An Isoform-specific Inhibitory Domain Regulates the LHX3 LIM Homeodomain Factor Holoprotein and the Production of a Functional Alternate Translation Form* 

Kyle W. Sloop, Conor J. Dwyer, and Simon J. Rhodes‡

From the Department of Biology, Indiana University-Purdue University Indianapolis, Indianapolis, Indiana 46202

Received for publication, May 1, 2001, and in revised form, July 5, 2001
Published, JBC Papers in Press, July 24, 2001, DOI 10.1074/jbc.M103888200

The LHX3 LIM homeodomain transcription factor is required for pituitary development and motor neuron specification. The Lhx3 gene encodes two isoforms, LHX3a and LHX3b, that differ in their amino-terminal sequences. Humans and mice with defective Lhx3 genes are deficient in gonadotrope, lactotrope, somatotrope, and thyrotopite pituitary cells. We show that, whereas Lhx3b is highly expressed in these Lhx3-dependent cell types, high levels of Lhx3a expression are restricted to a glycoprotein subunit-expressing thyrotropes and gonadotropes. Cross-species comparison reveals the LHX3b-specific domain is more conserved than the LHX3a-specific domain. We demonstrate that the LHX3b-specific domain is a transferable inhibitor that reduces gene activation and DNA binding by homeodomain proteins. In addition, we identify a novel LHX3 protein (M2-LHX3) and determine that this molecule is generated by an internal translation initiation codon. The LHX3a- and LHX3b-specific coding sequences regulate differential usage of this internal start codon. Further, we identify the major activation domain of LHX3 in the carboxyl terminus of the molecule. M2-LHX3 is active because it retains this domain and binds DNA better than LHX3a or LHX3b. Other LIM homeodomain genes, including Lhx4, generate similar truncated proteins. These studies describe how transcriptional regulatory genes can generate multiple functional proteins.

DNA sequencing projects have revealed that the human genome consists of approximately one third of the previously estimated total number of genes (1). Because of the limited number of human genes, it is likely that multiple strategies are used to generate proteins with sufficient regulatory capacity to coordinate cellular processes such as developmental and metabolic programs. The production of multiple proteins from a single gene vastly increases the functional complexity of the genome. Combinations of alternative RNA splicing, multiple promoter usage, RNA editing, alternate sites of translation initiation, and post-translational modifications enable expression of the genome to diverse “proteomes” necessary for the generation of unique cell types and tissues.

* This work was supported by grants (to S. J. R.) from the National Science Foundation and the National Research Initiative Competitive Grants Program/United States Department of Agriculture. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Biology, Indiana University-Purdue University Indianapolis, 725 W. Michigan St., Indianapolis, IN 46202-5132. Tel.: 317-278-1797; Fax: 317-274-2846; E-mail: srhodes@iupui.edu.

1 The abbreviations used are: HD, homeodomain; oGSU, α-glycoprotein subunit; EMSA, electrophoretic mobility shift assay; GH, growth hormone; GST, glutathione-S-transferase; PRL, prolactin; TSH, thyroid-stimulating hormone; RT, reverse transcription; PCR, polymerase chain reaction; DB, DNA binding domain; PGE, polyacrylamide gel electrophoresis; PGBE, pituitary glycoprotein hormone basal element; TEF, Thyrotopite embryonic factor.

LIM homeodomain (HD)1 transcription factors comprise a subfamily of HD proteins that contain two amino-terminal zinc finger-like LIM motifs, which mediate interactions with other transcription factors and co-regulatory proteins, and a characteristic DNA-binding HD. Gene targeting experiments in mice have demonstrated that LIM HD class transcription factors regulate many aspects of mammalian organogenesis and are critical to cell lineage specification processes (2). For example, the LHX3 and LHX4 LIM HD proteins are essential for proper development of the anterior lobe of the pituitary gland. These factors are necessary for the differentiation and proliferation of the specific anterior pituitary cell types (3). The Lhx3 gene alone may control several aspects of these processes because it codes for two alternate isoforms, LHX3a and LHX3b (4–7).

