Expression of thyroid hormone receptor isoforms in rodents

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CHAPTER 8

General discussion
§ 8. General discussion

8A. Zonal distribution

In the liver periportal hepatocytes (located around the afferent vessels) and pericentral hepatocytes (located around the efferent vessels of the liver acinus; figure 1) exhibit different metabolic activities and subcellular structures. This observation in the 1970's (1) led to the concept of metabolic zonation of the liver acinus. Oxidative energy metabolism, gluconeogenesis, urea synthesis and bile formation take place mainly in the periportal zone. Glycolysis linked to lipogenesis, glutamine synthesis and xenobiotic metabolism are predominant in the pericentral zone. Periportal to pericentral gradients of oxygen, hormones and metabolites, as well as zonal differences in the hepatic innervation, are accounting for the heterogeneous gene expression within the liver acinus (2,3). Zonation along the porto-central axis of the liver lobe can be stable or dynamic. Stable zonation does not change along the porto-central axis as a result of metabolic or hormonal changes, while a dynamic zonation does change as a result of these inputs.

![Figure 1. Arrangement of hepatocytes in the liver. Portal and central veins are showed by arrows](image)

8.A.2. Zonal distribution of TR
In the course of developing a novel set of antibodies directed against the different TR isoforms we discovered that TR expression in rat liver has a zonal distribution. The
specificity of the antibodies was validated on the basis of different criteria which will now be discussed. Our TRα1, TRα2 and TRβ1 antibodies recognize the TRα1, TRα2 and TRβ1 proteins respectively, as analyzed by Western blots (see §2 and §3). The specificity of the TRβ1 antiserum was supported by Western blot analysis after transfection of the full-length human TRβ1 cDNA into HeLa cells. Western blots revealed a 55 kD protein band corresponding to the TRβ1 isoform. No cross-reactivity was seen with either Vaccinia-expressed TRα1 or TRβ2 expressed in COS cells. The specificity of our antibodies was furthermore confirmed using livers of TR knock-out mice. The TRα1 antibody showed no signal at 47 kD in liver whole cell extracts (WCE) from a TRα1+/TRβ1− animals. The TRα2 antibody did not show the 58 kD band in knock-out compared to WT animals. Furthermore, no cross-reaction with TRα1 protein could be demonstrated, since Vaccinia-TRα1 was also not detected with the TRα2 antibody (see §3). Thereafter, at the expression of TR isoforms in the liver of knock-out animals using immunohistochemistry is studied. The specificity of the monoclonal antibodies was tested on liver slices from mice devoid of either TRα1 (TRα1−/−) or TRα2 (TRα2+/−/β1−/−). No staining was observed with the TRα1 monoclonal antibody in a TRα1−/− liver. This was not a general loss of TR since the TRβ1 polyclonal was still able to detect the TRβ1 protein in the TRα1−/− animals. TRα2 staining was absent in the TRα2−/−/TRβ1−/− animals. In these mice, TRα1 was still detected in WT. During these studies into the specificity of our three antibodies against the TR isoforms, we found evidence for a zonal distribution of these receptors. Immunohistochemistry using our antibodies on liver sections showed that TRβ1, TRα1 and TRα2 are expressed in a population of cells located around the central veins (pericentral), whereas no staining was seen around the portal veins (see §2 and §3). This expression pattern of TRβ1 overlaps with that of the T3-responsive gene GS, which we stained as a control on consecutive sections. However, the area of expression of the TRα1 and TRα2 proteins extends further along the porto-central axis which can be taken as a further proof of the specificity of our antibodies.

Zonal expression is not restricted to the liver. Indeed, zonal distribution of various members of the nuclear receptor superfamily has been reported in several organs; examples are TR2 and TR4 orphan receptors in mouse kidney and brain (4-6), glucocorticoid receptors in mouse hypothalamus (7) and inner ear (8), androgen receptors in mouse brain (9,10), peroxisome proliferator-activated receptors (PPAR's) in rat brain and kidney (11), and estrogen receptors (ER) in bovine ovary (12) and human fetal testis (13).

