Two Promoters Mediate Transcription from the Human LHX3 Gene: Involvement of Nuclear Factor I and Specificity Protein 1

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The LHX3 transcription factor is required for pituitary and nervous system development in mammals. Mutations in the human gene are associated with hormone-deficiency diseases. The gene generates two mRNAs, hLHX3a and hLHX3b, which encode three proteins with different properties. Here, the cis elements and trans-acting factors that regulate the basal transcription of the two mRNAs are characterized. A comparative approach was taken featuring analysis of seven mammalian Lhx3 genes, with a focus on the human gene. Two conserved, TATA-less, GC-rich promoters that are used to transcribe the mRNAs precede exons 1a and 1b of hLHX3. Transcription start sites were mapped for both promoters. Deletion experiments showed most activity for reporter genes containing the basal promoters in the context of ~2.0 kb of hLHX3a and 1.8 kb of intron 1a (hLHX3b). Transfection, site-directed mutation, electrophoretic mobility shift, Southwestern blot, and chromatin immunoprecipitation approaches were used to characterize the interaction of transcription factors with conserved elements in the promoters. Specificity protein 1 is a regulator of both promoters through interaction with GC boxes. In addition, a distal element within intron 1a that is recognized by nuclear factor I is critical for hLHX3b promoter function. We conclude that dual promoters allow regulated production of two hLHX3 mRNAs. (Endocrinology 147: 324–337, 2006)
allows regulation by nuclear factor I (NFI) family transcription factors.

Materials and Methods
Cloning of mammalian Lhx3 gene and cDNA sequences

Figures 1 and 2 were constructed using Adobe Illustrator software.

Figure 1. The human and mouse Lhx3 genes feature two GC-rich, TATA-less promoters with multiple transcription start sites. A. Structure of the human LHx3 gene. Exons are depicted by boxes with translated regions shown in black or hatched. Introns are indicated by lines. The major mRNA products and their protein derivatives are shown. B. Alignment of the proximal regions of the Lhx3a promoters of the human and mouse Lhx3 genes. Transcription start sites (TSS) for Lhx3a mRNAs were mapped by RLM-RACE and S1 nuclease assay experiments using human pituitary gland and mouse aT3-1 pituitary cell RNAs as substrates. Numbers are relative to the translation start codon C. The major transcription start site for the hLHX3b promoter was mapped by RLM-RACE using human pituitary RNA.

First 3-fold genome coverage (~12 million reads at the time of screening) was searched via basic local alignment tool nucleotide (BLASTN) using the full-length cDNA sequence of the hLHX3a cDNA. Trace files whose sequence showed highly significant (scores of >300) match to the cDNA, as well as the mate-pair end sequences from the respective clones, were collected in a directory and used to construct initial genomic contigs via phred (24) and phrap (25) algorithms. Contig sequences were masked for repetitive elements using RepeatMasker (Smit, A. F. A., and P. Green, unpublished results; http://ftp.genome.washington.edu/RM/RepeatMasker.htm) and used to search for overlapping trace files in the archive, which were added to the directory for reconstruction of contigs. The process was repeated until none of the contigs in the phrap output identified trace files not already in the directory. This resulted in construction of four contigs containing portions with high similarity to exons of the hLHX3 cDNA, leaving three gaps in the gene sequence. Primers then were designed to span the gaps by PCR, and sequence was obtained by amplification of bovine genomic DNA from the same animal used in the whole genome shotgun sequencing. The PCR products were sequenced with the amplification primers, nested primers, or both. The resulting 12,883-bp contig was edited by manual inspection using the Consed viewing program (25), and areas of low sequence quality or areas where read overlap was excluded from low-complexity sequence were targeted for finishing using additional PCR-based amplification and sequencing. To obtain confirming bovine Lhx3 cDNA sequence, primers were designed based on the cDNA sequence predicted from the first set of genomic trace files obtained. Primers 5′-gagactccttgccgagctcgc-3′ (1834 bp upstream), 5′-ggtagctagctgctgcgtc-3′ (1267 bp), and 5′-ggaacagccctgctgt-3′ (1481 bp) were designed from the hLHX3b promoter upstream in aT3-1 promoter region of the gene was amplified in approximately 500-bp increments using an antisense primer (5′-ctctgctccaggctgccaagtgt-3′) and one of the following sense primers: 5′-ggtgtagtgcgctcaggtcctg-3′ (580 bp upstream of exon 1a), 5′-gagactccttgccgagctcgc-3′ (1186 bp), 5′-ggaacagccctgctgt-3′ (1267 bp), and 5′-ggaacagccctgctgt-3′ (1481 bp). For the hLHX3b promoter upstream in intron 1a, three regions were amplified using an antisense primer (5′-cctcgcttccttgccgagctcgc-3′) and one of the following sense primers: 5′-ggtgtagtgcgctcaggtcctg-3′ (1834 bp upstream), 5′-ggtagctagctgctgcgtc-3′ (1267 bp), and 5′-gagactccttgccgagctcgc-3′ (1481 bp). PCR was performed with Pfu Ultra polymerase (Stratagene) and MasterAmp PCR optimization buffers (Epicentre, Madison, WI) (if required due to high GC content of the target sequences). To create luciferase reporter genes, fragments of the hLHX3a promoter upstream region or of the hLHX3b promoter/intron 1a region were cloned into the pGL2-basic plasmid (Promega, Madison, WI). All plasmids were confirmed by DNA sequencing (Biochemistry Biotechnology Facility, Indiana University School of Medicine).

