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

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Neuroendocrine control of the reproductive cascade is mediated by GnRH, which in mammals is produced by a subset of neurons scattered throughout the hypothalamus and forebrain. Utilizing a cultured cell model of GnRH neurons (GT1–7 cells), two regulatory regions in the rat GnRH 5′ flanking DNA were identified as essential for cell-type specificity: a 300-bp enhancer and a 173-bp conserved proximal promoter. Using transient transfections to compare expression in GT1–7 cells to a non-GnRH-expressing cell type (NIH 3T3), we show that the GnRH enhancer and the proximal promoter each play roles in conferring this specificity. Deletion of footprint 2 (FP2; −26 to −76) from the promoter when coupled to the GnRH enhancer diminishes reporter activity in GT1–7 cells more strongly than in NIH 3T3 cells. Furthermore, deletion of FP2 from the promoter when coupled to the heterologous Rous sarcoma virus 5′-long terminal repeat promoter abolishes the difference in reporter activity between GT1–7 and NIH 3T3 cells, suggesting that FP2 of the GnRH promoter is necessary for cell-specific expression. In addition, FP2 alone is sufficient to confer cell-specific expression and can interact with the GnRH enhancer to augment reporter gene expression specifically in GT1–7 cells. Finally, a 31-bp sequence from within FP2 (−63 to −33) synergistically activates transcription when coupled with the GnRH enhancer in GT1–7 cells but not in NIH 3T3 cells. Thus, this 31-bp region contains elements necessary for interaction between the GnRH enhancer and promoter. We show that two of five protein complexes that bind to the −63 to −33 region are GT1–7 cell specific, and both of them appear to be homeodomain proteins. The identification of a cell-specific element in the GnRH proximal promoter significantly advances our understanding of the transcriptional basis for neuron-specific GnRH gene expression. (Molecular Endocrinology 14: 1509–1522, 2000)

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INTRODUCTION

GnRH, an essential reproductive hormone expressed in a restricted subset of neurons scattered throughout the hypothalamus of mammals, is a decapeptide secreted in a pulsatile manner from axonal terminals at the median eminence (1, 2). GnRH neurons have a unique embryological origin, migrating from the olfactory placode where they arise at embryonic day 11 in the mouse (3). Currently, the only known unique identifier of GnRH neurons is GnRH itself. However, determinants of cell identity must be involved in the development and maintenance of GnRH gene expression. These genes could either encode specific transcriptional activators or a unique combination of more generally expressed transcription factors expressed only in GnRH neurons.

An excellent model system for examination of GnRH gene transcription is the GT1–7 cell line. The GT1–7 cell line was immortalized by targeting the oncogene, SV40 T antigen, to GnRH neurons using the 5′-flanking region of the rat GnRH gene in transgenic mice. The culturing of a hypothalamic tumor derived from such mice facilitated the clonal isolation of the GT1–1, GT1–3, and GT1–7 cell lines (4). These cell lines have been invaluable in studying GnRH gene expression, allowing a detailed analysis of the rat GnRH gene regulatory region. A 300-bp enhancer (−1863 to −1571) was identified by deletion analysis of a 3-kb 5′-regulatory region of the rat GnRH gene (5). Additionally, a conserved 173-bp promoter was identified by cross-species similarity (6). Fusion of the GnRH

enhancer to the GnRH promoter in a reporter gene plasmid recapitulates the activity seen with the 3-kb 5′-flanking region in transient transfection assays (5) and in transgenic mice (M. A. Lawson, S. B. Nelson, and P. L. Mellon, unpublished).

Many transcription factors have been found to interact with the rat GnRH regulatory regions including Oct-1, GATA-4, SCIP/Tst-1, and Otx2 (Refs. 7–10a). All of these proteins were identified utilizing the GT1–7 GnRH neuronal model system, and colocalization with GnRH has been confirmed *in vivo* by immunohistochemistry and/or *in situ* hybridization. Oct-1, a POU-homeodomain transcription factor, binds to two regions within the GnRH enhancer and two regions within the GnRH promoter (7, 11). GATA-4 binds to one site in the GnRH enhancer, and its expression colocalizes with GnRH neurons during embryonic development (8, 9, 12). *In vitro* synthesized SCIP binds to regions within the GnRH promoter and colocalizes with GnRH expression during mouse embryonic development (10). Otx2, a homeoprotein required for anterior head development (13–15) related to the Orthodenticle gene in *Drosophila,* binds to a single site in the promoter and colocalizes with GnRH in the embryo during migration and in the adult hypothalamus (10a). Although a few of the proteins that bind to the rat GnRH enhancer and promoter have now been identified, none of these proteins is restricted uniquely to the GnRH neurons, supporting the hypothesis that a unique combination of proteins control GnRH-specific expression.

Interactions between the rat GnRH enhancer and promoter are important for maintaining a high level of reporter gene transcription specifically in GT1–7 cells. The enhancer activates transcription of the heterologous Herpesvirus thymidine kinase promoter (TK) only 4 fold in GT1–7 cells, yet when placed upstream of the GnRH promoter, the enhancer activates transcription 55-fold (5). Therefore, interactions must exist between the enhancer and promoter to cause this specific increase in transcriptional activity in GT1–7 cells. We have devised a transient transfection paradigm to compare reporter gene expression between different cell lines. This approach has allowed us to determine that a 62-bp region of the GnRH promoter (footprint 2; FP2) is necessary and sufficient to confer neuronal specificity of GnRH gene expression *in vitro*. Additionally, a 31-bp element from within FP2 acts synergistically with the enhancer and binds two GT1–7 cell-specific protein complexes, both of which may be homeodomain proteins.

RESULTS

Individual Roles for the Enhancer and Promoter in GT1–7 Cell-Specific Expression of the Rat GnRH Gene

The rat GnRH regulatory region contains a 300-bp neuron-specific enhancer (−1863 to −1571) and a conserved 173-bp promoter. To test the participation of these two regions in conferring neuron-specific transcription, each element was placed into a heterologous context (Fig. 1). To accomplish this, chimeric regulatory regions were constructed using the GnRH enhancer and promoter and the enhancer and pro-

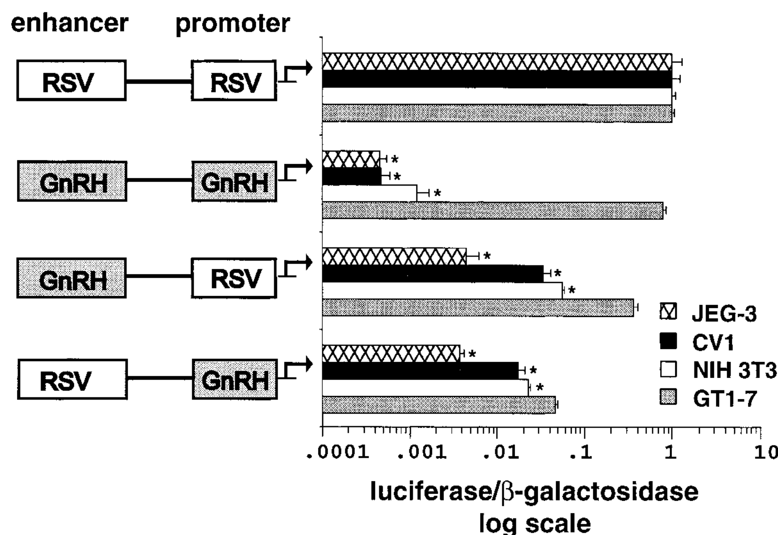


Fig. 1. The Rat GnRH Enhancer and Promoter Have Individual Roles in Cell Type-Specific Expression

Transient transfections were conducted in JEG-3, CV1, NIH 3T3, and GT1–7 cells (identified by *hatched, black, white*, and *gray bars,* respectively). *Diagrams at left* depict luciferase reporter plasmids: the RSV enhancer fused to the RSV promoter (RSVe/RSVp-luc), the GnRH enhancer (−1863 to −1571) fused to the GnRH promoter (−173 to +112) (rGnRHe/rGnRHp-luc), the GnRH enhancer fused to the RSV promoter (rGnRHe/RSVp-luc), and the RSV enhancer fused to the GnRH promoter (RSVe/rGnRHp-luc). The mean value for the RSVe/RSVp-luc divided by RSVe/RSVp-gal (internal control) is set to 1 for each cell type as shown. Error bars represent SEM of at least three experiments conducted in duplicate. *Asterisks* designate statistical differences from GT1–7 values of $P = 0.001$. Note that the x-axis is shown as a log scale to accommodate the large differences in expression.

