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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)

THE *Lhx3/P-LIM/LIM3* GENE encodes LIM-homeodomain transcription factors with essential roles in neuroendocrine development (reviewed in Ref. 1). During anterior pituitary development, *Lhx3* participates with other regulatory genes, such as *Sf1*, *Prop1*, *Pit1*, *Egr1*, *Pitx1*, *Pitx2*, and *Tpit*, to guide the specification of differentiated hormone-secreting cells (reviewed in Refs. 2–4). In mammals, the *Lhx3* gene is expressed in the embryonic nervous system and in the primordial and mature pituitary gland (5–8). Mice homozygous for a null *Lhx3* allele die after birth and feature incomplete structural development of the pituitary and defective motor neuron specification (9–11). In these animals, some anterior pituitary corticotrope cells are functional, but expression of the characteristic hormones of the gonadotrope, thyrotrope, somatotrope, and lactotrope cell types is not detectable, indicating that *Lhx3* is required for the eventual differentiation of these specialized lineages (9). These observations are consistent with molecular studies demonstrating that LHX3 and related LIM-homeodomain factors can activate anterior pituitary expressed genes, including those encoding αGSU, PRL, FSHβ, TSHβ, the GnRH receptor, and the Pit-1 transcription factor (*e.g.* Refs. 6 and 12–16). Furthermore, mutations in the human *LHX3* (*hLHX3*) gene that are predicted to lead to the production of disabled proteins cause recessive genetic diseases featuring combined pituitary hormone deficiency and other symptoms (17–19).

The *hLHX3* gene contains seven coding exons and six introns that span approximately 8.7 kb located within the subtelomeric region of chromosome 9 (Fig. 1A) (20). The mouse *Lhx3* gene has a similar organization (21). The single *hLHX3* gene produces two major mRNAs known as *hLHX3a* and *hLHX3b* (13). Translation from the first methionine codons of the *hLHX3a* and *hLHX3b* mRNAs generates the LHX3a and LHX3b protein isoforms (13). These proteins have identical LIM domains, a central DNA-binding homeodomain and a carboxyl terminus that contains the major activation domain but have distinct amino termini resulting from alternate use of 5′ exons in the gene (20, 22). A third protein isoform, M2-LHX3, is generated by preferential translation of the second in-frame methionine codon of the *hLHX3a* mRNA (22). The three LHX3 protein isoforms display different biochemical and functional properties (13, 14, 22, 23).

In this study, we investigated the transcriptional mechanisms by which mammalian *Lhx3* genes generate multiple mRNAs that encode proteins with diverse regulatory properties. Phylogenetic comparisons and functional tests were used to map two conserved, TATA-less, GC-rich promoters that guide transcription of the two mRNAs. Two kilobases of the *hLHX3a* promoter and 1.8 kb of intron 1a (a region that contains the *hLHX3b* promoter) mediate basal activity. The specificity protein 1 (Sp1) transcription factor binds to proximal GC boxes and is a strong regulator of both promoters. Furthermore, a critical upstream element within intron 1a

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Abbreviations: BLASTN, Basic local alignment tool nucleotide; ChIP, chromatin immunoprecipitation; NFI, nuclear factor I; PITX, paired-like homeodomain transcription factor; RLM-RACE, RNA ligase-mediated rapid amplification of cDNA ends; Sp1, specificity protein 1.

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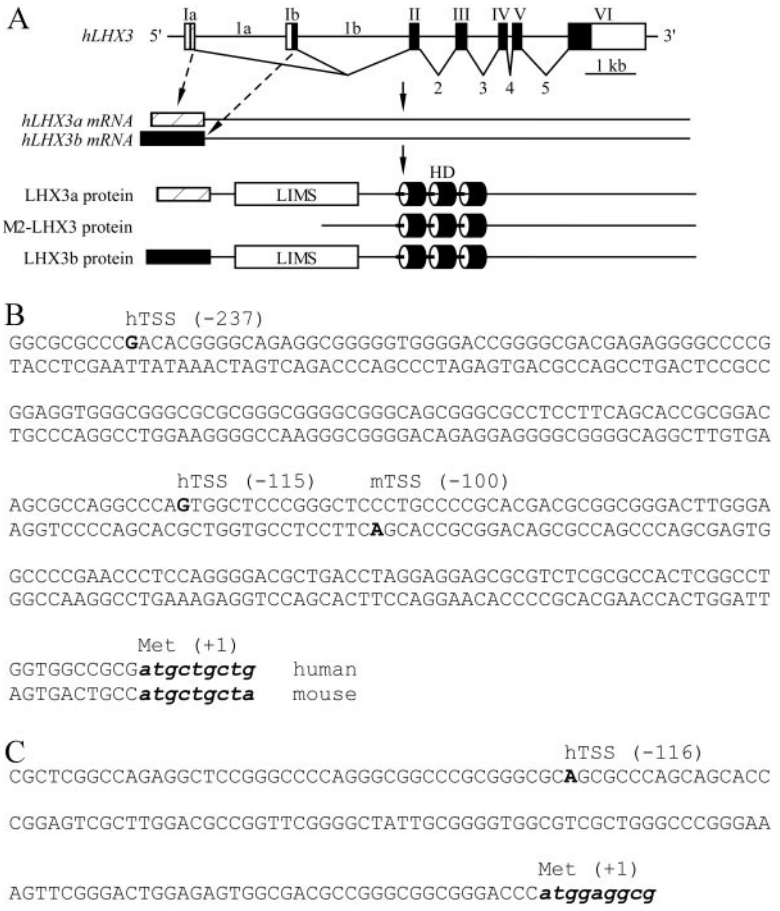

