Cloning and characterization of a complementary DNA for a thyroid hormone-responsive protein in mature rat cerebral tissue

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A gene responsive to thyroid hormone (TH) has been identified in the adult rat brain cerebral tissue. A cDNA probe differentially expressed in euthyroid, hypothyroid and hyperthyroid rat cerebral tissue, generated by reverse transcriptase–PCR differential display of mRNA, was used to screen the rat brain cDNA library. A 3.4 kb positive clone hybridized in Northern blots with a 3.8 kb mRNA that proved to be TH responsive (THR). The remaining coding sequence and a part of the 5′ untranslated region of this cDNA were obtained by 5′ rapid amplification of cDNA ends. The deduced amino acid sequence revealed that THR protein (THRP), a 68 kDa moiety, has 83 % sequence similarity with c-Abl interactor protein (Abi-2), which is a substrate for tyrosine kinase activity of c-Abl. The extensive similarity between the two proteins suggests a potential role for THRP as a substrate for c-Abl. Northern analysis showed that the expression of THR mRNA in hyperthyroid rats is 6-fold that in euthyroid rats. There is also a 4–6-fold increase in the concentration of THRP, as analysed by Western analysis. Owing to the extensive similarity between rat THRP and human Abi-2, a polyclonal anti- (human Abi-2) antibody was successfully used for Western analysis of proteins from the rat tissues. The observed increase in both the mRNA and the protein did not decline after β-adrenergic system blockade with propranolol, suggesting that the action of TH on the expression of this gene is not mediated through the β-adrenergic system. Immunohistochemical studies revealed that neuronal cells were particularly rich in THRP. Both THR mRNA and THRP are rapidly induced *in vivo* after intravenous administration of thyroxine. Tissue distribution studies indicated that the cerebral tissue was particularly enriched with THR mRNA and 68 kDa THRP. A cDNA clone for a THR gene could provide a useful tool to study the molecular mechanisms of TH effects on cerebral tissue in adult animals.

INTRODUCTION

Clinical thyroid dysfunction is frequently associated with cognitive and affective disorders [1], yet the molecular basis of thyroid hormone (TH) action in the adult brain remains largely unknown. The nuclear receptor-mediated gene induction is reported to be the major mechanism of TH action in a variety of tissues [2,3]. Moreover several TH-responsive (THR) genes have been reported in the central nervous system during embryonic and neonatal development [4–7]. However, a few THR genes have been identified in the adult rat cerebral cortex [8–11] with modest response to TH. This modest response and the paucity of THR genes in adult cerebral tissue has led to the belief that either the adult brain is poorly responsive to TH after its critical developmental period in early neonatal life, or the effect of TH on adult cerebral cortex is post-transcriptional or initiated at sites other than nuclear receptors. For instance, TH increases β-adrenergic receptor number by 51 % and can enhance adrenergic neurotransmission [12]. The presence of nuclear TH receptors of both alpha and beta subtypes in neurons and glial cells of adult rat brain [13,14] supports a direct role of TH on gene induction. We therefore attempted to identify THR genes in the cerebral cortex of adult rats by reverse transcriptase–PCR differential display of mRNA [15,16].

EXPERIMENTAL

Materials

Propranolol, 3,3′,5-L-tri-iodothyronine (T_3), L-thyroxine, methimazole, polyethylene glycol (25 kDa), calf serum, goat serum and ovalbumin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sodium pentobarbital was purchased from The Butler Company (Columbus, OH, U.S.A.). Sequencing and PCR primers were obtained from Life Technologies (Grand Island, NY, U.S.A.) and the *Taq* polymerase from Perkin Elmer Corp. (Norwalk, CT, U.S.A.). All reagents for polyacrylamide and agarose gels were purchased from Research Organics (Cleveland, OH, U.S.A.). Goat anti-(rabbit IgG) conjugated with FITC was purchased from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Animal groups

Male Fisher 344 rats at 4 months of age were obtained from Harlan Industries (Indianapolis, IN, U.S.A.). The rats were housed under standard conditions of our animal care facility. The rats were maintained on regular rat chow (Teklad, Milwaukee, WI, U.S.A.) and water *ad libitum*. Hyperthyroidism was induced by daily intraperitoneal injection of 15 μg of T_3/100 g of body weight for 10 days. For propranolol treatment, the T_3-treated animals were also injected with 15 mg of propranolol/kg of body weight (subcutaneously, twice a day). Hypothyroidism was induced by 0.025 % methimazole in drinking water for 4 weeks. For the time-course experiments, a group of adult euthyroid rats were injected intravenously with L-thyroxine (200 μg/100 g of body weight). The thyroxine was dissolved in physiological saline containing 1 % (w/v) BSA. Each rat received approx. 200 μl of this solution. Groups of rats ($n = 3$) were killed at various times after the injection. The rats

Abbreviations used: PEST region, sequence enriched in serine/threonine, glutamate/aspartate and proline residues; T_3, 3,3′,5-L-tri-iodothyronine; TH, thyroid hormone; THR, thyroid hormone responsive; THRP, thyroid hormone responsive protein.

