Expression of thyroid hormone receptors A and B in developing rat tissues; evidence for extensive posttranscriptional regulation

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Abstract

The perinatal changes in the pattern of expression of the thyroid hormone receptor (TR) isoforms TR α_1 , TR α_2 , TR β_1 , and TR β_2 were investigated using *in situ* hybridization and immunohistochemistry, and RT-PCR and western blotting as visualization and quantification techniques respectively. In liver, lung, and kidney, TR α mRNA was expressed in the stromal and TR β mRNA in the parenchymal component of the tissues. When compared with liver, TR α mRNA concentrations were tenfold higher in lung, kidney, and intestine, and 100-fold higher in brain, with TR α_2 mRNA concentrations exceeding those of TR α_1 5- to 10-fold. Tissue TR β_1 mRNA concentrations were similar in liver, lung, and brain, and 3- to 5-fold higher in kidney and intestine. None of the TR β_2 mRNA could be detected outside the pituitary. Tissue TR α_2 and TR β_1 protein levels reached adult levels at 5 days before birth, whereas TR α_1 protein peaked after birth. Because of the distinct time-course of thyroid hormone-binding receptors TR α_1 and TR β_1 , we speculate that an initiating, TR β_1 -mediated signaling from the parenchyma is followed by a TR α_1 -mediated response in the stroma. When compared with organs with a complementary parenchymal–stromal expression pattern, organs with extensive cellular co-expression of TR α and TR β (brain and intestinal epithelium) were characterized by a very low TR α protein: mRNA ratio, implying a low translational efficiency of TR mRNA or a high turnover of TR protein. The data indicate that the TR-dependent regulatory cascades are controlled differently in organs with a complementary tissue expression pattern and in those with cellular co-expression of the TR α and TR β genes.

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Introduction

Since it was discovered that metamorphosis can be induced in amphibians by feeding them thyroid gland extract (Gudernatsch 1912), it has become generally accepted that thyroid hormones play a crucial role in controlling growth, development, differentiation, and metabolism of virtually all tissues of vertebrates. The beneficial effects of thyroid hormones on perinatal organ development and maturation are most readily recognizable in brain (Oppenheimer & Schwartz 1997, Morreale de Escobar *et al.* 2004). However, since thyroid hormones can pass the placenta and accumulate in embryonic cells to functionally relevant concentrations well before the embryonic thyroid starts to function on its own (Morreale de Escobar *et al.* 1990), thyroid hormone-dependent instructive effects in earlier phases of organ development are likely (Glinioer 2001, van Tuyl *et al.* 2004).

The effects of thyroid hormones on gene expression are mediated via the thyroid hormone receptor (TR) genes α (*NRI1*) and β (*NRI2*), which are both the members of the steroid/TR superfamily of ligand-dependent transcription factors (Zhang & Lazar 2000, Yen 2001, Yen *et al.* 2006). The TR α and TR β genes generate, via alternative splicing, four thyroid hormone-binding receptors, namely TR α_1 and TR β_{1-3} (Williams 2000, Chassande 2003). In addition, several proteins, including TR α_2 , which do not bind thyroid hormone-response elements and/or thyroid hormones, are formed. Presumably, these proteins modulate thyroid hormone-mediated gene expression (Cheng 2000, Williams 2000, Plateroti *et al.* 2001, Yen 2001).

In rat, northern blot analysis showed that TR α mRNA concentration increased in brain, liver, and brown adipose tissue from 18 days of development onwards to

reach its highest level perinatally (Strait *et al.* 1990, Tuca *et al.* 1993). TR β mRNA also increased perinatally in brain, but was reported to reach its peak only in the second postnatal week (Strait *et al.* 1990). In contrast, TR β mRNA decreased perinatally in liver and brown adipose tissue (Strait *et al.* 1990, Tuca *et al.* 1993). In the developing nervous system of the rat, the highest cellular concentration of TR α mRNAs was found in the areas of neuronal differentiation such as the fetal neocortical plate, whereas TR β mRNAs were largely restricted to the zones with neuroblast proliferation, such as the germinal trigone and the cortical ventricular layer (Bradley *et al.* 1992). Although these findings suggested complementary and, perhaps, mutually exclusive roles of the TR α and TR β genes in organogenesis, they did not result in a specific hypothesis for the mechanism of action of thyroid hormones in development. Furthermore, these studies did not address TR expression at the protein level. We, therefore, studied the spatial and the temporal distribution of the mRNAs and proteins encoding TR α and TR β by *in situ* hybridization and immunohistochemistry. Since our *in situ* probes did not differentiate between the respective splice variants, quantitative PCR techniques were used to determine the tissue levels of TR α_1 , TR α_2 , TR β_1 , and TR β_2 mRNA. Since tissue TR mRNA level and nuclear thyroid hormone-binding capacity (i.e., functional TR protein) do not always correlate (Strait *et al.* 1990, Tuca *et al.* 1993), we also investigated tissue TR protein level by western blot analysis and immunohistochemistry, using our recently described antisera (Zandieh Doulabi *et al.* 2002, 2003). We report a highly specific, but heterogeneous spatial and temporal expression pattern of the TR α and TR β mRNAs, and report evidence for extensive posttranscriptional regulation in the perinatal period.