FIG. 1. The human and mouse *Lhx3* genes feature two GC-rich, TATA-less promoters with multiple transcription start sites. A, Structure of the human *LHX3* gene. Exons are depicted by *boxes* with translated regions shown in *black* or *hatched*. Introns are indicated by *lines*. The major mRNA products 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 αT3–1 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.

allows regulation by nuclear factor I (NFI) family transcription factors.

Materials and Methods

Cloning of mammalian Lhx3 gene and cDNA sequences

Fragments of the *hLHX3* gene (20) were amplified by touchdown PCR using bacterial artificial chromosome clone RPC11–83N9 (Sanger Centre, Cambridge, UK) or normal human genomic DNA as substrates. Primers were designed based on human genome sequence data accessed through GenBank at the National Center for Biological Information (NCBI). The upstream (*hLHX3a* promoter) region of the gene was amplified in approximately 500-bp increments using an antisense primer (5′-cctcctaggtcagcgtcccctg-3′) and one of the following sense primers: 5′-gtcttgagtcctcagcagtgct-3′ (580 bp upstream of exon 1a), 5′-gagactcacaggacaagacccttga-3′ (1.096 kb), 5′-agagctggatgccacctttagg-3′ (1.581 kb), 5′-tgcttcgtgtctcactcgagag-3′ (2.080 kb), 5′-tgcacacacaagcatctcactc-3′ (2.701 kb), 5′-cctgctccaggctgccaagtgt-3′ (3.240 kb), and 5′-gtagtccggaaagggccagtgt-3′ (~4.8 kb). For the *hLHX3b* promoter upstream in intron 1a, three regions were amplified using an antisense primer (5′-cgccactctccagtcccgaacttt-3′) and one of the following sense primers: 5′-gcgtgtgctccagctcaggcctct-3′ (1804 bp upstream), 5′-ggtaacaagtgctgtgcaaagtga-3′ (1267 bp), and 5′-agtgcccgtcagctcttgcacaca-3′ (418 kb). 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.html) 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 exclusively 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′-gagatccgctgtgtgcc-3′ and 5′-ccttgcagtacacgctctcc-3′ were designed from the putative exon 2 sequence and used to obtain a full coding sequence bovine *Lhx3b* cDNA clone via iterative screen (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 (Epicentre), and 5′-atgctgctggaaacggggctcga-3′ (monkey exon 1a), 5′-gaatctctcggcgcaggtccgcc-3′ (monkey exon 1b) or 5′-atgctgctggaaacggagctggc-3′ (pig exon 1a), and 5′-ggatctctcggcgcaggtcctcc-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 αT3–1 pregonadotrope cell (29) total RNA and primed with 5′-ggcagtcgctgcacttgagacactt-3′. Second-strand cDNA was generated using primers from the manufacturer and the following gene-specific primers: 5′-gagacgcgctcctccgaggtca-3′ (5′ RACE *Lhx3a*) and 5′-tctccagtcccgaacttt-3′ (5′ RACE *Lhx3b*).

S1 nuclease assays

S1 nuclease assays were performed as described (30). Briefly, ^{32}P end-radiolabeled single-stranded DNA probes for the *hLHX3a* and *hLHX3b* promoters were generated. S1 digestion reactions contained 15 μg of αT3–1 RNA hybridized with labeled probes. Radiolabeled DNA products were analyzed by electrophoresis through 12% polyacrylamide 8 M urea gels. A ^{32}P end-labeled 1-kb extension ladder (Invitrogen) was used as a molecular marker.

Cell culture and transfection

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 lysate 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 PROP1 (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 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 1×10^6 LβT2 cells were cross-linked with formaldehyde and then lysed. Cellular DNA was sonicated to fragments of 200-1000 bp. The supernatant from the sonicated lysate was then precleared 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′-agtcagacccagccctagagtga-3′ and 5′-actaatccagtggttcgtgcggg-3′ (mouse *Lhx3a* promoter region); 5′-aaaggcctggggctggtcctag-3′ and 5′-ggtcagggaacactagcttggag-3′ (NFI site region in mouse intron 1a/*Lhx3b* promoter); and 5′-ctcctgctcgaagtctagagc-3′ and 5′-gctggtgataagtggccttgg-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 QuikChange 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′-cagaggggtgaggttgggggctgccttgag-3′ (−291 Sp1 site in *hLHX3a* promoter); 5′-cccgggaggtggttgggcgcgcgggcgggg-3′ (−181 Sp1 site in *hLHX3a* promoter); 5′-gcgacccccggcccctcccttcccgttgccctttcccccggccg-3′ (−203/−185 Sp1 sites in intron 1a); and 5′-ggagggctggcgggatgccagcagggtgggccgcc-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 8×10^7 cpm/ml of a ^{32}P end-radiolabeled DNA probe (30–60 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 LβT2 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′-gaagtcttggtttcagcagccc-3′ and 5′-aatgggttgtgcaccttgcctt-3′ for the NFI-A isoform; 5′-gggaactggagtcaacttccca-3′ and 5′-ggtggagttcgagttgagatga-3′ for NFI-B; 5′-acttccaggagagctttgtcac-3′ and 5′-tgggggacgggctgttgaatg-3′ for NFI-C; and 5′-aagtactgatggggagcggctc-3′ and 5′-tgctggtggaaggaggggaggt-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 Ia, one for the mouse *Lhx3a* promoter and one for a *hLHX3b* promoter upstream of exon Ib 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

