

Acute Changes in Maternal Thyroid Hormone Induce Rapid and Transient Changes in Gene Expression in Fetal Rat Brain

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Despite clinical evidence that thyroid hormone is essential for brain development before birth, effects of thyroid hormone on the fetal brain have been largely unexplored. One mechanism of thyroid hormone action is regulation of gene expression, because thyroid hormone receptors (TRs) are ligand-activated transcription factors. We used differential display to identify genes affected by acute T₄ administration to the dam before the onset of fetal thyroid function. Eight of the 11 genes that we identified were selectively expressed in brain areas known to contain TRs, indicating that these genes were directly regulated by thyroid hormone. Using *in situ* hybridization, we confirmed

that the cortical expression of both neuroendocrine-specific protein (NSP) and Oct-1 was affected by changes in maternal thyroid status. Additionally, we demonstrated that both NSP and Oct-1 were expressed in the adult brain and that their responsiveness to thyroid hormone was retained. These data are the first to identify thyroid hormone-responsive genes in the fetal brain.

Key words: thyroid hormone; neuroendocrine-specific protein; NSP; Oct-1; POU-domain; cerebral cortex; brain development; differential display; congenital hypothyroidism

It is well known that thyroid hormone is essential for normal mammalian brain development (Oppenheimer and Schwartz, 1997). However, until recently it was generally believed that the effects of thyroid hormone on brain development occur only after birth (Fisher, 1999). Several recent clinical observations provide evidence to the contrary. First, thyroid hormone of maternal origin crosses the placenta and reaches the fetus (Vulsma et al., 1989; Contempre et al., 1993). In addition, the receptors for thyroid hormone (TRs) are expressed in the fetal brain before the onset of fetal thyroid function, and receptor occupancy is within the range known to elicit physiological effects (Bernal and Pekonen, 1984; Ferreira et al., 1988). Second, iodine therapy prevents neurological cretinism in regions of endemic goiter only if initiated before the beginning of the third trimester (Cao et al., 1994). Moreover, children born to pregnant women with untreated hypothyroidism during the second trimester exhibit measurable neurological deficits despite normal circulating thyroid hormone at birth (Haddow et al., 1999; Pop et al., 1999). These findings strongly suggest that thyroid hormone, perhaps of maternal origin, plays important roles in brain development before birth.

The present study was initiated to test the broad hypothesis that thyroid hormone of maternal origin can exert direct effects on brain development before birth. Our strategy for testing this hypothesis was based on the recognition that thyroid hormone of

maternal origin can reach the rat fetus (Obregon et al., 1984; Porterfield and Hendrich, 1992) and that TRs are expressed in the fetal rat brain before the onset of fetal thyroid function (Perez-Castillo et al., 1985; Bradley et al., 1992; Falcone et al., 1994). Therefore, considering that TRs are ligand-activated transcription factors (Lazar, 1993, 1994; Mangelsdorf and Evans, 1995), we predicted that thyroid hormone of maternal origin can affect fetal brain development by regulating the expression of specific genes.

The classes of genes regulated by thyroid hormone in the fetal brain were difficult to predict because thyroid hormone is known to influence many cellular and developmental processes in the postnatal brain (Dussault and Ruel, 1987), and the classes of genes supporting these processes may be widely varied. Therefore, we used the nonbiased method of differential display (Liang and Pardee, 1992) to identify putative thyroid hormone-responsive genes in the fetal cortex. To ensure that we identified genes regulated by thyroid hormone of maternal origin, we focused on the period of fetal development before the onset of fetal thyroid function (Fisher et al., 1977).

We now report the identification of several putative thyroid hormone-responsive genes that are selectively expressed in areas of the fetal brain that also express TRs. In addition, we have further characterized the response to thyroid hormone of two of these genes, one encoding neuroendocrine-specific protein (NSP) and one encoding Oct-1, in both the fetal and adult cortex.

MATERIALS AND METHODS

Animals. All animal procedures were performed in accordance with the National Institutes of Health Guidelines for animal research and were approved by the University of Massachusetts-Amherst Institutional Animal Care and Use Committee.

Experiment 1: Identification of thyroid hormone-responsive genes in the fetal cortex using differential display. Nulliparous female Sprague Dawley rats ($n = 10$; Charles River, Wilmington, MA) were surgically thyroidectomized, provided with 0.1% calcium chloride in the drinking water

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Table 1. Putative thyroid hormone-responsive genes identified by differential display (Experiment I)

cDNA fragment ^a	Match in GenBank	DNA identity %	GenBank accession no.	Reference	mRNA localization in GD16 fetus
2A	Cl-13/ NSP/ <i>s-rex_b</i>	97.7 85.6 98.4	X52817 L10333 U17604	Wieczorek and Hughes, 1991 Roebroek et al., 1993 Baka et al., 1996	Cortex, hippocampus, hypothalamus, diencephalon, midbrain, medulla, spinal cord, trigeminal ganglion
18C	Oct-1	99.0	U17013	J. N. Buskin, unpublished data	Cortex, hippocampus, diencephalon, midbrain, retina, liver
6C	None	N/A	AF133273 ^b		Cortex, hippocampus, diencephalon, midbrain, spinal cord, liver
15A	None	N/A	AF133274 ^b		Cortex, retina, liver
4C	None	N/A	AF133275 ^b		Cortex, midbrain, liver
5C	None	N/A	AF133276 ^b		Cortex, midbrain, liver
17C	BC1	98.0	M16113	DeChiara and Brosius, 1987	All tissues
17CB	Ribosomal protein L31	91.9	X04809	Tanaka et al., 1987	Cortex, hippocampus, midbrain, liver
18AL ^c		N/A			Global expression
18AS ^c		N/A			Global expression
14C ^c		N/A			Global expression

^a RNAimage primers used to generate cDNA fragments: number indicates H-AP forward arbitrary primer (1–8), letter indicates H-T₁₁M reverse primer (H-T₁₁A, H-T₁₁C, or H-T₁₁G), and a prefix of 1 indicates upregulation by thyroid hormone. For example, fragment 18C is upregulated by thyroid hormone and was generated using H-AP8 and H-T₁₁C primers.

^b Submitted to GenBank at the time of manuscript submission.

^c Fragments not sequenced. Global expression pattern revealed by *in situ* hybridization suggested that these fragments were falsely identified differential display products.

(Zoeller et al., 1988), and maintained on normal rat chow. Two weeks after thyroidectomy, the females were paired with males overnight; the presence of sperm in a vaginal smear the following morning indicated mating, and this day was considered gestational day (GD) 1. Dams were given subcutaneous injections of either thyroxine [T₄; 1.25 µg/100 gm body weight (BW); Sigma, St. Louis, MO] or saline at 9 A.M. and 4 P.M. on GD15. At 9 A.M. on GD16, dams were decapitated, and trunk blood was collected for measurement of serum T₄. Half of the fetuses from each litter were frozen intact on pulverized dry ice and stored at –80°C. Parasagittal sections of these GD16 fetuses were later collected for *in situ* hybridization. The cortex was dissected from the remaining fetuses, frozen on pulverized dry ice, and stored at –80°C until extraction of RNA.

Total RNA was isolated from the cortex of one GD16 fetus obtained from a dam with undetectable circulating total T₄ and from the cortex of one GD16 fetus from a dam with normal levels of total T₄ (23.1 ng/ml). Total RNA was extracted using an acid-phenol extraction procedure (Chomczynski and Sacchi, 1987). This RNA was subjected to reverse transcription (RT) and amplified by RT-PCR according to the manufacturer's instructions (RNAimage, GenHunter, Nashville, TN). The PCR products amplified from each RNA pool using 16 primer pair combinations were electrophoresed in adjacent lanes on a 6% denaturing polyacrylamide gel. The gel was dried and apposed overnight to BioMax autoradiographic film (Kodak, Rochester, NY). Gene fragments that on film appeared to be either upregulated or downregulated by T₄ injection were extracted from the gel, reamplified, and cloned into pCRII (Invitrogen, Carlsbad, CA) or pBluescript KS (Stratagene, La Jolla, CA) (Table 1). Sequencing was performed using ABI FS-Dye-Terminator chemistry (PE Applied Biosystems, Foster City, CA).

