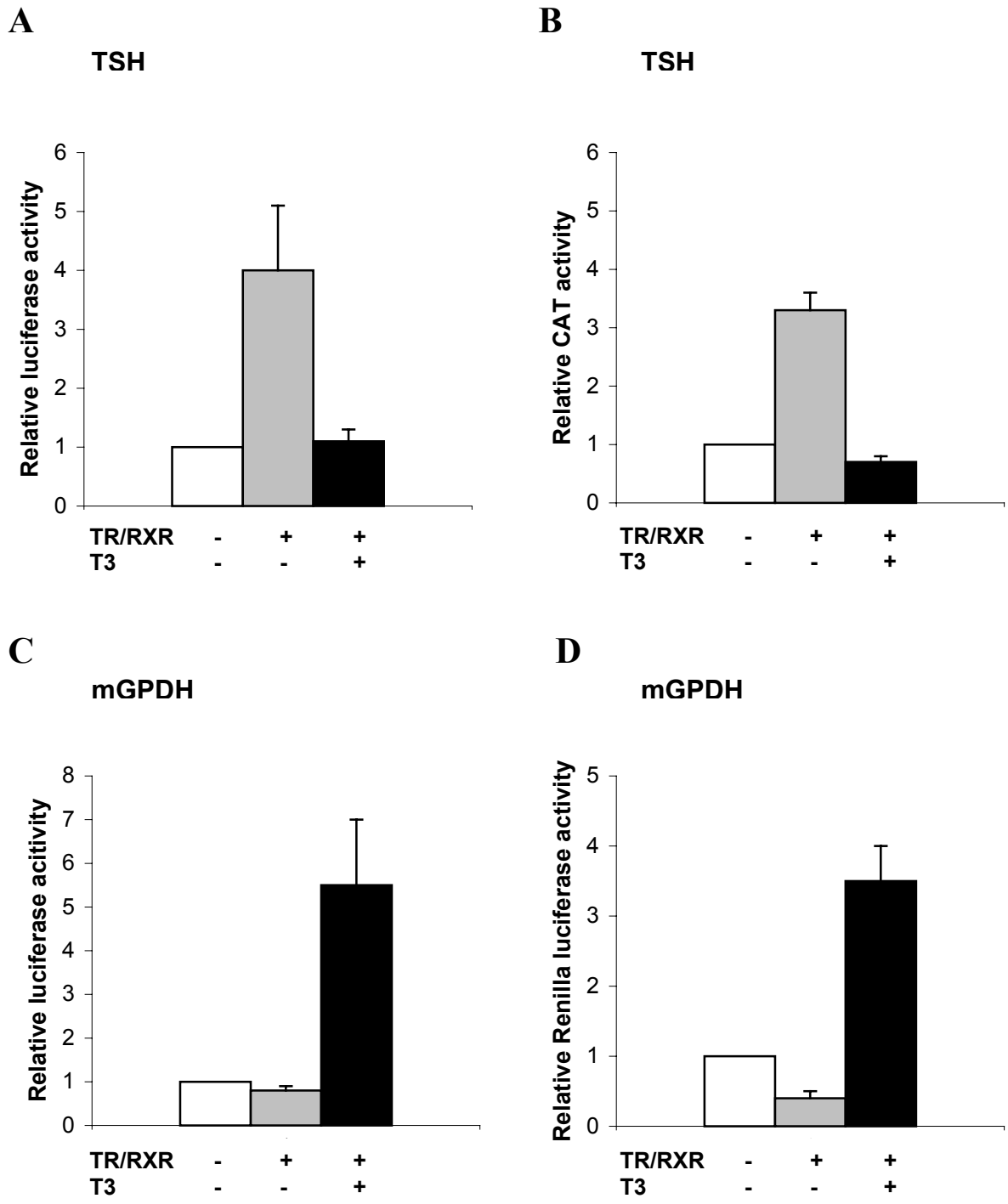


Fig. 18. T3-mediated regulation of promoter fragments of TSH (A+B) and mGPDH (C+D)

Promoter fragment of TSHα (-802/+22) was ligated into reporter plamids upstream of Firefly luciferase **(A)** or chloramphenicol acetyl transferase (CAT) **(B)**. Promoter fragment of mGPDH (-316/+109) was placed in a reporter plasmid bearing either Firefly luciferase **(C)** or Renilla luciferase **(D)**. HepG2 cells were cotransfected with 2 μg of either reporter gene construct and 0.4 μg of both TR and RXR and incubated in the absence or presence of 100 nM T3 for 24 h. Luciferase activity was measured and normalized to total protein concentration. CAT was assessed by real-time PCR and normalized to housekeeping gene S27. Data are mean +/- SD of at least at three independent experiments performed in triplicates.

4.6.2 Investigation of chromatin modification

Previous studies have proposed an important role for histone acetylation in modulating gene transcription by nuclear hormone receptors (McKenna and O'Malley 2002). It has been assumed that promoter repression by TR is connected with recruitment of histone deacetylases (HDACS), while transcriptional stimulation is associated with increased histone acetylation. To investigate if this mechanism applies to the positively promoter and a negatively regulated promoter used in these assays, the T3-induced pattern of acetylated H3 of the promoter constructs was assessed by chromatin immunoprecipitation. In this assay, acetylated chromatin is precipitated with anti-acetylated H3 antibody and the amount of precipitated DNA fragments can be detected by PCR. On the contrary, hypoacetylated chromatin is precipitated less efficiently, and less DNA is amplified.

Promoter fragments were transfected into HepG2 cells along with TR/RXR. After incubation of cells in the presence or absence of T3 for 24 h, extracts were taken and immunoprecipitated with anti-acetylated H3 antibody. Promoter sequences that co-precipitated were detected by PCR amplification (Fig. 19).

In case of the negatively regulated promoter, cotransfection of TR enhanced acetylation in the absence of T3. The supplement of T3 reversed this effect. After additional cotransfection of cofactors NCoR and SRC-1, these differences in acetylation were even more pronounced.

This picture was opposite for the mGPDH promoter. Here, deacetylation of the promoter occured ligand-independent, whereas the promoter became highly acetylated and therefore activated in the presence of T3. In concert with the negatively regulated promoter, it could be observed that cotransfection of NCoR and SRC-1 further enhanced the divergent acetylation status after addition of T3.

These results indicate that the state of acetylated histone H3 reflects the transcriptional effects seen in transient expression assays for T3-mediated negative and positive gene regulation.

Therefore, gene activity is correlated with chromatin structure.

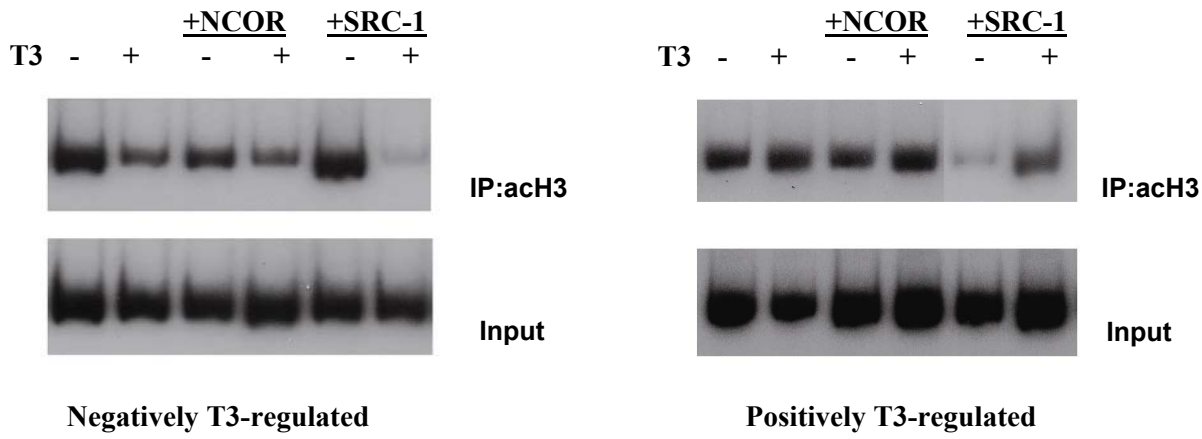


Fig. 19. ChIP analysis of T3-mediated promoter regulation

Promoter fragments (1 μg) of negatively (**A**) and positively regulated (**B**) genes were transfected into HepG2 cells. TR/RXR and cofactors (0.1 μg) were cotransfected and cells were incubated in the absence or presence of 100 nM T3 for 24 h. Chromatin was fixed by formaldehyde and preserved as input control or immunoprecipitated with anti-acetylated H3 antibody (acH3). Immunoprecipitated and input samples were subjected to PCR analysis with primer spanning the respective gene promoter.

4.6.3 Investigation of TSHα and mGPDH promoter in one-hybrid assay

To address the question of whether binding of TR to the promoter is important in the context of transcriptional regulation of the TSHα promoter, a one-hybrid assay was performed to detect TR binding. HepG2 cells were used for transfection experiments because of their ease of handling and lack of functional active endogenous nuclear receptors.

Full-length wild-type TRα fused to the transcriptional activation domain of VP16 generates a constitutively active receptor, even in the absence of T3. The TSH-luc vector was cotransfected with VP16-TR whereas control transfections were performed with empty VP16 vector. For comparison, the mGPDH promoter was used (Fig. 20). In case of the TSHα promoter, no activation by VP16-TR was observed, indicating a lack of TR binding to the promoter. In contrast, mGPDH promoter showed up-regulation after cotransfection of VP16-TR, consistent with one well-characterized pTRE.

Taken together, this data indicates that while TR binds to a regulatory element in the mGPDH promoter, a TRE-like sequence is missing in the TSHα promoter. Negative regulation of TSHα therefore involves a mechanism without direct binding of TR to the TSHα promoter.

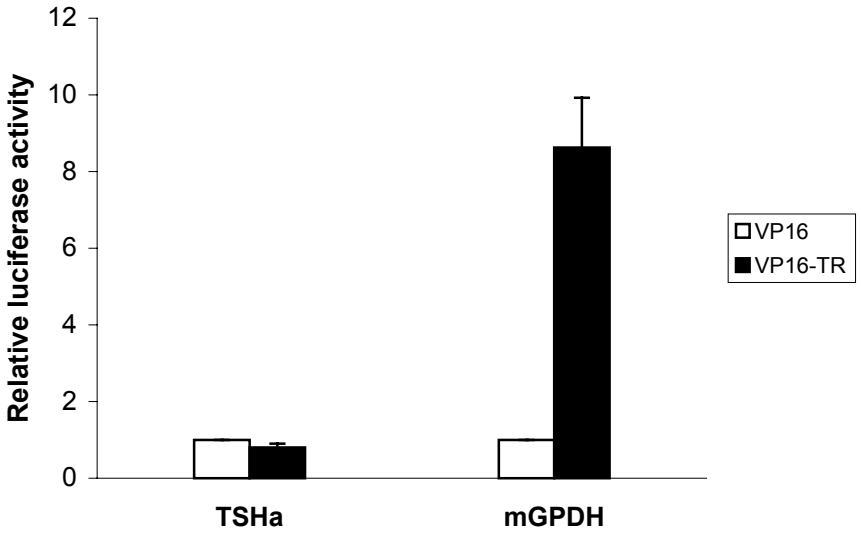


Fig. 20. One-hybrid assay of TSHα and mGPDH promoter

Promoter-reporter gene constructs for TSH or mGPDH promoter were cotransfected into HepG2 cells together with fusion protein of TR and the viral activator domain VP16 (VP16-TR) or the VP16 vector alone. Transfections were performed with 2 µg of either promoter construct together with 0.4 µg of VP16-TR or VP16-empty. Cell extracts were prepared after 24 h and assayed for luciferase activity, which was normalized to total protein concentration. Data are mean +/- SD of at least at three independent experiments performed in triplicates.

4.6.4 Effects of TR mutants on gene regulation in different assay systems

The significance of different receptor regions in terms of positive and negative regulation was examined by using various TR mutants, which possess functional disruptions in the investigated domains. Fig. 21 depicts the used TR mutant constructs. Two deletion constructs C1 and C2 were created lacking the N-terminal domain and DNA binding domain respectively as previously described (Sasaki et al., 1999). Analogous to already characterised substitutions in hTRβ, several mutations were generated in cTRα, disrupting the DNA binding domain (C71S, C89S) (Nagaya et al., 1992). Furthermore, mutant TRs with impaired CoR or CoA binding ability (P158K, C253K, E401A) were created, according to previous reports (Tagami et al., 1998; Nakano et al., 2004). T3 binding affinities of all mutated receptors were similar to wild type.

The functional characteristics of mutant receptors were first investigated based on the effect on expression of a reporter plasmid construct, containing a DR+4 TRE element upstream of a minimal tk promoter and the luciferase gene (DR+4-tk-luc) (Fig. 22).

Using the DR+4-luc construct, wild type TR as well as CoR binding mutants P158R and C253K mediated transcriptional activation in the presence of the ligand. P158R induced an even higher stimulation than wild type TR, probably due to the lack of CoR binding. Consistent with the notion that for positively regulated promoter binding of TR is necessary for stimulation, promoter activation could not be observed for DNA binding mutants C71S and C89S. Likewise, mutation E401A within the AF-2 domain led to significant loss of ligand-dependent activation, proving the important role of coactivator binding for T3-mediated gene activation. These controls confirmed the expected properties of the TR mutants in the context of positively regulated genes.

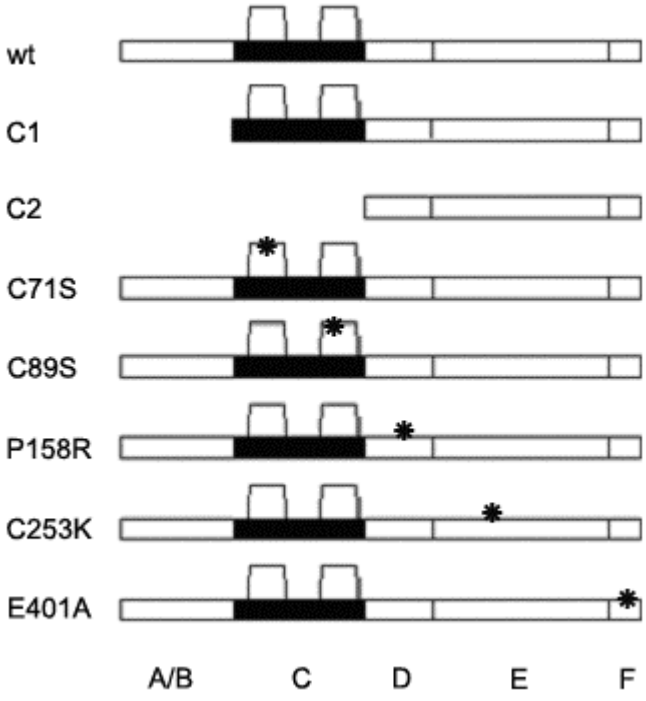


Fig. 21. Diagram of various TR mutants used in the present experiments

C1 and C2 are deletion mutants, while other TR mutants contain point mutations in various domains indicated by *.

