Ptx1, a *bicoid*-related homeo box transcription factor involved in transcription of the pro-opiomelanocortin gene

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The pituitary gland contains six distinct hormone-producing cell types that arise sequentially during organogenesis. The first cells to differentiate are those that express the pro-opiomelanocortin (POMC) gene in the anterior pituitary lobe. The other lineages, which appear later, include cells that are dependent on the POU factor Pit-1 and another POMC-expressing lineage in the intermediate pituitary lobe. Using AtT-20 cells as a model for early expression of POMC in the anterior pituitary, we have defined a regulatory element conferring cell specificity of transcription and cloned a cognate transcription factor. This factor, Ptx1 (pituitary homeo box 1), contains a homeo box related to those of the anterior-specific genes *bicoid* and *orthodenticle* in *Drosophila*, and Otx-1 and Otx-2 in mammals. Ptx1 activates transcription upon binding a sequence related to the *Drosophila bicoid* target sites. Ptx1 is the only nuclear factor of this DNA-binding specificity that is detected in AtT-20 cells, and it is expressed at high levels in a subset of adult anterior pituitary cells that express POMC. However, Ptx1 is expressed in most cells of Rathke's pouch at an early time during pituitary development and before final differentiation of hormone-producing cells. Thus, Ptx1 may have a role in differentiation of pituitary cells, and its early expression pattern suggests that it may have a role in pituitary formation. In the adult pituitary gland, Ptx1 appears to be recruited for cell-specific transcription of the POMC gene.

[*Key Words*: Pituitary; POMC; *bicoid*; homeo box; transcription; cell specificity]

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The determination of cell fate is a key regulatory process for development of complex organisms, and the pituitary gland has been a useful model to study this process. The mature gland contains a limited number of differentiated cells that can be identified easily on the basis of the hormone they contain (Fig. 1A). Furthermore, the hormone is a good marker to follow the appearance of each cell type during pituitary organogenesis (Voss et al. 1992). The first factor shown to affect pituitary cell differentiation is the Pit-1/GHF-1 factor, which contributes to cell-specific transcription of the growth hormone (GH) and prolactin (PRL) genes and is essential for maintenance of three cell lineages that express the GH, PRL, and TSH genes (Bodner et al. 1988; Ingraham et al. 1988; Li et al. 1990).

Current evidence suggests that the presumptive anterior pituitary cells are committed very early (elAmraoui et al. 1993) and that pituitary development is linked to pattern formation in the head and forebrain. The elegant surgical experiments of Couly and Le Douarin (Couly et al. 1985, 1987) using chick–quail chimeras have indicated that the presumptive territory of the adenohypophysis is located at the central anterior ridge of the neural plate and that it is contiguous with those of the hypothalamus (with which the pituitary has important developmental and regulatory interactions), infundibulum, and diencephalon. Thus, if a regulatory *Hox*-type code exists for anterior head structures (Puelles et al. 1993) as it has been documented for pattern formation in the trunk (Krumlauf 1994), one would anticipate that pituitary development will be linked to that of the head (Shawlot et al. 1995) and that the "pituitary segment", should it exist, would be the most anterior.

Head morphogenesis involves extensive tissue movements and, as a result of these, the pituitary anlage is first observed around day 9 in mouse embryo development (E9) as an invagination of the mouth ectodermal

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epithelium (Schwind 1928). This structure, known as Rathke's pouch, makes contact with the developing diencephalon. It has been suggested that this contact is essential for further pituitary development (Le Douarin et al. 1967a,b) and for activation of pro-opiomelanocortin (POMC) expression (Hayes et al. 1990). The first hormone-producing cells that differentiate express POMC, and they appear in the ventral part of the developing gland at about E12 in the mouse (Elkabes et al. 1989; Simmons et al. 1990). These cells are within the anterior pituitary and are known as corticotrophs because their major POMC-processing product is ACTH (Fig. 1A). Another POMC-expressing lineage develops later (E14.5) in the intermediate pituitary lobe, and these melanotroph cells process POMC into α-melanocyte-stimulating hormone (α-MSH) (Jacobson et al. 1994). Other hormone-producing cells appear later (GH and TSH around E15.5–16); however, these Pit-1-dependent lineages may be committed as early as E13.5–14 when Pit1 mRNA is first detected (Dollé et al. 1990). The Pit-1 transcription factor was shown to be required for development and maintenance of the thyrotroph (TSH), somatotroph (GH), and lactotroph (PRL) cells of the anterior pituitary (Li et al. 1990). Thus, the early commitment of POMC-expressing cells offers an opportunity to define mechanisms involved in the earlier steps of pituitary differentiation.

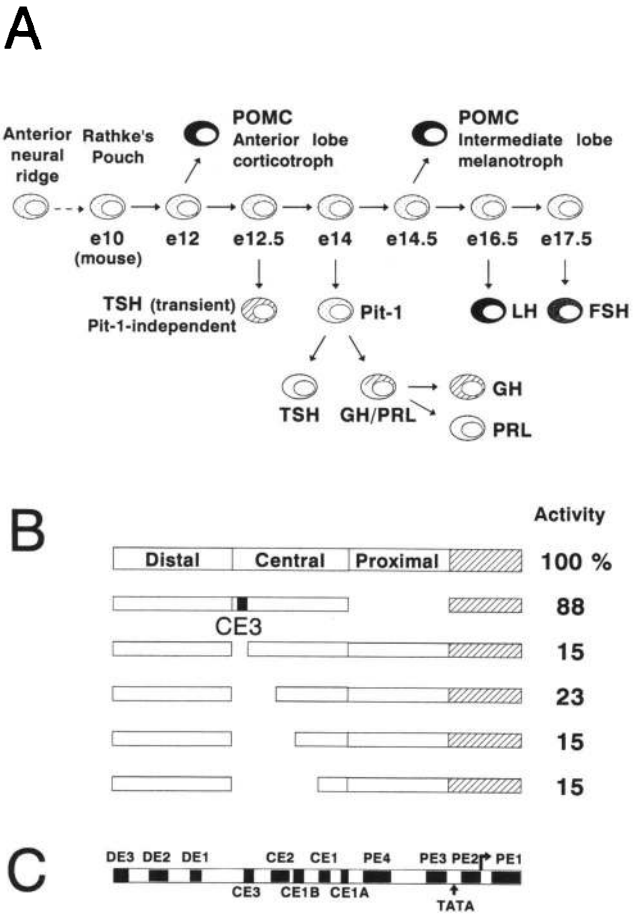

Figure 1. Differentiation of hormone-producing cells during development of the mouse pituitary and localization of pituitary-specific POMC promoter element. (*A*) The hormone-producing cells of the pituitary differentiate sequentially during organogenesis. The origin of the pituitary anlage, Rathke's pouch, has been mapped to the central anterior neural ridge (Couly et al. 1985). Later during organogenesis, each of the hormone-producing cells differentiates sequentially as indicated (Voss et al. 1992). Two different lineages of POMC-expressing cells arise independently: The first ones are the corticotrophs of the anterior pituitary that appear on E12 and the second are the melanotroph cells of the intermediate lobe that appear on E14.5. The first day of hormone gene expression is indicated for each lineage in mice (Elkabes et al. 1989; Dollé et al. 1990; Simmons et al. 1990; Japon et al. 1994). (*B*) The activity of rPOMC promoter deletions within the central domain of the promoter was measured by electroporation in AtT-20 cells of promoter fusions with the luciferase reporter gene. The promoter domains are aligned with the diagram in *C* below. (*C*) The position of the known regulatory elements of the rPOMC promoter (−480 bp to +63 bp) is shown. Previously identified elements of the promoter (Therrien et al. 1991, 1993) are listed above the diagram and elements identified in the present work are listed below the diagram. The position of TATA box and start site of transcription are indicated by arrows.

