Gonadotropin-releasing hormone (GnRH) is encoded by the proGnRH gene which contains four exons and three introns. In this study, two immortalized GnRH-expressing cell lines (Gn11 and NLT) were characterized. The NLT and Gn11 cells, derived from a same brain tumor in a transgenic mouse, display neuronal morphology and neuron-specific markers. However, NLT cells secrete much higher levels of GnRH than Gn11 cells. To delineate the mechanism underlying this difference, reverse transcriptase-polymerase chain reaction and RNase protection assays were performed to examine proGnRH gene expression. While the mature proGnRH mRNA was predominately expressed in NLT cells, Gn11 cells express an abundant short transcript. Sequence analysis revealed that this short transcript contains exons 1, 3, and 4, but not exon 2, which encodes the GnRH decapptide. RNase protection assays demonstrated that NLT cells express much higher levels of mature proGnRH mRNA than Gn11 cells. The lower level of GnRH secreting capacity in Gn11 cells is due, in part, to decreased expression of mature proGnRH mRNA. When proGnRH gene expression in the mouse brain was examined, the same short splicing variant was observed in the olfactory area and preoptic area-anterior hypothalamus. But the prevalent transcript in these regions was the mature proGnRH mRNA. In contrast, only the mature proGnRH mRNA was found in the caudal hypothalamus. These results suggest that alternative splicing may be one of the mechanisms regulating proGnRH gene expression in the animal brain.

GnRH neurons in the hypothalamus play an essential role in the regulation of mammalian reproduction. These neurons originate from precursor cells in the olfactory placode and migrate to their target sites during embryonic development (1, 2). The most prominent axonal projection of GnRH neurons is to the median eminence, where GnRH is released and transported via the hypothalamic hypophyseal portal vessels to the anterior pituitary to stimulate the synthesis and release of luteinizing hormone and follicle-stimulating hormone (3). The regulation of GnRH neuronal activities has been difficult to study, however, due to their scattered distribution and paucity in cell number (3–5). Recently, this laboratory and Mellon et al. (6, 7) have generated immortalized GnRH-expressing neuronal cell lines by targeted tumorigenesis. In our laboratory, targeting the expression of the simian virus 40 T antigen (SV40-Tag) to the GnRH neurons with the human GnRH gene 5′-upstream regulatory sequence resulted in the development of an olfactory tumor in one of the transgenic mice (7). Two GnRH immunoreactive cell lines (Gn11 and NLT) were subsequently derived from this tumor. Characterization of the NLT and Gn11 cells demonstrated that these cells display neuronal morphology and neuron-specific markers, such as microtubule-associated peptide 2 (MAP-2) and Tau protein (8, 9). Solution hybridization-RNase protection assays and RT-PCR analysis indicated that these cells express proGnRH mRNA and are able to synthesize and secrete GnRH as demonstrated by RIA. Therefore, these cell lines provide a suitable in vitro model for the study of GnRH neuronal activity and its regulation.

Although the NLT and Gn11 cells were derived from the same tumor, RIA measurement of GnRH concentrations demonstrated that the NLT cells secrete about 10 times higher levels of GnRH than the Gn11 cells. In an attempt to understand the molecular basis responsible for generating this difference, we characterized the expression of the proGnRH gene in these cell lines by RT-PCR and solution hybridization-RNase protection assays. In addition, the expression of proGnRH gene expression in the mouse olfactory and preoptic area-hypothalamus was also examined. Our results demonstrate that a splicing variant lacking exon 2 of the proGnRH gene was present in Gn11 cells but not in NLT cells. This same splicing variant was also found in the olfactory and preoptic area-anterior hypothalamus, but not in the caudal hypothalamus of the mouse brain. This is the first demonstration that alternative splicing of the primary proGnRH gene transcript occurs in the immortalized GnRH-expressing neuronal cell lines and in the animal forebrain.

MATERIALS AND METHODS
Cell Culture—Gn11 and NLT cells were maintained in Dulbecco’s modified essential medium supplemented with 10% fetal bovine serum. For GnRH measurement, culture medium was replaced 4 h before sample collection from dishes containing 90% confluent cells, and a...
0.5-ml medium sample was collected from each dish and kept at −80 °C for the GnRH RIA.

