Human mitochondrial phosphoenolpyruvate carboxykinase 2 gene

Structure, chromosomal localization and tissue-specific expression

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The mitochodrial (mt) phosphoenolpyruvate carboxykinase 2 (PCK2) gene was isolated by screening a human genomic library with a rat cytosolic (cy) PCK1 cDNA probe comprising sequences from exons 2–9 and by PCR amplification of human genomic DNA spanning consecutive exons with known primer pairs from mtPCK2 cDNA containing sequences from two putative neighbouring exons. The mtPCK2 gene spans approx. 10 kb and consists of ten exons and nine introns. All exon–intron junction sequences match the classical GT/AG rule. Northern blot analysis of poly(A)$^+$ and total RNA from various tissues revealed one mRNA species of approx. 2.4 kb. The gene is expressed in a variety of human tissues, mainly in liver, kidney, pancreas, intestine and fibroblasts. In contrast with the cytosolic isoenzyme, the mitochondrial form might not have a purely gluconeogenic function. The mtPCK2 gene maps to chromosome 14q11.2–q12, in contrast with the cyPCK1 gene located on 20q13.2–q13.31.

INTRODUCTION

Phosphoenolpyruvate carboykinase (PCK) (EC 4.1.1.32) catalyses the GTP-driven conversion of oxaloacetate to phosphoenolpyruvate. The enzyme is expressed in various organs, mainly in liver and kidney. It is located in both the cytosol (PCK1) and mitochondria (PCK2) [1,2]. In liver and kidney the relative intracellular distribution varies widely with the species. Thus in rat liver 80–90 % of total PCK activity is cytosolic and 10–20 % mitochondrial [3–5], in human liver 30–50 % is cytosolic and 50–70 % mitochondrial [6–8], in guinea pig liver 15–20 % is cytosolic and 80–85 % mitochondrial [2,4,9], and in chicken liver approx. 95 % is mitochondrial [10]. In rat, guinea pig and chicken kidney the cytosolic-to-mitochondrial activity ratios are 75:25 [4], 20:80 [9] and 40:60 [11] respectively.

The cytosolic form, cyPCK1, is regulated by a variety of hormonal stimuli [12], whereas the mitochondrial enzyme seems to be constitutive [11]. The regulation of the expression of the cyPCK1 gene has been studied most extensively in rat liver: glucagon induces its expression with the permissive action of glucocorticoids; insulin antagonizes the induction by glucagon [13–15]. Gene expression is modulated positively by physiological oxygen tensions [16,17]. Higher PCK1 activity and content [18,19] coincide with higher PCK mRNA levels in periportal hepatocytes [20–22]. The regulation of the expression of the mitochondrial form, mtPCK2, has been investigated in chicken kidney: starvation or acidosis did not alter mtPCK2 activity, whereas they enhanced cyPCK1 [11].

The two forms of PCK are encoded by separate genes: the two different cDNA species of human [23,24] and chicken [25–27] were isolated; they showed identities of 68 % and 60 % respectively. The cytosolic enzyme of rat [28], human [23] and chicken [26] contains 622 amino acid residues. The cognate rat cytosolic enzyme shows an amino acid identity with the human enzyme of 90 % and with the chicken enzyme of 83 %. The mitochondrial pre-enzyme possesses 640 residues in human [24] and 621 or 617 residues in chicken [27]; the size of the mature form is unknown in human and comprises 607 residues in chicken.

The cytosolic PCK1 gene of rat, human and chicken has been elucidated: in rat and human it is composed of ten exons and nine introns [28,29]; and in chicken it is composed of eight exons and seven introns [26]. The mitochodrial PCK2 gene has so far not been isolated in any species. Therefore the goal of the present investigation was to clone and characterize the human mitochondrial PCK2 gene, to establish its expression in major organs and to elucidate its chromosomal localization. A DNA fragment 15 kb in length was isolated from a human genomic library, which included exons 5–10 of the human mtPCK2. The missing exons and introns were prepared by PCR amplification of human genomic DNA between consecutive exons. The entire isolated mtPCK2 gene contained ten exons and nine introns and spanned approx. 10 kb from the translational start site to the stop codon. The mtPCK2 gene was localized by fluorescence *in situ* hybridization (FISH) to chromosome 14q11.2–q12. Northern blot analysis showed an mRNA of approx. 2.4 kb expressed mainly in liver and kidney but also in intestine, pancreas and fibroblasts.

EXPERIMENTAL

Materials

The human leucocyte genomic library EMBL-3 (HL 1006d) was from Clontech (Heidelberg, Germany), The oligonucleotides were synthesized by Nucleic Acids Products Supply (Göttingen, Germany). Restriction endonucleases, DNA polymerase (Klenow fragment), and DNA standards were obtained from MBI Fermentas (St. Leon-Rot, Germany); dideoxynucleotides were from Boehringer (Mannheim, Germany). T4 DNA ligase,

Abbreviations used: DAPI, 4′,6′-diamidino-2-phenylindoledihydrochloride; FISH, fluorescence *in situ* hybridization; cyPCK1, cytosolic phosphoenolpyruvate carboxykinase 1; mtPCK2, mitochondrial phosphoenolpyruvate carboxykinase 2; PEP, phosphoenolpyruvate; UTR, untranslated region.

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alkaline phosphatase and T4 DNA polynucleotide kinase were obtained from Biolabs New England (Schwalbach, Germany); [α-^{32}P]dCTP (specific radioactivity 111 Tbq/mmol) was obtained from ICN (Meckenheim, Germany). *Taq* DNA polymerase was from Eurogentec (Seraing, Belgium), Stratagene (Heidelberg, Germany) or Boehringer Mannheim. All other chemicals were of analytical grade and came from commercial suppliers.

