The expression of the β-amylloid precursor protein (APP), which plays a key role in the development of Alzheimer’s disease, is regulated by a variety of cellular mediators in a cell-dependent manner. In the present study, we present evidence that thyroid hormones negatively regulate the expression of the APP gene in neuroblastoma cells. Transient transfection studies using plasmids that contain progressive deletions of the 5′ region of the gene demonstrate that triiodothyronine (T3), the more active form of the thyroid hormones, represses APP promoter activity by a mechanism that requires binding of the nuclear T3 receptor (TR) to a specific sequence located in the first exon. The unliganded receptor increases promoter activity, and T3 reverses that activity to basal levels. The repressive effect of T3 does not exhibit TR isoform specificity, and it is equally mediated by TRα and TRβ. Gel mobility shift assays using in vitro synthesized nuclear receptors and nuclear extracts led to the identification of a negative thyroid hormone response element, at nucleotide position +80/−86, that preferentially binds heterodimers of TR with the retinoid X receptor. Insertion of sequences containing this element confers negative regulation by T3 to a heterologous TK promoter, thus indicating the functionality of the element.

The β-amylloid protein, the major component of the Alzheimer-associated plaques, is derived from a set of alternatively spliced β-amylloid precursor proteins (APP),1 which are encoded by a single gene located on human chromosome 21 (for a review see Ref. 1). Although at physiological levels APP appears to be involved in neurotrophic events (2), its overexpression might cause neuronal degeneration by a mechanism that probably involves an increased production of β-amylloid protein (3) and neurotoxicity (4). APP is ubiquitously expressed in mammalian tissues, and its expression can be regulated by a variety of stimuli, including nerve growth factor (5, 6), phorbol esters (7, 8), or retinoic acid (9), a ligand of the nuclear superfamily of steroid/thyroid hormone receptors.

An apparent relationship between thyroid status and Alzheimer’s disease has been suggested. Thyroid hormones, in particular T3, are essential for normal brain maturation and function (10), and their deficiency causes neurologic symptoms that in a way resemble those observed in Alzheimer’s patients. Moreover, although a strong link between thyroid hormones and Alzheimer has not been yet established, it has been suggested that a history of thyroid dysfunction may represent a risk factor for this pathology (11, 12). In addition, data from our laboratory (13) indicate that T3 affects splicing and secretion of APP isoforms in neuroblastoma cells.

Most of the effects of the thyroid hormone are mediated by binding and activation of nuclear thyroid hormone receptors (TRs). TR functions as a ligand-inducible transcription factor to increase or decrease the transcription of target genes by binding to specific DNA sequences called thyroid hormone response elements (TREs), which consist of hexameric half-sites of the consensus sequence AGGTCA arranged as palindromes or direct repeats (for a review, see Ref. 14). Unliganded TRs mediate transcriptional repression of most positive TREs due to binding of nuclear corepressors (15). Ligand binding induces a conformational change that causes the release of corepressors and the recruitment of coactivators, which bind to the AF-2 C-terminal domain (16), and allows transactivation.

The mechanisms involved in T3-dependent transcriptional repression remain less well defined. Negative response elements (nTREs) are located close to, and often downstream from, the transcriptional start site (17–20). The nTREs are frequently composed of more than one TR-binding site, each of them containing sequences that partially resemble the AGGTCA sequence described for positive TREs (17, 20). TRs can bind to these sequences as monomers, homodimers, or heterodimers with RXR (17, 20, 21). However, up to now, a consensus sequence for nTREs has not been yet established, and the precise role of RXR on the T3-induced repression remains unclear. In addition, TRs can also negatively affect the expression of certain genes, without requiring binding to DNA, by interfering with AP-1-induced transcriptional activation (22).