GnRH RIA—The levels of GnRH in the samples were determined by RIA as described (10). Cells in the culture dish were lysed for determination of protein concentrations as described (Sigma) (11). GnRH values were expressed as picograms/mg of protein. The sensitivity of the GnRH RIA was 0.2 pg/tube, and the interassay coefficient of variation at 20 pg/tube was 14%.

Mouse Brain Tissues—Adult CD1 mice maintained on a 12/12 h light/dark schedule (lights on at 07:00 h) were sacrificed by CO2 inhalation and cervical dislocation. The olfactory cortex was isolated to include both olfactory bulbs and the tissue rostral to the hypothalamic section with a dorsal incision 3 mm anterior to the preoptic area-anterior hypothalamus was dissected from the anterior edge of the mammillary bodies to the anterior of the optic chiasm, laterally 1 mm beyond the lateral aspect of the median eminence and 3 mm dorsally. The caudal hypothalamus included the rostral mammillary bodies to a point 1 mm caudal to the mammillary bodies, with the same dorsal, and lateral parameters as those taken for the rostral hypothalamus. The brain tissues were kept at −80 °C before RNA extraction.

Immunocytochemistry—Cells growing on glass coverslips were fixed, permeabilized, and stained as described by the manufacturer (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD). Primary antibodies were used at the following dilutions: anti-MAP-2 antibody 1:500, anti-Tau antibody 1:500 (Sigma). Labeled cells were examined on a microscope using a 1:500, anti-Tau antibody 1:500 (Sigma). Labeled cells for anti-MAP-2 antibodies were used at the following dilutions: 1:500, anti-Tau antibody 1:500 (Sigma). Labeled cells were examined on a microscope using a ×100 lens.

Extraction of Total Cellular RNA—RNA was extracted from cells and the brain tissues according to the manufacturer's manual (Molecular Research Center, Inc., Cincinnati, OH). The resultant RNA was suspended in 20 μl of RNase-free diethyl pyrocarbonate water for RNase protection assay and RT-PCR analysis.

RT-PCR—First-strand cDNA synthesis was performed by using oligo(dT)16 as the primer and Moloney murine leukemia virus reverse transcriptase (Boehringer Mannheim). PCR was performed by using primers complementary to sequences in exon 1 and exon 4 of the mouse proGnRH gene. The 5′ primer sequence was: 5′-GAAGTACTCAAC-CTACCAA-3′, and the 3′ primer was: 5′-GCCATACAGGGCTCAACG-CTC-3′.

Sequencing of the PCR Products—PCR reaction mixtures were separated by electrophoresis in a 1.5% agarose gel, and the two bands corresponding to 352 and 210 bp were cloned into pGEM-T plasmids (Promega, Madison, WI), and sequenced by Sanger's method using T7/SP6 primers and Sequenase® according to the manual (U.S. Biochemical Corp.).

Southern Blot Analysis—The PCR products were electrophoresed in a 1.5% agarose gel, and transferred to a nitrocellulose membrane. Probes for the Southern blot were obtained from the above pGEM-T plasmids through restriction enzyme digestion. The cDNA fragments were 32P-labeled using the Klenow enzyme and random hexanucleotide primer (Boehringer Mannheim).

Northern Blot Analysis—Fifteen μg of total RNA from NLT, Gn11, or CV-1 cells were electrophoresed on a 1.5% agarose gel and transferred to a nitrocellulose membrane. After hybridization to a 32P-labeled probe specific for Tau, the membrane was washed three times with 0.1 × SSC, 0.1% SDS at 45 °C. For autoradiography, the membrane was exposed to x-ray film with an intensifying screen at −80 °C for 1 day.

