Molecular cloning, sequencing and expression of the cDNA of the mitochondrial form of phosphoenolpyruvate carboxykinase from human liver

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In human liver, phosphoenolpyruvate carboxykinase (PCK; EC 4.1.1.32) is about equally distributed between cytosol and mitochondria in contrast with rat liver in which it is essentially a cytosolic enzyme. Recently, the isolation of the gene and cDNA of the human cytosolic enzyme has been reported [Ting, Burgess, Chamberlain, Keith, Falls and Meisler (1993) *Genomics* **16**, 698–706; Stoffel, Xiang, Espinosa, Cox, Le Beau and Bell (1993) *Hum. Mol. Genet.* **2**, 1–4]. It was the goal of this investigation to isolate the cDNA of the human mitochondrial form of hepatic PCK. A human liver cDNA library was screened with a rat cytosolic PCK cDNA probe comprising sequences from exons 2 to 9. A cDNA clone was isolated which had overall a 68 % DNA sequence and a 70 % deduced amino acid sequence identity with the human cytosolic PCK cDNA. Without the flanking 270 bases (= 90 amino acids) each at the 5' and 3' end, the sequence identity was 73 % on the DNA and 78 % on the amino acid level. The isolated cDNA had an open reading frame of 1920 bp; it was 54 bp (equivalent to 18 amino acids) longer than that of human or rat cytosolic PCK cDNA. The isolated cDNA was

cloned into the eukaryotic expression vector pcDNA1 and transfected into human embryonal kidney cells HEK293; PCK activity was increased by 3-fold in the mitochondria, which normally contain 70 % of total PCK activity, but not in the cytosol. The isolated cDNA was also transfected into cultured rat hepatocytes; again, PCK activity was enhanced by about 40-fold in the mitochondria, which normally possess only 10 % of total PCK activity, but not in the cytosol. In the rat hepatocytes only the endogenous cytosolic PCK and not the transfected mitochondrial PCK was induced 3-fold with glucagon. Comparison of the amino acid sequences deduced from the isolated cDNA with human and rat cytosolic PCK showed that the additional 18 amino acids were located at the N-terminus of the protein and probably constitute a mitochondrial targeting signal. Northern-blot analyses revealed the human mitochondrial PCK mRNA to be 2.25 kb long, about 0.6 kb shorter than the mRNA of the cytosolic PCK. Primer extension experiments showed that the 5'-untranslated region of mitochondrial PCK mRNA was 134 nucleotides in length.

INTRODUCTION

Rat liver is the most widely used model in biochemical hepatology. The results obtained with this model are often generalized, although there can be remarkable species differences, e.g. of subcellular enzyme distribution. Thus in rat liver about 90 % of the total activity of the key gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PCK) is cytosolic and 10 % mitochondrial [1,2], in human liver [3,4] 50 % is cytosolic and 50 % mitochondrial, in guinea-pig liver [5,6] 15 % is cytosolic and 85 % mitochondrial, and in chicken liver more than 95 % is mitochondrial [7]. Since, in rat liver, PCK, fructose 1,6-bisphosphatase and glucose 6-phosphatase are expressed mainly in the periportal zone and glucokinase and pyruvate kinase L mainly in the perivenous area, the model of 'metabolic zonation' proposes that the periportal hepatocytes possess the higher gluconeogenic capacity and the perivenous hepatocytes the higher glycolytic capacity (see refs. [8,9] for review). In rat liver, higher PCK activity and content [10,11] coincide with higher PCK mRNA levels in periportal hepatocytes [12,13]. Also in hamster and mouse liver, PCK mRNA is expressed to higher levels in periportal cells [14]. In contrast, in guinea pig [15] and man [16] total PCK activity appears to be distributed homogeneously; the mRNA distribution is not known. Enrichment of periportal and perivenous cells from guinea-pig liver by the digitonin/

collagenase perfusion technique showed that mitochondrial PCK was homogeneously distributed, whereas cytosolic PCK was zoned as in rat liver with higher activity in periportal hepatocytes [15].

Cytosolic PCK of the rat has been studied most intensively. The cDNA structure of the protein containing 622 amino acids and the gene structure comprising 10 exons, 9 introns and the 5'-regulatory region have been determined [17,18]. The regulation of the gene by glucagon and insulin [19,20] as well as by oxygen [21,22], and the zonation of its expression [12–14] have been investigated. Little is known about PCK in humans: a cDNA was isolated from a kidney library [23], and the corresponding gene, but without the putative exon 1 and the 5'-regulatory region, was cloned only during the course of the present study [24]. On the basis of a high identity with rat liver cytosolic PCK, both the cDNA and the gene appeared to represent the cytosolic form of the enzyme with 622 amino acids. Direct proof has not been obtained. The cDNA and gene structures of the mitochondrial form are unknown. Consequently the regulation of the human liver cytosolic and mitochondrial PCK genes and their zonal expression have not been studied.

Therefore it was the goal of the present investigation to clone and sequence the cDNA of mitochondrial PCK from a human liver cDNA library. The isolated cDNA had an open reading frame of 1920 bp (640 amino acids), which is 54 bp (18 amino

Abbreviations used: CMV, cytomegalovirus; DEPC, diethyl pyrocarbonate; DOTAP, *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl sulphate; HEK cells, human embryonal kidney cells; *K, phosphoenolpyruvate carboxykinase (EC 4.1.1.32).

