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Functional Relationship between LHX4 and *POU1F1* in Light of the *LHX4* Mutation Identified in Patients with Pituitary Defects

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Context: Pituitary development depends on the actions of a large number of transcription factors. Among them, LHX4 is believed to play a crucial role, as suggested by the dominantly inherited GH deficiency associated with the recently identified *LHX4* mutation, although the precise mechanism underlying this phenotype is still to be elucidated.

Objective: The objective of this study was to gain insight into both the function of LHX4 and the pathophysiology of the LHX4-related syndrome. We sought potential targets of this factor and assessed the abilities of various recombinant LHX4 isoforms expressed in Chinese hamster ovary cells to bind to and activate the *POU1F1* upstream regulatory sequence.

Results: We show that normal LHX4 binds to a human-specific element and subsequently activates transcription from the proximal upstream regulatory sequence of *POUIF1*, a gene encoding a POU homeodomain transcription factor known as the main regulator of *GH* expression. As shown in this cell system, the mutant LHX4 proteins predicted by the defect identified in patients fail to bind to and subsequently activate the *POU1F1* regulatory sequence, but do not impair the ability of normal LHX4 to activate this target.

Conclusions: Such findings are consistent with the existence, in humans, of an LHX4-driven pathway leading to the expression of *GH* through transcriptional activation of *POU1F1*. They argue against a dominant-negative effect of the mutant LHX4 proteins over normal LHX4. Finally, they provide a clear-cut evaluation of the functional consequences, at the molecular level, of the *LHX4* mutation, which, through disruption of the former pathway, would account for one key feature of the LHX4-related syndrome. **(*J Clin Endocrinol Metab* 90: 5456–5462, 2005)**

THE PITUITARY IS an endocrine gland that, in the human, is composed of two parts, the anterior and posterior lobes. The posterior pituitary, which derives from the ventral diencephalon, secretes the oxytocin and vasopressin hormones. The anterior pituitary gland, which arises from the oral ectoderm, is composed of five cell lineages: somatotrophs, lactotrophs, thyreotrophs, gonadotrophs, and corticotrophs, each of which is specialized in the secretion of one type of hormone, *i.e.* GH, prolactin (PRL), TSH, LH/FSH, and ACTH, respectively. Proper development of the pituitary gland depends on inductive signals emanating from the surrounding embryonic region and also on the actions of a large number of transcription factors that have a strictly regulated, both spatially and temporarily, expression pattern in the developing pituitary (1). The importance of these transcription factors in the pituitary organogenesis process has been originally unveiled through the study of appropriate models (natural mutants and/or genetically engineered mice) (2, 3). Molecular defects in several of these factors (*i.e. POU1F1*, *PROP1*, *HESX1*, *LHX3*, *LHX4*, *TPIT/TBX19*, and *SOX3*) were found to account for the pathogenesis of several pituitary disorders in humans (4–7).

In this regard, the human *LHX4* gene has recently been shown to be mutated in patients with a complex combined pituitary hormone deficiency syndrome (OMIM 606606) (8). More precisely, patients bearing a mutation in *LHX4* display short stature, which, when investigated, was shown to be due to GH deficiency. Interestingly the disease phenotype segregates in a dominant manner in that kindred, the affected members bearing the mutation in the heterozygous state. Genetically engineered mice, by contrast, are asymptomatic when bearing the targeted Lhx4 homeodomain disruption in the heterozygous state, whereas homozygous animals, which have an abnormal pituitary phenotype, die shortly after birth (9, 10).

To gain insight into the function of LHX4 and provide some explanation for the discrepancy about the mode of segregation as well as the severity of the pathological phenotype between genetically engineered mice and patients bearing the mutation in *LHX4*, we first sought potential targets for this transcription factor using a candidate gene approach based on the patients' phenotypic features. In this study we provide evidence supporting the existence of a molecular link between LHX4 and *POU1F1*, the gene encoding the main regulator of *GH* expression, while evaluating the functional consequences of the recently identified *LHX4* mutation.

Materials and Methods

Plasmid constructs

A DNA fragment spanning 850 bp of the *POU1F1* upstream sequence was generated by PCR amplification (standard reaction) using human

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Abbreviations: CHO, Chinese hamster ovary; DTT, dithiothreitol; GFP, green fluorescence protein; LIM, acronym standing for Lin-11, Isl-1, and Mec3; LMO, LIM only; mut, mutant; PRL, prolactin; wt, wild type.

