Alternative splicing of thyroid hormone receptor alpha transcripts during health and disease
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General Discussion
Chapter 6

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This thesis deals with the alternative splicing of thyroid hormone receptor alpha transcripts. After transcription, pre-mRNAs are processed within the spliceosome to remove the introns and ligate the exons to form mature mRNA. The use of an alternative splice site or alternative polyadenylation site can then lead to different mRNAs. The mature mRNA coding sequence is flanked by untranslated regions (UTRs) that are modified by a cap structure (5’end) and a poly(A) tail (3’end) both of which play a critical role in mRNA translation and stability (1). After transport from the nucleus into the cytoplasm the mRNA degradation process takes place. Usually the event starts by poly(A) tail shortening, followed by decapping and 5’-3’ decay or 3’-5’ decay by exonucleases and/or endonucleases. Alternatively, decay can take place via a deadenylation independent process. There are several reports stating that the presence or absence of specific sequence elements in the UTRs play a role in the stabilization, transport and translational efficiency of the mRNA (2;3). Here it will be discussed how these mechanisms controlling the mRNA processing could influence the splicing of TRα transcripts resulting in a change in the expression of the different TRα mRNA isoforms.

7.1 TRα transcription and processing during physiological conditions

Simultaneous measurement of TRα1 and TRα2 transcripts

A dual-colour PCR was developed using the LightCycler for the specific and simultaneous measurement of TRα1 and TRα2 in one tube (4). Figure 1 schematically shows the primer and probe design on the TRα gene. TRα1 and TRα2 share the forward primer and probe, located in exon 8. The TRα1 specific reverse primer and probe are located in the TRα1 specific part of exon 9 (just 3’ of the TRα2 specific alternative splice site) whereas the TRα2 reverse primer and probe are located in exon 10. The probes are designed according to the FRET principle which means that when the two probes (forward and reverse) bind in the near vicinity of each other, energy transfer occurs which leads to a specific fluorescent signal. The TRα1 and TRα2 specific reverse probes are labelled with two different fluorescins leading to a specific signal for TRα1 and TRα2 which are detected in two different channels of the LightCycler. With this technique sensitive measurements of TRα transcripts became possible (for example in HepG2 cells and in livers of mice), also allowing a reliable quantification of the TRα1:TRα2 ratio (5). Since both TRα1 and TRα2 are measured in the same tube at the same time the need
for adjustment to expression levels of housekeeping genes is made redundant. This is a
major advantage especially if housekeeping genes have unstable expression patterns. In
appendix I the expression of three often used housekeeping genes (β-actin, cyclophilin
and elongation factor 1 alpha) is described in tissue samples of patients who underwent
a variety of treatments and in rats during a 12-h light, 12-h dark cycle (6). Indeed β-actin
and cyclophilin levels not only changed significantly throughout the day in rats but were
also significantly affected by hormone treatment in human liver tissue.

Co-ordination of transcription, splicing and polyadenylation

There is increasing evidence that transcription, splicing and polyadenylation are
coupled. The RNA polymerase II (Pol II) itself can direct splicing and polyadenylation
factors to pre-mRNA via its C-terminal domain (7). On the other hand the transcriptional
co-activator PGC-1 which can bind Pol II and other transcriptional co-activators can
also associate with splicing factors and influence the splicing in a promoter-dependent
context (8). In this thesis an effect of PGC-1 on the splicing of TRα is described for the
first time, although no effect of PGC-1 on TRα transcription was observed. However,
the PGC-1 mutant 565-754 which only expresses the domains involved in splicing
showed the complete opposite effect on splicing of endogenous TRα transcripts in
HepG2 cells when compared to wild-type PGC-1. This indicates that the domains
involved in transcription may eventually also play a role in the splice site choice. The
mechanism of the effect on splicing is still unknown but there is good evidence that
it is indeed a transcription-dependent phenomenon. In the study of Monsalve et al.
PGC-1 only affected the splicing of a minigene that was driven by a promoter that contained a target region for PGC-1. No PGC-1 binding site can be found in the TRα promoter but this promoter is a target for the orphan nuclear receptor estrogen-related receptor α (ERRα). This receptor in turn is activated by and can associate with PGC-1 supporting a possible effect of PGC-1 on the transcription of TRα (9;10). Chromatin immunoprecipitation studies on the promoter of TRα could provide further evidence for a role of PGC-1 in TRα transcription and processing.

