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Human Propionyl-CoA Carboxylase β Subunit Gene: Exon-Intron Definition and Mutation Spectrum in Spanish and Latin American Propionic Acidemia Patients

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Summary

Propionyl-CoA carboxylase (PCC) is a mitochondrial biotin-dependent enzyme composed of an equal number of α and β subunits. Mutations in the PCCA (α subunit) or PCCB (β subunit) gene can cause the inherited metabolic disease propionic acidemia (PA), which can be life threatening in the neonatal period. Lack of data on the genomic structure of PCCB has been a significant impediment to full characterization of PCCB mutant chromosomes. In this study, we describe the genomic organization of the coding sequence of the human PCCB gene and the characterization of mutations causing PA in a total of 29 unrelated patients—21 from Spain and 8 from Latin America. The implementation of long-distance PCR has allowed us to amplify the regions encompassing the exon/intron boundaries and all the exons. The gene consists of 15 exons of 57–183 bp in size. All splice sites are consistent with the gt/ag rule. The availability of the intron sequences flanking each exon has provided the basis for implementation of screening for mutations in the PCCB gene. A total of 56/58 mutant chromosomes studied have been defined, with a total of 16 different mutations detected. The mutation spectrum includes one insertion/deletion, two insertions, 10 missense mutations, one nonsense mutation, and two splicing defects. Thirteen of these mutations correspond to those not described yet in other populations. The mutation profile found in the chromosomes from the Latin American patients basically resembles that of the Spanish patients.

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Introduction

Propionyl-CoA carboxylase (PCC; EC 6.4.1.3) is a mitochondrial, biotin-dependent enzyme involved in the degradation of several essential amino acids (threonine, valine, isoleucine, and methionine), fatty acids of odd-numbered chain lengths, and cholesterol (Fenton and Rosenberg 1995). It catalyzes the enzymatic carboxylation of propionyl-CoA to D-methylmalonyl-CoA in an ATP-dependent process. PCC is a heteropolymeric enzyme composed of two types of subunits, an α subunit (74 kD), containing the covalently attached biotin moiety, and a β subunit (55 kD). Genetic deficiency of PCC causes an inborn error of metabolism, termed "propionic acidemia" (PA). Two distinct genotypic forms of PA have been distinguished by somatic cell complementation: pccA (MIM 232000) and pccBC (MIM 232050), resulting from mutations in the PCCA or the PCCB gene, respectively. The disorder is clinically heterogeneous, with a severe ketoacidotic form that can be lethal in the neonatal period and a mild, late-onset form that includes developmental retardation and that may be associated with episodic ketoacidosis.

Human α-PCC (Lamhonwah et al. 1989; Stankovics and Ledley 1993) and β-PCC cDNAs (Ohura et al. 1993*b;* Lamhonwah et al. 1994) previously have been cloned and characterized. Recombinant α-PCC and β-PCC subunits have been expressed in both eukaryotic (Stankovics and Ledley 1993; Lamhonwah et al. 1994) and prokaryotic (Leon-Del-Rio and Gravel 1994; Kelson et al. 1996) expression systems. The PCCB cDNA contains an open reading frame of 1,617 nucleotides, encoding a 539–amino acid polypeptide. The PCCB gene is located on the long arm of human chromosome 3 (Kraus et al. 1986; Lamhonwah et al. 1986).

So far, mutations in the PCCB gene have been described only in a few patients, who, in most cases, carry mutations affecting the coding sequences. Until now, 13 putative disease-causing mutations, including RNA-splicing defects, have been reported, occurring in Caucasian and Japanese patients (Lamhonwah et al. 1990;

Tahara et al. 1990, 1993; Ohura et al. 1993*a,* 1993*b,* 1995; Gravel et al. 1994; Hoenicka et al. 1998). Several of these mutations lie in the same exon, which spans nucleotides 1199–1299, suggesting that this exon may be a hot spot for mutations (Tahara et al. 1990, 1993; Ohura et al. 1993*a*). The most frequent mutation found in Caucasian patients is an insertion/deletion (ins/del) (1218del14ins12) (Tahara et al. 1990, 1993; Pérez-Cerdá et al. 1994). Recently, we reported that c1170insT is a frequent mutation among Spanish patients (Hoenicka et al. 1998). In Japan, the single-base substitution T428I appears to be the most common disease-causing mutation (Ohura et al. 1993*a*), underscoring the possible independent origin of the mutations.

