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Expression of erbAα and β mRNAs in regions of adult rat brain

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Summary

The proto-oncogenes erbAα and erbAβ together encode three functional thyroid hormone receptors (erbAα1, β1, and β2), as well as two proteins (erbAα2 and α3) that do not bind T3. The erbAα2 protein has been shown to inhibit the T3 inductive effects of functional receptors, and α2 mRNA is expressed at high levels in adult rat brain. Thus, expression of erbAα2 may explain the observation that adult rat brain is not a T3 responsive organ, despite the presence of T3 receptors. However, expression of the different erbA mRNAs has not been studied within distinct regions of rat brain. To gain further insight into the roles of these molecules, we have used polymerase chain reaction to investigate the expression of all five erbA mRNAs within discrete regions of adult rat brain. The results indicate that all three erbAα mRNAs are expressed in all regions studied (brainstem, cerebellum, cortex, hippocampus, pituitary, quadrigeminal plate, striatum, and thalamus). All regions contained less erbAα3 RNA than either α1 or α2. Expression of α2 exceeded that of α1 in all regions except striatum. ErbAβ1 was expressed in all brain regions, whereas erbAβ2 was confined to the pituitary.

Introduction

Two closely related proto-oncogenes, erbAα and β, encode intranuclear thyroid hormone receptors (Sap et al., 1986; Weinberger et al., 1986). In the rat, alternative splicing of the erbAα gene primary transcript results in the production of at least three mRNAs, designated r-erbAα1, α2 (or vI), and α3 (or vII) (Thompson et al., 1987; Izumo and Mahdavi, 1988; Mitsuhashi et al., 1988a, b).

The respective proteins are identical through their first 370 amino acids, at which point α1 diverges completely from α2 and α3 (Fig. 1). The last 40 amino acids of the α1 protein are replaced by 122 amino acids in α2. The α3 protein is identical to α2 with the exception that it is missing the first 39 amino acids of this unique carboxy terminal extension. The area of divergence involves the ligand binding domain. The r-erbAα1 protein is a functional T3 receptor (Izumo and Mahdavi, 1988). In contrast, the r-erbAα2 and α3 proteins do not bind T3 (Izumo and Mahdavi, 1988; Lazar et al., 1988; Mitsuhashi et al., 1988b). However, α2 (and presumably α3) still binds DNA (Lazar et al., 1988). Moreover, the α2 protein has been shown to inhibit the T3 inductive effects of functional T3

