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α and β thyroid hormone receptor (TR) gene expression during auditory neurogenesis: Evidence for TR isoform-specific transcriptional regulation *in vivo*

(C-*erbA*α/C-*erbA*β/*in situ* hybridization/deafness/inner ear)

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ABSTRACT **Clinicians have long recognized that congenital deficiency of iodine (a component of thyroid hormone) somehow damages the human embryonic nervous system, causing sensori-neural deafness. Recently, a deletion encompassing most of the human β thyroid hormone receptor (TRβ) gene has been found in children who are neurologically normal except for one striking defect: profound sensori-neural deafness. We now show that the TRβ gene is prominently expressed very early in rat inner ear development. This expression is remarkable because both TRβ1 and TRβ2 mRNAs are restricted, as early as embryonic day 12.5, to that portion of the embryonic inner ear that gives rise to the cochlea, the structure responsible for converting sound into neural impulses. The timing of this expression, when correlated with human inner ear development, raises the possibility that TRs may act in human ontogenesis earlier than previously suspected. These results provide a rare correlation between a specific human neurologic deficit (deafness) and transcription factor expression in a highly discrete embryonic cell population (ventral otocyst). TRα gene expression is also prominent in the developing cochlea, but, in contrast to the restricted pattern of TRβ gene expression, TRα1 and TRα2 transcripts are also found in inner ear structures responsible for balance. Deafness in children homozygous for a large deletion in the TRβ gene suggests that cochlear α1 TRs cannot functionally compensate for the absence of TRβ1 and TRβ2. The developing inner ear may, therefore, represent an example of TR isoform-specific transcriptional regulation *in vivo*.**

Differential regulation of gene expression by transcription factors is widely viewed as one of the principal mechanisms guiding brain development (1, 2). Although numerous DNA binding proteins have been identified in neural tissue, the role of individual transcription factors in the differentiation of specific cell groups, like those populating the inner ear, is just beginning to be elucidated (3). Thyroid hormone receptors (TRs) comprise a group of transcription factors that likely plays an important role in the development of the mammalian nervous system (4–9). TR dysfunction has been implicated in profound human neurologic deficits, including irreversible mental retardation, severe movement disorders, and deafness (10). The mechanisms by which TRs contribute to these disorders are unknown.

TRs belong to a superfamily of ligand-responsive transcription factors that includes receptors for retinoids and steroids (11–14). Members of this receptor family induce or repress transcription by binding to cis-acting elements associated with target genes (11, 12). TRs are encoded by two genes, α and β c-*erbA* (15, 16). In rats, these genes give rise to three functional TRs, α1, β1, and β2, and to nonthyroid hormone binding isotypes, referred to here collectively as α2 (13, 14).

Populations with widespread deficiency of iodine, an essential component of thyroid hormone, have long been known to have a greatly increased incidence of congenital sensori-neural deafness (10, 17, 18). Moreover, experimentally induced hypothyroidism in neonatal rodents leads to inner ear malformation and deafness (19, 20). Most striking is the recent observation that children homozygous for a deletion encompassing much of their β TR (TRβ) gene have congenital sensori-neural deafness but are otherwise normal neurologically (21–24). Just where and when TRs may act during auditory ontogenesis is unknown. Hearing deficits may result from distant indirect effects of thyroid hormone or, alternatively, from the direct transcriptional regulation of target genes by TRs within the auditory system. To help distinguish between these possibilities, we examined the expression of TR mRNAs in the developing rat inner ear by *in situ* hybridization histochemistry. Prominent β1 and β2 TR mRNA expression in the developing rat inner ear is restricted to that portion that gives rise to hearing. Our results are consistent with a direct regulatory role for TRβ gene products in mammalian auditory ontogenesis.

MATERIALS AND METHODS

Embryonic staging, tissue preparation, cRNA probe templates, *in situ* hybridization histochemistry, and autoradiography are described in detail elsewhere (25). Briefly, templates for generating antisense cRNA probes were prepared by subcloning nucleotides 1437–1886 of rat c-*erbA*α1 (26), nucleotides 1923–2378 of rat c-*erbA*α2 (27), nucleotides −206–183 of rat c-*erbA*β1 (28), and nucleotides 110–491 of rat c-*erbA*β2 (29) into the *Hin*cII site of pGEM3Z (Promega). Sense control cRNA probes were generated from a template containing nucleotides 81–528 of the rat glucocorticoid receptor cDNA (30). Serial 12-μm-thick frozen tissue sections were collected from fetal and postnatal Sprague–Dawley rat pups. Following fixation, acetylation, and delipidation, sections were incubated with hybridization buffer containing ^{35}S-labeled cRNA probes derived from divergent (nonhomologous) regions of α1, α2, β1, and β2 rat TR and related cDNAs and incubated at 56°C for 24 hr. Following hybridization, nonspecifically hybridized probes were removed by immersing sections in RNase A and washing at a final stringency of 0.1× SSC (1× SSC = 0.15 M NaCl/15 mM sodium citrate) at 65°C. Probe hybridization specificity was established as described (25). Quantification of hybridization

