FEBS Letters 437 (1998) 216–220 FEBS 20993

# Human Prop-1: cloning, mapping, genomic structure

## Mutations in familial combined pituitary hormone deficiency[1]

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Received 7 September 1998

**Abstract Prop-1 is a newly isolated pituitary-specific *paired-like* homeodomain transcription factor whose cDNA sequence is well known in mouse. To study its involvement in human combined pituitary hormone deficiency (CPHD), we have isolated the human cDNA ortholog and determined the exon/intron organization and chromosomal localization of the human gene. A Prop-1 defect was characterized in three CPHD families. One missense mutation (R73C) involves a residue conserved in 95% of the more than 400 homeodomain proteins so far identified; in vitro splicing assays demonstrated the functional importance of the second defect, whereas the remaining mutation is a frameshift. Given the disease phenotype documented in the patients, these data, which will facilitate molecular investigations in other patients, demonstrate the crucial role of Prop-1 in the proper development of somatotrophs, lactotrophs, thyreotrophs and gonadotrophs.**



*Key words:* Prop-1; Combined pituitary hormone deficiency; Mutation; Chromosomal localization

### 1. Introduction

Short stature of genetic origin due to growth hormone deficiency (GHD) is a highly heterogeneous condition, at the clinical, biological and genetic level [1]; the GHD phenotype can be either isolated (IGHD) or associated with combined pituitary hormone deficiency (CPHD). In the latter case, the patients' phenotype is characterized by a deficiency of one or more of the other hormones produced by the anterior pituitary, i.e. prolactin, thyroid-stimulating hormone β (TSHβ), luteinizing and follicle stimulating hormones (LH and FSH), or adrenocorticotrophic hormone, produced by lactotrophs, thyreotrophs, gonadotrophs, or corticotrophs, respectively. Although the great majority of IGHD or CPHD cases are sporadic, familial cases have also been described, the disease phenotype being transmitted as an autosomal or X-linked trait [1].

The study of naturally occurring mouse mutants displaying a CPHD phenotype demonstrated both allelic and locus heterogeneity in this condition: the dwarf phenotype of two mouse strains, named Snell and Jackson, with CPHD characterized by absent GH, prolactin and TSHβ gene expression associated with pituitary hypoplasia, is due to mutations in the Pit-1 gene [2]. The corresponding protein, a member of the POU family of transcription factors, is involved in transcriptional activation of GH, prolactin, and TSHβ genes and in the differentiation and proliferation of somatotrophs, lactotrophs, and thyreotrophs [2]. Strikingly, a similar but not identical dwarf phenotype has been identified in a third murine strain, named Ames, in which the deficiency of GH, prolactin, and TSH is associated with decreased LH and FSH gene expression [3] and low LH and FSH plasma levels [4]. This non-allelic mutation, characterized by an absence of detectable Pit-1 transcripts in developing pituitaries [5,6], was recently shown to result from a defect in the gene encoding Prophet of Pit-1 (Prop-1) [7], a novel pituitary-specific *paired-like* homeodomain transcription factor [8].

In humans, numerous Pit-1 gene mutations have been described in patients with familial or sporadic CPHD [9]. Just recently, during the course of this study, four Prop-1 mutations have been reported in patients with CPHD [10,11]. To facilitate screening for mutations of Prop-1 in CPHD and to further test the involvement of this transcription factor in this disease phenotype, we have isolated the human ortholog of mouse Prop-1 cDNA, determined the exon/intron organization and chromosomal localization of the human gene, and investigated three candidate CPHD families.

### 2. Materials and methods

#### 2.1. Cloning and sequencing of the human Prop-1 cDNA and genomic DNA

To isolate the human (h) ortholog of mouse Prop-1 cDNA, cross-species PCR was performed using primers chosen from the mouse Prop-1 cDNA sequence (P1: 5′-CCGCCACCGCACCACCTTCAACCCA-3′, and P2: 5′-GGGGGCTGGGTGCAAGGTGGGGTAC-3′) with human genomic DNA as a template; as a control, similar experiments were carried out with mouse genomic DNA. Sequencing of the human PCR product led to the characterization of a 397-bp fragment of the hProp-1 coding sequence. Based on the latter sequence, both 5′ and 3′ portions of the hProp-1 cDNA sequence were obtained by RACE with the use of human pituitary 5′- and 3′-RACE-Ready cDNA (Clontech).