In this report, we present evidence that T3 reduces APP transcripts in N2a-β neuroblastoma cells. Transient transfection studies with different fragments of the 5′ region of the APP gene, together with gel mobility shift assays, show that the negative effect of T3 requires binding of TR to sequences located within the first exon of the APP gene, between positions +80 and +96. These sequences preferentially bind TR/RXR heterodimers, whereas monomers or homodimers are either not bound or bound with a very low affinity. Insertion of the +55/+102 region of the APP gene, which contains the +80/+96 element, confers negative regulation by T3 to a heterologous promoter. Taken together, our results reveal the existence of a

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The abbreviations used are: APP, β-amylloid precursor protein; T3, triiodothyronine; TR, T3 receptor; TRE, thyroid hormone response element; nTRE, negative TRE; RXR, retinoid X receptor; CAT, chloramphenicol acetyltransferase; bp, base pair(s); TK, thymidine kinase.
T3 Negatively Regulates APP Gene Expression

**Experimental Procedures**

**Cell Culture**—Murine N2a neuroblastoma cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum as described previously by Ortiz-Caro et al. (23). Previous to the experiments, the culture medium was replaced with a similar medium containing serum depleted of thyroid hormone by treatment with resin AG1X8 as described by Samuels et al. (24), and the cells were then incubated in this medium for an additional 24-h period before the beginning of the experiments. N2a-β cells, a subclone that constitutively expresses the β-isofrom of TR (TRβ), were grown as described previously by Lebel et al. (25), and the experiments were carried out in the same medium containing 0.5% thyroid hormone-depleted fetal calf serum.

**RNA Extraction and Hybridization**—Total RNA was extracted from the cell cultures by the guanidine thiocyanate method (26). The RNA (30 μg) was run in 1% formaldehyde-agarose gels and transferred to nylon-nitrocellulose membranes (Nytran) for Northern blot analysis. The RNA was stained with 0.02% methylene blue. The blots were hybridized, as described by Church and Gilbert (27), with a plasmid containing a human APP cDNA labeled by random oligonucleotide priming. Hybridizations were at 65 °C in PSE buffer (0.3 M sodium phosphate, pH 7.2, 7% SDS, 1 mM EDTA). Quantification of mRNA levels was carried out by densitometric scan of the autoradiograms. The values obtained were corrected by the amount of RNA applied in each lane, which was determined by densitometry of the stained membranes.

**Reporter Plasmids and Expression Vectors**—The chloramphenicol acetyl transferase (CAT) reporter plasmid containing the −1099 to +105 fragment of the human APP gene has been previously described (28). Progressive 5′ deletions to −487, −369, −155, and −75 bp were prepared by polymerase chain reaction and subcloned into the BamHI site of pB-LCAT5, a plasmid that lacks the AP-1 binding site present in the pUC backbone. The CD2as encoding TRα, RXRα, and v-ErbA as well as the cDNAs encoding TR mutants E401Q, E401K, and C-1 are inserted into the EcoI site of the expression vector pSG5, which contains the SV40 early promoter (29). The reporter construct TRE␮/TK-CAT consists of a single copy of the +55/+102 fragment of APP inserted in front of a TK promoter driving the expression of the CAT gene.

**DNA Transfection**—N2a cells were transfected by the calcium phosphate precipitation method with 1 μg of reporter plasmids and carrier DNA. One hundred nanograms of a luciferase reference vector was simultaneously used as an internal control of the transfection efficiency. In cotransfection experiments, 1 μg of reporter plasmid and 1 μg of the corresponding expression vector were used. After 16 h of incubation in the presence of calcium phosphate, a new medium containing 0.5% thyroid hormone-depleted serum was added, and the cells were then incubated for an additional period of 48 h in the presence or absence of 5 nM T3. Each treatment was performed in duplicate cultures that normally showed less than 5–15% variation in CAT activity, which was determined by incubation of 1C-labeled chloramphenicol with cell lysate. Each experiment was repeated at least two or three times with similar relative differences in regulated expression.

**Mobility Shift Assays**—Synthetic oligonucleotides containing the TRE-binding sequences of the human APP promoter, were end-labeled with [32P]ATP using T4-polymerase kinase and then incubated with in vitro translated receptors or with nuclear extracts obtained from N2a-β cells. cDNAs for TR and RXR in pSG5 were transcribed and translated in vitro with the TNT kit (Promega) following the manufacturer’s recommendations. The nuclear extracts were obtained by the method of Andrews and Faller (30). For gel retardation assays, translated receptors (2 μl) or nuclear extracts (5 μg) were incubated on ice for 15 min in a buffer (20 mM Tris HCl, pH 7.5, 75 mM KCl, 1 mM dithiothreitol, 5 μg/ml bovine serum albumin, 13% glycerol) containing 3 μg of poly(dI-dC) and then were incubated for 15–20 min at room temperature with approximately 70,000 cpm of the double-stranded labeled oligonucleotide. Unprogrammed reticulocyte lysate was used as a control for nonspecific binding. For competition experiments, increasing concentrations of unlabeled double-stranded oligonucleotide or an oligonucleotide containing the consensus sequence TRECAT (5′-AGGTCCAGGTACCT-3′), were added to the binding reaction mixture. For gel supershift, the reaction mixtures were incubated with 1 μl of specific anti-TR or anti-RXR antibodies for 30 min at 4 °C. DNA-protein complexes were resolved on 7% nondenaturing polyacrylamide gels containing 0.5% TBE buffer. The gels were dried and autoradiographed at −70 °C.

