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Phylogenetic Footprinting Reveals Evolutionarily Conserved Regions of the Gonadotropin-Releasing Hormone Gene that Enhance Cell-Specific Expression

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Reproductive function is controlled by the hypothalamic neuropeptide, GnRH, which serves as the central regulator of the hypothalamic-pituitary-gonadal axis. GnRH expression is limited to a small population of neurons in the hypothalamus. Targeting this minute population of neurons (as few as 800 in the mouse) requires regulatory elements upstream of the GnRH gene that remain to be fully characterized. Previously, we have identified an evolutionarily conserved promoter region (−173 to +1) and an enhancer (−1863 to −1571) in the rat gene that targets a subset of the GnRH neurons *in vivo*. In the present study, we used phylogenetic sequence comparison between human and rodents and analysis of the transcription factor clusters within conserved regions in an attempt to identify additional upstream regulatory elements. This approach led to the characterization of a new upstream enhancer that regulates expression of GnRH in a cell-specific manner. Within this upstream enhancer are nine binding sites for Octamer-binding transcription factor 1 (OCT1), known to be an important transcriptional regulator of GnRH gene expression. In addition, we have identified nuclear factor I (NF1) binding to multiple elements in the GnRH-regulatory regions, each in close proximity to OCT1. We show that OCT1 and NF1 physically and functionally interact. Moreover, the OCT1 and NF1 binding sites in the regulatory regions appear to be essential for appropriate GnRH gene expression. These findings indicate a role for this upstream enhancer and novel OCT1/NF1 complexes in neuron-restricted expression of the GnRH gene. (*Molecular Endocrinology* 18: 2950–2966, 2004)

ACCURATE EXPRESSION OF GnRH is essential for reproduction of species ranging from fish to human. GnRH is a decapeptide neurohormone that controls the activity of the hypothalamic-pituitary-gonadal axis. Expression of the GnRH gene is limited to a small population of neurons, as few as 800, scattered throughout the preoptic area, hypothalamus, and septum in the adult (1). Both *in vitro* and *in vivo* studies indicate that the restricted pattern of GnRH expression is conferred at the transcriptional level (2–7).

Although significant research examining the human and mouse GnRH gene regulatory regions has been performed, the rat GnRH gene has been investigated most extensively. The rat GnRH gene contains two well-characterized regulatory regions, a 300-bp enhancer located at −1863 to −1571 relative to the start site and a 173-bp promoter just 5′ of the transcriptional start site (2). Interestingly, although there are considerable differences between the human, mouse, and rat GnRH 5′-upstream regions, much of the sequence of these well-defined regulatory elements in the rat GnRH gene is evolutionarily conserved (6, 8, 9).

Studies using transgenic mice have provided insight into the mechanisms underlying restricted expression of the GnRH gene, as well as having revealed their complexity. Transgenic mouse lines carrying a transgene containing the rat GnRH enhancer and promoter (3) targeted a substantial number of GnRH neurons through development and in the adult. However, this combination of regulatory elements was not sufficient for targeting the entire population of GnRH neurons. In the case of the human gene, 3.8 kb of 5′-flanking region used in transgenes targeted reporter expression to the hypothalamus, but the percent efficiency was not directly addressed (10). However, 5.2 and 3.4 kb of the mouse upstream region (4, 5, 7) target transgene reporter expression to almost the entire GnRH neuronal population, whereas truncation to 2.1 kb drives transgene expression in only a subset and 1.7

Abbreviations: GST, Glutathione-*S*-transferase; HPV-16, human papilloma virus type 16; HSD, honestly significant difference; MMTV, mouse mammary tumor virus; NF1, nuclear factor 1; OCT1, Octamer-binding transcription factor 1; RSV, Rous sarcoma virus.

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kb fails to express at all (4). Thus, sequences distal to the characterized enhancer and promoter in the rat GnRH gene are likely involved in promoting complete and focused expression of GnRH.

In addition to the *cis*-regulatory elements of the GnRH promoter, several transcription factor complexes have been identified that contribute to the appropriate temporal/spatial expression of the GnRH gene. Interestingly, the transcription factors identified thus far that bind to the regulatory regions of the GnRH gene, including Octamer-binding transcription factor 1 (OCT1) (11), SCIP/OCT6 (12), BRN2 (13), GATA4 (14), CEBPβ (15), OTX2 (16), PBX1 and PREP1 (17), and DLX2 (Givens, M. L., N. Rave-Harel, and P. L. Mellon, manuscript submitted), are expressed in the GnRH neuron, but are also expressed in other tissues or brain regions. Therefore, it is likely that unique complexes, which may include additional, as yet unidentified transcription factors or cofactors, facilitate restricted expression of GnRH.

Using phylogenetic footprinting and transcription factor cluster analysis, we have identified a novel upstream enhancer that contributes to cell-specific expression of GnRH. Remarkably, this region contains nine binding sites for OCT1, a transcription factor previously shown to be important for activation of the well-characterized enhancer and promoter of the rat gene (11, 18). In addition, we find that this upstream enhancer contains multiple binding sites for nuclear factor 1 (NF1), each in close proximity to an OCT1 binding site. In fact, NF1 binds adjacent to OCT1 on elements located within each of the three conserved regulatory regions of the rat GnRH gene and physically and functionally interacts with OCT1. We also show that the binding sites for NF1 and OCT1 are necessary for proper expression of GnRH in a cultured cell model for the hypothalamic GnRH neuron. The identification of a novel upstream enhancer region containing conserved binding motifs indicates an important role for this region in cell-specific expression of the GnRH gene.

RESULTS

Phylogenetic Footprinting Reveals Three Blocks of Conserved Sequence in the GnRH-Regulatory Region

The specificity of GnRH gene expression is conferred at the transcriptional level (2–7). Thus, deciphering the transcriptional regulatory elements that contribute to restricted expression is essential for understanding regulation of the GnRH gene. The progressive release of the human and mouse genomic sequence has provided the opportunity to use phylogenetic footprinting to compare upstream promoter regions between species. Therefore, to identify putative regulatory regions upstream of the GnRH gene, we performed a pairwise BLAST alignment of the available 3 kb of the rat GnRH promoter and the corresponding human and mouse sequences. Three regions of significant homology emerged from the rat and human alignment (Fig. 1). The most proximal sequence homology corresponds to the well-characterized, highly conserved, 173-bp promoter (6, 8). A second region of homology in the rat GnRH sequence (−1930 to −1603), contains the functional enhancer sequence we have previously characterized (2, 3, 11, 19). By adjusting the parameters, an alignment of the human and mouse sequences with the rat enhancer sequence (−1863 to −1571) was obtained, demonstrating the high degree of conservation of this regulatory domain (Fig. 2A). Surprisingly, an additional region of homology, located from −2980 to −2631 in the rat sequence, is also conserved between the rat, mouse, and human genes. This upstream region is composed of two blocks of strong homology with the human sequence. One region, located from −2928 to −2794 in the rat gene, has 92% homology to the human sequence. A second region, located from −2734 to −2658 in the rat gene, has 78% homology to the human sequence. The comparative regions of homology between rat and mouse exist between −2980 and −2735 with 96% homology and from −2733 to −2633 with 84% homology. Upon closer examination, these regions of homology are separated only by a 16-bp insertion in the human and mouse sequence relative to the rat gene, equivalent to a 16-bp deletion within the rat sequence (Fig. 2B). Thus, a contiguous block of conserved sequence of 350 bp is found in the rat gene from −2980 to −2631, a similar size to the previously characterized enhancer.

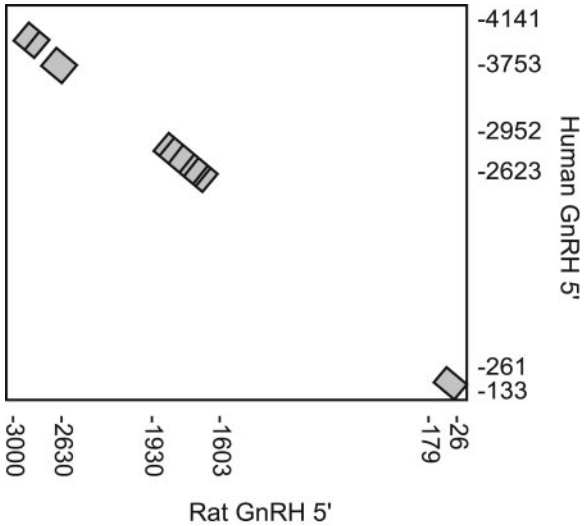


Fig. 1. Identification of Evolutionarily Conserved Regions Upstream of the GnRH Gene

Pairwise BLAST was used to compare the 3 kb upstream of the rat GnRH gene transcriptional start site (x-axis) to 5 kb upstream of the human GnRH gene transcriptional start site (y-axis). In the context of the rat GnRH gene, these three regions of homology represent the proximal promoter, the previously characterized enhancer, and a novel upstream enhancer.

