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Cloning of cDNA Encoding for a Novel Isozyme of Fructose 6-Phosphate,2-Kinase/Fructose 2,6-Bisphosphatase from Human Placenta[1]

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Two independent cDNA clones encoding fructose 6-phosphate,2-kinase/fructose 2,6-bisphosphatase were isolated from a human placental cDNA library. The deduced amino acid sequences showed that one of the clones, 2K-1, was almost identical to the rat testis isozyme and the other, 2K-3, was different from any known isozymes expressed in mammalian tissues. The results of Southern blot analysis suggested that clones 2K-1 and 2K-3 were encoded as single copy genes and located in different parts of the genome. Since open reading frames of the cDNA clones were not complete, we obtained the 5′-end of the clone 2K-3 cDNA using the 5′-rapid amplification of cDNA end method. The entire cDNA (HP; 1,756 bp) had a coding capacity of 519 amino acids (M_r=59,410), and putative phosphorylation sites for protein kinases A and C on the C terminus. Northern blot analysis using a fragment of the HP as a probe showed that a major band of 5.4 kb, significantly different in size from known isozyme mRNAs such as liver (2.1 kb), muscle (1.9 kb), heart (4.0 kb), and testis (2.0 kb), was present in poly(A)$^+$RNA preparations of human first trimester and term placentae. These results strongly suggested that this 5.4 kb mRNA codes a novel isozyme of fructose 6-phosphate,2-kinase/fructose 2,6-bisphosphatase.

Key words: cDNA cloning, human placental fructose 6-phosphate,2-kinase/fructose 2,6-bisphosphatase, isozyme, phosphorylation site.

The bifunctional enzyme fructose 6-phosphate,2-kinase [EC 2.7.1.105]/fructose 2,6-bisphosphatase [EC 3.1.3.46] (Fru6P2-kinase/Fru2,6BPase) catalyzes the synthesis and hydrolysis of fructose 2,6-bisphosphate, which is the most potent activator of phosphofructokinase (*1*), a key regulatory enzyme of glycolysis. There are several Fru6P2-kinase/Fru2,6BPase isozymes that differ in tissue distribution. The importance of the enzyme for the regulation of phosphofructokinase activity (and glycolysis) has been established in different tissues in various animals, including rat and human liver (*2-5*), rat skeletal muscle (*6, 7*), bovine heart (*8*), and rat testis (*9*).

The placenta is unique as an organ in that it has some of the characteristics of the liver, of the intestinal mucosa, of the lung, of the kidney, and the endocrine gland, and it differentiates and grows from embryonic tissue to reach maturity in a period of only months in human (*10*). The placenta is not only active and selective in its transfer of substances essential to the development of fetus, but also able to modify molecules of maternal, fetal and external origin. The placenta has a distinct metabolic identity. The human placenta has a high glycogen content and a high glucose-6-phosphate content early in pregnancy, but both of these decline toward term. Moreover, the placenta utilizes much glucose, about a third of all the glucose supplied to it by the maternal circulation, for its own metabolic needs; the exact fraction depends on the respective sizes and activities of the placenta and the fetus at the particular stage of gestation (*11*). However, it is still unknown how the placenta controls glucose utilization and the enzymes involved.

The glycolytic pathway is quantitatively the more significant route of glucose utilization in the human placenta. The placental glycolytic rate is accelerated by anoxia (*12*) and by maternal diabetes (*13, 14*). It is worthwhile to elucidate what type of Fru6P2-kinase/Fru2,6BPase isozyme exists in placental cells from the standpoint of the control of glucose utilization. Here we report the molecular cloning of cDNA encoding a novel isozyme of Fru6P2-kinase/Fru2,6BPase from human placenta.

