Alternative splicing of thyroid hormone receptor alpha transcripts during health and disease
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Introduction
Chapter 1

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1.3 Splicing of thyroid hormone receptor alpha transcripts
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Thyroid hormones ($T_3$: 3,5,3'triiodo-l-thyronine and $T_4$: thyroxine) play a critical role in development, metabolism, cellular proliferation and differentiation of many organs and tissues. Most of the effects of thyroid hormone are mediated by thyroid hormone receptors (TRs) which, along with the receptors for steroid hormones, retinoids and vitamin D, belong to the nuclear receptor superfamily. TRs are DNA-binding transcription factors that can activate or repress gene transcription by recruiting several protein complexes which modify the structure of chromatin. Two genes (TR$\alpha$ and TR$\beta$) encode a variety of TRs. In the case of TR$\alpha$, alternative splicing of the pre-mRNA results in the generation of two functionally different receptors. TR$\alpha$1 binds $T_3$ and activates transcription whereas TR$\alpha$2 does not bind $T_3$ but has a dominant negative effect in TR-mediated transcription. In this introduction we discuss three topics: the physiology of thyroid hormone receptors, the mechanism of (alternative) splicing of gene transcripts and finally the splicing of thyroid hormone receptor transcripts.

1.1 Thyroid hormone receptors

**Gene structures of thyroid hormone receptors (TRs)**

TRs are encoded by two different genomic loci, TR$\alpha$ by c-erbA or NR1A1 on chromosome 17q11.2 and TR$\beta$ by NR1A2 on chromosome 3p24.3 (1-3). The human TR$\alpha$ gene is organized in 10 exons and encodes for TR$\alpha$1, TR$\alpha$2 and their $\Delta$ variants (figure 1). An alternative $T_3$-binding protein (p43), related to TR$\alpha$1, was discovered in chicken erythroid cells (4). However no additional mRNA was identified that could lead to the specific synthesis of p43 and it is therefore believed that p43 is generated by an alternative translation initiation at an internal AUG in the TR$\alpha$1 mRNA (5). TR$\alpha$1 is generated by a messenger RNA (mRNA) that begins at exon 1 and terminates at a poly-A addition signal located in the 3' region of exon 9 (6). TR$\alpha$2 is derived from the RNA produced by an alternative splice site located 128 bp downstream of the start of exon 9 and the splice acceptor site of exon 10. The poly-A addition signal used to terminate this product is located 364 bp downstream of the start of exon 10. This event replaces the 40 final amino acids of TR$\alpha$1 with 120 amino acids specific for TR$\alpha$2 resulting in different C-terminal regions. $\Delta$TR$\alpha$ variants were originally described in mouse ES cells where short transcripts were found that derived from an alternative promoter located within intron 7 of the TR$\alpha$ gene (7). The transcripts encode isoforms of TR$\alpha$1 and TR$\alpha$2 which lack the DNA binding domain and part of the ligand binding domain.
The ΔTRα variants are therefore unable to bind DNA and T₃. Both ΔTRα variants are able to inhibit transcription mediated by TRα1 and TRβ1 in an in vitro transfection study although the inhibition of transcription was non-specific for ΔTRα2. Moreover TRα knockout mice which lack TRα1 and TRα2 but still contain ΔTRα1 and ΔTRα2 display a very severe phenotype with an impaired development of the small intestine and bone and these mice die shortly after weaning (8). Knocking out all TRα variants restores most of these defects. Another member of the thyroid/steroid hormone
receptor family which is encoded on the TRα genomic locus is Rev-ErbAα (Rev-ErbA) (9;10). Rev-ErbA is encoded by eight exons from the opposite DNA strand from which the TRα mRNAs are transcribed. As a result the mRNAs of Rev-ErbA and TRα2 (but not TRα1) are complementary for 269 nucleotides making specific base pairing interactions possible between the two transcripts. To date no ligand has been identified for Rev-ErbA and it is therefore referred to as an orphan receptor. c-erbA is the human cellular homolog of v-erbA which is one of the two oncogenes of the avian erythroblastosis virus (AEV), an acute chicken retrovirus capable of inducing erythroleukemia and sarcomas (2). v-erbA does not bind thyroid hormone but retains the ability to bind DNA in a sequence-specific fashion (11).

The TRβ gene consists of 11 exons of which exons 3-8 are common for all TRβs and show high homology to the TRα isoforms. It encodes TRβ1, TRβ2, TRβ3 and a truncated variant TRAβ3, all generated by alternative splicing and/or alternative promoter usage resulting in differences only in the N-terminal regions of the proteins (12;13). In this introduction we will mainly focus on the TRα isoforms.