To characterize the intron/exon organization of the human Prop-1 gene, human genomic DNA was subjected to PCR amplification in several overlapping fragments using primers designed from the human full-length cDNA coding sequence. The resulting PCR products were

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[1] Sequence data described in this paper have been deposited at the GenBank Data Library under accession numbers AF076215 and AF076214.


PII: S0014-5793(98)01234-4

cloned into PCR 2.1 Topo vector (Invitrogen) prior to sequencing. All sequences were determined on both strands.

*2.2. Chromosomal localization of the human Prop-1 gene*

The chromosomal localization of the human Prop-1 gene was first determined by using the PCRable DNA MSC human/rodent somatic cell hybrid mapping panel (Quantum) as template. PCR was performed with primers located in intron II (5′-AAAGACTGGAGCAGCACAGGACGCA-3′, 5′-GCAAGTAAGAAGTGTCAGCTTCCAC-3′). Amplification products were analyzed by electrophoresis on a 1% agarose gel stained with ethidium bromide.

Further localization was achieved by FISH analysis of human chromosome spreads, using a biotin-labeled plasmid clone containing 3.6 kb of the genomic Prop-1 sequence, as described [12].

*2.3. Subjects*

Three CPHD families were investigated. One originates from Tunisia (family A), the second from Egypt (family B), and the remaining one from Turkey (family C). All affected individuals were born to consanguineous unions. They presented with severe growth retardation (below −5 S.D. in height) associated with clinical evidence of hypogonadism (micropenis and microorchidy in patients AII1 and AII2; infantile gonads with pubertal delay at 15 and 17 years old in patients BII2 and BIII1, respectively; pubertal delay in patients BIII2, CII1 and CII2), and pituitary hypoplasia in patients AII1, AII2, BII3, CII1 and CII2. Endocrinological investigations performed in families A and C revealed the existence of CPHD involving GH, TSH, prolactin, FSH and LH, the cortisol levels being in the normal range. A combined GH, TSH, FSH and LH deficiency was documented in the patients belonging to family B.

*2.4. Prop-1 mutation screening*

Genomic DNA was isolated from blood samples obtained from each individual using standard techniques. Screening for mutations was carried out by direct sequencing of PCR products amplified from genomic DNA, using three primer sets (F1: 5′-GAGCTGCGGAAGCAGAGAAATCTCA-3′ and R1: 5′-AGAGGTAACTGTCTCACATCCCCAC-3′; F2: 5′-AAAGACTGGAGCAGCACAGGACGCA-3′ and R2: 5′-GCAAGTAAGAAGTGTCAGCTTCCAC-3′; F3: 5′-CTCTTGTCATTGGAGTAGGGTGTCA-3′ and R3: 5′-CAGACTTCCTCCACTAATCACCCCA-3′) flanking the three coding exons of Prop-1.

*2.5. Site-directed mutagenesis and splicing assays*

A PCR amplification product encompassing the whole Prop-1 coding sequence was generated from control genomic DNA using a forward primer located 64 bp upstream the translation start site and a reverse primer located 186 bp downstream of the termination codon. This product was subcloned into the SV40 promoter-based expression vector pECE [13] and subjected to site-directed mutagenesis using the QuikChange kit (Stratagene) with oligonucleotides designed to introduce the A-to-T substitution found in family C.

Transient expression of the wild-type and mutant constructs in COS-7 cells was performed as described elsewhere [14]. Approximately 24 h after transfection, total RNA was isolated and reverse-transcription-primed PCR (RT-PCR) was performed. The effect of the splice-acceptor-site mutation was studied by use of a sense primer located on exon 2 (5′-AGTGCAGTTGGAACAGCTGGAGTCA-3′) and an antisense primer located on exon 3 (5′-CAGACTTCCTCCACTAATCACCCCA-3′). Aliquots of RT-PCR from cells transfected with the wild-type and mutant constructs were analyzed by electrophoresis on a 1% agarose gel.