The anterior pituitary gland is composed of specialized cell types that control endocrine homeostasis by secreting hormones in a regulated fashion. The cell types of the mature gland are somatotropes that secrete GH, thyrotopes that release TSH, lactotropes that produce PRL, gonadotropes that synthesize luteinizing hormone and follicle-stimulating hormone, and corticotropes that secrete adrenocorticotropic. To form the pituitary gland and differentiate these cell types, neural ectoderm of the ventral diencephalon must first associate with a fold of ectoderm from the developing oral cavity. These early events, which establish the primordial pituitary structure known as Rathke’s pouch, require the actions of both the LHX3 and LHX4 transcription factors. The developing pituitaries of mice lacking both the Lhx3 and Lhx4 genes fail to progress beyond the rudimentary pouch stage (3). Upon formation of this rudimentary pituitary, Lhx3 is required for formation of a definitive Rathke’s pouch, pituitary cell commitment, and the emergence of the hormone-secreting cell lineages. In Lhx3 knockout mice, pituitary development is arrested after the initial formation of Rathke’s pouch, and the differentiated hormone-secreting cells, with the exception of a few corticotropes, are absent (8). Interestingly, the phenotype of Lhx3 null mice is consistent with the clinical description of human patients with mutations in this gene; these patients display combined pituitary hormone deficiency but possess normal corticotropes hormone function (9). Together, these studies indicate that Lhx3 is critical for both early structural events and for the specification and proliferation of the gonadotrope, lactotrope, somatotrope, and thyrotopite pituitary cell lineages.

The Lhx3 gene produces two isoforms, LHX3a and LHX3b,
that contain common LIM domains, HD, and carboxyl-terminal sequences but possess alternate amino-terminal domains (4–7). We have shown previously that the LHX3a isoform activates specific pituitary target genes, alone or in combination with the pituitary POU HD domain factor PTT-1, more effectively than the LHX3b isoform (5). The differences in the gene activation properties of these factors correlate with their abilities to bind DNA regulatory elements; the b-specific domain inhibits DNA binding of LHX3 to specific DNA sites (5).

Here, we investigate the regulatory properties of the LHX3a- and LHX3b-specific domains. The LHX3b-specific domain sequence is highly conserved in mammals. The Lhx3a and Lhx3b isoforms are differentially expressed in specialized pituitary cell types, suggesting cell-specific functions. The LHX3b-specific domain is a transferable inhibitory domain that can repress the function of HD transcription factors. In addition, we describe a novel, functional LHX3 molecule, M2-LHX3, that is specifically expressed from the Lhx3a transcript in pituitary cell lines, and examine the alternate protein translation mechanism utilized to generate this isoform. M2-LHX3 binds DNA regulatory elements better than LHX3a and LHX3b, and it utilizes a previously uncharacterized activation domain within its carboxyl terminus to induce transcription. The Lhx4 gene also produces an equivalent alternate protein, and we propose that other members of the LIM HD class of developmental regulatory genes use similar strategies to increase their coding capacities.

**EXPERIMENTAL PROCEDURES**

**Lhx3 Isoform Cloning from Mammalian Species**—Total RNA was extracted from rat, pig, and guinea pig pituitaries using Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) as described previously (5). Rhesus monkey pituitary RNA was a kind gift from Dr. T. Golos (Wisconsin Regional Primate Research Center). Complementary DNA was synthesized from RNAs using Superscript II reverse transcriptase (Life Technologies, Inc.) and 5′-ta(g/a)aggatca(g/a)cggtcca-3′ as a primer. PCR then was performed using the following primers: 5′-t(c/g)gatcttacaattaattaattttctgg-3′ (Lhx3a), 5′-c(g/a)cgatc(c/g)taacctgatcgtac-3′ (Lhx3b, Lhx3a), 5′-c(g/a)cgatcttaacctgtcgtatc-3′ (Lhx3b), and 5′-c(g/a)cgatc(c/g)taacctgatcgtac-3′ (Lhx3b, Lhx3a). PCR products were ligated into pCRII-TOPO (Invitrogen, Carlsbad, CA) and sequenced on both strands by automated DNA sequencing (Biochemistry Biotechnology Facility, Indiana University, Indianapolis, IN). Gene sequences were assembled and aligned using the Wisconsin Genetics GCG computer package.

**Lhx3 Isoform Expression in Pituitary Cell Lines**—Analysis of Lhx3a and Lhx3b expression in rodent pituitary cell lines was as described (5). cDNA was synthesized from mRNA-1 (kind gift of Dr. S. Camper, University of Michigan, Ann Arbor, MI), 235-1, and GC RNA, and PCR reaction products were analyzed on 11% acrylamide Tris borate gels. Recombinant proteins were synthesized in vitro from pcDNA3.1/Myc-Hisακ expression vectors by translation in rabbit reticulocyte lysates (Promega, Madison, WI), [35S]methionine (Amersham Pharmacia Biotech). Proteins were analyzed using SDS-PAGE followed by treatment with Amplify fluorography reagent (Amersham Pharmacia Biotech) and exposure to Biomax MR film (Eastman Kodak Co.).