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genomic DNA (Roche, Indianapolis, IN) as a template. The PCR product was cloned into the luciferase vector pGL3 by means of standard cloning techniques. This *POU1F1* upstream regulatory sequence construct was designated pGL3-POU1F1. The cDNAs coding for wild-type LHX4 (LHX4wt) and the mutant isoforms (LHX4mut1 and LHX4mut2) were cloned into the expression vector pTracer. The resulting expression vectors were designated pLHX4wt, pLHX4mut1, and pLHX4mut2, respectively. The pLMO-LHX4 construct (LMO for LIM only, where the "LIM" acronym derives from the defining members of the LIM-homeodomain family: Lin-11 from *Caenorhabditis elegans*, Isl-1 from rat, and Mec-3 from *C. elegaus*) was generated from the pLHX4wt construct by site-directed mutagenesis using the QuikChange site-directed mutagenesis system (Stratagene, Amsterdam, The Netherlands) to introduce a stop codon after the last amino acid of the second LIM domain. pLHX4-green fluorescence protein (GFP) and pLMO-LHX4-GFP GFP fusion constructs were generated using the expression vector pEGFP-N3 (Ozyme, Saint-Quentin-en-Yvelines, France). All primers used in amplification and mutagenesis steps are available upon request. In every case, resulting constructs (Fig. 1) were confirmed by sequencing the inserts and vector-flanking fragments.

Cell culture and transfection

Chinese hamster ovary (CHO) cells were obtained from American Type Culture Collection (Manassas, VA) and grown in Iscove's medium (Invitrogen Life Technologies, Inc., Cergy-Pontoise, France) containing 10% fetal calf serum (Invitrogen Life Technologies, Inc.) at 37 C. All transfections were performed at 60% confluence by the Lipofectamine Plus method (Invitrogen Life Technologies, Inc.) in OptiMEM medium according to the manufacturer's standard protocol.

Luciferase activity assays

For luciferase activity assays, 1.5×10^5 CHO cells were cultured for 24 h in 12-well culture plates and transfected with 25 ng of the *POU1F1* upstream regulatory sequence construct or the empty pGL3 vector together with either empty pTracer expression vector and/or the various LHX4-derived pTracer constructs (total amount of transfected expression vector/construct, 75 ng) according to the manufacturer's instructions (Lipofectamine, Invitrogen Life Technologies, Inc.). The cell extracts were prepared and assayed for luciferase activity using the Promega assay system (Madison, WI). Luciferase activity was normalized to protein concentration, which was measured using the Coomassie Plus Protein Assay Reagent Kit (Pierce Chemical Co., Rockford, IL) rather than to a second reporter plasmid, because, as shown by others (11), the use of such internal standards in transient experiments may lead to a systematic error. Each transfection experiment (in triplicate) was performed independently at least four times.

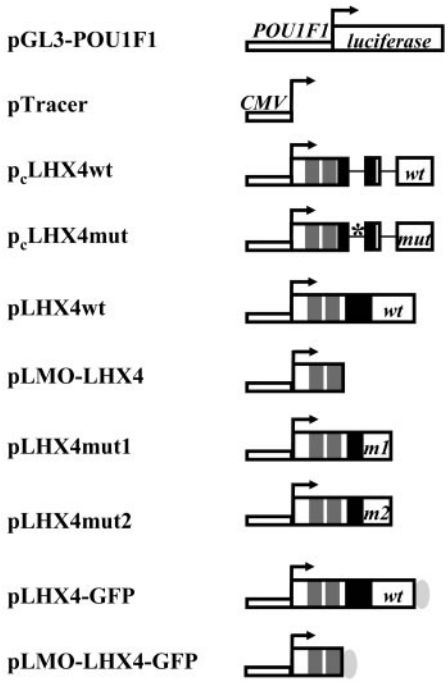

FIG. 1. Constructs used in this study. The small empty box in the *left* part of every illustration shows the promoter present in the expression vector used, which, as indicated for the empty vector (*i.e.* pTracer), is cytomegalovirus (CMV). *Thin lines* in the p$_c$LHX4wt and p$_c$LHX4mut constructs represent introns. The *asterisk* in the p$_c$LHX4mut construct indicates the presence of the splice site mutation. *Gray boxes* indicate the two LIM domains, whereas *black boxes* denote the homeodomain. *Round spots* stand for the GFP-coding sequence.