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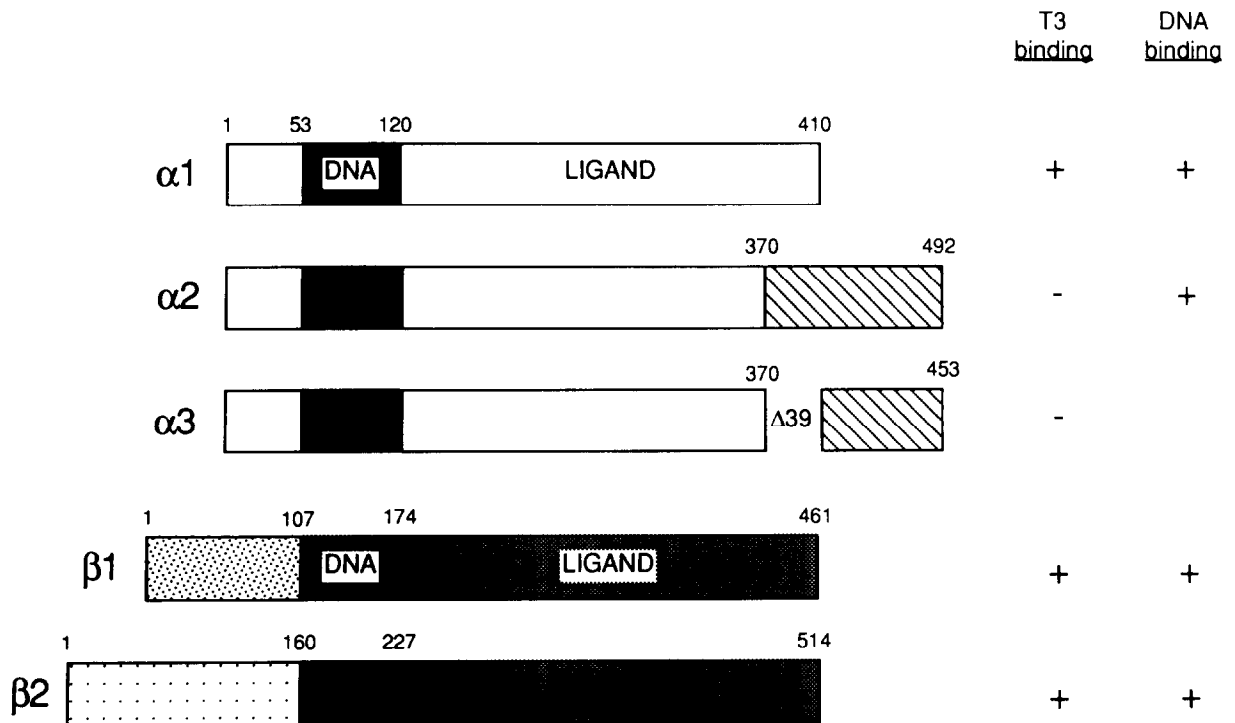
Fig. 1. Schematic comparison of rat erbAα and β deduced protein sequences. ErbAα1, α2, and α3 are identical through their first 370 amino acids, at which point α1 diverges completely from α2 and α3. ErbAα3 differs from α2 only in that it is missing amino acids 371–409 in the α2 sequence. ErbAα1 is a functional T3 receptor, but α2 and α3 do not bind T3. ErbAα2 binds DNA; α3 also presumably binds DNA, although to our knowledge this has not yet been studied. ErbAβ1 and β2 differ in their amino terminal regions, but are identical in their DNA and ligand binding domains. Both are functional T3 receptors. In general, no homology exists between the erbAα and β amino terminal domains. However, the DNA and ligand binding domains of α1 are approximately 85% identical to the analogous regions of β1 and β2.

receptors in a transient transfection system (Koenig et al., 1989). Data obtained from Northern analysis have demonstrated that the tissue distribution of the erbAα mRNAs in the rat is not uniform. For instance, the r-erbAα2/α3 mRNAs are expressed more in adult whole brain than in other organs (Lazar et al., 1988; Mitsuhashi et al., 1988b), while r-erbAα1 mRNA is most abundant in skeletal muscle and brown fat (Lazar et al., 1988).

Two distinct r-erbAβ mRNAs (β1 and β2) are also expressed in the rat (Hodin et al., 1989). Whereas r-erbAβ1 mRNA is widely distributed, r-erbAβ2 mRNA appears by Northern analysis to be present only in the anterior pituitary (Hodin et al., 1989). The resultant β1 and β2 proteins differ in their amino terminal domains, but are identical to one another in their DNA and ligand binding domains (Hodin et al., 1989) (Fig. 1). Both the β1

and β2 proteins are functional T3 receptors (Koenig et al., 1988; Hodin et al., 1989). The physiologic roles of the different α and β erbA proteins have not yet been established.

Adult whole rat brain is not thyroid hormone responsive (Barker and Klitgaard, 1952), despite the presence of thyroid hormone receptors (Oppenheimer and Schwartz, 1974). The reason for this is unknown, although it may be due to the relatively high degree of expression of erbAα2. Previous studies concerning the expression of the different α and β erbA mRNAs in rat brain have been limited to Northern analyses of the whole organ, pituitary, and hypothalamus (Thompson et al., 1987; Lazar et al., 1988; Mitsuhashi et al., 1988b; Hodin et al., 1989). Better understanding of the functions of the various erbA proteins, as well as the T3 unresponsive nature of rat brain, may be gained by evaluating the expression of the

various erbA mRNAs within the different regions of rat brain. We therefore assessed the distribution of the erbAα and β mRNAs in adult rat brain using the technique of polymerase chain reaction (PCR).