8.A.3. Zonal distribution of TR's: Physiological relevance

It is known that TRβ1 expression is dependent on T3 and its promotor contains a TRE. The liver is the main site of T3 production by local conversion of T4 and T3 catalyzed by the enzyme 5'-deiodinase type I(5'DI). In view of the pericentral distribution of TRβ1, could it be that 5'DI is also zonally distributed? We found that 5'DI expression also had a preferential pericentral distribution but that its zonation was broader than that of TRβ1. This became very clear when we
used a quantitative method (14) to measure the expression levels of TRβ1 in conjunction with those of 5'DI. This suggests that expression of TRβ1 is influenced by locally generated T3, but also that 5'DI expression might be dependent on a specific TR. Experimental studies in knock-out mice showed that thyroid hormone regulation of liver 5'DI is indeed specifically dependent on TRβ isoforms. In liver and kidney 5'DI mRNA and activity levels were reduced in TRβ− but not TRα− mice. Liver 5'DI remained weakly T3-inducible in TRβ− mice whereas induction by T3 was abolished in double mutant TRα1−/TRβ− mice (15). In §5 we showed that in the liver of TRα2−/TRβ− mice 5'DI enzyme and mRNA expression was lower than that of controls, and decreases by thyroid hormone deprivation and increases after T3 addition; this suggests that TRα1 can compensate for TRβ deficiency. This observation is supported by the existence of an overlap between both TR isoforms and 5'DI expression (see §1).

Are there other T3-responsive genes besides 5'DI that are predominantly regulated by a specific TR isoform?. A number of T3-responsive genes involved in metabolism are zonally expressed in the liver. Glutamine synthetase (GS) expression is restricted to the area around the central vein (16) in a stable fashion (17), whereas PEPCK expression has a dynamic zonation focused around the portal vein (18,19). Other T3-responsive genes such as "Spot 14" (20,21), cholesterol 7α-hydroxylase (CYP7a) (22-24), acetyl-co-enzym-A carboxylase (ACC) (20) are expressed pericentrally, whereas malic enzyme (ME) (25), and glucose 6-phosphate dehydrogenase (G6PD) (26,27) are expressed in both pericentral and periporal zones.

The TRβ1 expression pattern is the same as that for the "Spot 14" gene involved in lipogenesis. We showed in §5 at the mRNA level that induction of "Spot 14" gene expression failed in TRβ− mice and could not be compensated by other TR isoforms in line with a previous study (28).

The co-localization of TR isoform and target gene 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 data available. Malic enzyme is known to have a wide periporal to pericentral distribution in the liver lobe. The effect of thyroid hormone on ME mRNA appears to be dependent on the TRβ isoforms (29). Malic enzyme mRNA levels are lower in the TRβ− and TRβ−/TRα1− mice as compared to the WT mice. This difference does not exist between the TRα1− or TRα2−/TRβ− mice and their controls. ME mRNA levels were suppressed by thyroid hormone deprivation in all animals except in the TRβ− and the TRα1−/TRβ− mice, resulting in a higher expression level in these hypothyroid mice than in the corresponding WT mice. However, injection of T3 increased malic enzyme mRNA in all but the TRα1−/TRβ− animals. This suggests that malic enzyme does not only require TRβ for its regulation. Thus it seems that ME expression can be regulated by both TRα of TRβ isoforms with a preference for the TRβ isoform with a possible role for TRα2. Our data do not entirely exclude other mechanisms for TR isoform preference in target gene regulation, such as promoter context and co-factor availability. Another possible explanation could be that the promoter context...
determines the TR isoform that regulates expression of the target gene. Regulation of ME in mutant TRβ2* (PV) is derived from an RTH patient with a C-insertion in codon 448, resulting in a frame shift mutation in the C-terminal 16 amino acids in TRβ1 which significantly reduced mRNA levels of ME in heterozygous TRβ2*/+ and in TRβ2*/+ (30) and a upregulation of ME mRNA in TRα2*/+ mice (31). Competition between TRβ2* and TRα2*/+ for malic enzymes TRE or preference for a co-factor may explain those results.

In case of glutamine synthetase (GS), it is not possible to draw any conclusion regarding the TR isoform specificity due to lack of data concerning their expression in TR knock-out animals. However, we can hypothesize that (since GS has exactly the same zonal distribution as TRβ1) its expression is also regulated predominantly by this isoform since we showed in §5 that GS protein expression is almost abolished in TRα2*/+/TRβ1* animals.

Finally, isoform specific expression of T3-responsive genes is not restricted to the liver. Deiodinase type 3 (D3) is expressed in a cell type-specific manner in Purkinje cells of the chicken cerebellum (32) while 5'DI is zonally expressed mainly in the granular cell layer of cerebellum (33).

