Thyroid hormone negatively regulates the transcriptional activity of the APP gene.

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RUNNING TITLE:
T3 negatively regulates APP gene expression

SUMMARY
The expression of the β-amyloid 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 which 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, lead to the identification of a negative thyroid hormone response element (nTRE), at the nucleotides position +80/+96, that preferentially binds heterodimers of TR with the retinoid X receptor (RXR). Insertion of sequences containing this element confers negative regulation by T3 to a heterologous TK promoter, thus indicating the functionality of the element.

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The β-amyloid protein, the major component of the Alzheimer-associated plaques is derived from a set of alternatively spliced β-amyloid precursor proteins (APP) 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 likely involves an increased production of β-
amyloid protein (3) and neurotoxicity (4). APP is ubiquitously expressed in
mammalian tissues and its expression can be regulated by a variety of stimuli,
including NGF (5-7), βFGF (8), interleukin-1 (9), phorbol esters (10,11) or retinoic
acid (12), 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 (13), and its deficiency causes neurologic
symptoms, that in a way resemble those observed in Alzheimer´s patients.
Moreover, and 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 (14,15). In addition,
data from our laboratory (16), 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) (for a review see 17). In T3-activated genes TREs are normally
located in the 5'-flanking region and consist of hexameric half-sites of the
consensus sequence AGGTCA arranged as direct repeats spaced by four base
pairs (18), palindromes separated by 0-1 base pairs (19) or inverted palindromes
spaced by six base pairs (20). TR can potentially interact with these elements as
monomers, homodimers, and preferentially as heterodimers with the retinoid X
receptor (RXR), another member of the nuclear receptor superfamily.
Heterodimerization increases the binding affinity to certain TREs and the ligand-
mediated transactivation of positively regulated genes (21,22). Unliganded TRs
mediate transcriptional repression of most positive TREs due to binding of
nuclear corepressors (23). 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 (24), and allows transactivation.

The mechanisms involved in T3-dependent transcriptional repression
remain less well defined. Negative response elements (nTREs) are located
close, and often downstream, to the transcriptional start site (25-28). In addition,
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 (25,28). Moreover, TRs can bind to these sequences as monomers, homodimers or heterodimers with RXR (25,28,29). 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.

TRs can also negatively affect the expression of certain genes, without requiring binding to DNA, by interfering with AP-1-induced transcriptional activation (30). A ligand-dependent interaction between TR and CBP, an essential coactivator for the AP-1 complex, has been recently proposed to mediate this antagonism (31-32). The human APP promoter contains a number of sequences homologous to known transcription factors, among others two consensus AP-1 binding sites (33-36), which play a role in the regulation of the APP gene by phorbol esters in a human glial cell line (11).

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 functional nTRE, located in the first exon, which could mediate a repressive effect of thyroid hormone on APP gene expression.

EXPERIMENTAL PROCEDURES

Cell culture. - Murine N2a neuroblastoma cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum as previously described by Ortiz-Caro et al. (38). 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. (50), 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 subclon which constitutively expresses the β-isof orm of TR (TRβ), were obtained from Dr. Dussault’s laboratory. N2a-β cells were grown as previously described by Lebel et al. (37) and the experiments were carried out in the same medium containing 0.5% of thyroid hormone-depleted fetal calf serum.

**RNA extraction and hybridization** - Total RNA was extracted from the cell cultures by the guanidine thiocyanate method (51). 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 (52), with a plasmid containing a human APP cDNA labeled by random oligonucleotide priming. Hybridizations were at 65ºC in PSE buffer (0.3M sodium phosphate; pH=7.2, 7% SDS, 1mM 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/+105 fragment of the human APP gene has been previously described (53). Progressive 5' deletions to -487, -307, -15, +55 and +75 bp were prepared by polymerase chain reaction from the original -1099/+105 bp fragment, kindly provided by Dr. Lahiri’s laboratory, and subcloned into the BamH1 site of pBL-CAT8, a plasmid which lacks the AP-1 binding site present in the pUC backbone. The cDNAs encoding TRα, RXRα and v-erbA, as well as the cDNAs encoding TR mutants E401/Q, E401/K and C1, were the kind gift of Dr. D. Barettino (39). These different receptors are inserted into the EcoI site of the expression vector pSG5 which contains the SV40 early promoter. The reporter construct TREAPP/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 cultured in Dulbecco’s modified Eagle’s medium containing 10% of thyroid hormone depleted fetal calf serum were transfectcd by the calcium phosphate coprecipitation method with 1μg 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, 1µg of reporter plasmid and 1µg of the corresponding receptor expression vector were used. After 16h of incubation in the presence of calcium phosphate, the medium was discarded and washed with 5 ml of phosphate-buffered saline. A new medium containing 0.5% thyroid hormone depleted serum was added and the cells were then incubated for an additional period of 48 hours 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 [14C]-chloramphenicol with cell lysate protein. After autoradiography, the non-acetylated and acetylated [14C]-chloramphenicol was quantified and the data are expressed as the mean ± standard deviation of the percent of acetylated forms after each treatment. Each experiment was repeated at least 2-3 times with similar relative differences in regulated expression.