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The nucleotide sequence results reported here will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number U94904.

were anaesthetized with sodium pentobarbital (45 mg/kg) intraperitoneally and killed by decapitation. Tissues were harvested, rinsed in cold PBS and immediately frozen in liquid nitrogen.

Thyroxine rather than T_3 was used in the time course experiments because the transport of thyroxine to the brain is more efficient than that of T_3 [17], and up to 80 % of T_3 in the brain arises locally from thyroxine [18].

Cloning of THR cDNA by differential display of mRNA

Total cellular RNA from euthyroid, hyperthyroid and hypothyroid rat cerebra was analysed with reverse transcriptase–PCR-based differential display as described by Liang and Pardee [15] and modified by Liang et al. [16]. One of the cDNA bands that was differentially displayed as being very prominent in hyperthyroid animals was used in Northern blots of RNA from three different treatment groups in various thyroidal states. The Northern blot analysis confirmed that a mRNA species is overexpressed in hyperthyroid rat cerebrum. This particular cDNA clone was used to screen rat brain cDNA library Uni-ZAP XR (Stratagene, La Jolla, CA, U.S.A.). Plaque hybridization was performed by using the protocol supplied by the manufacturer. After secondary and tertiary screening from the positive plaques, pBluescript SK− phagemid was excised by using the supplier's excision protocol *in vivo*. These positive clones were tested by Northern blot analysis to establish that the differences observed in differential display were indicative of true conditions *in vivo*. A 3.4 kb clone tested positive in Northern blots and was labelled as THR cDNA. This clone was sequenced by the dideoxy chain-termination method of Sanger et al. [19] with the phage M13 universal and reverse primers and synthetic oligonucleotide primers, in both directions, with the use of a commercially available sequencing kit (USB, Cleveland, OH, U.S.A.). Nucleotide sequence analysis showed that the clone contains a single long open reading frame, but lacked the initiation codon. The remaining coding sequence, along with part of the 5′ untranslated region, was obtained by 5′ rapid amplification of cDNA ends [20–22] (Gibco BRL, Gaithersburg, MD, U.S.A.).

RNA isolation and Northern blot analysis

Total cellular RNA was extracted by the original procedure of Chomczinsky et al. [23]. RNA (20 μg) was subjected to electrophoresis in 1.0 % (w/v) agarose gels in the presence of 2.2 M formaldehyde [24,25], transferred to nylon membrane by diffusion blotting and hybridized overnight with ^{32}P-labelled cDNA probe [26]. The cDNA probe was a gel-purified (Qiagen, Chatsworth, CA, U.S.A.) 1 kb *Eco*RI fragment along the 5′ end of the 3.4 kb THR cDNA clone. The membrane was washed four times for 5 min each with 0.1 % SDS/2 × SSC. Stringency washes were with 0.1 % SDS/0.1 × SSC at 60 °C for 15 min each. After being rinsed in 0.1 % SDS/2 × SSC the membrane was exposed to X-ray film for autoradiography. The loading efficiency was determined by stripping and reprobing this same membrane with 18 S ribosomal cDNA. The concentration of THR mRNA was determined by densitometry with a personal densitometer from Molecular Dynamics (Sunnyvale, CA, U.S.A.). The summed absorbances of the bands was analysed after subtraction from background. The values are expressed as means ± S.E.M. of the ratio of THR mRNA to 18 S cDNA.

Protein preparation and Western blot analysis

The polyclonal rabbit anti-(human Abi-2) antibody [27], raised against glutathione S-transferase (GST)-conjugated Abi2Δ1–100, was kindly provided by Dr. A. Pandergast (Duke University, Durham, NC, U.S.A.). GST–Abi2Δ1–100 lacks 100 residues from the N-terminal end. Of the remaining 301 residues 295 are 98.5 % identical to 295 residues of THR protein (THRP) from positions 76–369. Owing to this extensive similarity in the two proteins, it was possible to use this polyclonal anti-(human Abi-2) antibody to characterize THRP in rat tissues by Western blotting and immunohistochemistry.