The recruitment of the Pit-1 factor for transcription of the GH and PRL genes, as well as for development of the cells expressing those genes, has supported the idea that factors responsible for cell-specific transcription of POMC may also be involved in differentiation of POMC-expressing cells. Using AtT-20 cells as a model of anterior pituitary corticotrophs, we have identified transcription factors and cognate DNA sequences that are required for corticotroph-specific transcription of POMC. We have shown that a factor [corticotroph upstream transcription element (CUTE)] of the helix–loop–helix (HLH) family that appears restricted to the corticotroph lineage is an important determinant of POMC specificity, although its activity is only exerted in synergy with another promoter element (Therrien et al. 1991, 1993). The present work has identified this other cell-specific element of the promoter and we have cloned and characterized a cDNA encoding a novel *bicoid*-related pituitary homeo box 1 factor (Ptx1) that binds the element and activates transcription. Ptx1 is the only factor exhibiting this DNA-binding specificity in AtT-20 cells, and it appears to play an important role for POMC transcription. Interestingly, Ptx1 is closely related to the mammalian *Otx* genes that are expressed in the rostral brain during development (Simeone et al. 1992, 1993) and that are homologous to the *Drosophila orthodenticle* (*Otd*) gene, which is essential for development of the head in *Drosophila* (Finkelstein et al. 1990a,b). Otx-2 was also shown recently to be essential for forebrain and midbrain development in mice (Acampora et al. 1995). Ptx1 mRNA appears early during formation of Rathke's pouch, but in adult tissues, high-level expression is maintained in a subset of anterior pituitary cells, the POMC-expressing corticotrophs. Thus, Ptx1 expression marks the developing pituitary, and the recruitment of this factor for corticotroph-specific expression of POMC provides a first model target gene for transcriptional regulation by *bicoid*-related homeoproteins.

Results

Previously, we have shown that pituitary-specific expression of the POMC gene is conferred by sequences within the upstream 300 bp of the rat promoter; this conclusion was supported by cell transfection experiments and in transgenic mice (Tremblay et al. 1988; Therrien et al. 1991, 1993). This 314 bp was divided into two domains (distal and central) that were shown to act in synergy (Figs. 1B and 2A). Each domain contains multiple regulatory elements (Fig. 1C) that are all required for full promoter activity (Therrien et al. 1991). Only a subset of these elements are recognized by cell-restricted transcription factors and exhibit cell-specific transcriptional activity. We have previously characterized one of these elements (DE2) in the distal domain of the promoter. This element is composite, and its most active part, named DE2-C, is a target for a corticotroph-restricted HLH factor that we have called CUTE (Therrien et al. 1993). However, the DE2 element is unable to activate transcription on its own (Fig. 2A). It requires elements within the central domain of the promoter for activity.

Cell-specific POMC regulatory element

This regulatory element was originally identified by genetic analysis. Indeed, 5′ deletions of the central domain indicated that its most upstream sequences were active in transcription (Fig. 1B). This deletion endpoint localized element CE3 of the promoter. To define the properties of this element, it was inserted in three copies upstream of the minimal POMC promoter and of a luciferase reporter, as shown in Figure 2A. In addition, two copies of the DE2C element were added upstream to determine whether CE3 is able to support synergy with the CUTE factors. The activity of these constructs was assayed by electroporation in POMC-expressing AtT-20 cells as well as in non-POMC expressing cells, pituitary GH-expressing GH3 cells, and fibroblast L cells. CE3 was found to exhibit activity only in AtT-20 cells, and addition of element DE2C resulted in synergistic activation (Fig. 2A). The association of the CE3 and DE2 elements was sufficient to generate an AtT-20-specific promoter that is as active as the wild-type POMC promoter. By comparison to the POMC promoter, this synthetic promoter exhibited even greater specificity, as it is completely devoid of residual activity in L and GH3 cells. Subelement DE2C is sufficient for synergism (Fig. 2A), but the entire DE2 element (Therrien et al. 1993) exhibited greater activity (data not shown).

However, CE3 is sufficient on its own to confer corticotroph specific activity, and it was shown to confer this property to a heterologous promoter. Fusion of the CE3 element to the yeast CYC1 promoter led to expression of a downstream hygromycin resistance reporter gene in AtT-20 cells and to the appearance of hygromycin-resistant colonies (Fig. 2B). Neither the CE3-containing nor the control plasmid yielded resistant colonies when transfected in L cells (data not shown). Thus, element CE3 exhibits all of the expected properties of a corti-

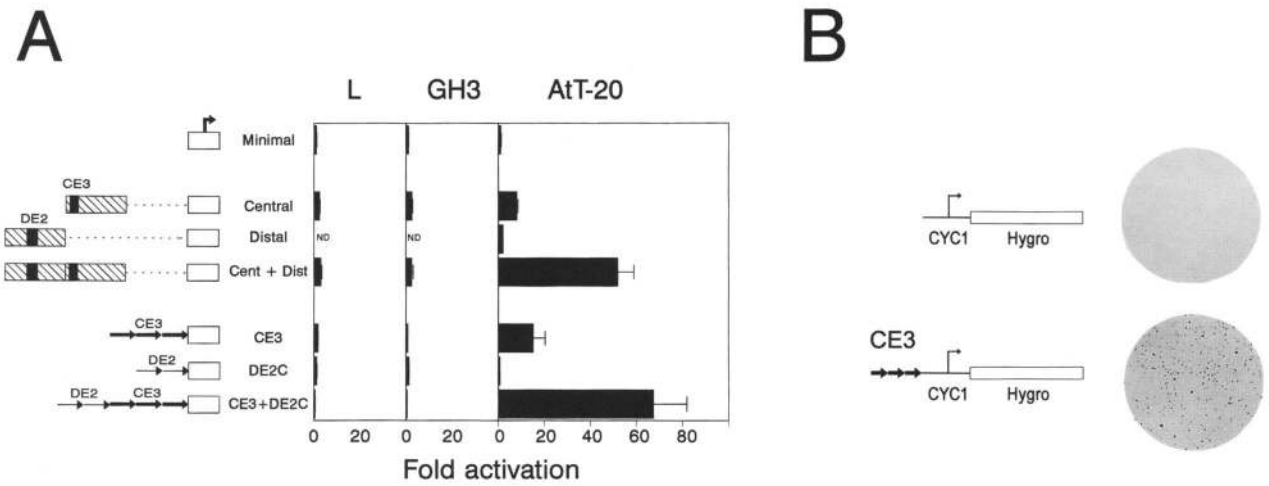

Figure 2. Identification of corticotroph-specific regulatory element. (*A*) Reconstitution of corticotroph-specific transcription with CE3 and DE2 oligonucleotides. The activity of the intact POMC promoter domains is compared to that of oligonucleotide reporter constructs. The cell specificity of reporter constructs was assessed by electroporation into POMC-expressing AtT-20 cells, growth hormone-expressing GH3 cells, and mouse L fibroblasts. Promoter elements were tested by fusion to the rPOMC minimal promoter (positions −34 to +63 bp) (Jeannotte et al. 1987). The DE2 element was characterized previously and shown to confer specificity (Therrien et al. 1993), and the central domain of the promoter was also defined in the same work. Activity is reported as fold activation relative to the minimal promoter construct (±S.E.M.). In AtT-20 cells, the activity of the reporter containing central and distal domains is similar to that of the intact POMC promoter (Therrien et al. 1993). (ND) Not determined. (*B*) The CE3 element is active on the heterologous yeast CYC1 promoter. The indicated reporter plasmids were constructed with the CE3 oligonucleotide and electroporated in AtT-20 cells. After 3 weeks of selection in the presence of 100 U/ml of hygromycin B, resistant colonies were stained with Coomassie blue. Neither construct produced resistant colonies in L cells (data not shown).