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The nucleotide sequence data reported in this paper have been deposited in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number X92720.

Dedicated to Professor Karl Decker, Freiburg, on the occasion of his 70th birthday.

acids) longer than the cytosolic PCK cDNA; it had a DNA and deduced amino acid sequence identity with human cytosolic PCK of 68 % and 70 % respectively. Expression of the isolated cDNA in the human embryonal kidney (HEK) cell line HEK293 and in cultured rat hepatocytes showed directly that it encoded the mitochondrial form of human PCK.

EXPERIMENTAL

Chemicals

The human liver cDNA library (HL1115b) was from Clontech (Heidelberg, Germany) and the pcDNAI expression vector from Invitrogen (Heidelberg, Germany). DNA polymerase (Klenow fragment), ADP, dideoxynucleotides, pyruvate kinase and lactate dehydrogenase were obtained from Boehringer (Mannheim, Germany). *Thermus aquaticus* (Taq) DNA polymerase and agarose were products of AGS (Heidelberg, Germany). T4 DNA ligase was supplied by New England Biolabs (Schwalbach, Germany). Calf intestinal alkaline phosphatase and sequencing kits were purchased from USB (Braunschweig, Germany). Restriction endonucleases were obtained from Gibco/BRL (Eggenstein, Germany), USB or Fermentas (St. Leon-Rot 1, Germany). [α - 32 P]dCTP (specific radioactivity 111 TBq/mmol), [γ - 32 P]ATP (specific radioactivity 167 TBq/mmol) and [[α - 35 S]thio]dATP (specific radioactivity 37 TBq/mmol) were bought from ICN (Meckenheim, Germany). All other chemicals were of analytical grade and came from commercial suppliers.

Screening procedures

A human liver cDNA library in phage λ gt11 was seeded on agar plates using *Escherichia coli* MC1061 as host strain. Resulting plaques containing recombinant phages were transferred to Nylon membrane (Hybond N; Amersham International). Phage DNA was denatured for 10 min in 1.5 M NaCl/0.5 M NaOH, neutralized for 10 min in 1.5 M NaCl/0.5 M Tris/HCl, pH 7, and rinsed in $3 \times$ SSC (where $1 \times$ SSC is 450 mM NaCl and 45 mM sodium citrate, pH 7) before being dried and finally immobilized on the membrane by UV light irradiation for 3 min at 1200 J (UV Stratalinker 1800; Stratagene, Heidelberg, Germany). Filters were hybridized with an [α - 32 P]dCTP-labelled rat cytosolic PCK cDNA probe containing a 1.2 kb *Pst*I cDNA fragment [12] from PC201 [18]. Hybridization was performed by standard protocols [25]. After hybridization, filters were washed twice in $2 \times$ SSC/0.1 % SDS (20 min at room temperature) and twice in $0.2 \times 1 \times$ SSC/0.1 % SDS (20 min at 65 °C). They were finally exposed to X-ray film at -70 °C usually overnight.

Purification of phage DNA and subcloning procedures

Phage plaques giving rise to positive autoradiographic signals were plugged off the agar, transferred to dilution buffer (0.1 M NaCl, 100 mM MgSO₄, 35 M Tris/HCl, pH 7.5, and 0.01 % gelatin) and reseeded on to agar plates. 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 *Bsi*WI restriction endonuclease. The insert was purified and subcloned into the *Sma*I site of both M13mp18 (for sequencing single-stranded DNA) and Bluescript (for sequencing double-stranded DNA) by standard procedures [25]. Plasmid DNA was transfected into *E. coli* CMK 603 and DH5 α respectively and purified for sequencing. The cloned DNA was sequenced from both directions by means of the dideoxy-termination method using a sequencing kit (USB) according to the supplier's instructions.

Expression of the cDNA in HEK293 cells and rat hepatocytes

The isolated human PCK cDNA was ligated into the *Eco*RI site of pcDNAI. The resulting plasmid was termed pcDNAI-PCK_{hm}. In this vector inserted DNA is expressed under the control of the human cytomegalovirus (CMV) promoter [26]. The plasmid was propagated in *E. coli* MC 1061. Transformants were checked for orientation of the inserted PCK cDNA by means of restriction-site analysis and sequencing. Two clones were isolated, which allows expression of PCK cDNA in sense and antisense orientation. For transfection experiments, plasmids were purified on a Qiagen tip 500 column (Qiagen, Düsseldorf, Germany). Transfections were performed by use of a mammalian transfection kit (Stratagene).

Expression in HEK293 cells

pcDNAI-PCK_{hm} (17 μ g) was dissolved in 607 μ l of sterile water; 68 μ l of 2 M CaCl₂ and 675 μ l of $2 \times$ concentrated Hepes-buffered saline (NaCl/Hepes) (280 mM NaCl, 1.5 mM Na₂HPO₄, 2H₂O, 50 mM Hepes, pH 7.3) were added. The mixture was left at room temperature for 20 min to allow formation of DNA-(Ca)₃(PO₄)₂ complexes. HEK293 cells at a density of 10^4 /cm² tissue culture bottle were grown for 24 h. Before transfection the cells were washed twice with PBS (140 mM NaCl, 2.7 mM KCl, 8 mM Na₂HPO₄, 1.5 mM K₂HPO₄, pH 6.8), and subsequently 13.5 ml of minimal essential medium and 675 μ l of modified bovine serum were added. The DNA suspension was added dropwise slowly to each bottle. Incubation was continued for 3 h at 37 °C and 3 % CO₂. Thereafter the medium was aspirated and the cells were washed twice with PBS. Then 20 ml of standard culture medium supplemented with 10 % fetal calf serum was added to the bottles and the cells were incubated for another 72 h.