Screening of a human leucocyte genomic library

Approx. 8.8×10^5 plaque-forming units of the human genomic library in EMBL-3 were screened on 20 agar plates (14.5 cm), with *Escherichia coli* NM538 as host strain. Resulting plaques containing recombinant phages were transferred to nylon membrane (Hybond N; Amersham, Braunschweig, Germany) and processed as described in [24]. Filters were hybridized with an [α-^{32}P]dCTP-labelled rat cytosolic PCK1 cDNA probe containing a 1.2 kb *Pst*I cDNA fragment [20] from PC201 [28]. Hybridization was performed by standard protocols [30]. Post-hybridization washes were twice in $2 \times$ SSC/0.1 % SDS (20 min at room temperature) and twice in $0.2 \times$ SSC/0.1 % SDS (20 min at 65 °C) (SSC is 0.15 M NaCl/0.015 M sodium citrate). Filters were finally exposed to X-ray film at -70 °C, usually overnight. The most intensely hybridizing plaques were selected and transferred into dilution buffer [0.1 M NaCl/100 mM $MgSO_4$/35 mM Tris/HCl (pH 7.5)/0.01 % gelatin] and reseeded on agar plates for further purification. Pure phage isolates were obtained after four rounds of plating. DNA from positive phage preparations was purified and the inserted DNA was released from recombinant phage DNA by digestion with *Sal*I restriction endonuclease. Three fragments of 11, 2.8 and 1.2 kb were obtained and purified by gel electrophoresis, then subcloned into the *Sal*I site of pBluescript by standard procedures [30]. Plasmid DNA was transfected into *E. coli* DH5α, then propagated and purified for sequencing. Cycle sequencing was performed with an automated DNA sequencer (Applied Biosystems Model 373 A; Perkin-Elmer) in accordance with the protocols provided by the manufacturer.

PCR reactions and cloning of PCR products

Genomic DNA was isolated from human peripheral white blood cells as described [31]. To isolate exons and introns not contained in the three DNA fragments obtained by screening the genomic library, overlapping PCR amplifications were performed with the genomic DNA isolated from leucocytes as template and specific primers containing sequences from opposite strands of two putative neighbouring exons of the cDNA previously isolated [24]. PCR amplification was performed in 100 μl of reaction mixture containing 50 ng of genomic DNA, $1 \times$ standard PCR buffer (Eurogentec), each of the four dNTPs at 200 μM, 20 pmol of each primer and 0.5 unit of Goldstar *Taq* DNA polymerase (Eurogentec). The PCR amplification (35 cycles) was performed in a Perkin-Elmer 2400 thermocycler. Each cycle consisted of 30 s of denaturation at 96 °C, 45 s of annealing at 60 °C and 2 min of extension at 72 °C. The amplified DNA fragments were sized by agarose gel electrophoresis that included DNA standards. The PCR fragments were subcloned directly into the TA3-pCRII cloning vector from Invitrogen (NV Leek, The Netherlands). Plasmid DNA was transfected into *E. coli* XL-1 blue and propagated. Plasmids were purified by Qiaprep Spin Plasmid Preparation Kit from Qiagen (Hilden, Germany) and sequenced.

Northern blot analysis

Total RNA from human liver and intestine, as well as from rat liver and from cultured human fibroblasts and HepG2 cells, was prepared as described [32]. Samples of normal human liver and intestine were obtained, with the permission of the ethical committee of the Medical School in Göttingen, from patients who had undergone partial resection of the liver or small intestine owing to liver or intestine metastases respectively. Northern blot analysis of these total RNA preparations was performed as described [24]. Hybond N nylon membrane with the transferred electrophoresed RNA was preincubated in 5 ml of hybridization solution containing $5 \times$ SSC, $5 \times$ Denhardt's solution ($1 \times$ Denhardt's is 0.02 % Ficoll 400/0.02 % polyvinylpyrrolidone/0.2 % BSA), 0.5 % SDS and 500 μg of salmon sperm DNA at 68 °C for 30 min. In addition, a blot of electrophoresed poly(A)$^+$ RNA, containing approx. 2 μg per lane from eight different human tissues, was commercially available as multiple tissue Northern blot (Human MTN, 7760-1; Clontech, Heidelberg, Germany). The MTN blot was prehybridized in 5 ml of express hybridization solution supplied by the manufacturer in a hybridization tube with continuous rolling in a Biometra hybidization box (OV 3) at 68 °C for 30 min. Both mtPCK2 cDNA [24] and cyPCK1 cDNA [23] were used as hybridization probes. Each cDNA (15 ng) was labelled by the random-primer method with [α-^{32}P]dCTP as the labelled nucleotide and the Klenow fragment of DNA polymerase (Megaprime; Amersham). The radioactively labelled DNA probes, each at 10^6 c.p.m./ml, were denatured for 5 min at 95 °C, then chilled quickly on ice and added to the hybridization solution. Blots were hybridized at 68 °C for 1 h, rinsed three times in $2 \times$ SSC/0.05 % SDS, then washed once for 40 min at room temperature and twice for 20 min in $0.1 \times$ SSC/0.1 % SDS at 50 °C. They were analysed overnight by a PhosphorImager (Molecular Dynamics, Krefeld, Germany). The data were scanned by IMAGE QUANT software from Molecular Dynamics.

Chromosome mapping by FISH

Mapping was performed by FISH of human mtPCK2 cDNA probes to human interphase lymphocyte chromosomes counterstained with propidium iodide and 4′,6′-diamidino-2-phenylindoledihydrochloride (DAPI) [33,33a]. Plasmids PCK12, PCK13 and PCK14, containing exons 5–10 and introns E–I, were used as probes. The probes were biotinylated by nick-translation and detected with avidin–FITC followed by biotinylated anti-avidin antibody and avidin–FITC. This FISH experiment was performed by Amplimmun (Karlsruhe, Germany).