CE3 exhibits all of the expected properties of a corticotroph-specific enhancer element, and together with element DE2, these elements account for the corticotroph specificity of the POMC promoter.

A novel bicoid*-related homeo box protein, Ptx1*

The CE3 element was used as a probe for the cloning of DNA-binding proteins from a cDNA expression library prepared in λgt11 using AtT-20 RNA. The initial screening yielded one clone that bound the CE3 but not a DE2 probe. The sequence of this 1.6-kb cDNA indicated an open reading frame of 945 bp (Fig. 3A,C). The protein encoded by this reading frame was shown by immunoprecipitation to comigrate with the homologous protein of AtT-20 cells (data not shown). Further cloning from a random-primed AtT-20 cDNA library yielded three clones that extended into the 5′-untranslated region of the cDNA. We named the encoded protein Ptx1, as sequence comparison with the data bases indicated that it is a novel protein that contains a homeo domain (Fig. 3).

Comparison of the Ptx1 homeo domain with others indicated the greatest homology with the *Caenorhabditis elegans unc-30* gene (Jin et al. 1994), with the mouse *Otx-1* and *Otx-2*, and with the *Drosophila Otd* homeo domains. The similarity included residue 9 (lysine) of the predicted third helix of the homeo domain (position 50 of the homeo domain), which defines the *bicoid* class of homeo domains (Fig. 3B). Significant homology is also observed with the homeo domains of Pax3, Phox2, and Mhox. All of these proteins fall into the large group of *paired*-related homeo genes, and the *bicoid*-related homeo box forms a subgroup within this family (Bürglin 1993). The *Hox*-type homeo domain usually contains a glutamine residue at position 9 of the third helix, whereas the *paired* proteins contain a serine (*Pax* subgroup), a glutamine (Mhox/Phox1-related), or a lysine (*bicoid* subgroup) (Bopp et al. 1986; Bürglin 1993). This amino acid difference was shown to be critical for specific DNA sequence recognition (Hanes et al. 1989; Wilson et al. 1993; Gehring et al. 1994). The Ptx1 homeo domain does not show as much similarity to the only other known vertebrate *bicoid*-related homeo protein *goosecoid* (*gsc*) (Blumberg et al. 1991); thus, Ptx1 defines a new family of *bicoid*-related homeo proteins that is related but distinct from Otx-1 and Otx-2. It is noteworthy that all *bicoid*-related genes are involved in formation of anterior structures both in *Drosophila* and mice (Cohen et al. 1990; Finkelstein et al. 1990a).

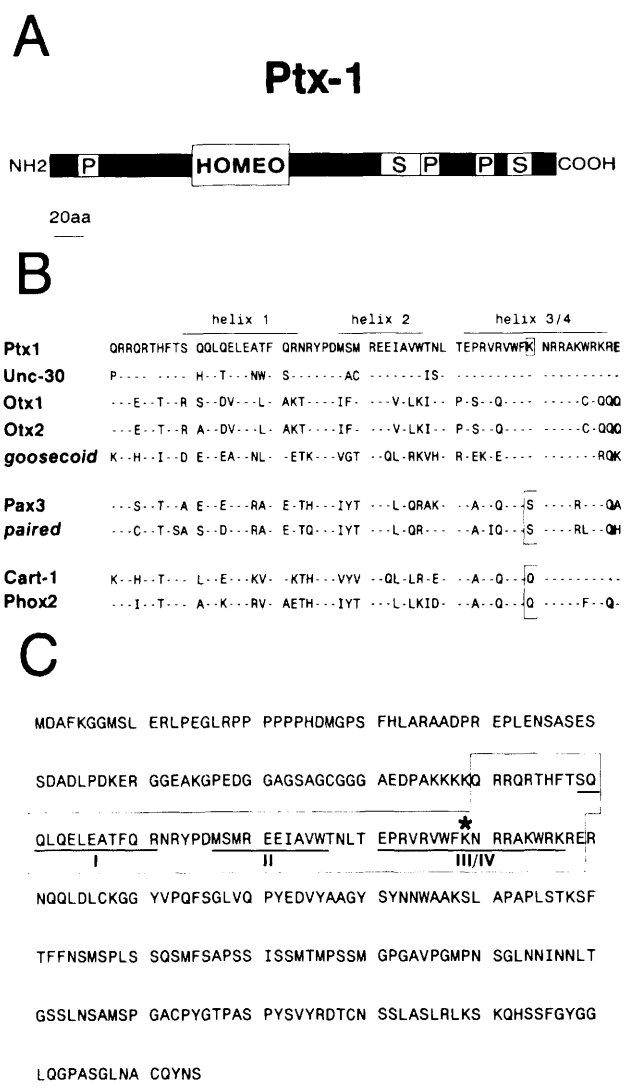

Figure 3. Structure of the Ptx1 homeo protein. (*A*) A schematic structure is shown for the predicted amino acid sequence of the Ptx1 protein. The only significant motifs that were identified in the cDNA include the homeo domain (HOMEO) and proline (P), or serine (S)-rich regions as shown. (*B*) Alignment of Ptx1 homeo domain with those of other mouse *bicoid*-related homeo genes (*Otx-1*, *Otx-2*, and *gsc*) and the closely related nematode gene *unc-30*. The comparison with other homologous *paired*-related homeo domains is also shown. Predicted α-helices are indicated and residue 9 of helix 3 is boxed. (*C*) Predicted protein sequence of Ptx1. The homeo domain is boxed, and the predicted helices are indicated (Bürglin 1993; Gehring et al. 1994). The lysine residue present at position 9 of helix 3, which is characteristic of *bicoid*-related homeo domain proteins (Hanes et al. 1989), is indicated by an asterisk.

Ptx1 is a transcription factor

To test the ability of Ptx1 to activate transcription, the Ptx1 cDNA was inserted in both orientations into a Rous sarcoma virus (RSV) expression vector and transfected along with various reporter constructs in L cells. Ptx1 overexpression led to significant activation of the POMC promoter that was lost in promoter deletions removing the CE3 element (Fig. 4A). Ptx1 *trans*-activation is specific to POMC promoter sequences, as the viral RSV and thymidine kinase (TK) promoters were not affected by Ptx1 overexpression (Fig. 4A). Other pituitary gene promoters, PRL, Pit-1, and αGSU, were also insensitive to Ptx1 overexpression (Fig. 4A). Ptx1 *trans*-activation could only be observed using CE3-containing reporters and not with any other POMC promoter element (Fig. 4B). A similar activation was observed with constructs containing CE3 or CE3 in association with DE2. Thus,

