

# Otx2 Induction of the Gonadotropin-releasing Hormone Promoter Is Modulated by Direct Interactions with Grg Co-repressors<sup>\*[5]</sup>

Received for publication, April 1, 2009. Published, JBC Papers in Press, April 28, 2009, DOI 10.1074/jbc.M109.002485

Rachel Larder and Pamela L. Mellon<sup>1</sup>

From the Department of Reproductive Medicine and Center for Reproductive Science and Medicine, University of California, San Diego, La Jolla, California 92093-0674

Hormonal communication between the hypothalamus, pituitary, and gonads orchestrates the development and regulation of mammalian reproductive function. In mice, gonadotropin-releasing hormone (GnRH) expression is limited to ~1000 neurons that originate in the olfactory placode then migrate to specific positions scattered throughout the hypothalamus. Coordination of the hypothalamic-pituitary-gonadal axis is dependent upon correct migration of GnRH neurons into the hypothalamus followed by the appropriate synthesis and pulsatile secretion of GnRH. Defects in any one of these processes can cause infertility. Recently, substantial progress has been made in identifying transcription factors, and their cofactors, that regulate not only adult expression of GnRH, but also the maturation of GnRH neurons. Here, we show that expression of Otx2, a homeodomain protein required for the formation of the forebrain, is dramatically up-regulated during GnRH neuronal maturation and that overexpression of Otx2 increases GnRH promoter activity in GnRH neuronal cell lines. Furthermore, Otx2 transcriptional activity is modulated by Grg4, a member of the Groucho-related-gene (Grg) family. Using mutational analysis, we show that a WRPW peptide motif within the Otx2 protein is required for physical interaction between Otx2 and Grg4. Without this physical interaction, Grg4 cannot repress Otx2-dependent activation of GnRH gene transcription. Taken together, these data show that Otx2 is important for GnRH expression and that direct interaction between Otx2 and Grg co-repressors regulates GnRH gene expression in hypothalamic neurons.

Normal reproductive function requires the precise orchestration and integration of hormonal regulation at all levels of the hypothalamic-pituitary-gonadal axis. Dysfunction at any of these levels leads to pathophysiologic disorders such as infertility, polycystic ovarian syndrome, and hypogonadotropic

hypogonadism. Gonadotropin-releasing hormone (GnRH),<sup>2</sup> the central regulator of the hypothalamic-pituitary-gonadal axis, is secreted in a pulsatile manner from a small, yet critical, population of neurons within the hypothalamus. The correct migration of GnRH neurons to the hypothalamus, from their origins within the olfactory placode, followed by the appropriate synthesis and pulsatile secretion of GnRH, is essential for effective coordination of the hypothalamic-pituitary-gonadal axis. Examination of the molecular mechanisms involved in GnRH neuron migration and transcription has benefited from the generation of immortalized GnRH neuronal cell lines (1, 2). The GT1–7 cell line represents a fully differentiated neuron, which accurately processes the pro-GnRH precursor and secretes GnRH in a pulsatile pattern (3, 4). The GN11 cell line represents a developmentally earlier, migratory version of GnRH neurons and expresses low levels of GnRH (2). Using these cell lines, substantial progress has been made in characterizing the evolutionary conserved enhancer and promoter elements that confer neuron specific activity as well as identifying transcription factors that regulate GnRH gene expression (5–11).

We previously identified a highly conserved Otx2 target sequence within the proximal promoter region of the rat GnRH gene and demonstrated that overexpression of Otx2 significantly increased GnRH promoter activity in GT1–7 cells via this bicoid-like binding site (7). Otx2, the vertebrate homologue of *Drosophila* orthodenticle, is expressed in a specific spatial and temporal manner during embryogenesis to define regional identities in the developing brain (12–16). In the adult brain, expression of Otx2 is restricted to the cerebellum and GnRH neurons within the hypothalamus (7, 17), however, mice homozygous for a null allele of Otx2 are embryonic lethal due to severe brain abnormalities (12, 18, 19), preventing analysis of the GnRH neurons. Alternative usage of Otx2 promoters during development allows expression levels of Otx2 to be tightly regulated (17); in addition interactions between Otx2 and cofactors provide a further level of control over Otx2 protein activity. Both Sox2 and Lhx1 have been shown to synergize with Otx2 to increase promoter activation (20–22), whereas mem-

\* This work was supported, in whole or in part, by National Institutes of Health Grants R01 DK044838 and HD020377 (to P. L. M.). This work was also supported by NICHD/NIH through a cooperative agreement (U54 HD012303) as part of the Specialized Cooperative Centers Program in Reproduction and Infertility Research (to P. L. M.).

[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Fig. S1.

<sup>1</sup> To whom correspondence should be addressed: 9500 Gilman Dr., La Jolla, CA 92093-0674. Tel.: 858-534-1312; Fax: 1-858-534-1438; E-mail: pmellon@ucsd.edu.

<sup>2</sup> The abbreviations used are: GnRH, gonadotropin-releasing hormone; Grg, Groucho-related-gene; EMSA, electrophoretic mobility shift assay; HDAC, histone deacetylase; IVT, *in vitro* translated; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; ANOVA, analysis of variance; RT, reverse transcription; TK, thymidine kinase.

bers of the Groucho-related gene (Grg) family have been hypothesized to switch Otx2 from an activator to a repressor (16).

Interestingly, we have previously shown that members of the Grg family of co-repressors are important regulators of GnRH transcription (10). Grg proteins are expressed in a wide variety of tissues, in both vertebrates and invertebrates, and are expressed at all stages of development (23–26). They interact with multiple transcription factors all of which are involved in mechanisms that regulate processes such as differentiation, cell specification, embryonic patterning, and apoptosis (27). Several models have been proposed to explain how Grg proteins may be acting to switch off transcription, including both HDAC-dependent and independent mechanisms (28–30). Grg proteins fall into one of two groups: “long” (Grg1, Grg2, Grg3, and Grg4) or “short” (Grg1-S, Grg3b, Grg5, and Grg6). Long Grgs consist of four distinct domains: an N-terminal glutamine-rich region (Q-domain), a glycine/proline-rich region (GP-domain), a serine/proline-rich region (SP-domain), and the C-terminal region with multiple tryptophan and aspartic acid repeats (WD40 repeats). All of the domains are important for interactions with transcription factors (31–33), the Q-domain is required for homo- and heterodimerization of Grg proteins (34), and the GP-domain is involved in the recruitment of histone deacetylases (HDACs) (35). Grg1-S, Grg3b, and Grg5 lack the NLS, SP-domain, and WD40 repeats (24, 26, 36, 37). The WD40 domain is conserved in Grg6, but the N terminus of the protein is truncated and has little similarity to the other Grgs. The short Grgs are proposed to function as dominant-negative forms of long Grgs; however, the exact mechanism by which they influence Grg-mediated repression remains to be resolved (38, 39).

Herein, we have examined how Otx2 activation of GnRH gene transcription is regulated by the physical interaction of Otx2 with Grg4, a long Grg co-repressor. We show that Otx2 expression is restricted to GT1–7 cells and that Otx2 interacts *in vivo* with the GnRH promoter. Furthermore, we describe the identification and characterization of Grg4 as a repressor of Otx2-induced activation of GnRH transcription. We report that Otx2-induced activation is repressed by Grg4 through an HDAC-independent mechanism and demonstrate that this repression is mediated by physical interaction between the two proteins via a specific amino acid motif within the Otx2 protein.

## EXPERIMENTAL PROCEDURES

**Western Blotting Analysis**—Nuclear proteins were isolated as previously described (40). Protein concentration was determined using Bio-Rad protein assay reagent: 20  $\mu$ g of nuclear extract was boiled for 5 min in 5 $\times$  Western loading buffer, fractionated on a 14% SDS-PAGE gel, and electroblotted for 2 h at 300 mA onto polyvinylidene difluoride (Millipore, Billerica, MA) in 1 $\times$  Tris-glycine/20% methanol. Blots were blocked overnight at 4 °C in 2% milk/1% bovine serum albumin then probed for 2 h at room temperature with rabbit-anti-human Otx2 antibody (Abcam, Cambridge, MA) diluted 1:500 in blocking buffer. Blots were then incubated with an anti-rabbit horseradish peroxidase-linked secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and bands were visualized

using the SuperSignal West Pico chemiluminescent substrate (Pierce).

**PCR**—Preparation of cDNA and RT-PCR were performed as previously described (41). Adult mouse hypothalamus and adult mouse eye cDNA were purchased from Zyagen (San Diego, CA). Standard PCR conditions were used and were identical for all primers. Quantitative RT-PCR amplification utilized GN11 or GT1–7 cDNA, IQ SYBR Green Supermix (Bio-Rad), specific primer sequences (Table 1), and an IQ5 Real-time PCR instrument (Bio-Rad). Each reaction consisted of 4.5  $\mu$ l of dH<sub>2</sub>O, 1.5  $\mu$ l of each forward and reverse primers (5  $\mu$ M), 12.5  $\mu$ l of IQ SYBR Green Supermix, and 5  $\mu$ l of cDNA (1:10 dilution). The IQ5 real-time PCR program was as follows: 95 °C for 3 min followed by 40 cycles of 10 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C. Standard curve used serially diluted plasmids containing the gene of interest (range of 1 ng to 10 fg).

**Chromatin Immunoprecipitation**—GN11 and GT1–7 cell chromatin was extracted as described previously (10), sonicated to between 300 and 500 bp, then precipitated with rabbit-anti-human Otx2 antibody (Abcam) or normal rabbit IgG (Santa Cruz Biotechnology) as control. Immunoprecipitated DNA was analyzed for the mouse GnRH promoter sequence by PCR using primers from –293 and +54 bp of the evolutionarily conserved murine sequence (Table 1).

**Cell Culture and Transient Transfections**—All cells were cultured in Dulbecco’s modified Eagle’s medium (Mediatech, Manassas, VA) containing 10% fetal calf serum (Gemini Bio-Products, Sacramento, CA), and 1% penicillin/streptomycin (Invitrogen) in a humidified 5% CO<sub>2</sub> incubator at 37 °C. Cells were seeded into 24-well plates and incubated overnight at 37 °C before being transiently transfected using FuGENE reagent (Roche Applied Science). Luciferase reporters were pGL3-GnRHe/p (rat GnRH enhancer (–1863 bp to –1571 bp) fused to the rat GnRH promoter (–173 bp to +112 bp)); pGL3–5kbGnRH (5 kb of the rat GnRH regulatory region); pGL3–152-Otx2-multimer (five copies of –153 bp to –145 bp of the rat GnRH promoter fused to a herpes simplex virus thymidine kinase (TK) promoter); and pGL3–198-Otx2-multimer (five copies of –199 bp to –191 bp of the rat GnRH promoter on a TK promoter). Expression plasmids included pSG5-wtOtx2, pSG5-mutOtx2, pKW2T-Grg4-FLAG, pKW2T-mutGrg4-FLAG, and pSG5-Grg5. Appropriate empty vectors served as negative controls. Cells were transfected with 100 ng of expression plasmid, 200 ng of luciferase-reporter plasmid, and 100 ng of the internal-control TK –109-bp promoter on  $\beta$ -galactosidase. Cells were harvested after 48 h, lysed, then assayed for luciferase and  $\beta$ -galactosidase as previously described (42). Luciferase values were divided by  $\beta$ -galactosidase values to control for transfection efficiency. All experiments were performed in triplicate and repeated a minimum of three times. Statistical significance was established as  $p < 0.05$  by ANOVA.

