

Characterization of human phosphoserine aminotransferase involved in the phosphorylated pathway of L-serine biosynthesis

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In the present study, we first report two forms of human phosphoserine aminotransferase (PSAT) cDNA (HsPSAT α and HsPSAT β). HsPSAT α has a predicted open reading frame comprising 324 amino acids, encoding a 35.2 kDa protein (PSAT α), whereas HsPSAT β consists of an open reading frame comprising 370 amino acids that encodes a 40 kDa protein (PSAT β). PSAT α is identical with PSAT β , except that it lacks 46 amino acids between Val²⁹⁰ and Ser³³⁷ of PSAT β , which is encoded by the entire exon 8 (138 bp). Both PSAT α and PSAT β can functionally rescue the deletion mutation of the *Saccharomyces cerevisiae* counterpart. Reverse transcriptase–PCR analysis revealed that the expression of PSAT β mRNA was more dominant when compared with PSAT α mRNA in all human cell lines tested. PSAT β was easily detected in proportion to the level of mRNA; however, PSAT α was detected only in K562 and HepG2 cells as a very faint band. The relative enzyme activity of glutathione S-transferase (GST)–PSAT β expressed in *Escherichia coli* appeared

to be 6.8 times higher than that of GST–PSAT α . PSAT mRNA was expressed at high levels (approx. 2.2 kb) in the brain, liver, kidney and pancreas, and very weakly expressed in the thymus, prostate, testis and colon. In U937 cells, the levels of PSAT mRNA and protein appeared to be up-regulated to support proliferation. Accumulation of PSAT mRNA reached a maximum in the S-phase of Jurkat T-cells. These results demonstrate that although two isoforms of human PSAT can be produced by alternative splicing, PSAT β rather than PSAT α is the physiologically functional enzyme required for the phosphorylated pathway, and indicate that the human PSAT gene is regulated depending on tissue specificity as well as cellular proliferation status with a maximum level expression in the S-phase.

Key words: alternative splicing, cell-cycle progression, human phosphoserine aminotransferase gene, L-serine synthesis, phosphoserine aminotransferase α and β , tissue specificity.

INTRODUCTION

L-serine serves as a building block for protein synthesis and can be modified in different metabolic pathways for the generation of several essential compounds, including glycine, D-serine, cysteine, serine phospholipids, sphingomyelins and cerebroside. It is also required for the synthesis of nucleotide precursors, such as purines and thymidine, which is directly linked to cellular replication [1]. Although L-serine is available from dietary sources, it can be synthesized from glycolytic intermediates by a phosphorylated pathway in mammals. The phosphorylated pathway starts at 3-phosphoglycerate and proceeds via three consecutive enzymic steps to L-serine. The 3-phosphoglycerate dehydrogenase (PHGDH; EC 1.1.1.95) catalyses the first step in the phosphorylated pathway by oxidizing 3-phosphoglycerate to 3-phosphohydroxypyruvate, using NAD⁺/NADH as a cofactor. Phosphoserine aminotransferase (PSAT; EC 2.6.1.52) catalyses the conversion of 3-phosphohydroxypyruvate into 3-phosphoserine that is dephosphorylated subsequently by phosphoserine phosphatase (EC 3.1.3.3) to form L-serine.

Several reports have indicated that an enhanced capacity of L-serine synthesis resulting from a significant increase in PHGDH activity may confer a growth advantage to tumour cells by its coupling with nucleotide biosynthesis, suggesting an involvement of L-serine metabolism in neoplasia [2,3]. It has also