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Abbreviations: E, embryonic; MMI, methimazole; P, postnatal; RT, reverse transcriptase; TRα, α thyroid hormone receptor; TRβ, β thyroid hormone receptor.

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histochemistry was performed by standardized image analysis as described (25). Animal experiments were performed in accordance with National Institutes of Health guidelines for the use of animals in research.

For RNA PCR, total RNA was reverse transcribed using a primer complementary to rat TRβ mRNA. The resulting cDNAs were amplified by PCR, using rat TRβ2-specific primers, and PCR products were analyzed on ethidium bromide-stained agarose gels. See the legend of Fig. 3 for primer sequences.

To induce hypothyroidism in pregnant rats, dams were given 0.025% methimazole (MMI) (Sigma) via drinking water beginning on embryonic day 8.5 (E8.5). Maternal administration of MMI has been shown to induce hypothyroidism in rat fetuses (31). One-half of the hypothyroid dams were made hyperthyroid by infusing 10 μg of L-thyroxine (T_4) (Sigma) per 100 g of body weight per day via subcutaneous Alzet osmotic pumps implanted on E16.5. Osmotic pumps containing vehicle (0.9% NaCl) were implanted in the remaining hypothyroid and control dams. Dams and their pups were sacrificed on E20.5, and dam serum thyroxine levels were measured by RIA (Diagnostic Products, Los Angeles).

RESULTS

Auditory and vestibular components of the mammalian inner ear are derived from paired dorsolateral ectodermal swellings called otic placodes (32). By rat E12.5, the otic placode has invaginated to form a "closed ovoid sac" (33) called the otocyst or otic vesicle (32). Although quite undifferentiated in form, the rodent otic vesicle is comprised of cells whose fates have already been specified. Cells populating the ventral pole of the otic vesicle give rise to the cochlea, the component of the inner ear responsible for hearing (32–34). The remainder of the otic vesicle differentiates into the vestibular apparatus, which mediates the sensation of balance, and the endolymphatic duct (32–34). By *in situ* hybridization histochemistry, TRβ1 and TRβ2 mRNA expression within the rat E12.5 otic vesicle was found to reflect this cochlear/vestibular division of cell fate. Whereas the dorsal otic vesicle has near-

