Chicken Ovalbumin Upstream Promoter-Transcription Factor (COUP-TF) Modulates Expression of the Purkinje Cell Protein-2 Gene

A POTENTIAL ROLE FOR COUP-TF IN REPRESSING PREMATURE THYROID HORMONE ACTION IN THE DEVELOPING BRAIN*

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The cerebellar Purkinje cell-specific PCP-2 gene is transcriptionally activated by thyroid hormone during the 2nd and 3rd weeks of postnatal life in the rat. In contrast, thyroid hormone has no detectable effects on PCP-2 expression in the fetal rat. We now present data that suggest that the orphan nuclear receptor chicken ovalbumin upstream promoter-transcription factor (COUP-TF) represses triiodothyronine (T3)-dependent transcriptional activation of PCP-2 in the immature Purkinje cell. Gel shift assays show that the PCP-2 A1TRE and adjoining sequences (−295/−199 region) bind to rat and mouse brain nucleoproteins in a developmentally regulated fashion and that one of these nucleoproteins could be the orphan nucleoprotein COUP-TF. In support of this hypothesis, in vitro translated COUP-TF binds to the −295/−199 region and COUP-TF represses T3-dependent activation of the PCP-2 promoter in transient transfection analyses. Finally, immunohistochemical studies reveal that COUP-TF is specifically expressed in the immature Purkinje cell and that this expression diminishes coincident with thyroid hormone induction of PCP-2 expression. Our findings are consistent with the hypothesis that the presence or absence of inhibitory proteins bound to the thyroid hormone response element of T3-responsive genes governs the responsiveness of these genes to thyroid hormone during brain development.

Thyroid hormone plays an important role in mammalian cerebellar development (1). Thyroid hormone affects development of a variety of cerebellar cells including the Purkinje cell (2). Purkinje cells are generated by embryonic (E)1 day 15 in the fetal rat and migrate to the cerebellar anlage before birth (3). Maturation of these cells is delayed until shortly after birth and is completed during the first few weeks of neonatal life (4).

Migration of immature Purkinje cells and formation of the fetal cerebellar anlage appear to be unaffected by the presence or absence of thyroid hormone (5). In contrast, however, absence of thyroid hormone during the first 2 weeks after birth leads to irreversible reductions in Purkinje cell dendritic growth (2, 6).

We have chosen the Purkinje cell-specific gene Purkinje cell protein-2 (PCP-2) as a model gene with which to study thyroid hormone effects on the developing Purkinje cell. Expression of PCP-2, like development of the Purkinje cell, is only transiently responsive to thyroid hormone stimulation (7, 8). PCP-2 expression is stimulated by thyroid hormone during the 2nd and 3rd weeks of postnatal life in the mouse and rat. Conversely, the gene is refractory to thyroid hormone stimulation in the fetus and early neonate and also in the adult. Other genes, including the cerebellar specific calbindin and myoinositol-1,4,5-triphosphate receptor genes and the oligodendrocyte-specific myelin basic protein gene respond to thyroid hormone in a similar, transient fashion during cerebellar development (7).

Studies from our laboratory have further revealed that the PCP-2 promoter can be directly regulated by T3 (7, 9). We have identified two thyroid hormone response elements (TREs) within the PCP-2 regulatory region, one situated in the upstream region (A1TRE, −295/−268) and a second apparently nonfunctional TRE in the first intron (10, 11).

Immature fetal and early neonatal Purkinje cells express the α isoform of the thyroid hormone receptor (12). Additionally, T3 is present in the late gestational and early neonatal rat brain (13). Nevertheless, the PCP-2 gene in the immature Purkinje cell is refractory to thyroid hormone stimulation (5, 7, 8). We have recently examined two potential mechanisms that could have accounted for the observed T3 nonresponsivity of the PCP-2 gene in the fetal rat: 1) that T3-dependent activation of PCP-2 specifically requires the β1 isoform of the thyroid hormone receptor; and 2) that fetal brain T3 levels are insufficient for activation of the PCP-2 gene.