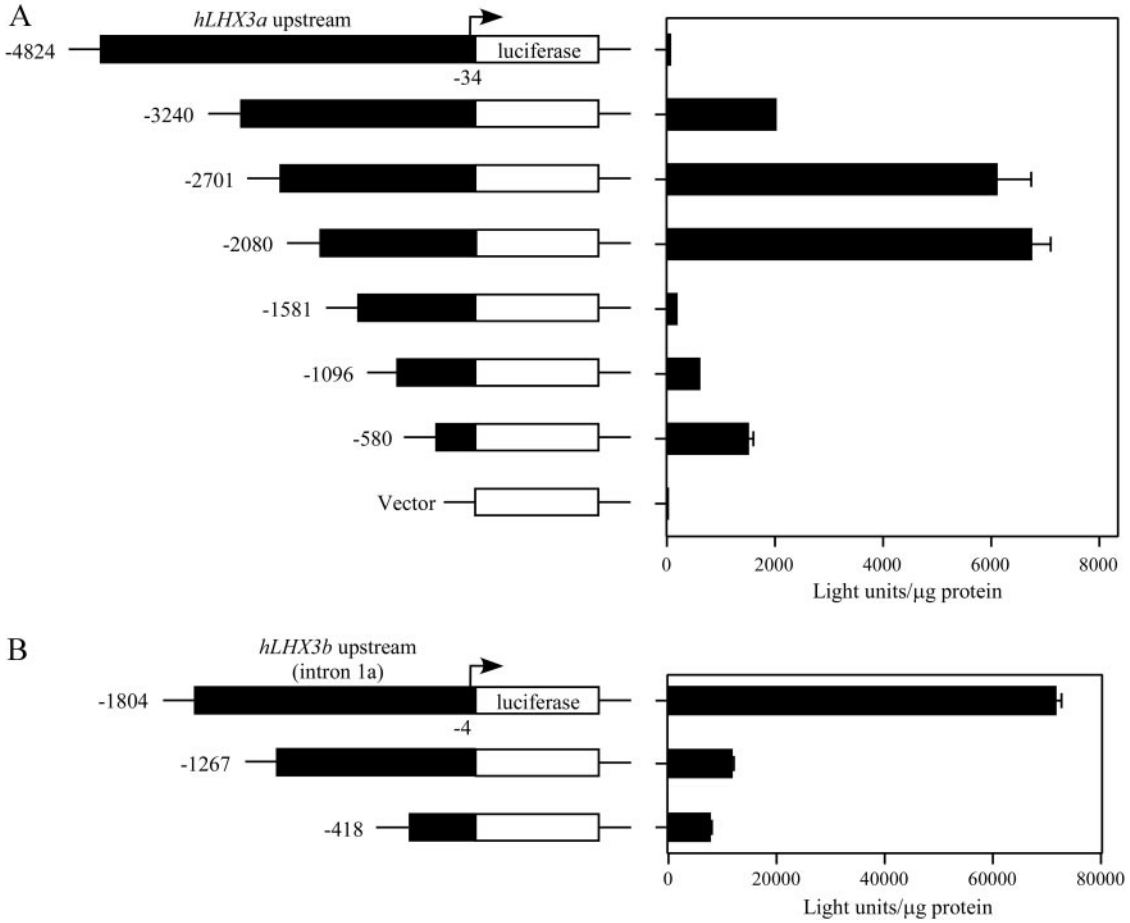

FIG. 2. The two *hLHX3* gene promoters are active in pituitary cell types that express LHX3. Molar equivalents of *luciferase* reporter genes with the indicated 5′ flanking regions of the *hLHX3a* (A) and *hLHX3b* (B) promoters were transiently transfected into mouse pituitary gonadotrope LβT2 cells and the basal activities determined. Similar data were obtained using mouse αT3–1 pregonadotrope pituitary cells. Promoter activity 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.

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*.