RESULTS

Isolation and analysis of a mtPCK2 gene clone from a genomic library

To isolate a clone encoding the human mtPCK2 gene, a genomic library in EMBL 3A phage was screened with a 1.2 kb fragment of rat cytosolic PCK1 cDNA (PC 201) comprising exons 3–9 as a probe [28]. A probe of cyPCK1 cDNA was used because at that time a mtPCK2 cDNA had not yet been isolated [24]. Out of approx. 8.8×10^5 phage clones, three were selected on the basis of their positive hybridization to the probe. Recombinant phage DNA containing 15 kb of insert, which encoded part of the human mtPCK2 gene, was isolated. The insert DNA was released from recombinant phage DNA by digestion with *Sal*I restriction endonuclease. Three fragments were isolated and subcloned into the *Sal*I site of pBluescript; they were named PCK11 (11 kb), PCK12 (2.8 kb) and PCK13 (1.2 kb). PCK11 was restricted with

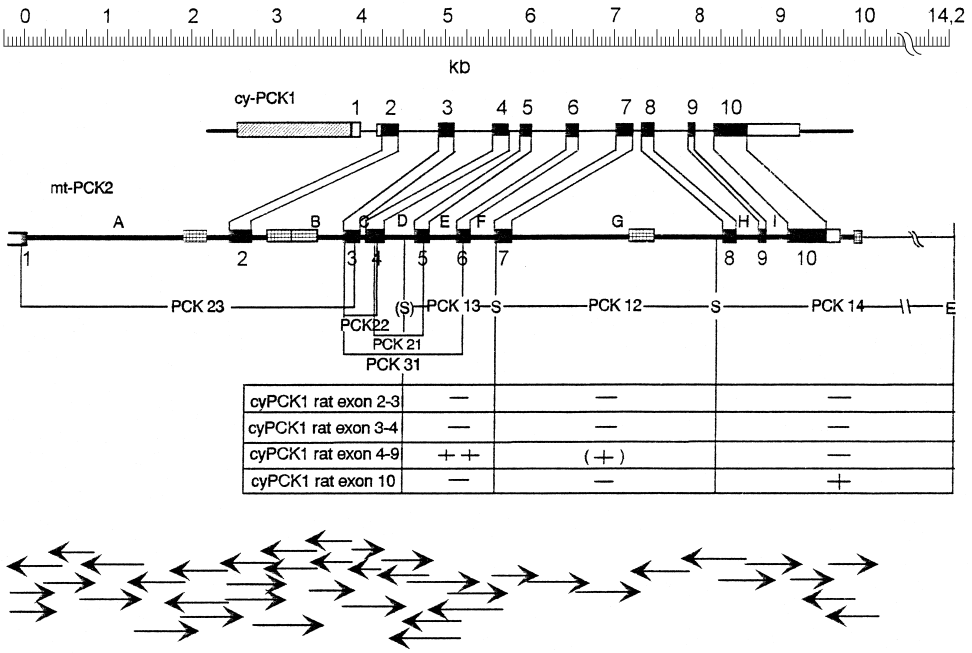

Figure 1 Comparison of the exon–intron structures of the human mitochondrial PCK2 and cytosolic PCK1 genes: sequencing strategy of the human mtPCK2 gene

The exons are denoted by solid boxes and the introns by thick connecting lines. The hatched box indicates the promoter region of the cyPCK1 gene. Open boxes represent the UTRs of exon 1 or exon 1 and 2 respectively, as well as of exon 10. *Alu* sequences are shown by stippled boxes. Abbreviations: S *Sal*I; E, *Eco*RI of the mtPCK2 gene; (S), *Sal*I site of the genomic library EMBL 3A phage. PCK13, PCK12 and PCK11 are the 1.2, 2.8 and 11 kb DNA fragments isolated from the genomic library by digestion with *Sal*I (see the Experimental section). PCK14 is a DNA subfragment of the 11 kb PCK11 DNA fragment obtained by digestion with *Eco*RI. PCK23, PCK22 and PCK21 are fragments of 4, 0.4 and 0.6 kb, respectively, synthesized by PCR using genomic leucocyte DNA as template with primers from the 5′ UTR and exon 3, exon 3 and exon 4, and exon 4 and exon 5 respectively (5′ primers are underlined and 3′ primers are overlined in the sequence in Figure 2; see Table 1). The DNA fragments isolated from the genomic library were characterized by Southern blot hybridization with radioactively labelled cDNA fragments from the indicated exons of rat cyPCK1 cDNA: ++, very intense signal; +, normal signal; (+), faint signal; −, no signal. Sequencing strategy is indicated by arrows showing the direction of sequencing.

*Eco*RI endonuclease. The two fragments obtained were subcloned into *Eco*RI–*Sal*I sites of pUC19 and named PCK14 (5.5 kb) (Figure 1) and PCK15 (5.5 kb) (results not shown).

The orientation of the fragments PCK12, PCK13 and PCK14 was studied by Southern blot analysis with radiolabelled rat cyPCK1 cDNA probes containing exons 2–3, exons 3–4, exons 4–9 and exon 10. PCK13 revealed an intense, and PCK12 a faint, hybridization signal with the rat exons 4–9 probe; they did not react with the rat exons 3–4, exons 2–3 or exon 10 probes. PCK12 gave only a weak signal, probably owing to its very high intron content, as established later. PCK14 hybridized strongly with the rat exon 10 probe. The Southern blot analysis showed that the 5′–3′ orientation of the fragments was PCK13, PCK12 and PCK14 and that probably exons 5–10 and introns E–I were present in the isolated insert DNA (Figure 1). Unidirectional sequencing of PCK13 for orientation indicated that it contained at its 5′ flank parts of intron D (250 bp) and the complete exon 5 (188 bp).

