Expression of thyroid hormone receptor isoforms in rodents

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CHAPTER I

INTRODUCTION
INTRODUCTION

The genomic actions of thyroid hormones are primarily the result of the interaction of $T_3$ with specific nuclear receptors. The $T_3$-receptor complex binds to regulatory regions of genes (thyroid hormone-response elements) and modifies gene expression. $T_3$-dependent gene expression requires, besides the receptor itself, a pre-receptor event (such as hormone availability) and a post-receptor event (e.g., interaction with DNA and proteins).

In part A of this introduction three topics will be discussed: pre receptor events - in this case hormone availability, post receptor events such as interaction with other proteins and DNA, and finally the receptor structure itself.

The three topics that will be discussed in part B are the biological relevance of thyroid hormone receptors, expression of thyroid hormone receptors and finally the scope of this thesis.

I.A. Overview of thyroid hormone metabolism and action

I.A.1. Thyroid hormone synthesis, secretion and availability

Thyroid hormones ($T_4$ and $T_3$) are involved in growth, differentiation, development and metabolism. TH synthesis and secretion (figure 1) is regulated by a feedback system that involves the hypothalamus, pituitary and thyroid gland (HPT-axis) (1). Thyrotropin releasing hormone (TRH) is synthesized in the paraventricular nucleus (PVN) of the hypothalamus and the anterior pituitary. TRH binds to its receptor in pituitary cells which leads to synthesis and release of thyroid stimulating hormone (TSH). After release into the circulation TSH binds to its receptor on the thyroid follicular cells where it stimulates the production and secretion of $T_4$ and $T_3$. However, the majority of the daily $T_3$ (the active form of thyroid hormone) production occurs outside the thyroid gland, mainly in the liver (2) in which $T_4$ to $T_3$ conversion is achieved via 5'-deiodination by type I deiodinase (5'DI). $T_3$ reaches its target genes via the blood circulation. Cellular uptake of TH occurs via plasma membrane thyroid hormone transporters, after which TH is available for binding to thyroid hormone receptor.

I.A.2. Mechanism of action of thyroid hormone receptors (TR)

TR proteins can bind to a specific DNA sequences called thyroid response elements (TRE) as monomers or homodimers but they bind preferentially as heterodimers with members of the RXR (retinoid X receptors) family. TR can inhibit or enhance gene expression depending on the nature of TREs, the hormonal state (availability of ligand) and the cellular environment which are schematically shown in figure 2 and discussed below.
Figure 1. Regulation of the hypothalamus-pituitary-thyroid (HPT-axis) and extrapituitary metabolism of thyroid hormones. In the liver, enzyme type I deiodinase (5'DI) converts T4 to the active metabolite T3 by outer ring deiodination and to the inactive metabolite rT3 by inner ring deiodination.

Arrows with + sign represent a positive feedback regulation and with a - sign indicates negative feed back. TRH, thyrotropin releasing hormone; TSH, thyroid stimulating hormone; T4, thyroxine; T3, 3,3',5-triiodo-L-thyronine; T4, L-thyroxine; rT3, reverse 3,3',5'-triiodo-L-thyronine; T2, 3,3'-diiodo-L-thyronine.
Introduction

Histone deacetylation

Repression

nucleosome

Activation

Figure 2. Model for activation and repression of the gene expression by thyroid hormone receptor. In the absence of $T_3$, TR/RXR recruits a co-repressor complex that has histone deacetylase (HDAC) activity. In the presence of $T_3$, TR/T3/RXR releases the co-repressor complex and recruits a co-activator complex that has histone acetyltransferase (HAT) activity. Enzymatic modification of nucleosomes by HDAC or HAT activity results in a closed or open chromatin structure that leads to transcription repression or activation. TBP, TATA binding protein; TAFs, TBP associated factors; RXR, retinoic $\alpha$-receptor; N-COR, nuclear receptor corepressor; POLII, RNA Polymerase II; TRAP, Thyroid hormone associated protein; P/CAF, P300/CBP associated proteins.
**I.A.2.1. Thyroid response elements (TREs)**

TR regulates gene expression by binding to TREs on target genes. TREs are built from two hexameric half-sites with the consensus sequence G/AGGTC/GA that are arranged in sequences: palindromic (AGGTCA TGACCT), inverted palindromic or direct repeat (DR) separated by four nucleotides (DR-4; AGGTCA CAGGAGGTCA). Most of the known TREs are referred to as positive TREs, in which gene transcription is repressed by unliganded TRs and activated by liganded TR.

Negative TREs are described in TSHα, TSHβ and TRH. Transcription of these target genes which are physiologically important for negative feedback regulation of the HPT-axis is by the thyroid hormone. Transcription can be stimulated not only by liganded but also by unliganded TR (3).

To explain this negative regulation two different models have been proposed (4-7) but the exact mechanism remains unclear.

Recent DNA microarray analysis of thyroid hormone targeted genes in the liver suggest that there are more negatively regulated genes than positive ones (8) in mouse liver which implies a substantial role for negative regulation of TR target genes also outside the pituitary gland.