Materials and methods

Isolations of RNA

Adult male Sprague-Dawley rats (250 g) were decapitated. Brainstem, cerebellum, cortex, hippocampus, pituitary, quadrigeminal plate, striatum, and thalamus were dissected from the whole brain. Tissues were snap frozen in liquid N_2, and then homogenized in 5 M guanidine isothiocyanate containing 5% β-mercaptoethanol, 50 mM Tris (pH 7.5), and 10 mM EDTA. RNA was prepared by precipitation with LiCl (Cathala et al., 1983). RNA quality was assessed by comparing the relative fluorescence of the 28 S and 18 S RNA bands following electrophoresis through a 1.2% agarose gel containing ethidium bromide. In all cases, the 28 S band exhibited 2–3 times the fluorescence of the 18 S band.

Generation of first strand cDNA

To synthesize first strand cDNA, primers complementary to the erbA coding strands were employed. These were nt 1723–1712 for r-erbAα1 (Thompson et al., 1987), and nt 1573–1562 (with respect to erbAα2) for r-erbAα2 and α3 (Mitsuhashi et al., 1988a). These sequences were chosen because they are similar to each other (α1 5'-GGAGGTTCTGAG; α2/α3 5'-GGTGGTCTTGAG) and also similar in distance from the region to be amplified (α1 91 nt; α2/α3 73 nt). Therefore, it was likely the reverse transcription reactions would be primed with similar efficiency for all three erbAα species. To synthesize r-erbAβ1 and β2 cDNA, a primer was chosen extending from nt 707–696 with respect to β1 (Koenig et al., 1988), which is equivalent to nt 673–662 of β2 (Hodin et al., 1989).

Reaction mixtures contained 150 ng of total RNA, 500 μM of each deoxynucleotide triphosphate, 1 μg of transcription primer, 50 mM Tris (pH 8.3), 50 mM KCl, 8 mM $MgCl_2$, and 100 mM dithiothreitol. Volumes were adjusted to 50 μl with water and 0.5 μl of AMV reverse transcriptase (Pharmacia) was then added. Human placental ribonuclease inhibitor was also added to a final concentration of 0.5 units/μl. Reactions were allowed to proceed for 1 h at 40°C. A mixture containing no RNA was used as a negative control.

PCR

For amplification of the top strand of the three erbAα cDNAs, a common primer 30 nt long having its 5' end at nt 1343 (Thompson et al., 1987) was used. To amplify the bottom strand of the α1 cDNA, a unique 30 nt primer having its 5' end at nt 1622 was used. A common primer 29 nt in size was employed to amplify the bottom strands of α2 and α3 that had its 5' end at nt 1484 of α2 (Mitsuhashi et al., 1988a). The expected sizes of the three PCR products were 280 bp for α1, 504 bp for α2, and 387 bp for α3.

The bottom strands of the erbAβ cDNAs were amplified by using a common primer 30 nt in size with its 5' end at nt 611 of β1 (Koenig et al., 1988). The primer for the top strand of β1 spanned nucleotides 340 to 369, and for β2, positions 210 to 239 (Hodin et al., 1989). Predicted sizes of the β1 and β2 products were 272 and 368 bp, respectively.

For PCR, 10 μl of the above transcription reactions were used. Reactions contained 200 ng of each primer. An additional 5 mM of each deoxynucleotide triphosphate was added. Buffer conditions were adjusted to contain 40 mM of KCl and 0.01% gelatin. Water was added to bring the final reaction volume to 50 μl. Lastly, 0.5 μl T. aquaticus DNA polymerase (Perkin Elmer Cetus) was added.

