Published in final edited form as:
J Biol Chem. 2004 July 16; 279(29): 30287–30297. doi:10.1074/jbc.M402960200.

TALE Homeodomain Proteins Regulate Gonadotropin-releasing Hormone Gene Expression Independently and via Interactions with Oct-1*

Naama Rave-Harel[a,b], **Marjory L. Givens**[a,c,d], **Shelley B. Nelson**[a,c,e], **Hao A. Duong**[a,f], **Djurdjica Coss**[a,g], **Melody E. Clark**[a,h], **Sara Barth Hall**[a], **Mark P. Kamps**[i], and **Pamela L. Mellon**[a,j,k]

[a] Department of Reproductive Medicine, University of California, San Diego, La Jolla, California 92903

[j] Department of Neurosciences, School of Medicine, University of California, San Diego, La Jolla, California 92903

[i] Department of Pathology, School of Medicine, University of California, San Diego, La Jolla, California 92903

Abstract

Gonadotropin-releasing hormone (GnRH) is the central regulator of reproductive function. Expression of the GnRH gene is confined to a rare population of neurons scattered throughout the hypothalamus. Restricted expression of the rat GnRH gene is driven by a multicomponent enhancer and an evolutionarily conserved promoter. Oct-1, a ubiquitous POU homeodomain transcription factor, was identified as an essential factor regulating GnRH transcription in the GT1-7 hypothalamic neuronal cell line. In this study, we conducted a two-hybrid interaction screen in yeast using a GT1-7 cDNA library to search for specific Oct-1 cofactors. Using this approach, we isolated Pbx1b, a TALE homeodomain transcription factor that specifically associates with Oct-1. We show that heterodimers containing Pbx/Prep1 or Pbx/Meis1 TALE homeodomain proteins bind to four functional elements within the GnRH regulatory region, each in close proximity to an Oct-1-binding site. Cotransfection experiments indicate that TALE proteins are essential for GnRH promoter activity in the GT1-7 cells. Moreover, Pbx1 and Oct-1, as well as Prep1 and Oct-1, form functional complexes that enhance GnRH gene expression. Finally, Pbx1 is expressed in GnRH neurons in embryonic as well as mature mice, suggesting that the associations between TALE homeodomain proteins and Oct-1 regulate neuron-specific expression of the GnRH gene *in vivo*.

Tissue-specific gene expression can be mediated, in the simplest case, by a transcription factor restricted to a particular cell type. Usually, however, tissue-specific expression is achieved

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

[k] 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.

[b] Fellow of the Lalor Foundation.

[c] Both authors contributed equally to this work.

[d] Supported in part by National Institutes of Health Training Grant DA07315.

[e] Supported in part by National Institutes of Health Training Grant T32 AG00216. Present address: Dept. of Development and Cell Biology, University of California, 4150 McGaugh Hall, Irvine, CA 92697-2300.

[f] Present address: Dept. of Chemistry and Biochemistry, UCLA, 607 Charles E. Young Dr. E., Los Angeles, CA 90095-1569.

[g] Supported by National Institutes of Health Grant F32 HD41301 and the Lalor Foundation.

[h] Recipient of an American Cancer Society fellowship. Present address: Arena Pharmaceuticals, Inc., 6166 Nancy Ridge Dr., San Diego, CA 92121.

through unique combinations of DNA elements binding more broadly expressed proteins. This type of transcriptional regulation, termed combinatorial control, provides an efficient mechanism integrating responses to a variety of signals using a relatively limited number of proteins (1).

We investigated the mechanisms underlying neuron-specific transcription of the gonadotropin-releasing hormone (GnRH)[1] gene. GnRH is the decapeptide hormone that regulates secretion of the pituitary gonadotropins, luteinizing hormone, and follicle-stimulating hormone, thereby mediating central nervous system control of reproductive function (2). The GnRH-secreting neurons are a small population of highly specialized cells dispersed throughout the hypothalamus that release GnRH in a pulsatile manner. We have previously developed an immortalized cultured cell model system for GnRH neurons, the GT1-7 cell line, by targeted oncogenesis (3). These cells are hypothalamic neurons that secrete GnRH and therefore provide an appropriate model for defining the molecular mechanisms for neuron-specific gene expression.

Previously, we established that neuron-specific activation of the rat GnRH gene is conferred, in culture and *in vivo*, by two upstream regulatory elements: an enhancer (−1863 to −1571) and an evolutionarily conserved promoter (−173 to +1) (4–6). Within these sequences is a complex arrangement of binding sites for *trans*-acting factors that potentiate transcription. However, single binding elements are not sufficient to confer GT1-7 cell-specific expression to reporter genes in transient transfection assays, demonstrating that coordinate action from multiple elements is required for GnRH transcription.

Several factors have been shown to regulate GnRH cell-specific transcription, including the homeodomain proteins Oct-1 (7), SCIP/Oct-6 (8), Brn2 (9), Dlx2 and Msx1,[2] and Otx2 (10); the zinc finger protein GATA-4 (11); nuclear factor-1 [3]; and CCAAT/enhancer-binding protein-β (12). In particular, the POU homeodomain protein Oct-1 was identified as an essential factor regulating basal and hormone-induced transcription of the GnRH gene (7,9,12,13). Oct-1 binds to five elements within the rat GnRH regulatory region and is necessary for enhancer activity in GT1-7 cells (7). Mutation of the Oct-1 site at −1781 to −1774, within the enhancer, decreases basal transcription to 5% (5). Thus, Oct-1 plays a critical role in GnRH gene expression.

A paradox that remains to be explained, however, is that Oct-1 and the other GnRH regulatory proteins identified thus far have been detected in a variety of cell types, whereas the GnRH gene is expressed only in hypothalamic GnRH neurons and the cell line representing them, GT1-7. A possible explanation is that GT1-7 cells may contain a neuron-specific Oct-1 coactivator. Oct-1 is not known to be a strong transcriptional activator by itself; however, in conjunction with coactivators, it can promote potent tissue-specific transactivation of target genes (14,15). Alternatively, Oct-1 may interact with other DNA-binding proteins that are neuron-specific (16).

In this study, we used a two-hybrid interaction screen in yeast to search for Oct-1 cofactors in GT1-7 cells. This approach led us to the isolation of the TALE homeodomain transcription factor Pbx1b. We show that TALE homeodomain proteins specifically associate with Oct-1. Furthermore, we demonstrate a role for this interaction in the activation of GnRH gene

[1]The abbreviations used are: GnRH, gonadotropin-releasing hormone; EMSA, electrophoretic mobility shift assay; GST, glutathione *S*-transferase; dnPrep1, dominant-negative Prep1; GnRHe, GnRH enhancer; RSVp, Rous sarcoma virus promoter; DAB, 3,3′-diaminobenzidine; dpc, days postcoitus; GnRHp, GnRH promoter; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.
[2]M. L. Givens, N. Rave-Harel, V. O. Goonewardena, R. Kurotani, C. H. Swan, J. L. R. Rubenstein, B. Robert, and P. L. Mellon, submitted for publication.
[3]M. L. Givens, R. Kurotani, N. Rave-Harel, N. L. G. Miller, and P. L. Mellon, submitted for publication.

expression. The findings in GT1-7 cells are likely relevant to the regulation of GnRH expression *in vivo* since Pbx1 is expressed in embryonic as well as mature GnRH neurons in mice.

Experimental Procedures

Two-hybrid Interaction Screen in Yeast

To isolate cDNAs encoding GT1-7 proteins that associate with Oct-1, 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 were the generous gifts of Dr. Michel Strubin and have been described previously (14). In this library vector, the hybrid proteins are expressed in yeast from a centromeric *TRP1* plasmid under the control of a galactose-inducible promoter. The yeast tester strain also contains a centromeric bait plasmid carrying the *URA3* gene and the human Oct-1 cDNA. A chromosomal copy of the selectable marker, the *HIS3* gene, under the control of six copies of the octamer motif serves as the genomic target for Oct-1. The GT1-7 cDNA library (3×10^6 primary transformants) was introduced into the reporter strain using the method of Schiestl and Gietz (17), 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 synthetic galactose medium lacking histidine and containing 10 mM 3-aminotriazole and incubated for 10 days. Eighty-nine *URA*$^+$*/TRP*$^+$ 3-aminotriazole-resistant colonies were plated on medium containing 5-fluoroorotic acid to select against cells expressing Oct-1 from the *URA3* plasmid. Seven *URA*$^-$*/TRP*$^+$ colonies that lost their ability to grow on 3-aminotriazole when their Oct-1 plasmid was removed were recovered from the yeast using the method of Robzyk and Kassir (18). The DNA sequences of the 22 library plasmids recovered from these colonies were determined by the chain termination method (19).

Oligonucleotides

The double-stranded consensus oligonucleotides used in the electrophoretic mobility shift assay (EMSA) analysis included the Oct-1 consensus oligonucleotide (TGTCGAATGCAAATCACTAGAA, top strand) (7), the PBX oligonucleotide (AGCGCGGGGCGCATCAATCAATTTCG, top strand), and the PBX-mut oligonucleotide (AGCGCGGGGCGCATCAATTAATTTCG, top strand) (20). The sequences of the GnRH oligonucleotides are shown in Fig. 3. The top and bottom strands of the −75 oligonucleotide correspond to GnRH sequences −109 to −94 and −104 to −89, respectively. All oligonucleotides were synthesized by Operon Technologies, Inc.

