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Ptx1 regulates SF-1 activity by an interaction that mimics the role of the ligand-binding domain

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Ptx1 (Pitx1) is a *bicoid*-related homeobox transcription factor expressed from the onset of pituitary development. It was shown to cooperate with cell-restricted factors, such as Pit1, NeuroD1/PanI and steroidogenic factor 1 (SF-1), to establish a combinatorial code conferring lineage- and promoter-specific gene transcription in the pituitary. Transcriptional synergism between Ptx1 and SF-1 on two SF-1 target genes, pituitary luteinizing hormone β and Müllerian-inhibiting substance (MIS), requires SF-1 binding to DNA and appears to result from direct physical interaction between these two proteins. The interaction between the C-terminus of Ptx1 and the N-terminal half of SF-1 results in transcriptional enhancement that equals the activity of a constitutively active SF-1 mutant and that may mimic the effect of a still unidentified SF-1 ligand. Thus, the unmasking of SF-1 activity by Ptx1 may represent a developmental mechanism to alleviate the need for SF-1 ligand in transcription and, possibly, at critical times during organogenesis.

Keywords: cofactor/homeobox/ligand/nuclear receptors/Pitx

Introduction

The Ptx family of transcription factors is a recently identified class of *bicoid*-related homeoproteins that are thought to be involved in the development of anterior structures and in mesoderm specification. To date, three Ptx genes have been cloned from various species (reviewed in Drouin *et al*., 1998a,b; Gage *et al*., 1999): Ptx1 (Pitx1) (Lamonerie *et al*., 1996; Muccielli *et al*., 1996; Szeto *et al*., 1996; Shang *et al*., 1997; Vorbruggen *et al*., 1997), Ptx2 also known as Pitx2 or Otlx2 (Muccielli *et al*., 1996; Semina *et al*., 1996; Gage and Camper, 1997) and Ptx3 (Semina *et al*., 1997; Smidt *et al*., 1997). Ptx1, the founding member of this family, was cloned initially as a regulator of pro-opiomelanocortin (POMC) gene expression in pituitary corticotrope cells (Lamonerie *et al*., 1996), but was later found to be a pan-pituitary regulator of transcription (Tremblay *et al*., 1998) and to be required for mandible and hindlimb development (Lanctôt *et al*., 1999b; Szeto *et al*., 1999) in agreement with its expression during development (Lanctôt *et al*., 1997). Ptx2 was first identified as the causative gene for Rieger's syndrome (Semina *et al*., 1996) and it was recently shown to be an effector for development of left–right asymmetry of lateral mesoderm plate derivatives such as heart and stomach (Logan *et al*., 1998; Piedra *et al*., 1998; Ryan *et al*., 1998; Yoshioka *et al*., 1998; Campione *et al*., 1999). Expression of Ptx1 and Ptx2 is detected in the stomodeum at embryonic day 8 (E8) and, subsequently, is maintained in all stomodeal derivatives, including Rathke's pouch, the pituitary anlage (Muccielli *et al*., 1996; Lanctôt *et al*., 1997; 1999a). Thus, Ptx1 and Ptx2 represent the earliest known markers of pituitary organogenesis and may have redundant activity in this tissue. However, antisense RNA experiments have shown that Lim3/Lhx3 gene expression, a gene crucial for proper pituitary development (Sheng *et al*., 1996), requires Ptx1, thus placing Ptx1 upstream of Lim3/Lhx3 in the cascade of regulators involved in pituitary development (Tremblay *et al*., 1998). In contrast, Ptx3 is not expressed in the pituitary but rather in midbrain dopaminergic neurons and in the eyes (Semina *et al*., 1997, 1998; Smidt *et al*., 1997). No target gene has yet been identified for Ptx3.

The Ptx1 transcription factor is present throughout pituitary development and in all adult pituitary cell lineages, albeit at different levels (Tremblay *et al*., 1998; Lanctôt *et al*., 1999a). Consistent with this observation, Ptx1 was found to activate transcription of a large set of pituitary target genes (Lamonerie *et al*., 1996; Szeto *et al*., 1996; Tremblay *et al*., 1998). Ptx1 was shown to be particularly important for transcription of genes specific to the gonadotrope lineage, the pituitary cell type in which Ptx1 protein is most abundant (Lanctôt *et al*., 1999a): these include the genes for glycoprotein hormone subunit α (αGSU) and luteinizing hormone β (LHβ) (Tremblay *et al*., 1998). However, since each Ptx1 target gene is expressed in a cell-restricted manner in the pituitary whereas Ptx1 is not, it is clear that Ptx1 alone cannot account for their lineage-specific expression. This specificity appears to be achieved by cooperation between Ptx1 and cell-restricted factors (Szeto *et al*., 1996; Poulin *et al*., 1997; Tremblay *et al*., 1998). For example, Ptx1 interacts with the somatotrope-, lactotrope- and thyrotrope-restricted factor Pit1, synergistically to activate the prolactin (PRL) promoter and, to a lesser extent, the growth hormone (GH) promoter (Szeto *et al*., 1996; Tremblay *et al*., 1998). In corticotrope cells, Ptx1 specifically cooperates with the basic helix–loop–helix (bHLH) heterodimer, NeuroD1/PanI, to activate the POMC promoter (Poulin *et al*., 1997). Finally, in gonadotropes, we have reported recently that Ptx1 activates the LHβ promoter in synergy with the orphan nuclear receptor steroidogenic factor 1 (SF-1) (Tremblay *et al*., 1998) and with the immediate early response gene product, Egr1 (Tremblay and Drouin, 1999); the latter appears to mediate gonadotropin-releasing hormone (GnRH)-induced signals. Thus,

Ptx1 appears to work in a combinatorial code to confer lineage- and promoter-specific gene transcription in the pituitary gland. In this code, the Ptx factors (Ptx1 and Ptx2) reflect the ectodermal (stomodeum) origin of the pituitary.

The orphan nuclear receptor SF-1 was first isolated as an essential regulator of the P450 hydroxylases in the adrenal gland and gonads (Lala *et al*., 1992; Ikeda *et al*., 1993; Parker and Schimmer, 1997). Its expression, however, is not limited to steroidogenic tissues as it is also found in the pituitary and ventromedial nucleus of the hypothalamus (VMH; Ikeda *et al*., 1994, 1995; Ingraham *et al*., 1994). SF-1 knockout mice lack adrenal glands and gonads (Luo *et al*., 1994; Sadovsky *et al*., 1995), exhibit VMH abnormalities, and male mice have female internal genitalia due to the absence of Müllerian-inhibiting substance (MIS), a key hormone involved in male sex differentiation (Luo *et al*., 1994). The MIS gene was shown to be a direct target of SF-1 action (Giuili *et al*., 1997). SF-1$^{-/-}$ mice also have markedly reduced mRNA levels for several pituitary-specific genes such as αGSU, LHβ, follicle-stimulating hormone β (FSHβ) and the GnRH receptor (Ikeda *et al*., 1995; Shinoda *et al*., 1995). Although SF-1 was cloned 6 years ago (Lala *et al*., 1992), true ligands for this nuclear receptor are not yet available, although Lala *et al*. (1997) reported that hydroxycholesterol (OHC) steroidogenic intermediates or their derivatives enhance SF-1-dependent transcription. However, the relevance of OHC as a biological SF-1 ligand could not be supported (Mellon and Bair, 1998). Although these OHCs are naturally present in steroidogenic tissues, they are not thought to be synthesized in pituitary gonadotrope cells that express SF-1. This suggests that a different set of ligand(s) or cofactor(s) is involved in the modulation of SF-1 activity in this tissue.

