Expression of thyroid hormone receptor isoforms in rodents

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CHAPTER 5

Zonal Distribution of Thyroid Hormone Receptors $\alpha_1$ and $\beta$ Govern Receptor Isoform Specificity in Hepatic Gene Regulation

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ABSTRACT

Regulation by thyroid hormone of several hepatic genes show dependence on specific thyroid hormone receptor isoforms, and it was recently shown that TRβ1 has a pericentral location in the rat liver lobule. Here, we show that TRα1 has a broader expression pattern than TRβ1 in the liver functional unit in both TRα1 overexpressing TRα2-/TRβ- mice and in WT controls. This suggested that the local availability in the liver unit of a specific TR isoform underlies the apparent receptor-specificity of target gene regulation noted earlier.

To test this hypothesis, the expression of three classical hepatic target genes (malic enzyme, "Spot 14" and type I iodothyronine deiodinase (5'D1)) under hypo- and hyperthyroid conditions in different TR knock-out strains (TRα1-, TRβ-, TRα1-/TRβ- and TRα2-/TRβ-). Responses to T3 in the different receptor deficient mice showed that the three target genes exhibited distinct dependencies on specific isoforms: "Spot 14" was specifically dependent on TRβ, 5'D1 was only partially dependent on TRβ whereas malic enzyme was regulated equally well by TRα1 and TRβ. The regulation of these target genes thus correlates with their own expression patterns in the liver unit and that of the TR isoforms that regulate them. This suggests that the different spatial expression patterns of TRα1 and TRβ1 in the liver lobe is the underlying reason for TR isoform specificity in T3 regulation of these and possibly also other target genes.

INTRODUCTION

The liver is a major target organ for thyroid hormone (T3) action. A large number of hepatic genes are regulated by T3 (1,2) via thyroid hormone receptors (TR), which are ligand dependent transcription factors belonging to the nuclear receptor superfamily (3). TR act by binding to specific DNA sequences, thyroid response elements (TREs), in the promoters of their target genes, thereby stimulating or repressing transcription (4). In absence of T3, TR can repress basal transcription of many positively regulated genes (5-7). The repressive effect can also be ligand dependent; as is the case for approximately half of the target genes in the liver (1,2).

TR isoforms are encoded by the distinct α and β genes, which give rise to the ligand binding receptors TRα1, TRβ1, TRβ2 and TRβ3, respectively (8-10). The α gene also encodes the TRα2 protein that does not bind T3 although it binds to DNA with reduced affinity; its physiological role is however unclear (11). TRα2 is co-expressed with TRα1 in most tissues but at 2-6 fold higher levels (12-14). Deletion of different TR isoforms by gene targeting in mice suggest that they have separate as well as overlapping functions in vivo(15-21). TRβ1 is the predominant TR isoform in mouse liver, constituting approximately 80% of the T3-binding proteins in this tissue (22). The function of the TRα2 gene is unclear but deletion of TRα2 results in mice that simultaneously overexpresses TRα1 as a consequence of the complex gene structure of the TRα gene. Furthermore, these mice exhibit a mixed hypo- and hyperthyroid
phenotype (11). The combined knock-out TRα2⁻/TRβ⁻ also overexpresses TRα1, resulting in suppression of deafness and a significant restoration of pituitary-thyroid control of thyroid hormone homeostasis seen in TRβ⁺ mice (23). We have previously shown that T₃-regulation of serum cholesterol levels is specifically dependent (24,25) on the TRβ gene. At the molecular level, different hepatic target genes display various levels of dependence on TRβ for normal regulation by T₃: cholesterol-7-alpha-hydroxylase (CYP7a) is specifically dependent on the TRβ gene for regulation by T₃ (24,25) whereas e.g. malic enzyme does not require TRβ (26). The mechanism for TR isoform specificity in regulation of hepatic genes is however unclear.