**Analysis of Endogenous GnRH mRNA Levels after Overexpression of Otx2**—GN11 and GT1–7 cells were transiently transfected with either pSG5 or pSG5-Otx2 using FuGENE. Total RNA was extracted 48 h later using TRIzol (Invitrogen) according to the manufacturer’s protocol. Preparation of cDNA and quantitative RT-PCR were performed as described above.

## Grg4 Represses Otx2 Activation of GnRH

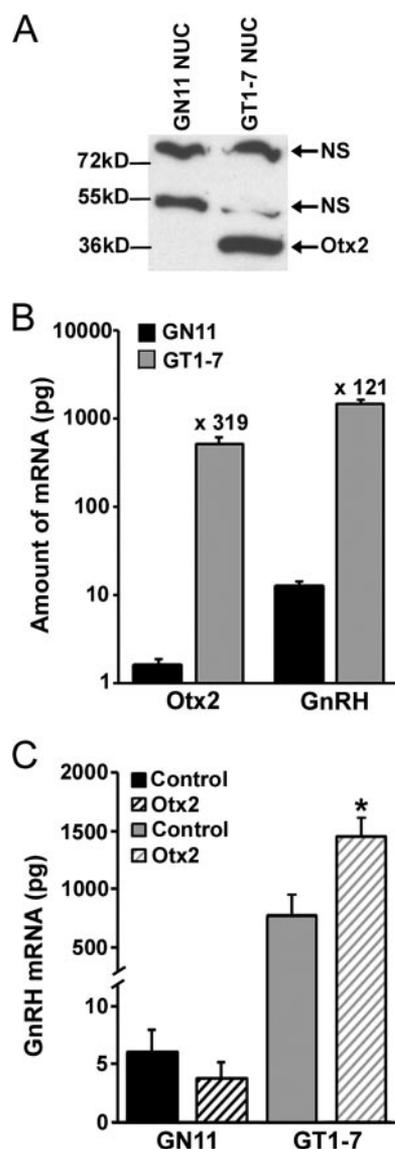
**EMSA—*In vitro* transcription and translation** was performed with the Promega TNT-coupled reticulocyte lysate system following the manufacturer's protocol. T7 polymerase was used to produce protein from pSG5-Otx2. EMSA oligonucleotides (Table 1) were annealed, end-labeled, and then purified as previously described (43). Binding reactions used 3 fmol of  $^{32}$ P-labeled oligonucleotide and either 1  $\mu$ g of GT1-7 nuclear protein, 1  $\mu$ l of *in vitro* translated Otx2, or 1  $\mu$ l of rabbit reticulocyte lysate. After addition of the probe, binding reactions were incubated for 10 min at room temperature prior to electrophoresis on a 5% polyacrylamide gel in 0.25 $\times$  Tris-Borate-EDTA. Competition assays were performed by preincubating the reactions with 100- to 500-fold excess of unlabeled oligonucleotide for 5 min prior to addition of probe. For supershift assays, 0.5  $\mu$ g of a goat-anti-human Otx2 antibody (Santa Cruz Biotechnology), or normal goat IgG control, was added to the reaction. Gels were electrophoresed at 250 V for 2 h then dried, under vacuum, and exposed to film.

**Bioinformatic Analysis**—Approximately 500 bp of GnRH upstream regulatory region sequence was obtained for rat, mouse, human, dog, horse, cow, shrew, and bat using Ensembl. Sequences were aligned using the ClustalW program and imported into GeneDoc for annotation.

**Co-immunoprecipitation of Endogenous Proteins from GT1-7 Cells**—Whole cell protein extracts were harvested by lysing cells in buffer (20 mM Tris, pH 7.5; 100 mM NaCl; 0.5% Nonidet P-40; 0.5 mM EDTA) for 10 min. After centrifugation, 200  $\mu$ g of extract was pre-cleared by incubation with 25  $\mu$ l of Protein G magnetic beads (PerkinElmer Life Sciences) for 1 h at 4  $^{\circ}$ C. Supernatant was incubated with 4  $\mu$ g of goat-anti-human Otx2 antibody (Santa Cruz Biotechnology) or normal goat IgG (Santa Cruz Biotechnology) for 1 h at 4  $^{\circ}$ C. Protein G magnetic beads (25  $\mu$ l) were added and incubated overnight at 4  $^{\circ}$ C. Beads were washed in 1 $\times$  phosphate-buffered saline, then re-suspended in 40  $\mu$ l of 2 $\times$  SDS Sample Loading Buffer/1%  $\beta$ -mercaptoethanol. After boiling for 5 min, 20  $\mu$ l of sample was electrophoresed on a 12% SDS-PAGE gel, transferred to polyvinylidene difluoride (Millipore), then probed as described with either rabbit-anti-human Otx2 antibody (Abcam, 1:500 in blocking solution) or rabbit-anti-mouse Grg4 antibody (Santa Cruz Biotechnology, 1:500 in blocking solution).

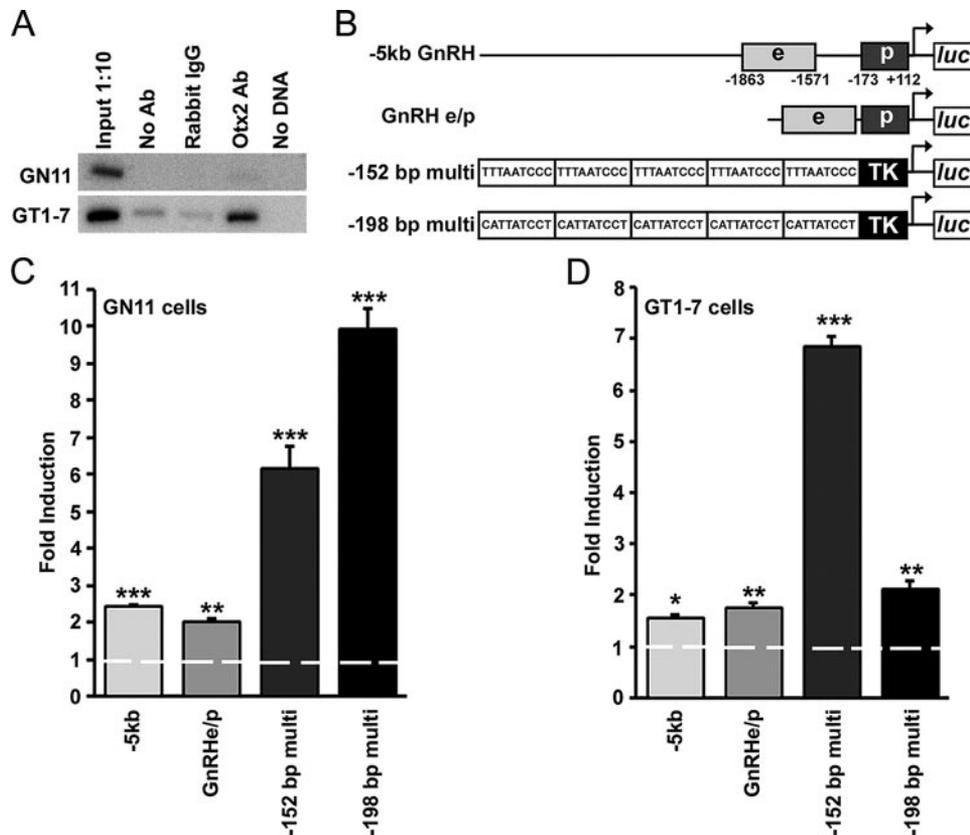
**Co-immunoprecipitation of Overexpressed Proteins from NIH3T3 Cells**—NIH3T3 cells were co-transfected with pKW2T-Grg4-FLAG and either pGFP-wtOtx2, pGFP-mutOtx2, or pGFP-NTermOtx2 using FuGENE (Roche Applied Science). Wild-type and mutated Otx2 plasmids were cloned using full-length inserts from their respective pSG5 plasmids with EcoRI. Forty-eight hours after transfection, whole cell extracts were prepared, and co-immunoprecipitation assays were performed, as described previously, using a goat-anti-rat Grg4 antibody (Santa Cruz Biotechnology). Blots were probed with either a rabbit polyclonal GFP antibody (Santa Cruz Biotechnology, 1:500 dilution in blocking solution) or anti-FLAG M2 monoclonal antibody conjugated to horseradish peroxidase (Sigma-Aldrich, 1:500 in blocking solution).

**Transient Transfection and Visualization of GFP and FLAG Expression in Cultured Cells**—NIH3T3 cells were plated on two-well glass chamber slides (Nalge Nunc International,



**FIGURE 1. Otx2 expression is dramatically increased in the mature GnRH neuronal GT1-7 cell line.** *A*, Western blotting analysis of nuclear protein from GN11 and GT1-7 cells. A band corresponding to Otx2 was detected at the expected size of  $\sim$ 37 kDa in nuclear extracts from GT1-7 cells. No Otx2 protein was detectable in GN11 nuclear protein extracts. NS, nonspecific band. *B*, quantitative RT-PCR analysis of Otx2 and GnRH mRNA extracted from GN11 and GT1-7 cells. Results are expressed as picograms of Otx2 or GnRH mRNA normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and are the mean of three separate experiments performed in triplicate. Results shown are average  $\pm$  S.E. Numbers above the GT1-7 bar indicate -fold increase in mRNA levels in GT1-7 cells compared with GN11 cells. *C*, quantitative RT-PCR analysis of GnRH mRNA extracted from GN11 and GT1-7 cells transfected with either an Otx2 expression plasmid (Otx2) or an "empty" vector (control). Overexpression of Otx2 in the more mature GT1-7 neuronal cell line induced GnRH mRNA levels  $\sim$ 1.9-fold. One-way ANOVA established statistical significance of Otx2 expression plasmid versus control as  $p < 0.05$  (\*). Results are expressed as picograms of GnRH mRNA normalized against GAPDH mRNA and are the mean of three separate experiments performed in triplicate. Results shown are average  $\pm$  S.E.

Naperville, IL) at 50,000 cells/ml; 16 h later, each well was transfected with 500 ng of pKW2T-Grg4-FLAG plasmid and pGFP-wtOtx2, pGFP-mutOtx2, or pGFP-NTermOtx2 using FuGENE (Roche Applied Science). After 48 h at 37  $^{\circ}$ C, cells were fixed in 3.7% formaldehyde with 0.5% Triton X-100 for 10 min at room temperature. After washing in phosphate-buffered saline, cells



**FIGURE 2. Otx2 induces GnRH promoter activity.** *A*, chromatin immunoprecipitation assays were performed in GN11 and GT1-7 cells using an antibody specific for Otx2 (representative shown of three replicates). Amplification of immunoprecipitated chromatin, using PCR primers specific to the GnRH promoter, revealed that Otx2 associates with the GnRH promoter in GT1-7 cells, but not in GN11 cells (*Otx2 Ab*). Rabbit IgG and no antibody (*no Ab*) were used as negative controls. *B*, schematic diagram showing the luciferase reporter constructs used in transient transfection assays. Numbers indicate the position of nucleotides relative to the start site: promoter (*p*), enhancer (*e*), thymidine kinase (*TK*), luciferase (*luc*). *C* and *D*, effect of overexpression of Otx2 on -5-kb GnRH, GnRHe/p, -152-bp Otx2-multimer, and -198-bp Otx2-multimer in GN11 (*C*) and GT1-7 (*D*) cells. The -fold induction compared with empty vector alone (*dashed line*) is indicated, corrected for  $\beta$ -galactosidase, which was used as an internal control. Overexpression of Otx2 resulted in significant -fold induction of all GnRH promoter and Otx2-multimer reporters tested. One-way ANOVA established statistical significance of Otx2 expression plasmid versus empty expression plasmid (control) as \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . All experiments were performed in triplicate and repeated three times. Results shown are average  $\pm$  S.E.

were incubated for 1 h at room temperature with an anti-FLAG-M2 monoclonal antibody conjugated to Cy3 (1:100 dilution in phosphate-buffered saline, Sigma-Aldrich). Cells were then washed in phosphate-buffered saline, and coverslips were mounted using Vectashield hardset mounting media with 4',6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Fluorescent cells were detected using a Nikon Eclipse TE2000-U microscope.