Materials and methods

Animals

Female Wistar rats in heat were selected and mated in the morning, the next day being designated embryonic day 1 (E1). Fetuses were harvested by caesarian section at E13, E14, E15, E16, E17, E18, E19, and E20; or were born naturally and killed 1 (P1) or 4 days (P4) later, or as adults. All experiments were performed under protocols approved by the University of Amsterdam Committee on Animal Research.

Tissue isolation and preparation

All tissues were fixed in phosphate-buffered 4% formaldehyde for *in situ* hybridization and in the ratio of methanol:acetone:water (2:2:1 (v/v)) for immunohistochemistry as described (van Tuyl *et al.* 2004).

In situ hybridization

In situ hybridization was carried out exactly as described (Moorman *et al.* 2000). *In situ* hybridization carried out according to this protocol produces semiquantitative data, that is, differences in absorbance between different tissues or cells within one section reflect differences in mRNA concentration between these tissues or cells (Jonker *et al.* 1997). This protocol is included in the method paper referenced to Moorman *et al.* (2000). The EcoRI–HindIII cDNA fragments of the clones rc-erbA- α (nt –29 to +1821) and rc-erbA- β (nt –37 to +2165) were used as probes to detect TR α and TR β mRNA respectively (Bradley *et al.* 1992). These fragments do not discriminate between TR isoforms. [α - 35 S]UTP-labeled antisense and sense probes were generated with Sp6 and T7 RNA polymerase respectively. We opted to use almost the entire TR mRNA sequence to make the *in situ* assay as sensitive as possible, because the hybridization signal is dependent on the length of the probe (before fragmentation). We then used PCR to quantify subtypes.

Quantitative PCR

At E15, E18, P1, and in the adult, TR mRNA levels were quantified in brain, pituitary gland, lung, liver, kidney, and intestines. For each stage, minimally three rats were assayed. TR mRNAs were quantified by establishing the efficiency of both the reverse-transcription and polymerase chain reaction steps of the assay. Large-scale mRNA synthesis from the respective TR cDNA fragments was performed with the MEGAshortscript T7 Kit (Ambion, Nieuwerkerk a/d IJssel, The Netherlands) and quantified by including a known amount of [α - 35 S]CTP. Reverse-transcriptase efficiency was estimated by comparing mRNA input (different amounts of the RNA transcripts) and DNA output, and amounted to 80–100%. TR α cDNA amplification was estimated by competitive RT-PCR as described (Moller & Jansson 1997), whereas TR β cDNA amplification was measured by real-time PCR (Lightcycler, Roche).

TR α

The sense primer was chosen in the common part of the TR α_1 and TR α_2 sequences, whereas the antisense primers were in the specific parts. For TR α_1 , a 343 bp fragment was formed (nt 1219–1562 of the mRNA) and for TR α_2 a 242 bp fragment (nt 1411–1653). After reverse transcription (RT) and PCR amplification, the concentration of TR α_1 and TR α_2 cDNA in the sample was estimated by titration of $8\text{--}5000 \times 10^{-4}$ amol (TR α_1) or $4\text{--}2500 \times 10^{-3}$ amol (TR α_2) of a 'competitor' fragment that differed from the parent fragments by missing an identical 82 bp 5' HincII fragment.

The amount of competimer added was determined by including a known amount of [α -³⁵S]dATP during large-scale PCR synthesis. TR α mRNA concentration in the sample was calculated from a double logarithmic plot of the TR α :competimer ratio and the competimer concentration (Moller & Jansson 1997).

TR β

The antisense primer was chosen in the common part of TR β ₁ and TR β ₂, whereas the sense primers were in the specific part. For TR β ₁, a 185 bp fragment was formed (nt 437–622 of the mRNA) and for TR β ₂, a 244 bp fragment (nt 343–587 of the mRNA). TR β cDNA concentration in the sample was deduced from a standard curve with 5–50 000 $\times 10^{-4}$ amol TR β ₁ or TR β ₂ DNA fragment. The TR β ₁ and TR β ₂ fragments were made by large scale PCR synthesis and quantified by including a known amount of [α -³²P]dATP. TR β ₂ levels were also quantified with fluorescence resonance energy transfer of hybridization probes nt 264–290 (red640-labeled) and nt 292–316 (fluorescein-labeled) on amplified fragment nt 241–556. Dilutions of pituitary gland mRNA with a predetermined TR β ₂ mRNA concentration were used to generate a standard curve.