moter elements of the Rous sarcoma virus (RSV) 5′-long terminal repeat (LTR). Specifically, the heterologous RSV enhancer was fused to the GnRH promoter (RSVe/rGnRHp-luc), and the GnRH enhancer was fused to the RSV promoter (rGnRHe/RSVp-luc). These regulatory regions were engineered upstream of the luciferase reporter gene in a reporter plasmid vector. As controls, reporter genes containing the RSV enhancer on the RSV promoter (RSVe/RSVp-luc) or the combination of the GnRH enhancer and promoter (rGnRHe/rGnRHp-luc) were also prepared. These plasmids were transfected into four different cell lines, NIH 3T3 (mouse fibroblasts), GT1–7 (mouse GnRH expressing neurons), JEG-3 (human placental), and CV1 (monkey kidney fibroblasts). To control for differences in transfection efficiency and relative expression level in various cell types, the RSV enhancer and the RSV promoter fused to the *Escherichia coli* β-galactosidase gene (RSVe/RSVp-gal) was used as an internal control in all transfections. The ratio of RSVe/RSVp-luc values to RSVe/RSVp-gal values was set to 1 for each cell type. The relative expression levels of the various reporter genes in the cell types indicated in Fig. 1 are depicted on a log scale since the differences are dramatic. There is a significantly higher level of reporter gene expression in GT1–7 cells than in the other three cell types with the rGnRHe/rGnRHp-luc, rGnRHe/RSVp-luc, and RSVe/rGnRHp-luc regulatory regions. These data show that the GnRH enhancer and the GnRH promoter each play roles in determining GT1–7 cell-specific expression and that the combination of the GnRH enhancer and promoter together yields a synergistic degree of specificity.

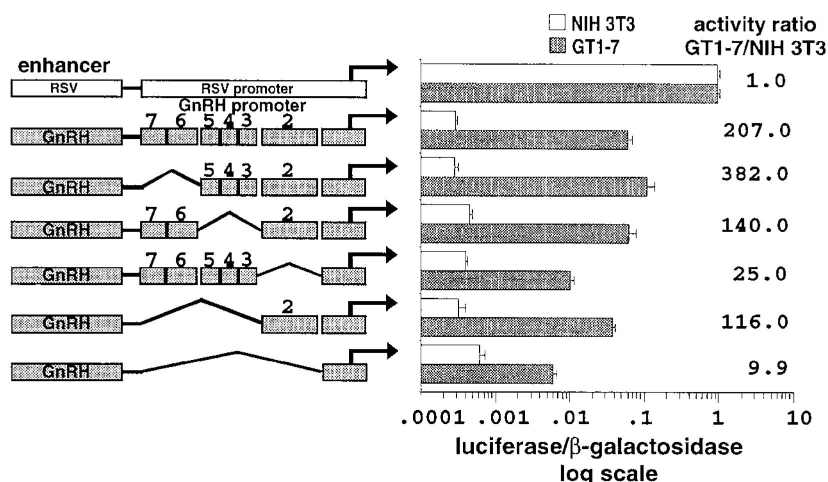
FP2 Is Necessary for Cell-Specific Expression

Previously, seven protein-binding regions within the rat GnRH promoter were identified by DNAse I protection assays (DNA footprinting) with GT1–7 nuclear extract (6, 16). Footprint 1 (FP1) contains the TATA box and transcriptional start site. Deletion of footprint 2 (FP2, −26 to −76) results in a 20-fold loss of reporter gene activity in GT1–7 cells. Footprint 3 (FP3, −79 to −85) does not bind proteins in electrophoretic mobility shift assays (EMSA), but a complex does form on this region with nuclear proteins from GT1–7 cells treated with human chorionic gonadotropin (17). Footprint 4 (FP4, −89 to −110) binds at least five protein complexes in EMSA, one of which is Oct-1 (11). Footprint 5 (FP5, −112 to −127) binds one protein complex in EMSA. Deletion of three footprints, 3, 4, and 5, results in a 50% reduction in reporter gene activity (6). Deletion of footprint 6 (FP6, −129 to −158) and footprint 7 (FP7, −161 to −173) also results in a 50% decrease in reporter gene activity (6). Additionally, it has been shown that Otx-2 binds to FP6 and a 4-bp mutation in the binding site reduces activity to 20% of wild type (10a). Thus, all of these regions contribute to activation of transcription of the GnRH gene.

To identify the cell-specific element(s), 5′ and internal deletions were created within the GnRH promoter and transfected into GT1–7 cells, and the results were compared with those from parallel transfections into NIH 3T3 cells. Subsections of the promoter containing various footprinted regions were placed downstream of the GnRH enhancer (Fig. 2A), as previously described by Eraly *et al.* (6), or the heterologous RSV enhancer (Fig. 2B). Here, we have used vectors containing the luciferase reporter gene instead of the chloramphenicol transferase reporter gene. When the 5′ and internally deleted promoter regions are placed downstream of the GnRH enhancer, reporter expression in GT1–7 cells was consistently higher than in NIH 3T3 cells (Fig. 2A). This result demonstrates that the GnRH enhancer can confer specificity in a heterologous context (5). The relative difference in reporter gene expression between the two cell types can be calculated by dividing the GT1–7 values by the NIH 3T3 values (Fig. 2A at *right*, relative activity). It is apparent from these comparisons that a large decrease in cell type specificity occurs when FP2 is individually deleted and when FP2 through FP7 are deleted (note the log scale). The role for FP2 in cell type specificity is further substantiated by transient transfections with the deletions placed downstream of the RSV enhancer (Fig. 2B). Here again, there is a higher level of reporter gene expression in GT1–7 cells compared with the NIH 3T3 cells with the full-length 173-bp promoter although the degree of difference is diminished due to the lack of the GnRH enhancer. The individual deletion of FP2 results in a loss of cell type specificity since both the GT1–7 cells and the NIH 3T3 cells express the reporter gene to the same degree. Additionally, when FP2 was reinserted, cell specificity reappears. It remains unclear why deletion of FP2 through FP7 does not completely abolish the cell-specific expression, but this result may indicate that there is some specificity inherent to the TATA box machinery.

FP2 (−82 to −21) or −63/−33 Can Interact with the GnRH Enhancer to Augment Expression

To test for interactions of FP2 with the GnRH enhancer, FP2 (−82 to −21) was inserted between the GnRH enhancer and the heterologous RSV promoter (rGnRHe/FP2/RSVp-luc). The insertion of FP2 into this context results in a large increase in reporter gene expression in GT1–7 cells in comparison to NIH 3T3 cells (Fig. 3A). To determine whether a smaller region of FP2 can confer cell type specificity, transcriptional analysis of a 4-fold multimerized −63 to −33 region (4×−63/−33) was conducted. The reporter plasmids contained the GnRH enhancer fused to 4×−63/−33 and the RSV promoter (rGnRHe/4X63/RSVp-luc). When transfected into GT1–7 cells, rGnRHe/4X63/RSVp-luc increased reporter gene expression compared with the rGnrHe/RSVp-luc (Fig. 3A). This increase was not as great as that seen with FP2, but it does suggest that 4× −63/−33 synergizes with the



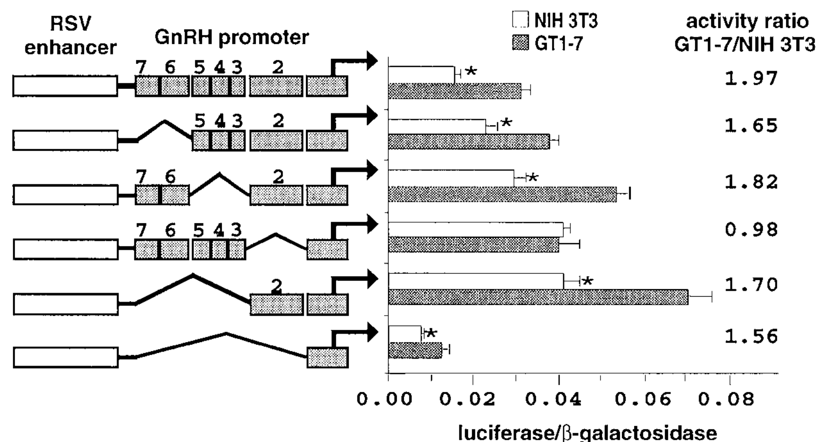


Fig. 2. Deletion of Footprint 2 Decreases the Ratio of GT1–7 to NIH 3T3 Activity When Coupled to the GnRH Enhancer and Eliminates the Cell Type-Specific Difference When Coupled to the RSV Enhancer

Transient transfections were conducted in NIH 3T3 and GT1–7 cells (identified by *white* and *gray bars,* respectively). The mean value for RSVe/RSVp-luc divided by RSVe/RSVp-gal is set to 1 for each cell type (bars shown in A but not in B). The activity ratios are the values in GT1–7 cells divided by the values in the NIH 3T3 cells. A, The GnRH enhancer fused to the full-length GnRH promoter or deletions of the GnRH promoter are shown at *left*. Values are depicted on a log scale. B, The RSV enhancer fused to the full-length GnRH promoter or deletions of the GnRH promoter are shown at *left*. *Asterisks* identify significant differences between the GT1–7 and NIH 3T3 values by paired *t* test, $P = 0.001$. *Error bars* represents SEM of at least three experiments conducted in duplicate.

