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Sequence differences between human muscle and liver cDNAs for UDPglucose pyrophosphorylase and kinetic properties of the recombinant enzymes expressed in *Escherichia coli*

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UDP-Glc pyrophosphorylase (EC 2.7.7.9) catalyses the interconversion of MgUTP plus Glc1*P* and UDP-Glc plus $MgPP_i$. Complementation of an *Escherichia coli* strain lacking this activity has allowed isolation of cDNA encoding this enzyme from a human muscle library. Two forms were identified and the nucleotide sequence of each was determined; they were found to differ only in the 5′ region and we suggest that these arise from the use of a different first exon in the two transcripts. These nucleotide sequences are different from that of the cDNA which was isolated previously from a human liver library [Peng, H.-L. & Chang, H.-Y. (1993) *FEBS Lett. 329*, 153–158] and it is proposed that these liver and muscle forms are derived from different genes. The cDNA for muscle form I, muscle form II, the liver form, and the liver form fused to part of the *lacZ* gene were expressed in *Escherichia coli* and the kinetic properties of each enzyme were characterised. Muscle form I and the LacZ/liver fusion enzyme exhibit Michaelis-Menten kinetics towards all substrates while muscle form II has a sigmoidal dependence of rate upon the concentration of $MgPP_i$. The liver form shows Michaelis-Menten kinetics towards MgUTP. For the remaining three substrates, complex kinetics were observed involving a combination of sigmoidicity at low substrate concentration and partial inhibition at high substrate concentration.

Keywords: cDNA sequence; enzyme kinetics; *galU*; human isoenzymes; UDPglucose pyrophosphorylase.

UDP-Glc is an important intermediate in mammalian carbohydrate interconversions. In muscle, its primary fate is conversion to glycogen while in lactating mammary gland it is converted to UDPgalactose, a precursor of lactose. In liver, glycogen is also a major product of UDP-Glc metabolism but smaller amounts are converted to UDPglucuronate, which then reacts with xenobiotic and endobiotic compounds to aid in their solubilisation and excretion.

UDP-Glc is formed in the reaction catalysed by UDP-Glc pyrophosphorylase (UDP-Glc PPase):

$$\text{MgUTP} + \text{Glc1}P \leftrightarrow \text{UDP-Glc} + \text{MgPP}_i .$$

The enzyme has been studied from several mammalian tissues including human liver (Knop and Hansen, 1970) and erythrocytes (Tsuboi et al., 1969) although the bulk of the published literature (see review of Turnquist and Hansen, 1973) concerns the bovine liver enzyme.

In view of its diverse metabolic roles, tissue-specific isoforms of UDP-Glc PPase would not be surprising. Aksamit and Ebner (1972) reported that multiple chromatographic forms of the enzyme are present in bovine mammary tissue although it was not determined whether these arise from variable oligomerisation or post-translational modification rather than from different genes. Shows et al. (1978) demonstrated two electrophoretic forms in human cells and showed that these are encoded by genes on chromosomes 1 and 2. However, the molecular differences between these two genes, and the properties of the resulting enzymes, have not been investigated. Here we describe two distinct forms isolated as clones from a human muscle cDNA library. These clones, and a liver form that has been isolated previously (Peng and Chang, 1993), result in enzymes with somewhat different kinetic properties when expressed in *Escherichia coli*.

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Abbreviations. UDP-Glc PPase, UDPglucose pyrophosphorylase; Glc1*P*, glucose 1-phosphate; Glc6*P*, glucose 6-phosphate; Glc(1,6)P_2, glucose 1,6-bisphosphate.

Enzymes. UDPglucose pyrophosphorylase, UTP:α-D-glucose-1-phosphate uridylyltransferase (EC 2.7.7.9); phosphoglucomutase, α-D-glucose 1,6-bisphosphate: α-D-glucose-1-phosphate phosphotransferase (EC 5.4.2.2; formerly 2.7.5.1); glucose-6-phosphate dehydrogenase, D-glucose-6-phosphate:$NADP^+$ oxidoreductase (EC 1.1.1.49).

Note. The nucleotide sequence and deduced amino acid sequence data published here have been submitted to the GenBank database and are available under the accession number U27460.

MATERIALS AND METHODS

cDNA library screening. A human skeletal muscle cDNA library, constructed in the *Eco*RI site of the λ ZAP vector, was purchased from Stratagene (La Jolla, CA). The λ library was converted into the phagemid form by *in vivo* excision as recommended by the supplier. The *E. coli* mutant strain HP069 (MC4100, *galU F′lac*) was used for the selection of clones with

the Gal$^+$ phenotype. MacConkey-galactose agar was prepared with MacConkey agar base (Difco, Detroit MI) supplemented with 0.2% galactose and 50 μg/ml ampicillin.

Recombinant DNA techniques and DNA sequencing. All restriction enzymes were obtained from Boehringer Mannheim. The cDNA was subcloned into M13 vectors and the sequence determined with the Sequenase kit (Amersham) with either universal M13 primer or synthetic oligonucleotides. The sequence was confirmed in both strands, and the question of compressions was resolved by sequencing with dITP. Nucleotide sequences were analysed with the DNASTAR program using a Macintosh LCII computer. The expression vector pTrc99a was obtained from Pharmacia P-L Biochemicals.