been shown that rat neoplastic tissues and rapidly proliferating plant tissues contain a higher PSAT activity than that found under normal conditions [4,5]. In healthy human beings, it is assumed that *de novo* synthesis of L-serine by the phosphorylate pathway is essential to supply the L-serine required in the brain, because the delivery of L-serine to the central nervous system (CNS) is insufficient due to the blood–brain barrier [6,7]. In relation to the role of L-serine in the CNS, L-serine and its downstream metabolite glycine serve as the neurotrophic factor to promote survival, dendritogenesis and electrophysiological development of neurons [8]. In addition, D-serine that can be formed directly from L-serine by serine racemase is known to act as a co-agonist of the *N*-methyl-D-aspartate receptor along with glutamate and glycine residues. Thus it plays a role in synapse refinement, neuronal plasticity and excitotoxicity [9,10]. The importance of L-serine synthesis by the phosphorylated pathway in the brain has also been illustrated by human inborn disorder with enzymic defects in PHGDH, which results in a severe neurological syndrome [11]. The cDNAs for both human PHGDH [12,13] and phosphoserine phosphatase [14] involved in the phosphorylated pathway of L-serine biosynthesis have been cloned and their nucleotide sequences have been determined. Northern-blot analysis, employing a PHGDH cDNA probe, has revealed that up-regulation in the level of PHGDH mRNA is responsible for the increased activity of PHGDH in tumour cells

Abbreviations used: CNS, central nervous system; FBS, foetal bovine serum; GST, glutathione S-transferase; HBSS, Hanks balanced salt solution; ORF, open reading frame; PHGDH, 3-phosphoglycerate dehydrogenase; PSAT, phosphoserine aminotransferase; RT, reverse transcriptase; TdR, thymidine deoxyribose.

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The nucleotide sequences for the human phosphoserine aminotransferase α and β cDNAs have been deposited in the GenBank[®] Nucleotide Sequence Database under the accession numbers AF113132 and AY131232 respectively.

of human and mouse origin [12]. Molecular characterization of the genetic deficiency of PHGDH activity causing severe neurological impairment has suggested that a single substitution of a valine residue at position 425 or 490 by a methionine residue may be responsible for the significant decrease in PHGDH activity [13]. However, the molecular basis for the change of PSAT activity associated with human neoplasia and serine-metabolism-related neurological disorder remains largely unknown, possibly due to the unavailability of the nucleotide sequence and enzymic characterization of human PSAT.

The aim of the present study was to investigate the nucleotide sequence and enzymic activity of two isoforms (PSAT α and PSAT β) of human PSAT, which can be generated by alternative splicing, and to demonstrate differential expression of PSAT depending on tissue specificity and cellular proliferative status.

EXPERIMENTAL

Kits, enzymes, reagents, human T-cell cDNA library, antibody and cells

The DNA sequencing kit (OmniBase™) was purchased from Promega (Madison, WI, U.S.A.). All restriction enzymes, DNA-modifying enzymes, including T4 DNA ligase and RNase, were purchased from Promega or Bethesda Research Laboratories (Gaithersburg, MD, U.S.A.). Radioactive materials, including [α -³²P]dCTP (approx. 3000 Ci/mmol), [γ -³⁵S]dATP (approx. 1000 Ci/mmol) and a random primer labelling kit were purchased from Amersham Biosciences (Arlington Heights, IL, U.S.A.). [³H]Thymidine deoxyribose (TdR; 2 Ci/mmol) and nylon membrane (GeneScreen Plus™) for Northern-blot analysis were obtained from NEN Biotechnology System (Boston, MA, U.S.A.). Bacterial media were from Difco (Detroit, MI, U.S.A.). A human T-cell cDNA library derived from Jurkat cell line (clone E6-1) and the plasmid pBluescript KS^{+/−} vector used for subcloning as well as DNA sequence analysis were purchased from Stratagene (La Jolla, CA, U.S.A.). Human multiple tissue Northern blot and ExpressHyb solution were purchased from Clontech Laboratories (Palo Alto, CA, U.S.A.). Rabbit antiserum raised against recombinant human PSAT α protein, which was expressed using the *Escherichia coli* system, was prepared essentially as described previously [16]. This polyclonal anti-PSAT α was capable of reacting with both PSAT α and PSAT β . Rabbit polyclonal anti-human cyclin A was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Human leukaemia (Jurkat, MOLT-3, K562, HL-60, U937 and THP-1), lymphoma (Sup-T1) and COLO 320DM cells were maintained in RPMI 1640 medium (Bethesda Research Laboratories), containing 10% (v/v) foetal bovine serum (FBS; Upstate Biotechnology, Lake Placid, NY, U.S.A.), 20 mM Hepes (pH 7.0), 5×10^{-5} M 2-mercaptoethanol and 100 μ g/ml gentamicin. The culture medium used for mouse NIH 3T3 and BW5147.G.1.4 was Dulbecco's modified Eagle's medium (Bethesda Research Laboratories), supplemented with 10% FBS, 20 mM Hepes (pH 7.0), 1 mM sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol and 100 μ g/ml gentamicin. Yeast strain BY4741 (*his3, leu2, met15, ura3, Δ ser1::Neo^R*) was obtained from A.T.C.C. (no. 4002440; Manassas, VA, U.S.A.).

