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P-OTX: A PIT-1-interacting homeodomain factor expressed during anterior pituitary gland development

(POU domain)

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ABSTRACT **A novel OTX-related homeodomain transcription factor has been identified on the basis of its ability to interact with the transactivation domain of the pituitary-specific POU domain protein, Pit-1. This factor, referred to as P-OTX (pituitary OTX-related factor), is expressed in primordial Rathke's pouch, oral epithelium, first branchial arch, duodenum, and hindlimb. In the developing anterior pituitary, it is expressed in all regions from which cells with distinct phenotypes will emerge in the mature gland. P-OTX is able to independently activate and to synergize with Pit-1 on pituitary-specific target gene promoters. Therefore, P-OTX may subserve functions in generating both precursor and specific cell phenotypes in the anterior pituitary gland and in several other organs.**

The anterior pituitary gland arises from a region of midline ectoderm that, upon head folding, comes into direct contact with neuroepithelium of the ventral diencephalon, creating a region of mesodermal incompetence between these two tissues. The primordium of the anterior pituitary gland first appears at mouse embryonic day 9 (E9) as an invagination of the somatic ectoderm in the roof of the oral cavity and is known as Rathke's pouch. Ultimately, after a series of proliferative and differentiation events, the mature anterior pituitary gland consists of five distinct cell types, each characterized by the unique hormone that it produces. The anterior pituitary gland presents a well-characterized model for investigating the molecular mechanisms by which a complex organ, containing distinct cell phenotypes, can arise from a single cell type (1–4).

Recently, several factors have been identified that are expressed in a restricted fashion, preceding, or coincident with, the initial formation of Rathke's pouch. These include P-Lim/literhx3/mLIM-3, a LIM homeodomain factor that is initially expressed during formation of Rathke's pouch (5–7); RPX/Hesx-1, a homeodomain factor that is initially expressed in the mesendoderm and anterior neural plate and subsequently only in Rathke's pouch and continues to be expressed in the developing pituitary until E14 (8, 9); and, several other homeodomain factors including six-3 (10), Hox7.1/Msx-1 (11), and Pax-6 (12).

The tissue-specific POU domain factor, Pit-1, is selectively expressed in the caudomedial region of the anterior lobe on E14.5 and continues to be expressed in the mature anterior pituitary gland (13). Pit-1 has proved to be critical in regulating expression of thyroid-stimulating hormone, growth hormone, and prolactin and for the terminal differentiation of the three cell types in which they are expressed—thyrotropes, somatotropes, and lactotropes, respectively (2). The Snell and Jackson genetic dwarf mice, which do not express functional Pit-1, never express the genes encoding these hormones (14) and, subsequently, exhibit a failure in the proliferation/survival of the thyrotrope, somatotrope, and lactotrope lineages, thereby providing genetic evidence for the requirement of Pit-1 in pituitary development (2, 15).

While Pit-1 is capable of activating distal target genes, clearly the actions of additional factors are required for effective cell-type-specific patterns of expression observed in the anterior pituitary gland (13, 16). Transactivation by Pit-1 requires specific N-terminal activation and synergy domains, the actions of which are dependent upon whether Pit-1 binds to a specific DNA site as a monomer or as a dimer (17). We, therefore, used a yeast two-hybrid screening approach to identify factors present in the pituitary that are capable of interacting with the N-terminal domain of Pit-1. In this manuscript, we report the identification of a novel factor capable of interacting with the N-terminal activation domain of Pit-1. This factor, P-OTX, has an OTX-related homeodomain and is expressed throughout pituitary development. P-OTX is capable of activating early and late markers of pituitary development, including several Pit-1 distal target genes, and acts synergistically with Pit-1 on some of these target genes.