GnRH enhancer. Thus, we can conclude that both FP2 and $4\times -63/-33$ can act synergistically with the GnRH enhancer to confer cell type specificity.

FP2, but Not −63/−33, Is Sufficient to Confer Neuron-Specific Expression

To determine whether the FP2 region can enhance neuronal specificity in a completely heterologous context, FP2 was inserted between the RSV enhancer and the RSV promoter (RSVe/FP2/RSVp-luc). Here we see a significant decrease in reporter gene expression in both cell types (Fig. 3B) possibly due to the additional 62 bp inserted between the RSV enhancer and promoter. Regardless of this overall decrease in expression, there is a higher level of reporter gene expression in GT1–7 cells compared with NIH 3T3 cells. Additionally, transfections into αT3–1 (a mouse pituitary gonadotrope cell line immortalized in the same manner as GT1–7 cells) and IMR-32 (a human neuroblastoma cell line), showed lower relative activity than expression in NIH 3T3 cells (data not shown), indicating that NIH 3T3 cells provide the best comparison with GT1–7 cells. Internal block mutations within FP2 in the context of the RSV enhancer and GnRH promoter did not result in a change in the relative difference between reporter

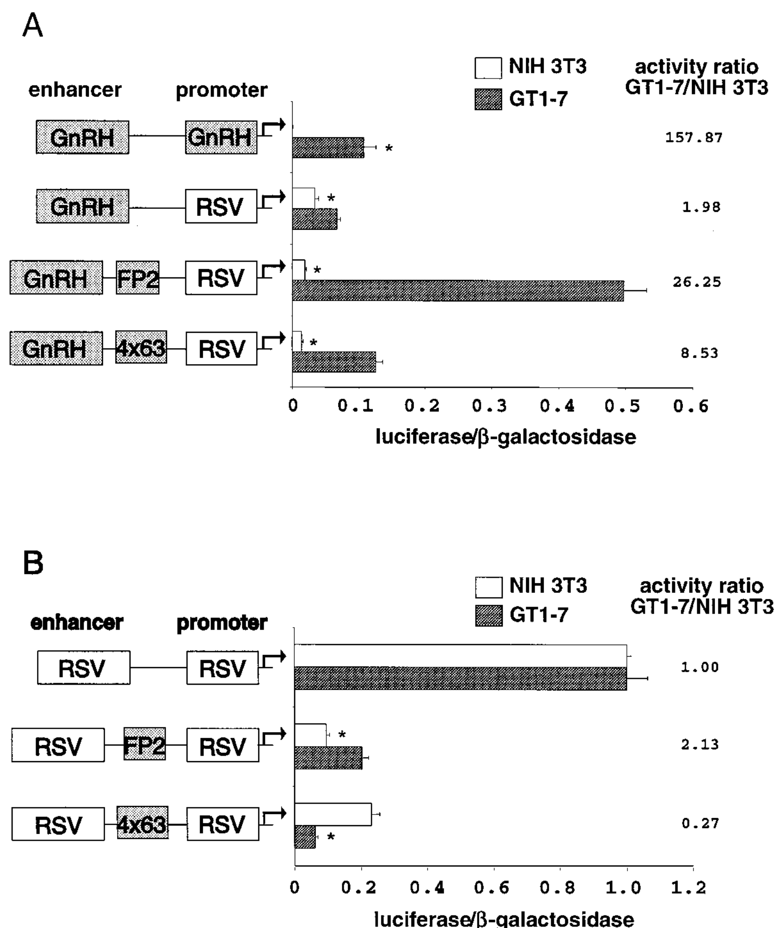


Fig. 3. Footprint 2 Is Sufficient to Confer Cell Type Specificity

A, Footprint 2 synergizes with the GnRH enhancer to augment reporter gene expression exclusively in GT1–7 cells. B, FP2 in a heterologous context is sufficient to increase cell type specificity but the multimerized −63/−33 element is not. Transient transfections were conducted in NIH 3T3 and GT1–7 cells (identified by *white* and *gray bars*, respectively). The mean value for the RSVe/RSVp-luc divided by RSVe/RSVp-gal is set to 1 for each cell type (shown in B, but not in A). The activity ratios are the values in GT1–7 cells divided by values in NIH 3T3 cells, shown at *right*. *Diagrams at left* depict luciferase reporter plasmids: A, the GnRH enhancer fused to the GnRH promoter, the GnRH enhancer fused to the RSV promoter, FP2 inserted between the GnRH enhancer and the RSV promoter, and 4×−63/−33 inserted between the GnRH enhancer and the RSV promoter; B, the RSV enhancer fused to the RSV promoter, FP2 inserted between the RSV enhancer and promoter, and 4×−63/−33 inserted between the RSV enhancer and promoter. *Asterisks* represent significant difference between values in GT1–7 and NIH 3T3 cells by paired t test, $P < 0.01$. *Error bars* represent SEM of at least three experiments conducted in duplicate.

expression in GT1–7 *vs.* NIH 3T3 cells (data not shown). Thus, specific sequences required for this GT1–7 cell-specific expression could not be further localized. To determine whether the smaller region of FP2 is able to confer cell type specificity, transcriptional analysis of the 4-fold multimerized −63 to −33 region was conducted. The reporter plasmids contained the RSV enhancer fused to 4× −63/−33 and the RSV promoter (RSVe/4X63/RSVp-luc). Reporter gene expression levels were higher in NIH 3T3 cells than in GT1–7 cells, demonstrating that the 4×−63/−33 is not able to act independently when placed in a heterologous context. Thus, we have identified FP2 as the smallest region (62 bp) of the GnRH promoter that is sufficient to autonomously confer cell-specific expression of the GnRH gene.

Complexes Specific to GT1–7 Nuclear Extract Bind the −63/−33 Element

Interactions between the GnRH enhancer and GnRH promoter are likely what confer the cell-specific expression of the GnRH gene based on our evidence (Figs. 1–3) and evidence obtained in transgenic mice (M. A. Lawson and P. L. Mellon, unpublished observations). Having demonstrated that both FP2 and the −63 to −33 promoter elements interact with the enhancer to specify reporter gene expression to GT1–7 cells, we next wanted to determine what proteins may bind to this region to confer cell specificity. We chose to analyze the −63 to −33 region because it contains fewer potential protein binding sites, while still maintaining the capability of interacting with the GnRH

