Maternal Hypothyroidism Selectively Affects the Expression of Neuroendocrine-Specific Protein A Messenger Ribonucleic Acid in the Proliferative Zone of the Fetal Rat Brain Cortex*

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ABSTRACT

Thyroid hormone is essential for mammalian brain development, but the mechanisms by which thyroid hormone exerts its effects, the developmental processes affected, and the timing of thyroid hormone effects are poorly understood. An important question is whether thyroid hormone of maternal origin is essential in guiding fetal brain development. In both humans and rats, thyroid hormone of maternal origin reaches the fetus before the onset of fetal thyroid function. Moreover, receptors for thyroid hormone (TRs) are present in the fetal brain and are occupied by thyroid hormone. Finally, a recent report strongly indicates that transient undiagnosed maternal hypothyroidism can lead to measurable neurological deficits in the offspring despite the lack of neonatal hypothyroidism. Considering that TRs are ligand-activated transcription factors, we recently initiated a project

HYROID HORMONE is essential for normal brain development (1-9). Much of the clinical literature supporting this concept is focused on the neurological consequences of untreated congenital hypothyroidism and/or neonatal hypothyroidism (10–12). However, recent studies indicate that thyroid hormone is also essential for brain development before birth and in the absence of congenital/ neonatal hypothyroidism. For example, neurological cretinism, which occurs in geographic areas of endemic goiter, is characterized by severe neurological deficits, but the individual may be euthyroid at birth (13-15). In addition, children born to women with untreated hypothyroidism during the second trimester exhibit an increased incidence of measurable neurological deficits despite having normal circulating thyroid hormone levels at birth (16, 17). Together, these studies suggest that maternal hypothyroidism may adversely affect brain development in ways that cannot be predicted by thyroid status at birth or repaired by thyroid hormone therapy after birth.

Several lines of evidence support the concept that thyroid hormone, perhaps of maternal origin, exerts receptor-mediated effects on the fetal brain. For example, receptors for to identify thyroid hormone-responsive genes in the fetal cortex before the onset of fetal thyroid function. One of the thyroid hormoneresponsive genes we identified, neuroendocrine-specific protein (NSP), is expressed as two separate transcripts, NSP-A and NSP-C. Only NSP-A is affected by maternal thyroid hormone. We now demonstrate that the messenger RNA encoding NSP-A is expressed exclusively in the proliferative zone of the fetal cortex, and that its expression is affected by maternal hypothyroidism. Moreover, as development proceeds, NSP-A becomes selectively expressed in Purkinje cells of the cerebellum, a well known thyroid hormone-responsive cell. These findings strongly support the concept that thyroid hormone of maternal origin exerts specific receptor-mediated effects on fetal brain development. (*Endocrinology* **142:** 390–399, 2001)

thyroid hormone (TRs) are expressed in the fetal brain before the onset of fetal thyroid function (18). Moreover, thyroid hormone of maternal origin reaches the fetal brain and is bound to the TR (7, 10, 19–22). Considering that TRs are ligand-dependent transcription factors (23, 24), we recently initiated a study to identify genes expressed in the fetal rat brain before the onset of fetal thyroid function that are affected by changes in maternal thyroid hormone (25). The ultimate goal of this project is to identify the developmental processes affected by maternal thyroid hormone, the timing of thyroid hormone effects, and the mechanisms by which thyroid hormone exerts these effects.

One of the genes expressed in fetal cortex whose expression was affected by manipulation of maternal thyroid status was the gene encoding neuroendocrine-specific protein (NSP) (25). Because its expression is strongly correlated with neuronal differentiation (26), NSP may be an important mediator of thyroid hormone effects on brain development. Our previous work demonstrated that there are two NSP transcripts expressed in the fetal cortex, a 3.5-kb transcript designated NSP-A and a 1.5-kb transcript designated NSP-C (27, 28); the NSP-A transcript was selectively affected by thyroid hormone. However, we mapped the distribution of NSP expression in the fetal cortex and throughout development using a complementary RNA (cRNA) probe that crosshybridized to the NSP-A and NSP-C transcripts. Therefore, we were unable to determine whether NSP-A and NSP-C exhibit different patterns of expression in the fetal brain. Moreover, we were unable to confirm that maternal hypo-

