Published in final edited form as:
J Biol Chem. 2005 September 2; 280(35): 30975–30983. doi:10.1074/jbc.M502315200.

The Groucho-related Gene Family Regulates the Gonadotropin-releasing Hormone Gene through Interaction with the Homeodomain Proteins MSX1 and OCT1*

Naama Rave-Harel[‡,§], **Nichol L. G. Miller**[‡,¶,‖], **Marjory L. Givens**[‡,‖,**], and **Pamela L. Mellon**[‡,‡‡,§§]

[‡]Department of Reproductive Medicine, University of California, San Diego, La Jolla, California, 92093-0674

[‡‡]Department of Neurosciences, University of California, San Diego, La Jolla, California, 92093-0674

Abstract

Gonadotropin-releasing hormone (GnRH) is exclusively expressed in a unique population of hypothalamic neurons that controls reproductive function. GnRH gene expression is highly dynamic. Its transcriptional activity is regulated in a complex spatiotemporal manner during embryonic development and postnatal life. Although a variety of transcription factors have been identified as regulators of GnRH transcription, most are promiscuous in their DNA-binding requirements, and none are solely expressed in GnRH neurons. Their specific activity is probably determined by interactions with distinct cofactors. Here we find that the Groucho-related gene (GRG) family of co-repressors is expressed in a model cell line for the GnRH neuron and co-expresses with GnRH during prenatal development. GRG proteins associate *in vivo* with the GnRH promoter. Furthermore, GRG proteins interact with two regulators of GnRH transcription, the homeodomain proteins MSX1 and OCT1. Co-transfection experiments indicate that GRG proteins regulate GnRH promoter activity. The long GRG forms enhance MSX1 repression and counteract OCT1 activation of the GnRH gene. In contrast, the short form, GRG5, has a dominant-negative effect on MSX1-dependent repression. Taken together, these data suggest that the dynamic switch between activation and repression of GnRH transcription is mediated by recruitment of the GRG co-regulators.

The formation of unique transcription factor complexes determines the intricate spatial and temporal expression of genes during development as well as in terminal differentiation (1). An example of combinatorial regulation by multiple factors can be seen in cell-specific transcription of the gonadotropin-releasing hormone (GnRH)[1] gene. GnRH, a central regulator

*This work was supported in part by National Institutes of Health Grant R01 DK44838 (to P. L. M.).

§§To whom correspondence should be addressed: Dept. of Reproductive Medicine, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093-0674. Tel.: 858-534-1312; Fax: 858-534-1438; pmellon@ucsd.edu..

§Supported by the Lalor Foundation. Present address: Dept. of Bio-technology and Food Engineering, Technion, Haifa 32000, Israel.
¶Supported in part by National Institutes of Health Grant GM08666.

‖These two authors contributed equally to this work.

**Supported in part by National Institutes of Health Grant DA07315. Present address: Emory University, School of Public Health, 1518 Clifton Rd., Atlanta, GA 30322.

[1]The abbreviations used are: GnRH, gonadotropin-releasing hormone; AES, amino-terminal enhancer of split subgroup; DIG, digoxigenin; eh1, engrailed homology domain; GRG, Groucho-related gene; HSD, honestly significant difference; NLS, nuclear localization signal; Q, glutamine-rich domain; RSV, Rous sarcoma virus; TLE, transducin-like enhancer of split; GST, glutathione *S*-transferase.

of the hypothalamic-pituitary-gonadal axis of the reproductive system, is expressed in a discrete population of neuronal cells (2). These neurons, scattered throughout the basal hypothalamus in the adult (3), release GnRH in a pulsatile manner.

Due to the difficulties in studying the small and dispersed population of GnRH neurons, cultured cell models for GnRH neurons, the GT1-7 and NLT/Gn11 cell lines, were developed by targeted oncogenesis (4,5). These model cell lines provided the first insight into the transcriptional regulation of GnRH expression. Using these models, evolutionarily conserved enhancer and promoter elements conferring neuron-specific activation in culture were identified (−1863 to −1571 and −173 to +1, respectively, in the rat sequence) (6-9). Furthermore, these regulatory sequences were sufficient for targeting a substantial population of GnRH neurons in transgenic mice (10-14).

Interestingly, GnRH promoter activity appears to change during development in accordance with the location of the GnRH neurons. Whereas a low level of expression is detected in the nasal region, promoter activity dramatically increases as the neurons enter the anterior forebrain (15). Moreover, GnRH gene expression increases gradually, shortly after birth, preceding the increase in GnRH secretion that drives puberty (16). These data emphasize the need for highly flexible transcriptional regulatory mechanisms.

Thus far, the identification of transcriptional activators has contributed valuable information regarding the up-regulation of GnRH gene expression (9,17-21). The majority of these activators are homeodomain proteins, which typically have promiscuous DNA-binding properties. Their specific activity may therefore be determined by interactions with particular cofactors (22). Such cofactors can enhance or, alternatively, inhibit the interactions between the homeodomain proteins and the transcriptional machinery as well as the chromatin template and consequently alter the function of the transcription factors themselves (23). In line with this concept, the POU domain protein OCT1, an essential activator of GnRH transcription in the GT1-7 cells (20,24), was also shown to function as a downstream regulator in hormone-induced repression of the GnRH gene (25-27). Furthermore, DLX2, an activator, and MSX1, a transcriptional repressor, were shown to functionally antagonize each other by competing for the ATTA elements within the GnRH regulatory region (28-30). The dynamic exchange between activation and repression, observed with OCT1 and DLX2/MSX1 is probably facilitated by specific co-regulators. Intriguingly, none of the transcription factors shown yet to regulate the GnRH gene is exclusively expressed in the GnRH neuron (31,32). Transcriptional cofactors in this rare cell type might therefore be involved in promoting tissue-specific expression.

In the current study, we searched for OCT1 cofactors in the GT1-7 cells. This approach led to the isolation of GRG5, a member of the Groucho-related gene (GRG) family of co-regulators. We show that GRG family members physically and functionally interact with the homeodomain proteins MSX1 and OCT1 to regulate GnRH gene expression. Furthermore, the *Grg* family is co-expressed with GnRH during prenatal stages in the mouse and may contribute to early regulation of GnRH gene expression *in vivo*.

MATERIALS AND METHODS

Two-hybrid Interaction Screen in Yeast

To isolate cDNAs encoding GT1-7 proteins that associate with OCT1, we first created a randomly primed GT1-7 cDNA library (using poly(A)$^+$ mRNA) fused C-terminally to the transactivation domain of the VP16 protein. The yeast strains and expression vectors (generous gift of Dr. Michel Strubin) have been previously described (19,33). The GT1-7 cDNA library was introduced into the reporter strain using the method of Schiestl and Gietz (34), except that

the lithium acetate solution contained 1 M sorbitol, and that sheared herring testis DNA (10 mg/ml) (Clontech) was used as carrier DNA. After induction of the library proteins, cells were plated on galactose synthetic medium lacking histidine, containing 10 mM 3-aminotriazole, and assayed for OCT1-mediated stimulation of the selectable *His* gene transcription as previously described (19). Plasmids were recovered from the yeast using the method of Robzyk and Kassir (35). The sequences of the GT1-7 cDNA clones were determined by the chain termination method.

Antibody Production and Immunocytochemistry

GRG5 full-length cDNA was cloned into a pET28 vector containing the *Staphylococcus aureus* protein A zz domain fused to a His_6 tag (zz-pET28) to produce a zz-tagged fragment. Recombinant protein was expressed in bacteria, isolated on Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen), and used to immunize a rabbit (Covance). Antibody was affinity-purified against the same GRG5 protein coupled to CNBr-Sepharose (Amersham Biosciences). Cells were plated onto glass coverslips in 24-well tissue culture dishes at a density of 150,000 cells/well. Immunostaining was performed as described previously (36) with minor differences. Cells were blocked with 10% goat serum, stained with GRG5 antibody (1 h, 1:500 dilution), and detected with rhodamine-conjugated anti-rabbit IgG (30 min, 1:100 dilution; Molecular Probes, Inc., Eugene, OR). Following washes, coverslips were mounted in Vectashield solution containing 4′,6-diamidino-2-phenylindole for nuclear staining (Vector) and visualized with a Zeiss Axioskop 2 microscope.

In Situ Hybridization and Immunohistochemistry

In situ hybridization and immunohistochemistry were carried out as detailed by Rave-Harel *et al*. (19) with certain modifications. Mouse embryos were removed at 13.5 days postcoitum, embedded in paraffin, and sectioned at 7–10-μm thickness. Slides containing embryo sections were deparaffinized with xylene washes, hydrated in ethanol/water solutions, and digested with proteinase K for 7 min at 37 °C, followed by postfixation in 10% neutral buffered formalin for 20 min at room temperature. The sections were washed with 1× phosphate-buffered saline and 2× SSC for 5 min and then hybridized with digoxigenin-labeled (DIG) sense and antisense probes for *Grg1* or *Grg5*. The hybridized DIG-labeled probe was detected using alkaline phosphatase-conjugated anti-DIG antibody (Roche Applied Science) at a dilution of 1:2000 and visualized with the chromogen combination 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

Antisense and sense probes were created by incubating 1 μg of linearized plasmid DNA with 10× DIG RNA labeling mix (Roche Applied Science) as well as 5× transcription buffer (Promega) and the RNA polymerase T7, T3, or Sp6 for 2 h at 37 °C. The antisense *Grg1* probe corresponds to the sequence encoded from the 3′ end of the cDNA until the endogenous BglII site, and the antisense *Grg5* sequence corresponds to the entire cDNA, +1120 to +1 of the murine gene.

