

Neuron-Specific Expression *in Vivo* by Defined Transcription Regulatory Elements of the GnRH Gene

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The GnRH-expressing neurons are the ultimate regulator of reproductive function. GnRH gene expression is limited to this small population of neurons in the hypothalamus. Transfections using 3 kb of the rat or mouse 5'-regulatory region provide specific gene expression in the hypothalamic cell line GT1-7. The combination of two elements, a 300-bp enhancer and a 173-bp promoter, recapitulates specificity in GT1-7 cells. It was not known whether these elements could specifically target gene expression throughout development in the whole animal. We demonstrate that the 3-kb rat GnRH regulatory region provides a higher degree of specificity than the equivalent mouse sequence in a mouse hypothalamic cell line.

Moreover, combination of the enhancer and the promoter of the rat gene targets expression to GnRH neurons in transgenic mice in a developmentally appropriate manner. Transgene expression is regulated by activin A, a known activator of GnRH gene expression. In contrast, the enhancer on a heterologous promoter produces inappropriate expression *in vivo*. We conclude that the enhancer and promoter regions of the rat GnRH gene are necessary for targeted expression to hypothalamic neurons and are sufficient to confer regulated, cell type-specific expression to a reporter gene *in vivo*. (*Endocrinology* 143: 1404–1412, 2002)

CONTROL OF REPRODUCTIVE function is dependent upon input from a variety of sources relaying information about reproductive fitness. Essential to the integration of this information are the GnRH neurosecretory neurons of the hypothalamus. GnRH neurons serve as the ultimate neuronal regulator of reproductive function via GnRH release into the pituitary circulation, exerting profound stimulatory effects on pituitary gonadotropin hormone synthesis and release and, consequently, on gonadal function. The expression of GnRH in the hypothalamus is limited to the GnRH neurons and is the distinguishing feature of this neuronal cell population. Understanding the cell-specific regulation of GnRH is essential to understanding the development of GnRH neurons and the mechanisms by which reproductive control is mediated through control of GnRH gene expression.

The mechanism of cell-specific expression of the GnRH gene has been extensively studied, yet is not fully understood. The production of specific GnRH-producing cell lines by targeted expression of the simian virus 40 T antigen in transgenic mice using the human or rat 5'-regulatory regions has demonstrated that the information necessary for targeted expression is contained within the proximal 1-kb (human) or 3-kb (rat) DNA sequence (1, 2). Although the human promoter has been shown to target reporter genes appropriately, the 3-kb mouse regulatory regions do not exclusively provide GnRH neuron-restricted expression in transgenic mice (3–5).

Both the rat and mouse GnRH 5'-regulatory regions have

been shown to direct high levels of reporter gene expression after transfection into the immortalized GnRH neuron cell line, GT1 (6, 7). Although further characterization of the human promoter has also been undertaken (5, 8), the rat regulatory region has been examined most closely. We have shown that high levels of specific expression of the rat GnRH gene in the GT1-7 cell line can be attributed to a 300-bp enhancer sequence that lies 1.5 kb upstream of the transcriptional start site (6). The promoter also plays a role in the cell-specific regulation of GnRH gene expression in GT1-7 cells (9, 10). Overall, the proximal 3 kb of the rat and mouse GnRH 5'-regulatory regions share highly homologous promoter sequences (11), but diverge in their remaining sequences. These differences suggest that some determinants of cell-specific expression may lie outside the promoter, presumably in the identified enhancer of the rat gene or in similar sequences in the mouse gene.

In the present study we have examined the relative specificity of mouse *vs.* rat GnRH genes in transfections and show that paradoxically the rat 3-kb 5'-proximal region exhibits a higher degree of specificity of expression in the mouse GT1-7 cells than does the equivalent region of the mouse gene. Further, we demonstrate in a transgenic mouse model that together the rat enhancer and promoter are sufficient for targeted expression of a reporter gene to the GnRH-expressing neurons of the hypothalamus. Expression of this reporter gene is temporally and anatomically appropriate in the developing and adult mouse, a targeting effect that is lost when the Rous sarcoma virus (RSV) promoter is substituted for the GnRH promoter. Additionally, high levels of reporter gene expression allow the quantification of reporter gene activity, allowing us to show that a known activator of GnRH gene

Abbreviations: DAB, 3,3'-Diaminobenzidine tetramethyl chloride; LTR, long terminal repeat; rh-activin A, recombinant human activin A; RSV, Rous sarcoma virus.

expression, activin A (12, 13), regulates the expression of the transgene. These studies show that the 300-bp enhancer and 173-bp promoter elements of the rat GnRH gene contain the genetic information necessary and sufficient for appropriate, regulated expression of GnRH in the adult hypothalamus and through neuronal migration during development.

Materials and Methods

Plasmids and transfection

The GnRHe/GnRHp-Gal and the GnRHe/RSVp-Gal plasmids were constructed using the minimal rat GnRH enhancer (–1871 to –1578 upstream of the transcriptional start), the rat GnRH promoter (–173 to +105), and the RSV 3′-long terminal repeat (3′LTR) promoter sequences (9395–9523 of the RSV viral sequence) described previously (9). The plasmids were constructed in the pGL3 luciferase reporter gene background (Promega Corp., Madison, WI). The luciferase-coding sequences were exchanged with the β -galactosidase-coding sequence derived from pSDK-Gal (provided by E. Turner). The β -galactosidase sequence was optimized for mammalian translation by the creation of Shine-Dalgarno and Kozak consensus sequences at the translational start site. The mouse and rat GnRH 5′-reporter genes were constructed using –2987 to +112 bp rat and –3026 bp mouse (provided by U. Chandran and D. DeFranco) sequences inserted into the β -galactosidase vector background described above. The RSV 3′LTR enhancer (9300–9400 of the RSV viral sequence) and promoter (9395–9523 of the RSV viral sequence) together were used to drive the control expression vectors.