The calcitonin-CGRP gene is a good example for coordination of splicing and polyadenylation (11). In thyroid C cells the calcitonin-CGRP pre-mRNA is processed to splice exons 1 to 3 and use exon 4 as the 3'terminal exon with concomitant polyadenylation. An intron element located downstream of exon 4 was shown to enhance polyadenylation of exon 4. Interestingly the factors that bound to this intron element included U1 snRNP, SRp20 and PTB which are all known splicing factors. On the other hand in neuronal cells the calcitonin-CGRP pre-mRNA is processed to exclude exon 4 and use exon 6 as the 3'terminal exon with concomitant polyadenylation. The splicing is mutually exclusive and it is thought that two sets of factors can assemble on the intron element. One complex promotes exon 4 inclusion and the other promotes exon 4 exclusion. The choice is dependent on the type of splicing proteins that assemble in these complexes and it is therefore likely that a few of these splicing factors have a tissue specific expression.

**Regulation of splicing factors**

During RNA processing general splicing factors bind to the pre-mRNA to allow correct splicing of the introns and form mature mRNA. In the case of alternative splice sites, binding of specific splicing proteins may eventually play a role in determining the direction of splicing. Several pathways may be involved in the modulation of action of the splicing factors, including hormonal activation. Insulin for example regulates the inclusion of the exon encoding protein kinase C (PKC) β2 mRNA which is important for increases in the glucose transport effect of insulin (12). Regulation of the alternative splicing leading to the exon inclusion occurs via the phosphatidylinositol 3-kinase signalling pathway through the phosphorylation state of SRp40, a splicing factor required for this insulin-regulated splice site selection. Similarly insulin can also regulate exon inclusion of the fibronectin transcript via an increase in the SRp40 concentration acting on the same purine-rich RNA element that was found in the PKC β2 RNA (13).
Several studies have shown that cells often respond to stress by changing the splice site selection (14). In a model for ischemia where cells in the brain were observed during several minutes without circulation and oxygen, some splicing proteins translocated from the nucleus to the cytosol (15). This coincided with a change in the alternative splice site selection of specific genes. A similar translocation effect was observed when primary neuronal cells were treated with increasing concentrations of calcium. Other stress factors such as osmotic shock and UV light can also induce accumulation of splicing factors such as hnRNP A1 in the cytosol of fibroblasts (16). In our studies we did not observe an effect of T₃ on the expression of the splicing factors SF2 and hnRNP A1 but we have not checked the cellular distribution of these splicing proteins and we can therefore not exclude the possibility that T₃ induced a translocation of (one of) these splicing proteins to the cytoplasm leading indirectly to a change in the TRα1:TRα2 ratio. Interestingly, T₃ can induce the expression of PGC-1 (17), a transcription factor with splice factor properties (8), and in this thesis we show that both T₃ and PGC-1 can alter the endogenous TRα1:TRα2 ratio in HepG2 cells towards TRα2. Further studies are needed to find out if the effect of T₃ on the splicing of TRα is indeed mediated by PGC-1.

The stability of mRNA transcripts

Regulation of mRNA stability is controlled by specific modifying elements within the mRNA which can bind mRNA nonspecific or specific proteins. Many mRNAs with short half lives, such as those encoding cytokines, growth factors and proto-oncogenes, contain adenylate/uridylate-rich elements (AREs) which mediate mRNA decay via binding to RNA-binding factors that recognize the ARE (18). Other RNA-binding proteins increase mRNA stability by recognizing different mRNA structures and so prevent endonuclease cleavage, for example in the case of the 3'UTR of transferrin receptor mRNA (19). In the case of the ferritin mRNA 5'UTR, interaction with specific proteins prevents binding of the ribosomal initiation complex and inhibits translation (20). Another example is poly(A) binding protein which binds to the poly(A) tail and has the ability to stabilize the mRNA and enhance translation (21;22).

TRα1 and TRα2 have different poly(A) start sites as well as unique 3'UTRs raising the possibility of a differential regulation of mRNA stability and turnover. Indeed it has been shown that TRα1 has a shorter half life (2-4 hours) than TRα2 (~8 hours) in several cell lines (23). This implicates that when TRα gene transcription decreases, TRα1
mRNA more rapidly disappears which might also be the case in our study where we treated mice with LPS and observed a reduction in both TRα1 and TRα2 mRNA levels as well as a decrease in the TRα1:TRα2 ratio in the liver. However we cannot exclude the possibility that LPS might have an effect on the stability of TRα mRNA as well. Studies using actinomycin D (which blocks transcription) or cycloheximide (which blocks protein synthesis) can usually give a good indication of the mRNA stability in cells. The use of these inhibitors helped to elucidate the elevation of TRα2 mRNA transcripts in X-ray-transformed rodent cells when compared to non-transformed rodent cells which appeared to be due to an increased stabilization of the TRα2 transcript (24). In another study it was shown that cycloheximide treatment resulted in an increase in TRα1 and a decrease in TRα2 mRNA (23). The effect of cycloheximide was not due to changes in synthesis or degradation and therefore the increase in the TRα1:TRα2 ratio was most probably caused by an effect of cycloheximide on the splicing process. However levels of Rev-erbA mRNA were also markedly increased due to cycloheximide treatment providing another explanation for a change in the splicing direction (discussed in more detail below).