After the *in situ* hybridization, slides were subjected to immunohistochemical analysis. These slides were soaked in buffer to stabilize and retrieve the antigen (1 mM citric acid, 8 mM sodium citrate) for 20 min at 65 °C. Immunohistochemistry was then performed as previously described (11). The GnRH peptide was recognized with the LR1 antibody at a dilution of 1:2000 (gift of R. Benoit) and visualized using the horseradish peroxidase-conjugated ABC kit (Vector).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation assays were carried out as described previously (37) with a few modifications. Chromatin of GT1-7 cells was cross-linked for 10 min using formaldehyde.

The resulting chromatin solution was precipitated with polyclonal GRG1 and GRG4 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or acetyl-specific histone 3 antibody (Upstate Biotechnology, Inc., Lake Placid, NY). The following day, chromatin-antibody complexes were isolated from the solution by incubation with 50 μl of protein A-Sepharose beads (50% slurry, preblocked with 100 μg/ml sonicated *Escherichia coli* DNA and 1 mg/ml of bovine serum albumin) while being rocked at 4 °C for 2 h. The beads were harvested and washed as described previously (37). Cross-linking was reversed by the addition of NaCl to final concentration of 300 mM and incubation overnight at 65 °C. Chromatin-antibody complexes were eluted from the Sepharose beads by the addition of 10% SDS and proteinase K and subsequent incubation at 37 °C. The DNA was extracted with phenol/chloroform, precipitated with ethanol, and dissolved in Tris-EDTA buffer. Immunoprecipitated DNA was analyzed for the GnRH promoter sequence by PCR using primers for the evolutionarily conserved murine sequence, −253 to −19.

Protein Production and GST Retention Assay

Expression vectors used for protein production included the full-length human *Oct1* and the POU domain of *Oct*1 in the PCR2.1 vector, the mouse *Grg4* in pGEM vector, the mouse *Grg5* and *Grg5*Δ*Q* in the cytomegalovirus vector (38), and the mouse *Msx1* in the Bluescript vector (39). *In vitro* transcription and translation were performed with the Promega TNT coupled reticulocyte lysate system in accordance with the manufacturer's protocol, employing Sp6, T7, or T3 polymerase. [^{35}S]Methionine was used for labeling the protein products. Translation mix containing no vector was used as a control for unprogrammed translation in the reticulocyte lysate. GST fusion OCT1 and GRG5 were created by cloning the human *Oct1* and the mouse *Grg5* cDNAs into the pGEX-4T1 vector. GST-GRG4 and GST-GRG4Q (40) as well as GST-MSX1 and GST-MSX1ΔHD (39) were previously described. GST fusion proteins expressed in bacteria were bound to glutathione-Sepharose 4B resin (Amersham Biosciences) as previously described (19). The interaction assay was performed in accordance with the method described by Zappavigna *et al.* (41). Samples were separated using a 10% SDS-gel, after which the gel was fixed, soaked in Amplify (Amersham Biosciences), dried, and exposed to Eastman Kodak Co. X-BioMax film at −80 °C.

Co-Immunoprecipitation

GT1-7 cells were transfected with a FLAG-MSX1 expression vector and used for preparation of nuclear extracts, as previously described (42). Protein G-Sepharose™ 4 Fast Flow beads were incubated with either mouse monoclonal FLAG antibody (M2; Sigma) or normal rabbit IgG (Santa Cruz Biotechnology) for 2 h at 4 °C while rotating. After washing away unbound antibody, the protein G-Sepharose-antibody conjugates were incubated overnight with GT1-7 nuclear extract expressing FLAG-MSX1 at 4 °C while rotating in binding buffer (20 mM HEPES, pH 7.8, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1 mM dithiothreitol, 5% glycerol, 1% Triton X-100). After washing in buffer containing normal and higher salt concentration (250 mM NaCl), SDS sample buffer was added, and samples were incubated at 90 °C for 5 min. Samples were run on a 12% SDS-PAGE gel and transferred to polyvinylidene difluoride. The membrane was blocked with 5% dry milk in TBS-T and probed with anti-GRG1 (anti-TLE1, M101; Santa Cruz Biotechnology). An anti-rabbit IgG-horseradish peroxidase-linked secondary antibody (Amer-sham Biosciences) was then applied, and the signal was visualized using the SuperSignal® West Pico chemiluminescent substrate (Pierce).

Cell Culture and Transfections

GT1-7 cells were grown in mono-layer culture in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, penicillin (100 units/ml), streptomycin (0.1 mg/ml), and 4.5 mg/ml glucose in an atmosphere with 5% CO_2. Cells were transfected with Fugene (Roche

Applied Science) in 24-well multidishes. The expression plasmids used for co-transfections included the mouse *Grg5* in the FLAG-cytomegalovirus vector (43), the mouse *Grg4* in the pCi vector, the human *Oct1* in the pcDNA1.1 vector, the mouse *Msx1* in the pCB6+− vector (39), and empty vectors as negative controls. The reporter plasmids contained the GnRH enhancer (−1863 to −1571) fused to the RSV promoter (GnRHe/RSVp), the RSV enhancer fused to the GnRH promoter (−173 to +112) (RSVe/GnRHp), the RSV enhancer fused to the RSV promoter (RSVe/RSVp), and four copies of the region from −1802 to −1762 of the GnRH enhancer fused to the RSV promoter, each in a pGL3 vector driving luciferase expression. Cells were transfected with 100 ng of expression plasmids, 400 ng of reporter plasmid, and 200 ng of the internal control, herpes simplex virus thymidine kinase −109 promoter on *β*-galactosidase. Cells were harvested 48 h after the transfection, lysed, and assayed for luciferase and *β*-galactosidase expression as previously described (18).

RESULTS

The Groucho-related Gene Family Is Expressed in GT1-7 Cells

OCT1 and the other transcription factors, characterized thus far as regulators of GnRH gene transcription, have promiscuous DNA-binding specificities and are not exclusively expressed in GnRH neurons. Therefore, we performed a yeast two-hybrid assay to identify OCT1 cofactors that might facilitate restricted expression of GnRH (19). Using the method of Strubin *et al.* (33) with a GT1-7 cDNA library, we isolated a member of the Groucho-related gene family of cofactors, GRG5. Groucho-related proteins (GRG in rodents and transducin-like enhancer of split, TLE, in humans) are the vertebrate homologues of the *Drosophila* Groucho (44,45). These proteins interact directly with sequence-specific transcription factors in addition to histones (46) and the basal transcription machinery (47) and function as transcriptional co-repressors (38,40,43,48-52). The GRG family includes several characterized members, GRG1–GRG6. GRG5, a truncated version of these co-regulators, belongs to the amino-terminal enhancer of split (AES) subgroup (53). Having identified GRG5 in our yeast two-hybrid screen, we looked for the presence of GRG family members in the GT1-7 cells. By Western blot analyses, GRG1–GRG5 were expressed in the nuclear extract of GT1-7 cells as well as protein extract from embryonic mouse aged 9.5 days postcoitum, which was used as a positive control (53) (Fig. 1*A*). In addition, using immunocytochemistry, GRG5 was localized in the nucleus of GT1-7 cells (Fig. 1*B*). We also detected the presence of the GRG family members in another cell model for the GnRH neuron, NLT, by reverse transcription-PCR (data not shown). Therefore, several members of the GRG family are expressed in our model GnRH neuronal cell lines and may be available for complex formation with OCT1 in those cells.

Grg1 and Grg5 Co-localize with the GnRH Neurons during Embryonic Development

As components of the Notch signaling cascade, the Groucho-related genes are expressed during embryonic segmentation, neurogenesis, and epithelial differentiation (54-56). Transcripts of *Grg* family members are present early in embryonic development starting on day 6.5 postcoitum in the mouse. At later embryonic stages (12–16 days postcoitum), *Grg* transcripts are present in the olfactory epithelium, olfactory lobe, the ventricular zone of the brain and spinal cord, the outer layers of the cerebral cortex, the lung and kidney epithelia, and salivary gland (53,57). Whereas specific patterns of expression are seen during development, *Grg* transcripts are ubiquitously expressed in the adult mouse (53,55,57).

With the aim of defining *Grg* expression in relation to the expression of GnRH, we performed double *in situ* hybridization and immunohistochemistry on parasagittal sections of mouse embryos 13.5 days postcoitum. On this day of prenatal development, the entire population of GnRH neurons has been established, and the cells are migratory, leaving the vomeronasal organ, crossing the cribriform plate to enter the rostroventral forebrain (3). GnRH promoter

activity is highly dynamic at this stage (15). It was therefore important to confirm that GRG family members, as candidate cofactors for regulating dynamic promoter activity, co-localize with GnRH neurons at this time point in development.

Grg transcripts were expressed in the vomeronasal organ, olfactory epithelium, and the primordium of the septum (Fig. 2). Co-expression of *Grg1* (*purple*) and GnRH (*brown*) was seen in neurons located in the nasal region (Fig. 2*A*, *expanded panel*) and forebrain. Similarly, neurons co-expressing *Grg5* and GnRH were identified in the primordium of the septum and crossing the cribriform plate (Fig. 2*B*, *expanded panel*). Thus, *Grg1* and *Grg5* co-expressed with GnRH during prenatal stages of development. Interestingly, *Oct1* was also shown to co-localize with migratory GnRH neurons at this embryonic stage (19).