Western blotting and immunohistochemistry

Extracts of brain, pituitary gland, thyroid, trachea, lung, heart, muscle, liver, intestines, kidneys, adrenals, testis, epididymal fat pad, bladder, and spleen from 3 E17, P4,

and adult rats were analyzed in triplicate by western blotting, using antisera specifically identifying TR α ₁, TR α ₂, and TR β ₁. The specificity of the antibodies used was described previously (Zandieh Doulabi *et al.* 2002, 2003). The extracts were prepared in 0.25 M sucrose, 40 mM dithiothreitol, 2% SDS, 1 mM EDTA, 62.5 mM Tris–HCl (pH 7.6), and 10% glycerol, separated on 10% (w/v) polyacrylamide gels, and blotted onto an Immobilon-P (Millipore) membrane. After staining with amido black to verify similar protein loading, the membrane was incubated in 10 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.5% Tween20, and 5% nonfat dried milk powder (Natriron, Nutricia) for 6 h, followed by an overnight incubation in a ratio of 1:1000 dilution of the respective antisera. Antibody binding was quantified using chemiluminescence (CDP-Star, Roche), in combination with the LUMI-imager F1 (Roche; Bakker 1998). In the absence of primary antisera, no staining was seen, whereas in their presence, bands at the anticipated *M_r* of the corresponding receptors appeared. However, in liver and kidney extracts, extra bands at both higher and lower *M_r* than found in other organs were observed for TR α ₁ and TR β ₁. These patterns were also found in organs that were rapidly isolated and analyzed immediately.

Immunoperoxidase staining was performed on dewaxed tissue sections. Antibody binding on sections was visualized by the indirect unlabeled antibody peroxidase anti-peroxidase (PAP) method (Sternberger *et al.* 1970). PAP immunocomplexes were purchased from Nordic (Tilburg, The Netherlands). If care is taken not to overdevelop

Table 1 Developmental changes in the expression of thyroid hormone receptors (TRs) in the central nervous tissue, lung, liver, kidney, adrenal, and intestines

Stage of development		TR α ₁		TR α ₂		TR β ₁		TR β ₂	
		mRNA	Protein	mRNA	Protein	mRNA	Protein	MRNA	Protein
Organ	Central nervous system								
	Early fetal	—	—	+	—	—	—	—	—
	Late fetal	—	—	+	—	—	++	—	—
Lung	Neonatal	—	—	++	—	++	++	—	—
	Early fetal	—	—	++	+	+	—	—	—
	Late fetal	—	+	++	—	++	+	—	—
Liver	Neonatal	—	+	+	—	+	+	—	—
	Early fetal	—	—	++	—	—	—	—	—
	Late fetal	+	+	+	+	++	++	—	—
Kidney	Neonatal	—	+	+	+	+	+	—	—
	Early fetal	—	—	+	—	—	—	—	—
	Late fetal	—	+	+	—	+	+	—	—
Adrenal	Neonatal	—	+	+	—	+	+	—	—
	Early fetal	+	—	+	—	—	—	—	—
	Late fetal	+	+	+	++	—	+	—	—
Intestines	Neonatal	—	+	—	++	—	—	—	—
	Early fetal	—	—	—	—	—	—	—	—
	Late fetal	—	+	++	—	+	+	—	—
	Neonatal	—	—	+	+	+	+	—	—

Early fetal, E12–E16; late fetal, E17–E22; neonatal, P0–P4. —, no expression detected; +, well detectable expression; ++, strong expression.

the enzymic staining, local differences in immunohistochemical staining intensity represent differences in protein concentration between these cells or tissues (van Straaten *et al.* 2006).

Results

Distribution of TR α and TR β

We describe the expression patterns TR α and TR β for each organ separately, going from mRNA to protein and from early fetal to postnatal. An overview of our findings is provided in Table 1.

Central nervous system

The staining pattern of TRs in the central nervous system has been described (Bradley *et al.* 1992). In agreement, TR α mRNA expression in the basal plate of the neural tube was already pronounced at E15 (Fig. 1A) and very strong in the cortical plate of the brain at E16 (Fig. 1C). In contrast, TR β mRNA expression was undetectable (Fig. 1B) or much weaker (Fig. 1D). Since the TR α_2 mRNA concentration exceeded that of TR α_1 more than 20-fold (Fig. 2A), it was responsible for the observed TR α mRNA expression pattern. The expression of TR α_2 and TR β_1 mRNA

followed a similar temporal pattern and was highest at P1 (Fig. 2A). TR β_2 mRNA was not detectable. Both before and shortly after birth, the TR α_1 and TR α_2 protein concentration in the brain (Fig. 1G, H, J and K) was very low when compared with that in the other organs (Fig. 3A–F). In contrast, TR β_1 protein was already present in fetal brain (E17) and abundant at P4 (Figs 1I, L and 3). These data show that the developing brain was characterized by a very low protein: mRNA ratio for TR α_1 and, in particular TR α_2 , whereas this ratio was much higher for TR β_1 . A summary of these findings can be found in Table 1.

Other intracranial organs

TR α and TR β expression colocalized at many sites in the central nervous tissue. In the inner ear, however, the strong signal of TR β mRNA in the sensory epithelium of the cochlea and the very weak signal in the cochlear nerve (Fig. 1F) contrasts with the opposite staining pattern TR α mRNA (Fig. 1E) and demonstrates the specificity of the *in situ* hybridization procedure. We have included this result as an example of the sometimes highly contrasting TR expression patterns in organs.