SF-1 binds to and activates the pituitary LHβ gene (Halvorson *et al*., 1996; Keri and Nilson, 1996). The LHβ promoter contains binding sites for Ptx1 and SF-1 that are 20 bp apart. These two regulatory elements and their relative position within the LHβ promoter are conserved in many species (Halvorson *et al*., 1996; Keri and Nilson, 1996; Tremblay *et al*., 1998). Although each factor individually can activate the LHβ promoter (Halvorson *et al*., 1996; Keri and Nilson, 1996; Tremblay *et al*., 1998), coexpression of both results in a strong synergistic activation (Tremblay *et al*., 1998). We now show that these two factors interact with each other through specific domains and that the resulting enhancement of transcription (observed on both LHβ and MIS promoters) mimics the activation of SF-1 by deletion of its ligand-binding domain (LBD) and possibly by its ligand. These data suggest that Ptx1 acts as a modulator of SF-1 activity.

Results

Specificity of Ptx1 and SF-1 synergism for activation of the LHβ promoter

We recently showed that Ptx1 cooperates with SF-1 synergistically to activate the LHβ promoter (Tremblay *et al*., 1998). To determine whether this synergism is specific to Ptx1 and SF-1, we tested the ability of two other *bicoid*-related homeoproteins, Otx1 and Otx2, and two nuclear receptors, the glucocorticoid receptor (GR) and Dax-1, to enhance transcription synergistically. Dax-1

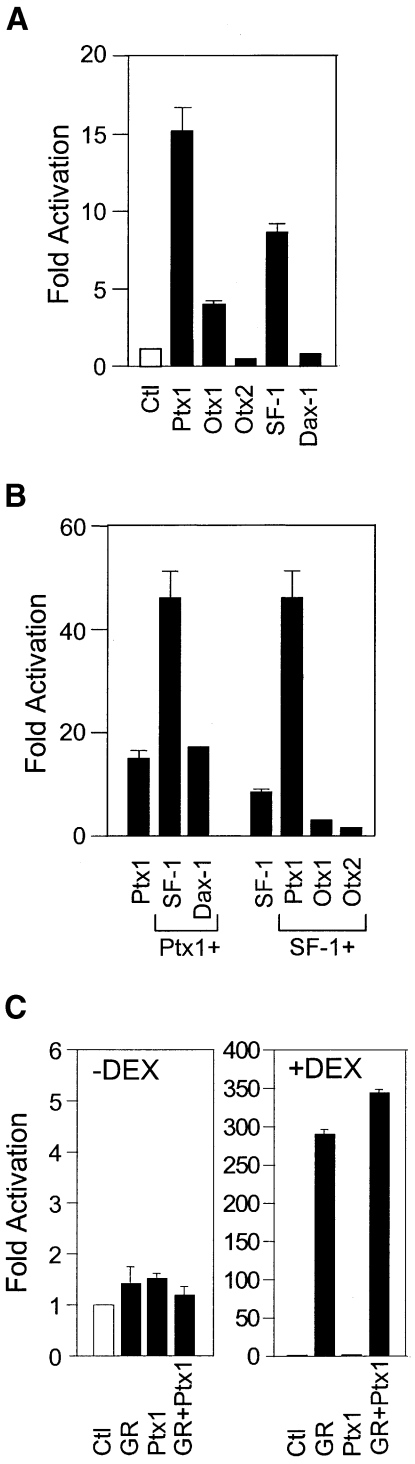

Fig. 1. Ptx1 and SF-1 synergize on the LHβ promoter. (**A**) The effects of Ptx1 and two other *bicoid*-related homeoproteins, Otx1 and Otx2, and of SF-1 and Dax-1, another nuclear receptor, were tested on the –776 bp bovine LHβ promoter. The LHβ reporter was co-transfected in CV-1 cells with a control plasmid (empty expression vector, open bar) or an expression vector for Ptx1, Otx1, Otx2, SF-1 and Dax-1 (solid bars). (**B**) Ptx1 and SF-1 transcriptionally cooperate. The combinations of Ptx1 with SF-1 or Dax-1, and of SF-1 with Ptx1, Otx1 or Otx2 were tested for their ability to synergize on the same reporter. Note the difference in scale in (A) and (B). (**C**) Ptx1 does not affect GR-dependent transcription. A GRE-containing reporter (Drouin *et al*., 1993) was transfected into CV-1 cells together with expression vectors for GR and/or Ptx1, and in the presence or absence of the synthetic glucocorticoid, dexamethasone (DEX) 10^{-7} M. Results are shown as fold activation (±SEM).

