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Robert D. Ward
University of Michigan

MinChul Cho
University of Michigan

S. A. Camper
University of Michigan

Shannon W. Davis
University of Michigan

Lori T. Raetzman
University of Michigan

See next page for additional authors

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Authors

Robert D. Ward, MinChul Cho, S. A. Camper, Shannon W. Davis, Lori T. Raetzman, Timothy P.L. Smith, Constance Esposito, Robert H. Lyons, Jan-Fang Cheng, Edward M. Rubin, and Simon J. Rhodes

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Comparative genomics reveals functional transcriptional control sequences in the *Prop1* gene

Robert D. Ward · Shannon W. Davis · MinChul Cho · Constance Esposito · Robert H. Lyons · Jan-Fang Cheng · Edward M. Rubin · Simon J. Rhodes · Lori T. Raetzman · Timothy P. L. Smith · Sally A. Camper

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Abstract Mutations in *PROP1* are a common genetic cause of multiple pituitary hormone deficiency (MPHD). We used a comparative genomics approach to predict the transcriptional regulatory domains of *Prop1* and tested them in cell culture and mice. A BAC transgene containing *Prop1* completely rescues the *Prop1* mutant phenotype, demonstrating that the regulatory elements necessary for proper *PROP1* transcription are contained within the BAC. We generated DNA sequences from the *PROP1* genes in lemur, pig, and five different primate species. Comparison of these with available human and mouse *PROP1* sequences identified three putative regulatory sequences that are highly conserved. These are located in the *PROP1* promoter proximal region, within the first intron of *PROP1*, and downstream of *PROP1*. Each of the conserved elements elicited orientation-specific enhancer activity in the context of the *Drosophila alcohol dehydrogenase* minimal promoter in both heterologous and pituitary-derived cells lines. The intronic element is sufficient to confer dorsal expansion of the pituitary expression domain of a transgene, suggesting that this element is important for the normal spatial expression of endogenous *Prop1* during pituitary development. This study illustrates the usefulness of a comparative genomics approach in the identification of regulatory elements that may be the site of mutations responsible for some cases of MPHD.

R. D. Ward · MinChul Cho · S. A. Camper
Graduate Program in Cellular and Molecular Biology, University of Michigan, Ann Arbor, Michigan, USA

S. W. Davis · L. T. Raetzman · S. A. Camper
Department of Human Genetics, University of Michigan, Ann Arbor, Michigan, USA

C. Esposito · R. H. Lyons
DNA Sequencing Core Facility, University of Michigan, Ann Arbor, Michigan, USA

J.-F. Cheng · E. M. Rubin
DOE Joint Genome Institute, U.S. Department of Energy, Walnut Creek, California, USA

S. J. Rhodes
Department of Biology, Indiana University-Purdue University Indianapolis (IUPUI), Indianapolis, Indiana, USA

T. P. L. Smith
U.S. Meat Animal Research Center, U.S. Department of Agriculture, Agricultural Research Service (USDA/ARS), Clay Center, Nebraska, USA

S. A. Camper (✉)
4909 Buhl Bldg., 1241 E. Catherine St., Ann Arbor
MI 48109-0618, USA
e-mail: scamper@umich.edu

Present Address:
L. T. Raetzman
Department Molecular and Integrative Physiology, University of Illinois, Urbana, Illinois, USA

Present Address:
R. D. Ward
Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas, USA

Introduction

All vertebrates have pituitary glands composed of specialized hormone-producing cells (Matsumoto and Ishii 1987). The individual hormones are evolutionarily conserved, although their function varies across the classes of *Animalia*. This conservation suggests that genetic regulation of pituitary function may be conserved.

In humans, growth insufficiency resulting from pituitary hormone deficiency is not infrequent, occurring in approximately 1 in 4000 live births (Procter et al. 1998; Vimpani et al. 1977). Growth hormone (GH) insufficiency is the most common type of dwarfism and usually results from mutations in the GH gene cluster (Braga et al. 1986; Mullis et al. 1990). Multiple pituitary hormone deficiencies (MPHD) result from mutations in transcription factors important for the normal development and function of the pituitary gland, including *POU1FI* (*PIT1*), *Prophet of PIT1* (*PROP1*), *HESX1*, *LHX3*, *LHX4*, and *SOX3* (Bhangoo et al. 2006; Cogan et al. 1998; Laumonnier et al. 2002; Machinis et al. 2001; Mendonca et al. 1999; Netchine et al. 2000; Pfäffle et al. 1992; Radovick et al. 1992; Tajima et al. 2003; Wu et al. 1998). The first transcription factor to be linked to MPHD was PIT1 (Tatsumi et al. 1992). Patients with mutations in *PIT1* generally have deficiencies in GH, prolactin (PRL), and thyroid-stimulating hormone (TSH) as well as profound pituitary hypoplasia (Cohen et al. 1996). Mutations in *PROP1* are a common genetic cause of familial MPHD. Patients with *PROP1* mutations exhibit progressive hormone loss with varying age of onset and severity (Bottner et al. 2004; Fluck et al. 1998). Most common are deficiencies in PRL, GH, and TSH as well as follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (Agarwal et al. 2000; Deladoey et al. 1999). Progressive adrenocorticotrophic hormone (ACTH) loss presents as late as the third decade of life (Bottner et al. 2004). Some MPHD cases, however, cannot be traced to mutations in the protein-coding or intron-exon splice sites in the DNA sequence of known genes important for pituitary development.

The mechanism of *PROP1* action has been studied extensively in two mouse models, the Ames dwarf (*Prop1*^{df}) and the *Prop1*^{null} (Nasonkin et al. 2004; Ward et al. 2005). In mice, *Prop1* is expressed throughout the developing Rathke's pouch in a dorsal to ventral expression gradient from about embryonic day 10 (e10) until about e16 (Sornson et al. 1996). *PROP1* transcripts are present in the adult pituitaries of human and pig, although the levels were not quantified relative to the embryonic pituitary (Nakamura et al. 1999a, 1999b; Skelly et al. 2000; Sloop et al. 2000; Usui et al. 2000). A number of *Prop1* downstream targets have been identified, including *Pit1, Hesx1, Tle3*, and *Notch2* (Brinkmeier et al. 2003; Gage et al. 1996a; Raetzman et al. 2004).

It is of great interest to identify the transcriptional regulators of *Prop1* because it is pituitary-specific, unlike many of the other key regulators of pituitary development: *Pitx1*, *Pitx2*, *Lhx3*, *Lhx4*, and *Hesx1*. Each of these genes is expressed prior to *Prop1* and is a candidate for transcriptional regulation of *Prop1*. *Prop1* expression is activated in *Pitx1* and *Pitx2* single mutants, but this may be due to the ability of *Pitx1* and *Pitx2* to compensate for each other (Suh et al. 2002; Szeto et al. 1999). *Prop1* expression also appears to be initiated normally in *Hesx1*- and *Lhx4*-deficient mice (Dasen et al. 2001; Raetzman et al. 2002). Thus, the spatial and temporal regulation of *Prop1* expression is not fully explained by these genes, suggesting that additional factors may be involved.

The regulation of gene expression involves the cooperation of a variety of transcription factors in a tissue-, temporal-, and/or spatial-specific fashion that interact with *cis*-acting regulatory elements in DNA sequences (Kleinjan and van Heyningen 2005). The identification of these elements can be difficult because they may be located at a considerable distance from the gene or even within the introns of neighboring genes (Bagheri-Fam et al. 2001; Lang et al. 2003; Lettice et al. 2003). The identification of these elements is facilitated by comparative genomics in the form of cross-species DNA comparisons. The alignment of the DNA sequences of orthologous genes from different species, both closely and distantly related, can reveal potential conserved regulatory elements that can then be analyzed *in vivo* (Boffelli et al. 2004; Nobrega and Pennacchio 2004; Pennacchio and Rubin 2001). Transcription factors involved in development, like *Prop1*, are often conserved among vertebrates, and their regulatory sequences are also likely to be conserved (Plessy et al. 2005).

In this study we report the sequence of the *PROP1* gene from several mammals and utilize cross-species PROP1 protein sequence comparison to verify the conservation of the functional domains of the protein and use genomic sequence comparison to identify putative transcriptional regulatory elements in the noncoding regions of the *Prop1* gene. This analysis revealed the presence of three conserved noncoding elements within and near the gene. Each of them exhibited orientation-dependent enhancer activity in tissue culture, and an element in intron 1 conferred tissue-specific and unique spatial expression in transgenic mice. These studies establish a functional role for the intronic element in *Prop1* gene regulation. Finally, using bacterial artificial chromosome (BAC) transgene rescue of the *Prop1* mutant phenotype, we demonstrate that all of the sequences necessary for functional expression of *Prop1* are located within the BAC.