The stability and degradation of specific mRNAs can be regulated by hormones. For example T₃ can stabilize malic enzyme mRNA in rat liver and reduce the half life of the TSHβ subunit mRNA in rat and murine pituitary cells (25;26). The latter was accompanied by a reduction in the length of the poly(A) tail. Since we have not checked if T₃ has an effect on the stability of (either one of the) TRα mRNA transcripts, we cannot rule out the possibility that a difference in the mRNA stability between TRα1 and TRα2 is the cause of a changed TRα1:TRα2 ratio after addition of T₃ in HepG2 cells.

The Rev-Erb mRNA is another factor that may contribute to the TRα1:TRα2 ratio by influencing mRNA stability. The 3'end of TRα2 and Rev-Erb are complementary and this could result in the formation of sense-antisense RNA duplexes which will interfere with the splicing of TRα transcripts or more specifically with the polyadenylation of TRα2. Indeed, evidence for such a regulatory system has been obtained by in vitro studies showing that addition of Rev-Erb mRNA to a splicing assay increases the formation of TRα1 (27). Correlation studies in cell lines also show a high TRα1:TRα2 ratio when a high Rev-Erb/TRα2 ratio is found (28). However, it remains to be demonstrated whether Rev-ErbA functions as a physiologically significant antisense regulator in vivo. Mice that lack the Rev-ErbAα gene show alterations in the development of granule and Purkinje cells, resembling the alterations observed in hypothyroid rats (29). However the
TRα1:TRα2 ratio in the cerebellum is not disturbed arguing against a role for Rev-Erb in the splicing process of TRα transcripts in vivo. Furthermore, we could not find a correlation between mRNA levels of Rev-Erb and the TRα1:TRα2 ratio in our mouse studies. We did observe a concomitant decrease in Rev-Erb mRNA levels and the TRα1:TRα2 ratio in the liver. However, the TRα1:TRα2 ratio returned to normal levels after 8 hours whereas Rev-Erb levels remained low, suggesting that other factors are involved.

**Possible models for the alternative splicing of TRα transcripts**

The difference in the balance in TRα1 to TRα2 mRNA transcripts could be the result of regulatory processes that are involved in the selection of the 3'-end of the mRNA. At the 3'-end of the TRα pre-transcript a choice can be made: (1) whether to process the transcript and (2) where on the transcript to place the 3'end. It is not clear if the primary event is at the level of splicing or polyadenylation or both. When the alternative

![Figure 2](image)

**Figure 2.** A schematic representation of the TRα gene and a model for competition between two 5'splice sites (A and C) or between a 5'splice site and an alternative polyadenylation site (B and C). The region specific for TRα1 is indicated in light grey and the region specific for TRα2 in dark grey. Splice sites and polyadenylation sites are indicated by black arrows. The splicing enhancer sequence SEα2 is indicated by a dashed box. Abbreviations: 5'ss: 5'splice site; SEα2: splicing enhancer alpha 2; PA: Poly(A) recognition site.
5'-splice site in exon 9 is used, TRα2—which is polyadenylated in exon 10—is generated, whereas skipping the alternative splice in exon 9 leads to the use of the poly(A) site in exon 9 generating TRα1 (figure 2). There are two possible models to accommodate these choices, namely competition between alternative 5'splice sites or between the 5'alternative splice site and the poly(A) recognition site which will be discussed below.

Alternative 5' splice site recognition

In the first model the splicing process precedes the polyadenylation and there is competition between the 5'splice site at the end of exon 9 and the alternative splice site present within exon 9 (figure 2A and C). The first component of the spliceosome that recognizes and discriminates among potential 5'splice sites in a pre-mRNA is U1 snRNP. In a model described by Eperon et al., splicing outcome depends on the number of sites that are occupied by U1 snRNP (30). When multiple sites are occupied the downstream site is used. Two well known splicing factors, SF2 and hnRNP A1, compete to bind the pre-mRNA; SF2 enhances U1 snRNP binding thereby causing a shift towards the downstream site (in our case leading to TRα1) whereas hnRNP A1 interferes with U1 snRNP binding leading to the use of the alternative splice site (TRα2). The ratio SF2/hnRNPA1 in a particular tissue could therefore determine the TRα1:TRα2 ratio in that tissue. In an elegant study Hanamura and co-authors determined the protein levels of SF2 and hnRNP A1 in several rat tissues (31) and we compared the hnRNP A1/SF2 protein ratio they found with the TRα1:TRα2 mRNA ratio that we observed in mouse tissues. There appears to be a good correlation between the two ratios. The hnRNP A1/SF2 ratio is high in brain where we find more TRα2 over TRα1 and in liver the hnRNP A1/SF2 ratio is low where we find similar amounts of TRα1 and TRα2. In testis the hnRNP A1/SF2 ratio is even lower than in liver, indicating that if there were a correlation in each tissue the TRα1:TRα2 ratio would be high. We have not measured TRα mRNA expression in testis, however Strait et al. reported that only TRα2 is expressed in this tissue in rats (32). This indicates that in addition to the tissue-specific expression levels of splicing factors other levels of regulation may play a role such as the cellular localization and/or phosphorylation of splicing factors (33;34). There is also the possibility that other splicing factors are involved and therefore ideally one should do proteomics in the liver of an LPS-treated mouse to test if the expression of splicing proteins alters due to LPS and if there is a correlation with the decreased TRα1:TRα2 ratio.
A competitive polyadenylation site

