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Cloning and gene structure of the rod cGMP phosphodiesterase delta subunit gene (*PDED*) in man and mouse

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Rod-specific cGMP phosphodiesterase (PDE) is a key enzyme of the phototransduction cascade, and mutations in its catalytic subunits have been associated with retinal degenerative diseases. The bovine δ-subunit solubilises the normally membrane-bound PDE and is the only subunit expressed in extraocular tissues. We isolated the human and mouse orthologs, and found 78% identity at the DNA level and 98% identity at the protein level. The *Caenorhabditis elegans* homolog shows 69% identity at the protein level. The human *PDED* gene consisted of 5 exons spanning at least 30 kb of genomic DNA. Northern blot analysis showed a 1.3 kb transcript in human retina, heart, brain, placenta, liver, and skeletal muscle. Fluorescence *in situ* hybridisation (FISH) and radiation hybrid mapping localised the human *PDED* gene to chromosome 2q37. A preliminary screen of all 5 exons in 20 unrelated patients with autosomal recessive retinitis pigmentosa revealed no *PDED* mutations.

Keywords: PDED; Pded; genomic structure; expression; chromosome 2q37; mutation analysis

Introduction

In vertebrate phototransduction, cGMP hydrolysis is initially activated by the G protein coupled receptor rhodopsin. The enzyme responsible for this hydrolytic activity is a phosphodiesterase (PDE) that specifically hydrolyses cGMP to 5'-GMP. The reduction of cytoplasmatic cGMP mediates the closure of cation-specific channels in the rod outer segment and leads to the hyperpolarization of the cell membrane.[1,2]

Photoreceptor phosphodiesterases (PDEs) are members of one of at least seven different gene families of cyclic nucleotide PDEs known in mammals. The major isozyme in rod photoreceptors is the membrane-associated PDE (PDE6).[3] This multi-subunit protein is attached to the disc membrane, but also exists as a soluble form. The enzyme consists of a heterodimeric catalytic complex: one α subunit and one β subunit, each associated with an inhibitory γ subunit.[4] The isozyme found in the cone outer segment is different, consisting of two identical α' subunits and one or two

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inhibitory γ subunits.[5] The attachment of the PDEs to the disc membranes in rods is mediated by the interaction of the C-terminal isoprenylated catalytic α and β subunits.[6,7] An additional fourth subunit of bovine rod PDE6, the δ subunit, has been cloned and characterised. The published bovine cDNA codes for a 17 kDa protein that was co-purified with the soluble form of both rod and cone phosphodiesterase. Functional studies suggest that the δ subunit binds to the isoprenylated catalytic subunits at their carboxy termini and solubilises the membrane-bound PDE.[8]

Mutations in genes coding for photoreceptor signal transduction proteins are candidates for retinal degeneration diseases. Autosomal recessive retinal degeneration in the *rd* mouse is caused by a mutation in the gene for the murine β subunit of the rod cGMP PDE.[9] In humans, a homozygous nonsense mutation in this gene (gene symbol *PDEB*), localised at 4p16.3,[10] is associated with autosomal recessive retinitis pigmentosa, a common hereditary photoreceptor dystrophy.[11,12] In contrast to these homozygous mutations in *PDEB*, a heterozygous missense mutation in *PDEB* is responsible for an autosomal dominant form of congenital stationary night blindness.[13] Another form of autosomal recessive retinitis pigmentosa is caused by mutations in the α subunit of the rod cGMP PDE (gene symbol *PDEA*),[14] which maps to 5q31.2–q34.[15] No mutations in the human γ and δ subunits have been thus far described.

We now report the cloning of the human and mouse cDNAs of the rod cGMP PDE δ subunit (gene symbol *PDED*/*Pded*). The map location and genomic organisation reported here should facilitate the screening of families with inherited retinal dystrophies and provide the basis for further investigation into the function of this highly conserved gene.

Materials and Methods

Genomic PAC Clones

A human PAC library derived from cultured fibroblasts[16] and gridded on filters was screened with an EST (GenBank acc. no. R81870) that showed high sequence identity with bovine *PDE* δ subunit (GenBank acc. no. U65073). The probe was labelled with [α-^{32}P]dCTP by random priming and two positive clones were isolated (LLNLP704M15186, LLNLP704I11616). Clones were provided by the Resource Centre of the German Human Genome Project (RZPD, Berlin).