Overexpression and purification of glutathione S-transferase (GST)–PSAT α and GST–PSAT β

*Bam*HI and *Eco*RI sites were created on each end of the coding region of PSAT α or PSAT β by PCR using the syn-

thetic primers, PSAT-2T forward primer 5'-ATTGGATCC-ATGGACGCCCCCAGGC-3' and PSAT-2T reverse primer 5'-CCCCTTAAGTCATAGCTGATGCATC-3' (*Bam*HI and *Eco*RI sites are underlined). PCR conditions were as follows: 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1.5 min and extension at 72 °C for 2 min. The amplified cDNA fragment of PSAT α or PSAT β was cloned in the *Bam*HI–*Eco*RI site of pGEX-2T (Amersham Biosciences), resulting in pGEX-2T-PSAT α and pGEX-2T-PSAT β , in which the cDNA was placed under a strong tac promoter in sense orientation. The plasmids were then introduced into *E. coli* DH5 α and transformants were selected on Luria–Bertani broth containing ampicillin plates. The synthesis of GST fusion proteins was induced by isopropyl β -D-thiogalactoside. The GST fusion proteins accumulated in the soluble fraction of *E. coli* DH5 α extracts were purified using glutathione–Sepharose 4B (Amersham Biosciences) according to the manufacturer's instructions.

Identification of PSAT enzyme activity by complementation test

The regions coding for PSAT α and PSAT β were excised from HsPSAT α and HsPSAT β by digestion with *Eco*RI and cloned into the *Eco*RI site of yeast expression vector pYES2 (Invitrogen, Carlsbad, CA, U.S.A.), resulting in the plasmids pYES2-PSAT α and pYES2-PSAT β . The yeast *Saccharomyces cerevisiae* BY4741 (*his3, leu2, met15, ura3, Δ ser1::Neo^R*) was transformed with pYES2-PSAT α , pYES2-PSAT β or the empty vector pYES2. Since the expression vector pYES2 contains the URA3 gene, individual transforms were selected on SGal minimal medium plate [2% (w/v) galactose, 0.67% yeast nitrogen base without amino acid, 0.002% histidine, 0.004% leucine, 0.002% methionine and 1.5% (w/v) agar]. Drop tests were performed at either complete medium (YPGal plate) or minimal medium (SGal plate). Complementation was monitored by analysing the growth of transformants on SGal plate. For confirmation of the transformant, PCR was performed with PSAT open reading frame (ORF)-specific primers (forward primer 5'-ACTACAAAGGAGTTGGCA-3' and reverse primer 5'-CAAGAACAGAGTATATCCTG-3') using standard conditions (94 °C for 1 min, 55 °C for 1.5 min and 72 °C for 2 min for 30 cycles).

PSAT activity assay

The assay for PSAT α and PSAT β was performed as described previously [17]. Assay mixtures contained 50 mM Tris/HCl (pH 8.2), 32 mM ammonium acetate, 2 mM glutamate, 2 mM NADH, 2.5 mM phosphohydroxypyruvate, 3 units of glutamate dehydrogenase and 20 μ g of total protein. The reaction was initiated at 25 °C by the addition of phosphohydroxypyruvate and the enzyme activity was measured by monitoring the NADH consumption spectrophotometrically at 340 nm.