Expression in cultured rat hepatocytes

Cationic liposomes {*N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl sulphate (DOTAP); Boehringer} were used for transfection experiments of primary rat hepatocyte cultures. Some 5×10^5 cells per dish (60 mm diameter) were seeded in 1.5 ml of Medium 199, containing 100 nM dexamethasone, 0.5 nM insulin and 4 % fetal calf serum, and 84 μ l of transfection buffer (2.5 μ g of pcDNAI-PCK_{hm} and 12.5 μ g of DOTAP added to 84 μ l of NaCl/Hepes). Cells were pre-cultured at 16 % O₂, 5 % CO₂ and 79 % N₂. After 7 h, 1 ml of Medium 199 containing 100 nM dexamethasone and 0.5 nM insulin was added to the cells and transfection proceeded for another 13 h. Then the medium was changed and cells were further cultured under the above conditions for 72 h. PCK activity was then induced for 4 h by adding fresh medium containing 1 nM glucagon.

Northern-blot analysis

Total RNA was isolated from human and rat liver as described [25]. Normal human liver tissue was obtained, with the consent of the ethical committee of the Medical School in Göttingen, from patients who had undergone partial liver resection due to liver metastases. Total human RNA (10 μ g) and total rat RNA (5 μ g) was separated by electrophoresis in a 1 % agarose/2.2 M formamide gel and transferred to Amersham Hybond N Nylon membrane. A 0.24–9.5 kb RNA ladder (BRL) was used as a size marker. Three different filters were prepared for hybridization with a 1.2 kb cDNA [18] fragment encoding the rat cytosolic PCK, with a 0.8 kb cDNA fragment encoding the 5' end of human mitochondrial PCK and with a 1.4 kb cDNA fragment

encoding the 3' end of human mitochondrial PCK. A 15 ng amount of each was labelled by the random-primer method using [α - 32 P]dCTP as the labelled nucleotide and the Klenow fragment of DNA polymerase [25]. Blots were hybridized with each 1×10^6 c.p.m./ml labelled probe in 5 ml of solution containing $5 \times$ SSC, $5 \times$ Denhardt's (where $1 \times$ Denhardt's is 0.02% Ficoll 400/0.02% polyvinylpyrrolidone/0.02% BSA), 0.5% SDS and 500 μ g of salmon sperm DNA at 60 °C overnight. Blots were washed twice in $2 \times$ SSC/0.1% SDS at room temperature for 15 min and twice in $0.2 \times$ SSC/0.1% SDS at 55 °C for 30 min and exposed to DuPont Cronex 4 medical X-ray film.

Primer extension

A 22-mer synthetic oligonucleotide (10 pmol) containing the sequence 5'-CTCAGCCCATGCCAGTTAAGCC-3' complementary to position +29 to +50 downstream of the translational start site of the human liver mitochondrial PCK cDNA (Figure 2) and a 24-mer synthetic oligonucleotide (10 pmol) containing the sequence 5'-CATTTCTGCAGAGTGCTGCTAAGG-3' complementary to position +3 to -21 of the translational start site of the human cytosolic PCK cDNA [23] were labelled at the 5' end using [γ - 32 P]ATP and T4 polynucleotide kinase. Labelled oligonucleotide (1×10^5 c.p.m.) was precipitated with ethanol together with 100 μ g of total RNA from human liver. The pellet was resolved in 6 μ l of Pipes buffer (40 mM Pipes, 1 mM EDTA, 0.4 mM NaCl, pH 6.4) and 24 μ l of deionized formamide. After denaturing for 10 min at 85 °C, the mixture was incubated at 55 °C overnight. Then the primer-extension reaction was performed in a mixture of 4 μ l of $5 \times$ concentrated reverse transcriptase buffer (BRL) (250 mM Tris/HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 2 μ l of 1 mM dithiothreitol, 2 μ l of a solution containing 10 mM each dATP, dCTP, dGTP and dTTP, 1 μ l (34 units) of RNase inhibitor (USB) and 1 μ l of a 1 mg/ml solution of actinomycin D. The final volume was adjusted to 20 μ l by adding DEPC/water. The reaction was started by the addition of 1 μ l (200 units) of Moloney murine leukaemia virus superscript reverse transcriptase (BRL) and continued at 42 °C for 1 h. Thereafter the RNA was digested with 1 μ g of RNase A1 and 1 μ g of T1 (100 units) for 30 min at 37 °C. After extraction with phenol/chloroform the cDNA was precipitated with ethanol and redissolved in 6 μ l of loading buffer (80% formamide, 10 mM EDTA, pH 8.0, 1 mg/ml xylene cyanol FF, 1 mg/ml Bromophenol Blue) and subsequently analysed on a 6% polyacrylamide/7 M urea sequencing gel. As a size marker, M13mp18 single-stranded DNA was sequenced with M13-40 primer and loaded on to the gel. Electrophoresis was performed for 2.5 h at 2400 V. The gel was washed, dried and exposed to DuPont Cronex 4 medical X-ray film for 3 days [25].