The liver is metabolically zonated: various metabolic reactions are spaced apart in different locations along the porto-central axis of the liver unit; thus, not all metabolic processes take place in every liver cell (27). For instance, malic enzyme displays a broad periportal to pericentral distribution, and its positive T₃-regulation is mediated by liganded TR binding to a TRE in its promoter (28-32). In contrast, "Spot 14" has a strict pericentral location (33,34). The gene contains TREs in the enhancer region and responds rapidly to T₃ with an increase in mRNA, and it has therefore been used as a paradigm for T₃ action on hepatic gene expression (35,36). 5' deiodinase-1 (5'DI) has a somewhat broader expression pattern than "Spot 14", but is mainly focused around the central vein (37). The human 5'DI gene contains TREs; in the mouse gene however, no TRE has yet been identified (38-40).

Recently we found by immunohistochemistry that TRβ1 is specifically expressed in the rat liver lobule, with a pericentral location matching that of several T₃ target genes (37). This has led us to hypothesize that local availability of the different TR isoforms in the liver cell governs the level of dependence on TRβ for normal T₃-regulation of a specific gene. We tested this hypothesis by comparing expression patterns of three classical hepatic T₃ target genes with those of TRα1 and TRβ under hypo- and hyperthyroid conditions in different TR deficient strains. The three positively T₃ regulated genes, malic enzyme, "Spot 14" and 5'DI, were chosen based on their different spatial distribution in the liver unit.

Here we show that TRα1 has a broader expression pattern than TRβ in the liver functional unit in both the TRα1 overexpressing TRα2⁻/TRβ⁻ and in wild-type (WT) controls.

Determination of responses to T₃ in the different knock-outs (TRα1⁻, TRβ⁻, TRα1⁻/TRβ⁻ and TRα2⁻/TRβ⁻) showed that "Spot 14" was specifically dependent on TRβ whereas malic enzyme was independent. 5'DI on the other hand was partially dependent on TRβ; absence of TRβ could be partially rescued by over-expression of TRα1 in the TRα2⁻/TRβ⁻ mice. This is in agreement with the spatial expression pattern of these three genes: "Spot 14" is pericentral as is TRβ, malic enzyme has a broad expression pattern similar to TRα1, and 5'DI is mainly expressed pericentrally but extend more periportally than "Spot 14" and TRβ1 do. Taken together, these results suggest that the spatial expression patterns of the individual TR isoforms in the liver lobe is the underlying reason for the apparent target gene specificity.
MATERIALS AND METHODS

Determination of mRNA levels in liver.
RNA was prepared from frozen liver. For the TRα1/β animals polyadenylated mRNA was prepared (41) from three separate liver pools from each animal group. For the TRα1 TRβ1 and TRα2/β animals total RNA was prepared using Ultraspec (BIOTECX, Houston, TX, USA) solution from four to six animals in each group, an equal amount of RNA from each animal in the group was then used for the pool. Northern blots were hybridized with cDNA probes for rat malic Enzyme (ME)(42), rat "Spot 14" (43) and mouse 5'deiodinase-1 (5'DI)(44). Hybridization with Glyceraldehyde-3-Phosphate Dehydrogenase (G3PDH) served as a control for equivalent loading of mRNA. Levels of mRNA expression were normalized to that of G3PDH using a Fuji BAS2500 (Fujifilm, Tokyo, Japan) for quantification. Determinations were done at least two times for each genotype with similar results.

Antibodies
To produce anti-TR antisera three linear synthetic peptides coupled to keyhole limpet haemocyanine (KLH) were used. These peptides, encoding for human TRβ1(aa 74-92; NH$_2$-VNDQSVSAIQTFQTEEKK-COOH), TRα1 (aa 402-410; NH$_2$-EVFEDQEVC
COOH) and TRα2 (aa 425-442; NH$_2$-SLRGPVLQHQSPKSPQR-COOH) were used to generate monoclonal antibodies. The anti-5'DI) antiserum was raised against a C-terminal peptide of rat 5'-DI(45) and was a kind gift of Dr. Jack Leonard (University of Massachusetts, Worcester, USA). Glutamine synthetase polyclonal antibodies were kind gift of Prof. W. H. Lammers (Department of Anatomy, Academic Medical Center, Amsterdam, The Netherlands).