PCR took place in a Perkin-Elmer thermal cycler. Each cycle consisted of denaturation at 94°C for 1 min 15 s and extension at 72°C for 4 min. A specific annealing step below 72°C was not needed. Amplification was allowed to proceed for 30 cycles. There was a final extension step at 72°C for 10 min. Products were assessed by ethidium bromide staining in 2% agarose gels. PCR of mock reverse transcription reactions failed to yield detectable products.

To quantify the erbAα cDNAs more accurately, PCR also was performed using the common top strand primer end-labeled with [32]P. This

16

primer spans a region that is identical for all erbAα cDNAs. Model PCR reactions first were performed using known amounts of cloned α1 and α2 cDNAs (0.01–100 pg, with 10 ng of plasmid vector DNA as carrier in each case). The PCR products were separated by agarose gel electrophoresis, excised from the gel, and the associated radioactivity was determined. Three such experiments revealed highly reproducible relationships between the amounts of input cDNA and PCR products. For erbAα1, this fit the equation α1 product = 2.85log[input cDNA] + 8.04, $r = 0.95$. For erbAα2 the equation was α2 product = 0.762-log[input cDNA] + 1.99, $r = 0.98$. To further substantiate this quantitation, the reverse transcription reaction was performed with 1, 10, 100, and 1000 ng of input cerebellar RNA. Radiolabeled PCR was performed, and the amounts of labeled α1 and α2 products were found to parallel the above equations. Radiolabeled PCR was then performed for each brain region using 150 ng of RNA. The radioactivity associated with each of the α1 and α2 product bands was determined, and was then converted to gravimetric amounts of input cDNA for each region using the above equations. The amount of input cDNA thus calculated for each brain region fell within the range of the standard curves for both α1 and α2. The radiolabeled PCR experiment was performed 3 times and results were analyzed by one tailed t-test.

Southern transfer

Southern transfer and hybridization were carried out per standard methods with nylon membranes (Ausubel et al., 1988). Probes consisted of synthetic oligonucleotides end-labeled with [γ-³²P]ATP which were complementary to sequences internal to the PCR primers. The erbAα1 probe spanned nt 1452–1438, the erbAα2 specific probe spanned nt 1098–1084, and a probe common to α2 and α3 spanned nt 1268–1254. An erbAβ1 specific probe spanned nt 427–413, and β2 probe nt 289–275.

Digestion of PCR products with mung bean nuclease

40 μl of PCR products were purified on Sephadex G25 spin columns that had been preequilibrated with mung bean nuclease buffer (50 mM NaOAc pH 5.0, 30 mM NaCl, 1 mM ZnSO₄). Each sample was divided into two equal portions, and 1 unit of mung bean nuclease (New England Biolabs.) was added to one of the samples. Both samples were incubated at 30°C for 30 min. Following phenol-chloroform extraction, the products were separated on a 2% agarose gel.

DNA sequencing

Dideoxynucleotide sequencing of double stranded DNA was performed with [α-³⁵S]dATP and Sequenase per the supplier's protocol (U.S. Biochemical) with minor modifications. Sequencing reactions included approximately 0.5 pmol template and 2.5 pmol primer. Template DNA was denatured in the presence of primer by being placed in boiling water for 3 min, then ice for 10 min. Sequencing labeling reactions were for 1 min at room temperature and termination reactions 5 min at 37°C. DNA templates were prepared by cutting and extracting PCR products from 2% low melting point agarose gels. PCR primers were used as sequencing primers.