The GT1-7, α T3-1 and NIH-3T3 cell lines were cultured in DMEM supplemented with 10% FBS (Omega Scientific, Tarzana, CA), penicillin, streptomycin, 3.5 mg/ml glucose, and 0.75% sodium bicarbonate and incubated in an atmosphere of 5% CO₂ at 37 C. For transient transfection assays, cells trypsinized from confluent plates were seeded at approximately 100,000 cells/cm² in 24-well plates and incubated overnight. Transfections were carried out using Eugene 6 reagent (Roche, Indianapolis, IN) in serum-containing medium according to the manufacturer's instructions. After 24 h, cells were harvested into 100 mM PBS containing 0.1% Triton X-100. Cell lysates were assayed directly for luciferase and β -galactosidase activities using the glow-type luciferase assay kit (Promega Corp.) and the Galacton-Plus kit (Tropix, Bedford, MA), respectively, according to the manufacturer's instructions. Luminescence was measured in a 96-well plate using 20 μ l lysate in a LB96V luminometer (EG&G Berthold, Gaithersburg, MD). The histogram shows normalized data from three independent determinations. Error bars represent the SEM. Data were analyzed using JMP software (SAS Institute, Inc., Cary, NC). ANOVA was carried out on data optimally transformed by the method of Box and Cox. *Post-hoc* comparisons were made using Dunnett's least significant difference test in comparison with a control and Bartlett's test of variance homogeneity. Significance was set at $P < 0.05$.

Transgenic animals

Transgenic animals were generated using the GnRHe/GnRHp-Gal and the GnRHe/RSVp-Gal reporter genes that had been digested out of the plasmid background and purified by sucrose gradient centrifugation. The DNA fragment solution was used for microinjection of eggs harvested from pseudopregnant females. Generation of the transgenic mice was carried out by the University of California-San Diego Transgenic Mouse Core Facility. The generation and further use of transgenic mice were performed according to institutional, local, state, federal, and NIH guidelines for the use of animals in research under an institutional animal care and use committee-approved protocol. Animals were screened by Southern blot or PCR of tail biopsy DNA with DNA and primers specific to the β -galactosidase-coding sequences.

Histochemical and immunohistochemical staining

Adult mice were anesthetized and perfused with a 100 mM PBS at pH 7.4 containing 4% paraformaldehyde and 0.2% saturated picric acid. Fixed tissue was harvested and equilibrated overnight in 100 mM PBS supplemented with 20% sucrose. For brain, 30- μ m sections were cut in

the coronal plane on a sliding microtome and transferred to β -galactosidase staining solution.

Females impregnated by transgenic males on d 13.5 postcoitus were anesthetized and killed by cervical dislocation. Individual embryos were dissected from the uterus, and they and their placentas were washed in PBS and processed for subsequent genomic DNA screening for the presence of the transgene. Embryos were fixed by rocking in the solution described above for 4 h, and then transferred to PBS supplemented with 20% sucrose and incubated overnight at 4 C. Fixed embryos were embedded in OCT medium (Sakura Finetech, Torrance, CA), and 10- μ m sagittal sections were taken and mounted onto glass slides.

For β -galactosidase histochemical staining, sections were submerged in a reaction mixture of 1 mg/ml 4-chloro-5-bromo-3-indolyl- β -galactoside, 4 mM K₄Fe(CN)₆·2H₂O, 4 mM K₃Fe(CN)₆, and 2 mM MgCl₂ in 100 mM PBS, pH 7.4. Tissue was allowed to stain for 4–16 h in a humidified atmosphere at room temperature. Alternatively, sections were allowed to stain for 16 h at 4 C. After staining, tissue was transferred to PBS overnight, then mounted and fixed onto glass slides by vacuum desiccation. Slides were then coverslipped or further processed for immunohistochemistry.

For immunohistochemistry, slide-mounted or free-floating tissue was incubated in 0.3% H₂O₂ for 10 min, rinsed in PBS, and incubated in 2% normal goat serum in 0.3% Triton containing a 1:10,000 dilution of GnRH LRI antibody (courtesy of R. Benoit) for 2 d. After rinsing in PBS, sections were processed for horseradish peroxidase/3,3′-diaminobenzidine tetramethyl chloride (DAB) using the ABC elite system (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's instructions. Stained slides were coverslipped and examined for DAB and β -galactosidase activity.

β -Galactosidase assay of tissue

For the analysis of β -galactosidase activity in freshly harvested tissue, mice were deeply anesthetized and decapitated. The hypothalami were dissected out by blocking the tissue with a coronal incision posterior to the optic chiasm and a parallel incision 5 mm anterior. Tissue was further blocked by sagittal incisions at the anterior commissure bilateral to the midline, and cortex was removed by a dorsal incision above the third ventricle. Equivalent portions of liver and whole ovaries were also harvested. The tissue blocks were homogenized in a 1-ml Dounce homogenizer (Kontes Co., Vineland, NJ) in PBS containing 0.1% Triton X-100 and 10 mM dithiothreitol, clarified by centrifugation at 10,000 \times g, and heat-inactivated by 30-min incubation at 48 C. Tissue extracts were further clarified by centrifugation, and 20 μ l were assayed for β -galactosidase activity as described above. Extracts were also assayed for protein content (Bio-Rad Laboratories, Inc., Hercules, CA). Luminescence assay results were normalized by the protein content of the extracts. Statistical analysis of four determinations was carried out as described above.

Hypothalamic explant culture

Adult male transgenic mice were deeply anesthetized and decapitated. Whole hypothalami were dissected and placed into Krebs-Ringer bicarbonate buffer (pH 7.4, 0.1% glucose). Blocking was conducted as described above, except the posterior border was defined by a coronal cut at the level of the pituitary stalk. The resulting blocks were sliced approximately 200 μ m thick in the coronal plane.

Sectioned hypothalami were preincubated in Krebs-Ringer bicarbonate buffer for 30 min before placement on a membrane culture insert in Ham's F-12 medium (Life Technologies, Inc., Grand Island, NY) supplemented with 25% horse serum (Omega, Inc., Tarzana, CA), penicillin, and streptomycin. Sections were moistened with medium to prevent drying and to promote gas exchange. Test medium was supplemented with 50 ng/ml recombinant human activin A (rh-activin A) provided by Genentech, Inc. (South San Francisco, CA).

After 24-h incubation, sections were homogenized as described above and assayed for β -galactosidase activity. Raw luminescent data corrected for background measurements were used in the comparisons. Data are plotted on a nominal histogram by expression level bins using Excel data analysis software and the control group distribution. This resulted in a bin size of 167 relative light units/group. The rh-activin-treated group was then distributed into the bins for comparison only.

Analyses of data were carried separately out using JMP statistical software (SAS Institute, Inc.). Nonparametric tests of significance included the Wilcoxon rank-sum test, followed by *post-hoc* two-way comparison of medians and χ^2 tests. Significance is reported at $P < 0.05$ for a one-tailed or χ^2 test.