Immunohistochemistry
Mice livers were fixed for 16 hours at 4°C in PFA. Paraffin-embedded sections (6 μm) were pasted on superfrost/Plus (Menzel-Gläser, Germany) slides and dewaxed prior to immunostaining. Sections were then placed in Tris buffered salt (TBS, (pH=8.0)) and microwaved for 10 minutes to unmask the antigenic epitope. After cooling down to room temperature, the sections were placed in a blocking solution for 60 minutes (2% low fat milk (Campina, Netherlands), 0.1% (w:v) saponin (Sigma, Germany) diluted in 0.2 M acetate buffer at pH=5.4 for TRα1 antiserum and (2% low fat milk (Campina, Netherlands) and 0.5% (w:v) Triton X-100 (Sigma, Germany) diluted in TBS at pH=8 for 5'DI, TRα2, TRβ1 and glutamine synthetase antiserum. The first antibodies were diluted (1:25 for TRs and 1:250 for 5'DI) in the blocking solution, and incubated one hour at room temperature and overnight at 4°C. The sections were washed with 0.5% (w:v) Triton X-100 in TBS (pH=8.0) for 10 minutes and then incubated with the second antibody, either goat antirabbit or goat antimouse (conjugated with alkaline phosphatase) diluted (1:500) in 0.5% (w:v) Triton X-100 in TBS (pH=8.0). After washing 10 minutes in TBS, the sections were placed in alkaline phosphatase buffer (50mM MgCl$_2$, 100mM NaCl, and 100 mM Tris pH=9.2) for 10 minutes. The substrate NBT/BCIP (Roche Diagnostics, Germany) and 0.1M
levamisole (Sigma, Germany) were diluted respectively 1:50 and 1:100 in alkaline phosphatase buffer and added to the sections for 30-60 minutes in the dark. The sections were washed in TBS and then placed in methanol (Merck, Germany) for at least 5 minutes to reduce the background. The sections were mounted with Kaiser's glycerol gelatin (Merck, Germany).

**Animals and experimental setup**

Altogether 125 male mice aged 2 to 3.5 months (12 TRα²⁻, 12 TRα¹ WT, 14 TRβ⁻, 14 TRβ WT, 16 TRα¹⁺/β⁻, 17 TRα¹/β WT mice, 17 TRα²⁺/β⁻ and 23 TRα²/β WT mice) were used. The studies were approved by the Institutional Animal Care and Use Committee. TRα¹, TRα² and TRβ deficient mice were genotyped by Southern blot or PCR analysis using PCR primers specific for the mutant TRα¹, TRα² and TRβ alleles, as described(11,15,21,23). The genetic background of the TRβ⁻ mice is a hybrid of 129/Sv x C57Bl/6J and that of TRα¹⁻ mice is a hybrid of 129/OlaHsd x Balb/c. The TRα¹/β (46) and the TRα²/β mice and their housing conditions were recently described (23). The wild type control and their respective compound knock-out strains used in this study were originally derived from crosses between heterozygotes for the respective single knock-out strains. The control strains therefore had the same, mixed genetic background as their respective receptor deficient strain. To minimize the risk for genetic drift between the WT and the knock-out strains, no more than 3 generations of breeding was done until new intercrosses were set up for regeneration of the respective WT and compound knock-out strains as described previously (23,46).

The mice were divided into 3 groups, each consisting of 4-6 knock-out (TRα¹⁻, TRβ⁻, TRα¹⁺/TRβ⁻ or TRα²⁺/TRβ⁻ and 4-6 of the respective WT animals. The experimental setup has recently been described in detail(24) and is summarized in figure 2. In brief, the mice were made hypothyroid by feeding a low iodine diet (LID) and inclusion of 0.05% methimazole and 1% potassium perchlorate in the drinking water (MMI) for 21 days when on LID diet. This treatment decreased serum free T₃ to about two pmol/l and serum free T4 to less than four pmol/l From day 35, one group of animals was injected daily with five μg T₃ for an additional 5-day period to induce hyperthyroidism. At the time of sacrifice, trunk blood was collected following decapitation. Livers were immediately frozen on dry ice. The animals were a subset of the experiment described previously, and their free T₃ and free T₄ levels were determined in detail (24,25).