Cell-fractionation procedures

All procedures were performed at 4 °C. HEK293 cells from one culture bottle or hepatocytes from one culture dish were scraped off into 800 μ l of 0.9% NaCl and 100 μ l of lysis buffer (2.53 mM $MnCl_2$, 9.72 mM dithiothreitol and 500 mM Tris/HCl, pH 8.1). A cell-fractionation procedure slightly modified from standard methods [27] yielded enriched mitochondria. Cells were homogenized with a motor-driven Teflon pestle at 3000 rev/min for 1 min and centrifuged at 800 g for 10 min. The resulting supernatant (1) was removed. Pellet 1 was resuspended in 450 μ l of 0.9% NaCl and 50 μ l of lysis buffer and homogenized as described above. This homogenate was centrifuged at 800 g for 10 min; the resulting pellet (2) was discarded and supernatant 2 was combined with supernatant 1. The pooled supernatants were

centrifuged at 8500 g for 15 min. The resulting supernatant (3) was used as a source of cytosol and pellet 3 as a source of mitochondria. The successful separation of mitochondria from cytosol was assessed by determination of lactate dehydrogenase activity; 99% of the total activity was in the cytosolic fraction compared with 1% in the mitochondrial fraction. Pellet 3 was dissolved in 800 μ l of buffer, and mitochondria were disrupted by the use of an UltraTurrax (IKA/Staufen) at maximal speed for 1 min. Some 500 μ l of this homogenate and 500 μ l of the cytosolic fraction were used to determine PCK activity.

Miscellaneous

PCK activity was determined essentially as described previously by measuring the product, phosphoenolpyruvate, with pyruvate kinase and lactate dehydrogenase [28]. Protein concentration was determined in 1–50 μ l of homogenates by use of a commercial kit (Bio-Rad, München, Germany). PCR protocols were adapted from standard procedures [25].

RESULTS AND DISCUSSION

Cloning and sequencing of a PCK cDNA from a human liver cDNA library

A rat liver cDNA probe, comprised of sequences of about 1.2 kb length from exon 2 to exon 9 of the 10 exons of rat cytosolic PCK [18], was used to screen a human liver cDNA library in phage λ gt11. Since it could be expected that the sequence identity between the rat cytosolic PCK cDNA and the human mitochondrial PCK cDNA would not be very high, only faint signals were pursued in the first screening cycle. This should favour the selection of the mitochondrial form and not the cytosolic form. A clone was found, which contained a 1.4 kb insertion hybridizing to the rat cytosolic PCK cDNA probe (Figure 1; Fr1). This fragment was isolated, cloned and sequenced in both directions. It carried a stretch of 18 dAMP residues at its 3' end and exhibited 67% DNA sequence identity with rat cytosolic PCK cDNA. The 5' end of fragment Fr1 matched position 822–830 (GAGATCATC) of the rat cytosolic PCK cDNA [18] corresponding to position 733–741 of the human mitochondrial PCK cDNA (see Figure 2), which lies in exon 5 of the gene [18], indicating that the isolated cDNA was incomplete, i.e. it was missing the 5' end including the start codon ATG and 5'-untranslated region. A PCR approach was chosen to synthesize

Figure 1 Strategy of cDNA isolation

After screening of a human liver cDNA library in λ gt11 a phage clone was isolated containing a 1.4 kb cDNA fragment (Fr1 = bases 733 to 2099 in Figure 2). In a second approach PCR was performed using the primer pair indicated by arrows and total DNA from the phage library as template. This procedure yielded a 0.8 kb cDNA fragment (Fr2 = bases 20 to 799 in Figure 2). This fragment was used as a hybridization probe in a second screening, which yielded a full-length 2.2 kb cDNA fragment (Fr3 = bases —67 to 2099 in Figure 2). For details see the text.

TGGAACA AAA T G TGGCGGAAGA C CGGAAAGAAC TGT GAT T CTTGAGA CAT CAAA AG
 ccggaattccggcccgcccttccatcactccccggctccgctcgggttctgcccaccccgagccctgccaggtgcc -66

AA AAA GTGACCT ACATTC TG CC TTAGC G ACT TGCA AAAT T C CA - A AACG TG A C TC G- AAA TTGT CA
 ATGGCGCATTGTACCGCCCTGGCTGCGGCTTAAGTGGCATGGGCTGAGCCCTTGGGCTGGCCATCATGCCGTAGCATCCAGACCTCGAGTGTCTT 99
 1 MetAlaAlaLeuTyrArgProGlyLeuArgLeuAsnTrpHisGlyLeuSerProLeuGlyTrpProSerCysArgSerIleGlnThrLeuArgValLeu

G-- AGC A AGC G - A G GA G G C C A T AC TGAG T G T TCA C CT
 AGTGGAGATCTGGGCCAGCTTCCCACTGGCATT-CGAGATTTTGTAGAGCACAGTGCCTCGCTGTGCCAACAGAGGGCATCCACATCTGTGATGGAAC 198
 34 SerGlyAspLeuGlyGlnLeuProThrGlyIle ArgAspPheValGluHisSerAlaArgLeuCysGlnProGluGlyIleHisIleCysAspGlyThr

AG G-G GG TT GG A A G AG A C A CGG GAAG TG C T T T T
 GAGGCTGAGAATACTGCCAC-ACTGACCTGCTGGAGCAGCGGCTCATCCGAAAGCTCCCCAAGTACAATACTGCTGGCTGGCCCGCACAGACCCC 297
 67 GluAlaGluAsnThrAlaThr rLeuThrLeuLeuGluGlnGlnGlyLeuIleArgLysLeuProLysTyrAsnAsnCysTrpLeuAlaArgThrAspPro