Results

ErbAα PCR

Total RNA was prepared from various regions (brainstem, cerebellum, cortex, hippocampus, pituitary, quadrigeminal plate, striatum, and thalamus) of adult male rat brains. This RNA was used to prepare first strand cDNA, which was then subjected to PCR to specifically amplify erbAα1, α2, and α3 sequences. Following amplification of the erbAα cDNAs, the products were separated on a 2% agarose gel. Bands of the expected sizes for α1, α2 and α3 were seen in all the brain areas examined (Fig. 2). To further confirm that the desired target cDNAs had been amplified, we performed Southern transfer of the PCR products onto nylon membranes, and probed sequentially with ³²P-labeled synthetic oligonucleotides that were complementary to sequences internal to the PCR primers. In all cases, the anticipated specificity was observed (data not shown).

An unexpected fourth band was noted in the ethidium bromide stained gel migrating below the α2 band (Fig. 2). The following three lines of evidence supported the conclusion that this band
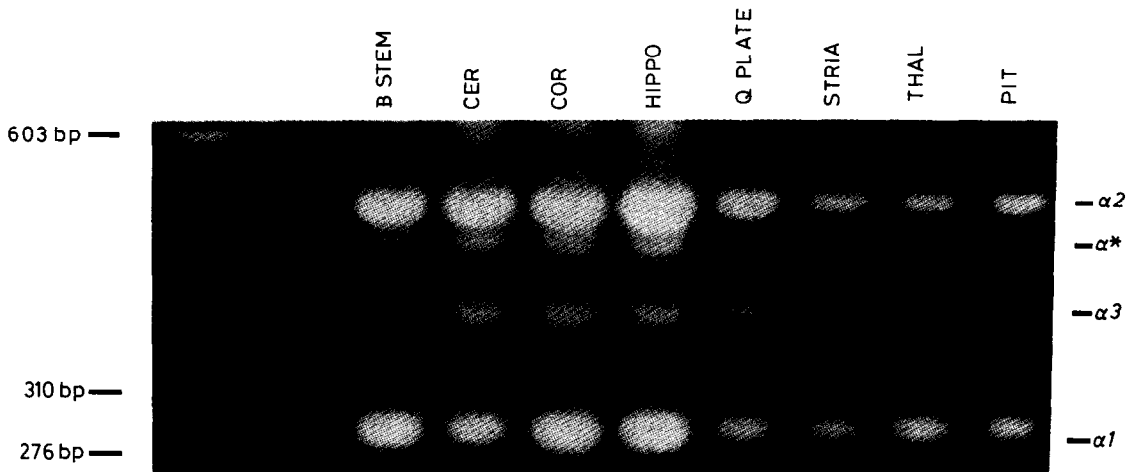
17

Fig. 2. Expression of erbAα mRNAs in brain regions. RNA isolated from various brain regions was used to synthesize first strand cDNA. This material was subjected to PCR using α1 and α2/α3 specific primers designed to generate products of distinct sizes (α1 280 bp, α2 504 bp, α3 387 bp). The PCR products were analyzed by electrophoresis through a 2% agarose gel and visualized using ethidium bromide staining. Note the unexpected band migrating ahead of α2. For explanation, see text and Fig. 3.

(designated α*) was due to the formation of an α2/α3 heteroduplex. First, when α* DNA was extracted from a low melting agarose gel and amplified with PCR with the α2/α3 primers, the α2 and α3 bands, in addition to α*, were regenerated (Fig. 3). Second, when these PCR products were incubated with mung bean nuclease, the α* band disappeared but the α2 and α3 bands were unaltered (Fig. 3). Finally, sequencing the DNA extracted from the α* band demonstrated a mixture of two nucleotide sequences consistent with the cDNA sequences of α2 and α3 (data not shown).