**Generation of Mutated Expression Plasmids**—Mutagenesis was performed using the QuikChange XL site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. Truncated Otx2 (NTermOtx2) was generated by PCR from the wild-type plasmid and corresponds to amino acids 1–142 of Otx2 (primers in Table 1).

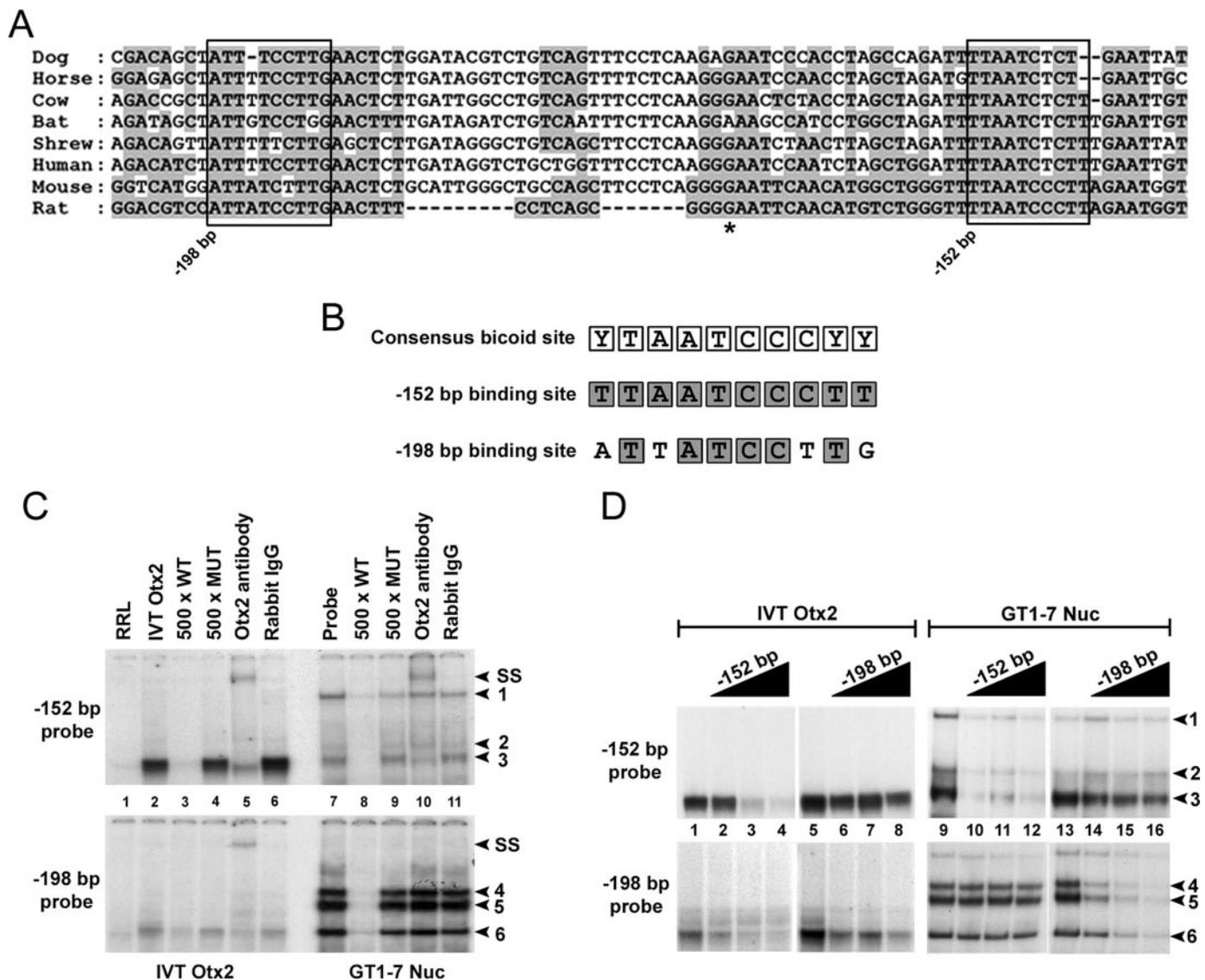
**Statistical Analysis**—The raw data were analyzed by one-way ANOVA, followed by post hoc comparisons with the Tukey-Kramer honestly significant difference test using the statistical package JMP 7.0 (SAS, Cary, NC). Significant differences were designated as  $p < 0.05$ .

## RESULTS

**Otx2 mRNA Expression Increases as GnRH Neurons Mature**—We speculate that the increase in GnRH gene expression that occurs during GnRH neuron development may be controlled in part by the relative expression levels of specific transcriptional regulators within the cell. During migration, GnRH neurons express low levels of GnRH and will likely express low levels of critical activating factors required to induce GnRH transcription. Conversely, high levels of these activators will be seen in GnRH neurons that have reached the hypothalamus and transcribe high levels of GnRH. To determine whether an increase in Otx2 expression correlates with an increase in GnRH transcription, we examined Otx2 mRNA and protein expression in the GnRH neuronal cell lines: GN11 (representing an early stage of development) and GT1-7 (representing a more mature hypothalamic neuron). These analyses revealed that an increase in Otx2 expression mirrors the increase in GnRH expression. Otx2 protein expression is readily detected by Western blotting analysis of GT1-7 cell protein but is undetectable in GN11 cells (Fig. 1A). Quantitative RT-PCR analysis determined that Otx2 mRNA levels are ~319-fold higher in mature GnRH neurons (Fig. 1B). This rise correlates with a ~121-fold increase in GnRH

expression. These data suggest that an increase in Otx2 expression during development may promote the more mature phenotype of the GnRH neuronal GT1-7 cell line, most notably, the increase in GnRH expression that is observed in these cells and their ability to secrete GnRH in a pulsatile manner. To test this hypothesis, we transfected GN11 and GT1-7 cells with either a control expression plasmid (pSG5) or an Otx2 expression plasmid (pSG5-Otx2) and measured the levels of endogenous GnRH mRNA. Interestingly, overexpression of Otx2 in the more mature GT1-7 neuronal cell line induced GnRH mRNA levels ~1.9-fold. In contrast, overexpression of Otx2 was insufficient to promote expression of endogenous GnRH in immature GN11 cells (Fig. 1C). These data corroborate our studies of the chromatin status of the GnRH regulatory region. Our analysis indicates that the GnRH regulatory region is in an active chromatin state in the more mature GT1-7 neuronal cell line as defined by DNase I sensitivity and Histone H3 acetylation and methylation assays (44–46). In contrast, it shows a predominantly closed status in non-neuronal NIH3T3 and

## Grg4 Represses Otx2 Activation of GnRH



**FIGURE 3. Otx2 preferentially binds to a bicoid-like target sequence within the rat GnRH promoter.** *A*, the rat GnRH regulatory sequence between  $-136$  and  $-206$  bp was aligned with sequences from the mouse, human, shrew, bat, cow, horse, and dog GnRH regulatory regions using the ClustalW program. The previously identified Otx2-binding sites are enclosed in *boxes*. \*, indicates  $-173$  bp of the rat promoter. Nucleotides that are conserved between all the species are enclosed in *gray boxes*. *B*, alignment of the two rat GnRH promoter Otx2-binding sites. The DNA sequence at each binding site was compared with a consensus Otx2-binding site sequence. The  $-152$ -bp site is a 100% match to the consensus sequence. The  $-198$ -bp site is a 60% match to the consensus sequence. Y indicates that either C or T can be present at that position. *Gray boxes* indicate a match to the consensus sequence. *C*, EMSA was performed with IVT Otx2 (*lanes 2–6*) or GT1–7 nuclear protein extracts (*lanes 7–11*). The DNA probes consisted of radiolabeled oligonucleotides containing the Otx2-binding sites identified at  $-152$  bp and  $-198$  bp of the rat GnRH promoter. A 500-fold excess of unlabeled wild-type (WT) or mutant (MUT) probe and an antibody against Otx2 were included in the reaction mixtures as indicated. Rabbit reticulocyte lysate (RRL) was used as a control to show nonspecific binding (*lane 1*). Otx2 antibody was able to shift a complex that formed on both probes after incubation with IVT Otx2. Several GT1–7 protein complexes form on each probe (*arrows 1–6*). A supershifted GT1–7 cell protein complex (SS) was only observed on the  $-152$ -bp probe after addition of an Otx2 antibody. *D*, comparison of the DNA-binding affinity of the Otx2 binding sites at  $-152$  bp and  $-198$  bp. Addition of increasing amounts of unlabeled  $-152$ -bp probe (100-fold, 250-fold, and 500-fold excess) eliminated binding of IVT Otx2 to the  $-198$ -bp probe (*lanes 1–4*). In contrast, the addition of increasing amounts of unlabeled  $-198$ -bp probe had no effect on binding to the  $-152$ -bp probe (*lanes 5–8*).

GN11 cells.<sup>3</sup> This inactive status likely prevents transcription factors, such as Otx2, from activating endogenous GnRH transcription in these cells.

*Otx2 Interacts with the GnRH Regulatory Region in GT1–7 Cells*—The DNA sequence driving expression of GnRH has been well characterized in the rat, and two key regions, the proximal promoter (+1 to  $-173$  bp) and a distal enhancer ( $-1571$  to  $-1863$  bp), have been identified as being critical for

proper GnRH transcription (47). Multiple binding sites for a number of different transcription factors have been identified within these regions and these sites are conserved across several vertebrate species (5, 6, 9, 48). Previously, we have reported that overexpression of Otx2, a homeodomain-containing transcription factor, significantly activates the rat GnRH promoter in GT1–7 cells via a conserved bicoid-like target sequence and that mutation of this site significantly reduces this activation (7). Recently, a second conserved Otx2-binding site was identified in the mouse GnRH promoter, and mutation of both of

<sup>3</sup> A. Iyer, N. Miller, and P. Mellon, submitted for publication.