Protein for all the three receptors was observed in the extracts of adult pituitary glands (Fig. 3A, D and I). TR β_1 protein was present in E18 and P4 pituitary

Figure 1 Thyroid hormone receptor expression in perinatal central nervous system. TR α mRNA appears in the basal plate of the E15 spinal cord (A) well before TR β mRNA (B). In the E16 brain, TR α mRNA expression in the cortical plate (C) is much stronger than TR β expression (D). In contrast, the TR β signal is intense in the cochlear sensory epithelium (F), whereas TR α expression dominates in the cochlear nerves (E). At E18 and P4, protein expression of TR α_1 and TR α_2 is very low (G, H, J and K). In contrast, TR β_1 protein is highly expressed in various structures of the brain (I and L). B, basal nuclei; C, cochlea; Ce, cerebellum; Co, cortex; CP, choroid plexus; N, nose. Bar, 100 μ m.

Figure 2 Quantification of thyroid hormone receptor mRNA levels during perinatal development. A, brain; B, lung; C, liver; D, kidney; E, small intestine; F, colon. TR α_1 (triangles), TR α_2 (squares), and TR β_1 (dots) mRNA concentrations are depicted $\pm$ S.E.M. The age of the animals is indicated on the X-axis, while the concentration of the mRNAs is shown on the Y-axis (left, TR α_1 (C–F) and TR α_2 , and right, TR α_1 (A and B) and TR β_1 concentration). Note that the scales of the Y-axes differ in the respective panels.

glands, with the highest concentration in the posterior lobe (Fig. 4C and F). Both TR α isoforms only became detectable after birth in the anterior and middle lobes (Fig. 4A, B, D and E).

Lung

The pulmonary epithelium is initially surrounded by a conspicuous mass of mesenchyme. Expression of TR α mRNA in this mesenchyme was first observed at E13 and became pronounced at E15 (Fig. 5A). TR β mRNA expression in the epithelium became more intense during the subsequent 'canalicular' phase of the lung development (E16–E18; Fig. 5B and D), when TR α mRNA expression remained distinctly present in the mesenchyme (Fig. 5C). Owing to the attenuation of the walls of the airways during the 'alveolar' phase of lung development (>E19), the complementary expression pattern of TR α and TR β became more and more

difficult to discern (Fig. 5E and F). TR β mRNA remained present in the wall of the pulmonary arteries (arrows Fig. 5D and F) and TR α mRNA in the myocardium of the pulmonary veins (arrows Fig. 5C). TR α_2 mRNA levels were approx. tenfold higher than TR α_1 mRNA levels and peaked at E18 and P1 (Fig. 2B), indicating that the TR α *in situ* hybridization signal actually represented TR α_2 mRNA. The TR β signal in the *in situ* hybridizations represented TR β_1 , because no TR β_2 mRNA could be detected. TR β_1 mRNA levels peaked at E18 (Fig. 2B).

Western blot analysis revealed the presence of TR α_1 protein at E17 and a marked increase in the concentration by P4 (Fig. 3A and B). At the same time points, the intensity of the TR α_2 -protein band was relatively weak and even appeared to decrease with development (Fig. 3D and E). TR β_1 protein levels were similar at E17 and P4 (Fig. 3G and H). Despite the positive western blots, we were not able to demonstrate

Figure 3 Western blot of thyroid hormone receptor protein levels during perinatal development. Representative example of three extracts assayed in triplicate. A–C, TR α_1 ; D–F, TR α_2 ; G–I, TR β_1 ; A, D and G: E17; B, C, E, F, H and I: P4. Ad, adult; MW, molecular weight. Organ abbreviations: A, adrenal gland; BL, bladder; B, brain; Fp, epididymal fat pad; He, heart; In, intestine; IL, ileum; Je, jejunum; K, kidneys; Li, liver; Lu, lung; M, muscle; Pg, pituitary gland; Sp, spleen; Te, testis; Th, thyroid gland; Tr, trachea.

either TR α_1 or TR α_2 protein by immunohistochemistry (Fig. 5G, H, J and K), indicating that the proteins were present at low levels in many cells. TR β_1 protein was predominantly expressed in lung epithelium (Fig. 5J and L). The protein: mRNA ratio for TR α_1 increased in development, and remained more or less constant for TR α_2 and TR β_1 .

Liver

Expression of TR α mRNA in the liver was first observed at E15 (Fig. 6A) and could be located in the stromal component, including the wall of the veins, from E17 onwards (Fig. 6C and E). Expression of TR β mRNA was also observed at E15, declined during the next 2 days to undetectable levels, but reappeared in the parenchymal tissue at E19 (Fig. 6B, D and F). At E20, the complementary expression of TR α in the stromal and of TR β in the parenchymal tissue had become very

striking (Fig. 6E and F). The concentration of TR α_1 and, in particular, TR α_2 mRNA, was very low when compared with other tissues during all ages investigated (Fig. 2C). As a result, the TR α_1 :TR α_2 ratio was the highest observed in any of the tissues investigated and was more than five times higher than that in the brain. TR β_1 mRNA levels peaked at E18 (Fig. 2C), whereas TR β_2 mRNA levels were undetectable.