Results

Rat GnRH promoter exhibits specificity of expression in culture

Previous studies of the rat GnRH regulatory sequences have demonstrated that reporter gene expression directed by the -2987 bp GnRH 5'-flanking region is highly restricted to the GT1-7 cell line (6). Others have reported targeted gene expression in transgenic mice bearing reporter genes directed by the mouse GnRH 5'-flanking region (3, 4, 14, 15). The most thorough analysis indicates that the mouse 5'-regulatory sequences lack sufficient information to limit expression of reporter gene expression to GnRH neurons (4). These results suggest that the rat and mouse GnRH genes may exhibit species-specific differences in cell type-restricted gene expression.

To test the hypothesis that the rat 5'-regulatory sequences exhibit greater specificity of expression than the corresponding mouse sequences, we examined reporter gene expression in cells transfected with plasmids under the regulatory control of the rat (2987 bp) or mouse (3026 bp) 5'-flanking regions. These regulatory regions direct the expression of the *Escherichia coli* β -galactosidase gene. To provide direct comparison of relative levels of expression in different cell types and to control for differences in expression levels due to transfection efficiency or reporter gene expression levels independent of transcriptional activity, a third reporter gene was constructed using the RSV 3'LTR, also directing expression of the β -galactosidase gene. In transient transfection assays, gene expression levels were normalized to an internal control plasmid identical to the RSV comparison plasmid, except bearing direct replacement of the firefly luciferase gene with the β -galactosidase gene. The plasmids used in this study are summarized in Fig. 1A. The plasmids were tested in GT1-7, α T3-1, and NIH-3T3 cells (Fig. 1B). In GT1-7 cells, similar levels of expression relative to RSV-LTR control plasmid levels in GT1-7 cells were observed. When tested in the pituitary gonadotrope cell line α T3-1, similar levels of expression relative to the RSV-LTR were also observed, although these levels were 1/100th the level of expression in the GT1-7 cell line. When tested in the mouse fibroblast cell line NIH-3T3, reporter expression directed by the mouse 5'-regulatory sequences was approximately 10-fold greater than the corresponding rat-directed reporter levels. We conclude from this analysis that the rat GnRH 5'-regulatory region exhibits a higher degree of specificity when tested *in vitro*.

Targeted transgene expression in vivo by defined minimal regulatory elements

Previous studies indicate that the approximately 300-bp GnRH enhancer and 173-bp promoter are sufficient for levels of targeted expression in cultured cells comparable to the 3-kb regulatory sequence (6, 9). These observations suggest that the minimal regulatory elements identified in the GT1

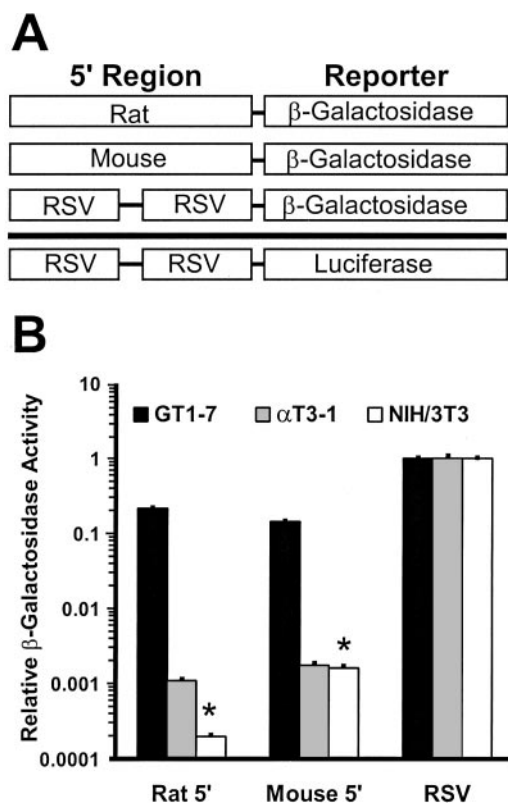


FIG. 1. Analysis of cell type-specific expression of the rat and mouse GnRH reporter genes. β -Galactosidase reporter gene plasmids were examined by transient cotransfection assay with the RSVe/RSVp-Luc internal control vector. As a comparison of the ratio of galactosidase to luciferase activity, an RSVe/RSVp-Gal was cotransfected with the RSVe/RSVp-Luc internal control in parallel. A, The structure of the reporter genes used in the comparison showing the composition of the 3-kb 5'-regulatory sequences. The RSV enhancer/RSV promoter plasmids, used as controls for both β -galactosidase and luciferase values, are also shown. All plasmids contained the same vector background, with the exception of the reporter gene-coding sequences. B, Results of the measurement of relative activity in the GnRH neuronal cell line GT1-7, the pituitary gonadotrope cell line α T3-1, and the fibroblast cell line NIH-3T3. The ratio of activity to the RSVe/RSVp-Luc to that to the RSVe/RSVp-Gal internal control cotransfection was set at 1, and the relative activities of the mouse and rat reporter genes are reported relative to this value for all cell types examined. Activity is plotted on a log scale. The asterisk indicates a significant difference between pairs as determined by ANOVA of Box-Cox transformed data and *post-hoc* pair comparisons using the Tukey-Kramer highest significant difference test for all pairs. Significance was set at $P < 0.05$.

cell model system may direct expression to GnRH-expressing neurons *in vivo*. To test this, transgenic mice were generated using a reporter gene consisting of the minimal enhancer and promoter of the rat GnRH gene directing expression of the *Escherichia coli* β -galactosidase gene (GnRHe/GnRHp-Gal, Fig. 2A). Ten transgenic lines were obtained that contained the integrated GnRHe/GnRHp-Gal transgene as determined by Southern blot analysis of genomic DNA isolated from tail biopsy (Table 1). Of these, seven were successfully bred to establish lines, and five of the seven were found to express the transgene. Histochemical staining for β -galactosidase activity (x-gal, blue precipitate) was used to assess which progeny expressed the transgene. Colocalization of transgene expression to GnRH-immuno-