EMSA

Nuclear extracts were prepared according to the method described by Schreiber *et al.* (21). Annealed wild-type and mutant oligonucleotides (20 ng or 1 pmol) containing sequences of the GnRH enhancer or promoter or consensus sequences were phosphorylated with [γ-^{32}P] dATP (6000 Ci/mmol; PerkinElmer Life Sciences) and polynucleotide kinase or filled in with [α-^{32}P]dATP (3000 Ci/mmol; PerkinElmer Life Sciences) and Klenow fragment using standard procedures (22). Probes were phenol/chloroform-extracted and passed over Sephadex G-25 micro-columns (Amersham Biosciences), and radioactivity was counted with a scintillation counter. Probes were diluted in 50 mM NaCl. Binding reactions were carried out under conditions described previously (7). Each probe (1 fmol) was incubated with 2 μg of GT1-7 crude nuclear extract in 20-μl reactions. Reactions were incubated at room temperature for 5 min and separated on 5% polyacrylamide gel in 0.25× Tris borate/EDTA as described previously (7). Competitions were performed by preincubating the reactions with a 100–200-fold excess of unlabeled oligonucleotide for 5 min on ice prior to adding the probe. Supershift assays were performed by adding 1 μl of antibody or rabbit IgG control to the complete reaction

and then incubating as described above. The antibodies used were from Santa Cruz Biotechnology, Inc., except anti-Meis1 antibody (a generous gift of Dr. Michael L. Cleary) and the antibody directed against all Pbx forms (21). Gels were run at 250 V for 2 h and then dried under a vacuum and exposed to film for 1–3 days.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation assays were performed as described previously (23), with a few modifications. Chromatin from GT1-7 or LβT2 cells was cross-linked for 10 min using formaldehyde. The resulting chromatin solution was precipitated with Pbx1, Prep1, and Oct-1 polyclonal antibodies. The following day, chromatin·antibody complexes were isolated from the solution by incubation with 50 μl of protein A-Sepharose beads (50% slurry, pre-blocked with 100 μg/ml sonicated *Escherichia coli* DNA and 1 mg/ml bovine serum albumin) while being rocked at 4 °C for 2 h. The beads were harvested and washed as described previously (23). Cross-linking was reversed by addition of NaCl to a final concentration of 300 mM and incubation overnight at 65 °C. Chromatin·antibody complexes were eluted from the Sepharose beads by 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. Immunoprecipitated DNA was analyzed for the GnRH promoter sequence by PCR.

Protein Production and GST Retention Assay

Expression vectors used for protein production included full-length human Oct-1 and the POU domain of Oct-1 in the pCR2.1 vector and the human Prep1, Meis1, and Pbx cDNAs (including various deletions) in pGEM vectors. *In vitro* transcription and translation were performed with the Promega TNT coupled reticulocyte lysate system following the manufacturer's protocol, employing SP6, T7, or T3 polymerase. [^{35}S]Methionine was used for labeling the protein products. Translation mixture without DNA was used as a control for unprogrammed translation in the reticulocyte lysate. GST-Oct-1 was created by cloning the human Oct-1 cDNA into the pGEX-4T1 vector. Bacteria transformed with the pGEX vectors were grown to an absorbance of 0.5 and then induced overnight with 0.2 mM isopropyl-β-D-thiogalactopyranoside. Bacterial pellets were sonicated in 0.1% Triton X-100 and 5 mM EDTA in 1× phosphate-buffered saline and centrifuged, and the supernatant was bound to glutathione-Sepharose 4B resin (Amersham Biosciences). The interaction assay was performed as described previously (24). 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 X-BioMax film at −80 °C or to a PhosphorImager (Bio-Rad) at room temperature.

Cell Culture and Transfection

All cells were grown in monolayer culture in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 4.5 mg/ml glucose in a 5% CO_2 atmosphere. Cells were transfected with FuGENE 6 (Roche Applied Science) in 24-well multidishes. GT1-7 cells from only passages 5–10 were used for the transactivation experiments. The expression plasmids used for cotransfections included human dominant-negative Prep1 (dnPrep1) in a pCMV vector; human Oct-1 Prep1, Pbx1b, and Meis1 in pcDNA1.1 vectors; and the negative control vectors pCMV and pcDNA1.1. The reporter plasmids contained the GnRH enhancer (GnRHe; −1863 to −1571) fused to the Rous sarcoma virus promoter (RSVp) (GnRHe-RSVp) and GnRHe-RSVp containing a TGAATGATAG → TGAATctTAG mutation in the −1749 Pbx/Prep1-binding site, each in a pGL3 vector driving luciferase expression. GT1-7 cells were transfected with 100 ng of expression plasmid, 400 ng of reporter plasmid, and 200 ng of internal control (herpes simplex virus thymidine kinase −109 promoter on β-galactosidase). Cells were harvested 48 h after transfection, lysed, and assayed for luciferase and β-galactosidase expression as described previously (25).

In Situ Hybridization and Immunohistochemistry

Adult mice were perfused with Zamboni's fixative (4% paraformaldehyde and 0.1 M sodium phosphate (pH 7.4)) as described in detail previously (26). Their brains were removed and post-fixed overnight. The forebrains and midbrains were then sliced coronally on a microtome in 30-μm sections and stored in potassium phosphate-buffered saline. One series of sections was incubated with anti-Pbx1 antibody at 1:1000 dilution and subsequently underwent a nickel reaction. Next, the sections were incubated with anti-GnRH antibody (LR1; a gift of R. Benoit) and subsequently underwent a 3,3′-diaminobenzidine (DAB) reaction (peroxidase substrate kit, Vector Laboratories). Sections were mounted, dried, cover-slipped with synthetic distyrene plasticizer xylene resin (DPX; Electron Microscopy Sciences), and visualized by light microscopy. These sections identify Pbx1 by black staining and GnRH by brown staining. Mouse embryos were removed at 13.5 days postcoitus (dpc) and embedded in optimal cutting temperature medium (OCT; VWR Scientific) or paraffin-embedded and sectioned at 7–10 μm thickness. The series of frozen sliced embryo sections carrying rat GnRHe-GnRHp driving the β-galactosidase transgene (6) was subjected to X-gal reaction (1 mg/ml X-gal dissolved in Me_2SO, 1× phosphate-buffered saline, 4 mM ferrocyanide, 4 mM ferricyanide, and 2 mM $MgCl_2$) and incubated with anti-Pbx1 antibody and DAB as described above. These sections identify Pbx1 by brown staining and β-galactosidase by blue staining. Paraffin-embedded wild-type embryos were deparaffinized with xylene washes, hydrated in ethanol/water solutions, and digested with proteinase K for 7 min at 37 °C, followed by post-fixation 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 sense and antisense probes for Pbx1 or Oct-1.

The digoxigenin-labeled probes were added to a slide at a dilution of 1:1000 in hybridization buffer (150 μl/section; 50% formamide, 10% dextran sulfate, 1 mg/ml yeast RNA, 50 × Denhardt's, 200 mM sodium chloride, 1 mM Tris base, 9 mM Tris hydrochloride, 5 mM sodium phosphate, 2.5 mM EDTA, in diethyl pyrocarbonate-treated water) and incubated overnight at 65 °C. Sections were washed four times at 65 °C with 50% formamide, 1× SSC, and 0.1% Tween 20 and then twice at room temperature with 100 mM maleic acid, 150 mM NaCl, and 0.1% Tween. The slides were blocked with 5% normal goat serum and 0.1% Tween 20 for 3–4 h. The hybridized digoxigenin-labeled probe was detected using alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Applied Science) at a dilution of 1:2000 and visualized with the chromogen combination 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium chloride. Antisense and sense probes were created by incubating 1 μg of linearized plasmid DNA with 10× digoxigenin RNA labeling mixture (Roche Applied Science) as well as 5× transcription buffer (Promega) and T7, T3, or SP6 RNA polymerase for 2 h at 37 °C.

Results

The Homeodomain Protein Pbx1b Interacts with Oct-1 in a Yeast Two-hybrid Screen

To understand the mechanism by which the ubiquitous transcription factor Oct-1 specifies GnRH expression, we searched for Oct-1 cofactors expressed in the GT1-7 cell line. For this purpose, we created a GT1-7 cDNA library with a C-terminal fusion to the VP16 transactivation domain for use in a yeast two-hybrid system developed especially to screen for Oct-1-interacting proteins, which led to the isolation of the B-cell-specific transcriptional coactivator OBF-1/Bob-1 (14). One of the cDNAs, isolated by this method, encoded a known transcription factor, mouse Pbx1b. Pbx1b belongs to the PBC subclass of the TALE (three-amino acid loop extension) homeodomain proteins. The mammalian Pbx family is composed of Pbx1a, Pbx1b, Pbx2, Pbx3a, and Pbx3b (Pbx1b and Pbx3b are alternatively spliced short forms) (27). These proteins have been shown to regulate gene expression through cooperative interaction with

other DNA-binding proteins such as Hox (27) and with the TALE homeodomain proteins Meis (28) and Prep (29). Therefore, we considered Pbx1b a suitable candidate for an Oct-1-interacting protein.

Multiple TALE Homeodomain Proteins Are Expressed in GT1-7 Cells

The Pbx proteins are expressed in many tissues and cell lines (30). Having identified Pbx1b in our yeast two-hybrid screen, we next examined which Pbx family members are expressed in our model cell line, the GT1-7 cells. The presence of Pbx mRNAs and proteins was determined in several cell lines and tissues by Northern and Western blot analyses. These analyses revealed that both variants of Pbx1 mRNAs were expressed in GT1-7 cells (data not shown). We further observed, using a series of Pbx-specific antibodies, that the Pbx1b protein was abundant in GT1-7 nuclear extracts compared with the other Pbx family members (Pbx1a, Pbx2, and Pbx3a) and with Pbx1b expression in mouse hypothalamus and the mouse fibroblast cell line NIH3T3 (Fig. 1A). The EL4 cell line served as a negative control for Pbx1b expression (30). Since Pbx transcriptional activity has been shown to be dependent on cooperative interaction with other TALE homeodomain proteins, Meis (28,31) and Prep (Pknox) (29,31), we investigated the presence of these Pbx partners in GT1-7 cells. Western blot analysis with specific antibodies showed that the Prep1 and Meis1 proteins were highly expressed in GT1-7 nuclear extracts compared with their expression in other cell lines (Fig. 1B). Thus, in addition to confirming the presence of Pbx proteins, two other TALE homeodomain proteins, Meis and Prep, were also detected in our cell model for GnRH neurons. These data suggest that TALE proteins (in particular, Pbx1b) are available for complex formation with Oct-1 in GT1-7 cells.