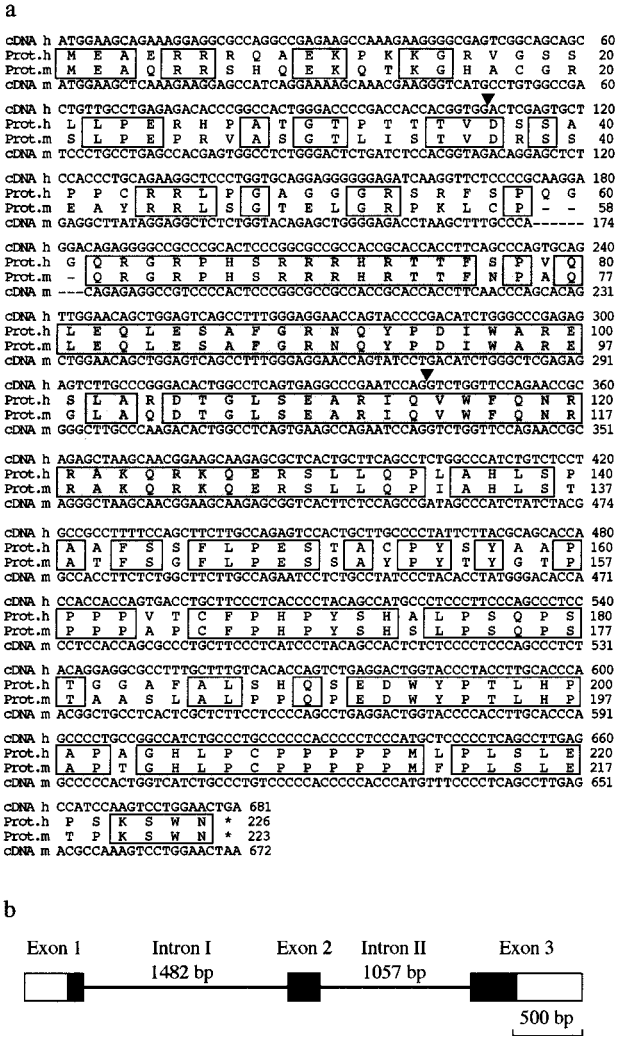


Fig. 1. Human Prop-1 cDNA coding sequence and genomic structure. a: Alignment of human (h) and mouse (m) Prop-1 cDNA and amino acid (Prot.) sequences, and location of exon-intron boundaries. The predicted amino acid sequence of human and mouse Prop-1 is indicated by one-letter abbreviations below the human and above the mouse cDNA sequence, respectively. Nucleotides and amino acids are numbered on the right (nucleotide +1 corresponds to A of the ATG translation start codon). Identical amino acid residues are boxed, and gaps introduced to optimize the alignment are indicated by dashes. The shaded sequence encodes the homeodomain. The dark triangles indicate the location of the two introns. b: Schematic representation of the genomic organization of the human Prop-1 gene. Exons are indicated by filled or empty boxes (translated or untranslated sequences, respectively). The exons and introns are drawn to scale; the exact sizes are given for the introns.

Table 1
Splice donor and acceptor sites and sizes of exons of the human Prop-1 gene

| Exon | | Splice donor and acceptor sites | |
|---|---|---|---|
| No. | Size (bp) | 3′ acceptor | 5′ donor |
| 1 | >418 | | GGTGG**gt**ragtgacc.... |
| 2 | 233 | ....ccgttctc**ag**ACTCG | TCCAG**gt**gatgctcc.... |
| 3 | >812 | ....cccttctc**ag**GTCTG | |
| | | Consensus | |
| | | (c/t)nc**ag** | **gt**ragt |

Exon sequences are in uppercase letters; intron sequences are in lowercase letters, with the conforming 5′ gt and 3′ ag sequences in bold letters.