**RESULTS**

**Zonal distribution of TR isoforms and overexpression of TRα¹ in TRα²⁺/TRβ⁻ mice**

Our recent data show that TRβ¹ has a zonal distribution in rat liver that coincides well with that of several known T₃ target genes (37). A comparison of TRα¹ of TRβ expression in WT mouse liver using our newly developed monoclonal antibodies
revealed a portal to central gradient for TRα1, however, this gradient is broader than that for TRβ1 (figure 1). Glutamine synthetase (GS) antiserum was used as a marker of the central veins of the liver lobes. As shown by immunohistochemistry TRα2 expression also showed a portal to central gradient similar to that for TRα1. Staining for 5′D1 showed a broader gradient of expression as compared to TRβ1, and is similar to that found in rat liver (37). In the TRα2/TRβ− mice, TRα1 protein was almost homogeneously distributed in the livers confirming overexpression of this protein. As could be expected no protein expression of TRβ1 or TRα2 was observed in the TRα2/TRβ− mice (figure 1), furthermore, there was no detectable 5′D1 protein in the TRα2/TRβ− mice, this was confirmed by the low 5′D1 mRNA levels and activity data (figure 4) for these animals when on normal diet. The weak residual staining of GS indicate that it is partially TRβ dependent.

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**Figure 1.** Zonal expression of TRβ1, TRα1, TRα2, Glutamine synthetase (GS) and 5′deiodinase-1 (5′D1) in liver from WT (first two columns) and TRα2/TRβ− mice (third column). Magnification 100x in first column and 40x in second and third columns.
**Altered expression of hepatic target genes**

We have previously shown that $T_3$ regulation of several hepatic genes show dependence on specific TR isoforms\(^2,24,25\). To investigate if the dependence is reflected by a coincidence between the spatial distribution of the gene product and the respective TR isoform in the liver lobe, we determined $T_3$-regulation of three positively regulated $T_3$-responsive genes (malic enzyme, "Spot 14" and 5'-deiodinase-1) that have different spatial expression patterns in liver of different TR deficient strains. The experimental setup is similar to that used earlier \(^24,25\) and is also described in figure 2.

![Figure 2](image.png)

**Figure 2.** Experimental setup.

A total of 125 male mice aged 2 to 3.5 months (12 TRα1, 12 TRα1 WT, 14 TRβ-, 14 TRβ WT, 16 TRα1/β-, 17 TRα1/β WT mice, 17 TRα2/β- and 23 TRα2/β WT mice were used. Prior to the experiment, animals were sacrificed to obtain basal thyroid hormone levels and liver tissue for mRNA and enzyme activity determinations. Thereafter, 4 to 6 knock-out and 3 to 6 of the respective WT animals were assigned to three groups: (A) LID, low iodine diet, (B) MMI plus 0.05% methimazole and 1% potassium perchlorate in the drinking water (MMI) and (C) $T_3$, daily injections of $T_3$ for the last 5 days. \(^\dagger\) Sacrifice of animals for collection of serum and tissue.

Malic enzyme is known to have a wide periportal to pericentral distribution in the liver lobe \(^28-32\). Malic enzyme mRNA levels were lower in the untreated TRβ- and the TRα1+/TRβ- as compared to the corresponding WT mice (figure 3, panel A). This difference was not seen between the TRα1- and TRα2+/TRβ- mice and their controls. Similarly, mRNA levels were suppressed by thyroid hormone deprivation in all animals except in the TRβ- and the TRα1+/TRβ- mice (MMI, figure 3, panel A), resulting in a higher expression level in these hypothyroid mice than in the corresponding WT mice. Injection of $T_3$ increased Malic enzyme mRNA to a level higher than the basal expression in all but the TRα1+/TRβ- animals ($T_3$, figure 3, panel A). This suggests that Malic enzyme does not require TRβ for $T_3$ upregulation of its expression.

"Spot 14" has been reported to have a tight pericentral localization\(^33,34\), and it has been suggested to play a role in hepatic lipogenesis has been suggested for it. "Spot
14" mRNA expression was decreased at least two-fold by thyroid hormone deprivation in TRα1−/− and all WT mice (MMI, figure 3 panel B), in contrast to all animals that lack TRβ; TRβ−/−, TRα1−/−/TRβ−/− and TRα2−/−/TRβ−/− mice. Similarly, the increase in "Spot 14" mRNA levels by T3 injections seen in the WT and TRα1−/− mice was absent in all mice lacking TRβ; (TRβ−/−, TRα1−/−/TRβ−/− and TRα2−/−/TRβ−/− strains) (T3, figure 3 panel A). Interestingly, the absence of T3 regulation in TRα2−/−/TRβ−/− animals suggests that TRα1 cannot substitute for lack of TRβ in this respect. These results indicate that "Spot 14" is specifically dependent on the TRβ gene for normal T3 regulation.