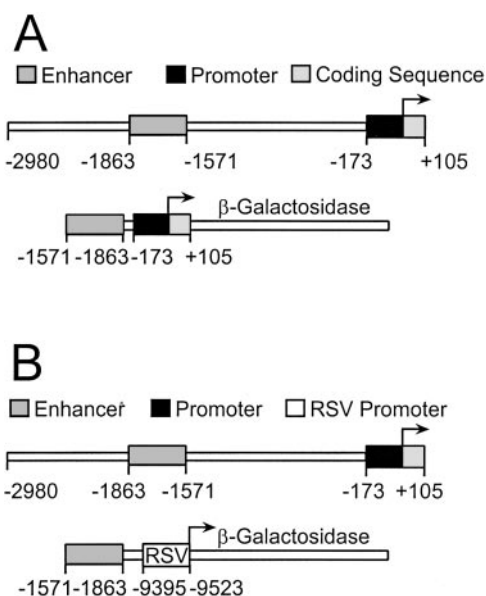


FIG. 2. A, Structure of the GnRHe/GnRHp-Gal reporter transgene used to generate the transgenic mouse lines. Numbers indicate the sequences included in the reporter gene plasmid and their relative positions in the 3-kb 5'-regulatory region of the rat GnRH gene. B, Structure of the GnRHe/RSVp-Gal reporter transgene used to generate transgenic mouse lines. Numbers indicate the sequences included in the reporter gene plasmid and their relative positions in the 3-kb 5'-regulatory region of the rat GnRH gene. RSV indicates the minimal promoter sequences of the RSV LTR.

TABLE 1. Summary of GnRHe/GnRHp-Gal transgenic mouse lines

Founder	Gender	GnRHe/GnRHp-Gal	
		Expression	Progeny
1	F	–	+
2	F	+	+
6	F	+	+
13	M	+	+
14	M	ND ^a	–
15	F	–	+
20	F	ND ^b	ND ^b
27	M	ND	ND
31	M	+	+ ^c
32	F	+	–

Expression was determined by examination of progeny or by examination of founder mice not successfully bred. All were maintained as heterozygous lines. ND, Not determined.

^a Founder died before screening.

^b Founder died giving birth.

^c Founder did not pass the transgene to progeny.

reactive neurons of the hypothalamus was determined by subsequent immunohistochemical staining for GnRH (DAB, brown precipitate).

Expression of the transgene was consistent among the five lines examined. A typical section containing the nucleus of the diagonal band of Broca from a heterozygous mouse from line 13 is shown in Fig. 3A. Strong blue and brown staining was visualized in cells staining for the presence of GnRH peptide (Fig. 3B). Cells positive for both β -galactosidase and GnRH could be observed along the entire continuum of the olfactory bulb (Fig. 3C) to the median eminence (Fig. 3D), consistent with the regional distribution of GnRH-expressing

neurons. Although strong β -galactosidase staining was restricted to cells costaining for GnRH expression, not all neurons staining for GnRH costained for GnRHe/GnRHp-Gal transgene expression (Fig. 3B). This result suggests that the targeted expression of the GnRHe/GnRHp-Gal transgene was limited to a subpopulation of GnRH neurons. We conclude from these observations the minimal 300-bp enhancer and 173-bp promoter of the rat GnRH gene is sufficient to limit expression of the β -galactosidase reporter gene to the GnRH neurons of the hypothalamus of transgenic mice.

Transgene expression in other tissues

In addition to examination of the expression of the GnRHe/GnRHp-Gal transgene in the hypothalamus by histochemical staining for β -galactosidase activity, we examined other tissues for the presence of GnRHe/GnRHp-Gal transgene expression by enzymatic assay. The results of three determinations are plotted in the histogram in Fig. 4. High levels of β -galactosidase were detected only in the hypothalami of transgenic mice. Although slightly elevated levels of β -galactosidase activity were detected in both liver and ovary of transgenic mice, these values were not statistically significant in comparison to tissue extracts derived from wild-type nontransgenic littermates. Fixed tissue was also further examined for the presence of cellular staining for β -galactosidase activity, and no staining was detected. We conclude that the transgene was most highly expressed in hypothalamic tissue of transgenic mice, reflecting the high specificity of expression indicated by our transfection analysis of cellular specificity of GnRH gene expression.

Transgene expression is regulated by activin

We have shown that rh-activin A is a positive regulator of GnRH gene expression *in vivo*, in cultured hypothalami, and in the GnRH-secreting cell line, GT1-7 (12). Based on these previous findings, we investigated whether GnRHe/GnRHp-Gal transgene expression could also be regulated by rh-activin A treatment. Hypothalamic slices from adult male transgenic mice were distributed between two groups and cultured *in vitro* in the presence of 50 ng/ml rh-activin A or control vehicle solution for 24 h. After incubation, slices were individually homogenized and assayed for β -galactosidase activity. The results of four independent studies are presented in Fig. 5. Details of the statistical analysis are described in *Materials and Methods*. Briefly, hypothalamic sections were randomly distributed between vehicle- and rh-activin A-treated groups. Because sections may contain few or many transgene-expressing neurons, a wide distribution of expression levels is expected. To compare treated and untreated hypothalami, data are grouped in nominal categories representing equal intervals of expression levels to create a histogram. The proportional value of slices expressing the transgene in a particular range is represented in each category. Differences in the distribution of control and treated groups from left to right indicate differences in the level of transgene expression from low to high levels. Because of the variable number of GnRH neurons in each slice, treated and untreated slices in each category were not analyzed for significance, as they will test as significant under