The lack of data on the PCCB gene structure has been a significant impediment to full characterization of the PCCB mutant chromosomes. In this article, we describe the genomic organization of the coding region of the PCCB gene. Each exon-intron boundary has been defined. The information on genomic structure has enabled us to perform a complete mutation analysis, for a total of 29 unrelated PA patients—21 from Spain and 8 from Latin America. A total of 16 different mutations were detected. Eleven of these are described for the first time. The mutation spectrum found in the chromosomes from Latin American patients resembles that of the Spanish patients. We speculate that the mutations could have originated in Europe and were carried to the New World by the founding families.

Subjects and Methods

Subjects

In this study we included 32 patients (including three pairs of siblings) from 29 unrelated families with documented PA due to a deficiency in the PCCB gene. Twenty-three patients from 21 unrelated families are from different regions of Spain. The remaining patients are from Latin American countries (7 from Chile, 1 from Brazil, and 1 from Ecuador).

For all the patients, some of whom have been reported elsewhere (Del Valle et al. 1982; Pérez-Cerdá et al. 1998), diagnosis of PA was based on clinical symptoms and biochemical analysis. The deficiency was confirmed by assaying PCC carboxylase activity in lymphocytes and/or skin fibroblasts, by use of the method described by Suormala et al. (1985). The cell cultures were maintained in Minimum Essential Medium (MEM; GIBCO-BRL) supplemented with 1% glutamine, 10% FCS, and antibiotics.

Assignment of cell lines to the major complementation groups was achieved by (1) estimation of PCC activity and the ratio between PCC and β-methylcrotonyl-CoA carboxylase activity in parents' fibroblasts (Wolf and Rosenberg 1978); (2) complementation studies that tested fusion of the patients' skin fibroblasts with representative cell lines from the major complementation groups, pccA (line 117, kindly provided by R. A. Gravel, Montreal) and pccBC (line GMO3590, from the National Institute of General Medical Sciences Human Genetic Mutant Cell Repository), by use of a solution of 50% PEG 4000 in MEM, in accordance with the procedure previously described by Gravel et al. (1994); and (3) ascertainment of the presence of functional α subunit in cultured fibroblasts labeled with ^{3}H-biotin, in accordance with the method of Lam Hon Wah et al. (1983). Fibroblasts, lymphocytes, peripheral blood leukocytes, and dried blood spots were also obtained from at least 20 unrelated controls and were used as RNA and/or DNA sources.

Exon-Intron Boundaries

Genomic DNA, isolated in accordance with the method described by Old (1986), was used as template for the analysis of PCCB gene organization. The exon-intron boundaries were defined by generation, by use of long-distance PCR, of overlapping intron-containing genomic fragments encompassing the entire coding sequence of the PCCB gene. Amplification reactions were performed by means of the XL PCR kit (Perkin Elmer), in a final volume of 50 μl, by use of 300 ng genomic DNA. The $Mg(OAC)_2$ concentration (1.2–1.5 mM) and annealing temperature (61°C–66°C) were optimized for each primer set used. Thirty cycles of two-step PCR amplification were performed, each consisting of 20 s of denaturation at 94°C and 6–10 min at the annealing temperature, with lengthening of the annealing step by 15-s increments for each of the last 15 cycles. After a final 10-min extension at 72°C, the reaction products were electrophoresed on 0.8% agarose gels, purified by Gene Clean, and direct sequenced with fluorescently labeled primers, by use of the fmol DNA sequencing kit (Promega). Sequences were analyzed on an automated DNA sequencer (ALF Express, Pharmacia). Primers derived from the PCCB cDNA sequence (Ohura et al. 1993*a;* Gravel et al. 1994) were used for the initial sequencing. The resulting sequence was compared with the cDNA sequence, to identify exon-intron boundaries. New intronic primers then were designed to obtain the remaining exon-intron junctions. Some of the sequences used to design the intronic primers were kindly provided by D. Leclerc and R. A. Gravel. The size of each PCR product was determined on an 0.8% agarose gel, by use of DNA Molecular Weight Marker II (Boehringer Mannheim).