Since expression of the thyroid hormone receptor isoform β1 in the brain immediately precedes T3-dependent activation of the PCP-2 gene (14), our laboratory had previously raised the possibility that the TRβ1 isoform is specifically required for T3-dependent activation of the PCP-2 gene (7). Sandhofer et al. (8), however, have recently shown that the ontogenic pattern of PCP-2 expression in TRβ null mice (15) is indistinguishable from that of wild type pups, a finding which effectively excludes the possibility that the TRβ gene is essential for appropriate developmental expression of the PCP-2 gene. Additionally, Forrest et al. (15) have not detected deficiencies in the cerebellar development of these mice. Thus, a lack of TRβ1 expression

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1 The abbreviations used are: E, embryonic; P, postnatal; PCP-2, Purkinje cell protein-2; T3, triiodothyronine; TRE, thyroid hormone response element; TR, thyroid hormone receptor; COUP-TF, chicken ovalbumin upstream promoter-transcription factor; BOR, retinoic acid receptor-related orphan nuclear receptor; RXR, retinoid X receptor; CAT, chloramphenicol acetyltransferase, COUP-RE, COUP-response element; T4 thyroxine; bp, base pair.

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cannot account for PCP-2 T3 nonresponsivity in the immature Purkinje cell.

Schwartz et al. (5) have recently determined that inadequate levels of brain T3 also cannot account for PCP-2 T3 nonresponsivity in the immature fetal Purkinje cell. The fetal thyroid begins secreting thyroid hormone on embryonic day 17 in the rat (16). Brain T3 levels rise approximately 10-fold from this stage of embryogenesis until birth (13). After birth, brain T3 levels continue to increase rapidly and reach adult normal levels by postnatal day 2 (see below). Although T3 is clearly present in the late gestational and early neonatal rat brain, hypothyroidism does not measurably affect PCP-2 expression until approximately 1 week after birth (7, 8). A potential explanation for this T3 nonresponsivity is that T3 levels in the late gestational and early neonatal rat brain are insufficient for T3-dependent activation of PCP-2 expression. Schwartz et al. (5), however, found that the PCP-2 gene remains unresponsive to T3 stimulation in the late gestational rat fetus even if fetal brain T3 levels are artificially boosted to adult normal levels. Thus, low brain T3 levels do not account for PCP-2 T3 nonresponsivity during late gestational cerebellar development.

We have recently demonstrated that thyroid hormone signaling can be inhibited by trans-acting factors that bind to specific cis-elements within the regulatory regions of thyroid hormone responsive genes (9). We observed that cis-elements within the PCP-2 upstream regulatory region inhibit T3-dependent activation of the PCP-2 promoter in transient transfection assays. These cis-elements (−267/−199 region) are located immediately 3′ to the PCP-2 TRE (−295/−268 region). Thyroid hormone signaling can also be inhibited by trans-acting factors that compete with thyroid hormone receptors for binding to thyroid hormone response elements (17–20).

The recent identification of the orphan nuclear receptor COUP-TF has focused attention on the role of these receptors in repressing early PCP-2 expression during early Purkinje cell development. COUP-TF could be an inhibitory trans-acting factor involved in regulating PCP-2 expression early in Purkinje cell development.

EXPERIMENTAL PROCEDURES

Animals and T3 Assay—Pregnant female Swiss Webster mice and Sprague-Dawley rats were purchased from Harlan (Madison, WI). Postnatal day 0 corresponds to the 1st day after birth. Excess T3 levels in the newborn rat brain were achieved by a single intraarterial injection of thyroxine (T4, 100 µg of T4/100 g body weight) to the dam on day 21 of gestation. Such treatment has previously been demonstrated to increase concentrations of T3 in the fetal brain (5, 21). Excess levels of hormone were maintained postnatally by injecting pups with thyroxine on a daily basis. Pups were injected daily with 1 µg of T4 through postnatal day 7. From P8 to P14 pups were injected daily with 2 µg of T4 and with 4 µg of T4 from P15 to P21. Pups receiving this regimen of treatment were born with levels of brain T3 greater than euthyroid adult levels and remained elevated in these treated pups as they continued to develop (data not shown). The concentration of brain T3 was assessed by the radioimmunoassay of Surks et al. (22). Extraction of the iodothyronines from the brains was performed as described by Morreale de Escobar et al. (16).