**TABLE 1**  
Sequences of oligonucleotides  
Mutated sequences are underlined.

Primers	DNA sequence
Q-RT-PCR Otx2 forward	5'–GCAGAGGTCCCTATCCCATGA–3'
Q-RT-PCR Otx2 reverse	5'–CTGGGTGGAAAGAGAAGCTG–3'
Q-RT-PCR GnRH forward	5'–TGCTGACTGTGTGTTTGGGAAGGCT–3'
Q-RT-PCR GnRH reverse	5'–TTTGTATCCACCTCCTTGCGACTCA–3'
Q-RT-PCR GapDH forward	5'–TGCACCACCAACTGCTTAG–3'
Q-RT-PCR GapDH reverse	5'–GGATGTCAGGGATGATGTTCC–3'
ChIP promoter forward	5'–CCAGCTTCCTCAGGGGAATT–3'
ChIP promoter reverse	5'–CCTGTTTGGATGTGAAAGTCAAAGGGA–3'
–152-bp EMSA forward	5'–CATGTCTGGGTTTAAATCCCTTAGAATGGT–3'
–152-bp EMSA reverse	5'–ACCATTCTAAGGGATTAAAACCCAGACATG–3'
–152-bp MUTANT EMSA forward	5'–CATGTCTGGGTTT <u>GC</u> CCCTTAGAATGGT–3'
–152-bp MUTANT EMSA reverse	5'–ACCATTCTAAGGG <u>CG</u> CAAACCCAGACATG–3'
–198-bp EMSA forward	5'–TCTAAGACGTCATTATCCCTTGAACCTTCC–3'
–198-bp EMSA reverse	5'–GGAAAGTTCAAGGATAATGGACGCTTAGA–3'
–198-bp MUTANT EMSA forward	5'–TCTAAGACGTCAG <u>GC</u> CCCTTGAACCTTCC–3'
–198-bp MUTANT EMSA reverse	5'–GGAAAGTTCAAGG <u>CG</u> CCCTTGGACGCTTAGA–3'
Grg1 forward	5'–AATGATGGCACCAAGCTCTG–3'
Grg1 reverse	5'–GTCACAGCTAAGCACTGATG–3'
Grg2 forward	5'–AGAGTCTGGTGGAAAGGAGGAC–3'
Grg2 reverse	5'–TCCACTACCAGTTATAGTC–3'
Grg3 forward	5'–GGGCCAGCTGTATAGACATC–3'
Grg3 reverse	5'–TTGTCACCAGACCGCTGTAC–3'
Grg4 forward	5'–TGATGCCCTCATTTGGACCAG–3'
Grg4 reverse	5'–GAGTCCAGTTTGCACGAAGA–3'
Grg5 forward	5'–CATGATGTTTCCGCAAAGCC–3'
Grg5 reverse	5'–GAGCTAAATCCGACTTCTCTC–3'
Grg6 forward	5'–TAGTCAGGAACCTCAAAGGC–3'
Grg6 reverse	5'–TGGATCCTGTGATGATGAGC–3'
GapDH forward	5'–ATGGGTGAAGTTCGGTGTGA–3'
GapDH reverse	5'–GCTTCCCCTTGATGACAAGC–3'
NTerm Otx2 forward	5'–TCTTATCTAAAGCAACCGCC–3'
NTerm Otx2 reverse	5'–AATGGTTGGGACTGAGGTACT–3'
Otx2 AAAA mutant forward	5'–AGCAGTGTCCAGTGTCTATC <u>CGGCCCGCGCGCTCCATCTCCCCACTGCTGAC</u> –3'
Otx2 AAAA mutant reverse	5'–GTCAGACAGTGGGGAGATGGAC <u>CGCGCCCGCGGATAGACACTGGAGCACTGCT</u> –3'
Grg4 GP deletion forward	5'–CAACTCCAAGCTCAGCATTATCCGTATCCCCATCAGCCA–3'
Grg4 GP deletion reverse	5'–TGGCTGATGGGGATACGGATAATGTCTGAGCTTGGAGTTG–3'

these sites in a GnRH transgene tested in transgenic mice resulted in reduced GnRH promoter activity within the brain (49). To establish whether Otx2 associates with the mouse GnRH regulatory region *in vivo*, we performed chromatin immunoprecipitation assays using an Otx2 antibody. Using PCR primers specific to the mouse GnRH promoter, amplification of the DNA immunoprecipitated by an Otx2 antibody revealed that Otx2 interacts *in vivo* with the GnRH promoter chromatin in live GT1–7 cells, lending further evidence to a role for Otx2 in regulation of the GnRH promoter in mature GnRH neurons (Fig. 2A).

**Overexpression of Otx2 Increases GnRH Promoter Activity**—To investigate the effects of Otx2 overexpression on GnRH promoter activity in both immature and mature GnRH neuronal cell lines, Otx2 was overexpressed in GN11 and GT1–7 cells with a luciferase reporter gene fused downstream of either –5 kb of the rat GnRH regulatory region or of the previously characterized rat GnRH enhancer and promoter (Fig. 2B). Additionally, to confirm Otx2 activation of the GnRH promoter was occurring via the two previously identified Otx2 sites (7, 49), we performed transient transfection assays with a luciferase reporter plasmid containing either five copies of the Otx2 site at –152 bp (5× ttaaatccc) or five copies of the Otx2 site first identified in the mouse at –319 bp, and conserved at –198 bp of the rat promoter (5× cattatcct). These multimerized binding elements were fused to a minimal TK promoter to obtain high levels of transcription (Fig. 2B). As seen in Fig. 2 (C and D), overexpression of Otx2 significantly increased the activity of all

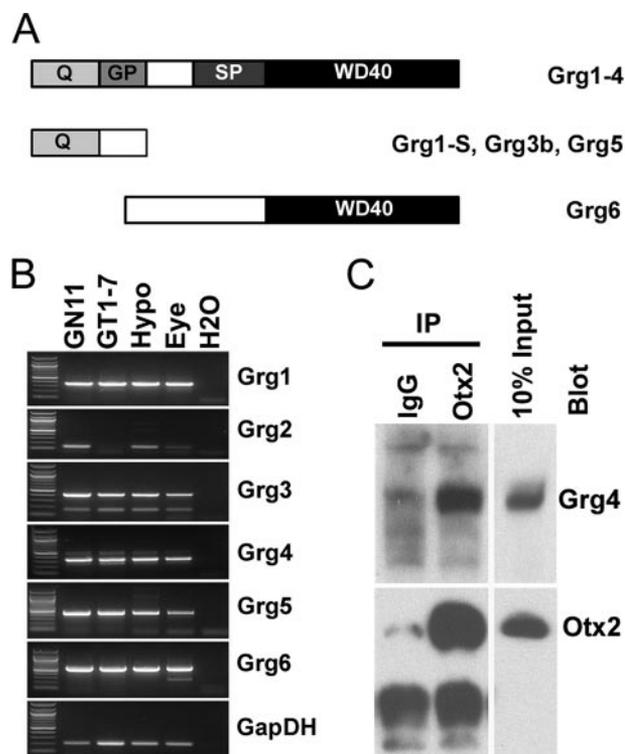
the luciferase reporters tested. The –5-kb reporter, which contains both of the previously identified Otx2 sites, and the GnRHe/p reporter, which only contains the –152-bp site, were activated to similar levels in both GN11 and GT1–7 cells, suggesting that the inclusion of a second Otx2-binding site at –198 bp does not enhance the response of the GnRH regulatory region to Otx2. The –152-bp multimer reporter showed a ~6-fold induction after overexpression of Otx2 in both cell lines. In contrast, the –198-bp multimer showed a robust, ~9.9-fold induction in GN11 cells but was only induced ~2.1-fold in GT1–7 cells. Although activation of the –198-bp multimer is significant in both cell lines, the decreased level of activation in GT1–7 cells suggests that other proteins, which are not expressed in GN11 cells, may have a stronger affinity for the DNA sequence and thus compete with Otx2 for binding to this site resulting in a significant, but less robust response in Otx2-induced promoter activity.

**Otx2 Preferentially Binds to a Bicoid-like Target Sequence at –152 bp**—Regions of the genome that are evolutionarily conserved likely serve important biological functions. We have previously reported that the well characterized 173-bp rat promoter is highly conserved between rat, mouse, and human (5, 50). To determine whether the DNA sequences contained within the two previously identified Otx2-binding sites are conserved, the rat GnRH regulatory sequence from –206 bp to –136 bp was aligned with the GnRH regulatory sequence from various mammals (Fig. 3A). The alignments show that, although the ~50 bp between the two sites shows poor conser-

## Grg4 Represses Otx2 Activation of GnRH

vation, with only 44–62% identity across the species analyzed, both of the binding sites are well conserved in mouse, human, shrew, bat, cow, horse, and dog ( $\geq 80\%$  identity) suggesting that both sequences are important for proper regulation of GnRH transcription. Further analysis of the binding sites revealed that the  $-152$ -bp site is a 100% match to a consensus Otx2-binding site identified by SELEX (Systemic Evolution of Ligands by EXponential enrichment) analysis (51). In contrast, the  $-198$ -bp site was only 60% identical (Fig. 3B). To analyze the affinity of Otx2 for these two binding sites, we performed EMSAs with probes containing either the  $-152$  bp or  $-198$  bp sequence (see Table 1). *In vitro* translated (IVT) Otx2 protein binds to both of the probes (Fig. 3C, lane 2, complex 3) and the addition of unlabeled WT probe (Fig. 3C, lane 3) significantly decreased complex formation, whereas competition with an unlabeled, mutated probe had no effect on (Fig. 3C, lane 4). Furthermore, incubation with an Otx2 antibody resulted in a supershift of this complex (Fig. 3C, lane 5, SS). Several GT1–7 nuclear proteins bind both probes (Fig. 3C, lanes 7, complexes 1–6), and incubation with an Otx2 antibody results in a supershift of complex 3 on the  $-152$ -bp probe (Fig. 3C, lane 10, SS). In contrast, the complexes that form on the  $-198$ -bp probe (Fig. 3C, lane 10, complexes 4–6) are unchanged by the addition of an Otx2 antibody. Competition assays determined that the  $-198$ -bp probe was unable to compete any of the protein complexes that formed on the  $-152$ -bp probe (Fig. 3D, lanes 5–8 and 13–16). The  $-152$ -bp probe was able to compete away the IVT Otx2 protein complex (Fig. 3D, lanes 1–4) but was unable to deplete any of the three GT1–7 nuclear protein complexes that formed on the  $-198$ -bp probe (Fig. 3D, lanes 9–12). These results suggest that, as predicted by sequence homology to the consensus, Otx2 has a greater affinity for the  $-152$ -bp site and indicate that, although Otx2 is capable of binding to the  $-198$ -bp site, other, unidentified GT1–7 nuclear proteins preferentially bind to this sequence.