GRG1 and GRG4 Interact in Vivo with the GnRH Regulatory Region

The data presented thus far indicate that GRG family members are present in GnRH neurons *in vitro* and *in vivo*. To test whether the GRG co-regulators associate with the factors bound to the GnRH regulatory region *in vivo*, we performed chromatin immunoprecipitation assays. We conducted these assays in the GT1-7 cells with antibodies recognizing GRG1 and GRG4. We also included an antibody that recognizes acetylated histone 3 in our chromatin immunoprecipitation analyses to verify an open chromatin template in GT1-7 cells. We chose to target the GnRH proximal promoter because it was well characterized in the rat gene and is highly conserved between rat and mouse (6,24). PCR amplification of the sequences, immunoprecipitated by the antibodies, showed that both GRG antibodies precipitated the mouse GnRH promoter region in GT1-7 cells (Fig. 3). Moreover, the acetylated histone 3 associated with the GnRH promoter in GT1-7 cells but not in the pituitary gonadotrope immortalized LβT2 cells that do not produce GnRH. This indicates that *in vivo*, GRG1 and GRG4 are members of the transcription factor complexes that form on the GnRH promoter in an open chromatin state. Thus, they may play a role in transcriptional regulation of GnRH.

The GRG Proteins Interact with OCT1 and MSX1 in Vitro

Two forms of GRG proteins have been described thus far, the long form (GRG1 through 4, and GRG6 in the mouse) and the short form (the AES proteins, such as GRG5). The long GRGs consist of five characteristic domains (Fig. 4*A*). The carboxyl-terminal region of these proteins contains multiple WD40 repeats that are important for interaction with proteins involved in repression (44,45). The amino terminus of these proteins contains a highly conserved glutamine-rich (Q) domain that is required for tetramerization of GRG family members. In addition, a nuclear localization signal (NLS) motif and phosphorylation sites are located in the central region. Two domains implicated in repression activity, rich in both glycine and proline (GP) or in serine and proline (SP) flank the NLS. The GRG short forms (such as GRG5) are composed of the N-terminal region including the Q domain and a portion of the GP domain but lack the NLS, SP domain, and the entire WD40 domain (Fig. 4*A*).

The GRG family has been shown to interact with a variety of DNA-binding proteins, including transcription factors, chromatin high mobility group factors (58,59), and histones (46). The WRPW peptide motif and the F*XIXX*IL peptide motif in the engrailed homology domain (eh1) of such factors have been shown to be important for recruitment of the GRG proteins (54,60, 61). Previously, we identified MSX1 as a transcriptional repressor that binds to the regulatory regions of the GnRH gene and functionally antagonizes DLX2 activity (30). MSX1 is a homeodomain protein that contains an eh1 domain (amino acids 50–72; Fig. 4*A*) (38), making it another plausible candidate for interaction with GRG proteins. Therefore, to explore the possible interaction with MSX1 as well as confirming the interaction between GRG and OCT1 observed in the yeast screen, we performed GST pull-down assays. GRG family members, OCT1 and MSX1, were expressed *in vitro* as ^{35}S-labeled proteins and incubated with GST

fusion proteins immobilized on glutathione-Sepharose beads. As seen in Fig. 4*B*, GST-GRG4 and GST-GRG5 interact with OCT1 as well as MSX1 (*OCT1* and *MSX1 panels*, *lanes 3* and *4*), whereas GST-OCT1 interacts with GRG4 and GRG5 (*GRG4* and *GRG5 panels*, *lane 2*). GST-MSX1 also interacts with GRG4 (Fig. 4*B*, *GRG4 panel*, *lane 6*). As a negative control, green fluorescent protein was added to the interaction assay and did not precipitate with any of the GST-tagged proteins (Fig. 4*B*, *GFP panel*). Previous studies have mapped the interaction between the eh1 domain of the homeodomain proteins and the GRG proteins to the WD40 domain of the long GRG family members (38). Interestingly, this domain is not found in GRG5. Thus, we were interested in determining the GRG5 domain important for interaction with MSX1 and OCT1. The GRG Q domain has been previously implicated in protein-protein interactions (40,48); we therefore used a mutant GRG5, lacking the Q domain (GRG5ΔQ) in our pull-down assay. As seen in Fig. 4*B*, deletion of the Q domain of GRG5 prevented interaction with GSTOCT1 and GST-MSX1 as well as with the GRG family members GRG5 and GRG4 (Fig. 4*B*, *GRG5ΔQ panel*). Using a GSTGRG4 protein construct containing only the Q domain (GRG4Q), we confirmed that the Q domain is sufficient for the interaction with both OCT1 and MSX1 (Fig. 4*B*, *OCT1* and *MSX1 panels*, *lane 5*).

We also investigated the interaction of wild-type GRG proteins with a mutant MSX1, lacking the homeodomain and C-terminal region (G-MSX1ΔHD), in order to determine which of the MSX1 domains was necessary. Fig. 4*B* indicates that the N-terminal region of MSX1 is sufficient for MSX1-GRG4 interaction (Fig. 4*B*, *GRG4 panel*, *lane 7*). This region retains the MSX1 eh1 domain (amino acids 50–72); therefore, the interaction between the mutant MSX1 and the long GRGs was expected. We next examined the association of the GRG proteins with the POU domain of OCT1. Our results show that the POU domain is sufficient for OCT1-GRG5 dimerization, but not for OCT1 dimerization with the long GRG4 (Fig. 4*B*, *POU panel*, *lanes 3* and *4*). In this context, we note that whereas no GRG-recruiting motif has yet been identified in the OCT1 peptide (38), a conserved glutamine-rich Q domain is present in both OCT1 and OCT2 (Fig. 4*A*) (62). Although dimerization of the POU domain in solution was previously established (63), we did not observe interaction of the POU domain with GST-OCT1. It is possible that the context of the full-length OCT1 disrupts the interaction between the POU domains, whereas homodimerization of OCT1 may depend on the N-terminal and C-terminal regions. As expected, both OCT1 and MSX1 form homodimers (Fig. 4*B*, *OCT1 panel*, *lane 2*, and *MSX1 panel*, *lane 6*) (39,62), and interestingly, OCT1 and MSX1 appear to form heterodimers as well (Fig. 4*B*, *OCT1 panel*, *lane 6*, and *MSX1 panel*, *lane 2*). OCT1-MSX1 heterodimerization seems to be dependent on regions outside of the OCT1 POU domain and the MSX1 N-terminal domain (Fig. 4*B*, *OCT1 panel*, *lane 7*, and *POU panel*, *lanes 6* and *7*). We also observed an interaction between OCT1 and DLX2 (data not shown). In summary, the interaction between the GRG family and OCT1 or MSX1 requires the Q domain of the GRG proteins and is independent of the homeodomain and the C-terminal regions of the MSX1 protein. Moreover, the POU domain of OCT1 is sufficient for interaction with GRG5 but not GRG4.

The GRG Proteins Interact with MSX1 in Vivo

To further verify the physiological significance of the interactions observed *in vitro*, we tested whether endogenous GRG proteins specifically associate with MSX1 in a cell model for GnRH neurons. Using a FLAG-tagged MSX1 protein expressed in GT1-7 cells, we co-immunoprecipitated the tagged protein in addition to a protein complex recognized by the antibody against GRG1, a representative of the GRG long forms. As seen in Fig. 4*C*, the immunoprecipitated complex formed a prominent band that co-migrated with the GRG1 protein expressed in unprecipitated GT1-7 extract. As a negative control, an equivalent amount of normal rabbit IgG was added to the interaction assay and did not precipitate the GRG1 protein. These data demonstrate that GRG proteins associate with MSX1 in GnRH-expressing

cells. Therefore, the interaction between this family of co-repressors and the MSX1 repressor is probably relevant to the transcriptional regulation of GnRH expression *in vivo*.

GRG Proteins Regulate GnRH Gene Expression

The GRG proteins do not bind directly to DNA but instead form transcriptionally repressive complexes with DNA bound factors (44,45). Given the presence of the GRG family members in the GT1-7 cells (Fig. 1), their interaction with the GnRH promoter (Fig. 3), and their interaction with previously identified transcriptional regulators (Fig. 4, *B* and *C*), we investigated their effect on GnRH promoter activity. Transient transfections were conducted in GT1-7 cells using luciferase reporter vectors. Overexpression of GRG4 led to a 40% reduction in GnRH enhancer and promoter reporter activity in the context of a heterologous RSV element (GnRHe/RSVp and RSVe/GnRHp) (Fig. 5*A*). However, no statistically significant effect was observed with GRG4 in the presence of the RSV enhancer/promoter reporter. This suggests that GRG4 acts as a co-repressor of GnRH promoter expression in culture. We then explored the activity of the short form of the GRG family, GRG5. Overexpression of GRG5 did not result in a statistically different change in reporter activity but consistently trended toward an increase (up to 132%) (Fig. 5*A*). Moreover, co-transfection of GRG5 reversed the repressive activity of GRG4 on the GnRHe/RSVp reporter (Fig. 5*B*). Therefore, it is possible that transcription factor complexes that repress GnRH activity are in fact inhibited by GRG5 overexpression in GT1-7 cells. These results are in agreement with the previous observation that GRG5 acts as a dominant negative to the repressive activity of the full-length GRGs (43,64).