In liver extracts, the TR α_1 antibody detected not only a band at the expected size (47 kDa) but also bands at higher and lower *M_s*. TR α_1 levels increased between E17 and P4, when concentrations reached adult values (Fig. 3A and B), whereas the TR α_2 protein did not change (Fig. 3D and E). Similarly, the 57 kDa TR β_1 band did not change in development, but bands of 30 and 110 kDa became increasingly prominent (Fig. 3G and H). Immunohistochemical staining revealed weak staining of TR α_1 protein in parenchymal cells surrounding the central veins, but after birth only (Fig. 6G

Figure 4 Thyroid hormone receptor expression in the pituitary gland. TR α_1 and TR α_2 proteins are not detectable in the E18 pituitary (A and B) and begin to accumulate postnatally (P4) in the anterior lobe (D and E). In contrast, TR β_1 protein was highly expressed at E18 (C) and P4 (F), in particular, in the middle lobe. A, anterior; I, intermediate; and P, posterior lobe of pituitary. Bar, 100 μ m.

and J; cf. (Zandieh-Doulabi *et al.* 2003), whereas TR α_2 protein had then become detectable in isolated liver cells (Fig. 6H and K). In fetal rat liver, TR β_1 protein was observed faintly in the parenchyma of the liver (Fig. 6I). After birth, expression had become much stronger in the hepatocytes surrounding the central veins (Fig. 6L; cf. (Zandieh Doulabi *et al.* 2002)). The protein: mRNA ratios for TR α_1 , TR α_2 , and TR β_1 did not change appreciably with development.

Kidney

The development of the definitive kidney is characterized by the penetration of the ureteric bud into the metanephric mass. The metanephric tubules expressed TR β mRNA (Fig. 7B, D and F), whereas the ureteric buds and surrounding mesenchyme expressed TR α mRNA (Fig. 7A, C and E). TR α_2 mRNA levels were more than ten times higher than TR α_1 levels (Fig. 2D). Both TR α_1 and TR α_2 mRNA concentrations peaked perinatally, whereas TR β_1 mRNA peaked just after birth. TR β_2 mRNA was undetectable.

TR α_1 protein levels increased perinatally, with a band of 30 kDa, which is also present in liver extracts, becoming more prominent with age (Fig. 3A and B). At E18, TR α_1 protein was weakly present in the nephric tubules (Fig. 7G), but had disappeared at P4 (Fig. 7J).

TR α_2 protein was not detectable in any cell type in the kidney (Fig. 7H and K). TR β_1 protein was detected in the glomeruli and tubules at E18 (Fig. 7I). After birth, the glomeruli were no longer positive (Fig. 7L). The protein:mRNA ratio for TR α_1 and TR α_2 did not change appreciably in development. If, however, the 30 kDa band is a proper TR α_1 gene product, the protein: mRNA ratio for TR α_1 does increase with development. The protein:mRNA ratio for TR β_1 declined with development, but in the adult, a 30 kDa band that was also seen in liver, had become very prominent. If this is a bona fide TR β_1 gene product, the ratio is substantially higher in the adult.

Adrenals

TR α mRNA, which was still found in the entire adrenal at E16 (Fig. 7A), had become confined to dispersed islands of cells in the medulla and the capsule at E18 (Fig. 7C). TR β mRNA levels were low and confined to the capsule (Fig. 7B and D). PCR analysis was not performed. Western blot analysis showed prominent bands for both TR α_1 and TR α_2 after birth (Fig. 3B and E). The TR α_2 antibody produced an additional 100 kDa band. This band was also observed in human (not shown), but not in rat pituitary gland. Only one band was observed for TR β_1 protein (Fig. 3F). The TR α_1 protein was diffusely distributed in the adrenal at E18 (Fig. 8A), but had become confined to the adrenal medulla at P4 (Fig. 8D). The distribution of TR α_2 protein was confined to well-defined islets of cells at E18 (Fig. 8B) and to the medulla after birth (Fig. 8E). The TR β_1 protein was diffusely distributed in the adrenal tissue and capsule at E18 (Fig. 8C), but became localized to the adrenal cortex after birth (Fig. 8F). The morphological data indicate that the TR α mRNA in the adrenal capsule does not generate detectable protein. In contrast, the low abundance of TR β mRNA and the readily detectable TR β_1 protein in sections indicate a high protein:mRNA ratio.

Intestines

The epithelial layer of the intestines (Fig. 9A–F) and the parenchymal cells of the pancreas (Fig. 9C and D) co-expressed TR α and TR β mRNA from E16 onward (Fig. 9A–F). Between E18 and E20, both TR α and TR β mRNAs became confined to the crypts. TR α_1 , TR α_2 , and TR β_1 mRNA levels followed a similar developmental pattern and peaked perinatally (Fig. 2E and F).