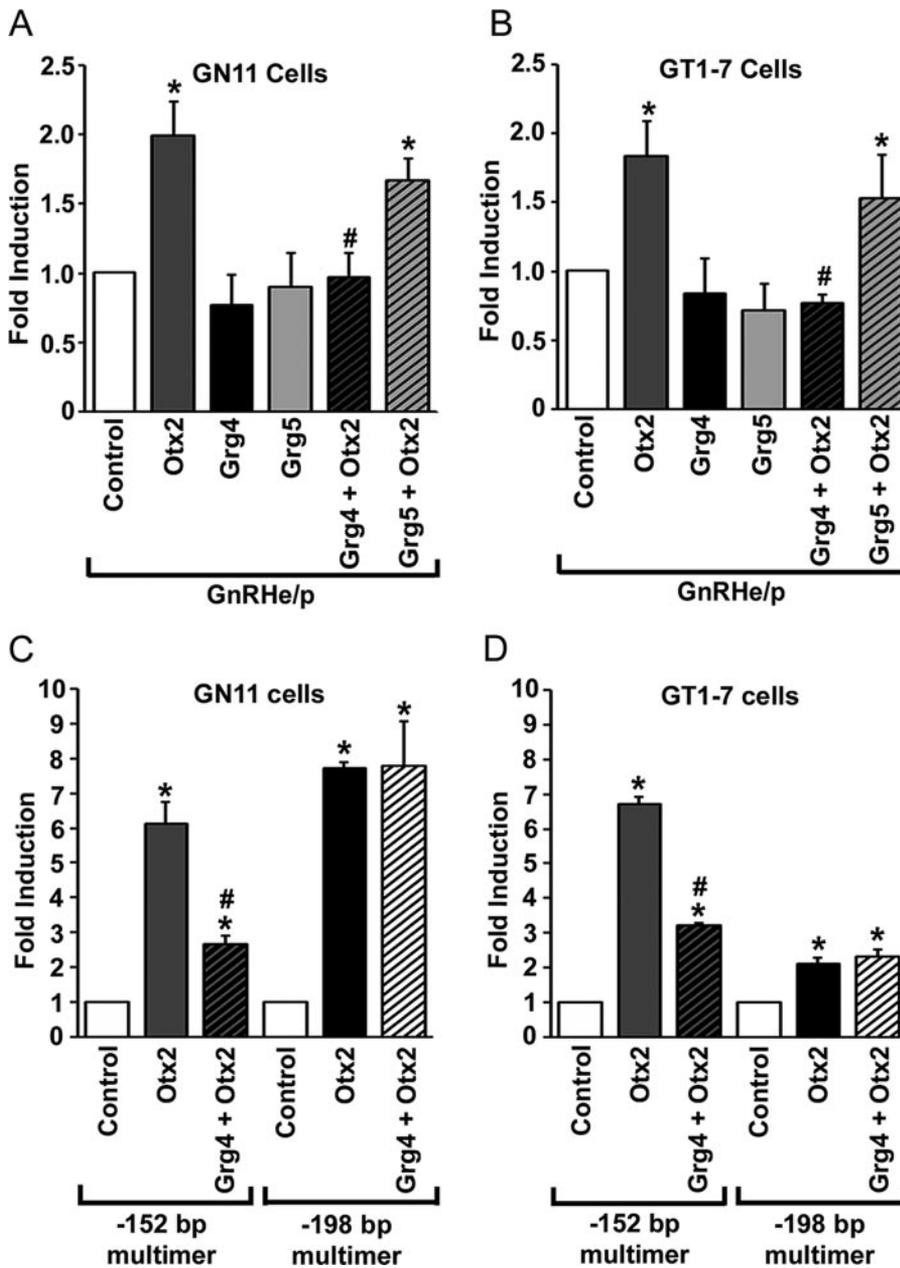
**Grg4 Physically Interacts with Otx2 in the GnRH Neuronal GT1–7 Cell Line**—We have previously reported that the Grg family of proteins (Fig. 4A) regulates GnRH expression via interactions with the homeodomain proteins Msx1 and Oct-1 (10). Additionally, we have shown that Grg1 and Grg5 mRNAs are co-expressed with GnRH protein during embryonic development and that several Groucho-related proteins (Grg1–5) are expressed in the mature GnRH neuronal GT1–7 cell line. Further investigation of Grg mRNA expression, using semi-quantitative RT-PCR analysis, reveals that all six Grg family members can be amplified from cDNA derived from GN11 cells, adult mouse hypothalamus, or adult mouse eye (Fig. 4B). All of the Grg family members, with the exception of Grg2, can also be amplified from cDNA derived from GT1–7 cells. Groucho-related co-repressors regulate multiple processes during embryogenesis and in the adult (52). During brain development, physical interaction between Otx2 and the WD40 domain of long Grg family members, is hypothesized to switch the role of Otx2 from that of an activator, to a repressor, allowing appropriate patterning of the midbrain-hindbrain regions to occur (16, 53). To explore whether long Grgs physically interact with Otx2 in GnRH neurons, we performed co-immunoprecipitation assays in GT1–7 cells using antibodies against



**FIGURE 4. Grg4 physically interacts with Otx2 in GT1–7 cells.** A, schematic showing the structure of Grg family proteins: glutamine-rich region (Q), glycine/proline-rich region (GP), serine/proline-rich region (SP), and tryptophan and aspartic acid repeats (WD40). B, cDNA from GN11 cells, GT1–7 cells, adult mouse hypothalamus, and adult mouse eye was analyzed by RT-PCR for expression of all Grg family members (Grgs 1–6). For details of primer sequences see Table 1. GAPDH (*GapDH*) was amplified as a positive control. C, representative co-immunoprecipitation assay performed using GT1–7 whole cell protein extracts. Endogenous Grg4 protein was co-immunoprecipitated from GT1–7 cells when proteins were immunoprecipitated (IP) with an Otx2 antibody. Otx2 and Grg4 were detected by Western blotting the immunoprecipitated proteins. Goat IgG was used as a control.

Otx2 and a representative long Grg (Grg4). Fig. 4C demonstrates that endogenous Otx2 and Grg4 proteins were co-immunoprecipitated from GT1–7 whole cell extracts when immunoprecipitation was performed using an Otx2 antibody. This indicates that Grg4 interacts with Otx2 in GnRH-expressing cells and suggests that members of the Grg family may modulate the role of Otx2 as an activator of GnRH transcription.

**Grg4 Represses Otx2-induced Activation of GnRH Expression via an HDAC-independent Mechanism**—Grg4 represses Oct-1 activation of the GnRH promoter and amplifies the repressive effects of Msx1 on GnRH transcription (10). Given this, along with our data showing that Otx2 and Grg4 physically interact in GT1–7 cells, it was important to investigate the functional consequences of an Otx2/Grg protein-protein interaction on GnRH promoter activity. Transient transfections performed in both GN11 and GT1–7 cells reveal that co-transfection of Grg4 with Otx2 results in complete ablation of the increase in GnRH promoter activity observed with Otx2 alone (Fig. 5, A and B). Interestingly, overexpression of Grg5 (confirmed by Western blotting analysis, see supplemental Fig. S1), a short Grg lacking the GP-, SP-, and WD40-domains (see Fig. 4A), had no significant effect on either GnRHe/p activity or Otx2-induced activation of GnRH transcription (Fig. 5, A and B). A reduction in Otx2-induced GnRH promoter activity is also seen when co-



**FIGURE 5. Grg4 represses Otx2-induced activation of GnRH expression.** GN11 (A) and GT1-7 (B) cells were co-transfected with the GnRHe/p-luciferase reporter and where indicated, Otx2, Grg4, or Grg5. GN11 (C) and GT1-7 (D) cells were co-transfected with either the  $-152$  bp Otx2 multimer-luciferase reporter ( $-152$ -bp multimer) or the  $-198$  bp Otx2 multimer-luciferase reporter ( $-198$ -bp multimer) and where indicated Otx2 or Grg4. The -fold induction compared with control (empty vector) is shown, corrected for  $\beta$ -galactosidase, which was used as an internal control. All transfections were performed in triplicate and repeated three times. Results shown are average  $\pm$  S.E. Statistical analysis was performed using one-way ANOVA: \*, significant difference ( $p < 0.01$ ) relative to control (empty vector); #, significant difference ( $p < 0.01$ ) relative to Otx2 expression vector alone.

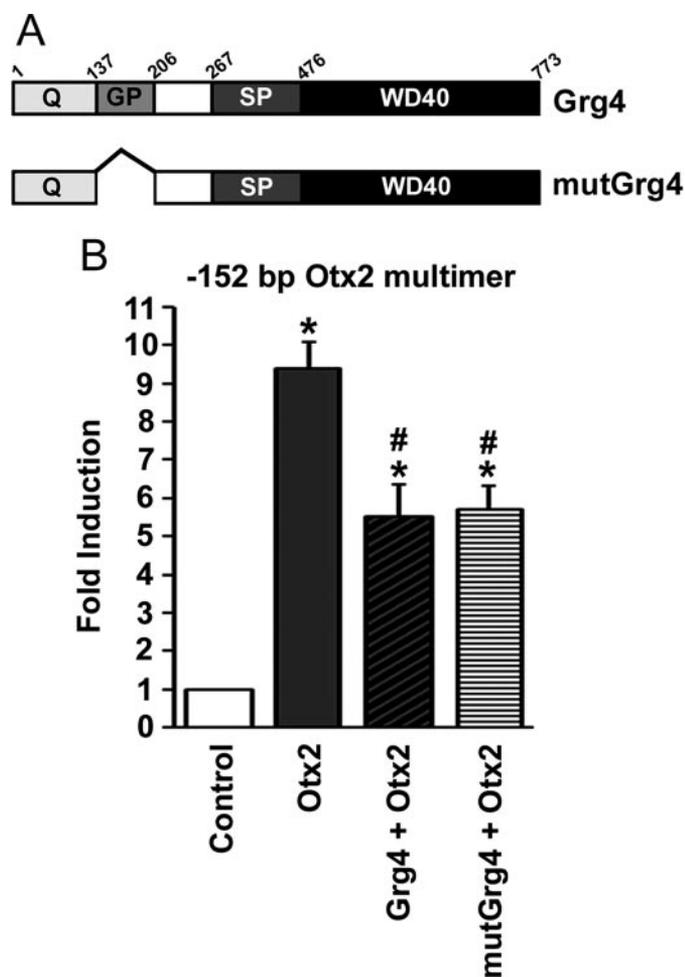
transfections of Grg4 and Otx2 are performed with the  $-152$  bp Otx2-multimer reporter (Fig. 5, C and D). However, unlike with the GnRHe/p reporter, Otx2 activation of the multimer is not completely ablated by Grg4, suggesting that Grg4 is also acting at other regions of the GnRH enhancer and promoter to achieve maximal repression of GnRH transcription. Surprisingly, Grg4 is unable to repress the increase in GnRH promoter activity seen when co-transfections are performed with the  $-198$ -bp Otx2-multimer reporter, suggesting that although both sites are capable of being activated by Otx2, the specific sequence

found at  $-198$  bp is insufficient to support negative regulation by Grg4 (Fig. 5, C and D).

Groucho-related co-repressors mediate embryonic patterning and neurogenesis via a variety of methods, including disruption of activator complexes, recruitment of histone deacetylases, and remodeling of chromatin (35, 54, 55). To determine whether Grg-mediated repression is occurring via an HDAC-dependent mechanism, we examined the effects of a Grg4 protein lacking the HDAC-recruiting GP-domain (Fig. 6A) on Otx2-induced activation of the  $-152$ -bp Otx2-multimer reporter. Both wild-type Grg4 (wtGrg4) and mutant Grg4 (mutGrg4) were able to significantly repress Otx2-induced promoter activity (Fig. 6B) indicating that Grg-mediated repression is occurring via an HDAC-independent mechanism.