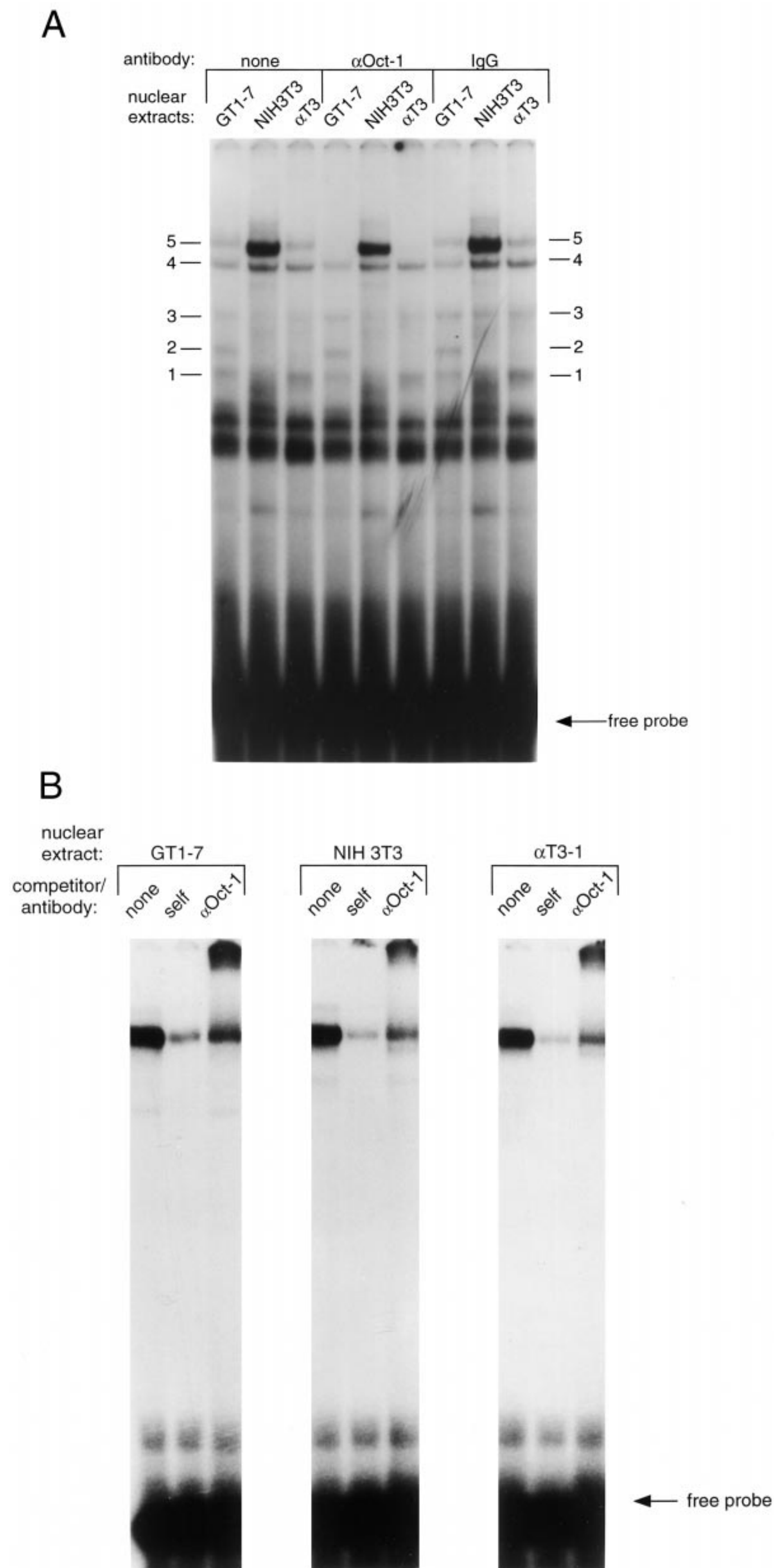


Fig. 4. Oct-1 Binding within Footprint 2

EMSA was conducted with three different nuclear extracts; GT1–7 (GnRH expressing cell line), NIH 3T3 (mouse fibroblast cell line), and αT3–1 (mouse pituitary gonadotrope cell line). Antibodies were added to the reactions, Oct-1 (antibody against Oct-1) and normal rabbit IgG (nonspecific antibody). *Arrow at bottom right* indicates migration of free probe. A, Oct-1 forms complex 5 in GT1–7 and αT3–1 nuclear extracts but not in NIH 3T3 nuclear extract using the −63/−33 probe. The specific complexes

enhancer to confer cell specificity. Previously, we have identified five GT1–7 nuclear protein complexes binding to −63/−33, one of which is Oct-1 (complex 5) (11). In these experiments, we used EMSA to compare the protein complexes bound to the −63/−33 element in GT1–7 nuclear extract with NIH 3T3 nuclear extract (Fig. 4A). Nuclear extract from αT3–1 cells was included as a second cell type that does not express GnRH. The low mobility complex formed with GT1–7 nuclear extract (complex 5) was previously identified as Oct-1 by antibody supershift analysis (11). In EMSA with NIH 3T3 nuclear extract, a complex comigrates with the Oct-1 band from the GT1–7 cells, but addition of an antibody against Oct-1 does not block binding of the complex. The Oct-1 antibody blocks binding of complex 5 in GT1–7 and αT3–1 nuclear extract. These data suggest that either Oct-1 does not bind to FP2 in NIH 3T3 cells or that another complex binding with NIH 3T3 nuclear extract masks the binding of Oct-1. Regardless, the major low mobility complex in NIH 3T3 extracts binding to −63/−33 does not contain a significant amount of Oct-1, which may account for the lower transcriptional activity observed in Figs. 1 and 2. To prove that functional Oct-1 is present in NIH 3T3 cells, we show that Oct-1 from NIH 3T3 and GT1–7 nuclear extracts binds appropriately to an octamer consensus sequence (Fig. 4B). Oct-1 binding to −63/−33 is not unique to GT1–7 nuclear extract since nuclear extract from αT3–1 cells forms the Oct-1 complex (Fig. 4A). Additionally, two complexes with a high mobility (complexes 1 and 2) are present in EMSA with GT1–7 nuclear extract but not with NIH 3T3. In αT3–1 nuclear extract, complex 1, but not complex 2, is present by EMSA. These differences in protein complexes binding to FP2 could be responsible for the cell type-specific interactions between the GnRH enhancer and promoter.

To determine whether complex 1 and 2 binding to −63/−33 are specific across various cell types, a panel of nuclear extracts was used in EMSA with −63/−33 as a probe (Fig. 5). The nuclear extracts were from the following cell lines: GT1–7, AtT-20 (mouse pituitary corticotrope), NLT (mouse GnRH-expressing tumor from the nasal region outside the CNS), NIH 3T3, Y1 (mouse adrenal), αT1–1 (mouse pituitary), αT3–1 (mouse pituitary gonadotrope), CV1 (monkey fibroblast), HeLa (human cervical fibroblast), and JEG-3 (human choriocarcinoma). From the panel of nuclear extracts, it appears that complex 2 is not present in any of the other cell types tested. In JEG-3 cells, a complex exists that migrates slightly faster than complex 2 from GT1–7 cells. It is possible that the protein from JEG-3 cells is a human homolog of the complex 2 protein in mice, but it is more likely that this complex

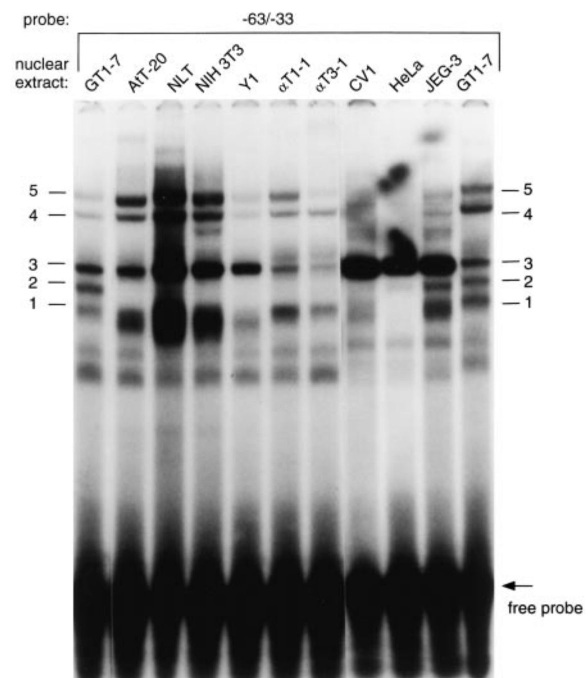


Fig. 5. Complex 1 and Complex 2 from GT1–7 Nuclear Extract Are Relatively Cell-Specific Complexes

An EMSA was conducted using the −63/−33 probe and nuclear extracts from the different cell types, indicated *above* each lane. The specific complexes labeled at *left* and *right* were previously identified by their ability to be competed by 100-fold excess of unlabeled oligonucleotide. *Arrow at bottom right* indicates migration of free probe.

from JEG-3 cells is a different protein from complex 2. Complex 1 is present in GT1–7 nuclear extract and appears to comigrate with complexes seen in NLT, αT3–1, and αT1–1 nuclear extracts. Thus, complex 1 is partially cell specific and complex 2 is unique to GT1–7 cells, in the tested cell types.