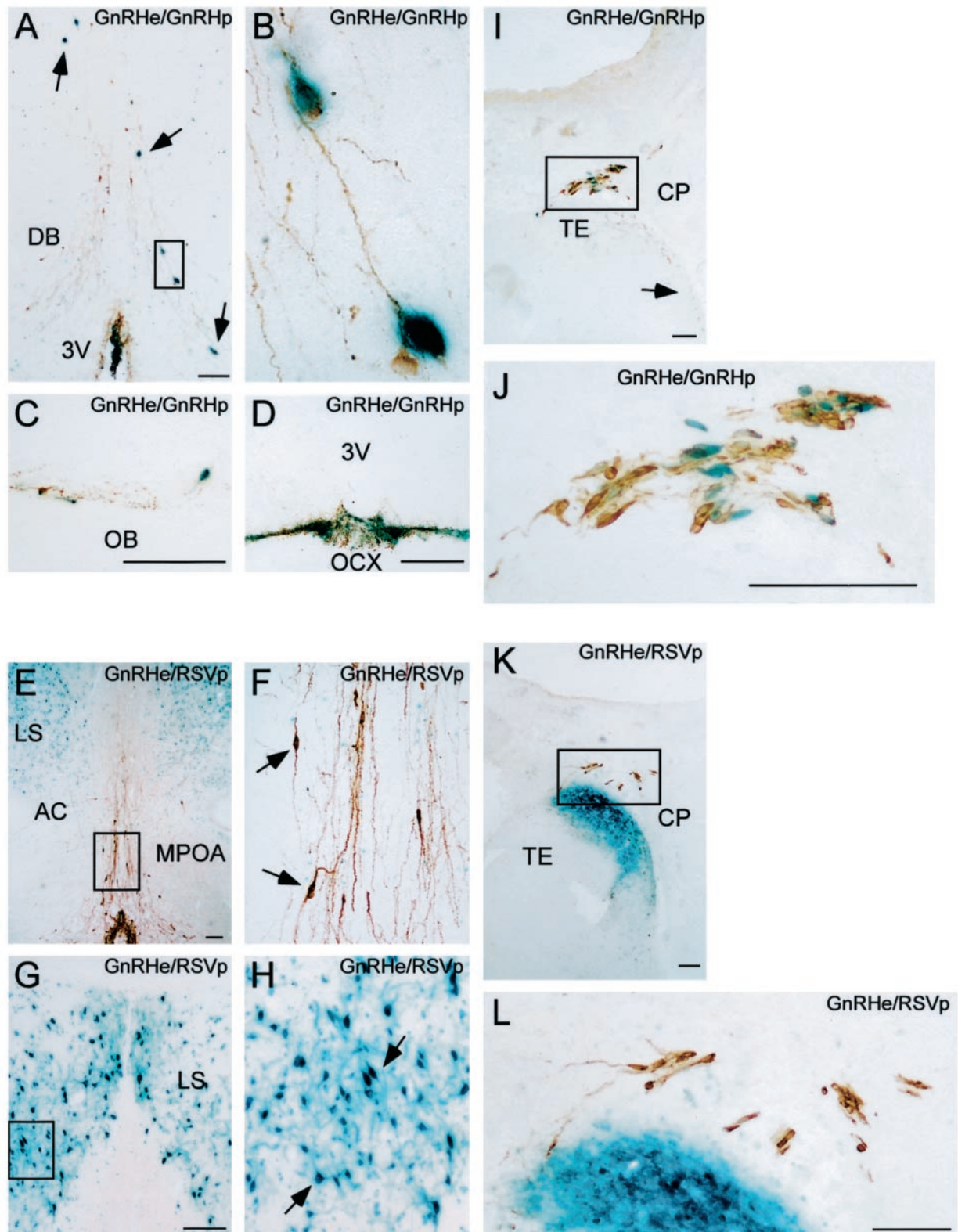


FIG. 3. Localization of β -galactosidase reporter gene expression and GnRH expression in hypothalamic neurons of transgenic mice. Colocalization of β -galactosidase reporter gene expression (blue staining; x-gal) and GnRH expression (brown staining; DAB) in hypothalamic neurons of GnRHe/GnRHp adult transgenic mice (A–D). A, Coronal section of the medial preoptic area and the nucleus of the diagonal band (DB) showing cells costaining for GnRH and β -galactosidase as well as cells staining for only GnRH. Intense staining is also visible near the third ventricle (3V) anterior to the arcuate nucleus. Low levels of staining are also visible as small spots near the diagonal band. It is not clear whether these are perikarya of non-GnRH cells or staining of transected fibers in the section. B, An expanded view of the cells boxed in A showing perikarya

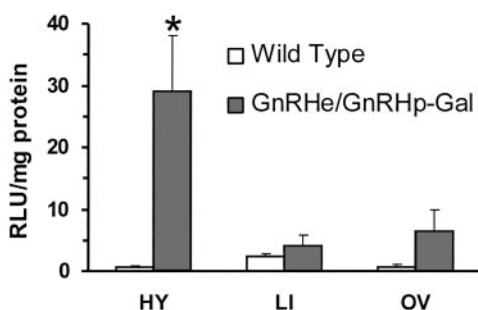


FIG. 4. Expression of the GnRHe/GnRHp-Gal transgene in the hypothalamus of transgenic mice measured by enzymatic assay of homogenized tissue samples. Tissue was harvested and analyzed as described in *Materials and Methods*. The histogram shows the relative activity of the reporter enzyme corrected for the protein content for three independent determinations, two animals of line 2 and one animal of line 13, compared with nontransgenic littermates. Wild-type, Nontransgenic wild-type littermate; GnRHe/GnRHp-Gal, GnRH transgenic mouse; HY, hypothalamus; LI, liver; OV, ovary. The asterisk indicates a significant difference from paired wild-type measurements as determined by ANOVA and *post-hoc* test of all pairs by Tukey-Kramer highest significant difference test. Significance is reported as $P < 0.05$. RLU, Relative light units.

any scenario. Rather, differences in the overall distribution were tested for significance by nonparametric analysis to detect a significant left to right shift in distribution in the rh-activin A-treated group, indicating an overall increase in transgene expression. Analysis of the results by Wilcoxon rank-sum test and *post-hoc* χ^2 test indicates that the median level of β -galactosidase activity was significantly greater in the rh-activin A treatment group than in the control group ($\chi^2 = 6.933$; $df = 1$; $P > \chi^2 = 0.008$). We conclude from this study that GnRHe/GnRHp-Gal transgene expression is positively regulated by activin A and that the GnRHe/GnRHp-Gal reporter gene is not only appropriately targeted to the GnRH cells of the hypothalamus, but is also appropriately regulated by factors known to regulate GnRH gene expression *in vivo* and in culture.

