ACADEMIC PRESS

Available online at www.sciencedirect.com

SCIENCE DIRECT®

DEVELOPMENTAL BIOLOGY

Developmental Biology 254 (2003) 149–160

www.elsevier.com/locate/ydbio

Alien/CSN2 gene expression is regulated by thyroid hormone in rat brain

Stephan P. Tenbaum,[a] Stefan Juenemann,[b] Thomas Schlitt,[b] Juan Bernal,[a]
Rainer Renkawitz,[b] Alberto Muñoz,[a] and Aria Baniahmad[b],*

[a] *Instituto de Investigaciones Biomédicas CSIC/UAM, C/ Arturo Duperier 4, 28029 Madrid, Spain*
[b] *Genetic Institute, Justus-Liebig-University, Heinrich-Buff-Ring 58-62, D-35392 Giessen, Germany*

Received for publication 25 February 2002, revised 8 October 2002, accepted 8 October 2002

Abstract

Alien has been described as a corepressor for the thyroid hormone receptor (TR). Corepressors are coregulators that mediate gene silencing of DNA-bound transcriptional repressors. We describe here that Alien gene expression in vivo is regulated by thyroid hormone both in the rat brain and in cultured cells. In situ hybridization revealed that Alien is widely expressed in the mouse embryo and also throughout the rat brain. Hypothyroid animals exhibit lower expression of both Alien mRNAs and protein levels as compared with normal animals. Accordingly, we show that Alien gene is inducible after thyroid hormone treatment both in vivo and in cell culture. In cultured cells, the hormonal induction is mediated by either TRα or TRβ, while cells lacking detectable amounts of functional TR lack hormonal induction of Alien. We have detected two Alien-specific mRNAs by Northern experiments and two Alien-specific proteins in vivo and in cell lines by Western analysis, one of the two forms representing the CSN2 subunit of the COP9 signalosome. Interestingly, both Alien mRNAs and both detected proteins are regulated by thyroid hormone in vivo and in cell lines. Furthermore, we provide evidence for the existence of at least two Alien genes in rodents. Taken together, we conclude that Alien gene expression is under control of TR and thyroid hormone. This suggests a negative feedback mechanism between TR and its own corepressor. Thus, the reduction of corepressor levels may represent a control mechanism of TR-mediated gene silencing.

Keywords: Corepressor; Alien; COP9 signalosome; Thyroid hormone; Brain; Hypothyroidism

Introduction

Alien has been described as a corepressor for the thyroid hormone receptor (TR) (Dressel et al., 1999), a member of the nuclear hormone receptor super family (Mangelsdorf et al., 1995). Nuclear hormone receptors are hormone-regulated transcription factors that can repress and activate gene expression by binding directly to DNA or by interacting with other transcription factors. Thereby, cofactors play an important role in mediating these transcriptional properties (for review, see McKenna and O'Malley, 2000; Ordentlich et al., 2001; Rosenfeld and Glass, 2001; Wolffe et al., 2000). TR bound to its DNA-regulatory elements is able to silence gene expression in the absence of hormone by formation of a receptor–corepressor complex. Thyroid hormone leads to target gene activation through binding and inducing a conformational change of the TR ligand binding domain, dissociation of corepressors, and recruitment of coactivators. Several classes of such hormone-sensitively interacting corepressor proteins for the thyroid hormone receptor have been described, including the homologous NCoR and SMRT class of corepressors (Chen and Evans, 1995; Hörlein et al., 1995), the nonhomologous protein Alien as a member of another class (Altincicek et al., 2000; Dressel et al., 1999; Polly et al., 2000), and the recently described corepressor Hairless as a member of yet another unrelated class of corepressors (Potter et al., 2001). Other corepressors, such as SUN-CoR, that bind constitutively to

---

* Corresponding author. Fax: +49-641-99-35469.
E-mail address: Aria.Baniahmad@gen.bio.uni-giessen.de (A. Baniahmad).

0012-1606/03/$ – see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0012-1606(02)00023-4

TR have also been described (Zamir et al., 1996; for review, see Burke and Baniahmad, 2000). The corepressor Alien interacts with TR in a hormone-sensitive manner in vitro and in vivo (Dressel et al., 1999). We also found that Alien interacts with the orphan receptor DAX-1 (Altincicek et al., 2000) that is expressed in adrenal gland and brain. Alien is localized in the cell nucleus and potentiates the transcriptional silencing mediated by TR. Interestingly, in contrast to the NCoR/SMRT class of corepressors, Alien does not bind to retinoic acid and retinoic X receptors. Alien mediates its repression function at least in part by recruiting histone deacetylase activity. Alien is a highly conserved protein between human and *Drosophila* sharing 90% identity (Dressel et al., 1999). Intriguingly, an Alien isoform, CSN2 (COP9-signalosome subunit 2; Deng et al., 2000) with a molecular weight of about 52 kDa, has been shown to be a subunit of an evolutionary conserved multimeric protein complex called COP9-signalosome (Chamovitz and Segal, 2001; Henke et al., 1999; Schwechheimer and Deng, 2000; Wei and Deng, 1999). This protein complex is involved in multiple cellular processes, including phosphorylation of both p53 and IκB, deneddylation of Cullin and protein degradation (Lyapina et al., 2001; Schwechheimer et al., 2001), mitogen-activated protein kinase (MAPK) signaling (Claret et al., 1996; Naumann et al., 1999; Spain et al., 1996), and cell cycle control (Bech-Otschir et al., 2001; Mahalingam et al., 1998; Mundt et al., 1999; Tomoda et al., 1999; Yang et al., 2002). However, regulation of Alien/CSN2 gene expression has not been addressed.

Thyroid hormone action is essential for mammalian brain maturation (Dussault and Ruel, 1987; Legrand, 1984; Porterfield and Hendrich, 1993). Lack of adequate levels of thyroid hormones, the active form 3,5,3′-triiodothyronine (T3), and the pro-hormone thyroxine (T4) during fetal and neonatal periods lead to multiple brain abnormalities and mental retardation in humans (DeLong, 1990; Legrand, 1984). Conditions like iodine deficiency, congenital hypothyroidism, maternal hypothyroxinemia, and prematurity diminish physiological levels of thyroid hormone and may compromise brain maturation. Crucial processes in mammalian brain development, such as axogenesis and dendritic arborization, myelination, lamination of the cerebral cortex, as well as neuronal cell migration are affected by hypothyroidism and result in structural abnormalities of the central nervous system (reviewed in Bernal and Nunez, 1995; Bernal, 2002). In the last years, a number of genes have been identified to be under the direct or indirect control of thyroid hormone in the brain (Bernal, 2002; Brent, 1994; Cuadrado et al., 1999; Munoz et al., 1991; Oppenheimer and Schwartz, 1997). Deregulation of these TR target genes may in part explain the symptoms of hypothyroidism in brain.

Thus, the function of TR in regulating T3 target genes is mediated at least partially by coactivators and corepressors. Because the cellular levels of corepressors control the receptor function, the regulation of corepressor gene expression is likely to be an important regulatory step of TR activity.

Here we show by in situ hybridization and Northern and Western blot analysis that the expression of Alien is down-regulated in the hypothyroid rat brain. In accordance with these findings, the expression of Alien mRNA and protein is induced by T3 in cultured neuroblastoma cells. Therefore, we conclude that the gene expression of the TR corepressor Alien is repressed by the unliganded TR. This suggests a negative feedback mechanism and contribution of Alien to TR action during hypothyroidism and normal brain development.

Material and methods

Plasmids

The in vitro transcription vector pT7-asAlien$_{419}$-SP6 was constructed by insertion of the 419-bp *Bgl*II/*Hin*dIII fragment of pAB-hAlien (Dressel et al., 1999) in antisense orientation into *Hin*dIII/*Bam*HI sites of pT7βSal (Norman et al., 1988).

Cell culture

Murine N2A neuroblastoma cells (ATCC: CCL-131) and its derivates N2A+TRα as well as N2A+TRβ cells (Lebel et al., 1994) were grown under standard conditions in DMEM supplemented with 25 mM Hepes, pH 7.4, and 10% (v/v) fetal calf serum (FCS). In case of hormone deprivation, 10% (v/v) FCS depleted of thyroid hormone by treatment with resin AG1X8 (Samuels et al., 1979) was supplemented.