MATERIALS AND METHODS

cDNA Library Construction and Cloning—Total RNA

[1] This study was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan.
The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide databases with the accession numbers D49817 and D49818.
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Abbreviations: Fru6P2-kinase/Fru2,6BPase, fructose 6-phosphate, 2-kinase/fructose 2,6-bisphosphatase; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA end; RT-PCR, reverse transcription-PCR.

was prepared from chorionic villi dissected from a single placenta obtained on elective abortion at week 11 of pregnancy (first trimester) by using an RNA extraction kit (Amersham), and poly(A)$^+$RNA was purified using Oligo-(dT)-cellulose Type 7 (Pharmacia LKB). The oligo(dT)- and random-primed cDNA library was constructed by using poly(A)$^+$RNA (5 μg), a cDNA synthesis kit from GIBCO BRL, a Protoclone λgt10 system from Promega, and a Gigapack II Gold Packaging Extract from Stratagene. The placental cDNA library (4×10^6 recombinants) was screened under low stringency conditions as described previously (*15*), using ^{32}P-labeled human liver Fru6P2-kinase/Fru2,6BPase cDNA (*4*) (a kind gift of Prof. K. Uyeda, University of Texas Southwestern Medical Center) and *Eco*RI/*Bam*HI fragment of frog liver Fru6P2-kinase/Fru2, 6BPase cDNA (*15*) as probes. Two cDNA clones with a positive signal, designated 2K-1 and 2K-3, were purified and subcloned into the *Not*I site of pBluescript II SK(+). In order to obtain the 5′-region, 5′-rapid amplification of cDNA ends (5′-RACE) (*16*) was performed according to the procedure described by the kit supplier. Human placenta 5′-RACE-Ready cDNA (Clontech) was utilized as a template in a primary polymerase chain reaction (PCR) with the primer (P-8: 5′-ATGGTACGCGGCTGCACGTGGATGTTC-3′, corresponding to #738-764 of HP cDNA) and the anchor primer supplied by Clontech. The primary PCR product was diluted and then used in a secondary PCR with a nested primer (P9: 5′-GGTAGTACACGATGCGGCTCTGGATGT-3′, corresponding to #707-733 of HP cDNA) and the anchor primer. The amplified PCR products were then subcloned into the pCRII vector (Invitrogen) and sequenced.

DNA Sequence Determination—DNA sequences of the cDNA inserts were determined directly from double-stranded plasmids by the dideoxynucleotide chain-termination method (*17*) with a PRISM Dye Terminator Cycle Sequencing kit (Applied Biosystems) and synthetic oligonucleotide primers using a Model 373A DNA Sequencing System (Applied Biosystems). DNA sequence data were analyzed using the GeneWorks Nucleic Acid and Protein Sequence Analysis Program (Intelligenetics).

Reverse Transcription-PCR (RT-PCR) Amplification—The poly(A)$^+$ RNA was reverse-transcribed with AMV reverse transcriptase using the gene-specific primer (P1: 5′-GGCAGATGACCAGCACATT-3′, corresponding to #1162-1180 of HP cDNA). PCR amplification was carried out using 25 ng of the reverse-transcribed RNA with the P2 primer (5′-CGGATCCATGATCACTGGCTCCAAG-3′, corresponding to #1125-1149 of HP cDNA and containing a *Bam*HI site) and the 2K5′ primer (5′-TGAATTCGTGTCGGGCGCAGCCGCGAAGATGCCGTT-3′, containing the *Eco*RI site and #1-26 of HP cDNA) using a Thermal Sequencer TSR-300 (Iwaki Glass). The PCR conditions were 45 s at 94°C, 45 s at 62°C, 120 s at 72°C.

Southern Blot Analysis—Each placental DNA sample (10 μg/lane) was digested with a restriction enzyme including *Xba*I, *Sac*I, *Pst*I, *Kpn*I, *Hin*dIII, *Eco*RI, or *Bam*HI, subjected to electrophoresis in 0.8% agarose, and blotted onto BYODINE B (Pall) nylon membrane filter. The membrane was hybridized at 42°C for 24 h with ^{32}P-labeled PCR fragments that were specific for the 3′-noncoding region of 2K-1 (corresponding to #728-887) and 2K-3 (corresponding to #915-1052). The blots were washed in 0.5×SSC, 0.1% SDS at 50°C and exposed to Kodak XAR film for 72 h at −70°C with an intensifying screen.