GnRH promoter is necessary for targeted expression *in vivo*

Our results presented in Fig. 1 indicate that the rat GnRH 5'-regulatory region exhibits a greater degree of specificity in transfections than the equivalent mouse 5'-regulatory region. Although the 300-bp enhancer of the rat gene is suf-

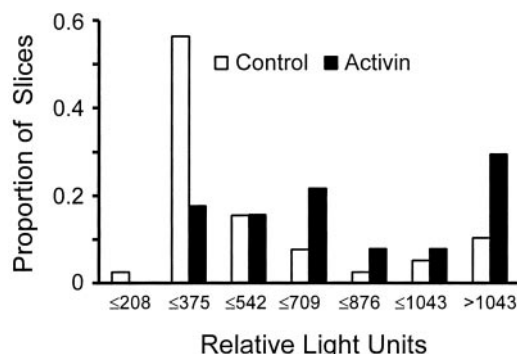


FIG. 5. Regulation of the GnRHe/GnRHp-Gal transgene in hypothalamic explant culture by activin A. Hypothalamic explants were cultured as described in *Materials and Methods*. After incubation in vehicle or the presence of 50 ng/ml rh-activin A, explants were homogenized, and reporter enzyme activity was measured by luminescent assay. The proportion of explants exhibiting enzymatic activity in four independent determinations is plotted on a distribution according to increasing ranges of expression levels of the transgene. The entire distribution of the data, rather than values in discrete categories, were analyzed for significant differences using the nonparametric Wilcoxon rank-sum test, followed by *post-hoc* two-way comparison of medians and χ^2 tests. The activin treatment caused a significant increase in the median value of the distribution, with a χ^2 value of 6.933, $df = 1$, and $P > \chi^2 = 0.008$. Individual categories are not marked for significance, as the entire distribution is the subject of the test.

ficient to confer cell type specificity of expression in transfections, the 173-bp promoter also contributes to specificity (6, 9, 10). To determine whether the promoter is necessary for appropriate, targeted expression in GnRH neurons, we generated additional transgenic lines of mice using a reporter gene under control of the 300-bp rat GnRH enhancer and the minimal RSV promoter sequences, GnRHe/RSVp-Gal, shown in Fig. 2B. Eleven founder animals were identified containing the GnRHe/RSVp-Gal reporter (Table 2). Transgenic mouse lines were established from founder animals and examined for appropriate expression. Strikingly, as shown in Fig. 3E, positive β -galactosidase staining in adult transgenic mouse brain was evident in the lateral septum, but not in the regions of the hypothalamus that normally contain GnRH neurons. Figure 3F represents a higher magnification of Fig. 3E and shows that β -galactosidase-positive cells did not stain for GnRH, as indicated by the lack of brown DAB

costaining for both the reporter gene and GnRH as well as staining for only GnRH. C, A section of the anterior olfactory bulb (OB) is shown in a dorsal-ventral orientation from left to right. Costaining for both GnRH and reporter gene expression is visible in three perikarya. D, Intense costaining for both GnRH and reporter gene expression at the optic chiasm (OCX), indicating the significant presence of transgene protein in fiber tracts leading from coexpressing cells elsewhere in the hypothalamus. Expression of the GnRHe/RSVp-Gal transgene in the hypothalami of adult transgenic mice (E–H). E, Pronounced staining is visible in the lateral septal (LS) region near the medial preoptic area (MPOA). AC, Anterior commissure. No staining is visible in the GnRH-immunoreactive cells of the diagonal band or in the arcuate nucleus. F, An expanded view of the cells boxed in E, showing low levels of staining in the medial septal region, but no colocalization with GnRH-immunoreactive cells. G, The same magnification photograph of the region dorsal of the section in E shows significant staining of stellate and bipolar cells of the lateral septal region. H, An expanded view of the cells boxed in G shows the intense staining of cells of the septo-hippocampal nucleus. Comparison of expression of the GnRHe/GnRHp-Gal and GnRHe/RSVp-Gal transgenes in postcoitus d 13.5 embryos (I–L). I, Colocalization of staining for GnRH and GnRHe/GnRHp-Gal reporter activity in cells migrating across the cribriform plate. GnRH neurons are stained in brown; transgene-expressing cells show blue staining for β -galactosidase activity. J, Higher magnification of boxed cells in I showing strict expression of the GnRHe/GnRHp-Gal transgene in GnRH-immunoreactive cells at the cribriform plate (CP). K, Intense staining in the telencephalon (TE) of GnRHe/RSVp-Gal reporter activity (blue) and the lack of staining in migrating GnRH-immunoreactive cells (brown). L, Higher magnification of boxed area in K showing the intense staining in the telencephalon and the lack of any staining in migrating GnRH neurons at the cribriform plate. The orientation of the sections is posterior-anterior, left to right. Black scale bars in all panels represent 100 μ m. B, F, and H are digital enlargements of the inset boxes of the respective parent panels.

TABLE 2. Summary of GnRHe/RSVp-Gal transgenic mouse lines

Founder	Gender	GnRHe/RSVp-Gal	
		Expression	Progeny
882	F	+	+
887	M	–	+
891	ND	ND	ND
900	F	+	+
902	ND	ND	ND
915	M	+	+
919	M	+	+
944	F	–	–
953	M	–	+
974	M	+	+
976	F	+	+

Expression was determined by examination of progeny or by examination of founder mice not successfully bred. All were maintained as heterozygous lines. ND, Not determined.

precipitate in the cell bodies and fibers. Staining for β -galactosidase was observed throughout the olfactory bulb to median eminence continuum, but also included regions posterior and caudal to the median eminence (data not shown). In addition, stained cells were morphologically distinct from the bipolar dorsal-ventral orientation of normal GnRH neurons (Fig. 3G). We conclude that the proximal promoter of the rat GnRH gene does contain determinants of cell-specific expression of the GnRH gene necessary for the appropriate targeted expression.

Transgene expression during GnRH neuron migration in the mouse embryo

The GnRH neurons are unique among neurosecretory cells, in that they arise in the olfactory placode, outside the central nervous system, and migrate during development to the hypothalamus. The GnRHe/GnRHp-Gal and GnRHe/RSVp-Gal transgenes exhibit different expression patterns in adult transgenic mice. However, because the GnRHe/GnRHp-Gal transgene did not target the entire population of GnRH neurons, the possibility exists that they may not represent the population of cells that migrates to the hypothalamus from the olfactory placode and may possibly represent a unique population of cells that arise within the developing forebrain.

To determine the source of the cell populations expressing either transgene, we examined sagittal sections of transgenic mouse embryos on postcoitus d 13. Expression of the GnRHe/GnRHp-Gal transgene was limited to the population of migratory GnRH neurons (Fig. 3I). The migrating GnRH neurons at the cribriform plate include cells that are strongly positive for both GnRH and β -galactosidase activities (Fig. 3J). Quantification of the proportion of cells staining for both GnRH and β -galactosidase was carried out across several sections of littermate embryos. Overall, 736 cells were counted. Of those, 168 cells (~23% of all migratory GnRH neurons) exhibited colocalization of β -galactosidase activity. An additional 14 cells (~2%) staining for β -galactosidase activity, but not for GnRH, were also found. Some low level of β -galactosidase staining in the developing telencephalon could be detected in some sections (*arrow* in Fig. 3I). This staining was much weaker and was not clearly associated with perikarya staining for GnRH, although low

levels of immunoreactive bodies could also be detected in the same area. It is not clear whether this staining represents GnRH neurons further along the migratory trajectory, or other GnRH-immunoreactive cells developing *in situ*. The presence of both transgene activity and GnRH immune reactivity suggests that these are cells expressing GnRH, albeit at levels significantly lower those observed in cells at the cribriform plate. Outside the developing forebrain, no significant staining was observed, indicating that transgene expression is restricted to the hypothalamic neuronal cell population.