By fluorescence, the amount of total erbAα PCR product was greater in brainstem, cerebellum, cortex, and hippocampus than in the other regions. However, the amounts of the α1, α2, and α3 PCR products relative to one another appeared similar from one region to the next, i.e., within any given area, α3 was the least abundant species, while α1 and α2 were similar to one another. Northern analysis, however, has previously shown that r-erbAα2 mRNA is present in excess over α1 mRNA in adult whole rat brain (Lazar et al., 1988; Mitsuhashi et al., 1988b). To further assess the relative amounts of α1 and α2 in each brain region, we performed PCR using the common top strand primer which had been end-labeled with [32]P. The bands were then cut from a 2% agarose

gel and the associated radioactivity determined. These data then were used as described in Materials and Methods to determine the gravimetric amounts of input erbAα1 and α2 cDNAs for each brain region subjected to PCR. The relative amounts of α1 and α2 input cDNAs are shown in Fig. 4. Although the absolute amounts of each differ from region to region, statistical analysis using the one tailed t-test showed significantly ($p < 0.05$) more α2 than α1 in all areas except striatum.

ErbAβ PCR

First strand cDNAs derived from the different brain region RNAs also were used to specifically amplify erbAβ1 and β2 sequences. Results of the erbAβ PCR are shown in Fig. 5. Bands of the predicted sizes for β1 and β2 were identified. As can be seen, β1 was expressed in all the areas investigated, while β2 was localized to the pituitary. Identities of the erbAβ PCR products were confirmed via Southern analysis using specific oligonucleotide probes complementary to regions internal to the primers (data not shown).

Discussion

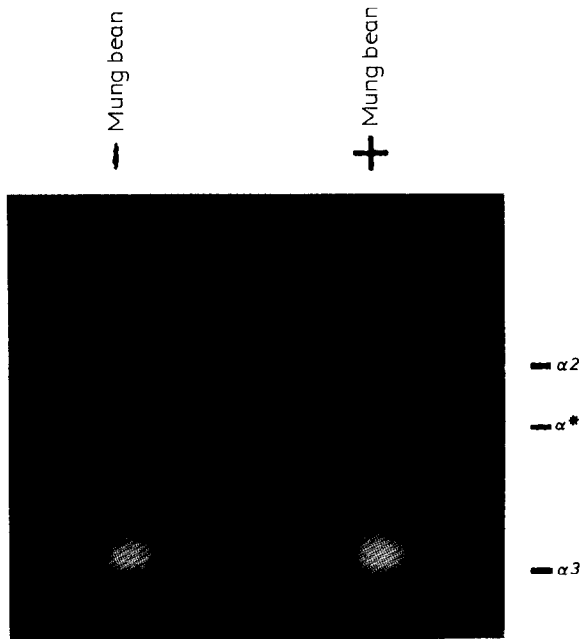The mechanism underlying the T3 unresponsiveness of adult rat brain, despite the presence of

18

Fig. 3. Mung bean nuclease digestion of erbAα*. An unexpected PCR product (α*), migrating between α2 and α3 (see Fig. 2), was identified, purified from a low melting agarose gel, and subjected to further PCR using the α2/α3 specific primers. The products of this amplification were divided into two aliquots, one of which was digested with mung bean nuclease (the other was a control). The aliquots were then subjected to electrophoresis through a 2% agarose gel. PCR of α* cDNA resulted in the generation of α2 and α3 products, as well as α* (left). Mung bean nuclease specifically digested α* (right).

thyroid hormone receptors, is unknown. Previous studies had identified only a single class of T3 receptor in rat brain, which was identical to that found in liver (Oppenheimer and Schwartz, 1978a). The existence in adult rat brain of alternative splice products of the erbAα primary transcript (α2 and α3 mRNAs) which produce proteins that do not bind T3 led us to postulate that they may play a role in the T3 unresponsiveness of that organ. That erbAα2 mRNA is expressed in whole brain in excess of αl (Lazar et al., 1988; Mitsuhashi et al., 1988b), together with the observation that the α2 protein can inhibit the T3 inductive effects of functional thyroid hormone receptors (Koenig et al., 1989), supported this possibility.