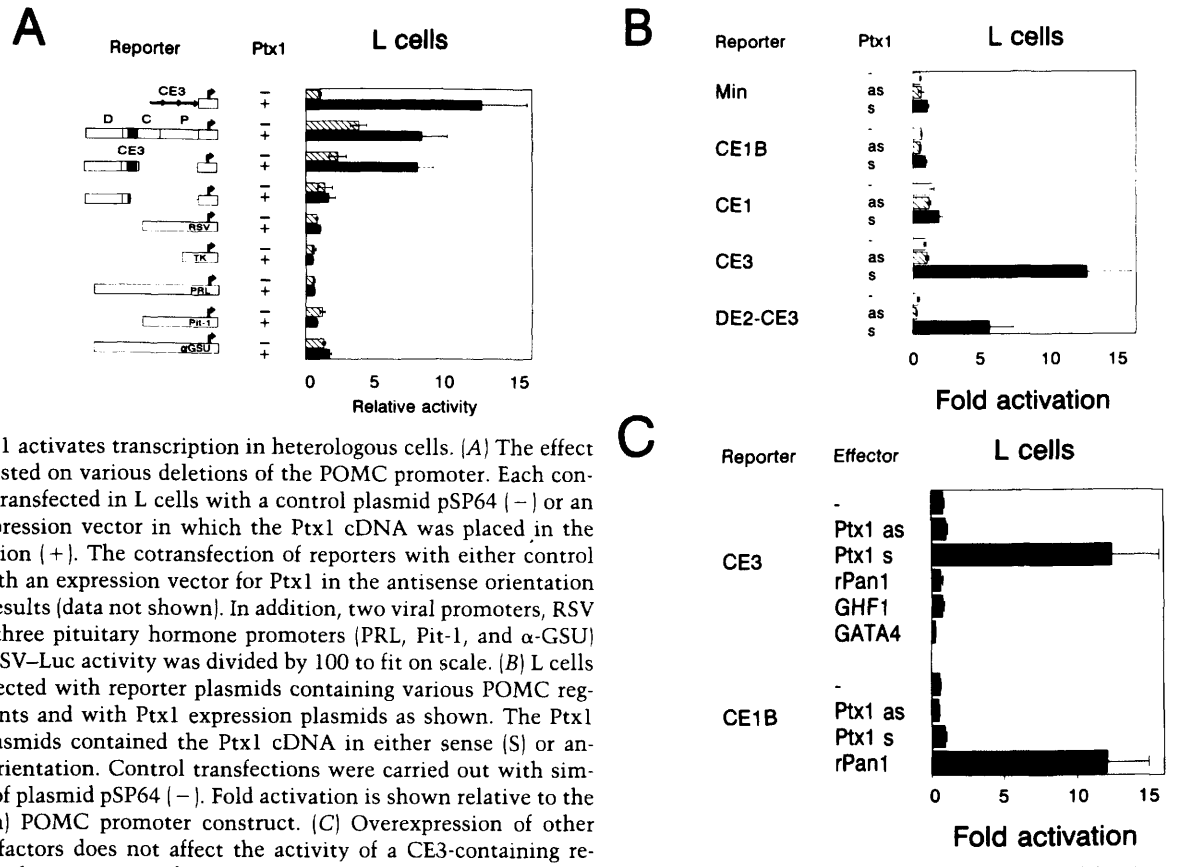

Figure 4. Ptx1 activates transcription in heterologous cells. (*A*) The effect of Ptx1 was tested on various deletions of the POMC promoter. Each construct was cotransfected in L cells with a control plasmid pSP64 (−) or an RSV–Ptx1 expression vector in which the Ptx1 cDNA was placed in the sense orientation (+). The cotransfection of reporters with either control plasmid or with an expression vector for Ptx1 in the antisense orientation gave similar results (data not shown). In addition, two viral promoters, RSV and TK, and three pituitary hormone promoters (PRL, Pit-1, and α-GSU) were tested. RSV–Luc activity was divided by 100 to fit on scale. (*B*) L cells were cotransfected with reporter plasmids containing various POMC regulatory elements and with Ptx1 expression plasmids as shown. The Ptx1 expression plasmids contained the Ptx1 cDNA in either sense (S) or antisense (AS) orientation. Control transfections were carried out with similar amounts of plasmid pSP64 (−). Fold activation is shown relative to the minimal (Min) POMC promoter construct. (*C*) Overexpression of other transcription factors does not affect the activity of a CE3-containing reporter. Indicated transcription factor expression vectors were transfected in L cells together with a CE3 reporter plasmid (*top*). A reporter containing the rPOMC CE1B E-box was used as control for overexpression of the rPan1 ubiquitous HLH factor (*bottom*). Activity is reported as fold activation relative to the CE3 construct.

the activation is solely dependent on the presence of the CE3 element. Overexpression of other transcription factors did not activate the CE3 reporter. Thus, the pituitary-specific POU factor Pit-1/GHF-1 that enhances GH and PRL gene activity (Bodner et al. 1988; Ingraham et al. 1988) did not activate the CE3 reporter (Fig. 4C) nor the POMC promoter (Therrien et al. 1993). Also, the cardiac-specific GATA-4 factor that was shown to stimulate ANF and BNP gene activity (Grépin et al. 1994) was inert in this system. Overexpression of the ubiquitous HLH factor, rPan1 (rodent homolog of E12) (Nelson et al. 1990), did not activate transcription from the CE3 reporter but did activate transcription from a reporter construct containing the CE1B E box of the POMC promoter (Fig. 4C). Taken together, these data indicate that the Ptx1 homeo protein activates transcription specifically in the presence of the CE3 target sequence.

Specific sequence recognition

The CE3 element contains sequences homologous to the *bicoid* target site of the drosophila *hunchback* gene (Fig. 5A; Driever et al. 1989). To test whether these sequences might be the target of Ptx1, mutant CE3 oligonucleotides were compared to the intact sequence for activity in AtT-20 cells and for *trans*-activation by Ptx1 in L cells (Fig. 5B). Mutant M1, which has transversions of 3 bp (Fig. 5A) within the *bicoid* target site homology, was completely devoid of activity in both assays. In contrast, mutant M3, which lies outside of the homology region, was fully active (Fig. 5B). In parallel, binding experiments were done by gel retardation using a CE3 probe and bacterially produced glutathione *S*-transferase (GST)-Ptx1 fusion protein (Smith et al. 1988). The GST–Ptx1 fusion protein binds the CE3 probe (Fig. 5C) but not other probes (DE2, CTF, Sp1; data not shown), and the GST protein itself does not bind CE3. GST–Ptx1 binding is competed by the wild-type CE3 or by active M3 CE3 mutant oligonucleotides but not by CE3 mutant M1, which is inactive, or by an unrelated oligonucleotide, PE2 (Fig. 5C). The thrombin cleavage product of the GST–Ptx1 fusion protein, which no longer contains GST peptides, displayed exactly the same binding properties as GST–Ptx1 (data not shown). The binding of Ptx1 to a single site in CE3 is consistent with the observation of only one specific complex in gel retardation (Fig. 5C) and with the binding properties of other Lys-9 homeo domains (Wilson et al. 1993). Thus, the binding properties