In conclusion, we have shown that TR is zonally expressed, and that local TRβ1 isoform expression governs the expression of specific hepatic genes. However, in the case of both TRα1 and TRα2 the isoform specificity is less clear since no TRα specific T3-responsive genes are yet identified in the liver.

The relationship between thyroid state and TR expression is reported in §6. We showed that thyroid hormone deficiency or excess is not associated with obvious changes in zonation. The zonal expression of TRβ1 and TRα1 remained unchanged, while that of TRα2 was slightly broader in hyperthyroid animals in the light period. The broader zonation of TRα2 in hyperthyroid rats suggests a specific role in protecting tissue from exposure to thyroid hormone.

8.B.1. Diurnal variation: introductory remarks
Most, if not all, biological functions including behavior are subject to well-controlled daily oscillations, not only in mammals, but also in plants and fish. In mammals these 24h rhythmic changes include sleep-wake cycles, urine production, heart beat frequency, blood pressure, body temperature, hormonal secretion and liver metabolism. When the diurnal changes are controlled by the endogenous time measuring system, they are referred to as circadian rhythms (34-38). It is important to mention here that peripheral organs such as liver and kidney also have an endogenous clock (39,40).

The suprachiasmatic nucleus (SCN) of the hypothalamus coordinates most circadian rhythms through neural, humoral and other signals evoked by feeding activity (41). However, daily systemic changes induced by food intake are referred to here as diurnal variation and not as circadian rhythm because they may not be always dependent on an endogenous clock.
8.B.2. Diurnal variation of TRs

In §2 we demonstrated the existence of diurnal variation of TRβ1 protein expression which peaks at ZT12.5 (ZT refers to Zeitgeber time or circadian time which starts at the beginning of the light period; ZT0). This peak is paralleled by an increase in the maximal T₃-binding capacity in liver nuclei at ZT12.5 whereas no changes were observed in the association constant (Kₐ value) of nuclear T₃-binding. In §3, using the same time points as those used in §2, we showed that at the protein level, TRα2 has a marked diurnal variation which peaks at ZT6 (in the light period). In the same experiment no statistical significant daily variation in TRα1 protein expression could be demonstrated. Note that ZT6 animals is in the resting period as such, they do not show feeding activity. Based on these two experiments it was however not possible to determine whether the diurnal variation of TRs is regulated by the biological clock in the SCN or by feeding activity.

In §4 we set out to determine the origin of TR diurnal variation and two sets of experiments were performed: in the first experiment, the biological clock was disabled by selective lesion of the SCN in the experimental animals. In intact rats, liver TRβ1 mRNA had no statistically significant diurnal variation although there was a peak at ZT10. In contrast, TRα1 and TRα2 mRNA expression in control rats was higher in the light period than in the dark period; these changes were abolished in the SCNx rats. Thus the SCN seems the major force regulating the diurnal variation of TRα isoforms expression in the liver, which therefore can be viewed as a real circadian rhythm.

To evaluate whether these effects could be indirectly explained by a disappearance of the rhythmic feeding behavior in SCNx rats, we performed a second experiment in which otherwise intact animals were subjected to a regular feeding schedule, i.e., with one meal every 4 hours. When we looked at TR mRNA expression in liver, we found an enhanced TRβ1 mRNA amplitude, whereas the amplitude of the diurnal variation of TRα2 and TRα1 was only slightly reduced.

Thus it can be concluded that in the liver, TRβ1 expression is clearly affected by food intake. On the other hand, diurnal changes in liver TRα1 and TRα2 are mainly controlled by the SCN, and not via the endogenous clock-mediated daily rhythm in food intake.

8.B.3. Diurnal variation of TR: physiological relevance

It is interesting to speculate on the physiological relevance of our study. Since the TRα gene was probably present earlier during the evolution than the TRβ gene (42), it can be postulated that TRα, being the first, is involved in the main overall - thyroid hormone-dependent - daily housekeeping tasks in the organ and therefore under regulation of the central biological clock. The TRβ1 gene, having developed later, could be thought to be more involved in fine-tuning of hepatic gene expression, for instance in response to feeding activity. It has been shown recently that restricted feeding induces shifts in the circadian rhythm of liver gene expression independent of the SCN clock (39), which agrees with our finding that the rhythm of the TRβ1 changes as a result of regular feeding (RF) but not as a result of an SCN lesion.
Several T₃-responsive genes show marked diurnal variations (Table 1) in the liver which can be dependent on one or more mechanisms mentioned above. For example, in the case of 5'DI we could not find a significant diurnal variation of enzyme at the protein level. However, there is data available on the circadian gene expression of 5'DI at the mRNA level in a micro-array data bank which shows a peak between ZT12-18 (http://expression.gnf.org/cgi-bin/circadian/index.cgi, microarray; 95552_at); note that this expression pattern overlaps with that of the TRβ1 protein in our study (43).