The missing 5′ part of the gene could be obtained either by another screening of the genomic library or by PCR amplification of genomic DNA with primers of neighbouring exons, the sequences of which were known from the previous isolation of the mtPCK2 cDNA [24].

Isolation and analysis of mtPCK2 gene fragments by PCR amplification of genomic DNA

By using genomic DNA from leucocytes as template, the fragment PCK21 containing intron D and the flanking parts of exons 4 and 5 were isolated by PCR amplification with the sense primer ON-PC-11 from exon 4 and the anti-sense primer ON-PC-3 from exon 5 (Table 1 and Figure 2). Similarly, the fragment PCK22 containing intron C and the flanking parts of exon 3 and exon 4 was PCR-amplified with the primer pairs ON-PC-32 from exon 3 and ON-PC-10 from exon 4; fragment PCK23 containing intron A, exon 2, intron B and the flanking parts of exon 1 and exon 3 was PCR-synthesized with the primer pair ON-PC-24 from the 5′ untranslated region (UTR) and ON-PC-33 from exon 3 (Table 1 and Figure 2). The PCR-amplified DNA fragments were isolated and subjected to electrophoretic analysis. All showed single bands: PCK21, PCK22 and PCK23 were 0.6, 0.4 and 4 kb in length respectively. The DNA fragments were isolated and subcloned into the TA3-pCRII cloning vector.

Sequence and exon/intron structure of the human mtPCK2 gene

The PCR-amplified DNA fragments PCK23, PCK22 and PCK21 and the DNA fragments PCK13, PCK12 and PCK14 isolated from a genomic library were sequenced with a gene-walking sequencing strategy in both directions (Figure 1), with 17mers to 24mers as primers (Table 1). The sequence from nucleotide +1 (44 bp upstream of the translation start site) to nucleotide +10047 (455 bp downstream of the stop codon) was determined (Figure 2). Co-linearity of the mtPCK2 gene clone and the PCR-synthesized mtPCK2 gene fragments was ensured by an overlap of PCK13 from the gene library and PCK21 from PCR amplification in 385 bp from positions 4430–4814 (Figures 1 and 2). Co-linearity was further substantiated by PCR-amplifying and

Table 1 Oligonucleotides used to sequence and PCR-amplify DNA fragments of human mtPCK2

Designations are based on: ON, oligonucleotide; PC, PCK. The position in the gene is based on a translation start site at position 45. The position in the cDNA is shown only for primers used to PCR-amplify DNA fragments PCK21, PCK22 and PCK23 as well as PCK31.

Designation	Position in gene	Position in cDNA	Base pairs	Primer sequence 5′–3′
Sense				
ON-PC-9	9184–9202		19	CTACAACTTCGGGCACTAC
ON-PC-11	4182–4199	Exon 4	18	CTGACTCGGCCTATGTGG
ON-PC-13	9510–9526		17	TGACAGAGCAGGTCAAC
ON-PC-24	1–21	5′ UTR	21	CTCCGCTCGGTTCCTGGCCAC
ON-PC-29	8799–8818		20	GTATACGAGGCCTTCAACTG
ON-PC-31	7437–7460		24	CTACTCAGGAGGCAGGAAAATTGC
ON-PC-32	3857–3877	Exon 3	21	AAGGATGTGGCACGAGTAGAG
ON-PC-32in	6562–6583		22	GTATTCTTTCCATGGCCTTCTG
ON-PC-34	449–470		21	GGTACTTCGAGAGGCAGCAGG
ON-PC-37	2673–2696		24	GCATCCACATCTGTGATGGAACTG
ON-PC-39	973–993		21	CTGATGCTCACGGAGAACTTC
ON-PC-42	3109–3130		22	GAATCACTTAAACCCAAGAGGC
ON-PC-43	1551–1572		22	GCAACCATTGTTGGTCCAAGTG
ON-PC-44	1969–1990		22	GTTCAAGCGATTCTCCTGCCTC
Anti-sense				
ON-PC-3	4814–4795	Exon 5	20	GTAGGGCAAAGCACTTCTTG
ON-PC-6	9421–9404		17	GAGATCAAGGCTCCTTC
ON-PC-10	4199–4183	Exon 4	17	CCACATAGGCCGAGTCA
ON-PC-16	9651–9628		24	GTGCCTTCCCTATTCCCAGATGAG
ON-PC-25A	4279–4256		24	CAGACACTTGACAAAGTCACCATC
ON-PC-26bi	2642–2619		24	CTACAAAATCTCGAATGCCAGTGG
ON-PC-30	8725–8702		24	CTGGTGAGCTTGACTGGTACTCTC
ON-PC-33	3906–3883	Exon 3	24	TGAGAAGGAGTTACAATCACCGTC
ON-PC-36	2160–2139		21	CGGTGGCTCATGCCTGTAATC
ON-PC-48	1620–1600		20	GAATAGGTGACTGGATGCTG
ON-PC-50	964–945		20	CACACAGTCTTGAAGACAGC
ON-PC-51	546–535		22	GGATATTTAACCTTCCACCTTC
ON-PC-52	5276–5254	Exon 6	23	CATCATAGCCAGGTTGGTCTTGC
Universal primer	Multiple cloning site		17	GTAAAACGACGGCCAGT
Reverse primer	Multiple cloning site		19	GTACCAGTATCGACAAAGG

then sequencing a gene fragment PCK31 spanning exons 3 and 6 with sense primer ON-PC-32 and anti-sense primer ON-PC-52 (Table 1); fragment PCK31 had the expected size of approx. 1400 bp as analysed by gel electrophoresis and its sequence was identical with that of the overlapping gene clone PCK13 and gene fragments PCK21 and PCK22 (Figures 1 and 2).