Experiment II: Verification of thyroid hormone-responsive genes in the fetal brain. Nulliparous female Sprague Dawley rats ($n = 44$; Zivic Miller, Pottersville, PA) were exposed to the goitrogen 2-mercapto-1-methylimidazole (MMI; Sigma; $n = 23$) to block the synthesis of thyroid hormone. MMI was dissolved to 0.02% in drinking water and provided fresh daily. Controls ($n = 21$) were provided with unaltered drinking water. After 2 weeks of MMI treatment, the females were mated as described above. Both hypothyroid (MMI-treated) dams and euthyroid controls (no MMI) were subdivided into five additional groups receiving either no injection or a single subcutaneous injection of T₄ (1.25 µg/100 gm BW; Sigma) at either 9 A.M. or 9 P.M. on either GD14 or GD15. These injections were therefore timed to occur 48, 36, 24, or 12 hr before animals were killed at 9 A.M. on GD16. At 9 A.M. on GD16, all dams were decapitated, and trunk blood was collected for measurement of serum T₄ and thyrotropin (TSH). All fetuses were frozen intact as

described above. Frontal sections through the cortex and thalamus of one fetus from each dam were collected (Altman and Bayer, 1995).

After *in situ* hybridization, the signal was measured over the cortex and thalamus of GD16 fetal brains using the thresholding function in which all pixels containing density values exceeding a minimum value were averaged over the specified brain area. The resulting values were averaged over four sections for each fetus, with one fetus per litter and four to five litters per treatment group. A two-way ANOVA, with main effects of MMI treatment and timing of acute T₄ exposure, was followed by Bonferroni's *t* test.

To confirm the *in situ* hybridization results, we performed Northern analysis on RNA extracted from fetal cortex derived from dams treated with either MMI alone or MMI plus T₄. The cortex was dissected from one GD16 fetus per litter, derived from uninjected MMI-treated dams ($n = 4$) and MMI-treated dams injected with T₄ at 9 P.M. on GD14 ($n = 5$). RNA was extracted from these tissue pools as described above. Four replicates of total RNA from each pool (20 µg RNA per lane) were electrophoresed with RNA molecular weight standards (Life Technologies, Gaithersburg, MD) on a 1.2% agarose/6.5% formaldehyde gel. RNA was transferred to a nylon Zeta-Probe membrane (Bio-Rad Laboratories, Hercules, CA) and cross-linked by baking. Cyclophilin and NSP probes were generated by both random primer labeling and nick translation in the presence of ³²P-dCTP according to the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN). Both types of cyclophilin and NSP probes were mixed and hybridized simultaneously to ensure adequate detection of the target mRNA. Membranes were briefly prehybridized, hybridized at high stringency with 10⁶ cpm probe/10⁶ cpm probe/1 ml hybridization buffer, and washed according to manufacturer's instructions. The membranes were then apposed to a storage phosphor screen (Molecular Dynamics, Sunnyvale, CA), scanned into a Storm 840 Phosphorimager at 200 µm resolution, and evaluated with ImageQuant (Molecular Dynamics). The density of each band was quantified and normalized with respect to cyclophilin to control for loading variations. Student's *t* test was used to compare normalized band densities between the two RNA pools. In a separate experiment, serial dilutions of total RNA demonstrated a linear relationship between the amount of total RNA loaded and intensity of the cyclophilin signal between 5 and 20 µg RNA (data not shown).

Experiment III: Developmental pattern of NSP and Oct-1 expression in the brain. We initiated this study to define changes in NSP and Oct-1 expression during development to ensure that the pattern of expression observed in adults represents continuous expression from embryonic life. Nulliparous female Sprague Dawley rats ($n = 6$; Zivic Miller) were

Table 2. Characteristics of plasmids used to prepare cRNA probes for *in situ* hybridization

cDNA	Vector	Insert length (bp)	Antisense ^a	Sense ^b	Reference
TR α 1	pGEM3Z	681	<i>Eco</i> RI/Sp6	<i>Hind</i> III/T7	Bradley et al., 1989
TR β 1	pGEM3Z	681	<i>Eco</i> RI/Sp6	<i>Hind</i> III/T7	Bradley et al., 1989
2A	pCRII	461	<i>Eco</i> RV/Sp6	<i>Bam</i> HI/T7	Table 1
18C	pCRII	146	<i>Bam</i> HI/T7	<i>Eco</i> RV/Sp6	Table 1
6C	pCRII	185	<i>Eco</i> RV/Sp6	<i>Bam</i> HI/T7	Table 1
15A	pCRII	423	<i>Eco</i> RV/Sp6	<i>Bam</i> HI/T7	Table 1
4C	pCRII	212	<i>Eco</i> RV/Sp6	<i>Bam</i> HI/T7	Table 1
5C	pCRII	212	<i>Eco</i> RV/Sp6	<i>Kpn</i> I/T7	Table 1
17C	pCRII	241	<i>Bam</i> HI/T7	<i>Eco</i> RV/Sp6	Table 1
17CB	pBluescript	172	<i>Eco</i> RI/T3	<i>Cl</i> A1/T7	Table 1
18AL	pCRII	100	<i>Eco</i> RV/Sp6	<i>Bam</i> HI/T7	Table 1
18AS	pCRII	100	<i>Bam</i> HI/T7	<i>Eco</i> RV/Sp6	Table 1
14C	pBluescript	220	<i>Bam</i> HI/T7	<i>Xho</i> I/T3	Table 1
TRH	pSp65	1200	<i>Hind</i> III/Sp6		Lechan et al., 1986

^a The enzyme used for linearizing the plasmid and the polymerase used to produce the complementary (antisense) RNA probe.

^b The enzyme used for linearizing the plasmid and the polymerase used to produce the control (sense) RNA probe.

maintained on rat chow and unaltered drinking water *ad libitum* and mated as described above. Five dams ($n = 1$ per GD) were decapitated at 12 P.M. on GD14, GD16, GD18, and GD21, and the fetuses were collected and sectioned as described in Experiment II. The remaining dam carried the pregnancy to term. The resulting pups [$n = 1$ per postnatal day (PND)] were decapitated at 12 P.M. on PND3, -6, -9, -11, -14, and -19, and the intact heads (PND11 or earlier) or brains (PND14–PND19) were frozen on pulverized dry ice and stored at -80°C . An adult male Sprague Dawley rat was used to determine the expression pattern in the adult brain. Coronal sections were collected through the brain at the level of the rostral dentate gyrus.