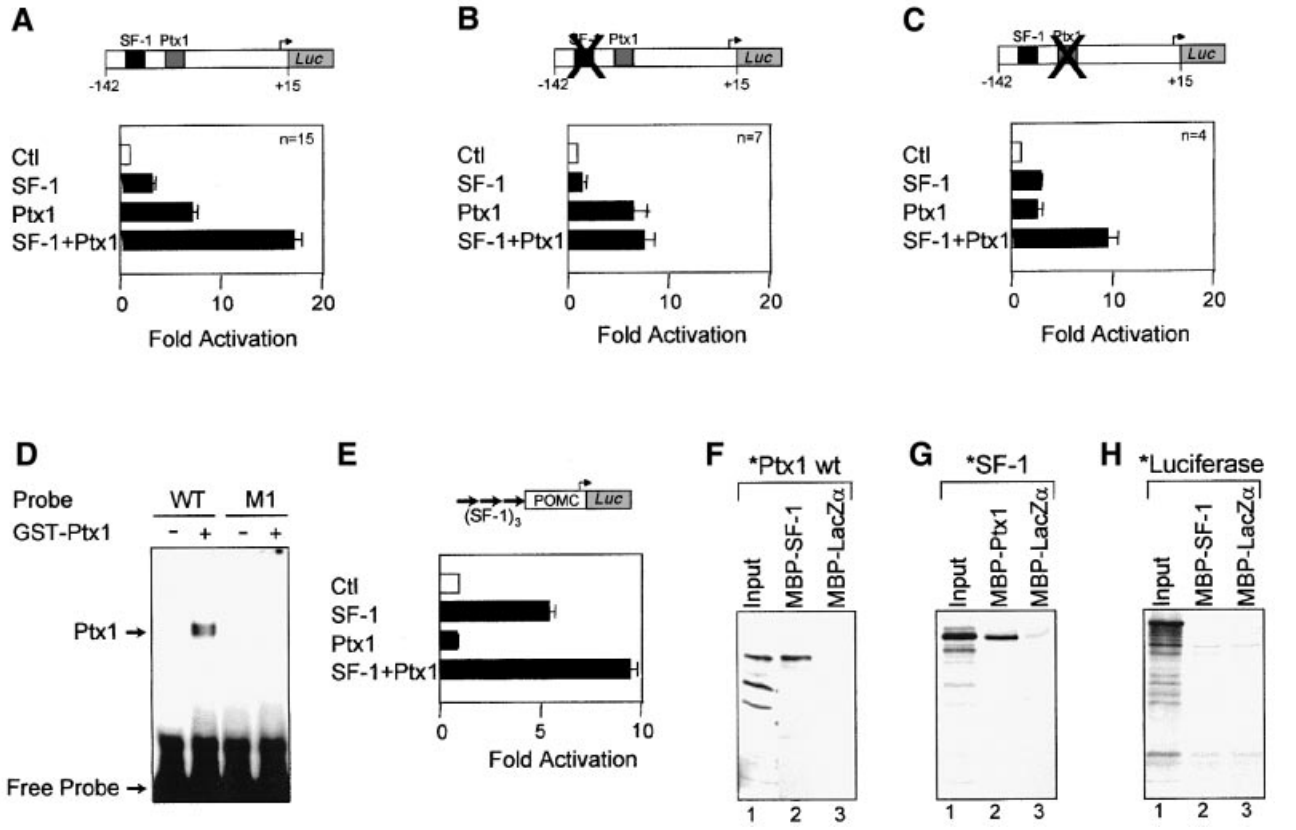

Fig. 2. The synergy between Ptx1 and SF-1 requires the SF-1- but not the Ptx1-binding site. Trans-activation by either Ptx1, SF-1 or both factors was tested on three –142 bp bovine LHβ reporters: (**A**) the wild-type promoter that contains binding sites for SF-1 and Ptx1; (**B**) a reporter with a mutation in the SF-1 site (Halvorson *et al*., 1996; Keri and Nilson, 1996); (**C**) a reporter with a mutation in the Ptx1 site (Lamonerie *et al*., 1996); and (**E**) on a different reporter plasmid containing three LHβ SF-1-binding sites upstream of a small POMC gene promoter (–34 bp to +63 bp). Promoter constructs were co-transfected in CV-1 cells with the indicated expression plasmids. Results are shown as fold activation (±SEM) for the indicated number of experiments (*n*), each performed in duplicate. (**D**) The Ptx1 site mutant does not bind GST–Ptx1 *in vitro*. Gel retardation was performed as described previously (Lamonerie *et al*., 1996) using oligonucleotide probes for either wild-type (WT) or mutant (M1) Ptx1-binding sites. Direct interaction between Ptx1 and SF-1 was shown in pull-down assays performed using immobilized, bacterially produced MBP fusion proteins (MBP–SF-1, MBP–Ptx1 and MBP–LacZα, as control) and *in vitro* translated ^{35}S-labeled Ptx1 (**F**), SF-1 (**G**) or luciferase (**H**). Bound proteins were separated by SDS–PAGE and visualized by autoradiography. An aliquot of input protein corresponding to 20% of labeled protein used in the assay is shown for comparison.

is co-expressed with SF-1 in pituitary gonadotropes (Ikeda *et al*., 1996) and, although Otx1 is expressed in the pituitary, Otx2 is not found in this tissue (Acampora *et al*., 1998). In contrast to Ptx1 and SF-1, Otx1 weakly activated the LHβ promoter whereas Otx2 and Dax-1 did not trans-activate at all (Figure 1A), despite the fact that both Otx1 and Otx2 bind the LHβ promoter Ptx1-binding site (Acampora *et al*., 1998; Drouin *et al*., 1998a,b; Tremblay *et al*., 1998). Moreover, when these factors were tested in combination, synergistic activation of the LHβ promoter was observed only between Ptx1 and SF-1 (Figure 1B). As for Otx1 and Otx2, SF-1 could not synergize with two other *paired*-class homeoproteins expressed in pituitary gonadotropes, Pax6 and Six3 (data not shown). Trans-activation by the GR was not affected by Ptx1 whether basal or hormone-stimulated transcription was assessed (Figure 1C). These results indicate that the cooperation between SF-1 and *bicoid*-related homeoproteins is restricted to the Ptx subfamily of factors. Consistent with this observation, Ptx2 and Ptx1b, a Ptx1 isoform, can also synergize with SF-1 (J.J.Tremblay, C.G.Goodyer and J.Drouin, in preparation). Interestingly, the Ptx1 and Ptx1b isoforms differ solely in their N-terminus. Since both Ptx1 isoforms can synergize with SF-1, this suggests that the N-terminal domain of Ptx1 is not likely to be involved in the synergy with SF-1.

Ptx1/SF-1 synergism requires an SF-1- but not a Ptx1-binding site

The LHβ promoter contains several consensus Ptx1-binding sites and an SF-1 element that are conserved across species (Halvorson *et al*., 1996; Keri and Nilson, 1996; Tremblay *et al*., 1998). Since we have shown recently that the proximal Ptx1-binding site present at –95 bp is sufficient for activation by Ptx1 (Tremblay *et al*., 1998), we generated a –142 bp LHβ promoter construct that retains the SF-1 site located at –120 bp and the proximal Ptx1-binding site. This shorter promoter fragment still exhibited transcriptional enhancement between the factors (Figure 2A) and it was used to define the mechanism of cooperation between SF-1 and Ptx1. We have shown previously that Ptx1/SF-1 synergism is lost when the SF-1-binding site is deleted (Tremblay *et al*., 1998). Similarly, we show here that mutagenesis of two nucleotides within the SF-1 element prevents synergism with Ptx1 (Figure 2B); this mutation previously was shown to impair SF-1 binding and trans-activation (Halvorson *et al*., 1996; Keri and Nilson, 1996). In contrast, a mutation of

the Ptx1-binding site did not prevent enhancement of SF-1 activity by Ptx1 (Figure 2C); this mutation was shown previously to abolish Ptx1-dependent transcription (Lamonerie *et al*., 1996) and it no longer bound Ptx1 *in vitro* (Figure 2D). The weak activation of this mutant promoter by Ptx1 could suggest the presence of a cryptic Ptx1-binding site in the reporter plasmid; since such a possibility is difficult to rule out completely, we used another reporter containing oligonucleotides for the SF-1-binding site placed upstream of an unrelated promoter, namely a short fragment of the POMC promoter. Ptx1 enhanced SF-1-dependent activity of this reporter but did not itself activate it (Figure 2E). Thus, these results suggest that Ptx1 binding to DNA is not strictly required for synergy with SF-1 and that a direct interaction between SF-1 and Ptx1 may occur.

Ptx1 and SF-1 interact directly

In order to test this hypothesis, we assessed whether the two factors interact directly using the pull-down assay. As shown in Figure 2F, an immobilized maltose-binding protein (MBP)–SF-1 fusion protein specifically retained *in vitro* translated ^{35}S-labeled Ptx1 whereas an unrelated MBP–LacZα fusion did not, indicating that the Ptx1–MBP–SF-1 interaction is not mediated by MBP itself. Reciprocal results were obtained when Ptx1 was linked to MBP and labeled SF-1 was used (Figure 2G). The Ptx1–SF-1 interaction further was shown to be specific since no interaction was observed with luciferase protein as control (Figure 2H).

Mapping of the Ptx1 domain interacting with SF-1

Trans-activation by Ptx1 mutants. The transcriptional properties of Ptx1 have not yet been dissected. In order to interpret a detailed analysis of Ptx1–SF-1 interaction, we first defined transcriptionally active domains of Ptx1 using a series of Ptx1 mutants (Figure 3A). The DNA-binding properties of the Ptx1 mutants were tested in gel shift assays (Figure 3B). Specific binding was observed with all mutants except those (ΔC_5, ΔC_6 and K139A) that affect the homeodomain (HD, Figure 3B). The expression of these three non-binding mutants was verified by Western blot using a Ptx1-specific antibody (data not shown). Both gel retardation and Western blot assays were done using nuclear extracts and they revealed similar nuclear expression levels for the various mutants.

