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Immunofluorescence localization of thyroid hormone receptor protein β1 and variant α2 in selected tissues: Cerebellar Purkinje cells as a model for β1 receptor-mediated developmental effects of thyroid hormone in brain

(c-erbA/triiodothyronine/immunohistochemistry/ontogeny/neurons)

KEVIN A. STRAIT*†, HAROLD L. SCHWARTZ*†, VIRGINIA S. SEYBOLD†, NICHOLAS C. LING‡, AND JACK H. OPPENHEIMER*†§

*Section of Endocrinology and Metabolism, Department of Medicine, and †Department of Cell Biology and Neuroanatomy, University of Minnesota, Minneapolis, MN 55455; and ‡Whittier Institute, La Jolla, CA 92073

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ABSTRACT **Rat c-erbAβ1 mRNA rises in cerebrum during the first 10 days of life, coincident with an increase in tissue triiodothyronine (T_3) levels and T_3-dependent brain development. These data suggest that the β1 receptor may mediate the T_3 effect. However, in cerebellum c-erbAβ1 mRNA levels were very low. Since cerebellar development, including dendritic arborization of Purkinje cells, is a T_3-sensitive process, we assessed the levels of the β1 receptor protein in cerebellum during development. Antisera to unique peptide regions of β1 were raised. Their specificity was demonstrated by specific immunoprecipitation of the *in vitro* translated product, 85% immunoprecipitation of the T_3 binding activity in hepatic nuclear extracts, and Western blot analysis of tissue extracts. Immunohistochemical studies using anti-β1 antiserum stained liver nuclei but not testis nuclei, which contain no T_3 binding activity or β1 mRNA. In cerebellar Purkinje cells, an immunofluorescent signal, localized to the nucleus and more intense than that seen in the liver, was observed. A positive but weaker signal was also present in the granule cells. Thus, we may infer that the cerebellum contains significant concentrations of β1 receptor protein despite the low β1 mRNA content. Both the intensity of staining in Purkinje cell nuclei and immunoprecipitable β1 receptor binding capacity rose in the neonatal period. Antiserum to the non-T_3 binding α2 variant protein was also prepared and a distinctive pattern of fluorescence was observed. Strong fluorescence was seen in the nuclei of granule cells, but none was seen in Purkinje cells. The α2 fluorescence in testis was high, consistent with the high levels of α2 mRNA in this tissue. The fluorescent signal appeared to originate primarily in dividing spermatogonia. Our findings support the concept that the β1 receptor plays a central role in T_3-induced brain development and strongly suggest that the Purkinje cell is a direct target for T_3.**

Thyroid hormone plays a critical role in the ontogeny of the central nervous system. In the rat, normal brain development requires the presence of thyroid hormone during the period between 10 and 15 days after birth (1, 2). The absence of thyroid hormone during this developmental period results in a marked reduction in the myelination of axons, arborization of dendrites, formation of microtubules, and migration of cells (for a review, see ref. 3). The presence of triiodothyronine (T_3) receptors in brain (4) suggests that the developmental changes represent a direct effect of thyroid hormone on brain cells.

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Recently, a number of cDNAs have been cloned (α1, β1, and β2), which encode thyroid hormone receptors capable of binding T_3 with the affinity and analogue specificity of the nuclear T_3 receptor (5–9). In addition, cDNAs for thyroid hormone receptor variants (α2) that are highly homologous to the other thyroid hormone receptors yet lack the ability to bind T_3 have also been cloned (7–9). Northern blot analysis of total RNA from adult brain revealed the presence of mRNAs for c-erbA α1, -α2, and -β1 (7, 10–12).