G CA GA C A T C C C AAGAG AA A A G - AT - AAAACA -CA C C T
 AAGGATGTGGCAGCAGTAGAGAGCAAGACGGTGATTGTAAGTCTTCTCAGCGGACACGGTACCCTCCCGCTGGTGGGGCTGTGGGCAGCTGGGC 396
 100 LysAspValAlaArgValGluSerLysThrValIleValThrProSerGlnArgAspThrValProLeuProProGlyGlyAlaCysGlyGlnLeuGly

CG AGAG AG TG AA GT CA CC C G A A T C CA C G
 AACTGGATGTCCCGAGCTGATTTCAGCGAGCTGTGGATGAGAGGTTTCCAGGCTGCATGCAGGGCCGACCATGTATGTGCTTCCATTGAGCATGGGT 495
 133 AsnTrpMetSerProAlaAspPheGlnArgAlaValAspGluArgPheProGlyCysMetGlnGlyArgThrMetTyrValLeuProPheSerMetGly

GC A T GAAG CA CG G G T GC C C G C G GA C G C C
 CCTGTGGGCTCCCGCTGTCCCGCATCGGGGTGAGCTCACTGACTCAGCCTATGTGGTGGCAAGCATGCGTATTATGACCCGACTGGGGACACCTGTG 594
 166 ProValGlySerProLeuSerArgIleGlyValGlnLeuThrAspSerAlaTyrValAlaSerMetArgIleMetThrArgLeuGlyThrProVal

GG A A C G G G A C C T T GTGC T C TTT AA CCTTG C A A C C
 CTTGAGCCCTGGGAGTGGTGAATTTGTCAGTGTCTGCACCTCGGTGGGCCAGCCCTGACAGGACAAGGGGAGCCAGTGAAGCAGTGGCCGTGCAAC 693
 199 LeuGlnAlaLeuGlyAspGlyAspPheValLysCysLeuHisSerValGlyGlnProLeuThrGlyGlnGlyGluProValSerGlnTrpProCysAsn

G CTG G C C C C T GCA A T T G C C G G C G
 CCAGAGAAAACCTGATTGGCCAGCTGCCCGACGAGCGGAGATCATCTCTTCCGCGAGCGGCTATGTTGGCAACCTCCCTGCTGGGCAAGAAGTGTCTT 792
 232 ProGluLysThrLeuIleGlyHisValProAspGlnArgGluIleIleSerPheGlySerGlyTyrGlyGlyAsnSerLeuLeuGlyLysLysCysPhe

T CA G G AGC AGA G A G T T A A AG TG AA TAC
 GCCTACGCATCGCTCTCGGCTGGCCCGGATGAGGGCTGGCTGGCAGAGCAGATGCTGATCCTGGGCATCACCAGCCCTGCAGGGAAGAAGCGCTA 891
 265 AlaLeuArgIleAlaSerArgLeuAlaArgAspGluGlyTrpLeuAlaGluHisMetLeuIleLeuGlyIleThrSerProAlaGlyLysLysAlaLeu

CTG G AT - C C G C AAC CAGC C C G G T C C C
 TGTGACGCCGC-CTTCCTAGTGCCTGTGGCAAGCAACCTGGCTATGATGCGCCCTGCACTGCCAGGTGGAAAGTGGAGTGTGTGGGGATGATATT 990
 298 CysAlaAlaAlaPheProSerAlaCysGlyLysThrAsnLeuAlaMetMetArgProAlaLeuProGlyTrpLysValGluCysValGlyAspAspIle

C A GCAC ATT AA A A T T C T C T G T A TG AG C
 GCTTGGATGAGGTTTGACAGTGAAGGTGCACTCCCGGCCATCAACCTGAGAAGCGGCTCTTTGGGGTTGCCCTGGTACCTCTGCCACCACCAATCCC 1089
 331 AlaTrpMetArgPheAspSerGluGlyArgLeuArgAlaIleAsnProGluAsnGlyPhePheGlyValAlaProGlyThrSerAlaThrThrAsnPro

T CAAG C AG A C C C G T A TG G AG T
 AAGCCCATGCTACAATCCAGAGTAACACTATTTTACCAATGTGGCTGAGACCAAGTATGTTGGCGTACTTGGGAGGGCATTGACCAGGCTCTTCCA 1188
 364 AsnAlaMetAlaThrIleGlnSerAsnThrIlePheThrAsnValAlaGluThrSerAspGlyGlyValTyrTrpGluGlyIleAspGlnProLeuPro

T A C C CA C G AA AAT GGAG GCT A AG TGG A T C C GA G C A T CA
 CCTGGTGTACTGTGACCTCTGGCTGGGCAAAACCTGGAAACCTGGTGACAAGGAGCCCTGTGCACATCCCAACTCTCGATTTGTGCCCGGCTCGC 1287
 397 ProGlyValThrValThrSerTrpLeuGlyLysProTrpLysProGlyAspLysGluProCysAlaHisProAsnSerArgPheCysAlaGlnLeuProArg

T TG T TCT G A T A G T A T TGCT T C T A C
 CAGTGGCCCATCATGAGCCAGCCTGGGAGGCCAGAGGGTGTCCCATTTGAGCCATCATCTTTGGTGGCCGAGACCCAAAGGGGTACCCCTGGTA 1386
 430 GlnCysProIleMetAspProAlaTrpGluAlaProGluGlyValProIleAspAlaIleIlePheGlyGlyArgArgProLysGlyValProLeuVal