Pbx1 and Prep1 Proteins Form Complexes with Oct-1 in Vitro

The isolation of Pbx1b in a yeast two-hybrid screen for Oct-1-interacting protein suggests that Pbx1 forms a protein complex with Oct-1 independent of DNA binding. To verify this interaction, as well as to explore a potential interaction between Oct-1 and the Pbx1 partner Prep1, a pull-down approach was utilized with GST fusion proteins (Fig. 1C). Bacterial extracts expressing GST-Oct-1 or GST were mixed with glutathione-Sepharose beads. The beads with absorbed fusion protein were subsequently incubated with *in vitro* translated ^{35}S-labeled proteins. As expected, according to the demonstrated interactions between POU domain proteins (16), GST-Oct-1 specifically bound to the Oct-1 protein. It also bound to the Pbx1b protein, but not to the green fluorescent protein, which was used as a negative control. Intriguingly, GST-Oct-1 also specifically interacted with Prep1 (Fig. 1C). No binding was observed when proteins were incubated with GST alone. These data confirm that the Pbx1b and Prep1 proteins interact with Oct-1 *in vitro*.

To ascertain the domains of Pbx1 and Prep1 that serve for the interaction with Oct-1, the pull-down experiments were repeated with various domains of Pbx1b and Prep1. Two Pbx1 mutant proteins were used to map the interface between Pbx1 and Oct-1. To test the contribution of the N-terminal domain of Pbx1b, the naturally occurring Pbx mutant E2A-Pbx1b (32) was used. This oncogenic fusion protein contains the transactivation domain of the E2A protein substituting for the first 88 residues of Pbx1 (Fig. 1D). In addition, we used a Pbx1b mutant protein lacking 75 N-terminal amino acids (PbxΔNT3). Fig. 1C shows that both Pbx1 mutant proteins specifically bound GST-Oct-1. These data indicate that the N-terminal region of Pbx1b is not essential for the interaction with Oct-1.

To determine which region of the Prep1 protein provides the major contribution to the protein-protein interaction with Oct-1, two deletions were made. The deletion in the N-terminal region removed the Meis homologous regions 1 and 2 (Prep1ΔHR), which were previously shown to be important for protein-protein interactions with Pbx proteins (37). The deletion in the C-terminal region removed the Prep1 homeodomain (Prep1ΔHD) (Fig. 1D). Fig. 1C indicates

that GST-Oct-1 specifically bound Prep1 lacking the N-terminal domain, but not the Prep1 lacking the homeodomain. These data suggest that sequences located in the DNA-binding domain of Prep1 serve for the interaction with Oct-1.

TALE Homeodomain Proteins Bind Functional Sites within the GnRH Regulatory Region

Pbx proteins and their cofactors Meis and Prep are DNA-binding TALE homeodomain transcription factors that bind cooperatively to defined consensus sequences. To further examine the role of these proteins in regulating GnRH gene expression, we searched for Pbx-binding sites in conserved regions of both the enhancer and promoter. Careful inspection of the GnRH enhancer sequence revealed eight candidate sequences partially homologous to different types of Pbx-binding elements (29). To test whether Pbx proteins and their cofactors could bind any of these sites, EMSAs were performed employing GT1-7 nuclear extracts. As a control, a consensus site for cooperative binding of Pbx/Hox and Pbx/Meis/Prep1 heterodimers (PBX oligonucleotide TTGATTGAT) was used (20).

One of the candidate sites in the GnRH enhancer (TGAaTGAtAG, −1749 to −1739) contained 8/10 matches to the optimal Pbx/Meis consensus sequence (TGATTGACAG) (29). When this motif was used as a probe (−1749), it was observed that an excess of the PBX consensus oligonucleotide, but not of the TTaATTGAT mutant oligonucleotide (PBX-mut), successfully competed the −1749 probe-specific complexes (Fig. 2A, *lanes 9* and *10*, respectively). Furthermore, when the PBX consensus oligonucleotide was used as a probe, two complexes of equivalent mobility to those observed with the −1749 probe were detected (Fig. 2A, *lane 1*). These complexes were competed by an excess of the unlabeled PBX consensus oligonucleotide and less efficiently by the −1749 oligonucleotide (Fig. 2A, *lanes 2* and *3*), but not by the PBX-mut oligonucleotide (*lane 4*). These results suggest that Pbx proteins and their cofactors are major components of the protein complexes that bind to the −1749 probe. The specific sequence bound by the −1749 complexes has been mapped previously to the TGAtAG motif by mutational analysis. However, methylation interference analysis also revealed the importance of the 5′-TGAa sequence for complex binding (11,33). Thus, the element bound by the −1749 complexes coincides with the Pbx site.

To confirm the presence of Pbx proteins in the two complexes (A1 and A2), a supershift assay was conducted. Incubation of the binding reaction with a polyclonal antibody that recognizes all members of the Pbx family produced a supershifted band and eliminated the formation of both complexes, whereas nonspecific immunoglobulin had no effect (Fig. 2A, *lanes 17* and *14*, respectively). Furthermore, incubation with Pbx1-specific antiserum caused a slight reduction in the upper (A1) and lower (A2) complexes (Fig. 2A, *lane 11*), whereas an antiserum directed against the long Pbx forms (Pbx1a, Pbx2, and Pbx3a) supershifted the upper band completely (*lane 12*). Pbx2-specific antiserum caused the formation of a faint supershift, whereas Pbx3-specific antiserum had no effect (Fig. 2A, *lanes 15* and *16*, respectively).

After identifying Pbx family members in complexes A1 and A2, we also tested whether the Pbx cofactors Prep1 and Meis1 were present in the complexes binding to the −1749 site. A polyclonal antibody against Prep1 eliminated the two complexes completely (Fig. 2A, *lane 19*). Although anti-Meis1 monoclonal antibody caused the formation of a supershifted complex, no significant changes in the binding of complexes A1 and A2 were observed (Fig. 2A, *lane 18*). These data were validated using *in vitro* translated proteins for reconstituting the binding activity. This analysis revealed that cooperative binding of Prep1, Pbx1a, and Pbx1b was sufficient for reconstitution of the complexes *in vitro* (data not shown). *In vitro* translated Pbx1·Meis1 complexes were detected on the −1749 site using the OCT-PBX probe (Fig. 2B, *lanes 3* and *4*; for probe sequence, see Fig. 3B); however, no trimeric Pbx·Meis1·Prep1 complexes were observed (Fig. 2B, *lane 6*). These data indicate that Pbx/Prep1 and Pbx/Meis1 heterodimers may compete with each other for binding to this site and prevent a detectable

change in binding in the presence of antibody recognizing Meis1, as shown in Fig. 2A (*lane 18*). We therefore conclude that the factors that bind to the −1749 element of the GnRH enhancer are Pbx/Prep1 and Pbx/Meis1 heterodimers; that the upper protein complex (A1) contains Prep1/Pbx1a, Meis1/Pbx1a, and possibly Prep1/Pbx2 and Meis1/Pbx2 heterodimers; and that the lower protein complex (A2) contains Prep1/Pbx1b and Meis1/Pbx1b heterodimers.

Further analysis revealed an additional Pbx/Prep1-binding site within the 3′-region (34) of the GnRH enhancer (−1603) and two Pbx/Prep1-binding sequences within the GnRH promoter, one located at −75 (in the upstream region of footprint 2) and one at −100 (in footprint 4) (4). Competitions of the complexes bound to these sites with the −1749 oligonucleotide are shown in Fig. 2C. All three elements have been shown to be essential for GnRH transcription since mutations of these sites decrease GnRH gene expression to 50% (−75 and −100 sites) and 10% (truncation of the region containing the −1603 site) of the wild-type level (34,35). Furthermore, the −1749 element has been shown previously to play an important role in GnRH enhancer activity, as a specific 2-bp mutation in this element reduces GnRH enhancer activity to 20% of the wild-type level (11). Therefore, we have determined the identity of the transcription factors in previously uncharacterized complexes that bind to regions of the GnRH enhancer and promoter shown to be essential for appropriate transcriptional activity. The Pbx/Prep1- and Oct-1-binding sites in the rat GnRH enhancer and promoter are illustrated in Fig. 3.

Pbx1 and Prep1 Interact in Vivo with the GnRH Regulatory Region

The EMSA data indicate *in vitro* binding of the TALE proteins Pbx1, Prep1, and Meis1 to the rat GnRH enhancer and promoter. To examine whether the TALE complexes associate with the GnRH regulatory region *in vivo*, a chromatin immunoprecipitation assay was utilized with antibodies recognizing the Pbx1 and Prep1 proteins. Since both Pbx/Prep1-binding sites within the GnRH promoter are highly conserved between rat and mouse (4), we were able to test transcription factor interaction with the endogenous mouse gene. PCR amplification of the DNA (immunoprecipitated by antibodies) showed that both antibodies precipitated the mouse GnRH promoter region in GT1-7 cells (Fig. 3C). Moreover, anti-Pbx1 and anti-Prep1 antibodies did not precipitate the GnRH promoter in the pituitary gonadotroph cell line LβT2, but does precipitate the follicle-stimulating hormone-β promoter in these cells (36). This suggests that Pbx1 and Prep1 associate with the GnRH promoter in GT1-7 cells, but not in LβT2 cells, which do not express GnRH. Taken together, these data indicate that, *in vivo* as well as *in vitro*, Pbx1 and Prep1 are components of the transcription factor complexes that form on the transcriptionally active GnRH promoter.