Previous studies from our laboratory, in which we measured the level of the two mRNAs encoding T_3 receptors (α1 and β1), indicate that during the first 10 days of neonatal life the level of β1 mRNA in brain increases 40-fold to reach adult levels (10). This marked increase in β1 mRNA occurs during the period of thyroid hormone-sensitive brain development and parallels the increase in serum and brain cytosolic T_3 levels (10, 13). In contrast, the α1 mRNA increased only 3-fold by day 10 but then fell by 50% at 15 days of age. Therefore, we hypothesized that coordinated increases in the expression of the β1 receptor and in tissue T_3 levels are components of a developmental program required for T_3-dependent brain development. Studies by Legrand (1) had also shown that thyroid hormone plays an important role in the morphologic development of the cerebellum by governing the rate of migration of neurons from within the external granular layer and the complexity of dendritic arborization of the Purkinje cells. We were therefore surprised that the level of the β1 mRNA per mg of DNA in cerebellum was only 1/27th that in the remainder of the brain, designated as "cerebrum." Moreover, Bradley *et al.* (14) using *in situ* hybridization methods could not detect β1 mRNA in any area of the cerebellum and found α1 mRNA only in the granular layer. Since the structural changes in cerebellum are characteristic of thyroid hormone-induced changes elsewhere in brain, these observations raised doubts about the hypothesis advanced. Our previous studies, using Northern blot analysis, indicated the presence of low levels of β1 mRNA in cerebellum. The ratio of nuclear T_3 binding capacity to α1 and β1 mRNA content was, however, substantially higher in cerebellum than in cerebrum (10). Therefore to test our hypothesis we wished (*i*) to assess the β1 receptor protein content in cerebellum as a whole and in Purkinje cells in particular and (*ii*) to determine whether the levels of both the β1 mRNA and β1 receptor protein in cerebellum exhibit the same neonatal surge previously demonstrated for the β1 mRNA in cerebrum (10).

To accomplish these objectives we developed and characterized a specific antiserum to the β1 receptor protein,

§To whom reprint requests should be addressed.

assessed its distribution in brain and selected other tissues, and quantitated the contribution of the β1 receptor to specific T_3 binding of nuclear extracts. In the course of these studies, we have also developed a specific antiserum to α2 protein. The α2 mRNA represents an alternative splice product of the c-*erbA*α gene and encodes a protein that does not bind T_3. We have established the presence of this protein in cells from several tissues, determined its cellular and subcellular localization, and compared its distribution with that of the β1 receptor. Development of a specific antibody to α1 protein is difficult because of the identity of α1 and α2 at the amino terminus and α1 and β1 at the carboxyl terminus.

METHODS

Animals. Male Sprague–Dawley rats (60 days old) or late gestational females were purchased from Bio-Lab (White Bear Lake, MN). Animals were killed by decapitation and their brains were rapidly removed, dissected on ice, and then frozen in liquid nitrogen and stored at −80°C until further use.

RNA Isolation and Measurement of c-erbA mRNA Content. Total RNA was extracted from the various brain regions by the method of Chirgwin *et al.* (15) followed by further purification by organic extractions and salt washings (16). The absolute mass of c-erbA mRNA in brain regions from 4-day-old pups and adult rats was measured as described (10).

Peptide Synthesis and Immunization. Computer analysis of the amino acid sequences for the c-erbAs deduced from their respective cDNAs was used to identify peptides that showed an absence of amino acid homology with the other c-erbA-encoded receptors and a secondary structure possessing the hydrophilicity (19) and structural (20) determinants of effective antigens. On the basis of these data we chose two peptides for synthesis: α2 peptide, amino acids 425–454 (SLRGPVLQHQSPKSPQQRLLELLHRSGILH); β1 peptide, amino acids 62–92 (TWASSIFHLDPDDVNDQSVSSAQTFQTEEKK). An amino-terminal tyrosine was added to each peptide to allow for labeling with ^{125}I (21). The chosen peptides were synthesized with an amidate carboxyl terminus by solid-phase methodology (22) on a *p*-methylbenzhydrylamine resin by a previously described procedure (23). The protected peptide resin was treated with hydrogen fluoride and the deprotected peptide product was purified by gel filtration, cation or anion exchange, and partition chromatography as described (23). The synthetic product had the correct amino acid composition based on amino acid analysis. One hundred micrograms of peptide conjugated to keyhole limpet hemocyanin (Sigma) was injected s.c. into New Zealand rabbits every 2 weeks. After 10 weeks, the animals were bled and the sera tested for anti-peptide reactivity.