Table 1

PCCB Primers Used for Exon Amplification

Exon	5′-Intron Primer (5′→3′)	Distance from Exon (bp)	3′-Intron Primer (5′→3′)	Distance from Exon (bp)	Length of PCR Product (bp)	EMBL Accession No.
1	$cDNA_1$: GCTCTAGACGCGCCGGCACAGCAA	...	AS1: ccgggcacgcagtgataggtc	65	272	AJ006487
2	S184: cttcaagccctcccatgaacagc	85	AS184: ggtaggccagctgagccacacct	56	261	AJ006488
3	S304: ggcatagtggccaaactcattag	71	AS304: agttttcattccccaagattggc	92	232	AJ006489
4	S373: cactgggttttcatctctagc	64	AS373: gtacctagctcagtgtcttgt	94	215	AJ006490
5	S430: ctgtggtattttgtgaatgtcg	74	AS430: gccaggccatggcttagagaa	65	253	AJ006491
6	S544: tatctttccacagataatgcctc	67	AS544: caacctagatctgggtcagga	91	269	AJ006492
7	S655: gaatcaactctaaggctgtgacc	70	AS655: aggctctttggcaatgccctct	65	249	AJ006493
8	S764: gaagcagcaactttgggctggc	63	AS764: cctgtcagggcagagaaaggcaa	67	251	AJ006494
9	S885: aagcccagcagggacagaactgg	128	AS885: caagtggtccagtgcttcctcac	92	302	AJ006495
10	S965: gaaatgtcattctgtagtgtcat	79	AS965: gacagaaaggataccatggacc	77	280	AJ006496
11	S1091: ggatggctgctgaggacaaa	85	AS1091: tgggaaaggcaactctcctagcc	44	237	AJ006497
12	S1120: CGTTTTGTCAGATTCTGTGATGC	317	AS1199: cactactgccttccagttccc	198	616	AJ006497
13	S1237: GCCAAGCTTCTCTACGCATTTG	442	AS1300: agctatgccttgagctctcgc	72	613	AJ006497
14	S1399: cagttcctccctctatgctatg	157	AS1399: cactagtcaacagattagactgc	119	376	AJ006498
15	S1499: ctaccatctctgtatcaggttg	134	$cDNA_2$: GCTCTAGACTTGGTTTCTTTTCCTTTGATTT	...	289	AJ006499

NOTE.—$cDNA_1$ and $cDNA_2$ are exon-based primers that were designed, by Ohura et al. (1993*a*), to be complementary to the 5′ and 3′ UTRs of the human PCCB gene. S1120 and S1237 are exonic primers that hybridize to the preceding exon.

Table 2

Alignment of the Exon and Intron Boundaries

Exon	Size (bp)	Intron	Exon				Intron	Score	
								3′ Acceptor Site	5′ Donor Site
1	⩾183	…	ATG Met 1	GCG	AAG	CGA Arg 61	gtgagtcctgaggggcctaa	…	76.5
2	120	tgatctttgttccattgtag	GGA Gly 62	AAG	AAT	AAG Lys 101	gtatttgttcaaatggtggt	89.0	74.3
3	69	tttttgcatttttctggtag	TTT Phe 102	CCT	AGT	CAG Gln 124	gtatttcataactccaatag	81.9	74.3
4	57	gatttgtattttcttttttag[a]	GAT Asp 125	TTT	TGC	AAA Lys 143	gtaagtgtttaatactcaaa[b]	91.3	87.6
5	114	agatttctctgctgtctcag	ATC Ile 144	ATG	TTT	CTG Leu 181	gtgagaaacctgttaataga	88.2	83.2
6	111	ttgcttttctgttttggcag	AGG Arg 182	AAT	GTA	AAG Lys 218	gtaagaaagaagggcctgtt	95.2	94.3
7	109	ctggaaatcttttatttcag	GAC Asp 219	ACC	TCA	G Gly 255	gtgagaggccttgaagatga	96.4	91.0
8	121	tatgctccctgttctcttag	GT Gly 255	GTG	CCC	AG Ser 295	gtgggttgtaggccggtgca	84.3	86.0
9	82	tgccttttttctgcctaaag	T Ser 295	GAC	CAC	TCT Ser 322	gtaagtgccacatctgtttg[a]	80.0	79.0
10	124	aagtaaatttattcctgcag	GTT Val 323	GTT	TCA	G Gly 364	gtaggatggagctcttataa	97.2	83.6
11	108	gataatctctctctttctag	GA Gly 364	TGC	CCT	G Gly 400	gtaagtttttgacagagtgg	88.0	92.1
12	101	taactcttcctcatgtctag[c]	GC Gly 400	ACA	AGG	AAG Lys 433	gtgaggacctcatgttggag[c]	83.7	92.1
13	99	agcatttggatctgttttag	GCC Ala 434	TAT	GCA	AAG Lys 466	gtgagggcctcttgcttttc	77.6	92.1
14	100	atgaactcctctaatcacag	GGC Gly 467	GCT	CGA	G Gly 500	gtaggggactgtggtgaaga	92.2	84.7
15	⩾122	ccttttctgtgcttcaccag	GG Gly 500	TTT	TTG Leu 539	TAA Stop	…	87.3	…