Experiment IV: Effect of thyroid hormone on NSP and Oct-1 expression in the adult brain. We tested whether NSP and Oct-1 expression is affected by thyroid status in adults because it allowed us to test the thyroid hormone responsiveness of these genes in the absence of the maternal system and provided information about the temporal window of sensitivity to thyroid hormone. We manipulated thyroid hormone status of adult male Sprague Dawley rats ($n = 23$, 155–190 gm; Zivic Miller), as described by Koller et al. (1987). Each animal received a single intraperitoneal injection of either saline or 6-(*n*-propyl)-2-thiouracil (PTU; 1 mg/100 gm BW; Sigma) (Dyess et al., 1988) to block both thyroid hormone synthesis and the conversion of T_4 to triiodothyronine (T_3) by type I 5'-deiodinase (Chopra, 1996; Leonard and Koehle, 1996). PTU-treated animals ($n = 12$) were maintained for 12 d after PTU treatment on 0.05% MMI in the drinking water, provided fresh daily, and at 9 A.M. they received daily subcutaneous injections of either T_3 (10 μg /100 gm BW; Sigma; $n = 6$) or 100 μl saline/100 gm BW ($n = 6$). The remaining rats ($n = 11$, no PTU) were maintained on unaltered drinking water and at 9 A.M. received daily subcutaneous injections of either 10 μg T_3 /100 gm BW ($n = 6$) or 100 μl saline/100 gm BW ($n = 5$).

After treatment, animals were decapitated at 1 P.M., and trunk blood was collected for measurement of serum total T_3 , total T_4 , and TSH. Additionally, the relative abundance of the mRNA encoding thyrotropin releasing hormone (TRH) was measured in the hypothalamic paraventricular nucleus (PVN). Brains were collected and immediately frozen as described above. Coronal sections were collected through the brain at the level of the PVN (Paxinos and Watson, 1986, their Fig. 25) and through the cortex and hippocampus at the transition from the frontal cortex to the occipital cortex (Paxinos and Watson, 1986, their Figs. 31–33). Sagittal sections were collected through the cerebellum (Paxinos and Watson, 1986, their Fig. 79).

After *in situ* hybridization, the signal was measured over the PVN (only for TRH), cortex, dentate gyrus, CA1, CA2, CA3, and cerebellum. Signals in the cortex and hippocampus were measured without using thresholding, whereas measurements in the PVN and cerebellum were obtained using thresholding as described above. These values were averaged over four sections for each brain region, with five to six animals

per treatment group. A two-way ANOVA, with main effects of MMI treatment and T_3 injection, was followed by Bonferroni's *t* test.

Radioimmunoassay. Total T_4 was measured according to the manufacturer's instructions using a T_4 RIA kit (ICN, Costa Mesa, CA). This assay was performed at 40% binding with detection limits of 10–200 ng/ml and an intra-assay variation of 3.5%. Total T_3 was measured according to the manufacturer's instructions using a T_3 radioimmunoassay (RIA) kit (ICN). This assay was performed at 49% binding with detection limits of 0.25–8 ng/ml and an intra-assay variation of 4.4%. Serum levels of TSH were measured using ^{125}I -rat TSH (Covance Laboratories, Vienna, VA) and the double antibody National Institute of Diabetes and Digestive and Kidney Diseases RIA reagents including RP-3 standards. This assay was performed at 25% binding with detection limits of 1–50 ng/ml and an intra-assay variation of 8.0%.

In situ hybridization. In all experiments, frozen tissues were sectioned at 12 μm in a cryostat (Reichert-Jung Frigocut 2800N). Sections were thaw-mounted onto gelatin-coated microscope slides and stored at -80°C until hybridization. *In situ* hybridization was performed as described previously (Scott et al., 1998), with two exceptions. First, the hybridization buffer also contained 0.1% sodium pyrophosphate. Second, the hybridization buffer contained 200 mM dithiothreitol rather than 50 mM.

Probes. cRNA probes were generated from cDNA constructs described in Table 2. Transcription reactions were performed in the presence of ^{33}P - α -UTP (Andotek, Irvine, CA), as described previously (Scott et al., 1998). In each case, the total concentration of UTP was held to 12 μM , including 6 μM ^{33}P - α -UTP.

Autoradiography and signal quantitation. Slides were arranged in x-ray cassettes and apposed to BioMax film (Kodak), where the duration of exposure was dependent on the specific activity of the probe and the abundance of the target message. ^{14}C standards (American Radiolabeled Chemicals, St. Louis, MO) were simultaneously apposed to the film to verify that the film was not overexposed. Hybridization signal was analyzed as described previously (Scott et al., 1998) using a PowerCenter 150 Macintosh computer and the public domain NIH Image program (W. Rasband, National Institute of Mental Health). This system was interfaced with a Dage-MTI 72 series video camera equipped with a Nikon macro lens mounted onto a bellows system over a light box.

Statistical analysis. Outliers, defined as those values exceeding 1.5 interquartile ranges from the upper and lower quartiles, were eliminated using a box and whisker plot (Statistix, Analytical Software, Tallahassee, FL). A two-way ANOVA (Systat, Systat, Inc., Evanston, IL) was performed on the remaining values, with main effects described in the specific experiment. *Post hoc* tests of differences among individual means were performed using a Bonferroni's *t* test, in which the mean squared error term from the ANOVA table was used as a point estimate of the pooled variance.