5' and 3'RACE and RT-PCR

RACE (rapid amplification of cDNA ends) experiments were performed with a cDNA amplification kit containing human retina (Marathon ready cDNA, Clontech). 5'RACE was performed using an adaptor-specific primer AP1 and a *PDED* specific primer (5'RACE: 5'-TAC CTG GCA GTC CTT GAT AGA GGC AGC-3'). 3'RACE was performed using AP1 and a *PDED* specific primer (3'RACE: 5'-TAC ACA GAG CTG GTC CCC TGG TGG C-3'). The PCRs were carried out as recommended by the manufacturer. PCR products were gel purified, cloned into pGEM-T vector (Promega) and sequenced.

Total RNA from adult mice (brain and spleen) was prepared using TRIZOL reagent (Gibco/BRL). First strand cDNA synthesis was carried out using random primers and reverse transcriptase (Pharmacia). The Marathon ready cDNA from 17-day mouse embryo (Clontech) was used for RT-PCR and RACE experiments. One to 10 ng of cDNA was used as a PCR template (PdedF: 5'-CTG TTC ACC TGA GGT GTC CG-3', PdedR: 5'AGG AGG CAG GCA GGT GAC AGG-3') and semi-nested PCR (PdedR2: 5'-AGC TGG TTT CCT GGT GCA CAG GTG GG-3') reactions with Advantage cDNA PCR Kit (Clontech). Cycling profiles included an initial denaturation step (94°C for 1 min) followed by 30 cycles at 94°C for 30 sec, 60°C for 30 sec, and 68°C for 3 min. The amplification products were cloned into pGEM-T vector (Promega) and sequenced.

Exon–Intron Structure

A random shotgun library of PAC LLNLP704M15186 was constructed by ligation of sonicated PAC DNA in a linearised M13mp18 blunt-ended vector; 20 000 clones were transferred on to nitrocellulose filters and hybridised with the human 800 bp *PDED* 5'RACE product. Positive M13 clones (120 total) were sequenced using ABI dye primer cycle sequencing chemistry on ABIPRISM 377 automated sequencers. Sequence tracts were assembled using FACTURA and INHERIT programs. The primers used to amplify exons 1–5 of *PDED* from genomic DNA are listed in Table 1.

RNA Isolation and Northern Analysis

A human multiple tissue northern blot was obtained from Clontech. Total RNA from human and bovine retina was prepared using TRIZOL Reagent (GIBCO/BRL). Poly-$(A)^+$ RNA was then isolated from total RNA using an Oligotex mRNA mini kit (Qiagen). Approximately 1.3 to 1.6 μg of poly$(A)^+$ RNA from each sample was electrophoresed on a 1% formaldehyde-agarose gel and transferred to Hybond-N membranes (Amersham). The filters were then hybridised with ^{32}P-labelled probes.

Radiation Hybrid Mapping

Radiation hybrid mapping was performed using the GeneBridge 4 radiation hybrid DNA panel consisting of 93 radiation-reduced human-hamster cell hybrids (UK HGPM Resource Centre).[17] The positive and negative cell hybrids were determined by PCR with primers from *PDED* exon 4 (PDED-Ex4-F: 5'-TTG GTG GTT CTC AGG GTA GG-3', PDED-Ex4-R: 5'-TTC TCA ACC GAG CTC TCT GC-3'). Results were analysed on the Whitehead Institute web site (http://www.hgmp.mrc.ac.uk/Whitehead/contigdb/tubeorderhgmpgenebridge.html).