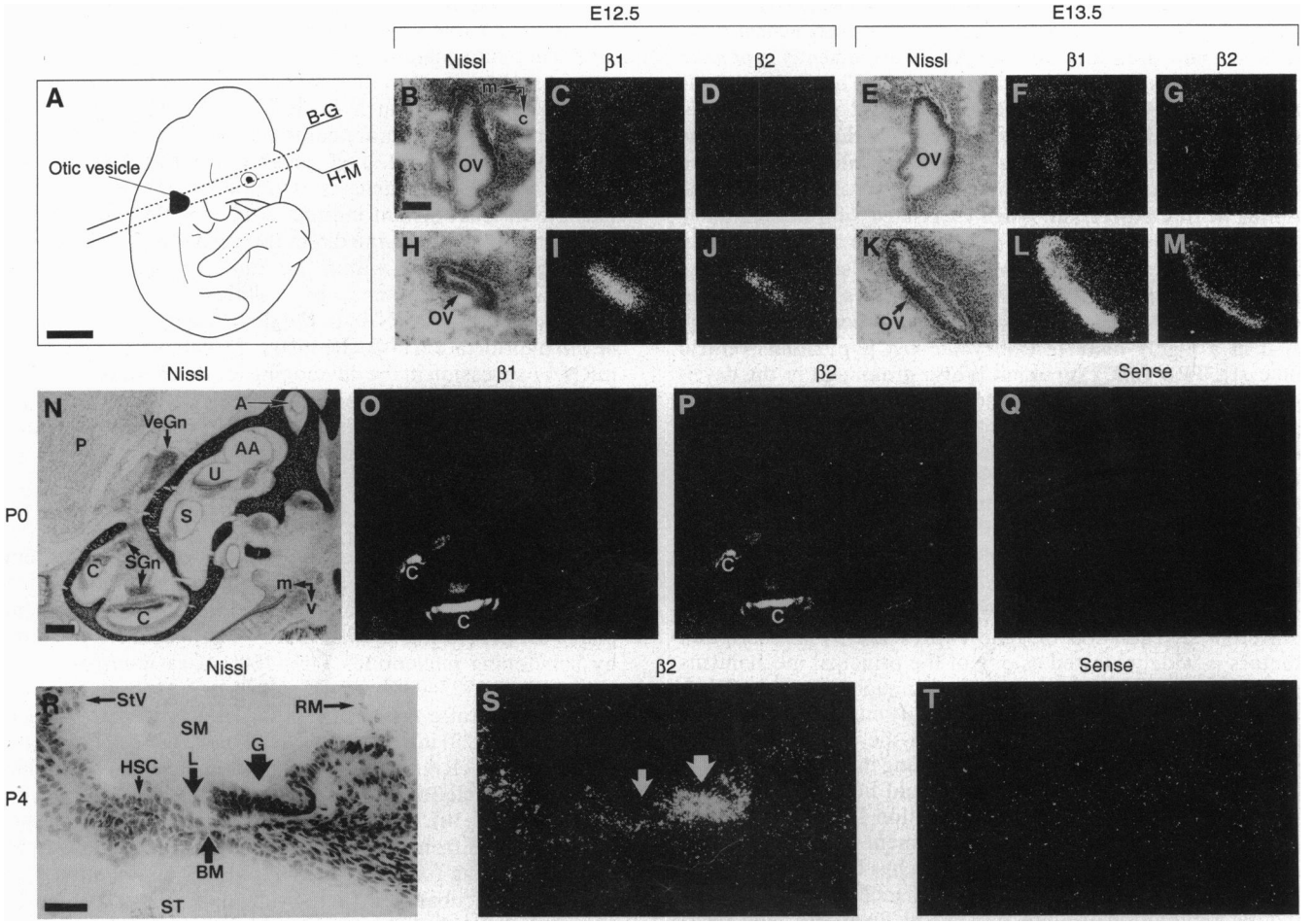

FIG. 1. Prominent TRβ1 and TRβ2 mRNA expression in the developing rat inner ear is restricted to the ventral otic vesicle and its cochlear derivatives. Serial adjacent sections from E12.5 and E13.5 otic vesicle and postnatal day 0 and 4 (P0 and P4) inner ear were incubated with rat TRβ1 and TRβ2 probes and a sense control probe. Following hybridization, sections were coated with nuclear emulsion. Sites of positive hybridization appear white in dark-field photos. (*A*) Schematic illustration of E12.5 rat fetus (after ref. 35). Dashed lines indicate transverse planes of otic vesicle sections shown in *B–G* (dorsal otic vesicle) and *H–M* (ventral otic vesicle). (*B–G*) Bright-field (Nissl) (*B* and *E*) and dark-field (*C* and *D*; *F* and *G*) photomicrographs of E12.5 and E13.5 dorsal otic vesicles. (*H–M*) Bright-field (*H* and *K*) and dark-field (*I* and *J*; *L* and *M*) photomicrographs of E12.5 and E13.5 ventral otic vesicles. (*N–Q*) Bright-field (*N*) and dark-field (*O–Q*) photomicrographs of coronal sections through P0 inner ear. (*R–T*) Bright-field (*R*) and dark-field (*S* and *T*) views of sagittal sections through P4 cochlea showing organ of Corti. Exposure time was 8 weeks. A, anterior semicircular canal; AA, ampulla of anterior semicircular canal; BM, basilar membrane; C, cochlear duct; G, greater epithelial ridge of organ of Corti; HSC, Hensen's supporting cells; L, lesser epithelial ridge of organ of Corti; OV, otic vesicle; P, pons; RM, Reissner's membrane; S, saccule; SGn, spiral (cochlear) ganglion; SM, scala media; ST, scala tympani; StV, stria vascularis; U, utricle; VeGn, vestibular ganglion. Directional arrows: c, caudal; m, medial; v, ventral. (Bars: *A* = 1 mm; *B–M* = 200 μm; *N–Q* = 400 μm; *R–T* = 50 μm.)