**In Vitro Transcription/Translation**—Radiolabeled human LHX3a, LHX3b, AN-LHX3h, a-LHX3b, b-LHX3a, PROP-1, α-PROP-1, and b-PROP-1 proteins were previously described (5). GST-a-LHX3b, GST-b-LHX3a, GST-AN-LHX3, and GST-M2-LHX3 vectors were constructed by cloning cDNAs into pGEX-KT. Recombinant proteins were expressed in *Escherichia coli* BL21 (DE3) pLysS and affinity-purified as described previously (15). Proteins were analyzed on 12% SDS-PAGE gels followed by staining with Coomassie Brilliant Blue.

**Electrophoretic Mobility Shift Analysis (EMSA)**—EMSA were performed as described (13) using purified recombinant proteins, in vitro translated proteins (5), nuclear extracts, or whole cell extracts (14). Total protein was determined by the Bradford method. Oligonucleotides representing the pituitary glycoprotein hormone basal element (PGBE) base pair -550 to -323 region of the murine αGSU promoter (5′-caactggaatctcataaatagtg-3′ and 5′-caactaataatgtaattctgg-3′), the LHX3 consensus binding site (5′-gcattgaatatttaatgaaatg-3′ and 5′-gatctaataatgtaattctgg-3′) or the PRDQ site, 5′-tccgactaatgtaattctgg-3′ and 5′-tctggaatctcataaatagtg-3′ were used as radiolabeled probes. Anti-LIM-3/LHX3 (Chemicon, Temecula, CA) or anti-Myc antibodies were added to confirm the specificity of interactions.

**RESULTS**

**The Amino Acid Sequence of the LHX3b-specific Domain Is Highly Conserved in Mammals**—To examine conservation of the alternate LHX3 amino-terminal domains, we used degenerate RT-PCR to clone cDNAs coding for these domains from several mammals. By contrast to the Lhx3a/LIM-3 genes of non-mammalian species, which have not been reported to encode multiple protein isoforms, LHX3a- and LHX3b-specific domains were identified in the rhesus monkey, pig, rat, and guinea pig (LHX3b only) (Fig. 1A). The LHX3b-specific domains from the species examined are more similar in length, composition, and sequence compared with the LHX3a-specific domains. Compared with the human LHX3 isoforms, the LHX3b-specific domains are ~90% identical across species, whereas the LHX3a-specific domains are ~52% identical.

**Lhx3a and Lhx3b Are Differentially Expressed in Rodent Cell Lines Representing Pituitary Cell Lineages**—We previously have used RT-PCR to show that Lhx3a is highly expressed in the αTSH cell line (5) and now demonstrate that high levels of this isoform also were present in cT3-1 cells (Fig. 1, B and C). The αTSH and cT3-1 cell lines are used to study pituitary thyrotrope and gonadotrope function, respectively (15). Both cell lines express the αGSU gene that codes for the common α
Regulation of LHX3 Function by Alternate Amino Termini

Amino acid conservation and pituitary cell expression of the Lhx3a and Lhx3b isoforms. A, alignment of alternate LHX3 amino termini from several mammalian species. Panel shows comparison of human a/b (GenBank accession nos. AF156888/AF156889), rhesus monkey a/b (AF370448/AF370449), porcine a/b (AF370444/AF370445), guinea pig a/b (AF370450), rat a/b (AF370446/AF370447), and murine a/b (L38248/L38249). B, expression of Lhx3 isoforms in rodent pituitary cell lines. RT-PCR was used to amplify specific regions of Lhx3a (139 base pairs (a), closed arrow) and Lhx3b (165 base pairs (b), open arrow) using cDNA from the indicated cell lines. Reaction products were separated by acrylamide gel electrophoresis. Negative control reactions (−) were performed in parallel in the absence of reverse transcriptase. M, markers. C, summary of Lhx3a and Lhx3b expression in cell lines representing pituitary cell lineages: GFT1 (pituitary precursor cell), AT20 (adrenocorticotropic hormone-producing corticotrope), αTSH (αGSU-producing thyrotrope), GC (GH-producing mammosomatotrope), and 235 (PRL-producing lactotrope).