Materials and methods

Protein and DNA sequence

A BAC clone of approximately 200 kb and containing the pig (*Sus scrofa*) *Prop1* gene was identified, and DNA was isolated from it. A shotgun sequencing library was pre-

pared from that BAC by using SeqWright (Houston, TX), and the resulting subclones were sequenced at the University of Michigan Sequencing Core using Applied Biosystems (Foster City, CA) sequencers (Model 3700) and BigDye V1.1 terminator chemistry, according to standard manufacturer's protocols. Sequence assembly of the shotgun sequence data was performed using phred and phrap (Ewing and Green 1998), and consed (Gordon et al. 1998), resulting in 6X draft sequence coverage. Limited finishing was performed based on the ''autofinish'' option in consed to close some gaps, especially those in the vicinity of the *Prop1* gene. A total of ten draft contigs of 1 kb or greater were obtained in the final assembly, accounting for 196 kb, or an estimated 98% of the original BAC. The sequences have a phred Q-score of 20 or higher, with the majority being Q40 or better. The BAC sequences were submitted to GenBank (NCBI; http://www.ncbi.nlm.nih.gov; accession number EF590118). One contig of approximately 26.3 kb contained the *Sus scrofa Prop1* gene, including 13.8 kb of 5′ flanking sequence, 3.7 kb encoding the *Prop1* gene, and 8.7 kb of 3′ flanking sequence. A set of lemur BACs containing the *Prop1* gene were isolated from a library derived from a cell line of the ring-tailed lemur (*Lemur catta*; AG07100C, Coriell Cell Repositories, Camden, NJ) using a human *PROP1* exon 3 probe. One lemur BAC, LBNL-2 102B17, was sequenced from ends of 3 kb subclones to approximately tenfold coverage using BigDye terminators (Applied Biosystems) and assembled into ordered and oriented contigs with the Phred-Phrap-Consed suite (Ewing and Green 1998; Gordon et al. 1998). The assembled BAC sequence was submitted to GenBank with accession number AC162436. *Prop1* gene sequence from gorilla (*Gorilla gorilla*), Black and Red Howler (*Alouatta belzebul*), Brown Capuchin (*Cebus apella*), and Gelada Baboon (*Theropithecus gelada*) was amplified from genomic DNA (gift from Dr Deborah Gumucio, University of Michigan, Ann Arbor, MI) by using the forward primer (5′-CCTGCTCCCAGGAGGGGATT-3′) that corresponded to the human *PROP1* 5′ flanking sequence that was highly conserved between mouse and human, and as the reverse primer (5′-AGGCTGGGGATCACCTTGGTG-3′) that corresponded to the 3′ UTR of the human *PROP1* gene. cDNA sequence was determined by using the high conservation between primate exon splice acceptor/donor sites to assemble the cDNA sequence from the gene sequence. The protein sequence was then translated from the cDNA as described above. The *Prop1* gene sequences were deposited in GenBank with accession numbers DQ177426 for gorilla, DQ177425 for capuchin monkey, DQ177427 for howler monkey, and DQ177424 for baboon.

The 20-kb human *PROP1* was obtained from the human chromosome 5 contig sequences from GenBank accession numbers NT086684 and NT023133. The 20-kb mouse (*Mus musculus*) *Prop1* sequence was obtained from the mouse chromosome 11 contig sequences from GenBank accession number NT096135. The *Prop1* genomic sequence for the chimpanzee (*Pan troglodytes*) was obtained via the Berkley Genome Pipeline from the genome VISTA analysis program [http://www.genome.lbl.gov/vista/index.shtml (Couronne et al. 2003)]. The cDNA and protein sequences were determined as described above. The *Prop1* genomic sequence for the rat and partial genomic sequence for the fugu (*Fugu rubripes*) and zebrafish (*Danio rerio*) were obtained by searching the UCSC genome browser [http://www.genome.ucsc.edu (Kent 2002)] for the closest matches to *PROP1*. The partial protein sequences for the fugu and zebrafish were determined by the translation of the partial gene sequence in all three frames to identify the PROP1 homedomain and transactivation domain sequence. The rat (*Rattus norvegicus*) cDNA sequence was obtained from GenBank (accession number NM153627) and translated as described above. *Prop1* genomic sequence was obtained for the dog (*Canis familiaris*; AF126157) and sheep (*Ovis aries*; AY533708) and PROP1 protein sequence for human (NP006252), pig (NP001001263), cow (*Bos taurus*; NP777103), sheep (NP001009767), mouse (P97458), dog (NP001018643), and partial protein sequence for the chicken (*Gallus gallus*; AB037110) was obtained from the NCBI website.

Sequence analysis

ClustalW alignment for protein and DNA sequences were done with the LASERGENE Navigator Meg align sequence alignment program (DNASTAR Inc., Madison, WI). Mouse, rat, human, and chimp chromosome comparisons were done using the University of California, Santa Cruz (UCSC) genome browser [http://www.genome.ucsc.edu (Kent et al. 2002)]. The pig *Prop1* BAC contigs were compared to the mouse genome using the genome VISTA program [http://www.genome.lbl.gov/vista/index.shtml (Couronne et al. 2003)]. The 20-kb *PROP1* genomic sequences for the human, lemur, pig, and mouse were analyzed with the mVISTA [http://www.genome.lbl.gov/vista/index.shtml (Bray et al. 2003)] comparative genomics program to determine the identity of the conserved noncoding elements.

Plasmid construction

The *CE-A/LacZ* plasmid was constructed for the targeted knockout of the *Prop1* gene (Nasonkin et al. 2004). The *CE-B + CE-A/LacZ* plasmid was constructed by digesting the *CE-A/LacZ* plasmid with *Xho*I and subcloning the region containing 3 kb of the *Prop1* 5′ flanking region, the *LacZ* coding region, and mouse protamine 1 splice and

polyadenylation regions into the *Xho*I site of pBluescript SK+ (Stratagene, La Jolla, CA). A 9.5-kb *Prop1* genomic clone (Nasonkin et al. 2004), which was generated from a P1 clone containing the entire *Prop1* gene, was used as a template to amplify the *CE-B* region with a series of primers that engineered flanking *loxP* sites (5′-ATAAC TTCGTATAGCATACATTATACGAAGTTAT-3′). The floxed *CE-B* fragment was subcloned into pGEM-T Easy (Promega, Madison, WI). This *CE-B loxP* construct was digested with *Not*I/*Xba*I and ligated into the *Not*I/*Xba*I sites of the pBluescript SK+ vector that contained the 3-kb *Prop1* 5′ flanking sequence with the *LacZpA* reporter.

The *αGsu-Prop1ΔCE-B* plasmid was made by creating a chimera of the *Prop1* intron 1 in which the *CE-B* region was deleted. First, the intron sequence for the 5′ flank of *CE-B* was amplified from the 9.5-kb *Prop1* genomic clone (Nasonkin et al. 2004) by using primers (5′-GGTTTGGGTG GCTAGCCATGGAA-3′ and 5′-TTCCCAAGCACCTCC TTCATATCCCACCCCCCAACTAAGCACCC-3′) that allowed this fragment to be annealed to the *CE-B* 3′ flanking sequence that was also amplified from the 9.5-kb *Prop1* plasmid with the primers 5′-CCTCCTATAAGCCTCAGA GCT-3′ and 5′-GGGTGCTTAGTTGGGGGGTGGGATAT GAAGGAGGTGCTTGG G-3′. These two PCR products were engineered with overlapping tails that could be annealed together to create a chimeric *Prop1* intron1 with the *CE-B* region deleted. This chimera was amplified with the primers 5′-GGTTTGGGTGGCTAGCCATGGAA-3′ and 5′-CCTCCTATAAGCCTCAGAGCT-3′, digested with *Nco*I and *Sac*I, and subcloned into the *αGsu-Prop1* plasmid (Cushman et al. 2001) to create the desired transgenic construct. This *αGsu-Prop1ΔCE-B* was subcloned into pBluescript SK+ by an *Eco*R1 partial digest. The *CE-B* + *αGsu-Prop1ΔCE-B* plasmid was made by amplifying the *CE-B loxP* region from the *CE-B* + *CE-A/LacZ* plasmid with the primers 5′-GGTATCGATTACCCTAGAGGGCAGTGCA GTGCCTG-3′ and 5′-GGAATCGATATCTCTTTGCTGT CTATCAATGACGT-3′ that engineered *Cla*I sites at the ends of the PCR product to allow the subcloning of this *CE-B loxP* region into the *Cla*I site of *αGsu-Prop1ΔCE-B*.

The 584-bp *CE-A* region was amplified from the 9.5-kb *Prop1* genomic clone with primers to engineer *Hin*dIII sites at the ends the sequence (5′-GTCTGGAAGCTTGC TGGTGAGGCTG-3′ and 5′-GGAAGCTTGTCTTGGAG AAGAGACCTCCTCCTGG-3′) and subcloned in both the forward and reverse orientations into the *Hin*dIII site of the pDeltaODLO 02 plasmid (Iniguez-Lluhi et al. 1997) obtained from Dr. Jorge Iniguez-Lluhi (University of Michigan, Ann Arbor, MI) that contained the *Drosophila* alcohol dehydrogenase (*ADH*) minimal promoter and the firefly luciferase reporter gene. The 508-bp *CE-B* region was PCR amplified from the 9.5-kb *Prop1* plasmid with primers that engineered *Eco*RI sites at the ends (5′-CGGAAGAATT CTGGTTGCCCAAGGTCC-3′ and 5′-GCCACTCGCAGA ATTCATTTC-3′) and subcloned into pBluescript (Stratagene) at the *Eco*RI site. The *CE-B* was then digested with *Sma*I/*Kpn*I and subcloned onto a version of pGL3basic (Promega) that contained the *TK* minimal promoter inserted into the *Bgl*I/*Hin*dIII sites. The *CE-B* region was released from this plasmid by digestion with *Xho*I and subcloned into the *Xho*I site of pDeltaODLO 02 in both the reverse and forward orientations. The 1196-bp *CE-C* region was amplified from the 9.5-kb plasmid (5′-GGA GTACTGGGACCCTTAAGGCCCTTGGGCTGCAGG-3′ and 5′-GGAGTACTGGAGTCTGAGACAGGAAGACTG AGAG-3′), cloned in to the pGEM-T Easy vector (Promega), digested with *Not*I, and subcloned into the *Not*I site of pDeltaODLO 02 in the forward and reverse orientations.

Cell culture

Monkey fibroblast CV-1 cells (American Type Culture Collection, Manassas, VA), mouse pituitary gonadotrope αT3-1 cells (Dr. Pamela Mellon, University of San Diego, La Jolla, CA), rat anterior pituitary GH3 cells, and mouse pituitary corticotrope AtT-20 cells were maintained at 37°C/5% CO_2 in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat inactivated fetal bovine serum (Hyclone, Logan, UT) and 100 units/ml penicillin-streptomycin (Invitrogen). The GH3 and AtT-20 cell lines were obtained from Dr. Audrey Seasholtz (University of Michigan, Ann Arbor, MI).