*Mutation of the Otx2 WSPA Motif Relieves Grg4-mediated Repression by Preventing Physical Interaction of the Two Proteins*—Grg proteins have been shown to interact with a variety of transcription factors (27). The WRPW peptide motif, first identified in Hairy-related proteins, and the FLXXIL peptide motif, commonly referred to as the engrailed homology domain, have both been shown to be important for recruitment of Grg proteins (56–58). Analysis of the Otx2 peptide sequence revealed the presence of a WSPA site (amino acids 152–155) with significant homology to both the Hairy-related WRPW motif and engrailed homology domain (Fig. 7A). To assess the importance of this motif for Grg4-mediated repression, we mutated the WSPA site and co-transfected mutant Otx2 (mutOtx2, WSPA mutated to AAAA) and Grg4 plasmids into GN11 cells, which do not express endogenous Otx2. Transfection of mutant Otx2 alone resulted in a  $\sim 3$ -fold activation of the GnRHe/p (Fig. 7B) indicating that, as expected, mutation of this domain did not affect the ability of the protein to activate transcription. Interestingly, the activation of the GnRH promoter by mutant Otx2 alone was significantly greater than that observed in response to wild-type Otx2 alone (wtOtx2,  $\sim 2$ -fold increase). Surprisingly, co-transfection of Grg4 together with mutOtx2 resulted in a significant, 41%

## Grg4 Represses Otx2 Activation of GnRH



**FIGURE 6. Grg4 represses Otx2-induced activation of the GnRH promoter by an HDAC-independent mechanism.** *A*, schematic showing the structure of Grg4 proteins used to investigate whether the GP-domain is required for Grg4-mediated repression: glutamine-rich region (Q), glycine/proline-rich region (GP), serine/proline-rich region (SP), and tryptophan and aspartic acid repeats (WD40). Numbers indicate the amino acid position relative to the start methionine. *B*, GN11 cells were co-transfected with the -152-bp multimer and either Otx2, Grg4, or mutant Grg4 (mutGrg4). The -fold induction compared with control (empty vector) is shown, corrected for  $\beta$ -galactosidase, which was used as an internal control. All transfections were performed in triplicate and repeated three times. Results shown are average  $\pm$  S.E. Statistical analysis was performed using one-way ANOVA: \*, significant difference ( $p < 0.01$ ) relative to control (empty vector); #, significant difference ( $p < 0.01$ ) relative to Otx2 expression vector alone.

decrease, in GnRH promoter activity compared with mutant Otx2 alone. The levels of activation seen after co-transfection of mutant Otx2 and Grg4 (~1.8-fold increase) were comparable to those with wild-type Otx2 alone (~2-fold increase); however, they were significantly higher than those observed after co-transfection of wild-type Otx2 and Grg4 (Fig. 7B). We hypothesized that the partial decrease in GnRH promoter activity with mutant Otx2 and Grg4 was due to Grg4 exerting its repressive effects elsewhere within the GnRH regulatory region given that Grg co-repressors have been reported to interact with other GnRH transcriptional activators (10). This hypothesis was tested by co-transfection of Grg4 and mutant Otx2 together with the -152-bp Otx2-multimer reporter. Grg4 was unable to significantly reduce mutant Otx2-induced activation of this reporter (Fig. 7C) confirming that the Grg4-

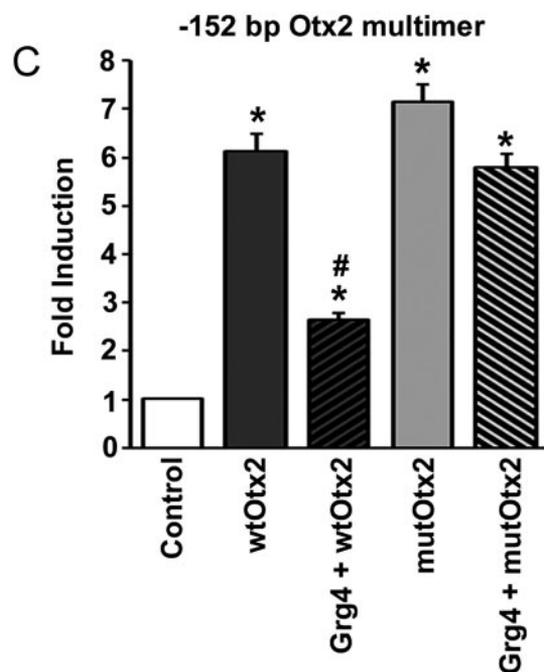
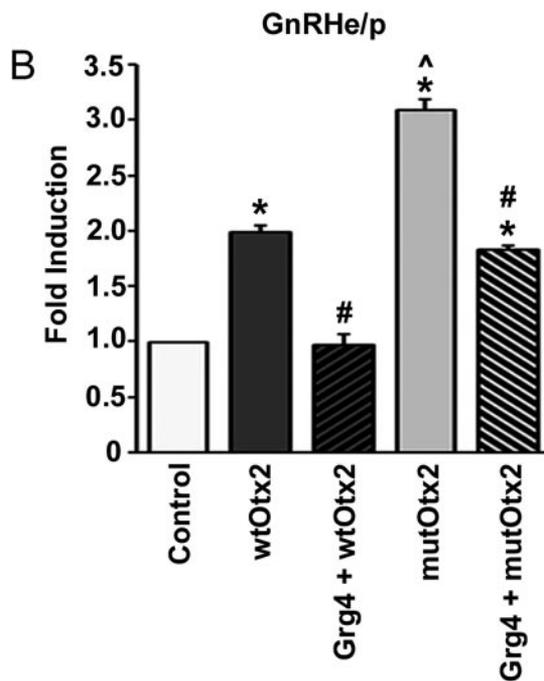
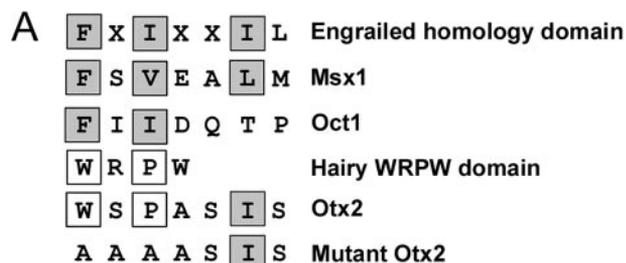
mediated partial decrease in Otx2-induced GnRH/p activity occurs due to Grg4 interacting with other transcriptional activators or repressors bound to the GnRH/p.

Finally, it was important to examine the ability of the mutant Otx2 protein to interact with Grg4 and its subcellular localization, because Otx2 can be localized to the inner nuclear layer in some cell types (59). Co-immunoprecipitation assays were performed with whole cell extract from NIH3T3 cells transfected with Grg4 and either wild-type Otx2, mutant Otx2 or a truncated Otx2 protein (NTermOtx2, Fig. 8A). Although all three Otx2 proteins co-localized with Grg4 in the nucleus (Fig. 8B), only wild-type Otx2 was capable of physically interacting with Grg4 (Fig. 8C). Unsurprisingly, an Otx2 protein lacking the C terminus 142 amino acids (NTermOtx2) was unable to interact with Grg4 (Fig. 8C). Furthermore, mutation of the first three amino acids of the Otx2 WSPA site was sufficient to prevent the two proteins from interacting (GFP-mutOtx2, Fig. 8C). Taken together, these results demonstrate that the Otx2 WSPA motif is critical for interaction with Grg4 and, therefore, crucial for the repressive role of Grgs on Otx2-induced activation of the GnRH promoter.

## DISCUSSION

Otx2 is required for regionalization and patterning of the developing brain and plays an important role in establishing the identity and fate of progenitor neurons. The interaction of Otx2 with cofactors, together with a specific spatial and temporal expression pattern, controls the effects of Otx2 on the activity of a given promoter (20–22). Reports detailing the co-localization of Otx2 and GnRH during both development and adulthood, along with data identifying Otx2-target binding sites within the GnRH regulatory region, have established Otx2 as an important factor in the regulation of GnRH transcription (7, 60). In this report, we have studied the regulation of GnRH expression by Otx2 in more detail and describe for the first time the identification and characterization of Grg proteins as co-repressors of Otx2-induced GnRH transcription.

Groucho-related co-repressors (Grg1, -2, -3, and -4) are thought to repress promoter activity by down-regulating the induction of target genes by transcriptional activators, enhancing the effects of transcriptional repressors or converting transcriptional activators to repressors (27). Our findings reveal that endogenous Grg4 and Otx2 proteins interact in GT1–7 cells and demonstrate that Grg4 represses Otx2-induced activation of both a GnRH reporter containing the rat enhancer and promoter regions and a reporter consisting of multiple copies of the Otx2 bicoid-like binding site. This suggests that Grg4 is not modulating GnRH transcription by down-regulating Otx2 expression, nor is it only augmenting the effects of known GnRH transcriptional repressors such as Msx1. Rather, these data indicate that Grg4 is either sequestering Otx2 away from the DNA, thus preventing activation of the promoter, or that interaction of Grg4 and Otx2 on the DNA blocks transcription from occurring. Because Grg4, like Otx2, has been shown to interact with the GnRH promoter chromatin *in vivo* (10), we hypothesize that, instead of sequestering Otx2 away from the DNA, Grg4 is recruited to DNA-bound Otx2 resulting in an attenuation of GnRH transcription. Long term repression by



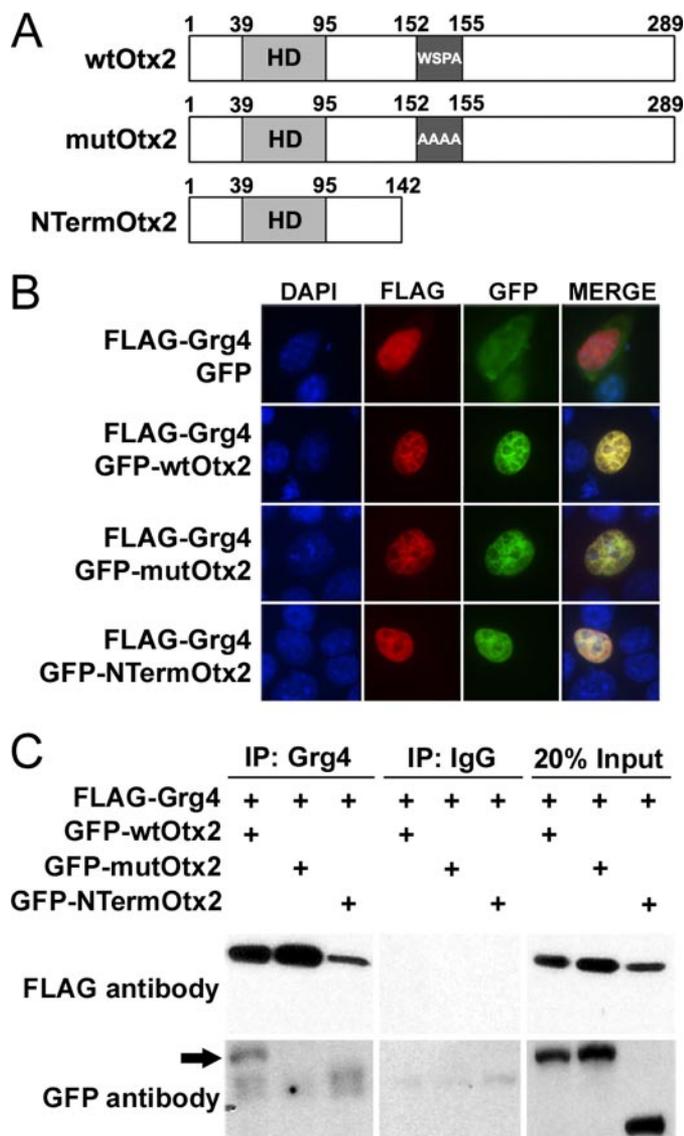
**FIGURE 7. Mutation of the Otx2 WSPA motif relieves Grg4-mediated repression of GnRH transcription.** *A*, analysis of Otx2 peptide sequence identifies a region between amino acids 152 and 155, which shows significant similarity to the WRPW motif found in Hairy-related proteins and that is known to be important for recruitment of Grg proteins. Gray boxes indicate similarity between amino acids, white boxes indicate identity. *B*, GN11 cells

Grgs is thought to involve the recruitment of HDACs, via the GP-domain of Grg protein, which is highly conserved between all the long Grgs (35). However, in this report, we demonstrate that a mutant Grg4 protein, in which the GP-domain has been deleted, remains capable of repressing Otx2-induced transcription as efficiently as wild-type Grg4. This suggests that, as in the case of Grg4-mediated repression of Pax2 and Oct-2 activation (61, 62), repression occurs via a HDAC-independent mechanism. In the case of Pax2, phosphorylation of Pax2 by c-Jun N-terminal kinase is blocked by association with Grg4, decreasing the transcriptional activity of Pax2. Analogously, Grg4 may inhibit Otx2 activity by preventing its post-translational modification. However, further investigation is required to determine whether protein modifications, such as phosphorylation, control the activity of Otx2.