Northern Blot Analysis—Total RNA was isolated by the single step acid guanidium thiocyanate-chloroform extraction method (*18*). Poly(A)$^+$RNA was isolated by using Oligotex-dT30 (Takara). For Northern blot analysis, poly(A)$^+$RNAs (4 μg) from first trimester and term placentas were fractionated on a 1.5% formaldehyde-agarose gel (*19*), transferred to BYODINE B nylon membrane filter, and fixed with Spectrolinker (Genentics). The filter was

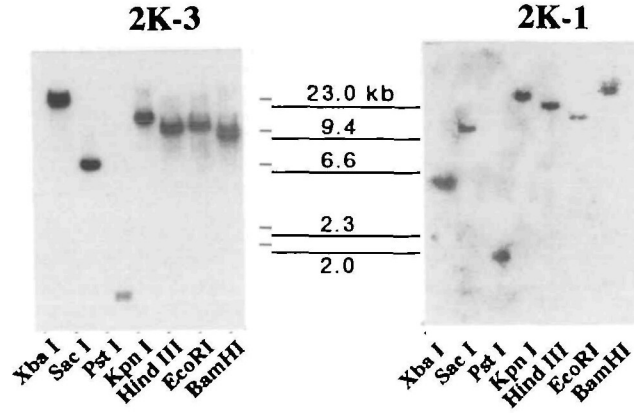

Fig. 2. **Southern blot analysis of genomic DNA isolated from human placental cells.** DNA (10 μg) were digested with the restriction enzymes as indicated, electrophoresed, and hybridized with the ^{32}P-labeled cDNA fragments of 2K-1 and 2K-3, respectively.

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2K-3     YYRYPKGESY QDLVQRLEPV IMELERQENV LVICHQAVMR CLLAYFLDKS
2K-1     RYRYPKGESY EDLVQRLEPV IMELERQENV LVICHQAVMR CLLAYFLDKA
Testis   RYRYPKGESY EDLVQRLEPV IMELERQENV LVICHQAVMR CLLAYFLDKA -405

2K-3     AEELPYLKCP LHTVLKLTPV AYGCKVESIY LNVESKLTHR ERSEDAKKGP
2K-1     AEELPYLKCP LHTVLKLTPV AYGCKVESIF LNVAAVNTHR DRPQNVDISR
Testis   AEELPYLKCP LHTVLKLTPV AYGCKVESIF LNVAAVNTHR DRPQNVDISR -455

2K-3     NPLMRRNSVT PLASPEPTKK PRINSFEEHV ASTSAALPSC LPPEVPTQLP
2K-1     PPEEALVTVP AHQ
Testis   PEEEALVTVP AHQ                                        -468

2K-3     GQNMKGSRSS ADSSRKH
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Fig. 1. **Comparison of C-terminal amino acid sequences of 2K-1, 2K-3, and rat testis Fru6P2-kinase/Fru2, 6BPase.** Residues identical to those of testis isozyme are boxed.