GRG4 Represses OCT1 Activation of GnRH Gene Expression

To verify the functional interaction between the GRG family and OCT1 in the regulation of GnRH transcription, we performed transient transfection assays in the GT1-7 cells. OCT1 is not known to be a strong transcriptional activator. Therefore, we created a reporter plasmid containing four copies of the essential OCT1 binding site in the enhancer (20) to increase the potential for OCT1 activation. This multibinding element was fused to the heterologous RSV promoter in order to obtain high levels of transcription. In this context, OCT1 activated reporter gene transcription by 170% (Fig. 6). Overexpression of GRG4 decreased reporter activity to 54%. Furthermore, OCT1-mediated activation was significantly decreased in the presence of GRG4. These data suggest that the GRG co-repressors can regulate OCT1-mediated activation of GnRH expression. Conversely, GRG5 had no significant effect on OCT1 activation of GnRH transcription (data not shown). Thus, it is possible that the interaction between OCT1 and GRG5 is involved in induced response rather than basal transcription. Alternatively, additional components, yet unidentified, may be required for function of the OCT1-GRG5 complex.

The GRG Family Regulates MSX1-mediated Repression of the GnRH Gene

MSX1 has been shown to repress GnRH promoter activity by binding to consensus homeodomain repeats (ATTA) in the enhancer and promoter (30). To determine the functional interaction between GRG5 and MSX1, we performed transient transfections in the GT1-7 cells using the GnRH enhancer coupled to the RSV promoter reporter. Overexpression of the MSX1 protein significantly reduced the activity of this reporter by 60% (Fig. 7). Interestingly, although GRG5 alone did not significantly affect GnRH expression, MSX1-dependent repression of the GnRH reporter was reversed by overexpression of GRG5 (Fig. 7*A*). These data suggest that GRG5 has a dominant negative effect on MSX1 repression. We then asked whether a long form of the GRG family, GRG4, might act as a co-repressor for MSX1 using the same vector. Overexpression of GRG4 significantly enhanced MSX1-mediated repression of GnRH reporter activity (Fig. 7*B*), indicating that GRG4 functions as a co-repressor of MSX1. As a control, overexpression of GRG5 or GRG4 had no effect on DLX2 activation through the

same ATTA sites, possibly due to the absence of an eh1 domain in DLX2 (data not shown). These data suggest that MSX1-dependent repression of GnRH expression is mediated through recruitment of GRG proteins to the GnRH regulatory region.

DISCUSSION

Cell-specific gene expression is often brought about by generally expressed transcription factors, forming a unique complex with specific co-regulators (65). Here we explore the role of the Groucho-related gene family of co-repressors in transcriptional regulation of the GnRH gene. We show that GRG family members, expressed in a specific pattern during early development (53,55,57), colocalize with migratory GnRH neurons (Fig. 2). Moreover, recruitment of the GRG proteins by the homeodomain transcription factors OCT1 or MSX1 leads to dynamic changes in GnRH gene expression in culture (Figs. 6 and 7). We therefore propose that GRG proteins function as co-regulators of GnRH transcription during prenatal development.

GRG proteins are thought to mediate long range repression. In other words, they are able to silence gene expression regardless of their distance, by kilobase pairs of DNA, from the enhancer region. This function may be accomplished by the formation of multiprotein complexes termed repressosomes (23). However, the mechanism by which the Groucho family represses transcription has not been clearly understood. The ability of GRG proteins to both oligomerize and interact with core histones (46) and, therefore, to polymerize along the template might explain their function in long range repression. Further, mounting evidence suggests that Groucho and its related proteins participate in chromatin remodeling by recruiting factors involved in histone deacetylation, such as histone deacetylase 1, Sin3, and RbAp48 (65,66). These findings connect GRG function with histone deacetylase activity and, thus, suggest that GRG repression occurs through remodeling of the chromatin structure. On the other hand, treatment with trichostatin A, an inhibitor of histone deacetylase activity, does not fully relieve the repressive activity of the GRG proteins (66,67). This indicates that chromatin remodeling is not the only means by which the Groucho family represses transcription but rather suggests GRG involvement in additional mechanisms of repression, such as inhibition of the basal transcriptional machinery. Interestingly, it was recently discovered that the GRG proteins interact directly with a component of the basal machinery, TFIIE (47), supporting a role for interaction with the basal machinery in GRG-mediated repression.

We have identified the transcription factors OCT1 and MSX1 as novel partners for the GRG proteins on the GnRH gene. Previous studies have mapped the interaction between the eh1 domain of the homeodomain proteins and the GRG proteins to the WD40 domain of the long GRG family members (38). Because the short form of the GRG family, GRG5, does not contain a WD40 domain (Fig. 4*A*), we explored the possibility that the Q domain might serve the same purpose. Using an *in vitro* protein interaction assay, we find that the Q domain of GRG5 is necessary, and that the Q domain of GRG4 is sufficient, for the interactions with both OCT1 and MSX1 (Fig. 4*B*). This is consistent with recent studies suggesting that the GRG Q domain functions in protein-protein interactions (40,48,68,69).

Intriguingly, OCT1 employs different domains for interaction with the long GRG form *versus* the short GRG form (Fig. 4*B*). The interaction between OCT1 and GRG5 maps to the POU domain, whereas GRG4 does not contact this domain. Identification of a Q domain, also serving as the activation domain, in the N-terminal region of OCT1 and -2 (62) suggests the possibility that the long GRG family members may interact with OCT1 through this region. Interestingly, the Q domain of OCT1 contains an eh1-like domain, FIISQTP (amino acids 206–212). This peptide has only one nonconserved change in amino acid, compared with the consensus F*X*I*XX*IL. This level of conservation is similar to the eh1-like domain of Dorsal

shown to mediate transcriptional repression and binding to Groucho (70). Thus, the interactions between the long GRGs and OCT1 may involve Q domain associations or, alternatively, requirement of the WD40 repeats by an eh1-like domain. Our results, which indicate sufficiency of the GRG4 Q-domain for interaction with OCT1, uphold the first possibility.

Given the common and promiscuous binding nature of the transcription factors OCT1 and MSX1, we hypothesized that their transcriptional activity might be regulated by the GRG co-factors. Indeed, we observed that the long GRGs enhance MSX1 repression and counteract OCT1 activation of the GnRH gene (Figs. 6 and 7). In contrast, the short form, GRG5, has a dominant-negative effect on MSX1-dependent repression (Fig. 7). Interestingly, clusters of binding sites for OCT1 and MSX1 are intermittently dispersed over the 3-kb regulatory region of the GnRH gene (9,30). This finding, together with the demonstrated physical (Fig. 4) and functional (Figs. 6 and 7) interaction between MSX1 or OCT1 and the GRG family, suggest that GRG proteins participate in long range repression of the *GnRH* gene.

Recent studies indicate that some DNA-binding proteins can function in both transactivation and transcriptional repression by recruiting specific co-repressor or co-activator complexes (40,48,71). This phenomenon may be attributed to the context of the binding site, both the cis- and trans-regulatory components. For example, the *Drosophila* protein, Tinman (NK-4) has been characterized as a transcriptional activator, which can form functional enhanceosome complexes with co-activators such as p300. However, it can also repress transcription of target genes through interaction with Groucho (48).

We hypothesize that such a phenomenon may also occur on the GnRH gene. In the case of OCT1, recruitment of PBX/PREP1 facilitates formation of activator complexes on the GnRH regulatory region (19), whereas the formation of repressive complexes would entail OCT1 recruitment of the GRG proteins (Fig. 8). Likewise, the antagonistic action of MSX1 and DLX2 through the same GnRH homeodomain elements may involve interactions with specific cofactors. MSX1 binding to these sites can recruit the GRG proteins to co-repress transcription of *GnRH*, whereas DLX2 binding may recruit co-activators to increase transcriptional activity. It is likely that the turnover of enhanceosome and repressosome complexes is coordinated on the OCT1 and MSX1 clustered sites, given their dispersed location and the ability of OCT1 to interact and cooperate with both activators and repressors (Fig. 4) (19,72-74). Perhaps GRG5, as a dominant-negative partner for the GRG co-repressors and MSX1, facilitates the transition from repressosome to enhanceosome. The dynamic formation of these enhanceosomes and repressosomes could serve as an efficient molecular response to developmental or environmental stimuli. In fact, such dynamic regulation of *GnRH* promoter activity has been reported during embryonic development (12,15), when we have found that *Msx1*, *Dlx2*, *Oct1*, *Pbx1*, *Grg1*, or *Grg5* and *GnRH* are co-expressed (Fig. 2) (19,30).

What is the physiological role of the Groucho-related proteins in GnRH regulation of the reproductive system? Interestingly, GRG5-null females display reproductive delay (75). Although this phenotype can be attributed to the pituitary abnormalities observed in these mice (76), giving the feedback loop regulation of the hypothalamic, pituitary, gonad axis, it may also represent an effect at the level of the GnRH neuron. In addition, transgenic expression of TLE1 (*e.g.* GRG1) in post-mitotic neurons inhibits neuronal development in the embryonic forebrain (77). These data suggest that the Groucho family may regulate differentiation of the GnRH neurons *in vivo*.