EMSA

Nuclear extracts were prepared as follows: 48 h after transfection, cells were resuspended and incubated for 15 min at 4 C in 400 μl buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA (pH 8), 0.1 mM EGTA, 1 mM dithiothreitol (DTT), and protease inhibitors]. Twenty-five microliters of buffer B (Nonidet P-40 10%) was added to CHO extracts; all extracts were centrifuged at 12,000 rpm for 30 sec. The pellets, which contain nuclear fractions, were then resuspended in 40 μl buffer C [20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA (pH 8), 1 mM DTT, and protease inhibitors], vortexed for 15 min at 4 C, and centrifuged at 12,000 rpm for 5 min; the resulting supernatants correspond to nuclear extracts. DNA binding reactions were performed at room temperature in a 20-μl reaction mixture containing 20 μg nuclear extracts, 5 μl 4× binding buffer (5% glycerol, 10 mM HEPES, 50 mM KCl, 0.1 μg heat-treated salmon sperm DNA, 50 μg BSA, and 1 mM DTT), 1 μg poly(dI-dC) (homopolymer of deoxyinosine and deoxycytidine residues) (Roche Applied Science), 1 ng probe end labeled with [γ-^{32}P]ATP (3000 μCi/mmol; Amersham Biosciences, Arlington Heights, IL), and T4 polynucleotide kinase (Invitrogen Life Technologies, Inc.). The sequences of the double-stranded oligonucleotides used as probes in the EMSA experiments are: wt, 5′-GTATGAATCATTAATTGACAACATATTTTC-3′; and mutant, 5′-GTATGAATCATCCCCTGACAACATATTTTC-3′. In the supershift (or immunodepletion) assay, 1 μl anti-LHX4 antibody (Chemicon International, Temecula, CA) was added to the mixture 20 min before migration.

Fluorescence analysis and microscopy

Cells transfected with either pLHX4-GFP or pLMO-LHX4-GFP were plated onto glass coverslips and fixed in 4% (vol/vol) formaldehyde/PBS 24 h after transfection. They were subsequently permeabilized in 0.1% Triton/PBS, and DNA was stained with 4′,6′-diamidino-2-phenylindole. Cells were then examined with a Leica DMR fluorescence microscope (Deerfield, IL).

Results

The human POU1F1 gene is a candidate direct target of LHX4

To begin to understand the molecular action of LHX4 in the developing pituitary, we sought transcriptional targets of this factor following a candidate gene approach based on 1) the GH defect of human patients bearing a mutation in *LHX4* and 2) upstream regulatory sequence data of genes with a pituitary-restricted pattern of expression. This approach indicated *POU1F1* as a potential direct target of LHX4; indeed, POU1F1 is known to regulate the expression of the *GH* gene. *In silico* analysis using VISTA tools (12) revealed the existence of a putative LIM homeodomain binding site within the proximal human *POU1F1* upstream regulatory sequence (Fig. 2A). Most importantly, this site (CATTAATT), which is highly homologous to the consensus AATTAATT sequence recognized by LIM homeodomain proteins (12), is located within a fragment that is not conserved between humans and

