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Cloning and Nucleotide Sequence of a cDNA Encoding the Rat Triosephosphate Isomerase

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A gene coding for triosephosphate isomerase (TPI) from a rat skeletal muscle cDNA library was cloned and its nucleotide sequence was determined. The 1,348-bp cDNA clone contains 24 bp 5' noncoding region, the entire 750 bp coding region corresponding to a protein of 249 amino acids, 547 bp 3' noncoding region and part of a poly(A) tail. It also contains a polyadenylation signal, AATAAA, starting from 17 bp upstream of the poly(A) tail. The calculated molecular weight of rat TPI is 27.8 kDa and the net charge is +4. The deduced amino acid sequence from rat TPI cDNA sequence has 93% and 94% homology with that of mouse and human clones, respectively. The amino acids at the residue of Asn12, Lys14, His96, Glu 166, His96, His101, Ala177, Tyr165, Glu130, Tyr209, and Ser212 in catalytic site are completely identical, confirming that the functional residues in TPI proteins are highly conserved throughout evolution. The most profound characteristic of rat TPI enzyme, compared with other TPIs, is that there are five cysteine substitutions at the residue of 21, 27, 159, 195 and 204. A Glu123 instead of Gly was found in rabbit, rhesus, mouse and human sequences. Through the method of RT-PCR, the mRNA transcription level of TPI gene was found to be different among various tissues and was highest in muscle.

Key words : TPI, Rat skeletal muscle, cDNA library, RT-PCR

INTRODUCTION

Triosephosphate isomerase (TPI, EC 5.3.1.1.) functions in glycolytic and gluconeogenic pathways by catalysing the interconversion of dihydroxyacetone phosphate and glyceraldehyde-3-phosphate (Noltmann, 1972; Gracy, 1982). TPI is a dimeric enzyme composed of two identical polypeptides and required for cell growth and maintenance (Brown *et al.*, 1985). Accordingly, TPI gene is expressed in all cell types and thus belongs to the so-called housekeeping gene category (Brown *et al.*, 1985).

TPI enzyme exists as multiple electrophoretic forms in various species and tissues, probably due to isoenzymes, allozymes and postsynthetic modifications of the enzyme (Gracy, 1982). The TPI nucleotide sequences of plants (Shih, 1994), animals (Maquat *et al.*, 1985; Straus and Gilbert, 1985), and microorganisms (Alber and Kawasaki, 1982; Kohl *et al.*, 1994; Pichersky *et al.*, 1984; Rentier-Delrue *et al.*, 1993; Shoemaker *et al.*, 1992; Swikels *et al.*, 1986) have been reported. The TPI proteins from thus far reported sources are highly conserved in the active site and subunit-subunit contact sites. A histidine residue at 96 and a glutamate residue at 166 play an important role in the catalytic function (Achari *et al.*, 1981; Alber *et al.*, 1981). Two asparagine residues at 16 and 72 function in dimerization of the enzyme (Gracy, 1982). The deamidation of the asparagine residues causes the enzyme to dissociate and to subject to proteolytic digestion (Gracy, 1982). Therefore, mutations in these highly conserved residues are detrimental to the function of the enzyme.

It was known that the hereditary TPI deficiencies in human involve the various mutations in TPI gene. Homozygous-deficient individuals usually have only 3 to 20% of normal TPI activity and suffer from chronic nonspherocytic hemolytic anemia, retardation of growth, increased susceptibility to bacterial infections, and pronounced neurological and muscular disorders (Valentine *et al.*, 1983). Recently, many studies have progressed toward the clinical usage as a target enzyme for the treatment of disease as well as model system of the housekeeping gene regulation (Shoemaker *et al.*, 1992; Brown *et al.*, 1985).

In this paper, we have isolated and sequenced a cDNA encoding the rat TPI from rat skeletal muscle cDNA plasmid library and examined the transcription level of the TPI among various tissues through RT-

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PCR technique.