Preparation of animals

Wistar rats and BALB/c mice maintained in the animal facilities of the Instituto de Investigaciones Biomédicas were used for the studies reported here. All efforts were made to minimize animal suffering and to reduce the number of animals used. The maintenance and handling of the animals were as recommended by the European Communities Council Directive of November 24th, 1986 (86/609/EEC). The induction of fetal and neonatal hypothyroidism in rats was previously described (Munoz et al., 1991). This protocol ensures that the animals are hypothyroid during the entire neonatal period. (Alvarez-Dolado et al., 1998). P0 animals were killed 8-12 h after birth. T4 was used for the in vivo hormonal treatments because it crosses the blood-brain barrier more efficiently than T3 and is converted to T3 in the brain (Dickson et al., 1987). T4 was administered as single daily intraperitoneal injections of 1.8 μg/100 g body weight starting 4 days before death. Rats were killed 24 h after the last T4 injection. T3 and T4 values for normal

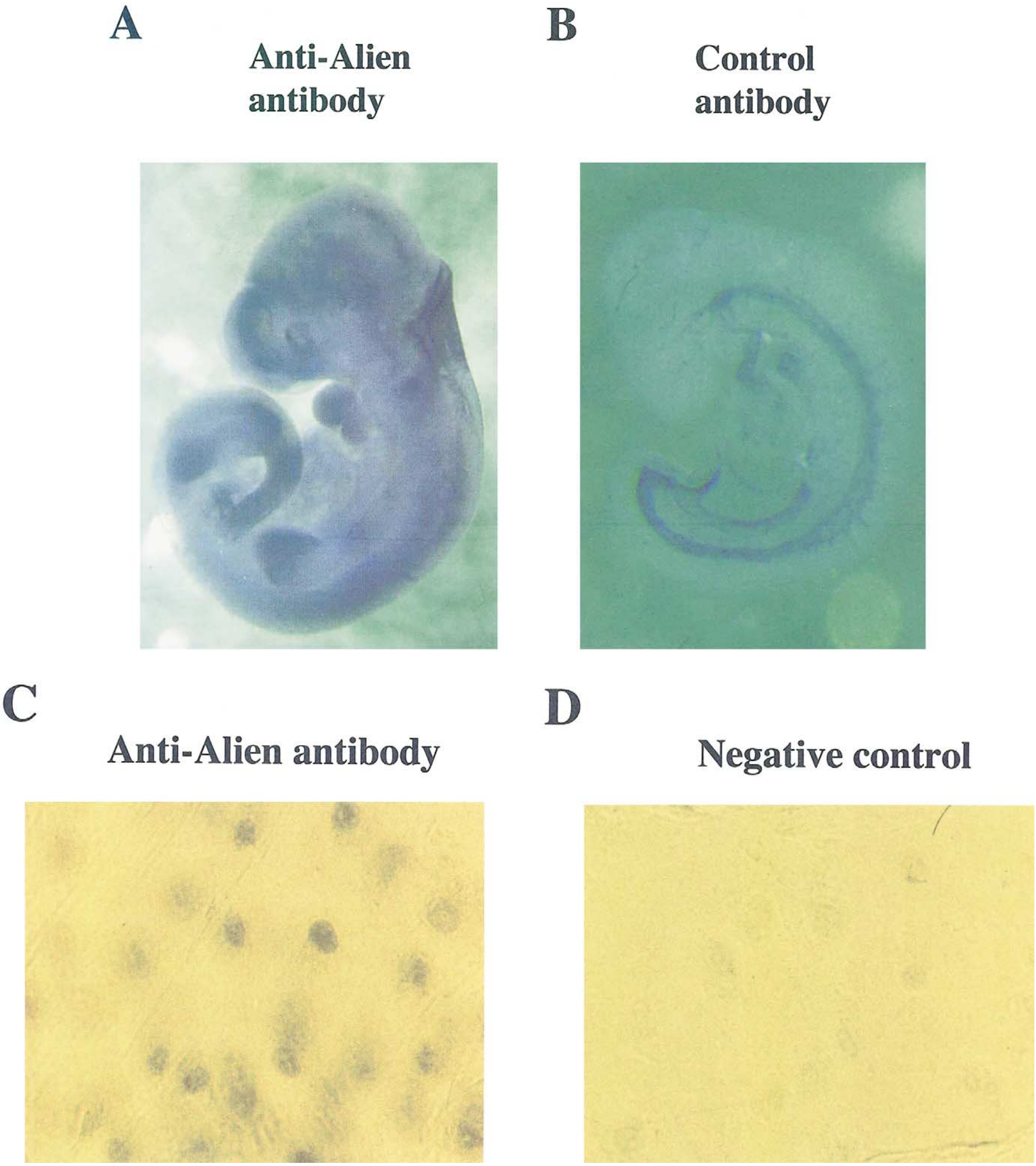


Fig. 1. Whole-mount immunostaining reveals ubiquitous expression of Alien. Mouse embryos at day E9.5 were immunostained with (A) affinity purified anti-Alien antibody or (B) as a control with an unrelated antibody against the affinity purified Willebrand factor (Control antibody). Control embryos incubated with control serum or with the peroxidase-coupled secondary antibody did not stain and remained entirely white (not shown). (C) Nuclear staining of mouse amnion tissue with the affinity purified anti-Alien antibody or control serum (D).

(control) and hypothyroid animals are as follows: T4 levels of brain tissue of control animals are 1.46–1.48 ng/g, while of hypothyroid animals, 0.022-0.024 ng/g; circulating levels: Plasma T3: control animals 62 ng/dl, while hypothyroid animals >15 ng/dl; plasma T4: control animals: 5 μg/dl, while hypothyroid animals had 0.2 μg/dl. For in situ hybridization studies, at least three animals were studied per experimental group to obtain representative values.

RNA extraction and Northern analysis

Total RNA preparation from N2A and derivate cells from 10-cm cell culture dishes was performed by using TRI REAGENT (Molecular Research Center Inc.) following the manufacturer's instructions. Northern blot experiments have been repeated twice. Total RNA from rat tissues was obtained by the guanidinium isothiocyanate-phenol-chloroform procedure (Chomczynski and Sacchi, 1987). For Northern analysis from a pool of rat brain tissue, polyA$^+$ RNA was then purified by affinity chromatography oligo(dT)-cellulose method (Sambrook et al., 1989). In this case, 6 μg of poly A$^+$ RNA pooled from brains of the different developmental ages (from eight animals in case of E19 and P0, seven for P5 and five for each P10 and P15) of control (C), hypothyroid (H), or T4-treated hypothyroid (H+T4) animals were loaded in each lane (Sambrook et al., 1989). Radioactive cDNA probes were prepared by the random priming procedure (Feinberg and Vogelstein, 1983). The hAlien cDNA (Dressel et al., 1999) was used for Northern hybridization.

Immunoblotting and in situ hybridization

Immunoblotting and in situ hybridization were performed as described earlier (Alvarez-Dolado et al., 1998, 2000; Gall and Isackson, 1989). To determine specificity and background of riboprobe hybridization, a representative amount of brain sections of each age and treatment was hybridized with Alien sense riboprobe following the above described protocol. After exposure of in situ hybridizations, the mounted sections were Nissl-stained with a 0.1% solution of toloudine-blue by using standard techniques, to visualize brain regions. For anatomical abbreviations, we followed those in Swanson (1992).

Whole-mount immunostaining

The whole-mount immunostaining was done as described elsewhere (Adams et al., 1999). Embryos were incubated with affinity purified polyclonal anti-Alien antibody or as controls only with the peroxidase coupled secondary antibody, and control flow through serum that did not result in staining, or the purified anti-Willebrand factor, kindly provided by Dr. U. Deutsch.

Genomic PCR

Genomic DNA from mouse liver was prepared by first crushing the tissue in liquid nitrogen. Subsequently, the genomic DNA from mouse liver and RMB3 cells was isolated by lysing cells using standard methods, including proteinase K incubation, phenol/chloroform treatment, and ethanol precipitation. For genomic Alien, first round PCR amplification was performed with the primer pair: exon 7: 5′- GGGG-AATTCACTGATGATGGAGAAGATGACC-3′ and exon 8: 5′-GGGGGAT-CCGTGAGTCAAACGGATTTATTCC-3′. The second nested PCR was performed with the primer pair: exon 7(2): 5′-GGGGAATTCGATTCAAATGTACACAG-CAC-3′ and exon 8(2): 5′-GGGGGATCCATTAGCATATT-GGCTAAGACC-3′. PCR products were sequenced directly by cyclic sequencing of the PCR products.