A Dominant-negative Form of Prep1 Interferes with GnRH Enhancer Activity

The data presented herein show that TALE proteins are present in the GnRH-expressing GT1-7 cells and that Pbx/Prep1 and Pbx/Meis1 heterodimers interact with the Pbx motifs within the GnRH regulatory region. Moreover, previous studies have shown that these Pbx motifs are important for appropriate transcriptional activity of the GnRH enhancer and promoter (11, 35). Therefore, it was important to determine whether TALE proteins are necessary for GnRH transcriptional activity in GT1-7 cells. For this purpose, we performed transient transfection experiments with reporter plasmids containing GnRHe-RSVp driving luciferase expression. Importantly, mutation of the Pbx/Prep1-binding sequence within the −1749 element (TGAATGATAG → TGAATctTAG) reduced GnRH enhancer activity to 30% of the wild-type level (Fig. 4A). These data are in agreement with previous results that show reduction to 20% of the wild-type level (11). We also cotransfected an expression plasmid encoding dnPrep1. dnPrep1 (illustrated in Fig. 1D) contains only the N-terminal domain, which is highly conserved between Prep1, the Meis family, and their single *Drosophila* homolog, HTH (28, 31,37), and serves for the interaction with Pbx proteins (37). The use of this N-terminal region as a dominant-negative is based on observations in *Drosophila*. The N-terminal domain of

HTH blocks endogenous HTH activity *in vivo* due to its ability to compete with HTH for the interaction with EXD (Pbx homolog) (38). Accordingly, we hypothesized that Pbx·dnPrep1 complexes within GT1-7 cells would interfere with the wild-type activity of the Pbx·Prep1 complexes in regulating GnRH gene transcription. Cotransfections of the enhancer-reporter plasmid (GnRHe-RSVp) with dnPrep1 caused an ~40% decrease in activity compared with cotransfection of the empty expression plasmid (pcDNA1.1) (Fig. 4A). The dnPrep1-mediated decrease in expression was eliminated by mutation of the −1749 Pbx/Prep1 element, indicating specific use of this GnRH enhancer site. These data demonstrate the ability of the GnRH enhancer Pbx/Prep1 motif to serve as a site for Pbx/Prep-mediated regulation in the context of the entire enhancer within the environment of the GT1-7 cell, thus revealing the functional role of the TALE proteins in GnRH gene transcription.

TALE Proteins and Oct-1 Activate Transcription through the GnRH Enhancer

Our findings thus far indicate that Oct-1 and TALE proteins physically interact *in vitro* and bind to several sites within the enhancer and promoter. To address the functional relevance of these interactions to GnRH gene transcription, we performed transient transfections in GT1-7 cells using the GnRHe-RSVp reporter. Overexpression of human Pbx1b, Prep1, Meis1, or Oct-1 individually with the reporter plasmid did not cause a significant increase in activity compared with the empty expression vector pcDNA1.1 (Fig. 4B) (data not shown). However, coexpression of Oct-1 with Prep1 or Pbx1b resulted in a significant activation (2.4- and 1.9-fold, respectively). Overexpression of Pbx1b and Prep1 also increased reporter activity by 2.1-fold. Interestingly, a reporter plasmid containing a mutation in the −1749 Pbx/Prep1-binding site was not significantly responsive to exogenously expressed proteins (Fig. 4B), demonstrating that the transactivation was specifically dependent on this motif. Taken together, these data suggest that TALE proteins and Oct-1 transactivate the GnRH enhancer cooperatively through the −1749 Pbx/Prep1-binding site, thus signifying a role for TALE·Oct-1 complexes in GnRH gene activation.

Interestingly, the Pbx/Prep-binding sites described herein all reside within close proximity to Oct-1-binding sites in the GnRH regulatory region. In fact, the Pbx/Prep-binding site at −100 partially overlaps the Oct-1-binding sequence (Fig. 3B). Similar co-location has been noted in several additional promoters (39–43). Despite these observations, protein-protein interaction in the context of DNA has not yet been explored. We performed EMSA using the OCT-PBX probe of the GnRH enhancer and a GT1-7 nuclear extract. Using increasing concentrations of nuclear extract, we observed the appearance of two slower mobility complexes, A and B, located above the Pbx1·Prep1 and Oct-1 complexes (Fig. 4C, *lanes 4* and *9*). Complex A was eliminated by addition of anti-Oct-1, anti-Prep1, or anti-Pbx1 antibody, but not by nonspecific immunoglobulin (Fig. 4C, *lanes 4–8*, respectively). Complex B was reduced by addition of anti-Oct-1 and anti-Prep1 antibodies, but not by anti-Pbx1 antibody (Fig. 4C, *lanes 5–7*, respectively), suggesting that it contains only Oct-1 and Prep1 proteins. These results indicate that Oct-1, Pbx1, and Prep1 can associate with the DNA concurrently as well as allow for the possibility that Oct-1 and Pbx1 or Prep1 can form heterodimeric complexes in the presence of DNA.

Pbx1 and Oct-1 Are Present in GnRH Neurons in Mouse

GnRH neurons arise in the olfactory placode at embryonic day 11 in the mouse embryo and then migrate across the nasal septum through the forebrain and into the hypothalamus (44, 45). Interestingly, according to a previous report, Pbx1 mRNA expression is also detected in the olfactory region and is restricted primarily to neuronal tissue in the early developing rat embryo (46). In addition, Pbx1b protein expression is already detected in all principal germ layers in 9.5 dpc mouse embryos (47). To verify the relevance of our findings in GT1-7 cells to GnRH expression *in vivo*, we tested whether Pbx1 is expressed in GnRH neurons in the

mouse. We analyzed mouse embryos at 13.5 dpc, at which time the entire population of GnRH neurons has been established and the cells are migratory (48). *In situ* hybridization and immunohistochemistry performed on parasagittal sections showed a trail of neurons expressing the GnRH peptide (*brown*) located from the olfactory epithelium to the cribriform plate, crossing into the anterior forebrain (Fig. 5A). Strong expression of Pbx1 mRNA (*purple*) was seen in the anterior forebrain, including the primordium of the septum and the developing diencephalon and preoptic area, consistent with the brain regions containing GnRH neurons during development and in the adult (44,48). Furthermore, expression of Pbx1 detected along the olfactory nerve co-localized with the migratory GnRH-positive neurons (Fig. 5C). We also verified that Oct-1 was present in GnRH-expressing neurons during development, again using *in situ* hybridization combined with immunohistochemistry on embryos at 13.5 dpc. At this stage, expression of Oct-1 mRNA could be seen in the anterior forebrain. including the subventricular zone of the medial and lateral ganglionic eminences as well as the developing neocortex. Strong expression of Oct-1 was also observed in the developing olfactory region, including the vomeronasal organ. Fig. 5B depicts clusters of cells expressing the Oct-1 transcript (*purple*) that are crossing the cribriform plate into the anterior forebrain. This same section was then incubated with an antibody recognizing the GnRH peptide (*brown*), which identified these same cell clusters as GnRH neurons (Fig. 5D). Therefore, Oct-1 and Pbx1 are expressed in GnRH-positive neurons at embryonic day 13.5 during development.

To verify expression at the protein level, transgenic mice carrying the rat GnRH enhancer plus the −173 proximal promoter fused to the *β*-galactosidase reporter gene were utilized (6). This transgenic line (GnRHe-GnRHp-Gal) was created in our laboratory to establish the specificity of the GnRH regulatory regions *in vivo*. Indeed, in these mice, *β*-galactosidase enzymatic activity is detected exclusively in GnRH neurons throughout development (6). We therefore used *β*-galactosidase staining as a marker for neurons expressing the GnRH gene. Immunohistochemical analysis with a Pbx1-specific antibody was used to visualize the Pbx1 proteins. Pbx1 expression (seen in *brown*, throughout the cells) was observed in a subset of GnRH-expressing neurons (*blue*) in mouse embryos (Fig. 5E). Having demonstrated co-localization at embryonic stages, we then wondered whether the Pbx1 protein is also present in fully differentiated GnRH neurons. Co-immunohistochemistry was conducted on wild-type adult mice. Nickel (*black*) was used to visualize the Pbx1 protein recognized by the antibody, and DAB (*brown*) was used to visualize GnRH-positive cells. This analysis revealed that Pbx1 was expressed in a subset of GnRH-expressing neurons scattered throughout the adult hypothalamus (Fig. 5, F and G). Thus, Pbx1 is expressed in embryonic as well as mature GnRH neurons and may regulate GnRH expression *in vivo*.

Discussion

As increasing numbers of transcription factors are identified, it appears that a greater diversity of gene expression can be accomplished via a combinatorial code, in which functional complexes apply different transcriptional effects on subgroups of DNA-binding elements (1). Here, we explore a novel interaction between proteins belonging to the POU and TALE homeodomain families of transcriptional regulators. We have demonstrated that the POU domain protein Oct-1 forms heterodimeric complexes with Pbx1 and Prep1. Moreover, we have shown that the interplay between Oct-1 and Pbx1/Prep1 contributes to transcriptional activation of the GnRH gene in hypothalamic neurons through specifically binding to functional elements within the GnRH regulatory regions.

Four Pbx/Prep1-binding sites and five Oct-1-binding sites are present within the GnRH enhancer and promoter. Interestingly, the Oct-1- and TALE-binding sites are paired in close proximity to each other (Fig. 3, A and B). The binding sites for Oct-1 and Pbx1/Prep1 in the −1603 enhancer element as well as in the −100 promoter element actually overlap each other,

whereas the −1749 enhancer TALE site and the −75 promoter TALE site are separated from Oct-1-binding sites by 25 and 21 bp, respectively. The observation of clustered Oct-1- and Pbx1/Prep1-binding sites in promoter regions has also been reported in the context of the urokinase enhancer (42), the Hoxb1 auto-regulatory enhancer (43), and the UDP-glucuronosyltransferase 2B15A promoter (49). Similarly, we observed four of these clustered binding elements dispersed throughout the GnRH enhancer and promoter regions. The placement of these Oct-1/Pbx1/Prep1 motifs in both the GnRH enhancer and promoter appears to facilitate interactions between these two regulatory regions, which are 1.5 kb apart. We observed that although a mutation in the −1749 Pbx/Prep1 element reduced GnRH enhancer activity to 20–30% on a heterologous promoter (Fig. 4A) (11), it did not reduce the level of transcriptional activity when situated on a plasmid containing both the GnRH enhancer and promoter regions (11). This difference might reflect compensation by the two promoter sites and suggests interplay between the enhancer and promoter.