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thyroidism could alter NSP-A expression in the fetal brain. Therefore, we generated probes specific for NSP-A and NSP-C and now report that in the fetal cortex the thyroid hormone-responsive NSP-A is expressed exclusively in the proliferative ventricular zone, and that in the adult cerebellum, this transcript is expressed exclusively in Purkinje cells. In addition, we have confirmed that the expression of NSP-A is affected by maternal hypothyroidism.

Materials and Methods

Animals

All animal procedures were performed in accordance with the NIH guidelines for animal research and were approved by the University of Massachusetts-Amherst institutional animal care and use committee. To test whether maternal hypothyroidism affects the expression of NSP-A in the fetal brain, we evaluated fetal brain tissues derived from two separate experiments. In the first experiment, tissues were obtained from a subset of animals described in our original report (25). Briefly, nulliparous female Sprague Dawley rats (n = 26; Zivic-Miller Laboratories, Inc., Pottersville, PA) were exposed to the goitrogen Methimazole (MMI) (Sigma, St. Louis, MO; n = 13) to block the synthesis of thyroid hormone. MMI was dissolved to 0.02% in drinking water and provided fresh daily. Controls (n = 13) were provided with unaltered drinking water. After 2 weeks of MMI treatment, the females were paired with males overnight; the presence of sperm in a vaginal smear the following morning indicated mating, and this day was defined as gestational day (G) 1. Both hypothyroid (MMI-treated) dams and euthyroid controls (no MMI) were subdivided into three additional groups receiving either no injection or a single sc injection of T₄ (12.5 μ g/kg BW; Sigma) at either 2100 h on G14 or 0900 h on G15. These injections were therefore timed to occur 36 or 24 h before the rats were killed at 0900 h on G16. This experimental design resulted in six groups of four or five litters each. All dams were killed by decapitation after CO2 inhalation. Fetuses were removed from the uterus, rapidly frozen on pulverized dry ice, and stored at -80 C until they were sectioned for in situ hybridization. RIA of total T₄ and TSH in serum of the dams confirmed the efficacy of our treatment (25).

In the second experiment, timed pregnant female Sprague Dawley rats (n = 24; Zivic-Miller Laboratories, Inc.) arrived at our facility on G2 and were maintained on drinking water containing either 0.04% 6-(n)-propylthiouracil (PTU) (Sigma; n = 12) with 3% sucrose to reduce the bitterness associated with PTU or 3% sucrose alone (n = 12). The goitrogen PTU blocks both thyroid hormone synthesis and the conversion of T_4 to T_3 by type 15'-deiodinase (29, 30). The two solutions were provided fresh daily for 14 days. On G15, all animals received a sc injection of either T_4 (50 µg/kg BW in 100 µl; Sigma; n = 12) or 100 µl saline (n = 12) at both 1000 and 1800 h. This paradigm produced four groups of six litters each: control, control+ T_4 , PTU+saline, and PTU+ T_4 . At 1000 h on G16, all dams were killed as described above, and trunk blood was collected for measurement of serum T_3 , T_4 , and TSH. All fetuses were frozen intact as described above and stored at -80 C until they were sectioned for *is situ* hybridization.

To characterize the spatial and temporal changes in expression of NSP-A and NSP-C throughout normal development, nulliparous female Sprague Dawley rats (n = 5; Zivic-Miller Laboratories, Inc.) were maintained on rat chow and water *ad libitum* and mated as described above. Dams were killed at 1200 h on G15, G16, G18, and G21, and the fetuses were collected as described above. The remaining dam carried the pregnancy to term. The resulting pups (n = 1/postnatal day, P) were killed as described above at 1200 h on P3, P9, P14, and P19, and the intact head (P3–P9) or brains (P14–P19) were frozen on pulverized dry ice and stored at -80 C.