Results

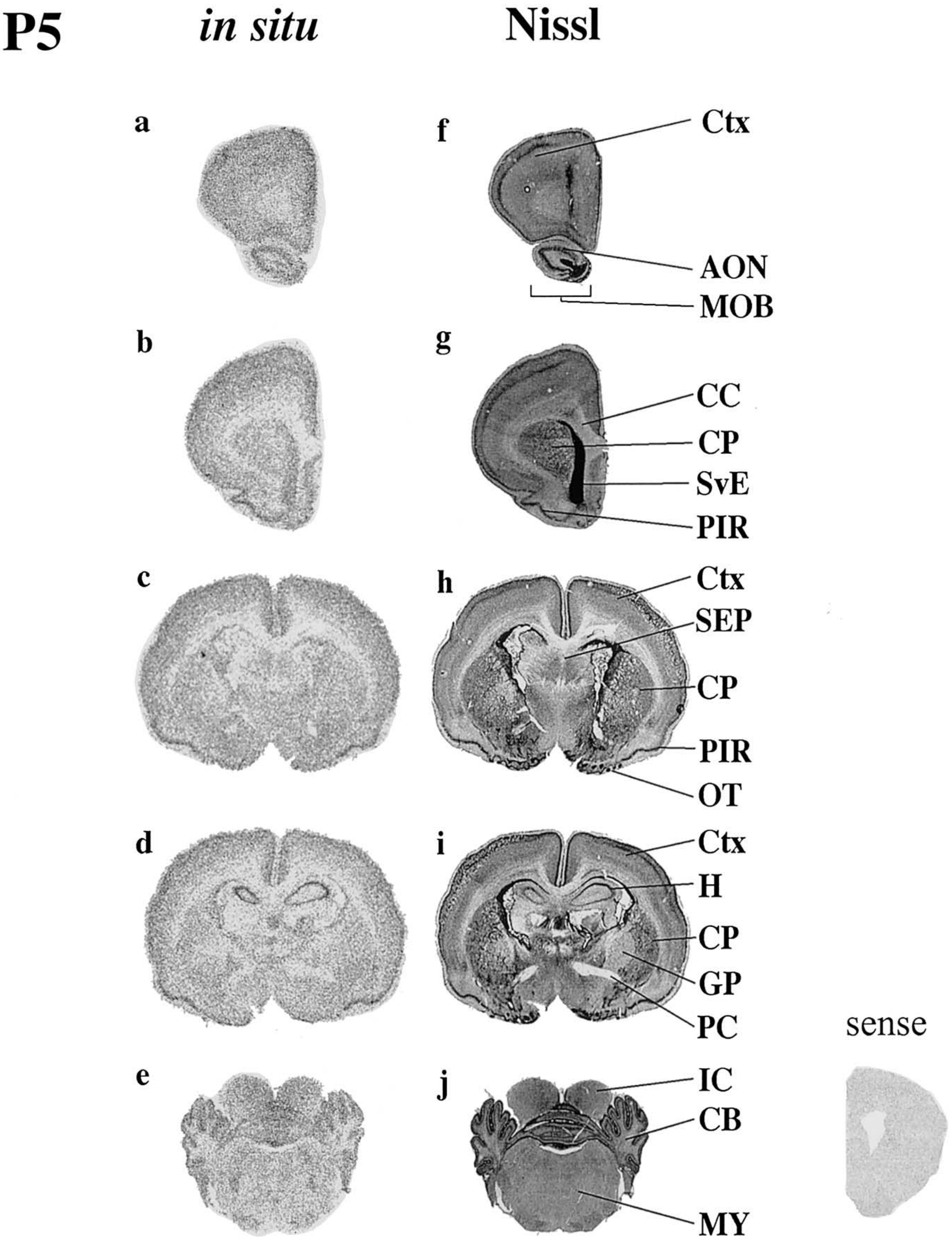
Expression pattern of Alien in mice

Data bank search for Alien cDNA (expressed sequence tags, EST) suggests that Alien is expressed in a large variety of different mouse tissues. This includes liver, kidney, mammary gland, macrophages, T-cells, lymph nodes, and placenta. Also, various embryonic stages express the Alien message (Schaefer et al., 1999). Using mouse embryos at embryonic day E9.5, we verified the expression pattern of the Alien protein using an affinity purified rabbit anti-Alien antibody (Goubeaud et al., 1996). Staining of embryos was observed throughout the embryo (Fig. 1A). As negative controls, the secondary peroxidase-coupled antibody alone and the control serum were used, that led to completely unstained embryos (not shown), and also the unrelated affinity purified rabbit antibody against the Willebrand Factor, which is expressed in endothelial cells (Coffin et al., 1991). Here, specifically, the vascular system is stained (Fig. 1B).

Furthermore, compared with the control, we detect nuclear staining with the affinity purified alien antibody shown for mouse amnion tissue (Fig. 1C and D), which is in accordance to previous results (Dressel et al., 1999).

These data suggest that Alien is expressed in a large number of tissues, including brain.

Fig. 2. Expression pattern of Alien in rat brain at postnatal day 5. In situ hybridization with radioactive Alien antisense riboprobe on rat brain sections of animals at postnatal day 5. Alien RNA is expressed throughout the brain with higher levels in the subventricular epithelium (SvE), the cerebral cortex (CTX), piriform cortex layer II (PIR), anterior olfactory nucleus (AON), and olfactory tubercle (OT), as well as in the caudate putamen (CP), globus pallidus (GP), pyramidal and granular layers of the hippocampus (H), and neuronal layers of the developing cerebellum (CB). No detectable expression was observed in other brain areas, such as corpus callosum (CC), septum (SEP), the anterior commissure (AC), and cerebellar white matter at P5. The brain sections are displayed in order from rostral to caudal. Further brain regions are indicated: main olfactory bulb (MOB), inferior colliculus (IC), and medulla (MY). Nissl staining of the same coronal brain sections is shown to visualize brain-specific areas. The control for specificity of the Alien riboprobe using sense-probe with rat brain section from postnatal day 5 is shown (sense).


P5
in situ
Nissl
a
f
Ctx
AON
MOB
b
g
CC
CP
SvE
PIR
c
h
Ctx
SEP
CP
PIR
OT
d
i
Ctx
H
CP
GP
PC
sense
e
j
IC
CB
MY

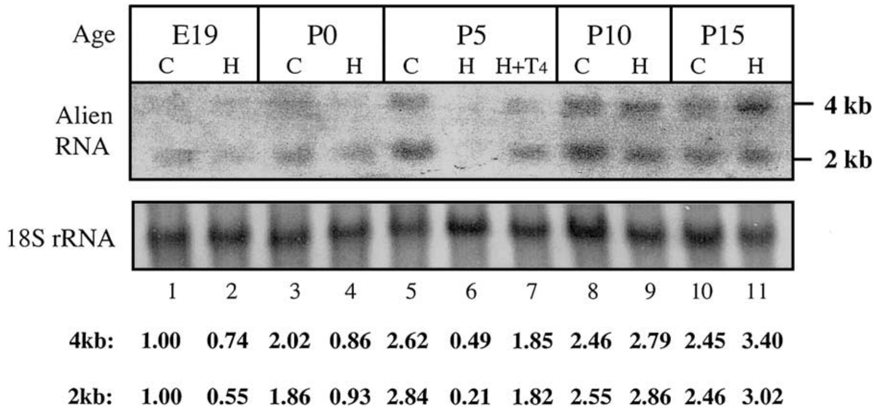


Fig. 3. Alien expression is induced by thyroid hormone in rat brain. Northern experiments were performed with polyA–mRNA isolated from rat brain pools at the indicated ages from embryonal day 19 (E19) up to postnatal day 15 (P15). Two Alien-specific bands were detected at 2 and 4kb. Normal, control rats (C) are compared with hypothyroid (H) rats. At P5, hypothyroid rats were treated with thyroxine (H+T4). Methylene-blue-stained 18S rRNA is displayed as a loading control. The 4- and 2-kb transcripts were quantified, normalized, and indicated arbitrarily as relative to Alien mRNA content of E19 control animals.

Alien expression is reduced in the hypothyroid brain

To determine the pattern of Alien expression in developing brain, we first performed radioactive in situ hybridization analysis using a specific antisense Alien riboprobe with rat brain sections. In 5-day-old animals (P5), Alien RNA is expressed throughout the brain (Fig. 2a–2j), fairly ubiquitous with higher levels in the subventricular epithelium (SvE), the cerebral cortex (CTX), piriform cortex (PIR) layer II, anterior olfactory nucleus (AON), and olfactory tubercle (OT), as well as in the caudate putamen (CP), globus pallidus (GP), and pyramidal and granular layers of the hippocampus (H). Neuronal layers of the developing cerebellum (CB) also showed high hybridization signal. No expression was observed in white matter areas, such as corpus callosum (CC), the anterior commissure (AC), and cerebellar white matter at P5 (Fig. 2).