Western blotting showed that TR α_1 protein levels decreased between 5 days before birth and 4 days after birth (Fig. 3A and C). The opposite was true for TR α_2 protein (Fig. 3D and F). Relatively low levels of TR β_1 protein were found both pre- and postnatally (Fig. 3G and I). The TR α_1 antiserum generated staining at the

Figure 5 Thyroid hormone receptor expression in perinatal lung. TR α mRNA was prominently present in the lung mesenchyme at E15 (A) and E18 (C). TR β mRNA in the pulmonary epithelium is visible at E15 (B) and pronounced at E18 (D). Note the complementarity of TR α and TR β mRNA expression (C and D). Thereafter (E and F: E20), the complementary distribution was no longer demonstrable. The pulmonary vein is identifiable by its expression of TR α (C), whereas the pulmonary artery is characterized by TR β expression in its myocardial wall (D and F; arrowheads). TR α_1 (G and J) and TR α_2 proteins (H and K) were undetectable at E18 (G and H) and P4 (J and K). TR β_1 protein was largely confined to the epithelium and detectable at both E18 (I) and P4 (L). Arrows, terminal bronchioli ('canaliculi'); asterisk, terminal bronchioli and alveoli. Bar, 100 μ m.

apical surface of the enterocytes at E18, but not at P4 (Fig. 9G and J). TR α_2 protein could not be detected (Fig. 9H and K). In contrast, strong staining for TR β_1 protein was observed in the smooth-muscle layer of the intestine at E18 (Fig. 9I) and P4 (Fig. 9L), as opposed to a relatively weak staining in the mucosa. The TR α mRNAs, therefore, generated very little protein in the epithelium. The protein:mRNA ratio for TR β_1 in the epithelium was also low. At the same time, high levels of TR β_1 protein were present in the smooth muscle layer, where the corresponding mRNA was barely detectable.

Comparison of TR protein in organs

We demonstrated the presence of TR α_1 and TR α_2 proteins on western blots of several other organs (Fig. 3, Table 1). Quantification showed that the concentrations of TR α_1 protein in lung, muscle, heart, kidney, adrenals, pituitary gland, spinal cord, testis, and fat pad were similar, that in brain, liver, and small intestine were approximately threefold lower, and that in trachea and red blood cells were twofold higher than average. Average concentrations of TR α_2 were found in liver, small intestine, and kidney, a very low concentration in red blood cells, approximately twofold lower than average concentrations in lung, trachea, muscle,

heart, spinal cord, and brain, and twofold higher than average in the fat pad, testis, and adrenals. The resulting TR α_1 :TR α_2 ratio, a parameter for tissue thyroid hormone responsiveness, was 3- to 10-fold lower than average in brain, liver, prenatal heart, lung, postnatal small intestine, adrenals, and fat pad; 2- to 10-fold higher than average in postnatal lung, trachea, muscle, and pituitary gland, and extremely high for blood. The TR α_1 :TR α_2 ratio declined with the development in liver and small intestine, and increased in lung, muscle, heart, and brain. The TR β_1 concentration was remarkably similar in all analyzed organs before birth and, after birth, relatively low in muscle, bone, fat pad, small intestine, and red blood cells, and relatively high in brain and spinal cord.

Comparison of the protein:mRNA ratio between organs

Protein synthesis is determined by the corresponding steady-state mRNA level and translational efficiency, whereas the steady-state protein level is determined by its synthesis (translation) and degradation. If we assume that no major changes occurred in the tissue concentration of polyA⁺ mRNA or total protein with time and that TR mRNAs are not sequestered from

Figure 6 Thyroid hormone receptor expression in perinatal liver. TR α mRNA was readily detectable in prenatal liver mesenchyme (A, E15; C, E17; E, E20). Expression declined in liver stroma, but became more prominent in the developing perivascular mesenchyme. TR β mRNA was also readily detectable at E15 (B), temporarily declined (D; E17), but had reappeared in the hepatocytes at E20 (F). Note the complementarities of TR α and TR β mRNA expressions (E and F). At E18 (G), TR α_1 protein expression was undetectable before birth (H, E18), but was observed in isolated liver cells after birth (K, P4). TR β_1 protein was present in most hepatocytes before birth (I), but had become confined to the pericentral hepatocytes by P4 (L). Li, liver; Lu, lung; G, gonad; P, portal vein; C, central vein. Bar, 100 μ m.

the translational machinery at a specific time point, protein:mRNA ratios inform us about the presence of a posttranscriptional level of regulation of gene expression (cf. Table 1). During lung development, the protein:mRNA ratio of TR α_1 increased, but the ratios of TR α_2 and TR β_1 remained more or less constant. Similarly, the protein:mRNA ratios for TR α_1 , TR α_2 , and TR β_1 did not change appreciably with liver and kidney development. During intestinal development, the TR α and TR β mRNAs generated very little protein in the epithelium, but the reverse was true for the smooth muscle layer, where a barely detectable TR β_1 mRNA generated a high concentration of protein. In the brain, the protein:mRNA ratio was very low for TR α_1 and TR α_2 , whereas that for TR β_1 was highest.