RNA Isolation and Northern Blot Analysis—Total RNA was extracted from the brains of individual rats (four per data point) by the method of Chomczynski and Sacchi (23) and purified by repetitive organic and salt washings (14). The relative mass of mRNA was determined by Northern blot analysis as described previously (14). Briefly, 10-µg aliquots of total RNA were electrophoresed on a 1% agarose formaldehyde gel and transferred to Magna nylon membranes (MSI, Northridge, MA). Hybridizations and washes were performed at 42 °C with a 32P-labeled PCP-2 DNA probe. The PCP-2 probe was a gift of Dr. Harry Orr (University of Minnesota). Blots were washed and subsequently exposed to phosphor screens (Molecular Dynamics, Sunnyvale, CA) and scanned (PhosphorImager 445 SI, Molecular Dynamics, Sunnyvale, CA). The images were quantified using ImageQuant 1.0 (Molecular Dynamics, Sunnyvale, CA). Samples were corrected for variations in loading by calculating the ethidium bromide staining of the 28 S and 18 S ribosomal RNA as described by Correa-Rotter et al. (24).

Nuclear Extract Preparation and In vitro Translational of Receptors—Mouse brain nuclear extracts were prepared from pooled brains (3–12 animals) of mice of the indicated ages using a modification of the Dignam procedure (10). Extractions were carried out in the presence of protease inhibitors as described previously (9). Protein concentrations were determined using the Bradford assay (25). In vitro translational receptors were prepared as described previously (10). Rat TRα1 cDNA was obtained from H. Towle (University of Minnesota), mouse RXRβ cDNA from K. Ozato (National Institutes of Health), and mouse COUP-TFI from L. Jonk (University of Groningen) (26).

Transient Transfection Assays—Neuro-2a cells were transfected transiently using the calcium phosphate method as described previously (9). Cells were cultured on 100-mm plates and cotransfected with 5 µg of 2PCP-2-CAT (9) and 5 µg of the indicated receptor expression vectors together with 100 ng of RSV-Luc and 10 µg of salmon testes DNA. Transfected cells were cultured in the absence or presence of 100 nM T3 and harvested after 36 h. Luciferase activity was determined using an aliquot of the cell extract and was used as a control in assessing transfection efficiency (7). CAT assays were carried out as described previously (9). Relative CAT activity is reported as CAT activity divided by the transfection efficiency as determined by the luciferase assay. Each set of transgenic embryos was transfected in duplicate three times and reported as mean ± S.D. Fold induction is reported as the relative CAT activity of transfected cells cultured in the presence of T3 divided by the relative CAT activity of transfected cells cultured in the absence of T3. The DNA's used were A1PCP-2-CAT (9), COUP-TFI pSGS (26), pSGS, and CDMDTRα1 (10). The empty expression vector pSGS was prepared by removing the COUP-TFI cDNA from COUP-TFI.
pSG5 by EcoRI restriction, removal of the insert, and religation of the vector.

Immunohistochemistry—Fresh mouse brains were fixed in neutral formalin for 1–4 h and then placed in 70% ethanol for at least 4 h. The brains were embedded in Amerfin (American Scientific Products, Minneapolis, MN). Sections of 8 μm each were cut, floated onto a organosilane-treated slide in a 50 °C water bath, and dried. Slides were subsequently heated at 60 °C for 30 min, deparaffinized in mixed xylenes twice for 10 min, and rehydrated in graded ethanol (100, 80, and 70%). The tissue sections were then placed in 0.01 M sodium citrate (pH 6.0), heated to near boiling in a microwave for 10 min, and slowly cooled to room temperature. The slides were then rinsed in water and soaked in phosphate-buffered saline for 10 min. This procedure has been previously used to aid in the immunohistochemical detection of antigens in fixed tissues (31).

Calbindin expression was detected using the Histomouse kit from Zymed Laboratories Inc. (South San Francisco, CA), and COUP-TF expression was detected using Zymed’s Histostain-SP kit. The Histomouse kit is designed for detection of mouse antigens using mouse monoclonal antibodies, and the Histostain-SP kit is designed for detection of mouse antigens using rabbit primary antibody. The enzyme used for generating a signal is horseradish peroxidase. The peroxidase-stained tissue sections were not counterstained. Anti-calbindin antibody (Sigma) was used at a 1:500 dilution; anti-COUPTF antibody was used at a 1:1000 dilution, and nonimmune rabbit serum was used at a 1:1000 dilution.