To investigate whether Alien expression could be affected by hypothyroidism, we performed Northern blot analysis comparing pools of rat brain tissue from normal and hypothyroid rats. In accordance with reports of other tissues (Altincicek et al., 2000; Schaefer et al., 1999), two transcripts of 2 and 4 kb were detected at all ages studied from embryonic day 19 (E19) to postnatal day 15 (P15) using Alien cDNA as probe (Fig. 3). In brains from euthyroid (control, C) rats, both RNAs were maximally expressed at P10 and P15. Interestingly, from embryonic day E19 to postnatal day P5, the levels of both Alien RNAs were significantly lower in hypothyroid animals, with a strong difference at day P5 between control (C) and hypothyroid (H) animals. This indicates that Alien gene expression in brain is under thyroid hormone control.

However, the differences between euthyroid and hypothyroid status spontaneously disappeared in later ages P10 and P15, reaching a similar expression level independent of the hypothyroid status. Comparing the strength of expression of both messages, a slight increase in the 4-kb message at postnatal day 15 was observed in hypothyroid animals (Fig. 3). This indicates that, at later ages of hypothyroidism, a slight change of the ratio between the two detectable 2- and 4-kb Alien transcripts occurs.

Due to the striking difference of Alien gene expression between normal and hypothyroid animals at postnatal day 5, we focused on that developmental day and tested whether thyroid hormone treatment can induce Alien expression in hypothyroid animals. At postnatal day P5, brain RNA was isolated from a pool of hypothyroid rats injected with thyroxine. Remarkably, thyroid hormone administration to hypothyroid rats caused partial normalization of the level of both Alien RNAs (Fig. 3). Thus, Alien mRNA expression is inducible by thyroid hormone in vivo.

An apparent downregulation of Alien message was observed at day P5, as assessed by Northern blotting, whereas at P15, only slight differences were detected between control and hypothyroid animals. We wanted to confirm these observations by in situ hybridization both at P5 and P15. Nissl staining (panel c & d, g & h) is shown to visualize specific brain areas (Fig. 4). Brain sections of day P5 from control and hypothyroid rats hybridized with Alien antisense riboprobe show lower Alien gene expression in hypothyroidism compared to the euthyroid, control animals (Fig. 4A, compare a-b and e-f). As negative control, the Alien sense riboprobe was used (Fig. 4C). At day P15, the regional pattern and level of RNA expression is roughly maintained (Fig. 4B, a and e). However, comparing these samples with P15 hypothyroid brains (Fig. 4B, b and f), reveals only slight differences in Alien RNA levels.

Taken together, these findings are in agreement with the Northern data (Fig. 3) that Alien RNAs are found to be downregulated in hypothyroid rats at P5. Also, the in situ hybridization experiments at day P15 are in agreement with the data obtained in Northern blot analysis (Fig. 3, lanes 6, 7, 10, and 11).

These results indicate that Alien expression is under

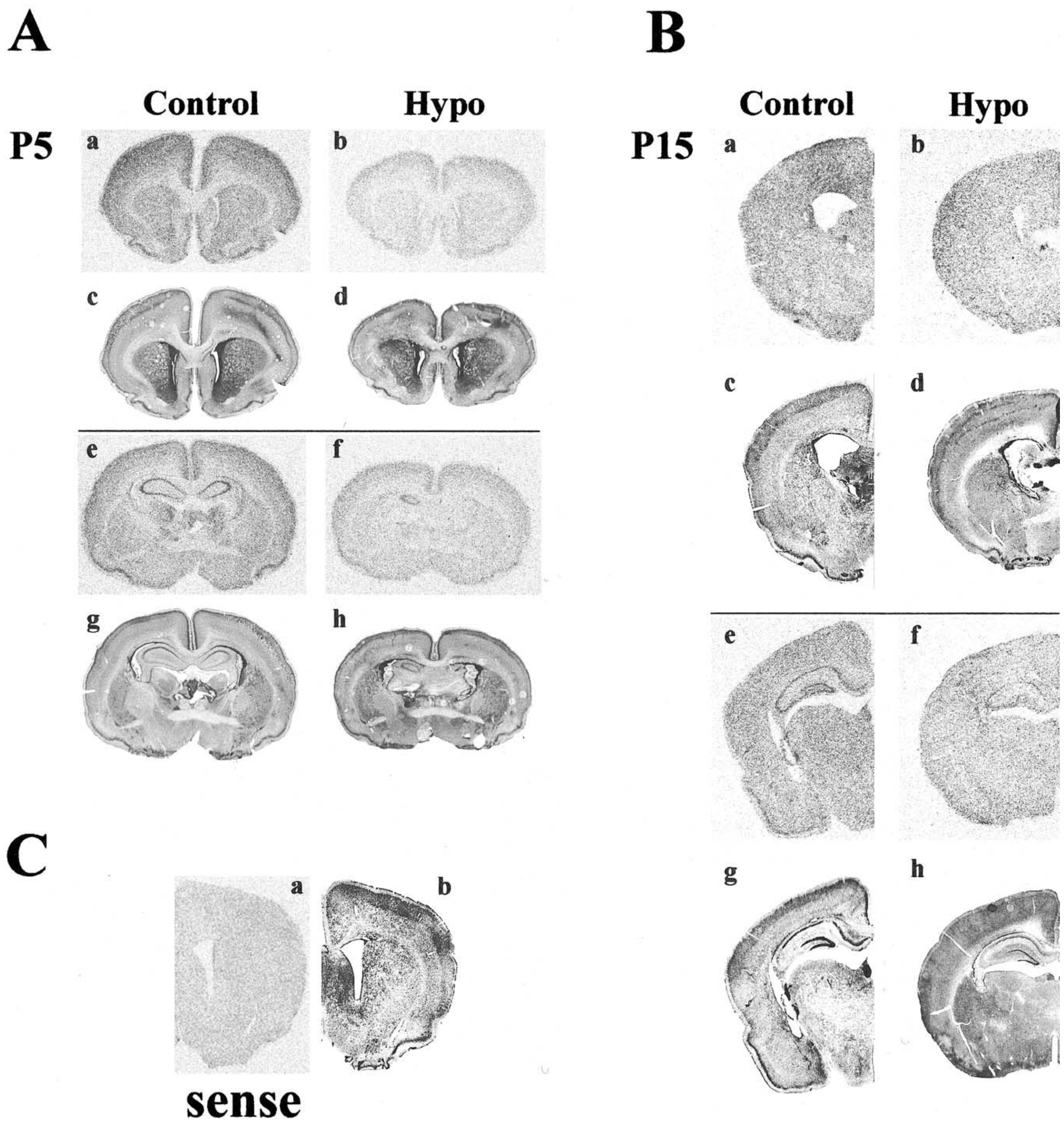


Fig. 4. Hypothyroid animals exhibit lower Alien gene expression in vivo Normal, control rat (Control) and hypothyroid (Hypo) rat brains were used for in situ hybridization with Alien antisense riboprobe of brain sections at both postnatal day 5 (A, P5) and P15 (B). Nissl staining is shown to visualize brain specific areas. As control for specific in situ hybridization, the Alien sense-riboprobe was used (C).

thyroid hormone regulation in the rat brain during the postnatal period in vivo.