Having isolated Pbx1b in our yeast two-hybrid screen for Oct-1 cofactors and subsequently identifying additional GT1-7 TALE proteins that form functional complexes with Pbx, we hypothesized that TALE·Oct-1 complexes may also be functional in transcriptional regulation. We further predicted their function as activators since most of the Oct-1- and TALE-binding sites within the GnRH enhancer and promoter are essential for transcriptional activation (7, 11,35). Indeed, the combination of Prep1 or Pbx1b with Oct-1 significantly increased GnRH reporter gene expression (Fig. 4B). Overexpression of Pbx1b and Prep1 together with Oct-1 did not result in transcriptional activation (data not shown), suggesting that these proteins do not stimulate transcription in the form of a DNA-bound ternary complex. However, the ability of Oct-1 and the TALE complexes to activate transcription through binding to their own sites, as well as through formation of TALE/Oct-1 heterodimers, may provide additional flexibility to the GnRH transcriptional machinery. Our *in vitro* mapping data show that Prep1 and Pbx1b interact with Oct-1 through their homeodomain and C-terminal regions, although they were previously shown to complex with each other through their N-terminal domains (37). Therefore, since Oct-1 and TALE proteins do not compete for the same interface, we believe that TALE·TALE and TALE·Oct-1 complexes can form and function concurrently. Importantly, our finding of cooperation between TALE and Oct-1 proteins is likely relevant to the transcriptional regulation of many genes since these factors are broadly expressed.

Oct-1 and TALE proteins may also be involved in the regulation of the GnRH gene by extracellular signals. Indeed, Oct-1 has been shown to interact with the glucocorticoid receptor on the GnRH promoter (13). Furthermore, both Oct-1 and Pbx1 are required for glucocorticoid-mediated repression of the prolactin promoter (39). Functional analysis established that the DNA-binding domain of the glucocorticoid receptor is necessary for synergy with both proteins in the context of different promoters (50,51). Oct-1 has also been implicated in repression of GnRH transcription by the nitric oxide pathway (12). Moreover, we recently identified Pbx1 and Prep1 as Smad cofactors and mediators of activin regulation of the follicle-stimulating hormone-β gene in the pituitary gonadotroph (36).

GnRH gene expression is the identifying characteristic of a small subset of hypothalamic neurons that control reproductive function. However, Oct-1, Pbx/Prep1, and the other proteins identified thus far that regulate GnRH transcription are also expressed in other tissues and brain regions. Thus, tissue-specific expression in this rare cell type may be promoted by a unique combination of these broadly expressed proteins. The contribution of Pbx proteins to tissue-specific expression in a combinatorial manner has been described by several studies (52,53). For example, the pancreatic cell-specific activity of the homeodomain factor Pdx1 changes due to interactions with TALE proteins. In exocrine cells, the elastase enhancer is regulated by a Pdx1·Meis2·Pbx1b complex, whereas in endocrine cells, Pdx1 binds alone (54). Interestingly, the Pbx1b protein is highly expressed in exocrine cells, but absent in endocrine

cells. Similarly, it is possible that a particular form of the Pbx protein family might play a role in the tissue-specific regulation of GnRH gene expression.

The cellular location of Pbx1 may also contribute to the specificity of GnRH gene expression. Unlike other homeodomain proteins, EXD and Pbx1 are often found in the cytoplasm and are translocated into specific nuclei at precise times during development (24,55,56). Recent reports suggest a mechanism for regulating Pbx/EXD target genes in which a protein-protein interaction between Pbx/EXD and their cofactors (Prep1/HTH) results in nuclear translocation (57). Endogenous Pbx1 was located in both the nucleus and cytoplasm of GT1-7 cells under our culture conditions (data not shown). Intriguingly, the subcellular localization of Pbx1 in the GnRH neuron *in vivo* appears to change during development. Although Pbx1 was detected throughout the cells in the embryo, it appeared to be expressed exclusively in the nuclei in adult neurons (Fig. 5). These data suggest that Pbx1-induced transcriptional activity might be required at a relatively late stage of brain development. However, Pbx1 may play an important role in composing the molecular identity of the GnRH neuron at embryonic stages.

Remarkably, Pbx1 is found in GnRH neurons throughout development. Our *in situ* hybridization and immunohistochemistry findings show expression of Pbx1 in GnRH neurons as early as 13.5 dpc in the mouse embryo, persisting until adulthood. Moreover, Pbx1 is also expressed in the pituitary at 13.5 dpc. In light of these observations, it will be interesting to examine the development of the reproductive system and its function in Pbx1-deficient mice. These mice die at 15/16 dpc, with severe abnormalities of multiple tissue and organs, including the skeleton (58), pancreas (59), liver (60), and kidney (61). Although they display defects in urogenital development (61), the development of the hypothalamus or pituitary has not been analyzed. Our characterization of Pbx1/Prep1 protein function in GnRH-expressing neurons as well as in the regulation of follicle-stimulating hormone-β expression in gonadotrophs (36) suggests a role for Pbx1 in proper functioning of the reproductive axis.

In conclusion, we have explored a novel interaction between the transcription factors Pbx/Prep1 and Oct-1. We show that these proteins bind to several clustered elements within the GnRH regulatory region that are essential for activation of GnRH gene expression in hypothalamic neurons. In addition to their role in the context of the GnRH gene, our findings may provide insight into the function of Oct-1 and TALE proteins in the regulation of other genes.

Acknowledgments

We thank Dr. Michel Strubin for providing the yeast strains and expression vectors required for the two-hybrid system and Dr. Michael L. Cleary for providing the anti-Meis1 antisera and the mouse Pbx1b cDNA. We thank Teri Williams, Brian Powl, Scott Anderson, Shannon Snyder, Laura Neely, and Rachel White for excellent technical assistance and Drs. Carolyn Kelley and Mark Lawson for early contributions to this work. We also thank members of the Mellon laboratory for reagents, discussions, and critical reading of the manuscript.

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. Mellon PL, Windle JJ, Goldsmith P, Pedula C, Roberts J, Weiner RI. Neuron 1990;5:1–10. [PubMed: 2196069]
4. Eraly SA, Mellon PL. Mol Endocrinol 1995;9:848–859. [PubMed: 7476968]
5. Whyte DB, Lawson MA, Belsham DD, Eraly SA, Bond CT, Adelman JP, Mellon PL. Mol Endocrinol 1995;9:467–477. [PubMed: 7659090]
6. Lawson MA, MacConell LA, Kim J, Powl BT, Nelson SB, Mellon PL. Endocrinology 2002;143:1404–1412. [PubMed: 11897697]

7. Clark ME, Mellon PL. Mol Cell Biol 1995;15:6169–6177. [PubMed: 7565769]
8. Wierman ME, Xiong X, Kepa JK, Spaulding AJ, Jacobsen BM, Fang Z, Nilaver G, Ojeda SR. Mol Cell Biol 1997;17:1652–1665. [PubMed: 9032292]
9. Wolfe A, Kim HH, Tobet S, Stafford DE, Radovick S. Mol Endocrinol 2002;16:435–449. [PubMed: 11875100]
10. Kelley CG, Lavorgna G, Clark ME, Boncinelli E, Mellon PL. Mol Endocrinol 2000;14:1246–1256. [PubMed: 10935548]
11. Lawson MA, Whyte DB, Mellon PL. Mol Cell Biol 1996;16:3596–3605. [PubMed: 8668176]
12. Belsham DD, Mellon PL. Mol Endocrinol 2000;14:212–228. [PubMed: 10674395]
13. Chandran UR, Warren BS, Baumann CT, Hager GL, DeFranco DB. J Biol Chem 1999;274:2372–2378. [PubMed: 9891005]
14. Strubin M, Newell JW, Matthias P. Cell 1995;80:497–506. [PubMed: 7859290]
15. Gstaiger M, Knoepfel L, Georgiev O, Schaffner W, Hovens CM. Nature 1995;373:360–362. [PubMed: 7779176]
16. Voss JW, Wilson L, Rosenfeld MG. Genes Dev 1991;5:1309–1320. [PubMed: 2065979]
17. Schiestl RH, Gietz RD. Curr Genet 1989;16:339–346. [PubMed: 2692852]
18. Robzyk K, Kassir Y. Nucleic Acids Res 1992;20:3790. [PubMed: 1641351]
19. Sanger F, Nicklen S, Coulson AR. Proc Natl Acad Sci U S A 1977;74:5463–5467. [PubMed: 271968]
20. Lu Q, Knoepfler PS, Scheele J, Wright DD, Kamps MP. Mol Cell Biol 1995;15:3786–3795. [PubMed: 7791786]
21. Schreiber E, Merchant RE, Wiestler OD, Fontana A. Neurosurgery (Baltimore) 1994;34:129–135.
22. Ausubel, FM.; Brent, R.; Kingston, RE.; Moore, DD.; Seidman, JG.; Smith, JA.; Struhl, K. Current Protocols in Molecular Biology. John Wiley & Sons, Inc.; New York: 1987.
23. Coss D, Jacobs SB, Bender CE, Mellon PL. J Biol Chem 2004;279:152–162. [PubMed: 14570911]
24. González-Crespo S, Morata G. Development (Cam) 1995;121:2117–2125.
25. Delegeane AM, Ferland LH, Mellon PL. Mol Cell Biol 1987;7:3994–4002. [PubMed: 2828923]
26. Simmons DM, Voss JW, Ingraham HA, Holloway JM, Broide RS, Rosenfeld MG, Swanson LW. Genes Dev 1990;4:695–711. [PubMed: 2379827]
27. Mann RS, Chan SK. Trends Genet 1996;12:258–262. [PubMed: 8763497]
28. Chang CP, Jacobs Y, Nakamura T, Jenkins NA, Copeland NG, Cleary ML. Mol Cell Biol 1997;17:5679–5687. [PubMed: 9315626]
29. Berthelsen J, Zappavigna V, Mavilio F, Blasi F. EMBO J 1998;17:1423–1433. [PubMed: 9482739]
30. Monica K, Galili N, Nourse J, Saltman D, Cleary ML. Mol Cell Biol 1991;11:6149–6157. [PubMed: 1682799]
31. Knoepfler PS, Calvo KR, Chen H, Antonarakis SE, Kamps MP. Proc Natl Acad Sci U S A 1997;94:14553–14558. [PubMed: 9405651]
32. Kamps MP, Murre C, Sun XH, Baltimore D. Cell 1990;60:547–555. [PubMed: 1967983]
33. Lawson MA, Buhain AR, Jovenal JC, Mellon PL. Mol Endocrinol 1998;12:364–377. [PubMed: 9514154]
34. Kelley CG, Givens ML, Rave-Harel N, Nelson SB, Anderson S, Mellon PL. Mol Endocrinol 2002;16:2413–2425. [PubMed: 12403831]
35. Eraly SA, Nelson SB, Huang KM, Mellon PL. Mol Endocrinol 1998;12:469–481. [PubMed: 9544983]
36. Bailey JS, Rave-Harel N, Coss D, McGillivray SM, Mellon PL. Mol Endocrinol 2004;18:1158–1170. [PubMed: 14764653]
37. Berthelsen J, Zappavigna V, Ferretti E, Mavilio F, Blasi F. EMBO J 1998;17:1434–1445. [PubMed: 9482740]
38. Ryoo HD, Marty T, Casares F, Affolter M, Mann RS. Development (Cam) 1999;126:5137–5148.
39. Subramaniam N, Cairns W, Okret S. J Biol Chem 1998;273:23567–23574. [PubMed: 9722596]
40. Carrier JS, Turgeon D, Journault K, Hum DW, Belanger A. Biochem Biophys Res Commun 2000;272:616–621. [PubMed: 10833461]