In conclusion, we have explored novel interactions between the Groucho family of co-repressors and the transcription factors OCT1 and MSX1. We show that GRG proteins interact with the GnRH regulatory region and mediate OCT1- or MSX1-dependent regulation of the

GnRH promoter. Moreover, we propose that these interactions occur during prenatal stages *in vivo* and may facilitate developmental regulation of *GnRH* gene expression.

Acknowledgments

We thank Corrinne Lobe for providing the *mGrg1* and *mGrg5* cDNA, Hans Clevers for providing the FLAG-*mGrg5* expression vector, Johan Ericson for providing the *Grg5* vectors for *in vitro* expression and *Grg4* cDNA, Abdallah Souabni for providing the GST-*Grg4* vectors, Cory Abate-Shen for the *Msx1* expression vector, and Shelley Nelson for providing the *mGrg4* expression vector. We thank Amnon Harel for advice and assistance in the production and purification of the GRG5 antibody and Douglass Forbes for use of the microscope. We thank Qingbo Tang for the creation of the *4xOct*/*RSVp* reporter plasmid. We thank Teri Williams, Brian Powl, Scott Anderson, Shannon Snyder, Laura Neely, and Rachel White for excellent technical assistance and Melody Clark for early contributions to this work. We thank Ze'ev Paroush and Stefano Stifani for discussions. We also thank Shelley Nelson and Djurdjica Coss for critical reading of the manuscript and members of the Mellon laboratory for discussions and support throughout this work.

REFERENCES

1. Wolberger C. Curr. Opin. Genet. Dev 1998;8:552–559. [PubMed: 9794820]
2. Vale W, Rivier C, Brown M. Annu. Rev. Physiol 1977;39:473–527. [PubMed: 15499]
3. Wray S, Grant P, Gainer H. Proc. Natl. Acad. Sci. U. S. A 1989;86:8132–8136. [PubMed: 2682637]
4. Mellon PL, Windle JJ, Goldsmith P, Pedula C, Roberts J, Weiner RI. Neuron 1990;5:1–10. [PubMed: 2196069]
5. Radovick S, Wray S, Lee E, Nicols DK, Nakayama Y, Weintraub BD, Westphal H, Cutler GB Jr. Wondisford FE. Proc. Natl. Acad. Sci. U. S. A 1991;88:3402–3406. [PubMed: 2014260]
6. Eraly SA, Mellon PL. Mol. Endocrinol 1995;9:848–859. [PubMed: 7476968]
7. Whyte DB, Lawson MA, Belsham DD, Eraly SA, Bond CT, Adelman JP, Mellon PL. Mol. Endocrinol 1995;9:467–477. [PubMed: 7659090]
8. Kepa JK, Spaulding AJ, Jacobsen BM, Fang Z, Xiong X, Radovick S, Wierman ME. Nucleic Acids Res 1996;24:3614–3620. [PubMed: 8836191]
9. Givens ML, Kurotani R, R.-H. N, Miller NLG, Mellon PL. Mol. Endocrinol 2004;18:2950–2966. [PubMed: 15319450]
10. Pape JR, Skynner MJ, Allen ND, Herbison AE. Mol. Endocrinol 1999;13:2203–2211. [PubMed: 10598592]
11. Lawson MA, MacConell LA, Kim J, Powl BT, Nelson SB, Mellon PL. Endocrinology 2002;143:1404–1412. [PubMed: 11897697]
12. Skynner MJ, Slater R, Sim JA, Allen ND, Herbison AE. J. Neurosci 1999;19:5955–5966. [PubMed: 10407034]
13. Suter KJ, Song WJ, Sampson TL, Wuarin JP, Saunders JT, Dudek FE, Moenter SM. Endocrinology 2000;141:412–419. [PubMed: 10614664]
14. Wolfe AM, Wray S, Westphal H, Radovick S. J. Biol. Chem 1996;271:20018–20023. [PubMed: 8702719]
15. Simonian SX, Herbison AE. Neuroendocrinology 2001;73:149–156. [PubMed: 11307033]
16. Ebling FJ, Cronin AS. Neuroreport 2000;11:R23–33. [PubMed: 11095489]
17. Kelley CG, Lavorgna G, Clark ME, Boncinelli E, Mellon PL. Mol. Endocrinol 2000;14:1246–1256. [PubMed: 10935548]
18. Lawson MA, Whyte DB, Mellon PL. Mol. Cell. Biol 1996;16:3596–3605. [PubMed: 8668176]
19. Rave-Harel N, Givens ML, Nelson SB, Duong HA, Coss D, Clark ME, Hall SB, Kamps MP, Mellon PL. J. Biol. Chem 2004;279:30287–30297. [PubMed: 15138251]
20. Clark ME, Mellon PL. Mol. Cell. Biol 1995;15:6169–6177. [PubMed: 7565769]
21. Wolfe A, Kim HH, Tobet S, Stafford DE, Radovick S. Mol. Endocrinol 2002;16:435–449. [PubMed: 11875100]
22. Biggin MD, McGinnis W. Development 1997;124:4425–4433. [PubMed: 9409661]
23. Courey AJ, Jia S. Genes Dev 2001;15:2786–2796. [PubMed: 11691830]

24. Eraly SA, Nelson SB, Huang KM, Mellon PL. Mol. Endocrinol 1998;12:469–481. [PubMed: 9544983]
25. Chandran UR, Attardi B, Friedman R, Zheng Z, Roberts JL, DeFranco DB. J. Biol. Chem 1996;271:20412–20420. [PubMed: 8702778]
26. Chandran UR, DeFranco DB. Behav. Brain Res 1999;105:29–36. [PubMed: 10553688]
27. Belsham DD, Mellon PL. Mol. Endocrinol 2000;14:212–228. [PubMed: 10674395]
28. Kelley CG, Givens ML, Rave-Harel N, Nelson SB, Anderson S, Mellon PL. Mol. Endocrinol 2002;16:2413–2425. [PubMed: 12403831]
29. Nelson SB, Lawson MA, Kelley CG, Mellon PL. Mol. Endocrinol 2000;14:1509–1522. [PubMed: 10976927]
30. Givens ML, Rave-Harel N, Goonewardena VD, Kurotani R, Berdy SE, Swan CH, Rubenstein JL, Robert B, Mellon PL. J. Biol. Chem 2005;280:19156–19165. [PubMed: 15743757]
31. He X, Treacy MN, Simmons DM, Ingraham HA, Swanson LW, Rosenfeld MG. Nature 1989;340:35–42. [PubMed: 2739723]
32. Monica K, Galili N, Nourse J, Saltman D, Cleary ML. Mol. Cell. Biol 1991;11:6149–6157. [PubMed: 1682799]
33. Strubin M, Newell JW, Matthias P. Cell 1995;80:497–506. [PubMed: 7859290]
34. Schiestl RH, Gietz RD. Curr. Genet 1989;16:339–346. [PubMed: 2692852]
35. Robzyk K, Kassir Y. Nucleic Acids Res 1992;20:3790. [PubMed: 1641351]
36. Jacobs SBR, Coss D, McGillivray SM, Mellon PL. Mol. Endocrinol 2003;17:1470–1483. [PubMed: 12730328]
37. Coss D, Jacobs SB, Bender CE, Mellon PL. J. Biol. Chem 2004;279:152–162. [PubMed: 14570911]
38. Muhr J, Andersson E, Persson M, Jessell TM, Ericson J. Cell 2001;104:861–873. [PubMed: 11290324]
39. Zhang H, Hu G, Wang H, Sciavolino P, Iler N, Shen MM, Abate-Shen C. Mol. Cell. Biol 1997;17:2920–2932. [PubMed: 9111364]
40. Eberhard D, Jimenez G, Heavey B, Busslinger M. EMBO J 2000;19:2292–2303. [PubMed: 10811620]
41. Zappavigna V, Sartori D, Mavilio F. Genes Dev 1994;8:732–744. [PubMed: 7926763]
42. Schreiber E, Merchant RE, Wiestler OD, Fontana A. Neurosurgery 1994;34:129–135. [PubMed: 8121549]
43. Roose J, Molenaar M, Peterson J, Hurenkamp J, Brantjes H, Moerer P, van de Wetering M, Destree O, Clevers H. Nature 1998;395:608–612. [PubMed: 9783587]
44. Chen G, Courey AJ. Gene 2000;249:1–16. [PubMed: 10831834]
45. Fisher AL, Caudy M. Genes Dev 1998;12:1931–1940. [PubMed: 9649497]
46. Palaparti A, Baratz A, Stifani S. J. Biol. Chem 1997;272:26604–26610. [PubMed: 9334241]
47. Yu X, Li P, Roeder RG, Wang Z. Mol. Cell. Biol 2001;21:4614–4625. [PubMed: 11416139]
48. Choi CY, Lee YM, Kim YH, Park T, Jeon BH, Schulz RA, Kim Y. J. Biol. Chem 1999;274:31543–31552. [PubMed: 10531357]
49. Jimenez G, Paroush Z, Ish-Horowicz D. Genes Dev 1997;11:3072–3082. [PubMed: 9367988]
50. Jimenez G, Verrijzer CP, Ish-Horowicz D. Mol. Cell. Biol 1999;19:2080–2087. [PubMed: 10022895]
51. Courey AJ, Huang JD. Biochim. Biophys. Acta 1995;1261:1–18. [PubMed: 7893745]
52. Aronson BD, Fisher AL, Blechman K, Caudy M, Gergen JP. Mol. Cell. Biol 1997;17:5581–5587. [PubMed: 9271433]
53. Mallo M, Franco del Amo F, Gridley T. Mech. Dev 1993;42:67–76. [PubMed: 8369224]
54. Paroush Z, Finley RL, Kidd T, Weinwright SM, Ingham PI, Brent RI, Ish-Horowicz D. Cell 1994;79:805–815. [PubMed: 8001118]
55. Dehni G, Liu Y, Husain J, Stifani S. Mech. Dev 1995;53:369–381. [PubMed: 8645603]
56. Grbavec D, Stifani S. Biochem. Biophys. Res. Commun 1996;223:701–705. [PubMed: 8687460]
57. Leon C, Lobe CG. Dev. Dyn 1997;208:11–24. [PubMed: 8989517]
58. Dintilhac A, Bernues J. J. Biol. Chem 2002;277:7021–7028. [PubMed: 11748221]