Solution Hybridization-RNA Protection Assay—The assay was performed with the RPA II ribonuclease protection assay kit (Ambion, Austin, TX). Template cDNA inserted in the pGEM-T plasmids was the same as that used for making Southern blot probes. The template plasmid was linearized with the restriction enzyme NcoI, and in vitro transcription was performed by using α-32P[UTP] and SP6 polymerase to generate the antisense mouse GnRH probe. As a control, mouse actin antisense probe was also synthesized. Solution hybridization was performed by mixing sample RNA (30–40 μg) with 4–6 × 105 cpm of α-32P[UTP]-labeled riboprobe in a final volume of 20 μl of hybridization solution. Hybridization was carried out at 45 °C overnight, and the mixture was digested with RNase at 37 °C for 1 h. The hybridized RNA fragments were precipitated, resuspended in 8 μl of gel loading buffer, and heated at 95 °C for 5 min before electrophoresis in a 6% denaturing polyacrylamide gel. The amount of radioactivity in the samples was counted using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

The NLT Cells Secrete More GnRH Peptide than the Gn11 Cells—Imortalized NLT and Gn11 GnRH-expressing neuronal cells were generated by targeting the expression of the SV40-Tag to GnRH neurons with the human GnRH gene promoter (7). The hybrid gene containing the human GnRH gene promoter from −1131 to +5 bp fused to the SV40-Tag coding region was injected into fertilized one-cell embryos to generate transgenic mice. An olfactory tumor containing Tag- and GnRH-immunopositive neurons developed in an F1 male. The tumor was dispersed with collagenase and cultured. Through serial dilution and cloning, a number of GnRH-immunopositive cells were obtained from this tumor. Immunocytochemical double labeling indicated the expression of both Tag and GnRH within these cells (7). In the present study, further characterization of two of these cell lines, referred to as NLT and Gn11, was performed.

The NLT and Gn11 cells have a doubling time about 24–48 h. Both cell types displayed neuron-like morphology with fusiform or multipolar processes and had extensive contacts as shown in Fig. 1a. Some thin processes extended far away from cell body and resembled neurosecretory axons by exhibiting varicosities, while other processes exhibited dendrite-like appearance. Immunocytochemical staining with polyclonal antibodies against either MAP-2 or Tau proteins, two neuron-specific markers (8, 9), demonstrated the expression of both antigens in NLT and Gn11 cells (Fig. 1a). Shown in Fig. 1b is a Northern blot analysis, indicating the expression of Tau mRNA in both NLT and Gn11 but not in CV1 cells.

RIA measurement of GnRH concentrations in the media collected from the culture dishes indicated that both NLT and Gn11 cells secrete GnRH. However, NLT cells secrete about 10 times higher levels of GnRH than Gn11 cells (Fig. 1c). These results suggest that although both the NLT and Gn11 cells are GnRH-expressing neuronal cells derived from the same tumor, a significant difference exists in their capacity to secrete GnRH.

Differential Expression of the ProGnRH Gene in NLT and Gn11 Cells—In an attempt to delineate the molecular basis responsible for generating this difference in GnRH secreting capacity between these two cell lines, we utilized RT-PCR to characterize proGnRH gene expression in these two cell lines. Interestingly, in Gn11 cells, two different species of PCR products were consistently observed, one at 352 base pairs, corresponding to the complete mouse hypothalamic GnRH cDNA, and a second more prominent species which was 142 base pairs shorter, at 210 base pairs long (Fig. 2). A Southern blot of the same agarose gel was performed and hybridized to the radiolabeled mouse hypothalamic GnRH cDNA probe. Both species of transcripts hybridized to the radiolabeled GnRH probe. In contrast, only the 352-bp transcript was present in the NLT cells (Fig. 2). This result suggests that in addition to the expression of the mature proGnRH mRNA which encodes GnRH and GAP, a short transcript is abundantly expressed in Gn11 but not in NLT cells.

Exon 2 of the ProGnRH Gene Is Spliced Out in the Short Transcript in Gn11 Cells—Alternative splicing of the primary proGnRH gene transcript has been demonstrated in various peripheral tissues (12–15). To test whether alternative splicing of the primary proGnRH transcript may account for the occurrence of the two different species of transcripts found in the Gn11 cells, these two species of transcripts were isolated and cloned into the pGEM-T vector (Promega). The cloned plasmids were then sequenced for AG1 restriction enzyme digestion. Southern blot analysis was performed with a probe that contained the mouse hypothalamic proGnRH cDNA sequence (exons 1–4). As expected, this probe hybridized to both the larger and smaller inserts (Fig. 3, left panel). However,
when a second probe which contained only the exon 2 sequence of the mouse proGnRH gene was hybridized to the same Southern blot, only the larger cDNA species hybridized to this probe (Fig. 3, right panel). The lack of hybridization of the smaller insert with the exon 2 probe indicates the absence of exon 2 sequence in this cDNA obtained from Gn11 cells.