RESULTS

Premature Elevations of Brain T3 Levels Do Not Result in Acceleration of PCP-2 Gene Expression in the Early Rat Neonate—Previous studies have shown that PCP-2 T3 nonresponsivity in the fetal rat is not due to insufficient levels of brain T3 (5). We now present a similar series of experiments designed to determine whether the PCP-2 T3 nonresponsivity in the early neonatal rat (7, 8) is due to insufficient levels of brain T3. Examination of brain T3 levels during development revealed that brain T3 does not reach adult normal levels until approximately postnatal day 2 in the rat (Fig. 1).

Fig. 1 further illustrates that for the first 5 days after birth, administration of excess T3 did not result in greater PCP-2 mRNA levels in T4-treated animals compared with euthyroid unmanipulated pups. Excess T3 did not significantly up-regulate PCP-2 expression until after postnatal day 5 when the levels of PCP-2 mRNA in the T4-treated animals began to exceed the values achieved in the euthyroid unmanipulated pups (day 10, p < 0.01). Thus, excess T3 is capable of accelerating the rate of brain PCP-2 expression above that produced by the euthyroid pups but not until after postnatal day 5. In conjunction with previously published results (7, 8), these data support the view that the PCP-2 gene is refractory to thyroid hormone from late gestation (5) until approximately 1 week after birth (Fig. 1). Of additional interest is the finding that on day 20 the levels of PCP-2 mRNA achieved are similar for the T3-treated and euthyroid animals, thus again demonstrating the transient effects of thyroid hormone on PCP-2 expression (7, 8).

Developmentally Regulated Mouse Brain Nucleoproteins Bind to the PCP-2 TRE—We recently reported data consistent with the presence of developmentally regulated inhibitors of thyroid hormone action in the immature Purkinje cell (9). These experiments revealed that rat brain nuclear proteins bound to the −295/−199 region of the PCP-2 gene and that the concentration of these nucleoproteins appeared to be present at the highest levels in fetal rat brain nuclear extracts.

In the present studies we have attempted to define more precisely the age-related course of nucleoprotein binding to the −295/−199 region as assessed by electrophoretic mobility shift assay (Fig. 2).2 Binding activity was strongest in the late gestational fetus (E18) and weakest in the mature adult brain (P90) (Fig. 2A). The binding activity declined with age and was reciprocally related to PCP-2 transcriptional activity during brain development (Fig. 1) (7, 8). The fall in binding activity was not due to the presence of an inhibitory factor in adult nuclear extracts since an equal mixture of fetal and adult nuclear extracts still formed binding complexes on the −295/−199 region of the PCP-2 gene and that the presence of developmentally regulated inhibitors of thyroid hormone action in the immature Purkinje cell (9).

2 All gel shift experiments were performed using nuclear extracts obtained from the brains of both rats and mice. Identical results were obtained using either rat or mouse extracts. Only the experiments using mouse nuclear extracts are presented in this article.
sites (AGGCCTTCTCAGGTCAGAGACCAGGAGA; the individual half-sites are underlined) (10). Mutation of either the 5' or 3' half-sites diminished but did not abolish the T3 responsivity of the A1TRE (10). Mutation of the central half-site abolished the T3 responsivity of the A1TRE (10).

Competition studies revealed that the A1TRE competed for fetal mouse brain nucleoprotein binding to the 2295/2199 region (Fig. 2C, lanes 2–4) as previously reported (9). Mutation of either the 5' or 3' half-sites did not abolish the ability of the A1TRE to compete with the 2295/2199 region for fetal mouse brain nucleoprotein binding (Fig. 2C, lanes 8–13). Mutation of the middle half-site, however, did abolish the ability of the A1TRE to compete for nucleoprotein binding (lanes 5–7). These findings suggested that the middle half-site of the A1TRE is required for the observed binding of mouse brain nucleoproteins to the 2295/2199 region.

Synthesized TREs containing the consensus sequence AGGTCA arranged as either a direct repeat with a 4-bp spacer (lanes 20–22) or as an inverted repeat with a 6-bp spacer (lanes 23–25) also competed for nucleoprotein binding (Fig. 2C). These findings suggest that the A1TRE binding nucleoproteins bind to TREs that differ in both sequence, half-site orientation, and spacing. However, the nucleoproteins do not require a functional TRE for binding since a defective A1TRE, containing both 5' and 3' half-site mutations, competes for nucleoprotein binding to the −295/−199 region (Fig. 2C, lanes 14–16). Finally, fetal nucleoprotein binding to the −295/−199 region is sequence-specific since a random sequence (lanes 17–19) and the binding site for another transcription factor (nuclear transcription factor-I, NF-I; lanes 26–28) do not compete.