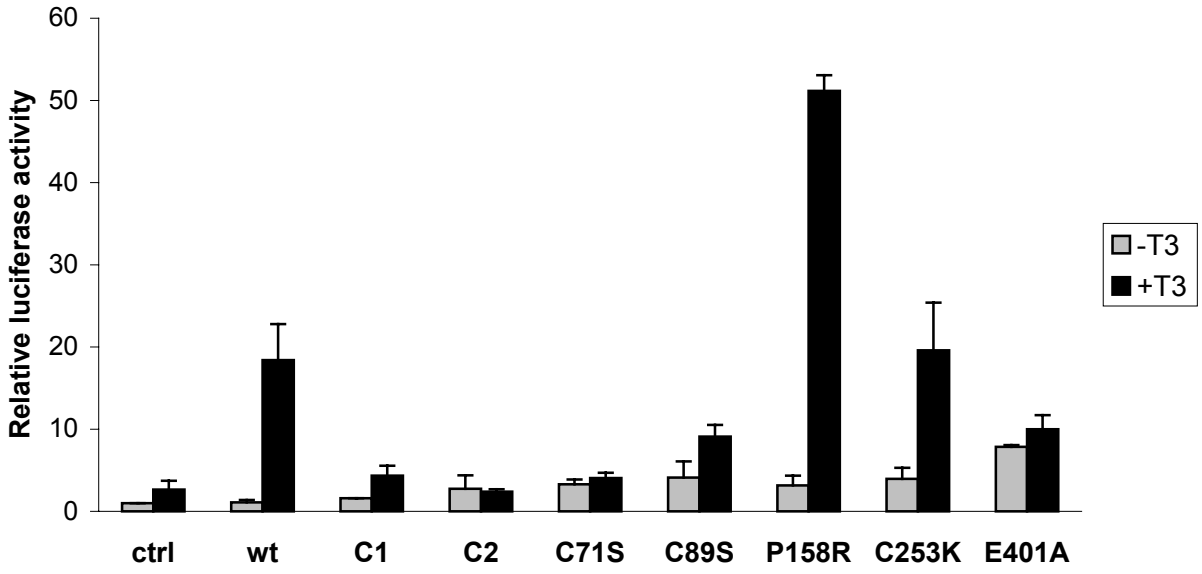


Fig. 22. Effects of the TRα mutants on T3-mediated positive regulation

DR+4-tk-luc reporter plasmid (4 μg) was transfected into HepG2 cells together with different TR mutants and RXR (each 0.4 μg). Cells were incubated in the absence or presence of 100 nM T3 for 24 h and assayed for luciferase activity, which was normalized to total protein concentration. Data are mean +/- SD of at least at three independent experiments performed in triplicates.

Having established the basic functional properties of the individual receptor mutants, the attention was next focused on understanding the role of DNA binding in mediating TR-dependent repression. Whether negative gene regulation can occur in the absence of direct binding of TR to the promoter is object of a controversial discussion.

A GAL4 two-hybrid assay was used to assess this question in a simplified system. TR wild type and the various mutants were expressed as fusion proteins with the DBD of the yeast GAL4 protein. Due to the GAL4 portion of the GAL4-TR construct, the endogenous TR binding domain is not required to efficiently pull the receptor to the promoter. Thus, this strategy allowed to directly test the effects of T3 when receptor is bound to the DNA as well as to assess the significance of different receptor regions.

The reporter gene UAS-tk-luc, which contains five GAL4 binding sites upstream of a minimal tk promoter and luciferase reporter gene, was used to assess consequences of T3 stimulation.

As illustrated in Fig. 23A, transient transfection of TR wild type resulted in a 138-fold increase of transcriptional activation upon ligand binding as compared to untreated cells. This strong activation was probably due to the abundance of GAL4 binding sites in the reporter plasmid. Of note, cotransfection of the different DBD mutants led to even higher activations. While the presence of the DBD deletion mutants C1 or C2 resulted in a T3-mediated 1000-fold activation of the reporter gene, zinc finger mutants C71S and C89S activated transcription 537- and 694-fold, respectively. In contrast, mutations impairing CoR binding (P158R, C253K) were found to reduce T3-dependent activation dramatically. Similarly, TR mutant E401A was not able to activate the reporter gene in response to T3. To verify comparable protein expression, Western blot analyses were performed on whole HepG2 cell extracts of transfected cells by using a specific TR antibody (Fig. 23B). Protein levels of C1 and C2 were not detectable since the used antibody was directed against the N-terminus of TR. However, all other GAL4 chimaric proteins were produced at equal levels. Therefore, differences in gene activation were not due to altered protein production.

Noteworthy, this data reveals that general restriction of TR to DNA in the presence of T3 results in gene activation. The increase of activation upon mutation of the DBD domain indicates a further functional activity of this region besides DNA binding. Finally, both CoA and CoR binding to TR seem to be essential for activation in response to T3.

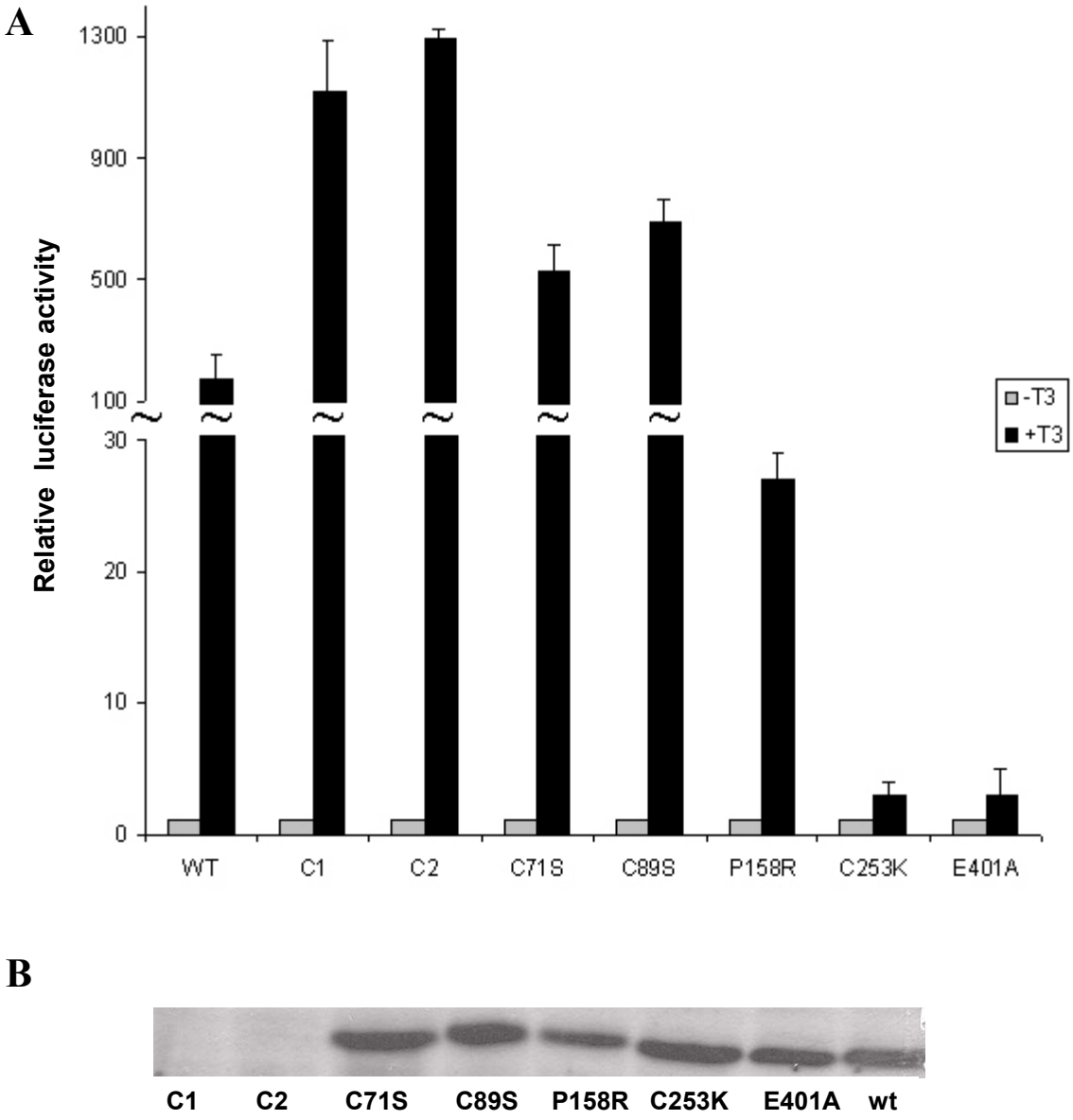


Fig. 23. Effects of TR and TR mutants in a GAL4 one-hybrid assay

(A) A chimeric protein of GAL4 DNA binding domain and TR wild type or TR mutants were cotransfected with a reporter vector, containing 5 binding sites for GAL4 upstream of a minimal tk promoter. Transfections were performed with 2 µg of reporter plasmid together with 0.4 µg of either GAL4-TR construct. Cell extracts were incubated in the absence or presence of 100 nM T3 for 24 h and assayed for luciferase activity, which was normalized to protein concentration. Data are mean +/- SD of at least three independent experiments performed in triplicates and are expressed as fold activation relative to the unstimulated expression of the TR construct.
(B) Western blot analysis with anti-TR antibody was performed to assess the level of TR protein expression in HepG2 cells.

Since the data revealed that the domains for CoA and CoR binding might be involved in gene activation, a further look was taken on the interaction between cofactors and the generated TR mutants. Cofactors cannot bind directly to DNA, but interact instead with DNA-bound transcription factors. As a result, they are recruited to the target gene promoter region where they integrate various activities, affecting the rate of transcription. Consequently, the GAL4 assay was used, in which cofactors were fused to the GAL4 DBD domain and restricted to the reporter plasmid bearing five GAL4 binding sites (5xUAS-tk-luc). Given that CoAs and CoRs possess domains of various functions, different GAL4 constructs were created.
NCoR contains three different nuclear receptor interaction domains, which have been reported to play a role in TR binding. NCoR-IDC (amino acid 2278 to 2444) consists of the interaction domain at the very C-terminus while NCoR-F1 (amino acid 1564 to 2444) includes all three domains. Either construct deletes the major repressing domains. In order to investigate SRC/TR interaction, the GAL4 binding domain was fused with two parts from SRC-1 protein: SRC-LXXLL (amino acid 493 to 1007) and SRC-Qrich (amino acid 1050 to 1185). The first construct contains the major nuclear receptor interaction domain whereas the latter includes a glutamine rich region, which is conserved between members of the SRC family.

In vitro interactions were tested between VP16-TR and different GAL4-NCoR/ -SRC-1 fusion constructs in the GAL4 two-hybrid assay.
As illustrated in Fig. 24, TR binds to NCoR-F1 in the absence of ligand. The results are consistent with the ability of NCoR to dissociate from TR in response to T3. However, VP16-TR did not interact with the smaller N-terminal NCoR-IDC fragment. In contrast, interaction between both SRC-Qrich and SRC-LXXLL and VP16-TR was seen in the presence of T3, consistent with the ability of T3 to recruit CoAs to the receptor. Controls showed that VP16-TR did not alter basic promoter activity in the presence or absence of T3.

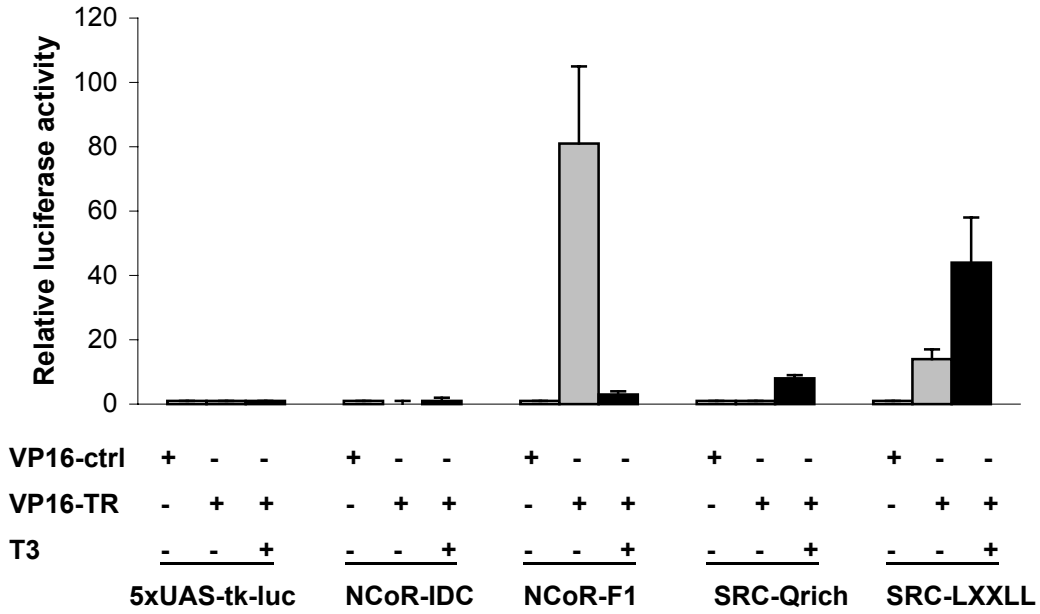


Fig. 24. T3-mediated interaction of TR and different cofactor fragments

Reporter plasmid 5xUAS-tk-luc and chimaric fusion protein of GAL4 DBD with corepressor NCoR (NCoR-IDC; NCoR-F1) or coactivator SRC-1 (SRC-Qrich; SRC-LXXLL) were transfected into HepG2 cells. Additionally, either expression plasmid for VP16-ctrl or VP16-TR was cotransfected. Transfections were performed with 2 µg of reporter plasmid together with 0.4 µg of any other component. Cell extracts were incubated in the absence or presence of 100 nM T3 for 24 h and assayed for luciferase activity, which was normalized to total protein concentration. Data are presented as fold induction of VP16-ctrl in the absence of T3 and are mean +/- SD of at least at three independent experiments performed in triplicates.