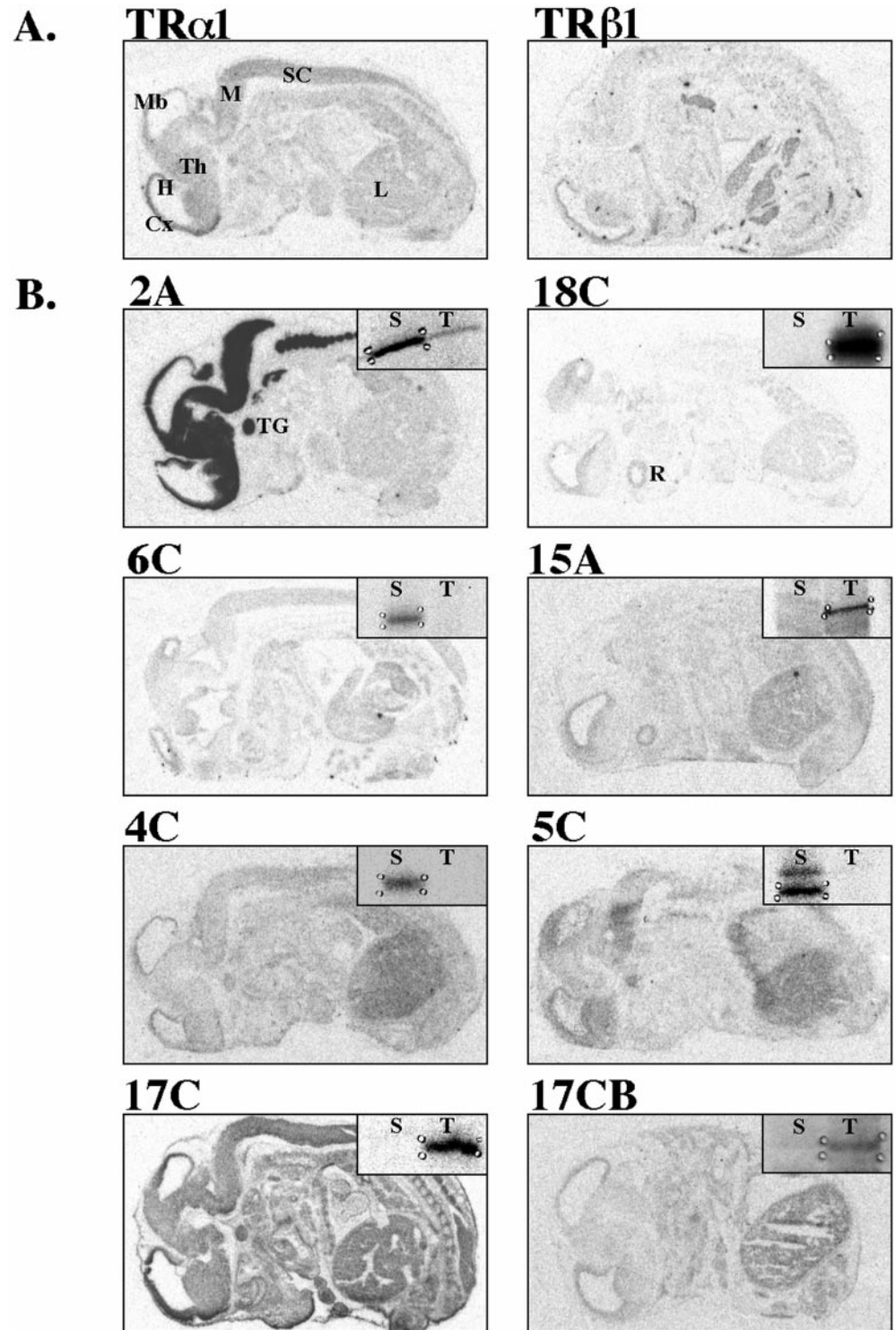


Figure 1. Distribution of putative thyroid hormone-responsive genes in the GD16 embryo. Images are derived from film autoradiograms after *in situ* hybridization to determine whether putative thyroid hormone-responsive mRNAs identified by differential display RT-PCR were expressed in areas known to contain TR mRNAs (Experiment 1). RNA probes were applied to sagittal sections of GD16 rat embryos. Sense controls were applied to adjacent sections and produced negligible hybridization signal (data not shown), with the exception of fragment 17C, which we did not examine further. *A*, Distribution of TR α 1 and TR β 1 mRNA. *B*, Distribution of putative thyroid hormone-responsive mRNAs as noted above panel. Film autoradiograms of gene fragments identified by differential display are shown in *insets*, illustrating direction of thyroid hormone effects. See Table 1 for RNAimage primers used to generate each fragment. Cx, Cortex; H, hippocampus; L, liver; M, medulla; Mb, midbrain; R, retina; S, saline-injected; SC, spinal cord; T, T₄-injected; TG, trigeminal ganglion; Th, thalamus. Scale bar, 0.5 cm.

RESULTS

Experiment 1: Identification of thyroid hormone-responsive genes in the GD16 cortex

We manipulated maternal thyroid status before the onset of fetal thyroid function to ensure that maternal T₄ was the sole source of thyroid hormone to the fetus. Using differential display, we identified 11 putative thyroid hormone-responsive genes in the fetal cortex generated from eight RNAimage primer combinations (Table 1). Seven of these genes appeared to be enhanced by T₄,

and four appeared to be suppressed by T₄. Before sequencing these gene fragments, we performed *in situ* hybridization to determine whether their expression pattern overlapped with that of TR α 1 or TR β 1. Among the original 11 genes, we found that eight exhibited anatomical patterns of expression overlapping with that of TR α 1 or TR β 1 (Fig. 1), indicating that they may be directly regulated by thyroid hormone. We chose to further evaluate three of these genes, two whose sequences matched entries in GenBank (Table 1). Fragment 2A was identical to NSP

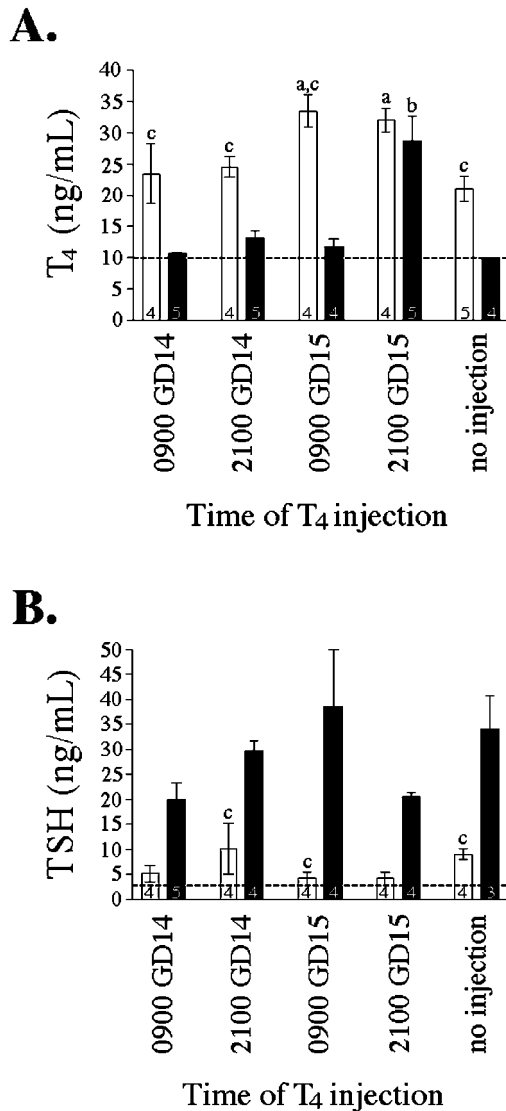


Figure 2. Effect of MMI and T₄ injections on serum levels of T₄ (A) and TSH (B) in GD16 pregnant females at the time they were decapitated (Experiment II). See Materials and Methods for details of thyroid hormone manipulation. Bars represent mean \pm SEM, with number of dams per group noted within each bar. All animals were killed at 9 A.M. (0900) on GD16. Groups differed in the timing of T₄ injection as shown below the ordinate. Open bars, Euthyroid dams (no MMI); closed bars, hypothyroid dams (MMI). ^aSignificantly different from euthyroid dams receiving no injection ($p < 0.05$). ^bSignificantly different from hypothyroid dams receiving no injection ($p < 0.05$). ^cSignificantly different from hypothyroid dams with identical timing of acute T₄ treatment ($p < 0.05$). Note: Serum hormone levels below detection limit were assigned the value of the detection limit (indicated by dashed line) for statistical purposes.

(Wieczorek and Hughes, 1991; van de Velde et al., 1994b; Baka et al., 1996) and was suppressed by T₄; fragment 18C was identical to Oct-1 (J. N. Buskin, unpublished observations) and was enhanced by T₄; and fragment 6C did not match the sequence of any GenBank entry and was suppressed by T₄.

Experiment II: Verification of maternal thyroid hormone effects on the expression of NSP, Oct-1, and 6C

We confirmed the efficacy of our thyroid hormone manipulation of pregnant female rats by measuring maternal serum total T₄ and TSH (Fig. 2). Treatment with the goitrogen MMI significantly

reduced circulating levels of maternal T₄ ($F_{(1,34)} = 59.957$; $p < 0.001$) and significantly elevated serum TSH ($F_{(1,30)} = 50.983$; $p < 0.001$). Treatment with thyroid hormone significantly elevated maternal serum T₄ ($F_{(4,34)} = 11.776$; $p < 0.001$) (Fig. 2). A single injection of 1.25 μ g T₄/100 gm BW transiently restored circulating T₄ to physiological levels and transiently suppressed TSH levels in hypothyroid dams, as predicted. However, physiological levels of T₄ were restored only 12 hr after T₄ injection in hypothyroid dams, but remained elevated in euthyroid dams for at least 24 hr (Fig. 2). Likewise, TSH suppression was evident for a shorter duration in hypothyroid dams. These data are consistent with previous work showing that thyroid hormone is cleared from the serum of hypothyroid dams more rapidly than from that of euthyroid dams (Versloot et al., 1998).