[a] Data from R. A. Gravel (personal communication).
[b] 5′ donor site reported by Ohura et al. (1995).
[c] 5′ donor site and 3′ acceptor site reported by Tahara et al. (1990).

PCR Amplification of Exons

$cDNA_1$ and $cDNA_2$ sequences correspond to primers designed previously, by Ohura et al. (1993*a*), to be complementary to the 5′ and 3′ UTRs, respectively, of the PCCB gene. The other primers basically were designed to amplify each PCCB exon and at least 40 bp of the surrounding intron sequence (table 1). PCR was performed in a final volume of 100 μl, by use of 0.5–1 μg genomic DNA, 0.2 μM each primer, 0.25 mM each dNTP, 1.5 mM $MgCl_2$, 10% dimethyl sulfoxide, and 0.5 units AmpliTaq (Perkin-Elmer). A "hot" start was performed, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, by use of a Peltier Thermal Cycler PTC-200. To amplify exon 1, the annealing tem-

Table 3

Mutations Identified in the PCCB Gene

Mutation[a]	Nucleotide Change	Effect on Coding Sequence[b]	Restriction-Site Change[c]	Frequency (%) of Allele	
				Spain	Latin America
ins/del	1218del14ins12	fs and stop codon	−*Msp*I	13/42 (31.0)	7/16 (43.7)
c1170insT	ins of T at 1170–1174	fs and stop codon	…	7/42 (16.7)	1/16 (6.2)
E168K	502G→A	Glu168Lys	−*Mbo*II (ACRS)	6/42 (14.3)	4/16 (25.0)
A497V	1490C→T	Ala497Val	−*Bso*FI	4/42 (9.5)	0/16 (0)
G198D	593G→A	Gly198Asp	+*Ava*II	2/42 (4.8)	0/16 (0)
R44P	131G→C	Arg44Pro	−*Msp*I	1/42 (2.4)	0/16 (0)
S106R	318C→A	Ser106Arg	−*Bst*UI (ACRS)	1/42 (2.4)	0/16 (0)
G131R	391G→C	Gly131Arg	−*Mnl*I	1/42 (2.4)	0/16 (0)
R165W	493C→T	Arg165Trp	+*Nla*III	0/42 (0)	1/16 (6.2)
R410W	1228C→T	Arg410Trp	−*Msp*I	1/42 (2.4)	1/16 (6.2)
1298-1299insA	ins of A at 1297–1299	fs and stop codon	…	0/42 (0)	1/16 (6.2)
R512C	1534C→T	Arg512Cys	−*Pml*I	1/42 (2.4)	0/16 (0)
L519P	1556T→C	Leu519Pro	+*Msp*I	1/42 (2.4)	0/16 (0)
W531X	1593G→A	Trp531Stop	−*Sty*I	1/42 (2.4)	0/16 (0)
IVS1+3G→C	183+3G→C	Splice mutation	−*Bsa*I (ACRS)	1/42 (2.4)	0/16 (0)
IVS10-11del6	1091-11del6	Splice mutation	…	1/42 (2.4)	0/16 (0)
Total no. (%) identified	…	…	…	41/42 (97.6)	15/16 (93.7)

[a] Mutations that have not been described yet in other populations are underlined. Designations correspond to those described by Antonarakis and the Nomenclature Working Group (1998), except for "ins/del" and "c1170insT," which had been named previously.

[b] fs = frameshift.

[c] ACRS = amplification created restriction site.

perature was 60°C. In some cases, PCR amplifications were performed directly by use of dried blood spots as the source of DNA (Pérez et al. 1993).

Mutation Analysis

Reverse transcriptase PCR (RT-PCR)–based methods were used initially, to identify mutations and to confirm the predicted consequences of splice mutations. The total fibroblast RNA (1 μg), isolated in accordance with the method of Chomczynski and Sacchi (1987), was used for first-strand cDNA synthesis. PCCB cDNA was amplified in five overlapping fragments of ~400 bp in length, by use of the primers and the amplification conditions described elsewhere (Ohura et al. 1993*a;* Gravel et al. 1994).