Alignment of the gene sequence with the cDNA sequence revealed that the human mtPCK2 gene contains ten exons and nine introns, like the human and rat cyPCK1 gene [28,29]. The overall exon plus intron size of the mtPCK2 gene is as almost twice that of the cyPCK1 gene, i.e. 9.9 kb compared with 5.3 kb (Figure 1, Table 2). The gene and cDNA sequences [24] differed at four positions, one in exon 3 (C at 3941 is T in cDNA), one in exon 4 (G at 4189 is A in cDNA) and two in exon 10 downstream from the stop codon (additional C at 9601 and additional T at 9673). The differences would alter residue 128 from Cys to Arg in exon 3, but they would leave residue 181 as Ser in exon 4; the alteration in exon 3 would be in a region without functional significance [24].

In general, the exon size of the human mtPCK2 gene is very similar to that of the human and rat cyPCK1 gene. Comparison of the human mtPCK2 with the human cyPCK1 gene revealed bigger differences in exons 1, 2 and 10 and smaller differences in exons 3, 7 and 8: exon 1 is larger by 69 bp and exons 2 and 10 are smaller by 18 and 501 bp respectively. Exon 3 is larger by 3 bp and exon 8 by 6 bp, but conversely exon 7 is smaller by 6 bp. Exons 4, 5, 6 and 9 are identical in size (Table 2). The translated sequence of the mtPCK2 gene is slightly longer than that of the cyPCK1 gene by only 54 bp, which probably code for the mitochondrial target sequence or parts thereof. Exon 1 of the mtPCK2 gene contains 29 translated bp compared with 0 bp of the cyPCK1 gene, and exon 2 contains 246 translated bp compared with 224 bp; the overall difference of 51 bp accounts for 17 residues of the putative mitochondrial target sequence. Exon 10 comprises 452 translated bp in each gene (Table 2).

The overall intron size of the human mtPCK2 gene is 7.9 kb and exceeds that of the human and rat cyPCK1 genes of 3.5 and 4.3 kb by 2.3-fold and 1.9-fold respectively (Table 2). Comparisons of the human mtPCK2 gene with the cyPCK1 gene shows very pronounced differences in introns A and G and remarkable differences in introns B, C and D: intron A is larger by approx. 10-fold and intron G by approx. 30-fold. Introns B and D are larger by 2–3-fold and intron C is approx. one-fifth the size. The remaining introns E, F, H and I differ by less than 2-fold (Table 2). All exons are flanked by the consensus splice signals (t/c)ag at the 5′ border and gt(a/g) at the 3′ border (Figure 2).

Intermediate and simple tandem repeat sequences

The mtPCK2 gene contains five *Alu* sequences: three are on the (+) strand approx. 4.3 and 2.7 kb apart, one is in intron B from positions 2900 to 3200, one is in intron G from 7270 to 7543 and the last is in the 3′ UTR from position 9976 to the end of the gene sequenced so far, having 80%, 84% and 83% identity respectively with the human *Alu* consensus sequence [34]. The