Since TR is not able to bind to the reporter gene construct directly (Fig. 24), the GAL4 assay is suitable to further investigate the interaction of cofactors and TR. Therefore, the NCoR-F1 and both SRC-1 fragments were chosen to study modulation of gene activity after cotransfection of different TR mutants. As shown in Fig. 25A, cotransfection of wild type TR to the GAL4-NCoR assay led to activation of the reporter gene whereas this activity was suppressed in the presence of T3 relative to the control.

Interestingly, introduction of the C71S and C89S mutations into the DBD of TR inverted promoter activity. In contrast to TR wild type, cotransfection of TR DBD mutants induced gene expression in response to T3. As seen with the native receptor, cotransfection of mutant E401A led to activation while regulation was reversed in the presence of T3. Corepressor binding mutants P158R and C253K lacked both basal activation and repression.

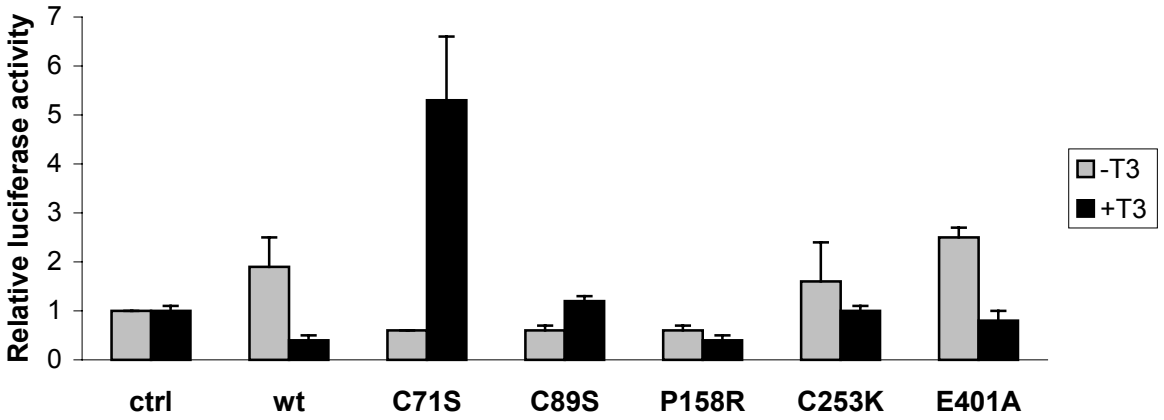
To further assess the T3-mediated effects in the GAL4-NCoR assay, additional NCoR was added, lacking a GAL4 DBD (Fig. 25B). Additional cotransfection of NCoR enhanced

activation 2.5-fold in the absence of ligand, which was blunted after supplement of T3. In comparison, soluble NCoR had no influence on gene expression in the absence of TR, indicating that the modulation of gene expression was not due to secondary squelching effects. The obtained results clearly indicate that TR can effect positive as well as negative gene regulation without directly binding to the DNA.

In transfection experiments with GAL4-SRC (LXXLL), the presence of wild type TR did not confer any alteration compared to the control activity. Contrary, TR DBD mutants C71S and C89S induced about 3-fold activation in the presence of T3. Coexpression of TR LBD mutants P158R, C253K and E401A however lacked T3-mediated activation, similar to the native receptor (Fig. 26A).
In an additional assay, interaction of TR wild type and TR mutants were investigated with the second GAL4-SRC fragment (Qrich). Relative to control, TR wild type induced T3-mediated repression. Once more, regulation of gene activity was different after introduction of mutations into the DBD domain of TR. Whereas TR wild type conferred gene suppression, mutants C71S and C89S abrogated down-regulation of gene expression in the presence of T3. As seen with the native receptor, the mutants with impaired cofactor binding (P158R, C253K and E401A) potently suppressed activity after addition of T3, although the extent of transcriptional response was different (Fig. 26B).

Since cotransfection of TR mutants C71S and C89S abrogated down-regulation in the assays, the effects of these mutants were tested on the negatively regulated promoter TSHα (Fig. 27). As shown already in Fig. 18, TSHα promoter is activated by unbound TR wild type and activation is suppressed in the presence of T3. By using C71S or C89S instead of TR wild type in this assay, a ligand-dependent down-regulation was not observed anymore.
This data demonstrates that even though TR does not bind directly to the TSHα promoter, an intact DBD is required for negative gene regulation of the T3-mediated negative regulation of TSHα.

A



B

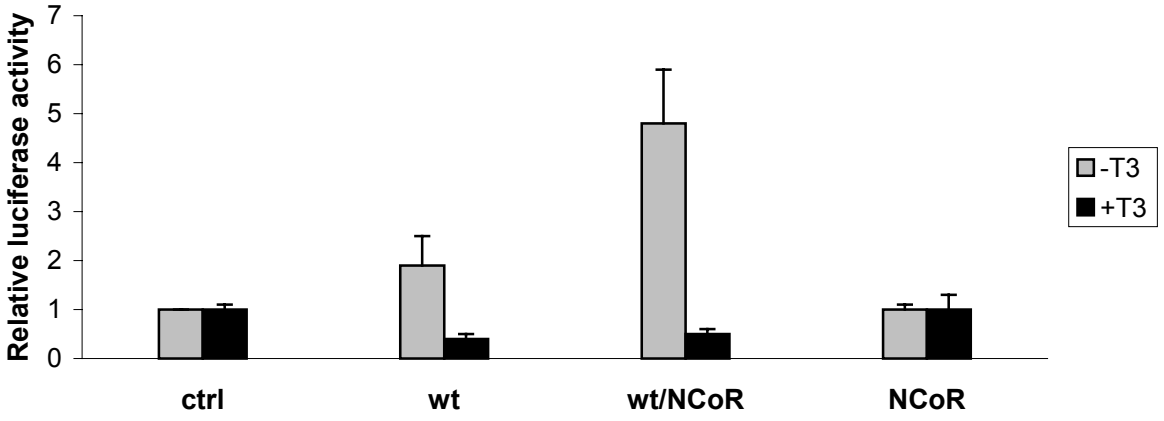


Fig. 25. NCoR interaction with TR wild type and TR mutants in mammalian two-hybrid assay

(A) Chimaric protein GAL4-NCoR was cotransfected with TR wild type (wt) or TR mutants and the 5xUAS-tk-luc reporter plasmid into HepG2 cells. Transfections were performed using 2 µg of reporter plasmid and 0.4 µg of expression plasmid for TR. Cell extracts were incubated in the absence or presence of 100 nM T3 for 24 h and assayed for luciferase activity, which was normalized to total protein concentration. **(B)** Expression plasmid for NCoR without GAL4 DBD was additionally cotransfected. Data are presented as fold induction of empty GAL4 vector (ctrl) in the absence of T3 and are mean +/- SD of at least at three independent experiments performed in triplicates.

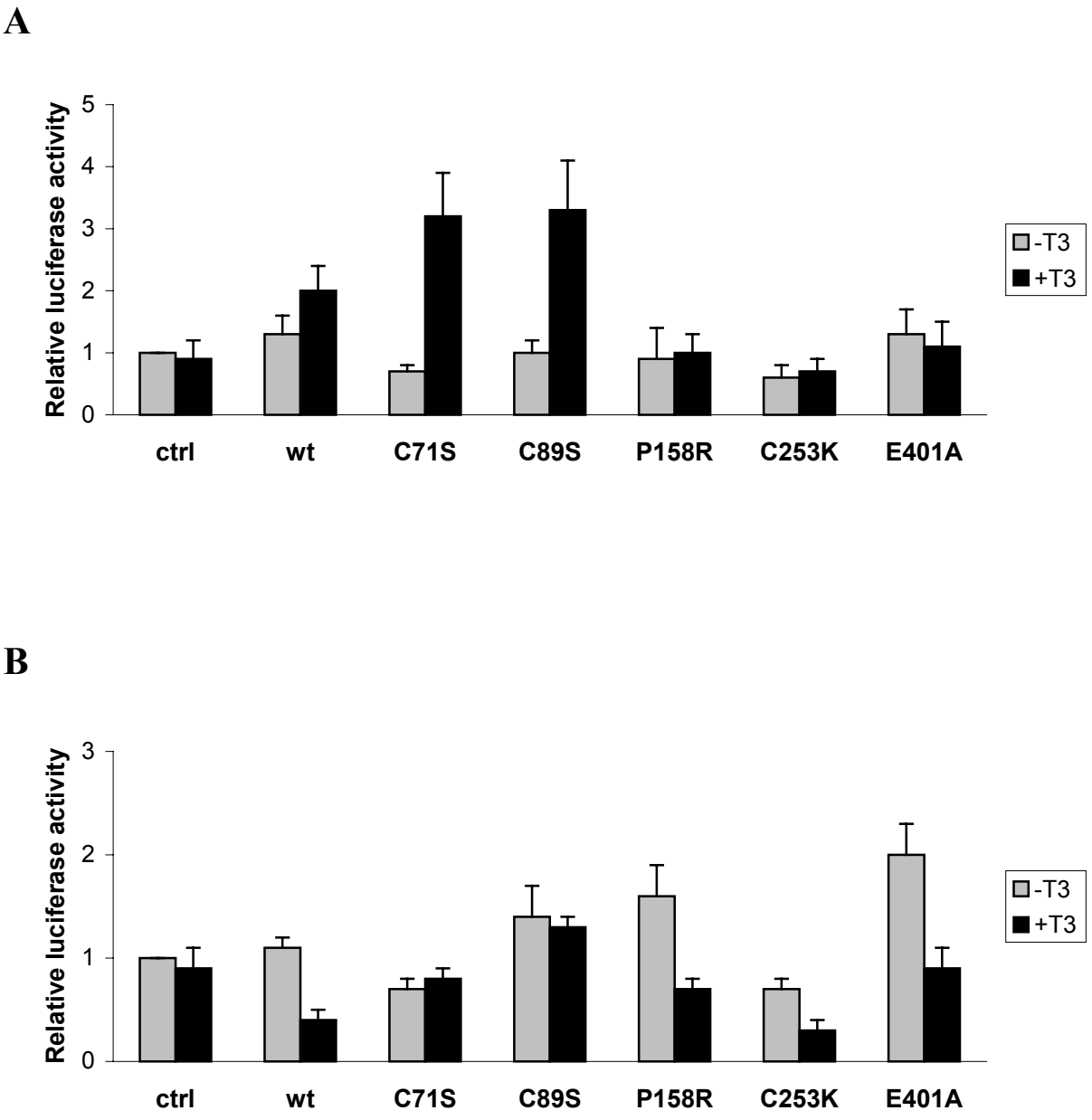


Fig. 26. SRC-1 interaction with TR wild type and TR mutants in mammalian two-hybrid assay

Chimaric protein GAL4-SRC (LXXLL) **(A)** or GAL4-SRC (Qrich) **(B)** was cotransfected with TR wild type (wt) or TR mutants and the 5xUAS-tk-luc reporter plasmid into HepG2 cells. Transfections were performed using 2 μg of reporter plasmid and 0.4 μg of expression plasmid for TR. Cell extracts were incubated in the absence or presence of 100 nM T3 for 24 h and assayed for luciferase activity, which was normalized to total protein concentration. Data are presented as fold induction of empty GAL4 vector (ctrl) in the absence of T3 and are mean +/- SD of at least at three independent experiments performed in triplicates.

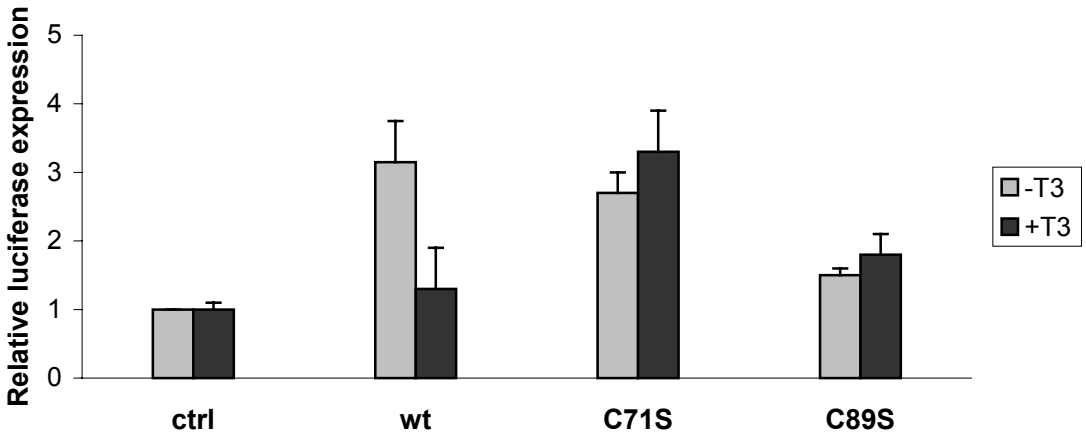


Fig. 27. Effects of TR DBD mutants on T3-dependent suppression of TSHα

HepG2 cells were cotransfected with 2 μg of promoter reporter gene construct and 0.4 μg of both TR and RXR and incubated in the absence or presence of 100 nM T3 for 24 h. Luciferase activity was measured as an indicator of the promoter activity and was normalized to total protein concentration. Data are presented as fold induction of empty GAL4 vector (ctrl) in the absence of T3 and are mean +/- SD of at least at three independent experiments performed in triplicates.

Taken together, the results obtained from this part of the study demonstrate clearly that TR is able to modulate gene expression without direct binding to target promoters.

The present data reveals that the DBD of TR has an additional function besides DNA binding, since the expressional readout altered always considerably after introduction of TR DBD mutants C71S and C89S in the assays.

Noteworthy, it could be shown that TSHα is negatively regulated in response to T3, but a nTRE in the TSHα promoter was not detected. This clearly demonstrates that the DBD of TR mediates negative regulation through a different mechanism than direct DNA binding. Furthermore, the data reveals that SRC-1 has different domains that are able to bind to TR in the presence of T3. However, while these domains both bind TR, they do not seem to exhibit the same function in the context of T3-mediated gene expression.

5 Discussion

Besides the classical mechanism via thyroid hormone receptor response elements, T3 regulates positive and negative gene expression through so far less understood molecular mechanisms. Questions concern the different kinetics of gene expression, regulation of genes lacking TREs and gene suppression in response to T3.
In this study, several aspects of T3-mediated gene regulation were analyzed. The functional analysis of cofactor PGC-1α in T3 regulation and the investigation of T3-mediated negative gene regulation are described and will be discussed.