**RESULTS**

**Negative Regulation of the APP mRNA Levels by Thyroid Hormone in N2a-β Cells**—APP mRNA levels were determined in N2a-β cells, which express high levels of TRβ (25), after treatment with 5 nM T3. Fig. 1 illustrates the results obtained in a representative Northern blot carried out with 30 μg of total RNA. A single band of 3.4 kilobases, which corresponds to APP mRNA was detected. Densitometric scanning of the bands showed that T3 decreased APP mRNA levels in a time-dependent manner. The negative effect of T3 was already detected at 24 h and became more evident after 48 h of treatment. No effects of T3 on APP mRNA levels were observed in parental N2a cells, which express very low levels of TR (23) (data not shown).

**TR-mediated Repression of APP Promoter Activity**—Transient transfection assays were carried out to determine whether or not T3 affects the transcriptional activity of the APP gene in neuroblastoma cells. N2a-β cells were transiently transfected with a chimeric plasmid containing the −1099 to +105 bp fragment of the human APP gene linked to the CAT reporter gene and then incubated for 48 h in the presence or absence of 5 nM T3. As shown in Fig. 2 (left panel), CAT activity was significantly inhibited in N2a-β cells incubated in the presence of T3, whereas this hormone did not affect the activity of the APP gene promoter in the parental N2a cell line.

To confirm the role of TR in this negative response, the unliganded parental N2a cells were co-transfected with the APP promoter-CAT construct and a vector expressing the α-isofrom of the thyroid hormone receptor (TRα). As shown in Fig. 2 (right panel), the unliganded TR increased basal promoter activity, and T3 effectively reversed this effect. The negative effect of T3 in N2a cells transiently transfected with TRα was very similar to that observed in N2a-β cells, thus showing that...
Fig. 2. Negative regulation of APP promoter activity by T3 in N2a cells. Left panel, CAT activity determined in N2a-β cells (which constitutively overexpresses TRβ) and in parental N2a cells. The cells were transfected with a CAT reporter plasmid containing the full-length (−1099 to +105) APP promoter, and CAT activity was measured after a 48-h period of incubation in the absence or presence of 5 nM T3. Right panel, CAT activity in N2a cells cotransfected with the reporter plasmid along with an expression vector for TRα or with an empty noncoding vector (pSG5-0). Data are expressed relative to the corresponding control values (100%) and are the mean ± S.D. of CAT activity determined in three independent transfections.

Fig. 3. Identification of DNA regions mediating the negative response of T3 on the APP promoter. N2a-β cells were transiently transfected with pBL-CAT plasmids containing progressive deletions of the APP promoter. A schematic representation showing the size of the APP constructs as well as the position of the two AP-1 sites is shown to the left of the graph. CAT activity was determined after 48 h of incubation in the presence or absence of T3. Data are expressed relative to CAT activity obtained with the construct containing the −1099 to +105 bp fragment and are the mean ± S.D. of CAT activities obtained from two separate experiments performed with duplicates.

Fig. 4. The AF-2 domain of TR is required for the negative response to T3. CAT activity of the reporter gene containing the −1099 to +105 bp region of the APP promoter was determined in parental N2a cells transfected with expression vectors encoding the wild type or mutant TRs after 48 h of incubation in the absence or presence of 5 nM T3. Data are expressed relative to the activity obtained in cells transfected with the empty noncoding pSG5 vector.

The inhibitory effect of T3 does not exhibit TR isoform specificity.

Identification of DNA Regions Mediating the Negative Regulation of APP Transcriptional Activity—To map the DNA sequences of the 5′-flanking region of the human APP gene involved in the T3-induced response, progressively deleted fragments (−1099, −487, −307, and −15) of the promoter were linked to the upstream region of the reporter CAT gene and transfected into N2a-β cells. As shown in Fig. 3, the negative effect of T3 was maintained even in cells transfected with the shortest (−15/+102 bp) fragment, which lacks the AP-1 sites. Similar results were obtained in N2a cells transfected with TRαs (data not shown). These results strongly suggest the existence of a TR-binding site in the −15 to +102 bp region of the gene.