Mutations also were detected by amplification of PCCB exons from genomic DNA of fibroblasts, followed by direct cycle sequencing, with the fmol sequencing system (Promega), of 100 ng of the PCR products, as described above. Prior to sequencing, the PCR-amplified fragments were purified by use of the Wizard PCR Preps DNA purification system (Promega).

Detection of certain mutations, as well as analysis of control chromosomes, was accomplished by restriction digestion analysis of PCR products, either directly or after creation of the restriction site during amplification. Digestion conditions were in accordance with the manufacturers' protocols. After digestion, fragments were electrophoresed on 4% Nusieve gels.

Western Blot Analysis

Western blot analysis was performed in accordance with standard procedures. The primary antibody used was affinity-purified anti–β-PCC produced in rabbit by immunization with purified β-PCC expressed in *Escherichia coli* as fusion protein maltose binding protein–PCCB, by use of the pMAL expression system, as described by Richard et al. (1997).

Results

Exon-Intron Boundaries

To determine the structure of the coding sequence of the PCCB gene, we performed long-distance PCR with genomic DNA and primers hybridizing to PCCB cDNA sequences. Following the initial reactions, new primers were designed on the basis of our increasing knowledge of the gene structure. Using this strategy, we successfully amplified genomic DNA fragments, from 2 kb to ~20 kb in size, that spanned the entire coding sequence. Alignment of these genomic PCR products demonstrated that the coding region of the PCCB gene consists of 15 exons of 57–183 bp in size. All exon-intron splice junctions conformed to the eukaryotic 5′ donor–3′ acceptor

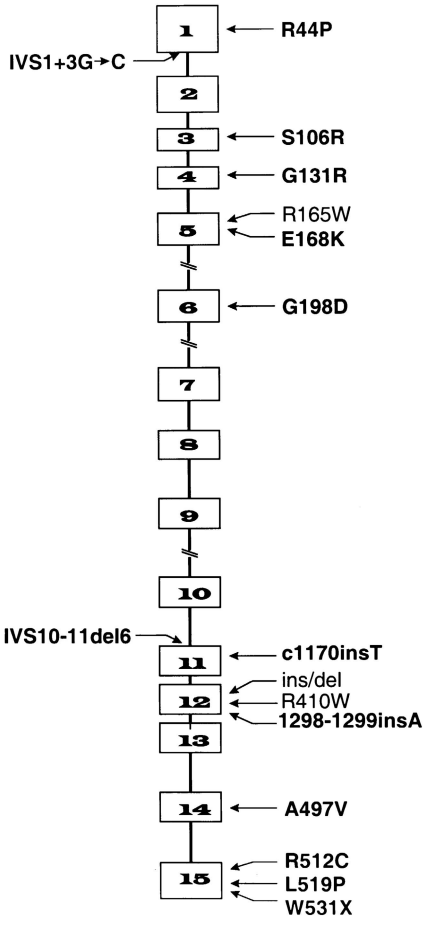

Figure 1 Mutations in the human PCCB gene. The locations of the PCCB mutations are shown in the schematic representation of the exonic structure of the gene. Mutations that have not been described yet in other populations are shown in boldface.

consensus splice junction gt/ag rule (Stephens and Schneider 1992). Junctions at introns 1, 2, 3, 4, 5, 6, 9, 12, and 13 were type 0 (splicing occurring between codons); junctions at introns 7, 10, 11, and 14 were type 1 (splicing occurring after the first base of the codon); and the junction at intron 8 was type 2 (splicing occurring after the second base of the codon). All exon-intron boundaries are listed in table 2. The splice sites were scored in accordance with the method described by Shapiro and Senepathy (1987): scores for donor sites varied within the range 74.3–94.3. The acceptor sites scored within the range 77.6–97.2.

To facilitate detection of mutations by restriction digestion and/or direct sequence analysis of genomic DNA, sets of intronic oligonucleotide primers were designed and tested for their ability to amplify individual exons and the immediately flanking intronic sequences (table 1). Amplification of exons 12 and 13 was performed with sense exonic primers (in capital letters in table 1), hybridized to the preceding exon and previously designed for a different use. The exon sequences are identical to those of the corresponding region in the cDNA.