However, previous investigations into the expression of erbA mRNAs in rat brain have been confined to Northern analyses of whole brain, hypothalamus, and pituitary (Thompson et al.,

1987; Lazar et al., 1988; Mitsuhashi et al., 1988b; Hodin et al., 1989). In addition, the probes used in these studies have not been able to distinguish expression of erbAα2 from erbAα3. Similarly, studies that have shown adult rat brain to be unresponsive to T3 have focused on the whole organ (Barker and Klitgaard, 1952), or have examined just cerebrum and cerebellum (Schwartz and Oppenheimer, 1978b). It is possible that regional effects of the erbA proteins and/or T3 may have gone undetected. For example, if a brain region expressed erbAαl but not α2 or α3 mRNA, this region might be predicted to be T3 responsive. Similarly, if a region expressed erbAα2 or α3, but not αl and β1, this might suggest a role for α2 or α3 in the central nervous system other than to inhibit thyroid hormone action. Therefore, we wished to determine whether expression of any of the erbA mRNAs was regulated in a regionally specific manner in adult rat brain. PCR seemed an ideal technique to study this question, since it is extremely sensitive, and previous Northern data of whole brain suggested that the erbA mRNAs are expressed at very low levels.

In these studies, we demonstrate that the three erbAα mRNAs are present in all the brain areas examined (brainstem, cerebellum, cortex, hippocampus, pituitary, quadrigeminal plate, striatum, and thalamus). Quantitative rather than qualitative differences are present. In each region, r-erbAα3 mRNA is the least abundant of the three. In addition, r-erbAα2 mRNA is expressed in abundance over αl mRNA in the various brain regions. The only exception is striatum, where the expression of α2 is similar to that of αl. Tools currently do not exist to determine whether expression of the erbA proteins parallels that of the respective mRNAs. Therefore, at present, we cannot find evidence to support the concept of regionally specific expression of any of the erbAα species. Although we have not examined individual nuclei within brain regions, we have no a priori reason to expect major differences in expression at this level. We also have not examined distinct cell types, e.g. neurons and glia, within each region. Previous studies have suggested that [125I]T3 binding activity is confined mostly to neurons (Kolodny et al., 1985), although there is not universal agreement upon this point (Yusta et
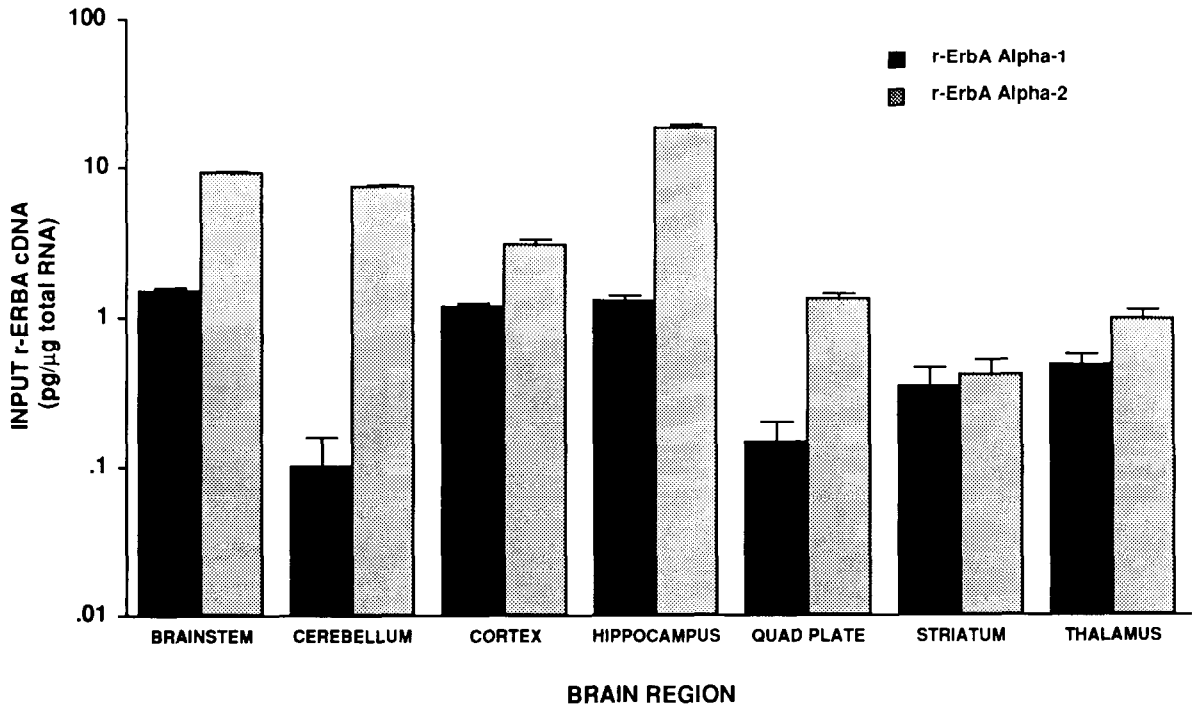
Fig. 4. Quantitation of erbAα1 and α2 mRNA expression in regions of adult rat brain. cDNAs from brain regions were subjected to PCR using a common ^{32}P-labeled primer. The products were separated by gel electrophoresis, and the associated radioactivity was determined. The amounts of starting α1 and α2 cDNAs were then determined by fitting the data to the equations described in Materials and Methods. The data represent the mean ± SEM for three experiments.