Reverse transcriptase (RT)–PCR

Total RNA was purified using TRIzol (Invitrogen) according to the manufacturer's instructions. The first-strand cDNAs were synthesized from 2 μ g of total RNA using SuperScriptII RT (Invitrogen). GAPDH was amplified with forward (5'-CCACTGGCGTCTTACCAC-3') and reverse (5'-CCTGCTTACCACCTTCTTG-3') primers. Both PSAT α and PSAT β were amplified with forward (5'-GCTTGGTTCTGGAGTGGATT-3') and reverse (5'-GTCCCACCAGCTT-TACAG-3') primers. PCR was performed at the following

conditions: 30 cycles at 94 °C for 1 min, 52 °C for 1.5 min and 72 °C for 2 min.

Induction of differentiation and retrodifferentiation of U937 cells

Differentiation of U937 cells was induced by adding PMA at a final concentration of 32 nM to the culture medium, and incubating the cells for 48 h as described previously [18]. To assess growth arrest of U937 cells during the induced differentiation, the incorporation of [³H]TdR into DNA by the cells treated with PMA was determined. U937 cells (10⁵) were added to each well of a 96-well plate with 32 nM PMA and pulsed for 4 h with 1 μCi of [³H]TdR at the times indicated. To induce retrodifferentiation, cells treated with 32 nM PMA for 48 h were layered over FBS, centrifuged, washed three times with 1 × Hanks balanced salt solution (HBSS) containing 2 % FBS, and then incubated in RPMI 1640 medium devoid of PMA for 13 days. Recovery of proliferating activity during retrodifferentiation was determined by incubation of the cells at a density of 10⁵/well of 96-well plates. At various times after incubation, 1 μCi of [³H]TdR was added per well for 4 h before the cells were harvested and assayed for incorporation of [³H]TdR by liquid scintillation.

Arrest of cell-cycle progression of Jurkat T-cells by hydroxyurea or nocadazole

Continuously proliferating Jurkat T-cells (4 × 10⁵ cells/ml) were cultured in RPMI 1640 medium with 1 mM hydroxyurea for 20 h to block the cell-cycle progression at the late G₁ phase [19], whereas Jurkat T-cells were cultured in the presence of 0.1 μg/ml nocadazole for 20 h to arrest at the G₂/M phase boundary [20]. To release the cells from the arrest points and to continue the cell-cycle progression, the cells treated with hydroxyurea or nocadazole were layered over FBS, centrifuged, washed three times with 1 × HBSS containing 2 % FBS, and then incubated in RPMI 1640 medium for 7 h.

Northern-blot analysis

Total RNA was extracted and isolated by solubilization in guanidinium thiocyanate as described elsewhere [21]. Equivalent amounts of RNA (10 or 15 μg) were subjected to electrophoresis on formaldehyde-agarose gels and transferred on to GeneScreen Plus™ membranes. The nylon membrane as well as human multiple tissue Northern blot was hybridized in ExpressHyb solution at 68 °C for 2 h with a cDNA probe radiolabelled with [α-³²P]dCTP, using the random-primer labelling method [22], and washed according to the manufacturer's instructions.

Cell lysate, protein quantification and Western-blot analysis

The cells were suspended in lysis buffer [20 mM Tris, 137 mM NaCl, 1 mM Na₃VO₄, 1 mM PMSF, 10 μg/ml aprotinin, 2.5 μg/ml *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) and 1 % (v/v) Nonidet P40, pH 8.0], disrupted by sonication and extracted at 4 °C for 30 min. After centrifugation at 19 000 g for 20 min, the supernatant was obtained as cell lysate. Protein quantification was performed using Micro bicinchoninic acid kit (Pierce, Rockford, IL, U.S.A.). Cell lysates (20 μg) were subjected to electrophoresis on a 4–12 % (w/v) NuPAGE gradient gel (Invitrogen Corporation/Novex, Carlsbad, CA, U.S.A.) and electrotransferred to Immobilon-P membrane. The membrane was allowed to react with individual primary antibodies and then

with horseradish peroxidase-conjugated donkey anti-rabbit IgG antibody. Detection of each protein was visualized using the ECL® Western blotting detection system according to the manufacturer's instructions.