T A TC G AA A C G G G - A A A G A G T T C A T
 TACGAGCCCTCAACTGGCGTCATGGGTGTTTGT-GGGCAGAGCCATGCGCTCTGAGTCCACTGCTGCAGCAGAACACAAAGGGAAGATCATATGCAC 1485
 463 TyrGluAlaPheAsnTrpArgHisGlyValPheValGlyArgArgMetArgSerGluSerThrAlaAlaAlaGluHisLysGlyLysIleIleMetHis

C C CA A CC T CCCA A CCA CA A A AAG
 GACCCATTTGCCATGCGGCCCTTTTTTGCTACAACCTCGGGCACTACCTGGAACACTGGCTGAGCATGGAAGGGCGCAAGGGGGCCAGCTGCCCCGT 1584
 496 AspProPheAlaMetArgProPhePheGlyTyrAsnPheGlyHisTyrLeuGluHisTrpLeuSerMetGluGlyArgLysGlyAlaGlnLeuProArg

AAG A - A AA C A CT CA G G G T AAC
 ATCTTCCATGTCAACTGGTTCGGGCTGACGAGGCAGGGC- TTCTGTGGCCAGGCTTTGGGAGAAATGCTCGGGTGTAGACTGGATCTGCGGGCGG 1683
 529 IlePheHisValAsnTrpPheArgArgAspGluAlaGlyHis PheLeuTrpProGlyPheGlyGluAsnAlaArgValLeuAspTrpIleCysArgArg

A C T AA A C CA AAC TC G A CTACA C C G AT C A C G AA GG GCAC CA TG TGG
 TTAGAGGGGGAGGACAGTGGCCGAGAGACCCATTGGGCTGGTGCCAAAGGAAGGAGCCTTGGATCTCAGCGGCTCAGAGCTATAGACACCACTCAG 1782
 562 LeuGluGlyGluAspSerAlaArgGluThrProIleGlyLeuValProLysGluGlyAlaLeuAspLeuSerGlyLeuArgAlaIleAspThrThrGln

T AG A T A CA GGAA CGA AG T GTG T A TGCC C C TGT AA CGA
 CTGTTCTCCCTCCCAAGGACTTCTGGGAACAGGAGGTTCTGTGACATTCCGAGCTACCTGACAGAGCAGGTCAACAGGATCTGCCAAAGAGGTGTTG 1881
 595 LeuPheSerLeuProLysAspPheTrpGluGlnGluValArgAspIleArgSerTyrLeuThrGluGlnValAsnGlnAspLeuProLysGluValLeu

AGA A CCTT T A CA A A AAG C G A
 GCTGAGCTTGAGGCCCTGGAGAGACGTGTGCACAAATGTGACCTGAGGCTAGTCTAGCAAGAGGACATAGCACCTCATCTGGGAATAGGGAAGGCA 1980
 628 AlaGluLeuGluAlaLeuGluArgValHisLysMetEnd

CCTTGCAGAAAATATGACCAATTGATATTAACATCTTCAATGTGCCATAGACCTTCCACAAAGACTGTCCAATAAAGAGATGCTTATCTATT 2079
 TTAATAAAAAAAAAAAAAA 2099

the 5' end of the PCK cDNA. Total recombinant phage DNA isolated from the cDNA library was used as template in the PCR amplification. By use of a primer pair, one of which contained the antisense sequence 5'-GTAGGGCAAAGCACTTCTTG-3' from the 5' region of the isolated 1.4 kb cDNA fragment corresponding to position 780–799 of the human mitochondrial cDNA (see Figure 2) and the other the sequence 5'-AGCG-ACCGGCGCTCAGCTGG-3' from the left arm of the cloning vector λ gt11, a PCR product of 0.8 kb was generated (Figure 1; Fr2).

This fragment was again cloned and sequenced in both directions. As was to be expected, it overlapped the 1.4 kb fragment in 67 bases. It had a sequence identity with the rat cytosolic PCK cDNA of 65% and terminated at position 20–25 (ACGGCC) relative to the start site of translation in rat cytosolic PCK cDNA. A 5'-stretched λ gt11 human liver cDNA library was rescreened using 0.8 kb cDNA fragment as a hybridization probe. A cDNA clone (Figure 1; Fr3) was isolated, subcloned and sequenced in both directions. The sequence of this fragment was identical with that of fragments Fr1 and Fr2. Fr3 had a length of 2166 bp and contained an open reading frame of 1920 bp including a translational start codon (Figure 2). The 5'-untranslated region comprised 67 bp and the 3'-untranslated region 179 bp. Two overlapping putative polyadenylation signals were located at positions 2056 and 2059 relative to the start site of translation (Figure 2). The sequence identity with the rat cytosolic PCK cDNA was 68%. This in a first approximation indicated that the isolated clone encoded PCK.