The level of expression could not be assessed as thoroughly for the N-terminal deletion mutants (ΔN_1 and ΔN_2) because the deleted region contains the epitopes against which Ptx1 antiserum was raised. In addition, the mutants behaved differently in gel retardation; indeed, they formed only a limited amount of DNA-bound complexes that migrate as would be expected of Ptx1 monomers (Figure 3B, lanes 4 and 5, asterisk). However, contrary to other Ptx1 proteins, they also exhibited slower

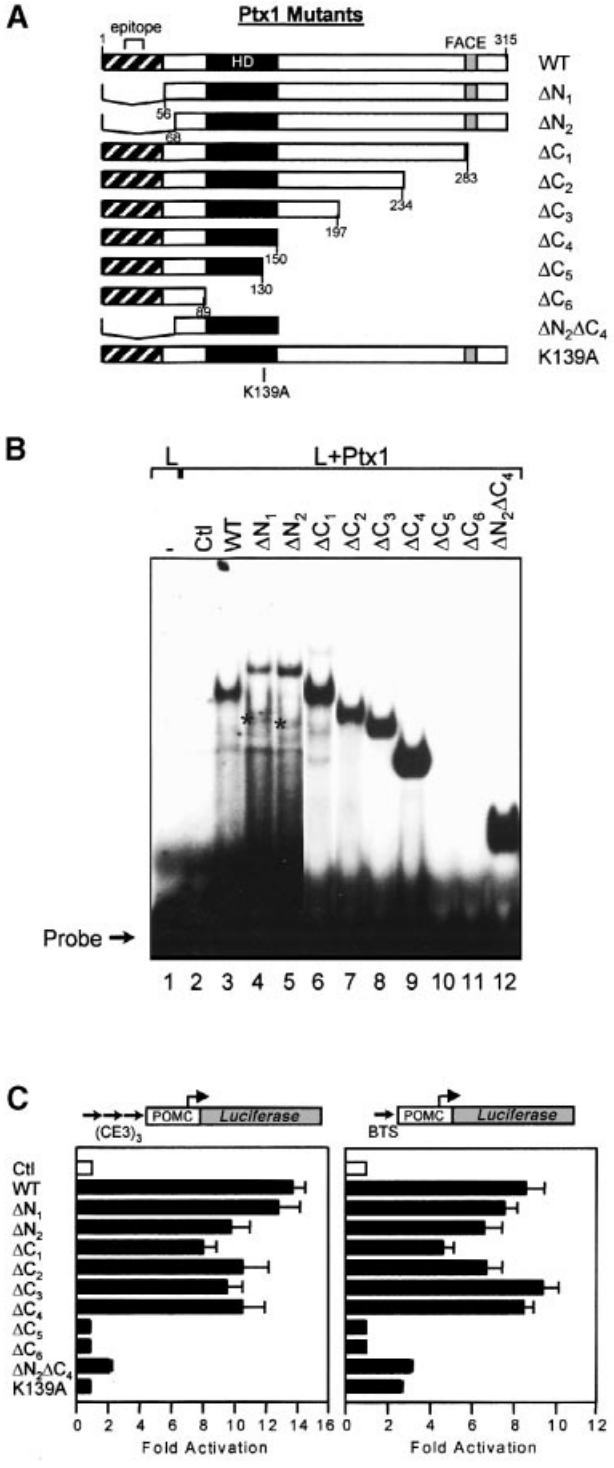

Fig. 3. Ptx1 contains two independent activation domains. (**A**) Schematic representation of Ptx1 mutants used in the present study. The hatched box delineates the Ptx1-specific (compared with the Ptx1b isoform) N-terminal domain, the black box represents the homeodomain (HD) and the gray-shaded box, the FACE domain. The FACE domain is a 14 amino acid region conserved within homeoproteins that share expression in craniofacial structures during development (Semina *et al*., 1996). A Ptx1-specific antiserum was raised against epitopes within the N-terminal region. (**B**) The DNA-binding properties and expression levels of the mutants were assessed in gel retardation assays using the Ptx1-binding site of the POMC gene (Lamonerie *et al*., 1996) as probe and nuclear extracts from L cells transfected with expression vectors for the various mutants. Asterisks in lanes 4 and 5 identify bands of the expected size for the two N-terminal mutants. (**C**) Trans-activation properties of Ptx1 mutants. The effect of each Ptx1 mutant was tested on two simple reporters by co-transfection in L cells. The reporters contain either three copies of the Ptx1-binding site of the POMC gene (Lamonerie *et al*., 1996) or one copy of the *bicoid* target site (BTS) found in the *Drosophila hunchback* gene (Driever and Nusslein-Volhard, 1989; Simeone *et al*., 1993) fused to the minimal POMC promoter (Therrien and Drouin, 1991) and cloned upstream of luciferase. Results are shown as fold activation (±SEM).

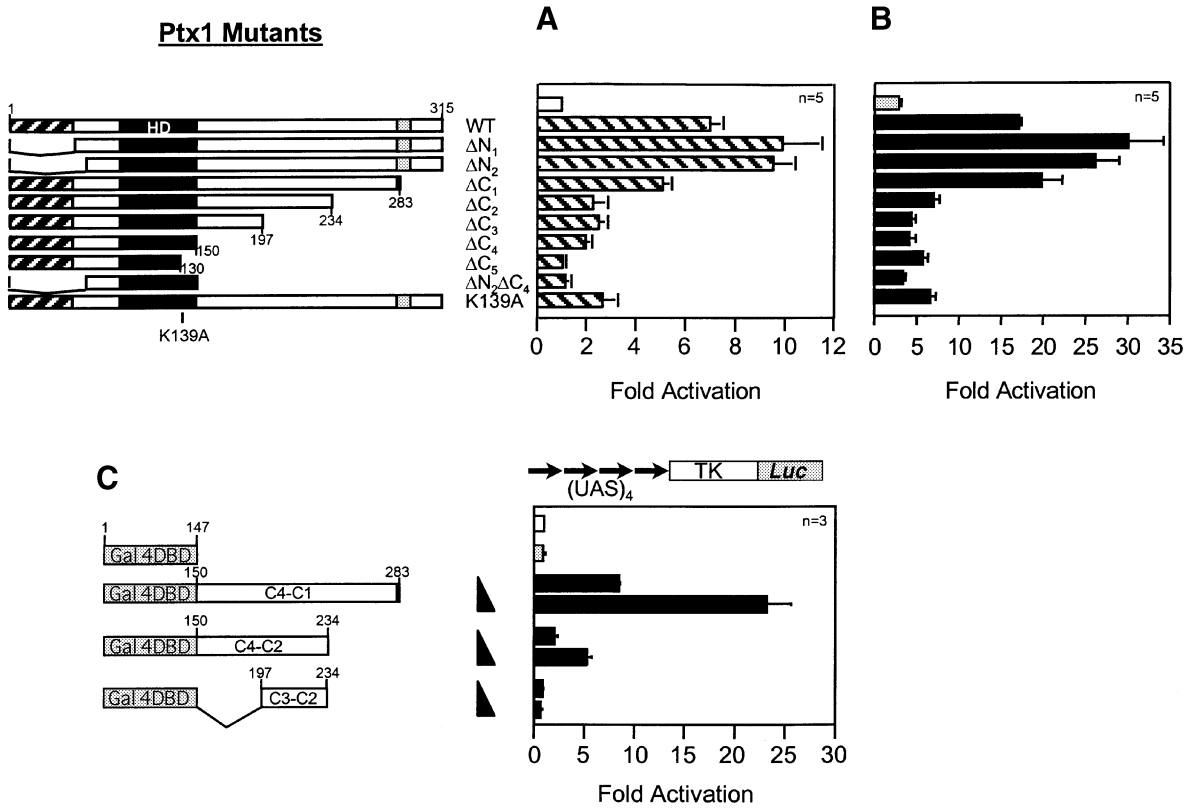

Fig. 4. The C-terminal activation domain of Ptx1 is required for synergy with SF-1. CV-1 cells were co-transfected with the bovine –142 bp LHβ reporter along with: (**A**) empty expression vector as control (open bar) or expression vectors for Ptx1 mutants (hatched bars) or (**B**) an SF-1 expression vector alone (gray bar) or in combination with the Ptx1 mutants (solid black bars). (**C**) The C-terminal trans-activation domain of Ptx1 was localized further using chimeras containing the Gal4DBD fused to Ptx1 fragments. The C4–C1, C4–C2 and C3–C2 chimeras contain Ptx1 sequences between the deletion endpoints illustrated above and they were assayed by co-transfection in CV-1 cells together with the indicated UAS reporter. Results are shown as fold activation ± SEM for the indicated number of experiments (*n*), each performed in duplicate.

migrating complexes that may correspond to Ptx1 dimers or to Ptx1 bound to another protein. Nonetheless, they showed transcriptional activity that was similar to that of wild-type Ptx1 (Figure 3C).