41. Mayer CM, Cai F, Cui H, Gillespie JM, MacMillan M, Belsham DD. Biochem Biophys Res Commun 2003;307:847–854. [PubMed: 12878188]
42. Palazzolo M, Berthelsen J, De Cesare D, Blasi F. Eur J Biochem 2000;267:5427–5437. [PubMed: 10951201]
43. Di Rocco G, Gavalas A, Popperl H, Krumlauf R, Mavilio F, Zappavigna V. J Biol Chem 2001;276:20506–20515. [PubMed: 11278854]
44. Schwanzel-Fukuda M, Pfaff DW. Nature 1989;338:161–164. [PubMed: 2645530]
45. Schwanzel-Fukuda M, Jorgenson KL, Bergen HT, Weesner GD, Pfaff DW. Endocr Rev 1992;13:623–634. [PubMed: 1459046]
46. Roberts VJ, van Dijk MA, Murre C. Mech Dev 1995;51:193–198. [PubMed: 7547467]
47. Schnabel CA, Selleri L, Jacobs Y, Warnke R, Cleary ML. Mech Dev 2001;100:131–135. [PubMed: 11118899]
48. Wray S, Grant P, Gainer H. Proc Natl Acad Sci U S A 1989;86:8132–8136. [PubMed: 2682637]
49. Turgeon D, Carrier JS, Levesque E, Beatty BG, Belanger A, Hum DW. J Mol Biol 2000;295:489–504. [PubMed: 10623541]
50. 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]
51. Subramaniam N, Campion J, Rafter I, Okret S. Biochem J 2003;370:1087–1095. [PubMed: 12487626]
52. Herzig S, Fuzesi L, Knepel W. J Biol Chem 2000;275:27989–27999. [PubMed: 10869353]
53. Penkov D, Tanaka S, Di Rocco G, Berthelsen J, Blasi F, Ramirez F. J Biol Chem 2000;275:16681–16689. [PubMed: 10748126]
54. Swift GH, Liu Y, Rose SD, Bischof LJ, Steelman S, Buchberg AM, Wright CV, MacDonald RJ. Mol Cell Biol 1998;18:5109–5120. [PubMed: 9710595]
55. González-Crespo S, Abu-Shaar M, Torres M, Martínez AC, Mann RS, Morata G. Nature 1998;394:196–200. [PubMed: 9671305]
56. Rieckhof GE, Casares F, Ryoo HD, Abu-Shaar M, Mann RS. Cell 1997;91:171–183. [PubMed: 9346235]
57. Berthelsen J, Kilstrup-Nielsen C, Blasi F, Mavilio F, Zappavigna V. Genes Dev 1999;13:946–953. [PubMed: 10215622]
58. Selleri L, Depew MJ, Jacobs Y, Chanda SK, Tsang KY, Cheah KS, Rubenstein JL, O'Gorman S, Cleary ML. Development (Cam) 2001;128:3543–3557.
59. Kim SK, Selleri L, Lee JS, Zhang AY, Gu X, Jacobs Y, Cleary ML. Nat Genet 2002;30:430–435. [PubMed: 11912494]
60. DiMartino JF, Selleri L, Traver D, Firpo MT, Rhee J, Warnke R, O'Gorman S, Weissman IL, Cleary ML. Blood 2001;98:618–626. [PubMed: 11468159]
61. Schnabel CA, Godin RE, Cleary ML. Dev Biol 2003;254:262–276. [PubMed: 12591246]

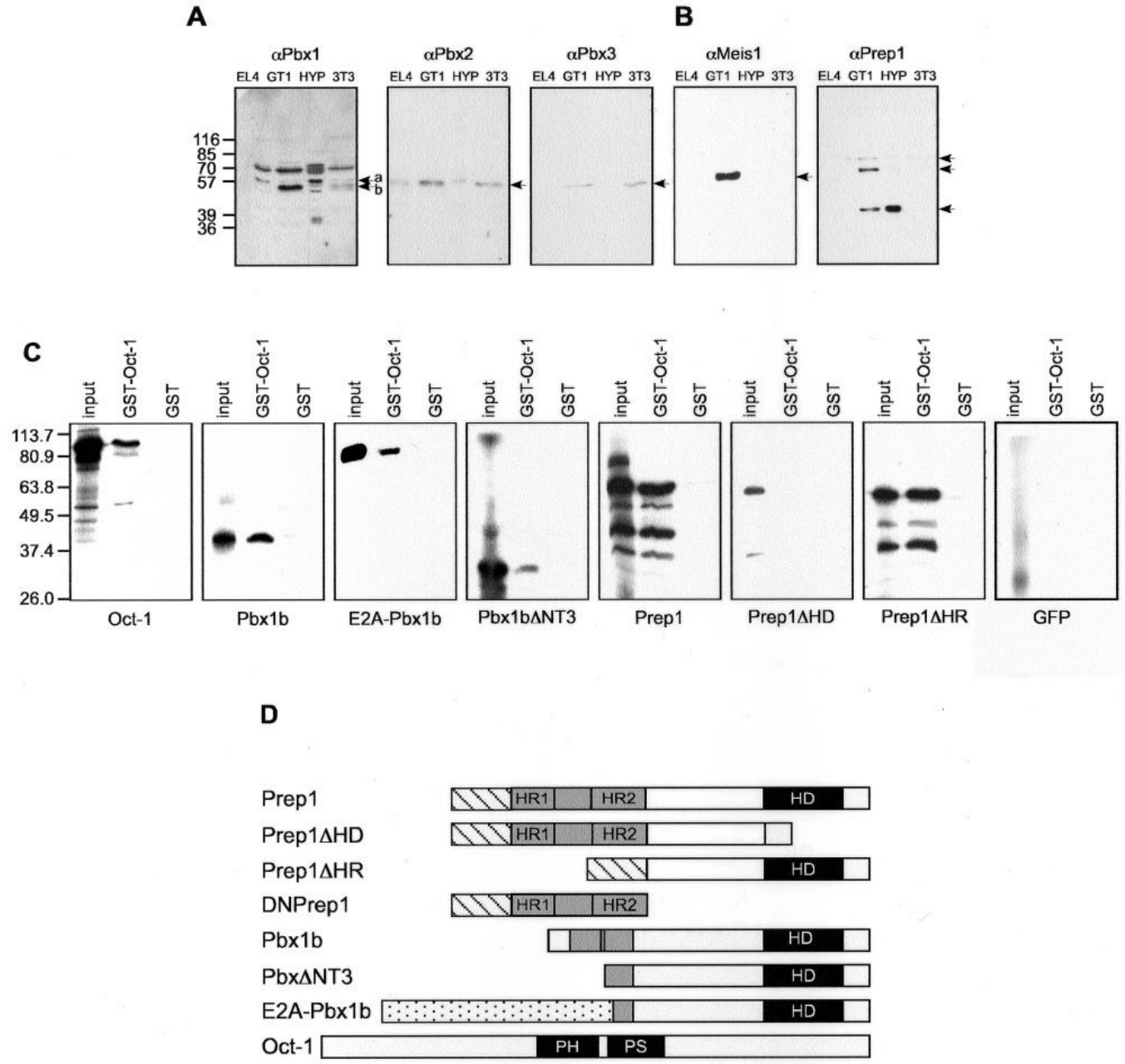

Fig. 1. TALE proteins present in GT1-7 cells specifically associate with Oct-1

A, Pbx proteins are expressed in GT1-7 cells. Western blots of nuclear extracts were probed with antibodies specific to Pbx1, Pbx2, and Pbx3. Each lane contained 12 μg of total protein from mouse hypothalamus (*HYP*) or the cell line indicated. *Numbers* on the left are molecular masses in kilodaltons. The *arrows* mark the positions of the identified proteins. The two Pbx1 forms, Pbx1a (*arrow a*) and Pbx1b (*arrow b*), are indicated. *B*, Pbx cofactors Prep1 and Meis1 are expressed in GT1-7 cells. Western blot analysis was performed using antibodies specific to Prep1 and Meis1. *C*, mapping the interaction between Prep1 and Pbx1b with Oct-1. *In vitro* translated ^{35}S-labeled 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 in the *input lanes* to visualize the protein products.