## 3. Results

### 3.1. Isolation of the human cDNA and gene encoding Prop-1

We performed cross-species PCR using primers (P1–P2) chosen from the mouse Prop-1 cDNA sequence with human or mouse genomic DNA as a template. This gave rise to PCR products the sizes of which were similar in both species and larger than expected for coding sequence, a result pointing to the presence of an intronic sequence. New PCR primers were subsequently used in 5′- and 3′-RACE experiments on human pituitary cDNA. This procedure led to the characterization of the full-length human Prop-1 coding sequence together with parts of the 5′- and 3′-untranslated regions (UTR) (GenBank accession number AF076215). While writing these data, this sequence was not present in any of the publicly available sequence databases. The 5′-UTR sequence includes 309 bp upstream of the translation start site which belongs to a good Kozak consensus sequence (5′-CGAGCCATGG-3′) [15]. 3′-RACE led to the identification of 473 nucleotides after the termination codon. The human coding cDNA sequence is presented in Fig. 1a; when compared with the mouse Prop-1 coding sequence [7], the human sequence was found to be highly conserved, demonstrating that this cDNA is indeed the human ortholog of the mouse Prop-1 cDNA: overall, the human and mouse coding sequences are 678 bp and 669 bp long, respectively; they are 78% identical at the nucleotide level and predict proteins that are 73% identical. As expected, maximum of identity was observed in the region encoding the homeodomain, the human sequence being 91% identical to the mouse Prop-1 sequence at the cDNA level and 93% identical at the protein level.

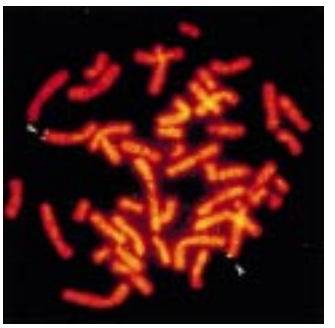

Fig. 2. FISH mapping the human Prop-1 gene. A specific signal is seen at 5q35 on both copies of chromosome 5 (arrowheads).

Human Prop-1 gene (Fig. 1b) is composed of at least 3 exons and 2 introns covering less than 4 kb of genomic DNA which was extensively sequenced (GenBank accession number AF076214). Sequences at the exon boundaries, as well as exon sizes are given in Table 1.

### 3.2. Chromosomal localization of the human Prop-1 gene

A primer set was used to screen a panel of 24 hybrid somatic cell lines (human/rodent) by PCR, each of them retaining one of the 24 human chromosomes. An amplification product of expected size and sequence was detected in only one hybrid cell line, demonstrating the location of the human