Mutation Spectrum

In this study, we included 29 unrelated PA patients—21 from Spain and 8 from Latin America (6 from Chile, 1 from Brazil, and 1 from Ecuador). All of them had been assigned to the major complementation group pccBC and, therefore, were selected as having a PCCB mutation, as described in Subjects and Methods.

All patients were screened for the most frequent mutations found previously in chromosomes from Spanish PA patients—namely, an ins/del (1218del14ins12) and c1170insT (1173-1174insT) (Pérez-Cerdá et al. 1994; Hoenicka et al. 1998). The ins/del was found to be present on 31% of the alleles from Spanish patients and 47% of the alleles from Latin American patients. However, the second most frequent mutation in Spain, c1170insT (frequency 16.7%), was found in only one chromosome, from a Chilean patient (frequency 6%) (table 3).

Mutation analysis of the remaining mutant chromosomes was performed by sequencing of the PCR products obtained, after amplification of the 15 PCCB exons, from genomic DNA and from those fragments derived from reverse-transcribed RNA (i.e., from RT-PCR). Most of the mutations were confirmed further by restriction-enzyme digestion.

Using this approach, we characterized the two mutant alleles in 27 patients, which account for 97.6% (41/42) of the mutant alleles from Spanish patients and 93.7% (15/16) of the mutant alleles from Latin American patients. Of these patients, ~38% are homozygous for one mutation, and close to 62% are compound heterozygotes for two different mutations. The mutation spectrum includes one ins/del, two insertions, 10 missense

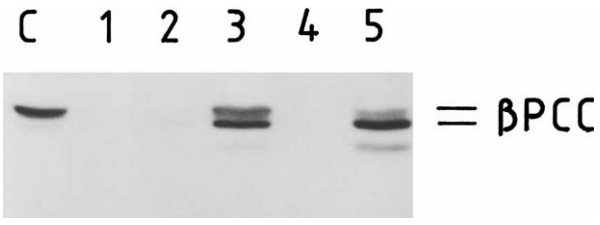

Figure 2 Western blot analysis of the β-PCC subunit from PA patients. Cell extracts of confluent fibroblasts containing equal amounts of total protein (120 μg) were immunoprecipitated with polyclonal anti–human β-PCC. Lane C, Control cell line. Lanes 1 and 3–5, Extracts from PCCB-deficient patients (SSL, PRG, MRA, and JGG, respectively). Lane 2, Extract from the PCCA-deficient patient, SAG (Richard et al. 1997).

Table 4

Genotypes and Western Blot Analysis of the PCCB-Deficient Patients

Patient(s)	Mutations[a]	Western Blot[b]	Origin (*n*)
ALT, CBV, MRA, LMC, DTV	ins/del, ins/del	−	Spain (3), Chile (2)
SSL, APJ	c1170insT, c1170insT	−	Spain (2)
PRG, NGA	E168K, E168K	+[c]	Spain (1), Chile (1)
SVL	A497V, A497V	+[c]	Spain (1)
CTG[d]	G198D, G198D	NS	Spain (1)
LGA, JGG, FM	ins/del, E168K	+[c]	Spain (2), Chile (1)
VMP	ins/del, A497V	+[c]	Spain (1)
AJA	ins/del, R44P	+[c]	Spain (1)
AG	ins/del, W531X	+[c]	Spain (1)
EAC	ins/del, R512C	−	Spain (1)
PPM	ins/del, R165W	+[c]	Ecuador (1)
PVD	ins/del, G131R	−	Spain (1)
DMS, HMM	c1170insT, E168K	+[c]	Spain (1), Chile (1)
PHH	c1170insT, L519P	+[c]	Spain (1)
CJF	A497V, S106R	NS	Spain (1)
MBG	E168K, IVS1+3G→C	+[c]	Spain (1)
OVE[e]	R410W, IVS10-11del6	+[f]	Spain (1)
HM	R410W, ND	+[f]	Brazil (1)
LVA	c1170insT, ND	NS	Spain (1)
MV	ins/del, 1298-1299insA	−	Chile (1)

[a] ND = not detected.

[b] A plus sign (+) indicates the presence of the β subunit, and a minus sign (−) indicates the absence of the β subunit. NS = not studied (fibroblasts not available).

[c] Two bands of β-PCC of normal and reduced size.

[d] Clinical report was published in the article by Del Valle et al. (1982).