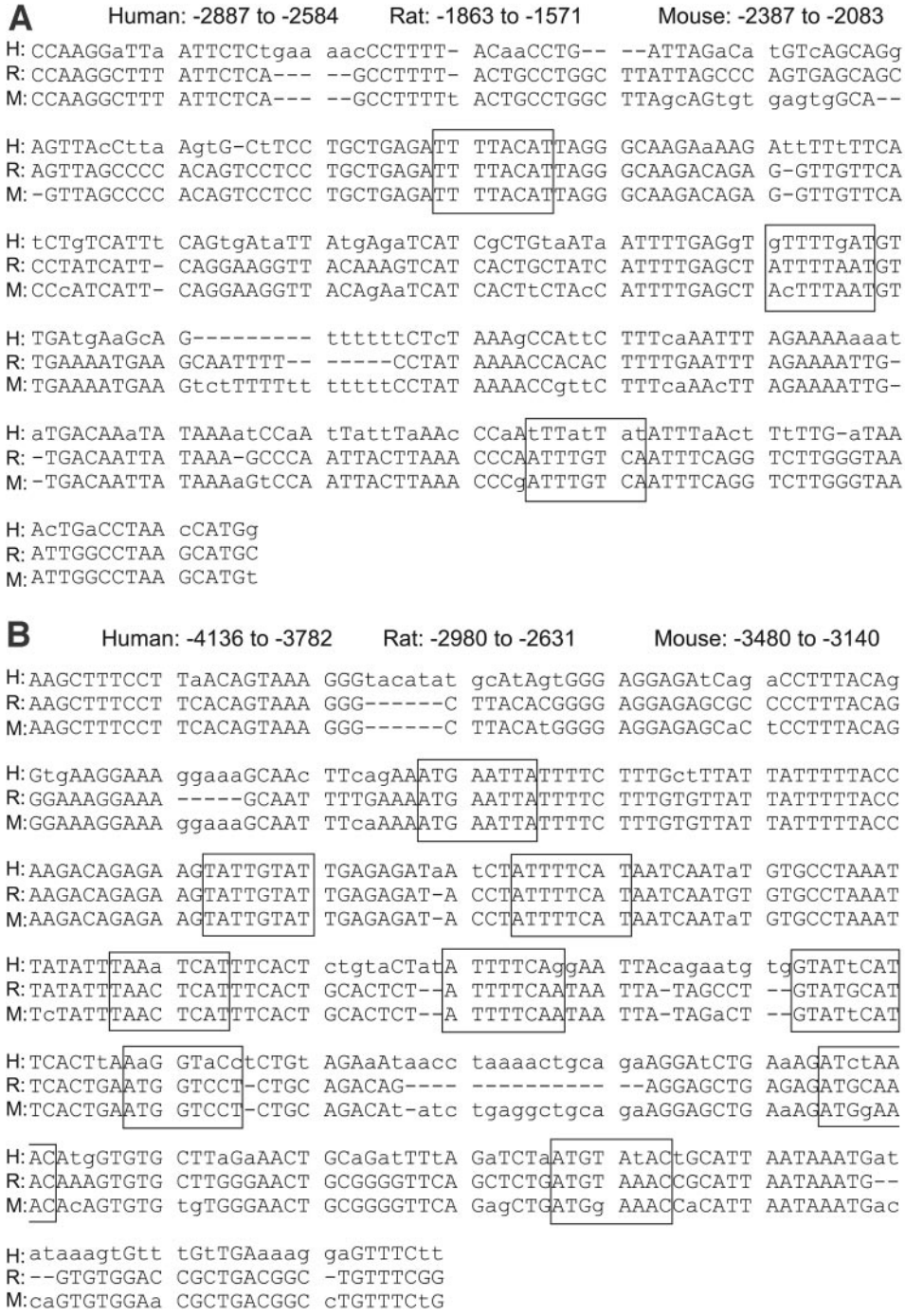


Fig. 2. Alignment of the Conserved Regions Upstream of the GnRH Promoter

The previously characterized approximately 300-bp enhancer (panel A) and the novel upstream conserved region of the rat GnRH gene (panel B) were aligned by pairwise BLAST with the homologous regions from the mouse and human sequences. Previously identified transcription factor binding sites for OCT1 that are conserved across species are *boxed*. The human and mouse sequences were obtained from the UCSC Genome Bioinformatics database. The UCSC mouse sequence is from the C57BL/6J strain, and the human sequence is from the International Human Genome Sequencing Consortium. The rat sequence is from Whyte *et al*. (2) and shows normal allelic variation from the sequence published by Kepa *et al.* (43). Three differences are found in the enhancer: the C shown in our sequence at −1820 is deleted in the published sequence; the A in our sequence at −1689 is a G in the published sequence; and one C is inserted between −1670 and −1669. Four single base pair insertions are found in the upstream region: one G is inserted in the published sequence between −2952 and −2951 in our sequence; one A is inserted between −2950 and −2951; one A is inserted between −2939 and −2938; and one G is inserted between −2925 and −2924 of our sequence.

Although phylogenetic mapping has been a successful approach for identifying novel regulatory elements, recent findings also suggest that the density of transcription factor binding sites within a promoter sequence can reveal potential *cis*-regulatory elements (20–22). To investigate whether the regions that are conserved between rodents and human contain a high density of transcription factor binding sites, we performed a phylogenetic comparison using the GenomatixSuite MatInspector program with a matrix for the transcription factors known to bind to the GnRH promoter (OCT1, SCIP/OCT6, BRN2, GATA4, CEBPβ, OTX2, PBX1, PREP1, and DLX2). Interestingly, clusters of transcription factor binding sites were observed in the regions of homology corresponding to the promoter, enhancer, and conserved upstream region in rat, mouse, and human (for example, see Fig. 2 for multiple OCT1 sites in the homologous regions). This indicates that not only is there evolutionary conservation of these regulatory domains but also that combinations of transcription factor binding sites are found in similar clusters within each of the conserved regions of the GnRH gene. These data suggest that this conserved upstream region may contain regulatory activity important for specific expression of the GnRH gene.

A Novel Conserved Upstream Region Confers Added Specificity to GnRH Gene Expression

Having identified an upstream region of the rat GnRH regulatory sequence that is highly conserved between rat, mouse, and human, and contains multiple potential binding sites for several transcription factors, we explored the possibility that this region might comprise a functional regulatory module. To test this hypothesis, we inserted the 350-bp region (−2980 to −2631) of the rat GnRH gene upstream of the previously defined, conserved enhancer and/or promoter elements in a luciferase reporter plasmid. Transient transfections were performed in the two GnRH neuronal cell lines, the differentiated GT1–7 cell line that expresses high levels of GnRH (23) and the developmentally immature GN11 cell line that expresses very low levels of GnRH (24), as well as control fibroblasts (NIH3T3 cells). Reporter plasmids containing combinations of the three regions of homology were used, namely, the GnRH promoter (P), enhancer (E), and upstream enhancer (UE), to test the functional activity and contribution of the conserved upstream region to cell-specific GnRH gene expression (Fig. 3). As previously shown, addition of the enhancer to the promoter dramatically increases expression in GT1–7 cells (2), but not in GN11 or NIH3T3 cells. This is reflected in the ratios of expression noted at the *right* of the figure (Fold Activity). Although the addition of the upstream enhancer to the previously characterized enhancer and promoter did not significantly increase reporter gene expression in GT1–7 cells, it increased the ratio of cell specificity by causing reduced expression in the GN11 and NIH3T3 cells; increasing the ratio of promoter activity between the GT1–7 cells and the other cell lines increased from 34.8 to 82.2 for GN11 and from 129.7 to 414.6 for NIH3T3. Interestingly, inclusion of the conserved upstream region on the promoter alone resulted in a modest increase in reporter activity (2.1-fold) over the promoter alone in GT1–7 cells. This increase was specific to GT1–7 cells as well, because addition of the conserved upstream region resulted in reduced expression in the GN11 and NIH3T3 cells. The ratio of expression in GT1–7 *vs.* GN11 increased from 2.5 to 6.0 and from 5.8 to 29.5 for the NIH3T3 comparison. The fact that the addition of the conserved upstream region on the promoter alone or the previously characterized enhancer with the GnRH promoter actually repressed reporter activity in NIH3T3 and GN11 cells, resulting in enhanced cell specificity, indicates that its activity may serve to focus and delimit GnRH gene expression.

The Conserved Upstream Region Functions as a Characteristic Enhancer

Common features of an enhancer are its ability to function in forward and reverse orientation, as well as to activate transcription on heterologous elements. Therefore, to further define the activity of the conserved upstream region, we tested this sequence for the characteristics typical of an enhancer (Fig. 4A). The 350-bp conserved upstream region was placed 5′ of a 128-bp heterologous promoter from the Rous sarcoma virus long terminal repeat (RSVp) in a luciferase vector and transiently transfected into the GT1–7 cells in parallel with the previously characterized GnRH enhancer on the RSV promoter and the RSV promoter alone. In this context, the conserved upstream region significantly increases reporter activity over the RSV promoter alone. In fact, the enhancer activity of the upstream region is similar in magnitude to the previously characterized enhancer in the context of the RSV promoter (Fig. 4A). Moreover, the upstream enhancer further augments the activity of the −1863 to −1571 enhancer on the heterologous promoter (Fig. 4B). The conserved upstream region can also function independent of its orientation, when placed upstream of the GnRH enhancer on a heterologous RSV promoter (Fig. 4B). This indicates that the conserved upstream region has the typical characteristics of an enhancer.

The Upstream Enhancer Contains Conserved Binding Sites for OCT1

Upon close examination of the nucleotide sequence comprising the upstream enhancer, several sequence motifs are found that were previously identified in the GnRH enhancer and promoter as binding important transcriptional regulators. They include several potential binding sites for the POU homeodomain transcription factor, OCT1 (Fig. 2, *boxed*). These putative octamer sites are repeated throughout the GnRH

