

GnRH Gene Expression: Lessons Learned from Immortalized Cells

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I. Development of Model Cell Lines for the GnRH Neuron

As the ultimate regulator of reproductive function, gonadotropin-releasing hormone (GnRH) is situated at the peak of the hypothalamic-pituitary-gonadal axis. GnRH neurons integrate information from the CNS and periphery to control reproductive maturation, cycles, and behavior. They are regulated by a variety of neurotransmitters, steroids, and peptide hormones. Studies of hypothalamic ultrastructure using double-staining techniques have indicated that dopamine, neurotensin, substance P, catecholamines, GABA, and estrogen-concentrating neurons, among others, may directly communicate with GnRH neurons.¹ Galanin and glutamate (NMDA) receptors are examples of receptors that can be colocalized with GnRH neurons.^{2,3} Unfortunately, the low number and scattered localization of GnRH neurons in the hypothalamus has made study of their cell and molecular biology difficult.¹ To study the mechanism of action of any modulating agent on the GnRH neuron, it is essential to have access to large, homogenous populations of GnRH neurons.

To develop a model system for the study of GnRH, oncogenesis was targeted to GnRH-secreting neurons by introducing a hybrid transgene composed of the rat GnRH regulatory region coupled to the coding region for SV40 T antigen (Tag) into transgenic mice.⁴ Tumors were produced from which GnRH-secreting cell lines (GT1) representing immortal, differentiated, hypothalamic neurons were developed.⁴ In similar work, Radovick and co-workers targeted oncogenesis with a 1.1 kb fragment of the human GnRH regulatory region and found migratory arrest of the GnRH neurons. A tumor from the region of the olfactory bulb produced a cell line, NLT, that makes low levels of GnRH, and may represent a developmentally earlier stage of GnRH neurons than the GT1 cells.⁵

GT1 cells express GnRH mRNA, accurately process the pro-GnRH precursor, and secrete GnRH in a pulsatile pattern.⁶⁻⁸ They express neuronal markers, including neurofilament and presynaptic vesicle proteins, but not glial markers.⁹ Additionally, GT1 cells extend neurites terminating in growth cones or forming gap junctions or synapses in culture.^{4,10} Intrahypothalamic injection of GT1 cells into the hypogonadal *hpg* mouse resulted in migration and extensive arborization of the GT1 cells and produced gonadal growth.¹¹ These studies support the validity of the GT1 cell line as a model for the hypothalamic GnRH neuron, and the use of targeted tumorigenesis for the development of differentiated cell lines in general.

II. Transcriptional Regulation of the GnRH Gene

The manifestation of differentiated cell function is principally dependent on the expression of gene products particular to that cell type. In the case of

the GnRH neurons, the expression of the GnRH gene distinguishes these neurosecretory cells from other hypothalamic neurons. Therefore, understanding the mechanisms by which GnRH gene expression is initiated and controlled is a prerequisite to understanding the development and regulation of this unique cell type. Initial studies showed that expression of a reporter gene under the control of 3 kb of the 5' region of the rat GnRH gene was restricted to the GT1 cell line in comparison to other neuronal and non-neuronal cell types.¹² This fundamental observation indicated that it would be possible to identify the determinants of neuron-specific GnRH gene expression and led to the description of the neuron-specific enhancer and proximal promoter sequences necessary for GnRH expression (Figure 12.1A). These regions, although closely integrated in function, are nevertheless distinct in their contributions to specificity and regulation of gene expression, and are discussed separately below. Most of the work on the specificity of GnRH gene expression has been conducted using the rat gene. Some analyses have been conducted on the mouse and human genes as well.^{13,14} The proximal promoter sequences are highly conserved between the rat and mouse genes, but these genes do not share identical enhancer-like elements, though some homologies can be detected in the upstream regions (P.L.M. unpublished observations). The following chapter will focus on results of studies with the rat GnRH gene.

A. The Neuron-Specific Enhancer of the GnRH Gene

1. Identification of the GnRH Enhancer

Two principle regions of the rat GnRH 5' regulatory region mediating transcriptional control of gene expression have been identified, the proximal -173 bp promoter, and a distal enhancer element encompassing -1863 to -1571 (Figure 12.1B).¹² In corroboration, Kepa et al. found that deletion from -3026 to -1031 decreased expression in GT1-7 cells, and Chandran et al. found a decrease after deletion from -3026 to -1580.^{15,16} It was further demonstrated that this enhancer was not functional in other cell types, indicating cell-type specificity.¹² DNase I footprinting shows that the enhancer binds multiple nuclear proteins over much of the 300 bp sequence,¹² and mutation of individual elements in this enhancer reduced GT1-cell-specific transcriptional activity.¹² Thus, the GnRH gene contains a complex multicomponent regulatory region. The high degree of interdependence between enhancer elements for activity may be a quality adapted for specifying expression to a rare cell type, since the simultaneous presence of interdependent DNA-binding proteins is required for significant activation.