In addition to the splice site competition model there could also be a competition between using the TRα2-specific 5'splice site and the polyadenylation signal of TRα1 (figure 2B or C). In this model exon 9 is defined as a composite exon whose 3'end is formed by either a 5'splice site or a poly(A) site based on circumstances (35). Which choice is made depends on how visible these sites are to the processing machinery and how efficiently the processing complex can assemble on a site (36). The strength of a 5'-splice site is usually determined by the concentration of splicing factors in the cell as well as additional stabilizing factors recruited onto splicing enhancer elements. Similarly the strength of a poly(A) site is determined by the concentration of polyadenylation factors in the cell and additional stabilizing factors recruited onto polyadenylation enhancer elements. Therefore both expression levels of basic splicing and polyadenylation factors as well as specific factors that bind on adjacent RNA motifs could play a role in the choice between the 5'-splice site or the poly(A) site.

Support for this model comes from evolutionary studies which show that TRα1 is restricted to vertebrates and TRα2 to mammals indicating that the TRα2-specific 5'splice site arose later during evolution (37). Indeed a single nucleotide difference in the TRα2 5'-splice site sequence between the mammalian and non-mammalian sequences is important for splice site activity. When in the mammalian 5'-splice site sequence the +6G (the nucleotide which differs between mammals and non-mammals) is mutated the TRα1:TRα2 ratio increases 7-fold and hardly any splicing towards TRα2 is observed (38). Moreover the TRα2-specific 5'splice site sequence differs from the consensus at the +5 position resulting in a suboptimal splice site. When the +5C was mutated to a +5G in a TRα minigene creating a 5'ss that matches the consensus sequence, the splicing direction completely switched to TRα2 with no use of the TRα1 polyadenylation site (38). This demonstrates that the strength of the TRα2 5'splice site is critical in determining the balance between TRα1 and TRα2 mRNA processing and that regulation can only occur with a suboptimal splice site. Recently a purine-rich element was identified 130 nt downstream of the TRα2-specific splice site and immediately upstream of the TRα1 stop codon which acts as a splicing enhancer (SEα2) (38). It is required for TRα2 splicing although when the TRα2-specific 5'-splice site was mutated to a consensus 5'ss sequence, SEα2 was not required. This suggests that this intronic splicing enhancer specifically regulates the suboptimal splicing of the TRα2-specific 5'ss. SEα2 functions
via interaction with trans-acting proteins including SF2, hnRNP F and hnRNP H. It is not clear which functions these three splicing proteins have. Does SF2 enhance splicing and do the hnRNP proteins prevent splicing as is the general antagonistic function of these protein families or do they all enhance splicing? If SF2 in this model would enhance the use of the TRα2-specific splice site, this would be in contrast to the previously discussed competition model between hnRNP A1 and SF2 where high levels of SF2 prevent the use of the alternative (TRα2-specific) 5'splice site. hnRNP F was shown to be involved in the polyadenylation process in differentiating B-lymphocytes, indicating that binding of hnRNP F to SEα2 could regulate polyadenylation of TRα1 (39). Interestingly, Hastings et al also studied the TRα1:TRα2 ratio in differentiating B-lymphocytes and found changes in the TRα1:TRα2 mRNA ratio correlating to levels of Rev-Erb mRNA (28). It would be worth checking if levels of hnRNP F were also changed and correlated to the TRα1:TRα2 ratio.

It has also been shown that the 70 kD subunit of U1snRNP can inhibit recognition of the poly(A) site (40). This would fit with the suboptimal 5'splice site in exon 9 which is not optimally bound by U1snRNP. When the splice site is mutated to a consensus splice site with a high U1snRNP affinity the poly(A) recognition would be totally blocked. Binding of splicing proteins to SEα2 could also enhance the affinity for U1snRNP at the 5'suboptimal splice in exon 9 resulting in a similar repression of poly(A) site use. This again is an argument against competing 5'splice sites because if the two competing 5'splice sites were equal in strength why would the downstream 5'splice site not be used anymore?