Tissue (1 g) was homogenized in 9 ml of PBS, divided into 1 ml portions and spun at maximum speed in a microcentrifuge. The supernatants were removed and stored at −70 °C. Protein concentration was determined by the procedure of Lowry et al. [28]. Proteins (6 μg) were subjected to PAGE [10 % (w/v) gel] under denaturing (SDS) and reducing conditions by the procedure of Laemmli et al. [29]. Before electrophoresis, tissue extracts were mixed with an equal volume of 2 × SDS sample buffer containing 5 % (v/v) 2-mercaptoethanol and boiled for 5 min. Proteins were transferred to Hybond-ECL (enhanced chemiluminescence) nitrocellulose membrane (Amersham, Arlington Heights, IL, U.S.A.) by the procedure of Towbin et al. [30]. The membrane was incubated with the anti-(human Abi-2) antibody at a final dilution of 1:1000 overnight at 4 °C. Horseradish peroxidase-linked goat anti-(rabbit IgG) was used at a final dilution of 1:10000 for 1 h at room temperature. Blots were developed by the enhanced chemiluminescence Western blotting technique (ECL kit) as described by the manufacturer (Amersham). The tissue content of THRP was determined by densitometry, with a personal densitometer from Molecular Dynamics. The summed absorbances of bands were analysed after subtraction from background.

Immunohistochemical analysis of rat brain

Immediately after removal, brains were embedded in Tissue Tek OCT compound (Baxter, McGraw Park, IL, U.S.A.) and stored at −44 °C until ready for cutting. Sections 6–8 μm thick were cut in a Cryostat at −21 °C, then placed on electrostatically charged slides and stored at −20 °C. Brain sections were thawed at room temperature and fixed in acetone for 5 min. After staining with haematoxylin for 20 s, the sections were rinsed briefly with water and then kept in PBS for 10–15 min. To avoid non-specific staining, the slides were incubated with blocking solution [10 % (v/v) calf serum/10 % (v/v) goat serum/1 % (w/v) polyethylene glycol (25 kDa) in PBS]. The brain sections were treated overnight with a 1:500 dilution of anti-(Abi-2) antibody. After being washed with PBS for 15 min, sections were treated for 30 min with a 1:50 dilution of goat anti-(rabbit IgG) conjugated with FITC. In control sections, anti-(Abi-2) antibody was eliminated from the staining protocol.

Statistical analysis

All results are reported as means ± S.E.M. The statistical analysis was performed by one-way ANOVA. $P < 0.05$ in Fisher protected least significant difference was taken as the limit of statistical significance.

RESULTS AND DISCUSSION

Isolation of cDNA encoding the rat THRP

A differentially expressed cDNA was used as a probe to screen rat brain cDNA library. A 3.4 kb clone was isolated. Nucleotide sequence analysis showed that the clone contains a single long open reading frame but lacked the initiation codon. The remaining 177 nt of the coding sequence along with 63 nt of 5′ untranslated region were obtained by 5′ rapid amplification of