To obtain the sequence of the bovine Lhx3 gene, the Trace Archive of the bovine genome project at the NCBI containing raw reads from the first 3-fold genome coverage (~12 million reads at the time of screening) was searched via basic local alignment tool nucleotide (BLASTN) using the full-length cDNA sequence of the hLHX3a cDNA. Trace files whose sequence showed highly significant (scores of >300) match to the cDNA, as well as the mate-pair end sequences from the respective clones, were collected in a directory and used to construct initial genomic contigs via phred (24) and phrap (25) algorithms. Contig sequences were masked for repetitive elements using RepeatMasker (Smit, A. F. A., and P. Green, unpublished results; http://ftp.genome.washington.edu/RM/RepeatMasker.htm) and used to search for overlapping trace files in the archive, which were added to the directory for reconstruction of contigs. The process was repeated until none of the contigs in the phrap output identified trace files not already in the directory. This resulted in construction of four contigs containing portions with high similarity to exons of the hLHX3 cDNA, leaving three gaps in the gene sequence. Primers then were designed to span the gaps by PCR, and sequence was obtained by amplification of bovine genomic DNA from the same animal used in the whole genome shotgun sequencing. The PCR products were sequenced with the amplification primers, nested primers, or both. The resulting 12,883-bp contig was edited by manual inspection using the Consed viewing program (25), and areas of low sequence quality or areas where read overlap was excluded from low-complexity sequence were targeted for finishing using additional PCR-based amplification and sequencing. To obtain confirming bovine Lhx3 cDNA sequence, primers were designed based on the cDNA sequence predicted from the first set of genomic trace files obtained. Primers 5′-gagactccttgccgagctcgc-3′ and 5′-ggtgtagtgcgctcaggtcctg-3′ were designed from the putative exon 2 sequence and used to obtain a full coding sequence bovine Lhx3 cDNA clone via iterative screening (26) of a pooled-tissue cDNA library that included pituitary gland (library 1BOV (27)). The clone obtained had an insert of 2,390 bp, and the complete insert was sequenced. The edited bovine Lhx3 gene and cDNA sequences have been submitted to GenBank with accession nos. AY923832 and AY923833.
respectively. Intron 1a sequences of the rhesus monkey and porcine Lhx3 genes were amplified by the PCR. Templates were 150 ng of genomic DNA from adult rhesus monkey (kindly provided by Dr. T. Golus, Wisconsin Primate Research Center, Madison, WI) or adult pig (female Yorkshire cross). Reactions included MasterAmp PCR optimization buffer G (Epitentre), and 5'-atcgctgtggagctggctca-3' (monkey exon 1a), 5'-gagaacctgccggagtccg-3' (pig exon 1a), and 5'-gatggctccggagctccgcccc-3' (pig exon 1b) primers were used. PCR products from multiple independent reactions were sequenced on both strands. Sequences were submitted to GenBank (accession nos. AY879262 for pig intron 1a and AY879263 for rhesus monkey intron 1a).

**RNA ligase-mediated rapid amplification of cDNA ends**

The transcription start sites of human and mouse Lhx3 genes were deduced by 5' RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) performed using the GeneRacer protocol (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer, as we have described (28). First-strand cDNA was generated from adult human pituitary gland RNA (13) or from pituitary aT3–1 pregonadotrope cell (29) total RNA and primed with 5'-ggctcctgcttgaggctgaact-3'. Second-strand cDNA was generated using primers from the manufacturer and the following gene-specific primers: 5'-gagaacgctgtcttgaggtc-3' (5' RACE Lhx3a) and 5'-ttctcctgcttgaggctgaact-3' (5' RACE Lhx3b).

**S1 nuclease assays**

S1 nuclease assays were performed as described (30). Briefly, 32P end-radiolabeled single-stranded DNA probes for the hLHX3a and hLHX3b promoters were generated. S1 digestion reactions contained 15 µg of aT3–1 RNA hybridized with labeled probes. Radiolabeled DNA products were analyzed by electrophoresis through 12% polyacrylamide 8 M urea gels. A 32P end-labeled 1-kb extension ladder (Invitrogen) was used as a molecular marker.

**Cell culture and transcription**

Human embryonic kidney (HEK) 293T and rodent pituitary cell lines were cultured and transfected as described (14). Typical transfections contained 2 µg of a luciferase reporter gene and 500 ng of an expression vector (if any). Control parallel samples received empty vector DNA. All assay groups were performed in triplicate. Forty-eight hr following transfection, cells were lysed in 25 mM Tris-Cl (pH 7.8), 2 mM dithiothreitol, 1% Triton X-100, 2 mM EDTA (pH 8.0), 10% glycerol. The lyase supernatant was assayed for luciferase activity using a luciferin substrate (Promega) and a Beckman Coulter luminometer (Fullerton, CA). Total cell protein was determined by the Bradford method (Bio-Rad, Hercules, CA), and luciferase activity was normalized to the amount of protein present. Expression vectors included human LHX3a, human LHX3b (13), human PRO1 (31), mouse SF1 (a gift from Dr. Holly Ingraham, University of California, San Francisco, CA), mouse EGR1 (a gift from Dr. Eileen Adamson, Burnham Institute, La Jolla, CA), rat Sp1 (a gift from Dr. Michael Wegner, University of Erlangen, Erlangen, Germany).