**I.A.2.2. Interactions of thyroid hormone receptor with other proteins**

**Co-repressors and associated proteins**

On positive TREs, unliganded TRs associate with co-repressors to repress transcription. Co-repressors such as SMRT (silencing mediator of retinoid and thyroid hormone receptor) or NCoR (nuclear receptor co-repressor) bind to TR in a domain called the hinge region (9,10). Repression also involves deacetylation of chromatin. NCoR/SMRT forms a multisubunit repressor complex with HDAC1 (histone deacetylase 1) and Sin3 proteins (11) (see figure 2) implying that in the absence of T³, co-repressors act as a hormone-dependent adaptor that associates with both Sin3 and HDAC1 through its repressor domain and with TR via a distinct interaction domain.

**Co-activators and associated proteins**

Co-activators enhance the hormone-dependent transcriptional activity of TR. In the presence of T³, the TR undergoes a conformational change, which results in the replacement of the co-repressor complex by a co-activator complex composed of p160 proteins (SRC and NcoA-1, TIF2, GRIP1, NcoA-2, p/CIP, ACTR, AIB1, TRAM-1), p300/CBP (CREB, p/CAF) and TRAP's (12-16). The TR-ligand-co-activator complex results in chromatin remodeling. Chromatin consists of repeating protein-DNA units called nucleosomes. The basic protein unit of nucleosomes are made up of globular and highly basic histones. The nucleosome core consists of two copies each of the histones H2A, H2B, H3 and H4 around which DNA is wrapped. Remodeling occurs due to a histone acetyl transferase activity (HAT) of the receptor bound co-activators which results in an ‘open’ transcriptionally active chromatin configuration. The ligand-occupied TR then associates with the multiprotein TRAP complex, which activates transcription by interacting with general transcription factors such as TFIIIB (figure 2).
I.A.3. Structures of thyroid hormone receptors (TR)

I.A.3.1. Structure of TR genes

TRs are encoded by two different gene loci, TRα (locus C-erbA-alpha on chromosome; human: 17q11.2, rat: 10 and mouse: 11 57.0 cM) and TRβ (locus C-erbA-BETA on chromosome: Human: 3p24.3, rat: 15 and mouse: 14 A3) (17). The TRα gene encodes for TRα1, TRα2 and their TRΔα variants as shown in figure 3. The TRα locus contains 10 exons and the differential splice site for TRα1 and TRα2 is located in exon 9. The TRα2 splice variant has no intact ligand binding domain and is therefore unable to bind thyroid hormone. It is however able to act as a constitutive repressor of transcription of genes controlled by nuclear receptor superfamily members such as TR, RAR (retinoid acid receptor) and RXR (18-23). An extra promoter located in intron 7 is responsible for the formation of TRΔα1 and TRΔα2 variants, which are also unable to bind hormone and DNA (24) and also able to act as constitutive repressors of other TR isoforms probably due to interfering by other pathways.

The TRβ locus encodes TRβ1, TRβ2, TRβ3 and also a truncated receptor TRΔβ3 (which is unable to bind thyroid hormone) which are all generated via alternative splicing and/or different promoter usage (17,25,26) as shown in figure 4. They differ only in the amino terminal part of the receptor. The TRβ locus contains 11 exons, of which exons 3-8 are common for all TRβs and also show high homology with TRα isoforms. Other exons which were later discovered are assigned as exon a and exon A and B. Alternative splicing of exons 1 and 2 results in the N-terminal part of TRβ1, exon a for TRβ2 and exons A and B for TRβ3 and its variant.
Figure 3. Schematic representation of the transcripts generated by the c-erbA locus. Unstained and black blocks: region common to α1 and α2 isoforms. Gray hatched blocks indicate region specific to 1-isoforms and black hatched blocks show region specific to 2-isoforms. Upstream region from the c-erbA gene and the 5'-end of the TR α mRNAs are not shown and 3'-untranslated regions of RNAs are shown as lines. For each isoform the length of mRNA transcript and protein size (estimated from mouse), are displayed with kb as kilo base pair and kD as kilo Da.

Figure 4. Schematic representation of the transcripts generated by the TRβ gene. Exon 3-8 common to all TRβ isoforms are shown as black blocks. Exon 1-2 common to TRβ1 isoform are shown as blocks with black lines. Exon a common to TRβ2 isoform are shown as light dotted blocks. Furthermore exon A common to TRβ3 and TRβ4 are displayed as gray blocks and exon B common to TRβ3 is represented as heavy dotted blocks. Black lines represents the introns. For each isoform the length of mRNA transcript and estimated protein size (rats) are displayed with kb as kilo base pair and kD as kilo Da.
1.A.3.2. Structure of TR proteins

The TRs have a domain organization similar to the other members of the nuclear receptor superfamily (figure 5). There are four domains namely: the A/B domain (the amino-terminal part which differs in TRs), the C domain (which contains the DNA binding domain), the D domain (hinge region) and the E/F domain (ligand binding domain) note that combined 3D structure of C, D and E/F domain are shown in figure 6. Each of these domains plays a different part in the interaction with proteins and DNA. Generally it is believed that the A/B domain together with the E domain is involved in the interaction with coactivator proteins. DNA binding of the TRs is regulated by the C and E domains and co-repressor interaction sites are mostly located in the D and E domains (figure 5). The Thyroid hormone receptor primary protein structure (sequence) is conserved between rodents and humans as seen in figures 7 and 9. Furthermore, as far as known TRα2 sequences are found only in mammals and not in avian or fish genomes which suggests "mammals specific" function of this isoform.