MATERIAL AND METHODS

Cloning of P-OTX, Colony Lift Filter Assay for β-Galactosidase, and *in Situ* Hybridization. A yeast two-hybrid system (Matchmaker, CLONTECH) was used to identify P-OTX cDNA in a pGAD10 adult mouse pituitary cDNA library using the Pit-1 N terminus (amino acids 1–128) in the pBGT9 vector as the bait. Colony lift filter assays were performed by transforming bait and prey into either yeast strain HF7c or YRG-2 and streaking individual colonies directly onto a filter (VWR Scientific, grade 410) layered over agar plates containing selection medium. Colonies were allowed to grow for 1–2 days at 30°C. The filters were then lifted and directly assayed for β-galactosidase activity. Whole-mount *in situ* hybridizations were performed as described (18) using T7 RNA polymerase to generate a cRNA probe corresponding to the sequences that encode amino acids 24–315 and the 3′ untranslated region of P-OTX. *In situ* hybridization of 20-μm sections of mouse embryos and adult pituitary gland fixed in buffered 10% formalin were performed as described (19).

***In Vitro* Protein–Protein Interaction, DNA Binding, and Transfection Assays.** A Pit-1 N-terminal cDNA fragment containing amino acids 1–128 was ligated into pGEX-2TK to yield a glutathione *S*-transferase (GST) fusion protein, and different regions of P-OTX suitable for *in vitro* protein translation were generated using PCR. [^{35}S]Methionine-labeled *in vitro*-translated protein was incubated for 20 min at 37°C with 2–3 μg of GST fusion protein bound to 25 μl of glutathione-

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Abbreviations: E, embryonic day; α-GSU, α-glycoprotein subunit; POMC, proopiomelanocortin.

Data deposition: The sequence reported in this paper has been deposited in the GenBank data base (accession no. U54499).

agarose beads in a total volume of 100 μl of 20 mM Hepes, pH 7.9/100 mM NaCl/1 mM EDTA/4 mM $MgCl_2$/1 mM dithiothreitol/0.02% Nonidet P-40/10% glycerol/0.5% nonfat dry milk, with ethidium bromide at 50 μg/ml to eliminate potential protein–DNA interactions (20). Protein-mediated gel shift assays, *in vitro* culture, and transient transfection of cells were performed as described (21, 22). Mouse α-glycoprotein subunit (α-GSU) promoter, rat growth hormone promoter (GH320), mouse *pit-1* promoter, rat prolactin promoter/enhancer (Prl), and rat proopiomelanocortin (POMC) reporter genes used in the transient transfections have been described (4, 6, 22–24). Cytomegalovirus promoter-driven rat Pit-1 and P-OTX expression vectors were used. P-OTX DNA was cloned as an *Eco*RI–*Sma*I fragment into the pCMV5 vector.

RESULTS

Cloning of P-OTX and Characterization of Its Interaction with Pit-1. The observation that distinct Pit-1 N-terminal domains may be critical in mediating gene activation (17) led us to search for potential Pit-1-associated factors. This search was initiated using a yeast two-hybrid system in which a GAL-4 DNA binding domain fused to the N terminus of Pit-1 was used as bait to screen a mouse pituitary cDNA library. A screen of 10^6 individual transformants identified 30 candidates. Specificity of the interaction with the Pit-1 N terminus was confirmed using additional bait constructs containing either the POU domain of Pit-1 or the N terminus of another POU domain factor, Brn-2 (Fig. 1*A*). The strongest interaction was observed with a prey construct containing a 1.0-kb insert that encoded a homeodomain protein. A full-length cDNA clone was isolated from a mouse pituitary λgt11 library. The transcript was determined to be approximately 2.5 kb by Northern blot analysis (data not shown). The cDNA clone contained a 945-nt open reading frame that predicted a novel 315-amino acid homeodomain protein (Fig. 1*B*). This homeodomain contained a WFK motif in the third helix, a characteristic shared by members of the bicoid-class family of homeodomains, including *Drosophila* orthodenticle, its vertebrate homologues (the OTX family), and *Caenorhabditis elegans* Unc-30. Homology with Unc-30 and the known OTX factors was limited to the homeodomain; the highest level of identity was seen with Unc-30 (85%, 51/60 amino acids in the homeodomain) (Fig. 1*C*). Comparison with other sequences in the GenBank and EMBL data bases did not reveal any significant homologies in other regions of the protein. We have named this factor, pituitary OTX-related protein (P-OTX) based on its relatedness to the orthodenticle family of homeodomains and the cDNA library in which it was identified.