Spot 14 expression peaks also in the evening, similar to TRβ1(ZT12-14). However, the primary input into the mRNA-Spot 14 diurnal rhythm seems to be the light-dark cycle, rather than periodic food intake. Synchronization of the rhythm by to the light-dark cycle suggests that the biological clock is also one of the determinants of rhythmic changes in hepatic gene expression (44). Although no statistically significant changes were found in diurnal variation of the TRβ1 mRNA, except a peak at ZT10, in the SCNx experiment, a central regulation of TRβ1 by the SCN cannot be ruled out.

Malic enzyme's diurnal variation (45) can also be manipulated by feeding (46). However the peak mRNA expression of malic enzyme (ZT22-02) does not overlap with that of the TRs discussed in this thesis but instead could agree with a dependence on TR expression.

The diurnal variation of GS protein expression peaks at ZT12.5 is similar to that of the TRβ1 protein, which suggests a direct involvement of TRβ1 in the regulation of GS expression.

**Table 1. Zonal expression and diurnal variation of thyroid hormone receptors and T₃-responsive genes in rodent liver.**

<table>
<thead>
<tr>
<th>Thyroid hormone receptors</th>
<th>Zonal expression</th>
<th>Diurnal variation mRNA</th>
<th>Diurnal variation protein</th>
<th>Peak diurnal variation mRNA</th>
<th>Peak diurnal variation protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRα1</td>
<td>pc</td>
<td>yes</td>
<td>ns</td>
<td>late light</td>
<td>begin dark</td>
</tr>
<tr>
<td>TRα2</td>
<td>pc</td>
<td>§3</td>
<td>yes</td>
<td>middle light</td>
<td>middle light</td>
</tr>
<tr>
<td>TRβ1</td>
<td>pc</td>
<td>§2</td>
<td>yes</td>
<td>middle light</td>
<td>begin dark</td>
</tr>
<tr>
<td>5'DI</td>
<td>pc</td>
<td>(1a)</td>
<td>no</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>S14</td>
<td>pc</td>
<td>(2b)</td>
<td>yes</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>ME</td>
<td>pc, pp</td>
<td>yes (1a)</td>
<td>yes</td>
<td>begin dark</td>
<td>begin dark</td>
</tr>
<tr>
<td>GS</td>
<td>pc</td>
<td>(1a)</td>
<td>yes</td>
<td>begin dark</td>
<td>begin dark</td>
</tr>
<tr>
<td>CYP7a</td>
<td>pc</td>
<td>(2b, 2a)</td>
<td>yes</td>
<td>begin dark</td>
<td>begin dark</td>
</tr>
<tr>
<td>ACC</td>
<td>pc</td>
<td>(2b)</td>
<td>yes</td>
<td>begin dark</td>
<td>begin dark</td>
</tr>
<tr>
<td>G6PD</td>
<td>pc, pp</td>
<td>yes (2b, 4a)</td>
<td>yes</td>
<td>middle dark</td>
<td>middle dark</td>
</tr>
<tr>
<td>PEPCK</td>
<td>pp</td>
<td>(1b)</td>
<td>yes</td>
<td>middle light</td>
<td>-</td>
</tr>
</tbody>
</table>

Abbreviations: 5'DI: 5'-deiodinase type I; S14: Spot14; ME: malic enzyme; GS: glutamine synthetase; CYP7a: cholesterol 7α-hydroxylase; ACC: Acetyl-coenzyme-A carboxylase; G6PD: glucose-6-phosphate dehydrogenase; PEPCK: phosphoenolpyruvate carboxy-kinase; ns: not significant; pp: periportal and pc: pericentral localization. *: enzyme activity.
8.B.4. Diurnal variation of TR in diseases
When the diurnal variation in TR protein expression was evaluated, it appeared that hypothyroidism results in a loss of diurnal variation for TRβ1, TRα1 and TRα2 isoforms. One may speculate that low levels of thyroid hormone result in a constantly elevated expression of all TR isoforms, so that every available hormone molecule will be bound. Therefore, a diurnal variation in TR levels is not beneficial during hypothyroidism since there would be too little receptor protein left at certain periods during the day to regulate T3-responsive genes.