5.1 GC cells maintain T3-mediated kinetics of gene expression

T3-mediated positive gene regulation is initiated by binding of TR to thyroid hormone response elements (TREs) in the promoter of target genes. Recent *in vivo* analysis revealed that while some genes are rapidly induced within 6 h, others are regulated after a lag period of 12-24 h (Weitzel et al., 2003). Analog expression patterns have been detected in different cell types (Helbing et al., 2003; Shih et al., 2004; Moeller et al., 2005). Of note, “late” induced genes seem to lack TREs, indicating a different activation mechanism in response to T3 in place of the standard model. The absence of common regulatory elements and the lag period, which precedes gene activation, suggest the existence of an intermediate factor. This factor might be up-regulated in the presence of T3 and subsequently regulates the expression of T3 target genes.
In order to analyze the different regulation patterns, a cell culture model was initially established, maintaining the *in vivo* investigated expression profiles. GH3 cells and its subclones have long been used to study the mechanism of thyroid hormone action. In contrast to other cell lines, they express functional thyroid receptors (DeFesi et al., 1985). Following the culture protocol, GH3 and GC cells didn't show the problem of rapidly progressing to hormone-autonomy after adaptation to serum-free cultivation, as it has been observed for GH3 cells (Riss et al., 1989). However, weaker T3 susceptibility was observed for GH3 cells than for GC cells, therefore the latter cell line was chosen for further studies. GC cells have been reported to maintain expression kinetics similar to those of hepatocytes (Miller et al., 2001). Gene expression patterns of several “early” and “late” induced genes were assessed and all of them showed comparable expression kinetics to rat liver (Fig. 5). Thus, the data

verifies that the GC cell line is a suitable cell line to study T3-mediated "early" and "late" induced gene expression.

5.2 T3-mediated mRNA up-regulation is due to *de novo* synthesis of mRNA

In addition to transcriptional mechanisms, the stability of mRNA is another important factor for controlling the level of gene expression (Brennan & Steitz, 2001). Many mRNAs do not have a fixed half-life time, but their stability is changed in response to environmental factors. In mammalian cells, mRNA half-life time can range from 20 min to 24 h and even small differences of mRNA stability alter the abundance of a given mRNA and therefore correlating protein expression dramatically (Wilusz et al., 2001). A variety of different external factors like hormones or growth factors have been shown to affect mRNA stability. Estrogen for example stabilizes the vitellogenin mRNA in amphibian liver (Brock et al., 1983). Therefore, it was tested whether T3 administration influences half-life time of mRNAs, which show an "early" or "late" induced expression in response to T3. As illustrated in Fig. 6 and 7, the half-life of PGC-1α or ANT2 mRNA was not altered in the presence of T3. Whereas ANT2 mRNA degrades as expected with a half-life time of 24 h, PGC-1α mRNA has a half-life time of ~ 90 minutes, consistent with data for other factors of the transcription machinery (Reichwald et al., 2000).
These results clearly demonstrate that an influence of T3 on the regulation of the investigated genes other than *de novo* mRNA transcription can be excluded.

5.3 T3 regulates PGC-1α gene expression via a TRE at – 4 kb

PGC-1α was originally identified as a transcriptional coactivator of the nuclear receptor PPARγ and regulates the activity of several nuclear receptors and other transcription factors (Puigeserver et al., 1998). PGC-1α is involved in the subsequent regulation of several T3-controlled metabolic pathways like fatty acid oxidation or gluconeogenesis (Knutti & Kralli, 2001). Hepatic PGC-1α mRNA is upregulated within 6 h after T3 treatment of hypothyroid rats (Weitzel et al., 2003). This rapid induction suggests that the gene is induced in a direct response to TR binding. To investigate the molecular mechanism underlying the regulation of PGC-1α by T3, the 5' flanking region of the PGC-1α promoter was analyzed for the presence of elements that bind TR. Using a one-hybrid assay it was revealed, that a - 4 kb region of the PGC-1α promoter confers luciferase activity (Fig. 8). A putative element at -4025/-4010 was identified and it was consistently shown, that TR binds to this element in

band shift assays (Fig. 9). The hormone response elements for TRs have been mainly found in the proximity of the transcription start site. The location of the PGC-1α TRE at a distance of 4 kb upstream from the start of transcription is yet not unprecedented. Thyroid response elements have been identified for example at a distant of - 6.5 kb for the tadpole xBTEB1 gene or at -9 kb in the rat Hr promoter (Furlow et al., 2002; Thompson et al., 1997).
This report shows for the first time, that PGC-1α is a direct target of T3, which regulates PGC-1α expression via TR binding to a TRE at – 4 kb upstream of the transcription start site.

5.4 Successful knockdown of PGC-1α in GC cells

Since late induced genes seem to lack TREs, several promoters were analyzed for other regulatory sequence elements, but a common one could not be detected (data not shown). Absence of a mutual sequence motif within late induced gene promoters favors a DNA binding independent regulation mechanism. Cofactors, which interact with a variety of different transcription factor and do not bind themselves to DNA, are therefore likely candidate factors mediating “late” induction. Its expression pattern and characteristics have implicated PGC-1α in the “late” induction of genes in response to T3 (Weitzel et al., 2003).
To determine a potential role of PGC-1α in mediating “late” induced gene expression, an RNAi technique was chosen to knockdown PGC-1α in GC cells. Success of RNAi depends on the identification of optimal transfection conditions as well as the half-life time of mRNA and protein of the specific target gene. It is assumed that the expression of target genes can be totally reduced by siRNA (McManus & Sharp, 2002).
In this work, the expression of PGC-1α was successfully inhibited in GC cells by transient transfection of siRNA oligonucleotides of 21 nucleotides length. A high proportion of transfected cells was guaranteed under optimized conditions. After transfection of 200 nM siRNA, relative PGC-1α expression was reduced by 5- to 10-fold. The response of GC cells to the selected siRNA was rather fast and efficient. As indicated, PGC-1α mRNA was already reduced 4 h after transfection, consistent with the rather short half-life of PGC-1α mRNA (see 4.2). Time course analysis showed, that that the level of PGC-1α mRNA remained low until the fifth day after transfection (Fig. 13A).
Protein stability seems to have a more important influence on the RNAi effect than the amount of present protein (Harboth et al., 2001). The half-life time of PGC-1α protein is 2.28 h as determined by Puigserver and colleagues (2001). In accordance to the fact that a short half-life of protein supports its knockdown, it was shown, that PGC-1α protein was

undetectable in Western blot experiments after siRNA transfection of GC cells (Fig. 13B). PGC-1α was therefore successfully down-regulated in the present study.

5.5 Knockdown of PGC-1α impairs several T3-induced metabolic pathways

Although recent data from PGC-1α deficient mice reveal that this cofactor might not be required for mitochondrial biogenesis per se, it is absolutely necessary for the expression of a large number of mitochondrial genes (Leone et al., 2005; Lin et al., 2004). Furthermore, PGC-1α is crucial in the control of hepatic gluconeogenesis, the most important source of *de novo* glucose production in higher organisms. Fasting activates PGC-1α in liver, which consequently coactivates key hepatic transcription factors, such as HNF-4α or FOXO1, thereby stimulating transcription from target promoters. Simultaneously, liver ceases using glucose as an energy supply and changes to β-oxidation of fatty acids. The increase in fatty acid β-oxidation is also under the control of PGC-1α (Herzig et al., 2001; Yoon et al., 2001; Puigserver et al., 2003). PGC-1α deficient mice show reduced mRNA levels of gluconeogenic and fatty acid oxidation enzymes (Koo et al., 2004).

Consistent with this data, reduced mRNA levels of key enzymes were observed in gluconeogenesis, fatty acid β-oxidation and mitochondrial biogenesis 24 h after knockdown of PGC-1α (Fig. 16). All of the described pathways are also regulated by T3.

Taken together, this report therefore accomplished to show that PGC-1α, as direct target of T3 action, is regulated within 6 h via its TRE at –4 kb and is consequently involved in the induction of different T3-regulated metabolic pathways (Fig. 28).

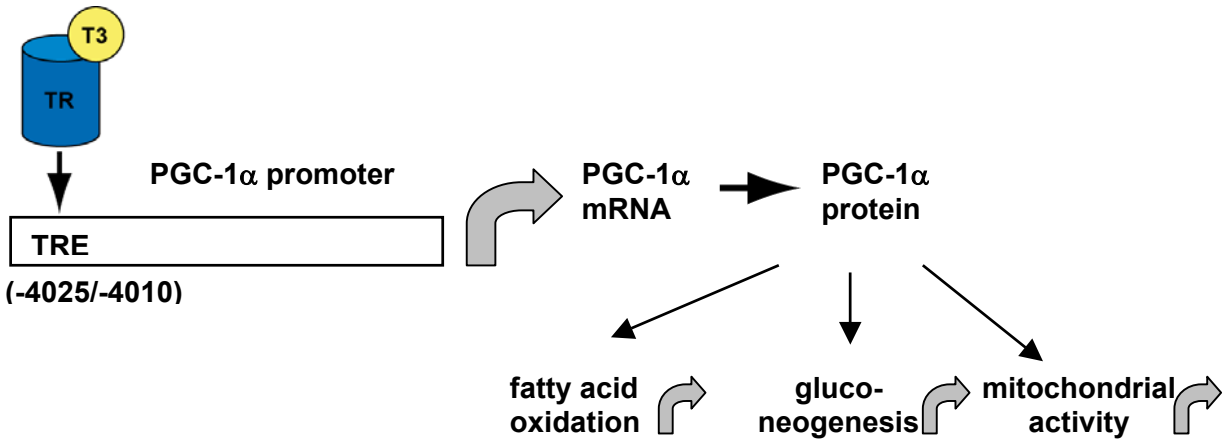


Fig. 28. Role of PGC-1α in T3-regulated metabolic pathways

PGC-1α, as direct target of T3 action, is regulated via its TRE at –4 kb and is consequently involved in the induction of different T3-regulated metabolic pathways.

5.6 Knockdown of PGC-1α does not effect T3-mediated "late" gene expression

T3-mediated gene expression patterns were investigated in siRNA-treated cells versus mock-incubated cells. The analysis focused on T3 target genes that had already been shown to be similarly regulated in GC cells and *in vivo* (see above). No alteration in expression patterns was detected in response to PGC-1α knockdown. Neither the time course nor the intensity of T3-mediated induction changed in siRNA versus mock treated cells (Fig. 17). To exclude a compensational up-regulation of other proteins, the mRNA levels of several transcription factors or cofactors like NRF-1, NRF-2 or PGC-1β were investigated (data not shown). However, a back-up system for the loss of PGC-1α was not detected, in line with results from knockout animals (Lin et al., 2005).

Taken together, these data indicate that T3-mediated gene expression patterns are most likely coordinated independently of PGC-1α. Nevertheless, the possibility cannot be excluded, that the sensitivity of the Western blot analysis was not high enough to pick up a small amount of PGC-1α protein that could have residual activity. Remaining PGC-1α protein in GC cells might be enough to preserve "late" induced gene expression. Interestingly, mitochondrial transcription factor Tfam showed the same magnitude of expression in the absence of T3 (Fig. 16). Since PGC-1α was shown to coactivate transcription factors that subsequently regulate Tfam (Virbasius & Scarpully, 1994), an unaltered expression of Tfam in siRNA treated cells might indicate the presence of residual PGC-1α protein. However, since mRNA levels of other target genes are reduced in cells where PGC-1α is down-regulated, it appears more likely that the expression of Tfam is ensured additionally by alternative mechanism(s).
Even though an altered gene expression pattern was not detected after knockdown of PGC-1α, it cannot be ruled out that the cofactor might play a role in the delayed regulation of other T3-target genes. However, recent data indicates that PGC-1α is less important for mitochondrial biogenesis than expected (Arany et al., 2005; Leone et al., 2005). Latest data from Wright and coworkers demonstrate that mitochondrial proteins with a short half-life increase as fast as, or faster than, PGC-1α protein after exercise in skeletal muscle. Therefore, enhanced expression of PGC-1α seems not to initiate the mitochondrial biogenesis but to sustain and enhance it (Wright et al., 2006).

Understanding how T3 induces different expression patterns is a problem unlikely to be straightforward or easy to solve, if one takes into account that several T3-mediated pre- and

post-transcriptional processes additionally influence activation. In view of the complex interactions and pathways of signal transduction and hormone action, it might be difficult to distinguish how one stimulus elicits a particular response.
However, the initial assumption that a direct activation via TR binding to a functional TRE is responsible for the "early" expression, whereas the "late" expression is initiated by a cofactor like compound seems further very likely. Future investigations should continue to investigate cofactors that show T3-mediated regulation within 6 h as requested for an intermediate factor. A following investigation should therefore target PGC-1β as a candidate factor. Taken into account that possibly more than one mechanism exist for these important metabolic pathways, NRF-1 is also considerable for further knockdown strategies.

In conclusion, the present data further confirmed that complex expression patterns of T3 *in vivo* are maintained in GC cells. It was demonstrated for the first time that T3-mediated regulation of cofactor PGC-1α involves a pTRE far upstream of the transcriptional start site. Therefore, the data revealed that PGC-1α gene expression within 6 h after administration of T3 is regulated via direct TR binding to the promoter. Knockdown of PGC-1α showed that the cofactor is dispensable for coordinating "late" gene induction in response to T3. It could be demonstrated however that the cofactor is involved in the induction of different T3-regulated metabolic pathways.
The successful establishment of the siRNA technique in GC cells should provide the framework to investigate other factors that might be important in T3-mediated "late" induction of genes.

5.7 TSHα suppression is cell unspecific and depends on altered histone acetylation

The present study further investigated several aspects of T3-mediated gene suppression.
T3 mediates negative regulation of gene expression as an essential part of its physiological action. Three major mechanisms have been proposed to explain transcriptional control of negatively regulated genes in response to T3 (reviewed in Lazar, 2003). Whether the regulation involves a DNA binding-dependent or independent mechanism remains a controversial issue (see Fig. 3).
In a first model, repression results from binding of TR to negative regulatory elements. In contrast to positive gene regulation, TR recruits CoAs in the absence of T3 whereas the liganded receptor tethers CoRs to the promoter. The proposed negative regulatory elements

are generally located close to, and often downstream of the transcriptional start site (Sasaki et al., 1999; Hashimoto et al., 2006; Kim et al., 2005; Villa et al., 2004). However, a consensus sequence for nTREs has not been established yet, and the precise mechanism involved in the inverted mechanism of CoA and CoR binding has remained unclear.