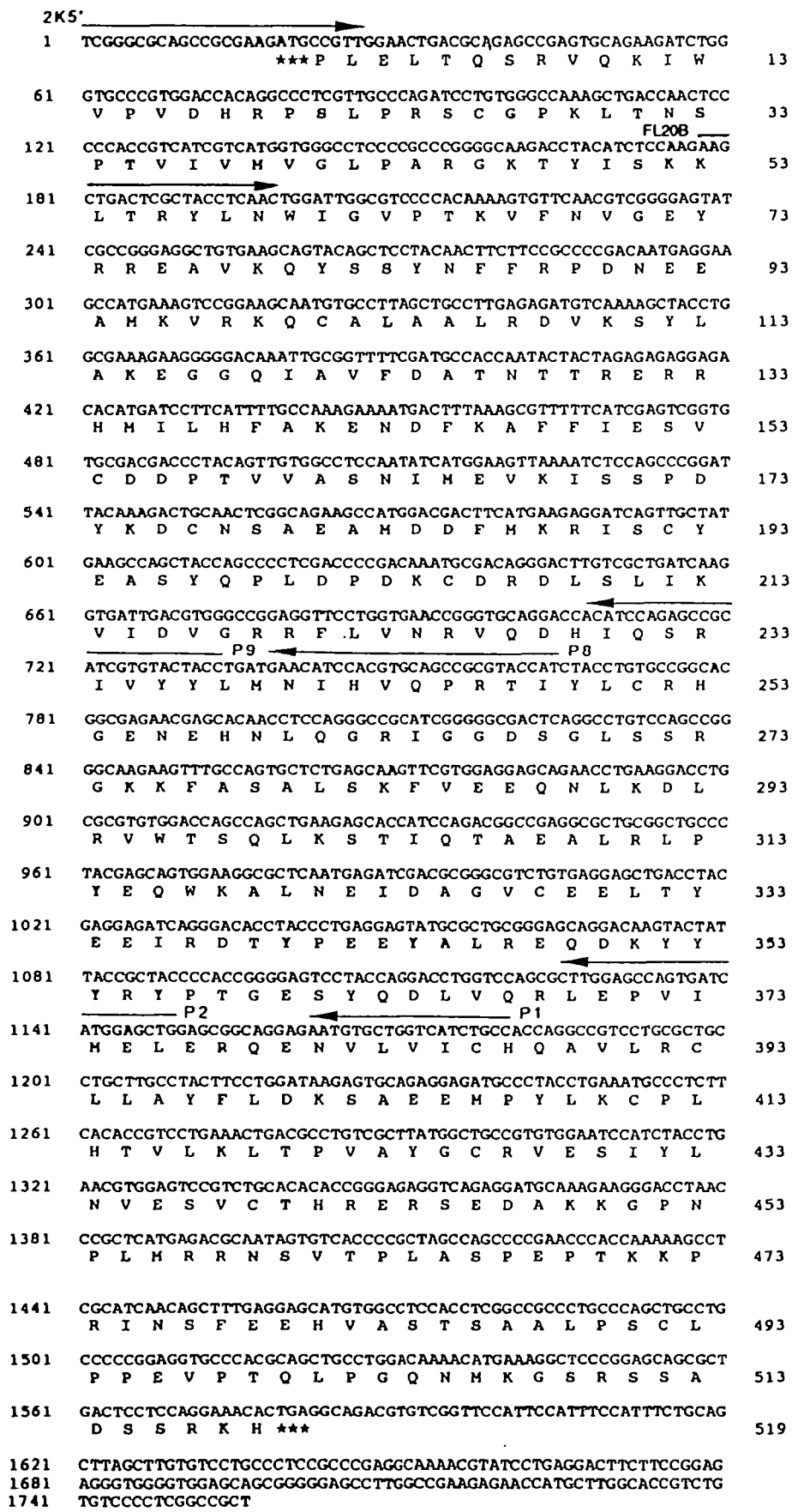

Fig. 3. **Nucleotide sequence and predicted amino acid sequence of HP.** The nucleotide sequence begins with a 5'-end of the cDNA insert; the translational initiation and termination codons are indicated with triple asterisks. Numbers on the left refer to the nucleotide sequence. Fragment AP-4 contains bases 1 to 733, and fragment 2K-3 contains bases 705 to 1756. Numbers on the right refer to the deduced amino acid sequence given in one-letter code below the nucleotides. The primers used in Fig. 3 are shown by arrows.

hybridized in 40% formamide-5×Denhardt's solution-10% dextran sulfate-0.2% SDS-0.1 mg denatured salmon sperm DNA per ml at 37°C for 36 h and washed at 48°C with 2×SSC-0.1% SDS. A ^{32}P-labeled 587-bp fragment corresponding to nucleotides 178 to 764 of HP produced by PCR using FL20B (corresponding to #178-198 of HP cDNA) and P8 as primers was used as the probe. The membrane was then exposed to Kodak XAR film for 36 h at room temperature.

RESULTS AND DISCUSSION

Four positive clones carrying human placental Fru6P2-kinase/Fru2,6BPase were isolated from a λgt10 cDNA library of human placental cells and designated as 2K-1, 2K-2, 2K-3, and 2K-4. By subcloning followed by sequencing of these inserts, 2K-2 and 2K-4 were shown to be identical to 2K-1 and 2K-3, respectively. Two kinds of clone, 2K-1 (887 bp) and 2K-3 (1,052 bp) were clearly distinguishable from each other, and both had truncated open reading frames past the 3′-noncoding sequences of the genes. As shown in Fig. 1, the deduced amino acid sequence of clone 2K-1 was identical with the rat testis isozyme except for two residues, but 2K-3 was distinct from any known isozyme, indicating that 2K-3 may be a novel clone of Fru6P2-kinase/Fru2,6BPase.