During the course of this study other laboratories isolated a PCK cDNA from a human kidney library and a human PCK gene, which, on the basis of an 85% sequence identity with rat cytosolic PCK cDNA, must encode the human cytosolic PCK [23,24]. Sequence alignment of the present cDNA with the human and rat cytosolic PCK cDNA revealed an overall sequence identity of 68% and 70% respectively (Figure 2). Without the coding 270 bp at the 5' and 3' ends, the sequence identity was 73 and 75% respectively. The coding region of the isolated cDNA contained 1920 bp compared with 1866 bp of the two cytosolic PCK cDNAs, i.e. it was 54 bp longer. This elongation could contain the information for a mitochondrial target sequence or parts thereof. In the 5'- and 3'-untranslated regions, sequence identity was only 38%. The 3' end contained 161 bp compared with 670 bp and 615 bp of the human and rat cytosolic PCK cDNA respectively. The high sequence identity of the isolated cDNA with cytosolic human PCK cDNA allows the conclusion that the cDNA encoded a PCK that was not identical with human cytosolic PCK and probably represented the mitochondrial isoenzyme. During the screening procedure no PCK clone was isolated that was identical with the sequence of human cytosolic PCK cDNA reported by another group [23].

Expression of the isolated human cDNA

The entire cDNA was cloned in sense orientation into the *EcoRI* site of the eukaryotic expression vector pcDNAI, which carries a CMV promoter [26]. This plasmid pcDNAI-PCK_{hm} (sense) was transfected into HEK293 cells. If the cDNA encoded the mitochondrial PCK isoenzyme, it should be expressed in mitochondria after transfection. Enzyme activity was determined

Table 1 Transfection of human HEK293 cells with pcDNAI-PCK_{hm}

HEK293 cells were transfected with the plasmid pcDNAI containing the isolated human cDNA in sense and antisense orientation (pcDNAI-PCK_{hm}). PCK activity was determined in the cytosol and mitochondria of the transfected HEK cells and for comparison also in non-transfected HEK cells and in freshly isolated rat hepatocytes. Values are means $\pm$ S.E.M. of eight transfection experiments.

	PCK activity (m-units/mg of protein)			
	Rat hepatocytes		HEK293	
	Cytosol	Mitochondria	Cytosol	Mitochondria
Non-transfected	34.2 $\pm$ 3.9	3.5 $\pm$ 0.8	0.8 $\pm$ 0.7	4.5 $\pm$ 1.8
Transfected				
Sense	—	—	1.3 $\pm$ 0.5	15.8 $\pm$ 3.9
Antisense	—	—	1.0 $\pm$ 0.3	4.0 $\pm$ 0.9

Table 2 Transfection of cultured rat hepatocytes with pcDNAI-PCK_{hm}

Cells were transfected with the plasmid pcDNAI containing the isolated human cDNA in sense orientation (pcDNAI-PCK_{hm}). PCK activity was determined in the cytosol and mitochondria. For comparison the endogenous PCK gene was activated by treatment of the cells with 1 nM glucagon for 4 h. Values are means from two transfection experiments. In contrast with the basal cytosolic PCK activity of freshly isolated rat hepatocytes (Table 1), as expected the basal activity in 24 h-cultured hepatocytes was much lower; however, it could be induced by glucagon to the same levels as in freshly isolated hepatocytes.

Hormone	PCK activity (m-units/mg of protein)			
	Non-transfected		Transfected	
	Cytosol	Mitochondria	Cytosol	Mitochondria
—	6.3	2.4	14.4	102.3
Glucagon	44.9	5.2	41.7	95.1

in cytosolic and mitochondrial subcellular fractions of the transfected and non-transfected control cells (Table 1). Non-transfected HEK293 cells exhibited only low PCK activity in the cytosol, but medium activity in the mitochondria. Transfected cells had a PCK activity in the control range in the cytosol but activity increased about 4-fold in the mitochondria. As a further control, HEK293 cells were transfected with the plasmid pcDNAI-PCK_{hm} (antisense), in which the PCK cDNA had been cloned in antisense orientation relative to the CMV promoter. These cells exhibited the same PCK activity in cytosol and mitochondria as the non-transfected cells. For comparison, PCK activity was also measured in freshly isolated rat hepatocytes, in which more than 85% of the enzyme activity was located in the cytosol (Table 1).

In rat hepatocyte cultures cytosolic PCK activity falls to less than 20% of the original during the first 24 h; it can be induced to about 5-fold higher levels by glucagon and this is due to transcriptional activation [21,29]. This model system was used as a control. Cultured rat hepatocytes were transfected with the

Figure 2 Nucleotide sequence and predicted amino acid sequence of the human mitochondrial PCK cDNA

The nucleotide sequence of human mitochondrial PCK cDNA (representing the sequence of Fr3 depicted in Figure 1) is compared with that of human cytosolic PCK cDNA, of which only the differences are shown: —, missing; A, T, G, C, replacement. The first 12 lower-case letters show the *EcoRI* site, into which cDNA was assembled in the λ phage cDNA library. The sequence identity of the coding region is 68%. Underlined sequence represents two overlapping polyadenylation signals.

Computer analysis revealed that the presequence of the isolated human mitochondrial PCK cDNA fulfilled the prerequisites for a mitochondrial targeting signal: it is void of hydrophilic amino

Figure 4 Northern-blot analyses of PCK mRNA from human liver

Total RNA from human liver (hl) (10 μ g) and rat liver (rl) (5 μ g) was transferred to a Hybond N filter (A, B, C, D). Filter A was hybridized with a radioactively labelled 1.2 kb fragment of rat liver cytosolic PCK cDNA (rlc), filter B with a 1.4 kb fragment of human liver mitochondrial PCK cDNA (hlm), filter C with a 0.8 kb fragment of human liver mitochondrial PCK cDNA and filter D with an equal mixture of the 1.4 kb fragment of human liver mitochondrial PCK cDNA and the 1.2 kb fragment of rat liver cytosolic PCK cDNA. A 0.24–9.5 kb RNA ladder was used as marker.

acids and has the potential to expose hydrophobic regions for transport through the mitochondrial membrane.