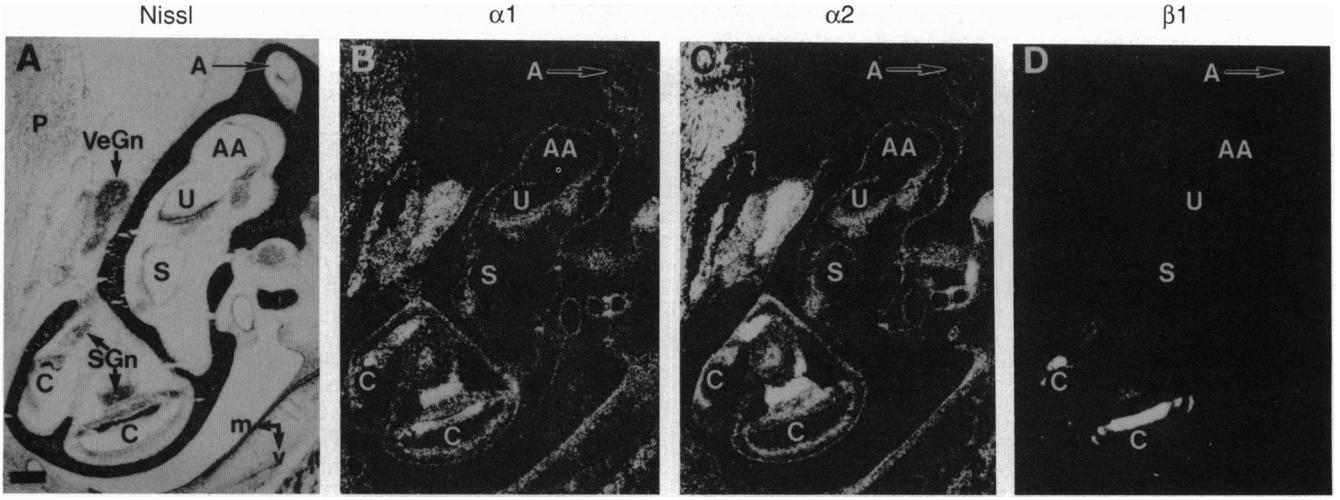

FIG. 2. TRα and TRβ genes are expressed in the developing rat inner ear. Bright-field (Nissl) (*A*) and dark-field (*B–D*) photomicrographs of serial adjacent coronal sections from P0 rat inner ear incubated with rat TRα1, TRα2 (c-*erbA*α2), and TRβ1 probes. Same field as shown in Fig. 1 *N–Q*. Following hybridization, sections were coated with nuclear emulsion. Sites of positive hybridization appear white in dark-field photos. Exposure time was 8 weeks. A, anterior semicircular canal; AA, ampulla of anterior semicircular canal; C, cochlear duct; P, pons; S, saccule; SGn, spiral (cochlear) ganglion; U, utricle; VeGn, vestibular ganglion. Directional arrows: m, medial; v, ventral. (Bar = 400 μm.)

background levels of TRβ1 and TRβ2 mRNAs (Fig. 1 *A–D*), the ventral otic vesicle contains readily detectable levels of TRβ1 and TRβ2 transcripts (Fig. 1 *H–J*). Between E12.5 and E13.5, this highly restricted expression pattern is maintained, and a prominent up-regulation of transcript levels occurs (Fig. 1 *K–M*).

Between E13.5 and the day of birth, the rat otic vesicle rapidly differentiates and forms the basic structure of the mature inner ear (32, 33). Consistent with their expression pattern early in embryogenesis, high levels of TRβ1 and TRβ2 mRNAs are confined to the sensory epithelium of the cochlear duct (Fig. 1 *N–Q*). Lower levels of TRβ1 and TRβ2 transcripts are detected in neurons of the spiral ganglion, which form the first link in the transmission of auditory neural impulses from the cochlea to the auditory cerebral cortex. The remaining vestibular structures of the inner ear, including the sensory epithelium of the saccule, utricle, and semicircular canals, show near-background labeling by TRβ1 and TRβ2 probes. Thus, from very early stages of inner ear development and throughout gestation, prominent TRβ1 and TRβ2 mRNA expression is closely associated with cochlear, but not vestibular, cell lineages.