The exon-intron architecture of the alternatively processed immunoglobulin transcripts resembles that of TRα. Two poly(A) sites are present in a different 3'-terminal exon on the primary transcripts with the first being found in mRNA encoding secreted immunoglobulins and the second being used in mRNAs producing the membrane-bound form. The use of an internal 5'splice site in exon 4 removes the secretory poly(A) site. Although no changes in the efficiency of splicing were observed, an increase in cleavage stimulatory factor (CstF) activity in plasma cells enhances the use of the first secretory poly(A) site due to binding to a specific sequence element. In this case competition between poly(A) sites and not splice site competition determines the 3'-terminal exon.

In the light of the above discussed models it seems most likely that competition between the alternative splice site and the poly(A) site in exon 9 results in the formation
of TRα2. The fact that when the alternative TRα2-specific 5'-splice site is mutated to a consensus 5'splice site only TRα2 is formed (independent of the presence of the intronic splicing enhancer SEα2) argues against a competition between 5'splice sites. On the other hand it is in favor of competition between a 5'splice site and usage of a poly(A) site because poly(A) site recognition is inhibited in the proximity of a 5'splice site. With a suboptimal 5'splice site competition is possible, however a strong 5'splice site (as in the case of the mutation to a consensus 5'splice site) completely abolishes poly(A) site recognition. Therefore the suboptimal nature of the alternative 5'splice site and the nearby intronic enhancer allow for regulation of the TRα RNA processing.

7.2 TRα transcription and processing during pathological conditions

Tissue responsiveness to thyroid hormone is dependent on 1) the amount of TH available dependent on transport of TH into the cell, the conversion of T₄ into T₃ by D1 and the degradation of T₃ into T₂ by D3, 2) the amount and affinity of TRs available to mediate the response to T₃ on gene expression. During pathological conditions tissue-specific TH responsiveness may change due to one or more disturbances in one or more of these processes. Here we will discuss only TRα transcription and processing during pathological conditions.

Thyroid hormone receptors in hypo- and hyperthyroid conditions

Thyroid hormone receptors have a developmental and tissue-specific pattern of expression. TRα is expressed in almost every tissue but seems to mediate thyroid hormone action in a subtype-specific manner, correlating to its level of expression or specific distribution pattern even within an organ (41;42). Thyroid status influences the expression of TR isoform mRNA and protein concentration in tissues. The TRβ gene can respond directly to T₃ since its promoter contains a TRE. The case for the TRα is less clear but there is evidence that it is also modulated by thyroid hormone (43).

Most studies concerned with the influence of thyroid hormone on the TR expression report an increase in TRα mRNA levels in liver, heart and pituitary during hypothyroidism and a decrease in TRα as a result of T₃ administration (32;43-45). A distinction can be made between studies which render animals hypothyroid and then supplement T₃ and studies that compare euthyroid animals with animals that are rendered either hypo- or hyperthyroid. In studies where thyroid hormone deprivation was induced with PTU and
animals subsequently received T₃ treatment the decrease in TRα mRNA expression after treatment with T₃ was very profound (43;46). Studies that compared euthyroid animals with hypothyroid or hyperthyroid animals are summarized in table 1. Hypothyroid animals in all studies showed a higher expression of TRα mRNA in liver and heart when compared to euthyroid animals (32;44;45). Only the pituitary showed no difference in TRα expression (47). Therefore one could conclude that thyroid hormone deprivation leads to an increase in TR expression in tissues to compensate for the low levels of circulating thyroid hormones. In the pituitary TRβ2 is the predominant isoform and several studies have shown that TRβ2 mRNA expression increases as a result of hypothyroidism (43;48). Restoration of euthyroidism subsequently decreases TR expression whereas an excess of thyroid hormones not always shows an effect on TR expression. Ercan-Fang et al. reported no changes in TRα in the pituitary after administration of T₃ (47). Hodin et al. on the other hand reported a large increase in TRβ1 expression in the pituitary after administration of T₃ which correlated to an increase in the T₃ nuclear binding capacity (43). Furthermore, Zandieh Doulabi et al. observed a decrease in TRα expression in liver when T₃ was given to euthyroid animals although this effect was significant only at 13:30 but not at 19:30 suggesting that this effect on TRα is subject to diurnal changes (45). When TRα and TRβ indeed serve different functions, such as interacting with different target genes, then the decrease in TRα could have consequences for thyroid hormone action.