The three-dimensional structure of the DNA and ligand binding domain as shown in figure 6 gives an impression of functional domains of TR and will be discussed in the next sections.

The A/B domains differ in size and in sequence between TRα and TRβ isoforms. The protein sequence of amino-terminal part of TRs is similar between TRα1 and TRα2, and differs between TRβ isoforms and is highly conserved between rat, human and mouse as seen in figure 7. The TRβ2 isoform in rats differs from those reported from mouse and human, since it is 38 amino acids longer as reflected in figure 7 and TRβ3 and TRΔβ3 are so far only shown in rats.

The role of the A/B domain in transcriptional activation is poorly understood due to differences in species and cell types. The most probable function of this domain lies in regulation of the interaction with other proteins such as transcription factors (27) or co-activators, since transcriptional activity is lost after deletion of the N-terminal amino acids 7-45 of the TRα1, TRβ1 and TRβ2 (27-30).

The C domain possesses two zinc fingers (31), each containing 4 cysteine residues coordinated by a zinc ion and 4 functional sites called P, D, T and A boxes (figure 8). The first zinc finger is important in the specific association with the hexameric TRE sequences. In the presence of DNA, the receptor dimerizes followed by placement of the first zinc finger of the DNA binding domain of each monomer in direct contact with the DNA major groove. The second zinc finger of each monomer is stabilized by direct interaction between the second zinc finger and the TRE. The P box is important in sequence specific recognition of response elements of other receptors besides TR such as ER (estrogen receptor), RXR, RAR and VDR (vitamin D receptor). The D-box is involved in distinguishing the spacing between half sites of hormone response elements. The T and A boxes are involved in RXR heterodimerization (32). Because of their critical role, changes in the zinc fingers or boxes directly result in abrogation of DNA-binding and transcriptional activity (33).

The hinge region (D domain) links the C domain to the ligand binding domain as can be seen in figure 6 and interacts with co-repressors (9). Mutation of this domain in the TRβ1 receptor abrogates the basal repression by the co-repressor (9). This indicates
that either the hinge region of the unliganded TR located in the first helix can provide a contact surface with co-repressor or that the hinge region has an allosteric effect on these interactions since the interaction with co-repressors is also associated with sequences in helices 3, 5 and 6 of LBD represented in figure 9 (10,34-36).

TRs possess some motifs called nuclear localization signals (NLS). The consensus sequences mediating nuclear translocation consist of two clusters of 2-3 basic amino acids separated by two non-basic amino acids (((K/R)(K/R))XX(K/R)(K/R)(K/R)). For the TRβ1 the KRLAKRK (aa-184-190) in domain D mediates the T₃-induced nuclear translocation (37). Since TRα isoforms also possess several NLS in the D and E domains it is possible that the same process is also applicable to these isoforms. Moreover, a recent study shows nucleocytoplasmic shuttling for TRα isoforms (38).

Ligand binding domain (LBD) or E domain is required for nuclear localization, homodimerization, and most importantly, ligand binding and the ligand-induced switch of the transcriptional activity. Molecular studies have established that the LXXLL motif within co-activators mediates the interaction with ligand-activated nuclear receptors (39). Biochemical and crystallographic analyses revealed that an LXXLL motif-containing α-helix from the co-activators interacts with a hydrophobic groove within the ligand-bound LBDs (40). This domain, as shown in figure 9, harbors different important sequences such as the so-called heptad repeats (hydrophobic amino acids that form the "leucine zipper" structure; aa334-341, aa349-356, aa356-363, aa365-372, aa372-379, aa379-386, aa392-399, aa399-406 and aa 421-428 in figure 9). These heptad repeats form hydrophobic interfaces for both TR homodimerization (41). One of these conserved regions, known as the ninth heptad (in figure 9; aa 421-428) is important for TR homodimerization and RXR-TR heterodimerization (42). Mutation in the ninth heptad in helix II decreases TR homodimerization, indicating possibility of the existence of other regions within LBD which are important for dimerization (33,43-45). Moreover, it seems that the ninth heptad is important for heterodimerization for TRα1 while for TRα2 the dimerization region lies in the DBD (46,47) (note that TRα2 and TRα1 differ from each other within the ninth heptad region).

Other important sequences lie in helix 6 (figure 9, amino acids 310-314). Within this helix TRΔα variants are generated as a result of splicing within exon 7 of the TRα locus. However, the sequence comparison shows no differences within species and between TRβ and TRα isoforms (figure 9). This suggests that the same variants could exist for TRβ isoforms which are not reported yet.