**Cell protein extraction**

Nuclear or whole cell protein extracts from HEK 293T and rodent pituitary cells were prepared as we have described (14, 32). Rat pituitary GH3 somatolactotrope nuclear extracts were purchased from Active Motif, Inc. (Carlsbad, CA).

**EMSAs**

EMSAs were performed as we have described (14) using radiolabeled double-stranded oligonucleotide probes with the results visualized by autoradiography or using a Storm phosphorimager (Amersham Biosciences, Piscataway, NJ). Cell extracts were prepared as described above. Human recombinant Sp1 protein was purchased from Promega. In some experiments, 2 µg of anti-Sp1, anti-NFI, or anti-SOX5 antibodies (Santa Cruz Biotechnology Santa Cruz, CA) were added to the binding reactions and incubated for an additional 30 min.

**Chromatin immunoprecipitation**

The chromatin immunoprecipitation (ChIP) method was adapted from that of Petz et al. (33) and was performed as we have described (14) using reagents from the ChIP Assay kit (Upstate Biotechnology, Lake Placid, NY). Approximately 1 x 10^6 L7T2 cells were cross-linked with formaldehyde and then lysed. Cellular DNA was sonicated to fragments of 200-100 bp. The supernatant from the sonicated lysate was then preclared with salmon sperm DNA/protein A agarose. Next, either Sp1- or NFI-containing complexes were immunoprecipitated using specific antibodies (Santa Cruz Biotechnology). Complexes were collected using protein A agarose. After washing and elution, cross-linking was reversed and DNA was extracted. The purified DNA was analyzed by PCR using the following primers: 5'-agaagctggagctggctca-3' and 5'-actaattaggctgcttgaggctgaact-3' (mouse Lhx3a promoter region); 5'-agggctcctgcttgaggctgaact-3' and 5'-tgctggctcctgcttgaggctgaact-3' (NFI site region in mouse intron 1a/Lhx3b promoter); and 5'-ttctcctgcttgaggctgaact-3' and 5'-ttctcctgcttgaggctgaact-3' (mouse β actin gene). PCR products were analyzed by agarose gel electrophoresis, and the identity of observed DNA fragments was confirmed by cloning into pTOPO vectors (Invitrogen) and DNA sequence analysis.

**Site-directed mutagenesis**

Site-directed mutagenesis was performed as described (32) using the QuickChange Site Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Sequences containing predicted transcription factor-binding sites were mutated using pGL2 plasmid substrates containing either intron 1a of the hLHX3 gene (i.e. the hLHX3b promoter) or ~2.7 kb of hLHX3a 5' flanking sequence. Mutagenic oligonucleotides were as follows: 5'-caagggctgcttgaggctgaact-3' (291 Sp1 site in hLHX3a promoter); 5'-ccggctgcttgaggctgaact-3' (181 Sp1 site in hLHX3a promoter); 5'-ggagggctgcttgaggctgaact-3' (203/185 Sp1 sites in intron 1a); and 5'-ggagggctgcttgaggctgaact-3' (NFI site in intron 1a).

**Southwestern analysis**

For Southwestern blotting experiments, pituitary protein extracts were separated by standard SDS-PAGE and transferred to nitrocellulose membranes by electrophoresis. Membranes were then incubated in TNED renaturation buffer (10 mM Tris-Cl pH 7.5, 0.1 mM EDTA pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 5% nonfat dry milk) at room temperature with slow rotation in a hybridization oven. The membranes were then incubated with approximately 8 x 10^6 cpm/ml of a 32P end-radiolabeled DNA probe (50–60 bp) overnight at room temperature in TNED buffer supplemented with 0.25% nonfat dried milk and 0.005 mg/ml sheared salmon sperm. After binding of the DNA probe, the protein blot membranes were washed three times for 5 min with 20 ml of TNED buffer plus 0.25% milk. Membranes were then air dried, and results were analyzed using a Storm Phosphorimager (Molecular Dynamics, Piscataway, NJ).

**RT-PCR of NFI isoforms**

Total RNA was isolated from mouse L7T2 gonadotrope pituitary and HEK 293T human embryonic kidney cell lines. RT of 1 µg of RNA was performed using the oligo-dT primers and the SuperScript First-Strand Synthesis System (Invitrogen). NFI transcripts were detected by primers that were designed to unique regions within the coding sequences of the four mouse/human NFI isoforms accessed through GenBank. These were 5'-gaagctggagctggctca-3' and 5'-aagatggctggagctca-3' for the NFI-A isoform; 5'-ggagggctgcttgaggctgaact-3' and 5'-ggagggctgcttgaggctgaact-3' for NFI-B; 5'-actcctgcttgaggctgaact-3' and 5'-ggagggctgcttgaggctgaact-3' for NFI-C; and 5'-ttctcctgcttgaggctgaact-3' and 5'-ttctcctgcttgaggctgaact-3' for NFI-X.

**Results**

Analysis of Lhx3 gene and cDNA nucleotide sequences from humans and mice suggested that the two major mRNAs are generated from two TATA-less promoters featuring high GC contents (Fig. 1 and data not shown). To characterize the
transcription start sites in the human and mouse Lhx3 genes, we performed RNA ligase-mediated RACE and S1 nuclease-mapping experiments using human pituitary gland RNA and mouse pituitary αT3–1 pregonadotrope cell (29) RNAs as substrates. These experiments revealed two major transcription start sites for a hLHX3a promoter upstream of exon 1a, one for the mouse Lhx3a promoter and one for a hLHX3b promoter upstream of exon 1b in intron 1a (Fig. 1, B and C, and data not shown).