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-

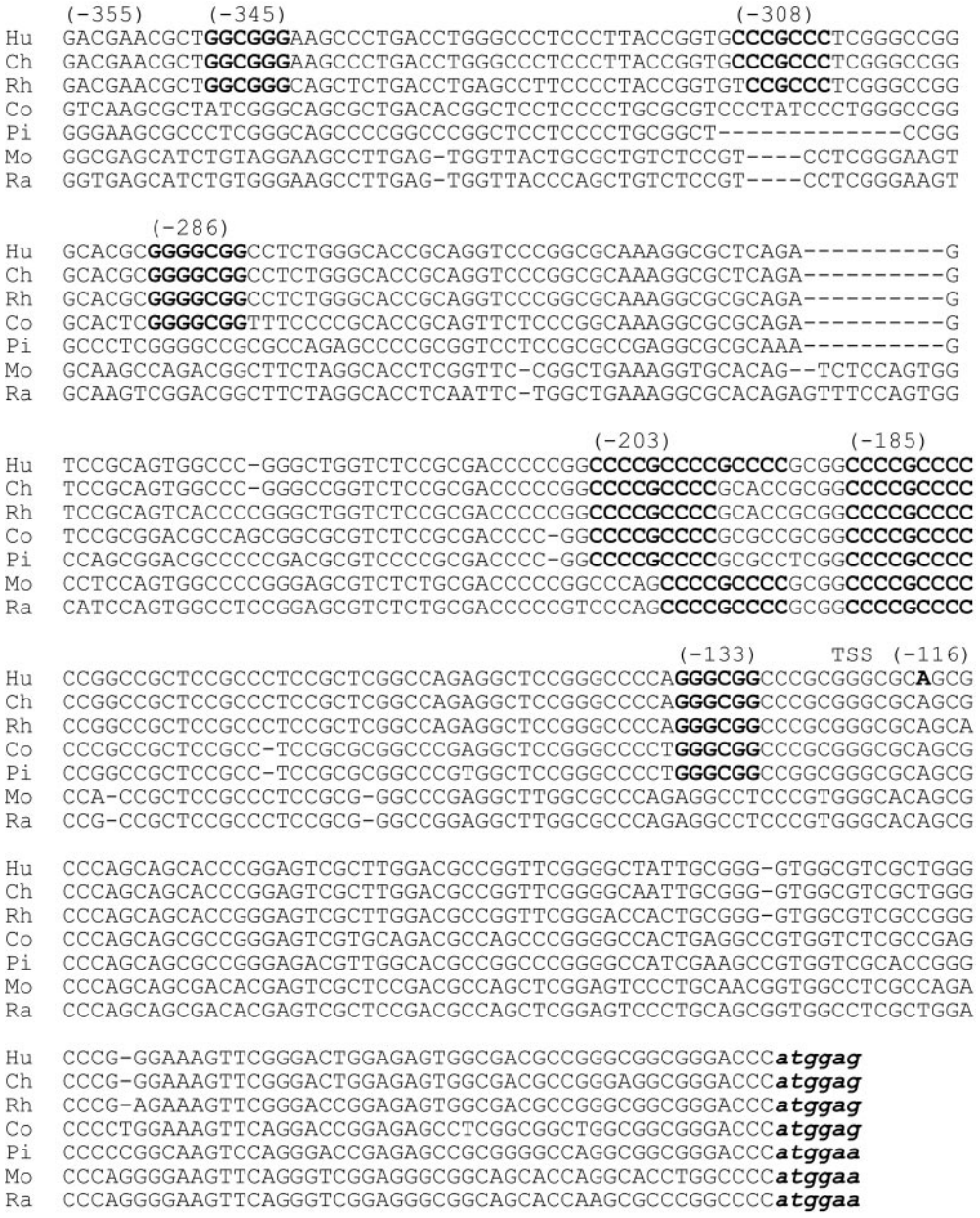

FIG. 4. Comparative sequence analysis of *Lhx3b* proximal promoter regions. The entire bovine (Co) *Lhx3* gene and intron 1a from the human (Hu), rhesus monkey (Rh), pig (Pi), and cow *Lhx3* genes were cloned and sequenced (see *Materials and Methods*). The chimp (Ch), mouse (Mo), and rat (Ra) *Lhx3b* 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*.