The LHX3b-specific Domain Inhibits DNA Binding of HD Transcription Factors—To determine the mechanism by which the LHX3b-specific domain negatively regulates transcriptional activation, we tested the ability of the LHX3a/b-chimeric proteins to bind specific DNA elements using EMSAs. First, in vitro translated LHX3a, LHX3b, a-LHX3a, b-LHX3a, and AN-LHX3 proteins were examined (Fig. 4, A and B). In these experiments, LHX3a and AN-LHX3 bound more strongly to the PGBE compared with LHX3b, a-LHX3b, and b-LHX3a (Fig. 4B). These results demonstrated that transfer of the LHX3b-specific domain to LHX3a inhibited its ability to bind DNA. As observed in the LHX3 Western blots shown in Fig. 2C, a faster migrating protein was present in LHX3a and AN-LHX3 protein translations (Fig. 4A), while this protein was only detected at trace levels in LHX3b, a-LHX3a, and a-LHX3b translations following extended film exposures (data not shown). Interestingly, EMSA analysis revealed the presence of an intense, faster migrating protein-DNA complex in the LHX3a and LHX3b reactions and a faint complex at this migratory position in LHX3a, a-LHX3b, and b-LHX3a reactions (Fig. 4B). To further characterize the regulatory properties of the a- and b-specific domains, we expressed recombinant LHX3 chimeric proteins in E. coli as fusions to the carboxyl terminus of GST. EMSA reactions using equivalent amounts of these purified proteins gave similar results to the data obtained with the in vitro translated proteins; transfer of the b-specific domain to LHX3a (GST-b-LHX3a) reduced binding of this molecule to DNA (data not shown). In summary, the b-specific domain inhibited binding of LHX3 to DNA regulatory sites. Additionally, transfer of the a-specific domain to LHX3b did not relieve the b-specific domain inhibitory effect. Removal of the a- and b-specific domains from LHX3 enabled binding of this protein to DNA sites similar to LHX3a binding.

Transfection assays demonstrated that transfer of the LHX3b-specific domain to PROP-1 (b-PROP-1) inhibited the ability of PROP-1 to activate a reporter gene (Fig. 3A). To examine the mechanism responsible for this inhibition, we generated in vitro translated LHX3a/b-PROP-1 chimeric pro-
teins (a-PROP-1, b-PROP-1) and performed EMSA experiments with a PRDQ9 DNA element (Fig. 4, C and D). Similar to transfer of the b-specific domain to LHX3a, the b-specific domain inhibited the ability of PROP-1 to bind DNA (Fig. 4 D). In these assays, transfer of the a-specific domain to PROP-1 (a-PROP-1) also reduced PROP-1 DNA binding, although not to the extent of the b-specific domain transfer (Fig. 4 D). Consistent with the transfection data, these results provide evidence that the LHX3b-specific domain negatively regulates LHX3 function by preventing HD binding to specific DNA regulatory elements.

Identification of a Novel LHX3 Protein—Our studies indicate the presence of an uncharacterized, faster migrating LHX3 species in Western blots of extracts from cells transfected with LHX3a and ΔN-LHX3 expression vectors, but not LHX3b vectors (Fig. 2 C). Because the carboxyl terminus of LHX3a was fused to a Myc epitope in these studies, Western experiments using an anti-Myc antibody indicated that the faster migrating LHX3 protein lacks amino-terminal residues. In addition, we observed a faster migrating protein form in in vitro translations of the Lhx3a and ΔN-Lhx3 cDNAs (Fig. 4A), and a more pronounced, rapidly migrating protein-DNA complex in EMSA reactions containing in vitro translated LHX3a and ΔN-LHX3 proteins (Fig. 4B, closed arrow). Because the lower molecular weight LHX3 molecule was generated in transfection experiments and in vitro translation reactions featuring expression of cDNAs (LHX3a and ΔN-LHX3), we suspected that this protein was formed by a post-transcriptional mechanism and not by alternative RNA splicing. In some studies, we used LHX3a and LHX3b cDNA expression vectors containing the 5’-untranslated sequences unique to each isoform (Fig. 5A). To determine whether translation initiation of the LHX3 isoforms was regulated by these upstream regions, we performed assays with expression vectors without these sequences (Figs. 2C and 5B). In both cases, significantly higher amounts of the faster migrating LHX3 protein were present in cells expressing
Lhx3a cDNAs compared with Lhx3b cDNAs. These observations suggest that the generation of this alternate, smaller form of LHX3 is inhibited by the Lhx3b-specific sequence.