To test the functional properties of the two identified hLHX3 gene promoters, we created luciferase reporter genes containing fragments of the hLHX3a and hLHX3b promoter 5′ flanking regions (Fig. 2). These reporter genes were transfected into cultured mouse pituitary LβT2 gonadotrope or αT3–1 pregonadotrope cells, and their activities were measured. Both of these cell types express the mouse Lhx3a and Lhx3b mRNA transcripts (14, 22). The hLHX3a promoter reporter genes were active in these pituitary cells with the 2701- and 2080-bp constructs displaying the highest activities (Fig. 2A and data not shown). The region between −4824 and −2701 in the hLHX3a upstream sequence appears to contain negatively acting elements. In addition, positive regulatory elements appear to lie between −2080 and −1581 bp of the hLHX3a promoter. The hLHX3b promoter reporter gene containing the entire intron 1a sequence (−1804 bp) was also active in the pituitary cells (Fig. 2B). Deletion of the distal region of this sequence to leave −1267 bp reduced the activity of the promoter to a level similar to that of a construct retaining only 418 bp of 5′ flanking sequence (Fig. 2B). This observation suggested that intron 1a contains proximal elements that are important for basal transcription of the hLHX3b promoter and additional regulatory elements located between −1804 and −1267 that confer higher levels of expression (see below).

We next used a comparative strategy to examine conservation of Lhx3 gene promoter sequences from primate, ungulate, and rodent mammals (Figs. 3 and 4). As part of these studies, the sequence of the entire bovine Lhx3 gene was determined. First, BLASTN searches of the NCBI Trace Archives containing whole genome shotgun reads from the bovine genome sequencing project were performed using the full-length cDNA sequence of the hLHX3a cDNA as a query. Recovered sequences were collected and aligned into four contigs containing portions with high similarity to exons of hLHX3 (see Materials and Methods), leaving three gaps in the gene sequence. The PCR was then used to span these gaps.
and to confirm all regions of low sequence quality or complexity by amplification and sequencing of bovine genomic DNA from the same animal used in the whole genome shotgun sequencing project. The resulting edited 12,883-bp gene contig was submitted to GenBank as accession no. AY923832. To confirm the exons predicted from the gene, a full-length bovine Lhx3b cDNA sequence was cloned by iterative screening of a multitissue cDNA library that included pituitary gland. The 2,390-bp bovine Lhx3b cDNA clone was submitted to GenBank as accession no. AY923833. This clone predicts a 403 amino acid protein with 95% primary sequence identity to human LHX3b (data not shown). The genome sequence encompasses the entire observed cDNA sequence, including 5,000 bp of sequence upstream from the first exon, and displays consensus splice boundary and polyA addition signal sequences (data not shown). The predicted bovine Lhx3a promoter 5' flanking region DNA sequence was aligned with the equivalent regions from the human, chimp, mouse, and rat genomes (Fig. 3). Chimp, mouse, and rat sequences were identified by BLASTN searches of NCBI databases. The Lhx3a promoter sequences are very GC rich (e.g. the human and bovine promoters have 79% and 76% GC content in this region, respectively) and lack obvious TATA boxes (Fig. 3).

Two GC boxes located at −181 bp and −165 bp of the human sequence (the LHX3a protein first codon is considered to be position +1) appear to be conserved in the examined mammalian sequences (Fig. 3). An additional element at −291 bp (in humans) is observed also in the chimp with the bovine sequence having a similar, more proximal element (Fig. 3).

To examine conservation of mammalian Lhx3b promoter sequences, the Lhx3 intron 1a sequences of the rhesus monkey and pig genomes were also cloned and sequenced (see Materials and Methods). The intron sequences were submitted to GenBank (accession nos. AY879263 for rhesus monkey and AY879262 for pig). As described above, the corresponding chimp, mouse, and rat sequences were obtained by BLASTN searches of GenBank databases. The aligned mammalian Lhx3 gene intron 1a DNA sequences display two regions of strong similarity: the proximal region around the transcription start site (Fig. 4). Similar to the Lhx3a promoter, the Lhx3b proximal regions are GC rich (e.g. the human and bovine promoters are ~80% GC content in this region) and have no obvious TATA elements. Six GC boxes are found in the human sequence (Fig. 4). Of these, the distal three sequences (~345, ~308, and ~286) are also found in the other primate sequences (chimp and rhesus), with the ~286 sequence also found in the cow. Two closely aligned central GC boxes (~203 and ~185) are found in all of the mammalian se-