A second model suggests that regulation is mediated by TR interaction with proteins bound to the promoter as opposed to direct TR binding to DNA sequences. Protein binding could alter the receptor conformation and result in reversed CoR/CoA recruitment upon ligand binding. Alternatively, TR might block signalling or compete for limited cofactors at the promoter.

In a third model, unliganded TR in solution activates gene expression by recruiting CoRs and HDACS from the promoter resulting in a net increase of histone acetylation and in transcriptional stimulation. In the presence of T3, CoRs dissociate from the receptor and return to the promoter, consequently repressing gene expression.

In addition, posttranslational modification of TR and cofactors may be a mechanism to mediate, at least in part, the specificity and selectivity in negative gene regulation. Consistently, it was reported that sumoylation and phosphorylation of nuclear receptors and cofactors modulate the regulation of target gene activity (Pascual et al., 2005; Bowan et al., 2000). TR signalling might be further influenced by interaction with various other signal pathways (Moeller et al., 2005; Hiroi et al., 2006).

To validate one of the described models for negative gene regulation, different experiments were carried out. Gene expression in response to T3 was investigated using the positively regulated mGPDH promoter and the negatively regulated TSHα promoter. TRα1 was chosen to study the general mechanism of T3-dependent negative regulation, since it is likely hat TRβ acts rather tissue-specific (Langlois et al., 1997).

The present study demonstrated that within rat and human cell lines from hypothalamus, liver or kidney, activation and repression remained the same for the respective promoter element (Fig. 18 and data not shown). Therefore, positive and negative gene regulation in response to T3 is generally not cell type dependent, but it is the configuration of the DNA, which accounts for either gene activation or suppression.

Since the existence of a negative regulatory element in the luciferase reporter gene has been reported (Maia et al., 1996), these experiments were compared using a second reporter gene system with chloramphenicol acetyl transferase (CAT) (Fig. 18). However, the use of either reporter system did not change the outcome in these studies. Of note, no alteration of the basic

luciferase reporter plasmid was detected after cotransfection of VP16-TR, which demonstrates the lack of a negative regulatory element in the luciferase gene.

It has been reported that T3-mediated gene regulation is associated with changes in histone acetylation (Lee et al., 2003; Sanchez-Pacheco & Aranda, 2003). Therefore, histone acetylation patterns of positively and negatively regulated promoters were compared in response to T3 (Fig. 19). The picture obtained for the negatively regulated promoter was inverted compared to the one received for mGPDH, reflecting the transcriptional effects seen in transient expression assays. Thus, for negatively regulated genes histone acetylation and deacetylation of the promoter appears to play a critical role in stimulation by the unoccupied receptor and in ligand-dependent suppression.

5.8 TSHα suppression is independent of direct TR binding to regulatory elements

Several reports have suggested that the β subunit of the TSH gene contains a nTRE immediately downstream of the transcriptional start site (Satoh et al., 1996; Sasaki et al., 1999). The "Z" region within this element has also been detected in the negatively regulated necdin gene (Nygard et al., 2006). However, very recent data indicates that this sequence is dispensable for T3-dependent inhibition in the TSHβ promoter, since deletion or mutation does not impair suppression (Matsushita et al., 2007). Additionally, the existence of a negative regulatory element in the TSHα subunit gene remains controversial. While Tagami and coworkers reported the absence of a nTRE in the TSHα promoter (Tagami et al., 1999), other studies suggested that TR interacts specifically with the promoter (Shibusawa et al., 2003a).

Binding of TR to DNA for the mGPDH and the TSHα was investigated in a one-hybrid assay (Fig. 20). Using the VP16-TR fusion protein, it was shown that mGPDH is activated independently of T3 administration. This expression results from binding of TR to a TRE in the mGPDH promoter, which has been already characterized (Weitzel et al., 2001a). However, no alteration in gene expression for the TSHα reporter gene construct was detected, demonstrating the lack of TR recognition sites in this promoter. The results of this study validate the idea that negative regulation in response to T3 does not require direct interaction of TR with promoter regulatory elements.

These results are therefore consistent with previous reports, proposing a DNA-binding independent mechanism for the TSHα subunit gene (Tagami et al., 1999).

The existence of pTRE-like sequences within negatively regulated genes has remained controversial, as they have been described for relatively few genes. Compared to interaction at pTREs, TR shows only weak binding to the described negative regulatory elements in EMSAs (Sasaki et al., 1999; Hashimoto et al., 2006). This casts doubt on their functional importance in T3-dependent negative regulation but rather suggests that these low affinity TREs could contribute to the overall transcriptional activity.

Similar to the thyroid receptor, negative regulation of the glucocorticoid receptor (GR), another member of the nuclear receptor superfamily, is poorly understood. Most genes that are negatively regulated by GR do not contain a classical glucocorticoid response element (GRE). Of note, a DNA binding-independent mechanism has been suggested for the glucocorticoid dependent repression. This regulation was first described for AP-1 and was thought to involve direct protein-protein interactions between GR and AP-1 (Yang-Yen et al., 1990; Nissen & Yamamoto, 2001). It has been further shown, that glucocorticoid-mediated activation can be dissociated from repression by introduction of point mutation A458T into GR. This receptor mutant is defective in DNA binding and transcriptional activation from classical GRE sites, whereas repressive effects on several target genes remain unaffected (Heck et al., 1994; Reichhardt et al., 2001). $GR^{dim/dim}$ mice, which carry this mutation, are viable, whereas GR knockouts mice are not, revealing the *in vivo* relevance of DNA binding-independent activities of GR (Cole et al., 1995; Reichhardt et al., 2001). However, the precise mechanism by which GR mediates negative repression remains elusive.
Together the results of this study and these reports strongly support the concept that negative regulation can occur independently of a DNA binding mechanism for nuclear receptors.

5.9 Restriction of TR to DNA induces transcriptional activation

The significance of the DBD and other receptor domains in terms of T3-mediated regulation was further investigated by using various TR mutants, which possess functional disruption in the investigated domains. As expected, introduction of point mutations in TR, impairing either DNA binding or cofactor interaction, strongly altered activation of the DR+4 reporter plasmid (Fig. 22). Receptors with mutated zinc fingers in the DBD or totally lacking this domain showed impaired activation in response to T3. Activation was also abrogated using the CoA binding mutant E401A, whereas no interference was observed for the CoR binding mutants. This data is consistent with the notion that DNA binding and interaction with coactivators is essential for positive regulation (reviewed in Yen, 2001).

Restraint of the TR wild type and TR mutants to DNA in the GAL4 assay led to activation of gene expression in the presence of T3 (Fig. 23). In contrast, liganded TR, not directly interacting with the DNA, was able to negatively regulate gene expression (Fig. 24). This data remarkably indicates that forced restriction of TR to DNA always mediates activation in response to T3 irrespective of the promoter context. The deletion or destruction of the DBD in TR dramatically increased activation in response to T3. These results point out that the DBD of TR is not only crucial for interaction with DNA. Seemingly, this domain serves as binding site for other proteins that influence transcriptional regulation.
Use of mutant E401A abrogated T3-mediated up-regulation, consistent with the notion that coactivator recruitment is critical for activation (reviewed in Yen, 2001).
Taken together this data clearly indicates that a forced restriction of TR to DNA always induces transcriptional activation. Therefore, the presented data further questions a DNA binding dependent mechanism for negative regulation.

5.10 DBD of TR has an additional function besides DNA binding

TR-mediated gene regulation without direct DNA interaction can be recapitulated using GAL4-NCoR and GAL4-SRC constructs in the context of a heterologous promoter.
As shown in Fig. 24, the activity of the luciferase reporter gene was stimulated by cotransfecting VP16-TR and NCoR-F1, and this effect was reversed in the presence of T3. The IDC region in the extreme C terminus of NCoR has been shown to interact with other nuclear receptors in a ligand-independent way (Oberfield et al., 1999). However, this fragment is not sufficient for binding of TR. While both SRC fragments recruit TR in the presence of T3, a higher activity was seen for interaction of SRC-LXXLL with VP16-TR. SRC-LXXLL contains the known nuclear receptor-interacting motif (Heery et al., 1997; McInerney et al., 1998). The Q-rich region of SRC comprises many glutamines and three amino acid motives, which are conserved between members of the SRC family. The region was shown to interact with the activation function AF-1 in the amino-terminal domain of nuclear receptors (Christiaens et al., 2002). Interestingly, AF-1 was reported to be missing in the TRα isoform of the thyroid receptor (Oberste-Berghaus et al., 1999). However, the results of this study indicate for the first time that TRα binds to the Q-rich region, although to a smaller extent.

The effect of mutations in different TR domains was assessed in a two-hybrid assay with NCoR-F1 and both SRC fragments (Fig. 25 and 26).

After cotransfection of TR wild type with GAL4-NCoR, a ligand-independent activation was observed, which was reversed in the presence of T3. However, TR mutations that selectively disrupted interaction with CoRs prevented basal activation and down-regulation in these experiments. Additional cotransfection of NCoR further enhanced activation (Fig 25B), but only in the presence of TR wild type, confirming that these effects are dependent upon interactions with the receptor. These experiments validate previous findings that corepressor enhance ligand-independent activation (Santos et al., 2006; Tagami et al., 1997). This data therefore contradicts recent reports by Nakano and coworkers (2004), who postulated that corepressors were not critical for gene activation in the absence of T3.

By studying cotransfection experiments of DBD mutants C71S and C89S with GAL4-NCoR, it was found that the intact DBD was critical for T3-mediated gene suppression, although TR did not interact directly with the DNA in this system (Fig. 25).
The importance of the DBD was further observed in cotransfections experiments of TR together with either GAL4-SRC (LXXLL) or GAL4-SRC (Qrich). TR wild type together with GAL4-SRC (LXXLL) did not modify basal activity after administration of T3, whereas down-regulation was observed for the GAL4-SRC (Qrich) fragment (Fig. 26). This indicates that even though TR binds to both regions in a ligand-dependent manner, the SRC domains seem to fulfil different tasks when interacting with the receptor. Introduction of point mutations in the DBD of TR mutants increased T3-mediated gene expression for both SRC fusion proteins in these cotransfection experiments. This data strongly supports the idea, that the DBD is not only important for DNA binding but also in terms of protein-protein interaction.

5.11 DBD of TR is crucial for protein-protein interaction

The ability of mutant C71S and C89S to exert T3-mediated negative regulation of the TSHα promoter was further investigated in this study. Tagami and colleagues demonstrated that a TR fragment lacking the DBD was sufficient to mediate negative regulation of the TSHα promoter. Mutant TR induced activation in the absence of ligand and the addition of T3 resulted in ligand-dependent repression (Tagami et al., 1999). In contrast, several other studies have reported that the presence and integrity of the C domain was critical to confer negative regulation (Shibusawa et al., 2003a; Nakano et al., 2004).
The data shown in this study reveal that an intact DNA binding region is in fact important for negative regulation of the TSHα promoter (Fig. 26). However, since no binding of the

receptor to the DNA in previous experiments could be detected (Fig. 20), the data indicates that an intact DBD could be rather crucial for protein-protein interaction than for DNA binding. Collectively, this study supports the notion that the DBD appears to bind proteins involved in mediating regulation of T3 target genes. These proteins may exert repressive functions since mutations disrupting the DBD resulted in enhanced gene transcription in response to T3.

The DBD is the most conserved domain among members of the nuclear receptor superfamily (Mangelsdorf et al., 1995). Extensive studies have shown that this domain is crucial for DNA binding as well as for receptor dimerization (Zhang et al., 2000). Regulation of gene expression by nuclear receptors is considered to result from recruitment of both CoRs and CoAs to the LBD. Much less is known about regulation by cofactors that bind to the DBD.
Lately some proteins have been described that interact with the C domains of nuclear receptors. The DBD has been shown to interact with other transcriptional factors such as NF-κB and AP- 1 (Tao et al., 2001) and to associate with tissue-specific coactivator GT198 or the histone acetyltransferase PCAF (Ko et al., 2002; Blanco et al., 1998). Recent reports indicate that the DBD of TR binds to the corepressors PSF and GRP1, and that these interactions are not modified by the presence of thyroid hormone (Mathur et al., 2001; Poirier et al., 2005). It was reported previously that the DBD of TR interacts with HDAC2 in a T3-dependent manner on the TSHβ promoter (Sasaki et al., 1999). Furthermore, TR associates with the transcription factor CREB through its DBD. As a result, TR is tethered to promoters containing CREB response elements (CRE) via the physical interaction with CREB, leading to the repression of cAMP-mediated transcription. Reciprocally, CREB antagonizes gene expression regulated by TR (Méndez-Pertuz et al., 2003; Furomoto et al., 2005).
These latter findings are of special interest since several lines of evidence point to a critical role for transcription factor CREB in negative regulation of the TSHα and TSHβ, which both contain CRE sites close to the transcription start site (Tagami et al., 1999; Méndez-Pertuz et al., 2003). Of note, PGC-1α also contains a CRE site at –146 bp upstream of the transcription start site (Herzig et al., 2001), and it was observed in this study that CREB influences regulation of the PGC-1α promoter in response to T3 (data not shown).

Shibusawa and colleagues generated a non-TRE binding TRβ mutant (GS125), in which two amino acids were mutated, preventing recognition of TRE sequences. They showed that the GS125 mutant impaired negative gene regulation in response to T3 (Shibusawa et al., 2003a).

However, mice carrying this mutant displayed some but not all abnormalities compared to common TR knockout models (Shibusawa et al., 2003b). This indicates that it is difficult to conclude that impaired negative regulation of DBD mutants depends on the inability of TR to bind to DNA.

The present study strongly indicates that changes in the conformation of the DBD impairs the recruitment of interacting proteins necessary for gene suppression rather than a required binding of TR to the promoter. Therefore, it contradicts previous studies, which concluded that loss of transcriptional activities was specifically caused by lack of DNA binding (Shibusawa et al., 2003a; Nakano et al., 2004). It can be speculated that the above described GS125 receptor mutant blocks the interaction with some cofactors off-DNA, whereas the binding of others is still assured. This assumption explains the partial impairment of negative gene regulation.