59. Brantjes H, Roose J, van De Wetering M, Clevers H. Nucleic Acids Res 2001;29:1410–1419. [PubMed: 11266540]
60. Fisher AL, Ohsako S, Caudy M. Mol. Cell. Biol 1996;16:2670–2677. [PubMed: 8649374]
61. Tolkunova EN, Fujioka M, Kobayashi M, Deka D, Jaynes JB. Mol. Cell. Biol 1998;18:2804–2814. [PubMed: 9566899]
62. Tanaka M, Clouston WM, Herr W. Mol. Cell. Biol 1994;14:6046–6055. [PubMed: 8065338]
63. Verrijzer CP, van Oosterhout JA, van der Vliet PC. Mol. Cell. Biol 1992;12:542–551. [PubMed: 1346336]
64. Ren B, Chee KJ, Kim TH, Maniatis T. Genes Dev 1999;13:125–137. [PubMed: 9887105]
65. Dasen JS, Barbera JP, Herman TS, Connell SO, Olson L, Ju B, Tollkuhn J, Baek SH, Rose DW, Rosenfeld MG. Genes Dev 2001;15:3193–3207. [PubMed: 11731482]
66. Chen G, Fernandez J, Mische S, Courey AJ. Genes Dev 1999;13:2218–2230. [PubMed: 10485845]
67. Yao J, Lai E, Stifani S. Mol. Cell. Biol 2001;21:1962–1972. [PubMed: 11238932]
68. Zhu CC, Dyer MA, Uchikawa M, Kondoh H, Lagutin OV, Oliver G. Development 2002;129:2835–2849. [PubMed: 12050133]
69. McLarren KW, Lo R, Grbavec D, Thirunavukkarasu K, Karsenty G, Stifani S. J. Biol. Chem 2000;275:530–538. [PubMed: 10617648]
70. Flores-Saaib RD, Jia S, Courey AJ. Development 2001;128:1869–1879. [PubMed: 11311166]
71. Dubnicoff T, Valentine SA, Chen G, Shi T, Lengyel JA, Paroush Z, Courey AJ. Genes Dev 1997;11:2952–2957. [PubMed: 9367978]
72. Prefontaine GG, Lemieux ME, Giffin W, Schild-Poulter C, Pope L, LaCasse E, Walker P, Hache RJ. Mol. Cell. Biol 1998;18:3416–3430. [PubMed: 9584182]
73. Chandran UR, Warren BS, Baumann CT, Hager GL, DeFranco DB. J. Biol. Chem 1999;274:2372–2378. [PubMed: 9891005]
74. Stern S, Tanaka M, Herr W. Nature 1989;341:624–630. [PubMed: 2571937]
75. Mallo M, Gendron-Maguire M, Harbison ML, Gridley T. Dev. Dyn 1995;204:338–347. [PubMed: 8573724]
76. Brinkmeier ML, Potok MA, Cha KB, Gridley T, Stifani S, Meeldijk J, Clevers H, Camper SA. Mol. Endocrinol 2003;17:2152–2161. [PubMed: 12907761]
77. Yao J, Liu Y, Lo R, Tretjakoff I, Peterson A, Stifani S. Mech. Dev 2000;93:105–115. [PubMed: 10781944]
78. Welting TJ, van Venrooij WJ, Pruijn GJ. Nucleic Acids Res 2004;32:2138–2146. [PubMed: 15096576]
79. Catron KM, Zhang H, Marshall SC, Inostroza JA, Wilson JM, Abate C. Mol. Cell. Biol 1995;15:861–871. [PubMed: 7823952]
80. Wong MW, Henry RW, Ma B, Kobayashi R, Klages N, Matthias P, Strubin M, Hernandez N. Mol. Cell. Biol 1998;18:368–377. [PubMed: 9418884]

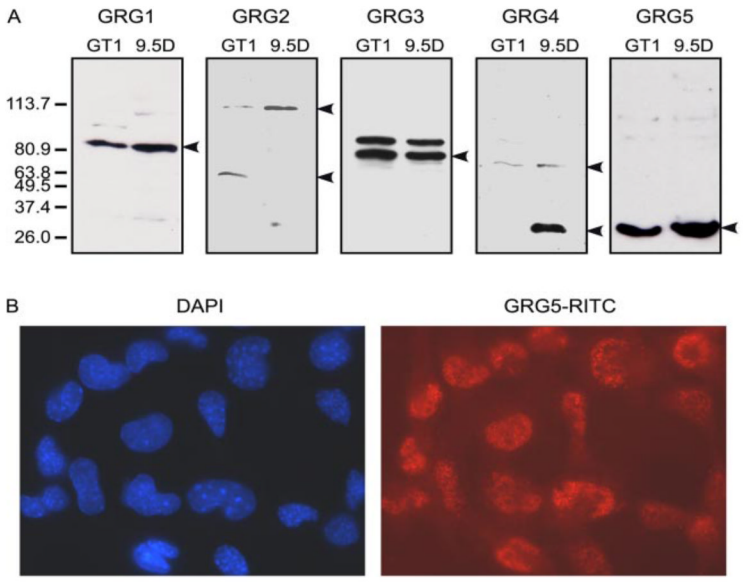

Fig. 1. Groucho-related proteins are expressed in GT1-7 cells

A, Western blots of nuclear extracts were probed with antibodies specific to GRG1, GRG2, GRG3, GRG4 (Santa Cruz Biotechnology), and GRG5. Each *lane* contained 12 μg of protein from GT1-7 cells or from 9.5-day postcoitum mouse embryo, as indicated *above* the gels (*GT1* and *9.5D*, respectively). The *arrows* mark the positions of the identified proteins. *B*, nuclear localization of GRG5 protein in GT1-7 cells. Anti-GRG5 polyclonal antibody was used for indirect immunofluorescence of GT1-7 cells. 4′,6-diamidino-2-phenylindole (*DAPI*) (*blue*) was used to visualize the nuclei, and rhodamine isothiocyanate (RITC; *red*) was used to visualize GRG5.