Overexpression of the human liver form of UDP-Glc PPase in *E. coli*. The human cDNA clone (pHC308) encoding the liver form of UDP-Glc PPase that was reported previously (Peng and Chang, 1993) did not yield a high level of expression in *E. coli*, presumably due to the presence of a long 5′ noncoding region. To improve expression, the entire coding sequence was synthesised by PCR using the high-fidelity, thermostable enzyme *Pfu* DNA polymerase (Stratagene). Two synthetic oligonucleotides, 5′-ATATAACCCGACCATGGCGAGATTT-3′ and 5′-CTTATCTTAGGGATCCAAGAT-3′ were used as the upstream and downstream primer, respectively. An *Nco*I site and a *Bam*HI site (underlined) were incorporated in these primers, which facilitated subcloning of the amplified DNA fragment. The reaction mixture contained 0.1 μg pHC308, 0.1 nmol of each primer, 250 μM of the four dNTPs, 1.5 mM $MgCl_2$ and 5 units *Pfu* DNA polymerase. After 32 cycles of 94°C (1 min), 55°C (1 min) and 72°C (2 min) the amplified DNA fragment was isolated from SeaPlaque low-melting temperature agarose gel (FMC Corp., Rockland ME). This was then digested with *Nco*I and *Bam*HI, and ligated into the corresponding restriction endonuclease sites of the expression vector pTrc99a. *E. coli* JM109 harbouring the resulting plasmid (pHC404) was used as the source of the human liver form of UDP-Glc PPase.

Enzyme preparation. The enzyme was partially purified from *E. coli* clones containing plasmid-encoded UDP-Glc PPase. In a typical preparation, cells were harvested from 2-l cultures in Luria-Bertani medium after overnight induction with 0.2 mM isopropyl β-D-thiogalactopyranoside at 37°C, and suspended in 60 ml ice-cold buffer A (50 mM Tris/HCl pH 7.8 containing 10 mM 2-mercaptoethanol and 10 mM $MgCl_2$). Buffer A was used throughout the purification and the temperature was maintained at 0−4°C. Cells were disrupted by sonication, the crude extract clarified by centrifugation, and nucleic acids removed by slow addition of 0.083 vol. 20% (mass/vol.) streptomycin sulfate. After centrifugation, the enzyme was precipitated from the supernatant by slow addition of 326 g/l solid ammonium sulfate, dissolved in 6 ml buffer A and dialysed twice against 1 l buffer A. Half of this solution was diluted with an equal volume of water and applied to a column (2.1×14.5 cm) of DEAE-cellulose (Whatman DE52) that had been equilibrated with buffer A. After washing with 10 ml half-strength buffer A and 90 ml full-strength buffer A, the column was developed with a 180-ml linear gradient of 0−0.25 M NaCl in buffer A at a flow rate of 3 ml/min; the enzyme eluted at approximately 0.1 M NaCl. The most active fractions were pooled, precipitated by slow addition of 436 g/l solid ammonium sulfate, and dissolved in 3 ml buffer A. When the enzyme was not to be used within a few days, an equal volume of glycerol was added and the solution stored at −20°C.

Enzyme assays. Tetrasodium pyrophosphate was purchased from Nacalai Tesque Inc. (Kyoto, Japan) and triethanolamine was from Ajax Chemicals (Auburn, Australia). Other substrates, coenzymes, coupling enzymes and buffers were obtained from Sigma. The kinetic properties were assessed for each of the substrates using assays based on those described by Tsuboi et al. (1969). Assays were conducted at 25°C in 0.1 M triethanolamine/HCl pH 8.0 (unless otherwise stated), in the presence of 1 mM dithiothreitol. In what is conventionally designated the forward direction (using Glc1*P* and MgUTP as substrates), the reaction was monitored at 340 nm by addition of NAD^+ (2 mM) and UDP-Glc dehydrogenase (0.25 U/ml). In the reverse reaction (UDP-Glc and $MgPP_i$ as substrates), the reaction was monitored at 340 nm by addition of phosphoglucomutase (5 U/ml) and its cofactor Glc(1,6)P_2 (2 μM), Glc6*P* dehydrogenase (5 U/ml) and $NADP^+$ (1 mM). For assays in both directions, $MgCl_2$ was added to a total concentration calculated so as to give a free magnesium ion concentration of 1 mM, using the stability constants cited by Aksamit and Ebner (1972) for the Mg complexes of the reactants.

Data analysis. Kinetic data were analysed using the nonlinear regression computer program DNRPEASY that is an adaptation of the DNRP53 program described elsewhere (Duggleby, 1984). The following equations were fitted to the data; where the results from several experiments were pooled, the combined analysis was performed as described by Duggleby (1990).

Michaelis-Menten kinetics:

$$v = V_m[S]/(K_m + [S]). \quad (1)$$

Two-substrate kinetics:

$$v = V_m[A][B]/([A][B] + K_a[B] + K_b[A] + K_{ia}K_b). \quad (2)$$

Competitive inhibition:

$$v = V_m[S]/(K_m(1 + [I]/K_i) + [S]). \quad (3)$$

Hill equation:

$$v = V_m/\{1 + (S_{0.5}/[S])^h\}. \quad (4)$$

Modified Monod-Wyman-Changeux model:

$$v = V_m([S]/K_s)(r + [S]/K_s)/\{L + (1 + [S]/K_s)^2\}. \quad (5)$$

RESULTS

Cloning of human cDNA encoding skeletal muscle UDP-Glc PPase. We have shown previously that an *E. coli* UDP-Glc-PPase-deficient mutant can be used as a tool for the isolation by complementation of cDNA clones encoding the enzyme (Peng and Chang, 1993). Taking advantage of this simple selection method, we have extended our investigation to explore the presence of different isoforms of the enzyme in skeletal muscle. Five phagemid clones that are capable of transforming *E. coli* HP069 into the GalU$^+$ phenotype were isolated from a human skeletal muscle cDNA library. The UDP-Glc PPase activity in cell lysates of these recombinant clones was determined and ranged over 2−7 U/mg protein, compared with 0.02 U/mg in *E. coli* JM109 (GalU$^+$) and no detectable activity in the GalU$^-$ host *E. coli* HP069. These results strongly suggest that these phagemid clones encode a UDP-Glc PPase.