**TR promoter context**

The promoter that initiates TRα transcription does not contain the typical TATA-like element that is found in most eukaryotic genes and which determines the transcription start site (14;15). Instead, the TRα promoter contains multiple initiation sites that are linked to the numerous transcription factor Sp1 sites found in this sequence region and therefore has the hallmark of a "housekeeping gene" promoter. The TRα promoter sequence does not harbour a thyroid hormone responsive element (TRE) but it is positively regulated by glucocorticoids through a glucocorticoid responsive element (GRE) (14). Furthermore a putative binding site for KROX20 (a transcriptional factor that plays a role in early development of the central nervous system) was found (15). The TRα promoter also contains a consensus SF-1 (Stereogenic Factor 1) responsive element (SFRE) which can mediate the effect of SF1 but is also a binding site for an orphan nuclear receptor, the estrogen receptor related receptor alpha (ERRα) (16). Moreover the TRα promoter has been reported a molecular target of ERRα in specific tissues. The Rev-Erb promoter contains a functional Rev-Erb responsive element which could represent auto regulation of Rev-Erb at the transcriptional level (17). The TRβ promoter contains a TATA box as well as two thyroid hormone responsive elements which can mediate responses to T₃ via TRα1 and TRβ1 (18;19).
TR translation products: structures and functions

Thyroid hormone receptors have a similar domain organization to the other members of the thyroid/steroid hormone receptor superfamily. This consists of a modular structure with the different regions corresponding to autonomous functional domains that can be interchanged between the related receptors without loss of function. A typical nuclear hormone receptor is made up of a variable N-terminal region (A/B), a conserved DNA-binding domain (DBD or region C), a hinge region (D) and a conserved E/F region that contains the ligand binding domain (LBD) (figure 2) (20). The A/F regions may contain autonomous transcriptional activation functions which contribute to constitutive ligand-independent activation by the receptor (AF-1 region) or strictly ligand dependent transactivation functions (AF-2). The various TR translation products are shown in figure 2. A short TRα1 protein isoform (p43) identified in chicken is synthesized by alternative translational initiation at an internal AUG in the mRNA encoding the full length TRα1 (4;21). P43 was localized in the matrix of mitochondria in rat liver where it could mediate the mitochondrial response to T₃ (5;22).

The A/B domain is similar for TRα1 and TRα2 but differs between TRα and TRβ as well as between the different TRβ isoforms. The function of the A/B domain remains largely unknown but since the A/B domain shows promoter and cell specific activity, it is suggested that it may contribute to specificity of action among isoforms (20). Furthermore, the A/B domain could be a target for phosphorylation which, for example, is the case for human TRβ1 which can be phosphorylated in vitro in response to T₃ and this may affect transcriptional activity (23;24). In TRα1 encoded by avian c-erbA, two serine residues in the A/B domain were identified that could be phosphorylated by protein kinase C (PKC), however the function of this phosphorylation remains unknown (25).

The DNA-binding domain confers the ability to recognize specific target sequences (thyroid response elements; TREs) on DNA and activate transcription. It is organized into two “zinc fingers” each containing 4 cysteine residues coordinated by a zinc ion (26). The amino acids that are required for DNA recognition are present in a region termed the “P box” located in the first finger whereas residues in the second finger form the “D box” which is involved in dimerization (20).

The hinge region is located between the DBD and LBD, allowing rotation of the DBD relative to the LBD. In TRs the hinge region harbours motifs called nuclear localization signals (NLS). For the TRβ1 one NLS (located between aa 184-190) has been shown
**Figure 2:** A schematic representation of a typical nuclear receptor (NR) and all TRα translation products. A typical nuclear receptor is composed of an N-terminal A/B domain required for transcriptional transactivation, a DNA-binding domain (C), hinge region (D) and ligand-binding domain (E/F) which is also involved in dimerization. The DNA-binding domain is coloured light grey. The region specific for TRα1 and ΔTRα1 is black stained and the region specific for TRα2 and ΔTRα2 is black-and-white. Different numbers represent amino acids on the TRα receptor. Mass is given in kiloDalton (kD).

To mediate T₃ induced nuclear translocation (27). TRα1 also possesses several NLS and both TRα1 and TRβ1 were shown to shuttle between the cytoplasm and the nucleus (27;28). Phosphorylation of TRα1 enhances nuclear retention and/or inhibits nuclear export (29). Another function of the hinge region is its involvement in transcriptional suppression by the binding and release of corepressors (11;30;31).