We measured the effects of maternal thyroid hormone manipulation on gene expression in the fetal brain using *in situ* hybridization because this approach allowed us to evaluate the potential site-specific effects of thyroid hormone on NSP, Oct-1, and 6C expression. Quantitative analysis of film autoradiograms after *in situ* hybridization revealed that both MMI ($F_{(1,31)} = 9.851$; $p < 0.004$) and maternal thyroid hormone ($F_{(4,31)} = 5.146$; $p < 0.003$) significantly affected NSP expression in the GD16 cortex (Fig. 3). Maternal thyroid hormone exposure significantly decreased NSP mRNA in the cortex of hypothyroid animals 36 hr after T₄ injection (9 P.M. on GD14). In contrast, thyroid hormone exposure significantly increased Oct-1 mRNA in the cortex of euthyroid animals 12 and 24 hr after T₄ injection (9 P.M. and 9 A.M., respectively, on GD15). These results are consistent with those of the original differential display where NSP expression was decreased by maternal thyroid hormone and Oct-1 expression was increased by maternal thyroid hormone. The expression of neither NSP nor Oct-1 was affected by thyroid hormone in the thalamus (data not shown). Additionally, 6C was not affected by thyroid hormone in either the cortex or the thalamus, suggesting that it was falsely identified as being thyroid hormone responsive in the differential display.

Previous work has shown that two mRNAs are transcribed from the NSP gene, a 1.5 kb transcript (NSP-C) and a 3.5 kb transcript (NSP-A) (Ninkina et al., 1997; Roebroek et al., 1998). Because these transcripts have a common 3' end identical to the NSP fragment amplified in the original differential display, our *in situ* hybridization probe hybridized to both transcripts. Therefore, we performed Northern analysis to determine whether both NSP transcripts are affected by maternal thyroid hormone. Northern analysis revealed that our probe hybridized to the predicted 1.5 and 3.5 kb transcripts (Fig. 4A). Expression of the 3.5 kb NSP-A transcript was significantly decreased ($30.7 \pm 13.0\%$) in fetuses derived from hypothyroid dams treated with T₄ (Fig. 4B). In contrast, expression of the 1.5 kb NSP-C transcript was unaffected by acute maternal thyroid hormone manipulation.

Experiment III: Developmental pattern of NSP and Oct-1 expression

After the initial observation that both NSP and Oct-1 were regulated by thyroid hormone in the GD16 cortex, we characterized the distribution and relative abundance of these mRNAs during development. We found that NSP expression was restricted to the nervous system at all times (Fig. 5). NSP mRNA was clearly detectable at GD14, the earliest developmental time evaluated. NSP expression was more robust in the intermediate zone of the cortex than in the ventricular zone [nomenclature derived from Boulder Committee (1970)]. Additionally, NSP

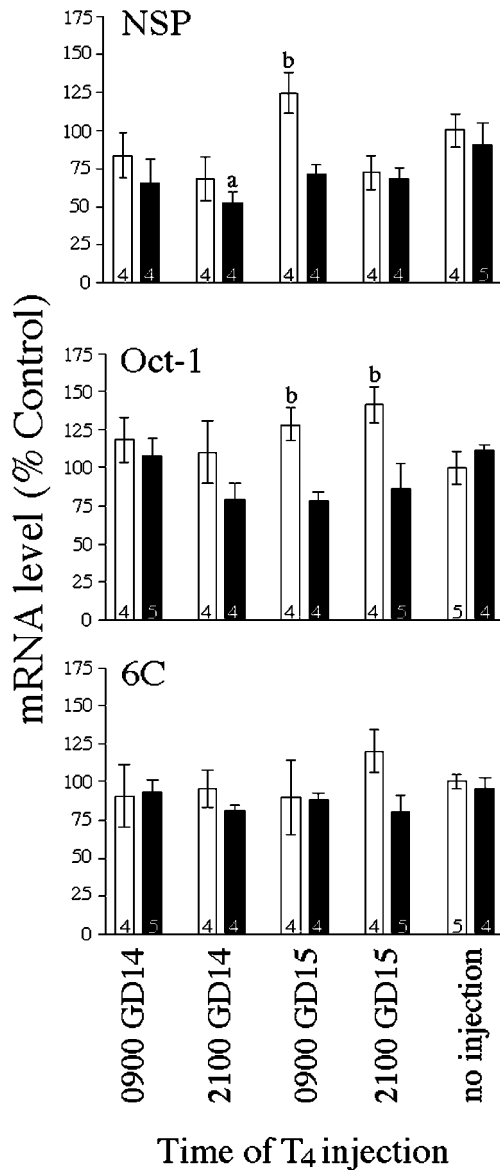


Figure 3. Effect of thyroid hormone manipulation on gene expression in the GD16 fetus (Experiment II). Quantitative analysis of film autoradiograms after *in situ* hybridization for NSP, Oct-1, and 6C are described in Materials and Methods. Bars represent mean \pm SEM of the film density (converted to % controls \pm CV) over the cortex, with number of dams per group noted within each bar. All animals were killed at 9 A.M. (0900) on GD16. Groups differed in the timing of T₄ injection as shown below the ordinate. *Open bars*, Euthyroid dams (no MMI); *closed bars*, hypothyroid dams (MMI). ^a Significantly different from hypothyroid dams receiving no injection ($p < 0.05$). ^b Significantly different from hypothyroid dams with identical timing of acute T₄ treatment ($p < 0.05$).

mRNA was more abundant during the fetal and neonatal period but became less abundant from approximately PND14 through adulthood. Regional differences in NSP expression were apparent throughout brain development. For example, NSP expression was predominant in the ventromedial hypothalamic nucleus beginning around the time of birth and persisting into adulthood. The hippocampus, medial habenular nucleus, and amygdala were also intensely labeled. The corpus callosum and other myelinated fiber bundles were never labeled.

Oct-1 mRNA was expressed both within the brain and outside

the nervous system (Fig. 6). Oct-1 expression was predominant in the ventricular zone of the cortex of fetal brains. Regional differences in Oct-1 expression levels were apparent throughout brain development. For example, Oct-1 expression was abundantly expressed in the thalamus from approximately GD16 until PND14. As development proceeded, Oct-1 expression became less robust and more spatially restricted, although expression was retained in the hippocampus.

Experiment IV: Effect of thyroid hormone on NSP and Oct-1 expression in the adult brain

The developmental study demonstrated that NSP and Oct-1 were continually expressed in the cortex from fetal life to adulthood. Therefore, we evaluated the effect of thyroid status on the expression of these genes in the adult brain to determine whether these genes retained their responsiveness to thyroid hormone. We confirmed the efficacy of our thyroid hormone manipulations of adult male rats by measuring serum T₃, T₄, and TSH and by measuring TRH mRNA in the PVN of the hypothalamus, which is known to be suppressed by thyroid hormone (Koller et al., 1987). Treatment with PTU and MMI significantly reduced circulating levels of T₄ ($F_{(1,18)} = 2349.446$; $p < 0.001$), elevated serum TSH ($F_{(1,17)} = 14.075$; $p < 0.002$), and elevated TRH mRNA in the PVN ($F_{(1,19)} = 10.845$; $p < 0.004$) (Table 3). Daily T₃ injections significantly reduced circulating levels of T₄ ($F_{(1,18)} = 2349.446$; $p < 0.001$), elevated T₃ ($F_{(1,19)} = 91.169$; $p < 0.001$), and suppressed TRH mRNA in the PVN ($F_{(1,19)} = 125.763$; $p < 0.001$), consistent with negative feedback of thyroid hormone on the pituitary and hypothalamus (Koller et al., 1987; Dyess et al., 1988; Zoeller et al., 1988).

Combined PTU and MMI treatment significantly elevated NSP mRNA in the dentate gyrus ($F_{(1,17)} = 6.719$; $p < 0.02$) and CA1 subfield of Ammon's horn ($F_{(1,18)} = 4.777$; $p < 0.05$) (Table 4). Additionally, T₃ treatment significantly decreased NSP mRNA in the dentate gyrus ($F_{(1,17)} = 6.682$; $p < 0.02$). NSP mRNA levels in the cortex, CA2, and CA3 subfields, and cerebellum were unaffected by treatments. In contrast, T₃ treatment significantly reduced Oct-1 mRNA in the cortex ($F_{(1,19)} = 19.362$; $p < 0.001$), dentate gyrus ($F_{(1,19)} = 15.843$; $p < 0.001$), CA2 ($F_{(1,17)} = 19.147$; $p < 0.001$), and CA3 ($F_{(1,19)} = 23.189$; $p < 0.001$) (Table 4). Oct-1 mRNA levels in the CA1 subfield and cerebellum were unaffected by treatments.