Discussion

Despite the detailed insights into the molecular mechanisms underlying the thyroid hormone- and TR-dependent modulation of gene expression that are now available (Cheng 2000, Zhang & Lazar 2000, Baxter *et al.* 2001, Yen 2001, O'Shea & Williams 2002,

Chassande 2003, Flamant & Samarut 2003) and the undisputed role of thyroid hormone in organogenesis (Oppenheimer & Schwartz 1997, Tata 1999), the mechanistic aspects of thyroid hormone action in development are still debated. Nevertheless, it is likely that epithelial-mesenchymal interactions play a role, because mesenchymal cells are thought to be primary determinants of epithelial cell fate during organogenesis (Brard 1990). Using *in situ* hybridization and immunohistochemistry as visualization techniques, and RT-PCR and western blotting as quantification techniques, we indeed observed a highly specific distribution pattern of the TR isoforms that appears to support such a mechanism of action for thyroid hormones. Expression of the TR α isoform was observed in the mesenchymal tissues and expression of the TR β isoform in the epithelial tissues of lung, liver, kidney, sensory system of the inner ear, and bone. As far as we know, this generalization has not been made so far. However, this generalization did not apply to the central nervous system and the intestine. In addition, the relation was obscured by an extensive posttranscriptional regulation of the expression of the TRs, with differences between organs and between different developmental stages of the same organ

Figure 7 Thyroid hormone receptor expression in perinatal kidney. TR β mRNA was present in the developing nephrons from E16 onwards (B, E16; D, E18; F, E20). TR α mRNA was present in the surrounding mesenchyme, but declined with development (A, E16; C, E18; E, E20). TR α_1 protein was seen in the collecting tubules at E18 (G), but had disappeared at P4 (J). TR α_2 protein was not detectable at either E18 (H) or P4 (K). At E18, TR β_1 protein was present in the glomeruli and, more abundantly, in the collecting tubules (I). After birth, TR β_1 protein had disappeared from the glomeruli (L). At E16, TR α mRNA was weakly expressed in the entire adrenal (A), but had become largely confined to the capsule at E18 (C). A weak staining for TR β mRNA was seen in the capsule of the adrenals (B and D). Ad, adrenal; K, kidney with U, ureter; C, cortex; G, glomerulus; and CT, collecting tubules. Bar, 100 μ m.

(cf. also (Strait *et al.* 1990, Lane *et al.* 1991, Rodd *et al.* 1992, Schwartz *et al.* 1992, Weiss *et al.* 1998)). In this respect, it is of interest that the protein:mRNA ratios that we derived for brain, liver, and kidney resemble those derived by Oppenheimer and Schwartz (Oppenheimer & Schwartz 1997) with respect to the TR α_1 , but, in contrast to their findings, we concluded that the TR β_1 protein:mRNA ratio is higher in brain than in liver.

The complementary expression pattern of TR α and TR β was most pronounced in developing lung, liver, and kidney, that is, in organs with distinct epithelial and mesenchymal components. Since most epithelia cannot differentiate when separated from their associated mesenchyme (Birchmeier & Birchmeier 1993), it is thought that epithelial differentiation is under control of its underlying mesenchyme. The respective expression patterns were much more pronounced at the mRNA than at the protein level, implying important posttranscriptional control mechanisms. Although both mRNA and protein levels in the organs mentioned reached maximal levels perinatally, it is of interest that for those organs, for which paired samples were available, tissue TR α_2 and TR β_1 protein peaked at 5 days before birth. In contrast, TR α_1 protein follows a similar time course as circulating thyroid hormones

(Dubois & Dussault 1977) to reach its highest level after birth. This latter temporal association is in line with the finding that unliganded TR α_1 is detrimental to postnatal development (Chassande 2003). Our data, therefore, suggest that an initiating, (unliganded) TR β -mediated role of the epithelium induces a TR α -mediated response of the mesenchyme.

On the other hand, extensive co-expression of TR α and TR β was observed in the developing brain and intestinal epithelium. Despite the high level of TR α_1 and particularly TR α_2 mRNA in these organs, TR α_1 protein levels were much lower, and TR α_2 protein levels were similar to those in the other organs investigated. In other words, organs with co-expression of TR α and TR β in the same cells seem to be characterized by a very low TR protein:mRNA ratio in the perinatal period. Thus, the protein:mRNA ratio for TR α_1 was more than tenfold lower and that of TR α_2 15- to 50-fold lower in perinatal brain than in perinatal liver, lung, or kidney. In contrast, the protein:mRNA ratio for TR β_1 was similar in these four organs in this period, but was very low in intestinal epithelium, especially before birth. This implies that in organs with co-expression of TR α and TR β , the translational efficiency of one of the TR mRNAs is much lower, and/or that the turnover of TR protein is much higher than that in cells without

Figure 8 Thyroid hormone receptor expression in the adrenal glands. *TR α_1* protein was present in most parenchymal cells of the adrenal at E18 (A), but had become confined to the adrenal medulla at P4 (D). *TR α_2* protein was confined to islets of cells at E18 (B) and to the adrenal medulla after birth (E). *TR β_1* protein was seen to be present in the adrenal and its capsule at E18 (C), but became concentrated in the adrenal cortex after birth (F). C, cortex and M, medulla of adrenal gland. Bar, 100 μ m.

co-expression. These data indicate that the TR-dependent regulatory cascades function differently in those organs in which the *TR α* and *TR β* genes are characterized by a complementary expression pattern and those in which they are co-expressed. Intriguingly, these posttranscriptional regulatory mechanisms, therefore, largely annul the apparent dominance of *TR α_2* expression in the central nervous system and that of *TR β_1* expression in the intestine.