Finally, the last α-helix in the LBD domain of TRα1 and TRβ isoforms (helix 12) is involved in mediating ligand-dependent transcriptional activation by an activation domain called the activation function-2 core(AF-2; a highly conserved LFLEVF motif in C-terminus). The binding of ligand results in conformational changes and repositioning of the AF-2 core which generates a coactivator binding surface and affects transcriptional activation by closing the ligand-binding pocket and forming a novel interface involving residues from the AF-2 itself and at least three other helices.
Figure 5. Structure of (A) thyroid hormone receptor isoforms and its functional domains and (B) nuclear receptors, including TR, can be divided into six regions (A–E) with three functional domains: (A/B) a co-activator interaction site, DBD, DNA-binding domain (C region) and LBD, ligand-binding domain (D–E). The lines above the domains point to sequences chosen to generate antibodies.
Figure 6. A 3D structure of DNA-binding domain (DBD), Hinge region and ligand-binding domain (LBD) of TR is shown. The Hinge region, position of zinc atoms in DBD and binding pocket for T₃ is shown by arrows.
7A

**Trulmouse**

MEQKPSKVEC GSDPEENSAR SPDGKRKKKN GCQPLSKSM

**Trurat**

MEQKPSKVEC GSDPEENSAR SPDGKRKKKN GCQPLSKSM

**Trulhu**

MEQKPSKVEC GSDPEENSAR SPDGKRKKKN GCQPLSKSM

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**Trulmouse**

EQQVVCSDKNA TGKYPCICIG EGCXQFFRRT IKNLHPVTGS CKYSCGIVL

**Trurat**

EQQVVCSDKNA TGKYPCICIG EGCXQFFRRT IKNLHPVTGS CKYSCGIVL

**Trulhu**

EQQVVCSDKNA TGKYPCICIG EGCXQFFRRT IKNLHPVTGS CKYSCGIVL

---

**Trulmouse**

KTRNQGACLC RRKSHAVGM AMDVLKIQE PVARRKMEQ NRERRRKEEM

**Trurat**

KTRQQGACLC RRKSHAVGM AMDVLKIQE PVARRKMEQ NRERRRKEEM

**Trulhu**

KTRNQGACLC RRKSHAVGM AMDVLKIQE PVARRKMEQ NRERRRKEEM

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**Trulmouse**

IKSLQQRPEP TPKEHLKHV ATEHPLSTNA QSSWKQRPK FLFIDIQQPS

**Trurat**

IKSLQQRPEP TPKEHLKHV ATEHPLSTNA QSSWKQRPK FLFIDIQQPS

**Trulhu**

IKSLQQRPEP TPKEHLKHV ATEHPLSTNA QSSWKQRPK FLFIDIQQPS

---

**Trulmouse**

IVSMDGQKV DILAFSEFTK IITPAITRVV DFARKLPMFS ELPCEDQIIL

**Trurat**

IVSMDGQKV DILAFSEFTK IITPAITRVV DFARKLPMFS ELPCEDQIIL

**Trulhu**

IVSMDGQKV DILAFSEFTK IITPAITRVV DFARKLPMFS ELPCEDQIIL

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**Trulmouse**

LGGCHEMAIS LRRAVYDEE SOTLTEGAM AVKREQLKNS GLGVSDAIF

**Trurat**

LGGCHEMAIS LRRAVYDEE SOTLTEGAM AVKREQLKNS GLGVSDAIF

**Trulhu**

LGGCHEMAIS LRRAVYDEE SOTLTEGAM AVKREQLKNS GLGVSDAIF

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**Trulmouse**

ELGKLSAFN LIDTWALLQ AVLMSTORS GLLCVKIEK SQEAYLAFE

**Trurat**

ELGKLSAFN LIDTWALLQ AVLMSTORS GLLCVKIEK SQEAYLAFE

**Trulhu**

ELGKLSAFN LIDTWALLQ AVLMSTORS GLLCVKIEK SQEAYLAFE

---

**Trulmouse**

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**Trurat**

HVVHRKHKNI PHWRKLLMK VTDDLPMIGAC HASSFLIIMKVC ECPTELEFPL

**Trulhu**

HVVHRKHKNI PHWRKLLMK VTDDLPMIGAC HASSFLIIMKVC ECPTELEFPL

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**Trulmouse**

FLEVFEDQEV

**Trurat**

FLEVFEDQEV

**Trulhu**

FLEVFEDQEV
TRu2 mouse
MEQKP5VEC GSDPENSA SPDGKRKRKN GQCPLKSSMS GYFSLDKD
TRu2rat
MEQKP5VEC GSDPENSA SPDGKRKRKN GQCPLKSSMS GYFSLDKD
TRu2hu
MEQKP5VEC GSDPENSA SPDGKRKRKN GQCSLKTSSMS GYFSLDKD

TRu2mouse
EQQCVGSDKA TGYHYRCIC TEGCGFFRRT IQQNLHPTY CKYDSCVVID
TRu2rat
EQQCVGSDKA TGYHYRCIC TEGCGFFRRT IQQNLHPTY CKYDSCVVID
TRu2hu
EQQCVGSDKA TGYHYRCIC TEGCGFFRRT IQQNLHPTY CKYDSCVVID

TRu2mouse
KTRNQGCQLC RFKKCIAVM AMDLVLDDSK RVKRIEQ MRERREKEEM
TRu2rat
KTRNQGCQLC RFKKCIAVM AMDLVLDDSK RVKRIEQ MRERREKEEM
TRu2hu
KTRNQGCQLC RFKKCIAVM AMDLVLDDSK RVKRIEQ MRERREKEEM