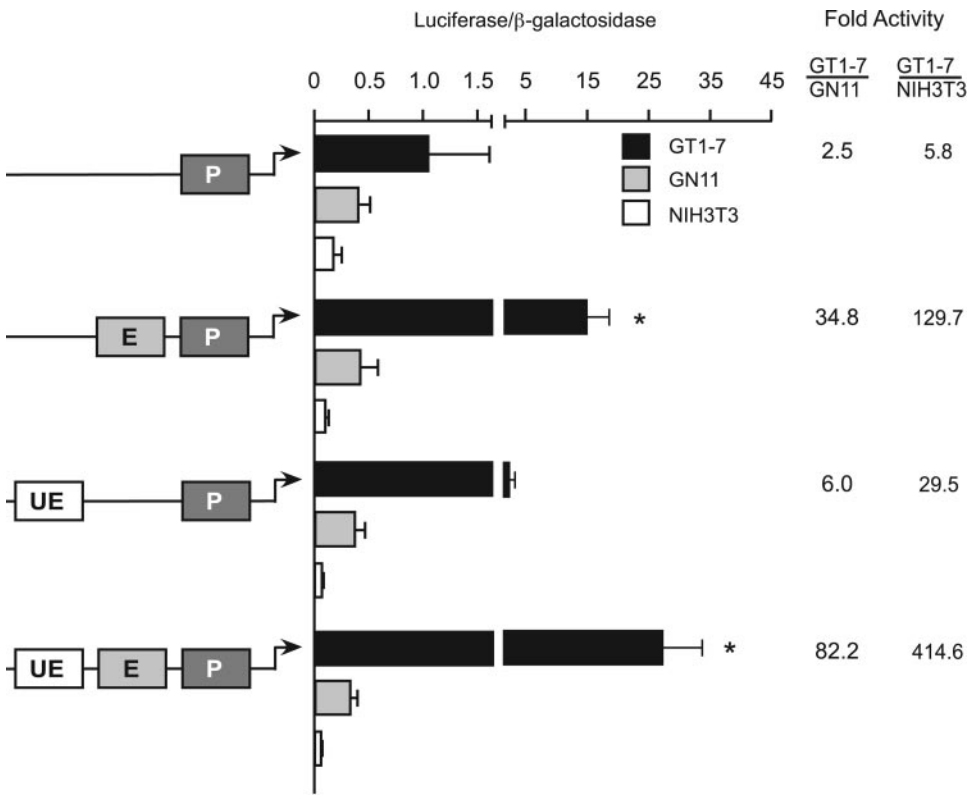


Fig. 3. The Conserved Upstream Region Confers Added Specificity to the Rat GnRH Regulatory Regions

Transient transfections were performed in GT1–7, GN11, and NIH3T3 cells using luciferase reporter plasmids containing the upstream enhancer (UE), enhancer (E), and promoter (P). The RSVe/RSVp-βgal plasmid was cotransfected to control for transfection efficiency. Luciferase values were normalized to β-gal activity. An RSVe/RSVp-luc plasmid was transfected in parallel to control for differences in metabolic rate and transfection efficiency between GT1–7, GN11, and NIH3T3 cell lines. Fold activity represents the relative activity of the GnRH reporter in GT1–7 *vs.* NIH3T3, or GT1–7 *vs.* GN11. The *asterisks* indicate statistical difference compared with the promoter alone in GT1–7 cells by Tukey-Kramer honestly significant difference (HSD) ($P < 0.05$).

upstream enhancer, enhancer, and promoter regions and are evolutionarily conserved. To confirm OCT1 binding to these DNA elements, we performed EMSAs using GT1–7 nuclear extract and radiolabeled probes corresponding to regions of the upstream enhancer. Figure 5 presents an EMSA analysis of one of these OCT1 binding sites (−2844/−2822). This site is a seven out of eight match for the octamer consensus and is conserved in the human and mouse sequence (Fig. 2 and Table 1). Two specific complexes are formed on this probe (labeled 1 and 2). Complex 2 binds specifically, is competed by an OCT consensus binding site (ATGCAAAT), and is supershifted by an anti-OCT1 antibody, indicating that complex 2 contains OCT1 (Fig. 5). Remarkably, OCT1 binds to all nine of the identified sites, as confirmed by antibody supershift and cross-competition of these sites for OCT1 binding (Table 1). These data indicate that the upstream enhancer binds OCT1 at multiple sites, as do the enhancer (three sites) and promoter (two sites). However, the upstream enhancer has a much higher density and number of OCT1 binding sites.

Characterization of a Second, Faster Mobility Complex that Binds Sites in the Upstream Enhancer

A second, faster mobility complex (*lower band*, complex 1 in Fig. 5) formed on all nine of the probes from the upstream enhancer that contained OCT1 binding sites. EMSA complexes with similar mobility to complex 1 had previously been observed in the analysis of OCT1 binding sites within the well-characterized GnRH enhancer and promoter (11, 25). We then assessed the other OCT1 binding sites in the enhancer and promoter to determine whether the faster mobility complex bound to these regions was complex 1 (Fig. 6A). A similar complex, which bound to the enhancer and promoter probes, comigrated and was cross-competed by unlabeled oligonucleotides from the upstream enhancer that bind both OCT1 and complex 1 (−2844/−2822) or an oligonucleotide sequence just 5′ of an OCT1 binding element (site 9) that binds complex 1 alone (−2701/−2678). Thus, in addition to the upstream enhancer region, complex 1 binds to probes

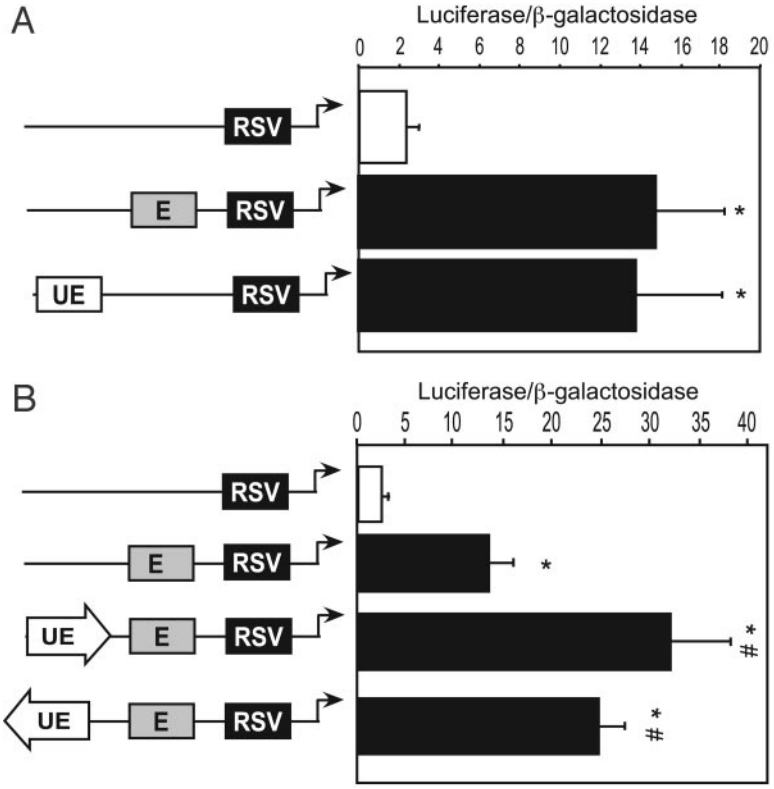


Fig. 4. The Conserved Upstream Region Displays Characteristic Enhancer Activity

A, Transient transfections were performed in GT1–7 cells with the GnRH conserved upstream region and the previously characterized enhancer fused to the RSV promoter (RSVp). B, The conserved upstream region was cloned into a luciferase reporter in forward (*right arrow*) and reverse (*left arrow*) orientation upstream of the previously characterized GnRH enhancer and the RSV promoter and transiently transfected into GT1–7 cells. RSVe/RSVp-βgal was used as an internal control. Values represent normalized reporter activity relative to RSVe/RSVp-luc. *, Statistical difference compared with the RSV promoter alone; # indicates statistical difference compared with the RSV promoter with the GnRH -1863 to -1571 enhancer in GT1–7 cells by Tukey-Kramer HSD (P value < 0.05).

corresponding to all five known OCT1 binding sites in the enhancer and promoter.

In addition to observing complex 1 binding to probes containing OCT1, we also noticed that when increased amounts of nuclear protein were added to the probes in EMSA, several slower mobility complexes were formed (Fig. 6A, band 3; and Fig. 6B, bands 3 and 4). Interestingly, the anti-OCT1 antibody supershifted these complexes in addition to shifting complex 2 (Fig. 6B, αOCT1). This suggests that OCT1 can form multiprotein complexes that bind to the regulatory regions of the GnRH gene.

Complex 1 Binding Requires Sequences that Flank the OCT1 Site

Having identified complex 1 binding to several regulatory regions of the GnRH gene, we sought to define the base pairs required for its binding. Close inspection of the DNA sequence within the upstream enhancer and enhancer revealed similarities in the sequence within close proximity to the multiple OCT1 binding sites. However, to determine the binding site requirements for complex 1, EMSA was performed with labeled probes containing scanning mutations throughout the sequence of two elements containing binding sites from the conserved upstream region or the enhancer. An increased concentration of protein extract was used to examine the sequences necessary for the binding of slower mobility complexes containing OCT1. In the case of the site analyzed in the upstream enhancer ($-2844/-2822$; site 3 in Table 1), the OCT1 complex showed reduced binding with mutations 3, 4, 6, and 7, whereas complex 1 had lower affinity for oligonucleotides containing mutations 1, 2, 3, 5, 7, 8, 9, and 11 (Fig. 7A). One of the enhancer sites ($-1796/-1762$; site 10 in Table 1) showed a similar pattern with an effect on OCT1 binding to mutations 8 through 11 and reduced binding of complex 1 with mutations 4, 5, 6, 8, 11, 12, and 13 (Fig. 7B). This analysis indicates that complex 1 binds to sequences that flank the OCT1 binding sites, utilizing contacts on both sides of the octamer sequence.