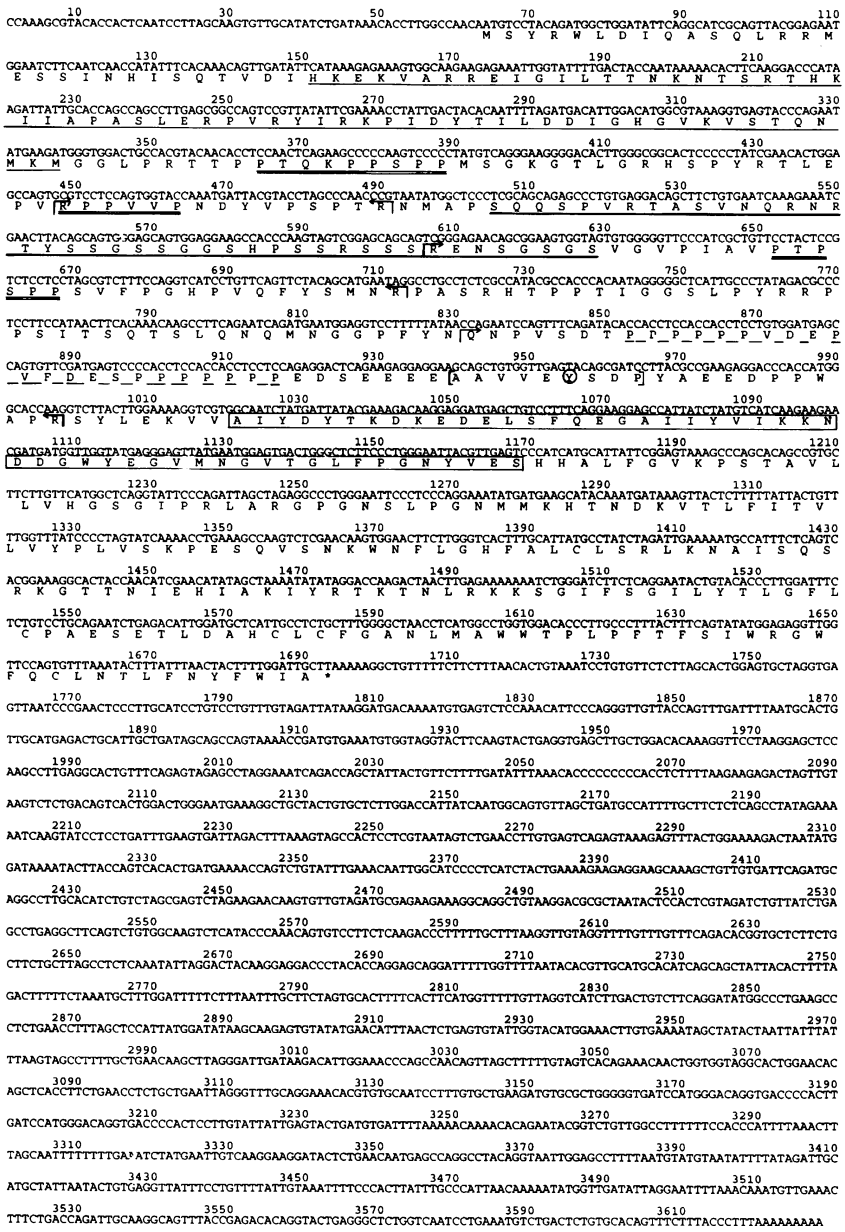

Figure 1 Nucleotide sequence and predicted amino acid sequence of the rat THR cDNA clone

Sequences closer to the C-terminus corresponding to the SH3 domain are boxed. The three potential SH3-binding sites are underlined with a thick line and a serine-rich region is underlined with a solid thin line. A potential tyrosine phosphorylation site is bracketed and the tyrosine residue is encircled. Three PEST regions are bracketed with arrows and a homeodomain homologous region at the N-terminus is underlined. A polyproline stretch is underlined with a broken line.

cDNA ends. The entire clone provides 3628 nt of sequence, including a 63 nt 5′ untranslated region, a 1629 nt coding region, and a 1927 nt 3′ untranslated region (Figure 1).

Deduced amino acid sequence of the rat THRP

THRP has an open reading frame of 543 residues that shows 83 % sequence similarity to Abi-2 (27), which is composed of 401 amino acids. Starting from amino acids 26 and 51 in THRP and Abi-2 respectively, 98.5 % similarity is observed over 344 residues. Before the region of 98.5 % similarity, the N-termini of the two proteins are entirely different. The N-terminus in THRP is 25 residues shorter than in Abi-2. Beyond the 98.5 % similar region, Abi-2 has only six more residues along the C-terminus, whereas the C-terminus of THRP has 176 additional residues that are lacking in Abi-2. On the basis of the structural similarities with Abi-2 and the remarkable conservation of all the domains in THRP required for a c-Abl substrate (Figure 2), it is likely that THRP is also a substrate for c-Abl tyrosine kinase and thus has a role in signal transduction.

THRP shows significant similarity (Table 1) to three other proteins, e.g. Abi-1 [31], Abi-3 (R. Ren, unpublished work) and Xlan4 [32]. These proteins, along with Abi-2, belong to a family of SH3-domain-containing proteins that exhibit similarity to homeodomain-containing proteins. The THRP has a number of interesting features: 49 residues, along the C-terminal region of

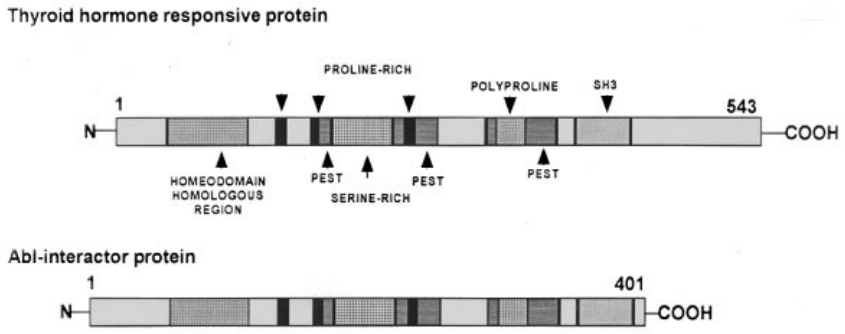

Figure 2 Diagrammatic representation of the structural features of THRP and a comparison with Abi-2 protein

Table 1 Comparison of THRP with SH3-domain-containing proteins that exhibit similarity to homeodomain-containing proteins

The Abi-3 sequence was submitted directly to the sequence database (R. Ren, unpublished work).