**Table 1.** The effect of thyroidal status on TRα mRNA expression

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Hypothyroidism</th>
<th>Hyperthyroidism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TRα₁</td>
<td>TRα₂</td>
<td>TRα₁</td>
</tr>
<tr>
<td>Liver</td>
<td>↑*</td>
<td>↑</td>
<td>=</td>
</tr>
<tr>
<td>13:30</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
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<tr>
<td>19:30</td>
<td>↑</td>
<td>↑*</td>
<td>↓*</td>
</tr>
<tr>
<td>Heart</td>
<td>↑</td>
<td>↑</td>
<td>=</td>
</tr>
<tr>
<td>Pituitary</td>
<td>=</td>
<td>=</td>
<td>↓</td>
</tr>
<tr>
<td>GH cells</td>
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<td></td>
<td>↓</td>
</tr>
<tr>
<td>HepG2 cells</td>
<td></td>
<td></td>
<td>↓*</td>
</tr>
</tbody>
</table>

↑: increased, =: no change and ↓: decreased relative to euthyroidism. * not significant
Table 1 also shows the effect of high levels of T₃ on TRα mRNA levels in cell lines. In our T₃-treated cells we found a significant decrease in the TRα1:TRα2 ratio which could result in a lower sensitivity to thyroid hormone action (5). Indeed Lazar and co-authors have observed a decrease in T₃ nuclear binding capacity in GH cells after stimulation with T₃ which was accompanied by a decrease in TRβ1 and TRα2 (49). There are no other studies except our study (5) that describe a direct measurement of the TRα1:TRα2 ratio. In the report of Hodin et al. (43) the expression level of TRα2 in heart is more decreased after T₃ treatment than the TRα1 expression and therefore the TRα1:TRα2 ratio is probably increased. However one should determine the TRα1:TRα2 ratio in each animal first before any definitive conclusions can be drawn. Taken together these results fit with a model of receptor autoregulation by thyroid hormone both at the transcription as well as the processing level of TRα.

**Thyroid hormone receptors during nonthyroidal illness**

Nonthyroidal illness (NTI) refers to changes in thyroid hormone parameters in patients with a wide variety of illnesses. Serum T₃ concentrations decrease and serum reverse triiodothyronine (rT₃) concentrations increase whereas TSH levels usually remain in the normal range (50). Similar changes in serum thyroid hormone levels are observed during fasting. Several *in vitro* and *in vivo* studies have shown that transport of T₄ into tissues is inhibited during NTI (51-54). The recent discovery of a very specific thyroid hormone transporter monocarboxylate transporter 8 (MCT8) further underlines the role of thyroid hormone transport in the cellular availability of thyroid hormones (55). Iodothyronine deiodinases D1 and D3 also contribute to the low serum thyroid hormone levels that are found during NTI. Critically ill patients who died in the ICU had lower hepatic D1 activity correlating to a lower T₃/rT₃ ratio and they also showed an induction in D3 activity (56). Moreover in chronically ill patients the low serum levels of thyroid hormone are related to the loss of TSH pulse amplitude and a reduced hypothalamic TRH expression (57;58).

The altered thyroid status during NTI can also change the expression of thyroid hormone receptors. In humans with chronic liver disease, reduced serum thyroid hormones were found to associate with an increase in TRα and TRβ mRNA in liver biopsies providing support for the maintenance of euthyroidism in tissues in the light of decreased serum thyroid hormone levels (59). In our studies we found an association between the TRα1:TRα2 ratio in liver biopsies of patients who had died on the ICU.
and severity of disease and age, indicating that in older and more severe ill patients the 
TRα1:TRα2 ratio was upregulated (Chapter 2). Moreover the TRα1:TRα2 ratio was also 
higher in patients who had needed renal replacement therapy as well as inotrope treat-
ment. In line with the study of Williams et al. (59), these results indicate a possible up-
regulation in the sensitivity to T3. A shortcoming of our study was our inability to compare 
the TR mRNA expression data with a control group of patients that had overcome 
their illness or simply healthy controls. Only one other study has been performed using 
human liver biopsies to measure TR expression. Chamba et al. have compared TRα and 
TRβ mRNA expression in normal human liver and liver affected by primary biliary 
cirrhosis, sclerosing cholangitis, cryptogenic cirrhosis and alcoholic cirrhosis (60). They 
were not able to detect TRα1 and TRβ1 mRNA expression and only a weak signal for 
TRα2 mRNA. Using polyclonal antibodies against TRs they could however detect TR 
protein expression but found no differences between normal and diseased liver.

An animal model for NTI is LPS-induced sickness. Administration of LPS results 
within two hours in systemic illness including hypothermia, which is a characteristic 
response in small rodents, and a release of pro-inflammatory cytokines such as IL-6, 
TNF-α and IL-1 in the liver (61). Serum thyroid hormones start to decrease 8 hours 
after injection whereas no changes in TSH are seen. Simultaneous changes in the TRβ 
mRNA expression and deiodinase activity in the hypothalamus, pituitary, thyroid and 
liver suggest that the whole HPT-axis is involved in the downregulation of thyroid 
hormone metabolism (Boelen, personal communications). TRα mRNA expression is 
also altered during NTI and precedes the changes in serum thyroid hormone levels. 
Table 2 summarizes the changes in TRα mRNA expression in the HPT-axis after 
LPS administration. Beigneux et al. reported a decrease in TR protein levels in liver 
after administration of LPS which was however not until 16 hours after LPS (62). A 
decrease in TR DNA binding was observed 4 hours after LPS and therefore preceded 
the decrease in TR protein levels. They suggested that the limited availability of the TR 
heterodimerization partner RXR might play a role in the decrease in TR DNA binding 
since all three RXR isoforms are downregulated at the protein level as early as 4 hours 
after LPS administration in hamster liver (63). We observed a decrease in both TRα 
mRNA and protein levels after 4 hours. However we have not measured the TRβ protein 
level which is the predominant TR isoform in liver. T3 target genes such as Spot 14, malic 
enzyme and D1 mRNA levels in liver and/or pituitary also decrease after LPS treatment 
but are preceded by the decrease in TR expression (62). From these studies it can be
Table 2. The effect of acute illness on TRα mRNA expression