Interestingly, although Grg4 was able to repress Otx2-induced activation of the GnRHe/p and the -152-bp multimer, it was unable to exert any repressive effects on Otx2 activation of the -198-bp multimer. Bioinformatic analysis reveals that both sites are conserved in various mammals. However, only the binding site at -152 bp is a 100% match to a consensus Otx2-binding site sequence. Otx2 has been shown to bind the core elements contained within both of the sites, but has a greater affinity for the TAATCCC element found at -152 bp (63), and our *in vitro* experiments confirmed this. Although we were able to demonstrate that overexpression of Otx2 significantly activates both multimers, and *in vitro* translated Otx2 protein binds to both sites, we did not see the formation of an Otx2 protein complex on the -198-bp probe when using GT1-7 cell nuclear extracts, suggesting that, although the site is capable of binding Otx2, and being activated by it, it preferentially binds other nuclear proteins in EMSAs. Although chromatin immunoprecipitation experiments demonstrated that Otx2 interacts with the GnRH promoter in GT1-7 cells, the proximity of the two sites prevented us from determining whether Otx2 is capable of binding to both sites *in vivo* and, if so, whether the -152-bp site was preferentially bound. We hypothesize that the differential effects of Grg4 on the two multimers may be explained by the affinity with which Otx2 binds to the DNA, as is observed with the interaction of Pax2 and Groucho (61). Weak binding of Otx2 to the DNA may affect the stability of the Otx2-Grg4 interaction and thus the ability of Grg4 to exert its repressive effects.

were transfected with the GnRHe/p-luciferase reporter and either wild-type Otx2 (wtOtx2), mutant Otx2 (mutOtx2), or Grg4. The WSPA site within the Otx2 protein has been mutated to AAAA in mutOtx2. The -fold induction compared with control (empty vector) is shown, corrected for  $\beta$ -galactosidase, which was used as an internal control. Statistical analysis was performed using one-way ANOVA: \*, significant difference ( $p < 0.01$ ) relative to control (empty vector); ^, significant difference ( $p < 0.01$ ) relative to wtOtx2 expression vector alone; #, significant difference ( $p < 0.01$ ) relative to either wtOtx2 or mutOtx2 expression vector alone. *C*, GN11 cells were co-transfected with the -152 bp Otx2 multimer-luciferase reporter (-152-bp multimer) and, where indicated, wild-type Otx2 (wtOtx2), mutant Otx2 (mutOtx2), or Grg4. The -fold induction compared with control (empty vector) is shown, corrected for  $\beta$ -galactosidase, which was used as an internal control. Statistical analysis was performed using one-way ANOVA: \*, significant difference ( $p < 0.01$ ) relative to empty expression vector; #, significant difference ( $p < 0.01$ ) relative to Otx2 expression vector alone. All transfections were performed in triplicate and repeated three times. Results shown are average  $\pm$  S.E.

## Grg4 Represses Otx2 Activation of GnRH



**FIGURE 8. Mutation of the Otx2 WSPA motif prevents physical interaction of Otx2 and Grg4.** *A*, schematic showing the structure of Otx2 proteins used to investigate binding between Grg4 and Otx2. The WSPA site has been mutated to AAAA in mutant Otx2 (*mutOtx2*). The C terminus 147 amino acids, containing the WSPA site, have been removed from the truncated Otx2 protein (*NTermOtx2*). Numbers indicate amino acid position relative to the start methionine; HD, homeodomain. *B*, NIH3T3 cells were transfected with Grg4-FLAG (*red*) and either GFP-empty vector, GFP-wtOtx2, GFP-mutOtx2, or GFP-NTermOtx2 (*green*). 4',6-diamidino-2-phenylindole (DAPI) staining (*blue*) was used to visualize the nuclei of fixed cells. All proteins, with the exception of that produced by GFP-empty vector, co-localized with Grg4-FLAG in the nucleus. *C*, representative co-immunoprecipitation assay performed using whole cell protein extracts from NIH3T3 cells transfected with FLAG-Grg4 and either GFP-wtOtx2, GFP-mutOtx2, or GFP-NTermOtx2. Proteins were immunoprecipitated (IP) with either a Grg4 antibody or rabbit IgG control. Otx2 and Grg4 were detected by Western blotting the immunoprecipitated proteins with a FLAG antibody, to detect Grg4, and a GFP antibody to detect Otx2. Wild-type Otx2 protein (indicated by an arrow), but not mutOtx2 or NTermOtx2, was co-immunoprecipitated from NIH3T3 cells when proteins were immunoprecipitated with a Grg4 antibody.

Previous studies have shown that Hairy-related proteins interact with Grg family members and that deletion of the Hairy protein WRPW motif is sufficient to prevent this physical interaction (57). Otx2 contains a sequence homologous to the WRPW motif, and we have shown that mutation of this domain relieves Grg4-mediated repression of Otx2-induced GnRH

transcription by preventing physical interaction of the two proteins. A recent report shows that the WD40 repeats of Grg4 are required for physical interaction of Grg4 and Otx2 (53). Therefore, it is likely that deletion or mutation of the Grg4 WD40-domain would also relieve Grg4-mediated repression of the GnRH promoter. Recently, Buscarlet *et al.* (56) reported that the anti-neurogenic properties of long Grg proteins are reliant upon the interaction of the Grg protein WD40-domain, with WRPW motif-containing proteins. Overexpression of long Grg proteins results in the inhibition of neuronal development *in vivo* and decreases neuronal differentiation of progenitor cells (64, 65). Interestingly, Otx2 has been reported to control the identity and fate of neuronal precursors by suppressing their differentiation (66). However, it has not been established whether this effect is mediated by a co-repressor, or is a consequence of Otx2 acting alone.

The anti-neurogenic properties of both long Grg proteins and Otx2 are of particular interest given that GnRH-producing neurons have a unique cellular origin within the olfactory placode. During embryogenesis, they migrate from the olfactory placode, across the cribriform plate, and into the basal forebrain to arrive at their final destination within the hypothalamus (67, 68). The factors that specify the onset of GnRH expression and the initiation of these neurons on their migratory pathway have yet to be determined, however, the birth of these cells may be mediated by the spatiotemporal expression of transcription factors. Given that Otx2 expression is observed within the olfactory placode at the time of GnRH neuronal birth, and co-localizes with GnRH during development and into adulthood, it would be interesting to determine the role of Otx2 in GnRH neuronal development and GnRH transcription. However, Otx2-null mice lack the entire forebrain and olfactory placode, preventing analysis of GnRH neurons. In contrast, heterozygotes display highly variable phenotypes with defects in craniofacial, ocular, and olfactory placode development (12, 18, 19). The varying degree of penetrance observed in these mice suggests that correct dosage of Otx2 is critical for normal development to occur, and this hypothesis is supported by data from human patients where a heterozygous loss-of-function mutation can have highly pleiotropic effects within an affected family. Furthermore, both duplication and deletion of the OTX2-containing region of human chromosome 14q22 lead to developmental abnormalities (69, 70), whereas specific mutations can cause a number of deficiencies including hypopituitarism, which includes low gonadotropins (71, 72). Ongoing studies are utilizing a GnRH-neuron-specific conditional Otx2 knockout approach to determine the role of Otx2 in GnRH neuronal development and regulation of GnRH transcription in the adult. Because expression of Grg family members also co-localizes with GnRH during development (10), it would also be interesting to study the influence of Otx2 and Grg protein interactions on the differentiation and migration of GnRH neurons. Unfortunately, the ability to study Grg protein function during development is also complicated due to the presence of multiple family members that are expressed in overlapping domains, making loss-of-function analyses difficult because of redundancy issues. Because long Grg proteins and Otx2 interact via the Grg WD40-domain, this may explain why no difference in

GnRH promoter activity was observed when co-transfecting Otx2 with Grg5, which lacks the GP-domain, SP-domain, and WD40 repeats. It also suggests that other long Grg family members could negatively regulate Otx2 activation of the GnRH promoter. Indeed, analysis of mRNA expression reveals that all of the Grg family members, apart from Grg2, are expressed in both GnRH neuronal cell lines, indicating that they may be interchangeable in repression of Otx2.

In conclusion, we have explored novel interactions between the homeodomain-containing protein Otx2 and members of the Groucho-related gene family of proteins. We have demonstrated that Grg4 physically interacts with Otx2 resulting in repression of GnRH promoter activity via an HDAC-independent mechanism. We hypothesize that modulation of the interactions of Otx2 with co-repressors and/or co-activators may contribute to the dynamic control of GnRH gene expression that is necessary during development and for reproduction. This modulation of Otx2 activity may be especially critical during puberty and in the control of the estrous cycle.

*Acknowledgments*—We thank Antonio Simeone for providing the pKW2T-Grg4-FLAG plasmid. We also thank Darren Logan for assistance with bioinformatic analysis and critical reading of the manuscript, Nichol Miller and Derina Sweeney for critical reading of the manuscript, and members of the Mellon laboratory for helpful discussion and support throughout this work. DNA sequencing was performed by the DNA-sequencing shared resource, UCSD Cancer Center, which is funded in part by NCI Cancer Center Support Grant P30 CA023100.