The LBD is required for binding of ligand and also mediates ligand-dependent transcriptional activity as well as homo- and heterodimerization (32). The LBDs of TRα and TRβ are markedly conserved and show only one amino acid residue difference (Ser 277 in TRα or Asn331 in TRβ) around the hormone binding pocket. This difference adds to the differences found between the two subtypes in regulation of transcription and binding to synthetic ligands (33). For example the presence of Asn331 instead of Ser277 in the TRβ1 results in a higher affinity of TRβ1 for the synthetic thyroid hormone analogue GC-1 due to a more stable interaction of the LBD with GC-1. A conserved region near the C-terminal end of the LBD, called the ninth heptad, is important for heterodimerization (34). Furthermore the LBD contains the activation domain 2 (AF-2) which is involved in interactions with coactivators and mediates ligand-dependent tran-
scriptional activation (35). Coactivators mediate the interactions of transcription factors
with the basal transcription machinery via their LXXLL motif (where L is leucine and X
is any amino acid), which is the crucial element that interacts with the AF-2 domain of
transcription factors (36;37).

**Binding of TRs to Thyroid Response Elements (TREs)**

TRs regulate gene expression through binding to specific DNA sequences known
as thyroid hormone response elements. TREs are usually composed of two or more
consensus motifs AGGTCA (referred to as “half sites”) (38). Specificity of target gene
recognition is determined, at least in part, by the orientation and spacing of two half
sites consisting of the consensus sequence AGGTCA (39). TRs preferentially bind to
two directly repeated AGGTCA sequences separated by four base pairs (DR4: AGGT-
CANNNNAGGTCA). Other configurations are palindromes (AGGTCATGACCT)
and inverted palindromes (TGACCTNNNNNNAGGTCA). In vitro studies using
fusion proteins of Rev-Erb and the N-terminus of the glucocorticoid receptor have
shown that Rev-Erb can bind to DR4 TREs but not to palindromes or inverted palin-
dromes (40). The DNA-binding domain of Rev-Erb can also bind in vitro to the TRE
of the rat TSHβ gene indicating a possibility that RevErb could also be involved in regu-
lating a subset of TREs.

TRs can bind to DNA as a monomer (only one TR), homomer (two TRs) or as a het-
erodimer with other members of the nuclear receptor superfamily, such as the retinoid
X receptor (RXR), retinoid acid receptor (RAR) and the chicken ovalalbumin upstream
promoter transcription factor (COUP-TF). Binding of a TR monomer or homodimer
is relatively weak due to rapid dissociation of the TR-TRE complex. The stability of the
complex is greatly enhanced when TR is bound to a TRE as a heterodimer and the RXR
is the preferent heterodimerization partner (41-43). The preferred DNA-binding form
is a heterodimer however at very high in vitro concentrations of TR only homodimers
are formed (41). TR homodimers have been shown to interact strongly with the SMRT
corepressor whereas TR/RXR heterodimers are inefficient at binding SMRT indicating
a role for homodimers in transcriptional repression (44).

In addition the sequence and topology of a TRE can determine the recruitment of
a homo- or heterodimer thereby also implying a specific role for the nature of the TRE
in recruitment of corepressors. A TRE is referred to as a positive TRE or a negative
TRE. In positively acting TREs gene transcription is activated by liganded TRs that are
associated with coactivators whereas binding of unliganded TRs, associated with corepressors, repress transcription. When binding of liganded TRs results in repression of transcription a TRE is called a negatively acting TRE. Negative TREs have been identified in the genes encoding the TSHα (45) and β (46) subunits, TRH and the β-amyloid precursor protein (APP) (47;48). For example in the case of the TSHβ gene the TRE recruits TRβ1 and mediates negative regulation by T₃. The mechanism, which is dependent on DNA binding, includes recruitment of histone deacetylases (HDACs) which are involved in transcriptional repression (by tightening nucleosome-DNA interactions thereby reducing access of the transcription factors to the chromatinated promoter (49)) (50;51). The properties of negative TREs are not completely understood but it is believed that the position of negative TREs (generally very close to transcription initiation sites) might play a role (44).

TRα2

Alternative splicing of the last exon of TRα1 results in the generation of the non-T₃-binding C-terminal variant TRα2 (52). TRα2 functions as a weak dominant negative inhibitor of thyroid hormone action (53;54). The nature of the dominant negative activity is discussed below.