Numbers on the left are molecular masses in kilodaltons. *D*, schematic diagram of the protein constructs used in our GST pull-down assay. Both the Prep1 and Pbx proteins contain N-terminal conserved domains that serve for heterodimerization with each other (*gray boxes*) and a TALE (three-amino acid loop extension) homeodomain (*HD; black boxes*). The Prep1 N-terminal non-conserved domain is depicted by the *hatched boxes*. The E2A-Pbx1b fusion protein contains the transactivation domain of E2A (*dotted box*) fused to the homeodomain and C-terminal part of Pbx1b. The DNA-binding domain of Oct-1 consists of the POU-specific domain and the POU homeodomain (*PS* and *PH*, respectively; *black boxes*).

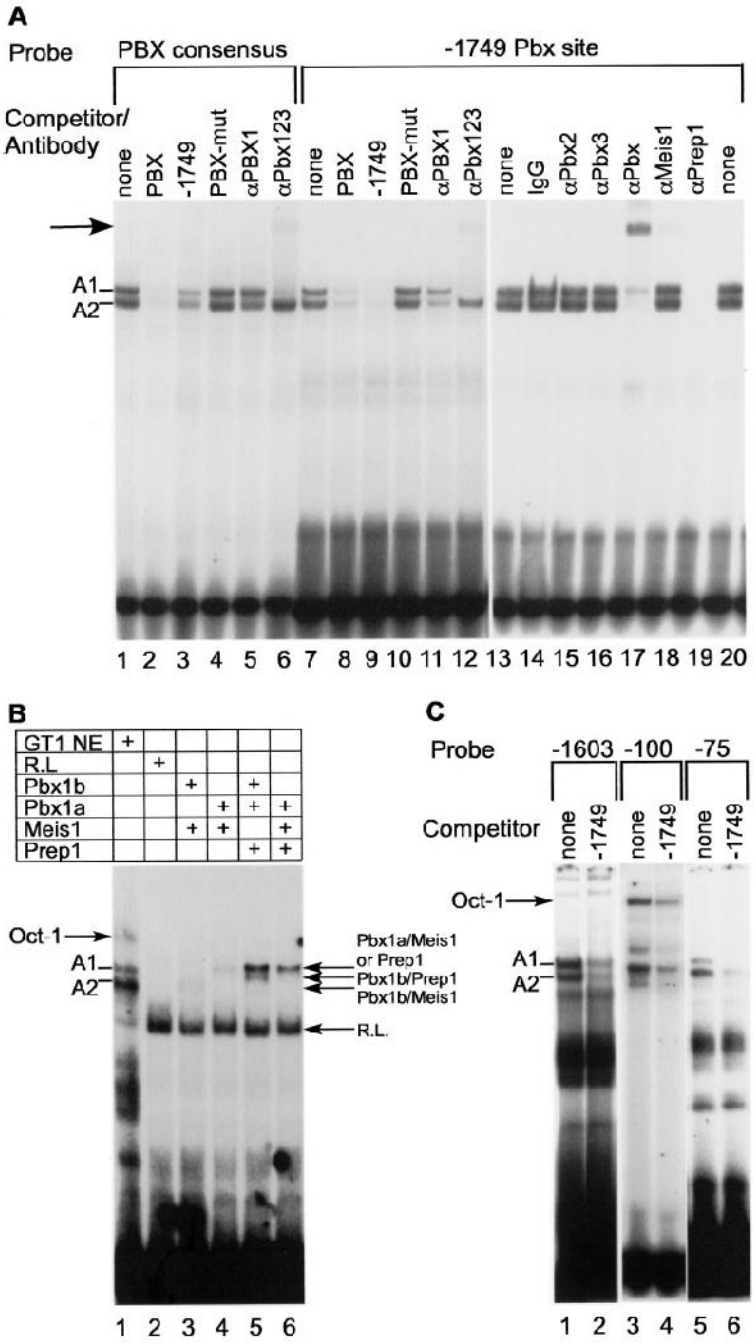

Fig. 2. Heterodimers of TALE homeodomain proteins bind four motifs within the GnRH enhancer and promoter

EMSA was performed on nuclear proteins isolated from the GT1-7 cell line. The DNA probes consisted of radiolabeled oligonucleotides containing the −1749 and −1603 sites of the enhancer, the −75 and −100 elements of the promoter, and a Pbx/Hox consensus site, PBX, which served as a control for Pbx-binding activity. *A*, TALE homeodomain proteins bind to the −1749 probe of the GnRH enhancer. The PBX probe is shown in *lanes 1–6*, and the −1749 probe is shown in *lanes 7–20*. A 100-fold excess of the unlabeled PBX oligonucleotide, the PBX-mut oligonucleotide, or the wild-type −1749 oligonucleotide used for competition and various antibodies used for supershift assays were included in the reaction mixtures as

indicated. The *arrow* mark the positions of supershifted complexes. *B*, comparison of the DNA-binding abilities of *in vitro* translated Pbx/Meis1 and Pbx/Prep1 using the OCT-PBX probe from the GnRH enhancer. EMSA was performed with GT1-7 nuclear extracts (*GT1 NE; lanes 1*) or with *in vitro* translated proteins as indicated. A control reaction containing the unprogrammed reticulocyte lysate (*R.L.*) is shown in *lane 2. C*, TALE homeodomain protein complexes bind to the −75, −100, and −1603 sites within the GnRH regulatory region. *Lanes 1* and *2*, the −1603 probe; *lanes 3* and *4*, the −100 probe; *lanes 5* and *6*, the −75 probe. A 100-fold excess of the unlabeled −1749 oligonucleotide was included in the reaction mixtures as indicated above the gel.

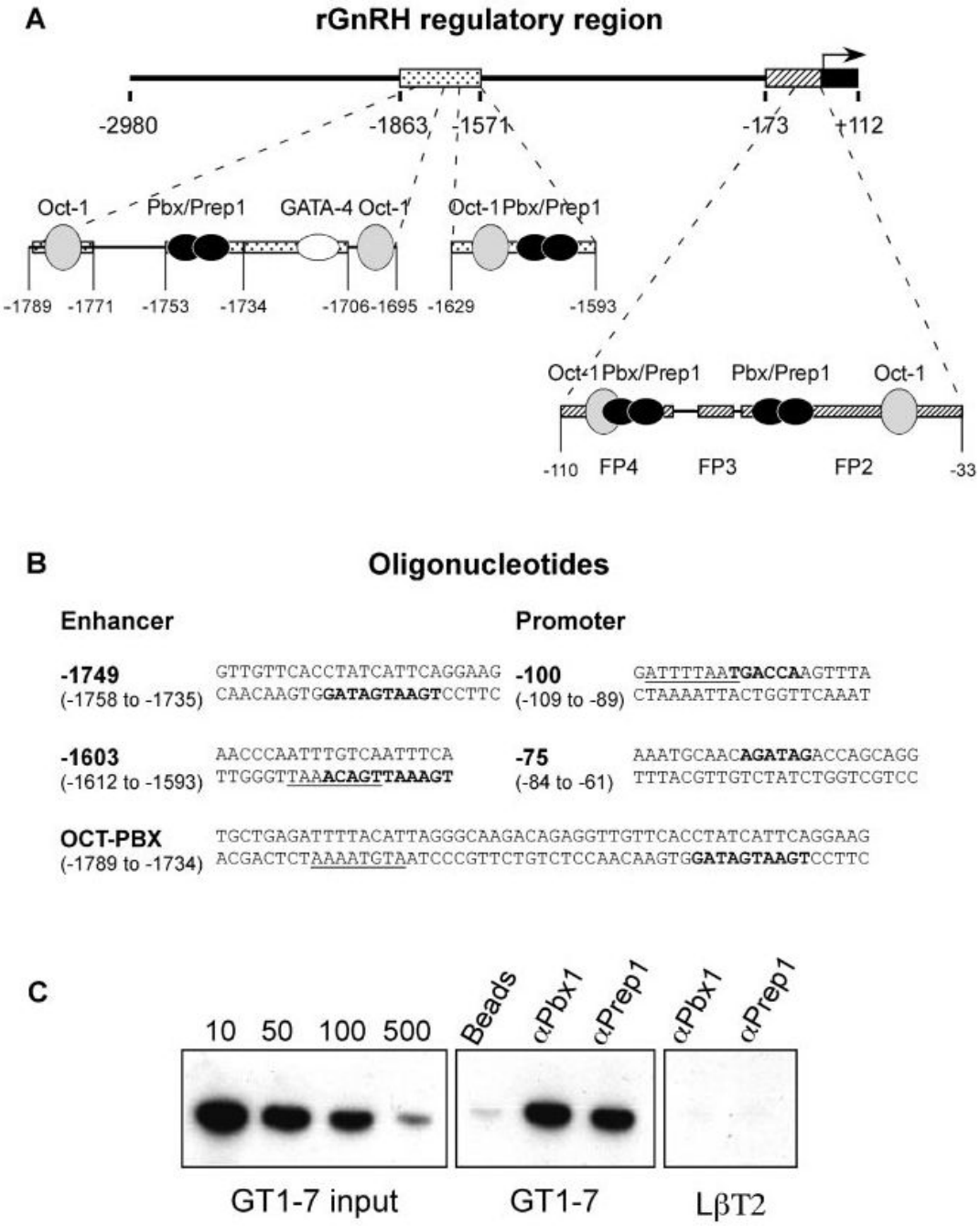

Fig. 3. TALE proteins interact with the GnRH promoter *in vivo*

A and *B*, the rat GnRH upstream regulatory region contains binding sites for the TALE and Oct-1 transcription factors. *A*, the *upper diagram* shows the positions of the elements in the rat sequence that confer neuron specificity, the enhancer (*dotted box*) and the proximal promoter (*hatched box*); the *lower diagrams* depict the central and proximal portions of the enhancer and the proximal portion of the promoter. The *boxes* enclose the regions footprinted (*FP*) by GT1-7 nuclear extract (5,35). The *ovals* illustrate the transcription factors: Oct-1 (*gray*), GATA-4 (*white*), and Pbx/Prep1 (*black*). *B*, the sequences of the rat GnRH enhancer and promoter elements that were used as oligonucleotide probes in EMSA are shown. The octamer motifs are *underlined*, and the Pbx-binding elements are in *boldface. C*, Pbx1 and

Prep1 interact with the GnRH promoter *in vivo*. A chromatin immunoprecipitation experiment was performed in GT1-7 cells using anti-Pbx1 and anti-Prep1 antibodies. The primers used for PCR amplification of the mouse GnRH promoter are located at −34 and −267 relative to the transcription start site and correspond to the conserved promoter elements depicted in the rat sequence. Immunoprecipitation of chromatin with either of the antibodies followed by PCR amplification gave the appropriate ~230-bp product. Immunoprecipitation with no antibody (*Beads*) was used as a negative control. Dilutions of 1:10 to 1:500 of the total input chromatin showed that PCR amplification of the immunoprecipitated chromatin was performed in the linear range. Immunoprecipitation of chromatin from LβT2 cells was used as an inactive GnRH promoter control. PCR amplification of the LβT2 chromatin total input was shown previously (36).