We observed an apparent discrepancy between the relatively weak staining of *TR α* proteins in histological sections of all organs except the adrenal, and their strong presence, in particularly *TR α_1* , on western blots. Although one explanation would be that the *TR α* s were not well accessible in the tissue sections of most organs, the use of a denaturing rather than a cross-linking fixative and absence of an effect of preprocessing the sections with antigen-retrieval techniques shows that, more likely, they are distributed over many more cells, hence that the cellular concentration remains too low to strongly stain these tissues. This interpretation suggests a more homogeneous distribution of *TR α* s than *TR β_1* in brain, lung, bone, and kidney. In agreement, the *TR α_1* and *TR α_2* proteins stained homogeneously in the anterior pituitary gland (Fig. 4), while the *TR α_1* protein had a much wider pericentral expression pattern in the liver than the

Figure 9 Thyroid hormone receptor expression in perinatal intestine. Note that *TR α* and *TR β* mRNAs are co-expressed in the epithelium of the intestines at E16 (A and B), E18 (C and D), and E20 (E and F). From E18 onwards, both mRNAs became concentrated in the crypts. Also note the expressions of *TR α* and *TR β* mRNA in the pancreatic epithelium (C and D). *TR α_1* protein staining was present in the apical part of the villar epithelium before (G), but not after birth (J). *TR α_2* protein was not detectable (H and K). *TR β_1* protein was seen predominantly in the smooth muscle layer and to a lesser extent in the epithelium of the intestine at E18 (I), and equally at both the locations after birth (L). C, crypt; V, villus; P, pancreas; SM, smooth-muscle layer. Bar, 100 μ m.

TR β_1 protein (Fig. 6; cf. also Zandieh Doulabi *et al.* 2002, 2003).

We detected 8.9 ± 2.4 amol TR β_2 mRNA in adult rat pituitary gland, but were unable to detect this TR β isoform outside the pituitary, implying that TR β_2 mRNA concentrations were more than 1000-fold lower. Nevertheless, we detected abundant staining in many organs with four rabbit antisera that were raised against a TR β_2 -specific oligopeptide (data not shown). In the pituitary, staining was most pronounced in the intermediate lobe of the pituitary, as was reported earlier (Li & Boyages 1997). The putative TR β_2 protein was 47 kDa, i.e., 11 kDa less than predicted by the reported open reading frame (Hodin *et al.* 1989). Although the size of this protein corresponds with the TR β_3 isoform (Williams 2000), the oligopeptide used for immunization is not present in TR β_3 . Possibly, TR β_2 uses a more downstream translation initiation site (cf. (Wood *et al.* 1994)). The pI of the putative TR β_2 protein was 7.6 in liver (corresponding with that of the unmodified TR β_2 protein) and 5.0 in the pituitary gland. Extremely low cellular levels of TR β_2 mRNA in association with easily detectable TR β_2 protein levels outside the pituitary gland were reported before (Lechan *et al.* 1993, Schwartz *et al.* 1994, Ercan-Fang *et al.* 1996, Oppenheimer & Schwartz 1997).

Presently, gene targeting is one of the most powerful approaches to establish causal relations between gene expression and phenotype. The analysis of single and compound mutations of the Thra and Thrb loci has revealed that TR α_1 and TR β_1 cooperate and can partially substitute for each other, and that the consequences of thyroid hormone deficiency differ from those of TR deficiency (O'Shea & Williams 2002). TR β_1 expression appears to be preferentially associated with the development of the cochlea and liver gene expression (Forrest *et al.* 1996, Weiss *et al.* 1998; cf. Figs 1 and 6), whereas TR β_2 appears to be responsible for the development and maintenance of the hypothalamic–pituitary axis (Abel *et al.* 1999). As suggested by the pronounced phenotype of hypothyroid neonates, unoccupied TR β s can interfere with normal development as well (Hashimoto *et al.* 2001). TR α_1 expression appears to be more closely associated with intestinal, cardiac, and bone development (Wikstrom *et al.* 1998, Plateroti *et al.* 1999, 2001). In agreement with our immunohistochemical data (cf. Figs 4 and 6 with Fig. 9), the effects of TR α deficiency become evident at weaning, that is, substantially later than those of TR β deficiency. However, the expression and function of TR α_1 is materially complicated by the other products of the Thra locus, TR α_2 , and the TR $\Delta\alpha$ isoforms (O'Shea & Williams 2002, Flamant & Samarut 2003). Thus, TR α_1 deficiency differs from TR $\alpha_{1/2}$ deficiency in the more severe phenotype of the latter,

with hypothyroidism, runting, and maturational delays in bone and intestinal development that become manifest at weaning (Fraichard *et al.* 1997). In contrast, TR $\alpha^{0/0}$ mice that lack all products from the Thra locus, including TR $\Delta\alpha$, have a milder phenotype than TR $\alpha_{1/2}$ -deficient mice (Macchia *et al.* 2001), due to the absence of the effects of TR $\Delta\alpha$ (Plateroti *et al.* 2001). Irrespective of these complexities, our study has shown that the developmental appearance of the respective TR proteins corresponds nicely with the phenotypes of mutations of the corresponding genes.

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