	Species	Tissue	Identity with THRP (%)	
			Overall	Specific region
Abi-1	Mouse	Leukaemia cell line	51.7	None
Abi-2	Human	Lymphoma cells	83	98.5*
Abi-3	Human	Brain cells	90.5	None
Xlan4	*Xenopus*	Oocytes	51	None

* Over 344 residues; see the Results section for details.

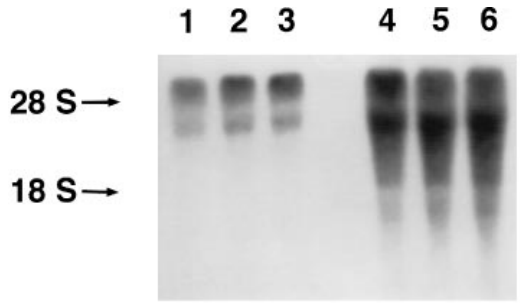

Figure 3 Representative Northern blot analysis of THR mRNA

Total cellular RNA (20 μg) from either euthyroid (lanes 1–3), or hyperthyroid (lanes 4–6) rat cerebra was probed with ^{32}P-labelled cDNA probe (see the Experimental section). The same blot was stripped and reprobed with ^{32}P-labelled 18 S ribosomal cDNA, as a loading control. A 3.8 kb mRNA showed an approx. 6-fold increase in hyperthyroid rats. The 6.5 kb mRNA seen was not THR.

the protein from position 320 to position 369, encode an SH3 domain (Figure 1). This SH3 domain shows 100 % identity with Abi-2 [27]. Several proline-rich stretches are found in THRP (Figure 1) that constitute potential binding sites for SH3 domain-containing proteins and contain the consensus PXXP sequence present in all high-affinity SH3 ligands [33,34]. A polyproline stretch, which could function as a transcriptional activation domain [35], is found upstream of the SH3 domain. In addition, three stretches of sequences enriched in serine/threonine, glutamate/aspartate and proline residues (PEST regions) have been identified in the central and C-terminal portions of THRP (Figure 1). PEST sequences have been implicated in increased susceptibility to protein degradation [36]. The N-terminus (residues 1–272) of THRP is extremely basic with a calculated pI of 11.4, whereas the C-terminal portion (residues 273–543) is more acidic with a pI of 5.9. A serine-rich region is found in the central portion of THRP (Figure 1). The protein contains 11 serine/threonine residues followed by proline, suggesting potential phosphorylation by proline-directed kinases [37]. Three sites conform to a cdc2 kinase consensus sequence Ser/Thr-Pro-X-basic [38]. Similarly to optimal peptide substrates for Abl, Fps and Src protein tyrosine kinases [39], THRP contains several tyrosine residues.

Interestingly, the basic N-terminal region of THRP exhibits 40–50 % similarity over a 53-residue stretch to the DNA-binding region of homeodomain proteins [40–42]. Homeodomain proteins have been implicated in specifying positional information in the embryo during development and in the control of cell lineages by regulating the expression of cell type-specific genes [41]. Noteworthy is the observation that all of the residues in the homeodomain that are implicated in contacting the DNA major groove are conserved in the basic region of THRP. Similarly, all of the residues that contact DNA backbone, with the exception of an invariant tryptophan, are present in this domain of THRP [42].

The SH3 domain, PEST sequences, serine-rich region, proline-rich stretches and phosphorylation sites are remarkably conserved between the rat THRP and human Abi-2 proteins (Figure 2).

Effect of TH on THRP gene expression

To confirm that the results of reverse transcriptase–PCR differential display of mRNA are indicative of a true situation *in vivo*, Northern blot analysis was performed. A 1 kb *Eco*RI fragment along the 5′ end of THR cDNA was used to probe the RNA species from euthyroid, hyperthyroid and hypothyroid rat cerebral tissue. A 3.8 kb mRNA species hybridized with the probe (Figure 3). The expression of this mRNA in T_3-treated rat brains, after correction with 18 S ribosomal RNA (2692.5 ± 185.5 arbitrary units), was almost 6-fold that in untreated controls (450.9 ± 51.7; $P < 0.001$). The change in hypothyroid rats compared with euthyroid rats did not reach statistical significance.