In order to determine the relational localization of genes encoding 2K-1 and 2K-3, we analyzed genomic DNA by Southern blotting using ^{32}P-labeled specific probes for 2K-1 and 2K-3, respectively. Figure 2 shows the restriction patterns of 2K-1 and 2K-3 with the enzymes *Xba*I, *Sac*I, *Pst*I, *Kpn*I, *Hin*dIII, *Eco*RI, and *Bam*HI. All these enzymes generated intense single bands, indicating that 2K-1 and 2K-3 are encoded as single copy genes. Moreover, since none of the patterns of any digest overlapped between 2K-1 and 2K-3, the genes encoding these two isozymes appeared to be located apart on the genome.

Since the 5′-end of the insert did not reach the initiation codon, the rest of the coding region of 2K-3 was extended upstream by the RACE method. Five clones (designated AP-2, AP-4, AP-5, AP-6, and AP-9) contained the putative initiation codon and the open reading frames overlapped with that of 2K-3. AP-4 was the largest clone among them and was identical with the consensus sequence of these AP-series, so AP-4 was regarded as representative of the clones. In order to confirm that 2K-3 and AP-4 were transcribed from a single mRNA molecule, nested RT-PCR was performed as described under "MATERIALS AND METHODS." An expected fragment (1,159 bp) generated with the primers P-2 and 2K5′ was purified and cloned into pGEM vector, and the insert was sequenced. The sequence data indicated that the clone 2K-3 and AP-4 had been generated from the same mRNA molecule, and overlapped by 29 nucleotides. Finally, the combined DNA sequence of 2K-3 and AP-4 was designated as HP, cDNA for a human placental Fru6P2-kinase/Fru2,6BPase.

The nucleotide sequence and deduced amino acid sequence of HP are shown in Fig. 3. The 1,563 bp sequence represents an open reading frame with the putative initiation codon and the termination codon. The predicted protein contains 519 amino acid residues with a calculated molecular weight of 59,410. The HP begins in the 5′-untranslated region, 18 nucleotides before the putative initiation codon, and through the entire open reading frame to the 3′-untranslated region of 178 nucleotides. Although further experiments are needed to establish the position of the initiation codon, the ATG codon (#19-21) of the HP cDNA is a likely candidate for the initiation codon, especially because the oligonucleotide sequence around this ATG which has a Kozak consensus sequence (*20*).

To study the *in vivo* expression of HP mRNA, Northern blot analysis was carried out with poly(A)$^+$RNA isolated from first trimester and term placentas using a ^{32}P-labeled 587-bp fragment of the HP, corresponding to nucleotides 178 to 764, produced by PCR using FL20B and P8 primers. It should be noted that homology of this fragment *vs.* corresponding fragments of known isozymes are 36, 36, 72, and 58% for muscle, liver, heart, and testis, respectively. As shown in Fig. 4, a single 5.4 kb band was detected for both first trimester and term placentas. This size of mRNA was significantly different from those of known isozyme mRNAs such as liver (2.1 kb) (*21*), skeletal muscle (1.9 kb) (*21*), heart (4.0 kb) (*8*), and testis (2.0 kb) (*9*). These results strongly suggested that this 5.4 kb mRNA codes a novel isozyme of Fru6P2-kinase/Fru2,6BPase. Study of the tissue-specific expression of the HP enzyme was not done because of lack of human tissue availability. In order to determine the tissue-specific expression of HP enzyme, we are now investigating whether HP-like enzyme is expressed in rat placenta.

A comparison of the amino acid sequence of HP with those of bovine heart (*8*), rat testis (*9*), human liver (*5*), rat liver (*3*), and rat skeletal muscle (*21*) enzymes showed overall similarities of 64, 63, 61, 62, and 61%, respectively (Fig. 5). The internal sequence of HP, in which conserved motif sequences for the substrate binding domains (*22-25*) are observed among several isozymes, was very similar to those of other enzymes (boxed in Fig. 5). The major differences among these sequences were observed in the N and C termini in which the regulatory phosphorylation sites are located. During the preparation of this manuscript, the structure of a core fragment of bovine brain Fru6P2-kinase/Fru2,6BPase subunit, whose molecular weight (120,000) (*26*) is twice that of all other isozymes, was reported (*27*). Interestingly, the amino acid sequences of HP and core fragment of bovine brain isozyme were almost