DISCUSSION

The present studies are the first to demonstrate that thyroid hormone can affect gene expression in the fetal rat brain. Moreover, because we manipulated thyroid status of pregnant rats before the onset of fetal thyroid function, our data clearly demonstrate that thyroid hormone specifically of maternal origin affects the expression of these genes. Considering recent observations that maternal thyroid hormone reaches the fetus (Vulsma et al., 1989; Contempre et al., 1993) and that children born to pregnant women with untreated hypothyroidism exhibit measurable neurological deficits (Haddow et al., 1999; Pop et al., 1999), our data strongly indicate that thyroid hormone of maternal origin affects fetal brain development by regulating the expression of specific genes.

Using differential display, we identified 11 putative thyroid hormone-responsive genes in the fetal brain using only eight primer pairs. Thus, a large number of genes expressed in the fetal brain may be affected by maternal thyroid hormone. Differential display is known to generate false-positives, as do other "subtrac-

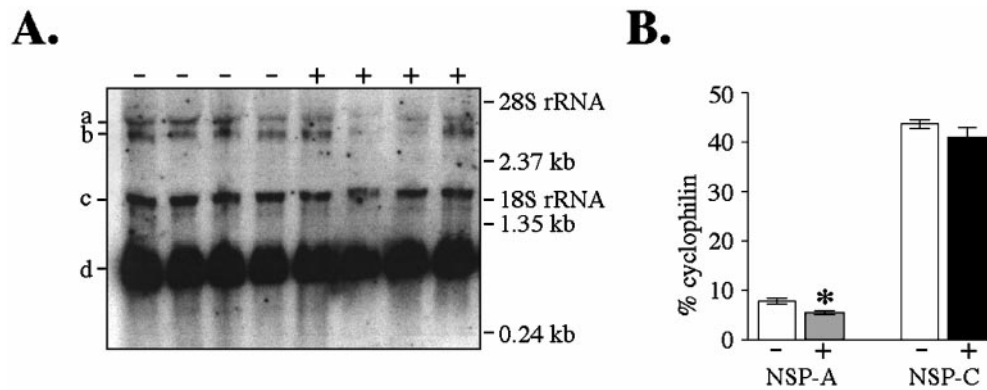


Figure 4. Effect of thyroid hormone manipulation on the expression of NSP variants using quantitative Northern analysis (Experiment II). *A*, Phosphorimage of Northern blot in which 20 μ g total RNA was hybridized with 32 P-labeled NSP cDNA. 32 P-labeled cyclophilin cDNA was hybridized simultaneously to control for loading variations. RNA was isolated from hypothyroid dams receiving either no injection (–) or a single T_4 injection at 9 P.M. on GD14 (+). Each sample corresponds to RNA pooled from four or five animals. Positions of the molecular weight standards and the 18S and 28S ribosomal RNAs are indicated.

B, Quantification of band density shown in *A* using ImageQuant software. NSP-A mRNA was decreased after acute maternal T_4 exposure. Bars represent mean band density \pm SEM (converted to % total cyclophilin). ^a Transcript of 3.5 kb hybridized to NSP probe, corresponding to NSP-A. ^b Transcript of 3.4 kb hybridized to cyclophilin probe. ^c Transcript of 1.5 kb hybridized to NSP probe, corresponding to NSP-C. ^d Transcript of 0.9 kb hybridized to cyclophilin probe. *Significantly different from hypothyroid dams receiving no injection ($p < 0.05$).

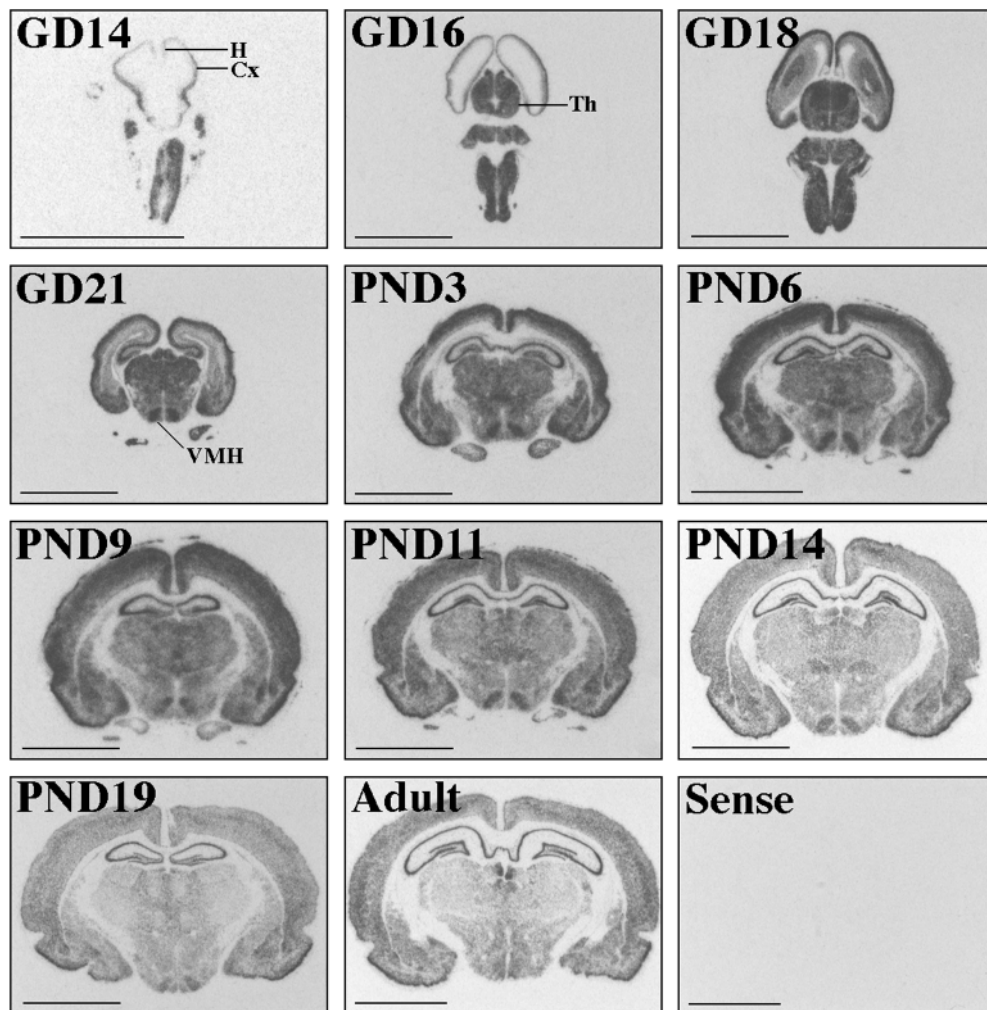


Figure 5. Developmental profile of NSP expression in the rat. Images are derived from film autoradiograms after *in situ* hybridization using the NSP cRNA probe (Experiment III). Age is noted in the top left corner of each panel. Cx, Cortex; H, hippocampus; Th, thalamus; VMH, ventromedial hypothalamus. Scale bar, 0.5 cm.