Q50 Homeodomain Transcription Factor Binding Sites Found within Footprint 2

To identify the protein(s) contained in complex 2, we examined the nucleotide sequence for known protein-binding elements. The −63 to −33 region contains a CAATTA region (Fig. 6A, *middle site*) that is homologous to a C/EBP and a homeodomain-binding site. Previously, we found that C/EBPβ, although present in GT1–7 cells, does not bind to this site, termed the middle site by Eraly *et al.* (11). Homeodomain transcription factors recognize an ATTA DNA core motif. Those homeodomains with a glutamine at position 50 (Q50) bind a CAATTA or CCATTA motif while a lysine

labeled at *left* and *right* were previously identified by their ability to be competed by 100-fold excess of unlabeled oligonucleotide. B, There are no differences in Oct-1 complex formation between GT1–7, NIH 3T3, and αT3–1 nuclear extracts with the octamer consensus probe. Competitions were conducted: none (no competitor) or self (100-fold excess unlabeled octamer consensus site).

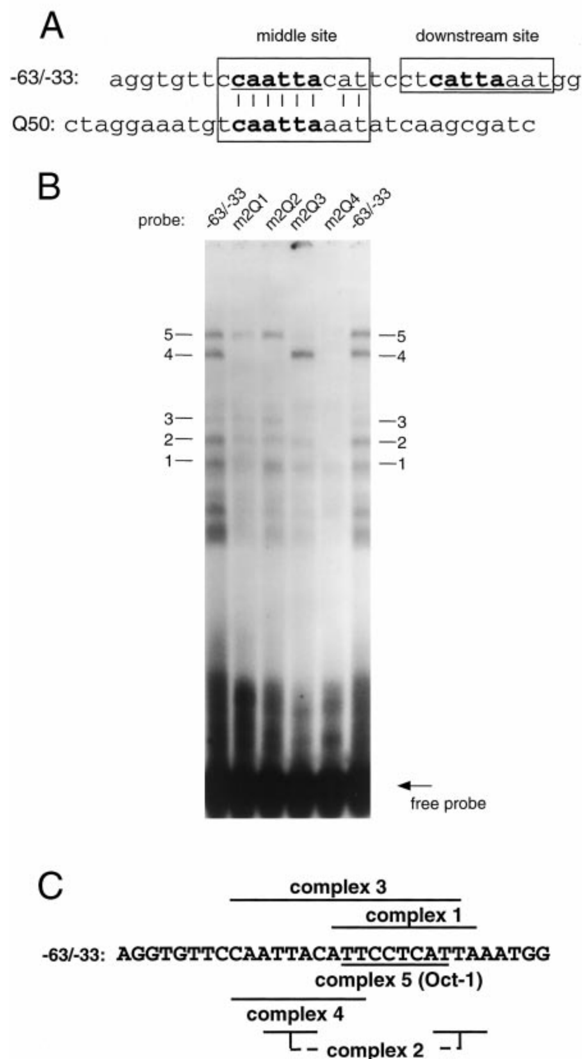


Fig. 6. Potential Q50 Homeodomain Protein Binding Sites in Footprint 2

A, The GnRH promoter −63/−33 region contains two possible Q50 homeodomain binding sites. The −63/−33 oligonucleotide is shown with *boxes* encompassing two regions of homology with the fushi tarazu/engrailed (ftz/en) consensus site (middle site and downstream site). *Bold sequences* correspond to the core Q50 homeodomain binding site, and *underlining* identifies regions of homology to a Q50 binding site. Alignment of the Q50 consensus binding site probe (Q50) is with the middle binding site within FP2 of the GnRH promoter (middle site). The Q50 oligonucleotide region *within the box* indicates the ftz/en consensus binding site and encompasses the middle site in the −63/−33 element. *Vertical lines* identify the bases that are conserved between the Q50 consensus site and −63/−33. B, EMSAs were conducted with GT1–7 nuclear extract and the indicated probes. The specific complexes labeled at *left* and *right* were previously identified by their ability to be competed by 100-fold excess of unlabeled oligonucleotide (not shown). *Arrow at bottom right* indicates migration of free probe. C, −63/−33 oligonucleotide sequence is shown with the likely complex binding sites. *Bars above* and *below the sequence* show the region likely bound by each complex listed.

at position 50 (K50) recognizes a GGATTA motif (18). By sequence comparison, it appears that both the middle and downstream sites contain Q50 homeodomain binding elements (Fig. 6A). Few of the specific Q50 homeodomain binding sites have been determined; the best characterized include engrailed (en), antennapedia (antp), and fushi tarazu (ftz) (19–21). Ftz is a member of the antp family of homeodomain proteins while en is a member of a separate family (22). En and ftz are Q50 homeodomain transcription factors that bind a core site of CAATTA, an 8 out of 10 match to the middle site in FP2 (Fig. 6A) (20, 21). The downstream site of the −63/−33 promoter element is also homologous to a Q50 homeodomain binding site that can be bound by ftz or antp, based on the core CAATTA motif (19). Thus, it is possible that members of the Q50 homeodomain protein family bind to one or both of these regions within the −63/−33 sequence.

Thus, it is important to determine whether the cell-specific complexes 1 or 2 are bound to the ATTA motifs. Previously, we conducted EMSA analysis of mutations within the −63/−33 probe to determine the regions binding each of the five protein complexes (11). In Table 1, we show the mutant oligonucleotide probe sequences below the wild-type −63/−33 oligonucleotide probe sequence. The mutant probes lack the binding of protein complexes to either the middle site (m2c block mutation) or to the downstream site (m2e block mutation and m2oct double point mutation) (11). Here we list the previous results in table form with the addition of a second block mutation in the middle site, m2d (data not shown), and four mutated probes corresponding to mutations in the Q50 consensus sites, m2Q1, m2Q2, m2Q3, and m2Q4 (EMSA shown in Fig. 6B). Complex 5 (Oct-1) and complex 1 are greatly reduced by the m2e and m2oct mutation, corroborating the binding of the corresponding proteins in the downstream site of FP2 (diagram in Fig. 6C). The new m2d mutation, as well as the previously documented m2c mutation, eliminates complex 4 binding, suggesting that complex 4 binds to the middle FP2 binding site. Complex 3 is eliminated by all of the block mutations including m2d, confirming that dramatic changes in the −63/−33 oligonucleotide disrupt complex 3. We previously indicated that m2c does not disrupt complex 2 formation (11), but a more detailed analysis shows a reduction in complex 2 formation on the m2c block mutation oligonucleotide probe (see also Fig. 7A).

Since the m2c mutation does not completely eliminate complex 2 formation, it is possible that complex 2 binds to a site at the downstream end of −63/−33. An ATTA motif is present in both the middle and downstream sites. To investigate the location of binding of complex 2, targeted mutations were created in the −63/−33 oligonucleotide probe (Table 1 for compiled data and mutation sequences). Mutation of the middle ATTA site to GGGG (m2Q1) results in decreases in complexes 1, 2, 3, and 5 while complex 4 is completely eliminated (Fig. 6B). A smaller mutation in the middle site, changing the first

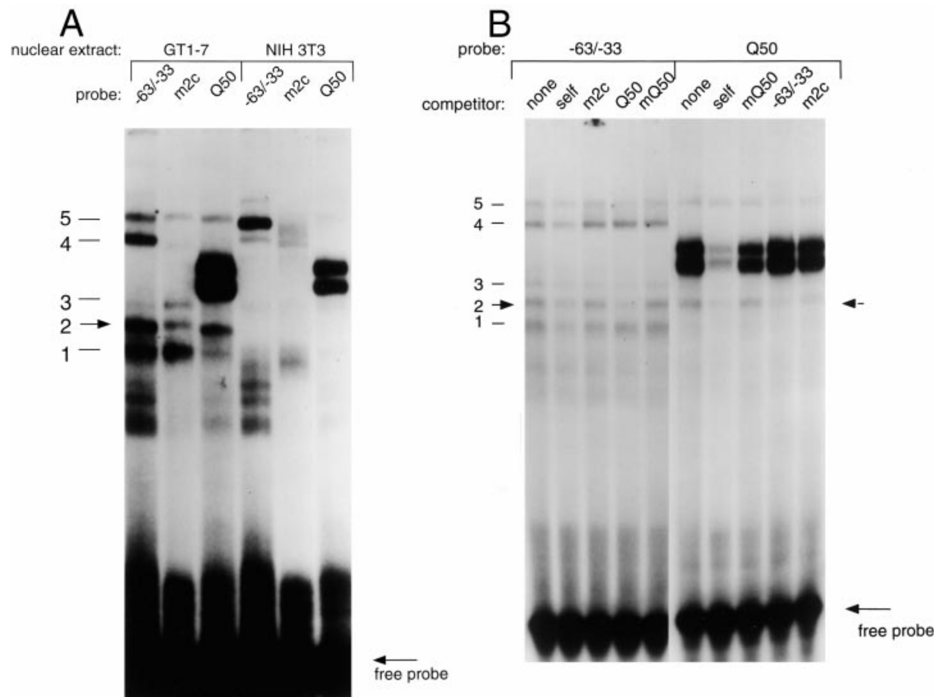


Fig. 7. The −63/−33 Region Binds a GT1–7 Cell-Specific Protein Related to the Q50 Homeodomain Family