COUP-TF Binding to the PCP-2 −295/−199 Region—The developmentally regulated −295/−199 region-binding nucleoproteins are present at their highest levels in the fetal and early neonatal mouse brains (Fig. 2). During this period the PCP-2 gene is unresponsive to T3 stimulation (5, 7, 8). The temporal association between the presence of these proteins and the absence of T3 stimulation of PCP-2 expression suggested that these proteins may play a role in repressing T3-dependent transactivation of PCP-2 in the fetus and early neonate. To test this hypothesis we attempted to identify the 2295/2199 region-binding nucleoproteins. A review of the literature raised the possibility that the orphan nuclear receptor COUP-TF could play such a role since this nucleoprotein 1) is expressed in the developing rat brain (26, 32, 33), 2) can bind to thyroid hormone response elements (30), and 3) can act as a repressor of thyroid hormone action by competing for TRE binding with the thyroid hormone receptor (30).

We tested for the presence of COUP-TF in the −295/−199 region-mouse brain nucleoprotein complex by supershift analysis. Three specific shift complexes were observed. The arrowhead refers to a nonspecific band observed in the probe alone lane. Quantitation of the shifted bands was determined by using a PhosphorImager and the ImageQuant computer program. Quantitation of each lane is contrasted to the gel shift of the E18 nuclear extract as fraction of E18. E, embryonic; P, postnatal. B, the −295/−199 region 32P-labeled DNA fragment was incubated with either 5 μg of E18 or P90 nuclear extract or 10 μg of E18 + E90 nuclear extract. C, the −295/−199 region 32P-labeled DNA fragment was incubated with 5 μg of E18 nuclear extract plus equivalent molar ratios of specific competitor DNAs. Arrows note the position of shifted bands. The three complexes are compressed into two bands in this panel due to a shorter time of electrophoresis.
SUPPLEMENTARY ONLINE DATA

Fig. 3. Fetal mouse brain nucleoproteins that bind to the −295/−199 region include the orphan nuclear receptor COUP-TF. A, the −295/−199 region 32P-labeled DNA fragment was incubated with 5 μg of E18 mouse brain nuclear extract and run on a polyacrylamide gel. Arrowheads mark the resulting shifted complexes and are referenced as complexes a, b and c. Arrows mark the positions of the complexes after supershifting. All lanes originate from the same gel and autoradiograph. B, the −295/−199 region 32P-labeled DNA fragment was incubated with in vitro translated TRα1 and RXRβ. These translation products bind to the A1TRE which is contained within the −295/−199 region (10). The arrow marks the position of the TRα/RXR supershift. C, 32P-labeled COUP-RE was incubated with in vitro translated COUP-TFI and RXRβ under the conditions described by Zou et al. (10). The arrowhead marks the position of the COUP-TFI supershift. * refers to a nonspecific band contributed by the rabbit reticulocyte lysate. D, 32P-labeled COUP-RE was incubated with in vitro translated COUP-TFI and RXRβ. The incubation conditions are those used by Sagami et al. (27).

supershift of two of the three complexes (Fig. 3A, lane 2, complexes a and c). Addition of antibody specific for the thyroid hormone receptor isoform α1 resulted in a supershift of the third complex (Fig. 3A, lane 3, complex b). Addition of both antibodies resulted in a supershift of all three complexes (Fig. 3A, lane 4). Addition of antibody specific for the β isoform of the thyroid hormone receptor did not result in a supershift (data not shown). These data suggest that both COUP-TF and TRα1 are present in fetal mouse brain nuclear extracts and form specific complexes on the −295/−199 region DNA target. The anti-COUP-TF and anti-TRα1 antibodies supershifted different complexes. This finding suggests that COUP-TF and TRα1 probably compete for the same binding site within the −295/−199 region.

Recent studies have shown that the orphan receptor ROR plays an important role in Purkinje cell development and in the expression of PCP-2 (34). ROR binds as a monomer to DNA sequences containing the consensus sequence TAAAGGTCA (35, 36). Recently, Schrader et al. (37) identified an ROR-binding site within the PCP-2 proximal promoter region. As the A1TRE also contains the sequence AGGGTCA, we queried whether ROR was one of the fetal mouse brain nucleoproteins bound to the −295/−199 region. However, addition of anti-ROR antibody did not result in a supershift, thus suggesting that ROR is not one of the −295/−199 region-binding nucleoproteins (Fig. 3A, lane 5).