The yeast two-hybrid interaction assays indicated that P-OTX interacted specifically with the N terminus of Pit-1 but not with the Pit-1 POU domain. To biochemically identify the region of P-OTX responsible for interacting with Pit-1, five specific regions of P-OTX were *in vitro*-translated and examined for potential direct protein–protein interactions with Pit-1. The region of P-OTX required for interacting with Pit-1 was localized to amino acids 151–281 in the C-terminal region (Fig. 1*D*). Inclusion of the homeodomain further enhanced the ability of P-OTX to interact with Pit-1.

Analysis of P-OTX Expression. Since P-OTX was isolated from an adult pituitary library based on its ability to interact with Pit-1, it was of interest to examine the ontogeny of P-OTX expression in the developing pituitary gland. At E9.5, P-OTX was expressed throughout the oral epithelium lining the roof of the buccal cavity and in Rathke's pouch ectoderm (Fig. 2). In addition, P-OTX was expressed in the epithelial layer of the mandibular component of the first branchial arch. This pattern contrasts with the more restricted expression of P-Lim, which is limited to Rathke's pouch (Fig. 2). P-OTX was subsequently expressed throughout the developing anterior pituitary gland,

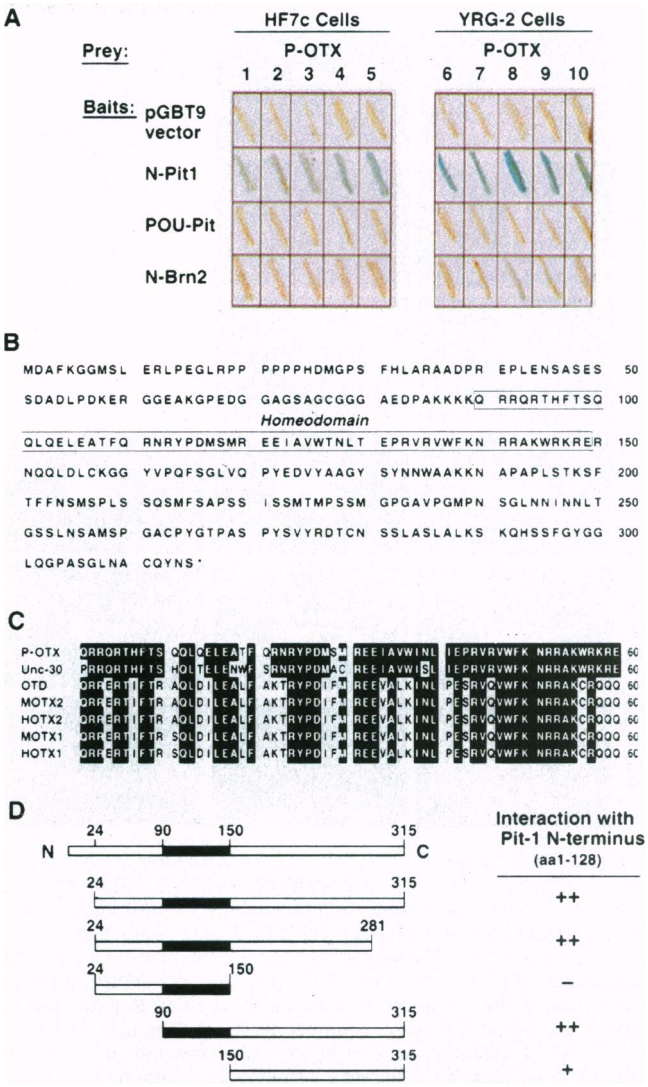

FIG. 1. Cloning of P-OTX and interactions with Pit-1. (*A*) The specificity of the specificity of P-OTX with the N terminus of Pit-1 was determined in two yeast strains (HF7c and YRG-2) using colony lift filter assays with different baits: pBGT9 vector (bait expression vector), N-Pit1 (N-terminal amino acids 1–128), POU-Pit1 (C-terminal amino acids 129–291), and N-Brn-2 (transactivation domain of Brn-2 POU domain factor, amino acids 1–262). P-OTX interacted specifically with N-Pit1 bait. (*B*) Protein sequence of P-OTX. (*C*) Sequence homology comparison of P-OTX with Unc-30, *Drosophila* orthodenticle (OTD), murine Otx-1 (MOTX1) and Otx-2 (MOTX2), and human Otx-1 (HOTX1) and Otx-2 (HOTX2). Residues homologous to P-OTX are highlighted by the black box and the asterisks above the residues WFK indicate the signature of OTX homeodomain transcription factors. (*D*) A schematic diagram of *in vitro*-translated P-OTX fragments used to identify the region of P-OTX required to interact with the Pit-1 N terminus (amino acids 1–128). The C terminus of P-OTX is required for interacting with Pit-1 N terminus.