Sequence analysis. DNA was prepared from these clones and their restriction enzyme sites were mapped. Two distinct groups were noted on the basis of the presence or absence of an Asp718 site. The nucleotide sequence of a representative phagemid of each of these groups, designated pHC377 (muscle form I) and pHC379 (muscle form II), was determined. Fig. 1A shows the sequence of pHC377, which comprises 1823 nucleotides. Fig. 1B compares the first 42 nucleotides of pHC379 with the corresponding region (nucleotides 70−111) of pHC377; beyond nucleotide 92, pHC379 has an identical sequence.

A

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   1                                                                CCCCCAATTTCCCAGCTGCTAAAGGAAGA
  30  GGAAGGTACCTGTGCGTGCACGCAGACGGGAAGGGCTGGGGAAGCGGGAGGACTGAGAAAACCAGATCTTAGCAAAGCA
   1   M   S   Q   D   G   A   S   Q   F   Q   E   V   I   R   Q   E   L   E   L   S
 109  ATG TCT CAA GAT GGT GCT TCT CAG TTC CAA GAA GTC ATT CGG CAA GAG CTA GAA TTA TCT
  21   V   K   K   E   L   E   K   I   L   T   T   A   S   S   H   E   F   E   H   T
 169  GTG AAG AAG GAA CTA GAA AAA ATA CTC ACC ACA GCA TCA TCA CAT GAA TTT GAG CAC ACC
  41   K   K   D   L   D   G   F   R   K   L   F   H   R   F   L   Q   E   K   G   P
 229  AAA AAA GAC CTG GAT GGA TTT CGG AAG CTA TTT CAT AGA TTT TTG CAA GAA AAG GGG CCT
  61   S   V   D   W   G   K   I   Q   R   P   P   E   D   S   I   Q   P   Y   E   K
 289  TCT GTG GAT TGG GGA AAA ATC CAG AGA CCC CCT GAA GAT TCG ATT CAA CCC TAT GAA AAG
  81   I   K   A   R   G   L   P   D   N   I   S   S   V   L   N   K   L   V   V   V
 349  ATA AAG GCC AGG GGC CTG CCT GAT AAT ATA TCT TCC GTG TTG AAC AAA CTA GTG GTG GTG
 101   K   L   N   G   G   L   G   T   S   M   G   C   K   G   P   K   S   L   I   G
 409  AAA CTC AAT GGT GGT TTG GGA ACC AGC ATG GGC TGC AAA GGC CCT AAA AGT CTG ATT GGT
 121   V   R   N   E   N   T   F   L   D   L   T   V   Q   Q   I   E   H   L   N   K
 469  GTG AGG AAT GAG AAT ACC TTT CTG GAT CTG ACT GTT CAG CAA ATT GAA CAT TTG AAC AAA
 141   T   Y   N   T   D   V   P   L   V   L   M   N   S   F   N   T   D   E   D   T
 529  ACC TAC AAT ACA GAT GTC CCT CTT GTT TTA ATG AAC TCT TTT AAC ACG GAT GAA GAT ACC
 161   K   K   I   L   Q   K   Y   N   H   C   R   V   K   I   Y   T   F   N   Q   S
 589  AAA AAA ATA CTA CAG AAG TAC AAT CAT TGT CGT GTG AAA ATC TAC ACT TTC AAT CAA AGC
 181   R   Y   P   R   I   N   K   E   S   L   R   P   V   A   K   D   V   S   Y   S
 649  AGG TAC CCG AGG ATT AAT AAA GAA TCT TTA CGG CCT GTA GCA AAG GAC GTG TCT TAC TCA
 201   G   E   N   T   E   A   W   Y   P   P   G   H   G   D   I   Y   A   S   F   Y
 709  GGG GAA AAT ACA GAA GCT TGG TAC CCT CCA GGT CAT GGT GAT ATT TAC GCC AGT TTC TAC
 221   N   S   G   L   L   D   T   F   I   G   E   G   K   E   Y   I   F   V   S   N
 769  AAC TCT GGA TTG CTT GAT ACC TTT ATA GGA GAA GGC AAA GAG TAT ATT TTT GTG TCT AAC
 241   I   D   N   L   G   A   T   V   D   L   Y   I   L   N   H   L   I   N   P   P
 829  ATA GAT AAT CTG GGT GCC ACA GTG GAT CTG TAT ATT CTT AAT CAT CTA ATC AAC CCA CCC
 261   N   G   K   R   C   E   F   V   M   E   V   T   N   K   T   R   A   D   V   K
 889  AAT GGA AAA CGC TGT GAA TTT GTC ATG GAA GTC ACA AAT AAA ACA CGT GCA GAT GTA AAG
 281   G   G   T   L   T   Q   Y   E   G   K   L   R   L   V   E   I   A   Q   V   P
 949  GGC GGG ACA CTC ACT CAA TAT GAA GGC AAA CTG AGA CTG GTG GAA ATT GCT CAA GTG CCA
 301   K   A   H   V   D   E   F   K   S   V   S   K   F   K   I   F   N   T   N   N
1009  AAA GCA CAT GTT GAC GAG TTC AAG TCT GTA TCA AAG TTC AAA ATA TTT AAT ACA AAC AAC
 321   L   W   I   S   L   A   A   V   K   R   L   Q   E   Q   N   A   I   D   M   E
1069  CTA TGG ATT TCT CTT GCA GCA GTT AAA AGA CTG CAG GAG CAA AAT GCC ATT GAC ATG GAA
 341   I   I   V   N   A   K   T   L   D   G   G   L   N   V   I   Q   L   E   T   A
1129  ATC ATT GTG AAT GCA AAG ACT TTG GAT GGA GGC CTG AAT GTC ATT CAA TTA GAA ACT GCA
 361   V   G   A   A   I   K   S   F   E   N   S   L   G   I   N   V   P   R   S   R
1189  GTA GGG GCT GCC ATC AAA AGC TTT GAG AAT TCT CTA GGT ATT AAT GTG CCA AGG AGC CGT
 381   F   L   P   V   K   T   T   S   D   L   L   L   V   M   S   N   L   Y   S   L
1249  TTT CTG CCT GTC AAA ACC ACA TCA GAT CTC TTG CTG GTG ATG TCA AAC CTC TAT AGT CTT
 401   N   A   G   S   L   T   M   S   E   K   R   E   F   P   T   V   P   L   V   K
1309  AAT GCA GGA TCT CTG ACA ATG AGT GAA AAG CGG GAA TTT CCT ACA GTG CCC TTG GTT AAA
 421   L   G   S   S   F   T   K   V   Q   D   Y   L   R   R   F   E   S   I   P   D
1369  TTA GGC AGT TCT TTT ACG AAG GTT CAA GAT TAT CTA AGA AGA TTT GAA AGT ATA CCA GAT
 441   M   L   E   L   D   H   L   T   V   S   G   D   V   T   F   G   K   N   V   S
1429  ATG CTT GAA TTG GAT CAC CTC ACA GTT TCA GGA GAT GTG ACA TTT GGA AAA AAT GTT TCA
 461   L   K   G   T   V   I   I   I   A   N   H   G   D   R   I   D   I   P   P   G
1489  TTA AAG GGA ACG GTT ATC ATC ATT GCA AAT CAT GGT GAC AGA ATT GAT ATC CCA CCT GGA
 481   A   V   L   E   N   K   I   V   S   G   N   L   R   I   L   D   H   *
1549  GCA GTA TTA GAG AAC AAG ATA GTG TCT GGA AAC CTT CGC ATC TTG GAC CAC TGA AATGAAA
1610  AATACTGTGGACACTTAAATAATGGGCTAGTTTCTTACAATGAAATGTTCTCTAGGATTTAGGCACTAAAAGGTACTTT
1689  ACTATGTTACTGTACCCTGCAGTGTTGATTTTTAAAATAGAGTTTTCTGCAGTATGCTTTTAGTCTAAGAAAAGCACAG
1768  ATGGTGCAATACTTTCCTTCTTTGAAGAGATCCCAAAGTTAGTTACTCTTAAGTGC
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B