Fig. 3B documents the capacity of anti-TRα1 antibody to specifically supershift TRα1/RXRβ heterodimers bound to the −295/−199 region. The inability of anti-COUP-TF antibody to supershift this complex demonstrates the specificity of this antibody (Fig. 3B). Fig. 3C documents the ability of anti-COUP-TF antibody to supershift in vitro translated COUP-TFI/RXRβ heterodimers bound to a COUP-response element (COUP-RE). Interestingly, we observed that formation of COUP-TFI/RXRβ heterodimers versus COUP-TFI homodimers/monomers on the COUP-RE was dependent on the assay conditions utilized (Fig. 3, C and D).

Fetal mouse brain nucleoproteins recognized by anti-COUP-TF antibody bind to the −295/−199 region DNA fragment. To determine whether in vitro translated COUP-TF binds to specific sequences within the −295/−199 region, we performed a competitive mobility shift assay. In this assay we incubated labeled COUP-RE with in vitro translated COUP-TFI and various amounts of cold competitor DNA. We observed that the PCP-2 A1 TRE (−295/−268) competed with the labeled COUP-RE for COUP-TFI binding (Fig. 4A, lanes 7–10). The affinity, however, of COUP-TFI for the COUP-RE is significantly greater than the affinity of COUP-TFI for the A1TRE (Fig. 4A, lanes 4–6 versus 7–10). In keeping with the reduced affinity of COUP-TFI translation products for the A1TRE, we observed little if any binding of COUP-TFI to the −295/−199 region DNA probe either as homo- or heterodimers (Fig. 4B). Finally, we found that neither the PCP-2 −267/−199 region (Fig. 4A, lanes 11–14) nor a nonspecific DNA fragment (Fig. 4A, lanes 15–18) competed with the labeled COUP-RE for COUP-TFI binding. These findings demonstrate that COUP-TF binds to the TRE located within the PCP-2 −295/−199 region.

COUP-TFI Represses T3-dependent Activation of the PCP-2 Promoter—The gel shift data suggest a role for COUP-TF in controlling T3 regulation of PCP-2 expression during specific stages of brain development. To determine whether COUP-TF can specifically repress expression of the PCP-2 promoter in vivo, we performed transient transfection experiments. The PCP-2 reporter construct used in these experiments contained 630 bp of PCP-2 sequence: approximately 240 bp of regulatory sequence upstream of the start site of transcription, the first exon, the first intron, and part of the second exon fused to chloramphenicol acetyltransferase (CAT) as a reporter. This construct (A1ΔPCP-2-CAT) contains the A1 TRE but not the previously described −267/−199 region and has been characterized in a separate series of experiments (9). Deletion of the −267/−199 region is required for T3-dependent activation of the PCP-2 promoter (9). The reporter construct, TK26pal, was used as a control for COUP-TF-mediated repression of T3-dependent transcriptional activation. TK26pal contains the palindromic TRE inserted into the herpes simplex virus thymidine kinase promoter. COUP-TF has been demonstrated to bind to the palindromic TRE with high affinity and can block T3-dependent transactivation mediated by this response element (30). A1ΔPCP-2-CAT and TK26pal were individually co-transfected into neuroblastoma Neuro-2a cells with a T3Ra1 expression vector and either the COUP-TFI expression vector (COUP-TFI pSG5) or the expression vector minus the COUP-TFI
coding sequence (pSG5). Transfected cells were then cultured in the presence or absence of T3. Analysis of reporter gene activity revealed that T3-dependent activation of the control TK28pal was significantly inhibited by co-transfection of the COUP-TFI expression vector (Fig. 5A). When the A1DPCP-2-CAT reporter construct was transfected into Neuro-2a cells, we found that T3 activated reporter gene activity approximately 10-fold (Fig. 5B). Co-transfection of the COUP-TFI expression vector COUP-TFI pSG5, however, significantly repressed (from approximately 10- to 4-fold activation) the T3-dependent activation of A1DPCP-2-CAT (Fig. 5B). These data demonstrate that COUP-TFI can repress T3-dependent activation of the PCP-2 promoter and are consistent with a role for COUP-TF in rendering the PCP-2 promoter refractory to thyroid hormone in developing Purkinje cells.