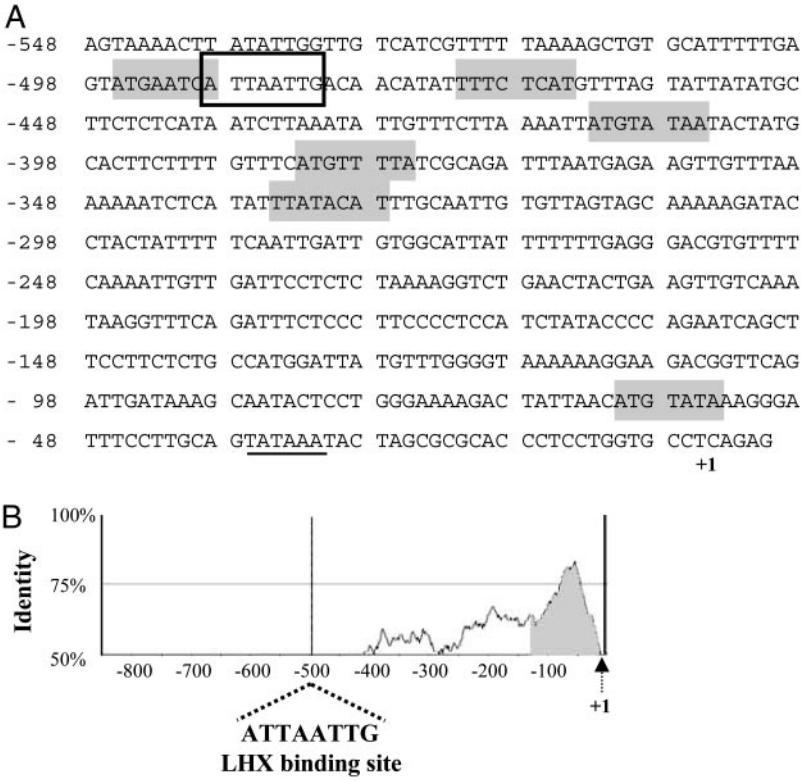

FIG. 2. The *POU1F1* upstream sequence contains a putative human-specific LHX4 binding element. A, Nucleotide sequence of the human *POU1F1* upstream region. The transcription start site (+1), the TATA box (*underlined*), and positions of binding sites for POU1F1 (*gray-shaded boxes*) are shown (22). The herein identified LHX4 binding site is depicted (*black frame*). B, The putative LHX4 binding site lies within a region that is not conserved between humans and mice. The *gray*-colored plot illustrates conserved sequence blocs in the upstream sequence of mouse and human *Pou1f1*/*POU1F1*.

mice (Fig. 2B). Interestingly, this human-specific *POU1F1* upstream regulatory sequence fragment contains putative binding sites for several other transcription factors, including POU1F1 (Fig. 2A).

LHX4 binds to and activates the POU1F1 upstream regulatory sequence

To test whether LHX4 can recognize and bind the *in silico*-identified LHX4 binding site within the *POU1F1* upstream regulatory sequence, we performed EMSA using nuclear extracts from CHO cells transfected with the pLHX4wt expression vector and a ^{32}P-labeled probe containing the LHX4 binding site. As shown in Fig. 3A (*left panel*), LHX4 can directly bind to the double-stranded probe *in vitro*, and binding to this probe is competed by an excess of unlabeled oligonucleotide. No such band pattern is obtained when an oligonucleotide probe mutated in the TAAT core sequence is used. We subsequently performed a similar EMSA experiment in the absence or presence of an antibody directed against LHX4. As shown in Fig. 3A (*right panel*), the intensity of the shifted band was dramatically reduced by addition of the anti-LHX4 antibody. This result, which indicates that binding of the antibody to LHX4 prevents its association with the labeled probe, confirms the presence of LHX4 in these protein:DNA complexes and the fact that LHX4 directly binds to the identified putative DNA target.

To assess the functional significance of this interaction, we then carried out luciferase activity assays. Figure 3B shows that overexpression of LHX4 in CHO cells causes activation of a reporter construct that contains an 850-bp *POU1F1* upstream sequence fragment encompassing the identified LHX4 binding site.

The LHX4 mutation abolishes the LHX4 binding and transactivating capacity

We have previously identified a molecular defect implicating *LHX4* in the pathogenesis of a complex GH deficiency syndrome (8). The identified acceptor splice site mutation (G to C transversion) affecting the AG invariant dinucleotide in intron IV was shown to result in the use of two cryptic acceptor splice sites located within the exon 5 sequence, with two types of transcripts thereby generated *in vitro*: one deleted of 12 nucleotides predicting a protein deleted of four highly conserved residues within the homeodomain sequence that have been shown to be essential for DNA binding specificity (LHX4mut1), and the other deleted of 17 nucleotides and predicting a truncated protein in which part of the homeodomain and the entire exon 6-encoded sequence is missing (LHX4mut2) (8).

To test the functional consequences of the *LHX4* defect, CHO cells were transfected with an expression vector containing the *LHX4* nucleotide sequence from exons 1–4 fused to the genomic fragment spanning intron IV (either normal or presenting the G to C transversion), exon 5, intron V, and exon 6 (*i.e.* p_cLHX4wt or p_cLHX4mut, respectively) (8). We first carried out luciferase activity assays to evaluate the transactivating capacity of mutant LHX4 proteins. As shown in Fig. 4A, LHX4 proteins encoded by the construct bearing the identified mutation failed to activate the *POU1F1* upstream sequence reporter plasmid. To test whether the loss of transactivating capacity was due to the loss of DNA binding capacity of the mutant LHX4 proteins, we then performed EMSA using nuclear extracts from cells transfected with either type (normal or mutant) of *LHX4* construct. As shown in Fig. 4B, normal LHX4 can directly bind to the

