Structure of the rat gonadotropin releasing hormone (rGnRH) gene promoter and functional analysis in hypothalamic cells

Jadwiga K.Kepa^{1,2}, Chun Wang^{1,2}, Christine I.Neeley^{1,2}, Mary V.Raynolds¹, David F.Gordon¹, William M.Wood¹ and Margaret E.Wierman^{1,2*}

¹Department of Medicine, University of Colorado Health Sciences Center and ²Research Service, Veterans Affairs Medical Center, Denver, CO 80220, USA

Received October 9, 1991; Revised and Accepted February 21, 1992

EMBL accession no. X62651

ABSTRACT

The gonadotropin releasing hormone (GnRH) gene encodes a protein which plays a critical role in mammalian reproductive physiology. Its expression is predominantly restricted to the hypothalamus although it has also been described in the placenta. To begin to determine the promoter elements important for tissue specific expression and to examine the mechanisms of developmental and hormonal regulation of the rat GnRH (rGnRH) gene, we cloned the rGnRH gene from a rat liver genomic DNA library. The nucleotide sequence of greater than 3 kb of 5'-flanking region was determined. The transcriptional initiation site in rat hypothalamic tissue and a mouse hypothalamic cell line were mapped by primer extension analysis and found to be different. In addition, transient transfection studies demonstrated that multiple regions of the distal promoter are important for tissue specific and basal promoter activity in hypothalamic cells. Furthermore, in these cells a potent activation region resides between -3026 and -1031 bp and suppressor region between -1031 and -903 bp upstream of the transcriptional start site. We conclude that different portions of the 5'-flanking region, which are activating and suppressing in nature, are critical for hypothalamic expression of the rGnRH gene.

INTRODUCTION

Gonadotropin releasing hormone (GnRH) plays a pivotal role in the regulation of the hypothalamic-pituitary-gonadal axis and its expression ultimately mediates gametogenesis and reproductive competence (1). The bioactive decapeptide directs the biosynthesis and release of the pituitary gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), which in turn stimulate the production of steroid hormones and gonadal peptides from the ovary and testis (1). GnRH is synthesized and secreted from approximately 800–1000 neurons in the hypothalamus (2–5). The transgenically derived GT1 cell lines created by

Mellon and colleagues (6) provide, for the first time, a uniform population of GnRH-secreting neurons with which to study hypothalamic gene expression.

In an attempt to study the pretranslational regulation of GnRH, a cDNA was initially isolated from a human placental cDNA library (7). The genes for the human (8–9), mouse (10), and rat (8,11) GnRH were subsequently cloned. However, the previous lack of GnRH expressing cell lines and limited tissue specific expression of GnRH hampered investigation in this area. The GnRH protein was demonstrated in the placenta of many species (12–14). GnRH was observed throughout gestation, where it may play a role in chorionic gonadotropin (cG) production (15–17). A GnRH-like activity has also been described in the gonad (18) and in mammary tissue (19). Although detectable in the hypothalamus, an RNA coding for GnRH has not been detected by Northern blot analysis in the placenta. Studies using the more sensitive techniques of solution hybridization (20) and polymerase chain reaction (PCR) (our unpublished observations) have demonstrated the presence of GnRH mRNA in human placenta and JEG-3 cells.

Because of the difficulty in directly studying physiologic regulation of GnRH gene expression, molecular techniques were used to examine the functional organization of the rGnRH gene promoter as it relates to expression in hypothalamic cells. This manuscript presents novel upstream sequence of the rGnRH gene and provides mapping of the transcriptional start site in rat hypothalamus and mouse hypothalamic-derived cultured GT1-7 cells. In addition, a functional analysis of the rGnRH promoter in a transiently transfected GT1-7 cells suggests the distal promoter contains potential activating and inhibitory regions important in hypothalamic GnRH gene expression.

MATERIALS AND METHODS

Isolation of genomic DNA clones and nucleotide sequence analysis

DNA fragments containing the GnRH gene were isolated from a lambda EMBL-3 rat liver genomic DNA library, obtained from

* To whom correspondence should be addressed at Section of Endocrinology (111H), VAMC, 1055 Clermont Street, Denver, CO 80220, USA

Clontech, (Palo Alto, CA). Approximately one million recombinant phages were screened (21) with a ^{32}P -labeled (22) human placental cDNA fragment (kindly provided by P. Seeborg). Sequence analysis was carried out by the dideoxynucleotide chain termination procedure (23) using Sequenase (United States Biochemicals). Genomic fragments were subcloned into pGEM based vectors (Promega) and sequenced using Sp6 and T7 oligomers as primers.