To further characterize the production of the smaller LHX3 protein, we performed time-course experiments to examine the accumulation of this LHX3 species (Fig. 5B). If the faster migrating LHX3 protein accumulated over time at the expense of the slower migrating protein, we would suspect that the production of this LHX3 molecule must result from proteolytic processing of full-length LHX3a. However, because the proteins accumulated at similar rates (Fig. 5B), we examined the LHX3a coding sequence for the presence of potential alternate AUG translation initiation sites. In addition, we compared the migratory position of a LHX3 protein lacking the amino terminus and both LIM domains (ΔLIM LHX3, removal of amino acids 1–149 of 397) with the migration of this uncharacterized LHX3 species. These experiments demonstrated that the newly identified LHX3 protein migrates with a slightly higher apparent molecular weight than that of the ΔLIM LHX3 (data not shown) and enabled us to hypothesize that this novel LHX3 protein results from the use of an alternate methionine (Met) initiation codon. Examination of the Lhx3a cDNA sequence revealed the presence of a potential in-frame ATG translation start site at position 134 of the LHX3a protein. Consistent with the hypothesis, site-directed mutagenesis of Met-134 of LHX3a (LHX3a mutM2) eliminated the production of the faster migrating LHX3 protein (Fig. 6A). In addition, mutation of the primary LHX3a and LHX3b start codons (LHX3a/b mutM1) led to the production of only this lower migrating LHX3 species. Further, this protein resolved to the same position as a protein generated by an expression vector encoding only amino acids 134–397 of LHX3a (Fig. 6A). We refer to this novel form of LHX3 as M2-LHX3. Interestingly, this Met (134 in LHX3a, 139 in LHX3b) is conserved in several LIM HD transcription factors and may represent a mechanism utilized by these factors to produce multiple protein isoforms with different gene regulatory capabilities (Fig. 6B). Indeed, Western analyses of cells transfected with a mouse LHX4 expression vector indicated the presence of a faster migrating LHX3 protein that appears to result from alternate translation initiation by Met-110 (Fig. 6C).

Translation of the M2-LHX3 Protein Is Regulated by the LHX3b-specific Coding Sequence—We initially observed that the M2-LHX3 protein was present in extracts from cells transfected with LHX3a expression vectors, whereas only trace amounts of this protein were found in assays examining LHX3b protein (Fig. 2A). Further, transfer of the LHX3b-specific coding sequence to LHX3a demonstrated that this cell line expresses high levels of Lhx3a

Fig. 4. The LHX3b-specific domain inhibits HD protein DNA binding. A, 32P-labeled in vitro translated chimeric LHX3 proteins separated by SDS-PAGE visualized by fluorography. B, EMSA using a PGBE (aDSU gene –350 to –23) probe incubated with in vitro translated LHX3 proteins. Protein-DNA complexes were separated from free probe (f) by electrophoresis. C, in vitro translated LHX3a/b-PROP-1 proteins. Proteins used in the reactions were analyzed by SDS-PAGE and fluorography. D, EMSA using a PRDQ9 probe incubated with in vitro translated LHX3a/b-PROP-1 proteins. Protein-DNA complexes were separated from free probe (f) by electrophoresis.

Fig. 5. Identification of a novel LHX3 protein. A, Western analysis using an anti-Myc monoclonal antibody of 293T cells transfected with control and Myc epitope-tagged LHX3a and LHX3b expression vectors containing 5′-untranslated regions (5′ UTR) demonstrates the presence of a faster migrating LHX3 protein. The migration of molecular size standards is indicated. B, Western analysis using an anti-Myc monoclonal antibody of pituitary GHFT1 cells transfected at various time points with Myc epitope-tagged LHX3a, LHX3b, and α-LNX3 expression vectors (lacking 5′-untranslated regions). C, control transfection. Extracts were made at 1, 2, 4, 10, and 24 h.
Regulation of LHX3 Function by Alternate Amino Termini

M2-LHX3 Activates the TSHβ and PRL Genes—To test the function of M2-LHX3, we performed transfection assays to examine the ability of this protein to activate the αGSU, PRL, and TSHβ genes. Previous studies have shown that LHX3a can activate the αGSU gene alone and the PRL and TSHβ genes in synergy with the PIT-1 POU HD protein (5, 13, 16–18, 22). Assays performed here indicate that the M2-LHX3 protein has a reduced ability to activate the αGSU gene (Fig. 8A) but can synergize with PIT-1 to induce transcription of the PRL and TSHβ promoters (Fig. 8, B and C).

M2-LHX3 Displays an Increased Ability to Bind DNA and Contains the Major LHX3 Transcriptional Activation Domain—To determine the mechanism by which M2-LHX3 induces transcription, we characterized its DNA binding and gene activation properties. EMSA experiments using purified, recombinant LHX3 proteins demonstrate that M2-LHX3 bound with greater affinity to the LHX3 consensus site than LHX3a or LHX3b (Fig. 9, A–C). The M2-LHX3 protein does not contain the amino-terminal LIM domains, and this result is consistent with previous reports describing the inhibitory nature of the LIM domains of some LIM HD proteins in DNA binding (13, 16, 22). A previous study has mapped a trans-activation domain in the second LIM domain of LHX3 (17). Most of this domain was absent from the M2-LHX3 protein. We therefore hypothesized that additional activation domains must be present. A systematic search for activation domains in all regions of the LHX3 protein isoforms was performed by assaying the transcriptional activity of LHX3 domains fused to the Gal4 DNA binding domain (Gal4-DB; Fig. 9, A and D). A sequence in the carboxyl terminus of LHX3 (referred to as the C2 region) was identified as the major activation domain. Deletion of the C2 domain from M2-LHX3 impairs its ability to activate the PRL promoter (Fig. 9E). Together, these data demonstrate that the M2-LHX3 molecule is capable of binding DNA regulatory elements and requires a trans-activation domain within its carboxyl terminus to induce transcription.