TRα2 forms a TRE-binding complex as a monomer and a homodimer but it binds DNA much weaker than TRα1. This is probably the result of the unique C-terminus of TRα2 which lacks the second half of the ninth heptad sequence (at amino acids 368-374 of TRα1) which is important for heterodimerization with RXR (55). TRα2 may therefore be unable to heterodimerize with RXR (or any of the other heterodimerization partners of TRα1 including TRα1 itself which was indeed reported by Katz et al (56)). However TRα2:RXR heterodimers have been reported, but these were only formed on a DR4 TRE (55;57;58). This discrepancy is explained by the nature of the DR4 TREs, e.g. certain flanking and spacer sequences of the TRE which can enhance binding of TRα2/RXR heterodimers. When the last 100 or 150 amino acids of the TRα1 C-terminus were fused to the C-terminus of TRα2ΔC (made by deletion of the last 20 of the 122 unique amino acids of TRα2), the heterodimerization property of TRα2ΔC was completely restored, however this was not the case when the TRα1 specific amino acids were fused to intact TRα2 (56). In addition Yang et al showed that restoration of the ninth heptad in TRα2, by insertion of 9 TRα1 specific amino acids after amino acid 370, is critical for restoring heterodimerization on the palindromic DNA (57). However for restoration of
heterodimerization on the inverted repeat, addition of a series of alanine residues functions as well. The partial lack of the ninth heptad is also involved in the very weak or lack of interaction that is reported for TRα2 with nuclear corepressors (58;59). When the ninth heptad is restored TRα2 is able to interact strongly with corepressors (60). These data clearly underline the role of the unique C-terminus of TRα2.

The dominant negative activity of TRα2 requires DNA binding but the relatively weak nature of this activity is only partly explained by the weak DNA binding (57). For example dephosphorylation of the TRα2C-terminus or rendering a nonphosphorylatable form of TRα2 completely restored its DNA binding as a monomer (61). Katz et al also showed that deletion of the last 20 of the 122 unique amino acids of TRα2 (creating a TRα2ΔC) increased the DNA-binding properties of TRα2ΔC to the level of TRα1. This indicates that the C-terminus of TRα2 is an inhibitory domain.

The fact that TRα2 has weak DNA binding activity and fails to interact properly with corepressors such as NcoR and SMRT indicates that TRα2 does not actively inhibit transcription (58). One of the mechanisms of the dominant negative activity of TRα2 is based on competition with the other TRs for binding to TREs. TRα2 is able to inhibit the binding of TRα1 monomers, homodimers and RXR-heterodimers although at a high ratio TRα2 to TRα1 (62). Moreover a modest inhibition by TRα2 was shown on TRα1 or TRβ1 mediated transcription on positive TREs but not on negative TREs (63). The nature of the TRE and its flanking sequences in the promoter of T₃ regulated genes is also important. For example TRα2 actively inhibits T₃/TRβ1 transactivation of a myelin basic protein promoter construct but not of a construct with the promoter of malic enzyme (64). Finally TRα2 has been shown to act in a dominant negative manner without binding to a TRE probably by titrating out essential (basal) transcription factors (65).

2.1 Splicing of gene transcripts

Constitutive and alternative splicing

Pre-mRNA splicing was discovered in 1977 and represents an essential step in the expression of genes which contain non-coding intron sequences (66;67). The introns are transcribed into pre-mRNA but are subsequently removed and the exons are ligated together to form translatable mRNA. Constitutive pre-mRNA splicing involves the initial recognition of conserved splice site sequences at the intron-exon boundaries by
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various proteins and splicing factors. The 5'splice site signal which is called the splice site donor is generally a variation of the consensus sequence CAG/GUAAGUA. The 3'splice site, or the splice site acceptor, consists of three components: a branch point sequence [YNCURAY (Y, pyrimidine; N, any nucleotide; R, purine)], a polypyrimidine-tract and a CAG sequence at the 3'-end of the intron (68). Auxiliary splicing enhancer sequences have been found in exons (exonic splicing enhancers) and in introns (intronic splicing enhancers). Splicing occurs in the spliceosome which is a 60S ribonucleoprotein complex (like a ribosome) consisting of snRNA-containing small nuclear ribonucleoprotein (snRNP) complexes, arginine/serine rich (SR) RNA-binding proteins and other non-SR protein splicing factors, including heterogeneous nuclear ribonucleoproteins (hnRNP), RNA helicases, kinases and other enzymes (69;70). The spliceosome assembles in a highly ordered and stepwise manner (71) (figure 3). First U1 snRNP binds with its 5'end to the complementary sequence at the 5'splice site at the pre-mRNA. Subsequently the U2 snRNP binds to the branch point and then the U4/U6 (these snRNPs form a tight complex) and U5 snRNP bind to form a splicing complex (the spliceosome). U1 and U4 are destabilized and the spliceosome is activated for catalysis. Splicing occurs in two ATP-independent trans-esterification reactions. During the first catalytical step a conserved nucleotide in the intron sequence attacks the phosphate at the 5'splice site and cleaves the 5'exon thereby producing a lariat intermediate (which has the shape of a lasso). The second step involves repositioning of the 3'hydroxyl of the 5'exon near the phosphate at the 3'splice site of the exon and cleavage of the 3'splice site of the pre-mRNA. The two exon structures are then joined together and the intron sequence is released as a lariat. The lariat which is still bound to the snRNPs is then disassembled in the nucleus and the snRNPs are recycled.