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                      M   S   R   F   V   Q   D   L   S   K   A   M   12
pHC379   1  CCT AAA ATG TCG AGA TTT GTA CAA GAT CTT AGC AAA GCA ATG   42
                                  | | | ||| ||| ||| ||| ||| ||| |||
pHC377  70  GAA GCG GGA GGA CTG AGA AAA CCA GAT CTT AGC AAA GCA ATG 111
                                                          M   1
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C

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pHC379 CCCCCAATTTCCCAGCTGCTAAAGGAAGAGGAAGGTACCTGTGCGTGCACGCAGACGGGA  60
M85693 ••••••••••••••••••••••••••••••••••••••••••••N••••••••••••••••• 161

pHC379 AGGGCTGGGGAAGCGGGAGGACTGAGAAAACC-AGATCTTAGCAAAGCAATGTCTCAAGA 119
M85693 ••••••••••••••••••••••••••••••••G•C•••••••••••••••••••••••••• 221
R58578                               ATC••••••••••••••••••••••••••••  30

pHC379 TGGTGCTTCTCAGTTCCAAGAAGTCATTCGGCAAGAGCTAGAATTATCTGTGAAGAAGGA 179
M85693 ••••••••••••••••••••••••••••••••••••••••••••••••••N••••••••••• 281
R58578 ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••  90

pHC379 ACTAGAAAAAATACTCACCACAGCATCATCACATGAATTT-GAGCACACCAAAAAAGACC 238
M85693 •••••••••••••••••••••••••••••••••••••••T•••••••N••••••G•G•• 341
R58578 ••••••••••••••••••••••••••••••••••••••••••••••••••••••••••••  146

pHC379 TGGATGGATTTCGGAAGCTATTTCATAGATTT                             270
M85693 •••G••••••••••••••••••••••••••                             373
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of human muscle UDP-Glc PPase. (A) The nucleotide sequence is numbered in the 5′ to 3′ direction, beginning at the first position of the *Eco*RI linker. The amino acid sequence is numbered from the first methionine at the putative translation initiation site. (B) The nucleotide sequences of the 5′-regions of the two muscle forms. (C) Alignment of the nucleotide sequences of pHC377 and two human expressed sequence tags. A dot indicates identity to pHC377 while a hyphen indicates a gap.