including the rostral tip, in which thyrotropes and corticotropes appear on E12–E12.5 (Fig. 2). At E15.5–E17.5, P-OTX transcripts were observed in all zones of the anterior lobe in which the five distinct cell types arise in a spatially and temporally specific fashion (Fig. 2). In contrast to Pit-1, P-OTX and P-Lim were expressed in the developing and mature intermediate lobe (Fig. 2).

To determine when P-OTX transcripts are initially expressed, whole-mount *in situ* hybridization analysis was performed on E7.5–E11.5 embryos. P-OTX transcripts were not observed in unturned E8 embryos (data not shown) but were evident immediately after "turning" of the embryo at E8.5 in

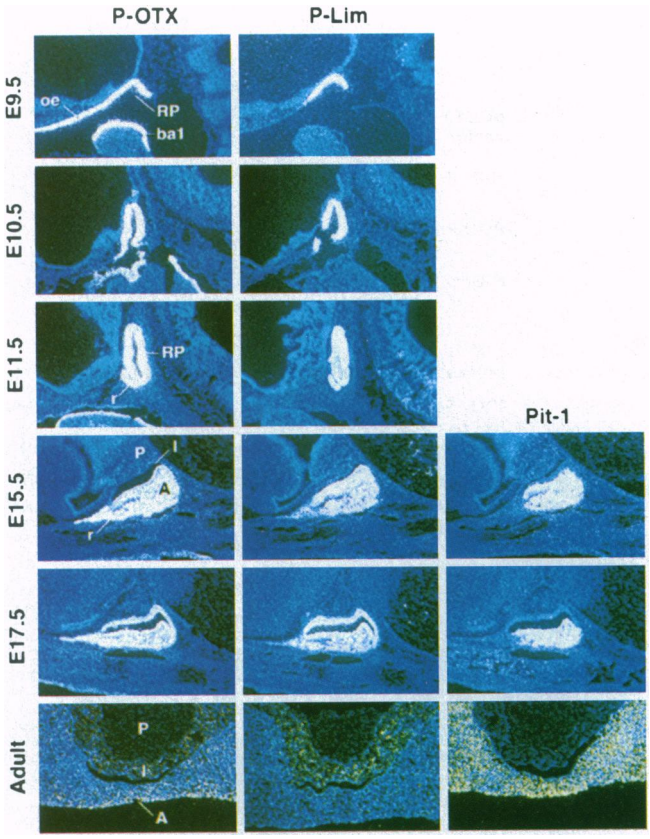

FIG. 2. Comparison of P-OTX, P-Lim, and Pit-1 expression in pituitary development. *In situ* hybridization of sagittal sections through developing embryonic pituitary and coronal sections through an adult pituitary by using cRNA probes specific for P-OTX, P-Lim, and Pit-1. On E9.5–E11.5, P-OTX-specific signals are observed in the oral epithelium (oe) lining the roof of the buccal cavity and mandibular component of the first branchial arch and throughout Rathke's pouch (RP) while P-Lim expression is limited to Rathke's pouch. At E15.5, P-OTX and P-Lim continued to be expressed throughout the developing anterior pituitary gland (A), including the rostral tip (r) but not in the posterior lobe (P). At E15.5, Pit-1-specific expression is detected throughout the developing anterior lobe but is absent from the rostral tip. At E17.5 and in the mature pituitary P-OTX and P-Lim signals are present in the anterior (A) and intermediate (I) lobes of the pituitary, while Pit-1 is expressed only in the anterior lobe. In the adult pituitary, the levels of P-OTX and P-Lim were consistently lower than that of Pit-1.