Primer extension

The 5'-ends of the transcripts were determined by extension of an oligonucleotide primer 5'-CCAGTGCTGGCTGGAGC-3' complementary to nucleotides 63 to 79 of exon II (19). The primer was 5'-end labeled using $\gamma[^{32}\text{P}]\text{-ATP}$ and T_4 polynucleotide kinase (24) to a specific activity of 10^9 cpm/ μg . The labeled primer was then annealed to 5 μg of total RNA from mouse GT1-7 hypothalamic cells or 20 μg polyA⁺ RNA from rat hypothalamic tissue. The GT1-7 cells produce abundant GnRH mRNA and thus 5 μg of total RNA was used. Rat hypothalami have little GnRH mRNA; thus total RNA isolated from individual rat hypothalami as described (25) were pooled and polyA⁺ RNA prepared by affinity chromatography on oligo dt cellulose (Type 7/Pharmacia) (26) and 20 μg of polyA⁺ RNA was used. The reaction was heated to 70°C for 5 min, and hybridized at 55°C for 2 hrs in a volume of 10 μl (27). Following hybridization, the primer was extended with 10 units of AMV reverse transcriptase in 20 mM Tris, HCl pH 8.0, 10 mM MgCl₂, 75 mM KCl, 10 mM DTT, 0.25 mM of all four deoxynucleoside triphosphates for 1 hour at 42°C. The extended products were precipitated with ethanol and electrophoresed on 6% polyacrylamide/8M urea gels and visualized by autoradiography. The sizes of the extended products were estimated by comparison with a sequencing ladder and pBR322/HpaII molecular weight markers.

Promoter reporter fusion constructs

The promoterless pA₃LUC and neutral promoter pRSVLUC control vectors were constructed as described previously (28–29). A HindIII fragment from the rGnRH promoter region containing nt –3026 to +116 was cloned into the HindIII site of the luciferase expression plasmid pA₃LUC, placing the promoter region DNA upstream of the luciferase (LUC) coding region (p-3026A₃LUC). pA₃LUC contains a trimerized SV40 polyadenylation signal located upstream of inserted promoter sequences which results in minimal background luciferase activity in the promoterless vector and prevents any cryptic read through (28–29). The large fragment of 5'-flanking DNA was used to

derive other 5' deletions using convenient restriction sites. All constructs are similar in their 3' extent to +116 bp.

Cell culture and transfection assays

GT1-7 cells, an immortalized mouse hypothalamic neuronal cell line (6), were grown in Dulbecco's modified Eagle medium (DMEM). All media was supplemented with 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 0.25 $\mu\text{g}/\mu\text{l}$ Fungizone. Transfections were carried out using electroporation (30) with 10 μg (GT1-7) of plasmid per 60 mm dish of 3×10^6 cells. Together with DNA, cells were placed in a cuvette and electroporated for 15–25 msec at 220 V and a capacitance of 250 μF . The cells were then incubated in fresh media in 60 mm dishes and harvested 16–18 hrs later. Cells were washed and resuspended in 100 mM potassium phosphate, pH 7.8, and 1 mM DTT; then lysed by freeze-thawing three times. Nuclei were then pelleted by centrifugation at $1000 \times g$ and supernatants assessed for luciferase activity in a buffer supplemented with 5 mM ATP and 15 mM MgSO₄ using a Monojet 2010 luminometer. D-luciferin (1 mM) (from Analytical Luminescence Laboratories, Inc.) was automatically injected into the cuvette and the resultant luciferase activities measured over 10^{-3} sec. Cell survival was typically 40–60% post-electroporation. Duplicate aliquots were averaged and to control for intra-assay transfection efficiency, protein determinations were used to standardize the luciferase activities. Lysate protein yield was 70 to 140 μg per 3×10^6 cells and the assay performed within 2–4 hours after the harvest of the cells. Resultant luciferase activities (31) were then normalized to the activity of a neutral promoter derived from the long

Figure 1. The structure of the rGnRH gene. Exons are numbered 1 to 4. Below the gene are shown two overlapping lambda recombinant clones containing 8–10 kb inserts from which the gene structure was determined. E = EcoRI sites.

Figure 2. Primer extension analysis of the 5'-end of the mouse GnRH gene in mouse GT1-7 hypothalamic cells (top) and rat GnRH gene in the hypothalamus (bottom). A 17mer oligonucleotide complementary to the second exon (5'-CC-AGTGCTGGCTGGAGC-3') was used as a primer to anneal with total or polyA⁺ RNA. The arrow in the top panel marks a predominant cDNA transcript corresponding to the reported human and mouse transcriptional start sites (30,32). The arrow in the bottom panel marks a predominant cDNA transcript corresponding to a transcriptional start that maps 7 nucleotides upstream to that reported in the rat (19).