<table>
<thead>
<tr>
<th>Tissue</th>
<th>TRα1</th>
<th>TRα2</th>
<th>TRα1/2</th>
<th>Reference</th>
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<td>↓</td>
<td>↓</td>
<td>(62)</td>
</tr>
<tr>
<td>Heart</td>
<td>↓</td>
<td>↓</td>
<td></td>
<td>Chapter 4</td>
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concluded that a rapid LPS-induced decrease in TR mRNA expression in the pituitary and liver is probably involved in the downregulation of thyroid hormone metabolism during NTI.

In addition to the decrease in TR expression the TRα1:TRα2 balance also changes during acute illness induced by LPS. Both in our study and in the study of Beigneux (62) the TRα1:TRα2 mRNA ratio in liver is decreased 4 hours after LPS administration and in both cases this is due to a faster decrease of TRα1 compared to TRα2. In the pituitary the decrease in the TRα1:TRα2 ratio is the result of a decrease in TRα1 only. A change in the TRα1:TRα2 ratio towards the dominant negative TRα2 may therefore add another level to the downregulation of thyroid hormone responsiveness during NTI.

Splicing in pathological conditions

Differences in splicing patterns that cause disease can be the result of mutations in splicing motifs or changes in the levels of splicing factors. Mutations that affect pre-mRNA splicing include mutations in the invariant splice site sequences (subclass I), mutations in variant motifs leading to changes in the strength of exon recognition motifs (subclass II) or intronic mutations which can generate cryptic donor or acceptor sites and can lead to partial inclusion of intronic sequences (subclass III) (64). One example of class I and class II mutations is found in patients with mutations in the ATP7A gene leading to a lethal disorder of copper metabolism (Menke disease, MD) or a milder form characterized by connective tissue abnormalities (occipital horn syndrome, OHS) (65). In MD the donor splice site of exon 6 of the ATP7A gene is disrupted leading to complete skipping of exon 6 and severe MD. In the same ATP7A gene a 3-bp deletion affecting the same donor site can also lead to incomplete exon skipping and the milder form OHS.
Mutations which cause cryptic splice sites in introns are found in the gene encoding the cystic fibrosis transmembrane conductance regulator and are associated with a wide variability in disease severity in patients suffering from cystic fibrosis (66;67).

There are also cases in which changes in the ratios of authentic protein isoforms cause disease due to a change in the levels of splicing proteins in certain tissues. A good example is the insulin receptor (IR) which is alternatively spliced into two isoforms: IR-A and IR-B. The IR-B isoform is predominantly found in the insulin-responsive tissues that are responsible for glucose homeostasis (adipose tissue, liver and skeletal muscle) and IR-A is found in brain, lymphocytes and spleen (68;69). Whereas IR-A has a higher affinity for insulin, IR-B has an overall faster signalling capacity (70). Dexamethasone can induce a switch from IR-A to IR-B in a hepatoma cell line which correlates to an increase in insulin sensitivity (71). Interestingly in patients with myotonic dystrophy type 1 (MD1) an unusual form of insulin resistance is found which is caused by aberrant regulation of the alternative splicing of the insulin receptor in skeletal muscle. Instead of the predominant IR-B isoform the nonmuscular IR-A isoform was found to predominate in skeletal muscle tissues of MD1 patients (72). This switch could be explained by increased levels of the splicing regulator CUG-BP that were also found in the skeletal muscle tissues. Overexpression of splicing factors from the SR family of splicing proteins is also associated with disease. In a mouse model of mammary gland tumorigenesis a profound change in the repertoire and levels of several SR proteins was found which was related to the relative levels of transmembrane glycoprotein CD44 transcripts (73). CD44 is normally involved in cell migration and attachment to extracellular matrix and surfaces of other cells but alternatively spliced isoforms of CD44 have been detected in many malignant tumors and correlated to poor patient survival (74).