5.12 Proposed mechanism for T3-mediated negative gene regulation

The new data presented in this study is consistent with just published results from Matsushita and coworkers (Matshushita et al., 2007). In previous studies, this group had postulated that DNA binding is necessary for T3-mediated negative gene regulation (Nakano et al., 2004). However, in this very recent paper they report that the interaction between the DBD of TR and other proteins is crucial in mediating negative gene regulation of TSHβ in response to T3. According to their data, the putative regulatory element in the TSHβ promoter is dispensable for T3-mediated suppression and transcription factor GATA2, but not unliganded TR, is the major transcriptional activator of the TSHβ gene. In their model, the DBD of TRβ interacts with GATA2 in a T3-independent manner. TR and GATA2 both associate with TR associated protein 220 (TRAP220). Coactivator TRAP220 lacks intrinsic histone acetyltransferase activity but is a component of the TRAP/SMCC complex that regulates the function of CTD in RNA pol-II (Ito & Roeder, 2001). It was speculated that T3 binding to TR destabilizes the interaction of TRAP/SMCC complex with GATA2, resulting in a dissociation of TRAP220. At the same time, liganded TR recruits HDACs or mediates dissociation of HAT-related compounds, leading to negative regulation of the TSHβ promoter.

This proposed model of negative regulation involving interaction between DNA bound GATA2 and TR through its DBD appears to be similar to the gene suppression of GR via interaction with NF-κB. Many inflammatory genes that are repressed by glucocorticoids are transcriptionally regulated by NF-κB (Barnes et al., 1997). Like TR, GR interacts with the

transcription factor through its DBD and inhibits NF-κB-mediated activation in the presence of the ligand (Scheinman et al., 1995). The crosstalk of GR and NF-κB also includes recruitment of a protein-complex almost identical to the TRAP/SMCC complex and histone deacetylation occurs in the presence of the ligand (Ito et al., 2000; Naar et al., 1999).
Gene suppression via protein-protein interaction between DNA-binding transcription factor and nuclear hormone receptors could therefore be a common regulation mechanism.

As consequence from the characterization of positive TRE sequences it was postulated, that negative regulation of transcription by T3 occurs via negative TRE sites. In practice, there have been several studies reporting the existence of nTREs, but the question has remained controversial as the consensus binding site is variable and nTREs have been only described for relatively few genes. Moreover, other experimental data suggest that suppression of genes in presence of T3 is DNA binding-independent.
Collectively the data in this study successfully revealed that the DBD of TR is not required for DNA binding in the context of negative regulation. Based on the present data and recent studies, it can be considered that the interaction between TR receptors and proteins like transcription factors is central to T3-mediated suppression of the TSHα gene.
However, it cannot be excluded that alternative mechanisms exist by which genes are negatively regulated in response to T3. Further studies have to address the question, if a suppression mechanism based on protein-protein interaction via the DBD of TR and GATA2 is also detectable for genes, for which negative regulatory elements have been reported.
Given the complexity of the system, the studies merit further evaluation to broaden our understanding of the T3-dependent gene repression mechanism.
However, in view of the results presented in this thesis and other recent reports, a DNA binding dependent mechanism for negative gene regulation of TSHα and TSHβ can be finally ruled out. The data strongly indicates a new proposed mechanism via interaction of the TR DBD with a second transcription factor, which is presented in Fig. 29.

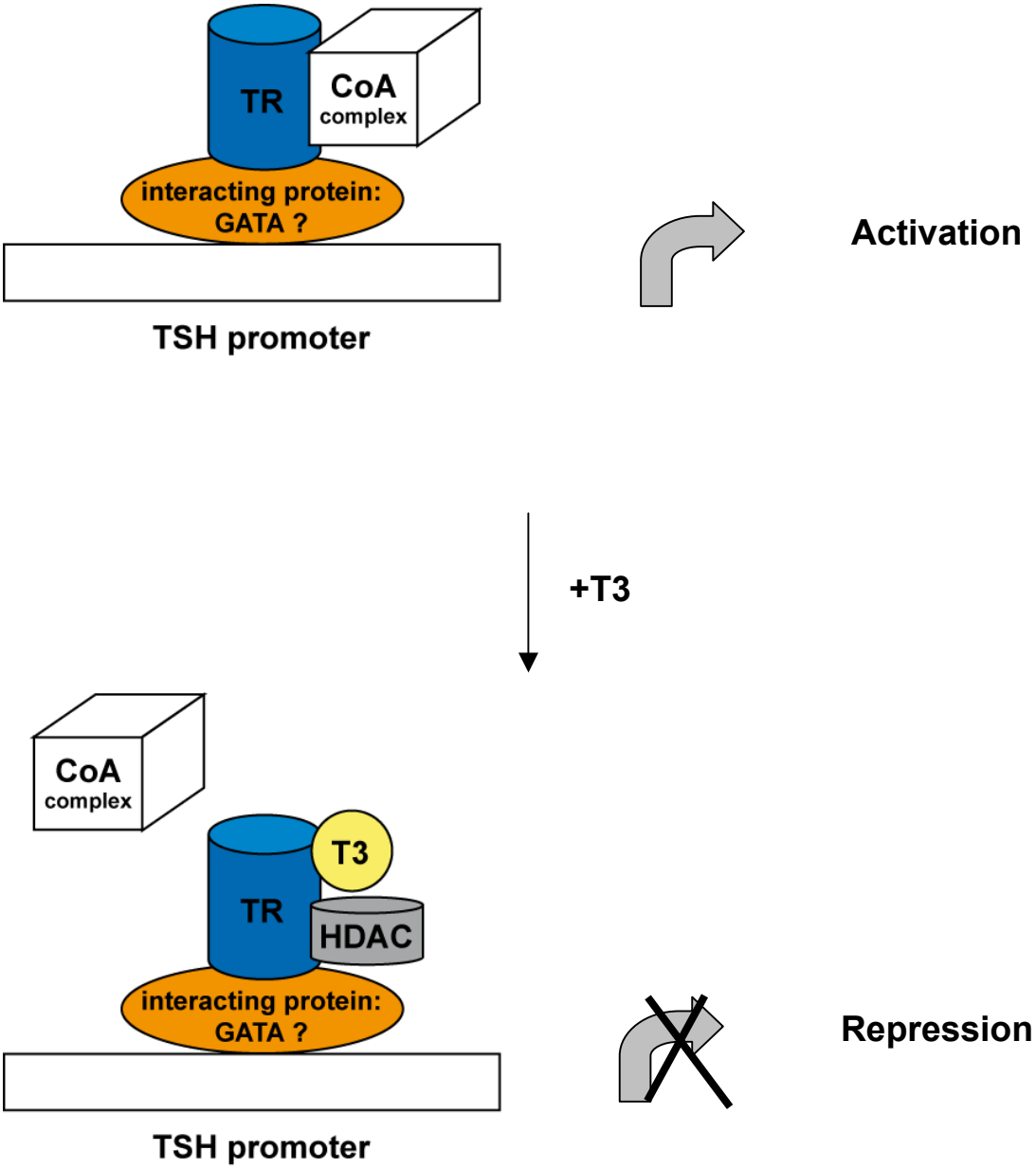


Fig. 29. Proposed mechanism for T3-mediated negative gene regulation

In the absence of T3, TR is recruited to the promoter via interaction of the TR DBD and GATA2. TR and GATA2 both associate with a CoA complex. Binding of T3 destabilizes the interaction with the CoA complex, which dissociates from the promoter. Liganded TR recruits HDACs, leading to negative regulation of the TSH promoter in the presence of T3.

6 Summary

Thyroid hormone (T3) controls growth, development and metabolism in virtually all mammalian tissues. The molecular actions of T3 are mediated by thyroid hormone receptors (TRs). TRs bind to DNA response elements (TREs) located in regulatory regions of target genes, thereby inducing gene expression upon ligand binding. However, several aspects of gene regulation in response to T3 indicate alternative regulation mechanisms. First, TREs appear to be absent in several T3 target genes, which show a "late" expression kinetic compared to "classical" T3 target genes. This indicates the possible involvement of a so far unknown intermediate regulation factor. Second, T3 action resembles effects regulated in fact by cofactor PGC-1α, which suggests the participation of PGC-1α in the control of T3-mediated gene regulation. In addition, PGC-1α was also postulated to mediate the "late" expression of T3 target genes without TREs. The molecular interaction of T3 and PGC-1α is still unelucidated. Third, molecular action of TR also involves suppression of important genes upon T3 binding. However, the molecular mechanism for negative gene regulation is yet undetermined. Therefore, the aim of this study was to investigate molecular mechanism(s) of T3 action besides the "classical" way via TREs.

To determine the role of PGC-1α as a possible intermediate regulation factor of T3 action, first a cell culture model was established, mimicking the same T3-mediated kinetics of gene expression as seen *in vivo*. Then, a knockdown strategy for PGC-1α via siRNA was chosen. By siRNA, the mRNA level of PGC-1α was reduced to 10% of its initial concentration and consequently PGC-1α protein was undetectable. The gene expression of a series of T3-controlled metabolic pathways was subsequently reduced, indicating PGC-1α as an intermediate regulation factor of T3 action. In accordance with this observation, sequence analysis of the human PGC-1α promoter revealed a putative TRE, which was confirmed in transient transfection experiments and electrophoretic mobility shift assays. However, the data also showed surprisingly that "late" expression kinetics of other genes in response to T3 were still maintained despite PGC-1α inactivation, suggesting that other intermediate regulation factors might be involved (e.g. PGC-1β or NRF-1).

Taken together, these experimental data accomplished to show that PGC-1α is a direct target of T3 action. PGC-1α is regulated in reponse to T3 within 6 h via a newly identified TRE at

4 kb upstream of the transcription start site. Subsequently, PGC-1α is involved in the induction of different T3-regulated metabolic pathways.

To investigate the mechanism of T3-mediated negative gene regulation, different one- and two-hybrid transfection assays were performed in various cell types. The histone acetylation pattern of T3 target genes was assessed by chromatin immunoprecipitation. The present data reveals that while T3-mediated gene activation correlates with histone acetylation, gene suppression implies deacetylation of histones. This indicates that the state of acetylated chromatin reflects the transcriptional effects seen in transient expression assays for T3-mediated negative and positive gene regulation. In addition, the study further demonstrates that negative gene regulation is independent of the used cell line.

To show the significance of various receptor regions in terms of repression and transcriptional activation, a number of different TR domain mutants were examined. Specifically, the function of the DNA binding domain was assessed since its role in T3-mediated gene suppression is object of controversial discussions. The here presented experimental data clearly reveals that in contrast to up-regulated genes, TR does not bind directly to the promoter of negatively regulated genes. Therefore, negative gene regulation is independent of TR binding to suggested negative regulatory elements in target promoters. Noteworthy, it could be successfully shown that the DNA binding domain of TR has in fact additional functions in the context of gene repression upon ligand binding. The data strongly indicates a mechanism in which T3-mediated gene suppression is regulated via direct interaction of the TR DNA binding domain with another up to now unknown regulatory protein instead of promoter DNA.

Taken together, the results of this study demonstrate comprehensively that T3 regulates gene expression by two additional mechanisms besides the “classical” model via binding of TR to pTREs. This report shows for the first time that PGC-1α is regulated via a TRE at -4 kb upstream of the transcription site. Knockdown of PGC-1α indicates an involvement of PGC-1α in the T3-mediated control of several metabolic pathways. Second, this study successfully shows that negative gene regulation upon ligand binding is mediated via a very different mechanism compared to gene activation in response to T3. The presented data contradict the hypothesized existence of negative regulatory elements in many reports. Instead the present study presents evidence that T3-mediated gene suppression involves an interaction mechanism between the DNA binding domain of TR and another regulatory protein (GATA2?). The search of this new regulatory protein is under investigation.

7 Abbreviations

A	adenine
AEBSF	4-(2-aminoethyl)benzylsulfonylflourid
ANT2	adenine nucleotide translocase 2
AOX	acyl coenzyme A oxidase
ATP	adenosin-5'-triphosphate
bp	basepair
BSA	bovine serum albumin
C	cytosine
°C	celsius degree
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
Ci	curie
CoA	coactivator
CoR	corepressor
CRE	cAMP response element
CREB	cAMP response element binding protein
C-terminus	carboxy -terminus
C	cytosine
Cyt c	cytochrome c
DBD	DNA binding domain
DEPC	diethylpyrocarbonate
D-MEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	deoxyribonucleotide triphosphate (dATP, dCTP, dGTP, dTTP)
DR	direct repeat
DTT	dithiothreitol
EMSA	electrophoretic mobility shift assay
EDTA	ethylendiamintetraacetat
FACS	fluorescence activated cell sorter
FCS	foetal calf serum

Fig.	figure
G	guanine
g	gram
G6-Pase	glucose-6-phosphatase
h	hour
HAT	histone acetyltransferase
HBS	hepes-buffered saline
HDAC	histone deacetylase
HEPES	*N*-2-hydroxyethyl-piperazine-*N*'-2-ethanesulfonic acid
HEK 293	human embryonic kidney cells
HepG2	human hepatocyte carcinoma cells
kb	kilobase
l	litre
LB	Luria Betani
LBD	ligand binding domain
M	molar
m	milli
μ	micro
min	minute
mRNA	messenger RNA
mGPDH	mitochondrial glycerol-3-phosphate dehydrogenase
NCoR	nuclear receptor corepressor
NRF	nuclear respiratory factor
N-terminus	amino-terminus
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGC-1α	peroxisome proliferator-activated γ coactivator 1α
RNA	ribonucleic acid
rpm	rounds per minute
RXR	retinoid X receptor
SDS	sodium dodecyl sulphate
SRC-1	steroid coactivator 1
T	thymine

T3	triiodothyronine
T4	thyroxine
TBE	tris-borat-EDTA-buffer
TE	Tris-EDTA-buffer
Tfam	mitochondrial transcription factor A
tk	thymidine kinase
TR	thyroid hormone receptor
TRE	thyroid hormone receptor element
TSH	thyrotropin
U	unit
V	volt
v/v	volume per volume
w/v	weight per volume

8 References

Alland L, Muhle R, Hou HJ, Potes J, Chin L, Schreiber-Agus N, and DePinho RA (1997). Role for N-CoR and histone deacetylase in Sin3-mediated transcriptional repression. Nature *387*: 49-55.

Arany Z, He H, Lin J, Hoyer K, Handschin C, Toka O, Ahmad F, Matsui T, Chin S, Wu PH, Rybkin II, Shelton JM, Manieri M, Cinti S, Schoen FJ, Bassel-Duby R, Rosenzweig A, Ingwall JS and Spiegelman BM (2005). Transcriptional coactivator PGC-1 alpha controls the energy state and contractile function of cardiac muscle. Cell Metab *1*: 259-271.