In situ hybridization

Frozen tissues were sectioned at 12 μ m in a cryostat (Reichert-Jung Frigocut 2800N, Leica Corp., Deerfield, IL). Frontal sections were collected from the cortex of one G16 fetus per dam, and sagittal sections were collected from the brains of animals in the developmental study. Sections were thaw-mounted onto gelatin-coated microscope slides and stored at -80 C until hybridization. *In situ* hybridization using the

NSP-A RNA probes was performed as described previously (25), except that the hybridization buffer contained 2×10^6 cpm probe/slide. *In situ* hybridization using the NSP-C oligodeoxynucleotide probe was performed as described previously (31), except that sections were immersed for 30 min in 4% formalin, and the hybridization buffer contained 200 mM dithiothreitol and 200,000 cpm probe/slide.

Probes

We cloned a fragment of the NSP-A transcript that does not overlap with that of NSP-C by standard PCR methods using primers designed to amplify a 202-bp region of NSP-A (1946-2147, accession no. U17604). Briefly, the forward (5'-AAGGCCTGTGAACCTGAC-3') and reverse (5'-GGCACGACCTCAGAACCAAG-3') NSP-A primers were synthesized by Life Technologies, Inc., Custom Primers (Life Technologies, Inc., Gaithersburg, MD). The NSP-A fragment was amplified from rat genomic DNA using 1 µм of each primer; 0.2 mм deoxy (d)-ATP, dCTP, dGTP, and dTTP; 1.0 mM MgCl₂; and 5 U Taq DNA polymerase (Life Technologies, Inc.). The PCR conditions after an initial denaturing step at 91 C for 5 min were 91 C for 1.5 min, 60 C for 2 min, and 72 C for 2 min, for 40 cycles. After a final extension at 72 C for 10 min, the PCR products were stored at 4 C. PCR products were separated on a 1.2% agarose gel and purified using GenElute spin columns according to the manufacturer's instructions (Sigma). The 202-bp NSP-A gene fragment was then ligated into pCRII (Invitrogen, Carlsbad, CA), and its authenticity was confirmed by sequence analysis using ABI FS-Dye-Terminator chemistry (PE Applied Biosystems, Foster City, CA). Both complementary and sense strand NSP-A RNA probes were generated in vitro in the presence of 1 μ g linearized plasmid; 500 μ M each of GTP, ATP, and CTP; and 12 μ M UTP (UTP and [³³P]UTP at a molar ratio of 1:1). The NSP-A complementary DNA (cDNA) was linearized with BamHI and transcribed in the presence of T7 polymerase for cRNA synthesis; it was linearized with EcoRV and transcribed in the presence of SP6 polymerase for sense strand RNA synthesis. In each case the DNA template was removed by DNase digestion, and the RNA probe was purified by standard phenol-chloroform extraction followed by two ethanol precipitations.

Life Technologies, Inc. Custom Primers synthesized the NSP-C oligodeoxynucleotide. The sequence was complementary to bases 1918– 1869 (accession no. L49143). For *in situ* hybridization, the oligodeoxynucleotide was 3'-end labeled in the presence of [³³P]dATP (ICN Biomedicals, Inc., Costa Mesa, CA) using terminal deoxynucleotidyl transferase (Roche Molecular Biochemicals, Indianapolis, IN) as previously described (32). The oligodeoxynucleotide probe was purified by phenol-chloroform extraction followed by two ethanol precipitations.