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Human liver      MSRFVQDLSKAMSQDGASQFQEVILQELELSVKKELEKILTTATSHEYEH   50
Bovine liver     ·····················R·················P···F··   50
Human muscle-I   ···········R·················S···F··   39
Human muscle-II  ·····················R·················S···F··   50

Human liver      TKKDLDGFRKLYHRFLQEKGPSVDWGKIQRPPEDSIQPYEKIKARGLPDN  100
Bovine liver     ··········F·····································  100
Human muscle-I   ··········F·····································   89
Human muscle-II  ··········F·····································  100

Human liver      ISSVLNKLVVVKLNGGLGTSMGCKGPKSLIGVRNENTFLDLTVQQIEHLN  150
Bovine liver     V···············································  150
Human muscle-I   ···············································  139
Human muscle-II  ···············································  150

Human liver      KSYNTDVPLVLMNSFNTDEDTKKILQKYNHCRVKIYTFNQSRYPRINKES  200
Bovine liver     ·T··············································  200
Human muscle-I   ·T··············································  189
Human muscle-II  ·T··············································  200

Human liver      LRPVAKDVSSSGESTEAWYPPGHGDIYASFYNSGLLDTFLEEGKEYIFVS  250
Bovine liver     ·L····N··Y···N·····················IG·········  250
Human muscle-I   ·········Y···N·····················IG·········  239
Human muscle-II  ·········Y···N·····················IG·········  250

Human liver      NIDNLGATVDLYILNHLINPPNGKRCEFVMEVTNKTRADVKGGTLTQYEG  300
Bovine liver     ···············M·····························  300
Human muscle-I   ···············································  289
Human muscle-II  ···············································  300

Human liver      KLRLVEIAQVPKAHVDEFKSVSKFKIFNTNNLWISLAAVKRLQEQNAIDM  350
Bovine liver     ···············································  350
Human muscle-I   ···············································  339
Human muscle-II  ···············································  350

Human liver      EIIVNPKTLDGGLNVIQLETAVGAAIKSFENSLGINVPRSRFLPVKTTSD  400
Bovine liver     ···············································  400
Human muscle-I   ·····A·····························  389
Human muscle-II  ·····A·····························  400

Human liver      LLLVMSNLYSLNAGSLTMSEKREFPTVPLVKLGSSFTKVQDYLRRFESIP  450
Bovine liver     ···············································  450
Human muscle-I   ···············································  439
Human muscle-II  ···············································  450

Human liver      DMLELDHLTVSGDVTFGKNVSLKGTVIIIANHGDRIDIPPGAVLENKIVS  500
Bovine liver     ···············································  500
Human muscle-I   ···············································  489
Human muscle-II  ···············································  500

Human liver      GNLRILDH                                          508
Bovine liver     ·····                                             508
Human muscle-I   ·····                                             497
Human muscle-II  ·····                                             508
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Fig. 2. Comparison of the deduced amino acid sequences of the human liver, bovine liver and two human muscle forms of UDP-Glc PPase. The human liver sequence (Peng and Chang, 1993) is shown in the first line; for the other proteins, a dot indicates identity to the human liver sequence.

pHC379 encodes a protein of 508 amino acid residues (Fig. 2) as in the liver form of the enzyme from which it differs at 10 positions. The overall nucleotide identity within the coding regions is 92.3%. The protein encoded by pHC377 is somewhat smaller (497 residues) as it uses as a translation initiation codon the second in-frame ATG normally found in the muscle II form.

The liver form that we identified previously (Peng and Chang, 1993) was isolated from a commercial cDNA library purchased from Stratagene. To confirm that this DNA is truly of human liver origin, we sought to identify this sequence in an independent human liver cDNA library, purchased from Clontech (cat. no. HL3006b). Approximately 10^7 plaque-forming units of this library were subjected to PCR (32 cycles of 94°C, 1 min; 55°C, 1 min; and 72°C, 2 min) using the synthetic oligonucleotides primers 5′-CAGAAGCTTGGTACCCCC-3′ and 5′-GATTAGATGATTAAGAAT-3′. These primers correspond to bases 719–736 and 874–857, respectively, of the muscle sequence (Fig. 1A). The amplified fragment was isolated from an agarose gel and its nucleotide sequence was determined as described by Cullmann et al. (1993). Among the eight nucleotides between positions 760 and 840 of the muscle cDNA that are different from those of the liver form, two were not identifiable unambiguously while the remaining six matched the liver sequence.

Kinetic properties. These cDNA clones were expressed in *E. coli* and the enzymes were partially purified and characterised with respect to their kinetic properties. Before presenting these results, the assay conditions will be described briefly.

Assay conditions. There have been conflicting reports of the kinetic properties of UDP-Glc PPase with respect to PP_i, with some authors describing a sigmoidal dependence of rate upon concentration (Villar-Palasi and Larner, 1960; Knop and Hansen, 1970). With the bovine enzyme, assayed in 0.1 M Tris/HCl pH 8.0, we also found a sigmoidal relationship (Fig. 3, circles). This appears to be due entirely to a competition between the buffer and PP_i for magnesium ions. Changing to triethanolamine/HCl pH 8.0, and maintaining a constant concentration of free

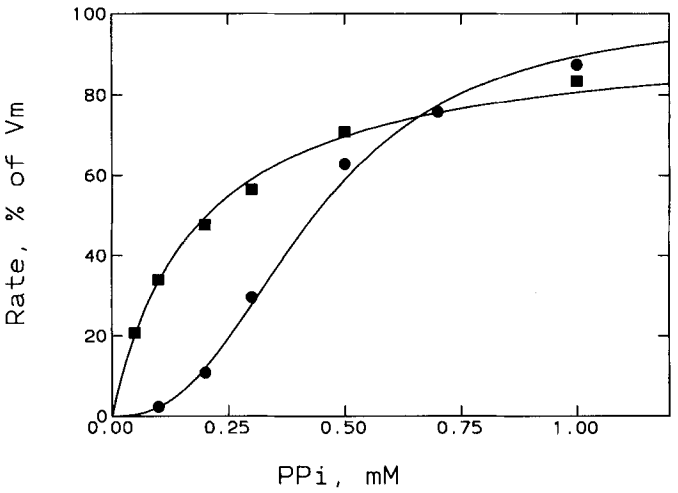

Fig. 3. Effect of the buffer on the apparent kinetics towards PP_i of bovine liver UDP-Glc PPase. The rate was measured over a range of PP_i concentrations using 0.1 M Tris/HCl (●) or 0.1 M triethanolamine/HCl (■) at pH 8.0. For Tris/HCl, the line represents the best fit of Eqn (4) to the data. In the case of 0.1 M triethanolamine/HCl, the concentrations of PP_i and $MgCl_2$ were varied simultaneously so as to maintain free $[Mg^{2+}]$ at 1 mM; the line represents the best fit of Eqn (1) to the data.