TRu2mouse
IRSLQQRPE P TPEEWDLIHV ATEAHSTNA QGSHWVQRAK FLPEDIQQSP
TRu2rat
IRSLQQRPE P TPEEWDLIHV ATEAHSTNA QGSHWVQRAK FLPEDIQQSP
TRu2hu
IRSLQQRPE P TPEEWDLIHV ATEAHSTNA QGSHWVQRAK FLPEDIQQSP

TRu2mouse
IVSMPDGDK V DLEAFSEFT K IITPAITR W DFAKKLPMF S ELPCEDQII
TRu2rat
IVSMPDGDK V DLEAFSEFT K IITPAITR W DFAKKLPMF S ELPCEDQII
TRu2hu
IVSMPDGDK V DLEAFSEFT K IITPAITR W DFAKKLPMF S ELPCEDQII

TRu2mouse
LGCCMCEIMS LRAAVRYDE S DTTITLGEM AVKRESQKNG GLGVSVDAIF
TRu2rat
LGCCMCEIMS LRAAVRYDE S DTTITLGEM AVKRESQKNG GLGVSVDAIF
TRu2hu
LGCCMCEIMS LRAAVRYDE S DTTITLGEM AVKRESQKNG GLGVSVDAIF

TRu2mouse
ELGKSLSAFN LDDITEVALLQ AVLMETERS GLLCVDKIEK SSEQYLFQ
TRu2rat
ELGKSLSAFN LDDITEVALLQ AVLMETERS GLLCVDKIEK SSEQYLFQ
TRu2hu
ELGKSLSAFN LDDITEVALLQ AVLMETERS GLLCVDKIEK SSEQYLFQ

TRu2mouse
HYVNNHKINPHFHFWKLLM EREVQSSILY KGAEEGRSPF GSLGVRHPEGQ
TRu2rat
HYVNNHKINPHFHFWKLLM EREVQSSILY KGAEEGRSPF GSLGVRHPEGQ
TRu2hu
HYVNNHKINPHFHFWKLLM EREVQSSILY KGAEEGRSPF GSLGVRHPEGQ

TRu2mouse
QLGMMHVVQG PQVQLEQQQL GEASSLRGPV LQHQPSPKSPQ QRLLELLHRS
TRu2rat
QLGMMHVVQG PQVQLEQQQL GEASSLRGPV LQHQPSPKSPQ QRLLELLHRS
TRu2hu
QLGMMHVVQG PQVQLEQQQL GEASSLRGPV LQHQPSPKSPQ QRLLELLHRS

TRu2mouse
GILHSRAVC GDSSEAISSL SSDEDEDE TE VEDLACK
TRu2rat
GILHSRAVC GDSSEAISSL SSDEDEDE VEDLANQA SP
TRu2hu
GILHSRAVC GDSSEAISSL SSSEPPEE VEDLANQA SP
Figure 7A-E. Full length sequence of (A) TRα1 between different species (mouse; gi: 72122, human; gi: 6018474 and rat; gi: 92878) and for (B) TRα2 (mouse; gi: 50389, human; gi: 472376 and rat; gi: x07409) and N-terminal sequence of TRβ isoforms: (C) TRβ1 (mouse; swissprot: p37242, human; swissprot: p10828 and rat; swissprot: p18113), (D) TRβ2 (mouse; swissprot: p37244, human; swissprot: p37243 and rat; swissprot: p37826), (E) TRβ3 (rat; gi: 11244755 only available sequence). N-terminal domain of TRα isoforms are shown in bold letters and their differences in C-terminus are represented in italics. Differences between species are underlined for TRα isoforms and are bold for TRβ. A continuous underline gives the position of epitope used for generating of antibodies against TR.
Figure 8. DNA-binding domain and Hinge region of human TRβ1. Schematic drawing of the two zinc fingers of human TR and the various sub-regions within the DNA-binding domains.