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**Fig. 3.** Comparative sequence analysis of Lhx3a proximal promoter regions. The entire bovine (Co) Lhx3 gene and the human (Hu) LHX3a promoter were cloned and sequenced (see Materials and Methods). The chimp (Ch), mouse (Mo), and rat (Ra) promoter sequences were obtained by BLASTN searches of GenBank databases at NCBI. The entire promoter is GC rich, but notable GC boxes are shown in **bold** and their position in nucleotides relative to the hLHX3a start codon (+1) are indicated. Transcription start site (TSS) positions are shown in **bold** and **labeled**, h, Human; m, mouse. Coding sequences are shown in *lowercase bold italics*.
quences, and a proximal element at −133 is found in all examined mammals, except rodents (Fig. 4). The observed clusters of GC boxes in the hLHX3 promoters led us to test the hypothesis that the Sp1 transcription factor can recognize these elements and regulate hLHX3 gene transcription. Luciferase reporter genes representing the most active 5′ flanking regions of the hLHX3a and hLHX3b promoters were transiently cotransfected into human embryonic kidney (HEK) 293T cells with Sp1 transcription factor expression vectors. In these experiments, the activity of the hLHX3a promoter was increased approximately 40-fold (Fig. 5A) and that of the hLHX3b promoter was boosted approximately 7-fold (Fig. 5B) in comparison with negative controls. For comparison, we tested whether other pituitary transcription factors could activate or repress transcription from these promoter constructs. LHX3a and EGR1 increased transcription from the hLHX3a promoter (5-fold and 4-fold, respectively; Fig. 5A). LHX3b, SF1, and PROP1 had very modest effects on the hLHX3a reporter gene (Fig. 5A). The hLHX3b reporter gene was moderately induced by LHX3a and inhibited by LHX3b, EGR1, and PROP1 (Fig. 5B).

To better characterize the effects of the Sp1 transcription factor, we performed EMSA experiments to test the Sp1 interaction properties of GC box-containing sequences in the Lhx3b proximal promoter regions. The entire bovine (Co) Lhx3 gene and intron 1a from the human (Hu), rhesus monkey (Rh), pig (Pi), and cow Lhx3 genes were cloned and sequenced (see Materials and Methods). The chimp (Ch), mouse (Mo), and rat (Ra) Lhx3b promoter sequences were obtained by BLASTN searches of GenBank databases at NCBI. Within the overall GC-rich promoter, GC boxes are shown in bold and their position in nucleotides relative to the start codon are annotated. The major transcription start site (TSS) position is labeled and protein-coding sequences are shown in lowercase bold italics.

Fig. 4. Comparative sequence analysis of Lhx3b proximal promoter regions. The entire bovine (Co) Lhx3 gene and intron 1a from the human (Hu), rhesus monkey (Rh), pig (Pi), and cow Lhx3 genes were cloned and sequenced (see Materials and Methods). The chimp (Ch), mouse (Mo), and rat (Ra) Lhx3b promoter sequences were obtained by BLASTN searches of GenBank databases at NCBI. Within the overall GC-rich promoter, GC boxes are shown in bold and their position in nucleotides relative to the start codon are annotated. The major transcription start site (TSS) position is labeled and protein-coding sequences are shown in lowercase bold italics.
We conclude that pituitary cell proteins recognize the GC boxes in the LHX3 promoters and that the −291 and −181 sites are likely weak and strong Sp1 interaction elements, respectively.

We similarly examined protein/DNA interaction properties with GC boxes in the LHX3b proximal promoters. In these studies, EMSA experiments using pituitary LβT2 or HEK cell extracts, anti-Sp1 antibodies, or purified Sp1 protein to probe protein/DNA interactions indicated that the −308 element can be weakly bound by Sp1 and the −203/−185 region is strongly recognized by Sp1 (Fig. 6B and data not shown). As for the hLHX3a promoter analysis, for some sites (−345 and −308), faster-migrating complexes that are not disrupted by anti-Sp1 were also observed.

To assess Sp1 association with the endogenous mouse Lhx3a gene promoter, we performed ChIP experiments. These studies demonstrate Sp1 occupancy of the proximal region of the Lhx3a promoter in LβT2 pituitary cells (Fig. 7A). Parallel negative controls showed no nonspecific recovery of an unrelated actin gene (Fig. 7A). To date, we have been unable to obtain similar data showing Sp1 association with the mouse Lhx3b gene promoter. Likely technical explanations for this result include the high-GC content of this genomic region, a condition that makes conventional PCR challenging.

We next examined the importance of the identified Sp1-binding sites in the transcriptional activity of the hLHX3 promoters. Mutation of either the −181 or −291 element within the hLHX3a promoter reduced the activity of the promoter in pituitary cells (Fig. 7B). Interestingly, these mutations reduce promoter activity to similar, low levels. This observation may indicate the elements in this region act together rather than in additive fashion. Similarly, mutation of the major Sp1-interacting region of the hLHX3b promoter (−203/−185) compromised activity by approximately 5-fold (Fig. 7C). We conclude that Sp1 is an important regulator of the hLHX3 gene promoters.