Fluorescence in situ *Hybridisation*

Human metaphase cells were prepared from phytohemaglutinin-stimulated peripheral blood lymphocytes according to

Table 1 Primer sequence for PCR amplification of human PDED exons

Exon	*Forward primer (5′->3′)*							*Reverse primer (5′->3′)*							*pos**	*bp*
1	AGA	AGG	GAT	CAG	AAG	CGG	GA	TCC	CAA	GTC	CTC	CCG	GCC	CC	–26/+21	264
2	CTT	TCA	GTG	CCT	TAT	TTT	TC	GCT	AGT	CTG	GCA	TTG	GTC	ACA	–19/+30	178
3	ATA	GTC	CTG	TGG	TTT	TCA	TT	TAT	TGT	TGC	CTT	TTG	TCC	TG	–35/+43	244
4	TTA	TAC	TGG	GGC	ACC	TGC	TC	AGA	CAC	ACT	TCA	TAA	TTT	CC	–26/+18	190
5	TGT	GGA	AAT	GAA	ATC	TTG	TG	ATC	AAA	CGT	GTG	GAG	GAA	A	–49/+66	236

*The genomic position of the forward (–) and reverse (+) primer in relation to exon boundaries.

standard procedures. Fluorescence *in situ* hybridisation (FISH) was performed with two PAC clones containing the human *PDED* gene (LLNLP704M15186, LLNLP704I11616). PAC-DNA was labelled by nick translation with biotin-14-dUTP (Gibco BRL) and preannealed with Cot-1 DNA (Gibco BRL). Detection and visualisation was done using the avidin-fluorescein isothiocyanate/antiavidin antibody system described elsewhere,[18,19] and chromosomes were identified by staining with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI).

SSCP Analysis

DNA from 20 unrelated autosomal recessive retinitis pigmentosa patients from Italy was investigated using PCR-SSCP analysis of all 5 *PDED* exons (Table 1) and all the *RHO* and *RDS* exons as well. Genomic DNA was amplified (30 cycles, 30 s at 95°C, 45 s at 60°C, 45 s at 72°C) after an initial denaturation for 2 min at 95°C. Reactions (12 µl) contained 50 mM KCL, 30 mM Tris-HEPES (pH 9), 5 mM $MgCl_2$, 0.005% gelatine, 3 mM DTT, 8% glycerol, 1% DMSO, 0.5 µM of each primer, 3.3 mM dNTP, 100 ng of genomic DNA and 0.4 U of Amplitaq (Perkin Elmer). SSCP was performed at 4°C using two different gel compositions: 9% acrylamide (1:60 bis), 8% glycerol, 0.5 × TBE, and 9% acrylamide (1:50 bis), 10% glycerol, 0.5 × TBE. The gels were silver stained as previously described.[20]

Results

Cloning of the Human PDED *and mouse* Pded *cDNA*

Screening human EST databases with the published bovine sequence (GenBank acc. no. U65073) produced approximately 20 different ESTs each from human and mouse. The human and murine ESTs cover the entire gene. None of the human ESTs has been mapped. A human spleen cDNA sequence (GenBank acc. no. AA343100) was used to design primers and to synthesise overlapping PCR products of 800 and 660 bp by 5' and 3'RACE, respectively, with human retina as a template (Figure 2B). Sequencing both cloned products (p5'RACE-1 and p3'RACE-1) revealed an ORF of 453 bp, a 5'-UTR of 129 bp and a 3'-UTR of 515 bp (Figure 1). Sequences from two positive mouse ESTs (GenBank acc. no. W13087, W30564) were used to design mouse *Pded* cDNA-specific primers. PCR fragments of approximately 800 bp were generated from mouse brain in the first round of the RT-PCR, and in mouse spleen and 17-day mouse embryo in a second semi-nested round of the RT-PCR. Sequencing of the cloned RT-PCR products yielded a 700 bp mouse cDNA, consisting of a 111 bp 5'-UTR, a 453 bp coding sequence, and a 136 bp 3'-UTR. We used the human 3'RACE primer for RACE-PCR with 17-day mouse embryo cDNA as a template. The cloned mouse 3'RACE product yielded part of the coding sequence (145 bp) and 466 bp of the downstream sequence (Figure 1).

Conservation of PDED *in Different Species*

PDED was found to be highly conserved between man, mouse and other species investigated. This conservation was examined using a genomic Southern blot containing DNA from a variety of species which was hybridised with the probe p5'RACE-1. Homologous sequences were detected for gorilla, cattle, pig, chicken and mouse (data not shown).