the region of the first branchial arch and in the ventral portion of the caudal-most region of the embryo (Fig. 3*a*). At E9.5, intense P-OTX expression was observed in the hindlimb buds, in the maxillary and mandibular components of the first branchial arch, and in Rathke's pouch (Fig. 3*b*). P-OTX expression was not observed in the forelimb bud. This pattern of expression continued through E10.5 (Fig. 3*c*). At E11.5, additional regions of hybridization were noted in the ventral mesenchyme covering the abdominal cavity and in the nasal epithelium (Fig. 3*d*). *In situ* hybridization of sagittally sectioned E17.5 embryos indicated that P-OTX was expressed in derivatives of the first branchial arch, including tongue and mandible, as well as in duodenum, salivary glands, nasal epithelium, and condensing cartilage in the hindlimb (Fig. 3 *e–i*).

P-OTX–Pit-1 Interactions in Gene Activation Events. Based on its ontogeny, pattern of expression, and interaction with Pit-1, it was of interest to explore the possibility that P-OTX could activate early and/or late target genes in pituitary development. To begin to explore this issue, we evaluated the ability of P-OTX to bind oligonucleotides containing the core OTX consensus DNA binding sequence (TAATCC) (25) from

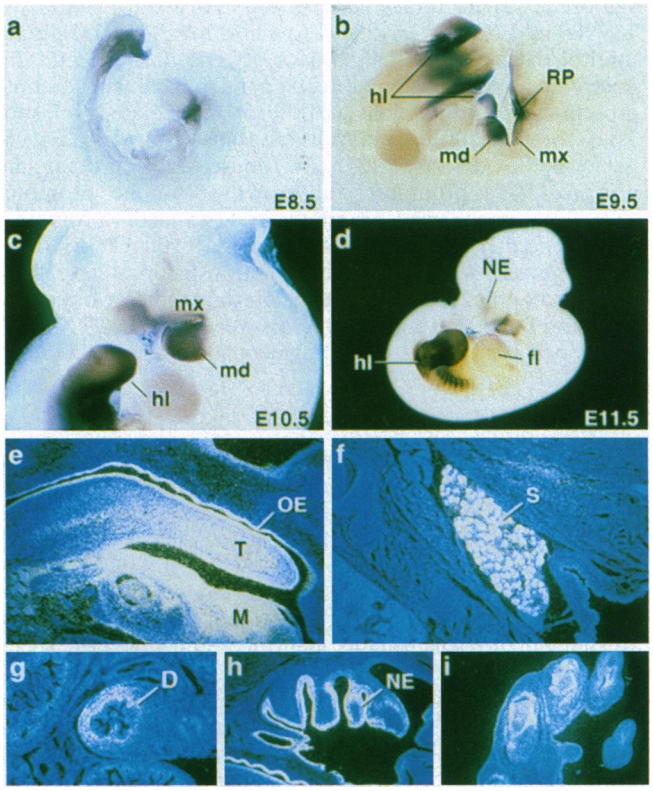

FIG. 3. Embryonic expression of P-OTX. (*a–d*) Whole mount *in situ* hybridization using a digoxygenin-labeled cRNA probe specific for P-OTX. Expression of P-OTX is first observed at E8.5 in the region of the first branchial arch and in the ventral portion of the caudal-most region of the embryo. At E9.5 and E10.5, P-OTX is detected in the hindlimb buds (hl), in the maxillary (mx) and mandibular (md) components of the first branchial arch, and in Rathke's pouch (RP). At E11.5, P-OTX signal is also detected in the nasal epithelium (NE) and the mesenchyme covering the abdominal cavity. (*e–i*) *In situ* hybridization of 20-μm sagittal sections from an E17.5 mouse embryo with a P-OTX cRNA probe. At E17.5, P-OTX expression was observed in the tongue (T) (*e*), oral epithelium (OE) (*e*), mandible (M), salivary gland (S) (*f*), duodenum (D) (*g*), nasal epithelium (NE) (*h*), and the precartilage condensations in the hindlimb (*i*).

the α-GSU gene (nt -375 to -399) and OTX-related sequences within the growth hormone promoter (nt -172 to -193), prolactin promoter (nt -1686 to -1707), and POMC promoter (nt -248 to -270 of the PP1 site and nt -288 to -311) regions. All of these sites are within regulatory regions that are required for cell-type-specific expression of these genes (23, 26–30). Gel mobility shift assays using these sites and bacterially expressed P-OTX demonstrated that P-OTX was able to interact with all of the elements tested (Fig. 4*A*), suggesting that sequences similar to the OTX consensus core motif, TAATCC, are important for P-OTX binding to DNA.