Probe specificity was evaluated by Northern analysis (Fig. 2). Total RNA was extracted from G16 cortical tissue using the method of Chomczynski and Sacchi (33). The RNA was electrophoresed with RNA molecular weight standards (Life Technologies, Inc.) on a 1.2% agarose/ 6.5% formaldehyde gel. RNA was transferred to a nylon Zeta-Probe membrane (Bio-Rad Laboratories, Inc., Hercules, CA) and cross-linked by baking. NSP-A cDNA was labeled in both random primer labeling and nick translation reactions in the presence of [32P]dCTP (ICN Biomedicals, Inc.) according to the manufacturer's instructions (Roche Molecular Biochemicals), and both types of probes were combined for hybridization. The NSP-C oligodeoxynucleotide was 3'-end labeled as described above, except that the reaction was performed in the presence of [32P]dCTP (ICN Biomedicals, Inc.). Membranes were briefly prehybridized, hybridized with either 500,000 (NSP-A cDNA) or $4 imes10^6$ (NSP-C oligodeoxynucleotide) cpm probe/ml hybridization buffer, and washed according to the manufacturer's instructions. The membranes were then apposed to a storage phosphor screen (Molecular Dynamics, Inc., Sunnyvale, CA) for either 6 days (NSP-A cDNA) or 23 h (NSP-C oligodeoxynucleotide). These screens were scanned into a Storm 840 PhosphorImager at 200-µm resolution and viewed using ImageQuant (Molecular Dynamics, Inc., Sunnyvale, CA).

Autoradiography and signal quantitation

After *in situ* hybridization, all slides were arranged in x-ray cassettes and apposed to BioMax film (Eastman Kodak Co., Rochester, NY) for either 1.5–3 weeks (NSP-A RNA probe) or 2 days (NSP-C oligodeoxynucleotide probe). ¹⁴C-Labeled standards (American Radiolabeled



FIG. 1. Effect of chronic PTU treatment and T_4 injections on serum levels of free T_3 (A), total T_4 (B), and TSH (C) in pregnant females at the time the rats were killed. See *Materials and Methods* for details of thyroid hormone manipulation. *Bars* represent the mean \pm SEM, with number of dams per group noted within each bar. All animals were killed at 1000 h on G16. Treatment groups are indicated *below* the ordinate. *, P < 0.05; **, P < 0.01 (significantly different from euthyroid dams receiving saline injection). Note that serum hormone levels below the detection limit were assigned the value of the detection limit (indicated by *dashed line*) for statistical purposes.

Chemicals, Inc., St. Louis, MO) were simultaneously apposed to the film to verify that the film was not overexposed. The hybridization signal was analyzed as described previously (34) using a Macintosh 7600 computer and the public domain NIH Image program (W. Rasband, NIMH). This system was interfaced with a Dage MTI 72 series video camera (DAGE-MTI, Michigan City, MI) equipped with a Nikon macro lens (Melville, NY) mounted onto a bellows system over a light box. The relative abundance of NSP-A and NSP-C messenger RNAs (mRNAs) was measured over the cortex of G16 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 six litters per treatment group.

Tissues that were used to characterize the spatial and temporal changes in expression of NSP-A and NSP-C transcripts throughout normal development were dipped in Kodak NTB-3 nuclear tract emulsion. These emulsion autoradiograms were developed in Dektol, fixed in Kodak fixer, and counterstained with methyl green (Sigma). Adjacent sections were counterstained with hematoxylin and eosin (Sigma).

RIA

Free T₃ was measured according to the manufacturer's instructions using a T₃ RIA kit (ICN Biomedicals, Inc.). This assay was performed at 17.8% binding with detection limits of 0.69–17.5 pg/ml and an intraassay variation of 17.9%. Total T₄ was measured according to the manufacturer's instructions using a T₄ RIA kit (ICN Biomedicals, Inc.). This assay was performed at 33.8% binding with detection limits of 2.0–20 μ g/dl and an intraassay variation of 19.9%. Serum levels of TSH were measured using [¹²⁵I]rat TSH (Covance Laboratories, Inc., Vienna, VA) and the double antibody NIDDK RIA reagents, including RP-3 standards. This assay was performed at 26.3% binding with detection limits of 1.0–30 ng/ml. All samples were measured in duplicate in the same assay.

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 was performed on hormone levels and imaging data using the StatView statistical package (Abacus Concepts, Berkeley, CA), with main effects of goitrogen treatment (MMI or PTU) and timing of acute T_4 exposure (MMI experiment) or T_4 injections (PTU experiment). The ANOVA was followed by *t* tests between individual means.