*Mobility shift assays* - Synthetic oligonucleotides containing the TR-binding sequences of the human APP promoter, were end labeled with [32P]ATP using T4-polynucleotide 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 (54). 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, 1mM DTT, 5 µg/ml BSA, 13% glycerol) containing 3 µg poly (dl-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 TRE_PAL (5´AGGTCATGACCT-3´), were added to the binding reaction mixture. For gel supershift, the reaction mixtures were incubated with 1 µl of specific anti-TR (αTR) or anti-RXR (αRXR) 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 the APP gene and high levels of TRβ (37), after treatment with 5 nM T3. Figure 1 illustrates the results obtained in a representative Northern blot carried out with 30 μg of total RNA. A single band of 3.4 kb, 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 hours, and became more evident after 48 hours of treatment. No effects of T3 on APP-mRNA levels were observed in parental N2a cells, which express very low levels of TR (38) (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 48h in the presence or absence of 5 nM T3. As shown in left panel of figure 2, 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, thus demonstrating that the T3-induced inhibition of the transcriptional activity requires the presence of adequate levels of TR.

To further confirm the role of TR in this negative response, the unresponsive parental N2a cells were co-transfected with the APP promoter-CAT construct and a vector expressing the α-isoform of the thyroid hormone receptor (TRα). As shown in the right panel of the figure, the unliganded TR increased basal promoter activity, and T3 effectively reversed this effect. Therefore, negative regulation by T3 appeared to be largely a consequence of reversal of the TR-induced activation rather than an active suppression of basal promoter activity. The negative effect of T3 in N2a cells transiently transfected with TRα was very similar to that observed in N2a-β cells. These results show that the inhibitory effect of T3 does not exhibit TR isoform specificity, since it was equally mediated by both α and β receptor isoforms.
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 figure 3, the negative effect of T3 was maintained even in cells transfected with the shortest (-15/+102 bp) fragment. Similar results were obtained in N2a cells transfected with TRα (data not shown). Since the repressive effect of T3 is mediated by DNA fragments in which the AP-1 sites have been lost, these results eliminate the possibility of an AP-1 mediated mechanism and 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 (39). 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 which fails to bind ligand, as well as with several TRα mutants affecting the AF-2 domain. The C-1 mutant carries a nine aminoacids C-terminal deletion like that found in v-erbA, and the E401/Q or E401/K mutants contain a point mutation of the Glu (E) residue at position 401. As illustrated in figure 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 E401/K mutants show a strongly reduced ligand binding affinity (39) 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 E401/Q mutant in which ligand binding affinity remains unaltered (39).

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 the upper panel of figure 5, a computer-assisted study of the nucleotide
sequence of this region, revealed the existence of three potential thyroid hormone response elements located at the nucleotides 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. A similar distribution of TR-binding sites has been previously described for the TSH, TRH and other genes negatively regulated by T3 (25,26,28).

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 figure 5, no specific retarded bands were detected when TR or RXR were used separately, and only the oligonucleotide (+75/+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. Figure 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 the previous figure, 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 specificity of the retarded band was assessed in the presence of increasing amounts of the unlabeled E3-containing oligonucleotide or the thyroid hormone consensus response element TREpal. Complex formation was progressively reduced by the unlabeled E3 oligonucleotide (5 to 50-fold excess), and it was practically abolished in the presence of the same amounts of TREpal. In addition, no competition was observed when an unrelated oligonucleotide was used. These results demonstrate that the E3 element of the APP gene specifically binds the thyroid hormone receptor, although with a lower affinity than the consensus TREpal. Figure 6B shows the results of a representative gel mobility shift assay carried out with nuclear extracts of N2a-β cells. Several retarded bands were detected. The most prominent band
presented a mobility identical to that obtained with the *in vitro* translated TR/RXR heterodimer (lane 3). The mobility of this band was slightly increased in the presence of T3. This band was specifically competed by an excess of both unlabeled E3 oligonucleotide or TREpal, but not by an unrelated oligonucleotide. The presence of TR and RXR in the complex was tested with specific antibodies against both receptor proteins. The retarded band was inhibited by the TR antibody (αTR) and supershifted by the RXR antibody (αRXR). These data show that the E3 element binds the endogenous TR/RXR heterodimers present in N2a-β cell nuclei. In addition to the major TR/RXR containing complex, nuclear extracts from N2a-β cells caused the formation of a minor retarded band of slower mobility. This unidentified band contains neither TR, nor heterodimers of RXR with other nuclear receptor, since it was unaffected by the antibodies.