Experiments described above indicate that the 5’ end of intron 1a (upstream of the hLHX3b promoter) contains a regulatory element that is critical for hLHX3b activity in pituitary cells (Fig. 2B). To further define this potential positive regulatory region, we first scanned the entire intron for trans-acting protein interactions using large (−400 bp), overlapping probes in EMSA experiments. All of the tested sequences displayed some protein binding, but the major complex-forming regions were −1504/−1084 and the two most proximal sequences that encompassed the GC boxes, including the identified Sp1 element (asterisks, Fig. 8A). Based on this observation and the functional data shown in Fig. 2B, we therefore concentrated on the −1504/−1084 region. EMSA experiments using shorter probes eventually refined the primary binding site within this region to an element located between −1444 and −1414 that interacted strongly with proteins from both LβT2 pituitary cells and HEK cells (Fig. 8, B and C). In Southwestern blot experiments, radiolabeled probes representing this intronic region interacted with proteins of approximately 60–65 kDa from cultured rodent GC, αT3–1, and LβT2 pituitary cells (Fig. 8D and data not shown). Sequence analyses of the −1444 to −1414 DNA sequence suggested the presence of a possible nuclear factor I (NFI) transcription factor-binding site. To test the possibility that NFI family proteins interacted with this region, we performed additional EMSA assays. Anti-NFI antibodies disrupted the protein/DNA complexes in EMSA experiments using LβT2 pituitary cell extracts, and a supershifted complex was observed (Fig. 8E). Antibodies to non-NFI proteins did not affect the protein/DNA complexes in parallel neg-
ative controls. To further examine NFI interaction with this element within mammalian Lhx3 genes, we performed ChIP experiments targeting the endogenous mouse intron 1a region. Consistent with the EMSA and Southwestern data, anti-NFI antibodies precipitated chromatin complexes containing this genomic sequence from LβT2 cells but not a region of an actin gene in parallel negative controls (Fig. 8F).

Mammalian NFI factors include the NFI-A, NFI-B, NFI-C, and NFI-X isoforms (reviewed in Ref. 34). To investigate which NFI family isoforms are expressed in the LβT2 pituitary cells used in our studies and in pituitary glands, we performed RT-PCR experiments using cDNA derived from LβT2, human adult pituitary, mouse adult pituitary, or HEK cells. Whereas all four NFI isoforms were expressed in the adult pituitary tissues and in HEK cells, only the A, C, and X isoform mRNAs were detected in the mouse LβT2 pituitary gonadotrope cells (Fig. 9A). During the course of our studies, another group reported a RT-PCR assay of NFI isoform expression in LβT2 cells with similar results, except that these authors did not detect NFI-A in their LβT2 cells (35). In Northern blot experiments using pituitary GC somatolacto-
tropo cell RNA, a separate laboratory detected expression of the A, C, and X NFI mRNAs (36). We conclude that all members of the NFI family are expressed in the pituitary and that subsets are found in differentiated pituitary cell types.

To better understand the interaction of NFI factors with the hLHX3 intron 1a element, we performed further structure/function studies of the DNA element. Inspection of the region of intron 1a revealed two NFI half-sites (beginning at 1441 bp) surrounding an E-box, i.e. a DNA sequence matching the CANNTG consensus that can be recognized by members of the basic helix-loop-helix transcription factor superfamily (Fig. 9B). This sequence is therefore positioned 1325 bp upstream of the major transcription start site. Upstream of these sequence features is an imperfect potential NFI half-site (Fig. 9B). Oligonucleotide probes representing the wild-type human sequence and variants with specific mutations in each of these sequence features were tested in EMSA experiments using LβT2 cell extracts. These experiments demonstrated that, whereas the two downstream NFI half-sites were critical for protein/DNA interaction, the E-box and the upstream element were not required for formation of the complex (Fig. 9C). Similar results were obtained in experiments using protein extracts from rat pituitary somatolactotrope GH3 cells (Fig. 9C). These observations are consistent with our initial mapping experiments (Fig. 8). Mutation of the downstream NFI half-sites of the intron 1a luciferase reporter gene severely compromised its activity in LβT2 cells (Fig. 9D). Intriguingly, mutation of the E-box similarly affected reporter gene function (Fig. 9D). It is also interesting to note that although both mutation of the NFI site and deletion of the region containing the site both significantly reduce activity of the Lhx3b promoter/intron 1a (Figs. 2 and 9), the mutation results in a more severe reduction in activity. One explanation for this is that a repressive element in the intron is also removed in the deletion experiment. The intron region containing the NFI site is conserved in primates, and a similar sequence is found in a 5′-shifted location in other examined species (Fig. 9E).