The ability of P-OTX to function as a transcription factor and its potential role in regulating distal target genes in the developing and mature pituitary gland was explored using transient cotransfection experiments in heterologous cell lines. P-OTX was capable of transactivating the α-GSU, POMC, growth hormone, and prolactin promoters (Fig. 4*B*) but was not able to effectively activate the Pit-1 and thyroid-stimulating hormone β promoters (data not shown). P-OTX exerted an additive effect with Pit-1 in activation of the growth hormone promoter. In contrast, on the prolactin enhancer/promoter reporter P-OTX exerted a minimal effect alone but, in conjunction with Pit-1, exerted a synergistic multiplicative effect. These data indicate that, in addition to its physical interaction with the Pit-1 N-terminal domain, P-OTX was capable of synergistic effects on specific targets. Therefore,

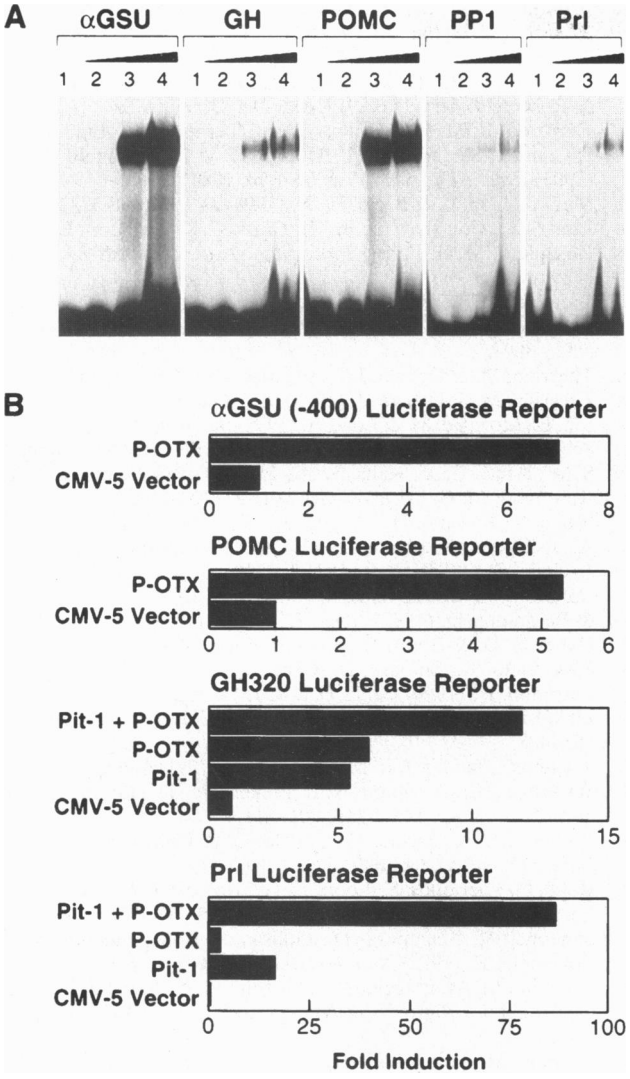

FIG. 4. P-OTX binds to DNA and activates expression of pituitary-specific genes. (*A*) Binding of P-OTX to DNA sites. Gel shift assays were used to examined binding of bacterially expressed P-OTX (amino acids 25–315) to synthetic sites from the rat α-GSU promoter (nt −375 to −399), and the murine growth hormone (nt −172 to −193), prolactin (nt −1686 to −1707), and POMC (nt −248 to −270 of the PP1 site and nt −288 to −311) promoters. Increasing amounts of bacterially expressed protein (1, 2.5, and 5 μl) were used in these studies. (*B*) P-OTX functions as a transcriptional activator. Transfections were performed in HeLa or CV-1 cells using cytomegalovirus expression vectors encoding P-OTX and/or Pit-1 and luciferase reporter constructs containing the α-GSU, POMC, and growth hormone (GH320) promoters and a prolactin promoter/enhancer reporter construct (Prl). Similar results were obtained in three or more experiments in several cell lines.