Barnes PJ and Karin M (1997). Nuclear factor-kappaB: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med *366*: 1066-1071.

Berry DL, Rose CS, Remo BF and Brown DD (1998). The expression pattern of the thyroid hormone response genes in remodelling tadpole tissues defines distinct growth and resorption gene expression programs. Dev Biol *203*: 24-35.

Blanco JC, Minucci S, Lu J, Yang XJ, K. Walker KK, Chen H, Evans RM, Nakatani Y and Ozato K (1998). The histone acetylase PCAF is a nuclear receptor coactivator. Genes Dev *12*: 1638-1651.

Boelen A, Wiersinga WM and Koehrle J (2006). Contributions of cytokines to nonthyroidal illness. Obesity and nutrition. Current Opinion in Endocrinology & Diabetes *13*: 444-450.

Bowan BG, Garrison N, Weigel NL, and O'Malley BW (2000). 8-Bromo-cyclic AMP induces phosphorylation of two sites in SRC-1 that facilitate ligand-independent activation of the chicken progesterone receptor and are critical for functional cooperation between SRC-1 and CREB binding protein. Mol Cell Biol *20*: 8720-8730.

Breen JJ, Hickok NJ and Gurr JA (1997). The rat TSHbeta gene contains distinct response elements for regulation by retinoids and thyroid hormone. Mol Cell Endocrinol *131*: 137-146.

Brennan CM and Steitz JA (2001). HuR and mRNA stability. Cell Mol Life Sci *58*: 266-277.

Brent GA, Larsen PR, Harney JW, Koenig RJ and Moore DD (1989). Functional characterization of the rat growth hormone promoter elements required for induction by thyroid hormone with and without a cotransfected beta type thyroid hormone receptor. J Biol Chem *264*: 178-182.

Brock ML and Shapiro DJ (1983). Estrogen stabilizes vitellogenin mRNA against cytoplasmic degradation.
Cell *34*: 207-214.

Christiaens V, Bevan CL, Callewaert L, Haelens A, Verrijdt G, Rombauts W and Claessens F. (2002). Characterization of the two coactivator-interacting surfaces of the androgen receptor and their relative role in transcriptional control.
J Biol Chem *271*: 49230-49237.

Cole TJ, Blendy JA, Monaghan AP, Krieglstein K, Schmid W, Aguzzi A, Fantuzzi G, Hummler E, Unsicker K and Schütz G (1995). Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation.
Genes Dev *9*: 1608-1621.

DeFesi CR, Fels EC and Surks MI (1985). L-Triiodothyronine (T3) stimulates growth of cultured GC cells by action early in the G1 period: evidence for mediation by the nuclear T3 receptor.
Endocrinology *116:* 2062-2069.

Denver RJ, Pavgi S and Shi YB (1997). Thyroid hormone-dependent gene expression program for Xenopus neural development.
J Biol Chem *272*: 8179-8188.

Dümmler K, Müller S and Seitz HJ (1996). Regulation of adenine nucleotide translocase and glycerol 3-phosphate dehydrogenase expression by thyroid hormones in different rat tissues.
Biochem J *317*: 913-918.

Elbashir SM, Harboth J, Lendeckel W, Yalcin A, Weber K and Tuschl T (2001). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells.
Nature *411*: 494–498.

Evans MJ and Scarpulla RC (1989). Interaction of nuclear factors with multiple sites in the somatic cytochrome c promoter. Characterization of upstream NRF-1, ATF, and intron Sp1 recognition sequences.
J Biol Chem *264*:14361-14368.

Feng X, Jiang Y, Meltzer P and Yen PM (2000). Thyroid hormone regulation of hepatic genes in vivo detected by complementary DNA microarray.
Mol Endocrinol *14*: 947-955.

Flamant F and Samarut J (2003). Thyroid hormone receptors: lessons from knockout and knock-in mutant mice.
Trends Endocrinol Metab *14*: 85–90.

Friesema EC, Jansen J, Milici C and Visser TJ (2005). Thyroid hormone transporters. Vitam Horm *70*:137-167.

Furlow JD and Kanamori A (2002). The transcription factor basic transcription element-binding protein 1 is a direct thyroid hormone response gene in the frog Xenopus laevis.
Endocrinology *143*: 3295-3305.

Furumoto H, Ying H, Chandramouli GVR, Zhao L, Walker RL, Meltzer PS, Willingham MC and Cheng SY (2005). An unliganded thyroid hormone ß receptor activates the cyclin D1/Cyclin-dependent kinase/retinoblastoma/E2F pathway and induces pituitary tumorigenesis.
Mol Cell Biol *1*: 124-135.

Gao G and Goldfarb M (1995). Heparin can activate a receptor tyrosine kinase.
EMBO J *14*: 2183-90.

Harborth J, Elbashir SM, Bechert K, Tuschl T and Weber K (2001). Identification of essential genes in cultured mammalian cells using small interfering RNAs.
J Cell Science *114*: 4557-4565.

Hashimoto K, Yamada M, Matsumoto S, Monden T, Satoh T and Mori M (2006). Mouse sterol response element binding protein-1c gene expression is negatively regulated by thyroid hormone.
Endocrinology *147*: 4292-4302.

Heck S, Kullmann M, Gast A, Ponta H, Rahmsdorf HJ, Herrlich P and Cato AC (1994). A distinct modulating domain in glucocorticoid receptor monomers in the repression of activity of the transcription factor AP-1.
EMBO J *13*: 4087-4095.

Heery DM, Kalkhoven E, Hoare S and Parker MG (1997). A signature motif in transcriptional co-activators mediates binding to the nuclear receptors.
Nature *387*:733-736.

Heinzel T, Lavinsky RM, Mullen TM, Söderstrom M, Laherty CD, Torchia J, Yang WM, Brard G, Ngo SD, Davie JR, Seto E, Eisenman RN, Rose DW, Glass CK and Rosenfeld MG (1997). A complex containing N-CoR, mSin3 and histone deacetylase mediates transcriptional repression.
Nature *387*: 43-48.

Helbing CC, Werry K, Crump D, Domanski D, Veldhoen N, and Bailey CM (2003). Expression profiles of novel thyroid hormone-responsive genes and proteins in the tail of Xenopus laevis tadpoles undergoing precocious metamorphosis.
Mol Endocrinol *17*: 1395-1409.

Herwig S and Strauss M (1997). The retinoblastoma protein: a master regulator of the cell cycle, differentiation and apoptosis.
Eur J Biochem *246*: 581-601.

Herzig S, Long F, Jhala US, Hedrick S, Quinn R, Bauer A, Rudolph D, Schutz G, Yoon C, Puigserver P, Spiegelman BM and Montminy M (2001). CREB regulates hepatic gluconeogenesis through the coactivator PGC-1.
Nature *413*: 179-183.

Hiroi Y, Kim HH, Ying H, Furuya F, Huang Z, Simoncini T, Noma K, Ngyuen NH, Scalan TS, Moskowitz MA, Cheng SY and Liao JK (2006). Rapid nongenomic actions of thyroid hormone.
Proc Natl Acad Sci *103*: 14104-14109.

Hodin RA, Lazar MA, Wintman BI, Darling DS, Koenig RJ, Larsen PR, Moore DD and Chin WW (1989). Identification of a thyroid hormone receptor that is pituitary-specific.
Science *244*: 76-79.

Hu X and Lazar MA (2000). Transcriptional repression by nuclear hormone receptors.
Trends Endocrinol Metab *11*: 6-10.

Huo L and Scarpulla RC (2001). Mitochondrial DNA instability and peri-implantation lethality associated with target disruption of nuclear respiratory factor 1 in mice.
Mol Cell Biol *21*: 644-654.

Ito K, Barnes PJ and Adcock IM (2000). Glucocorticoid receptor recruitment of histone deacetylase 2 inhibits interleukin-1beta-induced histone H4 acetylation on lysines 8 and 12.
Mol Cell Biol *20*: 6891-6903.

Ito M and Roeder RG (2001). The TRAP/SMCC/Mediator complex and thyroid hormone receptor function.
Trends Endocrinol Metab *12*: 127-134.

Jepsen K, Hermanson O, Onami TM, Gleiberman AS, Lunyak V, McEvilly RJ, Kurokawa R, Kumar V, Liu F, Seto E, Hedrick SM, Mandel G, Glass CK, Rose DW and Rosenfeld MG (2000). Combinatorial roles of the nuclear receptor corepressor in transcription and development.
Cell *102*: 753-763.

Jeyakumar M, Tanen MR and Bagchi MK (1997). Analysis of the functional role of steroid receptor coactivator-1 in ligand-induced transactivation by thyroid hormone receptor.
Mol Endocrinol *11*: 755-767.

Kim SW, Ho SC, Hong SJ, Kim KM, So EC, Christoffolete M and Harney JW (2005). A novel mechanism of thyroid hormone-dependent negative regulation by thyroid hormone receptor, nuclear receptor corepressor (NCoR), and GAGA-binding factor on the rat cD44 promoter.
J Biol Chem *280*: 14545-14555.

Knutti D and Kralli A (2001). PGC-1, a versatile coactivator.
Trends Endocrinol Metab *8*: 360-365.

Ko L, Caedona GR, Henrion-Caude A and Chin WW (2002). Identification and characterization of a tissue-specific coactivator, GT198, that interacts with the DNA-binding domains of nuclear receptors.
Mol Cell Biol *1*: 357-369.

Koibuchi N and Chin WW (2000). Thyroid hormone action and brain development.
Trends Endocrinol Metab *11*: 123-128.

Koo SH, Satoh H, Herzig S, Lee CH, Hedrick S, Kulkarni R, Evans RM, Olefsky J and Montminy M (2004). PGC-1 promotes insulin resistance in liver through PPAR-alpha-dependent induction of TRB-3.
Nat Med *10*: 530-534.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4.
Nature *227*: 680-685.

Langlois MF, Zanger K, Monden T, Safer JD, Hollenberg AN and Wondisford FE (1997).
A unique role of the beta-2 thyroid hormone receptor isoform in negative regulation by thyroid hormone. Mapping of a novel amino-terminal domain important for ligand-independent activation.
J Biol Chem *272*: 24927-24933.

Lazar MA (1993). Thyroid hormone receptors: multiple forms, multiple possibilities.
Endocr Rev 14: 184-193.

Lazar MA (2003). Thyroid hormone action: a binding contract.
J Clin Invest *112*: 497-499.

Lee KC, Li J, Cole PA, Wong J and Kraus WL (2003). Transcriptional activation by thyroid hormone receptor-beta involves chromatin remodeling, histone acetylation, and synergistic stimulation by p300 and steroid receptor coactivators.
Mol Endocrinol *17*: 908-22.

Lehman JJ, Barger PM, Kovacs A, Saffitz JE, Medeiros DM and Kelly DP (2000). Peroxisome proliferator–activated receptor γ coactivator-1 promotes cardiac mitochondrial biogenesis.
J Clin Invest *106*: 847-856.

Leo C and Chen JD (2000). The SRC family of nuclear receptor coactivators.
Gene *245*: 1-11.

Leone TC, Lehman JL, Finck BN, Schaeffer PJ, Wende AR, Boudina S, Courtois M, Wozniak DF, Sambandam N, Bernal-Mizrachi C, Chen Z, Holloszy JO, Medeiros DM, Schmidt RE, Saffitz JE, Abel ED, Semenkovich CF and Kelly DP (2005). PGC-1α Deficiency Causes Multi-System Energy Metabolic Derangements: Muscle Dysfunction, Abnormal Weight Control and Hepatic Steatosis.
PLoS Biol., 3, e101.10.1371/journal.pbio.0030101.

Lin J, Wu PH, Tarr PT, Lindenberg KS, St-Pierre J, Zhang CY, Mootha VK, Jäger S, Vianna CR, Reznick RM, Cui L, Manieri M, Donovan MX, Wu Z, Cooper MP, Fan MC, Rohas LM, Zavacki AM, Cinti S, Shulman GI, Lowell BB, Krainc D and Spiegelman BM (2004).
Defects in adaptive energy metabolism with CNS-linked hyperactivity in PGC-1alpha null mice.
Cell *119*: 121-135.

Lin J, Handschin C and Spiegelman BM (2005). Metabolic control through the PGC-1 family of transcription coactivators.
Cell Metab *6*: 361-370.

Livak KJ and Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_T$ method.
Methods *25*: 402-408.

Maia AL, Harney JW and Larsen PR (1996). Is there a negative TRE in the luciferase reporter cDNA?
Thyroid *4*: 325-328.

Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schütz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P and Evans RM (1995). The nuclear receptor superfamily: the second decade.
Cell *83*: 835-839.

Marimuthu A, Feng W, Tagami T, Nguyen H, Jameson JL, Fletterick RF, Baxter JD and West BL (2002). TR surfaces and conformations required to bind nuclear receptor corepressor.
Mol Endocrinol *16*: 271–286.

Mathur M, Tucker PW and Samuels HH (2001). PSF is a novel corepressor that mediates its effect through Sin3A and the DNA binding domain of nuclear hormone receptors.
Mol Cell Biol *7*: 2298-2311.

Matsushita A, Sasaki S, Kashowabara Y, Nagayama K, Ohba K, Iwaki H, Misawa H, Ishizuka K and Nakamura H (2007). Essential role of GATA2 in the negative regulation of thyrotropin {beta} gene by thyroid hormone and its receptors.
Mol Endocrinol *21*: 865-84.

McInerney EM, Rose DW, Flynn SE, Westin S, Mullen TM, Krones A, Insotroza J, Torchia J, Nolte RT, Assa-Munt N, Milbum MV, Glass CK and Rosenfeld MG (1998). Determinants of coactivator LXXLL motif specificity in nuclear receptor transcriptional activation.
Genes Dev *12*: 3357-3368.

McKenna NJ and O'Malley BW (2002). Combinatorial control of gene expression by nuclear receptors and coregulators.
Cell *108*: 465-474.

McManus MT and Sharp PA (2002). Gene silencing in mammals by small interfering RNAs.
Nat Rev Genet *3*: 737-747.

Méndez-Pertuz M, Sánchez-Pacheco A and Aranda A (2003). The thyroid hormone receptor antagonizes CREB-mediated transcription.
EMBO J *22*: 3102-3112.