## REFERENCES

- Mellon, P. L., Windle, J. J., Goldsmith, P. C., Padula, C. A., Roberts, J. L., and Weiner, R. I. (1990) *Neuron* **5**, 1–10
- Radovick, S., Wray, S., Lee, E., Nicols, D. K., Nakayama, Y., Weintraub, B. D., Westphal, H., Cutler, G. B., and Wondisford, F. E. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 3402–3406
- Wetsel, W. C., Mellon, P. L., Weiner, R. I., and Negro-Vilar, A. (1991) *Endocrinology* **129**, 1584–1595
- Wetsel, W. C., Valença, M. M., Merchenthaler, I., Liposits, Z., López, F. J., Weiner, R. I., Mellon, P. L., and Negro-Vilar, A. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 4149–4153
- Eraly, S. A., and Mellon, P. L. (1995) *Mol. Endocrinol.* **9**, 848–859
- Givens, M. L., Rave-Harel, N., Goonewardena, V. D., Kurotani, R., Berdy, S. E., Swan, C. H., Rubenstein, J. L., Robert, B., and Mellon, P. L. (2005) *J. Biol. Chem.* **280**, 19156–19165
- Kelley, C. G., Lavorgna, G., Clark, M. E., Boncinelli, E., and Mellon, P. L. (2000) *Mol. Endocrinol.* **14**, 1246–1256
- Kepa, J. K., Spaulding, A. J., Jacobsen, B. M., Fang, Z., Xiong, X., Radovick, S., and Wierman, M. E. (1996) *Nucleic Acids Res.* **24**, 3614–3620
- Lawson, M. A., Whyte, D. B., and Mellon, P. L. (1996) *Mol. Cell. Biol.* **16**, 3596–3605
- Rave-Harel, N., Miller, N. L., Givens, M. L., and Mellon, P. L. (2005) *J. Biol. Chem.* **280**, 30975–30983
- Whyte, D. B., Lawson, M. A., Belsham, D. D., Eraly, S. A., Bond, C. T., Adelman, J. P., and Mellon, P. L. (1995) *Mol. Endocrinol.* **9**, 467–477
- Acampora, D., Mazan, S., Lallemand, Y., Avantagegiato, V., Maury, M., Simeone, A., and Brûlet, P. (1995) *Development* **121**, 3279–3290
- Simeone, A., Acampora, D., Mallamaci, A., Stornaiuolo, A., D'Apice, M. R., Nigro, V., and Boncinelli, E. (1993) *EMBO J.* **12**, 2735–2774
- Frantz, G. D., Weimann, J. M., Levin, M. E., and McConnell, S. K. (1994) *J. Neurosci.* **14**, 5725–5740
- Rhinn, M., Dierich, A., Shawlot, W., Behringer, R. R., Le Meur, M., and Ang, S. L. (1998) *Development* **125**, 845–856
- Puelles, E., Annino, A., Tuorto, F., Usiello, A., Acampora, D., Czerny, T., Brodski, C., Ang, S. L., Wurst, W., and Simeone, A. (2004) *Development* **131**, 2037–2048
- Fossat, N., Courtois, V., Chatelain, G., Brun, G., and Lamonerie, T. (2005) *Dev. Dyn.* **233**, 154–160
- Ang, S. L., Jin, O., Rhinn, M., Daigle, N., Stevenson, L., and Rossant, J. (1996) *Development* **122**, 243–252
- Matsuo, I., Kuratani, S., Kimura, C., Takeda, N., and Aizawa, S. (1995) *Genes Dev.* **9**, 2646–2658
- Danno, H., Michiue, T., Hitachi, K., Yukita, A., Ishiura, S., and Asashima, M. (2008) *Proc. Natl. Acad. Sci. U. S. A.* **105**, 5408–5413
- Mochizuki, T., Karavanov, A. A., Curtiss, P. E., Ault, K. T., Sugimoto, N., Watabe, T., Shiokawa, K., Jamrich, M., Cho, K. W., Dawid, I. B., and Taira, M. (2000) *Dev. Biol.* **224**, 470–485
- Nakano, T., Murata, T., Matsuo, I., and Aizawa, S. (2000) *Biochem. Biophys. Res. Commun.* **267**, 64–70
- Koop, K. E., MacDonald, L. M., and Lobe, C. G. (1996) *Mech. Dev.* **59**, 73–87
- Leon, C., and Lobe, C. G. (1997) *Dev. Dyn.* **208**, 11–24
- Dehni, G., Liu, Y., Husain, J., and Stifani, S. (1995) *Mech. Dev.* **53**, 369–381
- Mallo, M., Franco del Amo, F., and Gridley, T. (1993) *Mech. Dev.* **42**, 67–76
- Gasperowicz, M., and Otto, F. (2005) *J. Cell. Biochem.* **95**, 670–687
- Cinnamon, E., and Paroush, Z. (2008) *Curr. Opin. Genet. Dev.* **18**, 435–440
- Buscarlet, M., and Stifani, S. (2007) *Trends. Cell Biol.* **17**, 353–361
- Jennings, B. H., and Ish-Horowitz, D. (2008) *Genome Biol.* **9**, 205
- Dasen, J. S., Barbera, J. P., Herman, T. S., Connell, S. O., Olson, L., Ju, B., Tollkuhn, J., Baek, S. H., Rose, D. W., and Rosenfeld, M. G. (2001) *Genes Dev.* **15**, 3193–3207
- Grbavec, D., and Stifani, S. (1996) *Biochem. Biophys. Res. Commun.* **223**, 701–705
- Zhu, C. C., Dyer, M. A., Uchikawa, M., Kondoh, H., Lagutin, O. V., and Oliver, G. (2002) *Development* **129**, 2835–2849
- Pinto, M., and Lobe, C. G. (1996) *J. Biol. Chem.* **271**, 33026–33031
- Chen, G., Fernandez, J., Mische, S., and Courey, A. J. (1999) *Genes Dev.* **13**, 2218–2230
- Miyasaka, H., Choudhury, B. K., Hou, E. W., and Li, S. S. (1993) *Eur. J. Biochem.* **216**, 343–352
- Lepourcelet, M., and Shivdasani, R. A. (2002) *J. Biol. Chem.* **277**, 47732–47740
- Swingler, T. E., Bess, K. L., Yao, J., Stifani, S., and Jayaraman, P. S. (2004) *J. Biol. Chem.* **279**, 34938–34947
- Wang, W., Wang, Y. G., Reginato, A. M., Glotzer, D. J., Fukai, N., Plotkina, S., Karsenty, G., and Olsen, B. R. (2004) *Dev. Biol.* **270**, 364–381
- Larder, R., Karali, D., Nelson, N., and Brown, P. (2006) *Endocrinology* **147**, 5676–5689
- Larder, R., Chang, L., Clinton, M., and Brown, P. (2004) *Biol. Reprod.* **71**, 828–836
- McGillivray, S. M., Bailey, J. S., Ramezani, R., Kirkwood, B. J., and Mellon, P. L. (2005) *Endocrinology* **146**, 2180–2185
- Cherrington, B. D., Bailey, J. S., Diaz, A. L., and Mellon, P. L. (2008) *Mol. Cell. Endocrinol.* **295**, 106–114
- Gross, D. S., and Garrard, W. T. (1988) *Annu. Rev. Biochem.* **57**, 159–197
- Kouzarides, T. (2007) *Cell* **128**, 693–705
- Li, B., Carey, M., and Workman, J. L. (2007) *Cell* **128**, 707–719
- Nelson, S. B., Eraly, S. A., and Mellon, P. L. (1998) *Mol. Cell. Endocrinol.* **140**, 151–155
- Rave-Harel, N., Givens, M. L., Nelson, S. B., Duong, H. A., Coss, D., Clark, M. E., Hall, S. B., Kamps, M. P., and Mellon, P. L. (2004) *J. Biol. Chem.* **279**, 30287–30297
- Kim, H. H., Wolfe, A., Cohen, R. N., Eames, S. C., Johnson, A. L., Wieland, C. N., and Radovick, S. (2007) *Mol. Endocrinol.* **21**, 457–471
- Givens, M. L., Kurotani, R., Rave-Harel, N., Miller, N. L., and Mellon, P. L. (2004) *Mol. Endocrinol.* **18**, 2950–2966
- Chatelain, G., Fossat, N., Brun, G., and Lamonerie, T. (2006) *J. Mol. Med.* **84**, 604–615

## Grg4 Represses Otx2 Activation of GnRH

52. Chen, G., and Courey, A. J. (2000) *Gene* **249**, 1–16
53. Heimbucher, T., Murko, C., Bajoghli, B., Aghaallaei, N., Huber, A., Stebegg, R., Eberhard, D., Fink, M., Simeone, A., and Czerny, T. (2007) *Mol. Cell. Biol.* **27**, 340–351
54. Yu, X., Li, P., Roeder, R. G., and Wang, Z. (2001) *Mol. Cell. Biol.* **21**, 4614–4625
55. Sekiya, T., and Zaret, K. S. (2007) *Mol. Cell* **28**, 291–303
56. Buscarlet, M., Perin, A., Laing, A., Brickman, J. M., and Stifani, S. (2008) *J. Biol. Chem.* **283**, 24881–24888
57. Paroush, Z., Finley, R. L., Kidd, T., Wainwright, S. M., Ingham, P. W., Brent, R., and Ish-Horowicz, D. (1994) *Cell* **79**, 805–815
58. Tolkunova, E. N., Fujioka, M., Kobayashi, M., Deka, D., and Jaynes, J. B. (1998) *Mol. Cell. Biol.* **18**, 2804–2814
59. Fossat, N., Le Greneur, C., Béby, F., Vincent, S., Godement, P., Chatelain, G., and Lamonerie, T. (2007) *BMC Dev. Biol.* **7**, 122
60. Mallamaci, A., Di Blas, E., Briata, P., Boncinelli, E., and Corte, G. (1996) *Mech. Dev.* **58**, 165–178
61. Cai, Y., Brophy, P. D., Levitan, I., Stifani, S., and Dressler, G. R. (2003) *EMBO J.* **22**, 5522–5529
62. Malin, S., Linderson, Y., Almqvist, J., Ernberg, I., Tallone, T., and Pettersson, S. (2005) *Nucleic Acids Res.* **33**, 4618–4625
63. Briata, P., Ilengo, C., Bobola, N., and Corte, G. (1999) *FEBS Lett.* **445**, 160–164
64. Yao, J., Liu, Y., Lo, R., Tretjakoff, I., Peterson, A., and Stifani, S. (2000) *Mech. Dev.* **93**, 105–115
65. Nuthall, H. N., Joachim, K., and Stifani, S. (2004) *Mol. Cell. Biol.* **24**, 8395–8407
66. Puelles, E., Acampora, D., Gogoi, R., Tuorto, F., Papalia, A., Guillemot, F., Ang, S. L., and Simeone, A. (2006) *J. Neurosci.* **26**, 5955–5964
67. Schwanzel-Fukuda, M., and Pfaff, D. W. (1989) *Nature* **338**, 161–164
68. Schwanzel-Fukuda, M., Jorgenson, K. L., Bergen, H. T., Weesner, G. D., and Pfaff, D. W. (1992) *Endocr. Rev.* **13**, 623–634
69. Ou, Z., Martin, D. M., Bedoyan, J. K., Cooper, M. L., Chinault, A. C., Stankiewicz, P., and Cheung, S. W. (2008) *Am. J. Med. Genet. A* **146A**, 2480–2489
70. Wyatt, A., Bakrania, P., Bunyan, D. J., Osborne, R. J., Crolla, J. A., Salt, A., Ayuso, C., Newbury-Ecob, R., Abou-Rayyah, Y., Collin, J. R., Robinson, D., and Ragge, N. (2008) *Hum. Mutat.* **29**, E278–283
71. Diaczok, D., Romero, C., Zunich, J., Marshall, I., and Radovick, S. (2008) *J. Clin. Endocrinol. Metab.* **93**, 4351–4359
72. Ragge, N. K., Brown, A. G., Poloschek, C. M., Lorenz, B., Henderson, R. A., Clarke, M. P., Russell-Eggitt, I., Fielder, A., Gerrelli, D., Martinez-Barbera, J. P., Ruddle, P., Hurst, J., Collin, J. R., Salt, A., Cooper, S. T., Thompson, P. J., Sisodiya, S. M., Williamson, K. A., Fitzpatrick, D. R., van Heyningen, V., and Hanson, I. M. (2005) *Am. J. Hum. Genet.* **76**, 1008–1022