The transcriptional properties of the Ptx1 mutants were tested using two simple promoters. The first one consisted of three copies of the POMC promoter Ptx1-binding site (Lamonerie *et al*., 1996), whereas the second contained a single copy of the *bicoid* target site (BTS) from *Drosophila hunchback* (Driever and Nusslein-Volhard, 1989; Simeone *et al*., 1993). As shown in Figure 3C, deletion of either the N- or the C-terminal domain did not affect the ability of Ptx1 to trans-activate the two reporters. However, a mutant in which both domains are deleted (mutant $\Delta N_2\Delta C_4$ which retains the HD and nuclear localization signal) no longer activated transcription. As expected, deletion within the HD (ΔC_5 and ΔC_6) or replacement of Lys139 by alanine (this is Lys50 of the HD) abolished transcriptional activity (Figure 3C). Taken together, these results suggest that Ptx1 contains two independent trans-activation domains located in the N- and C-terminal regions and that only one of these is required for trans-activation of simple reporters.

Synergism between Ptx1 and SF-1. The ability of these mutants to activate the –142 bp LHβ reporter and to synergize with SF-1 was then evaluated. Contrary to its activity on simple reporters (Figure 3C), the Ptx1 N-terminus did not appear sufficient for trans-activation on this promoter, as revealed by mutant ΔC_4 (Figure 4A). Mapping of Ptx1 C-terminal sequences required for activation of the LHβ promoter revealed that important sequences lie between the endpoint of mutant ΔC_2 and the C-terminus. These results suggest that either N- or C-terminal trans-activation domains of Ptx1 may be active depending on the promoter context. This idea was supported further by experiments showing that other pituitary hormone promoters (POMC, αGSU, GH and TSHβ) exhibit a pattern of activity for Ptx1 mutants similar to that of the simple promoters shown in Figure 3C (data not shown).

In order to delineate C-terminal sequences required for trans-activation activity, fusion proteins containing the Gal4 DNA-binding domain (DBD) and C-terminal fragments of Ptx1 were engineered and assayed by co-transfection with an upstream activator sequence (UAS)-containing reporter. Sequences between endpoints C4 and C1 were required for maximal transcriptional activity but a C4–C2 fusion retained partial activity (Figure 4C). The C3–C2 region was insufficient for transcriptional activity. Thus, sequences between endpoints C2 and C1 are critical for trans-activation, those same sequences that are required for transcriptional activity of the intact protein (Figure 4A).

When Ptx1 mutants were tested in the presence of SF-1, it appeared that a region of 49 amino acids, located between endpoints C1 (amino acid 283) and C2 (amino acid 234), was required for synergy since mutant ΔC_1 synergized with SF-1 whereas mutant ΔC_2 did not (Figure

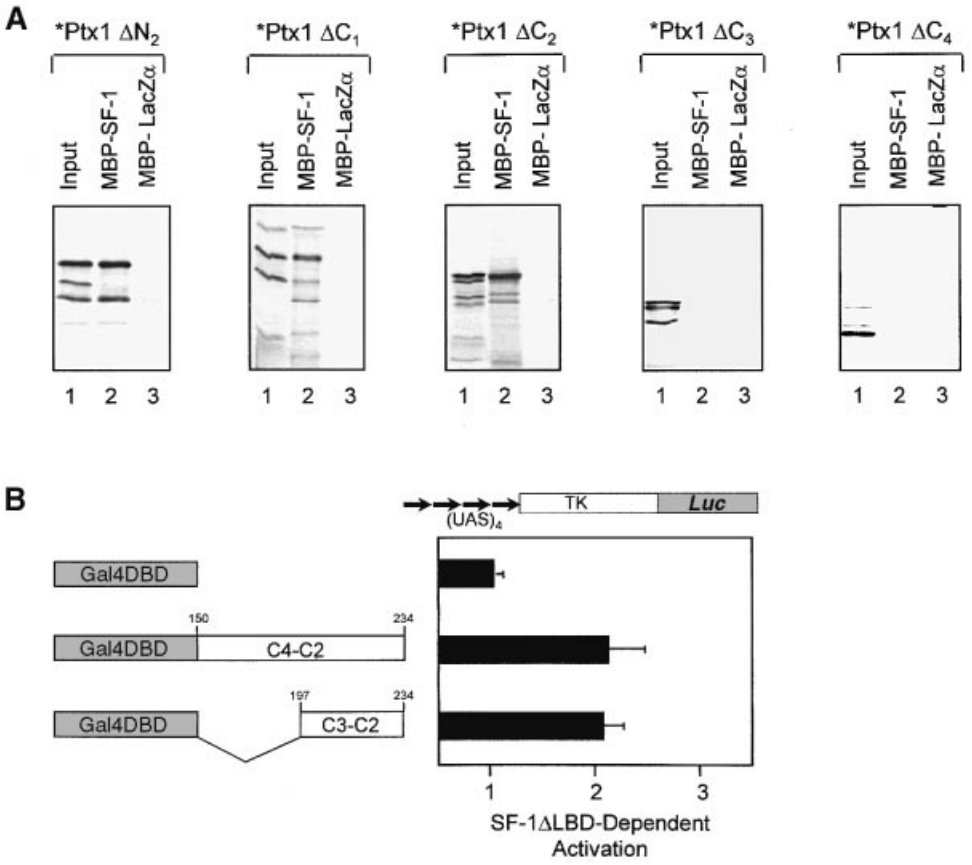

Fig. 5. Interaction domain with SF-1 maps to a 37 amino acid C-terminal region of Ptx1. (**A**) *In vitro* interaction. The indicated Ptx1 mutants (Figure 3A) were labeled by *in vitro* translation and tested for binding to MBP–SF-1 or to MBP–LacZα as control. Bound proteins were separated on SDS–PAGE and visualized by autoradiography. The input sample contains 20% of the labeled protein used in the assay. (**B**) *In vivo* interaction. A hybrid assay system in CV-1 cells was used to show *in vivo* interaction between small C-terminal fragments of Ptx1 fused to Gal4DBD and SF-1ΔLBD. The Gal4DBD fusions are the same as in Figure 4C. Results are shown as fold activation in the presence compared with the absence of SF-1ΔLBD (means of three experiments, each performed in duplicate ± SEM).

4B). Thus, Ptx1 sequences required for trans-activation (Figure 4A) were also required for synergism (Figure 4B).

The region of physical interaction between Ptx1 and SF-1 was identified next using the pull-down assay. As for trans-activation and synergism (Figure 4), the N-terminus was not required for physical interaction with SF-1 (mutant ΔN_2, Figure 5A). A 37 amino acid region, located in the C-terminus between residues 197 (ΔC_3) and 234 (ΔC_2), was essential for interaction with SF-1 since the former no longer interacted with MBP–SF-1 (Figure 5A). In order to confirm these data *in vivo*, the fusion constructs containing the Gal4DBD and C-terminal fragments of Ptx1 were assayed for interaction with SF-1 using a hybrid assay in mammalian cells (Figure 5B). These experiments clearly showed that the Ptx1 C-terminal sequences between endpoints C3 and C2 were sufficient for interaction with SF-1 since the Gal4DBD–C3–C2 fusion which is transcriptionally inert on its own (Figure 4C) exhibited as much SF-1-dependent enhancement of activity as did the longer Gal4DBD–C4–C2 fusion (Figure 5B). Taken together, these results indicate that different but contiguous subdomains of the Ptx1 C-terminus are involved in transcriptional activation (between amino acids 234 and 283) and in physical interaction (between amino acids 234 and 197) between the factors.