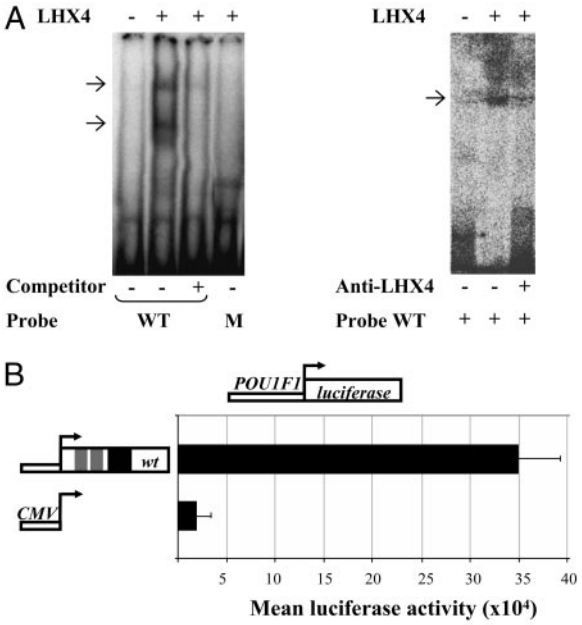

FIG. 3. The human *POU1F1* upstream regulatory sequence contains a LHX4 responsive element. A, Binding of LHX4 to the *POU1F1* upstream regulatory sequence assessed by EMSA. Nuclear extracts were obtained from CHO cells transfected with the empty expression vector or the pLHX4wt vector and were incubated with a radiolabeled probe containing the normal LHX4 binding site (WT) or a radiolabeled probe in which the LHX4 binding site was mutated (M). Competition experiments were performed using 100-fold excess of cold wt probe (*left panel*). To test whether LHX4 directly binds to the radiolabeled WT probe, a similar experiment was subsequently performed in the absence or presence of an antibody directed against LHX4. The intensity of the band corresponding to the complexes obtained with *LHX4*-transfected cells (medium lane) was dramatically reduced by the addition of the anti-LHX4 antibody, confirming the presence of LHX4 in these protein:DNA complexes (*right panel*). *Arrows* indicate the positions of specific bands. B, Functional significance of the LHX4-*POU1F1* interaction. CHO cells were transfected with the pPOU1F1 construct alone or together with either the empty expression vector (pTracer) or pLHX4wt, and the effect of LHX4 on the activation of the reporter gene was assessed. In addition, CHO cells were transfected with the empty reporter vector (pGL3) alone or together with pLHX4wt, which permitted us to confirm the specificity of the interaction with the *POU1F1* fragment (data not shown). One representative experiment (performed in triplicate) of four independent experiments is shown.