8.C. Modulation of TRs in disease
8.C.1. Introductory remarks
In the early 1970's specific binding sites for T3 were found in rat liver nuclei (47). Scatchard analysis of nuclear binding of T3 showed a single class of high affinity and low capacity binding sites (47-49). The association constant (Kd value) of binding of T3 to nuclei is similar in different tissues, but the number of binding sites varied per tissue (50,51). Several studies have evaluated relative changes in TR during thyroid excess or deficiency, using Scatchard analysis. In general, no changes in maximal T3-binding capacity (MBC) or Kd have been found in livers of hyperthyroid or hypothyroid rats (52) while changes have been reported in other tissues (53-55). Furthermore, a 30%-50% reduction of the number of T3 receptors in liver is found without changes in affinity constant after partial hepatectomy (56) and during starvation or food restriction (57-60).

After the discovery of genes encoding thyroid hormone receptors the TR expression in disease was studied at the mRNA and protein level (using antibodies against the TR). Alterations of thyroid hormone receptors at the mRNA and/or protein level (61,62) are reported during prolonged starvation, after injection of lipopolysaccharid (LPS) in animal models (63) and in patients in intensive care units (64). The studies show rather conflicting results. Starvation results in an increase of TRα2 mRNA in the liver of rats after a 48 hour period, whereas TRα1 and TRβ1 did not change. In the heart, no change in any of the TR isoform mRNAs was found (61). In another study (62), the concentration of the mRNA coding for the TRβ1, the total nuclear T3-binding capacity, and the fraction of the total binding capacity that can be immunoprecipitated with anti-TRβ1 were measured in rat liver after 72 hour fasting. No changes in TRβ1 mRNA concentration, a 60% fall in total binding capacity and decreased TRβ1 protein were observed indicating that the level of receptor mRNA does not always parallel the level of the TR protein. Other studies in diseased human liver (primary biliary cirrhosis, sclerosing cholangitis, cryogenic cirrhosis and alcoholic cirrhosis) showed no differences at the protein and mRNA level despite the presence of non-thyroidal illness (65).

8.C.2. Thyroid hormone receptors and hypo- and hyperthyroidism
We found that hypothyroidism resulted in increased TRβ1, TRα1 and TRα2 mRNA expression whereas hyperthyroidism resulted in a decreased TRα1 and TRα2 mRNA
expression and no significant difference in TRβ1 mRNA expression in either light or dark periods.

TRβ1 protein expression in hypothyroid rats was higher only during the light period, whereas in hyperthyroid animals it was higher during the light period and lower during the dark period (Table 2).

Table 2. Changes in expression of TR protein levels in different thyroid states and during congestive heart failure

<table>
<thead>
<tr>
<th>TRα1</th>
<th>TRα2</th>
<th>TRβ1</th>
</tr>
</thead>
<tbody>
<tr>
<td>hypothyroidism (liver)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>light period</td>
<td>NS</td>
<td>↑</td>
</tr>
<tr>
<td>dark period</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>hyperthyroidism (liver)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>light period</td>
<td>NS</td>
<td>↓</td>
</tr>
<tr>
<td>dark period</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>congestive heart failure (RV)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 weeks</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>4 weeks</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>congestive heart failure (LV)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 weeks</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>4 weeks</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

NS; not significant. ↓, Down-regulation; ↑, Up-regulation.

TRα1 protein expression was higher in hypothyroid rats during the light period and lower in hyperthyroid animals during the dark period. TRα2 protein expression was likewise higher in hypothyroid and lower in hyperthyroid animals at both time points. Interestingly, no change was seen in TRβ1 mRNA levels during hyperthyroidism compared to control animals, whereas in these same animals TRβ1 protein is up-regulated in the light period and downregulated in the dark period. Our results are generally in line with previous studies (66,67). However, the changes we found are not always observed in earlier studies mentioned in the previous section (8.c.1). Probable reasons are; A) that our antibodies are very specific and B), we have a better detection possibilities for lower mRNA levels (we used a robot for mRNA isolation and real-time PCR with a detection limit of about 1 attogram of cDNA).