[e] Clinical report was published in the article by Pérez-Cerdá et al. (1998).

[f] β-PCC smaller than normal size.

mutations, one nonsense mutation, and two splice-site alterations. The relative frequencies are shown in table 3. Mendelian inheritance of the mutations was confirmed by use of a restriction-digestion test, if available, or by sequencing of DNA from family members.

Figure 1 shows the 16 PCCB mutations and their locations. Ten of the mutations found in the coding sequence were novel. One is an insertion, 1298-1299insA, which inserts an extra A between positions 1297 and 1299 of exon 12, generating a frameshift and a stop codon seven triplets downstream in the new frame. The rest are missense and nonsense mutations (E168K, A497V, R44P, G131R, G198D, R512C, L519P, W531X, and G593A). To exclude the possibility of a polymorphism, each mutation also was analyzed in 40 normal chromosomes, by restriction digestion of the respective exons amplified by PCR. The R410W and R165W mutations have been described elsewhere, in a study of Japanese patients (Ohura et al. 1993*a*).

E168K is a 502G→A transition, located in exon 5, that replaces the acidic polar glutamic residue with the basic polar lysine residue. The allele frequency of this mutation is 14.3% in Spain and 25% in Latin America. This suggests that E168K may be a common mutation.

A497V is a 1490C→T transition that replaces the small nonpolar alanine residue by the larger nonpolar valine residue. This mutation has been detected in three Spanish PA patients who are from the same small village (Hoenicka et al. 1997). The resulting frequency of this mutation is 9.5% in Spain.

Each of the remaining mutations was represented by one or two alleles. The effect and nucleotide change of each mutation are shown in table 3. R44P is a 131G→C transversion occurring in exon 1, close to the putative cleavage site of the leader peptide. R512C, L519P, and W531X are clustered in exon 15 (fig. 1). W531X results in a 1593G→A transition that changes a tryptophan to a termination codon. Translation of this mutant mRNA would result in a protein truncated by nine amino acid residues in the C-terminal region.

Finally, as is shown in table 3 and figure 1, we also found two splice-site mutations. IVS10-11del6 causes an aberrant splicing of the PCCB mRNA, resulting in the skipping of exon 11 (authors' unpublished data), and the novel IVS1+3G→C disrupts the 5′ donor site of intron 1 and is predicted to affect the splicing efficiency of exon 1, although this could not be demonstrated, since the sense primer for RT-PCR hybridizes with this region.

Western Blot Analysis

To study the possible effect of the mutations on the size and/or quantity of the translated β-PCC, we per-

formed western blot analysis of our PCCB mutant cell lines (fig. 2 and table 4). The results indicated a total absence of immunoreactive β-PCC in all patients who were homozygous for the ins/del or c1170insT mutation and in those patients who were compound heterozygotes for the ins/del and 1298–1299insA, ins/del and R512C, or ins/del and G131R mutations. Cell lines from patients carrying the R410W mutation in combination with another, different mutation showed a band smaller than normal in size and quantity. For the remaining cell lines, we observed a reproducible pattern of two different bands, one apparently of normal size and the other smaller than normal. For the control cell lines, a unique band was obtained, and the PCCA mutant cell line that was included as a negative control showed absence of β-protein (fig. 2).

Discussion

The strategy of direct sequencing of long-distance PCR products obtained from genomic DNA has resulted in an effective method of determining the exon structure of the PCCB gene. The gene consists of 15 exons. Sequences flanking splice junctions have been determined. The 5′ donor and 3′ acceptor sites at the splice junctions correlated well with published consensus sequences (table 2).

The availability of gene sequence information provided the basis for implementation of a comprehensive screening for genetic alterations, by use of genomic DNA, in 29 unrelated PA patients—21 from Spain and 8 from Latin America. A total of 56/58 of the mutant chromosomes has been defined, with 16 different mutations detected. Thirteen of these mutations correspond to those not described yet in other populations. The population-frequency values for all these mutations conform to a spectrum of PCCB mutations consisting of four prevalent mutations, accounting for close to 71% of the total mutant alleles, and a high number of rarely occurring mutations. It is interesting to note that six of the single-base substitutions affect CpG sites, which sustain a higher mutation rate (Cooper and Youssoufian 1988).