-3026 **HindIII**
AAGCTTTCCTTCCACAGTAAAGGGCTTACAGCAGGGGAGGAGACGACCCCTTACAGGGGAAAGGAAAGCAATTTTGAAAATGAATTATTTTCT
-2932 TTGTGTTATTATTTTACCAAGACAGAGAAGTATTGTATTGAGAGATACCTATTTTCATAATCAATGTGTGCCTAAATTATATTTAACTCATTT
-2838 CACTGCACCTCTATTTTCAATAATTATAGCCTGTATGCATTCAGTGAATGGTCTCTGCAGACAGAGGAGCTGAGAGATGCAAAACAAAGTGTGCT
-2744 TGGGAAGTGCAGGGTTCAGCTCTGATGTAAACCGCATTAAATAAATGGTGTGGACCGCTGACCGGCTGTTTCGGCTCTTGCTTTTATAAGTCAGC
-2650 ACTTCTGCATTTCCACAGATTTTCCATGATGGTGCCATGCAGGAGTGGGCGCAGCTGCTCTTAATGAATTAGTTTGTGTGAAAAAGTCATTA
-2556 AAGCAAATTTACTTCTGGGTGTGGTTTCTGATGTTCTTCACAGACCACAGTCTCCAGCACTTTTGGTTCTACACTAGGGAAGACACCTGT
-2462 GACTTGATTGCTCTGTAGCTGATGAATATCACTGCTTGTGTTGTGTTGAGTGAGATCTAGGAGAGATTTCTAAGGGAAGGCGTTCCACATA
-2368 GTAAGACTCGCATTATCTCTAGCATGAAAGTGTGTATGTGAGTTAAATAGAACTCATGGGAGGACTGCAGAAAGGTTCTCAGCCTAAGTC
-2274 AGCTAGATGATTTCATTGATAAAATTCAGGTCTCTACTGGAGTAACTGTCTGAGACAATACATGTCAGGCTCTTATGTAGTCAAGTTCTCTCGG
-2180 AAACCATTGAAGGGATTTTGAATAAAAAATGTGTAAAGTCCTTATTATGATGGCTTCATTATTAATTAAGACCTATTTTCCCGAGTTCAT
-2086 TTTATGAATCTGTTATTTATGATATAAGTCTACCTCCAGGTGAACCTTTAAATCATCTTTTATACATGAAGAAATCTAGAATTATATTAAATTC
-1992 CCAAGGAGGTAGGTAGTATCTTTAAATGAAAGTATCAAACTACAAAGGAAAGTGGCTACATTATTTCAAGATAAAACATGTTCTCCAAGCT
-1898 TTATTCTCAGCCTTTTACTGCTGGCTTATTAGCCAGTGAGCAGCAGTTAGCCCCACAGTCTCTCTGCTGAGATTTTACATTAGGGCAAGACAG
-1804 AGGTGTTTCACCTATCATTCCAGGAAGGTACAAAGTCATCACTGTCTATCATTTTGAGCTATTTTAAATGTTGGAATTAAGGCAATTTTCTCTATA
-1710 AAACCACACTTTTGAATTTAGAAAAATGTGACAATTATAAAGCCCAATTACTTAAACCCAATTTGTCAATTCAGGTCTTGGGTAAATTTGGCCT
-1616 AAGCATGCAATCATGAGAGATCTAAAAATTTACCATTAAGCCCTCAAAGCCTCACTGACTGCTTAATGGTTTAAAGCCTATTGTAGTCTGTC
-1522 GGAAGTAAGGCAGATTGGTTTGCTGGTTCTTAGGACTCTGTGTGACCTAAGACAAGACTAAGTGTAAGTCTAGGCGGATCTGAAGTTTGTAGTCT
-1428 TGTGCGCCTATTCATAAACTATTGAAATGTTTTAATTTTTTTTAAATCAATACAGAAAACACTGAGGTAAGCAGTCCACTGACAAACAGGGCTCA
-1334 GACATCCTTGCCACTTTGTTAGTCCTAGCCAGAAACACAGTCTTTCTCTTAAGTGACATTTAGAAGCTCATCTCTAATAAACAAAAATACAGTG
-1240 GACAGTTTAAACAAAACCTGTTCCAGACTTAAGGAATCTTATAGAAAACCTAGCCTTACCCGCACACTTCAGAGAGAAGAGGTTCTTCTGCTGTT
-1146 TGTGTCAGAGTTAGGGAATCTACATAAAATGAATGTAGTCACCACAAAGTCCCTCTTCCCTACCTGTCTCAGGACAGAGCACAGATGACCTG
-1052 **StuI**
GGAAGCCAATGCTGCTTATTTAGGCTGATGTTATTACTCTTTACTTGAAACCCCTTGCAATGGAGTTATTTCAAAGAACACTGAGCAATGAGC
-958 **SspI**
GTAGTAGTGTGCCAGTAGTGTGCTGATCTCAGCTGTAAAAGATCAAAATATTGTGTTATTTTACAGTGTAGGTAGTGAAGAGATTTTACAA
-864 ATGTATTGTACACAGAGGTTTCATTCTGAAATAGAGGCAGCATCTGTCAAAACCTGTTATTCACTCAGTGACTCATCGAAAGCCACATATATAT
-770 ATAGCTAGATGCAAGAAGCTATTAATAGGCAGATAGCTTAGAAACAAC
-676 TTTGTGGATTATAGACAGAGTAAAGATAGCACAAATTTAGAGGCAGGCAGATGAACCTCAAGTTCAAGACCAGCCTGGTCTACATTCAGGA
-582 TAGCCAAGACTACACAGAGAAACCCAGTCTCGAAAAACAAAAACAAATTTAGTACATTTGTGTGTGCGCGCTGCACCTTTTCAGCACAGAGGTC
-488 **HaeIII**
AGAGGACAACTTGAGAGACTTAATTTCTTACTTACCTTGTGGGACCTGGATTGAATTCAGGCCGTGAGGATTGGCAGCAACCTCTAACCA
-394 TGAGCCATCTCACTAACCCCTCAGAGTTTTTAGATTGGGAACAACACTATGATTATCTGATTAAATGTTTCTCTTTACAGAGACCCAAATGCT
-300 AAGACTGGTGCTCAGGTACTTTGTCTCAGCTTTCTGTATTATCACCTAACTCCCTGTGAGTTTTGTAGTGTGTTTTAACTTACCAATCCCA
-206 **EcoRI** **PvuII**
GGACGTCCTATTATCCTTGAACCTTCTCAGCGGGGAATTCAACATGTCTGGGTTTTAATCCCTTAGAATGGTGGCTTCAGCTGTGAAAGTTTTA
-112 **BspMI**
GCTAAGATTTTAAATGACCAAGTTTAAAGAAAATGCAACAGATAGACCAGCAGGTGTTCAATTACATTCCTCATTAAATGGCTTTTGTGAGGGT
-18 **DraI**
TAAAGATACTATGGTCTATGTGCACTATGGTCACCAGCGGGGAAGACTTCAGTGTCCCAGAAAAAGAAATCATAAAAAGAAAGCTAGACAG
-77 **HindIII**
ACAGAAACATTGAAGTACTCAACCTACTGACAGAAAGCTTG