2. The Role of GATA Protein-Binding Elements in the GnRH Enhancer

The multicomponent neuron-specific enhancer of the GnRH gene contains two centrally located consensus GATA elements. The role of GATA factors

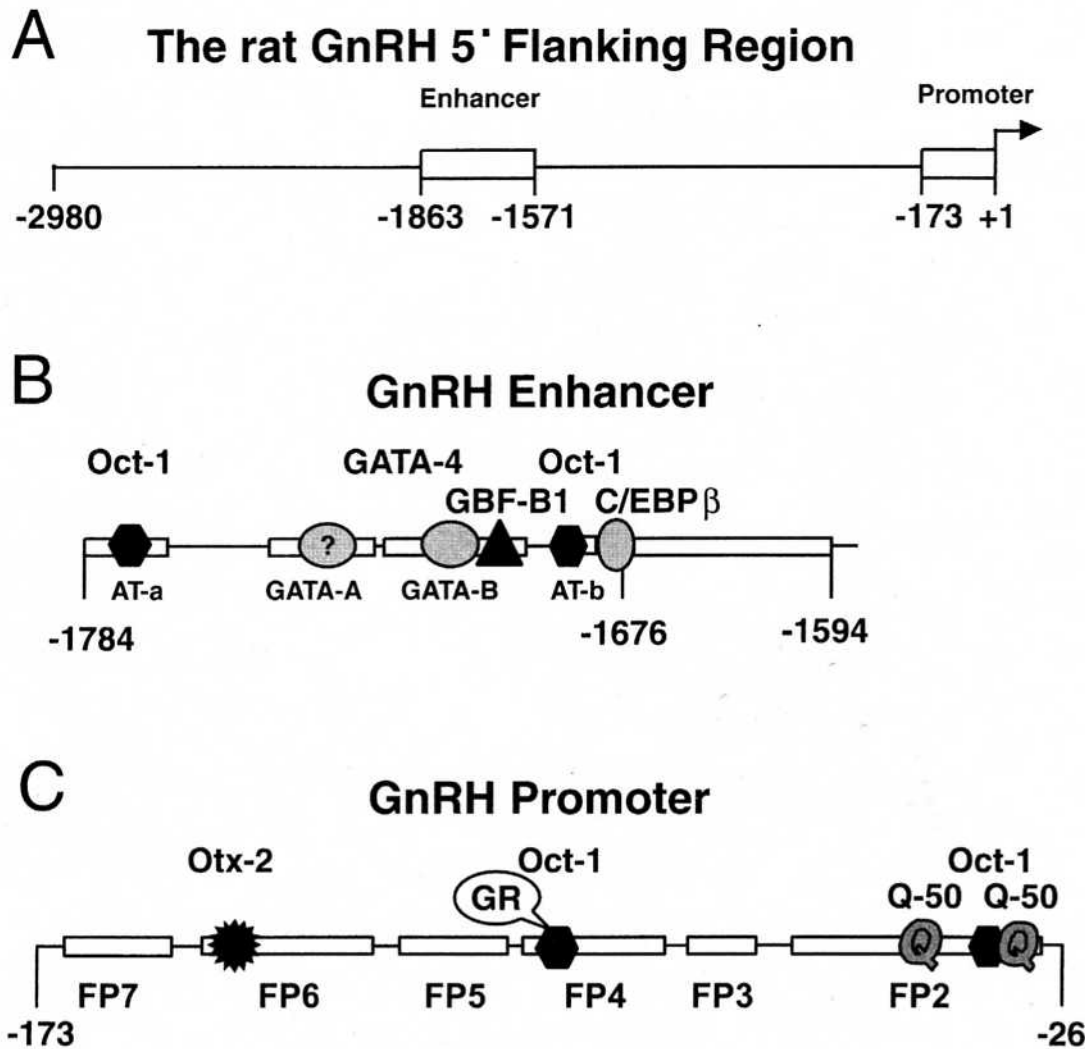


FIGURE 12.1

Structure of the Rat GnRH Gene Promoter. Representation of the 5' regulatory region of the rat GnRH gene promoter. (A) Overall structure of the GnRH regulatory region. Open boxes show the relative location of the distal neuron-specific enhancer and the -173 bp promoter sequences. (B) The structure of the neuron-specific enhancer showing the location of characterized transcription factor binding sites. Open boxes represent regions interacting with GT1 cell nuclear proteins as determined by DNase I protection assay. Factors with characterized binding sites are shown with names above. The question mark indicates the uncertainty of GATA-4 interaction at the GATA-A site. (C) The structure of the -173 bp promoter region. Open boxes indicate protein-bound regions as in (B). Glucocorticoid receptor (GR) interaction with the promoter through the Oct-1 site is indicated above the diagram. Not shown are the regions of SCIP interaction in FP3-5, FP6-7, and in the region between -314 and -343 or the distal GRE element, due to undefined sites of sequence binding.

in the differentiation of multiple, highly specialized tissues, has been well documented.¹⁷ GATA factors have been shown to be determinate for hematopoiesis, but are not known to specify neuronal lineages.¹⁸ Mutation of the GATA-B site decreases activity. However, GATA-A mutation only affects

expression when coupled with a heterologous promoter, indicating that the two sites are not equivalent.¹⁹ Electrophoretic mobility shift assays revealed two protein complexes binding on each GATA element, but the complexes were not related to each other in binding characteristics, consistent with the differing activities of the two elements. Although GATA-2 and GATA-4 mRNAs are found in GT1 cells, only GATA-4 protein is detected in shifted complexes, and only in one complex formed on the GATA-B site. However, mutation of either the GATA-A or -B site prevents induction by GATA-4, suggesting that GATA factors may interact with the GAT-A site under some conditions. Therefore, it is clear that GATA-4 is important in regulating the neuron-specific expression of the GnRH gene.¹⁹