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

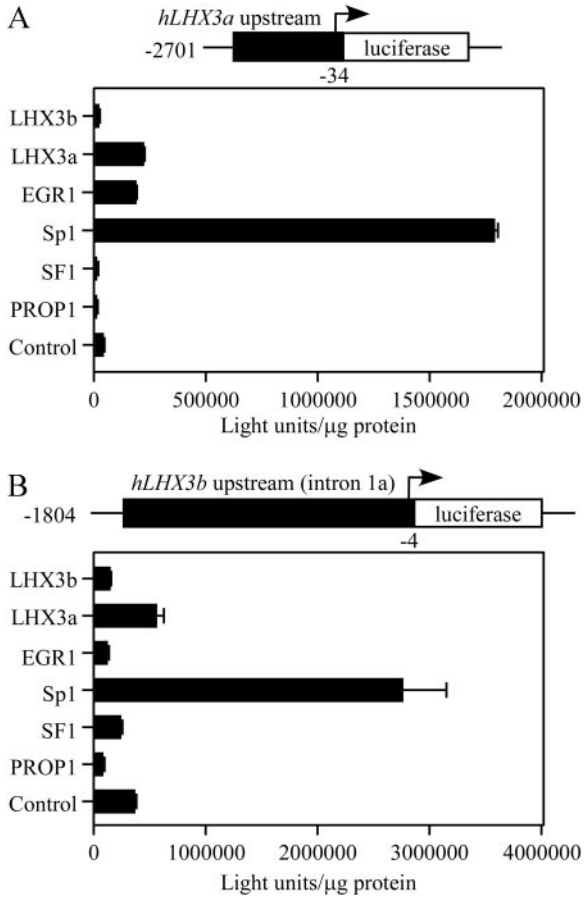

FIG. 5. The *hLHX3* promoters are activated by Sp1. *Luciferase* reporter genes with the indicated 5′ flanking regions of the *hLHX3a* (A) and *hLHX3b* (B) promoters were transiently cotransfected into HEK 293T cells with expression vectors for the indicated transcription factor cDNA. Negative controls (Control) received equivalent amounts of empty expression vector plasmid. Luciferase activities were determined 48 h posttransfection as described in Fig. 2.

hLHX3 promoters. After extended exposures of EMSA gels to film, the −291 element from the *hLHX3a* promoter formed a very weak complex with proteins from LβT2 pituitary cells that was disrupted by the addition of anti-Sp1 antibodies (data not shown). In addition, a faster-migrating complex was observed that was not affected by anti-Sp1 (Fig. 6A). This −291 sequence was bound by purified, recombinant Sp1 protein (Fig. 6A, right panel). The −181 *hLHX3a* GC box interacted with LβT2 cell proteins, and the interaction was prevented by the anti-Sp1 antibodies (Fig. 6A). Consistent with this observation, this site was strongly bound by recombinant Sp1 protein. The −165 element formed a faster-migrating complex with pituitary cell proteins, but separate experiments using anti-Sp1 antibodies or pure Sp1 protein indicate that this is likely not a high-affinity Sp1-binding site. We conclude that pituitary cell proteins recognize the GC boxes in the *hLHX3a* promoter 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 *hLHX3b* 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 occupation 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 reduce 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-