Figure 3. Sequence for the rat gene for GnRH from -3026 bp to +116 bp relative to the transcriptional start site. Rat B1 and B2 repeats are underlined with a double line. An arrowhead indicates the transcriptional start site as determined by primer extension. A CCAATT and two TTAA boxes are underlined with solid lines.

terminal repeat of the Rous Sarcoma Virus (pRSVLUC) transfected in parallel as described (29) to control for transfection efficiency between experiments.

RESULTS

Isolation of the rat GnRH gene

One million recombinant bacteriophage from a rat genomic DNA library were screened with a human placental cDNA fragment (7). Four positively hybridizing clones were plaque purified to homogeneity and two independent recombinants were found to overlap at a common 2.1 kb EcoRI fragment but differed in their respective 5' and 3' extents. Thus, one clone (lambda GnRH 24) encoded ~ 6 kb of 5'-flanking region as well as the first two exons of the gene, and another (lambda GnRH 1) encoded the entire GnRH gene and an additional 3–4 kb of 3'-flanking region (see Fig 1). Sequencing of the 2.1 kb overlapping fragment showed identity between the clones as well as near identity with the subsequently published rat sequence (11) and mouse sequence (10).

Structural features of the rat gene are as published by Adelman and coworkers (8,11). There are four exons and three introns, and the gene is approximately 4.5 kb in size (see Fig 1). The first exon encodes the 5' untranslated region. The remainder of the 5'UT, the signal peptide, the GnRH decapeptide and the amino terminal portion of the GnRH associated peptide (GAP) are encoded by the second exon. The third exon contains the GAP coding region followed by the fourth exon which encodes the carboxy terminal portion of GAP and the 3'-untranslated region.

Mapping of the transcriptional start site

To determine the site of transcriptional initiation, primer extension analysis was performed using total cellular RNA from both GT1-7 hypothalamic cultured cells and polyA⁺ RNA from rat hypothalamus. The length of the extended products was determined using parallel genomic sequencing reactions as well as ³²P-labeled pBR322/HpaII molecular weight markers. The results of the primer extensions are shown in Fig 2. A predominant extended product, specific to mouse GT1-7 neuronal cells, migrated at approximately 137 and 138 nucleotides. However, using rat hypothalamic polyA⁺ RNA, we observed a predominant extended product migrating at approximately 224 nucleotides and, in longer autoradiographic exposures, 2 minor

bands. RNA samples from rat muscle which does not produce GnRH were used as controls and did not produce any signal (not shown). These experiments map suggest that the first exon in the rat gene is 145 nucleotides which is longer than the first exon of the human or mouse of 61 and 58 nucleotides respectively.

Organization and nucleotide sequence of the 5'-flanking region

The nucleotide sequence of approximately 3 kb of the 5'-flanking region of the rat GnRH gene was determined and is shown in Fig 3. Two TATA-like regions, (TTTAA), differing somewhat from the consensus TATAA, were located at -19 and -90 of the rat sequence. A CAAT consensus sequence occurred at -56. When the proximal 5'-flanking region was compared to the sequence reported by Bond et al (11), several differences were noted. A T to C transition at position +3, an A to T conversion at position -11, a deleted T at position -54, an inserted T at position -456, a deleted G at position -505 and a G to A transition at position -580 were noted.