To confirm the above findings, these two clones were sequenced and the encoding nucleic acids deduced. As expected, sequencing revealed that the larger transcript expressed in NLT and Gn11 cells contains exons 1, 2, 3, and 4 of the proGnRH gene and that introns A, B, and C are spliced out from the primary transcript (Fig. 4a). The ATG (designated Met) in exon 2 of the proGnRH gene is used for translation initiation (Fig. 4b). However, in the smaller transcript present only in the Gn11 cell, exon 2 together with introns A and B are spliced out. Therefore, the shorter splicing variant in Gn11 cells contains exons 1, 3, and 4, but does not have exon 2 which encodes the signal sequence of GnRH, the GnRH decapeptide, and the first 11 amino acids of GAP. The ATG (designated Met) in exon 3 may be used for translation initiation. However, the Kozak sequence (16), which is a purine (G) at 3 nucleotides upstream of the ATG in the proGnRH mRNA (AGTGG/ACATG), is not present in the shorter splicing variant (CAAACAGATG).

The NLT Cells Express Higher Levels of proGnRH mRNA than Gn11 Cells—To investigate whether alternative splicing
Alternative Splicing of proGnRH Transcript

Alternative Splicing of the proGnRH Gene Transcript in the Mouse Brain—Although alternative splicing of the primary GnRH gene transcript has been demonstrated in various peripheral tissues, little is known about whether this phenomenon also occurs in the animal brain. To investigate this possibility, brain tissues from the mouse olfactory, preoptic area-anterior hypothalamus, and caudal hypothalamus, respectively. After RT-PCR and agarose gel electrophoresis, Southern blot hybridization was performed with a probe that contains the proGnRH cDNA. Both the larger and shorter transcripts were present in the olfactory (A) and preoptic area-anterior hypothalamus (B). However, the caudal hypothalamic area (C) expressed only the larger transcript. In the right panel, an exon 2 probe detected only the full-length proGnRH mRNA in the olfactory and preoptic area-anterior hypothalamus. proGnRH mRNA (Fig. 6C, left). The relative amount of the proGnRH mRNA versus the short splicing variant was estimated at approximately 20:1 by quantification with a PhosphorImager. When an exon 2 probe was used, only the proGnRH mRNA was detected in the olfactory, preoptic area-anterior hypothalamus and the caudal hypothalamus (Fig. 6A, A–C, right). The absence of hybridization of the smaller transcript in Fig. 6A and B, to the exon 2 probe indicates that exon 2 was spliced out in this species of the transcript. This result has been confirmed in two independent experiments. Therefore, these data indicate that alternative splicing of the proGnRH primary transcript also occurs in the mouse brain. In the caudal hypothalamus, processing of the primary proGnRH transcript gives rise to the mature proGnRH mRNA, which is then translated into proGnRH for subsequent processing to GnRH and GAP. In

Fig. 5. Quantification of proGnRH mRNA levels in NLT and Gn11 cells by RNAse protection assay. Template cDNA containing exons 1, 2, 3, and 4 of the mouse proGnRH gene was inserted in the pGEM-T plasmid between the Ncol and PstI sites. The template plasmid was linearized with restriction enzyme Ncol, and in vitro transcription was performed by using [α-32P]UTP and SP6 polymerase to generate antisense mouse GnRH probes. As a control, mouse actin antisense probe was also synthesized. The riboprobes were hybridized with 30–40 µg/tube of total cellular RNA obtained from either NLT or Gn11 cells. The hybridized RNA fragments were precipitated, electrophoresed in a 6% denaturing polyacrylamide gel, and quantified with a PhosphorImager. The positions of the proGnRH and actin mRNA signals are indicated.