“hybridization methods designed to identify differentially expressed genes (Wan and Erlander, 1997; Ledakis et al., 1998). However, only one of three genes chosen for further analysis was a false-positive. This success rate may have been caused in part by the anatomical screen used to determine whether genes identified by differential display were expressed in brain areas containing TRs. Although nearly all of the gene fragments generated from

the differential display were selectively expressed in brain areas and in organs that are responsive to thyroid hormone (Fig. 1), the genes chosen for further analysis were the most abundant and selectively expressed in TR-containing brain regions.

In principle, thyroid hormone of maternal origin could affect the expression of genes in the fetal brain by a direct action or by various indirect actions. However, several features of the present

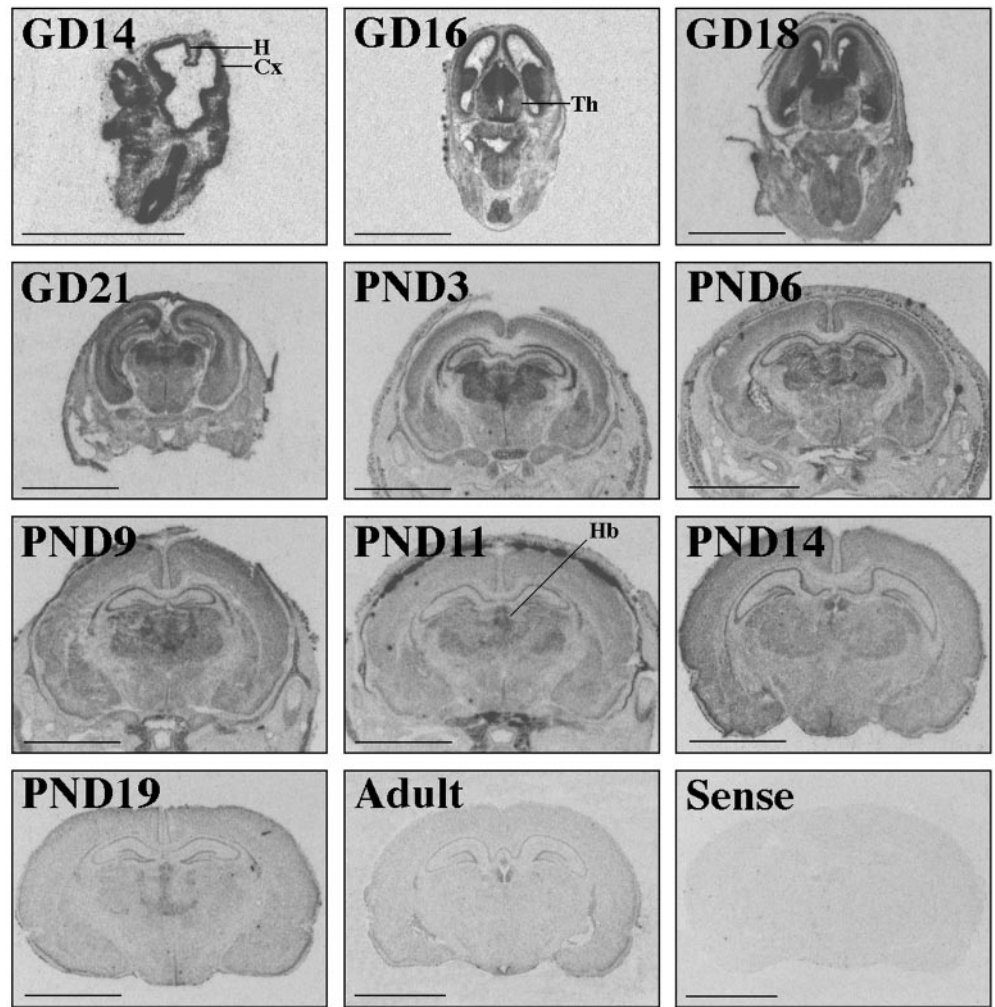


Figure 6. Developmental profile of Oct-1 expression in the rat. Images are derived from film autoradiograms after *in situ* hybridization using the Oct-1 cRNA probe (Experiment III). Age is noted in the *top left corner* of each panel. Cx, Cortex; H, hippocampus; Th, thalamus; Hb, habenula. Scale bar, 0.5 cm.

Table 3. Serum levels of total T₃, total T₄, and TSH, and expression levels of TRH mRNA in the PVN of adult thyroid hormone-manipulated animals (Experiment IV)

Thyroid status	T ₃ (ng/ml)	T ₄ (ng/ml)	TSH (ng/ml)	TRH (% control)
Control	0.790 ± 0.055 (5)	55.750 ± 1.335 (4)	12.013 ± 2.742 (5)	100.00 ± 2.171 (5)
T ₃	6.160 ± 0.517 (6)**	UD (6)	5.110 ± 1.371 (5)	85.50 ± 2.185 (6)**
MMI + PTU	0.590 ± 0.042 (6)	UD (6)	29.436 ± 6.903 (6)*	111.37 ± 1.640 (6)**
MMI + PTU + T ₃	6.620 ± 1.009 (6)**	UD (6)	4.161 ± 0.828 (5)	85.87 ± 3.535 (6)**

Serum T₃, T₄, and TSH values represent mean ± SEM (*n*). TRH values represent mean ± SEM (*n*) of film density (converted to % control ± CV) over the PVN.

* Significantly different from control euthyroid rats receiving saline injections (*p* < 0.05).

** Significantly different from control euthyroid rats receiving saline injections (*p* < 0.01).

UD, Undetectable. Serum hormone levels were assigned the value of the lower assay detection limit for statistical purposes.

results suggest that maternal thyroid hormone affects gene expression by a direct action. First, the dose of thyroid hormone given to the dam did not produce supraphysiological levels of T₄ in maternal blood. Therefore, the observed effects were not caused by thyrotoxic effects of thyroid hormone. Second, the single injection of T₄ produced rapid, transient, and selective effects on gene expression in the fetal brain. The abundance of the NSP mRNA was suppressed within 36 hr, whereas that of Oct-1 mRNA was elevated within 12 hr. These effects were observed only in the cortex, not in the thalamus, and 6C was unaffected in both brain regions. In contrast, nonspecific or metabolic effects of thyroid hormone would more likely produce

similar effects on the abundance of various genes and would not likely be limited to specific brain regions. Finally, we clearly demonstrated that NSP and Oct-1 expression can be regulated by T₃ in the adult brain. Therefore, the effects of thyroid hormone on the expression of these two genes is not dependent on the maternal system or on injecting T₄. Taken together, these data provide strong support for the concept that thyroid hormone of maternal origin can directly affect the expression of genes in the fetal brain.