Figure 9. Hinge region, ligand binding domain and alignment of TR genes α and β from various species. The genes for both TR subtypes from human, rat, chick, and frog were aligned using Clustal W. Consensus residues appear in normal type, variant or species-specific residues appear in reduced italic type. Positions that show a distinct subtype-specific difference (i.e. maintained in three of the four species shown here) are shaded. The α-gene consensus sequence appears in dark gray, the β-gene consensus sequence appears in light gray. Residues that make ligand contacts, denoted by a dark sphere, are uniformly conserved across species. Only S277/N331, which indirectly contributes to ligand interaction, differs between the two subtypes. The only residue that differs between the rat and human -genes is located at position 224. The conclusions here are supported by the inclusion of other species (mouse, sheep, flounder, zebrafish) in the alignment. The specific gene sequences used to construct the figure are: TRα1 human (gi: 135702), TRα2 rat (gi: 57390), TRα chick (gi: 135710), TRα xenla (gi: 135707), TRβ1 human (gi: 586092), TRβ1 rat (gi: 586094), TRβ1 chick (gi: 2507416) and TRβ1 xenla (gi: 214831). The ninth heptad is shown in the box and the line within the box points the position were TRα1 splices from TRα2. The variant box points the position were TR.Aα variants are spliced. AF-2 domain in helix 12 is also shown in the box. Modified from Wagner, R.L et al, 2002.
Even though the sequence comparison shows great similarity between species and between TRβ and TRα, in ligand-binding capacity there is a difference between those isoforms. In the hormone-binding pocket, few differences exist between the two receptors. In the hydrophobic region, the conformations are virtually identical. In the polar pocket, however, the position of a structural element of the LBD - the β-hairpin between S3 and S4 (residues Asn 331 to Glu 333 of hTRβ, residues Ser 277 to Glu 279 of rTRα), as seen in figure 10 in hTRβ in complex with TRIAC - differs between the two receptors resulting in a closer shift toward a ligand with helices H3 and H5–H6. Most of the residues in the polar pocket of both structures also adjust to adopt the same conformations and make the same interactions with the ligand, with Arg266 (Arg320 in the hTRβ; helix H5–H6) forming a charge pair (3.4 Å) with the negatively charged acetic acid group of TRIAC. However, in rTRα, Arg228 (located on H3) rotates about the C-C bond toward the ligand and forms a hydrogen bond to Ser277 (2.8 Å). In the hTRβ, the analogous residue Arg282 points away from the ligand. The alternate conformation of Arg228/282 results from both structural and sequence differences between the subtypes (42). This also shows that TR isoforms can have a different affinity for thyroid hormone analogs. Indeed, there are several TRβ and TRα selective analogs available (48,49).

**Figure 10.** Ribbon drawing structure of the hTRβ in complex with TRIAC. Modified from Wagner, R.L. et al., 2002. Secondary structure elements are labeled as shown in figure 9.
I.B. **Biological functions and distribution of thyroid hormone receptors**

I.B. 1. **Biological functions of TR**

The previous section showed that the affinity of thyroid hormone analogs differs between TR isoforms. TR isoforms also show differences in specific expression and preferential gene regulation as seen in table 1 and table 2 and discussed in the next sections. Table 1 shows the differences in levels of TR (mRNA and protein) between different tissues and shows also that the expression of the TR can be regulated via post transcriptional modifications, since mRNA and protein concentrations do not always correlate (22,50-54). Table 2 shows isoform specific TR functions in different organs. Hepatic gene regulation for example is mostly regulated by TRβ instead of TRα isoforms while cardiac gene expression is mediated primarily by TRα instead of TRβ isoforms. Because TR are not always expressed in the same way, mutation or deletion of a TR have a different impact on an organism.

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**Table 1.** Protein and mRNA concentration in rat tissues. (Adapted from Ercan-Feng *et al*, 1996.)

<table>
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<td>Liver</td>
<td>3900</td>
<td>5.1</td>
<td>765</td>
</tr>
<tr>
<td>Brain</td>
<td>819</td>
<td>20.2</td>
<td>40.5</td>
</tr>
<tr>
<td>Kidney</td>
<td>578</td>
<td>13.5</td>
<td>42.8</td>
</tr>
<tr>
<td>Heart</td>
<td>1460</td>
<td>7.37</td>
<td>198</td>
</tr>
<tr>
<td><strong>TRβ2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pituitary</td>
<td>5240</td>
<td>0.631</td>
<td>8300</td>
</tr>
<tr>
<td>Liver</td>
<td>829</td>
<td>&lt;0.00689</td>
<td>&gt;120,000</td>
</tr>
<tr>
<td>Brain</td>
<td>328</td>
<td>&lt;0.00684</td>
<td>&gt;48,000</td>
</tr>
<tr>
<td>Kidney</td>
<td>178</td>
<td>&lt;0.00501</td>
<td>&gt;35,500</td>
</tr>
<tr>
<td>Heart</td>
<td>506</td>
<td>&lt;0.00453</td>
<td>&gt;112,000</td>
</tr>
</tbody>
</table>
Table 2. Isoform specific TR functions in different organs.

<table>
<thead>
<tr>
<th>Thyroid hormone target</th>
<th>Receptor isoforms mediating actions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatic gene regulation</td>
<td>TRβ&gt;TRα</td>
</tr>
<tr>
<td>Bone development</td>
<td>TRα&gt;TRβ</td>
</tr>
<tr>
<td>TSH suppression</td>
<td>TRβ&gt;TRα</td>
</tr>
<tr>
<td>Ligand-independent TSH-elevation</td>
<td>TRα&gt;TRβ</td>
</tr>
<tr>
<td>Cochlear development and function</td>
<td>TRβ</td>
</tr>
<tr>
<td>Maturation of small intestine</td>
<td>TRα/TRβ</td>
</tr>
<tr>
<td>Cardiac gene expression</td>
<td>TRα&gt;TRβ</td>
</tr>
<tr>
<td>Heart rate</td>
<td>TRα</td>
</tr>
<tr>
<td>Retinal development</td>
<td>TRβ</td>
</tr>
<tr>
<td>Growth</td>
<td>TRα&gt;TRβ</td>
</tr>
<tr>
<td>Immune function</td>
<td>TRα&gt;TRβ</td>
</tr>
<tr>
<td>Temperature regulation</td>
<td>TRα&gt;TRβ</td>
</tr>
</tbody>
</table>