A comparison of the human and mouse genes showed 78% identity at the DNA level, with 98% identity at the protein level; three amino acid substitutions were detected (Figure 1). The degree of homology between the human and the bovine gene was even higher: 88% identity at the DNA and 99% identity at the protein levels. In addition, a BLAST database search with the human *PDED* cDNA identified a *Caenorhabditis elegans* homolog (GenBank acc. no. U14635) of *PDED*, which showed 69% identity at the protein level. GenBank entries containing human and murine *PDED*/*Pded* sequences (AF022912, AF045999, AJ001626, AF046000) are documented. No other homologies to known proteins, protein domains, or functional units were found.

Genomic Structure of PDED

A shotgun approach was used to determine the genomic structure of *PDED*. Exon–intron boundaries were determined by sequencing 120 *PDED* positive shotgun clones from PAC clone LLNLP704M15186.

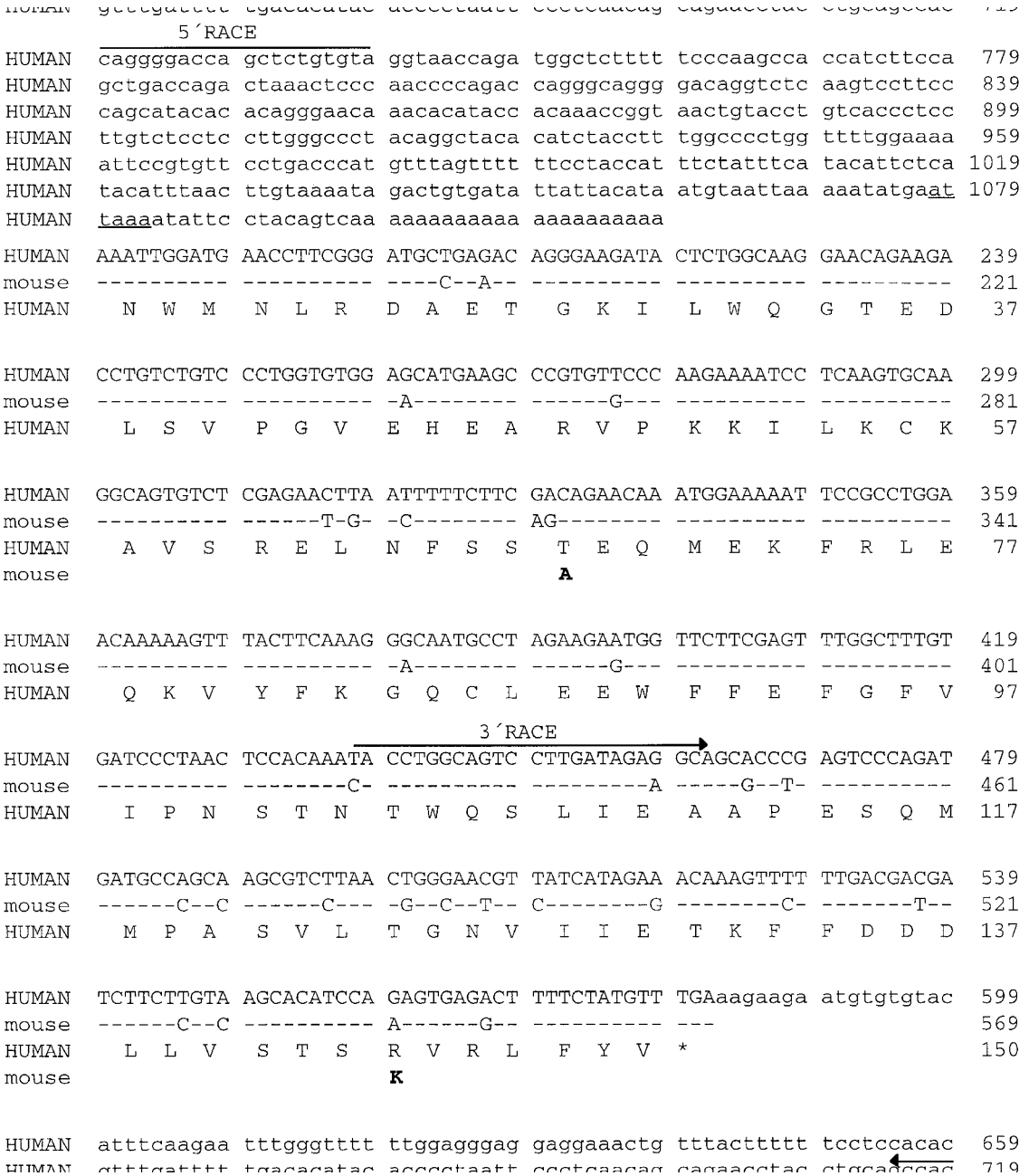

Figure 1 *Alignment of nucleotide and amino acid sequences of human* PDED *and mouse* Pded. *The human sequence is a composite of 5'RACE and 3'RACE products (5' and 3'RACE primers are indicated by arrows). The mouse sequence was obtained by RT-PCR from a 17-day mouse embryo. Nucleotides and amino acids are numbered at the right. Nucleotides of the coding sequences are in capitals, nucleotides of the untranslated regions are in lower case. Identical nucleotides in the mouse sequence are shown by blanks, and amino acid differences in the mouse are designated by bold letters. The poly(A) signal is underlined.*