MATERIALS AND METHODS

cDNA library construction and screening of rat TPI cDNA

Total cytoplasmic RNA from rat skeletal muscle was prepared by the acid guanidium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987; Oh *et al.*, 1995; Park *et al.*, 1995). Poly (A)+ mRNA was purified by poly A Tract® mRNA isolation system from Promega. The cDNA was made using Zap-cDNA synthesis kit (Stratagene, La Jolla, CA, USA). The size-fractinated cDNA was ligated into the EcoRI-and XhoI-cut pJG4-5 library vector that contains a Gal1 promoter, SV40 T antigen nuclear localization sequence, the B42 activation domain and the HA1 epitope at the NH_2-terminus of restriction site. The ligated DNA was transformed into Sure cells (Stratagene, La Jolla, CA, USA) by electroporation, using a Bio-Rad electroporator according to the manufacturer's instructions. The transformed colonies of 9.7×10^6 were obtained from the plates. The cDNA was screened by hybridization to the mouse counterpart ^{32}P end-labelled probe (5'-AGCC-CTGGCATGATCAAGGACTTAGGA-3') according to the Maniatis *et al.* (1982).

Analysis of the nucleotide sequence

Plasmid DNA encoding TPI was identified by double strand DNA sequencing (dideoxy-chain termination method: Sanger *et al.*, 1977) using a Sequenase® kit (Amersham, Buckinghamshire, England) and primers as follows : 5'-TACCCTTATGATGTGCCA-3', 5'-ATGATCA-AGGACTTAGGA-3', 5'-AAGCTT GCGAGTTTTTT-3', 5'-AAGACTGCAACCCCT-3', 5'-TTCTTGTCACTTTGG-3', 5'-GGACAGCTAGTTCTA-3' and 5'-ATCCAGCTTC-TGCCT-3'. The template DNA was prepared using miniplasmid kit (Bio101, USA). Analysis of sequencing data was performed by using a BLAST program of NCBI data base.

Reverse transcriptase-polymerase chain reaction

Total RNA and mRNA from rat was extracted as described above. All oligonucleotides were supplied by the Central Laboratory in College of Pharmacy, Ewha Womans University. The primers used were as follows : 5'-TGTGCAATGGCGCCTTCCAGG -3'(TPI sense), 5'-AGGCCCAGGTGTTTATTTCCC-3'(TPI antisense), 5'-TA-CAATGAGCTGCGTGTGGC-3'(β-actin sense), 5'-AT-GTCACGCAC GATTTCCC-3'(β-actin antisense). Reverse transcriptase (RT) reaction was performed according to the manufacturer's instruction of reverse transcription system (Promega, Madison, WI, USA). The RT reaction mixture was consisted of 4 μl of 25 mM $MgCl_2$, 2 μl of 10X RT buffer, 2 μl of 10 mM dNTP mixture, 0.5 μl (20 U) of rRNAsinR ribonuclease inhibitor, 1 μl (0.5 mg) of oligo (dT) 15 primer, 1 μg of RNA substrate and 15U of AMV reverse transcriptase adjusting the net volume to 20 μl (Goodmann and MacDonald, 1979). The reaction mixture was incubated for 15 min. at 42°C, 5 min. at 95°C, and then transferred into 4°C. Five μl of the solution was transferred to 50 μl of polymerase chain reaction (PCR) mixture containing 1X PCR buffer, 5 μl of 2.5 mM dNTPs (Perkin Elmer, Foster, CA, USA), 10 pmole of PCR primers, 5 μl of RT product adjusting the net volume to 50 μl. Five unit of thermostable DNA polymerase (Advanced Biotechnologies; AB, Surrey, KT22, 7BA, UK) was added to the mixture. The mixture was cycled 25 times at 95°C for 1min., 50°C for 1 min., and 72°C for 1.5 min., followed by a final period of 7 min. at 72°C incubation in the GeneAmp® PCR system 2400 (Perkin Elmer, Foster, CA, USA). After the reaction, 10 μl of the RT-PCR products were analysized by 1% agarose gel (Ultra pure electrophoesis grade, Bethesda Research Laboratories;BRL, Gaithersburg, MD, USA) and visualized by ethidium bromide staining. λ/Hind III were used as a marker and purchased from New England Biolabs (NEB, Beverly, MA, USA).