Alternative splicing is the process by which multiple forms of mRNA can be formed from one pre-mRNA molecule. Figure 4 shows the different types of alternative splicing. The most common type of alternative splicing is inclusion or exclusion of one or more exons or introns within the constant framework provided by the constitutive exons and introns (72;73). When an exon or intron is excluded it is called retained. Another alternative splicing event includes the usage of an alternative 5' donor or 3'acceptor splice site. This will lead to the excision of introns of different lengths and complementary variations in exon size. In the case of a cassette exon, the exon is included or excluded independently of other exons. Mutually exclusive splicing involves two or more exons of which only one is included in the mature mRNA and thus they are
Figure 3: A schematic representation of the constitutive splicing process. Exons (white) and introns (grey) are represented by boxes. After recognition of the 5' splice site by U1 and binding of U2 at the branch point near the 3' splice site the spliceosome assembles by recruiting U4/U6 and U5. After several rearrangements the 5' splice site is attacked by a nucleotide in the intron sequence (black circle) and is cleaved. The 5' end of the intron joins with this nucleotide and a lariat (lasso) structure is formed. Then the end of the first exon binds to the beginning of the next exon thereby cleaving the 3' splice site. The snRNP-bound lariat is disassembled and degraded in the nucleus and the snRNPs are recycled. Abbreviations: U1 to U6 are small nuclear ribonucleoproteins (snRNPs).

never spliced together. Multiple promoters or multiple poly(A) sites lead to alternative 5' or 3' sequences. If the variable sequences are located in the coding region this will subsequently lead to variations in amino-acid sequence in the N- or C-terminus of the protein. On the other hand variations in the untranslated sequences may influence RNA stability, transport or translational efficiency. Taken together alternative splicing can create multiple mRNAs derived from one pre-mRNA and this may provide an important mechanism for generating functional protein diversity.

**Splice site selection and splicing factors**

Selection of the correct splice site mainly involves the coordinate recognition of nearby 3' and 5' splice sites, usually across an exon (called exon definition) (74;75). However if two potential 5' splice sites are occupied by U1 snRNPs (the first snRNP
that recognizes and binds the 5'splice sites after which assembly of the spliceosome can start, splicing not necessarily follows and therefore additional factors must participate in the selection of the splice site (76-78). One such group of proteins that plays a role in selection of 5'splice sites is the family of SR proteins that are required for constitutive splicing (79;80) but also influence alternative splicing by facilitating assembly of the spliceosome (81;82). SR proteins are located in nuclear speckles which are highly dynamic structures that respond specifically to activation of nearby genes by release of their splicing factors (83;84). SR proteins are phosphorylated and regulate alternative splicing in a concentration dependent manner (80;85).

**Figure 4:** Types of alternative splicing events. Introns are shown as lines and exons are shown as boxes. Alternatively spliced exons/ introns are in black- and- white. Splicing patterns are indicated above and below by the diagonal dashed lines. Alternative splicing can occur due to an alternative 5'or 3'splice site. It can lead to the inclusion or exclusion of an internal exon (retained intron) also independent of the other exons (cassette exon). In mutually exclusive splicing, two or more alternative exons are involved and only one exon can be included in the mature mRNA. At the 5'and 3'end alternative splicing can occur in association with selection of alternative promoters and the use of alternative polyadenylation sites.
SR proteins, of which ASF/SF2 was the first protein found and the best characterized member of the family, share two characteristics in their sequence: the RNA recognition motif (RRM) near the amino terminus and a domain rich in arginines and serines (SR or RS rich region which is the eponym for the family) at the carboxyl terminus. SR proteins can affect the use of the 5'splice site and generally they stimulate the use of the proximal (downstream) site (figure 5) (79;86;87). They recognize specific enhancer sequences in the exons and they promote binding of U1snRNP to the 5'splice site (77;88).