(GenBank acc. no. AF039216)

The *PDED* gene consisted of 5 exons spanning at least 30 kb of genomic DNA (Figure 2A). All of the introns had canonical splice donor and acceptor sites (Table 2). Exon 1 contained the 5'UTR of *PDED* mRNA and the first 17 amino acid residues, including the protein initiation codon. Exons 2, 3 and 4 coded for 30, 42 and 35 amino acid residues, respectively. Exon 5 spanned the sequence encoding the last 26 amino acid residues and the entire 3'UTR.

For restriction mapping, PAC clone LLNLP704M15186 was digested and hybridised with the probes p5'RACE-1 and p3'RACE-1 (Figure 2B). Three *Eco*RI (14 kb, 13.5 kb, 3.8 kb), two *Hind*III (12 kb, 4.2 kb) and four *Pst*I fragments (7.7 kb, 7.7 kb, 3.5 kb, 580 bp) were obtained. The positive 4.2 kb *Hind*III and 3.5 kb *Pst*I fragments were subcloned and sequenced. The 4.2 kb *Hind*III fragment contained the cDNA sequence from bp 1 to 179 of the *PDED* gene (human 5'UTR and exon 1) and an additional 3 kb of upstream sequence. A 743 bp sequence starting 22 bp upstream of exon 1 (Figure 2A) had a GC content of 69%. No canonical TATA box was present; the only prominent promotor elements identified by sequence analysis were one GC box at position –100 and one CAAT box at position –108 (data not shown). The 3.5 kb *Pst*I fragment contained the cDNA sequence