Evaluation of Antibodies. For initial screening, various dilutions of the antisera were incubated overnight at 4°C with 40,000 cpm of ^{125}I-labeled peptide in 25 mM Tris·HCl, pH 8.0/0.025% bovine serum albumin, incubated for 2 hr with beads coated with anti-rabbit IgG (Kallestad Laboratories, Austin, TX), and washed to determine adherent radioactivity. Sera of 3 of the 10 animals injected with each peptide showed reactivity at 1:8000 dilutions. Positive sera were further tested for specificity by incubation with ^{35}S-labeled *in vitro* translation products of erbA cDNAs as described (24). Preimmune serum, absence of primary antiserum and the use of alternative translation products, and unlabeled peptide were used as controls. Positive sera precipitated only their respective translation products. The IgG fraction of the anti-β1 antiserum was purified by octanoic acid precipitation (25) and resuspended in phosphate-buffered saline (PBS; 20 mM sodium phosphate, pH 7.4/0.15 M NaCl) at a protein concentration of 0.2 μg/μl. Western blot analysis of nuclear extracts of liver was performed as described (26) with 400 μg of protein per lane.

Extraction and Immunoprecipitation of Nuclear T_3 Binding. T_3 binding proteins were extracted from tissues as described (4). Each nuclear extract (0.45 ml) was incubated at 4°C overnight with 50 μl of anti-β1 IgG, the same volume of IgG prepared from preimmune serum or buffer. The immune complex was cleared by incubation with bovine serum albumin-blocked Pansorbin for 20 min on ice, centrifuged for 15 min at 10,000 × *g*, and the supernatant was removed. The binding capacity remaining in the supernatants was determined by saturation analysis (27).

Immunohistochemical Studies. Animals were killed by decapitation, the tissues were rapidly removed, cut into gross sections, embedded in OCT mounting medium, and rapidly frozen in liquid nitrogen. Five-micrometer-thick sections were cut on a cryostat and mounted on glass slides coated with gelatin/chrome alum by warming to 37°C for 1 min. The slides were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, washed with 3× PBS for 5 min followed by two more washes in 1× PBS for 5 min, and dehydrated by a series of ethanol washes (30%, 60%, 80%, 95%, and 100%) for 2 min each and stored at −20°C. Before staining, slides were warmed to room temperature for 10 min and incubated with 40 μl of a 1:50 dilution of the antiserum for 45 min in a humid chamber at room temperature. The slides were washed twice in PBS for 2 min and blotted dry. Forty microliters of a 1:32 dilution of fluorescein-conjugated goat anti-rabbit IgG (Kallestad Laboratories) was pipetted onto the section and left for 45 min. Again, the slides were washed twice in PBS and mounted for microscopy.

RESULTS

Comparison of c-erbA mRNA Content in Regions of the 4-Day Neonatal and Adult Rat Brain. We first analyzed total RNA from 4-day-old neonate and adult rat brain frontal cortex, cortex, cerebellum, brainstem, and diencephalon (Table 1). Four-day-old pups were chosen to facilitate manual dissection of the brain region. The level of β1 mRNA in neonate and adult animals displayed considerably more variation when one brain region was compared to another than did the α1 and α2 mRNAs. The ratio of α1 to α2 in all brain regions examined was remarkably constant, suggesting similar processing of the α gene transcripts in all regions of the brain. In each of the brain regions examined, the level of the β1 mRNA increased ≈4-fold in the transition from the 4-day-old neonate to the adult, whereas levels of α1 and α2 mRNA during this period showed a 50% decrease, consistent with the results observed in whole cerebrum during this same interval (10).