Rapid induction of Alien mRNA in neuroblastoma cells

To investigate the time response of thyroid hormone-mediated induction of Alien messages, we used N2A neuroblastoma cell clones stably expressing TRα or TRβ (Lebel et al., 1994; Pastor et al., 1994). Cells were treated with T3 for various times before cell harvest, RNA isolation, and test for endogenous Alien expression with Northern experiments (Fig. 5A–C). Both cell clones expressing either TRβ (Fig. 5A) or TRα (Fig. 5B) show rapid induction of both Alien 2- and 4-kb messages within 2 h. The nature of the 4-kb band is still unclear, while the 2-kb transcript corresponds to the mouse Alien gene on chromosome 2

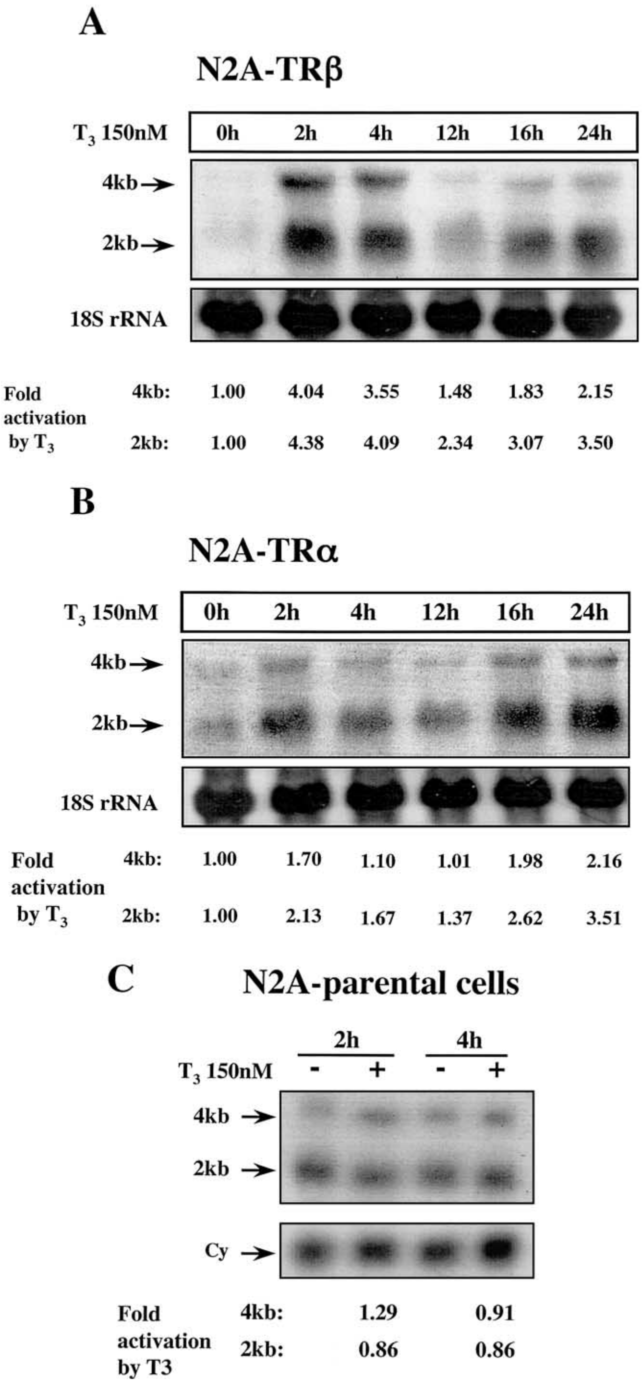


Fig. 5. Alien message is induced by thyroid hormone. Neuroblastoma cells (N2A-cell line) stably expressing TRα, TRβ, or the parental N2A cells were treated the indicated times with thyroid hormone (T3) at a final concentration of 150 nM prior harvest and analyses of Alien gene expression by Northern blotting. From (A) N2A stably expressing TRβ, (B) stably expressing TRα, and (C) the parental N2A cells, total RNA was isolated and hybridized with the Alien cDNA probe. Two Alien-specific messages at 2 and 4 kb are inducible by treatment with T3 in the TR- expressing but not in the parental N2A cells (C). Fold induction by T3 represents the quantification of the Alien-specific messages relative to untreated cells and normalized to that of control RNA (Cy: cyclophilin).

(Schaefer et al., 1999) homologous to that on human chromosome 15. The TRβ-expressing cells exhibit a stronger induction compared with the TRα-expressing N2A cells. As control, parental N2A cells lacking significant amounts of functional TRs exhibit no significant induction of the two Alien transcripts after 2 and 4 h of thyroid hormone treatment (Fig. 5C). Interestingly, Alien mRNA expression is reduced after 12 h of T3 treatment in both TR-expressing N2A cell lines before the expression is increased slightly at the 24-h time point (Fig. 5A and B). This interesting draw-back effect may be linked to corepressor induction and therefore to reduced thyroid hormone mediated response or another yet unknown feedback mechanism.

Taken together, these data suggest that Alien gene expression is rapidly induced by thyroid hormone treatment.

Alien protein is induced by thyroid hormone both in vivo and in TR expressing cells

To test whether Alien protein levels increase upon T3 treatment, protein extracts from pools of euthyroid and hypothyroid rat brain (cerebrum) were tested in Western blot experiments with anti-Alien peptide antibody (Dressel et al., 1999). As seen in Fig. 6A, the anti-Alien peptide antibody detects two bands, a weaker band migrating at about 41 kDa and a stronger band at 54 kDa, respectively. Therefore, we speculate on the existence of two Alien forms. We address the lower migrating band at 41 kDa as Alienα and the detected band at 54 kDa as Alienβ. Both detected protein bands are reduced in hypothyroid compared with normal, euthyroid (control) rat brain, while the Coomassie-stained PVDF membrane shows equal loading as control. Quantification of the bands revealed a 6.6-fold higher Alienα and a 2.1-fold higher Alienβ expression in control animals compared with hypothyroid animals (Fig. 6A). Similar results were obtained from N2A-TRα cells (not shown). Lower levels of Alien protein in hypothyroid animals are in accordance with the lower Alien mRNA levels shown in Northern and in situ experiments. Thus, anti-Alien antibody recognized two bands that exhibit reduced expression in hypothyroid rat brain.

Similar experiments were performed with N2A neuroblastoma cells expressing TRβ (Fig. 6B; Lebel et al., 1994). Western analyses with anti-Alien peptide antibody also revealed two detected bands in these cells. In contrast to the primary brain tissue, the lower migrating band at 41 kDa (Alienα) is much stronger compared with the slower migrating band at 54 kDa (Alienβ). Based on the molecular weight, Alienβ is likely to be the CSN2 subunit of the COP9-signalosome. Importantly, comparing the expression with and without thyroid hormone treatment, both bands detected by the Alien antibody show a strong induction after thyroid hormone treatment (Fig. 6B). As hormonal control, we have tested the parental neuroblastoma cells lacking detectable amounts of functional TRs (Fig. 6C). These cells lack T3-mediated induction of Alien proteins. As loading