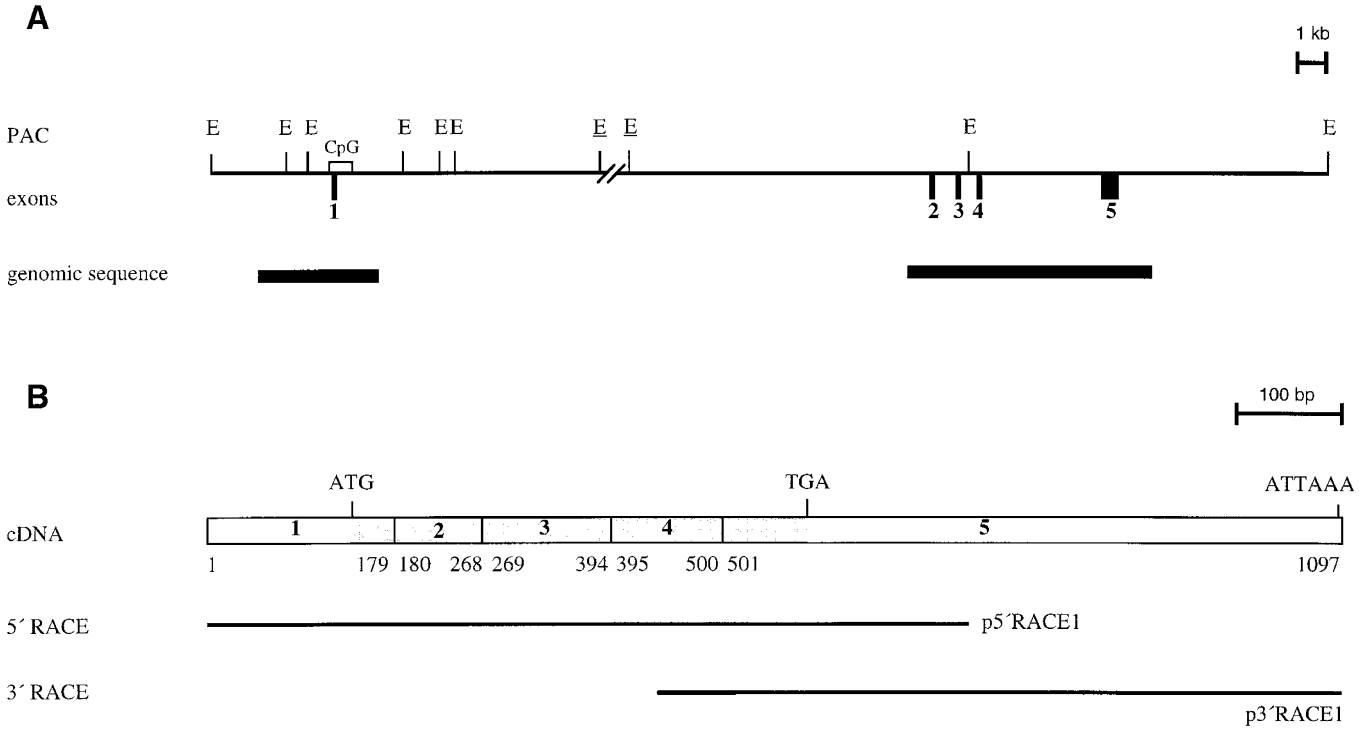

Figure 2 *Genomic structure and cDNA cloning of the* PDED *gene. (***A***) Genomic structure of the* PDED *gene and partial restriction map of PAC LLNLP704M15186 spanning the* PDED *gene is in scale, the two underlined* Eco*RI sites (E) could be identical. Exons are indicated by black boxes, the CpG island is shown by an open box. Black bars give the extent of the genomic regions sequenced. E,* Eco*RI. (***B***) The* PDED *cDNA is in scale with the ORF shaded and translation start, stop codon and polyadenylation signal marked. Numbers below correspond to nucleotide positions at the beginning and end of each exon. Cloned human RACE products are shown at the bottom.*

Table 2 Intron–exon organization of the human *PDED* gene

Exon	*Nucleotide No*	*bp*	*3′Splice junction**	*5′Splice junction**
1	1–179	179	–	ACTgtatccgtccgggag
2	180–268	89	gtcatatttgatcagAAA	AAGgtactttccaaccct
3	269–394	126	tttttctcttaccagCCC	AAGgtattctgctgccta
4	395–500	106	tccttcttccttcagAAT	AACgtgagtgagcaggtc
5	501–1097	597	tcttcttacttacagTGG	–

*The coding sequence is in capitals, the intron sequence in lower-case.
(GenBank acc. no. AF042833: AF042834; AF042835)

from bp 715 to 1097 (human 3'UTR) and additional 1.7 kb of downstream sequence.

Expression of PDED *mRNA*

Using the p5'RACE-1 clone as the hybridisation probe, a transcript of 1.3 kb was observed in northern blots of both human and bovine retina (Figure 3B). The same message of 1.3 kb was detected in heart, brain, placenta, liver, and skeletal muscle, with a weak hybridisation signal in lung and pancreas. No transcript was detected in kidney (Figure 3A).