Fig. 4. Processing of the proGnRH gene primary transcript in Gn11 and NLT cells. a, a schematic diagram of the amino acid sequence encoded by the mature proGnRH mRNA. The relative positions of the signal peptide (−21 to −1), GnRH decapetide (+1 to +10, underlined), and GAP (+14 to +69) are shown, b, the larger transcript present in both NLT and Gn11 cells, and the short splicing variant present only in Gn11 cell, were cloned into the pGEM-T plasmid. The two cDNA clones were sequenced by Sanger’s method using T7 and SP6 primers. In Gn11 and NLT cells, processing of the proGnRH gene primary transcript gives rise to the mature proGnRH mRNA containing exons 1, 2, 3, and 4. The ATG in exon 2 at −21 is used for translation initiation. In Gn11 cells, in addition to the mature proGnRH mRNA, processing the primary transcript yields a short splicing variant in which intron A, exon 2, and intron B are spliced out.

of the primary GnRH gene transcript in Gn11 cells may result in a decrease in the level of mature proGnRH mRNA. RNAse protection assays were performed to quantify proGnRH mRNA levels in both NLT and Gn11 cells. 32P-labeled antisense mouse proGnRH mRNA probe was obtained by in vitro transcription of a cDNA template containing exons 1, 2, 3, and 4 of the mouse proGnRH gene. As a control, the actin probe was also prepared at the same time. As shown in Fig. 5, the NLT cells express high levels of proGnRH mRNA in the RNAse protection assay. With the assay sensitivity at 50 fg/well level, proGnRH mRNA in Gn11 cells was undetectable, although RT-PCR revealed its expression in this cell line. In contrast, the actin mRNA levels were comparable between the two cell lines. This finding suggests that the low level of GnRH secreting capacity in Gn11 cells is due, in part, to decreased expression of the proGnRH mRNA.

The positions of the proGnRH and actin mRNA signals are indicated.

Total RNA was extracted from the mouse olfactory, preoptic area-anterior hypothalamus, and caudal hypothalamus, respectively. After RT-PCR and agarose gel electrophoresis, Southern blot hybridization was performed with a probe that contains the proGnRH cDNA. Both the larger and shorter transcripts were present in the olfactory (A) and preoptic area-anterior hypothalamus (B). However, the caudal hypothalamic area (C) expressed only the larger transcript. In the right panel, an exon 2 probe detected only the full-length proGnRH mRNA in the olfactory and preoptic area-anterior hypothalamus.
Alternative Splicing of proGnRH Transcript

the olfactory and preoptic area-anterior hypothalamus, the primary transcript is alternatively processed to generate either proGnRH mRNA or the shorter splicing variant.

DISCUSSION

Our data indicate that the NLT and Gn11 cells are neuronal in phenotype, express proGnRH mRNA, and secrete GnRH into the medium. Since one major difficulty in the study of GnRH neuronal activities is their low abundance and scattered distribution, these cell lines provide a convenient in vitro model for the study of GnRH secretion and regulation of gene expression. Using the Gn11 and NLT cell lines as a model, we found that although both cell lines were derived from a same tumor in a transgenic mouse, they display heterogeneity in the secretion of GnRH and expression of proGnRH gene. Our data provide evidence that the lower GnRH secreting capacity found in the Gn11 cells is due, in part, to the prevalence of a splicing variant lacking exon 2 which encodes the GnRH decapeptide. Moreover, we extended this finding to the animal forebrain by demonstrating that this splicing variant is also expressed in the olfactory and preoptic area/anterior hypothalamus, although the prevalent form of transcripts in these areas is the mature proGnRH mRNA. Therefore, these results suggest that alternative splicing of the primary transcript may contribute to the regulation of proGnRH gene expression in the animal forebrain.