Preparation of Specific c-erbAβ1 and -α2 Antisera. Both the α2 and β1 peptides resulted in detectable levels of antibodies 8 weeks after their initial injection, as determined by the ability to precipitate their respective ^{125}I-labeled peptide. Several rabbits produced antisera capable of precipitating >90% of the appropriate labeled peptide at a 1:8000 dilution, with <1% of irrelevant ^{125}I-labeled peptides precipitated by these antisera. Preimmune sera from the same animals also precipitated <1% of the labeled α2 and β1 peptides. All sera selectively immunoprecipitated ^{35}S-labeled translation products.

Western blots of nuclear extracts from liver showed signals with the β1 antiserum at M_r 49,000 and 52,000, which were absent when incubated with preimmune serum or in the presence of excess peptide (data not shown). The molecular weights of the proteins present in liver nuclear extracts are similar to those reported for the c-erbA translation products (5, 6) and the isolated T_3 receptor (28, 29). No signal was seen with the anti-β1 antiserum in testicular extracts, consistent with the reported absence of β1 mRNA from this tissue (10). With the anti-α2 antiserum, a single band of M_r 61,000, approximating the previously reported molecular weight (7), was observed in testicular extracts. In liver, in addition to the

Table 1. Levels of c-erbA mRNAs in regions of 4-day-old and adult brains

	α1		α2		β1	
	4 day	Adult	4 day	Adult	4 day	Adult
Frontal cortex	9.90 ± 2.8	5.31 ± 1.0	54.38 ± 3.9	32 ± 2.7	2.13 ± 0.37	9.71 ± 3.40
Cortex	6.85 ± 0.28	3.57 ± 1.2	41.57 ± 3.8	18.2 ± 4.7	1.37 ± 0.05	5.32 ± 1.13
Brain stem	3.58 ± 0.75	1.55 ± 0.17	14.9 ± 1.1	9.8 ± 0.43	0.21 ± 0.06	0.84 ± 0.21
Cerebellum	4.89 ± 0.51	2.62 ± 0.21	30.8 ± 2.4	15.1 ± 2.0	0.04 ± 0.01	0.17 ± 0.05
Diencephalon	3.57 ± 0.76	2.73 ± 0.48	20.1 ± 1.9	15.6 ± 1.9	0.42 ± 0.11	1.85 ± 0.21

The contents of the individual c-erbA mRNAs in several regions of euthyroid 4-day-old neonate and adult (2 month) rat brain (fmol per mg of DNA) were determined by Northern blot analysis in conjunction with solution hybridization as described (10). All measurements of mRNA levels were from four animals per group (means ± SD).

M_r 61,000 band, two smaller bands of M_r 24,000 and M_r 20,000 were seen. None of these three bands was observed when preimmune serum was used. These smaller bands may represent proteolytic products of the receptor or variant proteins translated from alternative in-frame AUG start sites.

Immunoprecipitation of T_3 Nuclear Binding Activity in Liver, Cerebrum, and Cerebellum. Incubation with a purified IgG fraction of the anti-β1 antiserum reduced the T_3 binding capacity in nuclear extracts from liver by an average of 85% in three separate experiments (Fig. 1*A*) and from nuclear extracts of cerebrum (Fig. 1*B*) and cerebellum (Fig. 1*C*) by only 30%. IgG from preimmune serum was without effect in any of the extracts. Increasing the amount of IgG added by 4-fold or incubating the supernatant with a second aliquot of IgG did not further reduce T_3 binding capacity in extracts of liver, cerebrum, or cerebellum (data not shown). Although the precise nature of the unprecipitated T_3 binding proteins remains to be defined, the high percentage of immunoprecipitation from liver extracts is consistent with the predominance of the β1 mRNA in this tissue (10).