Considering the evidence that thyroid hormone exerts a direct action on the expression of NSP and Oct-1, it was surprising that the administration of MMI did not affect their expression in the

Table 4. Effect of thyroid hormone on NSP and Oct-1 mRNA in the adult brain (Experiment IV)

Thyroid status	Brain region	NSP mRNA	Oct-1 mRNA
Control	Cortex	100.00 ± 2.44 (5)	100.00 ± 0.45 (5)
T ₃		99.12 ± 1.85 (6)	92.70 ± 1.58 (6)**
MMI + PTU		104.00 ± 2.58 (6)	99.45 ± 0.77 (6)
MMI + PTU + T ₃	Dentate gyrus	101.01 ± 1.22 (6)	95.71 ± 1.40 (6)*
Control		100.00 ± 1.73 (5)	100.00 ± 1.66 (5)
T ₃		98.51 ± 0.71 (5)	96.01 ± 1.48 (6)*
MMI + PTU	CA1 ^a	105.09 ± 1.08 (5)*	101.09 ± 0.52 (6)
MMI + PTU + T ₃		100.01 ± 1.27 (6)	95.99 ± 0.81 (6)*
Control		100.00 ± 1.09 (4)	100.00 ± 1.55 (4)
T ₃	CA2 ^a	105.27 ± 1.75 (6)	97.94 ± 1.57 (6)
MMI + PTU		108.80 ± 2.08 (6)*	101.97 ± 1.11 (6)
MMI + PTU + T ₃		104.17 ± 1.27 (6)	97.42 ± 1.13 (6)
Control	CA3 ^a	100.00 ± .67 (3)	100.00 ± 1.10 (5)
T ₃		101.36 ± 1.65 (6)	95.82 ± 0.98 (6)*
MMI + PTU		105.93 ± 1.54 (5)	99.37 ± 0.20 (4)
MMI + PTU + T ₃	Cerebellum	100.20 ± 1.15 (6)	95.11 ± 10.4 (6)**
Control		100.00 ± 0.85 (4)	100.00 ± 0.26 (5)
T ₃		100.64 ± 0.68 (4)	94.00 ± 1.86 (6)**
MMI + PTU	Cerebellum	105.51 ± 1.81 (6)	98.90 ± 0.92 (6)
MMI + PTU + T ₃		101.30 ± 1.26 (6)	93.32 ± 0.81 (6)**
Control		100.00 ± 1.64 (4)	100.00 ± 0.40 (4)
T ₃	Cerebellum	97.58 ± 0.61 (6)	100.14 ± 0.62 (6)
MMI + PTU		97.86 ± 0.31 (5)	101.60 ± 1.01 (6)
MMI + PTU + T ₃		95.2 ± 1.48 (6)	100.29 ± 0.17 (6)

Quantitative analysis of autoradiograms after *in situ* hybridization for NSP and Oct-1 mRNA in the cortex, hippocampus, and cerebellum of thyroid hormone-manipulated adult rats. Values shown represent mean ± SEM (*n*) of film density (converted to % controls ± CV) over the cortex, hippocampus, or cerebellum.

* Significantly different from control euthyroid rats receiving saline injections ($p < 0.05$).

** Significantly different from control euthyroid rats receiving saline injections ($p < 0.01$).

^a Subfields of Ammon's horn.

fetal brain, as measured by *in situ* hybridization. In the case of NSP, this may be related to the transcript-specific regulation by thyroid hormone. Specifically, the NSP fragment amplified in the differential display hybridized to two transcripts, a 1.5 kb transcript designated NSP-C and a 3.5 kb transcript designated NSP-A (van de Velde et al., 1994b; Ninkina et al., 1997). Northern analysis of RNA extracted from fetuses derived from MMI-treated dams showed that T₄ suppressed only NSP-A mRNA. Because NSP-A expression is much lower than that of NSP-C but our probe hybridized to both transcripts, we may not have detected the selective effects of MMI on NSP-A expression. Evaluation of the same tissues with probes specific for NSP-A and NSP-C has confirmed this interpretation (A. L. S. Dowling, unpublished observations).

It must be noted, however, that hypothyroxinemia itself induces a number of compensatory mechanisms that ameliorate the consequences of low thyroid hormone levels in the fetal brain. These compensatory mechanisms may account for the unremarkable effects of MMI on the expression of NSP and Oct-1. For example, thyroid hormone uptake into tissues is enhanced in hypothyroid animals (Everts et al., 1994a,b, 1995; Moreau et al., 1999), as is the conversion of T₄ to T₃ in fetal tissue (Ruiz de Ona et al., 1988; Calvo et al., 1990). We observed the effects of these compensatory mechanisms in that MMI-treated dams exhibited elevated T₄ and suppressed TSH for a shorter duration after a single injection of T₄. In the original differential display, we eliminated the potential confounding effects of compensatory mechanisms by comparing RNA pools extracted from fetuses

derived from thyroidectomized dams only. Therefore, conditions under which genes were identified in the differential display represented only a subset of conditions tested in our follow-up studies.

The functional characteristics of NSP and Oct-1 may provide insight into thyroid hormone effects on the prenatal brain. NSP is a neuron-specific protein (Wieczorek and Hughes, 1991) integrated into the endoplasmic reticulum (van de Velde et al., 1994a,b), and its expression is correlated with neuronal differentiation (Hens et al., 1998). NSP mRNA is localized to the axonal pole of neuronal cell bodies (Baka et al., 1996; Ninkina et al., 1997) and may play a role in protein packaging and/or trafficking (Senden et al., 1996). As shown here, expression of NSP appears to be regionally and developmentally regulated. Overall, the abundance of NSP mRNA declines during development in all areas studied. However, NSP expression is robust in both the hippocampus and the cortex throughout development. In these areas, NSP expression is consistently high from GD14, the earliest time evaluated, to adulthood. At early developmental stages, NSP mRNA appears to be more abundant in the intermediate zone of the cortex and less abundant in the periventricular zone. As the brain matures and cortical layers develop, NSP expression in the cortex takes on a laminar appearance. The regulation of NSP-A by thyroid hormone during early cortical development and its differential expression in the intermediate zone of the GD16 cortex supports the concept that NSP is involved in neuronal differentiation and that this role is modulated by thyroid hormone.

Oct-1 is a POU-domain protein (He et al., 1989; Treacy and Rosenfeld, 1992), named for the initial genes identified within this family, Pit-1, Oct-1/2, and Unc-86. Downregulation of Oct-1 expression is associated with cell cycle arrest and differentiation (Lakin et al., 1995), suggesting a role for Oct-1 in proliferation. Like NSP, the expression of Oct-1 appeared to be regionally and developmentally regulated, and the abundance of Oct-1 mRNA declined during brain development. In contrast to NSP, Oct-1 mRNA was detected both within and outside the nervous system and in early development was selectively expressed in the periventricular zone of the cortex. This zone contains proliferating neurons (Caviness et al., 1995) that express only TR β 1 (Bradley et al., 1992). Because Oct-1 has been shown to stimulate TR β 1 expression (Nagasawa et al., 1997), thyroid hormone may act through TR β 1 to enhance Oct-1 expression and maintain TR β 1 expression in these proliferating neurons. Both TR β 1 (Bradley et al., 1992) and Oct-1 expression diminish once these neurons leave the ventricular zone and begin to differentiate.

It is interesting to note that both NSP and Oct-1 expression retained their responsiveness to thyroid hormone in the adult brain. However, NSP expression was affected by thyroid hormone only in the hippocampus where it was enhanced by hypothyroidism. In contrast, Oct-1 expression was suppressed by thyroid hormone in both the cortex and the hippocampus. Considering that the specific effect of thyroid hormone on gene expression may depend on the cofactor recruited to the hormone receptor complex (Koenig, 1998), the change in direction of regulation of Oct-1 by thyroid hormone during development may be related to developmental changes in coactivator and corepressor expression. In addition, the retention of thyroid hormone responsiveness in adulthood demonstrates that these genes do not exhibit a “critical period” of thyroid hormone sensitivity. Finally, the observation that chemical thyroidectomy does not fully abolish Oct-1 expression suggests that NSP and Oct-1 expression are not fully dependent on thyroid status, but their expression is modulated nonetheless.

In conclusion, these studies show that thyroid hormone of maternal origin can selectively regulate the expression of genes in the fetal brain. Furthermore, these genes may retain their sensitivity to thyroid hormone in the adult brain. One can reasonably speculate that maternal thyroid hormone could influence gene expression in the fetal brain by a direct mechanism and various indirect mechanisms. Our data support the concept that thyroid hormone regulates the expression of NSP and Oct-1 by a direct action. Our use of differential display in combination with an acute administration of a physiological dose of thyroid hormone may have enhanced our ability to identify genes directly regulated by thyroid hormone.

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