Results

Manipulation of thyroid status in the dams used in these experiments produced the expected effects on circulating levels of thyroid hormone. MMI-treated dams exhibited significantly lower circulating T_4 levels and significantly elevated serum TSH compared with euthyroid controls (25). T_4 injection transiently normalized serum T_4 and TSH in MMI-treated dams. Likewise, dams treated with PTU exhibited



FIG. 2. Validation of probe specificity for NSP-A cDNA and NSP-C oligodeoxynucleotide. A, Schematic diagram of NSP-A and NSP-C mRNAs. Locations of NSP-A and NSP-C probes at the 5'-ends of their respective mRNAs are noted by *striped bars*. ATG, Translation initiation; STOP, translation stop codon. B, Northern blots in which 20 μ g total RNA from the G16 cortex were hybridized with either ³²P-Iabeled NSP-A cDNA or NSP-C oligodeoxynucleotide. Note the size difference and relative abundance of the two transcripts.

significantly lower circulating levels of free T₃ [F_(1,16) 64.514; P < 0.001] and total T₄ [F_(1,19) 9.683; P < 0.006] and increased levels of TSH [F_(1,17) 13453.487; P < 0.001; Fig. 1]. Free T₃ levels were significantly elevated by T₄ injection in PTU-treated animals, but were unaffected by T₄ in euthyroid animals (Fig. 1). However, total T₄ [F_(1,19) 140.105; P < 0.001] was elevated, and TSH was suppressed [F_(1,17) 13453.487; P < 0.001] by the T₄ injection in both PTU-treated and control dams.

The specificity of the NSP-A and NSP-C probes was confirmed by Northern analysis (Fig. 2). The NSP-A complementary RNA probe hybridized to a single size class of RNA of approximately 3.5 kb, whereas the NSP-C oligodeooxynucleotide hybridized to a single size class of RNA of approximately 1.5 kb. Quantitative analysis of film autoradiograms after *in situ* hybridization of NSP-A revealed that chronic MMI [F_(1,18) 9.181; *P* < 0.008; Fig. 3A] or PTU [F_(1,19) 5.586; *P* < 0.03; Fig. 4] significantly increased NSP-A expression in the G16 cortex. There was no effect of T₄ injection on NSP-A expression within the time period examined. NSP-C expression was not affected by treatment in the G16 cortex (Fig. 3B). Because MMI did not affect the expression of NSP-C mRNA (25), we did not analyze the effect of PTU on NSP-C expression.

To gain additional information about the potential role that the thyroid hormone-responsive NSP-A may play in brain development, we characterized the differential distribution of NSP-A and NSP-C mRNAs during development (Fig. 5). Both transcripts were clearly detected on G15, the earliest time point examined, and they increased in abun-



Time of T₄Injection

FIG. 3. Effects of MMI treatment and T_4 injections on NSP-A (A) and NSP-C (B) expression in the G16 fetus. The *left* and *center panels* represent film autoradiograms presented in pseudocolor (*red* > *yellow* > *blue* > *black*) to illustrate the effects of MMI treatment on NSP-A and NSP-C expression. The *right panels* are bar graphs depicting the relative abundance of NSP-A or NSP-C mRNA in the G16 cortex as reflected by the mean \pm SEM of the film density (converted to a percentage of the control value; see text). The numbers of dams per group are noted at the *bottom of each bar*. Groups differed in the timing of T_4 injection as shown *below the ordinate*. \Box , euthyroid dams (no MMI); \blacksquare , hypothyroid dams (MMI). Sense controls produced no detectable signal (not shown). V, Ventricle; Cx, cortex. a, Significantly different from euthyroid animals receiving T_4 injection at 2100 h on G14 (P < 0.05).



