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

Received for publication, March 17, 2004, and in revised form, May 10, 2004 Published, JBC Papers in Press, May 11, 2004, DOI 10.1074/jbc.M402960200

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

From the Departments of aReproductive Medicine, iPathology, School of Medicine, University of California, San Diego, La Jolla, California 92930

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 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) 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 (10), and Otx2 (10); the zinc finger protein GATA-4 (11); nuclear factor-1 (12); and CCAAT/enhancer-binding protein-β (12). In particular, the POU homeodomain protein Oct-1 was identified as an essential

* This work was supported in part by National Institutes of Health Grant R01 DK44838 (to P. L. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Fellow of the Lalor Foundation.

‡ Both authors contributed equally to this work.

§ Supported in part by the National Institutes of Health Training Grant DA07315.

‖ Supported in part by National Institutes of Health Grant T32 AG00216. T32 AG00216. Present address: 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; E-mail: pmellon@ucsd.edu.

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-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.
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 genes are 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 transcription 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 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 translational domain of the VPH protein. The yeast strains and expression vectors were the generous gifts of Dr. Michel Strubin to the transactivation domain of the VP16 protein. The yeast strains and expression vectors were the generous gifts of Dr. Michel Strubin to the transactivation domain of the VP16 protein. The yeast strains and expression vectors were the generous gifts of Dr. Michel Strubin to the transactivation domain of the VP16 protein. The yeast strains and expression vectors were the generous gifts of Dr. Michel Strubin to the transactivation domain of the VP16 protein. The yeast strains and expression vectors were the generous gifts of Dr. Michel Strubin to the transactivation domain of the VP16 protein. The yeast strains and expression vectors were the generous gifts of Dr. Michel Strubin to the transactivation domain of the VP16 protein. The yeast strains and expression vectors were the generous gifts of Dr. Michel Strubin to the transactivation domain of the VP16 protein. The yeast strains and expression vectors were the generous gifts of Dr. Michel Strubin to the transactivation domain of the VP16 protein. The yeast strains and expression vectors were the generous gifts of Dr. Michel Strubin to the transactivation domain of the VP16 protein. The yeast strains and expression vectors were the generous gifts of Dr. Michel Strubin to the transactivation domain of the VP16 protein. The yeast strains and expression vectors were the generous gifts of Dr. Michel Strubin to the transactivation domain of the VP16 protein. The yeast strains and expression vectors were the generous gifts of Dr. Michel Strubin to the transactivation domain of the VP16 pro...
TALE Proteins and Oct-1 Regulate GnRH Transcription

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 OBFB1-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 (Pknoko) (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 [35S]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 Pbx1 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 (Pbx1NT3). 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 homodomain (Prep1ΔHD) (Fig. 1D). Fig. 1C indicates that GST-Oct-1 specifically bound Prep1 lacking the N-terminal domain, but not the Prep1 lacking the homodomain. 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 tran-
scription 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 (P BX oligonucleotide TTGATTGAT) was used (20).

One of the candidate sites in the GnRH enhancer (TGAAGT-ATGAG, −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).
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 TGAAGT 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.
plexes 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.

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 (boxed 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/Prep1-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).
TALE Proteins and Oct-1 Regulate GnRH Transcription

GnRH Enhancer Activity

A

Expression Plasmids

Empty

dnPrep1

Reporter

GnRHe/RSvp

GnRHe/RSvp

−1749 mutant

Luciferase/galactosidase

B

Expression Plasmids

GT1-7 NE (µg) 2 4 8 16 16 16 16

Antibody

GT1-7 NE (µg)

Oct-1

Pbx1/

Prep1

C

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 → TGAATcTAG) 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 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 transfctions 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 complexes are labeled arrows mark the positions of the identified complexes. The slower mobility complexes are labeled A and B.
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 cribiform 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 cribiform 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 /H11002 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). Overexpre-