**Discussion**

In this study, we present the first characterization of the mechanisms that regulate transcription of the Lhx3 gene from any species. Two conserved, TATA-less, GC-rich promoters located upstream of exons 1a and 1b of mammalian Lhx3...
FIG. 8. A distal upstream region of intron 1a recognized by NFI factors is critical for activity of the hLHX3b promoter. A, Experiments shown in Fig. 2B demonstrated that an element between −1804- and −1267-bp of intron 1a is critical for activity of the hLHX3b promoter. To identify regions within the entire intron 1a sequence that bind cellular factors that may regulate transcription, approximately 400-bp overlapping fragments were used as probes in scanning EMSAs with HEK cell extracts. All regions displayed some protein binding, but three regions were strongly recognized (asterisks), including the −1504 to −1084 region. B and C, Protein binding within the −1504 to −1084 region was further refined by EMSA using probes of the indicated sizes until a 30-bp sequence encompassing −1444 to −1414 was identified. H, HEK cell protein; L, LβT2 pituitary cell protein. D, Southwestern blot experiments using DNA probes including the −1454 to −1414 sequence interacted with proteins of approximately 60–65 kDa on blots of protein extracts from cultured rodent pituitary cells. E, Anti-NFI antibodies disrupt protein/DNA complexes (arrow) in EMSA experiments using DNA probes containing the −1454 to −1414 DNA sequence and LβT2 pituitary cell extracts. The asterisk indicates a supershifted complex. Anti-SOX5 antibodies did not affect the protein/DNA complexes in parallel negative controls. F, ChIP experiments demonstrate NFI occupation of the −1454 to −1414 region of the endogenous mouse Lhx3 intron 1a in LβT2 pituitary cells. A gel showing separation of the amplified genomic DNA region fragments is shown. anti-NFI mLhx3, Anti-NFI antibody reaction used as a substrate in a PCR for the mLhx3b promoter/intron 1a (closed arrow); anti-SP1 mLAct, anti-NFI antibody reaction used as a substrate in a PCR for a region of the β-actin gene (negative control); In, input positive control; M, molecular markers; Neg, negative control (no substrate); PI, preimmune negative control; Pos mLAct, β-actin fragment amplification from input DNA (open arrow).
FIG. 9. Regulation of the hLHX3b promoter by NFI factors through interaction with a conserved, critical distal element within intron 1a. A, NFI factors are expressed in pituitary LβT2 gonadotropes and adult pituitary glands. RT-PCR analysis of NFI isoform mRNA expression. Isoform-specific primers were used in PCR containing either HEK 293T, LβT2, adult human pituitary, or adult mouse pituitary cDNA. M, Marker (in base pairs); Neg, negative control reactions lacking reverse transcriptase but including the tested cDNA and primers for the A or B isoform. B, The human −1454 to −1426 region contains three putative NFI recognition half-sites (bold, with two consensus proximal sites underlined) and a putative E-box (line over text). Oligonucleotides representing this sequence with specific mutations of each of these elements were synthesized (M1, M2, M3, and M4). C, EMSA experiments using LβT2 pituitary cell protein extracts and the oligonucleotide probes shown in C reveal that the two proximal NFI half-sites are most important for binding. Similar results were obtained in experiments using GH3 pituitary cell extracts (only bound complexes are shown). D, The NFI site is important for the basal activities of the hLHX3b promoter. Wild-type and mutated luciferase reporter genes containing intron 1a/hLHX3b promoter were transiently transfected into pituitary gonadotrope LβT2 cells and activities were determined. Promoter function was assayed by measuring luciferase activity 48 h after transfection. Activities are mean (light units/10 sec/μg total protein) of triplicate assays ± SEM. A representative experiment of at least three experiments is depicted. E, Conservation of the −1454 to −1413-bp region of intron 1a of mammalian Lhx3 genes. The entire Lhx3 gene was cloned from cattle and intron 1a was cloned from pig and rhesus monkey genomic DNA (see Materials and Methods). Alignment of the −1454 region of intron 1a in the human LHX3 gene with other species. Ch, Chimp; Co, cow; Hu, human; Mo, mouse; Pi, pig; Ra, rat; Rh, rhesus monkey.
genes function to initiate basal transcription of the Lhx3a and Lhx3b mRNAs. Interestingly, the proximal Lhx3b promoter is more strongly conserved in mammals than is the Lhx3a promoter (Garcia, M., and S. J. Rhodes, unpublished data). To date, molecular assays have demonstrated that the LHX3a protein is significantly more active than LHX3b in DNA binding and pituitary gene activation assays because of repressive properties conferred by the LHX3b-specific amino terminus (13, 22, 23). However, the observation that the hLHX3b promoter is conserved, together with the previous report that the LHX3b-specific amino terminus protein sequence is better conserved than that of the LHX3a-specific amino-terminal domain (22), suggests that LHX3b plays important and, perhaps, unique roles in neuroendocrine development.

The TATA-binding protein-associated factor components of the TFIIID complex are required for basal transcription at TATA-less promoters but TATA-binding protein itself may not always be required for initiation from this type of promoter (37). Classically, TATA-less promoter organizations were associated with housekeeping genes, which lacked precise temporal and spatial expression patterns. However, it is becoming apparent that TATA-less promoters may be more common than TATA-containing promoters (38) and that TATA-less promoters are often a feature of tissue-specific and regulatory genes (e.g. Refs. 39 and 40). The results described here for the hLHX3 gene are consistent with these findings.

We have shown that some of the conserved GC boxes within the hLHX3 promoters are Sp1-binding sites, that expression of Sp1 results in increased promoter activity, and that the Sp1 sites are important contributors to basal promoter function (Figs. 5–7). An organization including the presence of multiple Sp1 binding sites in a GC-rich, TATA-less promoter exhibiting several initiation sites has been observed for other tissue-specific genes that encode regulatory transcription factors. For example, the mouse and human Wilm’s tumor suppressor gene (wt1), the expression of which is regulated spatially and temporally during urogenital development, has all of these features (41).

Mice lacking the Sp1 gene die in utero by embryonic d 10 demonstrating that Sp1 is critical for development. However, in these animals expression of suggested Sp1 target genes is nevertheless detectable (42). Although our data here demonstrate that Sp1 proteins in pituitary cells do occupy functionally important, GC-rich elements within the hLHX3a and hLHX3b promoters, it is possible that, at specific times, other members of the Sp protein family might interact with these and other hLHX3 promoter elements. Mammalian genomes encode multiple Sp1-related/XKLF transcription factors (43) and some members of this family exhibit restricted expression patterns and play roles in the development of specific tissues (e.g. Ref. 44).