Sagittal sections taken through the P4 cochlea reveal that TRβ mRNA levels are most prominent within the greater epithelial ridge (Kolliker's organ) of the immature organ of Corti (Fig. 1 *R–T*) (36). The greater epithelial ridge includes primordial inner hair cells, which, along with outer hair cells, will eventually transduce sound into neural impulses. Other cells of the greater epithelial ridge include precursors of supporting cells as well as those cells thought to secrete the tectorial membrane (37). Hypothyroid developing rats have been shown to have structural derangements in their tectorial membranes and cochlear hair cells (19). Other P4 cochlear structures have low TRβ labeling, including the lesser epithelial ridge (includes inner hair cells), Hensen's supporting cells, stria vascularis, and the vestibular membrane. The P4 spiral ganglion shows near-background labeling by TRβ1 and TRβ2 probes. By P11, TRβ mRNA levels have begun to decrease toward background levels. Onset of auditory function occurs during the second week postnatally in the rat (36).

Fig. 2 compares TRα and TRβ transcript distributions in serial adjacent coronal sections taken through the P0 inner ear. Both genes are prominently expressed in the cochlear sensory epithelium. Whereas high levels of TRβ mRNAs are confined to the cochlea, TRα1 and TRα2 transcripts are distributed throughout cochlear and vestibular components of the developing inner ear. TRα1 and TRα2 mRNAs have very similar patterns of expression. Vestibular structures labeled by TRα probes include the saccule, utricle, anterior semicircular canal, and the vestibular ganglion. Widespread expression of the TRα gene in the developing inner ear is

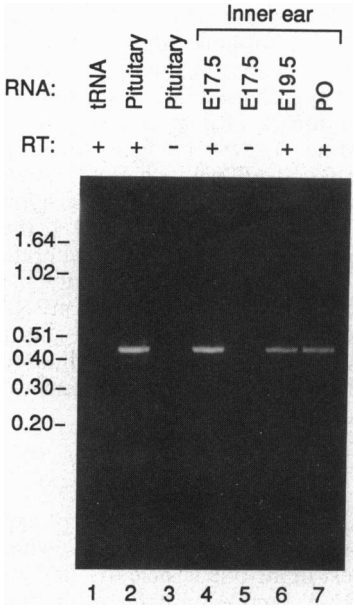

FIG. 3. Detection of rat TRβ2 mRNA sequences in the developing inner ear by PCR. Total RNA isolated from adult rat pituitary and inner ear was reverse transcribed and amplified by nested PCR using rat TRβ2-specific 5′ primers. Primers were complementary to published sequences 353–376 [reverse transcriptase (RT) reaction, PCR] (28) and 303–326 (PCR) (28) and identical to sequences 78–100 (PCR) (29) and 110–133 (PCR) (29). The resulting PCR products were analyzed on an ethidium bromide-stained 2.5% agarose gel. Age and tissue source of RNA are shown in the top row. The presence (+) or absence (−) of RT is indicated in the second row. Yeast tRNA (lane 1) served as a negative control. Positions of DNA size markers (kb) are indicated at the left. In the presence of RT, an expected 447-bp fragment was amplified from pituitary RNA. Bands of identical size were produced from pituitary and inner ear RNA, indicating rat TRβ2 transcripts are comparable in these tissues over the sequence amplified.

Table 1. Resistance of rat fetal cochlear sensory epithelial TR mRNA levels to alterations in maternal thyroid hormone status

Treatment	Maternal plasma T_4, $\mu g/dl$	Rat E20.5 cochlear sensory epithelium TR mRNA			
		$\alpha 1$	$\alpha 2$	$\beta 1$	$\beta 2$
MMI	0.63 ± 0.18	100 ± 16.82	100 ± 22.81	100 ± 10.73	100 ± 13.13
MMI + T_4	11.68 ± 2.91	93.2 ± 16.32	88.2 ± 5.39	96.8 ± 26.14	101.2 ± 18.53

Pregnant dams were made hypothyroid with MMI or hyperthyroid with MMI plus thyroxine (MMI + T_4). Dam plasma thyroxine levels were measured by RIA ($n = 4$) and are expressed in $\mu g/dl$ ± SD. Euthyroid control dams had a plasma thyroxine level of 2.49 ± 0.39 ($n = 4$). Rat TR mRNA levels were measured from film autoradiograms of hybridized coronal sections by standardized image analysis ($n = 6$) (25). Values are expressed as a percentage of the MMI-treated pup values ± SD.

consistent with the extensive distribution of TRα transcripts throughout the developing (25, 38) and adult (39) rat central nervous system.