Comparison of the GnRH promoter with the sequence data bases demonstrated a truncated copy each of the mouse B1 and B2 repeat between -637 and -385. There was also an alternating purine-pyrimidine (APP) sequence between -778 and -723 consisting of 28 pairs of ATs (32), which is a significant increase over the 6 pairs reported by Bond et al (11). In contrast to the human GnRH promoter sequence recently reported by Radovick and colleagues (33), there were no consensus steroid hormone response elements present in the rat sequence. Several half sites, however, which conform to the TGACC consensus for the ER/TRE subfamily of hormone response elements described by Umesono et al (34) were noted at -2687, -1479, -1059, -492, and -99. The 5'-flanking region of rat GnRH gene also contained two TAACC sequences (-401 to -377) which are identical to the consensus retinoic acid response element (RARE) motifs of the mouse (35), and human (36) B1 laminin gene promoter.

Comparison with reported consensus sequences of neuronal specific transcription factors revealed several putative DNA binding sites for such factors may be present in the rGnRH promoter. Motifs corresponding to sites specific for members

Table 1. Activities of luciferase constructs after gene transfer into different cells.

cell type	pA ₃ LUC	Absolute Luciferase Activity ^a pRSVLUC	p-3026GnRHLUC	Relative Luciferase Activity ^b p-3026GnRHLUC
GT1-7 ^c	38	3.4 × 10 ⁶	536,393	157,763
	64	2.5 × 10 ⁶	379,988	151,995
	46	3.2 × 10 ⁶	613,312	191,660
HeLa ^c	560	1.6 × 10 ⁶	1,040	650
	381	2.6 × 10 ⁶	596	230
	259	2.8 × 10 ⁶	724	258

^aLight units (LU) per 10 µg of protein

^bRelative light units per 10⁶ RSVLUC

^cEach line represents the mean results from a different cell preparation
Ten µg of each plasmid was transfected by electroporation. Cells were harvested after 16–18 hours of incubation and luciferase activity measured (see Methods).

Figure 4. Rat GnRH promoter deletions in a luciferase expression vector. 5'-deletional constructs were prepared using convenient restriction enzyme sites to examine areas important for basal promoter activity. The 3' extent of all inserts was to the HindIII site at +116. DNA fragments were inserted into the SmaI-HindIII site in pA₃LUC.

of the POU-domain (37) class of DNA binding proteins were observed. An Oct-1 binding motif (38) was seen at -2264 . The consensus sequence for Tst-1, GAYTYANA, initially described in the testis, certain neuronal subsets in the brain (39) and myelinating glia (40) occurred at -154 and -2009 . Additionally a consensus motif for Pit-1 (GHF-1), documented to regulate pituitary growth hormone and prolactin gene expression and differentiation, was present at -1420 and -2440 (41–42).

Tissue specificity and promoter activity of the distal rGnRH 5'-flanking region

Initial studies with the $-3026/+116$ construct in HeLa cells (a non-GnRH producing cell line) and the neuronal cell line, GT1-7, which expresses abundant GnRH mRNA and protein (6,43) demonstrated tissue specific expression of the rGnRH promoter activity (see Table 1). The $-3026/+116$ construct demonstrated promoter activity 400-fold higher in GT1-7 cells than non-GnRH producing cell lines. Smaller constructs (i.e., $-171/+116$) exhibited basal activity 5–10-fold higher than in HeLa cells (data not shown).

Experiments then investigated the basal activity of the 5'-flanking regions of the rGnRH promoter in hypothalamic GT1-7 cells. Design of the deletional constructs is shown in Fig 4. All constructs have identical 3' extents to $+116$ bp in the first exon. The promoterless vector had very low activity in the assay (20–30 LU). The largest test construct (p-3026A₃LUC) exhibited extremely high luciferase activity ($.5-1.5 \times 10^5$ LU). Deletion from -3026 to -1031 resulted in a dramatic 50-fold decrease in promoter activity suggesting removal of an important activating region (see Fig 5). Removal of the sequence from -1031 to -903 resulted in a 7-fold increase in luciferase activity suggesting the removal of an inhibitory element. Further deletion to -424 decreased promoter activity a further 3.6-fold. Additional deletion to -171 resulted in another 5-fold decline in luciferase activity. Truncation to -73 was associated with a further 8-fold decline in promoter activity. Finally, the smallest fragment with the greatest 5' truncation to position -16 exhibited luciferase activity 5-fold over the promoterless vector.

Figure 5. Basal promoter activity of the rGnRH gene 5'-flanking region measured by LUC activity assay in GT1-7 cells. The rGnRH promoter regions were inserted upstream of the LUC fusion gene in pA₃LUC. After transient introduction into GT1-7 cells by electroporation, LUC activity was measured. Each bar represents the average $\pm$ SEM of luciferase activity obtained from 'n' transfections.