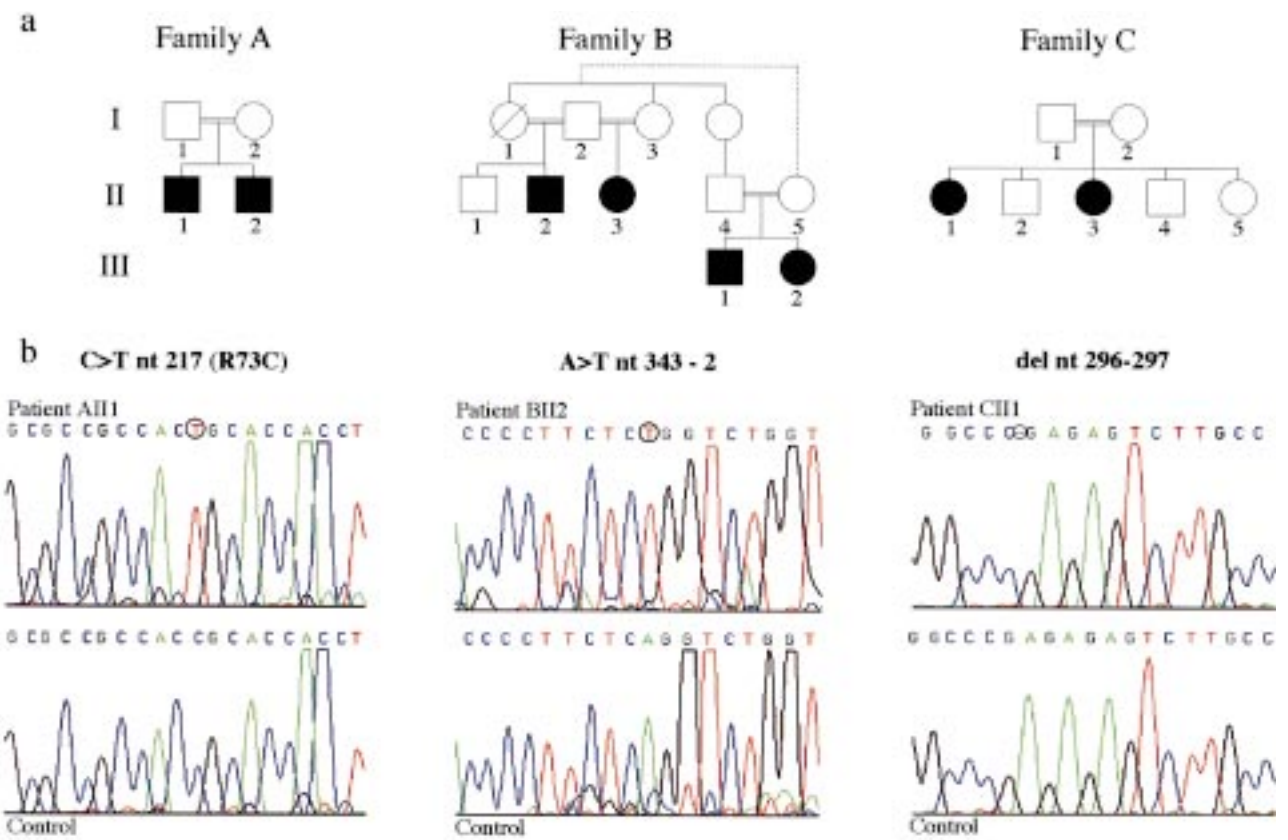


Fig. 3. Mutation analysis in families with CPHD. a: Genealogical trees of families A, B and C with CPHD. Affected individuals are shown as filled-in symbols. Consanguinity was documented in all families. b: Sequence chromatograms of affected individuals from families A (AII1), B (BII2) and C (CII1) (top), and control subjects (bottom). Mutations are encircled.

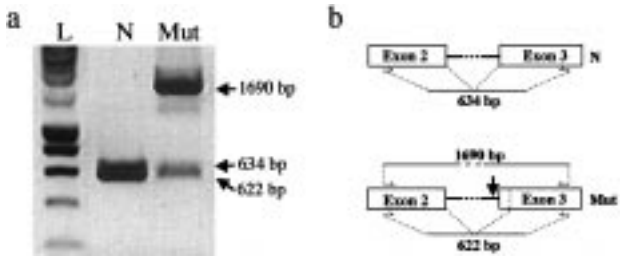


Fig. 4. Functional consequences of the A-to-T substitution identified in Family B on Prop-1 RNA splicing. a: RT-PCR amplification of RNA from COS-7 cells transfected with the normal (lane N) or mutant (lane Mut) Prop-1 gene. Lane L: Molecular size ladder (Smart Ladder Eurogentec). b: Schematic representations of the splicing mechanisms leading to normal (top) and abnormal (bottom) Prop-1 transcripts. Normal splicing (N) of intron II results in the generation of a 634-bp product, whereas the T-to-C substitution of the last dinucleotide of intron II (vertical arrow) results in two abnormal Prop-1 messengers (Mut), the major one (1690 bp) retaining the intronic sequence, and the minor one (622 bp) resulting from the use of a cryptic splice-acceptor site located in exon 3 (vertical dotted line within exon 3).

Prop-1 gene on chromosome 5 (data not shown). The gene was sublocalized by fluorescence in situ hybridization. Spots of specific hybridization were observed on metaphase spreads at band q35 (Fig. 2).

### *3.3. Mutation analysis in CPHD families*

In family A (Fig. 3a), both patients (AII1 and AII2) displayed a homozygous C-to-T transition (C217T) at codon 73 in exon 2 (Fig. 3b); this nucleotide change predicts a missense mutation (R73C) located in the N-terminal region of the homeodomain. As expected for recessively inherited diseases occurring in consanguineous families, both parents (AI1 and AI2) were found to be heterozygous for the mutant allele.