A 6.5 kb mRNA was also identified on Northern blots (Figure 3). This mRNA was not TH-sensitive. The entire THRP cDNA encompasses 3628 nt and therefore the THRP mRNA corre-

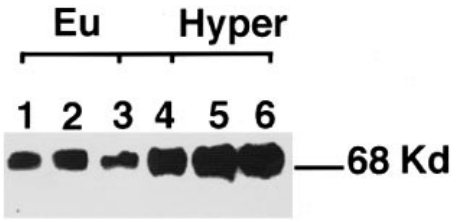

Figure 4 Representative Western blot analysis

Total proteins (6 μg) from either euthyroid (lanes 1–3) or hyperthyroid (lanes 4–6) rat cerebra were probed with polyclonal anti-(Abi-2) antibody (see the Experimental section). A single 68 kDa band is seen in cerebral tissue. The density of this band in T_3-treated rats was at least 4-fold that in euthyroid rats. (See text for details.) Abbreviation: Kd, kDa.

sponds to the 3.8 kb transcript. The 6.5 kb transcript might be the product of a related gene or an alternative splice form of THRP gene. Further studies are needed to address these possibilities. Similarly, the Abi-2 cDNA probe has been previously shown to recognize a 7 kb and a 1.9 kb mRNA in human tissues [27]. The 1.9 kb transcript corresponds to Abi-2, whereas the 7 kb transcript was assumed to be the product of a related gene or alternative splice form [27].

To explore the role of adrenergic transmission in the mechanism of action of the effect of TH on the expression of THR mRNA, rats were injected with T_3 in conjunction with propranolol, a β-adrenergic receptor blocker. There was no difference between the levels of THR mRNA from T_3-treated animals and those in the animals treated with T_3 as well as propranolol. The THR mRNA concentrations in rats treated with T_3 and propranolol concomitantly was 2152.6 ± 195 (arbitrary units). This was 4-fold increase compared with euthyroid control rat (524.9 ± 25.5; $P < 0.01$). Although the dose of propranolol used is shown to be sufficient for reducing the heart rate by 25% [43], the adequacy of adrenergic blockade was not confirmed independently in our rats.

To investigate the effects of TH on the levels of THRP, 6 μg of total proteins from cerebra of either euthyroid or hyperthyroid rats was analysed by Western blotting. A 68 kDa protein, in both euthyroid and hyperthyroid rats, interacted with the antibody (Figure 4). Densitometric analysis revealed a 4.4-fold increase in the levels of THRP in hyperthyroid rats (3620.0 ± 210.5 arbitrary units) compared with euthyroid rats (815.7 ± 153.6; $P < 0.001$).

The elevated levels of THRP observed in hyperthyroid rat brains, compared with euthyroid rats, were not abolished after treatment with propranolol. The mean concentration of THRP (in arbitrary units) found in rats treated with T_3 and propranolol concomitantly was 2829.3 ± 124.4. These levels of THRP were significantly higher than those found in euthyroid rats (1246.0 ± 93.7; $P < 0.001$).

The apparent molecular mass of THRP is close to that (60 kDa) estimated from the deduced amino acid sequence of the protein. In human B lymphoid cell lysates (only human tissue was tested in immunoblots), the anti-(Abi-2) antibody was shown to recognize a major 75 kDa and a minor 55 kDa protein [27]. It remains to be seen whether THRP in the rat and Abi-2 in the human are encoded by two independent genes that have evolved differently from a single parent gene over the course of evolution or are the result of differential splicing of mRNA derived from a single common gene. Chromosomal localization of THR gene and Abi-2 gene will provide further information about the relationship between the two proteins.