Formation of the slower mobility complexes on both sites was greatly reduced on the mutant probes that had low affinity OCT1 binding and partially diminished

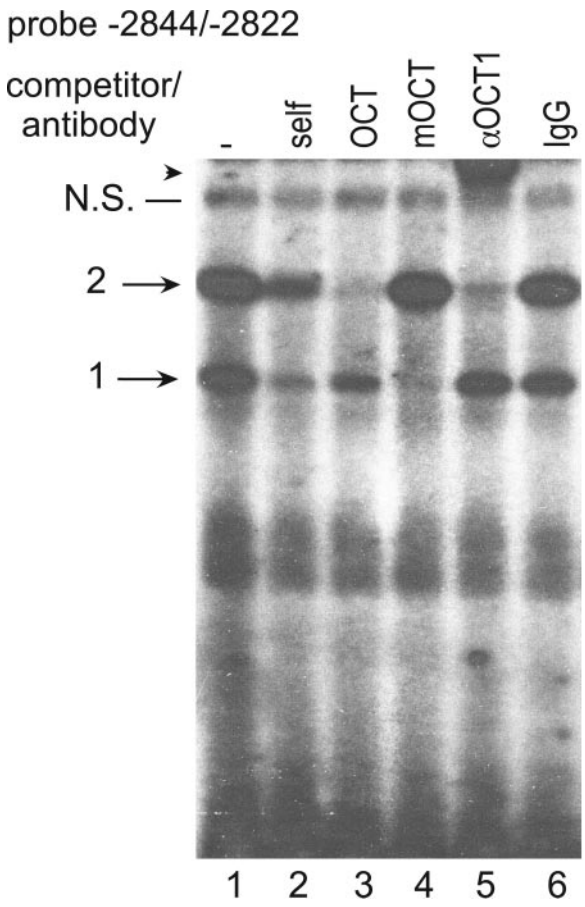


Fig. 5. OCT1 Binds to the Upstream Enhancer

EMSA with 2 μg of GT1–7 nuclear extract indicates binding of two complexes (labeled 1 and 2) to a radiolabeled probe (−2844/−2822) representing a region of the upstream enhancer that contains one of the nine OCT1 consensus binding sites (Table 1). Lane 1 represents the protein complex formation, whereas self-competition (lane 2), OCT1 consensus competition (OCT; lane 3), OCT1 mutant competition (mOCT; lane 4), anti-OCT1 antibody (αOCT1; lane 5), and an IgG control (lane 6) are shown in the subsequent lanes. The *arrowhead* marks the antibody supershifted complex. N.S., Nonspecific binding.

on the mutant probes that affected complex 1 binding (Fig. 7, A and B, bands 3 and 4). It is intriguing to note that mutant 7 in site 3 (Table 1) from the upstream enhancer significantly reduced OCT1 binding even though the sequence mutated in this oligonucleotide probe is outside of the 8-bp consensus for OCT1 binding. This mutation significantly reduced complex 1 binding and the slower mobility complex formation as well. Similarly, mutant 11 in the enhancer site (site 10, Table 1), residing only 1 bp within the consensus octamer sequence, significantly diminished OCT1 binding and affected complex 1 and the slower mobility complex formation on the DNA. Thus, sequences outside of the canonical octamer consensus site appear to be important for OCT1 binding as well as the slower mobility complex formation. This indicates that the slower mobility complexes require the same base pairs for binding as the OCT1 complex, consistent with the observation that they are shifted by an anti-OCT1 antibody, and are influenced by the binding affinity of complex 1.

Complex 1 Bound to the Upstream Enhancer, Enhancer, and Promoter of the GnRH Gene Contains Nuclear Factor 1

Interestingly, an unlabeled OCT1 mutant probe competed significantly for the binding of complex 1 whereas the consensus OCT1 probe competed minimally (Fig. 5, lane 4). The difference between the consensus and mutant probe is a mutation in the last 2 bp of the OCT1 binding site from CGAATGCAAATCAC to CGAATGCAAGCCAC. This change in the context of the 22-bp probe created a high-affinity binding site (AGCCA) for NF1 (26). NF1 has been shown to bind to a palindrome sequence, TTGGC(N_5)GCCAA (27). However, NF1 can also bind to variations of this sequence and half-sites of the palindrome (26, 28, 29). Figure 7 also indicated that the binding site for complex 1 flanks that of OCT1. Similarly, NF1 has been shown to flank the binding site of OCT1 in other contexts such as the human papillomavirus type 16 (HPV-16) promoter (28). We therefore pursued the possibility that this faster mobility complex might contain NF1.

Using a radiolabeled probe corresponding to a region of the upstream enhancer that binds both OCT1 and the putative NF1 complex (site 3, Table 1), we performed EMSA analysis with GT1–7 nuclear extract and antibodies recognizing OCT1 and NF1 (Fig. 8A). Because NF1 is capable of interacting with OCT1 and binding to DNA as a part of a multiprotein complex (28, 30), we used excess protein extract in this assay to determine whether the slower mobility complexes formed on this site contain OCT1 and NF1. Using these conditions, four complexes (labeled 1–4) formed on this site. Complex 1 was self-competed and shifted by an antibody recognizing NF1 (Fig. 8A, lanes 2 and 4). Complex 2 is OCT1 (Fig. 5 and Fig. 8A, lane 3), and complexes 3 and 4 were self-competed and completely shifted by an anti-OCT1 antibody (Fig. 6B). However, the antibody recognizing NF1 did not markedly shift the slower mobility complexes (Fig. 8A, lane 4). None of these complexes was affected by a nonspecific antibody control (Fig. 8A, lane 5). This indicates that NF1 can bind to the GnRH upstream element alone and is the identity of complex 1.

To examine whether similar complexes formed on the enhancer and promoter were in fact NF1, we performed EMSA with a radiolabeled oligonucleotide probe (−1767 to −1789) corresponding to the OCT1 binding site in the previously characterized enhancer, termed AT-a, shown to be essential for maximal GnRH expression (11) (Fig. 6 and Table 1). Three complexes formed on this probe when increased nuclear extract was added. The faster mobility complex 1 was self-competed (lane 2) and shifted by an antibody recog-

Table 1. OCT1/NF1 Binding Sites in the rat GnRH-Regulatory Regions

Site No.	Region	Octamer Site	Octamer Sequence ATGCAAAT	Consensus Match (8/8)			Probe	Competed by	Probe Binds OCT1	Probe Binds NF1
				R	M	H				
1	Upstream	−2905/−2898	ATGAATTA	4	4	4	−2918/−2896	OCT. 1–9	+	+
2	Upstream	−2853/−2860	ATACAATA	5	5	5	−2862/−2839	OCT. 1–9	+	+
3	Upstream	−2833/−2840	ATGAAAAT	7	7	7	−2844/−2822	OCT. 1–9, 10, 11, 13, 14	+	+
4	Upstream	−2800/−2807	ATGAGTTA	3	3	3	−2819/−2797	OCT. 1–9, 11	+	+
5	Upstream	−2779/−2786	TTGAAAAT	6	6	6	−2795/−2774	OCT. 1–9	+	+
6	Upstream	−2759/−2766	ATGCATAC	6	6	5	−2780/−2760	OCT. 1–9	+	+
7	Upstream	−2751/−2744	ATGGTCCT	4	4	3	−2764/−2739	OCT. 1–9	+	+
8	Upstream	−2721/−2714	ATGCAAAC	7	6	5	−2725/−2700	OCT. 1–9	+	+
9	Upstream	−2679/−2672	ATGTAAAC	6	6	5	−2680/−2657	OCT. 1–9	+	+
10	Enhancer (AT-a)	−1774/−1781	ATGTAAAA	6	6	6	−1789/−1762	OCT. 3, 11, 13, 14	+	+
11	Enhancer (AT-b)	−1694/−1701	ATTAAAAT	6	5	5	−1704/−1689	OCT. 3, 4, 10, 13, 14	+	+
12	Enhancer	−1600/−1607	TGACAAAT	5	5	3	−1612/−1593	3, 4, 8, 14	+	+
13	Promoter (FP4)	−99/−106	ATTAAAAT	6	4	6	−109/−89	OCT. 3, 10, 11, 14	+	+
14	Promoter (FP2)	−47/−54	ATGTAATT	6	6	6	−63/−33	OCT. 3, 10, 11, 14	+	+

EMSA analysis was performed using radiolabeled oligonucleotide probes for the regions corresponding to the OCT1/NF1 binding sites in the rat GnRH-regulatory regions. One hundred-fold molar excess of unlabeled oligonucleotide sequences were used for cross-competition (sites 1–14). OCT represents an octamer consensus oligonucleotide. Antibodies recognizing OCT1 and NF1 were used for antibody supershifts. R, Rat; M, mouse; H, human.

nizing NF1 (Fig. 8B, lane 3). Complex 2 was supershifted by an anti-OCT1 antibody (Fig. 8B, lane 4). The slower mobility complex (complex 3) was also self-competed (Fig. 8B, lane 2), and shifted by an anti-OCT1 antibody (Fig. 8B, lane 4), although not markedly affected by an antibody recognizing NF1 (Fig. 8B, lane 3). Similar results were observed using probes from the promoter region (data not shown). Therefore, OCT1 and NF1 both bind to octamer-containing elements from the GnRH enhancer and promoter.

NF1 Family Members Expressed in GT1–7 Cells

The NF1 family comprises four genes, *NF1A*, *B*, *C*, and *X* (29, 31). However, several splice variants exist for each family member (32). Having discovered this transcription factor binding to the upstream enhancer, enhancer, and promoter, we examined the expression pattern of *NF1A*, *B*, *C* , and *X* by RT-PCR in the model GnRH cell lines: GT1–7, the more mature GnRH-producing cell line and GN11 and NLT, two immature representatives of the GnRH neuron derived from the same tumor (24). We also examined *NF1* expression in the pituitary gonadotrope cell line, LβT2, as well as mouse fibroblasts (NIH3T3). Figure 9A shows that *NF1* family members *A*, *B*, *C*, and *X* are expressed in the GT1–7, GN11, and NLT cells, whereas NIH3T3 cells express all of the *NF1* family members and LβT2 cells do not express *NF1A* and *B*. Therefore, both of the GnRH model cell lines express mRNAs from all four genes of the *NF1* family.

NF1 Family Members Interact with OCT1

The results presented thus far indicate that OCT1 binds as a monomer and is also a component in multiprotein complexes that bind to several elements within the regulatory regions of the GnRH gene (Figs. 5 and 6). Further, NF1 binds to these same DNA elements and requires the sequence that flanks OCT1 for binding. Due to the proximity of NF1 and OCT1 binding sites in the context of the GnRH gene and their reported functional interaction in the context of viral promoters (28, 33), we hypothesized that OCT1 and NF1 might physically interact. To test this hypothesis, we performed glutathione-S-transferase (GST) pull down assays using *in vitro* translated, ^{35}S-labeled variants of the NF1 family members and GST-tagged OCT1. Figure 9B demonstrates the interaction between OCT1 and NF1. Green fluorescent protein was used as a negative control and OCT1 as a positive control (34) for interaction with GST-OCT1. GST alone did not interact with any of the *in vitro* translated input. Thus, OCT1 interacts with NF1 family members *in vitro*. Moreover, this interaction does not require a physical association with the binding sites for OCT1 or NF1.