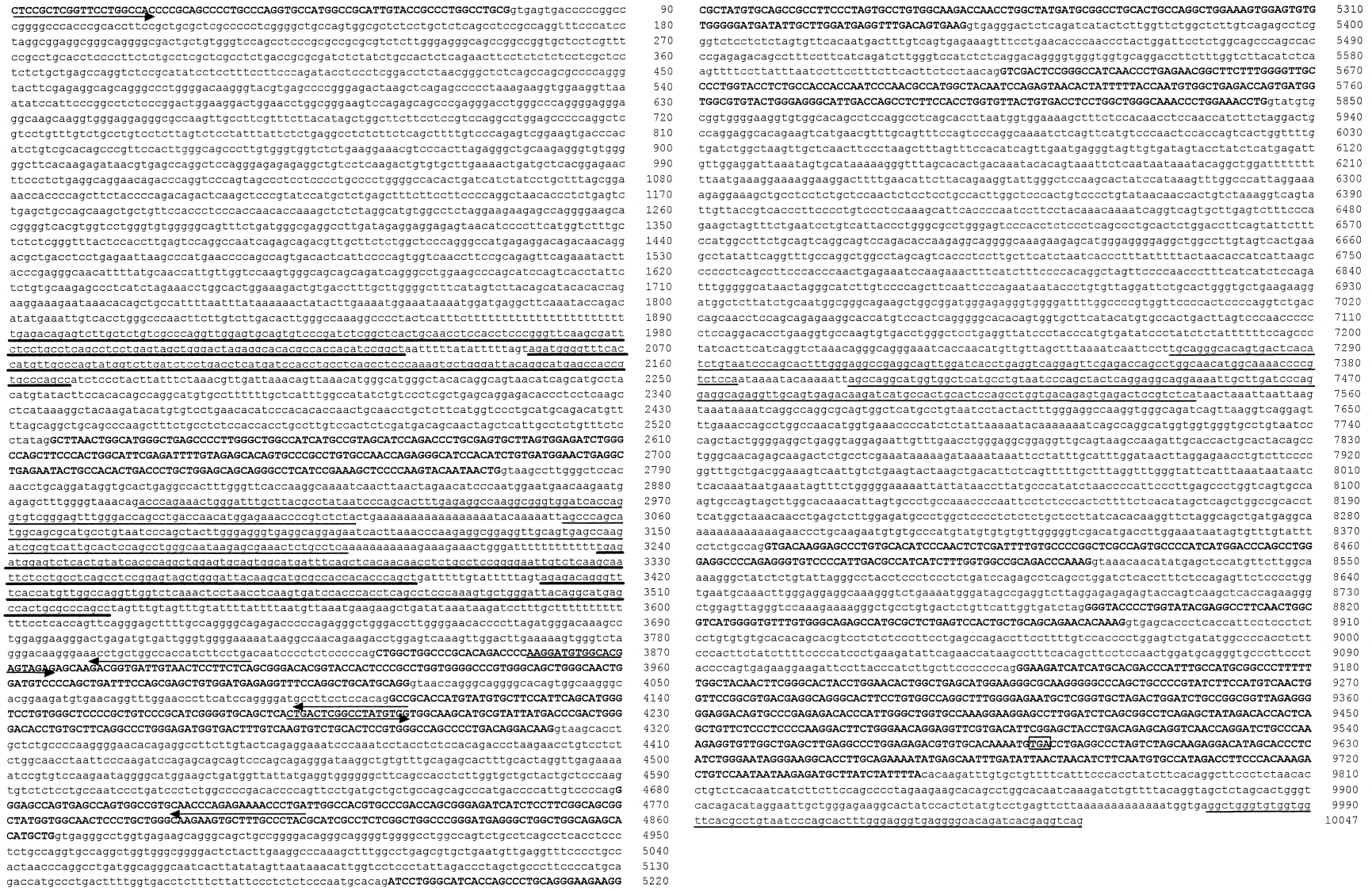

Figure 2 Sequence of human mitochondrial PCK2 gene

Exonic sequences are indicated in bold capital letters, introns in lower-case letters. The translational start site is at position 45. The stop codon is boxed. Two overlapping polyadenylation signals are at positions 9727–9734. *Alu* sequences on the (+) strand are underlined; those on the (−) strand are underlined in bold. Primer sequences used to synthesize DNA fragments PCK21, PCK22 and PCK23 are marked by arrows, 5′ primers under and 3′ primers over the sequence (see Figure 1).

Table 2 Comparison of exon–intron sizes of human mtPCK2, human cyPCK1 and rat cyPCK1

Abbreviations: aa, amino acids; tr, translated; utr, untranslated. Numbers in bold indicate translated nucleotides.

Exon/intron	Human mtPCK2 (bp)	Human cyPCK1 (bp)	Rat cyPCK1 (bp)
1 utr	134	94	102
1 tr	**29**	0	0
A	2453	168	265
2 utr	0	40	41
2 tr	**246**	**224**	**224**
B	1062	435	371
3	**185**	**182**	**182**
C	87	440	594
4	**204**	**204**	**204**
D	369	128	232
5	**188**	**188**	**188**
E	319	350	566
6	**163**	**163**	**163**
F	275	440	603
7	**219**	**225**	**225**
G	2537	88	90
8	**138**	**132**	**132**
H	269	424	582
9	**96**	**96**	**96**
I	254	214	214
10 tr	**452**	**452**	**452**
10 utr	160	661	612
Translated nucleotides	**1920**	**1866**	**1866**
Total exons + introns	9839	5345	6138
Protein	**Human mtPCK2 (aa)**	**Human cyPCK1 (aa)**	**Rat cyPCK1 (aa)**
Expressed (pre)protein	640	622	622

other two are on the (−) strand approx. 1.3 kb apart, one in intron A from 2169 to 1891, the other in intron B from 3525 to 3237, having 93 % and 87 % identity respectively with the human *Alu* consensus sequence (Figure 2). Thus in intron B two *Alu* sequences are linked tail-to-tail (Figure 2).

Simple tandem repeats (microsatellites) composed of $(AT)_n$ or $(AG)_n$, $(AC)_n$, $(GT)_n$, $(GC)_n$ and $(TC)_n$ with $n \geqslant 5$, such as the $GT(AT)_5(GT)_{16}$ found in the 3′ UTR of the human cytosolic PCK1 gene [29], are not present in the mitochondrial PCK2 gene (Figure 2).

Chromosomal location of the human mtPCK2 gene

The genomic PCK2 clones PCK12, PCK13 and PCK14 were used to locate the gene on metaphase chromosomes of human lymphocytes. FISH was performed with biotinylated PCK12, PCK13 and PCK14 probes together. The biotinylated probes were detected with avidin–FITC. In two independent experiments, 30 mitoses were exploited; in 24 of them two signals were obtained on the two homologous chromosomes 14 (Figure 3). Counterstaining with DAPI allowed karyotyping of the chromosomes. The human mtPCK2 gene was located on chromosome 14q11.2–q12; in contrast, the human cyPCK1 gene maps to chromosome 20q11.13 [23,29].

Tissue-specific expression of the human mtPCK2 gene

Northern blot analysis of poly(A)$^+$ RNA and of total RNA from various human organs was performed with radiolabelled cDNA probes to determine the tissue distribution of both human mitochondrial PCK2 and cytosolic PCK1 mRNA. A single mRNA of approx. 2.4 kb was detected with a mtPCK2 cDNA probe. This mtPCK2 gene transcript was strongly expressed in liver, kidney, small intestine and pancreas as well as in fibroblasts; it was weakly expressed in brain, heart and placenta and not at all in skeletal muscle (Figures 4 and 5). Probing with a cyPCK1 cDNA revealed a single mRNA of approx. 2.8 kb, mainly in liver but also in kidney and small intestine (Figures 4 and 5). Rat liver, in contrast with human liver, contained only cyPCK1 mRNA; the human hepatoma cells HepG2 expressed only mtPCK2 mRNA (Figure 5).