Chromosomal Location

The PACs LLNLP704M15186 and LLNLP704I11616 were used for FISH analysis of metaphase chromosomes. The hybridisation resulted in specific labelling of the telomeric end of the long arm of chromosome 2, corresponding to band 2q37 (Figure 4). To determine the precise chromosomal localisation of the *PDED* gene, we used radiation hybrid mapping with the GeneBridge 4 DNA panel, which places the *PDED* gene at a distance of 3.5 cR from D2S427 on the long arm of chromosome 2 within 2q37.1.

Mutation Analysis

A preliminary SSCP screening for mutations performed on 20 unrelated patients affected by autosomal recessive retinitis pigmentosa failed to identify any mutations or polymorphisms in any of the 5 exons of the human *PDED* gene (data not shown). Mutations in

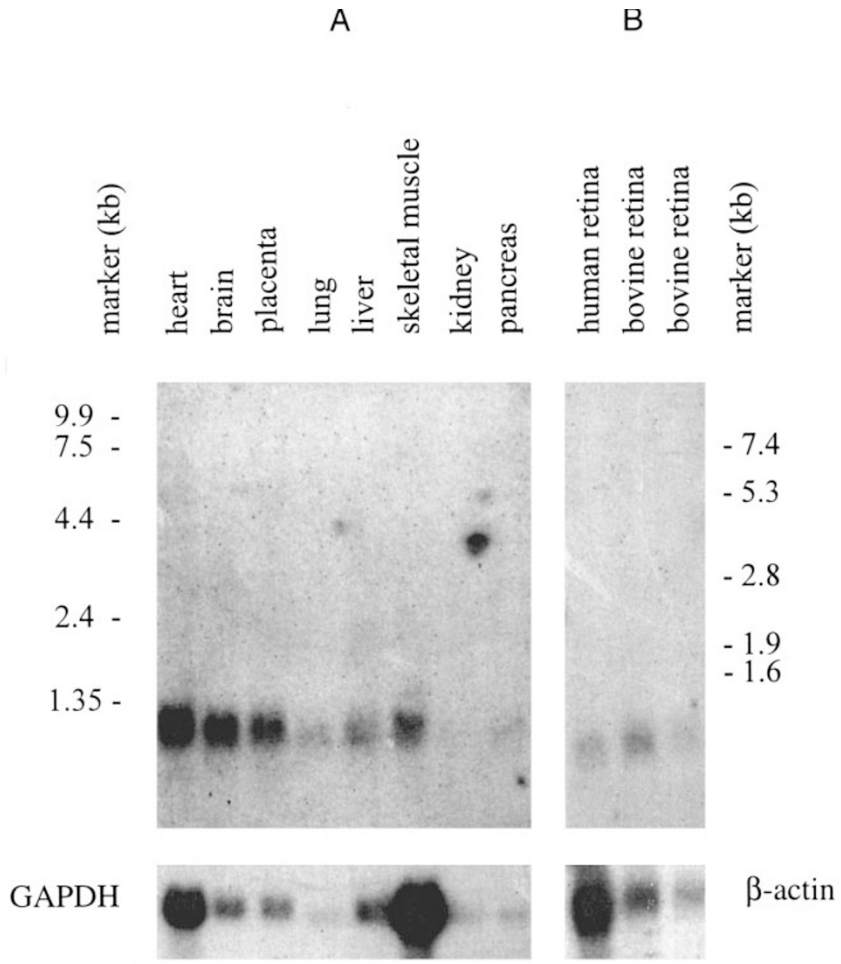

Figure 3 *Analysis of* PDED *expression.* (**A**) *A human adult multiple tissue northern blot was hybridised with the probe p5'RACE-1 (exposure: 18d). The* PDED *transcript (1.3 kb) is strongly expressed in heart, brain, placenta, liver and skeletal muscle. The hybridisation signals in lung and pancreas are weak and no signal is seen in kidney.* (**B**) *A northern blot of 1.3 to 1.6 μg of poly (A)$^+$ RNA from human and bovine retina was hybridised with the probe p5'RACE-1 (exposure: 18d). An identical transcript of 1.3 kb is expressed in human and bovine retina. The filters were rehybridised with GAPDH (exposure: 30 min) and β-actin (exposure: 18d).*