*Functional analysis of the E3 element* - The nucleotide sequence of the E3 element contains three motifs, (+80/+85), (+85/+90), and (+91/+96), 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 non-responsive 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-CAT reporter gene), and an expression vector for TRα. As shown in figure 7, the activity of the TK promoter was not affected by TR either in the presence or the absence of T3. In contrast, the insertion of the APP promoter fragment into the upstream region of the construct conferred T3 responsiveness to the TK promoter. The regulation was identical to that found with the APP promoter, where unliganded TR significantly increased CAT activity, and T3 reverted the increased activity to the uninduced basal levels.

**DISCUSSION**

In this report we present evidence that T3, the most active form of thyroid hormones, negatively regulate APP gene expression in a rat neuroblastoma cell line. The repressive effect of T3 on APP transcriptional activity was observed in N2a-β cells, a subclon 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 (30), have been described to mediate 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 -345 and -38 (33-36). Our results show that the progressive removal of the AP-1 sites did not significantly affect the repressive effect of T3 on the APP promoter, thus discarding the participation of a mechanism involving transcriptional antagonism between TR and the AP-1 complex. These results, together with the requirement of TR, suggested a mechanism in which the negative effect of T3 could be directly mediated by receptor binding to specific DNA sequences.

Negative TREs are normally located in the proximal promoter or even within the first exon, downstream of the transcriptional start site. Moreover, the negatively regulated genes frequently exhibit more than one nTRE which cooperate to allow for an integrated negative regulation of the promoter (28). The finding that T3 effectively repressed the activity of a minimal APP promoter fragment suggested the existence of a negative TRE in the proximity of the main transcriptional start site. Sequence analysis of the promoter region comprised between nucleotides -15 and +102 revealed the presence of three potential TR binding sites. The promoter fragments -4/+9 and +20/+30 contain sequences that strongly resemble the previously established AGGTCA consensus TRE half-site, one arranged as an inverted palindromic repeat, and the other as an overlapping direct repeat. The high homology (75%) observed between these elements and the consensus sequence, suggested that they could act as response elements. However, analysis of the binding properties of these elements, revealed that they are unable to bind TR or TR/RXR. Only the sequence located between nucleotides +80 and +96 binds the receptor significantly. Therefore, although the possibility that the more upstream sequences could contribute to modulate TR binding to the more downstream element cannot be dismissed, our results strongly suggest that the negative
effect of T3 on the APP gene might be mediated by a unique nTRE present within the first exon at positions +80/+96.

The putative nTRE of the APP gene contains at least two hemi-sites, each resembling the consensus TR binding motifs. A central core of nucleotides might be interpreted as a third hemi-site, 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 (40). 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 (25,26,28). The preferential binding of TR/RXR heterodimers firmly supports a structure containing two hemi-sites spaced by 5, or more likely, 4 base pairs. In both cases the downstream hemi-site (+91/+96 bp) is identical to the sequence found by Desvergne et al. in the rat malic enzyme TRE (18). The first hemi-site, 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. (41) -AGGT(C/A)A- and Norman et al. (42) -GGG(T/A)C(G/C)-, respectively.