The Repressive Effect of T3 Requires the AF-2 Domain of the Thyroid Hormone Receptor—Ligand-dependent transactivation function of the TR is associated with an autonomous and highly conserved C-terminal region of the receptor referred to as AF-2 (29). To determine whether this transcriptional domain could also play a role in the ligand-dependent repression of the APP promoter, we examined the response to T3 in N2a cells transfected with v-ErbA, a natural AF-2 mutant of TRα that fails to bind ligand, as well as with several TRα mutants affecting the AF-2 domain. The C-1 mutant carries a 9-amino acid C-terminal deletion like that found in v-ErbA, and the E401Q and E401K mutants contain a point mutation of the Glu (E) residue at position 401. As illustrated in Fig. 4, the wild-type receptor and the AF-2 mutants increased with a similar potency the activity of the −1099/+105 APP promoter construct in the absence of ligand. T3 effectively reversed the promoter activation induced by the wild-type TR, but it was unable to reduce significantly the constitutive induction caused by the AF-2 mutant receptors. The C-1 and E401K mutants show a strongly reduced ligand binding affinity (29), which, as in the case of v-ErbA, could explain the lack of response to T3. However, the effect of T3 was also abolished in the E401Q mutant, in which ligand binding affinity remains unaltered (29).

Identification of TR-binding Elements in the −15 to +102 region of the APP Gene—For a more detailed study of the DNA sequences involved in this T3-induced response, we further analyzed the −15/+102 bp region of the gene. As depicted in Fig. 5 (upper panel), a computer-assisted study of the nucleotide sequence of this region revealed the existence of three potential thyroid hormone response elements located at the nucleotide positions −4 to +9 (E1), +20 to +30 (E2), and +80 to +96 (E3). The first motif overlaps the major transcriptional start point, and the other two, E2 and E3, are located within the first exon of the gene.

To determine whether the potential response elements of the APP gene are able to bind TR, we conducted gel mobility shift assays with in vitro translated receptors and oligonucleotides containing the E1, E2, and E3 sequences. As illustrated in Fig. 5, no specific retarded bands were detected when TR or RXR were used separately, and only the oligonucleotide (+73/+101) containing the E3 motif was able to specifically bind TR/RXR heterodimers. No detectable complexes were established between the heterodimer and the probes containing the E1 (−10/−15) or E2 (+13/+37) sequences.

To further analyze the E3 domain, the only motif that effectively bound TR/RXR, we performed new gel mobility shift assays, using both in vitro translated receptors and nuclear extracts obtained from control and T3-treated N2a-β cells. Fig.
6A shows binding of the in vitro translated protein preparations. A specific band running in a position that is compatible with a mobility complex containing the heterodimer TR/RXR was detected. In agreement with the results observed in Fig. 5, bands corresponding to monomers or homodimers of TR or RXR were not detected. TR/RXR binding to the oligonucleotide containing the E3 motif appeared not to be significantly affected by T3, which only induced a slight increase in the mobility of the retarded band. The retarded band was competed by the unlabeled E3-containing oligonucleotide as well as by the thyroid hormone consensus response element TREpal. No competition was observed when an unrelated oligonucleotide was used.

**Functional Analysis of the E3 Element**—The nucleotide sequence of the E3 element contains three motifs 180/185, 185/190, and 191/196, each resembling the consensus core sequence AGGTCA established for the positive TREs. To confirm the functionality of this potential nTRE, we studied whether this element is able to confer T3 responsiveness to a heterologous nonresponsive promoter. For this purpose, N2a cells were transiently cotransfected with a TK-CAT construct or the TREAPP/TK-CAT (a chimeric plasmid containing the +55 to +102 bp fragment of the human APP gene linked to the TK-
T3 Negatively Regulates APP Gene Expression

In this report, we present evidence that T3 negatively regulates APP gene expression in a rat neuroblastoma cell line. The repressive effect of T3 was observed in N2a-β cells, a subclone that constitutively expresses high levels of TRβ, but not in the parental cell line, which contains low levels of thyroid hormone receptors. This suggests that the negative effect of T3 on the APP gene will occur specifically in cells and brain regions expressing high TR levels.