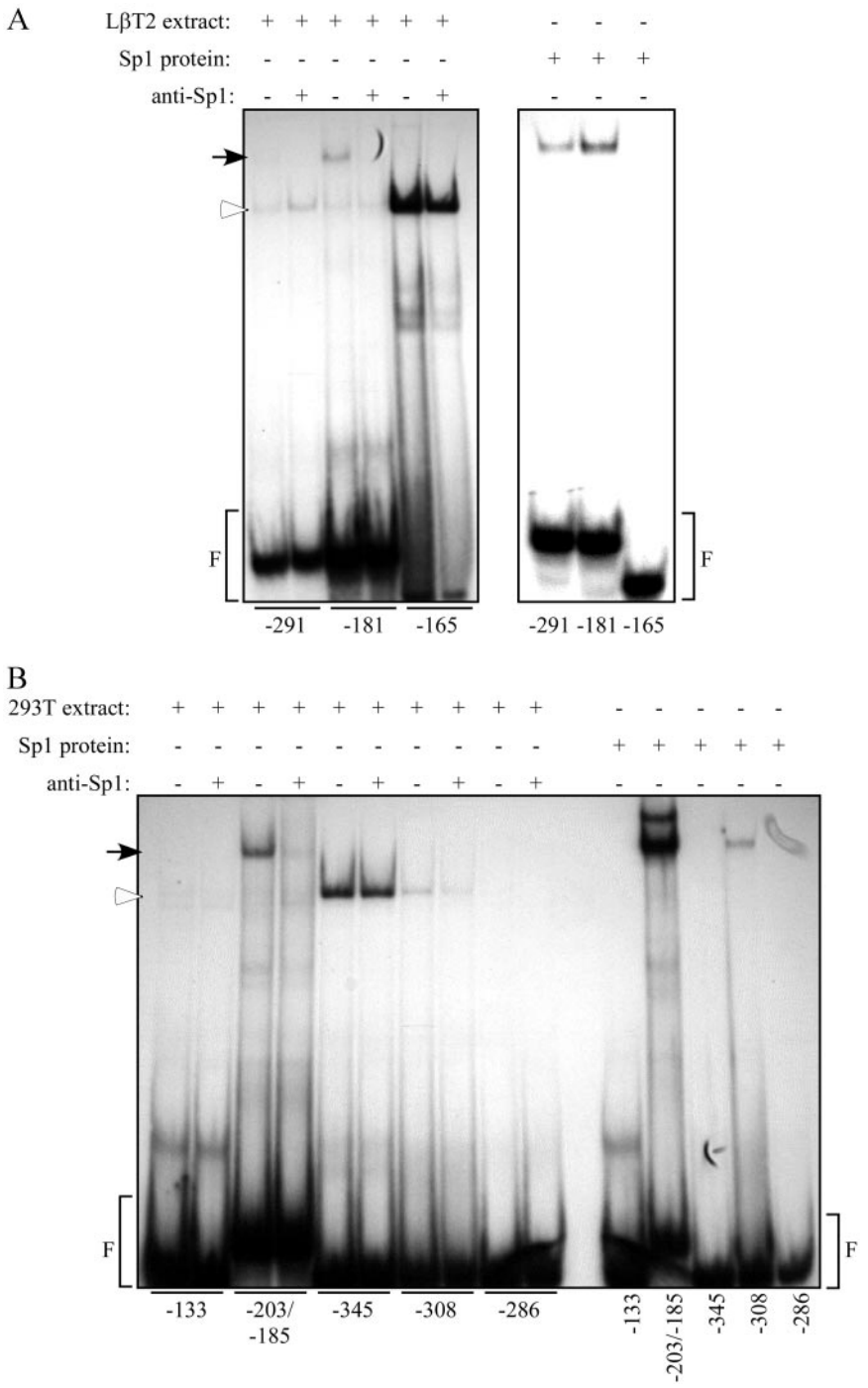

FIG. 6. Sp1-binding sites in the *hLHX3a* and *hLHX3b* proximal promoters. EMSA experiments were performed. A, Radiolabeled DNA probes representing the −289-, −179-, and −162-bp regions were incubated with protein extracts from pituitary gonadotrope LβT2 cells (left panel) and the resulting complexes were separated by electrophoresis. Anti-Sp1 antibodies were used to disrupt complexes containing Sp1 (*black arrow*) as indicated. Some sites produce faster-migrating complexes that do not contain Sp1 (*open arrowhead*). The −291 element forms a very weak complex with LβT2 proteins that can only be seen on extended exposures (not shown). In the right panel, purified recombinant Sp1 protein was used. F, free, unbound DNA. B, Radiolabeled DNA probes representing the indicated regions of the *hLHX3b* promoter were incubated with protein extracts from HEK 293T cells or purified recombinant Sp1 protein and the resulting complexes separated by electrophoresis. Anti-Sp1 antibodies were used to disrupt cell extract complexes containing Sp1 (*black arrow*). Some DNA elements form smaller complexes that do not contain Sp1 (*open arrowhead*). Similar data were obtained using pituitary LβT2 protein extracts (not shown).

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 pituitary gonadotrope 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-