Dominant-Negative NF1 Affects OCT1-Dependent Activation

A recent study found that NF1 physically and functionally associates with the homeodomain protein, TTF1 (35). That study used a dominant-negative, chimeric protein (NF1A/EN) containing the N-terminal region of the murine NF1A protein capable of protein dimerization and DNA binding fused to the N-terminal region of the *Drosophila* Engrailed. Thus, dominant-negative NF1 can form nonfunctional dimeric complexes through interaction with the N-terminal region of the chimeric protein. We used this dominant-negative hybrid protein to assess the contribution of NF1 and to

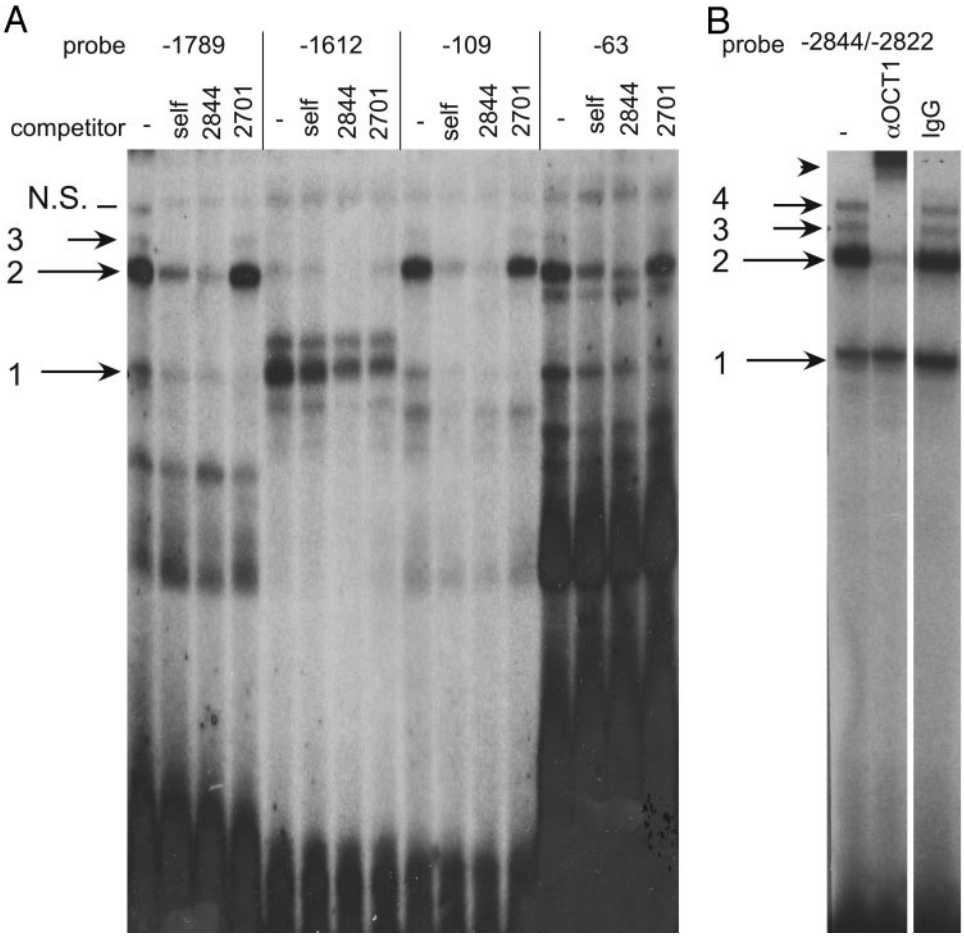


Fig. 6. Complex 1 Binds to the Enhancer and Promoter of the Rat GnRH Gene

A, Radiolabeled probes for two OCT1 sites in the GnRH enhancer [−1789/−1762 (AT-a) site 10 in Table 1; and −1612/−1593 site 12 in Table 1] (11) and two sites in the promoter [−109/−89 (FP-4, site 13 in Table 1) and −63/−33 (FP-2, site 14 in Table 1)] (18, 25) were incubated with 10 μg of GT1–7 nuclear extract in EMSA. Competition was performed with 100× of unlabeled oligonucleotides representing regions of the upstream enhancer that bind OCT1 and complex 1 (−2844/−2822) or complex 1 alone (−2701/−2678). The *arrows* indicate complex 2 and complex 1, whereas the *short arrow* indicates the slower mobility complex 3. N.S., Nonspecific binding. B, The slower mobility complexes bound to the GnRH probes contain OCT1. A radiolabeled probe corresponding to −2844/−2822 in the upstream enhancer was incubated with 10 μg of GT1–7 nuclear extract in EMSA. Protein binding is indicated in the first lane with *arrows* marking four complexes. The supershift with an antibody recognizing OCT1 (αOCT1) and nonspecific antibody control (IgG) are represented in subsequent lanes. The *arrowhead* marks the supershifted complex.

explore the effect on transactivation potential through interaction with the homeodomain protein OCT1 in transient transfection of GT1–7 cells. For this purpose, we used a reporter vector containing a multimer of the enhancer OCT1/NF1 binding site (−1805 to −1766, AT-a) driving luciferase expression. This element was chosen because it has been shown to be essential for GnRH expression (11) and is responsive to OCT1-mediated activation whereas the entire GnRH regulatory sequence is not (17). Interestingly, addition of increasing amounts of dominant-negative NF1 repressed reporter activity (Fig. 9C), indicating that NF1 contributes to GnRH promoter activity. Overexpression of OCT1 resulted in an increase in reporter activity, but this increase was diminished by dominant-negative NF1. These results suggest that a functional interaction occurs between OCT1 and NF1.

Mutations Disrupting the NF1/OCT1 Binding Sites Decrease Promoter Activity

To address the functional relevance of mutations that affect the DNA binding of OCT1 and NF1 complexes seen in Fig. 7, namely mutant 7 in the −2844/−2822 element of the upstream enhancer and mutant 11 in the −1796/−1762 element of the enhancer, these same mutations were introduced into the reporter plasmids containing the upstream enhancer, enhancer, and the short RSV promoter. These mutant plasmids were then transiently transfected into GT1–7