Transient transfection studies demonstrated that T3 represses APP promoter activity in N2a-β cells. Several mechanisms, involving either binding to specific nTREs or interference with other positive transcription factors, in particular the AP-1 complex (22), have been described as mediating negative regulation of gene expression by T3. The APP promoter has the typical structure of a housekeeping gene, lacking the TATA and CAAT elements but containing two AP-1 sites, which are located upstream of the transcription start point, at nucleotide positions −15 and −30. The putative nTRE of the APP gene contains at least two hemisites, each resembling the consensus TR binding motifs. A central core of nucleotides might be interpreted as a third hemisite or alternatively as a spacer. It is well known that orientation and spacing as well as the primary nucleotide sequence of core DNA-binding motifs strongly contribute to dictate the selective positive or negative transcriptional actions induced by the ligand-dependent family of transcription factors (35). The APP element binds heterodimers of TR with RXR but does not bind TR monomers or homodimers. This result is different from that obtained with other negative responses to T3, where the monomeric TR forms appear to play an essential role (17, 18, 20). The preferential binding of TR/RXR heterodimers firmly supports a structure containing two hemisites spaced by five or, more likely, four base pairs. In both cases the downstream hemisite (−91/−96 bp) is identical to the sequence found by Desvergne et al. in the rat malic enzyme TRE (36). The first hemisite, in turn, can be defined either at nucleotides positions +80/+85 (5-bp spacer model) or +81/+86 (4-bp spacer), both resembling the consensus sequences described in the rat growth hormone TRE by Brent et al. (37) (−AGGT/C/AAA−) and Norman et al. (38) (−GGG/T/A/C/G/C−), respectively.

Binding of unliganded receptors to nTREs can lead to a constitutive activation of gene transcription (39–41). In N2a cells, unliganded TRα stimulates APP promoter activity, and as occurs with a number of negatively regulated genes (17, 20, 41), T3 reverses the activation induced by the transfected unliganded receptor. The molecular mechanisms responsible for constitutive activation are still unknown. However, it has been recently reported that unliganded TR might stimulate the basal activity of negatively regulated promoter in a manner that requires the association of corepressors (42). The addition of T3 might reverse basal activity, perhaps by dissociation of those corepressors. Experiments with AF-2-defective mutants show that this receptor region is required for the T3-dependent repression of the APP promoter in N2a cells. A point mutation, E401Q, which affects T3-dependent activation without altering T3 binding (29), severely impaired the repressive activity of T3 on the APP promoter. These data indicate that the AF-2 function is required not only for ligand-dependent stimulatory responses mediated by positive response elements but also for the repressive effect of T3 on the APP promoter. Since the AF-2 region appears to be mainly involved in the binding of coactivators (43), our results strongly suggest that these proteins might play a role in the regulation of the APP gene by T3. In contrast, TR mutants lacking the AF-2 function activated transcription in a ligand-independent manner as efficiently as wild-type TR, indicating that the C-terminal region of TR is dispensable for T3-independent transactivation of the APP promoter and that different domains are required for the ligand-dependent and ligand-independent actions of TR on this promoter.
Finally, our data show that the TR/RXR-binding element of the APP promoter was able to confer negative regulation by T3 to the heterologous thymidine kinase promoter. Furthermore, this element also conferred stimulation by the unliganded receptor. These results confirm the functionality of the binding element and strongly suggest that this nTRE alone is sufficient to mediate both ligand-dependent and ligand-independent actions of the thyroid hormone receptor on the APP gene.

In summary, this report describes a negative regulation of APP gene expression by the thyroid hormones in neuroblastoma cells. This effect requires a nTRE, which binds TR/RXR heterodimers, located in the first exon downstream of the main transcriptional start site and is equally mediated by T3α and T3β. In addition to the repressive effect of the thyroid hormone on APP mRNA levels and APP promoter activity, we have previously demonstrated that thyroid hormones specifically alter the pattern of intracellular and secreted APP isoforms (13). Our results together with the reduction of thyroid hormone receptor levels observed in Alzheimer hippocampal cells (44) and the effects exerted by estradiol or retinoic acid on APP expression and metabolism (9, 45) strongly suggest that members of the nuclear superfamily of receptors and their ligands might play an important role in Alzheimer’s disease.

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