FIG. 4. Effect of chronic PTU treatment and T_4 injection on NSP-A expression in the G16 fetus. The *left* and *center panels* represent film autoradiograms in pseudocolor (red > yellow > blue > black) to illustrate effects of PTU treatment on NSP-A expression. Note the higher level of NSP-A expression in fetal cortex derived from a hypothyroid dam. The *right panel* is a bar graph depicting the relative levels of NSP-A mRNA in the G16 cortex in the four treatment groups. *Bars* represent the mean \pm SEM of the film density (converted to a percentage of the control value) over the cortex, with the number of dams per group noted *within each bar*. Treatment groups are indicated *below the ordinate*. Sense controls produced negligible hybridization signal (not shown). V, Ventricle; Cx, cortex. *, P < 0.05 (significantly different from euthyroid animals receiving no injection).

dance progressively through postnatal day 19. Film autoradiograms of frontal sections examined by in situ hybridization on G16 (Fig. 3, A and B) indicated that NSP-A and NSP-C transcripts were expressed in different parts of the cortex at this time. This was confirmed by analysis of the liquid emulsion-coated slides of G16 sections (Fig. 6). The emulsion autoradiograms demonstrated that NSP-A mRNA is selectively expressed in the proliferative ventricular zone of the G16 cortex. The abundance of NSP-A expression in this layer diminishes by G18 when cortical proliferation is nearly complete and the ventricular zone disappears (35). As development proceeds, NSP-A expression becomes expressed in the outer layers of the cortex (Figs. 6 and 5) and even takes on a laminar appearance during postnatal development. In contrast, NSP-C appears to be expressed exclusively in the outer, intermediate zone of the cortex on G16 (Fig. 6) and, as development proceeds, exhibits robust and continuous expression in these outer layers. The film autoradiograms also indicated that NSP-A and NSP-C are differentially expressed in the nasal epithelium, olfactory bulb and cerebellum (Fig. 5). We further characterized the expression of NSP-A and NSP-C in the developing cerebellum (Fig. 7). NSP-A was exclusively expressed in cerebellar Purkinje cells throughout development, whereas NSP-C was expressed in granule cells, but was absent from Purkinje cells.

Discussion

Recent studies indicate that maternal hypothyroidism can affect neurological development of the fetus in the absence of congenital/neonatal hypothyroidism of the offspring (see introduction). In fact, there is presently a debate about whether thyroid status should be routinely screened in pregnant women regardless of prior evidence of hypothyroidism (36). However, virtually nothing is known about the developmental processes regulated by thyroid hormone in the fetus at midgestation or about the mechanisms by which thyroid hormone may regulate these processes. The present results unequivocally localizes NSP-A expression to the proliferative zone of the fetal cortex and demonstrates that maternal hypothyroidism selectively alters its expression in this important epithelium. Maternal thyroid status was manipulated by two separate goitrogens, MMI and PTU, and effects on NSP-A expression were observed before the onset of fetal thyroid function on gestational day 17 (37). Therefore, these data strongly suggest that thyroid hormone of maternal origin can affect some aspect of cortical neurogenesis. Moreover, our present findings also demonstrate that the thyroid hormone-responsive NSP-A is selectively expressed in cerebellar Purkinje cells, which are known to be thyroid hormone responsive during development and in the adult (38).

We previously reported that the NSP-A transcript was expressed in the intermediate zone of the fetal cortex (25). However, our previous conclusion was based on results using a cRNA probe that hybridized to both NSP-A and NSP-C mRNAs. Apparently, this probe gave us misleading results, because the transcript encoding NSP-C is much more abundant than that encoding NSP-A. Therefore, the signal for the NSP-C transcript in liquid emulsion was robust before the signal for NSP-A was visible, which gave us the impression that both NSP transcripts were expressed in the intermediate zone. The preparation and use of specific probes for NSP-A and NSP-C allowed us to clearly determine that NSP-A is exclusively expressed in the ventricular zone of the fetal cortex. We also found previously that a single injection of T₄ significantly reduced the abundance of NSP-A mRNA in the G16 cortex (25). This conclusion was based on Northern analysis and *in situ* hybridization. However, we also observed a trend for NSP-C to be reduced by T₄ that may have contributed to the reduction in NSP-A/C we observed in the *in situ* hybridization. The use of probes specific for the two NSP transcripts in the present experiments precludes the possibility of this kind of cross-contamination. Using these specific probes, we now find no evidence for an effect of T₄ on NSP-A expression within 24–36 h. The ability of a single