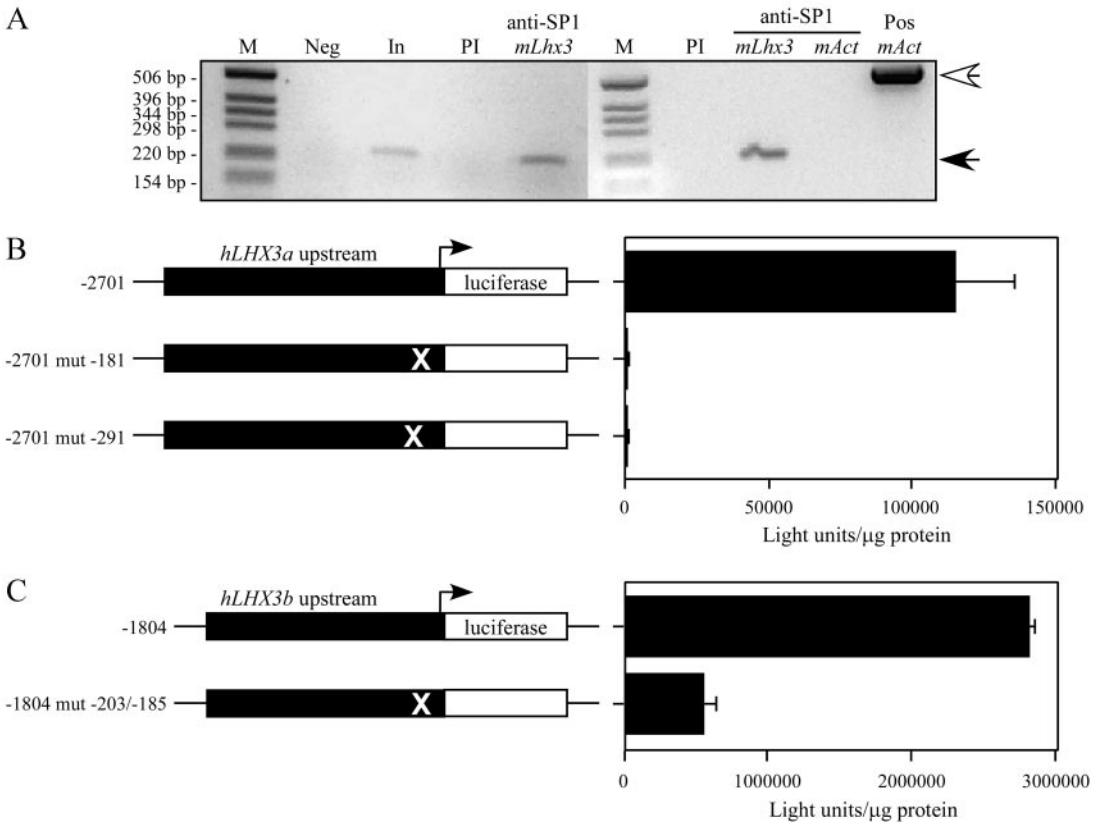

FIG. 7. Sp1 regulation of human and mouse *Lhx3* gene promoters. A, ChIP experiments demonstrate Sp1 occupation of the proximal region of the endogenous mouse *Lhx3a* promoter in LβT2 pituitary cells. A gel displaying separation of the amplified genomic DNA region fragments is shown. anti-SP1 *mAct*, Anti-Sp1 antibody reaction used as a substrate in a PCR for a region of the *β-actin* gene (negative control); anti-SP1 *mLhx3*, anti-Sp1 antibody reaction used as a substrate in a PCR for the *mLhx3a* promoter (*closed arrow*); In, input positive control; M, molecular markers (in base pairs); Neg, negative control (no substrate); PI, preimmune negative control; Pos *mAct*, *β-actin* fragment amplification (*open arrow*) from input DNA. B, A wild-type *hLHX3a* promoter *luciferase* reporter gene or equivalent constructs with mutations of the indicated Sp1-binding GC boxes were transiently transfected into pituitary LβT2 cells, and their activities were determined. Promoter activity 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. C, A similar approach was used to determine the importance of the proximal Sp1-binding sites in the *hLHX3b* promoter.

trope 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 −1454 to −1426 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*

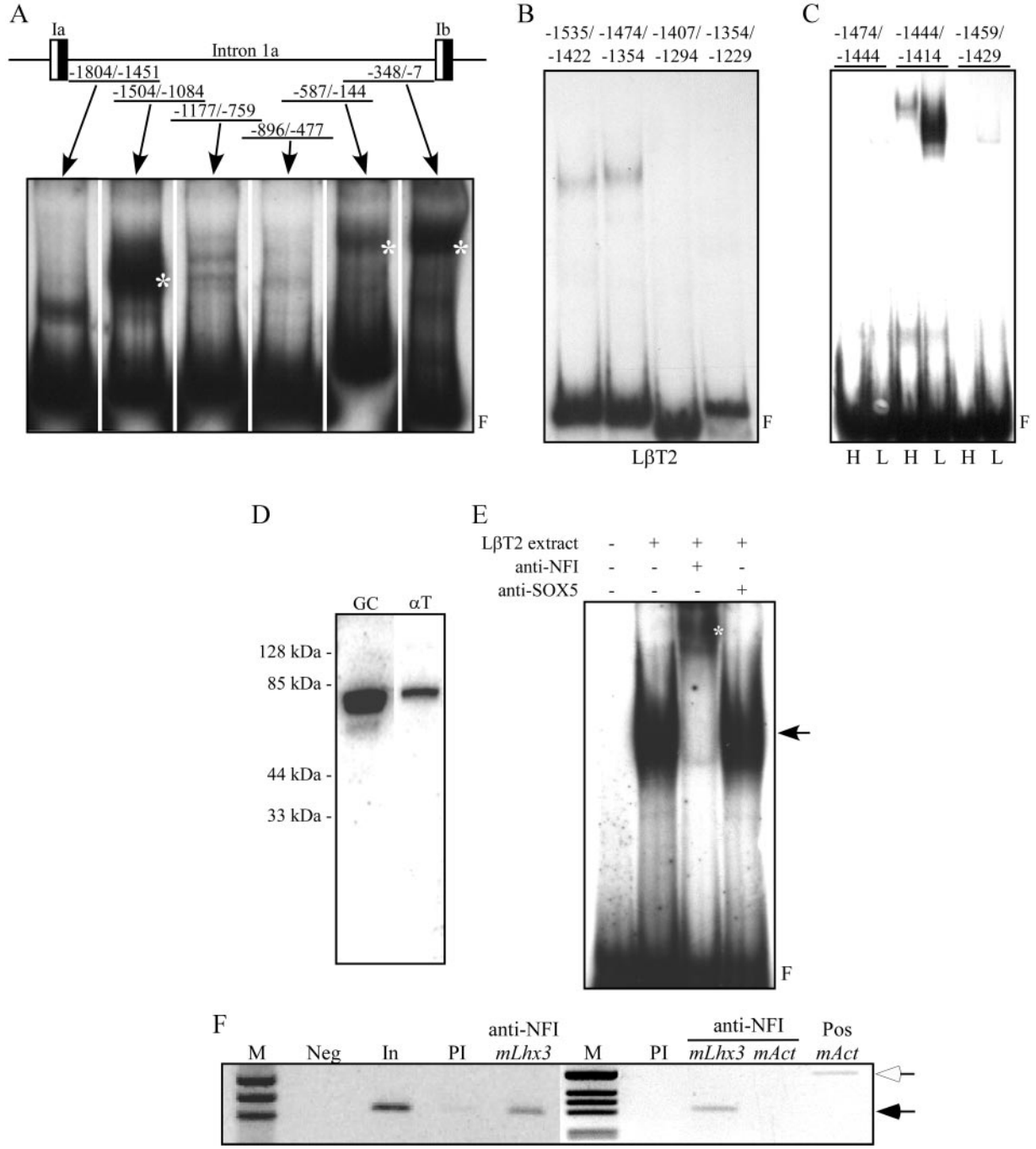

FIG. 8. A distal upstream region of intron 1a recognized by NFI factors is critical for activity of the *hLHX3b* promoter. A, Experiments shown in Fig. 2B demonstrated that an element between −1804- and −1267-bp of intron 1a is critical for activity of the *hLHX3b* promoter. To identify regions within 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, Lβ*T2* 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 Lβ*T2* 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 Lβ*T2* 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 *mAct*, 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 *mAct*, *β-actin* fragment amplification from input DNA (*open arrow*).