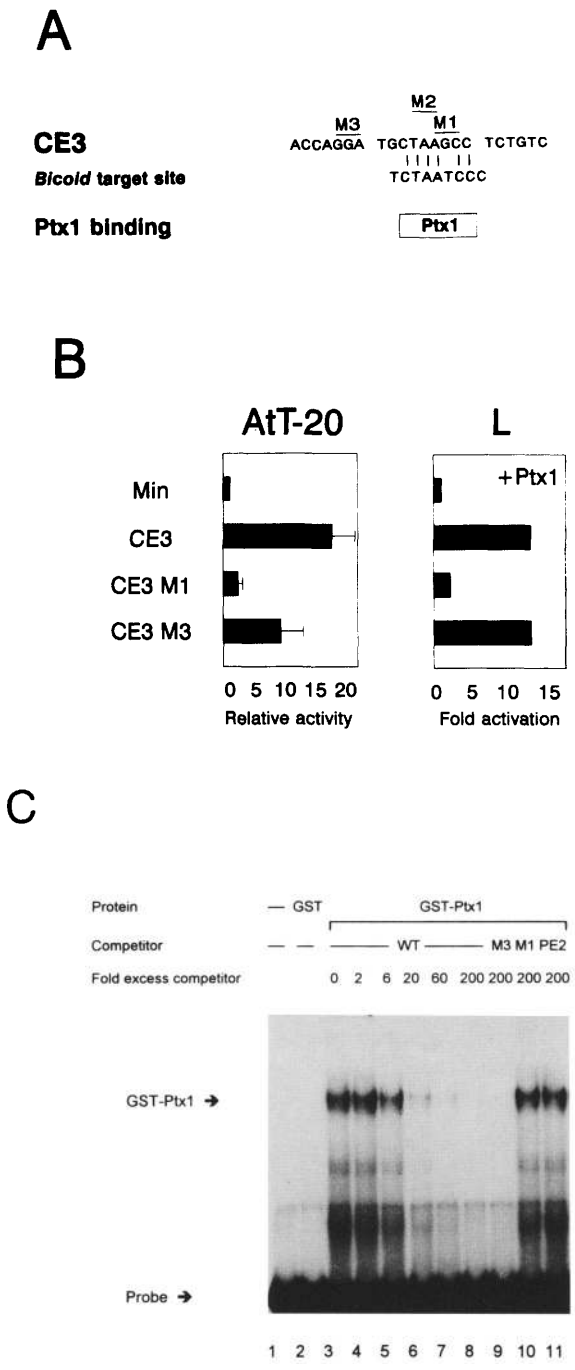

Figure 5. Ptx1 target site. (*A*) Sequence alignment of CE3 element with the *Drosophila bicoid* target site (Driever et al. 1989). The positions of the three mutations used in the present work are shown. The site of in vitro Ptx1 binding is indicated. (*B*) Activity of Ptx1 binding site mutant oligonucleotides. The activity of mutants M1 and M3 of the CE3 element was tested by electroporation in AtT-20 cells and by cotransfection with Ptx1 expression vector in L cells. (*C*) The binding of a GST–Ptx1 fusion protein to the labeled CE3 oligonucleotide was analyzed by gel retardation. The CE3 probe was either incubated with no protein (-), 300 ng of GST protein (GST), or 50 ng of GST–Ptx1 fusion protein (GST–Ptx1). The binding was challenged by the indicated fold molar excess of wild-type CE3 (WT), active mutant (M3), inactive mutant (M1), and unrelated (PE2) oligonucleotides.

of cloned Ptx1 are consistent with the in vivo activity of the CE3 mutants (Fig. 5).

Ptx1 activates POMC transcription

AtT-20 cells may express other *bicoid*-related homeo box transcription factors in addition to Ptx1. To test this possibility, we did Northern blots with AtT-20 and pituitary RNAs using probes for mouse Otx-1 and Otx-2 (Simeone et al. 1992): These experiments indicated that neither gene is expressed in these tissues (data not shown). In addition, in situ hybridization has not shown expression of *gsc* in the developing pituitary (Gaunt et al. 1993; C. Lanctôt and J. Drouin, unpubl.). The previously described factors of this family are therefore not expressed in the pituitary.

The presence of DNA-binding Ptx1 in AtT-20 cell extracts was tested directly by gel retardation (Fig. 6). A single band of appropriate DNA-binding specificity was observed (Fig. 6A); this binding was competed by the CE3 probe itself or by the M3 mutant (not shown) but not by the inactive mutant M1 sequence or by a Pit-1-binding site (data not shown). This binding activity is absent from L cell extracts as might be expected (Fig. 6A). Similar results were obtained using a *Drosophila bicoid* target site oligonucleotide (not shown). The Ptx1 DNA-binding activity is supershifted completely in retardation assays by addition of an antiserum against bacterially produced GST–Ptx1 (Fig. 6B), whereas the migration of another DNA-binding activity (Sp1-like) was not affected under the same conditions. Thus, Ptx1 appears to be the only DNA-binding protein of *bicoid*-related specificity that is active in AtT-20 cells and that may account for the activity of the CE3 element.

The importance of Ptx1 and its target site in POMC promoter activity was tested directly in an antisense RNA experiment using a mutant of the Ptx1-binding site, mutant M2 (Fig. 5A). The M2 mutation led to an important drop in POMC promoter activity in AtT-20 cells (Fig. 7). To test the contribution of Ptx1 to POMC transcription, a Ptx1 antisense expression vector was cotransfected into AtT-20 cells together with a POMC–hGH reporter. Overexpression of the Ptx1 antisense RNA led to a significant decrease in POMC–hGH expression, whereas overexpression of Ptx1 itself further enhanced the activity of the reporter, suggesting that Ptx1 activity is limiting in AtT-20 cells (Fig. 7). Overexpression of Ptx1 had a rapid effect on reporter activity, whereas it took longer for overexpression of Ptx1 antisense RNA to affect reporter activity. This would be consistent with a slow turnover of Ptx1 protein. Ptx1 antisense expression led to a significant decrease in Ptx1 DNA-binding activity without affecting other DNA-binding proteins (Sp1-related) in the same extracts (Fig. 7, inset). Consistent with the purported role of Ptx1, mutagenesis of its binding site (mutant M2) prevented the effect of the Ptx1 antisense RNA or of Ptx1 overexpression on POMC–hGH reporter activity (Fig. 7). Thus, the POMC promoter contains a specific Ptx1-binding site within the CE3 element, and this site and the cognate

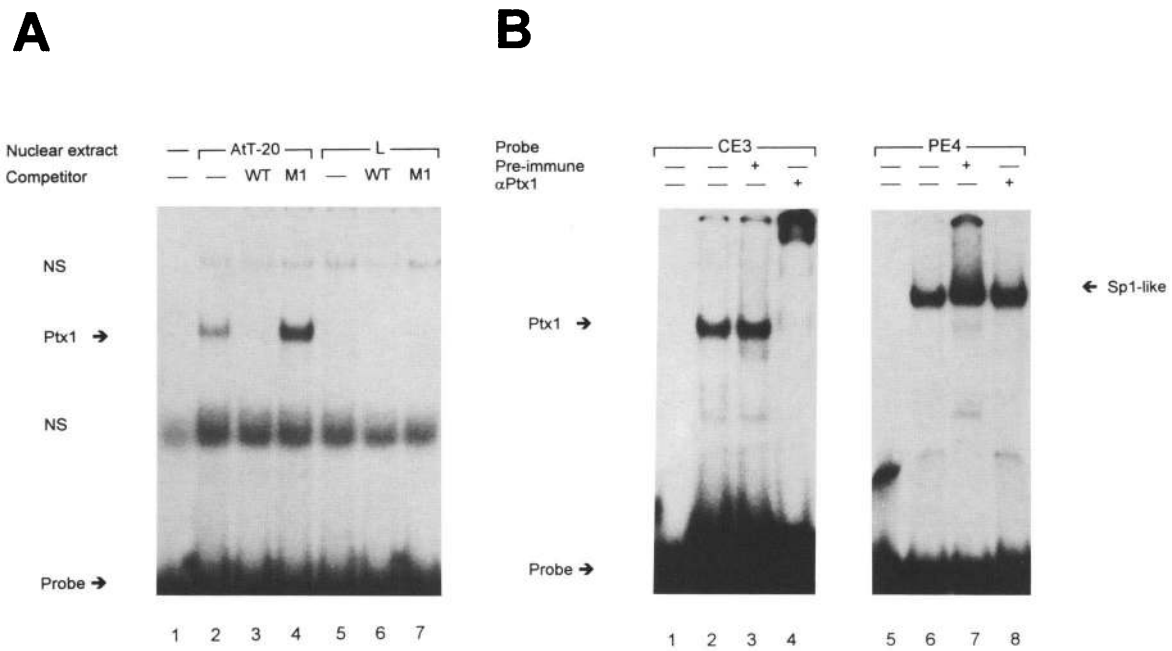

Figure 6. The Ptx1 protein is present in AtT-20 cells. (*A*) Gel retardation analysis of nuclear proteins that bind the CE3 probe. A single band of specific CE3 binding (labeled Ptx1) was observed in AtT-20, but not in L cells: This binding was competed by a 100-fold molar excess of the CE3 probe (WT) but not by the inactive mutant probe M1. The M1 mutation is shown in Fig. 5A. (*B*) The CE3-binding protein of AtT-20 cells (labeled Ptx1) was retarded further in gel retardation assays by addition of an antiserum against GST–Ptx1 (αPtx1) but not by addition of preimmune serum. Using the same nuclear extracts and conditions, another DNA-binding protein (Sp1-like) binding the PE4 element of the POMC gene (Therrien et al. 1991) was not affected by the antiserum.