FIG. 5. Distribution of NSP-A and NSP-C mRNA during development in the euthyroid rat. Images are derived from film autoradiograms after in situ hybridization. NSP-A RNA probes or NSP-C oligodeoxynucleotide probes were applied to tissues on G15, G16, G21, P9, P14, or P19. The distribution of NSP-A mRNA appears to be more restricted in the cortex on G16 and G21 (C and F) and in the nasal epithelium (E) and olfactory bulb (E) compared with NSP-C. Finally, NSP-A is clearly expressed in different parts of the cerebellum on P9 (G), P14 (I), and P19 (K) compared with NSP-C. C, Cerebellum; Cx, cortex; D, dorsal root ganglion; H, Cx, cortex, D, dorsar root ganglion; H, hippocampus; M, medulla; Mb, mid-brain; N, nasal epithelium; R, retina; SC, spinal cord; TG, trigeminal gangli-on; Th, thalamus. *Scale bar*, 0.2 cm. Note the transcript-specific distribution patterns in the cortex on G21 and in the cerebellum on P9, P14, and P19. Sense controls produced negligible hybridization signal (data not shown).





FIG. 6. Distribution of NSP-A and NSP-C mRNA in the cortex during prenatal development in the euthyroid rat. Images are derived either from hematoxylin- and eosin-stained sections (A, D, and G) or from emulsion autoradiograms in darkfield after *in situ* hybridization. NSP-A RNA probes (B, E, and H) or NSP-C oligodeoxynucleotide probes (C, F, and I) were applied to sagittal sections of G16, G18, or G21 cortex. Note that NSP-A expression is observed exclusively in the ventricular zone on G16 and G18. IZ, Intermediate zone of cortex; V, lateral ventricle; VZ, ventricular zone of cortex. *Scale bar*, 0.2 mm. Sense controls produced negligible hybridization signal (data not shown).

injection of T_4 to reduce cellular levels of a specific mRNA within a given time will necessarily depend on the overall degree to which T_4 reduces its transcription and the half-life of the mRNA in the cell. Therefore, it is likely that a single injection of T_4 , regardless of dose, does not suppress NSP-A expression in the G16 cortex to the extent that *in situ* hybridization can resolve it within 24 h. This does not affect gene expression in the G16 cortex, because we previously showed that Oct-1 expression was significantly elevated within 12 h of the T_4 injection (25). Clearly, the logistical issues surrounding the measurement of gene repression.

The two experiments we now describe differed in the timing of goitrogen treatment in that MMI was initiated 2 weeks before the females were mated, whereas PTU was initiated in pregnant females on G2 (see *Materials and Methods*). Considering that NSP-A expression was affected by the two treatments to the same extent, the present results suggest that goitrogen treatment initiated before conception does not produce more severe effects on gene expression in the G16 fetus than goitrogen treatment initiated after conception. This is important in part because it may indicate that hypothyroidism before pregnancy does affect the course of brain

development. However, it is also important because it represents a refinement in our model of gestational hypothyroidism and the consequences on gene expression in and development of the fetal brain. This model allows us to focus on the effects of maternal thyroid status on fetal brain development despite the fact that both MMI and PTU can cross the placenta (39, 40). Our reasoning is that fetal thyroid function does not begin in the rat until G17 (37), so the only source of thyroid hormone to the fetus on G16 is the maternal system. Therefore, this model allows us to study the effects of maternal hypothyroidism on fetal brain development without the confounding influence of effects on the fetal thyroid.