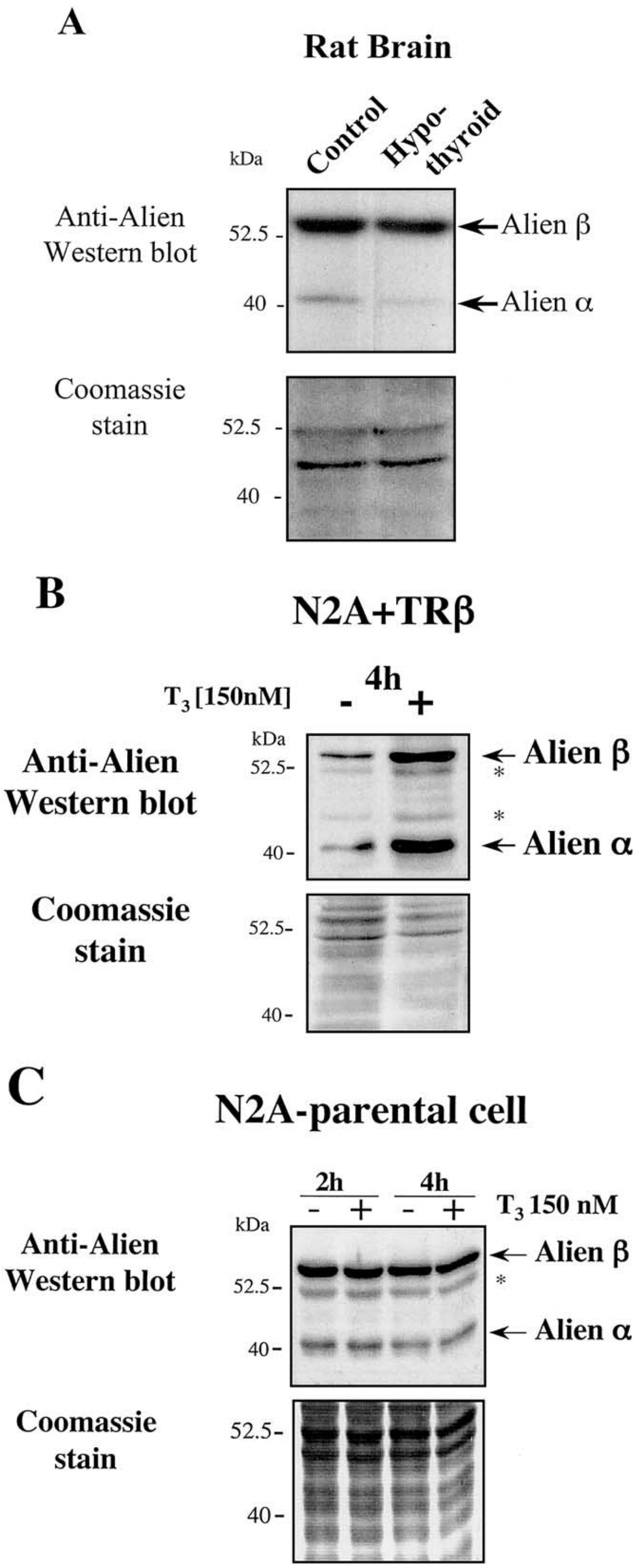


Fig. 6. Alien proteins are induced by thyroid hormone in vivo and in cell lines. (A) Total protein isolated from rat brain of control or hypothyroid animals was analyzed by Western blotting with the anti-Alien antibody. Two bands detected are migrating at about 41 (Alienα) and 54 kDa (Alienβ), respectively. Control animals show a 6.6-fold higher Alienα and a 2.1-fold higher Alienβ expression compared with hypothyroid animals quantified densitometrically. As loading control, the Coomassie staining of the PVDF membranes is shown. Western analysis was performed with either N2A cells expressing TRβ (B) or with the parental N2A cells (C). *, indicates bands that were not detected in brain tissue.

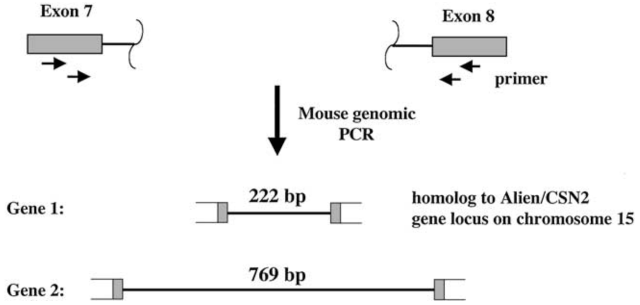


Fig. 7. Two different introns are identified between exon 7 and exon 8 of Alien gene in mouse genome. Schematic view of the mouse genomic PCR using two rounds of PCR with nested primers. Two specific PCR products were obtained which contained parts of the exons but different introns with the size of 222 and 769 bp, respectively. Sequencing analysis revealed that the 222-bp intron is highly homologue to the human Alien intron, while the 769-bp intron is highly homologue to a mouse contig clone (see text).

control, the Coomassie staining of the PVDF membrane is shown.

Thus, both detected Alien protein isoforms are induced by T3 in neuroblastoma cells.

Evidence for at least two Alien genes in the mouse genome

Homology search of Alien cDNA with human data base revealed the existence of at least two genes, one localized on chromosome 15, the other on chromosome 9. In addition, part of the Alien cDNA revealed sequence homologies on chromosome 2. To gain insight about the gene number coding for Alien in mouse genome and to obtain a possible hint of the origin of the two Alien mRNA messages, we first blasted the human genome sequence of chromosome 15 against the mouse data bank. We found sequence homologies only between human exons to the known mouse exons. However, no sequence similarities to the human introns were found in the mouse data bank.

Therefore, we set up genomic PCR using primers and nested primers against exons 7 and 8 with mouse genomic DNA. Interestingly, we revealed that two different sequences are flanked by exons 7 and 8, indicating that two different intron sequences have been isolated with a length of 222 and 769 bp, respectively (Fig. 7). Homology search with the 222-bp mouse intron sequence in the data bank showed strong homologies (97%) with the human intron, while no match was found with the second 769-bp intron sequence. However, this intron matched with sequences (98%) to the mouse Contig clone, Contig_114886.

Thus, our genomic PCR analyses and in silico searches suggest the existence of two introns, and therefore we hypothesize the existence of two Alien genes also in the mouse genome.

Discussion

Thyroid hormone plays a crucial role during brain development by regulating the expression of target genes. In this study, we used Northern blot and in situ hybridization assays in order to determine the ontogenic pattern of expression of Alien RNA in the developing rat brain and to investigate whether it is affected by hypothyroidism.

Alien RNA displayed a ubiquitous expression in the rat brain. This is in line with our findings that all tissues and cell lines analyzed so far express Alien to a certain degree (Altincicek et al., 2000; Schaefer et al., 1999, and unpublished results).

Alien was found fairly ubiquitously expressed predominantly in the cerebral cortex, pyramidal and granular layers of the hippocampus, subventricular epithelium, piriform cortex layer II, and external germinal layer as well as internal granular layer of the cerebellum, regions composed mainly of neurons, whereas those rich in fibers and glial cell populations, such as corpus callosum, septum, anterior commisure, and white matter of the cerebellum, show no Alien expression. Thus, the general pattern of Alien RNA distribution in the rat brain is suggestive of a preferential neuronal expression. However, basal expression in glial cells in vivo cannot be ruled out, and in line with that, cultured glia-derived cell lines, such as rat C6 and mouse B3.1, express Alien RNAs (data not shown).

Northern blot analysis revealed a relatively low Alien expression at late embryonic stages and an increase up to postnatal day 10 in normal, control animals. Hypothyroidism does not change the developmental profile of Alien expression, but induces a delay respectively to control conditions. The period of which thyroid hormone is known to be important in brain development is the very early postnatal period (Bernal, 2002). The thyroid hormone regulation of Alien occurs within this period. Similar to Alien, other TR target genes, such as reelin, laminin, and COX-1, are regulated within only a few days in postnatal brain development. A delay in expression and hormone-independent recovery has been described for most T3-regulated genes in brain, such as myelin proteins, reelin, or cerebellar genes (Rodriguez-Peña et al., 1993; Oppenheimer and Schwartz, 1997; Alvarez-Dolado et al., 1999; Bernal, 2002). Importantly, administration of T4 to hypothyroid animals partially recovered the amount of Alien RNA at P5. These findings strongly suggest that the expression of Alien RNA is dependent on thyroid hormone during brain development.

The pattern and time course of Alien expression during brain maturation correlate with that of thyroid hormone receptors, whose numbers increase at the end of the embryonic period, and are maximum by the end of the second postnatal week. Furthermore, the onset of Alien expression coincides with the period of maximum neuronal differentiation (Ferreiro et al., 1990; Mellstrom et al., 1991; Bradley et al., 1992). The overall decrease of the Alien mRNA in the hypothyroid brain is in agreement with the widespread presence of thyroid hormone receptors and additionally suggests a lack of modulation by local factors.

We recently demonstrated that Alien acts as transcriptional corepressor mediating gene silencing of TR (Dressel et al., 1999). Both Alien forms Alienα and Alienβ interact with TR (Dressel et al., 1999, and data not shown). Therefore, it may be speculated that downregulation of Alien in hypothyroidism during the crucial period of T3 action in brain maturation may contribute to abnormal TR function, and thus, could underlie to a certain extent the aberrant gene expression, taking place in the hypothyroid brain. On the other hand, if the manifestations of hypothyroidism are due to repression by unliganded TR (Forrest and Vennström, 2000; Morte et al., 2002), downregulation of Alien might attenuate such a repression, and this may represent a compensatory mechanism. Therefore, we conclude that the T3 regulation of Alien gene expression represents a negative feedback mechanism.

Additionally, changes in Alien expression might affect COP9-signalosome complex activity in the developing brain. Supporting this hypothesis, the Alienβ isoform CSN2 with a molecular weight of 52 kDa has been shown to be a subunit of the CSN and to be a limiting factor in COP9-signalosome assembly (Naumann et al., 1999). Based on these findings, we speculate that the hormonal regulation of Alien gene expression may also affect the divers functionality of the signalosome.

Transcriptional corepressors are important for adequate target gene silencing by interaction with transcription factors. In several cases, aberrant interaction of these regulatory proteins can lead to severe pathophysiological manifestations. On one hand, reduction of corepressor interactions is implicated in physiological disorders. Naturally occurring mutants of the orphan receptor DAX-1 involved in congenital hypogonadism in human lack binding of the Alien corepressor. In these cases, DAX-1 fails to silence target genes important for developmental processes (Altincicek et al., 2000; Crawford et al., 1998; Muscatelli et al., 1994).