Another class of proteins, which counteracts the activity of SR proteins by favouring the use of the distal 5' splice site (figure 5), comprises the hnRNP A/B proteins (89;90). hnRNP A1, the best characterized member of the group, contains a carboxy-terminal glycine-rich domain and two RRMs and was shown to bind pre-mRNA (87;91). hnRNP proteins shuttle between the cytoplasm and the nucleus and their accumulation in the nucleus is transcription dependent. During its transit through the nuclear pores to the cytoplasm it is bound to mRNA in an hnRNP-mRNA complex (92;93).

**hnRNP A1 and SF2 in alternative splicing**

One of the models for regulation of alternative splicing is based on the antagonistic effects of hnRNP A1 and SF2. In vitro splicing assays with adenovirus pre-mRNA and different amounts of SF2 and hnRNP A1 demonstrated that an excess of hnRNP A1 over SF2 results in activation of the distal 5'splice site (ss) whereas an excess of SF2 over hnRNP A1 generally favours the proximal 5'ss (89). The opposing effects of SF2 and hnRNP A1 were also observed in Hela cells that were transfected with several reporter genes (90). The reporter gene was spliced at the proximal 5'ss after overexpression of SF2 and at the distal 5'ss after overexpression of hnRNP A1. Moreover the ratio of hnRNP A1 to SF2 was also shown to control splice site choice of the alternative 3'splice (94). Competitive binding between SF2 and hnRNP A1 and affinity of U1snRNP for 5'ss at the pre-mRNA probably underlies this antagonism (figure 5) (95).

In terms of a mechanism for alternative splice site selection, SF2 enhances U1 snRNP binding at all 5'ss of the pre-mRNA and/or interferes with hnRNP A1 binding. This results in a double occupancy of alternative 5'ss by U1 snRNP which favours splicing via the 5'ss nearest to the 3' ss (which is the proximal downstream 5'ss) (77;87). In contrast, hnRNP A1 binds indiscriminately to the pre-mRNA which interferes with U1snRNP binding and thus the likelihood of simultaneous occupancy of 5'ss. As a result, the upstream distal 5'ss is used. The use of specific splice sites thus depends on the indi-
individual probabilities that they are occupied, i.e., on their affinity for U1 snRNP, rather than their position. Tissue specific variations in the total and relative amounts of SF2 and hnRNP A1 have been described and the ratio between SF2 and hnRNP A1 varies between tissues, developmental stages and stages of adenoviral infection. This may be important in the regulation of alternative splicing in a tissue-specific or developmentally regulated manner (85).

Figure 5: Model for the antagonistic actions of SF2 and hnRNP A1 in 5'splice site selection. Alternative 5'splice sites are displayed as black boxes; 3'splice sites are light grey. Introns are shown as lines. The splicing patterns are indicated above and below by the diagonal dashed lines. The upper panel shows a situation in which high concentrations of SF2 lead to double occupancy of the 5'splice sites by U1. In this case the downstream (proximal from the 3'splice site) site is used for splicing. The lower panel shows a situation with low concentrations of SF2 or high concentrations of hnRNP A1. U1 binding is reduced by competition with hnRNP A1 and this will lead to the use of the upstream or distal site. Abbreviations: U1=U1 snRNP
Transcription and splicing

Splicing does not occur as an isolated event; it interacts dynamically with both the transcription process and other RNA processing events such as capping and polyadenylation (96). The carboxyterminal domain (CTD) of the large subunit of RNA polymerase II (Pol II) plays a critical role in these interactions as was shown by McCracken et al (97). They showed that when the CTD of pol II is shortened polyadenylation and splicing is abrogated whereas the transcriptional activity of Pol II is not affected. Cramer et al reported that differences in the promoter structure of a transiently transfected fibronectin construct can lead to differences in the alternative splicing of the fibronectin ED I exon (98). Moreover they have shown that overexpression of SF2 has no effect on the alternative splicing when the construct is driven by a β-actin promoter but the splicing direction is affected when transcription is driven by a number of other promoters (99). Therefore several mechanisms have been proposed including the recruitment of splicing factors to the site of transcription by the promoter itself (100;101) or the promoter modulates the regulation of Pol II elongation or processivity (102). Recruitment of splicing factors by the promoter might involve direct interaction between splicing factors and transcription factors that bind to the promoter or to the transcriptional enhancer sites. One study which favours this hypothesis reports of a functional interaction between SF2 and the transcriptional coactivator p52 both in vitro and in vivo as well as colocalization of SF2 and p52 in the nucleus of Hela cells (103). Most importantly p52 not only acted as a transcriptional activator but it also modulated SF2-mediated pre-mRNA splicing.