mRNA (5). The available anti-LHX3 antiserum did not allow effective detection of LHX3 molecules in extracts from these cells in Western experiments (data not shown). However, EMSA analysis of αTSH cell extracts indicated that multiple complexes bound to both the αGSU PGBE and the LHX3 consensus binding sites (Fig. 7, A and B). These experiments were performed in parallel with EMSA reactions comprising protein extracts from 293T cells transfected with LHX3a-Myc, LHX3b-Myc, or M2-LHX3-Myc expression vectors. Comparison of the migratory positions of bound complexes revealed the presence of a complex in the αTSH reaction that resolved to a similar position to that of the M2-LHX3-Myc complex (Fig. 7, A and B). Importantly, this complex was specifically disrupted by an anti-LIM-3/LHX3 antibody, indicating that the M2-LHX3 protein is present in αTSH cells. Similar data were obtained in experiments using the anti-Myc epitope antibody to characterize the M2-LHX3 protein in transfected cells (data not shown). Because the M2-LHX3 isoform lacks the LIM domains (which have been demonstrated to inhibit DNA binding of many LIM HD proteins (see Refs. 13, 16, and 22)), it displays an increased binding to these DNA sites (see below) and is, therefore, more readily detected than the full-length LHX3 molecule in these assays. Together, these experiments demonstrate that the faster migrating LHX3 protein originates from an alternate start methionine, and that this LHX3 protein is present in LHX3a-expressing pituitary cells.

**Fig. 6.** A novel LHX3 protein (M2-LHX3) is generated by translation of a conserved alternate initiation codon and its production is inhibited by the LHX3b-specific domain coding sequence. A, Western analysis using an anti-Myc monoclonal antibody of 293T cells transfected with Myc epitope-tagged LHX3a, LHX3b, ΔN-LHX3, LHX3a mut M2 (Met-134 → Ala), LHX3b mutM2 (Met-139 → Ala), LHX3a mutM1 (Met-1 → Stop), LHX3b mutM1 (Met-1 → Stop), and M2-LHX3 (amino acids 134–397 of LHX3a) expression vectors. The migration of molecular size standards is indicated. B, alignment demonstrating potential alternate translation initiation methionines in LIM homeodomain proteins. Panel shows comparison of human LHX3 (hLHX3), porcine LHX3 (pLHX3), murine LHX3 (mLHX3), chicken LIM3 (cLIM3), Xenopus LIM3 (xLIM3), zebrafish LIM3 (zLIM3), Drosophila LIM3 (dLIM3), murine LH2 (mLH2), murine LH9 (mLH9), Caenorhabditis elegans LIN-1 (LIN-11), and Drosophila arrowhead (dAWH). Dots indicate identity; dashes denote gaps introduced to optimize alignment. C, Western analysis using an anti-Myc monoclonal antibody of 293T cells transfected with Myc epitope-tagged LHX3a, LHX3b, ΔN-LHX3, LHX3b mutM1 (Met-1 → Stop), LHX3a mutM1 (Met-1 → Stop), and M2-LHX3 (amino acids 134–397 of LHX3a) expression vectors. The migration of molecular size standards is indicated.