On the other hand, enhanced interaction of corepressor complexes with silencer proteins has been linked to the human syndrome of thyroid hormone resistance (RTH) (reviewed in Tenbaum and Baniahmad, 1997; Burke and Baniahmad, 2000). RTH displays a mostly dominantly genetically inherited disorder based on mutations of the TRβ gene. The main characteristic of RTH is the lack or reduction of response to thyroid hormone of target tissues. The main clinical indications are elevated level of plasma thyroid hormones and inappropriate thyrotropin levels. As symptoms, goiter, attention deficit, learning disabilities, and hearing defects, impaired bone maturation, and mental retardation were observed. Furthermore, speech impediment, frequent ear, nose, and throat infections have been described. Most of these symptoms show that TRβ plays a very important role in brain development.

In addition, it has been shown that TR$\alpha^{-/-}\beta^{-/-}$ null mutant mice with complete absence of thyroid hormone receptors result in an only mild phenotype (Göthe et al., 1999) compared with the profound deficiencies induced by severe hypothyroidism or thyroid hormone resistance in human and in animals. One possible explanation for these findings is that the observed manifestations are due to repressor activity of unliganded thyroid hormone receptors (Forrest and Vennstrom, 2000; Morte et al., 2002).

In line with that, mutated TRβ, involved in the RTH syndrome, was shown to have lower dissociation capability of corepressors than wild type TRβ (Yoh et al., 1997). Therefore, target genes required for normal development are not fully activated or remain repressed. A negative feedback loop that leads to reduction of corepressor levels would attenuate the repression and would represent an intrinsic control mechanism of gene silencing mediated by TR.

Acknowledgments

We thank Ana Cuadrado for her constructive help. We are also grateful to Fernando Núñez and Pablo Señor for animal care and to Margarita González for her excellent technical assistance. Part of this work is included in the Ph.D. thesis of S.P.T. This work was supported by grants from the Deutscher Akademischer Austauschdienst (to A.B. and S.P.T.), the "Programa de Acciones Especiales y Acciones de Política Científica" (APC1999-0172) of the Ministerio de Educación y Cultura of Spain (to S.P.T.), the Grant SAF2001-2291 from the Ministerio de Ciencia y Tecnologia to A.M., and the SFB 397 from the Deutsche Forschungsgemeinschaft (to A.B.).

References

Adams, R.H., Wilkinson, G.A., Weiss, C., Diella, F., Gale, N.W., Deutsch, U., Risau, W., Klein, R., 1999. Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. Genes Dev. 13, 295–306.

Altincicek, B., Tenbaum, S.P., Dressel, U., Thormeyer, D., Renkawitz, R., Baniahmad, A., 2000. Interaction of the corepressor Alien with DAX-1 is abrogated by mutations of DAX-1 involved in adrenal hypoplasia congenita. J. Biol. Chem. 275, 7662–7667.

Alvarez-Dolado, M., Gonzalez-Sancho, J.M., Bernal, J., Munoz, A., 1998. Developmental expression of the tenascin-C is altered by hypothyroidism in the rat brain. Neuroscience 84, 309–322.

Alvarez-Dolado, M., Ruiz, M., Del Rio, J.A., Alcantara, S., Burgaya, F., Sheldon, M., Nakajima, K., Bernal, J., Howell, B.W., Curran, T., Soriano, E., Munoz, A., 1999. Thyroid hormone regulates reelin and dab1 expression during brain development. J. Neurosci. 19, 6979–6993.

Alvarez-Dolado, M., Cuadrado, A., Navarro-Yubero, C., Sonderegger, P., Furley, A.J., Bernal, J., Munoz, A., 2000. Regulation of the L1 cell adhesion molecule by thyroid hormone in the developing brain. Mol. Cell. Neurosci. 16, 499–514.

Bech-Otschir, D., Kraft, R., Huang, X., Henklein, P., Kapelari, B., Pollmann, C., Dubiel, W., 2001. COP9 signalosome-specific phosphorylation targets p53 to degradation by the ubiquitin system. EMBO J. 20, 1630–1639.

Bernal, J., 2002. Thyroid hormones and brain development, in: Pfaff, D., Arnold, A., Etgen, A., Fahrbach, S., Moss, R., Rubin, R. (Eds.), Hormones, Brain and Behavior, Academic Press, San Diego, in press.

Bernal, J., Nunez, J., 1995. Thyroid hormones and brain development. Eur. J. Endocrinol. 133, 390–398.

Bradley, D.J., Towle, H.C., Young, W.S., 3rd., 1992. Spatial and temporal expression of alpha- and beta-thyroid hormone receptor mRNAs, including the beta 2- subtype, in the developing mammalian nervous system. J. Neurosci. 12, 2288–2302.

Brent, G.A., 1994. The molecular basis of thyroid hormone action. N. Engl. J. Med. 331, 847–853.

Burke, L.J., Baniahmad, A., 2000. Co-repressors 2000. FASEB J. 14, 1876–1888.

Chamovitz, D.A., Segal, D., 2001. JAB1/CSN5 and the COP9-signalosome. A complex situation. EMBO Rep. 2, 96–101.

Chen, J.D., Evans, R.M., 1995. A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377, 454–457.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

Claret, F.X., Hibi, M., Dhut, S., Toda, T., Karin, M., 1996. A new group of conserved coactivators that increase the specificity of AP-1 transcription factors. Nature 383, 453–457.

Coffin, J.D., Harrison, J., Schwartz, S., Heimark, R., 1991. Angioblast differentiation and morphogenesis of the vascular endothelium in the mouse embryo. Dev. Biol. 148, 51–62.

Crawford, P.A., Dorn, C., Sadovsky, Y., Milbrandt, J., 1998. Nuclear receptor DAX-1 recruits nuclear receptor corepressor N-CoR to steroidogenic factor 1. Mol. Cell. Biol. 18, 2949–2956.

Cuadrado, A., Bernal, J., Munoz, A., 1999. Identification of the mammalian homolog of the splicing regulator Suppressor-of-white-apricot as a thyroid hormone regulated gene. Brain Res. Mol. Brain Res. 71, 332–340.

DeLong, G.R., 1990. The effect of iodine deficiency on neuromuscular development. IID Newslett. 6, 1–12.

Deng, X.W., Dubiel, W., Wei, N., Hofmann, K., Mundt, K., Colicelli, J., Kato, J., Naumann, M., Segal, D., Seeger, M., Carr, A., Glickman, M., Chamovitz, D.A., 2000. Unified nomenclature for the COP9 signalosome and its subunits: an essential regulator of development. Trends Genet. 16, 202–203.

Dickson, P.W., Aldred, A.R., Menting, J.G., Marley, P.D., Sawyer, W.H., Schreiber, G., 1987. Thyroxine transport in choroid plexus. J. Biol. Chem. 262, 13907–13915.

Dressel, U., Thormeyer, D., Altincicek, B., Paululat, A., Eggert, M., Schneider, S., Tenbaum, S.P., Renkawitz, R., Baniahmad, A., 1999. Alien, a highly conserved protein with characteristics of a corepressor for members of the nuclear hormone receptor superfamily. Mol. Cell. Biol. 19, 3383–3394.

Dussault, J.H., Ruel, J., 1987. Thyroid hormones and brain development. Annu. Rev. Physiol. 49, 321–334.

Feinberg, A.P., Vogelstein, B., 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132, 6–13.

Ferreiro, B., Pastor, R., Bernal, J., 1990. T3 receptor occupancy and T3 levels in plasma and cytosol during rat brain development. Acta Endocrinol. (Copenh.) 123, 95–99.

Forrest, D., Vennstrom, B., 2000. Functions of thyroid hormone receptors in mice. Thyroid 10, 41–52.

Gall, C.M., Isackson, P.J., 1989. Limbic seizures increase neuronal production of messenger RNA for nerve growth factor. Science 245, 758–761.

Göthe, S., Wang, Z., Ng, L., Kindblom, J.M., Barros, A.C., Ohlsson, C., Vennström, B., Forrest, D., 1999. Mice devoid of all known thyroid hormone receptors are viable but exhibit disorders of the pituitary-thyroid axis, growth, and bone maturation. Genes Dev. 13, 1329–1341.

Goubeaud, A., Knirr, S., Renkawitz-Pohl, R., Paululat, A., 1996. The Drosophila gene alien is expressed in the muscle attachment sites during embryogenesis and encodes a protein highly conserved between plants, Drosophila and vertebrates. Mech. Dev. 57, 59–68.

Henke, W., Ferrell, K., Bech-Otschir, D., Seeger, M., Schade, R., Jungblut, P., Naumann, M., Dubiel, W., 1999. Comparison of human COP9 signalsome and 26S proteasome lid. Mol. Biol. Rep. 26, 29–34.