al., 1988). Therefore, it would be of interest to study expression of the individual erbAα mRNA species within distinct cell types of the rat brain. PCR may have sufficient sensitivity to make this feasible.

The recent identification of r-erbAβ2 and its reported pituitary specificity (Hodin et al., 1989)

also prompted us to examine the expression of the r-erbAβ mRNAs in adult rat brain. This seemed especially important because the previous study of β2 expression (Hodin et al.) utilized Northern analysis of total RNA, which is a very insensitive technique. Moreover, the only specific brain regions that were examined were pituitary and hy-
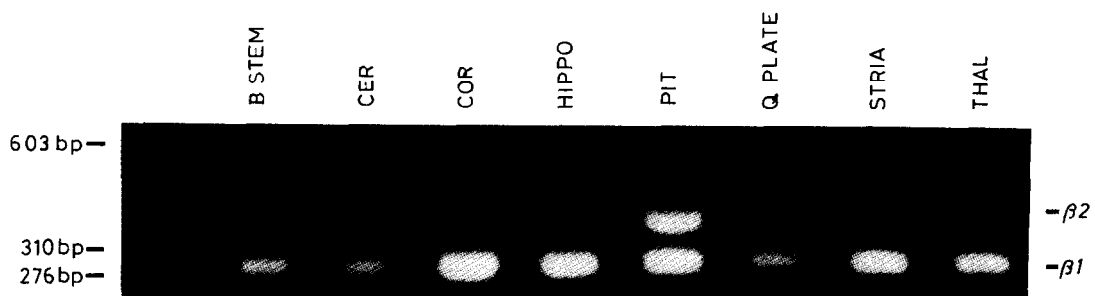

Fig. 5. Expression of erbAβ mRNAs in brain regions. RNA isolated from various brain regions was used to synthesize first strand cDNA. This material was subjected to PCR using β1 and β2 specific primers designed to generate products of distinct sizes (β1 272 bp, β2 368 bp). The PCR products were analyzed by electrophoresis through a 2% agarose gel then visualized by ethidium bromide staining.

pothalamus. Our PCR data indicate that whereas $\beta 1$ mRNA is present in all the areas investigated, expression of erbA$\beta 2$ mRNA is confined exclusively to the pituitary. Considering the sensitivity of PCR, it would seem reasonable to conclude that $\beta 2$ indeed is not expressed in any brain region except the pituitary. As such, it remains the only erbA mRNA species that is expressed absolutely in a regionally specific manner. While these data lend further support to the idea that $\beta 2$ plays some unique role in pituitary physiology, the exact function of this T3 receptor remains to be elucidated.

Acknowledgements

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