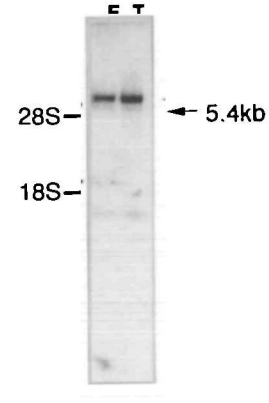

Fig. 4. Northern blot analysis of placental poly(A)$^+$RNA. Poly(A)$^+$RNAs (4 μg) from first trimester placenta (F) and term placenta (T) were fractionated by electrophoresis and hybridized with ^{32}P-labeled 587-bp fragment of the HP.

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Placenta -         PLE LTQSRVQKIW VPVDHRPSLP RSCGPKLTNS PTVIVMVGLP ARGKTYISKK LTRYLNWIGV -63
Heart    -     SGNPASS SE*NNNSYET KASLRISEKK C*WASYM*** **L***I*** *****V*** ********** 
Testis   -     ASPRE*T QNPLKKIWMP YSNGRPALHA SQR*VCM**C **L******* ********** ********** 
Liver(H) - SREMGELTQT RL*KIWIPHS SGSSRLQRRR G*SI*QF*** **MVI***** ********T* *********T
Liver(R) - SREMGELTQT RL*KIWIPHS SSSSVLQRRR G*SI*QF*** **MVI***** ********T* *********T
Muscle   -                        EEKA*KR TASI*QF*** **MVI***** ********T* *********T

Placenta - PTKVFNVGEY RREAVKQYSS YNFFRPDNEE AMKVRKQCAL AALRDVKSYL AKEGGQIAVF DATNTTRERR -133
Heart    - ******L*V* **Q***S*K* *D***H**** ***I******* V**K***A** TE*S****** ********** 
Testis   - **RE****Q* **DM**T*K* FE**L***** GLKI******* ***N**RKF* SE***HV*** ********** 
Liver(H) - ******L*Q* *****-S*SN *E**L***M* *LQI******* ***K**HN** SH*E*HV*** ********** 
Liver(R) - ******L*Q* *****-S*RN *E******T* *QLI******* ***K**HK** SR*E*HV*** ********** 
Muscle   - ******L*Q* *****-S*RN *E******T* *QLI******* ***K**HK** SR*E*HV*** **********

Placenta - HMILHFAKEN DFKAFFIFSV CDDPTVVASN IMEVKISSPD YKDCNSAEAM DDFMKRISCY ESAYQPLDPD -203
Heart    - DL**N**E** SF*V**VE** ****D*I*A* *L***V**** *PER*RENV* ***L***E** KVT******* 
Testis   - A**FN*GEQ* GY*T**VE*I *V**E*I*A* *VQ**LG*** *VNRDSD**T E**MR**E** *NS*ES**EE 
Liver(H) - SL**Q****H GY*V***E*I CNDPGII*E* *RQ**LG*** *I**DREKVL E**L***E** *VN*Q***EE 
Liver(R) - SL**Q****H GY*V***E*I CNDPEII*E* *KQ**LG*** *I**DQEKVL E**L***E** *IN*Q***EE 
Muscle   - SL**Q****H GY*V***E*I CNDPEII*E* *KQ**LG*** *I**DQEKVL E**L***E** *IN*Q***EE

Placenta - KCDRDLSLIK VIDVGRRFLV NRVQDHIQSR IVYYLMNIHV QPRTIYLCRH GENEHNLQGR IGGDSGLSSR -273
Heart    - SH*K***F** **N**Q**** *K***Y***K ********** H********* **S*F**L*K ********V* 
Testis   - -Q*****Y** IMD**QSYV* ***A****** ********** T**S******* **S*L**K** ****P***P* 
Liver(H) - -L*SH**Y** IFD**T*YM* ******I*** TV******** T**S******* **S*L*IR** ********V* 
Liver(R) - -L*SH**Y** IFD**T*YM* ******V*** TA******** T**S******* **S*L**R** ********A* 
Muscle   - -L*SH**Y** IFD**T*YM* ******V*** TA******** T**S******* **S*L**R** ********A*