Miller LD, Park KS, Guo QM, Alkharouf NW, Malek RL, Lee NH, Liu ET and Cheng SY (2001). Silencing of Wnt signaling and activation of multiple metabolic pathways in response to thyroid hormone-stimulated cell proliferation.
Mol Cell Biol *21***:** 6626-6639.

Miyamoto T, Suzuki S and DeGroot LJ (1994). Differential binding and activation of thyroid hormone response elements by TR alpha 1 and RXR alpha-trap heterodimers.
Mol Cell Endocrinol *102*: 111-117.

Moeller LC, Dumitrescu AM and Refetoff S (2005). Cytosolic action of thyroid hormone leads to induction of hypoxia-inducible factor-1alpha and glycolytic genes.
Mol Endocrinol *19*: 2955-2963.

Naar AM, Beaurang PA, Zhou S, Abraham S, Solomon W and Tjian R (1999). Composite co-activator ARC mediates chromatin-directed transcriptional activation.
Nature *398*: 828-32.

Nagaya T, Madison LD and Jameson JL (1992). Thyroid hormone receptor mutants that cause resistance to thyroid hormone. Evidence for receptor competition for DNA sequences in target genes.
J Biol Chem *267*: 13014-13019.

Nagy L and Schwabe JW (2004). Mechanism of the nuclear receptor molecular switch. Trends Biochem Sci *29*: 317-324.

Nakano K, Matsushita A, Sasaki S, Misawa H, Nishiyama K, Kashibara Y and Nakamura H (2004). Thyroid-hormone-dependent negative regulation of thyrotropin β gene by thyroid hormone receptors: study with a new experimental system using CV1 cells.
Biochem J *378*: 549-557.

Nissen RM and Yamamoto KR (2000). The glucocorticoid receptor inhibits NFκB by interfering with serine-2 phosphorylation of the RNA polymerase II carboxy-terminal domain. Genes Dev *14*: 2314-2329.

Nygard M, Becker N, Demeneix B, Petterson K and Bondesson M (2006). Thyroid hormone-mediated negative transcriptional regulation of Necdin expression.
J Mol Endocrinol *36*: 517-530.

Oberfield JL, Collins JL, Holmes CP, Goreham DM, Cooper JP, Cobb JE, Lenhard JM, Hull-Ryde EA, Mohr CP, Blanchard SG, Parks DJ, Moore LB, Lehmann JM, Plunket K, Miller AB, Milburn MV, Kliewer SA and Willson TM (1999). A peroxisome proliferator-activated receptor gamma ligand inhibits adipocyte differentiation.
Proc Natl Acad Sci *96*: 6102-6106.

Oberste-Berghaus C, Zanger K, Hashimoto K, Cohen RN, Hollenberg AN and Wondisford FE (2000). Thyroid hormone-independent interaction between the thyroid hormone receptor beta2 amino terminus and coactivators.
J Biol Chem *275*: 1787-1792.

Opitz R, Lutz I, Guyen NH, Scanlan TS and Klos W (2006). Analysis of thyroid hormone receptor betaA mRNA expression in Xenopus laevis tadpoles as a means to detect agonism and antagonism of thyroid hormone action.
Toxicol Appl Pharmacol *212*: 1-13.

Oppenheimer JH and Schwartz HL (1997). Molecular basis of thyroid hormone-dependent brain development.
Endocr Rev *18*: 462-475.

Ota Y and Mariash CN (2003). Paradoxical triiodothyronine suppression of S14 transcription in permanent hepatic cell lines.
Thyroid *13*: 437-445.

Pascual G, Fong AL, Ogawa S, Gamliel A, Li AC, Perissi V, Rose DW, Willson TM, Rosenfeld MG and Glass CK (2005). A SUMOylation-dependent pathway mediates transrepression of inflammatory response genes by PPAR-gamma.
Nature *437*: 759-763.

Pérez-Juste G, García-Silva S and Aranda A (2000). An element in the region responsible for premature termination of transcription mediates repression of c-myc gene expression by thyroid hormone in neuroblastoma cells.
J Biol Chem *275*: 1307-1314.

Petty KJ, Desvergne B, Mitsuhashi T and Nikodem VM (1990). Identification of a thyroid hormone response element in the malic enzyme gene.
J Biol Chem *265*: 7395-7400.

Pfahl M (1993). Nuclear receptor/AP-1 interaction.
Endocr Rev *14*: 651-658.

Poirier MB, Hamann G, Domingue ME, Roy M, Bardati T and Langlois MF (2005). General receptor for phosphoinositides 1, a novel repressor of thyroid hormone receptor action that prevents deoxyribonucleic acid binding.
Mol Endocrinol *8*: 1991-2005.

Puigserver P, Wu Z, Park CW, Graves R, Wright M and Spiegelman BM (1998). A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis.
Cell *92*: 829–839.

Puigserver P, Rhee J, Lin J, Wu Z, Yoon JC, Zhang CY, Krauss S, Mootha VK, Lowell BB and Spiegelman BM (2001). Cytokine stimulation of energy expenditure through p38 MAP kinase activation of PPARgamma coactivator-1.
Mol Cell *8*: 971-982.

Puigserver P, Rhee J, Donovan J, Walkey CJ, Yoon JC, Oriente F, Kitamura Y, Altomonte J, Dong H, Accili D and Spiegelman BM (2003). Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction.
Nature *423*: 550-555.

Rachez C and Freedman LP (2001). Mediator complexes and transcription.
Curr Opin Cell Biol *13*: 274-280.

Reichardt HM, Tuckermann JP, Göttlicher M, Vujic M, Weih F, Angel P, Herrlich P and Schütz G (2001). Repression of inflammatory responses in the absence of DNA binding by the glucocorticoid receptor.
EMBO J *24*: 7168-7173.

Reichwald K, Thiesen J, Wiehe T, Weitzel J, Poustka WA, Rosenthal A, Platzer M, Strätling WH and Kioschis P (2001). Comparative sequence analysis of the MECP2-locus in human and mouse reveals new transcribed regions.
Mamm Genome *11*: 182-190.

Ribeiro RC, Apriletti JW, Yen PM, Chin WW and Baxter JD (1994). Heterodimerization and deoxyribonucleic acid-binding properties of a retinoid X receptor-related factor.
Endocrinology *135*: 2076-2085.

Riss TL, Stewart BH and Sirbasku DA (1989). Rat pituitary tumor cells in serum-free culture. Selection of thyroid hormone-responsive and autonomous cells.
In Vitro Cell Dev Biol *25*: 27-35.

Rodríguez-Peña A, Escrivá H, Handler AC and Vallejo CG (2002). Thyroid hormone increases transcription of GA-binding protein/nuclear respiratory factor-2 alpha-subunit in rat liver.
FEBS Lett *514*: 309-314.

Sakurai A, Nakai A and DeGroot LJ (1989). Expression of three forms of thyroid hormone receptor in human tissues.
Mol Endocrinol *3*: 392-399.

Sanchez-Pacheco A, Palomino T and Aranda A (1995). Negative regulation of expression of the pituitary-specific transcription factor GHF-1/Pit-1 by thyroid hormones through interference with promoter enhancer elements.
Mol Cell Biol *15*: 6322-6330.

Sanchez-Pacheco A and Aranda A (2003). Binding of the thyroid hormone receptor to a negative element in the basal growth hormone promoter is associated with histone acetylation.
J Biol Chem *278:* 39383-39391.

Santos GM, Afonso V, Barra GB, Togashi M, Webb P, Neves FA, Lomri N and Lomri A (2006). Negative regulation of superoxide dismutase-1 promoter by thyroid hormone.
Mol Pharmacol *70* :793-800.

Sap J, Magistris LD, Stunnenberg H and Vennström B (1990). A major thyroid hormone response element in the third intron of the rat growth hormone gene.
EMBO J *9*: 887-896.

Sasaki S, Lesoon-Wood LA, Dey A, Kuwata T, Weintraub BD, Humphrey G, Yang WM, Seto E, Yen PM, Howard BH and Ozato K (1999). Ligand-induced recruitment of a histone deacetylase in the negative-feedback regulation of the thyrotropin beta gene.
EMBO J *18*: 5389-5398.

Satoh T, Yamada M, Iwasaki T and Mori M (1996). Negative regulation of the gene for the preprothyrotropin-releasing hormone from the mouse by thyroid hormone requires additional factors in conjunction with thyroid hormone receptors.
J Biol Chem *271*: 27919-27926.

Scheinman RI, Gualberto A, Jewell CM, Cidlowski JA and Baldwin AS Jr. (1995). Characterization of mechanisms involved in transrepression of NFkappaB by activated glucocorticoid receptors.
Mol Cell Biol *15*: 943-953.

Shibusawa N, Hollenberg AN and Wondisford FE (2003a). Thyroid hormone receptor DNA binding is required for both positive and negative gene regulation.
J Biol Chem *278*: 732-738.

Shibusawa N, Hashimoto K, Nikrodhanond AA, Liberman MC, Applebury ML, Liao XH, Robbins JT, Refetoff S, Cohen RN and Wondisford FE (2003b). Thyroid hormone receptor in the absence of thyroid hormone receptor-binding in vivo.
J Clin Invest *112*: 588-597.

Shih CH, Chen SL, Yen CC, Huang YH, Chen CD, Lee YS and Lin KH (2004). Thyroid hormone receptor-dependenttranscriptional regulation of fibrinogen and coagulation proteins.
Endocrinology *145*: 673-683.

Tagami T, Madison LD, Nagaya T and Jameson JL (1997). Nuclear receptor corepressors activate rather than suppress basal transcription of genes that are negatively regulated by thyroid hormone.
Mol Cell Biol *17*: 2642-2648.

Tagami T and Jameson JL (1998). Nuclear corepressors enhance the dominant negative activity of mutant receptors that cause resistance to thyroid hormone.
Endocrinology *139*: 640-650.

Tagami T, Park Y and Jameson J.L (1999). Mechanisms that mediate negative regulation of the thyroid-stimulating hormone alpha gene by the thyroid hormone receptor.
J Biol Chem *274*: 22345-22353.

Tao Y, Williams-Skipp C and Scheinman RI (2001). Mapping of glucocorticoid receptor DNA binding domain surfaces contributing to transrepression of NF-κB and induction of apoptosis.
J Biol Chem *276***:** 2329-2332.

Thompson CC and Bottcher MC (1997). The product of a thyroid hormone-responsive gene interacts with thyroid hormone receptors.
Proc Natl Acad Sci *94*: 8527-8532.

Tiraby C, Tavernier G, Lefort C, Larrouy D, Bouillaud F, Ricquier D and Langin D (2003). Acquirement of brown fat cell features by human white adipocytes.
J Biol Chem *278*: 33370-33376.

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Umesono K and Evans RM (1989).
Determinants of target gene specificity for steroid/thyroid hormone receptors.
Cell *57*: 1139-1146.

Villa A, Santiago J, Belandia B and Pascual A (2004). A response unit in the first exon of the beta-amyloid precursor protein gene containing thyroid hormone receptor and Sp1 binding sites mediates negative regulation by 3,5,3'-triiodothyronine.
Mol Endocrinol *18*: 863-873.

Virbasius JV and Scarpulla RC (1994). Activation of the human mitochondrial transcription factor A gene by nuclear respiratory factors: a potential regulatory link between nuclear and mitochondrial gene expression in organelle biogenesis.
Proc Natl Acad Sci *91*: 1309-1313.

Weitzel, JM, Kutz S, Radtke C, Grott S and Seitz HJ (2001a). Hormonal regulation of multiple promoters of the rat mitochondrial glycerol-3-phosphate dehydrogenase gene: identification of a complex hormone-response element in the ubiquitous promoter B.
Eur J Biochem *268*: 4095-4103.

Weitzel JM, Radtke C and Seitz HJ (2001b). Two thyroid hormone mediated gene expression patterns *in vivo* identified by cDNA expression arrays in rat.
Nucleic Acids Research *29*: 5148-5155.

Weitzel JM, Hamann S, Jauk M, Lacey M, Filbry A, Radtke C, Iwen KAH, Kutz S, Harneit A, Lizardi PM and Seitz HJ (2003). Hepatic gene expression patterns in thyroid hormone-treated hypothyroid rats.
J Mol Endocrinol *31*: 291-303.

Wilusz CJ, Wormington M and SW Peltz (2001). The cap-to-tail guide to mRNA turnover.
Nat Rev Mol Cell Biol *2*: 237-246.

Wright DC, Han DH, Garcia-Roves PM, Geiger PC, Jones TE and Holloszy JO (2006). Exercise-induced mitochondrial biogenesis begins before the increase in muscle PGC-1alpha expression.
J Biol Chem *282*: 194-199.

Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC and Spiegelman BM (1999). Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1.
Cell *98*: 115-124.

Yang-Yen HF, Chambard JC, Sun YL, Smeal T, Schmidt TJ, Drouin J and Karin M (1990). Transcriptional interference between c-Jun and the glucocorticoid receptor: mutual inhibition of DNA binding due to direct protein-protein interaction.
Cell *62*: 1205-1215.

Yen PM (2001). Physiological and molecular basis of thyroid hormone action.
Physiol Rev *81*: 1097-1142.

Yoon JC, Puigserver P, Chen G, Donovan J, Wu Z, Rhee J, Adelmant G, Stafford J, Kahn CR, Granner DK, Newgard CB and Spiegelman BM (2001). Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1.
Nature *413*: 131-138.

Zaid A, Li R, Luciakova K, Barath P, Nery S and Nelson BD (1999). On the role of the general transcription factor Sp1 in the activation and repression of diverse mammalian oxidative phosphorylation genes.
J Bioenerg Biomembr *31*: 129-135.

Zhang J and Lazar MA (2000). The mechanism of action of thyroid hormones.
Annu Rev Physiol *62*: 439-466.

Zhang W, Brooks RL, Silversides DW, West BL, Leidig F, Baxter JD and Eberhardt NL (1992). Negative thyroid hormone control of human growth hormone gene expression is mediated by 3'-untranslated/3'-flanking DNA.
J Biol Chem *267*: 15056-15063.

Zhang Y, Ma K, Song S, Elam MB, Cook GA and Park EA (2004). Peroxisomal proliferator-activated receptor-gamma coactivator-1 alpha (PGC-1 alpha) enhances the thyroid hormone induction of carnitine palmitoyltransferase I (CPT-I alpha).
J Biol Chem *279*: 53963-53971.

Zilz ND, Murray MB and Towle HC (1990). Identification of multiple thyroid hormone response elements located far upstream from the rat S14 promoter.
J Biol Chem *265*: 8136-8143.

9 Appendix

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