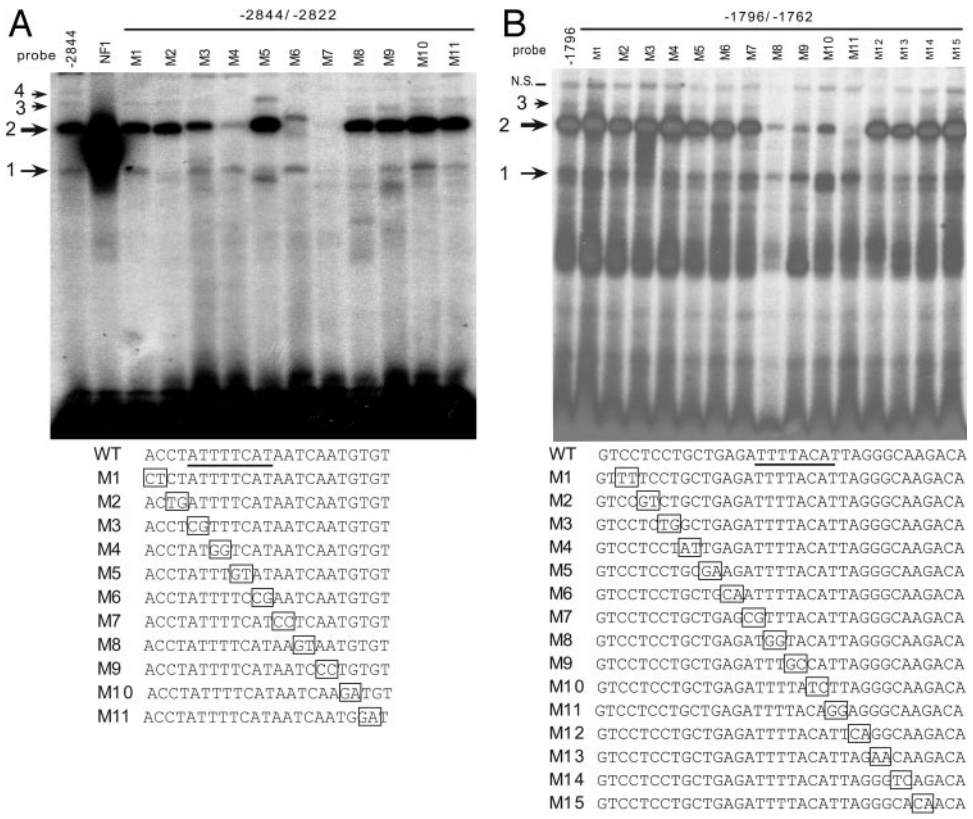


Fig. 7. Analysis of the Sequence Requirements for Complex 1 and OCT1 Binding

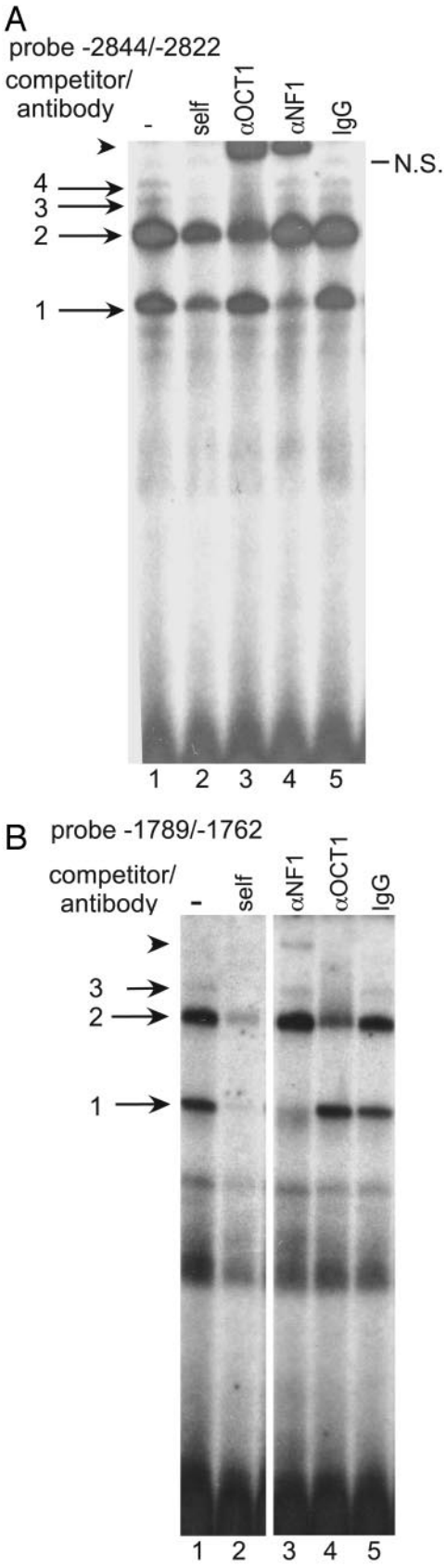
A, Scanning mutations (2 bp) (*boxed*) were created in the oligonucleotide probe corresponding to one of the nine conserved OCT1 binding sites in the upstream enhancer (−2844/−2822; site 3 in Table 1). Radiolabeled wild-type and mutated probes were incubated with 10 μg of GT1–7 nuclear extract for EMSA analysis. The NF1 oligonucleotide represents a putative consensus site for NF1 (*Materials and Methods*). B, Scanning mutations (*boxed*) were created in the oligonucleotide sequence representing one of the three OCT1 binding elements in the enhancer region (−1796/−1762; site 10 in Table 1). These wild-type and mutant probes were radiolabeled for use in EMSA with 10 μg of GT1–7 nuclear extract. The *thick arrow* indicates the complex containing OCT1 (complex 2), and the *thin arrow* indicates complex 1. Slower mobility complexes are indicated with *short arrows* (complexes 3 and 4). N.S., Nonspecific binding.

cells (Fig. 10A). Interestingly, the mutation of this single OCT1/NF1 element in the upstream enhancer (M7) significantly reduced reporter activity (23% of wild type) compared with the wild-type plasmid, whereas mutation of this single OCT1/NF1 element in the enhancer (M11) resulted in an even greater reduction in reporter activity (4% of wild type) (Fig. 10A). A combination of mutant 7 in the upstream enhancer and mutant 11 in the enhancer resulted in very low activity, although still well above background (3% of wild-type activity), despite the presence of 10 other NF1/OCT1 elements that were not mutated. These results suggest that the OCT1/NF1 complexes that form on the GnRH regulatory elements contribute substantially to promoter activity and that mutation of individual sites is sufficient to dramatically reduce expression (Fig. 10A).

We then asked what effect these mutations in the enhancer and upstream enhancer OCT1/NF1 elements might have in the context of the GnRH promoter. To address the role of OCT1/NF1 elements in the promoter, mutations were introduced in site 14 (Table 1) that have been previously shown to affect complexes now known to contain OCT1 and NF1 (M2Q3) (25). Like the mutations in the enhancer and upstream enhancer OCT1/NF1 sites, the mutation in the promoter is outside of the canonical octamer binding sequence but affects OCT1 binding. In the context of the reporter containing all three regulatory regions, only the enhancer element mutation (M11) caused a statistically significant reduction in expression, although the promoter element mutation (M2Q3) trended substantially lower (Fig. 10B). Each of the double combination mutations resulted in significantly reduced expression, and the triple mutation reduced expression dramatically to 4% of wild type. Therefore, the OCT1/NF1 binding elements within the regulatory region of the GnRH gene are essential for appropriate promoter activity.

DISCUSSION

Appropriate expression of GnRH is essential for the function of the reproductive system. Although there



are significant differences in the upstream sequences of the GnRH gene in mouse, rat, and human, the spatio/temporal expression of this gene across species is very similar (36). Previously, the promoter sequence characterized in the rat gene was found to be conserved in the mouse and human GnRH promoters (6, 8, 9). Moreover, transgenes containing upstream regulatory sequence from the human or rat GnRH genes driving reporter expression can adequately target a large population of the GnRH neurons in transgenic mice (3–5, 7, 10). These findings provide evidence that not only is there homology in the DNA-regulatory regions of the GnRH gene, but that there is also a conservation of the protein transcription factors that promote restricted expression of the GnRH gene.

Although extensive analysis of the GnRH promoter regions in the GT1–7 model cell line and transgenic mice have contributed to a better understanding of the regulation of this gene, there is still much to be learned. Cross-species comparison, using phylogenetic footprinting, is a valuable approach for identifying putative regulatory regions upstream of the GnRH gene. Having been maintained throughout evolution, it is likely that these conserved elements play an important role in gene regulation. Furthermore, the preservation of clustered transcription factor binding sites suggests that these evolutionarily conserved motifs likely encode *cis*-regulatory regions relevant for understanding the regulation of the gene.

Examination of the mouse GnRH sequence using progressive deletion of the 5′-promoter region in transgenic mice revealed two enhancer regions important for appropriate targeting and transgene reporter expression, between −5.2 kb and −2.1 kb as well as −2.1 and −1.7 (4). Alignment with the novel upstream enhancer and the previously characterized enhancer in the rat sequence indicate that the homologous upstream enhancer and enhancer lie within those key regions: −3.4 kb to −3.0 kb and −2.4 to −2.0 kb, respectively, in the mouse gene. Interestingly, the

Fig. 8. Complex 1 Contains NF1

A, The radiolabeled probe represents the −2844/−2822 sequence and was incubated with 10 μg of GT1–7 nuclear extract rather than 2 μg. Four major complexes are formed, represented by *arrows* (lanes 1–4). Lane 1 shows the complex formation. Self-competition is seen in lane 2; antibodies recognizing OCT1 (αOCT1) and NF1 (αNF1) are shown in lanes 3 and 4, respectively; and an IgG control is seen in lane 5. The *arrowhead* marks the supershifted complexes. N.S., Nonspecific binding. B, EMSA analysis was performed on a radiolabeled probe corresponding to one of the three OCT1 binding sites in the GnRH enhancer (−1789/−1762; AT-a; site 10 in Table 1) (11) with 10 μg of GT1–7 nuclear extract. Lane 2 represents self-competition. An antibody supershift was performed with antibodies recognizing NF1 (lane 3), and OCT1 (lane 4) as well as an IgG control (lane 5). The *arrowhead* indicates the supershifted complex.

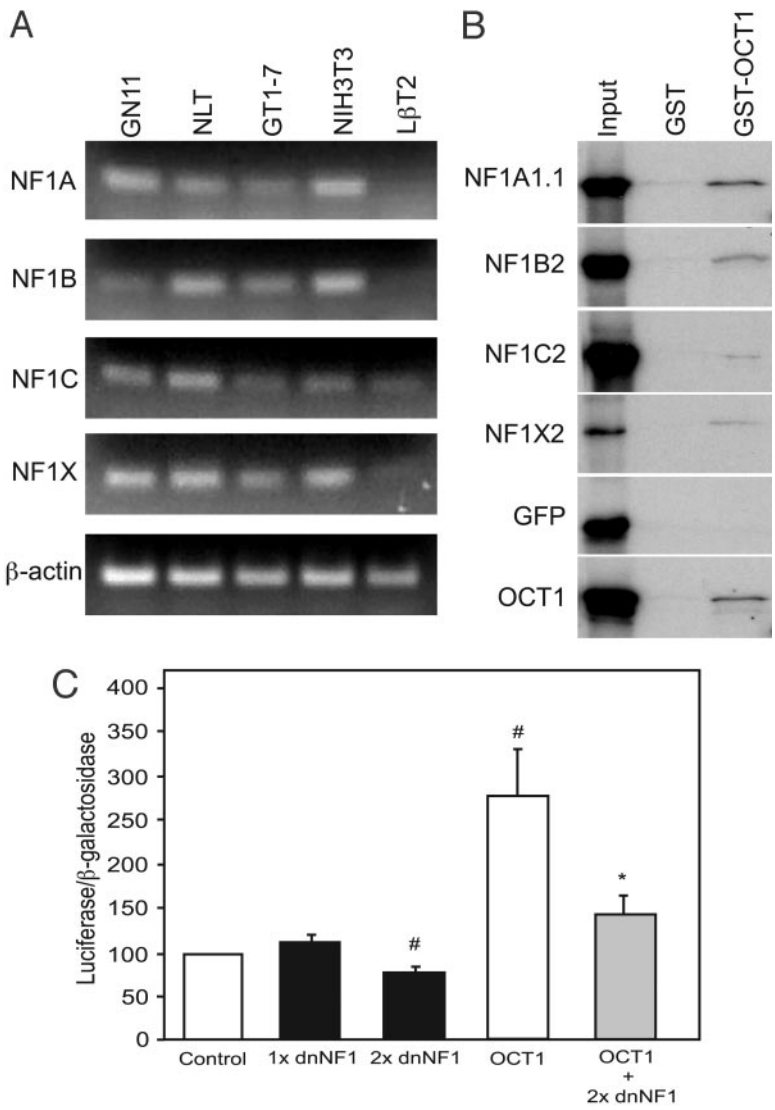


Fig. 9. NF1 is Expressed in GT1–7 Cells and Interacts Physically and Functionally with OCT1

A, NF1 family members are expressed in GnRH-expressing cell lines. RT-PCR was performed using RNA from GN11, NLT, GT1–7, NIH3T3, and LβT2 cells and run on an agarose gel. Primers for each *NF1* family member correspond to the unspliced, N-terminal region of the cDNA. β-Actin was used to control for equal loading. B and C, OCT1 and NF1 physically and functionally interact. B, GST-pull down assays were performed using GST-OCT1 and *in vitro* translated, radiolabeled variants of the NF1 family. The input lane represents 10% of the protein added to the pull-down assay. The GST lane represents nonspecific interactions, and the GST-OCT1 lane denotes specific complex formation with input proteins. Green fluorescent protein (GFP) serves as a negative control and OCT1 as a positive control for GST-OCT1 interactions. C, Transient transfections were performed in GT1–7 cells using a multimer of the enhancer AT-a site (-1805 to -1766) fused to the RSV promoter driving luciferase expression. Dominant-negative NF1 expression vector (100 ng) (NF1A/EN) is represented as 1×. Luciferase values were normalized to the RSVe/RSVp-β-galactosidase internal control and compared with the normalized values of the expression vector control, set at 100% activity. The numbers plotted represent percent activity relative to the empty vector controls. #, Statistical difference from the control whereas the *asterisk* represents statistical difference between OCT1 and OCT1 plus the dominant-negative NF1 by Tukey-Kramer HSD ($P < 0.05$).