A, The GT1–7 specific complex bound to the Q50 consensus site comigrates with complex 2 from GT1–7 nuclear extract. EMSAs were conducted as described in *Materials and Methods* with −63/−33, m2c, and Q50 probes, as indicated. The five specific complexes bound in GT1–7 nuclear extract to −63/−33 are indicated at the *left*. The Q50-specific band is indicated by the *arrow at left* and is absent in the lanes with NIH 3T3 extract (A, *right*). The *arrow at bottom right* indicates migration of free probe. Nuclear extracts from GT1–7 cells (*left*) and NIH 3T3 cells (*right*) were used in the EMSA. B, Complex 2 is competed by the Q50 probe. EMSA was conducted using GT1–7 nuclear extract. Competitions with 100-fold molar excess of the indicated oligonucleotide were conducted; none (no competitor added), self (same oligonucleotide as the indicated probe), m2c (−63/−33 with m2c mutation), Q50 (oligonucleotide with ftz/en consensus binding site) and Q50mut (Q50 oligonucleotide with mutation in homeodomain consensus site).

Table 1. The Binding of Complexes to Mutated Oligonucleotides within the −63/−33 Element

	−63 −33	DNA-Binding Complex 1	2	3	4	5
WT	AGGTGTTCCAATTACATTCCTCATTAAATGG	+++	+++	+++	+++	+++
m2c	AGGTG**GCGGCCGC**ACATTCCTCATTAAATGG	+++	+	−	−	++
m2d	AGGTGTTCCA**GCGGCCGC**CCTCATTAAATGG	+	+++	−	−	++
m2e	AGGTGTTCCAATTACATT**GCGGCCGC**AATGG	−	+++	−	+++	−
m2oct	AGGTGTTCCAATTACAT**G**CCTCA**G**TAAATGG	−	++	++	++	−
m2Q1	AGGTGTTCCA**GGGG**CATTCCTCATTAAATGG	+	+	+	−	+
m2Q2	AGGTGTTCCA**G**TT**G**CATTCCTCATTAAATGG	++	+	+	−	−
m2Q3	AGGTGTTCCAATTACATTCCTC**G**TT**G**AATGG	+	+	+	+++	−
m2Q4	AGGTGTTCCA**G**TT**G**CATTCCTC**G**TT**G**AATGG	+	−	−	−	−

Wild-type and mutant oligonucleotide probes are depicted as used for the EMSA in Fig. 6B and in previous publications (11). At right, the band intensity of each complex is indicated (+++ is maximum, + is minimum, and − is no band present) from Fig. 6B and Eraly *et al.* (11), with the numbers 1 to 5 indicating the five protein complexes.

and fourth A's to G's (m2Q2), results in a decrease in complex 2 binding and elimination of complex 4. The same mutation in the downstream ATTA site (m2Q3) decreases binding of complexes 1 and 2 and eliminates binding of complex 5 (Oct-1), as expected (see above). Since complex 4 is retained by the probe containing the downstream mutation, it is clear that this complex requires an intact middle ATTA site although it is not a cell-specific complex (see Fig. 5). Complex 2 appears to bind to the CAATTA motif in the middle site (−55 to −50)

and an ATTA motif (−41 to −38) present in the downstream site. Conversely, complex 2 may also be maintained by protein-protein interactions even when its DNA recognition sequence has been disrupted. Complex 1 is relatively cell specific (see Fig. 5) and binds the identical site as Oct-1, as is shown by its retention on the probe with the point mutations of the middle site and its reduction by mutations in the downstream site. Finally, an oligonucleotide probe containing the point mutations in both middle and downstream sites (m2Q4) reduces binding of complex 1 and eliminates binding of the remaining complexes (Fig. 6B). A diagram summarizing the proteins binding to −63/−33 is shown in Fig. 6C.

−63/−33 Binds a Complex That Comigrates with a Complex Binding to a Q50 Homeodomain Consensus Site

To determine whether proteins expressed in GT1–7 cells can bind a consensus Q50 binding site (represented by the binding site for en and ftz, termed Q50), EMSA was conducted with −63/−33, m2c, and Q50 as probes (Fig. 7A). In GT1–7 nuclear extract, the Q50 probe is bound by a protein complex that comigrates with complex 2 on the probe −63/−33. This complex is not present in NIH 3T3 cell nuclear extract (Fig. 7A, *arrow*) or other cell types (see Fig. 5). In EMSA with nuclear extracts from hypothalamus and forebrain, but not cerebellum, a complex is observed to comigrate with complex 2 using the Q50 probe (data not shown). Furthermore, the Q50 oligonucleotide competes for the binding of complex 2 bound to the −63/−33 probe while a mutated Q50 oligonucleotide (Q50mut) does not compete for the complex (Fig. 7B). Additionally, −63/−33 competes for binding of the specific complex bound to Q50 (*arrow*) while the m2c oligonucleotide (disruption of the middle site) does not compete (Fig. 7B). The two slower mobility complexes, which are abundant in both GT1–7 and NIH 3T3 nuclear extract, are not competed for by the −63/−33 oligonucleotide. These data suggest that a Q50 homeodomain transcription member binds to −63/−33 specifically in GT1–7 nuclear extract, perhaps modulating the cell-specific interactions between the GnRH enhancer and promoter.

DISCUSSION

Specification of the expression of individual genes during the complex processes of development and cellular differentiation is one of the most challenging problems in the field. This central question becomes all the more compelling when the tissue involved is the brain and the target is a unique, well defined set of neurons. Little is known about the neuronal regulatory elements and transcription factors that restrict the expression of individual genes to exclusive populations of neurons. The study of expression of the GnRH gene in the GT1–7 cell line provides an opportunity to elucidate the molecular mechanisms for neuron-specific gene expression.

In the simplest cases, cell type-specific gene expression can be traced to the presence of transcription factors unique to the individual cell types (23–25). As more is known about tissue-specific control regions, it has been discovered that they often comprise complexes of interacting elements and regulatory proteins with or without uniquely tissue-specific factors (26–28). These more complex control regions may have evolved to integrate diverse spatial and temporal information in determining cell fate. The GnRH gene thus far falls into the latter class in that the proteins identified to date are expressed in many cell types.

The GnRH regulatory region comprises an enhancer and a proximal promoter, which combined, can confer uniquely targeted expression in transgenic animals, but the enhancer on a heterologous (RSV) promoter is inadequate (M. A. Lawson and P. L. Mellon, unpublished results). Thus, the enhancer must act coordinately with the elements in the promoter. This high degree of interdependence between the regulatory elements may be a quality adapted for specifying expression to a very rare cell type, since activation requires the simultaneous presence of multiple specific proteins, some of which bind to both the enhancer and the promoter (11, 16).

The synergistic activation of transcription by the combination of the GnRH enhancer and promoter regulatory regions is specific to GT1–7 cells and GnRH neurons. Thus, the GT1–7 cells express the proteins necessary to facilitate the interaction between the enhancer and promoter, whereas other cell lines, such as NIH 3T3 cells, do not. Using transient transfections, we previously found that the GnRH enhancer can confer cell-type specificity without the GnRH proximal promoter (5). In this study, we have demonstrated that the promoter can also confer a degree of cell type specificity in the absence of the GnRH enhancer. FP2 strongly contributes to cell type specificity, since deletion of FP2, when the GnRH promoter is coupled with the RSV enhancer, abolishes preferential reporter gene expression in GT1–7 cells (Fig. 2B). The specificity of FP2 action is further demonstrated by fusion to the GnRH enhancer or the RSV enhancer, upstream of the RSV promoter. In these instances, FP2 increased the activity ratio by only 2-fold in NIH 3T3 cells but by 15-fold in GT1–7 cells (Fig. 3, A and B). Finally, we have identified a subregion of FP2, −63/−33, that specifically interacts with the GnRH enhancer in GT1–7 cells.