COUP-TF Modulation of T3 Action in the Brain

COUP-TF Expression in Developing Purkinje Cells—The finding that one of the rat brain nucleoproteins that bind to the −295/−199 region is COUP-TF supported the hypothesis that COUP-TF plays a role in repressing T3-dependent transcriptional activation of PCP-2 during Purkinje cell development. As PCP-2 is expressed only in cerebellar Purkinje cells, a direct test of the hypothesis required determining whether COUP-TF is expressed in Purkinje cells and if so whether the expression is developmentally regulated. We therefore initiated studies to determine whether COUP-TF is expressed in Purkinje cells in an age-dependent fashion.

Purkinje cells originate from the germinal zone of the rhombic lip and are generated by embryonic day 15 in the rat (38). Immature Purkinje cells migrate to the cerebellar anlage shortly before birth and form the Purkinje cell layer. As the calbindin gene is expressed exclusively within Purkinje cells in the cerebellum (39), immunohistochemical detection of the calbindin protein allows identification of developing Purkinje cells. To assess the expression of COUP-TF within developing Purkinje cells, we harvested brains from mice of the ages indicated in Fig. 6. The brains were processed and sectioned for use in immunohistochemistry. Sequential sections of tissue were individually stained with either anti-calbindin or anti-COUP-TF antibody.

We observed that COUP-TF was expressed in the nuclei of a specific population of cells within the cerebellar anlage on embryonic day 18 (E18) fetuses (Fig. 6). Even though the immature Purkinje cell does not yet express the calbindin marker (Fig. 6), the position of COUP-TF-positive cells in the cerebellar anlage is consistent with the position of migrating immature Purkinje cells (40). Immature Purkinje cells can be directly identified by postnatal day 0 (P0) onward as these cells have begun to express the calbindin protein (Fig. 6).

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COUP-TF Modulation of T3 Action in the Brain

COUP-TF (Fig. 5, P9 and P12). An extranuclear ring of staining was detected around the nuclei of some Purkinje cells from older animals (Fig. 6, P12). This pattern of staining could either reflect exclusion of COUP-TF from the Purkinje cell nuclei in these older animals or could represent an experimental artifact. Such a staining pattern was not seen, however, in sections incubated with nonimmune serum as a negative control (data not shown). Thus, the immunohistochemical studies revealed that COUP-TF is expressed in Purkinje cell nuclei during the period of development when PCP-2 expression is refractory to T3 and that nuclear COUP-TF expression diminishes at approximately the time when T3 accelerates PCP-2 expression.

**DISCUSSION**

Thyroid hormone facilitates normal development of the cerebellar Purkinje cell. Thyroid hormone mediates this control in part by modulating the transcription of specific genes expressed in the Purkinje cell at a developmentally appropriate time. Activation of these genes, however, is not solely dependent on the expression of T3 receptors or the attainment of critical levels of brain T3. Indeed, we have demonstrated that these genes are refractory to T3 in the immature Purkinje cell. Thus, the Purkinje cell appears to exert a local, cell-specific control over thyroid hormone action during a discrete phase of development. Release of this control during the next stage of Purkinje cell differentiation allows thyroid hormone to regulate gene transcription at a developmentally appropriate time.

Our findings suggest that in the immature Purkinje cell such local control may be mediated by inhibitory transcription factors. Specifically, we have implicated the orphan nuclear receptor COUP-TF as a potential inhibitor of thyroid hormone action in the immature Purkinje cell. The predominant patterns of COUP-TF expression within the developing rat and mouse have prompted others to suggest a role for COUP-TF in the hormonal control of gene expression during mammalian development (42). Indeed, COUP-TFII has already been implicated in the control of thyroid hormone action in developing muscle by regulating thyroid hormone activation of specific myoD family members (43). COUP-TFII has recently been shown to be required for appropriate development of a subset of neurons in the peripheral nervous system (44). Inactivation of COUP-TFI resulted in defective morphogenesis of the glossopharyngeal ganglion and aberrant nerve projection and arborization.

COUP-TF is widely expressed in the developing mouse brain (26, 32, 41, 45) including the cerebellar primordia (33). These patterns of expression are consistent with an important role for COUP-TF in brain development. There are few examples, however, of COUP-TF-regulated neural genes. COUP-TF has been demonstrated to repress transcription of the human transferrin gene in neuronal cells (46), block retinoic acid-induced differentiation of neuronal teratocarcinoma PCC7 cells (47), and regulate retinoic acid-dependent induction of the neural specific arrestin gene (33).