Another transcriptional coactivator which may provide a link between splicing and transcription is the peroxisome proliferator activated receptor (PPAR) gamma coactivator (PGC-1) which, in addition to the binding sites to Pol II and transcriptional co-activators, contains some RNA processing motifs that are characteristic of splicing proteins (104-106) (figure 6). PGC-1 harbours an activation domain (AD) at its N-terminus that contains binding sites for other coactivators such as steroid receptor coactivator 1 (SRC-1) and CREB-binding protein p300 (CBP/p300) (107). A leucine-rich motif (LxxLL motif) mediates ligand dependent interactions with certain nuclear receptors such as TR, PPARα, estrogen receptor (ER) and GR (106;108-110). The central region of PGC-1 (200-400) contains a negative regulatory element which represses the function of the N-terminal AD. A proline-rich region that overlaps with this inhibitory domain interacts with transcription factors such as PPARγ in a ligand-independent manner and with
nuclear respiratory factor one (NRF-1) (104;107;111). At the C-terminal end PGC-1 contains two conserved motifs: a serine/arginine-rich region (RS) and an RRM. The RS domain is characteristic for SR proteins and the RRM shows homology to the corresponding domains found in SR and hnRNP proteins which confer RNA binding activity. Indeed PGC-1 was found to co-localize with SR proteins in nuclear speckles and associates with the splicing factors and the elongation form of RNA polymerase II (112). These associations were dependent on the RS/RRM containing C-terminus of PGC-1. PGC-1 was shown to alter the splicing pattern of a fibronectin minigene in transiently transfected cells depending on the promoter structure (112). These findings suggest a role for PGC-1 as a DNA binding transcription factor to regulate post-transcriptional processes such as splicing (figure 7).

**Figure 6:** Schematic representation of the structure and functional domains of PGC-1. PGC-1 contains an activation domain, an LXXLL motif, an inhibitory domain and two conserved motifs: a serine/arginine rich region (SR) and a putative RNA recognition motif (RRM). The activation domain interacts with other transcriptional coactivators such as SRC-1 and CBP. The LXXLL motif mediates ligand dependent interactions with nuclear receptors. The inhibitory domain suppresses the activation domain and part of this domain interacts with PPARγ in a ligand-dependent manner. A third region mediates interactions with MEF2C and has no other known functions. The last domain containing the RS and RRM motifs mediates interactions with SR splicing factors and U1. PGC-1 can interact with the initiation form of Pol II at its N-terminal end and with the elongation form of Pol II at its C-terminal end. Abbreviations: CBP: CREB binding protein; SRC-1: steroid receptor coactivator 1; MEF2C: myocyte enhancer factor 2C; NRF-1: nuclear respiratory factor 1; NR: nuclear receptor; PPARγ: peroxisome proliferator-activated receptor; RS: arginine/serine rich region; RRM: RNA recognition motif. The number of amino acids are indicated at the beginning and the end of the protein.
Chapter 1

Initiation

Activation transcription

Elongation and Splicing

Figure 7: Schematic representation of the initiation and elongation of transcription coupled to splicing. Exons are shown as boxes and introns as lines. In the upper panel the transcription initiation complex assembles with Polymerase II, transcription factors (TFs), PGC-1 and histone acetylase transferases (HATs) at the promoter of a gene. During elongation, depicted in the lower panel, Pol II can associate with splicing factors such as the SR proteins and PGC-1. This complex may influence the splice site direction of alternatively spliced genes.
1.3 Splicing of the thyroid hormone receptor alpha transcripts

The balance of TRalpha 1 to TRalpha 2

An alternative 5'splice site is located in exon 9 of the TRα transcript. When this alternative splice site is used, TRα2 is generated and the transcript ends at a poly(A) signal located in exon 10 (figure 8). If the alternative splice site is not used, a poly(A) signal in exon 9 terminates the transcript resulting in the generation of TRα1.

![Diagram of TRα gene splicing](image)

**Figure 8:** A schematic representation of the last 3 exons (boxes) and introns (lines) of the TRα gene. The TRα1-specific part of exon 9 is indicated in light grey and the TRα2-specific exon 10 is indicated in dark grey. In the lower panel the TRα1 and TRα2 transcripts are depicted and the spliced fragments are represented by dashed lines. The splice sites of intron 9 are indicated by black arrows.