Ptx1 factor are essential determinants of POMC transcription.

Ptx1 is expressed in the anterior pituitary

The pattern of Ptx1 gene expression was initially assessed by Northern blot. Northern blot analysis of RNA from AtT-20 and L cells revealed only one mRNA of ~2.5 kb in AtT-20 but not L cells (Fig. 8A). Ptx1 mRNA was also detected in normal mouse pituitary but not in many other mouse tissues, including brain, lung, kidneys, heart, liver, and pancreas. A weak signal was observed in intestinal RNA. Thus, the pattern of Ptx1 expression is highly restricted in adult mice.

Ptx1 mRNA was revealed by in situ hybridization in the adult mouse pituitary (Fig. 8B). By comparison to a Ptx1 sense probe used as negative control, low-level hybridization was seen throughout the anterior and intermediate pituitary lobes but not in the posterior lobe. In addition, the anterior lobe contained scattered cells that are strongly positive for Ptx1 (Fig. 8B, C). We used immunocytochemistry against ACTH to reveal corticotrophs and in situ hybridization to reveal Ptx1 mRNA. It appeared that the ACTH-positive cells of the anterior pituitary express Ptx1 at high levels (Fig. 8D). Further work will be required to determine whether the low-signal of Ptx1 hybridization represents bona fide Ptx1 mRNA or another related species. Nonetheless, it is clear from these data that intermediate lobe melanotroph cells do not express Ptx1 at the same level as corticotrophs. Although unexpected, this result may reflect the differential control of POMC transcription by various hormones in corticotrophs and melanotrophs (Jacobson et al. 1994).

Ptx1 is expressed early during pituitary organogenesis, as its mRNA was present throughout Rathke's pouch at E10.5 (Fig. 8E). Ptx1 mRNA was never detected in the infundibulum or in the posterior lobe that develops from it later. Preliminary data suggest that the pattern of Ptx1 expression becomes restricted within the developing pituitary gland after day E16 (not shown).

Discussion

The analysis of transcriptional mechanisms responsible for cell-specific activation of the POMC gene in the pituitary gland has led us to clone a novel homeo box transcription factor, Ptx1 (Fig. 3), that is related to Otx-1 and Otx-2, a pair of homeo box genes specific to the forebrain. Both, Otx-1/2 and Ptx1 are related to the *Drosophila* maternal zygotic determination gene *bicoid* and, in particular, they have similar sequence specificity for DNA binding (Figs. 5 and 6). Thus, POMC constitutes the first identified target gene for a vertebrate homeo box transcription factor of the *bicoid* family.

Ptx1, a homeo domain transcription factor related to bicoid

The Ptx1 homeo domain has significant homology to other homeo domains, particularly with *unc-30, Otx-1,*

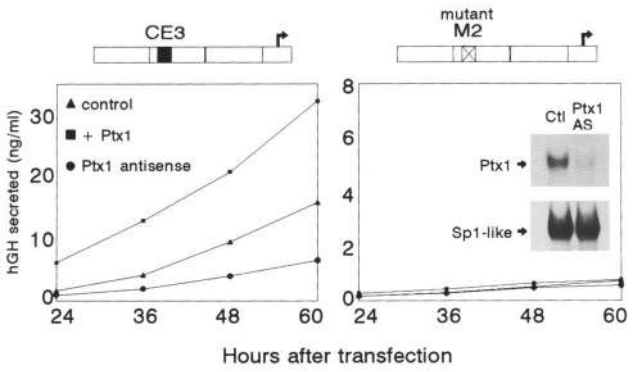

Figure 7. Blockade of POMC promoter activity by Ptx1 antisense RNA in AtT-20 cells. Two POMC (−480 to +63 bp)–hGH reporter constructs were used to test the involvement of Ptx1 in promoter activity. Mutant M2 (*right*) of the Ptx1-binding site (Fig. 5A) was used along with the intact promoter (*left*). Control samples were coelectroporated with empty expression vector; other samples contained either Ptx1 expression vector in sense (+Ptx1) or antisense orientation (Ptx1 antisense). The data from a representative transfection experiment performed in duplicate are shown. Similar experiments were performed four times with similar results, and cells stably transformed with the expression vectors were obtained. Reporter activities showed even greater differences in the stable lines (data not shown) than in transient transfection and in gel retardation experiments; (*inset*) nuclear extracts from cells stably transfected with the antisense constructs showed a significant decrease in Ptx1 DNA-binding activity relative to another DNA-binding activity (Sp1-like).

Otx-2, *Pax3*, *Phox2*, and *Mhox* (Fig. 3B). It thus falls within the large *paired* group of homeo boxes; within this group, it is part of the small *bicoid*-related subgroup that is mostly characterized by the Lys-9 of helix 3 (Bürglin 1993). The presence of this Lys residue within the motif VWFKNRR is typical of the *bicoid* class of homeo proteins that only includes Otx-1, Otx-2, and *gsc* in vertebrates (Blumberg et al. 1991; Simeone et al. 1992). Homology between the *gsc* and Ptx1 homeo domains is significantly less than those of Otx-1 and Otx-2 (Fig. 3B). Ptx1 is entirely different from Otx-1 and Otx-2 or from *Drosophila Otd* outside the homeo domain (data not shown). The presence of proline-rich regions is the only feature that is also common to *Otd*. The Ptx1 sequence has three proline-rich and two serine-rich domains (Fig: 3A,C), and these may function as trans-activation domains (Gerber et al. 1994). Thus, Ptx1 constitutes a novel gene product, and taken together with its POMC target gene, they provide a prototype to decipher the transcriptional actions of *bicoid*-related homeo proteins in a vertebrate system. The great similarity between *unc-30* and Ptx1 homeo domains raises the possibility that they may be homologs. However, there is no functional similarity between the two, as *unc-30* is specifically expressed in a small group of GABA-ergic neurons in *C. elegans* (Jin et al. 1994) and Ptx1 does not appear to be expressed in neuronal tissues (Fig. 8A; data not shown).

The presence of a Lys residue at position 50 of the homeo domain (position 9 of helix 3) is consistent with its recognition site within CE3 (Hanes et al. 1989, 1991; Wilson et al. 1993; Gehring et al. 1994), as this site shares homology with the *Drosophila bicoid* binding site (Fig. 5A). Furthermore, nucleotides shown to be essential for recognition by homeo boxes containing a lysine at position 9 of the third helix are conserved in this segment (Wilson et al. 1993). The inactivity of mutants M1 (Fig. 5B) and M2 (Fig. 7) supports the importance of those residues. In addition to the sequence of the Ptx1-binding site, the binding of GST–Ptx1 (Fig. 5C) is consistent with binding of a Ptx1 monomer. This is also consistent with the results of Wilson et al. (1993), who found by in vitro site selection that Lys-9-containing homeo domains preferred monomer binding in contrast to Ser-9 or Gln-9 homeo domains, which readily bound DNA as dimers. Thus, Ptx1 appeared to interact with its POMC target as a monomer. Nonetheless, its action within the POMC promoter context depends on interactions with other factors bound to the promoter (Therrien et al. 1993; T. Lamonerie, J.J. Tremblay, and J. Drouin, unpubl.).

Ptx1 is recruited for POMC transcription

The POMC promoter has a complex organization (Fig. 1C), and its activation requires the concurrent action of many transcription factors (Therrien et al. 1991). Although multiple ubiquitous transcription factors appear to be required for maximal activity of the POMC promoter, thus far only two factors appear to impart cell specificity: They include a previously described HLH factor of restricted corticotroph distribution, CUTE (Therrien et al. 1993), and Ptx1, which seems to be highly expressed in a subset of cells in the adult anterior pituitary (Fig. 8). Whereas the CUTE target site DE-2C has no transcriptional activity on its own and is only active in synergy, the Ptx1 target CE3 does exhibit intrinsic corticotroph-specific activity (Fig. 2). Thus, the CE3 element constitutes the angular stone of the POMC promoter, and its cognate factor Ptx1 appears to be the most important factor for corticotroph specificity of POMC expression, in agreement with the Ptx1 antisense experiments (Fig. 7) and with the restricted distribution of Ptx1 mRNA (Fig. 8).