Cellular Distribution of β1 and α2 Protein in Liver and Testis. In liver, the level of the β1 mRNA is 4 times that of the α1 and α2 mRNA (10). β1 antiserum showed strong nuclear reactivity in liver cells (Fig. 2*A*). This is in accord with the substantial level of β1 mRNA in liver (10) and our ability to precipitate a majority of T_3 binding from nuclear extracts of liver by using the β1 antiserum (Fig. 1*A*). Immunoreactivity was confined to the nucleus, consistent with previous reports of the subcellular localization of T_3 binding activity (30). Anti-α2 antiserum (Fig. 2*B*) produced a fluorescent signal in liver sections that could not be distinguished from that of the preimmune serum despite the weak but positive signal we had observed on Western blots. This may be a function of differential sensitivities and the large amount (400 μg) of protein loaded on the gels. An identical lack of nuclear fluorescence was observed when the β1 antiserum was preincubated with the β1 peptide (data not shown).

Adult testis lacks both α1 and β1 mRNAs and T_3 receptor binding (10). Testis does contain detectable levels of the nonbinding variant α2 mRNA (7, 10). Immunofluorescent studies in testis using the α2 antiserum produce intense nuclear staining (Fig. 2*D*), presumably as a reflection of the high level of α2 mRNA. The fluorescence seen with anti-β1 antiserum in testis (Fig. 2*C*) was extremely low and did not differ from that seen with preimmune serum. Preincubation of the α2 antiserum with α2 peptide blocked the fluorescent staining in testis to the level seen in Fig. 2*C* with anti-β1 antiserum. The finding of α2 immunoreactivity in testis demonstrates that the α2 mRNA is translated into protein. Closer inspection of the testicular cells, which react positively with the α2 antiserum, suggests that the α2 protein is present only in the nuclei of spermatogonia during mitosis. As the spermatogonia mature into spermatids they appear to lose reactivity for the α2 protein.

Cellular Distribution of β1 and α2 Protein in Cerebellum. Immunofluorescent studies of cerebellum also show that the β1 and α2 proteins are confined to the nuclei of cells (Fig. 3). Purkinje cells exhibited the most intense nuclear staining for the β1 protein (Fig. 3 *A* and *B*). No fluorescence was seen in the Purkinje cells when cerebellar sections were exposed to serum that had been preincubated with β1 peptide (Fig. 3*C*). In addition to the Purkinje cells, there was also weak staining of the nuclei of the granule cells by the anti-β1 antiserum (Fig. 3 *A* and *B*), which while less intense than that of the Purkinje cells was clearly stronger than that observed in sections exposed to serum preincubated with peptide (Fig. 3*C*). No

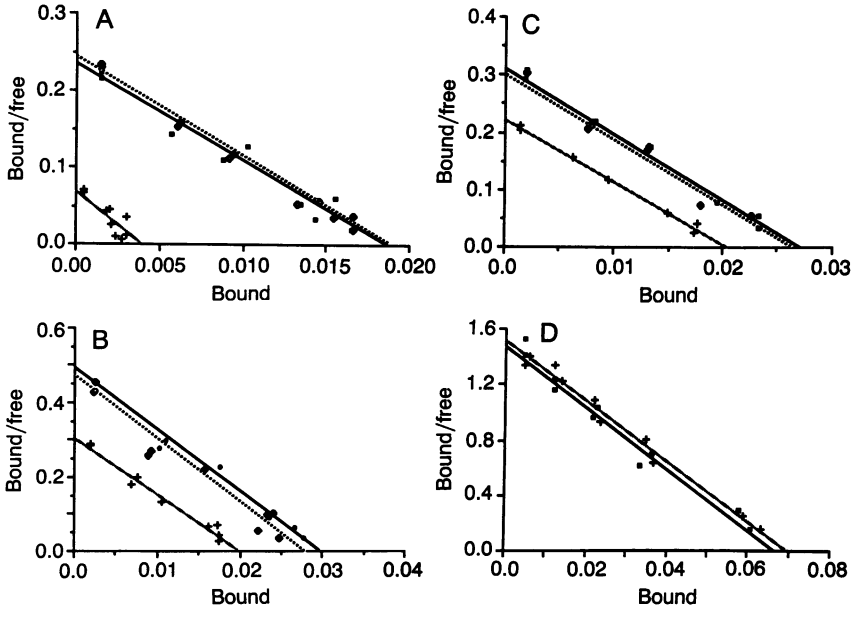

FIG. 1. Immunoprecipitation of T_3 binding proteins from nuclear extracts. T_3 binding proteins were immunoprecipitated from nuclear extracts of adult liver (*A*), adult cerebrum (*B*) and cerebellum (*C*), and whole brain of 19-day fetus (*D*). Nuclear extracts were incubated with the IgG fraction of anti-β1 antiserum (+), preimmune serum (◇), or buffer (■). Immune complexes were removed by a subsequent incubation with Pansorbin. The residual T_3 binding capacity (nM) remaining in the supernatant was assayed by saturation analysis (4).