TRβ2 mRNAs were originally thought to be pituitary-specific in the adult rat (29). Our observations of TRβ2 expression in the rat inner ear were, therefore, unexpected. To confirm these findings, we used a primer complementary to the rat pituitary TRβ2 sequence to reverse transcribe rat pituitary or rat fetal inner ear RNA. The resulting cDNAs were then amplified by nested PCR (Fig. 3). In control reactions using tRNA as a template, or, in the absence of RT, no specific PCR products were generated (Fig. 3, lanes 1, 3, and 5). In the presence of either adult rat pituitary or developing rat inner ear RNA, however, a single band of ≈447 bp was generated (Fig. 3, lanes 2, 4, 6, and 7). This band size matches that predicted from the pituitary TRβ2 sequence (29) and confirms that, over the region amplified, inner ear and pituitary TRβ2 transcripts are comparable.

Previous studies have shown that, in adult rat pituitary, TRβ1 mRNA levels rise and TRβ2 mRNA levels fall in response to thyroid hormone administration (40). We therefore investigated whether deafness and abnormal inner ear development in hypothyroid rats may result, in part, from up- or down-regulation of TRβ gene expression within the developing inner ear. To test this hypothesis we treated pregnant rats from E8.5 to E20.5 with MMI, an antithyroid drug known to cross the placenta and cause hypothyroidism in fetal rats (31). As summarized in Table 1, the TRβ2 developmental program proved resistant to alterations in thyroid hormone levels. Similar insensitivity to hypo- and hyperthyroidism was found for $\alpha 1$, $\alpha 2$, and $\beta 1$ TR mRNAs in the E20.5 cochlear sensory epithelium. These results are consistent with previous demonstrations in the whole developing rat brain that TR mRNA levels are resistant to alterations in thyroid hormone status (41).

DISCUSSION

Our results show that the TRβ gene is expressed in the anlagen of cell groups responsible for transducing sound into neural impulses. In the otic vesicle, we find prominent TRβ1 and TRβ2 mRNA expression only within that region of the otic vesicle that gives rise to the cochlea. Other regions of the otic vesicle that give rise to the vestibular apparatus, but not the cochlea, have near-background labeling. In two human conditions, developmental iodine deficiency and homozygosity for a large deletion in the TRβ gene, deafness occurs, but vestibular dysfunction has generally not been noted (17, 21–24). Combining our results with these clinical observations provides a correlation between transcription factor expression within a discrete embryonic cell population (ventral otocyst) and a specific human neurologic deficit (deafness).

In contrast to the restricted distribution of TRβ1 and TRβ2 transcripts in the developing inner ear, TRα1 and TRα2 mRNAs are widely expressed in cochlear and vestibular anlagen. The evolutionary conservation of two distinct classes of TR genes (14), combined with their differential expression during development (25, 38, 42–44), suggests α and β TRs have different biological roles. Despite this, isoform-specific actions of TRs have not yet been identified *in vivo* (14). Deafness in children homozygous for a large deletion in the TRβ gene (21–24) raises the possibility that cochlear α TRs cannot functionally compensate for the absence of TRβ1 and TRβ2. This possible failure of TRα1 to "cross-regulate" cochlear TRβ target genes suggests that the inner ear may serve as a model of TR isoform-specific transcriptional regulation *in vivo*.

Although the human TRβ gene has been extensively analyzed (16, 45–57), the distal extent of the large TRβ gene deletion noted above has not been mapped (24). Therefore, this deletion may encompass other loci in addition to the TRβ gene. Our results suggest that deafness in patients homozygous for this deletion could arise from the loss of the TRβ gene itself.

Early inner ear development is conserved between humans and rodents and serves as an important feature in defining stages of mammalian embryogenesis (32, 35). The E12.5 rat otic vesicle corresponds approximately to a human otic vesicle in a 4-week-old human embryo (Carnegie stage 13) (32). Prominent TRβ mRNA expression in the E12.5 rat otic vesicle raises the possibility that onset of TR action may occur weeks earlier in human development than the 10–18 weeks of gestation previously suspected (17, 18). We propose that TRβ mRNAs may serve as a very early marker of those cell lineages eventually populating the cochlear sensory epithelium and spiral ganglion.

Our data suggest that there are tissue-specific thyroid hormone response genes in the mammalian inner ear. The eventual identification of these target genes should help pinpoint the precise molecular role of α and β TRs in cochlear neurogenesis.

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