The distribution of Ptx1 mRNA in adult pituitary is not uniform: It is predominantly expressed in ACTH-containing corticotroph cells of the anterior lobe (Fig. 8D), and this is consistent with a proposed role of Ptx1 in cell-specific control of POMC transcription. Significantly, Ptx1 is not expressed at such levels in the intermediate lobe (Fig. 8B,C), where the other POMC-expressing cells, the melanotrophs, are found (Fig. 1A). This suggests that POMC transcription may not be regulated by the same factors in the two POMC-expressing lineages and that Ptx1 may only be involved in the corticotrophs. Little data are available on POMC promoter recognition in melanotrophs, as there are no representative cell lines. However, most hormonal regulators of POMC transcrip-

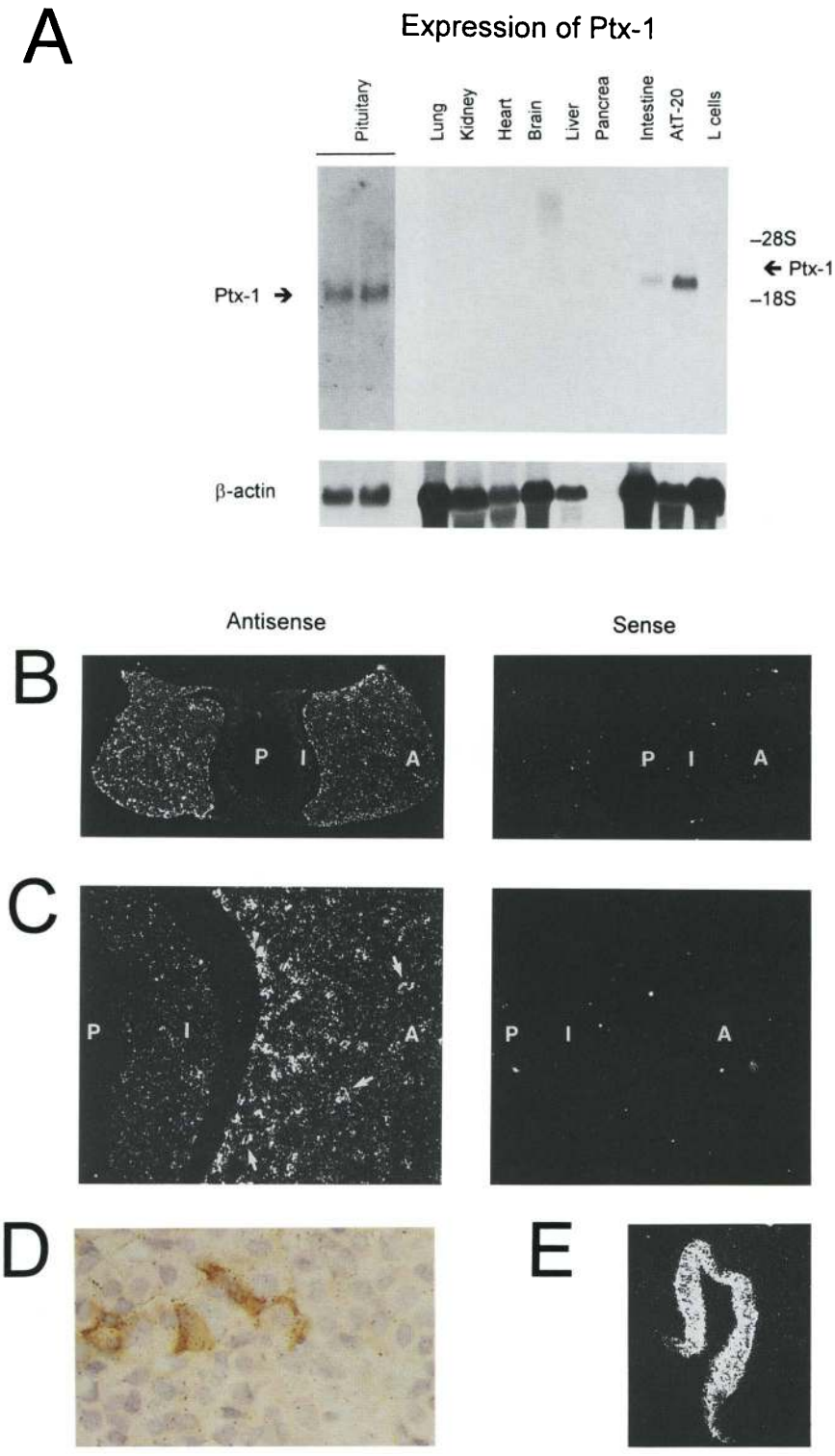

Figure 8. Expression of Ptx1. (*A*) Northern blot was used to reveal the Ptx1 mRNA in RNA extracted from various adult mouse tissues and in L and AtT-20 cells. The same blot was also hybridized with a β-actin probe as shown. (*B*) In situ hybridization analysis of Ptx1 mRNA in adult mouse pituitary. Antisense and sense ^{35}S-labeled Ptx1 probes were used. The 573-bp cRNA probe spanned the homeo domain. The anterior (A), intermediate (I), and posterior (P) lobes are indicated. Note scattered positive cells in anterior lobe. (*C*) Enlargement of photomicrograph shown in *B* indicating the presence of highly positive cells (arrows). (*D*) Colocalization of ACTH-positive cells and Ptx1 expression. ACTH-positive cells were detected by immunocytochemistry using a monoclonal antibody (Cortex Biotech) and peroxidase-coupled anti-mouse IgG. Ptx1 in situ hybridization was performed using a cRNA fragment probe derived from the 5′-untranslated region of the Ptx1 cDNA. (*E*) Ptx1 mRNA was detected throughout Rathke's pouch by in situ hybridization of mid-sagittal sections of paraffin-embedded mouse embryos at 10.5 days postcoitum.

tion exert specific effects on only one lobe (Jacobson et al. 1994) in agreement with the notion of differential control of POMC transcription. Also, both temporal and spatial differences (Fig. 1A) in the differentiation of each POMC-expressing lineage support the idea of cell-specific transcriptional regulatory mechanisms.

The low signal observed in in situ hybridization throughout the anterior and intermediate lobes probably reveals Ptx1 mRNA. This signal was observed with two different Ptx1 cRNA probes: one spanning the coding region (Fig. 8B,C) and another one containing mainly 3′-untranslated sequences (data not shown). In both cases,

the sense probe control showed virtually no signal. Thus, low-level Ptx1 mRNA expression may occur throughout the pituitary. We do not know yet whether Ptx1 mRNA is translated in cells other than corticotrophs. It may be remembered that Pit-1 mRNA is found in all pituitary cell types but only translated in a subset of cells (Simmons et al. 1990). Nonetheless, the inability of Ptx1 to alter expression of other pituitary-specific promoters like those of PRL, Pit-1, and α-GSU (Fig. 4A), taken together with the predominant expression of Ptx1 in corticotroph cells (Fig. 8D), supports the model of a recruitment of this homeo box transcription factor for control of POMC transcription.