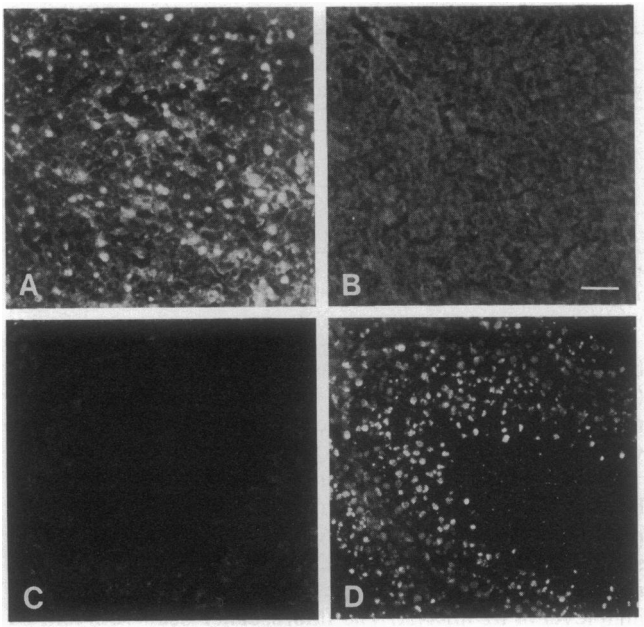

FIG. 2. Photomicrographs of indirect immunofluorescence in sections of adult (60 day) liver and testis. (*Upper*) Five-micrometer-thick sections from liver incubated with anti-β1 antiserum (*A*) or anti-α2 antiserum (*B*). (*Lower*) Five-micrometer-thick sections of adult testis incubated with anti-β1 antiserum (*C*) or anti-α2 antiserum (*D*). Fluorescent staining is primarily located within the nucleus. Preincubation of the antisera with 4 μg of their respective peptides resulted in a lack of staining in liver similar to what is shown for anti-α2 antiserum (*B*), and in the testis similar to anti-β1 antiserum (*C*) (data not shown). (Bar = 100 μm.)

staining was present for β1 protein in the medullary layer, which contains axonal fibers and glial cells. In similar sections of cerebellum, the α2 protein was clearly present in the nuclei of the granule cells but was conspicuously absent from the Purkinje cells (Fig. 3 *D* and *E*). α2 staining was also absent from the medullary layer. Finally, preincubation of the α2 antiserum with α2 peptide dramatically reduced the fluorescent staining (Fig. 3*F*) to levels observed with preimmune serum (data not shown).

Additional studies compared the immunofluorescent intensity of the β1 signal in the cerebellum of 19-day gestational fetus and adults. Despite the quantitative limitations inherent in this technique, visual inspection of the sections clearly showed that the nuclear signal per cell increased in the transition from the neonatal to the adult animal (data not shown). These observations were supported by quantitative immunoprecipitation studies of nuclear extracts from brains of 19-day fetuses using the anti-β1 antiserum (Fig. 1*D*). Unlike the extracts from adult cerebrum and cerebellum (Fig. 1 *B* and *C*) in which the β1 antiserum precipitates 30% of T_3 binding activity, the antiserum had no effect on T_3 binding capacity in the fetal extracts (Fig. 1*D*). These findings indicate that the β1 receptor protein increases with the increase in β1 mRNA during brain development.