The footprint 2 region of the GnRH gene proximal promoter is complex (reviewed in Ref. 16). It was shown previously that deletion of the FP2 element results in a 20-fold loss of transcriptional activation, the most dramatic loss in activation of any of the promoter deletions (6). Although data from DNase I footprint analysis of block mutations in the promoter suggests the binding of three protein complexes,

EMSA reveals five individual complexes within the middle and downstream site (11). This region also confers responsiveness to both phorbol esters and glucocorticoids. Previous data also show the induction of a slower mobility complex by the phorbol ester TPA, a protein kinase C activator (6). Furthermore, the glucocorticoid receptor binds to the 5′-region of FP2 in the equivalent area in the mouse GnRH promoter (29). Thus, the GnRH proximal promoter element, FP2, confers neuronal specificity to GnRH gene expression, as well as responsiveness to hormones and second messengers.

Although FP2 is necessary for conferring several responses to GnRH gene expression, little is known about the proteins that bind to this region. Previously, we had identified Oct-1 binding to the 3′-portion of FP2 (11). Oct-1 may play a role in cell-specific activation of GnRH expression through unique interactions with other ubiquitous transcription factors, through interactions with a GnRH neuron-specific activator, or through interactions coupling the GnRH enhancer to the GnRH promoter. The data presented here show that FP2 of the GnRH promoter can greatly augment transcriptional activation by the GnRH enhancer, thereby demonstrating cross-talk between FP2 and the GnRH enhancer. Oct-1 may be one of the regulators required for this interaction, particularly in consideration of its critical role in transcriptional activation through binding sites in the enhancer (7).

To identify potential cell-specific proteins in GT1–7 cells, we compared EMSA between GT1–7 and various other cell types. Three differences exist between GT1–7 and NIH 3T3 nuclear factors binding to the −63/−33 region of the promoter that could play a role in cell-specific expression of GnRH. First, Oct-1 containing complexes are not detected by EMSA that include the −63/−33 region of the GnRH promoter and nuclear extract from NIH 3T3 cells, in contrast to assays containing nuclear extract from GT1–7 cells. Rather, in assays utilizing nuclear proteins derived from NIH 3T3 cells, a strong, comigrating, but as yet unidentified, complex forms. It is possible that this complex masks Oct-1 binding or that the formation of the strong, low-mobility complex occludes Oct-1 binding by occupying nucleotide sequences necessary for Oct-1 interaction with the −63/−33 site. As mentioned earlier, binding sites for Oct-1 are also present in the GnRH enhancer, and it is possible that the synergy between the two elements could be due to Oct-1 or an Oct-1 binding partner. Although formation of Oct-1 complexes on the −63/−33 site with NIH 3T3 nuclear extract is not observed, Oct-1 complexes from pituitary-derived αT3–1 nuclear extracts are detected. Furthermore, expression through the GnRH enhancer and promoter in αT3–1 cells is lower than in NIH 3T3 cells (data not shown), indicating that the ability of Oct-1 to bind −63/−33 alone is not sufficient to confer cell-specific expression. This observation suggests that other factors dependent on Oct-1 interaction may be important for cell-specific activation of the GnRH promoter rather than Oct-1 itself.

A second candidate for cell-specific activation is complex 1. This complex is not present in NIH 3T3 nuclear extract whereas it appears to be present in αT3–1 nuclear extract by EMSA comigration experiments. Again, since αT3–1 cells express lower levels of reporter gene driven by the GnRH enhancer and promoter than NIH 3T3 cells, the presence of complex 1 is not sufficient to facilitate cell-specific interactions between the GnRH promoter and enhancer. A third candidate, that which forms complex 2, is relatively unique to GT1–7 cells by EMSA and may account for the differences seen in transcriptional regulation between the cell types. As described earlier, the GnRH enhancer fused to the 4×−63/−33 region results in a higher reporter expression in GT1–7 cells compared with NIH 3T3 cells. To further support this idea, the m2c mutation in the −63/−33 EMSA probe decreased binding of complex 2 (Fig. 7A). When the m2c mutation is present in the context of the whole promoter and enhancer, reporter gene expression is only 25% of wild type, the most significant decrease of all of the FP2 mutations examined to date (11). Thus, it is likely that complex 2 plays a role in maintaining cell type specificity and in transcriptional activation of GnRH expression.

Complex 2 is our best candidate for a cell-specific protein binding to FP2. Expression of complex 2 appears to be restricted to GT1–7 cells and to bind a potential Q50 homeodomain transcription factor site, CAATTA. This site is also present in repeating elements in the 3′-region of the GnRH enhancer. EMSA experiments have confirmed that complexes that form on these elements in GT1–7 nuclear extracts comigrate with complex 2 (data not shown, C. G. Kelley and P. L. Mellon, personal communication). This similarity suggests that complex 2 may also bind to the GnRH enhancer and could subsequently foster interactions between the two regulatory regions.

All homeodomain transcription factors bind to the core motif ATTA, which makes it difficult to identify the protein binding by simple binding site homology. Additionally, only a few specific homeodomain DNA-binding sites have been well characterized. To date, antennapedia, engrailed, and fushi tarazu are the only homeodomain proteins known to bind to a CAATTA site (21), and the crystal structure of engrailed bound to TAATTA has been shown (30). In EMSA experiments we have identified a complex that binds specifically with GT1–7 nuclear extract to both the −63/−33 probe and the CAATTA site. We have not yet identified the protein(s) in this complex since supershift antibodies are not available for Q50 homeodomain candidates, antennapedia, engrailed, or fushi tarazu. We have found engrailed 2 RNA and protein in GT1–7 neurons (data not shown) but engrailed 2 has not been shown to be expressed in regions known to contain GnRH neurons (31, 32). Furthermore, Hox proteins, mammalian homologs of antennapedia, are not ex-

pressed in the forebrain, and fushi tarazu has no known mammalian homolog. Thus, complex 2 binds to a core homeodomain site in the GnRH promoter and may be a novel or previously identified member of the Q50 homeodomain family. Future experiments will focus on identification of this cell-specific regulator of GnRH gene expression.

In conclusion, we have identified a 31-bp region (−63 to −33) of the rat GnRH promoter that interacts with the GnRH enhancer to increase cell-specific transcription. This region binds Oct-1, which may interact with other ubiquitous or specific transcription factors to control the cell-specific expression. Binding of Oct-1 to the GnRH enhancer and to FP2 of the promoter also may be crucial for the interaction between these two DNA regulatory elements. The data presented here substantiate the assertion that the interactions occurring between the GnRH enhancer and GnRH promoter are necessary for cell-specific reporter gene expression. We have identified cell-specific complexes binding the GnRH promoter that may play a role in interactions between the GnRH enhancer and promoter to control cell-specific expression of GnRH. Here, we suggest that a Q50 homeodomain transcription factor binds to a region of the GnRH promoter that is crucial for neuron-specific interactions with the GnRH enhancer. This protein may interact with other proteins, such as Oct-1 bound to the GnRH promoter and/or enhancer to confer cell-specific expression. Further investigations of cognate binding proteins and their protein-protein interactions will help to clarify the role of the FP2 element in GnRH transcription, elucidating the molecular mechanisms underlying the interactions between the enhancer and promoter of the GnRH gene.

MATERIALS AND METHODS

Plasmids and Cloning

The rGnRHe/rGnRHp-luc plasmid contains the rat GnRH enhancer (−1571 to −1863; in reverse orientation) and rat GnRH promoter (−173 to +112) in the pGL3 basic vector, controlling luciferase gene expression. Deletions of the rat GnRH promoter were created as previously described (11). Briefly, deletion of FP6 and FP7 retains −126 to +112; deletion of FP2 retains −173 to −70 and −28 to +112; deletion of FP3, 4, 5, 6, and 7 retains −82 to +112; and deletion of FP2, 3, 4, 5, 6, and 7 retains −28 to +112. In addition, the plasmid with the internal deletion of FP3, 4, and 5 retains the −173 to −128 and −74 to +112 region. These regions were subcloned from the CAT vectors into the pGL3 vector (Promega Corp., Madison, WI), containing the rat GnRH enhancer. The RSVe/rGnRHp-luc plasmid was created by placing the enhancer region of the RSV 5′-LTR and the rat GnRH promoter (−173 to +112) upstream of the luciferase gene in pGL3 basic. To place the RSV enhancer adjacent to the promoter deletions, the promoter deletions were transferred from the pGL3 vector containing the rGnRH enhancer to the pGL3 vector containing the RSV enhancer. The rGnRHe/RSVp-luc plasmid contains the rat GnRH enhancer (−1571 to −1863) and the promoter region from the RSV 5′-LTR in the pGL3 basic vector. RSVe/RSVp-luc contains the enhancer and promoter regions of the RSV 5′-LTR in the pGL3 basic vector. RSVe/RSVp-gal was created by removing the luciferase gene from the RSVe/RSVp-luc plasmid and replacing it with β-galactosidase from pSV-β-galactosidase (Promega Corp.).