GnRH neuronal population targeted using the -2.1-kb promoter sequence driving transgene expression was reduced by 40% (4), suggesting that the mouse region homologous to the rat upstream enhancer and the 5′-portion of the enhancer play a role in neuron-specific expression of the mouse gene *in vivo*. In support of this concept, a separate study using approximately -3.5 kb of the mouse promoter observed expression of their transgenic marker in 84–94% of GnRH-positive cells *in vivo* (7). Furthermore, a study in which transcriptional activity of the mouse GnRH regulatory regions was assessed in hypothalamic tissue homogenates, a method preventing determination of the efficiency and accuracy of targeting, but allowing quan-

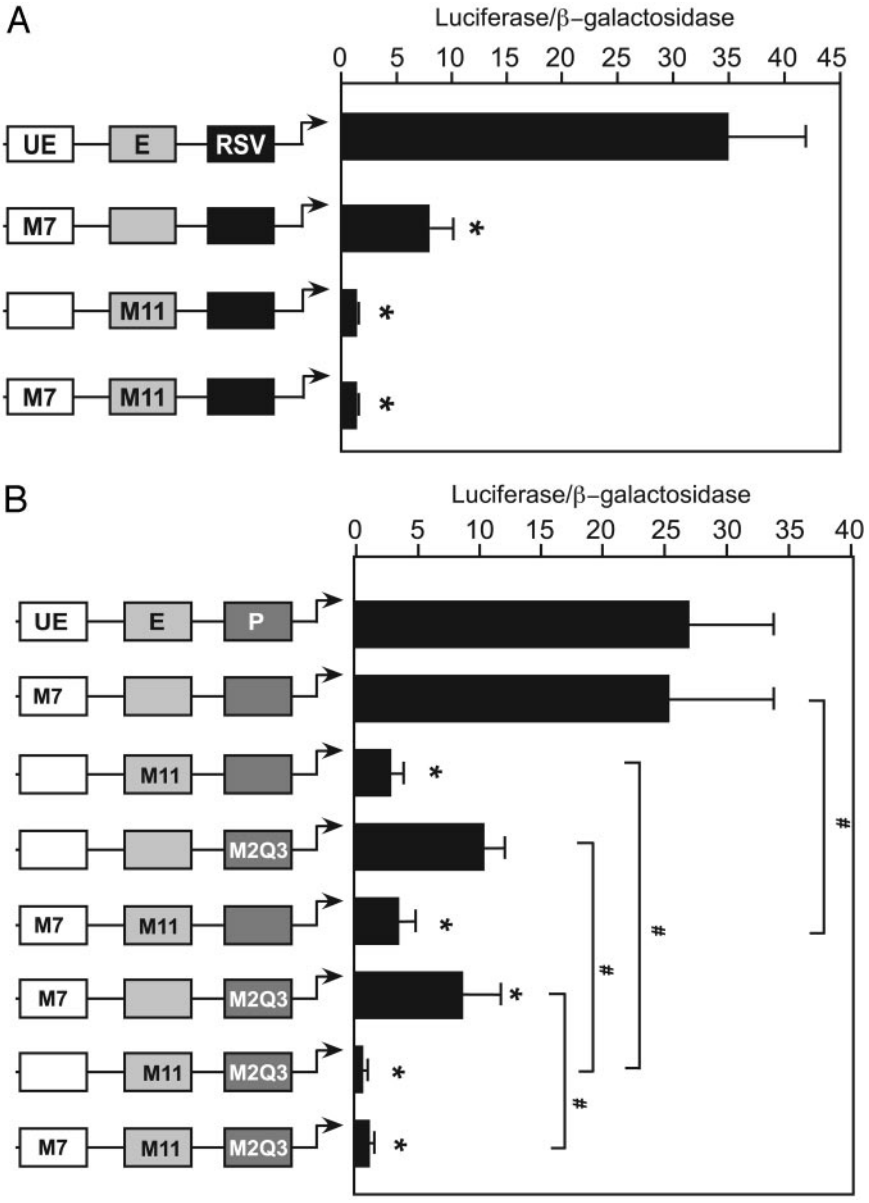


Fig. 10. The NF1/OCT1 Binding Sites Are Important for GnRH Gene Activation

A, Transient transfections were performed in GT1–7 cells using wild-type or mutant reporters containing the upstream enhancer (UE) and the enhancer (E) on the RSV promoter. Mutations are M7 (Fig. 7A) in one of the OCT1/NF1 elements in the upstream enhancer (site 3 in Table 1), and M11 (Fig. 7B) in one of the OCT1/NF1 elements in the enhancer (site 10 in Table 1). B, The wild-type or mutant GnRH upstream enhancer/enhancer/promoter reporter plasmids were transiently transfected into GT1–7 cells. The M2Q3 mutation (25) is in one of two OCT1/NF1 elements in the promoter (site 14 in Table 1). *, Values that statistically differ from the wild type; #, statistical difference between the *bracketed bars* by Tukey-Kramer HSD ($P < 0.05$).

tification, showed that a transgene with −3446 bp upstream was expressed at higher levels than −2078 or −1005 and that deletion to −1005 allowed substantial misexpression in the ovary (9). These studies indicate that the upstream enhancer and enhancer sequences are within the essential regulatory regions in the murine GnRH promoter. Moreover, these data support our findings with the rat GnRH gene, utilizing transfections into a cultured cell model of the hypothalamic neurons, and suggest that this conserved upstream region functions as an enhancer *in vivo* as well by facilitating restricted expression of the GnRH gene.

It is particularly interesting to note that transient transfection of the less mature model for the GnRH neuron (GN11 cells) did not yield a response similar to transfection of the GT1–7 cells (Fig. 3). Actually, GN11 cells expressed the GnRH regulatory sequences at levels as low as the NIH3T3 cell line. If, in fact, the GN11 cells represent an immature, migratory GnRH

neuron that has reduced GnRH transcriptional activity, our transfection results are consistent with this interpretation. Alternatively, it is possible that this developmentally immature cell line might use distinct regulatory regions, outside of those we have analyzed herein.

A more detailed analysis of the rat upstream enhancer revealed several binding sites for the transcription factor OCT1, also previously observed in the more proximal regulatory regions. As with the enhancer and promoter, these binding sites are conserved in the human and mouse GnRH sequences. OCT1, in particular, has been characterized as an important transcription factor that participates in basal and hormone-induced transcription of the GnRH gene (11, 13, 15, 37). For example, mutation of the OCT1 binding site located in the rat GnRH enhancer (−1774/−1781) results in 95% reduction of basal GnRH reporter activity in GT1–7 cells (11). Although OCT1 appears to play an important role in GnRH promoter expression, OCT1 is not restricted to the GnRH neuron. Thus, OCT1 may be a part of multiprotein complexes that can confer specificity. In this study, we have identified a protein partner for OCT1 in the GnRH neuronal cells, NF1, that appears to play a cooperative role in the binding and transcriptional activation by OCT1.

Our findings indicate that OCT1 and NF1 physically and functionally interact and bind to adjacent sites in the upstream enhancer, enhancer, and the promoter of the GnRH gene (Figs. 7, 8, and 9). Furthermore, slower mobility complexes containing OCT1 require sequence that flanks the OCT1 binding site and corresponds to the NF1 binding sequence. A functional interaction between NF1 and OCT1 has been described in the context of several upstream promoter regions such as the viral promoters, mouse mammary tumor virus (MMTV) and HPV type 16 (28, 33). In these studies, NF1 binding sites are located in close proximity to OCT1 consensus sites, as we have reported here. In the context of the MMTV promoter, mutation of the NF1 binding site not only diminishes NF1 binding but also the binding stability and transcriptional activation by OCT1 (33). Again, we have found similar results in the context of the GnRH promoter in which OCT1 binding and slower mobility complex formation are notably decreased when NF1 binding is hindered (Fig. 7A, mutant 7, and Fig. 7B, mutant 11), resulting in a considerable reduction of transcriptional activity (Fig. 10). Moreover, the proximity of the NF1 and OCT1 binding sites on the HPV-16 element was shown, by EMSA, to be a determining factor in higher order complex formation. The addition of only 2 bp separating the consensus binding sites is sufficient to disrupt the OCT1/NF1 complex (28). We observed a similar phenomenon with the disruption of complexes 3 and 4 binding to the upstream enhancer (site 3 in Table 1) of the GnRH gene when the OCT1 and NF1 binding sites were separated in EMSA analyses (data not shown). This suggests that these two proteins can interact with each other while bound to the DNA. Therefore, it appears that NF1 is important for stabilization of OCT1 on the DNA, and for formation of multiprotein complexes containing OCT1, as well as for the resultant transcriptional activity. Intriguingly, we were unable to observe a consistent, notable change in complex formation of the slower mobility complexes in the presence of the antibody recognizing NF1. One potential explanation is that these slower mobility complexes may contain OCT1 and a variant of the NF1 family not recognized by the antibody. This alternative is supported by the fact that the slower mobility complexes are shifted by an anti-OCT1 antibody, require the octamer site, and are influenced by the base pairs used by NF1. Moreover, we observed that the antibody against NF1 did recognize NF1C2 but not NF1A1.1, NF1B2, or NF1X2 by Western blot analyses with extracts overexpressing these variants (data not shown). Alternatively, the epitope recognized by the NF1 antibody may be masked due to interaction with other proteins in these complexes. Thus, we cannot rule out the possibility that the slower mobility complexes may contain NF1; however, these complexes clearly require OCT1.