Transient transfection and Dual-Luciferase® Reporter Assay

Cells were plated onto 24-well plastic plates (Fisher Scientific, Fair Lawn, NJ) at a density of 0.4×10^5 cells/well for CV-1, 0.7×10^5 cells/well for αT3-1, 1.0×10^5 cells/well for GH3, and 1.2×10^5 cells/well for AtT-20 cells, such that cells were 40%–60% confluent the next day. DNA cocktails totaling 0.3 μg/well [0.08 μg enhancer construct, 0.218 μg pBluescript SK+, 0.002 μg (cytomegalovirus) *CMV-Renilla* luciferase (Promega) internal control in 400 μl serum-free DMEM] were transfected into cultured cells using Fugene 6 (Roche, Indianapolis, IN) at a 12:5 ratio according to the manufacturer's protocol. The pDeltaODLO 02 plasmid that contains the *ADH* minimal promoter with the firefly luciferase reporter gene was used as a negative control and determined as basal level. Forty-eight hours after transfection, Dual-Luciferase Reporter Assay (Promega) was performed according to the manufacturer's protocol and measured using the Lmax Micro plate Illuminometer (Molecular Devices, Sunnyvale, CA) with the SOFTmax Pro software (Molecular Devices). The

results were normalized to the *CMV-Renilla* luciferase internal control. All assays were done in triplicate and the results were repeated a total of three times. Results were averaged and expressed as percent activity over basal.

Maintenance, generation, and genotyping of transgenic mice

To generate the BAC transgenic mice, BAC RP23-250I22 (supplied by Pieter J. de Jong, Childrens Hospital Oakland Research Institute) was purified over a Nucleobond AX column (BD (Biosciences) and injected into pronuclei of fertilized eggs generated from a cross between DF/B-*Prop1*$^{df/+}$ males of mixed genetic background (Buckwalter et al. 1991) and (SJL/J × C57BL/6J) F_1 females. BAC transgenic; *Prop1*$^{df/+}$ mice were crossed to N6-B6-*Prop1*$^{+/-}$ mice (Nasonkin et al. 2004) to generate BAC transgenic; *Prop1*$^{df/-}$ offspring. These mice and all littermates were weighed, photographed, and genotyped at weaning.

The presence of the BAC was assessed by PCR using primers designed to amplify products that span the junction between the BAC backbone and the mouse genomic DNA (Sp6 end 5′-CATATTTTCCCCATCCACCACCAT-3′ and 5′-TTCCCGCAAGAGCAAACACAAC-3′; T7 end 5′-CC GGAAGGAGCTGACTGGGTTGA-3′ and 5′-TGGGCAT TGAGCTTTCTGGGTTTT-3′). Previously described primers were used to genotype the *Prop1*^{df} allele (Cushman et al. 2001) and *Prop1* null allele (Nasonkin et al. 2004).

The *αGSU Prop1* transgenic mouse lines TgN(*Cga-Prop1*)^{D4Sac} and TgN(*Cga-Prop1*)^{D6Sac} (Cushman et al. 2001; Vesper et al. 2006) have been maintained in the mouse facility at the University of Michigan. Newborns were obtained by mating transgenic males of the D4 or D6 *αGsu-Prop1* transgenic lines with C57BL/6J females (The Jackson Laboratory, Bar Harbor, ME). Genomic DNA was prepared from tail biopsies of all progeny born and then screened for the *αGsu-Prop1* transgene as previously described (Cushman et al. 2001).

To create transient transgenic mice with various *Prop1* plasmids, inserts were released from the plasmid vector sequences and purified for microinjection. The 7.7-kb *αGsu-Prop1ΔCE-B* fragment was generated by digestion of the *αGsu-Prop1ΔCE-B* plasmid with *Not*I/*Cla*I. The 8.0-kb *CE-B + αGsu-Prop1ΔCE-B* fragment was generated by the digestion of the *CE-B + αGsu-Prop1ΔCE-B* plasmid with *Not*I/*Apa*I. Both inserts were isolated by agarose gel electrophoresis and purified with the Nucleospin Extract Kit (Clontech, Mountain View, CA). Microinjection and transplantation were performed as previously reported (Cushman et al. 2001). Genomic DNA was prepared from tail biopsies of all progeny born and then screened for the transgene using the same genotyping strategy as for the *αGsu-Prop1* (Cushman et al., 2001).

The 7-kb *CE-A/LacZ* fragment was generated by digestion of the *CE-A/LacZ* plasmid with *Xho*I. The 8.5-kb *CE-B + CE-A/LacZ* plasmid was generated by digestion of the *CE-B + CE-A/LacZ* plasmid with *Not*I/*Sca*I. Microinjection and transplantation were performed as described above. To detect both transgenes, a 250-bp product was amplified from the genomic DNA using the *Prop1*-specific primer (5′-GTGAGAAAACAGGTATCTAGCT-3′) and the *LacZ*-specific primer (5′-CCACTTTGCGTTTCTTGG-3′). Reactions were performed for 33 cycles of PCR conditions: 93°C for 3 min × 1, (94°C for 30 sec, 55°C for 45 sec, 72°C for 20 sec), 72°C for 5 min.

All mice were housed in a 12-h light, 12-h dark cycle with unlimited access to tap water and Purina 5008 or 5020 chow. All procedures using mice were approved by the University of Michigan Committee on Use and Care of Animals, and all experiments were conducted in accordance with the principles and procedures outlined in the NIH Guidelines for the Care and Use of Experimental Animals.

Analysis of transgenic animals

Embryos were harvested on e12.5 from surrogate mothers carrying *CE-B + CE-A/LacZ* transient transgenics and quick frozen on dry ice. Cryosections of 12–15 μm were prepared on slides and fixed in 0.5% glutaraldehyde, 1.25 mM EGTA, 2 mM $MgCl_2$, and PBS (pH 7.2) for 5 min at room temperature, washed three times in 0.02% NP-40 (Amersham Pharmacia Biotech, Inc., Piscataway, NJ), 100 mM sodium phosphate, 2 mM $MgCl_2$ wash buffer, and stained for β-galactosidase activity overnight at 37°C in a solution of 1 mg/ml X-gal (Roche), 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, and 0.02% NP-40 in PBS.

P1 heads from *αGsu-Prop1*, *αGsu-Prop1ΔCE-B*, *CE-B+αGsu-Prop1ΔCE-B*, and nontransgenic controls; e12.5 embryos from *CE-A/LacZ*, *CE-B+CE-A/LacZ*, and controls; or e12.5/e14.5 embryos from wild-type animals were harvested and fixed for 2-24 h in 4% paraformaldehyde in PBS (pH 7.2) followed by PBS wash, dehydration in a graded series of ethanol, and paraffin embedding. Six-micrometer sections were prepared on slides and washed in 0.3% Triton X-100 (Sigma, St. Louis, MO) in PBS (pH 7.2) for 15 min at room temperature, permeablized by proteinase K digestion (0.8 μg/ml in 100 mM Tris-HCl, 50 mM EDTA pH 8.0) for 15 min at 37°C, followed by a 5-min fixation in 4% paraformaldehyde in PBS (pH 7.2). To acetylate sections, tissues were exposed to 0.1 M triethanolamine, 0.25% acetic anhydride solution for 10 min. Tissues were prehybridized in hybridization buffer [50% formamide, 5× SSC, 2% blocking powder (Roche Molecular Biochemicals), 0.1% Triton X-100 (Sigma), 0.5% CHAPS (Sigma), 1 mg/ml yeast tRNA, 5 mM EDTA (pH 8.0), and 50 μg/ml heparin]. Tissues were then hybridized

overnight at 55°C with the either the *Prop1* or *mP1* probe diluted in hybridization buffer.

The *Prop1* riboprobe was generated as previously described (Cushman et al. 2001). The *mP1* riboprobe was generated by subcloning the *mP1* polyA region from a modified version of the pnlacF plasmid into pBluescript SK+ (Stratagene) at the *Bam*HI and *Bgl*II sites (Peschon et al. 1987). The clone was linearized by digestion with *Bam*HI to generate the antisense probe. The *Prop1* and *mP1* riboprobes were generated and labeled with digoxignenin (Roche Molecular Biochemicals) following standard procedures (Mannheim 1996).

Nontransgenic P1 pituitaries were analyzed for αGSU expression with a polyclonal rabbit anti-rat αGSU antibody (1:1800; National Institute of Diabetes and Digestive Kidney Diseases, Torrance, CA) and detected with a biotin-conjugated anti-rabbit IgG (1:400; Vector Laboratories, Burlingame, CA) using the Vectastain ABC kit (according to manufacturer's protocol; Vector Laboratories).

PIT1 immunohistochemistry was preformed on 6-μm paraffin sections of dissected pituitary tissue as described (Charles et al. 2005).

All images were captured with a Leitz DMRB microscope (W. Nuhsbaum, Inc., McHenry, IL) and an Optronics (Goleta, CA) camera.

Results

PROP1 protein conservation

In humans, *PROP1* comprises three exons that encode a 226-amino-acid protein that contains a DNA-binding homeodomain and a transactivation domain at the C-terminus. Previous studies comparing the bovine PROP1 protein sequence to that of other mammals revealed that the homeodomain is highly conserved whereas the N-terminus is not (Guy et al. 2004; Showalter et al. 2002). To obtain PROP1 protein sequences from several species for comparison, we designed primers to regions of the *PROP1* gene sequence exhibiting high conservation between mouse and human. We used these primers to amplify genomic DNA from capuchin monkey, howler monkey, gorilla, and baboon. We sequenced the amplification products, aligned the genomic sequences, and compared them. The splice junctions are highly conserved, which permitted prediction of the cDNA and protein sequence (see *Materials and methods*).

Human PROP1 protein sequence was compared to the PROP1 protein sequence of various primate species, which are close relatives of humans, ranging from the hominoid primate clade, which shared a common ancestor with humans about 6–8 million years ago, to old-world and new-world monkeys which diverged approximately 25 and 40 million years ago, respectively (Nei et al. 2001; Nobrega and Pennacchio 2004). Human PROP1 protein sequence was also compared to the PROP1 protein sequence of more distantly related mammals. We selected the lemur (*Lemur catta*), which is a prosimian of intermediate evolutionary distance, having diverged from human about 60 million years ago (Boffelli et al. 2003), and more distantly related mammalian species such as artiodactyls and rodents, which are thought to have shared a common ancestor with humans over 80 million years ago (Nei et al. 2001; Nobrega and Pennacchio 2004). In addition, we compared partial protein sequences for nonmammalian vertebrates such as chicken and fish, which diverged from humans approximately 300 and 400-450 million years ago, respectively (Aparicio et al. 1995; Nei et al. 2001; Nobrega and Pennacchio 2004) (Table 1).