RESULTS AND DISCUSSION

Sequence analysis of rat TPI gene cDNA

The complete nucleotide sequence of a cDNA clone corresponding to the TPI from rat skeletal muscle was determined by the dideoxy-chain termination sequencing method and is shown in Fig. 1. The 1, 348-bp cDNA clone contains 24 bp 5' noncoding region, the entire 750 bp coding region corresponding to a protein of 249 amino acids, 547 bp 3' noncoding region and part of poly(A) tail. It also contains a putative transcript cleavage polyadenylation signal, AATAAA, starting from 17 bp upstream of the poly(A) tail. The length of 3' noncoding region among various TPI mRNAs is divergent. The calculated molecular weight and the net protein charge of rat TPI are 27.8 kDa and +4, respectively. The predicted molecular weight of rat TPI is similar with that of other TPIs (Fothergill-Gilmore and Michels, 1993). The deduced amino acid sequence from the rat TPI cDNA sequence has 93% homology with mouse (Cheng *et al.*, 1990) and 94% with human (Maquat *et al.*, 1985), suggesting that TPI proteins are highly conserved throughout evolution. In Fig. 2, we have shown a comparison of the deduced amino acid sequences of rat TPI with those of other various organisms, which all of the sequences are withdrawn from Genbank database of NCBI using the BLAST network service. Rat TPI shows 93-95% homology with that of rabbit,

Fig. 1. Nucleotide sequence and deduced amino acid sequence of the triosephosphate isomerase from rat skeletal muscle. The nucleotides are numbered (top), and the deduced amino acids are numbered (bottom) beginning with the initiation methionine. The stop codon is indicated with an asterisk (*). The nucleotide sequence data published here have been deposited with GenBank data bank and are available under the accession number L36250.

Rhesus macaque, Mus musculus, and Homo sapience, whereas 46-66% identity with that of nonmammalian organism, such as *Drosophila melanogaster, Zea mays, Oryza sativa, Coptis japonica, Saccharomyces cerevisiae, and Escherichia coli.* Although animal TPIs have some differences compared with nonanimal TPIs, there are six highly conserved regions consisting of residue 11-14, 73-78, 95-100, 162-174, 209-212 and 226-238 (Fig. 2). Moreover, the catalytically active-site residues consisting of Asn12, Lys14, His96, and Glu166 (Banner *et al.*, 1975), short α-helix residues consisting of His96 and His101, and active-site loop closing site related residues consisting of Ala177, Tyr165, Glu130, Tyr209 and Ser212 are completely conserved, suggesting that those residues are functionally important among all TPI proteins. The calculated net charge of monomeric enzyme from the amino acid composition of rat TPI is +4 whereas that of *Leishmania mexicana mexicana* TPI is +2 (Kohl *et al.*, 1994). But the most prominent difference of rat TPI is that there are 5 cysteine substitutions at the residue of 21, 27, 159, 195 and 204. Therefore, rat TPI is a cysteine rich enzyme compared with that of other closely related animals, plants, and microorganisms. These additional Cys residues may form disulfide bond and change the enzymatic activity as well as the structure. Cys residues at 159, 195, and 204 are substitutes for Ser residue which is found in all other mammalian sequences compared (Fig. 2). Since only one nucleotide sequence differs in the genetic codes for Ser (TCC) and Cys (TGC), there is a possibility that the difference may be due to some artifact of cDNA cloning.