Although no systematic effort was undertaken to analyze other brain sections, we noted widespread distribution of nuclear α2 and β1 protein. Of interest was the finding that the nuclei of the large and easily identified motor neuron of the seventh facial nerve in the brainstem showed intense fluorescence with the anti-β1 antiserum but none with the anti-α2 antibody (data not shown). Surrounding cells reacted with the α2 antibody but not with the β1 antibody. Thus, the Purkinje cell is not unique with respect to the distribution of α2 and β1 protein.

DISCUSSION

The specificity of the antisera used in this study is apparent from (*i*) the selective immunoprecipitation of the translational product of the corresponding cDNA; (*ii*) the immunoprecipitation of binding activity from nuclear extracts; (*iii*) demonstration on Western blots of a major band with a molecular weight corresponding to that predicted for the full-length protein; and (*iv*) the nuclear localization of the signal in tissues containing the corresponding mRNAs. However, in view of the fact that additional although less intense bands were seen on Western blots, we cannot exclude the possibility that alternative start sites may have resulted in smaller proteins as suggested by Bigler and Eisenman (31) or that posttranslational modification may have generated alternative forms of the proteins with different molecular weights. The demonstration by Goldberg *et al.* (32) of receptor phosphorylation supports such a possibility. Regardless of these considerations, the immunoreactivity observed reflects the expression of the corresponding mRNA in each tissue.

The data presented support the hypothesis that the c-erbAβ1 protein plays a critical role in thyroid hormone-dependent development of rat brain. Our immunofluorescence studies in cerebellum also show that there are β1

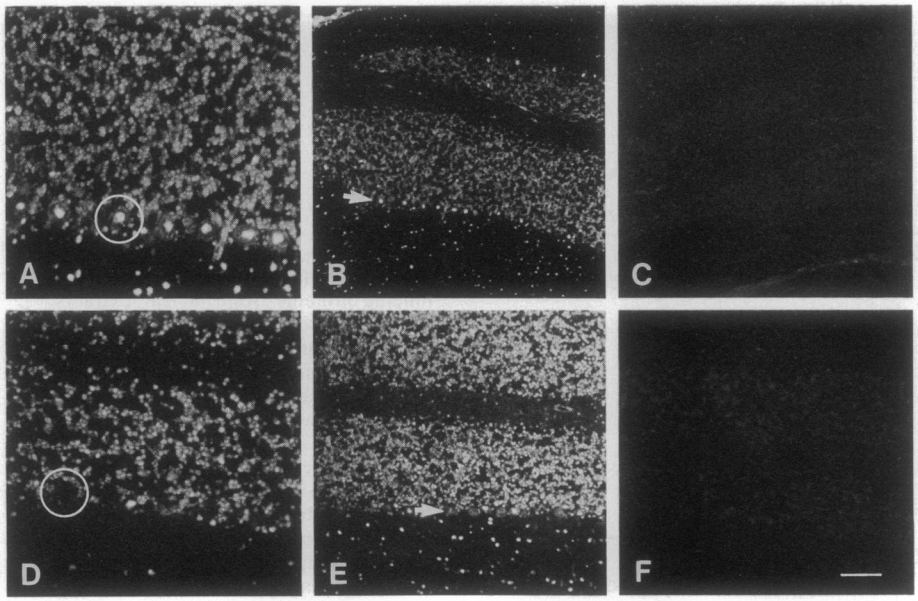

FIG. 3. Photomicrograph of indirect immunofluorescence of anti-β1 and anti-α2 antisera in cerebellum of 60-day-old rats. (*Upper*) Incubations of 5-μm-thick sections of cerebellum with anti-β1 antiserum (*A* and *B*) and an adjacent section exposed to anti-β1 antiserum that had been preincubated with 4 μg of the β1 peptide (*C*). (*Lower*) Cerebellar sections (5 μm thick) incubated with anti-α2 antiserum (*D* and *E*) and an adjacent section incubated with anti-α2 antiserum that had been preincubated with 4 μg of the α2 peptide (*F*). Note the intense staining by the anti-β1 antiserum in the Purkinje cell layer (circles, arrows), which is absent when the anti-α2 antiserum is used. (*B*, *C*, *E*, and *F*, bar = 100 μm; *A* and *D*, bar = 50 μm.)