We compared the PROP1 protein sequence for 13 mammals via a clustalW alignment (Fig. 1). Previous PROP1 protein comparison illustrated high conservation within the homeodomain (Guy et al. 2004). In addition, two basic regions were identified within the homeodomain, B1 and B2, which are important for nuclear localization and DNA binding of PROP1 (Guy et al. 2004) (Fig. 1, boxed areas). The B1 and B2 regions are 100% identical between human, pig (*Sus scrofa*), cow (*Bos Taurus*), dog (*Canis familiaris*), mouse (*Mus musculus*), and rat (*Rattus norvegicus*) (Guy et al. 2004). Our analysis shows that the homeodomain is 100% conserved between humans, chimpanzees (*Pan troglodytes*), gorillas, and baboons (Fig. 1, shaded area and Table 1). In fact, the homeodomain region is over 93% conserved between mouse and human (Fig. 1, Table 1) and over 93% conserved between chicken (*Gallus gallus*) and human (Table 1). Even very distantly related vertebrate fish such as fugu (*Fugu rubripes*) and zebrafish (*Danio rerio*) show extensive conservation with human in the PROP1 homeodomain region (Table 1). Although we were surprised that the predicted fish proteins are much more divergent from the mammalian proteins than the bird (chicken), an extensive cladistic analysis of the paired domains of fish and mammalian genes support the idea that the fish sequences we present are the likely orthologs of the mammalian sequences (data not shown). In addition, all of the species that we analyzed had 100% identity in the B1 and B2 regions with the exception of the chicken, in which only partial sequence was available (data not shown), and the lemur, which had an in-frame deletion of R71 (Fig. 1, indicated by white box). This is consistent with the expectation that the homeodomain region is an important functional region of the PROP1 protein because it has evolved slowly compared to the other domains. To date all of the mutations in the human population that are known to cause MPHD as a result of *PROP1* deficiency are predicted

Table 1 The PROP1 homeodomain is highly conserved among vertebrate species

	Description	Organism	Divergence from Human (MYA)[a]	PROP1 Protein Comparison (% ID to Human)			
				N-terminus	Homeodomain	Transactivation	Total protein
Mammals	Hominoid	Chimpanzee	6–8	100	100	96.9	98.7
		Gorilla		95.6	100	98	97.4
	Old-world	Baboon	~25	91.2	100	95.9	95.6
	New-world	Howler	~40	77.9	98.3	89.8	88.5
		Capuchin		80.9	98.3	87.8	88.5
	Prosimian	Lemur	~60	66.2	90	84.7	80.2
	Artiodactyl	Pig	~80	66.2	96.7	82.7	81.5
		Cow		47.1	96.7	80.6	74.9
		Sheep		50	95	81.6	75.3
	Carnivore	Dog		57.4	93.3	85.7	79.3
	Rodent	Rat		51.5	90	71.4	70
		Mouse		48.5	93.3	76.5	72.7
Non-mammals	Bird	Chicken	~300	n/a[b]	93.6[c]	69.4	n/a
	Fish	Fugu	400–450	n/a	81.7	25.5	n/a
		Zebrafish		n/a	76.7	26.5	n/a
		Tetraodon		n/a	78.3	29.6	n/a

[a] MYA = million years ago

[b] n/a = not available

[c] partial protein sequence of the chicken PROP1 lacks first few residues of the homeodomain

to eliminate function of the homeodomain, with the exception of the recently discovered nonsense mutation that occurs in the transactivation domain. The homeodomain mutations include various missense mutations (Fig. 1) as well as deletions, truncations, nonsense mutations, and splicing mutations (Cushman and Camper 2001; Parks et al. 1999; Reynaud et al. 2005). The homeodomain also contains the point mutation of the Ames dwarf mouse (Sornson et al. 1996) (Fig. 1). The transactivation domain is also well conserved among mammals, ranging from approximately 97%-98% identity between human and other hominoids to 76.5% identity between human and mouse (Fig. 1, Table 1). The N-terminus is the least conserved domain of PROP1, with only approximately 80% and approximately 78% identity between human and the closely related capuchin and howler primates, respectively, and less than 50% identity between human and mouse (Fig. 1).

There are polymorphisms that occur within the PROP1 protein of humans, as well as in that of sheep, mice, and cows. (Fig. 1). Allelic variants occur in the transactivation domain in sheep (*Ovis aries*), T181A (Guy et al. 2004), cow, H173R (Showalter et al. 2002), and mouse, G155A, S171A, and 208/209 P insertion (Sornson et al. 1996). There is also a mouse polymorphic deletion of one CA within a CA repeat in the mouse UTR (Sornson et al. 1996). The human polymorphisms S20N, A51G, and G60E are located in the N-terminus, while A142T (Nakamura et al. 1999b), is located in the transactivation domain. The 21 additional human polymorphisms located in the noncoding sequence and silent mutations within exons 1 and 2 are listed at the SNP NCBI site (http://www.ncbi.nlm.nih.gov/). Although none of these polymorphisms have been implicated in disease, they may play a role in the genetic variation of quantitative traits within the animal kingdom.

PROP1 sequence comparison reveals three conserved noncoding elements

Previous studies using 5′ RACE located the human *PROP1* transcription initiation site 309 nucleotides upstream of the translation initiation site (Duquesnoy et al. 1998). We predicted the transcription initiation site of the mouse *Prop1* by comparing the mouse genomic sequence with the consensus 5′ sequence of four mouse *Prop1* cDNA clones obtained from full-length cap-trapper cDNA libraries (Carninci et al. 2003). This revealed that the transcription initiation site is located 353 nucleotides upstream of the translation start site. This is 126 nucleotides upstream of the previous transcription initiation site annotated by the GenBank sequence NM008936 (http://www.ncbi.nlm.nih.gov/). Our results indicate a similar length for the human and mouse 5′ UTR, but the sequence of this region is poorly conserved (data not shown).

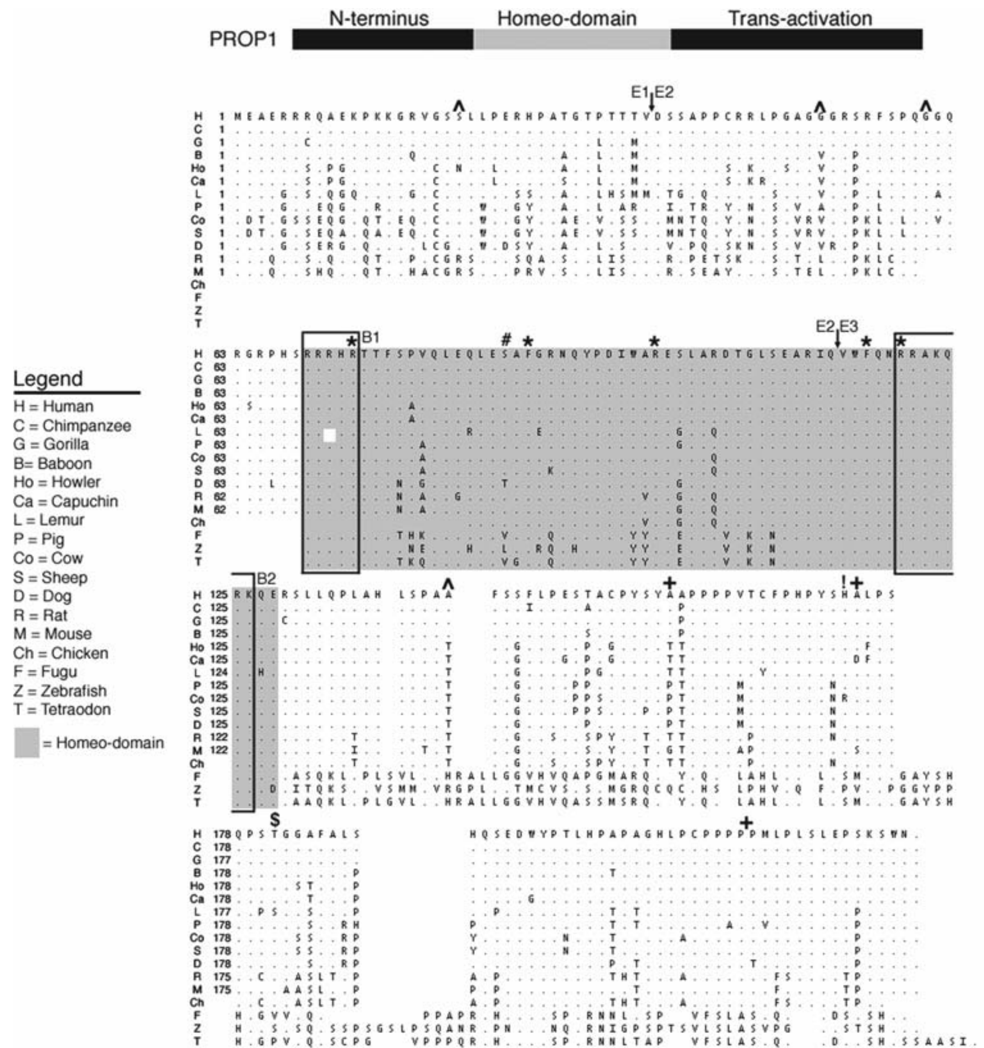

Fig. 1 PROP1 protein comparison reveals lack of conservation in the amino terminal domain. The PROP1 protein sequence alignment for 12 mammals shows very high conservation in the homeodomain region (shaded area) and in the transactivation domain in the carboxy terminus. The B1 and B2 regions within the homeodomain are boxed. The white box indicates the deleted amino acid within the B1 domain of the lemur. Mutations in those amino acids within the homeodomain region that are known to cause MPHD in humans (*) and mice (#) are indicated. Allelic variants in the sheep ($), cow (!), mouse (+), and human (^) are indicated. Human SNPs for *PROP1* were obtained from NCBI website (http://www.ncbi.nlm.nih.gov/). Arrows indicate the exon boundaries. E1, exon 1; E2, exon 2; E3, exon 3