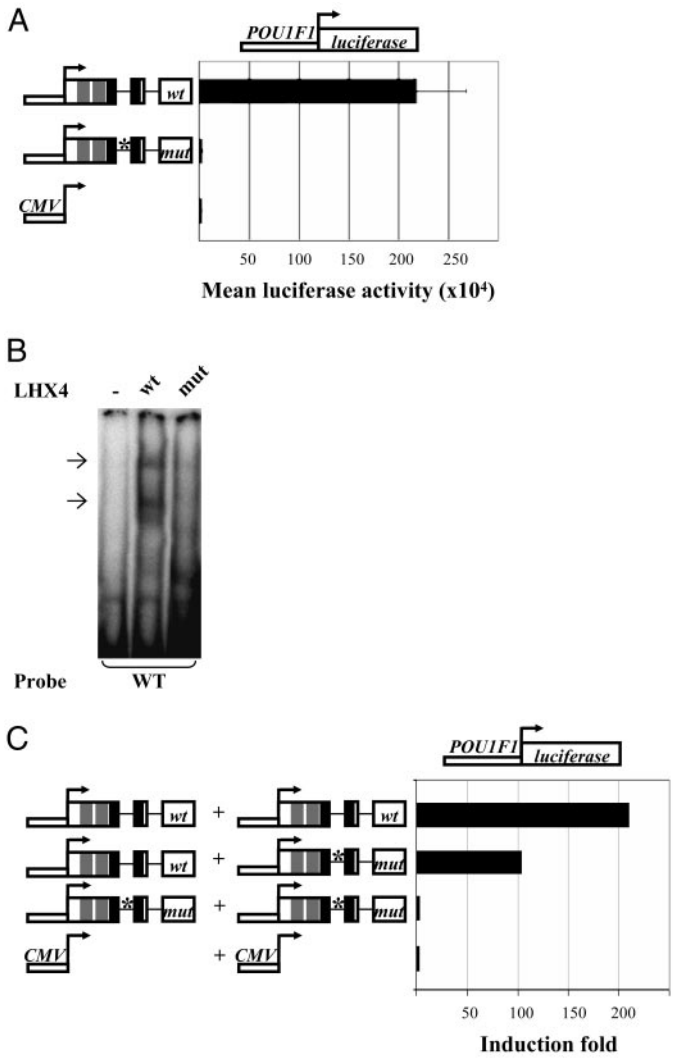

FIG. 4. Functional consequences of the *LHX4* molecular defect. A, Loss of transactivating capacity of mutant LHX4 proteins. CHO cells were transfected with pPOU1F1 together with the empty expression vector (pTracer), the normal LHX4-encoding construct (p_cLHX4wt), or the construct predicted to encode mut1 and mut2 (p_cLHX4mut), and luciferase activity assays were carried out as previously. One representative experiment (performed in triplicate) of four independent experiments is shown. B, The *LHX4* mutation abolishes the binding capacity of LHX4. EMSA with nuclear extracts obtained from CHO cells transfected with the empty expression vector (pTracer), the normal LHX4-encoding construct (p_cLHX4wt), or the construct predicted to encode mut1 and mut2 (p_cLHX4mut) (8) (lanes 1, 2, and 3, respectively) and were incubated with a radiolabeled probe containing the normal LHX4 binding site. *Arrows* indicate positions of specific bands. C, No dominant-negative effect of the mutant proteins over the normal LHX4 protein in this system. Cotransfection of p_cLHX4mut, encoding both mutant isoforms, and p_cLHX4wt does not significantly impair the transactivating capacity of normal LHX4. One representative experiment (performed in triplicate) of four independent experiments is shown.

double-stranded probe corresponding to the LHX4 recognition site within the *POU1F1* upstream regulatory sequence, whereas mutant LHX4 proteins display a drastic decrease in their DNA binding capacity.

Presence of mutant LHX4 proteins does not impair the capacity of normal LHX4 to activate the POU1F1 regulatory sequence

Although the probands were born to a consanguineous union, the disease phenotype was transmitted as a dominant trait (8). To investigate the molecular mechanism underlying this segregation pattern, we assayed transcription from the *POU1F1* upstream regulatory sequence in the presence of both wild-type and mutant LHX4 proteins. As shown in Fig. 4C, cotransfection of equal quantities of p_cLHX4wt and p_cLHX4mut did not inhibit transactivation of the *POU1F1* upstream regulatory sequence, suggesting the absence of dominant-negative effect of the mutant proteins over the normal one. Overall, these findings suggest that haploinsufficiency of normal LHX4 would account for the GH defect of the patient.

Transactivating capacity of mutant LHX4 and LHX4-LMO proteins

Because p_cLHX4mut is predicted to encode two LHX4 isoforms, we tested the capacity of each isoform to activate this regulatory sequence (Fig. 5A). As expected, both LHX4mut1, encoded by pLHX4mut1 (containing the normal LHX4 cDNA sequence deleted of 12 nucleotides), and LHX4mut2, encoded by pLHX4mut1 (containing the normal LHX4 cDNA sequence deleted of 17 nucleotides), fail to activate the *POU1F1* upstream regulatory sequence. In addition, cotransfection of either of these constructs with pLHX4wt (containing the normal LHX4 cDNA sequence) has the same effect on transcription from the *POU1F1* upstream regulatory sequence as cotransfection of the empty pTracer vector, with pLHX4wt. We then compared the transactivating capacities of these defective isoforms, both of which are predicted to have an identical NH_2 part containing the LIM1 and LIM2 domains, with that of a peptide composed exclusively of the NH_2 part of LHX4 (LMO-LHX4). As was the case for LHX4mut1 and LHX4mut2 proteins, in CHO cells, overexpression of LMO-LHX4 failed to activate expression from the *POU1F1* upstream regulatory sequence and did not interfere with the activity of normal LHX4 (Fig. 5A).

Subcellular localization of LHX4-LMO

To provide an explanation for the absence of interaction between normal LHX4 proteins and LMO-LHX4-like molecules in this cell system, we constructed GFP fusion proteins and tested their subcellular localization. As expected, the full-length LHX4 protein localizes to the nucleus and, interestingly, so does LMO-LHX4 (Fig. 5B).