The time points at which to measure the TR isoform protein and mRNA levels chosen for the hyper- and hypothyroid experiments were the same as discussed in §2 and §3 because they showed the highest difference in total nuclear T₃-binding capacity in liver. However, we found a discrepancy between mRNA and protein levels of TR (see also 8.c.1). One possibility is the fact that mRNA expression increases a few hours before the protein increases and we would then miss the mRNA peak due to our experimental set up. Another possibility could be a change in stability of the receptor protein as result of phosphorylation (68-71) or differences in proteasome degradation pathways of TRβ1 (72).

8.C.3. Thyroid hormone receptors and heart failure

Thyroid hormone lowers systemic vascular resistance, increases blood volume and has inotropic and chronotropic effects on the heart. Altogether, this results in an
increased cardiac output. Thyroid hormone also increases protein synthesis in the heart by regulating genes such as the myosin heavy chain (MHC) isoforms involved in systolic and diastolic function (73) and sarcoplasmic reticulum Ca2\(^{-}\)-ATPase-2a (SERCA2a) which is involved in regulation of diastolic relaxation of heart (74). With respect to presence of TR in the heart, it has been noted that TR isoforms are specifically linked to certain genes as has been for TRβ1 in relation to the MHCβ regulation (75). On the other hand there may be a ventricle-specific regulatory mechanisms of TRs in the heart. The overlap between the effect of deleting TRα or TRβ1 in knock-out animals on the regulation of SERCA2a and MHCα/β does not imply the isoform specificity but instead, hints at a preference of MHCβ regulation for the TRα1 isoform (76,77).

Right ventricular hypertrophy was induced in rats (see §7) by monocrotalin (a pyrolizidine alkaloid) injection that induces chronic pulmonary hypertension followed by right ventricle hypertrophy and subsequently congestive heart failure (CHF). TR isoform expression during CHF was indeed ventricle specific (Table 2). We showed that changes in TRβ1 protein expression occur in the right ventricle whereas changes in the TRα isoforms are restricted to left ventricle during CHF. Changes were also found in the expression of SERCA2a and the MHC isoforms such that in both ventricles, the SERCA2a mRNA levels were not affected after two weeks but decreased after four weeks during CHF. A shift in the expression of the MHC isoforms mRNA levels from MHCα to MHCβ isoforms in both ventricles was observed even in the almost complete absence of MHCα isoform in CHF rats. These results support the idea of a lack of connection between particular target genes and a particular TR isoform. Parallel to changes in TR isoform protein expression in CHF rats we found that the animals have non-thyroidal illness. Therefore, the down-regulation of the T\(_3\)-responsive genes may not only be the effect of a decrease in TR protein levels but also of a decrease in thyroid hormone levels.

A differential expression of TR isoforms by thyroid hormone at the mRNA level in heart has been observed before. Hypothyroidism results in an up-regulation of TRα isoform mRNA and down-regulation of TRβ1 mRNA whereas hyperthyroidism reverses these patterns (78). Furthermore, physiological hypertrophy shows an up-regulation of TRβ1 mRNA expression only whereas pathological left ventricular hypertrophy results in down-regulation of all TR isoforms at the mRNA level (75). However, in these previous studies, only one ventricle or part of a left ventricle was used. We are the first to show TR expression at protein level in the individual ventricles and at two time points during development of the hypertrophy. Our results on TR isoform protein expression are in line with human study that showed TR mRNA expression and changes in (LV) in dilated cardiomyopathy (79).

8.D. Perspectives
8.D.1. Zonal distribution of TR

It is clear from the studies presented above that a zonal expression of TRβ1, TRα1 and TRα2 exists in rodent liver. We showed also that TRα1 and TRα2 expression was broader than that of TRβ1. However, our studies are mainly descriptive in
nature and were performed in rodents. It would also be interesting to confirm the zonal distribution of TR in human liver and to study the zonal expression of the other thyroid hormone receptors TRβ2 and TRβ3 in rodents and in humans. Studying the mechanism underlying the zonal expression of the TR isoforms and T₃-responsive genes can be accomplished using novel techniques such as laser microdissection microscopy, which allows to selectively isolate cells around porto-central axis combined with either RT-PCR or microarrays.