We compared the 5q35.3 chromosome region containing human *PROP1* with the orthologous region in mouse using the UCSC genome browser (http://www.genome.ucsc.edu/), (Fig. 2A) (Kent et al. 2002). The nearest known 5′ neighboring gene to *PROP1* is *N4BP3*, located 118 kb upstream of *PROP1*, and the nearest known 3′ neighboring gene, *AK126616*, is 34 kb downstream. In comparison, the nearest 5′ and 3′ genes to the mouse *Prop1* gene are located 15 kb (*Olfr1378*) and 7 kb (*4933414115Rik*) in distance, respectively. This analysis also reveals that the mouse genomic region orthologous to the approximately 620-kb flanking region 5′ of the human *PROP1* gene is inverted and separated from mouse *Prop1* by a series of olfactory genes that map to human chromosomes 16 and 17 (Fig. 2A, lined box). An approximately 760-kb region located approximately 750 kb 5′ to the human *PROP1* gene is present in the reverse orientation 3′ to the mouse *Prop1* gene (Fig 2A, gray box). Additional comparisons of the *Prop1* locus between human and chimpanzee and between rat and mouse reveal gene order conservation among primates and among rodents (data not shown). A genome VISTA comparison of the pig *Prop1* BAC to the mouse genome indicated gene order conservation (Couronne et al. 2003), suggesting that the region surrounding the pig *Prop1* locus is more similar to the mouse *Prop1* locus than to the human (Fig. 2B). These disruptions in the gene order between the orthologous regions surrounding the human and mouse or pig *PROP1* genes may obscure the identification of conserved elements located at great distances from the gene, but comparative genomics is useful for the identification of putative regulators within 26 kb that extends 5′ and 3′ from mouse *Prop1* to the nearest-neighboring genes.

To determine whether all of the elements necessary for appropriate regulation of *Prop1* transcription are contained within a reasonably close distance to the gene, we sought to rescue the *Prop1* dwarf phenotype using a mouse BAC containing *Prop1*. *Prop1* mutants have profound, proportional dwarfism evident within the first two weeks of life, and adult mutants are approximately one third the size of their normal littermates (Buckwalter et al. 1991). Further-

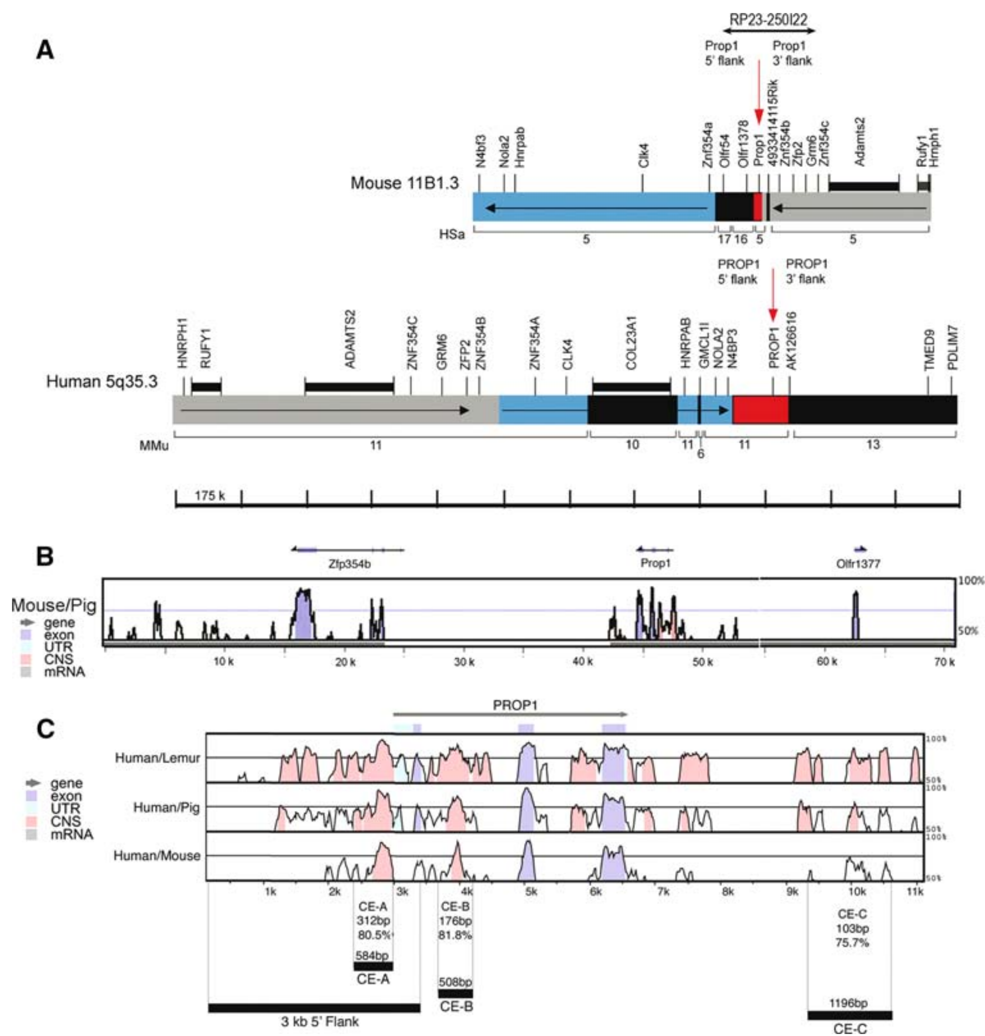

Fig. 2 Mammalian genomic DNA sequence comparisons reveal the presence of several highly conserved elements. **A** The approximate position of BAC RP23-250122, used for transgene correction of the *Prop1*-deficient phenotype, is indicated at the top (double arrow), aligned with the genome sequence from the mouse. Orthologous regions of the human chromosome 5q35.3 and the mouse chromosome 11B1.3 reveal disruptions in gene order. An approximately 760-kb region that is about 750 upstream of human *PROP1* is inverted and located approximately 10 kb 3′ of the mouse *Prop1* (gray box, arrow). An approximately 620-kb region 5′ to the human *PROP1* is inverted and located about 50 kb 5′ to the mouse *Prop1* (blue box, arrow). The nearest genes 5′ and 3′ to both the human and the mouse *PROP1* genes are shown. Large genes are indicated by black bars. The *PROP1* gene locus is indicated by the red box and red arrow. Scale bar is marked in 175 kilobase (kb) increments. The arrow indicates the orientation of the *PROP1* gene. MMu, mouse orthologs to the human 5q35.3 are indicated by a bracket and mouse chromosome number. HSa, human orthologs to the mouse 11B1.3 are indicated by a bracket and human chromosome number. **B** Genome VISTA plot comparing the pig *Prop1* BAC to the mouse genome revealing gene order conservation. Colored peaks represent greater than 75% identity over 100 bp. The mouse *Zfp354b*, *Prop1*, and *Olfr1378* genes are indicated by the arrows. **C** Pairwise comparisons are presented for human vs. lemur, pig, and mouse. Colored peaks represent greater than 75% identity over 100 bp. The regions of gene used for the analysis of the conserved noncoding regions are indicated by the black bars and dotted lines below the respective peaks. The *PROP1* gene is indicated by the gray arrow. **B, C** *x*-axis, kilobases; *y*-axis, percent identity ranging from 50% to 100%. Light blue, untranslated regions (UTR); dark blue, coding regions; pink, conserved noncoding regions; bp, base pairs; CE-A, conserved noncoding element A; CE-B, conserved noncoding element B; CE-C, conserved noncoding element C (URL: http://www-gsd.lbl.gov/VISTA/)

more, their pituitaries have little or no GH, TSH, and PRL and exhibit anterior lobe hypoplasia and overall pituitary dysmorphology (Gage et al. 1996b; Nasonkin et al. 2004; Ward et al. 2005). The mouse BAC RP23-250I22 is about 195 kb long and contains genomic sequence extending from within the *Olfr54* gene at the 5′ end of *Prop1* to the *Znf354c* gene at the 3′ of *Prop1* (Fig. 2A, double arrow). Therefore, this BAC contains additional sequence and predicted genes past the immediate-neighboring genes for *Prop1*. We injected this BAC into pronuclei of eggs derived from a cross of wild-type and *Prop1*$^{+/df}$ mice. Seven transgenic lines were established, of which three were

founded by *Prop1*$^{+/df}$ mice and four by *Prop1*$^{+/+}$ mice. Two BAC transgenic *Prop1*$^{+/+}$ lines were crossed to *Prop1*$^{+/df}$ mice to generate additional lines of BAC transgene; *Prop1*$^{+/df}$. These transgenic heterozygotes were crossed to *Prop1*$^{+/-}$ mice to generate BAC transgene; *Prop1*$^{df/-}$ mice and other assorted genotypes. *Prop1*$^{df/-}$ mice that harbor the *Prop1*-containing BAC did not have a dwarf phenotype but instead were equivalent in size to *Prop1*$^{+/+}$and *Prop1*$^{+/-}$ littermates (Fig. 3A, B). Of the five BAC transgenic lines tested, three produced multiple offspring ($n = 2$-4) in which the presence of the BAC rescued the *Prop1*$^{df/-}$ dwarf phenotype. Among these lines there were no cases of BAC transgene; *Prop1*$^{df/-}$ mice that were smaller than normal. One line produced multiple offspring ($n = 2$) in which the presence of the BAC did not rescue the *Prop1*$^{df/-}$ dwarf phenotype, suggesting that the BAC was not intact or integrated in a region of the genome incompatible with functionally appropriate expression. One line did not produce the desired combination of the BAC transgene with *Prop1*$^{df/-}$ in 30 pups examined, suggesting that the BAC integrated on chromosome 11.