DISCUSSION

Information concerning additional 5'-flanking sequence, transcriptional initiation site mapping and functional organization of the rat GnRH promoter have not been previously reported. Thus, we cloned the rGnRH gene to begin to examine the mechanisms which control rGnRH expression. To determine the transcriptional start site, primer extension analysis was performed. Previously, Adelman and coworkers (11) approximated the initiation site by successive generation of PCR products and suggested three potential but only one major start site. Our studies suggest one major site of initiation which differs by seven bases than that previously reported (11). The minor bands we observed may represent additional minor start sites in the rat GnRH gene since they were not observed in primer extension studies with GT1-7 cells or muscle. Studies in the mouse GT1-7 neuronal cell line confirm a start site identical to that in the mouse hypothalamus as suggested by Roberts and coworkers (44) and in the human hypothalamus reported by Radovick et al (9). Therefore, there are different transcriptional initiation sites and sizes of the 5'-untranslated sequence between the rat and the mouse or human GnRH gene and these differences may be important in species specific expression or translational control. We, like others (9), were unable to map the transcriptional start site in human placenta by primer extension analysis. Whether this is due to the low abundance of the mRNA in the placenta or use of a transcriptional start site too distant to be determined by our primer extension approach remains to be elucidated.

Initial studies demonstrated tissue specificity of rGnRH promoter activity in GnRH producing greater than non-GnRH producing cell lines. We then utilized restriction mapping and sequence information to begin to dissect the functional organization of the rGnRH promoter by 5'-deletional analysis. In GT1-7 cells, constructs containing a large fragment of the 5'-flanking cDNA (3 kb) exhibited high basal promoter activity. Deletion to -1031 decreased promoter activity 50-fold suggesting the removal of an activating region which is important for function in hypothalamic cells. Deletion of the region between -1031 and -903 resulted in a 7-fold increase in promoter activity suggesting the removal of inhibitory elements. Truncation of the more proximal promoter resulted in a 3.6-fold (to -424), a 5-fold (to -171) and 8-fold (to -73) further decline in promoter activity. Additional mutational experiments are underway to further define the sequence specificity of these activating and suppressor regions from hypothalamic cells. Although the genes for other hypothalamic releasing factors, thyrotropin releasing hormone (TRH) (45–46); growth hormone releasing hormone (GHRH) (47); and corticotropin releasing hormone (CRF) (48), have been recently cloned, there are no data as yet to assess the functional organization of the promoters of these genes. It will be important to determine whether there is a similar or different architecture of these promoters to target hypothalamic expression.

Considering the information derived from the deletional studies of the rGnRH promoter, a detailed examination of the sequence of the 5'-flanking region revealed several areas of potential interest for tissue specific expression and developmental regulation of rGnRH. Recently, investigators have described a family of POU-domain nuclear transcription factors (42,49–50). Pit-1 (GHF-1) (41–42) is expressed within the brain during development but then is targeted to the pituitary cells which express prolactin, growth hormone and thyroid stimulating hormone. Tst-1 is another member of the POU-domain family

found to inhibit the transcription of the gene encoding the protein PO, a Schwann cell surface adhesion molecule (39–40). The presence of Pit-1, Tst-1, and Oct-1 consensus sites at locations in the rGnRH sequence which when truncated decrease basal promoter activity in our studies raises the question of whether nuclear transcription factors of the POU-domain family may regulate GnRH expression in different tissues or across development. Experiments are currently underway to assess the functional importance of these sequences.

Additional sequence analysis revealed a large region of alternating purine-pyrimidine (APP) sequence in the rGnRH promoter. Similar sequences, which are thought to form Z-DNA, have been observed in the rat growth hormone (51), prolactin (52), and somatostatin genes (53). A role of Z-DNA in gene expression has not been established, although APP sequences in plasmid DNA have been associated with both gene activation (54) and repression (52) in mammalian cell transient expression assays. In our studies, deletion of the region containing the APP sequences –778 to –723 in the rGnRH promoter resulted in a 3.6-fold decrease in luciferase activity. Future experiments will determine whether these sequences are directly responsible for the observed changes in promoter activity.

To date, the exact mechanisms of steroid hormone regulation of rGnRH gene expression are unclear to date. Perfect consensus retinoic acid response elements (RAREs) were observed in the 5'-flanking sequence of the rGnRH promoter. Developmentally regulated expression by different forms of the retinoic acid receptor has been seen in the apolipoprotein A1 gene (55) and thus retinoic acid may play a role in rGnRH expression. In contrast to the human GnRH promoter, the rodent species have no palindromic sequences for other members of the steroid hormone family, however, several half sites for the ER/TR subfamily as described by Evans and coworkers (34) are present in the 5'-flanking region sequence. These data suggest that steroids may not regulate directly rat GnRH gene expression or do so through as yet undefined sequences. To support this possibility, investigators have recently documented that regulation by steroid hormones may not always occur through a palindromic sequence (34,56–58).