The GATA-B site of the GnRH enhancer also binds a second protein, GBF-B1. Methylation-interference and missing-contact probing showed that GBF-B1 contacts the complementary base pairs of the underlined bases 5'ATGATAG 3' rather than the overlapping GATA site. Mutations eliminating the binding of GATA-4 do not affect the binding of GBF-B1, and vice versa. In GT1 cells, specific mutation of the GBF-B1 site reduces enhancer activity. Elimination of GBF-B1 binding enhances GATA-4 responsiveness, indicating antagonism between the two factors.²⁰

The presence of GATA-4 in GT1 cells is novel for a neuronal cell type. Expression of GATA-4 has been reported in the nasopharyngeal arch at embryonic day 9.5, coinciding with the formation of the olfactory placode, which is derived from the arch and gives rise to GnRH neurons.^{21,22} This suggests that GATA-4 might play a role in differentiation of the GnRH neuron. In day 12 embryos, GATA 4 protein can be detected in cells along the migratory path of GnRH neurons, ranging from the vomeronasal organ to the cribriform plate. This cell population also stains for GnRH in adjacent sections.²³ Interestingly, GATA-4 is not detectable by these methods in embryonic day 15.5 or adult GnRH neurons, although GBF-B1-like binding activity is present in adult hypothalamic nuclear extracts. The presence of GATA-4 is consistent with the unique developmental origin of GnRH neurons and provides support for its role in cell fate specification of GnRH neurons.²³

3. The Role of the POU-Homeodomain Transcription Factor Oct-1 in the GnRH Enhancer

The two AT-rich regions centered in the enhancer, AT-a and AT-b, are both 6/8 matches to the conserved octamer-binding site, ATGCAAAT, for POU-homeodomain proteins.²⁴ POU-homeodomain proteins form a large family prevalently expressed in the brain.²⁵ Mutation of AT-a greatly decreases GnRH transcription, while mutation of AT-b does not. Electrophoretic mobility shift assays indicate that the factor binding to the AT-a and AT-b sites is equivalent between the sites and dependent upon the octamer consensus binding sequence. An enhancer carrying an AT-a mutation retains only 5 percent activity. In GT1 cells, Oct-1, Oct-2, Brain-3, SCIP/Testes-1, Brain-2,

and Brain-4 mRNAs can be detected.²⁶ Only Oct-1 is present in the specific shifted complex. Thus, Oct-1 is likely to be the primary POU-homeodomain transcription factor binding to the AT-a and AT-b sites in the GnRH enhancer.²⁶