TRα>TRβ: predominant role for particular isoform in this case for TRα isoform

1.B.1.1. Resistance to thyroid hormone

Only one mutation in the TR is enough to cause big changes in an organism. One of the most important manifestations of such a mutation is the syndrome of resistance to thyroid hormone (RTH). This syndrome is manifest in patients which harbor mutations in TRβ isoforms, leading to dominant negative transcriptional activities. These patients have elevated circulating of thyroid hormones and elevated TSH levels (55). Additionally, other clinical features are described such as goiter, attention-deficit disorder, tachycardia, delayed bone growth and development and hearing abnormality.

At the molecular level, most mutations causing RTH found so far are in co-repressor binding sites, which leads to mild resistance to TH. Other types of mutations reduce or abrogate the binding of either the co-activator or the ligand T₃, which in itself reduces co-activator binding. These mutant receptors with dominant negative properties are unable to down-regulate TSH production as a response to excess TH (56-58). The accompanying increase in circulating TH results in a mixed hypo- and hyperthyroid appearance at tissue level since tissues that do not express the mutant phenotype (like the heart) will react to the increased TH levels. This explains the
seemingly opposite clinical features such as goiter, attention-deficit disorder and growth retardation in childhood (all associated with hypothyroidism) on the one hand and features such as tachycardia and increased metabolic rate (associated with hyperthyroidism) on the other hand (59).

An interesting fact is that so far no patients with RTH are reported with mutations in the TRα1 isoform. It is possible that the mutations in TRα isoforms are undetected either because of absence of clinical features or this mutation could be lethal during the prenatal development. Use of TR knock-out mice could give more insight in to those possibilities.

1.B.1.2. Targeted mutations and isform predominant functions of TR

Nearly all combinations of TR knock-out mice (missing either all isoforms or a single isoforms) have been reported. Table 3 shows that all genotypes of TR knock-out mice are mostly viable.

The TRα1 knock-out is fertile, shows a mild hypothyroid phenotype reflecting in reduced free T4 and normal free T3 levels due to reduced mRNA levels of TSHα gene. This mice also have a reduced body temperature and reduced heart rate which cannot be compensated by thyroid hormone treatment (60).

Selective ablation of TRα2 results in overexpression of TRα1, low levels of thyroid hormones and normal levels of TSH. Interestingly, the phenotype of the TRα2 mutant mice also shows signs of hyperthyroidism such as decreased body weight, elevated heart rate and raised body temperature (61). This data suggests that the balance in TRα1:TRα2 provides an additional level of tuning hormone responsiveness in specific tissues.

The combined TRα1/TRα2 knock-out results in a severe hypothyroid phenotype. The thyroid gland develops abnormally and homozygous mutants are profoundly hypothyroid (reduced hormone levels up to 10% of wild type (WT) mice and a 3-fold decrease of TSHβ mRNA expression in pituitary). These mice also have an arrested maturation of the intestine and reduced bone growth; the animals die within a few weeks after birth (24) and both male and female mice are infertile. These data indicate the importance of truncated TRα2 in intestine development and a role for TRα isoforms in reproductive system (62).

The selective inactivation of the TRβ gene results in a hyperplasia in thyroid, increased serum thyroid hormones and TSH common to RTH. The TSH elevation in these mice is accompanied by an increase of both TSHα and TSHβ mRNA in the pituitary. These data indicates that RTH results from a defective thyrotroph function instead of a malformation of the thyroid gland. These mice also showed impaired T3-dependent regulation of cholesterol metabolism, defects in the cochlear function and defects in the retinal photoreceptor (63-65).

Inactivation of the TRβ2 gene results also in a hyperplasia in thyroid, increased serum thyroid hormones and TSH common to RTH. The TSH elevation in these mice is accompanied by an increase of TSHβ mRNA in the pituitary. These data indicates that TRβ2 inactivation alone is sufficient to cause RTH. These mice also showed impaired T3-dependent regulation of cholesterol metabolism, and defects in retinal
photoreceptor development. Interestingly these mice showed no signs of cochlear function, indicating isoform specific function of TRβ (66).

Mice which lack all isoforms (TRβ’/TRα00), all isoforms except the TRΔα variants (TRβ’/TRα’) and all isoforms except TRα2 and TRΔα variants (TRβ’/TRαI) show similar but more severe thyroidal function problems (RTH) when compared to the TRβ knock-out and additional defects in bone development and intestinal maturation (67-69).

The TR knock-out animal models emphasize two important features. Firstly, the mice without all known isoforms are still viable. The existence of an unknown receptor isoform is one possibility which is not yet clarified. Secondly, the deletion of an isoform can be partly compensated by other receptors. Furthermore, as summarized in table 2, the isoform predominant effects, for example in the hepatic gene regulation, shows a larger role for TRβ than for TRα isoforms which can not always be explained on the basis of their expression and tissue preference since both isoforms are expressed in the liver.