**Fig. 7.** M2-LHX3 is expressed in pituitary cells. A, an EMSA using a LHX3 consensus binding site probe was performed with nuclear extracts of either human kidney 293T cells transfected with LHX3 expression vectors or extracts of mouse pituitary αTSH cells. Radiolabeled probe was incubated with the indicated extracts and the resulting complexes were separated from free probe (p) by electrophoresis. Anti-LIM-3/LHX3 antibody (αLHX3) was added to some reactions. M2-LHX3-DNA complexes are indicated by an arrow. B, EMSA using an αGSU promoter PGBE probe.
The two LHX3 isoforms, LHX3a and LHX3b, are generated from renal organ development (35). Previously, it has been shown that PITX2 isoforms in zebrafish have distinct patterns of expression (28). Similarly, assays demonstrate that SKN-1a can induce transcription from the cytokeratin 10 gene whereas SKN-1i cannot (28). Similarly, patients with LHX3 gene mutations are deficient in each of the anterior pituitary hormones except adrenocorticotropic (9). To gain insight into which LHX3 isoform is critical for differentiation of each pituitary cell type, we examined rodent pituitary cell lines for the presence of Lhx3a and Lhx3b. Differential expression of the isoforms in these cell lines suggests that the factors play different gene regulatory roles in the development of the distinct cell types. Consistent with previous reports describing activation of the aGSU gene by Lhx3a (5, 13, 16–18), Lhx3a expression was identified in cell lines representing the gonadotrope and thyrotrope lineages. Although genes specifically regulated by the LHX3b isoform have yet to be identified, Lhx3b expression in cell lines representing the gonadotrope, lactotrope, somatotrope, and thyrotrope lineages is consistent with data from the Lhx3a null mice and suggests important functional roles for this factor in regulation of genes critical to development of these cells. Analysis of the alternate a- and b-specific amino-terminal domains in several mammalian species reveals that the amino acid sequence of the b-specific domain is more highly conserved than the a-specific domain. Together, the broad pattern of LHX3b pituitary cell type expression and the high degree of b-specific domain amino acid conservation suggest the LHX3b isoform is critical to anterior pituitary gland formation.

We have shown previously that the LHX3b-specific domain prevents LHX3 from activating the aGSU gene by inhibiting its binding to the PGBE (5). To further characterize the intrinsic regulatory function of this domain, we transferred the LHX3b-specific domain to related and unrelated DNA regulatory proteins. Transfer of the b-specific domain to LHX3a and LHX4, and PROP-1 impairs the trans-activation capabilities of these HD proteins. However, transfer of this domain to the non-HD proline and acidic amino acid-rich domain basic region leucine zipper transcription factor TEF does not affect its ability to activate the TSHβ gene. This contrasts with experiments where transfer of the amino-terminal SKN-1i domain to TEF inhibits its function by preventing DNA binding (28). Importantly, transfer of the LHX3a-specific domain to LHX3b did not confer the ability to activate the tested LHX3a-responsive genes. Transfer of the LHX3b-specific domain to HD-containing proteins, such as LHX3a and PROP-1, inhibits the DNA binding ability of these molecules. The inhibitory nature of the b-specific domain appears to be an intrinsic mechanism that prevents DNA binding of a HD protein. Although corepressor proteins may interact with the LHX3b-specific domain to prevent gene trans-activation, EMSAs using purified LHX3 chimeric proteins suggest that the b-specific domain impacts structural constraints upon these factors that impair HD accessibility to target DNA elements. We previously have demonstrated that LHX3b operates similarly in both pituitary and heterologous cells (5, 18). However, it also is possible that pituitary-specific coactivator or corepressor proteins interact with the LHX3b-specific domain in a highly coordinated fash-
Fig. 9. M2-LHX3 has increased DNA binding activity and retains the major LHX3 activation domain. A, schematic diagram of LHX3 proteins showing the tested regions of the molecules. B, expression of recombinant human LHX3 proteins. SDS-polyacrylamide gel analysis of affinity-purified GST-LHX3 fusion proteins. A Coomassie Blue stain of the protein gel is shown. C, EMSA analysis of binding of LHX3 proteins to a radiolabeled LHX3 consensus binding site. D, mapping of trans-activation domains within LHX3 molecules. Regions of LHX3 (indicated in panel A) were fused to the Gal4-DB, and the resulting expression vectors were tested for their ability to activate a luciferase reporter gene containing five upstream activating sequence (UAS) elements in transfections of human 293T cells. The LIM domains have some activation function, but the major trans-activation domain (C2) is in the carboxyl terminus. A representative experiment of at least three experiments is depicted. E, the C2 trans-activation domain is required for M2-LHX3 transcriptional synergy with Pit-1. Expression vectors encoding truncations of M2-LHX3 were generated. 293T cells were transfected with a PRL enhancer/promoter luciferase reporter gene and the indicated expression vectors. Promoter activity was assayed as described in Fig. 1. A representative experiment of at least four experiments is depicted.

ion to enable function of this isoform during pituitary development.