B. The GnRH Promoter

1. Identification of Functional GnRH Promoter Elements

The promoter-proximal 173 bp of the GnRH gene is highly conserved between rodents and primates. In the rat promoter, seven footprinted regions (FP1-7) are bound by multiple nuclear proteins found in GT1 cells, and each contributes to transcriptional activity in GT1 cells (Figure 12.1C).²⁷ The 51 bp FP2 region confers repression via protein kinase C activation.^{27,28} Footprint 2 is composed of at least three independent protein-binding sites, and each subelement contributes to transcriptional activity.²⁹ The enhancer and the promoter each play roles in conferring GT1 cell specificity. Deletion of FP2 from the promoter diminishes reporter activity in GT1-7 cells more strongly than in NIH 3T3 cells. Moreover, FP2 coupled to the RSV promoter confers GT1 cell-specific expression, indicating a role in expression specificity. Finally, a 31 bp sequence within FP2 (−63 to −33) synergistically activates transcription when coupled with the GnRH enhancer in GT1-7 cells, but not in NIH 3T3 cells.³⁰ Thus, this 31 bp region contains elements necessary for interaction between the GnRH enhancer and promoter.

2. POU-Homeodomain Proteins and Promoter Function

Both FP2 and FP4 contain octamer-binding sites like those identified in the enhancer, and they interact with Oct-1 protein. Functional activity of the reporter is dependent on this interaction. Thus, Oct-1 plays a critical role in the regulation of GnRH transcription, binding functional elements in both the distal enhancer and the promoter-proximal conserved region.²⁹ Interestingly, Wierman et al.³¹ have found that cotransfection of another member of the POU-domain family, SCIP, can down regulate GnRH gene expression. GnRH and SCIP colocalize in the rat hypothalamus. Three regions containing octamer-binding sites are the most relevant to repression, −73/−216 (FP3-5), −126/−171 (FP6 and 7), and −343/−314. Though bacterially expressed SCIP binds these regions *in vitro*, mutations of the octamer sites do not prevent response to repression by SCIP cotransfection.

Lastly, two of five protein complexes that bind to the −63 to −33 region of FP2 are GT1 cell-restricted, and both appear to be homeodomain proteins of the Q50 class. Two adjacent sites each bind a putative Q50 protein that has not been fully characterized. Both sites are completely conserved in mouse and human GnRH promoters.³⁰ The identification of this putative Q50 factor will greatly increase our understanding of the transcriptional basis for neuron-specific GnRH gene expression.

3. The Role of Otx2 in Promoter Activity

A bicoid-related Otx protein binding sequence occurs within the GnRH promoter at FP6.³² Proper expression of Otx2 is an absolute requirement for normal facial and forebrain development in the mouse. The Otx-binding site is required for specific expression of the GnRH promoter in GT1 cells and the binding sequence, -163 to -133, can confer specificity to a neutral RSV promoter in GT1 cells. Overexpression of Otx2 in GT1 cells induces the GnRH promoter, an effect that depends on the Otx binding site. The Otx2 protein is present in GnRH neurons of the adult mouse hypothalamus and during migration in development, suggesting an important role in the specification of GnRH gene expression.³³

III. Regulation of GnRH by Neurotransmitters, Growth Factors, and Hormones

A. Repression of GnRH Gene Expression by NMDA

The excitatory amino acids of the glutamate subtype N-methyl-D-aspartic acid (NMDA), kainic acid, and quisqualic acid activate GnRH release via different sets of receptors in rats.^{34,35} Induction of GnRH secretion by NMDA occurs in GT1 cells as well.³⁶ Nitric oxide (NO) also stimulates release of GnRH *in vivo*,^{34,37} and has been reported to control the release of GnRH in GT1 cells, but the results are conflicting.^{38,39} GnRH mRNA levels in GT1 cells decrease transiently upon addition of NMDA, release of NO, or increase in cGMP.⁴⁰

NMDA receptors (NR1, NR2A, and NR2D) and the neuronal and endothelial NO synthases (NOS) are present in GT1-7 cells. These findings implicate the action of a NMDA receptor-> NOS-> guanylyl cyclase-> cGMP-dependent protein kinase pathway leading to repression of GnRH mRNA. Pharmacological inhibition of the components of this pathway have established the involvement of each signaling molecule, and linearity of this regulatory pathway.⁴¹

The activity of the intact 3 kb of GnRH 5' flanking sequence is repressed by NMDA, NO, or cGMP in transient transfection assays in GT1-7 cells. The elements that confer repression were determined to be the enhancer AT-b site, known to bind POU-homeodomain proteins Oct-1 and an adjacent element at -1676 with homology to the C/EBP protein family consensus sequence.⁴² Both Oct-1 and C/EBP β bind to these sites *in vitro*. In nuclear extracts of GT1-7 cells treated with an NO donor, the relative binding affinity of Oct-1 was increased in a phosphorylation-dependent manner, suggesting that modulation of these factors controls enhancer activity. Thus Oct-1 and C/EBP β are both transcriptional regulators involved in the repression of GnRH gene expression by the glutamate/NO/cGMP signal transduction pathway.