In summary, we have cloned the rGnRH gene locus, extended the sequence information of the distal 5' promoter, determined the transcription initiation site and demonstrated functional regions important in the expression of GnRH in hypothalamic cells. These initial studies will allow us to begin to dissect the developmental, tissue specific and hormonal regulation of GnRH gene expression which is critical to reproductive competence and survival of the species.

ACKNOWLEDGEMENTS

We would like to thank Wei Sun for technical assistance and Ms. Gloria Smith for preparation of this manuscript. This work was supported by NIH HD25275 (MEW), Veterans Merit Review (MEW), the Lalor Foundation (JKK), and the University of Colorado Cancer Center (JKK). MEW is a Research Associate with the Veterans Affairs. The sequence reported in this paper is deposited in the Genebank sequence data base, accession number X62651. We also thank the Tissue Culture Core Laboratory of the University of Colorado Cancer Center (NH CA46934) for media. We are grateful to Peter Seeburg for providing the human placental GnRH cDNA and Pamela Mellon for providing the GT1-7 cell line.

REFERENCES

- Seeburg, P.H., Mason, A.J., Stewart, T.A. and Nikolics, K. (1987) *Recent Progress in Hormone Research* 43, 69–98.
- Pfaff, D.W. (1986) *J Neuroscience Res* 16, 109–115.
- Liposits, Z., Sétáló, G. and Flerkő, B. (1984) *Neuroscience* 13, 513–525.
- Merchenthaler, I., Görcs, T., Sétáló, G., Petrusz, P. and Flerkő, B. (1984) *Cell Tissue Res* 237, 15–29.
- Wray, S. and Hoffman, G.A. (1986) *J Comp Neurol* 252, 522–531.
- Mellon, P.L., Windle, J.J., Goldsmith, P.C., Padula, C.A., Roberts, J.L. and Weiner, R.I. (1990) *Neuron* 5, 1–10.
- Seeburg, P.H. and Adelman, J.P. (1984) *Nature* 311, 666–668.
- Adelman, J.P., Mason, A.J., Hayflick, J.S. and Seeburg, P.H. (1986) *Proc Natl Acad Sci* 83, 179–183.
- Radovick, S., Wondisford, F.E., Nakayama, Y., Yamada, M., Cutler, G.B. Jr and Weintraub, B.D. (1990) *Molecular Endocrinology* 4, 476–480.
- Mason, A.J., Hayflick, J.S., Zoeller, R.T., Young, W.S. III, Phillips, H.S., Nikolics, K. and Seeburg, P.H. (1986) *Science* 234, 1366–1371.
- Bond, C.T., Hayflick, J.S., Seeburg, P.H. and Adelman, J.P. (1989) *Molecular Endocrinology* 3, 1257–1262.
- Gibbons, J.M. Jr, Mitnick, M. and Chieffo, V. (1975) *Am J Obstet Gynecol* 121, 127–131.
- Khodr, G.S. and Siler-Khodr, T.M. (1980) *Science* 207, 315–317.
- Lee, J.-N., Seppala, M. and Chard, T. (1981) *Acta Endocrinologica* 96, 394–397.
- Siler-Khodr, T.M. and Khodr, G.S. (1978) *Am J Obstet Gynecol* 130, 216–219.
- Barnea, E.R. and Kaplan, M. (1989) *J Clin Endocrinol Metab* 69, 215–217.
- Kim, S.J., Namkoong, S.E., Lee, J.W., Jung, J.K., Kang, B.C. and Park, J.S. (1987) *Placenta* 8, 257–264.
- Bhasin, S., Weber, D., Peterson, M. and Swerdloff, R. (1983) *Endocrinology* 112, 1144–1146.
- Harris, N., Dutlow, C., Eidne, K., Dong, K.-W., Roberts, J. and Millar, R. (1991) *Cancer Research* 51, 2577–2581.
- Kelly, A.C., Rodgers, A., Dong, K.-W., Barrezueta, N.-X., Blum, N. and Roberts, J.L. (1991) *DNA and Cell Biol* 10, 411–421.
- Maniatis, T., Fritsch, E.F. and Sambrook, J. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor University Press, Cold Spring Harbor.
- Feinberg, A.P. and Vogelstein, B. (1983) *Anal Biochem* 132, 6–13.
- Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc Natl Acad Sci* 74, 5463–5467.
- Maxam, A.M. and Gilbert, W. (1977) *Proc Natl Acad Sci* 74, 560–564.
- Gharib, S.D., Wierman, M.E., Badger, T.M. and Chin, W.W. (1987) *J Clin Invest* 80, 294–299.
- Wei, L.L., Krett, N.L., Francis, M.D., Gordon, D.F., Wood, W.M., O'Malley, B.W. and Horwitz, K.B. (1988) *Mol Endocrinol* 2, 62–72.
- Calzone, F.J., Britten, R.J. and Davidson, E.H. (1987) *Methods in Enzymology*. Academic Press.
- Maxwell, I.H., Harrison, G.S., Wood, W.M. and Maxwell, F. (1989) *Biotechniques* 7, 276–280.
- Wood, W.M., Kao, M.Y., Gordon, D.F. and Ridgway, E.C. (1989) *J Biol Chem* 264, 14840–14847.
- Maxwell, I.H. and Maxwell, F. (1988) *DNA* 7, 557–562.
- de Wet, J.R., Wood, K.V., De Luca, M., Helsinki, D.R. and Subramani, S. (1987) *Mol Cell Biol* 7, 725–737.
- Rich, A., Nordheim, A. and Wang, A.H.-J. (1984) *Annu Rev Biochem* 53, 791–846.
- Radovick, S., Ticknor, C.M., Nakayama, Y., Notides, A.C., Rahman, A., Weintraub, B.D., Cutler, G.B. Jr. and Wondisford, F.E. (1991) *J Clin Invest* 88, 1649–1655.
- Umesono, K., Murakami, K.K., Thompson, C.C. and Evans, R.M. (1991) *Cell* 65, 1255–1266.
- Vasios, G.W., Gold, I.D., Petkovich, M., Chambon, P. and Gudas, L.J. (1989) *Proc Natl Acad Sci* 86, 9099–9103.
- Vuolteenaho, R., Chow, L.T. and Tryggvason, K. (1990) *J Biol Chem* 265, 15611–15616.
- He, X., Treacy, M.N., Simmons, D.M., Ingraham, H.A., Swanson, L.W. and Rosenfeld, M.G. (1989) *Nature* 340, 35–42.
- O'Neill, E.A., Fletcher, C., Burrow, C.R., Heintz, N. and Roeder, R.G. (1988) *Science* 241, 1210–1213.
- Monuki, E.S., Kuhn, R., Weinmaster, G., Trapp, B.D. and Lemke, G. (1990) *Science* 249, 1300–1303.
- He, X., Guerrero, I., Simmons, D.M., Park, R.E., Lin, C.R., Swanson, L.W. and Rosenfeld, M.G. (1991) *Mol Cell Biol* 11, 1739–1744.
- Bodner, M., Castrillo, J.-L., Theil, L.E., Deerinck, T., Ellisman, M. and Karin, M. (1988) *Cell* 55, 505–518.