In liver biopsies of critically ill patients we did not find an association between the expression of the SF2 and hnRNPA1 splicing factors and type or severity of disease. Similarly, in mice that were treated with LPS, SF2 levels did not significantly change in hypothalamus, pituitary and liver. We can however not exclude the possibility that other members of the SR family may be affected by LPS treatment. It would be nice to study protein expression levels of several splicing factors in livers treated with LPS to find out which factors are altered by LPS treatment and are possibly related to the alternative splicing of TRα pre-mRNA.
7.3 Future perspectives

The importance of a balance in TRα transcripts

Although the role of thyroid hormone in health and disease is well known, the physiological roles and specific functions of the individual thyroid hormone receptor isoforms remain largely unidentified. The generation of genetically modified mice has provided us with a new understanding of the complexity of TH action in vivo and the roles of the TR isoforms. TRα2 is only a weak antagonist of TRα1 but when the balance between TRα1 and TRα2 is disrupted, as in TRα2/− mice which lack TRα2 and overexpress TRα1, the phenotype shows features of both hypothyroidism and hyperthyroidism suggesting disturbed homeostasis (75). TRα2 is thus regarded as a receptor that could adjust the activity of the functional receptor TRα1 to metabolic needs, possibly regulating thyroid hormone sensitivity. Mice that completely lack TRα also have an increased sensitivity to thyroid hormone in heart and liver whereas no compensatory increase in TRβ1 was observed (76). However in the pituitary of these mice a compensatory increase in the mRNA levels of the steroid receptor coactivator-1 (SRC-1) was found (77). Since SRC-1 mediates TR-dependent thyroid hormone action this increase could also be responsible for the increased sensitivity to thyroid hormone which is observed in TRα0/0 mice at least in the pituitary. If SRC-1 is increased in other tissues of TRα0/0 mice as well remains to be established. Therefore, although the lack of a competing TRα1 aporeceptor cannot be ruled out, it is more likely that the lack of a constitutive silencing effect of TRα2 enhances the responsiveness to TH in liver and heart of TRα0/0 mice. In this light decreasing the TRα1:TRα2 ratio represents a mechanism to lower thyroid hormone sensitivity which may be useful to save energy during illness or to dampen the T3 response during hyperthyroid conditions (5). On the other hand an increase in the TRα1:TRα2 ratio may enhance T3 responsiveness during hypothyroid like conditions which is indeed observed in the heart of hypothyroid mice (77) and in liver tissue of critically ill patients (chapter III). To further explore the role of a balance in TRα1:TRα2 several experiments could be undertaken. In the first place the exact role of TRα2 must be studied during pathological conditions. To this end mice lacking TRα2 could be treated with LPS to see if they become more sensitive to LPS due to the lack of TRα2. Similarly the lack of TRα2 would probably lead to an earlier death in TRα2/− mice than in WT mice during fasting. Since the TRα2/− mice show growth retardation and signs of a dysfunctional thyroid, generation of a conditional
TRα2/− knock-out mouse for these experiments might circumvent a possible bias in these experiments due to abnormal development.

**Modulation of the splicing process**

An elegant way to further study the splicing process of TRα would be to do a comparison of the expression of splicing proteins between tissues with a high or low ratio of TRα1 to TRα2. For example in the hypothalamus a very low (0.02) TRα1:TRα2 ratio is found whereas in liver and heart the levels of TRα1 and TRα2 are similar. It would be interesting to know if expression levels of specific splicing proteins are associated with a high or low TRα1:TRα2 ratio. This could for instance be achieved by proteomics. When these splicing factors are known we could then further analyze the TRα1:TRα2 ratio in mice which lack these splicing factors or in our *in vitro* HepG2 system by overexpressing these splicing factors.

In the case of PGC-1, further experiments could focus on how PGC-1 regulates the TRα1:TRα2 ratio, for example in response to metabolic signals. PGC-1 is induced by metabolic signals such as cold in brown adipose tissue, fasting in liver and heart and exercise in skeletal muscle (78) and several studies have shown that PGC-1 then activates gene expression in response to these metabolic stimuli (79;80). Notably no studies have yet been performed on how metabolic signals might influence the splicing process via splicing factors, including PGC-1. In livers of rats that were fasted for 48 hours for example, the TRα1:TRα2 ratio was significantly decreased (81). Since PGC-1 is induced by fasting, taken together with the fact that PGC-1 can cause a decrease in the TRα1:TRα2 ratio in HepG2 cells, a model could be envisaged in which PGC-1 affects the splicing of TRα in response to fasting via binding to the promoter of the TRα gene (for example as a coactivator of ERRα which is known to activate the TRα promoter (10)). It could be directly involved by binding to the RNA transcript via its RNA recognition motif or PGC-1 could attract splicing proteins via its SR domain. This is still very speculative and further studies are needed to determine if PGC-1 is indeed directly involved in the processing of TRα transcripts.
Chapter 6

Reference List


Chapter 6


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General Discussion


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