Ptx1 is expressed early in pituitary development

Homeo box genes have not yet been implicated in pituitary transcription, and the known head-specific homeo proteins like Otx-1 and Otx-2, and Emx1 and Emx2 did not appear to be expressed in the anterior pituitary (Simeone et al. 1992, 1993). The expression of Ptx1 throughout Rathke's pouch (Fig. 8E) before expression of POMC suggests that this homeo protein may play another role before being recruited in the corticotroph lineage. For example, the POU–homeo factor Pit-1/GHF-1 was shown to be essential for gene transcription and differentiation of a subset of pituitary cells (Dollé et al. 1990; Li et al. 1990), and another homeo protein first identified as a transcription factor of the insulin gene was later found to be essential for pancreas development (Jonsson et al. 1994). Because it would generally be assumed that only a subset of cells from Rathke's pouch would ultimately give rise to corticotrophs, the expression of Ptx1 throughout the pouch supports the hypothesis of an early role. This is not incompatible with recruitment of Ptx1 for corticotroph-specific functions later in pituitary development.

The major role ascribed so far to homeo proteins has been in pattern formation (Krumlauf 1994). Thus, Ptx1 may define a segment from which the pituitary arises at mid-gestation (~E9) when Ratkhe's pouch forms. In this respect, it is noteworthy that we do not know yet whether the forebrain and other anterior structures arise from a segmented plan as for the trunk, although this hypothesis has been proposed (Puelles et al. 1993). If a head segmentation plan did exist, the pituitary fate could be linked to those of nasofrontal and mandibular structures as indicated by the fate map of the central anterior neural ridge in chicken (Couly et al. 1985, 1987) and in mice (Osumi–Yamashita et al. 1994).

In summary, the work presented here has documented an important transcriptional regulatory role for Ptx1 in the corticotroph cells of the adult pituitary and identified the target gene POMC for this novel *bicoid*-related homeo gene. The homology between Ptx1 and a small group of head-specific *bicoid*-related homeo box proteins, as well as its expression very early in pituitary development, suggests that Ptx1 may also have a role in pattern formation of the head and pituitary, and/or in differentiation of pituitary cells.

Materials and methods

Plasmids and oligonucleotides

Reporter plasmids were generated as follows: Trimers of various oligonucleotides were cloned at the *Bam*HI site of a pXP1-derived vector (Nordeen 1988) containing the minimal rPOMC promoter (−34 to +63 bp) (Jeannotte et al. 1987). These plasmids were recombined with a donor plasmid containing two copies of the DE2 or DE2C oligonucleotides (Therrien et al. 1993). Oligonucleotides were synthesized with an Applied Biosystem synthesizer. Positions of oligonucleotides in the POMC promoter are as follows: CE1, −216 to −188 bp; CE1B, −230 to −221 bp; CE3, −322 to −287 bp. RSV expression constructs were made by replacing the *Xba*I–*Kpn*I fragment from pRSV–Luc (De Wet et al. 1987) by a multiple cloning site oligonucleotide and by insertion of cDNA inserts in-frame with the first 16 amino acids of the firefly luciferase gene. The pGEX-2T vector (Pharmacia) was used to generate GST fusion proteins. The M2 promoter mutant was produced by deletion of 3 bp at a *Cel*I site.

cDNA library, screening, and DNA sequencing

A cDNA library from poly(A)$^+$ RNA from AtT-20 cells was built in phage λgt11 using the Superscript kit (BRL) according to the manufacturer's indications. Phage plaque screening was carried out as described (Singh et al. 1989) using 5′-end-labeled double-stranded oligonucleotides as probes. Phage inserts were subcloned at the *Eco*RI site of the Bluescript plasmid (Stratagene). Phage inserts and all reporter plasmids as well as expression vectors were sequenced by the dideoxy method (Sanger et al. 1977) using the T7 polymerase kit from Pharmacia. Sequence comparison to data libraries was done with the BLAST program (Altschul et al. 1990).

Cell culture and transient transfection assays

Murine corticotroph AtT-20, rat somatotroph GH3, and murine fibroblastic L cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum as described previously (Therrien et al. 1993). For electroporation, AtT-20 and GH3 cells were harvested by gentle trypsin treatment, washed in warm DMEM without serum, and resuspended in DMEM at 10^7 cells/ml. Aliquots of 350 μl were mixed with 50 μl of a DNA solution containing 5 μg of reporter gene, 0–10 μg of effector plasmid, 2.5 μg of RSV–GH internal control plasmid, and carrier DNA up to a total of 30 μg. Electroporation was done with the Bio-Rad Gene Pulser at 220 V, 960 μF. Cells were extracted for the luciferase assay as described previously after 16 hr (Therrien et al. 1991). L cells were transfected by the calcium phosphate method (Chen et al. 1987). Precipitate containing 3 μg of reporter plasmid, 0–5 μg of effector plasmid, 0.5 μg of RSV–GH internal control plasmid, and carrier DNA up to a total of 10 μg was applied on 10^5 cells in 35-mm petri dishes. After 16 hr, medium was changed, and cells were harvested 24 hr later.

Gel retardation assays

GST fusion proteins were produced according to the manufacturer's instructions (Pharmacia Biotech), and the indicated amount of purified proteins were used in gel retardation experiments. Thrombin-cleaved Ptx1 was produced by incubation of glutathione–Sepharose-bound GST–Ptx1 with 0.2 NIH unit of thrombin per milligram of fusion protein overnight at 22°C with gentle agitation. The reaction was spun down at 500*g* and the

cleaved protein was recovered in the supernatant and stored in aliquots at −70°C after quantitation. Samples were run for 2 hr at 200 V on 5% polyacrylamide gels in 40 mM Tris–HCl/195 mM glycine (pH 8.5) at 4°C.

Nuclear microextracts from AtT-20 or L cells were prepared as described previously (Therrien et al. 1993). Binding reactions were performed using ~10 fmoles of 5′-end-labeled oligonucleotide probes and 5 μg of extracts. They were incubated on ice for 60 min in a total volume of 20 μl containing 25 mM *N*-2-hydroxyethylpiperazine-*N*′2-ethane-sulfonic acid (HEPES at pH 7.9), 84 mM KCl, 5 mM DTT, 10% glycerol, 1 μg of poly[d(I-C)] used as nonspecific competitor DNA. For competition experiments, probe and competitor DNA were mixed together before the addition of proteins. For antibody supershift experiments, the antibody (αPtx1) was added to the nuclear extracts and incubated for 60 min on ice before addition of the probe. The binding reaction was incubated further for 60 min on ice.

RNA extraction and analysis

Cytoplasmic RNA was extracted from cell lines as follows: Exponentially growing cells were harvested in cold PBS with 0.5 mM EDTA, then lysed in 150 mM NaCl, 50 mM Tris–HCl (pH 7.9), 12 mM Mg acetate, 150 mM sucrose, and 0.5% NP-40. After pelleting nuclei 1% Sarkosyl and 25 mM EDTA were added to supernatant, and RNA was purified by phenol/chloroform extraction and ethanol precipitation. Total RNA from mouse tissues was extracted as described (Chirgwin et al. 1979). Northern blotting of formaldehyde–agarose gels on Hybond-N membranes (Amersham) was performed as described (Maniatis et al. 1982). Purified cDNA fragments labeled by random priming (Feinberg et al. 1983) were used as probes. Hybridization was carried out as described (Maniatis et al. 1982).

In situ hybridization

Sections (5 μm) of paraformaldehyde-fixed and paraffin-embedded tissues or embryos were used for in situ hybridization (Wilkinson 1992) carried out at 50°C using ^{35}S-labeled probes. The slides were washed under stringent conditions (65°C, 1× SSC, 50% formamide) and treated with RNase A (20 μg/ml, 30 min, 37°C). Two different Ptx1 cRNA fragments were used and produced similar patterns: a 573-bp fragment overlapping the homeo domain and a 310-bp fragment mainly from the 3′-untranslated region, which did not contain the homeo domain. ACTH immunoreactivity was detected with a mAb against amino-terminal ACTH (Cortex Biochem, CA) used at 5 μg/ml and anti-mouse IgG coupled to horseradish peroxidase. Slides were fixed in 4% paraformaldehyde for 10 min before proceeding with in situ hybridization.

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