Some of the identified GC elements within the hLHX3 promoters conform to consensus sites for the EGR1/NGF1A/KROX24 zinc finger transcription factor. EGR1 plays a direct role in the transcriptional control of the LHβ subunit gene (reviewed in Ref. 45), and gene inactivation experiments demonstrate its importance in pituitary somatotrope and gonadotrope cell development (46, 47). In transfection experiments, EGR1 boosted transcription from the hLHX3a promoter and reduced the activity of a hLHX3b reporter gene (Fig. 5), suggesting that EGR1 may differentially regulate hLHX3 promoter activities. The activity of EGR1 is often a response to environmental signals such as growth factors, neurotransmitters, and hormones, and the hLHX3 GC boxes may allow control of hLHX3 promoter activities through competitive interplay among factors such as EGR1, Sp1, and the Sp1-related Sp3 protein, as has been described for other promoters (e.g. Refs. 45 and 48–50).

We have shown in this report that a conserved, positively acting cis-element lies at approximately −1442 bp of intron 1a in the hLHX3b promoter. EMSA, Western blot, and ChIP assays indicate that NFI proteins interact with this region. NFI/CTF family transcription factors (34) have been suggested to contribute to both basal and tissue-restricted gene activation and repression, including in the pituitary and nervous systems (e.g. Refs. 35, 36, and 51–53). NFI and Sp1/Sp3-binding sites have been found in other genes with expression patterns in endocrine tissues, such as the ADAMTS-1 gene (54). Intriguingly, considering the involvement of Sp1 in hLHX3 promoter regulation, NFI family members have also been demonstrated to interfere with Sp1 activities in some promoters (55). All four of the four major NFI isoforms are found in pituitary cell types (Refs. 35 and 36 and this study). To test the potential roles of individual NFI proteins in hLHX3b promoter activation, we have performed transfection experiments in pituitary cells with expression vectors for all four NFI factors and a hLHX3b reporter gene. In these assays, overexpression of NFI did not significantly affect the activity of the promoter (Garcia, M., and S. J. Rhodes, unpublished observations), likely due to the presence of endogenous NFI proteins in the transfected cells. However, mutation of the NFI element compromised the activity of the hLHX3b promoter in pituitary gonadotrope cells (Fig. 9D). Interestingly, a mutation of an E-box-like sequence that did not affect gonadotrope cell NFI protein binding also strongly reduced the activity of the promoter (Fig. 9A), suggesting that this sequence is important for NFI-mediated transcription but not DNA interaction or that this sequence is recognized by other proteins that migrate within the same DNA/protein complex in EMSA experiments.

The embryonic expression patterns of the four murine NFI genes in many tissues including the pituitary suggest roles for the NFI factors in developmental regulation (Ref. 56 and Lyons, G., personal communication). Gene ablation experiments in mice are beginning to dissect the unique roles of individual NFI genes. Loss of the Nfia gene results in peri-natal death associated with nervous system defects (57, 58). Mice lacking functional Nfib genes also are not viable and have incomplete nervous system and lung development (59). The Nfic gene appears to play functions in mouse tooth root development (60). To date, Nfi gene knockout animals have not displayed overt pituitary-associated phenotypes, but the roles of NFI factors in pituitary development may have yet to be revealed due to compensation or redundancy phenomena.

In this study, we have characterized the proximal elements that coordinate the basal production of the two major Lhx3 gene mRNAs and have identified two classes of trans-acting...
factors that regulate this activity. Our studies indicate that both positive and negative cis-acting sequences function within the approximately −4.8 kb upstream of the hLHX3a promoter (Fig. 2A). A recent annotation of the human genome draft (accessed at the NCBI) indicates that the Q5CN6I1/SOXN gene encoding the quiescin Q6-like 1 protein, a putative sulfuryl hydroxylase (61), may be located as close as 1.2 kb from the hLHX3a transcription initiation sites. The close location of this gene is an important consideration in interpretation of future studies investigating transcriptional control elements that lie upstream of hLHX3. Furthermore, the basal promoters described here have some activity in nonpituitary cells: additional gene regulatory pathways in addition to those characterized herein are required to correctly guide the restricted temporal and spatial expression of the LHX3 mRNA. Future experiments will map the genomic regions required for these repressive and/or activating pathways and to understand how the promoters are differentially or coordinately controlled.

Autoregulation appears to be an important mechanism by which pituitary transcription factor genes participate in the establishment of stable cell fates during development (e.g. Refs. 62 and 63). The experiments shown in Fig. 5A suggest that this may also be true for the hLHX3a promoter. In addition, recent reports have demonstrated that paired-like homeodomain (PITX) class transcription factor genes act upstream of Lhx3 in pituitary development (64, 65). In preliminary experiments, we have tested the responses of the hLHX3a and hLHX3b promoters described in this report in cotransfection assays using rodent PITX1 and PITX2c expression vectors. In the presence of either PITX1 or PITX2c transcription from the −2.7 kb hLHX3a promoter is moderately increased (2- to 3-fold). The intron 1a/hLHX3b promoter is induced to a similar degree by PITX2c, but is not affected by PITX1 (Yaden, B. C., and S. J. Rhodes, unpublished observations). These data suggest that PITX proteins might exert some of their effects by actions at proximal Lhx3 promoters, but further studies will be required to examine whether additional direct and indirect mechanisms mediate the induction of Lhx3 by PITX-dependent pathways.

To date, the mutations in the hLHX3 gene that have been associated with combined pituitary hormone-deficiency diseases are located within the protein-coding regions of the gene (19). The transcriptional regulatory elements that we have characterized here provide candidate regions for diagnostic analyses looking for genetic lesions causing combined pituitary hormone-deficiency diseases of unknown etiology.

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