The BAC transgene; *Prop1*$^{df/-}$ mice exhibiting phenotypic rescue of body size have a pituitary gland morphology indistinguishable from that of wild-type littermates, while nontransgenic *Prop1*$^{df/-}$ mice have obviously hypoplastic anterior lobes (Fig. 3C). In addition, the BAC transgene; *Prop1*$^{df/-}$ pituitaries contained cells that express PIT1 (Fig. 3D-F), GH, PRL, and TSH (data not shown), which are essentially absent in the *Prop1*$^{df/-}$ dwarf pituitaries. Therefore, the *Prop1*-containing BAC is able to restore the pituitary cell types that are absent in the pituitaries of *Prop1*$^{df/-}$ mice, as well as pituitary size, somatic growth, and function of pituitary target organs.

We compared a 20-kb region of the human *PROP1* genomic sequence with similar portions of genomic DNA from three mammalian species: lemur, pig, and mouse. We discovered regions in the noncoding sequence of *PROP1* that are highly conserved over evolutionary time (Fig. 2C). Approximately 10 kb of this sequence overlapped in all four mammals and was analyzed by the mVISTA sequence comparison program (Bray et al. 2003), which displays regions of sequence conservation between two species that are at least 100 bp long with 75% identity (Fig. 2C). The human *PROP1* gene is approximately 4 kb long and is located in a region of the genome that contains relatively few genes. Exons 2 and 3, which encode the HD and TA domains (Fig. 1), are highly conserved among all four species (Fig. 2C, blue peaks). Exon 1, however, is relatively divergent among these species (Fig. 2C). The mVISTA plot also reveals three conserved noncoding sequences among human, lemur, pig, and mouse (Fig. 2C, pink peaks). The first conserved element A (*CE-A*) is located in the 5′ flanking sequence 9 bp upstream of the mouse transcription initiation site and is 80.5% identical between human and mouse over 312 bp (Fig. 2C). *CE-B* is located within intron 1 of *Prop1* and is 81.8% identical

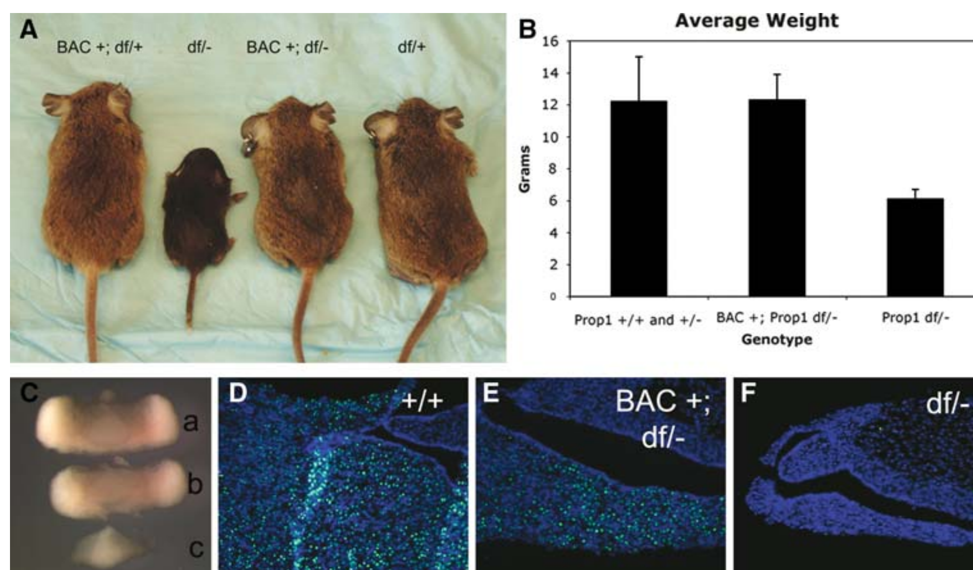

Fig. 3 A *Prop1* BAC transgene rescues the *Prop1* mutant phenotype. **A** Four sample mice and corresponding genotypes. *Prop1*$^{df/-}$ mice are dwarf, but the presence of the BAC transgene rescues the dwarf phenotype. **B** The average weight of mice of each genotype is graphed for all progeny of a cross of BAC transgenic; *Prop1*$^{df/+}$ × *Prop1*$^{+/-}$. Error bars represent the standard deviation. For *Prop1*$^{+/+}$ and *Prop1*$^{+/-}$ mice, 116 mice were weighed at weaning, while 19 and 11 were weighed for *Prop1*$^{df/-}$ and BAC transgenic; *Prop1*$^{df/-}$, respectively. **C** Dissected pituitaries from P21 mice. The genotypes are as follows: a = *Prop1*$^{+/+}$, b = BAC transgenic; *Prop1*$^{df/-}$, c = *Prop1*$^{df/-}$. **D-F** PIT1 immunohistochemistry. The genotypes for each image are as follows: **D** *Prop1*$^{+/+}$, **E** BAC transgenic; *Prop1*$^{df/-}$, **F** *Prop1*$^{df/-}$

between human and mouse over 176 bp (Fig. 2C). The third conserved region, *CE-C*, is the smallest conserved noncoding element and is only 75.7% identical between human and mouse with a length of approximately 100 bp (Fig. 2C).

The Prop1 CE-A and CE-B regions show enhancer activity in tissue culture

Various *in vitro* and *in vivo* methods have been used to test the biological function of conserved elements discovered by comparative genomics (Boffelli et al. 2003; Lettice et al. 2003; Nobrega et al. 2003; Zerucha et al. 2000). *CE-A* has no promoter activity in a heterologous CV1 monkey kidney or the αT3 mouse gonadotrope-like cell lines (data not shown). Therefore, *CE-A*, *CE-B*, and *CE-C* were analyzed for enhancer activity in tissue culture by inserting each of them in both the forward and the reverse orientation upstream of the *Drosophila* alcohol dehydrogenase (*ADH*) minimal promoter driving a luciferase (*Luc*) reporter gene (Fig. 4A, construct #1). The cell lines used for the transfection assays included the CV-1, αT3, GH3 rat somatotrope-like, and AtT-20 mouse corticotrope-like cell lines. The enhancer activity of these elements is reported as the fold increase in luciferase activity over the basal level of the *ADH-Luc* vector alone.

A 584-bp *Prop1* 5′ flanking segment containing the *CE-A* element from the mouse *Prop1* gene was cloned into the *ADH-Luc* vector in both forward and reverse orientations (Figs. 2C and 4A, constructs #2 and #3, respectively). On average, the *CE-A (F)/ADH* construct displayed about a 13.5-fold increase in activity in the CV-1 cells and approximately 4-5-fold increase in activity in the GH3, αT3-1, and AtT-20 cell lines (Fig. 4B). When the *CE-A* was tested in the reverse orientation (Fig. 4A, construct #3), the *CE-A (R)/ADH* showed no activity in any of the cell lines tested, indicating that the effect is orientation-dependent (Fig. 4B).

A 508-bp segment from intron 1 of the mouse *Prop1* gene containing the *CE-B* element was also tested for enhancer activity in the *ADH-Luc* reporter construct (Figs. 2C and 4A, constructs #4 and #5, respectively). The *CE-B (F)/ADH* construct elicited, on average, a fourfold increase in luciferase activity compared to the *ADH-Luc* alone in the CV-1 cells (Fig. 4B). However, the *CE-B (F)/ADH* had very low activity in the GH3 cells (less than a twofold average increase over basal with a range of 1.1–2.6-fold) and no activity in either the αT3-1 or the AtT-20 lines (Fig. 4B). The *CE-B (R)/ADH* construct showed no enhancer activity in any of the cell lines tested (Fig. 4B).

A 1196-bp segment from the 3′ flanking sequence of the mouse *Prop1* gene containing *CE-C* was inserted into the *ADH-Luc* reporter construct (Figs. 2C and 4A, constructs #6 and #7, respectively). A larger section was used in the analysis of the *CE-C* element because there were several smaller peaks on the mVISTA plot between human and mouse that showed significant conservation between human and pig and between human and lemur that flanked the *CE-C* segment (Fig. 2C). The *CE-C* in either orientation appeared to have no enhancer activity in the αT3-1 or the GH3 cell lines but did have a low level of activity in the forward orientation when tested in the CV-1 and AtT-20 cell lines (Fig. 4B).

In summary, the 5′ element *CE-A* had the highest activity with the *ADH-Luc* reporter. The *CE-B* intronic element and the *CE-C* 3′ element had low orientation-dependent enhancer activity in the CV-1 cells and very low, if any, activity in the pituitary-derived cell lines. This was not surprising because these pituitary cell types are derived from differentiated pituitary cells and do not express endogenous *Prop1*. These pituitary-like cells may not have all the components necessary to allow for the proper function of all of the *Prop1* enhancer elements or they may contain factors that repress the function of these elements.