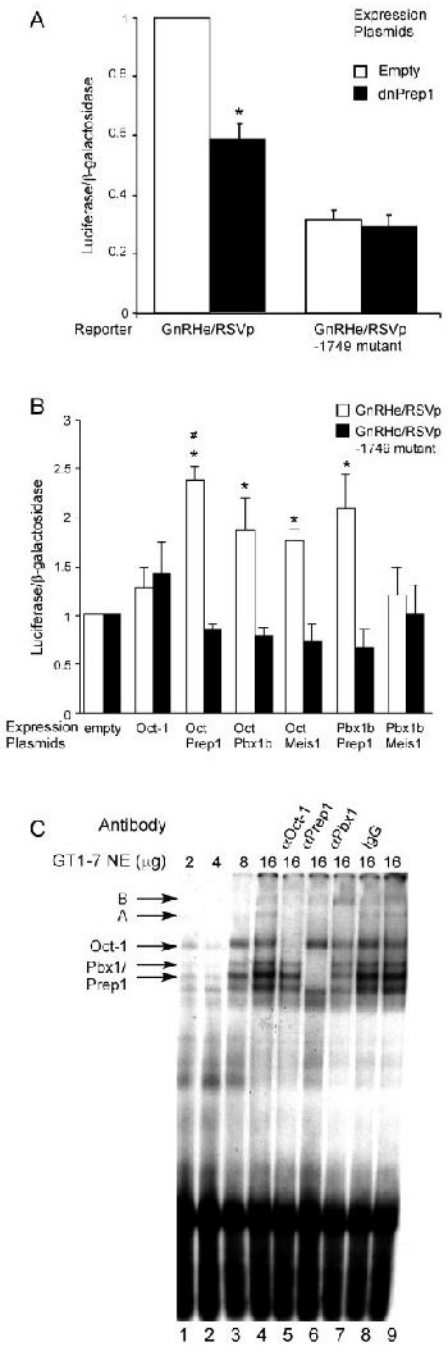

Fig. 4. TALE proteins in combination with Oct-1 regulate GnRH enhancer activity

A and *B*, transient transfections were conducted in GT1-7 cells with the GnRH enhancer (GnRHe-RSVp) and the GnRH enhancer containing a mutation of the −1749 Pbx/Prep1-binding element (GnRHe–1749mut-RSVp) driving luciferase expression. *A*, the dominant-negative form of Prep1 interferes with GnRH enhancer activity. The cells were cotransfected with the indicated expression vectors: pcDNA1.1 (empty vector, negative control; *white bars*) and human dnPrep1 (*black bars*). The luciferase activity of the empty expression vector (pcDNA1.1) normalized to the activity of the cotransfected *β*-galactosidase internal control was set at 1 for each experiment. The normalized values for the expression plasmids are reported relative to the value of pcDNA1.1. *Error bars* represent S.E. *, $p < 0.05$ (Tukey-

Kramer Honestly Significant Difference). The data shown are from five independent experiments, each performed in triplicate. *B*, TALE proteins in combination with Oct-1 activate transcription of the GnRH enhancer. Transient transfections were performed with expression vectors, all in the pcDNA1.1 backbone, as indicated. *, $p < 0.05$ for the wild-type *versus* mutant reporter (*t* test); #, $p < 0.1$ for the empty expression vector (Tukey-Kramer HSD). The data shown are from four independent experiments, each performed in triplicate. *C*, TALE proteins and Oct-1 associate with DNA concurrently. The OCT-PBX DNA probe of the GnRH enhancer (Fig. 3B) was incubated with GT1-7 nuclear extract (*GT1-7 NE*) and antibodies as indicated. The *arrows* mark the positions of the identified complexes. The slower mobility complexes are labeled *A* and *B*.

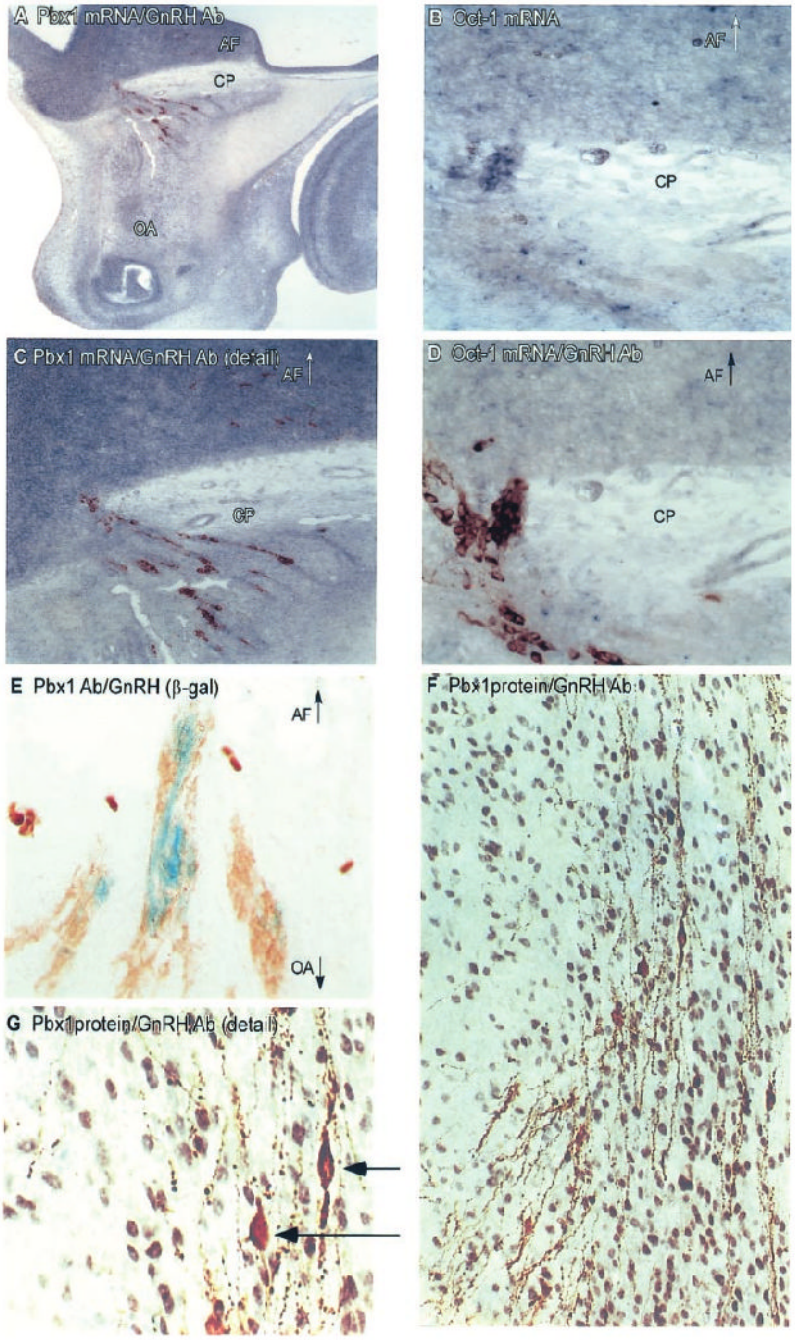

Fig. 5. Pbx1 and Oct-1 co-localize with GnRH neurons

A and *C*, Pbx1 co-localizes with GnRH neurons in 13.5 dpc mouse embryos. *In situ* hybridization/immunohistochemical analysis of mouse embryos was carried out using an antisense probe specific for Pbx1 (*blue*). Subsequently, anti-GnRH antibody (*Ab*) was incubated with the tissue section and visualized with DAB (*brown*). *A* and *C* are ×10 and ×40 magnifications of the same section, respectively. *B* and *D*, Oct-1 co-localizes with GnRH neurons in embryonic day 13.5 mouse embryos. *In situ* hybridization and immunohistochemical analysis were performed with an antisense probe specific for Oct-1 (*blue*; shown in *B*) and then anti-GnRH antibody (*brown*; shown in *D*) as described under "Experimental Procedures." *E*, the Pbx1 protein co-localizes with GnRH neurons in 13.5 dpc

mouse embryos. Transgenic animals carrying GnRHe-GnRHp driving the *β*-galactosidase (*β-gal*) reporter gene targeted LacZ expression to GnRH neurons (*blue*) (6). An antibody specific to Pbx1 was incubated with the tissue and visualized with DAB (*brown* precipitates). *F* and *G*, Pbx1 co-localizes with GnRH neurons in adult mice. Co-immunohistochemistry was conducted on a wild-type adult female mouse brain. Anti-Pbx1 antibody was incubated with the tissue section and visualized with nickel (*black*). Subsequently, anti-GnRH antibody was incubated with the tissue and visualized with DAB (*brown*). Co-localization of GnRH and Pbx1 was detected in hypothalamic neurons of the medial preoptic area and the organum vasculosum of the lamina terminalis. *F* and *G* are is ×10 and ×40 magnifications of the same section, respectively. *Arrows* mark the positions of some co-localized neurons. Sense probes for Oct-1 and Pbx1 showed no specific hybridization. *OA*, olfactory area, *CP*, cribriform plate; *AF*, anterior forebrain.