PCK protein should contain binding sites for a GTP-phosphoryl group and a guanine group as well as for phosphoenolpyruvate [31] and, moreover, an essential hyper-reactive cysteine thiol group, the exact function of which is not known [32]. The GTP-phosphoryl-binding site at positions 250–261 of human mitochondrial PCK and the hyper-reactive thiol at position 306 including the flanking hydrophobic micro-environment from position 299 to 314 are totally conserved in the five different PCKs from three species (Figure 3). The guanine-binding site with a consensus sequence NKXW at position 406–409 is maintained as such in the cytosolic PCKs and in a modified form as GKXW in the mitochondrial PCKs (Figure 3). The phosphoenolpyruvate-binding site, which has been very tentatively assigned the consensus sequence P(X)_{9–10}A(X)_{4–5}P(X)₅C(X)_{4–5}E [32], at position 31–58 is also conserved in the five PCKs (Figure 3).

Analysis of the mRNA of human liver mitochondrial PCK

To determine the size of the mRNA of human mitochondrial PCK, Northern-blot studies were performed with total human and rat liver RNA using as hybridization probes a 1.2 kb cDNA fragment coding for rat cytosolic PCK and two fragments of the human mitochondrial PCK cDNA, the one ranging from position 20 to 799 and the other from 733 to 2099 (Figure 2). The rat liver 1.2 kb cDNA probe hybridized to one specific mRNA yielding a band of 2.75 kb in length both in human and rat liver total RNA (Figure 4, filter A). This band corresponded to the mRNA of cytosolic PCK, which in rat liver was reported to have a length of 2.8 kb [18]. Using either the 1.4 kb cDNA fragment, containing the last two-thirds, i.e. the 3' portion of the cDNA of human mitochondrial PCK, or the 0.8 kb cDNA fragment, containing the first third, i.e. the 5' portion, as hybridization probes (Figure 4, filters B and C), only in human and not in rat

Figure 5 Primer extension

A 5'-end-labelled 22 bp oligonucleotide complementary to position +29 to +50 relative to the translational start site of human liver mitochondrial PCK cDNA (MIT) or a 24 bp oligonucleotide complementary to position +3 to –21 relative to the translational start site of human liver cytosolic PCK cDNA (CYT) were hybridized to 100 μ g of total RNA of human liver and extended with Moloney murine leukaemia virus superscript reverse transcriptase. M13mp18 was sequenced with the M13-40 primer as a size marker. The products were analysed on a 6% polyacrylamide/7 M urea gel. Numbers on the left and right indicate the length (bp) of sequenced marker and extended products respectively.

liver RNA a specific band was detected with a length of 2.25 kb. This band corresponded to the mRNA of human mitochondrial PCK, since no further bands were visible in the RNA lane. Although the 1.4 kb mitochondrial probe had 71% identity with the cDNA of rat cytosolic PCK and the 0.8 kb mitochondrial probe was 67% identical with this cDNA, they did not hybridize to the cytosolic PCK mRNA. To confirm the difference in size of the mRNAs of human cytosolic and mitochondrial PCK, hybridization was performed together with the 1.2 kb cDNA detecting the cytosolic mRNA and the 1.4 kb cDNA detecting the mitochondrial mRNA (Figure 4, filter D). In human liver total RNA, both the mitochondrial and the cytosolic PCK mRNA were detected, whereas in rat liver RNA only the cytosolic form was visible. Since mRNA abundance does not need to be proportional to enzyme activity, those results are in line with the observed equal distribution of PCK activity in human liver in the cytosol and the mitochondria [3,4] and the essentially cytosolic localization of the enzyme in rat liver [1,2].

To ascertain whether the isolated cDNA encoding human liver mitochondrial PCK represented the whole length of the corresponding mRNA, a 22 bp oligonucleotide matching from position 29 to 50 downstream of the translational start site on the mitochondrial PCK cDNA (Figure 2) was hybridized to 100 μ g of total RNA from human liver and extended by Moloney murine leukaemia virus superscript reverse transcriptase. After digestion with RNase A1 and T1 the extended product was analysed on a 6% polyacrylamide sequencing gel (Figure 5). M13mp18 single-stranded DNA sequenced with M13 sequencing

primer was run alongside as size marker. The extended product was 184 nucleotides long (Figure 5) indicating that, after subtracting 50 nucleotides from the coding sequence, the 5'-untranslated region of human mitochondrial PCK mRNA comprised 134 nucleotides. Since the isolated cDNA contained only 67 bp upstream of the translation start site (Figure 2), it missed the first 67 bp of the 5'-untranslated region. For comparison, primer extension experiments were performed also for human cytosolic PCK using a specific oligonucleotide from the cytosolic cDNA ranging from position +3 downstream to position -21 upstream of the translational start site [23]. The extended product was 136 nucleotides in length (Figure 5). Thus the so far unknown 5'-untranslated region of the human liver cytosolic PCK mRNA was 133 nucleotides long, i.e. the previously reported cDNA [23] containing 121 untranslated bp was also incomplete, missing 12 bp.

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