Thyroid hormone regulation of liver 5′deiodinase-1 (5′D1) has previously been shown to be specifically dependent on TRβ (47). 5′D1 expression starts slightly earlier along the porto-central axis than TRβ1 expression (figure 1). Here, we used TRα2−/−/TRβ−/− animals to investigate if 5′D1 regulation is specifically dependent on the TRβ gene, as was the case for "Spot 14", or if TRα1 overexpression can compensate for absence of TRβ. 5′D1 enzyme activity (figure 4, panel A) were lower in the untreated
TRα2⁻/TRβ⁻ than in the WT animals. Hypothyroidism decreased enzyme activity more potently in WT than in the TRα2⁻/TRβ⁻ animals (MMI Fig. 4, panel A). Whereas, T₃ increased 5'D1 enzyme activity (T₃ figure 4, panel A) both in the mutant and WT animals, although the increase was lower in the former strain. The results on 5'D1 enzyme activity were confirmed by determinations of 5'D1 mRNA levels (figure 4, panel B). These results suggests that although T₃ regulation of 5'D1 expression is dependent on TRβ in the normal animal, TRα1 can, when overexpressed as in the TRα2⁻/TRβ⁻ mice, compensate for TRβ deficiency. Thus, 5'D1 is not specifically dependent on the TRβ gene.

![Figure 4](image)

**A. TRα2/β 5'DI-1 activity**

<table>
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<tr>
<th>TREATMENT</th>
<th>Normal diet</th>
<th>MMI</th>
<th>MMI+T3</th>
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<tr>
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</tr>
<tr>
<td>G3PDH</td>
<td></td>
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<tr>
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<td>0.0 0.2</td>
<td>2.2 1.1</td>
</tr>
</tbody>
</table>

**B. TRα2/β 5'DI-1 mRNA**

![Figure 3](image)

**Figure 4.** Liver 5'deiodinase-1 (5'DI) enzyme activity and mRNA determinations in TRα2⁻/TRβ⁻ and WT mice. 5'DI enzyme activity (panel A) was determined as described in Materials and Methods and 5'DI mRNA levels (panel B) as in figure 3.
DISCUSSION

It has been estimated that up to 14% of hepatic genes could be regulated by T₃ (1,2). Since TRβ is the major TR isoform in the liver (22) it is not surprising that many of the T₃-regulated genes are dependent on this receptor for hormonal regulation. However, not all genes are fully dependent on TRβ. Some are fully hormone regulated in TRβ deficient mice whereas others fall between the two extremes. An obvious explanation for this would be that TRα1 can substitute for TRβ in some instances but not in others; the mechanism for this is however unclear. In the current study, we hypothesized that the local availability of TR isoforms in the liver cell varies between different parts of the liver unit, and that this governs the receptor isoform specificity of target gene regulation. Since it is known that several proteins, and metabolic processes have a zonal distribution over the liver unit, the regulation of a specific target gene could be governed by the presence or absence of TRβ in a given area of the liver unit.

To test the validity of this model, we first determined the spatial distribution of TRβ1 and TRα1 in livers from WT and TRα2/TRβ mice (figure 1). We found that TRβ1 was tightly pericent rally located in the liver unit similar as in our recent study in rats (37). TRα1 however, expressed at normal or elevated levels, had a wider distribution extending further away from the central vein. These observations provided a basis for exploring target gene specificity for TRs in the liver. Secondly, to determine TRβ dependence, we studied the transcriptional responses to hypo- and hyperthyroidism of three classical, positively regulated target genes: malic enzyme, "Spot 14" and 5'DI. By using different TR deficient mouse strains, we could determine TR isoform dependence in T₃ regulation of the target genes. Malic enzyme has a wide periportal to pericentral distribution, and was not dependent on a specific TR for T₃ induction (figure 3, panel A), although absence of both TRα1 and TRβ abrogated hormonal induction. Basal expression and ligand independent suppression of malic enzyme on the other hand, showed partial dependence on TRβ (figure 3, panel A). "Spot 14" is concentrated pericentrally, similarly as TRβ1, and our experiments showed that T₃ was unable to increase "Spot 14" mRNA level in any of the strains deficient of TRβ (figure 3 panel B). "Spot 14" responds rapidly to T₃ and the mRNA levels decrease after long term treatment (2), this and the fact that liver sampling was done 24 hours after the last T₃ injection thus explains the modest increase in "Spot 14" mRNA after T₃ administration. Interestingly the TRβ1 overexpression in TRα2/+/TRβ mice were unable to rescue T₃ responsiveness of "Spot 14", suggesting that "spot 14" is specifically dependent on TRβ.