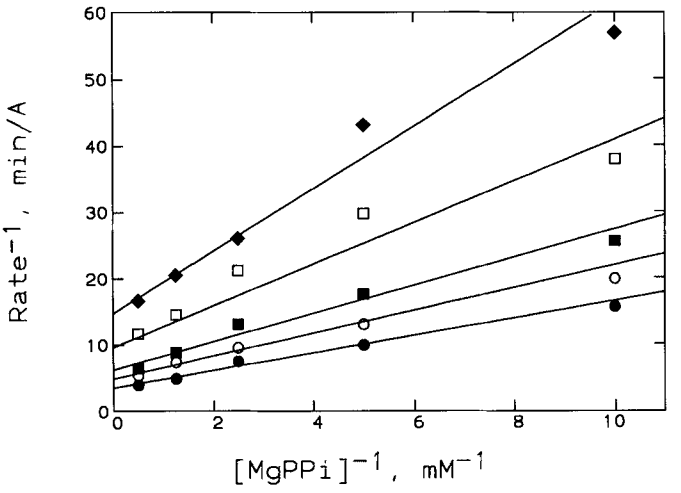

Fig. 4. Kinetic properties of the human muscle UDP-Glc PPase form I with respect to $MgPP_i$ and UDP-Glc. The rate was measured over a range of $MgPP_i$ concentrations with fixed UDP-Glc concentrations (μM) of 300 (●), 100 (○), 60 (■), 30 (□) and 15 (◆). The results are plotted in double reciprocal form and the lines represent the best fit of Eqn (2) to the data. A represents the absorbance at 340 nm.

magnesium ion while varying the concentration of $MgPP_i$, gave a saturation curve that is hyperbolic (Fig. 3, squares). As will be seen later, the human liver and muscle II forms of the enzyme show non-hyperbolic kinetics with respect to $MgPP_i$ that we believe represent true properties of the enzyme rather than an artefact of the assay.

Muscle form I. The human muscle I form of the enzyme exhibited Michaelis-Menten kinetics with respect to each of the four substrates. UDP-Glc PPase is thought to have an ordered sequential mechanism (Tsuboi et al., 1969; Aksamit and Ebner, 1972). As expected for this mechanism, the human muscle I form yielded an intersecting pattern in double-reciprocal plots obtained by varying the concentrations of both UDP-Glc and $MgPP_i$ (Fig. 4). From these data, Michaelis constants for each substrate and an inhibition constant for UDP-Glc were determined. In the forward direction, varying the concentration of each substrate (MgUTP and Glc1*P*) gave hyperbolic curves from which Michaelis constants were determined. The inhibition constant for MgUTP was determined by using it as a product inhibitor of the reverse reaction; this inhibition was competitive with UDP-Glc as the varied substrate. The kinetic constants are summarised in Table 1.

Liver form. The kinetics of the human liver enzyme in the reverse reaction are illustrated in Fig. 5 with $MgPP_i$ as the varied substrate. Double-reciprocal plots (Fig. 5A) are markedly curved, especially at low concentrations of UDP-Glc (diamonds). This curvature results from a combination of two effects. First, there is some substrate inhibition as can be seen in Fig. 5B; this is most readily apparent at an intermediate UDP-Glc concentration (squares) of 70 μM. This substrate inhibition is partial in the sense that the rate falls to a finite plateau rather than to zero as the concentration of $MgPP_i$ is raised. The curvature seen in Fig. 5A is partly due to sigmoidicity in the rate versus concentration curve at low concentrations of $MgPP_i$, as shown in Fig. 5C. Although the effect is not large (described by a Hill coefficient of 1.415 ± 0.064), the data clearly do not follow a hyperbola as shown by the broken line in Fig. 5C that represents the best-fit of the Michaelis-Menten equation to this subset of the data.

A similar pattern to that described for $MgPP_i$ emerges with UDP-Glc as the varied substrate; that is, a sigmoidal curve with partial substrate inhibition that becomes close to hyperbolic at high concentrations of $MgPP_i$.

The kinetics in the forward reaction have been less extensively characterised. For MgUTP, the kinetics appear to be hyperbolic with a K_m of 419 ± 32 μM which is approximately half

Table 1. Kinetic constants of three forms of human UDP-Glc PPase. The constants were obtained by fitting the appropriate equation to the data by nonlinear regression and are reported as the best fit value ± standard error.