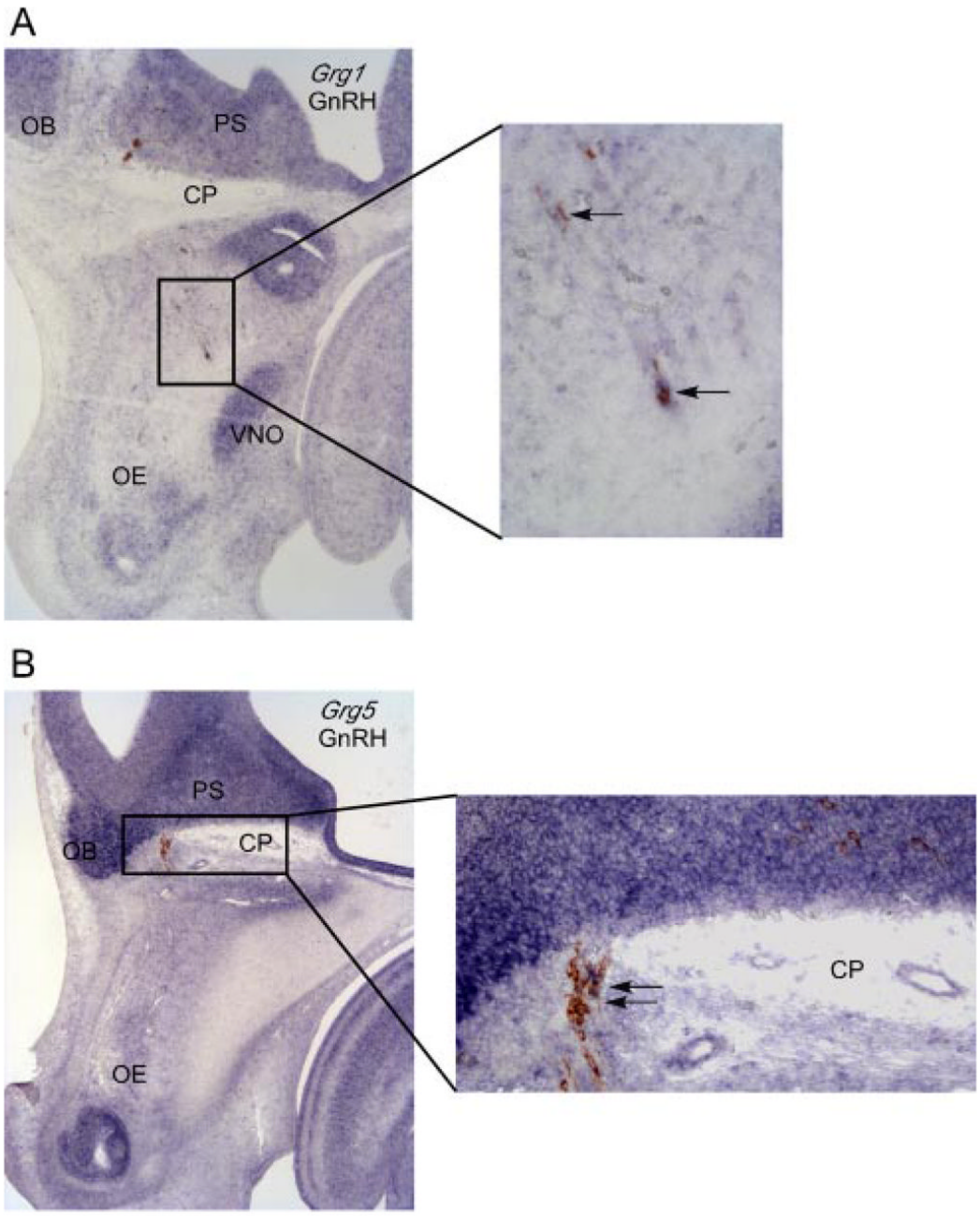

Fig. 2. Expression of *Grg1* and *Grg5* in relation to the migratory GnRH neurons *in vivo*

In situ hybridization/immunohistochemical analyses on embryonic day 13.5 mouse embryos were carried out using an antisense probe specific for *Grg1* (*A*)or *Grg5* (*B*). Expression of the *Grg* transcripts (*purple*) is shown in relation to the GnRH-positive cells (*brown*). The *expanded panels* represent ×20 magnification of the denoted region. *OB*, olfactory bulb; *PS*, primordium of the septum; *CP*, cribriform plate; *OE*, olfactory epithelium; *VNO*, vomeronasal organ.

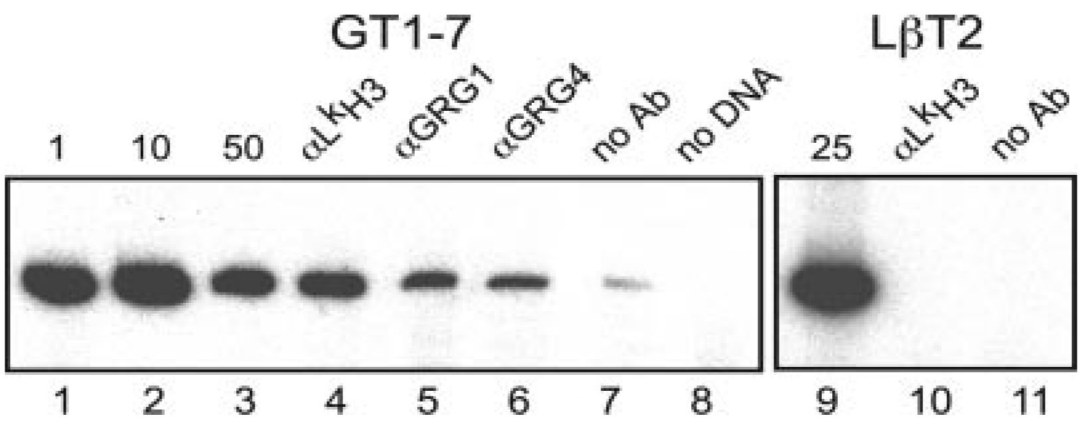

Fig. 3. GRG1 and GRG4 interact *in vivo* with the GnRH promoter

A chromatin immunoprecipitation experiment was performed in GT1-7 cells using antibodies recognizing GRG1 and GRG4. PCR amplification of the chromatin, immunoprecipitated with these antibodies, gave a 220-bp product corresponding to the GnRH promoter. Immunoprecipitations with no antibody were used as negative controls. Dilutions of 1:10, 1:50, and 1:25 of the total input as well as a concentrated aliquot are also shown. Immunoprecipitation with acetylated histone 3 (*α-L^{K}H3*) was used to test for an open chromatin state in the GT1-7 cells, in comparison with L*β*T2 cells.

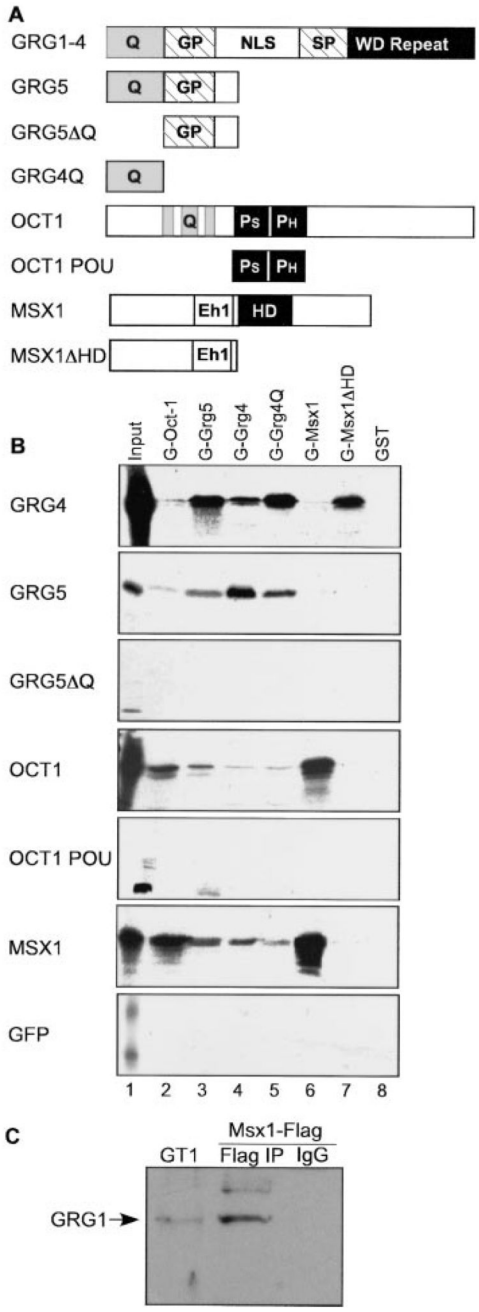

Fig. 4. GRG proteins specifically associate with OCT1 and MSX1

A, schematic diagram of the polypeptides used in the GST pull-down assays. The *upper scheme* illustrates the structure of the long GRG forms, GRG1 to -4. These proteins are characterized by a conserved N-terminal Q domain (*gray*) and a conserved C-terminal WD repeat domain (*black*). These regions are separated by domains that have been implicated in transcriptional repression (*GP* and *SP*, *stripes*) and nuclear localization (*NLS*, *white*). GRG5 is a truncated form of the GRG family, lacking the NLS, SP domain, and the entire WD40 domain. The OCT1 protein contains a POU domain, which is constituted of a POU homeodomain (*PH*) and a POU-specific domain (*PS*). It also includes three conserved Q motifs, located at amino acid positions 181–196, 205–220, and 229–241 in the human sequence. MSX1

protein contains a homeodomain (*HD*) and an eh1 domain. *B*, mapping the interactions between the GRG proteins, MSX1 and OCT1. ^{35}S-Labeled *in vitro* translated proteins were used for binding assays with GST or GST fusion proteins adsorbed to glutathione-Sepharose beads. One-tenth of each of the *in vitro* translated proteins used for binding was run on the input lanes to visualize the protein products. Since GST pull-down is not a quantitative assay, every band (weak or strong) is considered as a real interaction. Our inability to detect the mutual interaction between GST-MSX1 and GRG5 may be explained by interference of the GST part of the fusion protein with the interaction with the *in vitro* translated protein. This phenomenon has been described by others (78). *C*, GRG proteins interact with MSX1 *in vivo*. GT1-7 nuclear extracts expressing FLAG-MSX1 fusion protein (as labeled *above* the *gel*) were used in co-immunoprecipitation (*IP*) assays employing either a FLAG monoclonal antibody or as a control, normal rabbit IgG. Western blot of the proteins precipitated by this assay were performed with an antibody specific to GRG1. The *left lane* represents expression of the endogenous GRG1 protein in GT1-7 nuclear extract without immunoprecipitation.