We have previously investigated the importance of different TR isoforms for T₃ regulation of 5'DI (47), and found that TRβ1 is primarily responsible for regulating 5'DI expression. 5'DI is localized around the central vein in rat and mouse liver, but with a wider extension portally than TRβ (figure 1, and (37)). Overexpression of TRα1 in TRα2 TRβ mice however partially rescued 5'DI expression and activity (figure 4 panels A and B) suggesting that 5'DI expression was not dependent on the
Our data does not entirely exclude other possible mechanisms, such as promoter context and co-factor availability, for TR isoform preferences in target gene regulation. One possibility is that the promoter context determines the TR isoform that regulates expression of the target gene. In vitro, TRα1 and TRβ have certain preferences for DNA binding motifs in e.g. the PCP and TRH genes (48,49). However, in most genes both isoforms transactivate through highly similar elements. Furthermore, the three genes tested here have all been shown inducible by both isoforms in vitro. The rat "Spot 14" enhancer, as well as the isolated far upstream TRE, could be activated equally well by both TRα and TRβ in several cell types (50), which is in sharp contrast to our demonstration that "Spot 14" mRNA upregulation was specifically dependent on the TRβ gene. The TRE in the rat malic enzyme promoter, as well as the TRE in human 5'D1 promoter, bind both TRα and TRβ in vitro (38,51). Availability of co-factors may also contribute to TR isoform dependence for target genes, TRα1 and TRβ could use different co-factors for modulation of gene expression e.g. due to their distinct N-terminal regions (52). The availability of specific co-factors in a particular cell type would therefore govern which TR regulates that particular gene.

A role for TRα2 in regulation of hepatic genes cannot be completely ruled out. Recently it was suggested that deletion of TRα2, in mice ablated of all TRα products (TRα0 (26)), increases sensitivity to T3 in liver. It was suggested that TRα2 modulates the activity of TRβ on hepatic target genes explaining e.g. the observed increase in T3-dependent regulation of malic enzyme in these mice (26), thus indicating a role for TRα2 in suppressing T3-induction of malic enzyme. Our present results do not address this issue directly, due to differences in experimental design. The greater increase in response to T3 in malic enzyme mRNA observed for the TRα0 as compared to WT was however not seen in the TRα2+/TRβ− mice that expresses TRα1 at levels similar to those for TRβ in WT or TRα0 mice. If TRα2 suppresses TRβ, as opposed to TRα1 remains to be clarified.

Furthermore, our previous results showing that in TRα2+/TRβ− mice the overexpression of TRα1 does not rescue the abnormal serum cholesterol regulation in absence of TRβ indicates that TRα2 does not have a profound role in all liver processes. It has also been shown that TRα2 does not bind T3, although it binds DNA with reduced affinity (12,13), indicating that TRα2 may mediate T3 effects on hepatic target genes indirectly. In summary, this suggests that the ability or inability of TRα1 to substitute for TRβ in gene regulation is not caused by deletion of TRα2 per se. TRα2 may however have other roles in modulating T3 receptor activity (11). The reason for co-localization of target gene and TR isoform raises intriguing questions. Why is TRβ1 only expressed around the central vein, in a fashion similar to that for "Spot 14"? Are the same transcription factors involved in their regulation? These questions can currently not be answered with the scarce knowledge available on the determinants of TR isoform expression in different tissues or cells. Nevertheless, the current results on isoform dependence of T3 regulation of three differentially distributed target genes and the apparent different zonality of the two
TR isoforms in liver, suggest that isoform dependence is governed by spatial co-localization of TR isoform and target gene.

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