The Prop1 CE-A element is not sufficient for LacZ expression in transgenic mice

The *CE-A* and *CE-B* regions were also analyzed in transgenic mice to determine whether they function *in vivo*. *CE-A/LacZ* (Table 2), which consists of a 3-kb region immediately 5′ to the *Prop1 ATG* containing the 5′ UTR, the *Prop1* transcription initiation site, and the *CE-A* region, was analyzed for the ability to drive the expression of a nuclear localized *E. coli* β-galactosidase-mouse protamine 1 reporter construct (*LacZ-pA*) in transgenic mice (Fig. 2C). Transgenic founders were bred to produce lines for embryo analysis, or transient transgenic embryos were harvested and analyzed for the transgenic transcript via *in situ* hybridization (ISH) at e12.5 because this is the time of peak *Prop1* expression (Sornson et al. 1996). We used the well-characterized *αGsu-LacZ* and *αGsu-Prop1* transgenes as positive controls for detecting β-galactosidase activity in the pituitary gland by X-gal staining and for mouse protamine ISH, respectively (Cushman et al. 2001; Kendall et al. 1994). The promoter and enhancer sequences of the pituitary glycoprotein hormone α-subunit gene (*Cga* for chorionic subunit α or *αGsu*) are sufficient to drive expression of *LacZ* and other reporters in a manner that is developmentally, hormonally, and tissue-specifically correct (Kendall et al. 1994). Both the *αGsu-LacZ* transgenic mice and the *αGsu-Prop1* transgenic mice contain the mouse protamine splice sites and polyadenylylation sequences that can be used as a transgene-specific tag in any organ except the testis, which expresses the endogenous protamine gene. None of the five transgenic embryos

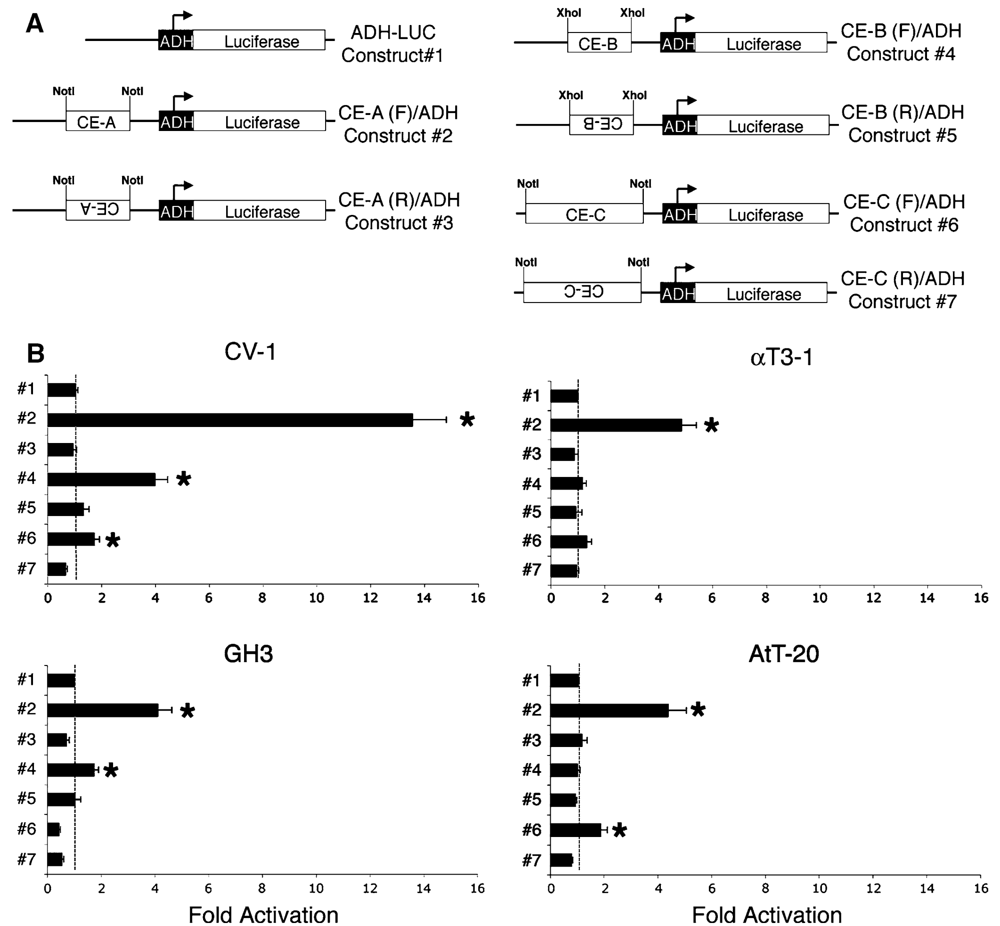

Fig. 4 Enhancer study of *Prop1* conserved noncoding elements from the mouse gene in monkey kidney CV-1, GH3 (rat somato-lactotropes), αT3-1 (mouse gonadotrope-like), and AtT-20 (mouse corticotrope-like) cells. **A** Diagram of the conserved elements upstream of the *ADH-Luc* reporter construct. **B** Luciferase activity as fold activation over basal shown for the constructs depicted in (**A**) for CV-1, GH3, αT3-1, and AtT-20 cells reveal orientation-dependent enhancer activity for *CE-A* in all lines and for *CE-B* in CV-1 and GH3 lines. ADH, alcohol dehydrogenase minimal promoter; LUC, luciferase reporter gene; F, forward orientation; R, reverse orientation. The asterisk designates constructs with activity significantly above baseline

exhibited detectable transcription of the *CE-A/LacZ* transgene compared with positive controls. Thus, the 3-kb 5′ flanking region of *Prop1* is not sufficient for transgene expression. The intronic *CE-B* element was tested in conjunction with *CE-A* for *in vivo Prop1* expression. Transgenic mice were made with the construct *CE-B+CE-A/LacZ*, which contains *CE-B* upstream of the *Prop1* 3-kb 5′ flanking region (Fig. 2C, Table 2). Fifteen transient transgenic embryos were harvested at e12.5 and assayed for expression of the transgene by either X-gal staining or ISH for *mP1*. None of the 15 embryos analyzed had detectable expression of the transgene, although the positive controls did exhibit expression. Thus, the combination of *CE-B* and *CE-A* is also not sufficient for expression *in vivo*.

The Prop1 CE-B putative enhancer is important for spatial expression in transgenic mice

The *CE-B* region was tested for enhancer function in transgenic mice in the context of the *αGsu* promoter. The first clues to a possible role for *CE-B* in the expression of *Prop1* came from the analysis of the *αGsu-Prop1* transgenic mice (Table 2). Endogenous *Prop1* is normally expressed from e9 until e15.5 in the mouse in a dorsal (highest) to ventral (lowest) expression gradient (Fig. 5A) (Sornson et al. 1996). In contrast, *αGsu* is expressed during pituitary development in the caudomedial and ventral portions of the pituitary where the thyrotropes and gonadotropes arise, and its expression continues in these two cell types throughout adulthood (Fig. 5B) (Japon et al. 1994; Kendall et al., 1994; Raetzman et al. 2002). Ten stable lines were created with the *αGsu-Prop1* transgene and six expressed the transgene in the adult pituitary. Two lines were maintained and analyzed (Cushman et al. 2001). These studies revealed that the *αGsu-Prop1* construct yields efficient overexpression of *Prop1* beyond e15.5 (Cushman et al. 2001). Expression of the *αGsu-Prop1* transgene is expanded dorsally beyond the normal expression domain of endogenous *αGsu* and beyond that observed for other *αGsu* driving expression of *LacZ* or other reporters. This dorsal expression is observed in pituitaries at postnatal day 1 (P1, day of birth) in 2/2 stable transgenic lines and at e18.5 in 3/3 transient transgenics that were created with the same *αGsu-Prop1* construct. This expression pattern appears to be a combination of both endogenous *αGsu* and *Prop1* (compare Fig. 5C with B and A). This result suggests that the *CE-B* region is important for the dorsal expansion of the *αGsu-Prop1* transgene and

Table 2 Transgenic constructs used to test the *in vivo* function of the conserved non-coding elements of the mouse *Prop 1* gene

Construct Name	Construct Diagram	# Lines	Age	Detection Method	# Lines w/ Exp.	Expression Pattern
CE-A/LacZ	3 kb; Prop1 5' Flank; CE-A; UTR[c]; LacZ; pA[d]	5 transient[a]	E12.5	*mP1 ISH*[f]	0/5	ND[e]
CE-B + CE-A/LacZ	CE-B; 3 kb; Prop1 5' Flank; CE-A; UTR; LacZ; pA	15 transient	E12.5	(7) XGal (8) *mP1 ISH*	0/15	ND
αGSU-Prop1	5 kb *αGSU* promoter; CE-B; pA; Prop1	10 stable[b] 7 transient	P1 e18.5	*Prop1 ISH*	6/10 3/7	2/2 (stable) A.L./I.L.[g] 3/3 (transient) A.L./I.L.
αGSU-Prop1ΔCE-B	5 kb *αGSU* promoter; (ΔCE-B); pA; Prop1	8 transient	P1	*Prop1 ISH*	6/8	5/6 ventral portion of A.L.[h]
CE-B + αGSU-Prop1ΔCE-B	CE-B; 5 kb *αGSU* promoter; (ΔCE-B); pA; Prop1	9 transient	P1	*Prop1 ISH*	7/9	6/7 A.L./I.L.

Footnotes to table:
[a] transient = transient transgenic lines
[b] stable = stable transgenic lines
[c] UTR = *Prop1* untranslated region
[d] pA = mouse Protamine polyA region
[e] ND = not detected
[f] *ISH* = *in situ* histochemistry
[g] A.L./I.L. = expression in anterior lobe and intermediate lobe
[h] A.L. = anterior lobe

thus important for the spatial expression of endogenous *Prop1*.

We tested the significance of *CE-B* for dorsalized transgene expression by deleting *CE-B* from intron 1 to produce the construct *αGsu-Prop1ΔCE-B* (Table 2). Transient transgenics bearing this construct were harvested at P1 and assayed for the expression of the transgene using ISH for *Prop1*. Six of the eight transgenic mice analyzed exhibited transgene expression. One had very weak expression and was eliminated from further consideration. The remaining five had strong expression. In these five transgenic mice, the expression pattern of the *αGsu-Prop1ΔCE-B* transgene is restricted to the ventral aspects of the pituitary, typical for the *αGsu* promoter and enhancer (compare Fig. 5D with C). This result indicates that *CE-B* was necessary for the dorsal expression of the *αGsu-Prop1* construct.