An intronic point mutation (A-to-T substitution) involving the last dinucleotide of the splice-acceptor site preceding exon 3 was identified in family B (Fig. 3a and b). The four patients (BII2, BII3, BIII1 and BIII2) were found to be homozygous for this mutation, whereas one healthy sibling (BII1) was heterozygous, a result consistent with the consanguinity documented in this family. To determine the consequences of this splice-acceptor-site mutation on the processing of Prop-1 transcripts, COS-7 cells were transfected with normal or mutant human Prop-1 genes. RT-PCR of RNA isolated from cells transfected with the wild-type construct yielded a 634-bp amplicon consistent with normal splicing of intron II (Fig. 4a and b). In contrast, RT-PCR of RNA isolated from cells transfected with the mutant construct produced large amounts of a larger PCR product (1690 bp) together with a weakly expressed species (622 bp) (Fig. 4a). Sequence determination of these products revealed that the mutation abolishes normal splicing: it generates a major transcript (1690 bp) retaining intron II, and a minor transcript (622 bp) that results from the utilization of a cryptic splice site within exon 3 (5′-TCTGGTTCCAG-3′), thereby causing the loss of the first 12 nucleotides of this exon (Fig. 4b).

In family C (Fig. 3a), a 2-bp deletion (del nt 296–297) was detected in exon 2 (Fig. 3b). This causes a frameshift, which predicts a premature TGA stop codon at position 109. Similarly, in this consanguineous family, both patients (CII1 and CII3) were found to be homozygous for this mutation, whereas both parents (CI1, CI2) and two healthy siblings (CII4 and CII5) were heterozygous, the healthy brother (CII2) carrying two normal Prop-1 alleles.

To further test whether the three mutations are responsible for the CPHD phenotype, 21 unrelated control individuals were screened for these Prop-1 nucleotide variations. None of their 42 chromosomes contained such mutations. However, in the course of this study, we detected three intragenic polymorphic sites. Two of them are transcribed: one is a synonymous substitution located at nucleotide 27 of the coding sequence (codon 9) in the first exon (GCT→GCC); the other one, which is located in exon 3 outside the homeodomain, is a G-to-A transition at nucleotide 424 resulting in the A142T substitution. The remaining polymorphic site involves the third nucleotide of intron I that can be either an adenine or a guanine residue. The frequency of these Prop-1 variants among the 42 control chromosomes studied is provided in Table 2. In the control population, the thymine residue at codon 9 was always associated with an adenine residue at the third nucleotide of intron I (Table 2), a result demonstrating a complete linkage disequilibrium between these two intragenic loci.

## 4. Discussion

We have cloned, mapped, determined the structure for, and sought CPHD-associated mutations in the human gene encoding Prop-1, a novel pituitary-specific transcription factor recently characterized in mice [7]. Two nucleotide variations were identified in the human Prop-1 coding sequence of unrelated controls, one leading to an amino acid substitution at codon 142 (alanine or threonine); this amino acid change, which is located outside the homeodomain, is considered as a polymorphism, since it was present in 19% of the 42 chromosomes studied. The human amino acid sequence concurs with a recent study in which an alanine residue was present at position 142 [10], whereas the reported mouse Prop-1 sequence contained a threonine residue at this position [7], an observation further supporting that residue 142 is indeed polymorphic. The human gene spans a genomic fragment of about 4 kb of chromosome 5 in a region (q35) syntenic to the mouse chromosome 11 which was shown to contain the Prop-1 gene [7,16]. In comparing the human Prop-1 cDNA and genomic sequences, we were able to determine the intron-exon boundaries; the human Prop-1 gene includes three trans-

Table 2
Frequency of Prop-1 variants in a control population

| Location | Nucleotide change[a] | Amino acid change | Allele frequency in control population |
|---|---|---|---|
| Exon 1 | T→C at nt 27 | A9A | 20/42 chromosomes (47.6%) |
| Intron I | A→G at nt 109+3 | – | 20/42 chromosomes (47.6%) |
| Exon 3 | G→A at nt 424 | A142T | 8/42 chromosomes (19%) |

[a]Nucleotide numbering refers to the adenine residue of the ATG translation start codon as nucleotide number one.

lated exons, each intron-exon boundary containing the gt-ag consensus sequences for eukaryotic donor and acceptor splice sites [17].