Although disrupting the stability of the OCT1/NF1 complexes in the upstream enhancer, the enhancer, and the promoter consistently reduced reporter activity, not all of the OCT1/NF1 binding sites appear to contribute equally to GnRH gene expression. Remarkably, mutation of a single enhancer or proximal promoter OCT1/NF1 site resulted in a substantial decrease in reporter activity despite the presence of so many other sites for the same transcription factors (Fig. 10). However, a mutation made in a single OCT1/NF1 site in the upstream enhancer had a more moderate effect. This may be due to the multiplicity of closely clustered sites in the upstream enhancer. Alternatively, it is possible that the functional differences between the many OCT1/NF1 sites are due to variations in the flanking sequences that recruit specific cofactors to promote a transcriptional response, or due to their positions relative to additional binding sites for transcription factors. It is also plausible that some of the OCT1/NF1 sites, perhaps less important for basal activity, may actually mediate a response from extracellular stimuli such as phorbol ester, steroid hormones, or nitric oxide. Indeed, the −1694/−1701 OCT1 site is important for *N*-methyl-D-aspartate and nitric oxide responsiveness of the rat GnRH gene (15). In addition, OCT1 has been shown to interact with the glucocorticoid receptor on the GnRH promoter (37) and both NF1 and OCT1 have been shown to participate in the transcriptional response to glucocorticoids in the context of the MMTV promoter (33).

Despite the fact that all of the NF1 family members are expressed in the model cell lines for the GnRH neuron (Fig. 9A), several splice variants of each member of the NF1 family are known to exist (32). Thus, it is possible that a novel NF1 splice variant may be present in the GnRH neurons that might contribute to specificity. Further analysis will be necessary to fully

define the role of NF1 in regulating GnRH gene expression.

In summary, we have identified an upstream enhancer of the GnRH gene that is conserved from rodents to human. This conserved upstream region not only enhances promoter activity, but also does so specifically in mature GnRH neurons. Within this region, we have identified several binding sites for the transcription factor OCT1 that are also conserved and important for regulation of the GnRH gene. In addition, we describe a role for NF1 in regulation of this gene as it facilitates OCT1 activity. Future analysis will be directed toward defining the role of this upstream enhancer in targeting to the GnRH neuronal population *in vivo* as well as further exploration of the role of NF1.

MATERIALS AND METHODS

Sequence Alignment

The 5′-flanking regions of the human, rat, and mouse GnRH genes were aligned by a pairwise BLAST using the following parameters: blastn matrix, 1, −2; extension, 2; gap x _dropoff, 50; expect, 10; wordsize, 11. Alternatively, the blastn matrix was adjusted to 2, −2. The low resolution BLAST image was reproduced in Canvas 8. Transcription factor cluster analysis was performed using the Genomatix publicly available software (http://www.genomatix.de/). Transcription factor binding sites were selected using weight matrices for the following factors from the MatInspector (38) library: V$CEBPB.01, V$DLX1.01, V$GATA2.01, V$GATA2.02, V$HOXA9.01, V$MEIS1_HOXA9.01, V$NF1.01, V$NF1.02, V$OCT.01, V$OCT1.01, V$OCT1.02, V$OCT1.04, V$OCT1.05, V$OCT1.06, V$PBX1.01, V$PBX1_MEIS1.01, V$PBX1_MEIS1.02, V$PBX1_MEIS1.03, V$TST1.01.

Plasmids

The GnRH upstream enhancer (UE) region from −2980 to −2631 was amplified by PCR using the following primers; 5′-GGGGTACCGCTTTCCTTCACAGTAAAGG-3′ and 5′-CGACGCGTCGTCAGCGGTCCACACCATTTA-3′. The fragment was then transferred into the *Kpn*I and *Mlu*I site upstream of GnRHe/GnRHp and GnRHe/RSVp reporter plasmids (19) to create GnRHue/GnRHe/GnRHp and GnRHue/GnRHe/RSVp, respectively. GnRHue/GnRHp and GnRHue/RSVp were generated by removing the GnRH enhancer fragments from the GnRHue/GnRHe/GnRHp and GnRHue/GnRHe/RSVp plasmids, respectively. Briefly, the fragments were removed from GnRHue/GnRHe/GnRHp with *Mlu*I and *Xba*I, and from GnRHue/GnRHe/RSVp with *Mlu*I and *Hin*dIII and self-ligated using T_4 DNA ligase. RSVp is 128 bp from the proximal promoter of the RSV long terminal repeat from positions 9395–9523 (3). Mutations were made using the QuikChange Site-Directed Mutagenesis Kit as directed by the manufacturer (Stratagene, La Jolla, CA). The sequence of all of the plasmids was confirmed by the UCSD Cancer Center DNA Sequencing Shared Resource Core.

Cell Culture and Transfections

GT1–7, GN11, and NIH3T3 cells were maintained in 10-cm diameter culture plates in DMEM culture medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (0.1 mg/ml) under a 5% CO_2/95% air atmosphere at 37 C. Transient transfections were performed using FuGENE 6 Transfection Reagent (Roche Diagnostic Corp., Indianapolis, IN) according to the manufacturer's protocol. GT1–7 were plated at 9×10^4 cells per well, GN11 at 6.5×10^4 cells per well, and NIH3T3 cells at 5×10^4 cells per well in 24-well plates and cultured for 16 h before transfection. Each luciferase reporter plasmid (400 ng) and 200 ng of RSVe/RSVp-β-gal reporter were transfected into each well as an internal control. For cotransfection experiments, 100 ng of expression vector, NF1A/EN or OCT1, was transfected with 400 ng of the luciferase reporter containing a multimer of the enhancer AT-a site (−1805 to −1766) fused to the RSV promoter as well as the RSVe/RSVp-β-gal internal control. Transfected cells were incubated for 48 h before harvesting. Luciferase activity was measured using a microplate luminometer (EG&G Berthold, Wildbad, Germany) by a 100-μl injection of the reaction buffer containing 100 mM Tris-HCl (pH 7.8), 15 mM $MgSO_4$, 10 mM ATP, and 65 μM luciferin. β-gal assays were performed according to the manufacture's protocol (Tropix, Bedford, MA). Raw luciferase values were normalized to the RSVe/RSVp-β-gal internal control and compared with normalized RSVe/RSVp-luciferase values to evaluate promoter activity in various cell lines.

Nuclear Extracts and EMSA

Nuclear extracts for EMSA were prepared according to Lee *et al.* (39). Annealed oligonucleotides (1 pmol) (QIAGEN, Alameda, CA) were phosphorylated with [γ^{32}P]ATP (6000 Ci/mmol; ICN Biomedicals, Inc., Aurora, IL) and T_4 polynucleotide kinase (New England Biolabs, Inc, Beverly, MA). The probes were purified using MicroSpin G-25 columns (Amersham Biosciences Corp., Piscataway, NJ). Each binding reaction contained 1 fmol probe and 2 or 10 μg nuclear extracts in 10 mM HEPES (pH 7.8), 50 mM KCl, 1 mM EDTA, 5 mM spermidine, 5 μg BSA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonylfluoride, 0.5 μg poly dI-dC, 6% glycerol, and 2% Ficoll. The binding reaction was incubated 5–30 min at room temperature after addition of probes. The DNA-protein complexes were electrophoresed for 2 h at 250 V and separated on 5% acrylamide-*N*,*N*-methylene bisacrylamide (30:1) gels. For competition reactions, 100 fmol (100 × labeled probe) of unlabeled annealed double-stranded oligonucleotides were added to the reaction. Supershift assays were performed with 0.2 μg of anti-OCT1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and 1 μl of anti-NF1 antibody (kindly provided by Dr. Naoko Tanese). The NF1 consensus oligonucleotide sequence is 5′-AGTTTGGCAGGGAGCCAAGTT-3′.

RT-PCR

Total RNA extraction was carried out as described by the method of Chomczynski and Sacchi (40). The reverse transcriptase reaction was performed according to the methods of Pernasetti *et al.* (41). cDNA was obtained by reverse transcribing 5 μg RNA from GN11, NLT, GT1–7, NIH3T3, and LβT2 cells using Oligo$(dT)_{12–18}$ primer and Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). PCR analysis was performed with 35 cycles of 45 sec at 95 C, 1 min at an annealing temperature of 60 C, and 1 min at 72 C, followed by an elongation step for 10 min at 72 C. Specific primers for *NFIA*, *NFIB*, *NFIC*, *NFIX*, and *β-actin* were used in the PCR reaction. Primers for the *NFI* genes were created by the Gronostajki Lab, http://elegans.swmed.edu/Worm_labs/Gronostajski/ and amplify regions of the *NF1* sequences that are not alternatively spliced. PCR products were viewed by electrophoresis on an ethidium-stained, 1.5% agarose gel.

Protein Production and GST-Retention Assay

Expression vectors used for protein production included the full-length human OCT1, the murine NF1 family members, NF1A1.1, NF1B2, NF1C2, and NF1X2. *In vitro* transcription and translation were performed according to protocol with the Promega TNT Coupled Reticulocyte Lysate System (Promega Corp., Madison, WI). Protein products were labeled using [^{35}S]methionine. Translation mix without DNA was used in the assays to control for endogenous translation in the reticulocyte lysate. The GST-tagged proteins, OCT1, and GST alone were generated as previously described (17). The interaction between GST-tagged proteins and *in vitro* translated, ^{35}S-radiolabeled proteins was performed as described previously (42). Protein interactions were visualized by separation on a 10% sodium dodecyl sulfate gel and exposed to Kodak X-BioMax film (Eastman Kodak, Rochester, NY).

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