The developmental significance of the present findings will require in part that we identify the specific subpopulation of neuroblasts that selectively express NSP-A. The ventricular zone of the fetal cortex is a pseudostratified germinal epithelium containing cells that are actively dividing and cells that have stopped dividing and are beginning to differentiate (35, 41–44). Therefore, it will be important to determine whether NSP-A is expressed in actively proliferating neuroblasts or in those neurons that have begun to differentiate but have not yet left the ventricular zone. Neuroendocrine-specific proteins are anchored in the membrane of



Р19

Α

P3

P9

P14

P19

FIG. 7. Distribution of NSP-A and NSP-C mRNA in the cerebellum during development in the euthyroid rat. Images are derived either from hematoxylin- and eosin-stained sections (*left column*) or from emulsion autoradiograms after *in situ* hybridization. NSP-A RNA probes or NSP-C oligodeoxynucleotide probes were applied to sagittal sections of P3, P9, P14, or P19 cerebellum. E, External germinal layer; I, internal granule layer; M, molecular cell layer; P, Purkinje cell. *Scale bar*, 0.2 mm in first four rows, 0.05 mm in last row. *Arrows* identify Purkinje cells. Note that NSP-A mRNA is localized to the Purkinje cell layer of the developing cerebellum, whereas NSP-C mRNA is localized to both the internal and external granule cell layers. Sense controls produced negligible hybridization signal (data not shown).

(C

the endoplasmic reticulum (45, 46, 28) and may be important for neuronal differentiation and axonal guidance (47). Thus, it is possible that the extended N-terminus of NSP-A (27) confers a function to this protein that is important in cortical neurogenesis. Although the magnitude of the effect of maternal goitrogen treatment on NSP-A expression was relatively small, it is likely that this effect is an underestimation of the actual effect. NSP-A-positive cells are quite restricted in their distribution, producing a very narrow signal on film that saturates quickly.

We found in the present study that NSP-A mRNA is expressed in several brain areas that differ from those in which NSP-C is expressed throughout development. For example, the film autoradiograms indicate that NSP-A is expressed in restricted regions of the nasal epithelium, olfactory bulb, and cerebellum compared with NSP-C (compare E with F, and G, I, and K with H, J, and L in Fig. 5). We further characterized the differential distribution of NSP-A and NSP-C expression in the cerebellum (Fig. 7) and found that during postnatal development, NSP-A expression is always restricted to Purkinje cells, and NSP-C expression is always absent from Purkinje cells. This may be particularly important because neonatal hypothyroidism permanently affects the maturation of Purkinje cells by decreasing arborization and numbers of dendritic spines (48). Because the Purkinje cells are the major efferent neurons from the cerebellar cortex (49), these morphological effects of hypothyroidism may contribute to the motor deficits associated with neonatal hypothyroidism (50, 51). The selective expression of NSP-A in Purkinje cells and its regulation by thyroid hormone (Dowling, A. L. S., in preparation) clearly suggest that NSP-A may play a role in the effects of Purkinje cell morphology and on animal behavior.

The present findings demonstrate that NSP-A exhibits a different pattern of expression in the developing brain compared with that of NSP-C. This is especially important in the early period of cortex development, when NSP-A is selectively expressed in the germinal ventricular zone. In addition, NSP-A is selectively expressed in cerebellar Purkinje cells. Finally, the present findings demonstrate that induction of maternal hypothyroidism with MMI or PTU affects NSP-A expression in the proliferative zone of the fetal cortex before the onset of fetal thyroid function. It is important to recognize that this observation does not allow us to conclude whether thyroid hormone exerts a direct effect on NSP-A expression. One can imagine several indirect mechanisms by which thyroid hormone could affect NSP-A expression in the fetal cortex. However, it is possible that thyroid hormone from the maternal system can reach the neuroblasts of the fetal cortex, activate the thyroid hormone receptor, and directly repress NSP-A expression. Thyroid hormone from the maternal circulation can cross the placenta and gain access to fetal tissues (52, 53, 20). In addition, the β 1 thyroid hormone receptor is selectively expressed in the proliferative zone of the fetal cortex on G16 (18). Moreover, considering that maternal hypothyroidism affects the expression of NSP-A, but not that of NSP-C, and that thyroid hormone appears to suppress NSP-A expression, but enhance Oct-1 expression, in the same tissue (25), it appears that thyroid hormone exerts specific effects on gene expression in the fetal brain.

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