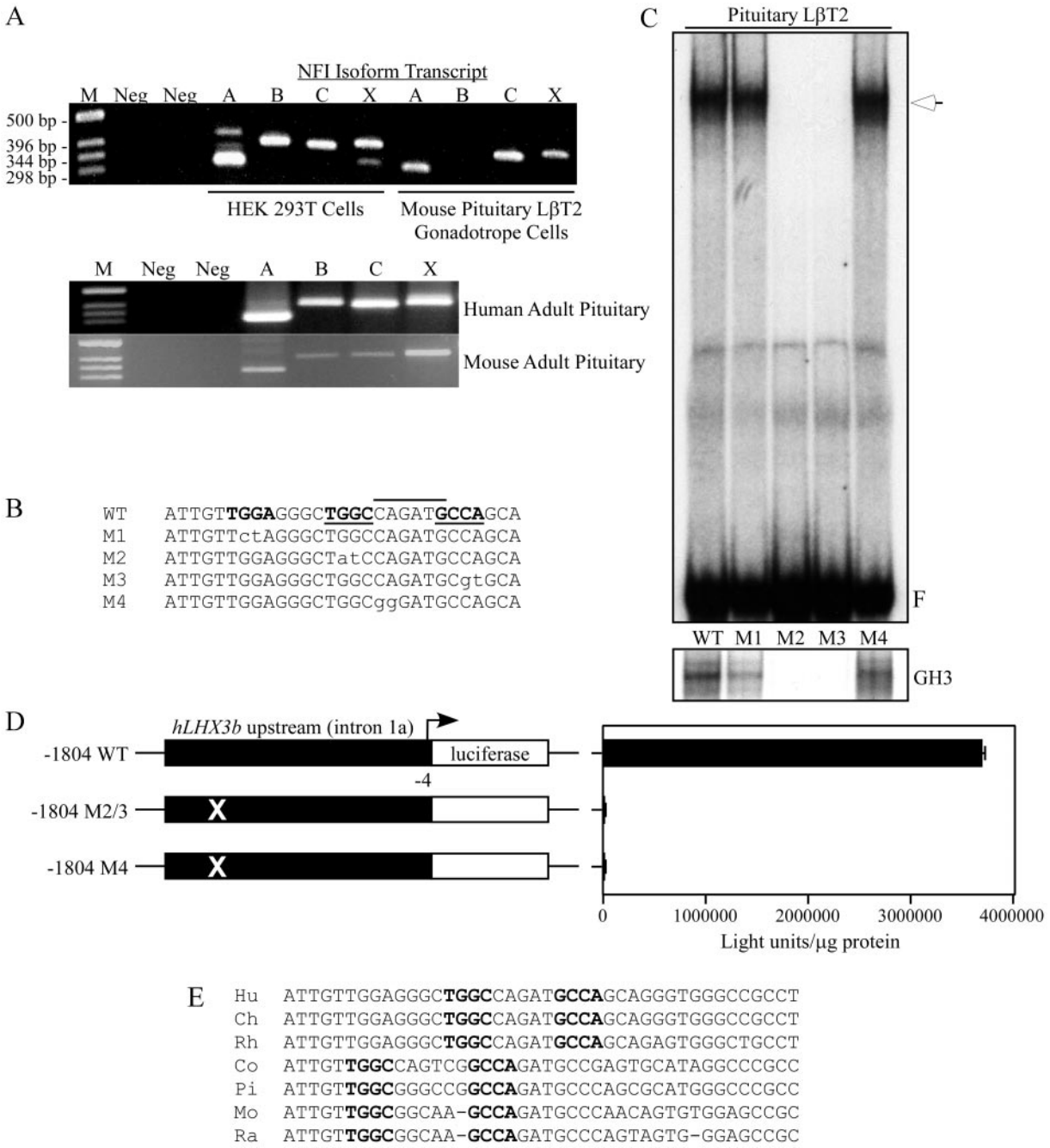

FIG. 9. Regulation of the *hLHX3b* promoter by NFI factors through interaction with a conserved, critical distal element within intron 1a. A, NFI factors are expressed in pituitary LβT2 gonadotropes and adult pituitary glands. RT-PCR analysis of NFI isoform mRNA expression. Isoform-specific primers were 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 TFIID 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, Southwestern 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* promoter 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 perinatal 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 *QSCN6L1/SOXN* gene encoding the quiescin Q6-like 1 protein, a putative sulfhydryl oxidase (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* mRNAs. 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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