Contrary to positive TREs, which normally mediate repression by TR in the absence of T3, binding of unliganded receptors to nTREs can lead to a constitutive activation of gene transcription (43-45). 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 (46). Addition of T3 might reverse basal activity perhaps by dissociation of those corepressors. In N2a cells, unliganded TRα stimulates APP promoter activity, and as occurs with a number of negatively regulated genes (25,28,45), T3 reverts the activation induced by the transfected unliganded receptor, although it does not suppress the basal transcriptional activity of the APP promoter below the original levels. This regulation is different from that observed in the keratin gene promoter, in which T3 not only reverses the ligand-independent effect of TR, but also suppresses the basal transcriptional activity below basal levels (44).
Ligand-dependent transcriptional activation by nuclear receptors is mediated by a domain termed AF-2 (activation function-2), that in TR is located within the distal carboxyl terminus of the ligand-binding domain (39). The experiments with mutant TRs show that the AF-2 region is required for the T3-dependent repression of the APP promoter in N2a cells. A point mutation E401/Q which affects T3-dependent activation without altering T3 binding (39) 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 (47), 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 the APP gene expression by thyroid hormone 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 TRα and TRβ. In addition to the repressive effect of the thyroid hormone on APPmRNA levels and APP promoter activity, we have also observed that the thyroid hormone affects splicing, and secretion of APP (16). Our results together with the reduction of thyroid hormone receptor levels observed in Alzheimer hippocampal cells (48) and the effects exerted by estradiol or retinoic acid on APP expression and metabolism (12,49), strongly suggest that members of the nuclear superfamily of receptors, and their ligands, might play an important role in Alzheimer's disease.
REFERENCES


**LEGEND TO THE FIGURES**

**FIG. 1.** T3 reduces APP mRNA levels in N2a-β neuroblastoma cells.

Cells were incubated with 5 nM T3 in a medium containing resin-treated serum. At the indicated times, cells were harvested and 30 μg of total RNA were processed for Northern blot analysis as described under "Materials and methods". APP-mRNA levels were determined by densitometric scan of autoradiograms and corrected by the amount of RNA applied. The top panel shows the results obtained from two separate experiments performed in duplicate. Data are the mean ± S.D. of four determinations. APP-mRNA bands from a representative autoradiogram and the
18S rRNA from the stained blots are illustrated in the medium and lower panels, respectively.

FIG. 2 **Negative regulation of APP promoter activity by T3 in N2a cells.**

Left panel illustrates the 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 48h period of incubation in the absence or presence of 5 nM T3. Right panel shows CAT activity in N2a cells cotransfected with the reporter plasmid along with an expression vector for TRα, or with an empty non-coding vector (pSG5-0). Data are expressed relative to the corresponding control values (100%), and are 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 at the left of the graph. CAT activity was determined after 48h 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 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 hours 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.
FIG. 5 Gel retardation analysis of TRα, RXR and TRα/RXR binding to nucleotide sequences of the -15 to +102 region of the APP promoter.

Illustrated at the top of the figure is the nucleotide sequence of the -15 to +102 bp region. Potential TR-binding sites are indicated by bold characters. Nucleotide identity with the consensus sequence (AGGTCA) is indicated by small black squares on the characters. The arrows indicate the position and orientation of potential half-sites. The oligonucleotides used in the gel retardation assays (-10/+15; +13/+37; and +75/+101) are also indicated. Panel B shows the results obtained in a representative assay carried out with in vitro translated TR and RXR. First and second columns of each group are controls obtained with the corresponding unlabeled probe or lysate alone, respectively.

FIG. 6 Characterization of the E3 TR-binding site of the APP gene.

In the upper panels, gel-mobility shift assays were carried out with a radiolabeled probe (+75/+101) containing the binding site and surrounding nucleotides and in vitro translated TR and RXR (2 µl). When indicated the proteins were incubated with 200 nM T3. A 5- and 50-fold excess of the unlabeled probe, the palindromic element TREpal, or an unrelated oligonucleotide was used for competition studies. In the lower panel, the assays were performed with the labeled oligonucleotide and 5 µg of nuclear extracts obtained from control N2a-β cells and from cells treated with T3. The composition of the retarded bands was tested by incubation with 1 µl of anti-TR (αTR) or anti-RXR (αRXR) antibodies. Competition experiments were carried out in the presence of a 50-fold excess of the same oligonucleotides used in the upper panel. The mobility of the TR/RXR heterodimers, as well as a not identified band (n.i.) are indicated by arrows.

FIG. 7 A fragment of the APP gene containing the E3 element confers T3 responsiveness to a heterologous TK promoter.

N2a cells were transfected with a TK-CAT reporter gene, or with the same construct containing the +55/+102 bp region of the APP gene inserted upstream of the TK promoter (TREAPP-TK-CAT). The reporter vectors were cotransfected with a TRα expression vector (pSG5-TR), or with a non-coding vector (pSG5-0). CAT activity was determined after 48 hours of incubation in the presence or absence of 5 nM
T3. Results are expressed relative to the value obtained in cells transfected with the TRE<sub>APP</sub>-TK-CAT construct in the absence of T3. Data are the mean CAT activities obtained from two independent experiments.

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