Ptx1 modulates SF-1 activity

Although SF-1 is expressed in the pituitary and is involved in the regulation of the LHβ gene (Ikeda *et al*., 1995; Halvorson *et al*., 1996; Keri and Nilson, 1996), the ligand that may modulate its activity in this tissue remains unknown. However, Shen *et al*. (1994) have shown that deletion of half of the putative ligand-binding domain of SF-1 (SF-1ΔLBD) increased transcriptional activity of SF-1, and suggested that this mimicked the effect of ligand. To explore this possibility, we compared the ability of SF-1ΔLBD and SF-1 to activate the LHβ promoter and to synergize with Ptx1. SF-1ΔLBD was markedly more active on the LHβ promoter than SF-1, in agreement with the model that the SF-1 LBD represses trans-activation function (Figure 6A). Interestingly, SF-1ΔLBD did not synergize with Ptx1. In fact, the activity of SF-1ΔLBD was identical to the synergistic action of SF-1 and Ptx1, suggesting that the Ptx1–SF-1 interaction has the same unmasking effect as deletion of the SF-1 LBD. The absence of synergism between SF-1ΔLBD and Ptx1 was not due to a loss of interaction between them since they still interact in the pull-down assay (Figure 6B).

Since the unmasking effect of deleting the SF-1 LBD previously was shown to operate on the MIS promoter (Shen *et al*., 1994), we tested whether Ptx1 may have a

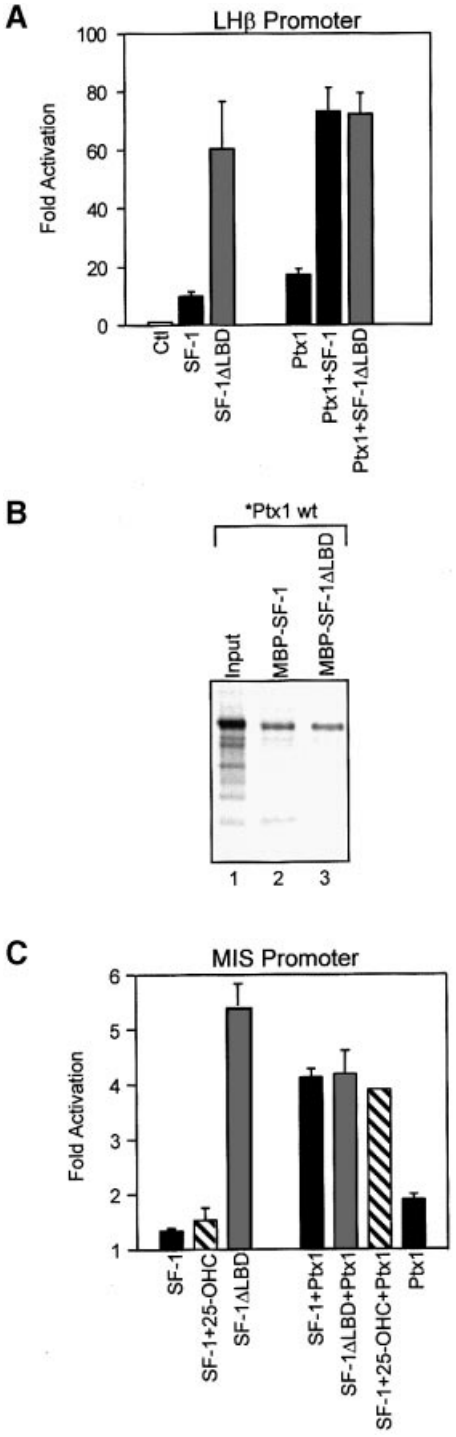

Fig. 6. Ptx1 interaction unmasks SF-1 activity. (**A**) Wild-type SF-1 (SF-1) or an SF-1 mutant deleted of its LBD (SF-1ΔLBD) were tested either alone or in combination with Ptx1 for activation of the bovine LHβ reporter. Results are shown as fold activation (±SEM). (**B**) Ptx1 still interacts with SF-1ΔLBD. Pull-down assays were performed using the full-length ^{35}S-labeled Ptx1 protein and immobilized MBP–SF-1 or MBP–SF-1ΔLBD. Protein complexes were separated on SDS–PAGE and visualized by autoradiography. (**C**) Synergism between Ptx1 and SF-1 on the MIS promoter which does not have a Ptx1-binding site. The experiment was performed as in (A). In addition, the putative SF-1 ligand, 25-hydroxycholesterol, was tested at a concentration of 10^{-5} M (Lala *et al*., 1997).

similar effect in this system as well. Indeed, we found that the cooperation between Ptx1 and SF-1 on this promoter was very similar to that on the LHβ promoter (Figure 6C), despite the absence of a Ptx1-binding site in the MIS promoter. The effect of a recent and weak candidate SF-1 ligand (Lala *et al*., 1997) was assessed in this system despite its dubious biological relevance (Mellon and Bair, 1998). 25-OH-cholesterol only weakly activated SF-1 and much less than observed with the SF-1 LBD deletion or the synergistic activation by Ptx1 (Figure 6C). Taken together, these data suggest that Ptx1 (or another member of the Ptx family) acts as a modulating cofactor of SF-1 and that this action may mimic, at least partly, the role of the SF-1 LBD.

Discussion

So far, only one class of putative ligands, all OHCs (Lala *et al*., 1997), have been proposed as SF-1 ligands, and their biological relevance appears questionable (Mellon and Bair, 1998). The present work revealed that enhancement of SF-1-dependent transcription can be achieved by interaction with a transcription factor that acts as a modulatory cofactor in the absence of ligand. In the context of pituitary development, the Ptx1/SF-1 synergism is consistent with the establishment of a regulatory code during organogenesis and cell differentiation in which a pan-pituitary factor, Ptx1 (or the related and redundant factor Ptx2), collaborates with a lineage-restricted factor, SF-1, for activation of a cell-specific program of gene expression (Tremblay *et al*., 1998). In such a model, the requirement for an SF-1 ligand may be bypassed by another regulatory process, the tissue-restricted expression of Ptx1 (and/or Ptx2) that is established at an earlier developmental stage.

Organization of Ptx1 transcription factor

Deletion analysis of Ptx1 revealed two independent activation domains located on each side of the HD (Figure 3C). The Ptx1 N- and C-termini both contain proline- and serine-rich motifs (Lamonerie *et al*., 1996, and Figure 7A) that are known to function as activation domains in other transcription factors (Gerber *et al*., 1994). The activity of these domains is, however, dependent on promoter context, as only the C-terminal activation domain is contributing to Ptx1-dependent activation of the LHβ promoter (Figure 4A). Such promoter dependence has been documented for other transcription factors, such as, for example, the retinoic acid and estrogen receptors (Tora *et al*., 1989; Nagpal *et al*., 1992). The Ptx1 C-terminal activation domain appears to be located between amino acids 197 and 283, with most of the activity dependent on the region between amino acids 234 and 283 (Figures 4C and 7A). Interestingly, this region does not include the FACE motif, a stretch of 14 amino acids conserved within homeoproteins that share expression in craniofacial structures during development (Semina *et al*., 1996). No function has yet been ascribed to this motif. The same Ptx1 C-terminal activation domain was found to be essential for synergism with SF-1 (Figure 4B).