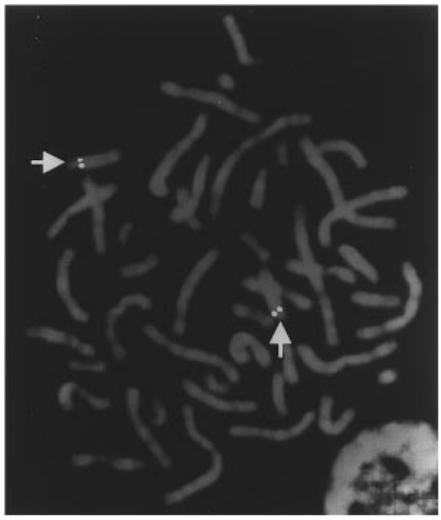

Figure 3 Chromosomal location of the human mtPCK2 gene on human chromosome 14q11.2–q12.0

FISH was performed with biotinylated mtPCK2 genomic DNA probes PCK12, PCK13 and PCK14 (see Figure 1) to metaphase chromosomes of human lymphocytes counterstained with DAPI. The typical fluorescence signals are shown by arrows; they are visible as two bright spots on the q arm of chromosome 14.

DISCUSSION

Structure of the human mtPCK2 gene

The human mtPCK2 gene is the third to be cloned and fully sequenced after the rat [28] and the human [29] cyPCK1 genes. The rat mtPCK2 gene (or cDNA) has not yet been cloned. The three mammalian PCK genes so far isolated have the same gene structure of ten exons and nine introns. The sizes of the exons of the rat and human cyPCK1 genes are identical, with the exception of the flanking exons 1, 2 and 10, which contain the 5′ UTR and the 3′ UTR. The sizes of the internal exons or of the translated sequences of the flanking exons respectively of the human mtPCK2 gene are identical (exons 4, 5, 6, 9 and 10), slightly larger (exons 1, 3 and 8) or smaller (exons 2 and 7) compared with the cyPCK1 genes. The sizes of the introns constitute a major difference; whereas the largest introns of the cyPCK1 genes have only approx. 400 bp, the three largest introns (A, B and G) of the human mtPCK2 gene are approx. 2500, 1100 and 2500 bp in length (Table 2, Figure 2). The similar gene structures and the high nucleotide and amino acid sequence identities of 68–70 % with the two cytosolic PCK1 and the mitochondrial PCK2 genes [24] suggest that they are derived from a common ancestor gene.

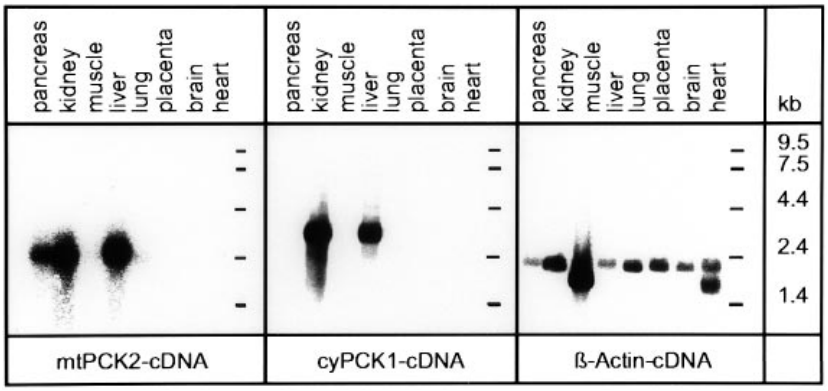

Figure 4 Northern blot analysis of poly(A$^+$) RNA from different human tissues

Northern blot analysis was performed with a Multiple Tissue Northern blot from Clontech, with both radiolabelled human mtPCK2 and radiolabelled human cyPCK1 cDNA. Hybridization with [α-^{32}P]dCTP-labelled mtPCK2 cDNA revealed a high level of a 2.4 kb transcript in liver and kidney, a lower level of transcript in pancreas and a very low level of transcript in lung, placenta, heart and brain. A second hybridization of the same blot with the cyPCK1 cDNA showed a high level of 2.8 kb transcript in liver and kidney only. A third hybridization of the same blot was performed with a β-actin cDNA control probe to prove the quality and equal quantity of tissue RNA species. Heart and skeletal muscle contain two forms of β-actin, 2.0 and 1.8 kb [45,46].

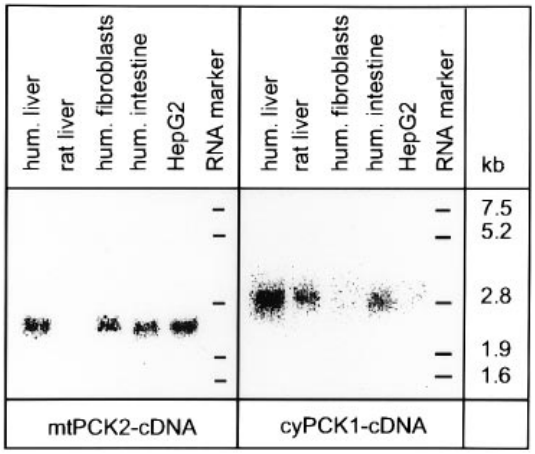

Figure 5 Northern blot analysis of total RNA from different human tissues

RNA was isolated from different human tissues, rat liver and HepG2 cell cultures as described in the Experimental section. Northern blots were hybridized with [α-^{32}P]dCTP-labelled human mtPCK2 and cyPCK1 cDNA. RNA application was controlled by ethidium bromide staining. A 2.4 kb transcript was demonstrated in human liver, small intestine, fibroblasts and HepG2 cells, but not in rat liver, by hybridization with human mtPCK2 cDNA. A 2.8 kb transcript was detected in human liver and intestine and in rat liver, but not in human fibroblasts or HepG2 cells, by hybridization with human cyPCK1 cDNA.