### Table 3. Genotypes, thyroid status and viability of TR knock-out mice

<table>
<thead>
<tr>
<th>Knockout</th>
<th>Deleted TR</th>
<th>Expressed TR</th>
<th>Thyroid status</th>
<th>Viability</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRα’’</td>
<td>TRα1, TRα2</td>
<td>TRα1, TRΔα2 and TRα1, TRΔα2 and β isoforms</td>
<td>Severe hypothyroid</td>
<td>PWL</td>
<td>(62)</td>
</tr>
<tr>
<td>TRαI’’</td>
<td>TRα1, TRΔα1</td>
<td>TRα2, TRΔα2 and β isoforms</td>
<td>Mild hypothyroid</td>
<td>yes</td>
<td>(60)</td>
</tr>
<tr>
<td>TRα2’’</td>
<td>TRα2, TRΔα2</td>
<td>TRα, TRΔα1 and β isoforms</td>
<td>Mixed hypothyroid</td>
<td>yes</td>
<td>(61)</td>
</tr>
<tr>
<td>TRα00</td>
<td>All α isoforms</td>
<td>All β isoforms</td>
<td>Euthyroid</td>
<td>yes</td>
<td>(63)</td>
</tr>
<tr>
<td>TRβ’’</td>
<td>TRβ2</td>
<td>All α isoforms, TRβ1 if exist TR(Δ)β3</td>
<td>RTH</td>
<td>yes</td>
<td>(69)</td>
</tr>
<tr>
<td>TRβ’/TRα00</td>
<td>All known isoforms</td>
<td>-</td>
<td>Profound RTH</td>
<td>yes</td>
<td>(69)</td>
</tr>
<tr>
<td>TRβ’/TRα’’</td>
<td>All β isoforms</td>
<td>TRΔα1, TRΔα2</td>
<td>Profound RTH</td>
<td>PWL</td>
<td>(68)</td>
</tr>
<tr>
<td>TRβ’/TRαI’’</td>
<td>All β isoforms</td>
<td>TRα1, TRα2</td>
<td>RTH</td>
<td>yes</td>
<td>(67)</td>
</tr>
</tbody>
</table>

RTH: Resistance to thyroid hormone. PWL: post weaning lethality.

### I.B.2. Distribution of Thyroid hormone receptors

The observation that T3 treatment of hypothyroid rats resulted in an induction of mRNA synthesis in the liver was the first evidence of the involvement of TH in the regulation of hepatic target genes (70,71). The presence of specific nuclear binding sites in different tissues detected using radiolabeled TH provided the first evidence of thyroid hormone receptors (72). Specific thyroid hormone receptor binding sites were found almost in all T3-responsive genes in all species (72-79) as reviewed (80).
After the discovery of TR encoding genes (17,18,24,26) and based on their mRNA expression, the developmental and tissue specific patterns of TR were reported. TRα1, TRα2 and TRβ1 are expressed in almost all tissues (81) and it seems that in almost all tissues the TRα isoforms are most predominant during prenatal development followed by TRβ isoforms (82,83). In adult organisms, TRα isoforms mRNA have the highest expression in skeletal muscle (82-88). The TRΔα mRNA are expressed in the intestine in which TRΔα1 is mainly expressed in the epithelium and lamina propria of the distal ileum and TRΔα2 is most expressed in lamina propria of the proximal jejunum (62,89). TRβ1 mRNA is most highly expressed in the liver (82,83), kidney and brain and pituitary (82,83). The TRβ2 mRNA expression is limited to the brain, pituitary (82,90-94), retina (95) and TRβ3 and TRβ3 mRNA expression is restricted to the liver, spleen, heart and lung (26). Genes which are regulated by TR are not always distributed homogeneously. For example in the pituitary, both TSHα and TSHβ mRNA are only expressed in thyrotroph cells. Furthermore, as discussed in previous section, TRα1 is not able to fully compensate the loss of either TRβ2 or TRβ1 in pituitary which leads to RTH. It is thus possible that certain genes could be regulated differently by TR isoforms in certain zones within the tissue. In the liver, T3-dependent "Spot 14" gene involved in lipogenesis is expressed only in a subpopulation of cells within the tissue (96,97).

1.C. Scope of thesis
Based on the previous discussion we can conclude that the understanding of the physiology of thyroid hormone requires an understanding of not only the molecular mechanism of TR action but also of this mechanism at the tissue level (or maybe in a subpopulation of cells within that tissue). Furthermore, there are genes whose regulation is dependent on specific TR isoform, so the question arises whether local expression of TRs contributes to this isoform specific gene regulation.
To test the possibility of this postulate, the liver was used as a target study organ since it is a major thyroid hormone target and all TRs are expressed in this organ. We generated polyclonal and monoclonal antibodies against TR and we validated their specificity as described in chapters 2 and 3 and focused on TR expression at the protein level. We investigated the diurnal variations of TR expression in relation to food intake and the biological clock in chapter 4. We investigated how TR governs isoform specificity in hepatic gene regulation in chapter 5. We looked at the thyroidal state and its relation to TR expression, localization and daily TR variation in chapter 6 and finally we focused on TR expression and its post-receptor effects in the failing heart in chapter 7.
Chapter  I

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