Mechanism of Ptx1/SF-1 synergism

We have shown that synergism between Ptx1 and SF-1 is specific to the Ptx family of *bicoid*-related homeoproteins

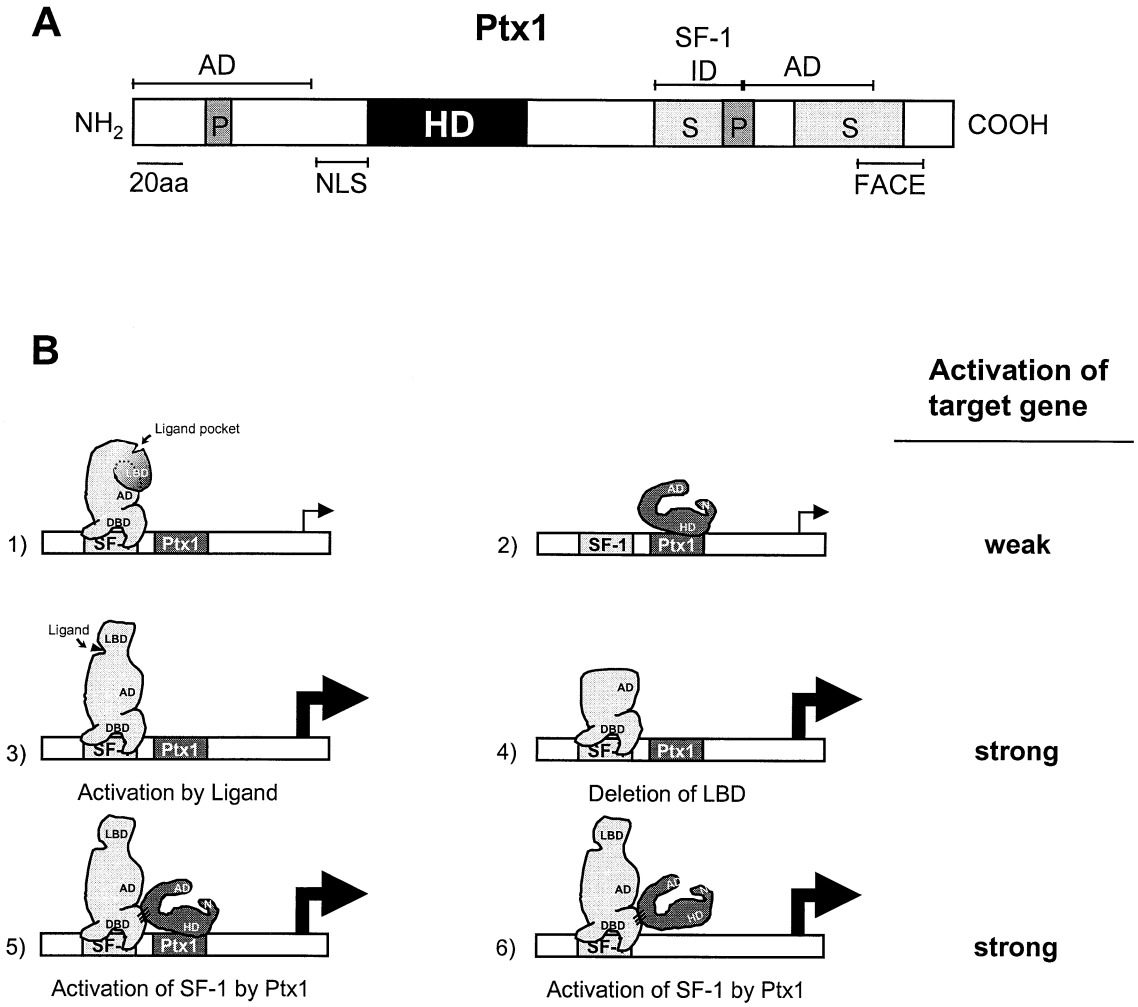

Fig. 7. Ptx1 modulates SF-1 activity in the pituitary. (**A**) Schematic representation showing the position of the homeodomain (HD), of proline (P)- and serine (S)-rich motifs, and of the FACE domain as in Figure 3. The position of a putative nuclear localization signal (NLS) is also shown along with the position of two transcriptional activation domains (AD) and of an SF-1-interaction domain (SF-1 ID) identified in the present work. The putative NLS was maintained in all Ptx1 mutants described herein. In contrast, a mutant protein deleted of this region (between amino acids 68 and 84) was no longer detectable in nuclear extracts by gel shift assay (data not shown). (**B**) Model for Ptx1/SF-1 synergism. The weak transcriptional effects of SF-1 (1) and Ptx1 (2) depends on binding to their cognate sites. A putative ligand would activate SF-1 through LBD binding and unmasking of the activation domain (3). A similar effect appears to be achieved by deletion of the LBD (4). The interaction of Ptx1 with SF-1 also appears to unmask transcriptional activity and thus bypasses the need for ligand (5). Although the Ptx1/SF-1 synergism results in the strongest activation of transcription when binding sites for both factors are present in the target promoter (5), synergism is also observed in the absence of a Ptx1-binding site (6).

since Otx1 and Otx2, two closely related factors, failed to synergize with SF-1 (Figure 1). Consistent with this, two other Ptx family members, Ptx1b (a Ptx1 isoform) and Ptx2, also synergize with SF-1 (J.J.Tremblay, C.G.Goodyer and J.Drouin, in preparation). We have shown previously that pituitary gonadotropes contain almost exclusively Ptx1 protein despite the presence of Ptx1b and Ptx2 mRNA in these cells (Tremblay *et al*., 1998), indicating that Ptx1 is most likely to cooperate with SF-1 for the control of LHβ transcription. It is noteworthy that Otx1 does not synergize with SF-1 (Figure 1B) despite the similarity of Ptx and Otx DNA binding specificity (Drouin *et al*., 1998a,b) and the activation of the LHβ promoter by Otx1 (Figure 1A, and Acampora *et al*., 1998). The physical interaction between Ptx1 and SF-1 was mapped to a 37 amino acid segment of Ptx1, between amino acids 197 and 234 (Figure 5), that lies just upstream of the primary trans-activation domain (Figure 7A). This segment is highly conserved between Ptx1 and Ptx2, with 29 conserved residues, but not at all in Otx1 or Otx2 (data not shown). The cooperation between Ptx1 (a homeoprotein) and SF-1 (a nuclear receptor) is reminiscent of the interaction between Ftz-F1, the *Drosophila* homolog of SF-1, and the homeoprotein Ftz (Guichet *et al*., 1997; Yu *et al*., 1997). Despite the apparent conservation of a mechanism for synergism, the protein interfaces involved in these interactions do not reveal significant conservation of primary sequences.

The direct interaction between Ptx1 and SF-1 raises the formal possibility that DNA binding of either factor may be sufficient for synergism, as was shown for the interaction between myogenic bHLH factors and MEF2 (Molkentin *et al*., 1995). In contrast, we found that DNA binding was required for SF-1 but not as stringently for Ptx1 (Figures 2 and 6C). These observations suggest that Ptx1 can modulate SF-1 activity by a DNA-independent

protein–protein interaction. We have documented this interaction *in vitro* (Figures 2F–H and 5A) and we have shown that a 37 amino acid Ptx1 polypeptide is sufficient for SF-1 interaction *in vivo* when it is fused to the heterologous DBD of Gal4 (Figure 5B). Furthermore, we have shown Ptx1/SF-1 synergism on promoters that are devoid of Ptx1-binding sites (Figures 2C and E, and 6C), in particular on the MIS promoter that is an SF-1 target (Shen *et al*., 1994; Giuili *et al*., 1997). Indeed, Ptx1 greatly enhanced SF-1-dependent activation of the MIS promoter (Figure 6C).