The human mtPCK2 gene contains five short interspersed elements approx. 300 bp in length, so-called *Alu* sequences, four in introns A, B and G and one in the 3′ flanking region (Figures 1 and 2). In contrast, the human cyPCK1 gene is devoid of *Alu* sequences; apparently none of the small introns of 400 bp and less (Table 1) has allowed the retrotransposition of an *Alu* element. The presence of five *Alu* sequences within little more than 10000 bp is above average: statistically not more than two *Alu* repeats would be expected for the size of the gene.

The human mtPCK2 gene does not contain microsatellites composed of the dinucleotide tandem repeats $(AT)_n$, $(AG)_n$, $(AC)_n$, $(GT)_n$, $(GC)_n$ or $(TC)_n$ with $n \geqslant 5$, in contrast with the cyPCK1 gene, in which a polymorphism for the $(GT)(AT)_5(GT)_{16}$ repeat was observed in the 3′ UTR. This microsatellite was used in linkage studies showing that the cyPCK1 gene was not tightly linked to the MODY gene (maturity-onset diabetes of the young, a form of non-insulin-dependent diabetes mellitus type 2) [23,29].

Chromosomal localization of the human mtPCK2 gene

The human mtPCK2 gene was located on the long arm of chromosome 14q11.2–q12 (Figure 3). In contrast, the human cyPCK1 gene was found on the long arm of chromosome 20q13.2–q13.31 [23,29]. The human cyPCK1 gene is part of a linkage group, also conserved on mouse chromosome 2, comprising phospholipase C1 and adenosine deaminase on the centromere and guanine nucleotide-binding protein α-stimulating polypeptide-1 (Gαs) on the telomere side [29]. The human gene locus on chromosome 14q11.2–q12 corresponds to locus q18.5 on mouse chromosome 14. The location of the mtPCK2 gene in the human and mouse genomes would then be within a linkage group flanked by the gene for apurinic apyrimidinic endonuclease and T-cell receptor alpha subunit (see the Genome Databank, release 9/22/97).

Function of human mitochondrial PCK2

Cytosolic PCK1 is generally accepted to be the key enzyme of gluconeogenesis in the liver. Its inducibility by glucagon, which is antagonized by insulin, is in line with this notion. Mitochondrial PCK2 might also be a gluconeogenic enzyme in the liver. However, its constitutive expression and especially the lack of inducibility by glucagon might be at variance with this view. It has been proposed that cyPCK1 would be best suited for gluconeogenesis from amino acids and that mtPCK2 would best serve gluconeogenesis from lactate [11]. Gluconeogenesis requires NADH and phosphoenolpyruvate (PEP) in the cytosol. With amino acids as substrates, NADH and the PEP precursor oxaloacetate are generated in the mitochondria and transferred to the cytosol as malate, from which NADH and oxaloacetate are regenerated, so that the latter can be converted by cyPCK1 to PEP. With lactate as substrate, NADH is generated in the cytosol but the PEP precursor oxaloacetate is formed in the mitochondria, where it could be converted by mtPCK2 to PEP, which could then be transferred to the cytosol ([35], pp. 15.19–15.21; [36], pp. 603–604).

Because mtPCK2 expression is essentially constitutive, the proposal would imply that gluconeogenesis from lactate, which is constantly produced by the erythrocytes [37], must be viewed as an almost continuous process. In contrast, gluconeogenesis from amino acids, which are supplied from food after a meal or, during fasting, mainly from skeletal muscles [37], would have to be regarded as a discontinuous process requiring the hormone-regulated adaptation of cyPCK1.

However, gluconeogenesis from lactate might also be possible with cyPCK1, if oxaloacetate generated in the mitochondria were transferred to the cytosol after transamination to aspartate, which does not require NADH ([35], pp. 15.19–15.21). Then, even in liver, mtPCK2 would not be a gluconeogenic enzyme and would serve other functions similar to those in the non-gluconeogenic fibroblasts. Here, mtPCK2 might operate in the reverse direction from PEP to oxaloacetate by using glycolysis-generated PEP imported from the cytosol to replenish the citrate cycle. Because the reported cases of mtPCK2 deficiency do not unequivocally support the gluconeogenic role of the enzyme (see below), the function of mtPCK2 in gluconeogenic organs such as liver and kidney and in non-gluconeogenic organs such as brain and connective tissue remains unclear.

Deficiencies in human cytosolic PCK1 and mitochondrial PCK2

Cytosolic PCK1 in liver is generally accepted as being a key gluconeogenic enzyme; mitochondrial PCK2 might also have a major role in gluconeogenesis (see above). A deficiency of cyPCK1 or of mtPCK2 would then be expected to cause a major disturbance of glucose homeostasis. Only one case of cyPCK1 deficiency has been described so far: a boy whose cyPCK1 activity was virtually zero at a normal total PCK activity in liver, and who suffered from severe, persistent neonatal hypoglycaemia, died at the age of 2 years 10 months [38]. Four cases of total PCK deficiency have been reported: all, one girl and three boys, whose total, i.e. cytosolic plus mitochondrial, PCK activity was below 10% of normal, also suffered from severe hypoglycaemia and died before the age of 2 years [39–41]. Two cases of mtPCK2 deficiency have been documented: in both, a boy and a girl, the defect was detected by analysis of fibroblasts, the residual activities were 18% and 16% respectively [42,43]. The boy initially presented at 4 months with failure to thrive, fasting hypoglycemia not responsive to glucagon, lacticacidaemia and hepatomegaly; the girl at 3 months also presented with failure to thrive and disordered liver function tests, she collapsed with profound hypoglycaemia and died of hepatocellular failure and septicaemia. In a later study it was suggested that the lowered mtPCK2 activity was not the primary defect [44]. Thus it remains to be established whether a primary mtPCK2 deficiency causes a disease characterized by hypoglycaemia.

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