Reaction	Constant	Value for		
		muscle form I	muscle form II	LacZ/liver form
		μM		
Forward	K_m(MgUTP)	917 ± 147	301 ± 15	563 ± 115
	K_m(Glc1*P*)	404 ± 51	207 ± 28	172 ± 10
	K_i(MgUTP)	979 ± 80	216 ± 21	643 ± 47
Reverse	K_m(UDP-Glc)	63 ± 6	41 ± 2	49 ± 4
	K_m($MgPP_i$)	384 ± 36	sigmoidal	166 ± 13
	K_i(UDP-Glc)	52 ± 8	not determined	13 ± 4

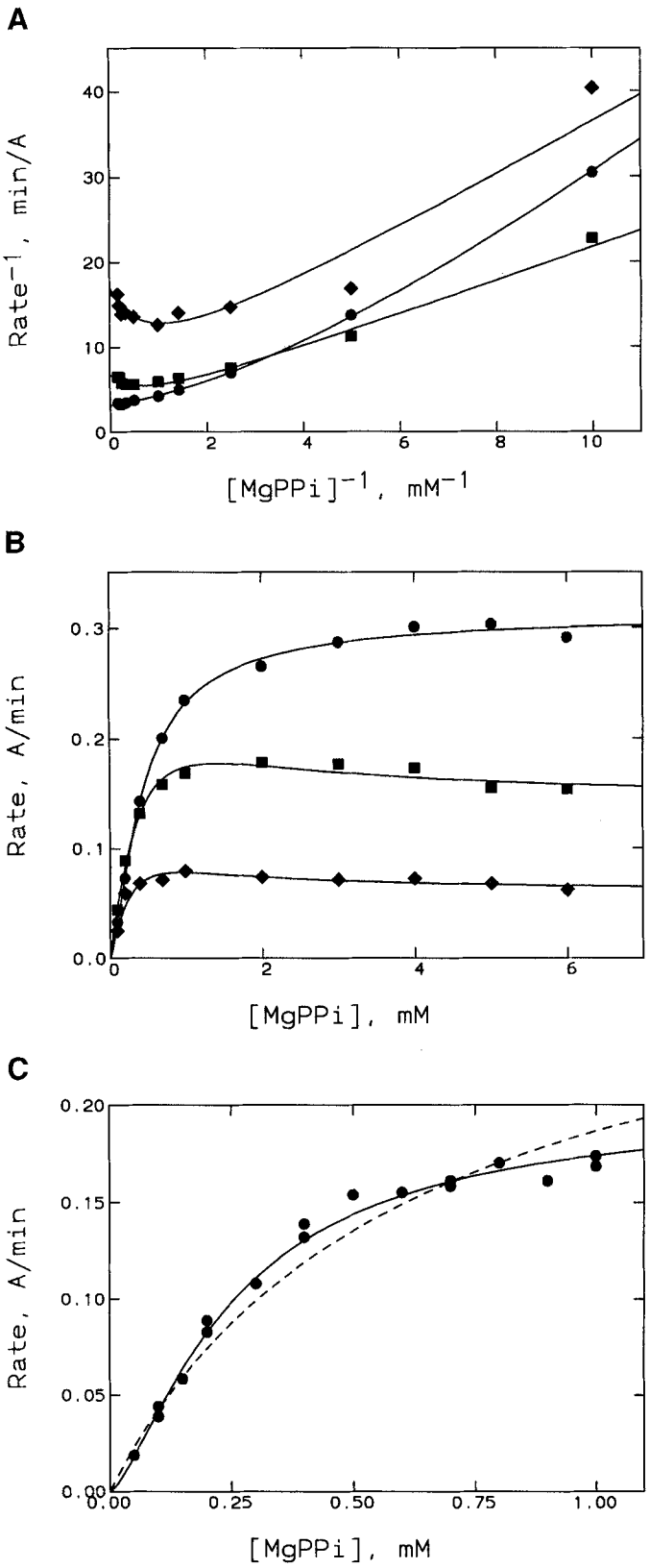

Fig. 5. Kinetic properties of the human liver UDP-Glc PPase with respect to $MgPP_i$ and UDP-Glc. (A, B) The rate was measured over a range of $MgPP_i$ concentrations with fixed UDP-Glc concentrations (μM) of 300 (●), 70 (■) and 20 (◆). The results are plotted with (A) or without (B) double-reciprocal transformation; the lines represent individual best fits of Eqn (5) to each set of data. (C) The results obtained at 70 μM UDP-Glc shown in greater detail; (——) the best fit of Eqn (4) to the data; (– – –) best fit of Eqn (1) to the data. A represents the absorbance at 340 nm.

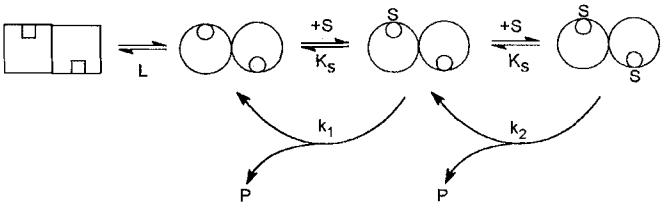

Fig. 6. Kinetic model for UDP-Glc PPase. The two-subunit Monod-Wyman-Changeux model was modified by allowing for different rate constants for catalysis when one (k_1) or both (k_2) active sites are occupied.

the value observed for the muscle form I under identical conditions. For Glc1*P*, the saturation curve is non-hyperbolic and is described by Eqn (5).

LacZ/Liver fusion enzyme. As noted earlier, one of the differences between muscle form I and the liver form is in the N-terminal region of the protein and it seems probable that the kinetic complexity of the liver form may arise, in part, from the way in which this region folds. This hypothesis was supported by experiments on a fusion protein in which the first nine residues of the liver sequence (MSRFVQDLS) are replaced with the first 39 residues of the pBluescript *lacZ* gene product (MTMITPSSKLTLTKGNKSWSSTAVAAALELVDPPGCRNS) plus the linker sequence ARG. The kinetic properties of this fusion protein are summarised in Table 1. Although these properties differ from those of muscle form I, no kinetic anomalies were observed and the enzyme followed Michaelis-Menten kinetics with respect to all substrates.