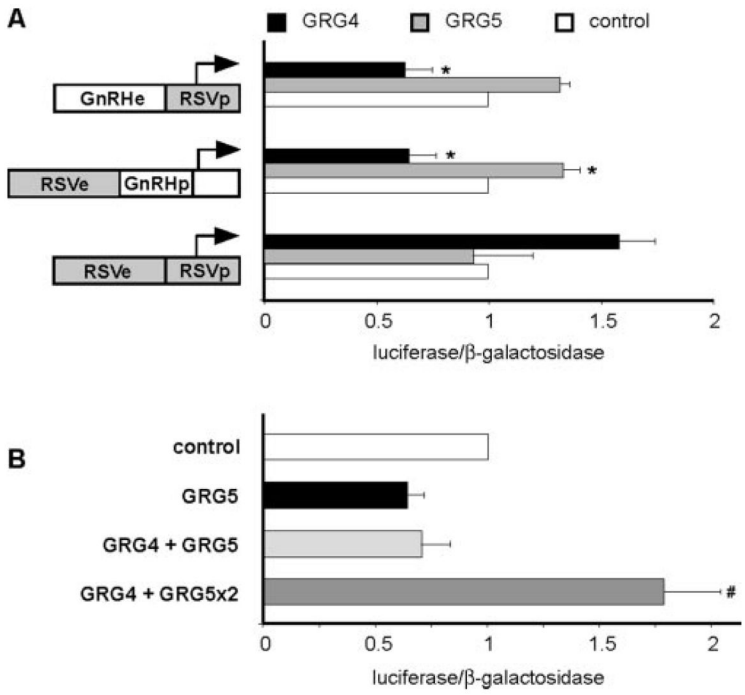

Fig. 5. GRG4 and GRG5 regulate GnRH gene reporter activity

Transient transfections were conducted in GT1-7 cells using various GnRH reporters, driving luciferase expression. The cells were co-transfected with expression vectors for the mouse GRG4 and/or GRG5 proteins. The internal control was a herpes simplex virus thymidine kinase −109 promoter regulating *β*-galactosidase expression. The activity of the empty expression vector, normalized to the activity of the co-transfected internal control, was set at 1 for each experiment. *Error bars* represent S.E. *A*, GRG4 represses GnRH reporter activity. GRG4 and GRG5 transcriptional activity (*black* and *gray*, respectively) is compared with the activity of the empty vector (*white*). The *asterisks* represent significance ($p < 0.05$ by analysis of variance, Tukey-Kramer honestly significant difference (HSD) test). *B*, GRG5 reverses the repression activity of GRG4. The activity of GRG4 by itself as well as in combination with increasing amounts of GRG5 was measured using the GnRH enhancer/RSV promoter reporter. The *pound sign* represent significance ($p < 0.05$ *versus* GRG4 by analysis of variance, Tukey-Kramer HSD test).

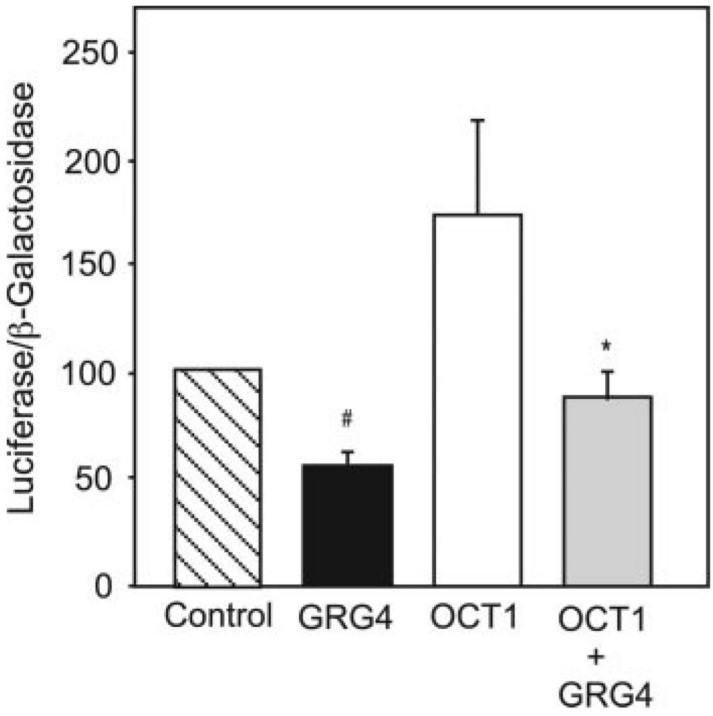

Fig. 6. GRG4 represses OCT1 activation of GnRH expression

Transient transfections were conducted in GT1-7 cells as described in Fig. 4, with four copies of the major OCT1 binding site (−1783/−1771) in the GnRH enhancer fused to the RSV promoter, controlling luciferase expression. The *error bars* represent S.E. The *asterisks* represent $p < 0.05$ *versus* GRG4 by Student's *t* test. The *pound sign* represents significance ($p < 0.05$ *versus* the empty vector by analysis of variance using the *post hoc* Tukey-Kramer HSD test).

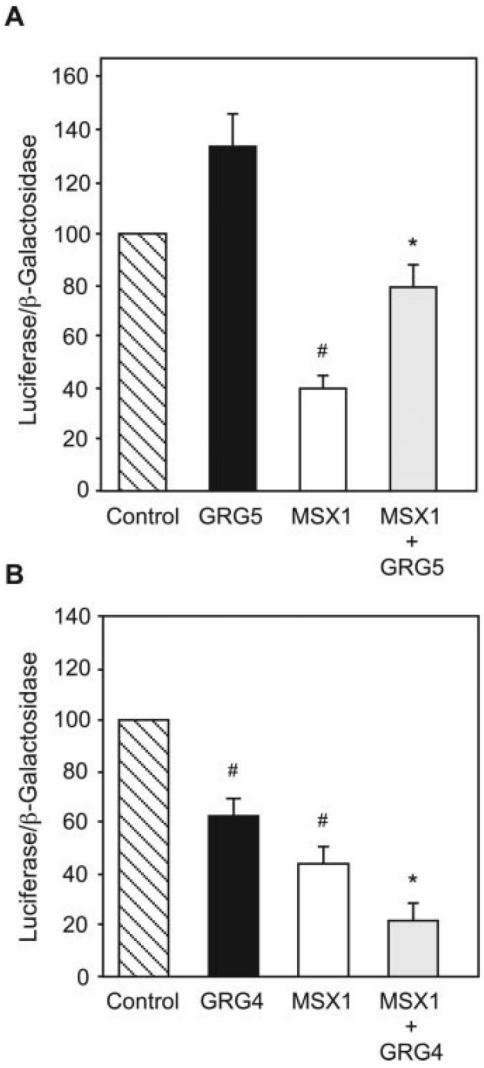

Fig. 7. The GRG family regulates MSX1-mediated repression of GnRH gene expression

Transient transfections were conducted in GT1-7 cells as described in the legend to Fig. 4, with the GnRH enhancer fused to the RSV promoter, controlling luciferase expression. The *error bars* represent S.E. *A*, GRG5 reverses MSX1 repression of GnRH activity. The *asterisks* represent significance ($p < 0.05$) *versus* MSX1, and the *pound sign* represents significance ($p < 0.05$) as compared with the empty vector by analysis of variance, Tukey-Kramer HSD test. *B*, GRG4 augments MSX1-dependent repression of GnRH gene expression. The *asterisks* represent a significant difference ($p < 0.05$) relative to the MSX1 vector.

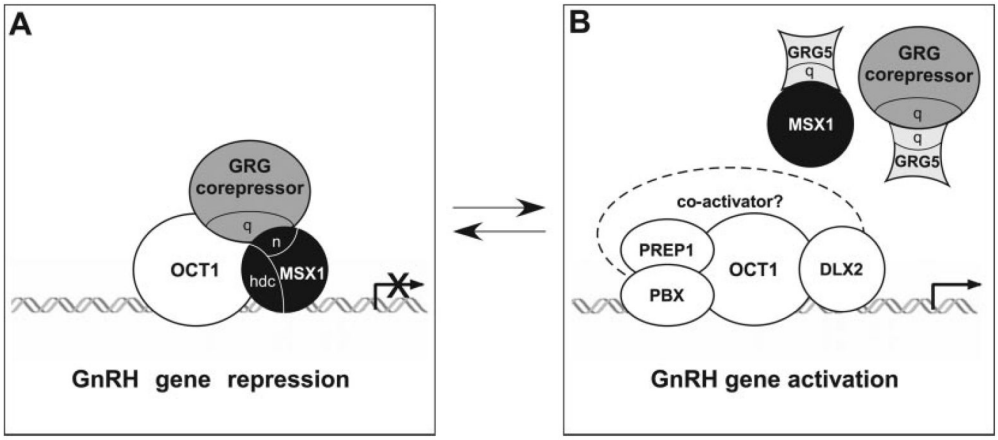

Fig. 8. Protein complexes regulating GnRH gene transcriptional repression and activation

The DNA segment represents the GnRH promoter region, which contains clusters of binding sites for OCT1, MSX1/DLX2, and the TALE proteins, PBX/PREP1. The *oval shapes* illustrate the transcriptional activators (*white*), repressors (*black*), and cofactors (*gray*) regulating GnRH transcription. The basal transcription machinery is not depicted for simplicity. *A*, recruitment of the GRG co-repressors by MSX1 and OCT1 may lead to the formation of a repressosome. Both MSX1 and the GRG long forms have been shown to interact with the basal transcription units TFIIA/B and TFIIE, respectively (47,79). OCT1 may function as a repressor in this setting. Domains that serve for protein-protein interaction, analyzed in our present study, are shown. *hdc*, homeodomain plus the carboxyl terminus; *n*, amino-terminal domain; *q*, glutamine-rich domain. *B*, GRG5 relieves repression through sequestering MSX and GRG co-repressors. The combinatorial action of OCT1, PBX/PREP1, and DLX2, possibly together with coactivators, leads to transcriptional activation. It is plausible that OCT1 mediates the connection to the basal transcription machinery by direct interaction with SNAPc (80). This whole complex may be considered an enhanceosome.