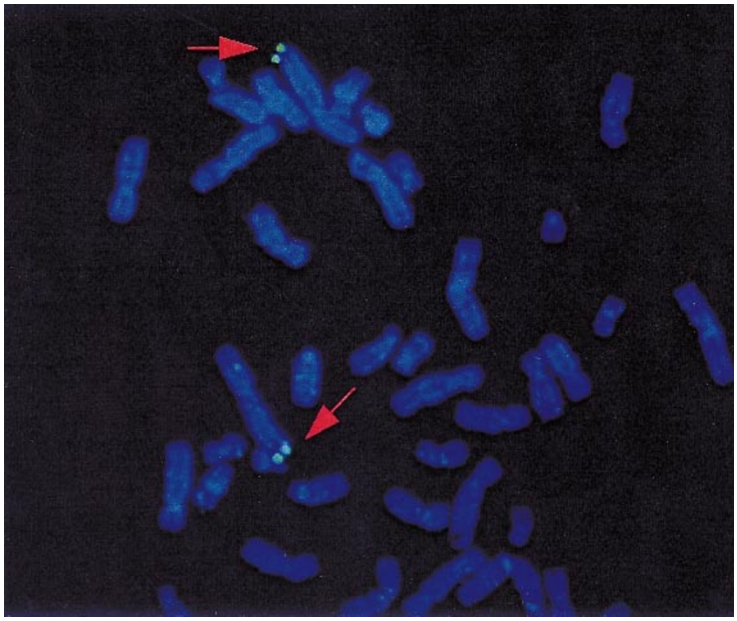

Figure 4 *Localisation of the human* PDED *gene to chromosome 2q37 using PAC clone LLNLP704M15186 as a probe. Fluorescent signals on DAPI stained chromosomes are indicated by arrows.*

the rhodopsin (*RHO*) and peripherin (*RDS*) genes were also excluded by SSCP screening in all 20 patients.

Discussion

The hallmarks of a housekeeping gene are a CpG island around the transcription starting point, no typical TATA box and ubiquitous expression.[21] The GC-rich region around the *PDED* transcription start fulfils the criteria of a CpG island.[22] The only prominent promotor elements identified by sequence analysis of the 3 kb sequence upstream of exon 1 were a GC and a CAAT box. Eight different adult tissues studied including retina expressed the *PDED* transcript at high levels (Figure 3), and the gene is highly represented in EST libraries. Thus, *PDED* appears to be a typical housekeeping gene.

The presence of a single band at 1.3 kb on northern blots, consistent with the predicted cDNA length derived from sequence prediction and RACE experiments, indicated that the complete cDNA was cloned. The putative translation start is marked by the methionine at nucleotide position 130, which coincides with the first methionine in both bovine and mouse sequences. An ideal Kozak consensus sequence[23] was not found in man or mouse. A single full length *PDED* homolog (with 103/148 amino acid identity) for *Caenorhabditis elegans* was also found. There are only a few proteins known with a sequence conservation much higher than 70% between worm and man, such as vab-3/PAX-6 or the Pou domaine of unc-86/BRN-3.[24,25] This indicates a fundamental function of the PDED protein in a wide variety of, or possibly all cell types. It remains to be elucidated whether its function relies on PDE protein interactions alone, or whether other proteins are involved as well.

The bovine homolog was originally described as a subunit of the rod-specific isoform of phosphodiesterase (PDE6), although widespread expression of the bovine gene had also been demonstrated. Rod specificity has been claimed on the basis of immunochemistry results, which showed a signal at the outer rod segments, but no signal in cones.[8] This implies that the PDE6 interaction serves a specialised function in the eye and makes *PDED* a candidate for mutations in retinal disease. Although a preliminary mutation screen on 20 unrelated patients with recessive retinitis pigmentosa did not show sequence alterations in any of the 5 exons, there is a high degree of genetic heterogeneity in retinal disease. Thus, various dominant and recessive forms of both retinal degeneration and stationary retinal disease, including the nearby Oguchi form of stationary night blindness[26] could be further candidates for investigation. The housekeeping nature of *PDED* could also mean that disruption causes embryonic lethality, but, as the example of *RPGR* has

shown,[27] retinal disease can be the only symptom of a ubiquitously-expressed gene.

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