receptors in the nuclei of the Purkinje cells. During the period of thyroid hormone-dependent brain development the cerebellum shares in the β1 mRNA increase previously noted for cerebrum (10). Furthermore, our immunohistochemical and immunoprecipitation studies show that the β1 protein increases as the level of the β1 mRNA increases. Our findings accord with the recent report of Forrest *et al.* (33), who also demonstrated a sharp increase in c-erbAβ expression in embryonic chicken brain during the critical hormone-dependent period of development. Whereas c-erbAα expression was ubiquitous during chicken development, the timing and tissue specificity of c-erbAβ expression in other tissues, such as lung, correlates with thyroid hormone-dependent developmental changes.

The previous failure of Bradley *et al.* (14) to demonstrate β1 mRNA in cerebellum was probably due to the very low level of the mRNA coupled with the limited sensitivity of *in situ* hybridization. Our previous measurements indicated that the β1 mRNA content per mg of DNA in cerebellum is only 1/27th that in cerebrum (10). The mol of β1 protein per mol of RNA can be calculated from the nuclear T_3 binding capacity previously determined (10), the percentage of total T_3 binding capacity immunoprecipitated with the β1 antibody in the present study (Fig. 1), and levels of β1 mRNA expressed as fmol per mg of DNA (10). These calculations reveal a ratio of 47 for cerebrum and 320 for cerebellum. Thus, there is a nearly 7-fold greater number of β1 receptor molecules for each mRNA molecule in cerebellum as compared to the rest of the brain. Similar calculations for liver result in a β1 protein/RNA ratio of 810. These results emphasize the enormous variation in the mRNA expression as protein in various tissues and brain regions. These considerations resolve the dilemma of an abundance of β1 protein, as determined by immunoprecipitation and immunofluorescence, with extremely low levels of β1 mRNA.

In testis, the complete absence of β1 mRNA and T_3 receptor, as determined by saturation studies, was reflected in the absence of β1 protein (Fig. 2*C*). A recently published immunofluorescent study by Macchia *et al.* (34) shows β1 protein present in the nuclei of cells of the testis; levels of β1 protein are on a par with the liver. This finding contrasts with previous reports that testis contains no β1 mRNA (10, 12, 17) and no detectable T_3 binding activity (10). Previous studies have demonstrated the presence of α2 mRNA in testis (7, 10). The present immunofluorescent studies of testis clearly show intense staining for α2 protein confined to the nucleus of the spermatagonia (Fig. 2*D*). If this protein has any function, it clearly cannot be that of "dominant negative" regulation (18) of the α1 and β1 T_3 receptors, which are not present. Our findings that the α2 receptor variant is found in dividing spermatogonia but not spermatids raises the alternative possibility that α2 protein may play a role in cell division.

Immunohistochemical studies using the specific α2 antiserum, although of intrinsic interest, provide only suggestive information about the level of the α1 receptor in tissues and cells. Data in our previously reported study (10) as well as results reported in Table 1 of this communication clearly indicate that the ratio of the α1 and α2 mRNA in brain remains constant during development and in various topographical regions of brain. This may be a reflection of the fact that the α1 and α2 mRNAs arise from a common gene via alternative processing. In addition, Bradley *et al.* (14) showed that α1 and α2 mRNAs were always found in the same cells of the brain. Therefore, if the α2 receptor variant protein is not present in a cell such as the Purkinje (Fig. 3*D*), it appears reasonable to infer that this same cell does not contain the α1 mRNA or α1 protein. If this can be confirmed by direct protein assays, one would have to conclude that some aspects of the T_3-dependent developmental process do not require the expression of the α gene.

In essence, our studies suggest that the Purkinje cell constitutes a direct target for the developmental actions of thyroid hormone in the brain and may serve as a useful model for further studies of the molecular details of this process.

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