Placenta - GKKFASALSK FVEEQNLKDL RVWTSQLKST IQTAEALRLP YEQWKALNEI DAGVCEELTY EEIRDTYPEE -343
Heart    - **Q**Q**R* *L***EIA** K*******R* *****S*GVT *****I**** *******M** A**QEQ**D* 
Testis   - *RE*SKH*AQ *ISD**IK** K*****M*R* *******SV* *****V**** *******M** ***Q*H**L* 
Liver(H) - **QY*Y**AN *IQS*GISS* K****RM*R* *******GV* ********** *******M** ***QEH**** 
Liver(R) - **QY*Y**AN *IRS*GISS* K****HM*R* *******GV* ********** *******M** ***QEH**** 
Muscle   - **QY*Y**AN *IRS*GISS* K****HM*R* *******GV* ********** *******M** ***QEH****

Placenta - YALREQDKYY YRYPTGESYQ DLVQRLEPVI MELERQENVL VICHQAVLRC LLAYFLDKSA EEMPYLKCPL -413
Heart    - F***DEE**L ****G***** ********** ******G*** **S****M** ********G* D*L***R*** 
Testis   - F***D*****R ****K****E ********** ********** *******M** ********A* **L******* 
Liver(H) - F***D*****R ****K****E ********** ********** *******M** *********S D*L******* 
Liver(R) - F***D*****R ****K****E ********** ********** *******M** *********S D*L******* 
Muscle   - F***D*****R ****K****E ********** ********** *******M** *********S D*L*******

Placenta - HTVLKLTPVA YGCRVESIYL NVESVCTHRE RSEDAKKGPN PLMRRNSVTP LASPEPTKKP RINSFEEHVA -483
Heart    - **IF****** ***K**T*K* ***A*N***D KPINNFPKSQ TPV*MRRNSF TPLSSSNTIR *PRNYSVGSR 
Testis   - ********** ***K****F* **AA*N***D *PQNVDISRP SEEALVTVPA HQ
Liver(H) - ********** ***K****** ***A*N***E KP*NVDITRE PEEALDTVPA HY
Liver(R) - ********** ********** ***A*N***D KP*NVDITRE AEEALDTVPA HY
Muscle   - ********** ********** ***A*N***D KP*NVDITRE AEEALDTVPA HY

Placenta - STSAALPSCL PPEVPTQLPG QNMKGSRSSA DSSRKH                                    -519
Heart    - PLQPLS*LRA LDTQEGADQP KTQAETSRA* HRLPSPAPPT SPS
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Fig. 5. **Comparison of amino acid sequences (in one-letter code) of HP (Placenta), bovine heart (Heart), rat testis (Testis), human liver [Liver (H)], rat liver [Liver (R)], and rat skeletal muscle (Muscle) Fru6P2-kinase/Fru2,6BPase.** Residues identical with those of HP are indicated by asterisks, and gaps are shown as dashes. Active sites are boxed, and phosphorylation sites are underlined.

identical. However, we can not discuss the relationship between HP enzyme and the brain enzyme, since the characteristics of mRNA coding this brain enzyme have not been reported.

Analysis of the deduced amino acid sequence of the HP indicated that a consensus phosphorylation site for a cAMP-dependent protein kinase was located in the C-terminal region (Ser-460) of the protein. Furthermore, it was revealed that six positions capable of being phosphorylated by protein kinase C were located at residues 51, 128, 271, 440, 470, and 516. The phosphorylation by protein kinase A of the liver enzyme at the N-terminal region results in inhibition of the Fru6P2-kinase and activation of the Fru2, 6BPase (*28-30*). The heart isozyme, however, is phosphorylated by both cAMP-dependent protein kinase and protein kinase C, both resulting in activation rather than inhibition of the kinase activity. The phosphorylation sites for both protein kinases of the bovine heart enzyme are

located near each other in the C-terminal region (Ser-466 and Thr-475) (*31*). Although it is possible that the HP isozyme is also regulated by the same mechanism as the heart enzyme, whether the phosphorylation occurs in placenta is uncertain. The placenta is fast-growing tissue, like many tumor cells, and highly glycolytic. The human placental Fru6P2-kinase/Fru2,6BPase has not been characterized before. Since there are phosphorylation sites at similar locations to those in the heart enzyme, a similar regulatory mechanism to that in the heart may occur in placenta.

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