In two patients, one of the two mutant alleles could not be characterized, suggesting that the mutations must lie in the promoter region or elsewhere within the intronic sequences. For the other patients, we can infer, on the basis of different evidence, that the mutations that were found are the likely cause of the deficiency. These mutations are the only substitutions found in the entire coding sequence and the exon-intron boundaries. Several of them (A497V, E168K, the ins/del, and c1170insT) appear to be common alleles. All mutations are absent from normal chromosomes, and Mendelian inheritance has been confirmed. Finally, to differentiate between polymorphism and mutation, we also analyzed the possible effect of the different changes on the stability and/or function of the protein and the conservation of the amino acid residue in β-PCC from different organisms. The c1170insT, ins/del, and 1298-1299insA mutations generate a frameshift with a considerable reduction of the protein sequence, resulting in the predicted consequence of a truncated protein. Supporting this prediction, western blotting of fibroblasts from patients who are homozygous for both mutations or compound heterozygotes for the ins/del and 1298-1299insA mutations revealed a total absence of β-PCC. This effect could be the result of a rapid posttranslational degradation of the protein. The prediction of the effect of missense mutations is more difficult. Point mutations, such as R512C, R44P, and G131R, could induce a big change in the direct environment of the mutated residue and could dramatically influence protein stability or function (Filippis et al. 1994). The total absence of immunoreactive protein, observed in the western blot of patients who carry G131R or R512C in combination with mutations producing undetectable levels of β-PCC, such as the ins/del or c1170insT, support the hypothesis that certain point mutations can be severe with regard to protein stability.

On the other hand, mutations A497V, G131R, L519P, R512C, G198D, and E168K are nonconservative changes affecting amino acids that are highly conserved in human (Swiss-Prot accession no. P05166; EMBL accession no. X73424), rat (Swiss-Prot P07633; EMBL M14634), *Bacillus subtilis* (Swiss-Prot P54541; EMBL D84432), *Saccharomyces cerevisiae* (Swiss-Prot P53003; EMBL X92557), and *Mycobacterium leprae* (Swiss-Prot P53002; EMBL U00012). More controversial is the effect of the R44P mutation. Until now, the exact length of the cleavable NH_2-terminal leader peptide had not been determined; however, a highly conserved amino acid motif had been identified for the proteolitic cleavage of several mitochondrial targeted proteins (Hendrick et al. 1989). According to these published consensus sequences, R44P is a mutation affecting the mature protein, very close to the cleavage site.

With regard to the two mutations affecting splice sites, we observed an aberrant splicing pattern for IVS10-11del6, which might result in the loss of 36 amino acids in β-PCC (authors' unpublished data). The IVS1+3G→C mutation is predicted to affect the splicing efficiency of exon 1; however, the possible effect of this mutation, on posttranslational mitochondrial transport and processing of the protein, remains to be clarified.

Finally, the fact that three different mutations—R512C, L519P, and W531X—are located in the same exon suggests the presence of a hot spot for mutations similar to that described elsewhere for exon 12 (Tahara et al. 1990, 1993; Ohura et al. 1993*a*). Ex-

pression analysis of all these mutations will reveal their functional consequences for the mutant protein and their involvement in the disease phenotype.

The similarity of the mutation profile reported for the chromosomes from both the Spanish and the Latin American patients suggests that the PA mutations probably were introduced in the New World after the historic migration of southern European settlers, as has been described for other genetic diseases, such as phenylketonuria (Desviat et al. 1995). The presence of mutations such as R410W and R165W in Japanese patients could be explained by a recurrence mechanism (Pérez-Cerdá et al. 1998), because both of these mutations affect a CpG dinucleotide. The higher frequency of the ins/del and E168K mutations in Latin America could be explained by a number of different factors, including genetic drift, or could be due to the specific geographic origin of the European ancestors of this group of probands. The elucidation of the mutation spectrum in different European populations will help to clarify this point.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

European Molecular Biology Laboratory (EMBL), European Bioinformatics Institute, http://www.ebi.ac.uk (for examples of highly conserved proteins in human [X73424], rat [M14634], *B. subtilis* [D84332], *S. cerevisiae* [X92557], and *M. leprae* [U00012] and for PCCB primers used for exon amplification [AJ006487–AJ006499])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for pccA [MIM 232000] and pccBC [MIM 232050])

Swiss-Prot, http://expasy.hcuge.ch/sprot/ (for examples of highly conserved proteins in human [P05166], rat [P07633], *B. subtilis* [P54541], *S. cerevisiae* [P53003], and *M. leprae* [P53002])

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