Muscle form II. To dissect further the relative contributions to the kinetic differences of the various sequence variations between the liver and muscle I forms, the properties of the muscle II forms were investigated (Table 1). This form was found to exhibit Michaelis-Menten kinetics with respect to all substrates except $MgPP_i$, for which the kinetics were sigmoidal. Thus it appears that this particular kinetic anomaly is associated with the N-terminal region while the non-hyperbolic kinetics of the liver form with respect to the other substrates are controlled by sequence differences in the remainder of the protein.

DISCUSSION

Complementation of an *E. coli* GalU$^-$ mutant has proved to be a useful means of isolating cDNAs encoding UDP-Glc PPase. Because it relies on expression of active enzyme, any cDNA clones with major 5′-truncations are eliminated. Using this method we have isolated previously a clone from a human liver cDNA library and here we report the detection of two forms from a human muscle cDNA library.

Comparing these sequences with those contained in the GenBank and EMBL databases revealed a number of human expressed sequence tags that matched well with sections of our clones, and two of these (M85693 from brain and R58578 from heart) are almost identical to pHC379 over a region spanning the first 270 bases (Fig. 1C); thus, there is evidence for the expression of the muscle II form in at least two other tissues.

The 5′-end of pHC379 is very similar to that of the liver form that we have described previously (Peng and Chang, 1993). By contrast, the cDNA for the two muscle forms of UDP-Glc PPase are totally different before nucleotide 93 but from that point onwards, the sequences are identical. This is unlikely to occur if the two cDNAs arise from different genes and we suggest that they may result from the use of a different first exon in these two transcripts. A similar situation has been reported

(Putt et al., 1993) to occur in the preceding enzyme of UDP-Glc formation, phosphoglucomutase.

It is of interest that there is a closer relationship between the amino acid sequences of bovine liver and human muscle forms (6 differences) than between the bovine liver and human liver forms (13 differences). In addition, the 3′ untranslated region of the human muscle cDNA, but not the human liver form, exhibits significant similarity to the corresponding region of the bovine liver cDNA. For example, the 15 nucleotides immediately following the termination codon of the two transcripts are identical. Together, these data strongly suggest that duplication of the human UDP-Glc PPase gene into the liver and muscle isoforms has occurred before the diversification of the evolutionary lines leading to the human and bovine species.

Human liver UDP-Glc PPase shows complex kinetics with respect to all substrates except MgUTP. A possible explanation of the kinetic behaviour is given by the model shown as Fig. 6. This is an adaptation of the two-subunit Monod-Wyman-Changeux allosteric model with the added complexity that the catalytic rate constant depends on whether one or both active sites are occupied. Although the enzyme is represented as a dimer in Fig. 6, we acknowledge that the active form of the enzyme is probably the octamer (Levine et al., 1969). The rate equation for this model is given as Eqn (5) where r is the ratio k_1/k_2. We wish to emphasise that this is only one possible interpretation of the curves; there is not enough information at present to be confident that this model is correct, although we note that it does describe the individual curves extremely well.

Some authors have reported that the kinetics of mammalian UDP-Glc PPase with respect to PP_i follow a sigmoidal saturation curve (Villar-Palasi and Larner, 1960; Knop and Hansen, 1970). This may be due partly to the failure to appreciate that the true substrate is $MgPP_i$, coupled with the use of a buffer that can complex magnesium ion. However, Steelman and Ebner (1966) described non-hyperbolic kinetics for the bovine mammary gland enzyme that cannot be explained simply as an experimental artefact. Thus it appears that non-hyperbolic kinetics similar to that which we have observed for the human liver and muscle II forms may be an intrinsic property of the enzyme, at least in some circumstances.

The metabolic significance of the kinetic anomalies that we have observed is unclear, but they tend to make the enzyme more responsive to changes in substrate concentration. For example, at a UDP-Glc concentration of 70 μM, the liver enzyme goes from 20% to 80% of maximum activity over a 6-fold concentration range of $MgPP_i$, in contrast to muscle form I which requires a 16-fold range. We are conscious that the properties that we have measured are those of human enzymes expressed in *E. coli* and that post-translational modifications that could affect these properties may be different. Thus, we are reluctant to extrapolate these findings to the situation that might occur in human tissues.

The cDNAs that we have isolated have been designated as the liver, muscle I and muscle II forms but we emphasise that this indicates only the libraries from which they were isolated. For example, the 'liver' form could conceivably be the predominant one in muscle; the fact that we did not detect it in the muscle cDNA library may simply result from the relatively small number of positive clones that were observed and characterised. In addition, this 'liver' transcript may be preferentially degraded and therefore would not be detected by complementation. It has been shown previously by Northern analysis (Peng and Chang, 1993) that the UDP-Glc PPase cDNA is expressed at high levels in muscle but the probe used in these experiments was not specific enough to identify which of the three forms was present. Measurements of the relative abundance of the three cDNAs in various tissues will help to resolve whether any of them can correctly be described as muscle or liver forms.

It is not yet known whether the 'muscle' and 'liver' forms of the cDNA are derived from two independent genes or different alleles of a single gene but the diversity of their nucleotide and amino acid sequences favours the former possibility. Support for this hypothesis comes from the observation that two different loci for UDP-Glc PPase have been demonstrated and assigned to chromosomes 1 and 2 (van Someren et al., 1974; Shows et al., 1978). We have recently isolated genomic DNA corresponding to the muscle form of the enzyme and its genetic locus is currently being determined.

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