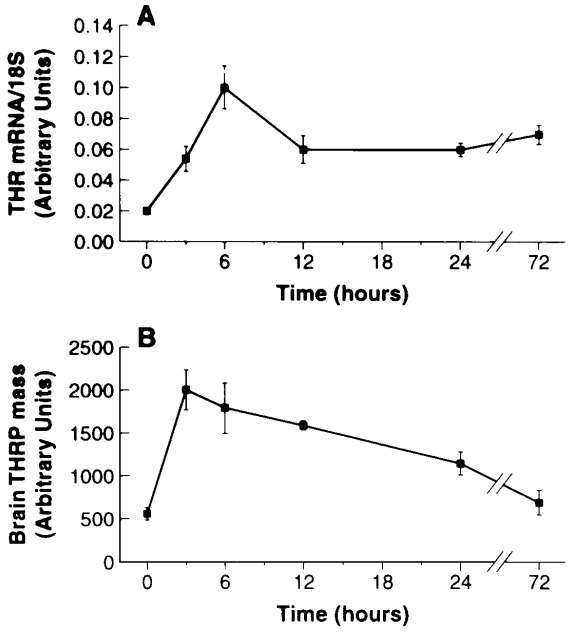

Figure 5 Time course of induction of THR mRNA (A) and THRP (B) by TH

Northern and Western blot analyses were performed on total cellular RNA (20 μg) and total proteins (6 μg) extracted from cerebra of these animals killed at various time points after intravenous injection of thyroxine (200 μg/100 g). The concentrations of THR mRNA (**A**) and THRP (**B**) were determined by densitometry of Northern and Western blots respectively. Three animals were killed for each time point. A significant increase in both THR mRNA and THRP was seen at 3 h.

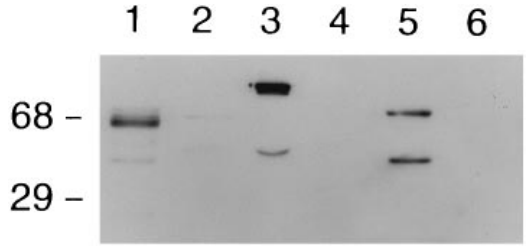

Figure 6 Tissue distribution of THRP in the rat

Western blot analysis. Total proteins (6 μg) from brain (lane 1), testicle (lane 2), liver (lane 3), kidney (lane 4), heart (lane 5) and lung (lane 6) were probed with anti-(Abi-2) antibody. The 68 kDa protein was found only in the brain.

Time course of induction of the gene for THR

Northern blot analysis was performed on total cellular RNA from cerebra of rats killed at various time points after the intravenous injection of 200 μg of thyroxine/100 g body weight (Figure 5A). It is evident that a significant increase in THR mRNA was observed as early as 3 h after thyroxine injection. Significantly increased levels of THR mRNA were observed as late as 72 h (Figure 5A).

Western blot analysis was performed on total proteins from cerebra of animals killed at various time points after the intravenous administration of thyroxine (Figure 5B). A rapid accumulation of THRP was observed within 3 h of thyroxine administration. Thereafter there was a gradual decline towards the baseline. The rise in THRP content after thyroxine treatment