The description of a Prop-1 missense mutation in the Ames dwarf mouse strain [7] characterized by CPHD associated with hypoplastic anterior pituitary, prompted us to examine the human ortholog as a CPHD candidate gene. Eight patients belonging to three independent families were examined. Since all affected individuals were born to consanguineous unions and parents showed no symptoms, an autosomal recessive mode of inheritance was considered. We found one missense mutation, one frameshift mutation, and one splice-site mutation, all highly likely to be responsible for the disease phenotype. Indeed, all three mutations, which were absent from 42 control chromosomes, are located in the gene fragment encoding the homeodomain of the Prop-1 protein. Their segregation with affection status is complete in each family, in keeping with the recessive transmission of the disease phenotype. The R73C missense mutation replaces a charged residue by a neutral amino acid in a region highly conserved among all members of the *paired-like* class of homeodomains. Moreover, this arginine residue, which is invariant in all these proteins, is conserved in 95% of the more than 400 homeodomain proteins so far identified [8,18]. As this amino acid substitution results from a C-to-T transition at a CpG doublet, it may correspond to a mutational hot spot, a hypothesis that could be tested by the molecular study of additional CPHD cases. It is striking to note that the 2-bp deletion identified in one family involves a dinucleotide (AG) that belongs to a series of three tandem repeats; since the same molecular defect was identified in other CPHD families [10,11], it is highly likely to represent a mutational hot spot, such repeats, like classical microsatellite loci, being prone to mutation by slipped strand mispairing. To address this question, the Prop-1 intragenic polymorphic sites identified in this study may be compared among those families to determine whether the mutation is recurrent or results from a founder effect. The nucleotide variation identified in our third CPHD kindred is a splice-site mutation in the conserved 3′ region of the intron preceding exon 3. In vitro splicing assays indeed demonstrated that the latter mutation generates two abnormal Prop-1 transcripts: the major one, which retains intron II, would result in a severely truncated protein with a deletion of about 50% of the Prop-1 protein including a large fragment of the homeodomain; the minor abnormal Prop-1 transcript is generated through the use of a cryptic splice site located in exon 3 [19]; if translated, it would result in the deletion of 4 residues of the homeodomain, two of which being invariant in more than 95% of all homeoproteins so far identified [8,18]. Altogether, these data strongly suggest that this mutation substantially alters the function of the Prop-1 gene.

A total of 6 different Prop-1 mutations have been identified in the CPHD families so far investigated ([10,11], and this study). Strikingly, all these mutations are clustered in the gene fragment encoding a key functional domain, the homeodomain. These patients displayed a disease phenotype similar to that of patients with a Pit-1 defect. However, Prop-1-deficient patients are also gonadotropin deficient; Prop-1, which is known to be essential for the determination of the somatotroph, lactotroph, and thyreotroph Pit-1-dependent lineages [5,20], therefore appears to be critical to the proper development of gonadotrophs.

The genetic heterogeneity of CPHD in humans is demonstrated by the existence of deleterious mutations in Pit-1 and Prop-1; it is likely that the recent description of CPHD phenotypes in murine strains generated by targeted disruption of other pituitary-specific transcription factors [21–25] will provide a basis for the molecular characterization of other types of combined hormone pituitary deficiencies in humans. This was recently illustrated by the association of forebrain midline defects with pituitary dysplasia in Hesx-1 null mutant mice, a result that led to the identification of Hesx-1 mutations in patients with septo-optic dysplasia [26]. Such studies will also contribute to elucidate the hierarchy of genetic factors involved in pituitary development.

*Acknowledgements:* This work was supported by the Institut National de la Santé et de la Recherche Médicale and by grants from the Assistance Publique/Hôpitaux de Paris (CRC), the Programme Hospitalier de Recherche Clinique (PHRC), the Association Française contre les Myopathies (AFM) and the Association pour la Recherche sur le Cancer (ARC).

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