Fig. 2. Comparison of the predicted amino acid sequence of the rat triosephosphate isomerase with different TPI sequences obtained from the GenBank database. Alignment was done by the BLAST network service of NCBI database. Asterisk(*) indicates an identical residue, and underline indicates a similar one. Gaps(-) have been introduced into each sequence to improve alignment. The wave line region, highly conserved region; vertical arrow, the residues in catalytic site; closed triangle, 5 substituents cysteine residue found in rat TPI; open circle, subunit interface of asparagine residues which cause multiple electrophoretic form; open triangle, closer relating residue in active site loop; closed circle, 2 His residues in short α-helix

RT-PCR analysis of rat TPI mRNA from various tissues

To investigate the transcription level of TPI gene among various tissues, we have isolated poly(A) mRNA from brain, heart, kidney, liver, lung, and skeletal muscle of Splague-Dawley strain rat according to the method described in materials and method. β-actin was also amplified by RT-PCR using the primers corresponding the residues of 193-212 and 539-557 of mouse sequences to quantitate mRNA levels and exclude the possibility of mRNA degradation. To compare the quantity of TPI mRNA from various tissues using RT-PCR, the product of

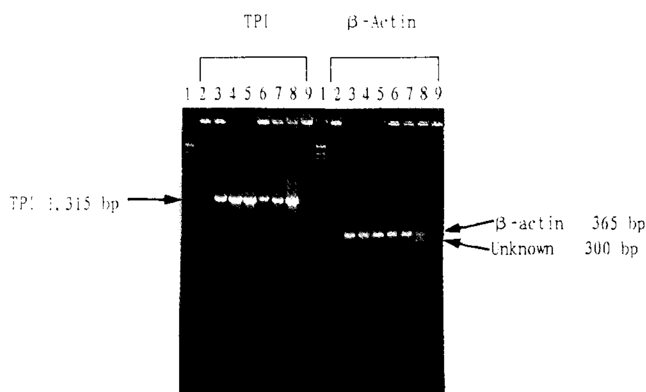

Fig. 3. Differential expression of TPI mRNA in the rat tissues. RT-PCR amplification was performed on 1 μg of poly (A)+ RNA extracted from brain, heart, kidney, liver, lung, and skeletal muscle. Each 10 μl of 50 μl RT-PCR mixture are loaded on 1% agarose gel, and electrophoresed. : Lane 1, λ-Hind III marker; Lane 2 and 9, control without extension primers; Lane 3, brain; Lane 4, heart; Lane 5, kidney; Lane 6, liver; Lane 7, lung; Lane 8, muscle. The expected RT-PCR fragment of TPI and β-actin are 1,315 and 365 bp, respectively.

this experiment should not be saturated after the final PCR cycles. Therefore, we employed only 25 cycles to amplify TPI cDNA using the primers corresponding to the nucleotide base 19-38 and 1312-1332. The expected cDNA fragments of TPI and β-actin were 1315 bp and 365 bp, respectively. As expected in housekeeping gene, TPI mRNAs exist in all tissues as shown in Fig. 3. However, TPI transcription level seems to be different among various tissues although the quantity of TPI mRNAs by RT-PCR can not be calculated exactly in this experiment. It seems that TPI mRNA was most abundant in muscle and reduced in order of kidney≒heart, brain≒lung and liver. The constant level of β-actin transcript suggests that the same amount of mRNA was used in RT-PCR reactions. It appears that more active transcription of TPI has been occurred in highly ATP-consuming organs. It may require Northern blot analysis to see more accurate quantitative comparisons among the organs having similar level of TPI transcripts. In our experimental condition, the quantity and the size of control β-actin cDNA from various tissues have been almost identical except those from muscle. Another 300 bp of β-actin fragment in addition to the 365 bp fragment which has been detected in other tissues has been found only in muscle. However, the sum of the intensity of two fragments seems to be same as compared to that of β-actin fragments from other tissues. The additional RT-PCR fragment of 300 bp might be the alternatively spliced product of β-actin gene or unknown isoform of β-actin. However, it remains to be clarified in the future.

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