B. Nuclear Receptor Regulation of GnRH Gene Expression

Although some evidence for the presence of receptors for estrogen in GnRH neurons exists, no direct regulation of GnRH by estrogen action has been documented.⁴³ However, glucocorticoid repression of GnRH transcription has been localized to the mouse GnRH promoter.¹⁶ Mouse GnRH gene transcription is repressed by glucocorticoids in transfection assays, and glucocorticoid receptor is present in GT1-7 cells. This effect is conferred by two regions within the mouse promoter termed the proximal and distal nGREs. Glucocorticoid receptor and Oct-1 are present in the complex that binds to the distal nGRE, which corresponds to FP4 in the rat gene. In contrast, glucocorticoid receptor, but not Oct-1, is present on the proximal nGRE which corresponds to the upstream and middle protein binding sites within the large FP2 in rat (-76 to -26).

C. Activin Regulation of GnRH Gene Expression

Activin is essential for the regulation of normal mammalian reproductive function at both the pituitary and gonadal levels. However, its central actions in the control of the hypothalamic-pituitary-gonadal axis remain largely unexplored. It is known that activin modulates GnRH protein secretion from GT1 cells.⁴⁴ Furthermore, intracerebroventricular injections of activin A increased GnRH mRNA levels in the medial preoptic area of adult male rats,⁴⁵ and activin treatment of hemi-hypothalami, explanted from male rats, stimulated GnRH release.⁴⁶ There is a close anatomical relationship between activin-containing neuronal fibers and GnRH somata and fibers in the hypothalamus.^{47,48} GT1 cells express mRNAs encoding activin receptor types I, IB, and II. Activin increases both GnRH protein secretion and GnRH mRNA expression in the GT1 cells. Furthermore, activin increased GnRH gene expression, suggesting that activin regulates GnRH gene expression at the level of transcription. Activin treatment of male rat hypothalamic explants increases GnRH protein secretion. Collectively, this evidence supports the role of activin in direct regulation of GnRH neurons.⁴⁹

IV. Summary

Overall, progress in understanding the regulation of GnRH gene expression has advanced substantially since the introduction of transformed cell lines derived from GnRH neurons. Our knowledge of GnRH gene expression has greatly expanded through the molecular characterization of the GnRH tissue-specific enhancer and its cognate DNA-binding proteins, including an octamer-binding POU-homeodomain protein, C/EBP β , and the zinc-finger cell type-specific protein, GATA-4. A key role for the evolutionarily conserved promoter in neuron-specific expression has also been shown. Interaction of

nuclear receptors with POU-homeodomain proteins are essential for full promoter activity, but much remains to be discovered concerning the other factors binding both the enhancer and the promoter sequences. Further, the NMDA, nitric oxide, cGMP neurotransmitter pathway for repression of the GnRH gene, the first demonstration of a neuron-specific gene regulation by this pathway, has also been characterized. Thus, through the use of the GT1 cell line, our knowledge of the molecular regulation of the GnRH gene has progressed as far or farther than that of any neuron-specific gene in the CNS. In addition to our transcriptional studies, the GT1 cell line has facilitated significant new understanding of the regulation of GnRH secretion.

In addition to the further characterization of the molecular components involved in regulating GnRH gene expression and hormone secretion, the lessons learned in the GT1 cell line can now serve as a basis for further study in whole animal models of GnRH action. The ability to utilize transgenic mouse models with directed alterations of systems identified *in vitro* will facilitate the study of GnRH gene regulation in the context of a complete physiological system. More classical approaches are aided by knowledge of factors acting directly on the GnRH neuron, thereby focusing studies necessarily undertaken in a complex hormonal environment. The fruitful approach of utilizing *in vitro* model systems for the study of the unique developmental and regulatory aspects of neuronal systems has been a key contribution to our knowledge of neuronal cell function.

V. Acknowledgments

Supported by NIH grant R01 DK44838 to P.L.M. The authors thank Carolyn Kelley, Naama Rave-Harel, Melody Clark, and Shelley Nelson for helpful discussions.

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