Various lines of evidence indicate that proGnRH gene expression is regulated in a tissue-specific pattern. In various peripheral tissues, including the placenta, mammary gland, testes, and ovary (12–14, 17), the expression and processing of the primary transcript is significantly different from that in the hypothalamus in that a greater proportion of the transcripts in these tissues contains intron A. Moreover, a major transcriptional start site upstream of that used in the hypothalamus is utilized for transcription of the proGnRH gene in these tissues (14, 17). In the immature T lymphocyte cell line Nb2, in addition to the mature GnRH mRNA, the alternatively spliced form of transcript is also found, which lacks exon 2 that encodes the GnRH decapeptide (15). Because the splicing process affects the coding capacity of transcripts directly, its efficiency and accuracy are obviously critical for normal functions of GnRH neurons. Our present data demonstrate that even among GnRH neurons located in different regions of the central nervous system, the primary transcript is differentially processed. For GnRH neurons located in the olfactory area where Gn11 and NLT cells were derived, and those in the preoptic area/anterior hypothalamus, in addition to the prevalent mature mRNA, a short splicing variant lacking exon 2 of proGnRH gene is also produced, whereas only the mature proGnRH mRNA is generated by GnRH neurons located in the caudal hypothalamus. This finding is in agreement with the emerging evidence that the expression of proGnRH gene is differentially regulated in different regions of the central nervous system. For example, in the human brain, in situ hybridization studies suggest the presence of three distinct subtypes of GnRH neurons with pronounced differences in morphology, labeling density, and location (18). The number of GnRH neurons detected by in situ hybridization and immunocytochemistry has also been shown to vary in response to ovariotomy (19, 20), steroid treatment (21), and during the estrous cycle (22, 23). Recent studies of the rat preovulatory luteinizing hormone surge demonstrate that the number of GnRH-expressing cells fluctuates during the periovulatory period, and peak numbers of GnRH-expressing cells are attained at different time points in the preoptic area versus GnRH neurons in the more rostral regions (24). Taken together, these studies support the presence of heterogeneous populations of GnRH neurons in the mammalian central nervous system. Our data suggest that alternative splicing may be one of the mechanisms by which heterogeneous populations of GnRH neurons can be generated in the animal brain.

Studies of RNA splicing mechanisms suggest that differences in the activities/amounts of general splicing factors or the presence of specialized proteins may participate in the regulation of alternative splicing (25). In addition, a number of cis-acting sequences that influence splice site recognition have been identified, which include intron size, exon sequence, alternative branch points, pyrimidine content of 3' acceptor sites, and secondary structure of the pre-mRNA (26). The difference in processing the primary proGnRH gene transcript between the Gn11 versus NLT cells and between the GnRH neurons in the caudal hypothalamus versus those in the olfactory and preoptic areas may reflect the differential capacity of different GnRH neurons to recognize exon 2 in the proGnRH pre-mRNA. In the Gn11 cells and GnRH neurons located in the olfactory area and preoptic area-anterior hypothalamus, the splicing machinery either recognizes exon 2 in the primary transcript to generate the mature mRNA or ignores it, resulting in the formation of a shorter species of transcript lacking exon 2. Whether the difference in splicing of the primary transcript is due to the presence or absence of specific splicing factors in the Gn11 cells versus NLT cells or due to the difference in the activities in general splicing factors assembled in the spliceosomes inside the nucleus remains to be determined.

Although the shorter transcript found in the Gn11 cells and in GnRH neurons in the forebrain lacks the normal ATG translation initiation codon located in exon 2, examination of exon 3 sequence indicates that another ATG translation initiation codon is present in exon 3. The methionine at position +25 of the proGnRH peptide may act as a translation initiation signal for the remaining 45 amino acids of the GAP. However, the Kozak sequence (16), which is preserved as a purine (G) located at 3 nucleotides upstream of the ATG in the larger transcript, is not present in the smaller transcript. Whether this splicing variant is translated into a protein in Gn11 cells and the translational efficiency has yet to be determined.

Since the NLT cells express higher levels of mature proGnRH mRNA and secrete larger quantities of GnRH, these cells have the advantage over the Gn11 cells for studying the regulation of proGnRH gene expression and GnRH secretion by various factors. Recent studies from this laboratory using the NLT cell line have demonstrated that these cells express type-I receptor for insulin-like growth factor (27) and the receptor for epidermal growth factor (28). These findings are interesting since both insulin-like growth factor-I and epidermal growth factor have been shown to regulate GnRH neuronal activities in vivo and have been suggested to play an important role in the control of pubertal development (29, 30). However, to date, the molecular mechanisms by which these growth factors regulate GnRH neuronal activities remain largely unknown, due to the fact that less than 1500 GnRH neurons are present in the animal brain (4, 5). Therefore, the availability of NLT cells should greatly enhance our ability to explore the molecular mechanisms that mediate the regulation of proGnRH gene expression and GnRH secretion.

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An Alternative Gonadotropin-releasing Hormone (GnRH) RNA Splicing Product Found in Cultured GnRH Neurons and Mouse Hypothalamus
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