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 ×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.
sion 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-α-islet2-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/IITH) results in nuclear translocation (57). Endogenous Pbx1 was located in 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 hypophalamus 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 Strublin 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. (1996) Curr. Opin. Genet. Dev. 8, 552–559
2. Vale, W., Rivier, C., and Brown, M. (1977) Annu. Rev. Physiol. 39, 473–527
3. Mellon, P. L., Windle, J. J., Goldsmith, P., Pedula, C., Roberts, J., and Weiner, R. I. (1996) Neuron 16, 143–154
4. Erly, S. A., and Mellon, P. L. (1995) Mol. Endocrinol. 9, 848–859
5. Whyte, D. B., Lawson, M. A., Belsham, D. D., Erly, S. A., Bond, C. T., and Mellon, P. L. (1995) Mol. Endocrinol. 9, 467–477
6. Lawson, M. A., MacConell, L. A., Kim, J., Pawl, B. T., Nelson, S. B., and Mellon, P. L. (2002) Endocrinology 143, 1404–1412
7. Clark, M. E., and Mellon, P. L. (1993) Mol. Cell. Biol. 13, 6160–6177
8. Wierman, M. E., Xiong, X., Kepa, J. K., Spaulding, A. J., Jacobsen, B. M., Fung, Z., Nilaver, G., and Ojeda, S. R. (1997) Mol. Cell. Biol. 17, 1652–1665
9. Wulf, A., Kim, H. H., Tobet, S., Stafford, D. E., and Radovich, S. (2003) Mol. Endocrinol. 16, 435–449
10. Kelley, C. G., Lavoroga, G., Clark, M. E., Boncinelli, E., and Mellon, P. L. (2000) Mol. Endocrinol. 14, 1246–1256
11. Lawson, M. A., Whyte, D. B., and Mellon, P. L. (1996) Mol. Cell. Biol. 16, 3596–3605
12. Belsham, D. D., and Mellon, P. L. (2000) Mol. Endocrinol. 14, 212–228
13. Chandran, U. R., Warren, B. S., Baumann, C. T., Hager, G. L., and DeFranco, D. B. (1999) J. Biol. Chem. 274, 2372–2378
14. Strubin, M., Newell, J. W., and Matthias, P. (1995) Cell 80, 497–506
15. Grogger, M., Knoepfler, P. S., and Schaffner, W., and Hovens, C. M. (1995) Nature 373, 360–362
16. Voss, J. W., Wilson, L., and Rosenfeld, M. G. (1991) Genes Dev. 5, 1309–1320
17. Schiestl, R. H., and Gietz, R. D. (1989) Curr. Genet. 16, 339–346
18. Robyk, K., and Kastan, Y. (1992) Nucleic Acids Res. 20, 3790
19. Sanger, F., Nicklen, S., and Coulson, A. R. (1977)Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
20. Lu, Q., Knoepfler, P. S., Scheele, J., Wright, D. D., and Kamps, M. P. (1995) Mol. Cell. Biol. 15, 3786–3795
21. Schreiber, E., Merchant, R. E., Wiestler, O. D., and Fontana, A. (1994) Neurosurgery (Baltim.) 34, 129–135
22. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1987)Current Protocols in Molecular Biology, John Wiley & Sons, Inc., New York
23. Coss, D., Jacobs, S. B., Bender, C. E., and Mellon, P. L. (2004) J. Biol. Chem. 279, 152–162
24. Gonzales-Crespo, S., and Moruta, G. (1995) Development (Camb.) 121, 2117–2125
25. Delegeane, A. M., Ferland, L. H., and Mellon, P. L. (1987) Mol. Cell. Biol. 7, 3994–4002
TALE Proteins and Oct-1 Regulate GnRH Transcription

30297

26. Simmons, D. M., Voss, J. W., Ingram, H. A., Holloway, J. M., Breude, R. S., Rosenfeld, M. G., and Swanson, L. W. (1990) Genes Dev. 4, 695–711
27. Mann, R. S., and Chan, S. K. (1996) Trends Endocrinol. 12, 258–262
28. Chang, C. P., Jacobs, Y., Nakamura, T., Jenkins, N. A., Copeland, N. G., and Cleary, M. L. (1997) Mol. Cell. Biol. 17, 5679–5687
29. Berthelsen, J., Zappavigna, V., Mavilio, F., and Blasi, F. (1998) EMBO J. 17, 1423–1433
30. Monica, K., Galili, N., Nourse, J., Saltman, D., and Cleary, M. L. (1991) Mol. Cell. Biol. 11, 6139–6157
31. Knoepfler, P. S., Calvo, K. E., Chen, H., Antonarakis, S. E., and Kamps, M. P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 14553–14558
32. Chang, C. P., Jacobs, Y., Nakamura, T., Jenkins, N. A., Copeland, N. G., and Cleary, M. L. (1997) Mol. Cell. Biol. 17, 5679–5687
33. Penkov, D., Tanaka, S., Di Rocco, G., Berthelsen, J., Blasi, F., and Ramirez, F. (2000) J. Biol. Chem. 275, 27989–27999
34. Eraly, S. A., Nelson, S. B., Huang, H. M., and Mellon, P. L. (2002) Mol. Endocrinol. 16, 2413–2425
35. Kelley, C. G., Givens, M. L., Rave-Harel, N., Nelson, S. B., Anderson, S., and Mellon, P. L. (2002) Mol. Endocrinol. 16, 2413–2425
36. Bailey, J. S., Rave-Harel, N., Coss, D., McGillivray, S. M., and Mellon, P. L. (2004) Mol. Endocrinol. 18, 1158–1170
37. Selleri, L., Depew, M. J., Cai, F., Cui, H., Gillespie, J. M., MacMillan, M., and Belsham, D. D. (2003) Biochem. Biophys. Res. Commun. 307, 847–854
38. Palazzolo, M., Berthelsen, J., De Cesare, D., and Blasi, F. (2000) Eur. J. Biochem. 267, 5427–5437
39. Bailey, J. S., Rave-Harel, N., Coss, D., McGillivray, S. M., and Mellon, P. L. (2004) Mol. Endocrinol. 18, 1158–1170
40. Hoffman, P. F., Rave-Harel, N., Coss, D., McGillivray, S. M., and Mellon, P. L. (2004) Mol. Endocrinol. 18, 1158–1170
41. Schnabel, C. A., Godin, R. E., and Cleary, M. L. (2003) Dev. Biol. 264, 262–276
TALE Homeodomain Proteins Regulate Gonadotropin-releasing Hormone Gene Expression Independently and via Interactions with Oct-1
Naama Rave-Harel, Marjory L. Givens, Shelley B. Nelson, Hao A. Duong, Djurdjica Coss, Melody E. Clark, Sara Barth Hall, Mark P. Kamps and Pamela L. Mellon

J. Biol. Chem. 2004, 279:30287-30297.
doi: 10.1074/jbc.M402960200 originally published online May 11, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M402960200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 60 references, 26 of which can be accessed free at
http://www.jbc.org/content/279/29/30287.full.html#ref-list-1