P-OTX may be capable of exerting effects both early in pituitary development, when the α-GSU gene is activated, and later in conjunction with Pit-1, in the initial activation of specific distal target genes.

DISCUSSION

Members of the mammalian POU domain gene family appear to regulate penultimate terminal differentiation and proliferation/survival events (2, 31–33), but it is likely that they require synergistic and combined actions with additional factors to mediate effective activation of distal target genes. As has been shown, the binding of Pit-1 as a monomer or dimer to specific sites on the prolactin and growth hormone promoters, respectively, dictates which N-terminal synergy domain is utilized for effective target gene activation, suggesting an additional regulatory strategy for Pit-1 to differentially activate target genes in distinct cell types. This additional strategy most likely involves recruitment of factors that interact with the Pit-1 N-terminal domain through protein–protein interaction (17). A search for proteins interacting with the Pit-1 N terminus led to the identification of a novel factor, P-OTX, that contains a homeodomain that is most closely related to that of *C. elegans* Unc-30 and the mammalian OTX proteins. These homeodomains all contain the WFK motif in the third DNA-binding helix, which has been demonstrated to be important for determining DNA binding specificity of the *Drosophila* bicoid gene product (34). Outside of the homeodomain, the P-OTX sequence diverges from other previously identified OTX proteins.

Deletion of the founding member of the OTX family, the *Drosophila* orthodenticle gene (*otd*), resulted in both neural and epidermal defects in the ventromedial regions of the embryo (35–40). Mammalian Otx2 is widely expressed in embryonic ectoderm, becoming restricted to anterior regions, with a posterior boundary between the presumptive mesencephalon and metencephalon mice (41). Treatment of embryos with agents that inhibit or alter Otx2 expression indicated a role for Otx2 in determination of anterior brain structures (42, 43). Indeed, homozygous mutant mice null for Otx2 expression did not develop rostral head structures anterior to rhombomere 3 (44–46). Moreover, it is pertinent that Unc-30, which is the factor most closely related to P-OTX, functions in terminal differentiation of specific neurons in *C. elegans* (47), implying that members of this family regulate both early and late developmental events.

Recent identification of factors that are expressed in a restricted fashion preceding or coincident with the initial formation of Rathke's pouch, such as RPX (8) and P-Lim (5–7), suggest that the coordinate and timely regulation of gene expression by homeodomain and LIM homeodomain proteins is important during the development of the anterior pituitary. In this paper, we have described the expression pattern of P-OTX, a novel homeodomain factor, that is also expressed throughout pituitary development beginning with the formation of Rathke's pouch. In contrast to RPX, whose expression is extinguished by E14.5, P-OTX and P-Lim expression continues in the mature anterior pituitary gland. Although P-Lim and P-OTX exhibit restricted patterns of expression outside of the pituitary, their expression uniquely overlaps, both temporally and spatially, in Rathke's pouch and the developing pituitary. Therefore, it is tempting to speculate that a functional relationship could exist between P-Lim and P-OTX in the early formation of Rathke's pouch and in the development of the five cell types found in the mature gland.

The ability of P-OTX to activate early markers of organ commitment (α-GSU) and markers of specific cell types (e.g., POMC in corticotropes and growth hormone in somatotropes) and at later times to interact with and synergize with Pit-1 (e.g., prolactin expression in lactotropes) may implicate it in the specification of patterns of gene expression that determine distinct cell phenotypes. Based on the roles of *Drosophila* otd and murine Otx2 in development of anterior head structures and Unc-30 in terminal differentiation of specific neuronal phenotypes, it is tempting to speculate that P-OTX may exert equally important functions in the development of structures arising from oral epithelium, in particular the anterior pituitary that arises from a subset of the somatic ectoderm on the roof of the oral cavity.

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