In the present study we propose that COUP-TF expression in the immature Purkinje cell renders the PCP-2 gene refractory to thyroid hormone stimulation during the early period of Purkinje cell development. We advance a simple model of inhibition in which COUP-TF competes with thyroid hormone receptors for binding to the PCP-2 A1TRE. This model is in keeping with several reports documenting a competitive mechanism for COUP-TF-mediated repression of hormonal transcriptional activation (reviewed by Tsai and Tsai (42)). Both COUP-TF and T3 receptors bind to the A1TRE (Fig. 4) and are present within the immature Purkinje cell (Fig. 6 (12)). Additionally, gel shift analyses showed that anti-COUP and anti-TR1 antibodies supershift different fetal nucleoprotein–295/–199 region complexes (Fig. 3). These findings suggest that COUP-TF and TR1 compete for binding to the same cis-element. If so, it is likely that as the relative concentrations of COUP-TF, T3, and thyroid hormone receptor change during development, the outcome of competition for binding to the A1TRE will also change. Thus, our finding that COUP-TF expression can still be detected in Purkinje cells that have begun to express PCP-2 in a T3-dependent fashion (Fig. 1 and Fig. 6, P7) is consistent with the competitive hypothesis.

**In vitro** translated COUP-TFI binds to the A1TRE with lower affinity than COUP-TF to the COUP-RE (Fig. 4, A and B). The COUP-TF found in fetal mouse brain nuclear extracts, however, bound avidly to the 295/199 region (Figs. 2 and 3). These findings suggest that COUP-TF and TR1 compete for binding to the same cis-element. If so, it is likely that as the relative concentrations of COUP-TF, T3, and thyroid hormone receptor change during development, the outcome of competition for binding to the A1TRE will also change. Thus, our finding that COUP-TF expression can still be detected in Purkinje cells that have begun to express PCP-2 in a T3-dependent fashion (Fig. 1 and Fig. 6, P7) is consistent with the competitive hypothesis.

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**FIG. 6.** Immunohistochemical analysis of COUP-TF expression in developing Purkinje cells. Sequential, 8-μm sections of brains from mice of the indicated ages were incubated with either anti-calbindin, anti-COUP-TF antisera, or nonimmune rabbit serum followed by peroxidase-
hanced COUP-TF binding to the −295/−199 region in vitro (Fig. 4B). Supporting the hypothesis of a heterodimeric partner, however, is the finding that both the A1TRE (−295/−268) and the −267/−199 region compete for fetal nucleoprotein binding to the −295/−199 region in gel shift analyses (9). It is thus possible that a nucleoprotein bound to the −267/−199 region facilitates COUP-TF binding to the A1TRE. This hypothesis is in keeping with the T3 response silencing function we have previously reported for the −267/−199 region (9). The presence or absence of this putative heterodimeric partner may also influence the T3-dependent response of PCP-2 during Purkinje cell development.

COUP-TF may also regulate the T3-dependent transcriptional activation of other Purkinje cell-specific genes such as calbindin and the myoinositol-1,4,5-triphosphate receptor. Like PCP-2, both of these genes exhibit a phasic pattern of thyroid hormone responsiveness (7). Whether COUP-TF plays a role in modulating thyroid hormone action in other brain cells remains unclear. The ubiquitous pattern of COUP-TF expression during brain development makes it likely, however, that COUP-TF modulation of thyroid hormone action in the brain is not limited to the Purkinje cell. The transient sensitivity of T3-dependent genes to T3 during brain development contrasts sharply with the continual, nonphasic T3-dependent regulation of lipogenic (48) or thyrotropin subunit genes (49). These genes may not be subject to the same control of thyroid hormone action as the genes regulated by thyroid hormone during development.

The postnatal rise in brain T3 results in widespread distribution of hormone throughout the brain. We propose, however, that the rise in brain T3 may not be the ultimate driving force behind the initiation of all T3-dependent developmental processes. Rather, gatekeeper transcription factors like COUP-TF may control thyroid hormone action in individual cells during discrete stages of cell development. Developmental changes in the expression of these factors may ultimately allow initiation of the T3 response. Local control of thyroid hormone action may thus provide cell- and stage-specific precision in the timing of thyroid hormone signaling during brain development.

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