The generation of knockout mice which lack TRα1 or its splicing variant TRα2 has helped us to understand the function and physiological relevance of TRα2 and the balance in TRα1:TRα2. The first reported TRα-knockout mouse, TRα⁻/⁻ did not survive beyond five weeks and this was due to the maintenance of the ΔTRα1 and ΔTRα2 (8). These truncated isoforms can repress the transactivation functions of other TRs and interfere with normal postnatal intestine development causing premature death. Mice that are completely deficient in TRα isoforms (TRα 0/0) have an increased sensitivity to thyroid hormone (113). They maintain normal thyroid-stimulating (TSH) concentrations despite low serum T4 levels. The increased sensitivity was mainly seen in tissues expressing TRβ isoforms suggesting that it was the abrogation of the silencing effect of TRα2 that caused the increased responsiveness to T3. TRα1 knockout mice do not over express TRα2 (or TRβ1) and show no changes in sensitivity to thyroid
hormone (114). Mice that lack TRα2 as a result over express TRα1 and this leads to features of hyperthyroidism, such as increased heart rate, weight loss and elevated body temperature but also to features of hypothyroidism such as low serum thyroid hormone levels with an inappropriately normal TSH concentration (115). These tissue-specific differences in thyroid hormone responsiveness may depend on the amount of TRα1 expressed and thus suggest that the TRα1:TRα2 ratio may be involved in thyroid hormone responsiveness in specific tissues. A pathological condition which is associated with a decrease in responsiveness to T₃ is fasting. When rats are subjected to fasting over a 48-hour period, the maximal binding capacity of the TR in liver decreases to 70% of the fed state and the TRα1:TRα2 ratio decreases threefold (116). In conclusion these studies suggest that differences in thyroid hormone responsiveness depend on both the amount of TRα1 expressed and the TRα1:TRα2 ratio which may regulate thyroid hormone responsiveness in specific tissues.

Rev-ErbA

Two studies have shown that in tissues where the Rev-ErbA mRNA levels are high, the TRα1:TRα2 ratio is also relatively high (9). Furthermore Rev-ErbA levels were shown to increase during adipocyte differentiation which is correlated to an increase in the TRα1:TRα2 ratio (117). In other conditions where the mRNA levels of Rev-ErbA increased as a result of a block on protein synthesis, the TRα1:TRα2 ratio also increased due to an increase in TRα1 and a decrease in TRα2 which was not related to transcription or mRNA stability (10).

The mRNA encoding Rev-ErbA is partly complementary to TRα2 mRNA. As a result the mRNAs of Rev-ErbA and TRα2 could hybridize and block the splicing of TRα2 pre-mRNA thereby favouring the formation of TRα1. Indeed, in an in vitro splicing assay, splicing of TRα2 was inhibited by the addition of antisense RNAs containing the 3’end of Rev-ErbA (118). Overexpression of Rev-ErbA mRNA in cells transiently transfected with a TRα minigene containing the last 4 exons and introns of TRα can also increase the TRα1:TRα2 ratio independent of translation of Rev-ErbA sequences (119). Therefore Rev-ErbA could play a regulatory role in the splicing of TRα by inhibition of splicing to TRα2.
Aim of this thesis

The level of TRα1 and TRα2 mRNA transcripts as well as the ratio of TRα1 to TRα2 varies between different tissues both in humans and in rodents. We therefore set out experiments to test whether the splicing of TRα pre-mRNA might be regulated. For this purpose we used a cell line which endogenously expresses TRα and tested the splicing direction of TRα under several conditions. In the next place we asked ourselves, if the splicing is regulated, what are then the modulators of this regulation during health and/or disease? To address this question we studied the balance in TRα1 to TRα2 in liver biopsies of patients who had died on the ICU in relation to the hormonal status and the extent of illness in each patient. Furthermore we used a mouse model of NTI to study the alternative splicing of TRα1 and TRα2 in different tissues after lipopolysaccharide (LPS) administration. In the last place we wondered what the mechanism of the regulation of alternative splicing might be. One possible candidate is the coactivator PGC-1 which might have an effect on transcription as well as on the splicing process via its N-terminal RNA processing domains. Therefore we used a transient transfection assay with a TRα minigene that contains the last 4 exons and introns of TRα including the alternative splice site and studied the TRα1:TRα2 mRNA ratio. We co-transfected a plasmid expressing full length PGC-1 or PGC-1 containing several deletions in its N-terminal domain, and studied the difference in splicing of the TRα transcripts.

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Introduction