42. Ingraham, H.A., Chen, R., Mangalam, H.J., Elsholtz, H.P., Flynn, S.E. Lin, C.R., Simmons, D.M., Swanson, L. and Rosenfeld, M.G. (1988) *Cell* 55, 519–529.
43. Wetsel, W.C., Mellon, P.L., Weiner, R.I. and Negro-Vilar, A. (1991) *Endocrinology* 129, 1584–1595.
44. Yeo, T.T. and Dong, K.-W. (1991) *The Endocrine Society Abstract* #1716.
45. Lee, S.L., Stewart, K. and Goodman, R.H. (1988) *J Biol Chem* 263, 16604–16609.
46. Yamada, M., Radovick, S., Wondisford, F.E., Nakayama, Y., Weintraub and Wilber, J.F. (1990) *Endocrinology* 4, 551–556.
47. Suhr, S.T., Rahal, J.O. and Mayo, K.E. (1989) *Mol Endocrinol* 3, 1693–1700.
48. Vamvakopoulos, N.D., Karl, M., Mayol, V., Gomez, T., Stratakis, C.A., Margioris, A. and Chrousos, G.P. (1990) *FEBS Lett.* 267, 1–5.
49. He, X. and Rosenfeld, M.G. (1991) *Neuron* 7, 183–196.
50. Treacy, M.N., He, X. and Rosenfeld, M.G. (1991) *Nature* 350, 577–584.
51. Thomas, M.J., Freeland, T.M. and Strobl, J.S. (1990) *Mol Cell Biol* 10, 5378–5387.
52. Naylor, L.H. and Clark, E.M. (1990) *Nucleic Acids Res* 18, 1595–1601.
53. Hayes, T.E. and Dixon, J.E. (1985) *J Biol Chem* 260, 8145–8156.
54. Hamada, H., Seidman, M., Howard, B. and Goodman, C.M. (1984) *Mol Cell Biol* 4, 2622–2630.
55. Rottman, J.N., Widom, R.L., Nadal-Ginard, B., Mahdavi, V. and Karathanasis, S.K. (1991) *Mol Cell Biol* 11, 3814–3820.
56. Mitchell, P.J., Wang, C. and Tijan, R. (1987) *Cell* 50, 847–886.
57. Akerblom, I.E., Slater, E.P., Beato, M., Baxter, J.D. and Mellon, P.L. (1988) *Science* 241, 350–353.
58. Crone, D.E., Kim, H.-S. and Spindler, S.R. (1990) *J Biol Chem* 265, 10851–10856.