To determine whether the position of the *CE-B* relative to the *αGsu* promoter is critical, the *CE-B* region was replaced in the *αGsu-Prop1ΔCE-B* construct upstream of the *αGsu* promoter to create a new construct, *CE-B + αGsu-Prop1ΔCE-B* (Table 2). The *CE-B + αGsu-Prop1ΔCE-B* transgenic mice were harvested at P1 and assayed for expression of the transgene. The *CE-B + αGsu-Prop1ΔCE-B* transgene expression was expanded to the dorsal aspect of the pituitary in six of seven transient lines (Fig. 5E), with the remaining line having only weak expression of the transgene. Finally, an ISH for *Prop1* on nontransgenic pituitaries gave no signal (Fig. 5F), thus verifying that the patterns of expression seen in Figs. 5C, D, F are due to the specific transgene. The transgenic analysis provided *in vivo* evidence that the *CE-B* contained within the intron 1 of *Prop1* is sufficient to confer spatial expression information in a position-independent manner in the context of the transgene.

Discussion

We sought to identify *cis*-acting DNA sequences important for mouse *Prop1* expression because regulation of *Prop1* is important for normal pituitary development and function. To accomplish this, we obtained genomic sequence from lemur and pig (*Sus scrofa*) *PROP1* BACs and compared these to *PROP1* sequences available online (Ahituv et al. 2004; Aparicio et al. 1995; Boffelli et al. 2003, 2004; Nobrega and Pennacchio 2004; Williams et al. 2003). We also generated *PROP1* genomic sequence for the first time for five different primate species to include in the comparison. We identified three conserved noncoding elements (CE) that are larger than 100 bp with greater than 75% identity between human and mouse and tested them for function in cell culture and transgenic mice. The three re-

gions that fit these criteria are *CE-A*, a 300-bp region in the *Prop1* promoter proximal region; *CE-B*, a 200-bp region within the first intron of *Prop1*; and *CE-C*, a 103-bp region within the 3′ flanking sequence.

Transfection of cultured cells has been successful for demonstrating the function of some elements (Nishimura et al. 2000; Surinya et al. 1998; Swamynathan and Piatigorsky 2002), but there are examples of important regulatory sequences that are not identified with this approach (Lang et al. 2003; Lettice et al. 2003; Nobrega et al. 2003; Zerucha et al. 2000). *Prop1* is expressed in a distinct spatial-, temporal-, and tissue-specific fashion during development, and the endogenous gene is not expressed in any of the available pituitary cell lines. Nevertheless, *CE-A* exhibited orientation-dependent activity in all cell lines. *CE-B*, located within intron 1 of *Prop1*, also appeared to have orientation-dependent enhancer activity in CV-1 cells and GH3 cells, although at a much lower level. There was

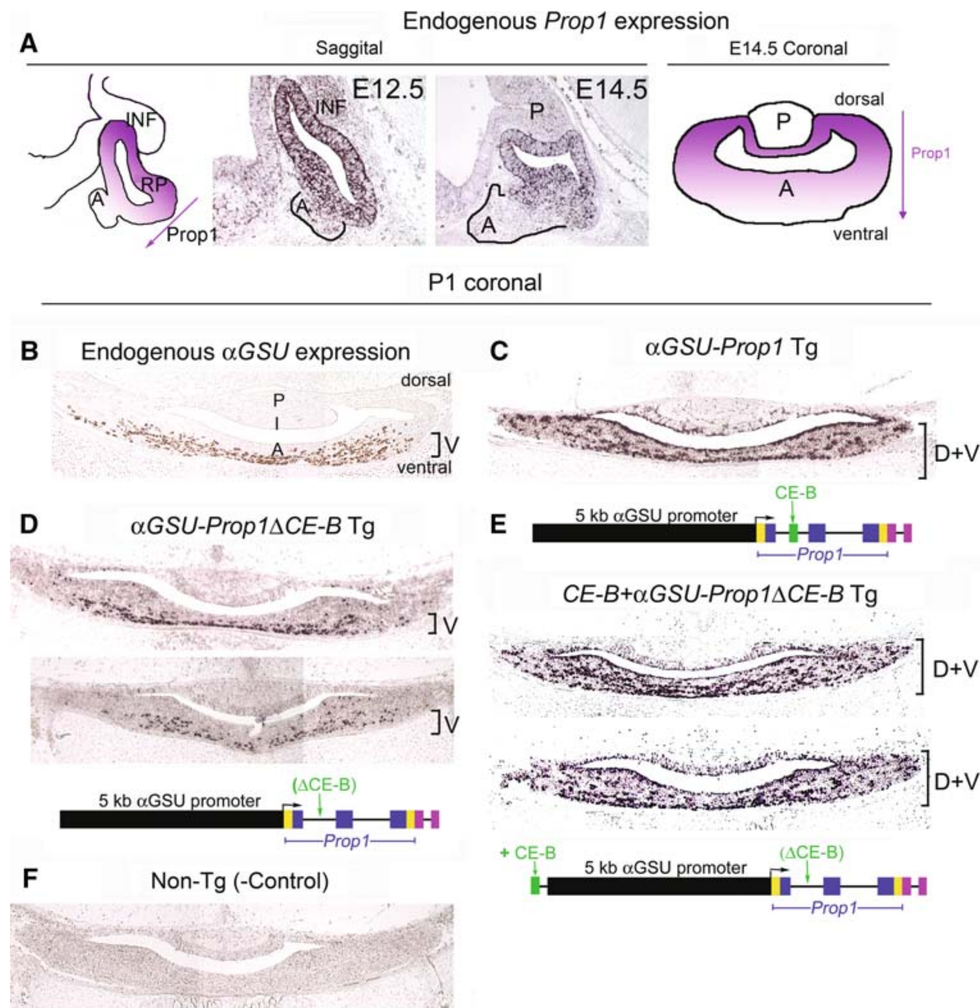

Fig. 5 Transgenic mice reveal functional properties of *CE-B*. **A** Endogenous *Prop1* expression illustrated diagrammatically and with *Prop1 in situ* hybridization in sagittal e12.5 and e14.5 mouse and coronal e14.5 pituitaries. A dorsal-to-ventral expression gradient is apparent. The anterior lobe is marked by a black line. **B** Immunohistochemical staining with αGSU antibody (brown DAB chromagen) reveals normal *αGsu* expression in the caudomedial and ventral regions of a P1 coronal pituitary expression via IHC. **C** *In situ* hybridization for *Prop1* transcripts [purple AP chromagen] reveals that the *αGsu-Prop1* transgenic construct expression is expanded dorsally in P1 coronal pituitary sections relative to endogenous *αGsu* expression. **D** *Prop1 in situ* hybridization shows that the *αGsu-Prop1ΔCE-B* transgenic construct expression (purple AP chromagen) was restored to the caudomedial and ventral regions in P1 coronal pituitaries. **E** *Prop1 in situ* hybridization shows that the *CE-B* + *αGsu Prop1ΔCE-B* transgenic expression (purple AP chromagen) was expanded to the dorsal aspects of the pituitary at P1. **C–E** Representations of the transgenic constructs are shown beneath the appropriate studies. Brackets mark the region for each pituitary in which the transgene expression was detected. **F** *In situ* hybridization for *Prop1* in a nontransgenic pituitary gives no signal. DAB, 3,3′ diaminobenzidine; AP, alkaline phosphatase; A, anterior lobe; P, posterior lobe; I, intermediate lobe; RP, Rathke's pouch; INF, infindibulum; v, ventral expression pattern; d + v, dorsal and ventral expression pattern

no enhancer activity in either the AtT-20 line or the αT3-1 line. The *CE-C* putative regulatory element had low enhancer activity in only the CV-1 and AtT-20 cell lines. There are many possible explanations for the weak activity of *CE-B* and *CE-C* in cell culture, but the cell culture assays did detect enhancer function. The pituitary-derived cell lines were developed in the different hormone-producing cell lineages, e.g., the αT3-1 cells are gonadotrope-like, whereas GH3 cells are somatotrope-like. Therefore, the differential enhancer activities of the different constructs in these cell lines may be examples of context-specific activity. In addition, the cell lines may be more representative of differentiated cell types and, because *Prop1* is expressed significantly only during early pituitary development in the rodent (Sornson et al., 1996), these cells may not contain the transcription factors and cofactors necessary for the full activity of the putative enhancers. These enhancers also are orientation-specific. However, other examples of orientation-dependent enhancers have been reported (Cheng et al. 2004; Falvo et al. 2000; Nishimura et al. 2000; Surinya et al. 1998; Swamynathan and Piatigorsky 2002; Wei and Brennan 2000).

Our transgenic experiments shed some light on the function of the *CE-B* region. In the context of the *αGsu* promoter, the *CE-B* element results in dorsal expansion of transgene expression. Although this construct used a heterologous promoter, which allows for the expression of the transgene after the endogenous *Prop1* expression is extinguished, these results provide evidence that the *CE-B* region in intron 1 of *Prop1* is important for spatial expression. The *CE-B* region in conjunction with the *αGsu* promoter will be useful for driving the expression of transgenes in the more dorsal aspects of the developing pituitary. Other studies have shown that the Rbp-Jκ DNA binding protein, which is the primary mediator of Notch signaling, can directly bind to intron 1 of *Prop1* and is important for the maintenance of *Prop1* expression (Zhu et al. 2006). Taken together, these data suggest an *in vivo* role for the *CE-B* in the regulation of *Prop1* expression.

Two kilobase pairs of the *Prop1* promoter proximal region (*CE-A*) is inadequate for reporter gene expression in transgenic mice, even in the context of *CE-B*. This indicates that additional sequences are necessary for *Prop1* expression in mice. The BAC rescue of *Prop1*$^{-/df}$ mice demonstrates that all of the elements necessary for transcriptional regulation of *Prop1* are contained within the BAC. We predict that the remaining critical sequences for *Prop1* expression are within the region immediately surrounding *Prop1*, within 15 kb upstream and approximately 26 kb downstream, because there is a disruption in gene order between human and mouse or pig. These critical control sequences are not readily identifiable by genomic sequence comparisons.

In summary, we identified a region, contained within intron 1 of *Prop1*, which is necessary and sufficient for the spatial expression of *Prop1* in the context of a heterologous pituitary specific promoter. While additional regulatory elements remain to be identified by other approaches, the intronic element is worth screening for mutations in unexplained cases of MPHD patients, especially those that appear heterozygous for mutations in *PROP1*.

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