Ptx1 as modulatory cofactor of SF-1 activity

Although SF-1 plays critical roles in several tissues, we still have a very fragmentary understanding of the control of its activity. The ligand that may control SF-1 activity is still unknown, but recent work has shown that some of the sex-specific roles of SF-1 in controlling MIS gene activity may depend on protein–protein interaction between SF-1 and the product of the Wilms' tumor (WT-1) gene (Nachtigal *et al*., 1998). As reported here, the interaction between Ptx1 and SF-1 may fulfill an equivalent function in the pituitary.

The unliganded SF-1 LBD acts as a repressor of SF-1 activity (Shen *et al*., 1994), and deletion of its C-terminal half unmasks SF-1 transcriptional activity. This LBD C-terminal half includes an AF-2 function (amino acids 452–458) that may be ligand dependent (Crawford *et al*., 1997) and a region (amino acids 437–447) for interaction with the transcriptional repressor DAX-1, which in turn recruits the co-repressor N-Cor (Crawford *et al*., 1998). In addition, SF-1 has another trans-activation domain that is SRC-1 dependent; unlike other nuclear receptors that have an AF-1 domain in the N-terminus, this SF-1 activation domain is in the proximal half of the LBD. It includes an SRC-1 interaction sequence and it is retained in SF-1ΔLBD (Crawford *et al*., 1997). It is thus possible that SF-1 activity is revealed by a putative ligand that unmasks activation functions as well as relieves a repressor activity that may involve an associated repressor such as DAX-1, or that interaction with another factor such as Ptx1 may achieve a similar effect. Our studies of the LHβ promoter activation by SF-1 have supported this model by showing enhanced activity of SF-1ΔLBD compared with SF-1 (Figure 6A). Significantly, the synergy observed between Ptx1 and SF-1 on the LHβ and MIS promoters (Figure 7B, models 5 and 6, respectively) resulted in the same activity as that of SF-1ΔLBD (Figure 7, model 4), suggesting that Ptx1 interaction has a similar effect on SF-1 as deletion of the LBD regulatory C-terminus. Thus, we propose that Ptx1 (or another Ptx family member) may function as a modulatory cofactor of SF-1 by unmasking its activation domain. It cannot be excluded at this point that the Ptx interaction may release an SF1-associated repressor, but DAX-1 cannot be implicated in our experiments since it is not expressed in CV-1 cells. The Ptx1 unmasking effect does not operate on all nuclear receptor LBDs as no effect was observed on GR (Figure 1C). The similarity between the LBD deletion and Ptx1 unmasking effects suggests that both revealed the activity of the SF-1 activation function located between residues 187 and 245 (Crawford *et al*., 1997). Since ligand-induced activation would be expected also to activate the AF-2 domain, it cannot be formally excluded that a more efficient SF-1 ligand (than those currently available) might further increase the activity of SF-1- and Ptx1-dependent promoters.

Materials and methods

Cell culture and transfection assays

Murine fibroblast L and African green monkey kidney fibroblast-like CV-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum. CV-1 and L cells were transfected by the calcium phosphate method (De Bold *et al*., 1983). Briefly, CV-1 and L cells were plated at 30 000 and 75 000 cells/well, respectively, in 12-well plates 24 h prior to transfection. Transfection was carried out using 1.5 μg of reporter plasmid, 0.25–1 μg of effector plasmid(s), 0.25 μg of Rous sarcoma virus (RSV)-hGH (human growth hormone) as internal control and a carrier DNA (Sp64 or Bluescript) up to a total of 5 μg/well. Cells were rinsed 12–16 h later and harvested the next morning. An aliquot of media was assayed for hGH by radioimmunoassay (Immunocorp, Montréal). A 100 μl aliquot of lysis buffer containing 100 mM Tris–HCl pH 8.0, 0.5% NP-40 and 5 mM dithiothreitol (DTT) was applied directly on the cells, incubated at room temperature with agitation for 15 min and 80 μl was assayed for luciferase activity using a LB953 Berthold Luminometer. Data are presented as means ± SEM of 4–17 experiments, each performed in duplicate.

Nuclear extracts and gel retardation assay

Nuclear microextracts were prepared from transfected L cells (Therrien and Drouin, 1993). Ptx1 gel retardation assays were done as outlined by Lamonerie *et al*. (1996). Ptx1 wild-type and mutant M1 oligonucleotides used in gel retardation assays were described previously (Lamonerie *et al*., 1996).

Plasmids and oligonucleotides

N- and C-terminal deletions of Ptx1 as well as SF-1ΔLBD (Shen *et al*., 1994) were generated by PCR. The DAX-1 cDNA was obtained by RT–PCR using first strand cDNA from rat testis and subsequently subcloned in an RSV-driven expression vector described elsewhere (Tremblay *et al*., 1998). Three copies of a double-stranded oligonucleotide containing the SF-1-binding site from the LHβ promoter (5′-GATCCTTCCCTGACCTTGTCTGTGA-3′ and 5′-GATCTCACAGACAAGGTCAGGGAAG-3′) 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). The Ptx1 fragments used in the Gal4DBD–Ptx1 fusions were generated by PCR with primers containing restriction sites and subsequently subcloned in-frame in the corresponding sites of a Gal4DBD vector. Site-directed mutagenesis was used to convert the lysine at position 139 of Ptx1 (residue 50 of the HD) to an alanine using the pALTER (Promega) system according to the manufacturer's recommendations. LHβ promoter deletion to –142 bp was generated by PCR. The resulting *Kpn*I–*Hin*dIII fragment was subcloned in the corresponding sites of a modified pGL2-Basic vector (Promega) which previously was deleted of its *Sma*I site. Mutations of the SF-1- and Ptx1-binding sites were also generated by PCR using the following primers showing the mutation in bold: (i) SF-1 site mutation (with *Kpn*I site added): 5′-ATGGTA^{-142}CCACTCTTGCCTCTCCCTGA**AA**TTGTCTGCCTCTCGCCC-3′$^{-104}$; and (ii) Ptx1 site mutation (natural *Sma*I site just 5′ of the Ptx1-binding site): $^{-101}$5′-GGGGA**TTG**TAGTGTCCAGGTTAC-3′$^{-79}$. The resulting PCR products subsequently were subcloned in the corresponding sites of the modified pGL2-Basic. The GRE reporter and GR expression plasmids were described previously (Drouin *et al*., 1993; Philips *et al*., 1997). Oligonucleotides were obtained from Perkin-Elmer. All mutations and deletions were confirmed by DNA sequencing.

Recombinant protein production

Escherichia coli strain BL21 was transformed with MBP fusion vectors (MBP–SF-1, MBP–Ptx1, MBP–SF-1ΔLBD and MBP–LacZα) derived from pMal-c (New England Biolabs). Colonies were grown in 1000 ml of 2× YT to an OD_{600} of 0.4–0.6. Induction of the expression of recombinant proteins and their purification were performed as recommended by the manufacturer. ^{35}S-labeled *in vitro* translated Ptx1 (wild-type and mutants), SF-1 and luciferase were obtained using the

TNT-coupled transcription–translation rabbit reticulocyte lysate system (Promega).

Protein–protein interaction assay

Protein–protein interaction assays were performed using 1 μg of MBP fusion proteins coupled to amylose–Sepharose beads (New England Biolabs) and 4–6 μl of *in vitro* translated ^{35}S-labeled protein as described in Durocher *et al*. (1997).

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