Hörlein, A.J., Naar, A.M., Heinzel, T., Torchia, J., Gloss, B., Kurokawa, R., Ryan, A., Kamei, Y., Soderstrom, M., Glass, C.K., et al., 1995. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature 377, 397–404.

Lebel, J.M., Dussault, J.H., Puymirat, J., 1994. Overexpression of the beta 1 thyroid receptor induces differentiation in neuro-2a cells. Proc. Natl. Acad. Sci. USA 91, 2644–2648.

Legrand, J., 1984. Effects of thyroid hormones on central nervous system development, in: Yanai, J.E. (Ed.), Neurobehavioural Teratology, Elservier Science Publishers, Amsterdam, pp. 331–363.

Lyapina, S., Cope, G., Shevchenko, A., Serino, G., Tsuge, T., Zhou, C., Wolf, D.A., Wei, N., Deshaies, R.J., 2001. Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome. Science 292, 1382–1385.

Mahalingam, S., Ayyavoo, V., Patel, M., Kieber-Emmons, T., Kao, G.D., Muschel, R. J., Weiner, D.B., 1998. HIV-1 Vpr interacts with a human 34-kDa mov34 homologue, a cellular factor linked to the G2/M phase transition of the mammalian cell cycle. Proc. Natl. Acad. Sci. USA 95, 3419–3424.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., et al., 1995. The nuclear receptor superfamily: the second decade. Cell 83, 835–839.

McKenna, N.J., O'Malley, B.W., 2000. From ligand to response: generating diversity in nuclear receptor coregulator function. J. Steroid Biochem. Mol. Biol. 74, 351–356.

Mellstrom, B., Naranjo, J.R., Santos, A., Gonzalez, A.M., Bernal, J., 1991. Independent expression of the alpha and beta c-erbA genes in developing rat brain. Mol. Endocrinol. 5, 1339–1350.

Morte, B., Manzano, J., Scanlan, T.S., Vennström, B., Bernal, J., 2002. Deletion of the thyroid hormone receptor alpha1 prevents the alterations of the cerebellum induced by hypothyroidism. Proc. Natl. Acad. Sci. USA 99, 3985–3989.

Mundt, K.E., Porte, J., Murray, J.M., Brikos, C., Christensen, P.U., Caspari, T., Hagan, I.M., Millar, J.B., Simanis, V., Hofmann, K., Carr, A.M., 1999. The COP9-signalosome complex is conserved in fission yeast and has a role in S phase. Curr. Biol. 9, 1427–1430.

Munoz, A., Rodriguez-Pena, A., Perez-Castillo, A., Ferreiro, B., Sutcliffe, J.G., Bernal, J., 1991. Effects of neonatal hypothyroidism on rat brain gene expression. Mol. Endocrinol. 5, 273–280.

Muscatelli, F., Strom, T.M., Walker, A.P., Zanaria, E., Recan, D., Meindl, A., Bardoni, B., Guioli, S., Zehetner, G., Rabl, W., et al., 1994. Mutations in the DAX-1 gene give rise to both X-linked adrenal hypoplasia congenita and hypogonadotropic hypogonadism. Nature 372, 672–676.

Naumann, M., Bech-Otschir, D., Huang, X., Ferrell, K., Dubiel, W., 1999. COP9 signalosome-directed c-Jun activation/stabilization is independent of JNK. J. Biol. Chem. 274, 35297–35300.

Norman, C., Runswick, M., Pollock, R., Treisman, R., 1988. Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the c-fos serum response element. Cell 55, 989–1003.

Oppenheimer, J.H., Schwartz, H.L., 1997. Molecular basis of thyroid hormone- dependent brain development. Endocr. Rev. 18, 462–475.

Ordentlich, P., Downes, M., Evans, R.M., 2001. Corepressors and nuclear hormone receptor function. Curr. Top. Microbiol. Immunol. 254, 101–116.

Pastor, R., Bernal, J., Rodríguez-Peña, A., 1994. Unliganded c-ErbA/thyroid hormone receptor induces TrkB expression in neuroblastoma cells. Oncogene 9, 1081–1089.

Polly, P., Herdick, M., Moehren, U., Baniahmad, A., Heinzel, T., Carlberg, C., 2000. VDR-Alien: a novel, DNA-selective vitamin D(3) receptor-corepressor partnership. FASEB J. 14, 1455–1463.

Porterfield, S.P., Hendrich, C.E., 1993. The role of thyroid hormones in prenatal and neonatal neurological development—current perspectives. Endocr. Rev. 14, 94–106.

Potter, G.B., Beaudoin, G.M., 3rd, DeRenzo, C.L., Zarach, J.M., Chen, S.H., Thompson, C.C., 2001. The hairless gene mutated in congenital hair loss disorders encodes a novel nuclear receptor corepressor. Genes Dev. 15, 2687–2701.

Rodriguez-Peña, A., Ibarrola, N., Iniguez, M.A., Munoz, A., Bernal, J., 1993. Neonatal hypothyroidism affects the timely expression of myelin-associated glycoprotein in the rat brain. J. Clin. Invest. 91, 812–818.

Rosenfeld, M.G., Glass, C.K., 2001. Coregulator codes of transcriptional regulation by nuclear receptors. J. Biol. Chem. 276, 36865–36868.

Sambrook, J., Fritsch, E.F., Maniatis, T.E., 1989. Molecular Cloning: A Laboratory Manual, 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Samuels, H.H., Stanley, F., Casanova, J., 1979. Depletion of L-3,5,3′-triiodothyronine and L-thyroxine in euthyroid calf serum for use in cell culture studies of the action of thyroid hormone. Endocrinology 105, 80–85.

Schaefer, L., Beermann, M.L., Miller, J.B., 1999. Coding sequence, genomic organization, chromosomal localization, and expression pattern of the signalosome component Cops2: the mouse homologue of Drosophila alien. Genomics 56, 310–316.

Schwechheimer, C., Deng, X.W., 2000. The COP/DET/FUS proteins-regulators of eukaryotic growth and development. Semin. Cell Dev. Biol. 11, 495–503.

Schwechheimer, C., Serino, G., Callis, J., Crosby, W.L., Lyapina, S., Deshaies, R.J., Gray, W.M., Estelle, M., Deng, X.W., 2001. Interactions of the COP9 signalosome with the E3 ubiquitin ligase SCFTIRI in mediating auxin response. Science 292, 1379–1382.

Spain, B.H., Bowdish, K.S., Pacal, A.R., Staub, S.F., Koo, D., Chang, C.Y., Xie, W., Colicelli, J., 1996. Two human cDNAs, including a homolog of Arabidopsis FUS6 (COP11), suppress G-protein- and mitogen-activated protein kinase-mediated signal transduction in yeast and mammalian cells. Mol. Cell. Biol. 16, 6698–6706.

Swanson, L.W., 1992. Brain Maps: Structure of the Rat Brain. Elservier Science Publishers B. V., Amsterdam.

Tenbaum, S., Baniahmad, A., 1997. Nuclear receptors: structure, function and involvement in disease. Int. J. Biochem. Cell Biol. 29, 1325–1341.

Tomoda, K., Kubota, Y., Kato, J., 1999. Degradation of the cyclin-dependent- kinase inhibitor p27Kip1 is instigated by Jab1. Nature 398, 160–165.

Wei, N., Deng, X.W., 1999. Making sense of the COP9 signalosome. A regulatory protein complex conserved from Arabidopsis to human. Trends Genet. 15, 98–103.

Wolffe, A.P., Collingwood, T.N., Li, Q., Yee, J., Urnov, F., Shi, Y.B., 2000. Thyroid hormone receptor, v-ErbA, and chromatin. Vitam. Horm. 58, 449–492.

Yang, X., Menon, S., Lykke-Andersen, K., Tsuge, T., Di, X., Wang, X., Rodriguez- Suarez, R.J., Zhang, H., Wei, N., 2002. The COP9 signalosome inhibits p27(kip1) degradation and impedes G1-S phase progression via deneddylation of SCF Cull. Curr. Biol. 12, 667–672.

Yoh, S.M., Chatterjee, V.K., Privalsky, M.L., 1997. Thyroid hormone resistance syndrome manifests as an aberrant interaction between mutant T3 receptors and transcriptional corepressors. Mol. Endocrinol. 11, 470–480.

Zamir, I., Harding, H.P., Atkins, G.B., Horlein, A., Glass, C.K., Rosenfeld, M.G., Lazar, M.A., 1996. A nuclear hormone receptor corepressor mediates transcriptional silencing by receptors with distinct repression domains. Mol. Cell. Biol. 16, 5458–5465.