In contrast, the GnRHe/RSVp-Gal transgene was widely expressed in the telencephalon, but was conspicuously absent from the population of migrating GnRH-expressing cells derived from the olfactory placode (Fig. 3, K and L). Intense staining was observed in the telencephalon extending down through the developing hypothalamus. No β -galactosidase staining in GnRH-immunoreactive cells could be detected even in areas of the telencephalon where transgene staining was significant. In addition to the staining shown in Fig. 3K, some weak β -galactosidase staining not clearly associated with cellular structures was observed in the diencephalon and lower spine, although staining in these regions was diffuse and not readily observed across multiple sections or embryos.

We conclude from these studies that the GnRHe/GnRHp-Gal transgene appropriately targets the migratory GnRH cell population, and that the minimal targeting elements used in the construction of the transgene direct appropriate developmental expression. Additionally, the necessity of the promoter sequences to mediate the activity of the neuron-specific GnRH enhancer is clearly demonstrated by the misappropriate expression of the GnRHe/RSVp-Gal transgene in both the developing and the adult mouse.

Discussion

Previous studies examining the targeting of various reporter genes under control of the human and mouse GnRH 5'-regulatory regions to the GnRH neurons of transgenic mice have suggested that the sequences used may be insufficient for targeted expression *in vivo* (3–5). In this and previous reports, we have shown that the rat enhancer and promoter bear strong determinants of cell type specificity in GT1-7 cells (6, 9). Mouse and human 5'-regulatory sequences contain regions of similarity to previously described neuron-specific enhancer in the rat gene, as determined by Clustal W alignment (16) and BlastN searching. However, no demonstration of enhancer-like activity has been presented, and the rat enhancer is not completely replicated in these sequences. Therefore, the differences in enhancer or enhancer-like elements may provide a basis for differential expression of the mouse and rat genes.

We began our study by examining the relative specificity of the mouse and rat GnRH 3-kb 5'-regulatory regions. Both regions direct high expression in the GnRH-secreting cell line GT1-7. The two regulatory regions were expressed at greatly reduced, but equivalent, levels in the gonadotrope cell line α T3-1. This observation is consistent with data presented by others indicating that GnRH is expressed at low levels in this

cell line and in cultured primary gonadotropes (17). When expression was tested in the fibroblast cell line NIH-3T3, the mouse gene showed promiscuity in its expression when directly compared with the rat. This concurs with observations reported in studies demonstrating the inability of the mouse 3-kb promoter-proximal sequences to restrict transgene expression to the GnRH-secreting cells of the hypothalamus (3, 4).

The specificity of the rat GnRH gene in GT1-7 cells is recapitulated by the minimal 300-bp enhancer on the highly conserved 173-bp promoter (6, 9). Further, these sequences not only direct high levels of expression in the GT1-7 GnRH neuronal cell line, but also contain the determinants of cell-specific expression, as they are expressed several orders of magnitude less strongly in other cell types as we show here with the full 3-kb regions and as shown previously with the minimal enhancer and promoter (9). However, it remained to be established whether this restricted expression demonstrated in cell lines would remain valid when tested in the whole animal. In fact, these minimal regulatory sequences alone are sufficient *in vivo* for appropriately targeted temporal and anatomical expression of a β -galactosidase reporter gene in transgenic mice. Although not all neurons expressing GnRH were targeted by the reporter, the targeted cells were of the appropriate developmental origin. Our results contrast with those using the mouse gene in a similar study (3) in which 85% of the mouse GnRH neurons were targeted, but expression was also found outside the GnRH cell population. In our case, 23% of the cells were targeted, but expression was limited to the GnRH neurons. It may be that the transgene-expressing cells represent a distinct subpopulation of GnRH neurons, or that the transgene is not capable of exhaustive targeting of the GnRH-expressing neurons. Although the sensitivity of the x-gal histochemical stain may not be sufficient to show the true extent of colocalization with GnRH, our results are nonetheless comparable to those of others using the same technique of colocalization of transgene expression with GnRH neurons. We cannot completely rule out low levels of expression of the GnRHe/GnRHp-Gal transgene in discrete subpopulations of cells in nonhypothalamic tissues. Some activity may be present in other tissues, but the levels are insignificant (Fig. 4). The high level of sensitivity of the assay and our ability to detect high levels of expression in the hypothalamus of transgenic mice indicates that expression in even a small population could be detected. Therefore, our observations indicate that significant expression of the transgene is probably limited to the GnRH neurons of the hypothalamus, but that the transgene is not capable of targeting the total GnRH cell population.

The promoter contributes moderately to the specificity of expression in cell lines (9). By exchanging the GnRH promoter for the minimal RSV promoter, we show that the promoter is required *in vivo* for appropriate targeted expression and that the targeted cells are of a distinct origin. Should the promoter not be required for specificity, we would have expected to see expression of the GnRHe/RSVp-Gal transgene in GnRH-expressing cells. Instead, the transgene directed expression to non-GnRH cells exclusively, indicating that the ubiquitously active minimal RSV promoter is restricted by the enhancer, but does not allow directed expres-

sion to GnRH neurons. The importance of the promoter in targeting is consistent with the role of the downstream promoter of the human GnRH gene in conferring cell-specific expression *in vitro* (18). Overall, this and other studies indicate that determinants of specificity lie in the 173-bp promoter sequences, as it is this region that shares extensive homology between species (11). Appropriate targeting *in vivo* by the human approximately 1.1-kb promoter has been reported and corroborated by studies *in vitro* (5, 18). In studies with the human gene, it is clear that upstream sequences in addition to the promoter are necessary for appropriate expression *in vivo*. Overall, interaction between upstream sequences and the promoter are necessary for appropriate expression of GnRH *in vivo*.