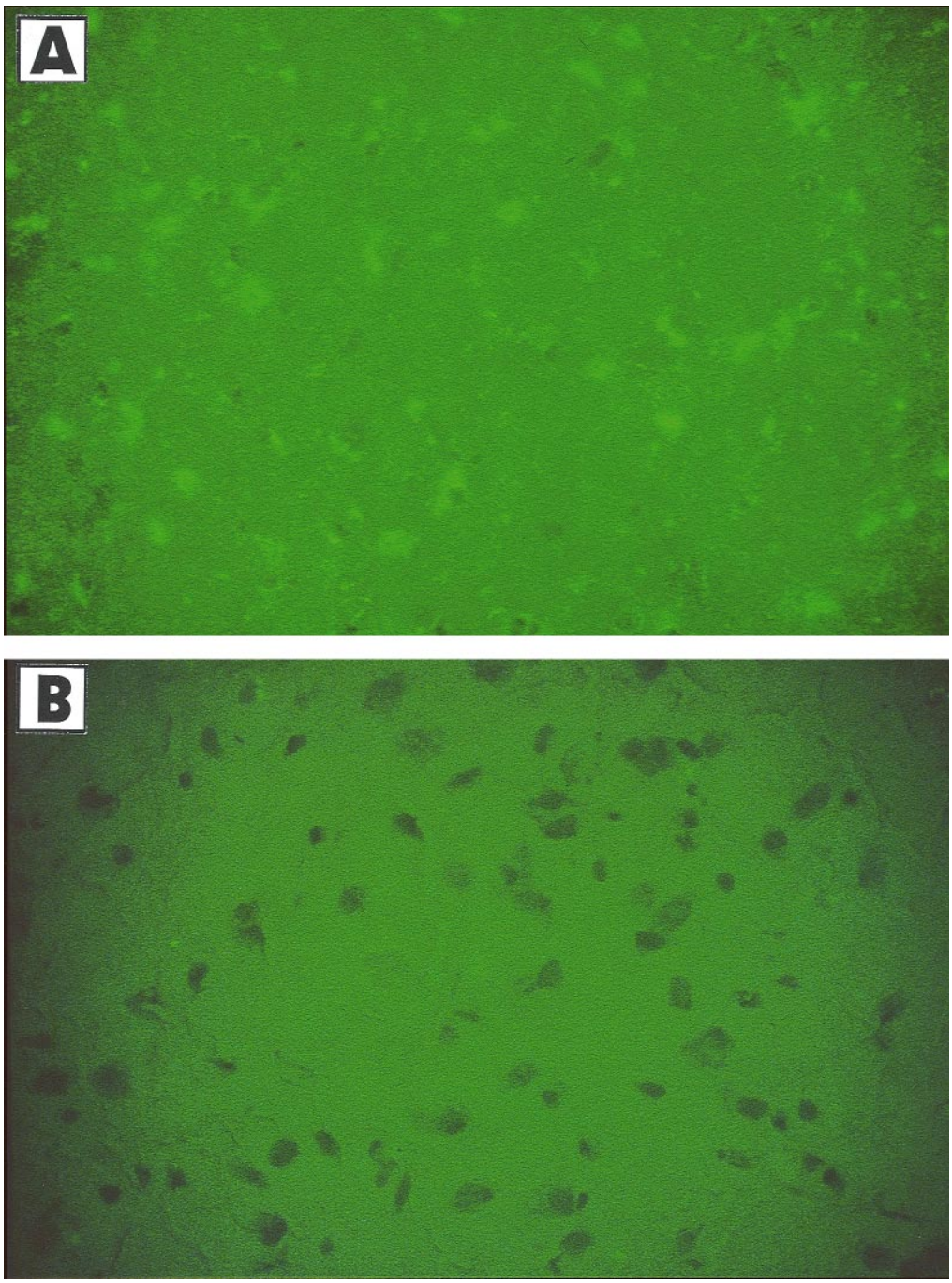

Figure 7 Localization of THRP in the rat brain

Frozen brain sections were analysed by immunohistochemistry. (**A**) Section treated with anti-(Abi-2) antibody. In the control (**B**), anti-(Abi-2) antibody was eliminated from the staining protocol. The positive fluorescence is noted in neuronal cell bodies. Magnification $\times 158$.

peaked earlier than that of THR mRNA (Figure 5). This suggests that the turnover kinetics of THRP is faster than that of THR mRNA.

Distribution of THRP and THR mRNA in rat tissues

To investigate the expression pattern of THR mRNA, Northern blot analysis was performed on total cellular RNA from several rat tissues including brain, testicle, liver, kidney, heart and lung. THR mRNA was found only in the brain.

To investigate the expression pattern of THRP, Western blot analysis was performed on proteins from several rat tissues (Figure 6). A 68 kDa protein, THRP was predominantly found in the brain only. The liver showed 97 and 51 kDa forms; the heart had 70 and 44 kDa forms. Because THRP belongs to a family of proteins, it seems possible that in the liver and heart a

variant form of THRP is expressed. None of these proteins was observed in testicle, kidney or lung.

Immunohistochemical analysis of THRP in the rat

Frozen rat brains were analysed by immunohistochemistry with anti-(Abi-2) antibody to determine the localization of THRP in the rat brain. Figure 7(A) clearly shows that THRP is localized in the neurons. The control (Figure 7B) is a consecutive section of the brain stained without anti-(Abi-2) antibody.

At present, in the absence of a genomic clone and thus promoter sequences of THRP, and lacking demonstration of thyroid response element consensus sequences, it cannot be ascertained whether TH has a direct effect on THRP expression. It is possible that TH responsiveness of this protein is an epiphenomenon to another metabolic change.

The sequence similarity between THRP and Abi-2 and Abi-3 (Table 1), and to much smaller extent with Abi-1, make it difficult to decide which molecular species is effectively studied. The lack of sufficient sequence information for Abi-3 makes it difficult to compare THRP and Abi-3 on the same Northern blots. The difference in mRNA sizes between THRP (3.8 kb) and Abi-2 (1.9 kb) easily distinguishes the two species of the mRNA.

The identification of THRP in cerebral tissue of mature adult rats provides a new tool in the investigation of the effects of TH on the central nervous system. The precise biological role of this protein in the brain is not known at present.

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Received 18 March 1997/25 June 1997; accepted 26 June 1997