Studies on the zonal distribution of downstream effectors of TR can give valuable information about various T₃-responsive genes. For example, the lipogenic glucose 6-phosphate dehydrogenase (G6PD) enzyme shows a broader pericentral expression pattern than TRβ1 in the liver (25). It implies involvement of other TR isoforms. Indeed, treatment with thyroid hormone induces higher G6PD mRNA expression in TRβ⁻⁻ mouse compared to wild type animals (28). Another example is PEPCK expression, which is restricted to around the portal veins of the liver lobe while TR expression appears to be restricted to an area around the central veins of the liver lobe. Since we do not know the exact sensitivity of our antibodies it is possible that the zone of TRα isoform expression is broader than seen in §3. The TRα would then overlap the PEPCK area and be able to regulate this well known T₃-dependent gene. But how does the TR regulate the expression of PEPCK? Negative regulation may be responsible here. Microarray analysis showed that there are more genes that are negatively regulated by TR in liver than ones that are regulated in a positive fashion (80). The latter observation is further supported by a study which showed that the gene expression in TRβ⁻⁻ hypothyroid mice compared to the WT showed a lower expression of PEPCK mRNA and that thyroid hormone treatment after both 2 hour (short term) and 5 days (long term) still induced this ratio of the gene transcription (28).

8.D.2. Diurnal variations of TR

Further studies should clarify whether the circadian rhythm of TRα isoforms in the liver is generated hormonally or by autonomic innervation of the liver. In case of TRβ1 more studies are needed to investigate the relationship between feeding activity and TRβ1-dependent gene regulation. For instance, increased carbohydrate intake results in a broader zonation of the "Spot 14" gene. If TRβ1 is the only TR involved in the regulation of "Spot 14" gene expression then we would expect a broader zonation of TRβ1 protein in the liver in response to carbohydrate intake. A limitation of our study probably lies in the choice of the time points at which we analyzed the diurnal variation of TRβ1 in the SCN lesion experiment. It is possible that we just missed the peak of expression of TRβ1. Therefore, for this reason, central regulation of TRβ1 in addition to feeding activity can not be ruled out. If we assume that TRβ1 solely regulates the diurnal variation of the "Spot 14" gene, then this central regulation of TRβ1 fits with the observation that the SCN is involved in regulation of the "Spot 14" gene (44).

Few studies have dealt with a possible diurnal expression of TRβ2 at either protein or mRNA level but the interesting fact remains that the diurnal variations in G6PD
and ME expression overlap with those reported for the TRβ2 mRNA in mouse liver at ZT 20 (98795_at Original Affymetrix Annotation). A careful study of the zonal expression and diurnal variation of TRβ2 can clarify the possible role of this TR isoform in the regulation of those genes. Since no data are available on the regulation of T3-responsive genes at the ZT12.5 when TRβ1 and TRα1 are highly expressed, our studies provide a unique opportunity to investigate the regulation of these genes by TR in vivo.

New studies are also needed to investigate the diurnal rhythm of TR isoforms in relation to the regulation of T3-responsive genes. PEPCK activity for example, shows a 24-hour circadian rhythm, which is eliminated after inactivation of the SCN. It has also been suggested that autonomic input into the liver may regulate PEPCK activity (81). Not only a restricted feeding regimen results in a shift of the diurnal peak of PEPCK activity (82) but also a protein meal presented in dark or light period (83). PEPCK expression is highest in afternoon when animals mostly rest (84). Thus if the TRs are involved in expression of PEPCK there is an indirect role for TRα isoforms and a possible negative regulation.

Finally, there is the role of reverb-α gene and its relation to the endogenous circadian clock of the liver (85) which by its relation to the TRα isoforms deserves more attention. It has been shown in vitro that reverb-α is involved in the splicing of the TRα1 and α2 isoforms and that its diurnal variation peaks around ZT8-10 (86) which coincides with the peak in TRα1 isoform mRNA expression.

8.D.3. Modulation of TRs in disease
Further studies could be aimed at clarifying the significance of the observed changes in TR expression in hyper- and hypothyroid animals in relation to T3-responsive genes in the liver. In the heart, preliminary results show the existence of a zonal distribution for the TR isoforms as well. Although we showed changes in TR expression in ventricular hypertrophy, further studies are needed to identify which cell population in the heart is most affected during the development of heart failure.
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