The sensitivity of the assay for β -galactosidase activity allowed us to further our studies of the expression of the GnRHe/GnRHp-Gal reporter gene to include examination of the modulation of expression. Although expression of the GnRHe/GnRHp-Gal gene is restricted to a few cells of the hypothalamus, the levels are sufficient to allow measurement by chemiluminescent assay. The TGF β family member activin A is a positive regulator of rat GnRH gene expression that has been demonstrated both in culture using the GT1-7 cell line and *in vivo* (12, 19). IGF-I has been shown to regulate the human GnRH promoter in cell culture, but these studies have not been extended to the mouse or rat genes (8). We demonstrate that activin A increases reporter gene expression in adult hypothalami. The ability to monitor expression a small number of cells in harvested tissue will allow further studies of transgene regulation *in vivo*.

In summary, we have demonstrated that the minimal regulatory elements directing expression of the rat GnRH gene identified in the GT1-7 GnRH neuronal cell line are sufficient for targeted reporter gene expression in GnRH neurons in transgenic mice. Although the entire population of GnRH neurons is not targeted, it is evident from our studies that expression of the transgene in the hypothalamus is limited to the GnRH cell population. We have further demonstrated the necessity of the 173-bp promoter for targeted expression. The necessity of the promoter is significant, as it is not the principal element required for targeting to the appropriate cell line in culture. Although the entire population of GnRH neurons is not targeted by the GnRHe/GnRHp-Gal transgene, expression is both anatomically and temporally correct. The technique of targeted reporter gene expression in transgenic mice will facilitate identification of the factor(s) determining cell type-specific expression. Additionally, the ability to target expression to the GnRH neurons will allow the development of model systems useful for analysis of GnRH gene regulation *in vivo* and provide a mechanism by which selective modification of GnRH neurons can be performed.

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References

- Mellon PL, Eraly SA, Belsham DD, Lawson MA, Clark ME, Whyte DB, Windle JJ 1995 An immortal cell culture model of hypothalamic gonadotropin-releasing hormone neurons. *Methods Enzymol* 7:303–310
- Radovick S, Wray S, Lee E, Nicols DK, Nakayama Y, Weintraub BD, Westphal H, Cutler Jr GB, Wondisford FE 1991 Migratory arrest of gonadotropin-releasing hormone neurons in transgenic mice. *Proc Natl Acad Sci USA* 88:3402–3406
- Skynner MJ, Slater R, Sim JA, Allen ND, Herbison AE 1999 Promoter transgenics reveal multiple gonadotropin-releasing hormone-I-expressing cell populations of different embryological origin in mouse brain. *J Neurosci* 19:5955–5966
- Pape JR, Skynner MJ, Allen ND, Herbison AE 1999 Transgenics identify distal 5'- and 3'-sequences specifying gonadotropin-releasing hormone expression in adult mice. *Mol Endocrinol* 13:2203–2211
- Wolfe AM, Wray S, Westphal H, Radovick S 1996 Cell-specific expression of the human gonadotropin-releasing hormone gene in transgenic animals. *J Biol Chem* 271:20018–20023
- Whyte DB, Lawson MA, Belsham DD, Eraly SA, Bond CT, Adelman JP, Mellon PL 1995 A neuron-specific enhancer targets expression of the gonadotropin-releasing hormone gene to hypothalamic neurosecretory neurons. *Mol Endocrinol* 9:467–477
- Yu K-L, Yeo TTS, Dong K-W, Jakubowski M, Lackner-Arkin C, Blum M, Roberts JL 1994 Second messenger regulation of mouse gonadotropin-releasing hormone gene expression in immortalized mouse hypothalamic GT1-3 cells. *Mol Cell Endocrinol* 102:85–92
- Zhen S, Zakaria M, Wolfe A, Radovick S 1997 Regulation of gonadotropin-releasing hormone (GnRH) gene expression by insulin-like growth factor 1 in a cultured GnRH-expressing neuronal cell line. *Mol Endocrinol* 11:1145–1155
- Nelson SB, Lawson MA, Kelley CG, Mellon PL 2000 Neuron-specific expression of the rat gonadotropin-releasing hormone gene is conferred by interactions of a defined promoter element with the enhancer in GT1-7 cells. *Mol Endocrinol* 14:1509–1522
- Kelley CG, Lavorgna G, Clark ME, Boncinelli E, Mellon PL 2000 The Otx2 homeoprotein regulates expression from the gonadotropin-releasing hormone proximal promoter. *Mol Endocrinol* 14:1246–1256
- Eraly SA, Mellon PL 1995 Regulation of GnRH transcription by protein kinase C is mediated by evolutionarily conserved, promoter-proximal elements. *Mol Endocrinol* 9:848–859
- MacConell LA, Lawson MA, Mellon PL, Roberts VJ 1999 Activin A regulation of gonadotropin-releasing hormone synthesis and release in vitro. *Neuroendocrinology* 70:246–254
- Gonzalez-Manchon C, Bilezikjian LM, Corrigan AZ, Mellon PL, Vale W 1991 Activin-A modulates GnRH secretion from a GnRH-secreting neuronal cell line. *Neuroendocrinology* 54:373–377
- Spergel DJ, Krüth U, Hanley DF, Sprengel R, Seeburg PH 1999 GABA- and glutamate-activated channels in green fluorescent protein-tagged gonadotropin-releasing hormone neurons in transgenic mice. *J Neurosci* 19:2037–2050
- Suter KJ, Song WJ, Sampson TL, Wuarin JP, Saunders JT, Dudek FE, Moenter SM 2000 Genetic targeting of green fluorescent protein to gonadotropin-releasing hormone neurons: characterization of whole-cell electrophysiological properties and morphology. *Endocrinology* 141:412–419
- Thompson JD, Higgins DG, Gibson TJ 1994 Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673–4680
- Krsmanovic LZ, Martinez-Fuentes AJ, Arora KK, Mores N, Tomic M, Stojilkovic SS, Catt KJ 1995 Local regulation of gonadotroph function by pituitary gonadotropin-releasing hormone. *Endocrinology* 141:1187–1195
- Dong K-W, Yu K-L, Chen Z-G, Chen Y-D, Roberts J-L 1997 Characterization of multiple promoters directing tissue-specific expression of the human gonadotropin-releasing hormone gene. *Endocrinology* 138:2754–2762
- MacConell LA, Widger AE, Barth-Hall S, Roberts VJ 1998 Expression of activin and follistatin in the rat hypothalamus. *Endocrine* 9:233–241