To create rGnRHe/FP2/RSVp-luc, the FP2 region (−82 to −21) was inserted between the rat GnRH enhancer and RSV promoter in the rGnRHe/RSVp-luc vector. RSVe/FP2/RSVp-luc contains RSVe/RSVp-luc with FP2 (−82 to −21) inserted in the polylinker between the RSV enhancer and RSV promoter. The −63/−33 region was multimerized by using a synthesized oligonucleotide with the following sequence: 5′-CTAGAAGGTGTTCCAATTACATTCCTCATTAAATGG3-′ and 5′-CTAGTCCATTTAATGAGGAATGTAATTGGAACACCTT-3′. The oligonucleotide was annealed and inserted into $pBSK^+$. To create the four multimer site, multiple rounds of digestion and ligation were conducted. The 4× −63/−33 multimer was then inserted between the enhancer and promoter of rGnRHe/RSVp-luc and RSVe/RSVp-luc to create rGnRHe/4×63/RSVp-luc and RSVe/4×63/RSVp-luc, respectively.

Cell Culture and Transfections

GT1–7 and NIH 3T3 cells were cultured in DMEM containing 10% FCS (Omega Scientific, Tarzana, CA), Penn/Strep, glucose, and sodium bicarbonate. These cells were incubated in 5% CO_2 at 37 C. For transient transfections, GT1–7 cells were split 1:3 and NIH 3T3 were split 1:25 from 100% confluent plates in 6-cm plates. Cells were incubated overnight and were transfected with calcium phosphate (33). Briefly, 7.2 μg of reporter DNA and 2.2 μg of internal control DNA were added to a 15-ml conical tube; 2× HBS (0.5 ml) was added to the DNA and briefly vortexed, and 0.25 M calcium chloride (0.5) was added drop wise into the tube while vortexing at low speed. The precipitate was incubated 5 min at room temperature before addition of 0.5 ml to one 6-cm plate of GT1–7 cells and one 6-cm plate of NIH 3T3 cells. Sixteen hours later the cells were washed two times with PBS, and DMEM 10% FCS was replaced. Cells were incubated 24 h longer and harvested.

Harvesting cells entailed washing the cells three times with PBS and adding harvesting buffer (0.5 ml; 0.15 M NaCl, 1 mM EDTA, and 40 mM Tris-HCl, pH 7.4). Cells were scraped from the plate and placed in 1.5-ml tubes and spun for 30 sec at 14,000 rpm. Buffer was removed, lysis buffer (50 μl; 100 mM potassium phosphate, pH 7.8, 0.2% Triton X-100) was added, and cells were resuspended by vortexing. Cells were spun for 5 min, and supernatant was placed into a new tube and assayed for luciferase and β-galactosidase activity (Galacto-Light Plus Kit, Tropix, Inc., Bedford, MA). For the luciferase assay, cell lysate (10 μl) was assayed in a 96-well plate, which was read in a luminometer (Microlumat Plus; Microplate Luminometer LB96V; EG&G Berthold, Gaithersburg, MD) using luciferin assay buffer (100 μl; 100 mM Tris, pH 7.8, 15 mM $MgSO_4$, 10 mM ATP and 65 μM luciferin). For the β-galactosidase assay, cell lysate (10 μl) and 0.25 M Tris, pH 7.8 (10 μ l), were combined and incubated at 48 C for 50 min. This was transferred to a 96-well plate, and diluted Galacton-Plus Substrate (70 μl; 1:100 in Galacto-Light Reaction Buffer Diluent) was added to each well. The plate was incubated for 15 min and assayed using the luminometer. Light Emission Accelerator (100 μl) was injected into each well and read for 10 sec after a 2-sec delay. The luciferase and β-galactosidase values for a nontransfected plate of cells were subtracted from each transfected plate value. Then luciferase values were divided by the β-galactosidase values to control for transfection efficiency.

Normalizing Transfection Data and Statistics

To control for differences in expression between the different cell types, each experiment was normalized. The RSV en-

hancer fused to the RSV promoter driving luciferase (RSVe/RSVp-luc) was transfected in duplicate in each experiment. The internal control, RSVe/RSVp fused to β-galactosidase (RSVe/RSVp-gal), was used as an internal control for each transfected plate of cells. The RSVe/RSVp-luc luciferase values were divided by the RSVe/RSVp-gal β-galactosidase values and averaged. The average was set to 1, and the values for the other plates were normalized to this value in the individual cell types. Thus, the values from the individual cell types can be directly compared. The mean of at least three experiments is depicted. The error bars represent SEM. In Fig. 1, a single-factor ANOVA and Dunnett's Least Significant Difference (LSD) Test were used to determine significant difference. In Figs. 2 and 3, significance was measured by paired *t* test analysis, $P \leq 0.05$ as indicated by *asterisks*.

Oligonucleotides

The −63/−33 oligonucleotide corresponds to the sequences −63 to −38 and −59 to −33. The m2c, m2d, and m2e oligonucleotides are identical to the −63/−33 oligonucleotide except for the substitution of 5′-GCGGCCGC-3′ at −58 to −51, −53 to −46, and −45 to −38, respectively. The m2oct oligonucleotide is identical to −63/−33 with a substitution of a G for the T at positions −47 and −40. The m2Q oligonucleotides are identical to −63/−33 with the substitution of G's for the ATTA from −50 to −53 (m2Q1), a G for the A at positions −50 and −53 (m2Q2), a G for the A at positions −38 and −41 (m2Q3), and a G for the A at positions −38, −41, −50, and −53 (m2Q4). The fushi tarazu/engrailed (ftz/en) consensus binding oligonucleotide (Q50) corresponds to the sequences CTAGGAAATGTCAATTA-AATATCAAG (top strand), and GATCGCTTGATATTTAATTGA-CATTC (bottom strand). The ftz/en consensus mutant (Q50mut) oligonucleotide corresponds to the sequences CTAGGAAAT-GTCAGGGAATATCAAG (top strand) and GATCGCTTGATAT-TCCCCTGACATTTC (bottom strand). Oligonucleotides were synthesized by Operon Technologies (Alameda, CA) and were annealed in 50 mM NaCl by heating to 95 C for 5 min and slowly cooling to room temperature.

EMSA

Nuclear extracts were prepared according to the method described by Schreiber *et al.* (34). Annealed wild-type and mutant oligonucleotides (1 pmol) containing sequences of the GnRH promoter and consensus sequences were filled in with α[^{32}P]dATP (3000 Ci/mmol Dupont NEN Life Science Products, Boston, MA) and Klenow using standard procedures (35). Probes were phenol/chloroform extracted and passed over G-50 micro columns (Amersham Pharmacia Biotech, Piscataway NJ). Probes were counted in a scintillation counter and diluted in 50 mM NaCl. The competitor oligonucleotide was end-filled with Klenow. Binding reactions were carried out in 10 mM HEPES-KOH, pH 7.8, 50 mM KCl, 1 mM EDTA, 5 mM spermidine, 5 mM DTT, 0.2 mg/ml BSA, 0.5 mM phenylmethylsulfonylfluoride, 12.5–25 μg/ml polydeoxyinosinic-deoxycytidylic acid, 10% (vol/vol) glycerol, and 20 mg/ml Ficoll. One femtomole of each probe was incubated with 2 μg of GT1–7 crude nuclear extract in 20 μl reactions. Reactions were incubated at room temperature for 5 min, loaded, with current on, into a 5% polyacrylamide gel [30:1 acrylamide/bisacrylamide, 0.25 × TBE (130 mM Tris, 45 mM boric acid, 2.5 mM EDTA), 5% glycerol], and electrophoresed for 2 to 3 h at 175 V. Gels were prerun for 30 min in 0.25 × TBE. After electrophoresis, gels were dried and subjected to autoradiography. Competition reactions were performed by preincubating the reactions with the specified amount of excess unlabeled oligonucleotide for 20 min before the addition of probe.

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