An active, short isoform (M2-LHX3) is produced from the LHX3 gene. This is the first study to identify this type of protein product from a LIM HD gene. We demonstrate that this protein is generated by the use of an internal start methionine at codon 134 (AUG134) in LHX3a. Interestingly, the conserved b-specific sequence restricts usage of this translation initiation codon. One of the important Kozak translation initiation consensus residues is a G at the +4 position (36). Sequence analysis reveals the first LHX3b AUG (M1) contains a G at this position, whereas LHX3a M1 has a less favorable C at this site. Examination of the +4 position of the LHX3 isoforms from mammalian species identified in this study shows strict conservation of the G and C residues in the Lhx3b and Lhx3a sequences, respectively. We suggest, that without a G at the +4 position, leaky ribosomal scanning of the LHX3a M1 occurs, which allows usage of AUG134 and production of the M2-LHX3 protein. Indeed, the first translation initiation site in the mouse Lhx4 mRNA also does not contain a G at this position, which may allow production of the M2-LHX4 protein. A similar mechanism appears to enable generation of multiple PIT-1 (34) and EGR3 transcription factor (29) isoforms.

The M2-LHX3 protein is present in the aTSH cell line and can bind to the PGE location in the αGSU gene. This element is required to directly restrict expression of the αGSU gene to pituitary gonadotropes and thyrotropes (37). LHX2 and LHX3 previously have been shown to specifically bind to the PGE in EMSA experiments (11, 13, 23). Several of these studies demonstrate that multiple complexes bind to the PGE in experiments using protein extracts from pituitary cell lines (11, 23). It has been suggested that the unidentified complexes contain LHX2 or LHX3 and different combinations of LHX2/LHX3-interacting proteins (23). However, these complexes have not been disrupted or characterized with antibodies against LHX2/LHX3-interacting proteins such as NLI, SLB, or MRG1 (11, 23). Our data suggest that the EMSA complexes described in these previous reports may be composed of full-length LHX2 or LHX3a in the higher migrating complexes and M2-LHX3 (or M2-LHX2) in the lower migrating complexes. In addition, the alternate translation initiation codon identified in this study is conserved in other LIM HD transcription factors and may be utilized to expand the regulatory properties of these genes. Indeed, our experiments demonstrate that this mechanism enables the production of a M2-LHX4 isoform.

M2-LHX3 is capable of binding DNA regulatory elements and inducing gene transcription because this molecule contains the major LHX3 trans-activation domain. However, the functional significance of the M2-LHX3 and M2-LHX4 proteins is unclear. Although it has been technically challenging to detect M2-LHX3 proteins in pituitary cell extracts using available antisera (data not shown), the increased DNA binding capacity of M2-LHX3 suggests that it may play important regulatory roles. Indeed, the M2-LHX3 molecule can synergize with PIT-1 to activate the PRL and TSHβ genes. Importantly, this activity requires a novel activation domain (C2) located within the carboxyl terminus. Identification of the C2 domain provides an explanation of data described in previous reports showing that LHX3a proteins with pituitary disease-causing LIM2 mutations still retain some ability to synergize with PIT-1 to activate the PRL promoter (18, 23). Alternately, because the M2-molecules do not contain intact LIM domains that are required for some LIM HD functions (9, 18, 23), these forms of the LHX3 and LHX4 proteins might play dominant negative regulatory roles. In transfection assays, the M2-LHX3 has a reduced ability to activate the αGSU promoter, a result similar to experiments examining the functions of LHX2 and LHX3 molecules with mutated or deleted LIM domains (13, 17, 18). M2-LHX3
does not contain the LIM2 trans-activation domain (17) that appears to be important for LHX3a aGSU induction. However, the C2 activation domain located within the carboxyl terminus is required to regulate the PRL gene. This domain also is necessary for activation of the aGSU promoter. The LHX3 molecule therefore contains multiple activation domains that are required for distinct activities.

The studies presented in this report describe mechanisms utilized by specialized cell types to produce multiple regulatory proteins with distinct functions from a single gene. Elaborate regulation of the LHX3 gene at the levels of transcription and translation expands the functional capacity of this gene by generating at least three protein isoforms. Understanding LHX3 isofrom expression and function furthers our knowledge of the pituitary cell proteome and suggests that these factors play distinct roles during mammalian pituitary development and function.

Acknowledgments—We are grateful to Drs. S. Camper, T. Golos, S. Konieczny, R. Maurer, A. Russo, and E. Taparowsky for materials, and to D. Crowell and L. Cushman for useful advice. We also thank G. Parker for assistance.

REFERENCES


* K. W. Sloop and S. J. Rhodes, unpublished data.
An Isoform-specific Inhibitory Domain Regulates the LHX3 LIM Homeodomain Factor Holoprotein and the Production of a Functional Alternate Translation Form
Kyle W. Sloop, Conor J. Dwyer and Simon J. Rhodes

doi: 10.1074/jbc.M103888200 originally published online July 24, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103888200

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

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

This article cites 37 references, 20 of which can be accessed free at http://www.jbc.org/content/276/39/36311.full.html#ref-list-1