Discussion

LHX4 is a LIM homeodomain protein the precise role of which, at the molecular level, is still to be determined. In mice, Lhx4 seems to be necessary for survival, because mice invalidated for Lhx4 die shortly after birth (9); in the pituitary, it has been shown to participate in the expansion of the different cell lineages (10, 13), and interestingly, Lhx4-invalidated mice have reduced staining for *Pou1f1* (10); this could, however, reflect a decreased number of *Pou1f1*-expressing cells rather than reduced transcriptional activity. The human ortholog, LHX4, has been implicated in the pathogenesis of a syndromic short stature (OMIM 606606), and when investigated, short stature was shown to be due to GH deficiency (8). Activation of *Gh/GH* gene expression requires the action of Pou1f1/POU1F1 in somatotrophs (14) (OMIM 173110). Although the *cis* and *trans* elements conferring the regulated, both pre- and postnatally, *Pou1f1* expression in the mouse pituitary have been widely studied (15), little is known about the regulatory mechanisms involved in the expression of the human gene. We hypothesized that LHX4 would positively control *POU1F1* during development, and that impairment of this interaction would underlie the LHX4-associated GH defect.

We identified a palindromic TTAATT putative LHX4 binding site at position −478 bp (the transcription start site is at position +1) of the human *POU1F1* upstream sequence.

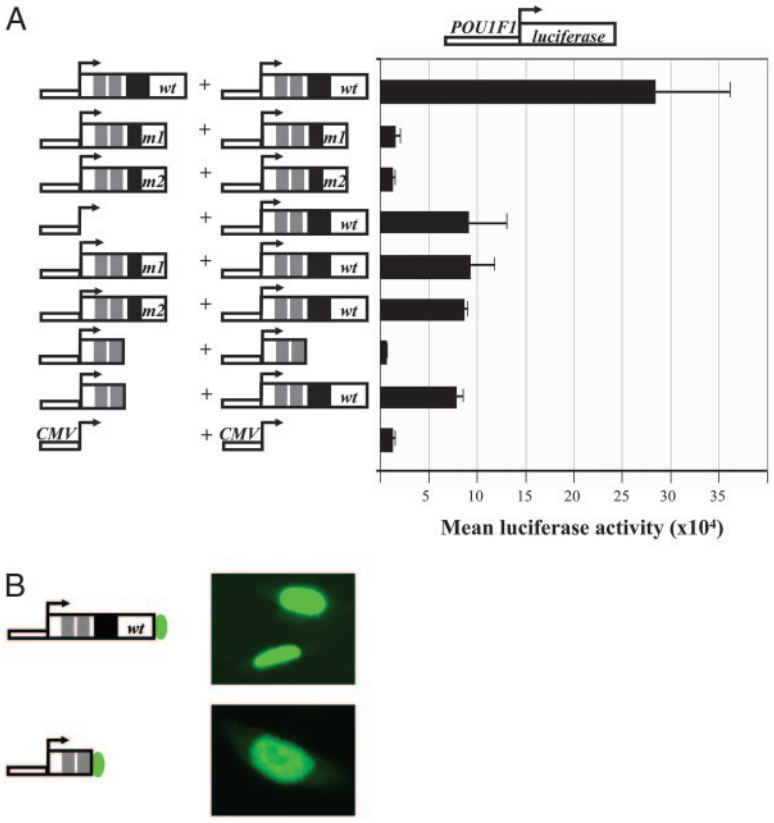

FIG. 5. Transactivating capacity and subcellular localization of LHX4-derived proteins. A, CHO cells were transfected with pPOU1F1 together with either the empty expression vector or constructs encoding different isoforms/derivatives of LHX4. One representative experiment of four independent experiments (performed in triplicate) is shown. B, Subcellular localization of LHX4 derivatives. CHO cells expressing LHX4-GFP or LMO-LHX4-GFP were visualized directly with GFP fluorescence.

Interestingly, this site lies within a region that is not conserved between human and mouse. Recent studies, however, indicate that the conserved fraction of the genome may be substantially smaller than the functional fraction (16). We, therefore, performed assays that showed that this sequence can bind LHX4 to consequently drive expression from the *POU1F1* upstream regulatory sequence. Noteworthy, this LHX4 binding site is located within a DNA sequence fragment containing several putative POU1F1 binding sites; in keeping with an *in vivo* activity of this human-specific *POU1F1* genomic region, such density of POU1F1 recognition sites is also encountered within the distal enhancer of the mouse *Pou1f1* gene (17) and the human *PRL* gene (18), both of which have been shown to be active *in vivo*.

To evaluate whether impairment of this LHX4-*POU1F1* interaction could indeed explain the deficit in GH associated with the *LHX4* splice site mutation (8), we tested the binding and transactivating properties of the mutant proteins. We indeed showed that the G to C transversion at the invariant dinucleotide of the acceptor splice site abolishes the binding capacity of LHX4, which, consequently, cannot activate the *POU1F1* upstream regulatory sequence. Interestingly, the patients bear the mutation in the heterozygous state, raising the question of the mechanism underlying this dominant transmission pattern, especially because LHX4 has two LIM domains (LIM domains, although not shown for LHX4, are known to mediate protein:protein interactions), both of which are maintained in the mutant isoforms. We provided *in vitro* evidence that dominance would be due to haploinsufficiency, rather than a dominant-negative effect of the mutant proteins over normal LHX4.

We also compared these mutant proteins to an artificially designed, LHX4-derived protein retaining only the LIM domains and farther NH_2 sequence with regard to the capacity to activate expression from the *POU1F1* upstream regulatory sequence fragment and to interfere with the function of normal LHX4. As expected, the effect of the predicted mutant LHX4 proteins in both regards was comparable to that of LMO-LHX4. Actually, LMO proteins are known to interact with several transcription factors and in particular with LIM homeodomain proteins via interaction with LIM-associated cofactors (19). In this regard, one possibility is that the absence of dominant-negative effect of LMO-LHX4 over wild-type LHX4, and subsequently of mutant LHX4 over normal LHX4, is due to the absence of appropriate adaptor molecules in our system, and one cannot exclude that such effect is observed in another cell system or under different experimental conditions. The nuclear localization of LMO-LHX4 proteins, besides providing evidence that the LIM domains can target LHX4 in the nucleus, allows us to exclude different compartmentalization as a possible cause for the absence of dominant-negative effect of the LMO-like LHX4 proteins over normal LHX4. Nonetheless, given the nature of the targeted modification, mice bearing the disrupted *Lhx4* allele are expected to express LMO-LHX4-like peptides, like human patients, and the observation that heterozygous animals are normal argues against a dominant-negative effect of the mutant proteins in humans. Although this discrepancy of a recessive *vs.* a dominant transmission pattern between humans and mice would be commonly encountered and sometimes reflects nothing more than incomplete study (20), in that case it could indeed be explained by the existence of a species-specific regulation of the *POU1F1* gene expression such as that proposed in this study.

It is important to keep in mind that the pituitary phenotype of human patients bearing a mutation in *LHX4* is characterized by a deficit in GH, TSH, and ACTH (8). In this regard, it is worth noting that *POU1F1* molecular defects have to date always been associated with a deficit in GH, TSH, and PRL, but not in ACTH (4). This remark raises two interesting issues. First, the ACTH deficiency of LHX4 patients cannot be explained by a defect in POU1F1 expression; by other means, it is well established that most transcription factors have numerous downstream target genes, which could, at least partially, underlie their pleiotropic effects. Therefore, the ACTH deficiency in patients with the *LHX4* mutation could be due to dysfunction of another, as yet unknown, LHX4-dependent pathway, one that does not involve POU1F1. The second issue is in regard to the PRL deficit clearly shown in patients bearing a mutation in *POU1F1*, but to date not documented in patients bearing the *LHX4* mutation. This observation suggests that in humans, LHX4 would play a key role in the regulation of *POU1F1* expression in somatotrophs and thyreotrophs, but not in lactotrophs. Such a regulation scheme could be explained by two hypotheses. 1) LHX4 would be present in somatotroph but absent from lactotroph precursors, when POU1F1 is required for terminal differentiation of lactotrophs. In this respect, in human pituitary adenomas, another pituitary factor, *PITX2,* has been shown to be differentially expressed in these closely related lineages, *i.e.* lactotrophs and somatotrophs (21). 2) Despite the presence of LHX4 in lactotroph precursors, the expression of *POU1F1* in this cell type would mainly depend on the presence of other factors potentially competing with LHX4 for binding to this site.

Overall, we identified a human-specific regulatory element in the *POU1F1* upstream regulatory sequence, conferring responsiveness to the LIM homeodomain transcription factor LHX4, and we showed that the molecular defect implicating LHX4 in the pathogenesis of syndromic short stature impairs the capacity of this transcription factor to bind and regulate *POU1F1*, the latter, of note, being the main activator of *GH* expression.

Acknowledgments

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