Flow-cytometry analysis

Approximately 1 × 10⁶ cells were suspended in 100 μl of PBS without Ca²⁺ or Mg²⁺ and 200 μl of 95 % (v/v) ethanol was added with vortex-mixing. The cells were incubated at 4 °C for at least 1 h, washed with PBS and resuspended with 12.5 μg of RNase in 250 μl of 1.12 % (w/v) sodium citrate buffer (pH 8.45). Incubation was continued at 37 °C for 30 min before staining the cellular DNA with 250 μl of propidium iodide, at 50 μg/ml, for 30 min at 25 °C. The stained cells were analysed on a FACScan Flow cytometer for relative DNA content based on increase in red colour using the doublet discrimination method [21].

RESULTS

Identification of two forms of human PSAT cDNA

We have initiated a human T-cell cDNA project that is aimed at investigating the expression profile of activated human T-cells and to identify novel, previously uncharacterized genes. One out of 300 cDNA clones, which were randomly sequenced from a λ Zap cDNA library of human Jurkat T-cells, was found to be 1065 bp in size and shared a significant sequence similarity to the rabbit PSAT gene [15]. The amino acid sequence of human PSAT appeared to be shorter than those from other sources, including rabbit, due to the presence of a missing area in the C-terminal regions. Although the overall N-terminal sequence of the human PSAT showed 82.4 % similarity with the rabbit counterpart, the similarity appeared to be 94.2 % when the missing area was not included for the alignment, suggesting that this human PSAT may not be a catalytically active enzyme. To test this hypothesis, we sequentially isolated a long form of human PSAT by RT-PCR using mRNA from Jurkat T cells. We were able to isolate 1260 bp cDNA, corresponding to a long form of human PSAT that possesses the C-terminal missing area compared with our short-form PSAT. During the course of the present study, an independent sequence (2.229 kb) representing a long form of human PSAT appeared in the GenBank® (accession no. NM_058179). The short and long human PSAT cDNAs were designated as HsPSAT α and HsPSAT β respectively. As determined by DNA sequence analysis, the first ATG codon, located at position 34 in HsPSAT α and at position 46 in HsPSAT β , lies within a good consensus initiation sequence; a purine (A) is present at the -3 position, and a (G) is present at position +4 [23]. The ORF of HsPSAT α continues to the first stop codon (TGA) at position 1007 bp to comprise 975 nt and encodes a protein of 324 amino acids (PSAT α) with a predicted molecular mass of 35.2 kDa, whereas that of HsPSAT β consists of an ORF comprising 1113 nt, encoding a protein of 370 amino acids (PSAT β) with a predicted molecular mass of 40 kDa. The nucleotide and deduced amino acid sequences of PSAT α and PSAT β are presented in Figure 1, and these sequence data have been submitted to the GenBank® database under the accession number AF113132 for HsPSAT α and AY131232 for HsPSAT β .

To determine clearly the basis for the α and β forms of PSAT, the nucleotide sequence of human genomic DNA was analysed. As shown in Figures 2(A) and 2(B), the ORF of PSAT α is identical with PSAT β , except that it lacks 46 amino acids between Val²⁹⁰ and Ser³³⁷ of PSAT β , which is encoded by the entire exon 8

PSAT α	CCTTGGTGACTCACCGCCCTCGCCGCCACCATTGGACGCCCCAGGCAGGTGGTCAACTTTGGCCCTGGTCCCGCC	78
PSAT β	<u>CGCTTGGTCTCCTTGGCTGACTCACCGCCCTCGCCGCCACCATTGGACGCCCCAGGCAGGTGGTCAACTTTGGCCCTGGTCCCGCC</u>	90
1	M D A P R Q V V N F G P G P A	
PSAT α	AAGCTGCCGCACTCAGTGTGTAGAGATACAAAAGGAATTATTAGACTACAAAGGAGTTGGCATTAGTGTCTTGAATGAGTCACAGG	168
PSAT β	AAGCTGCCGCACTCAGTGTGTAGAGATACAAAAGGAATTATTAGACTACAAAGGAGTTGGCATTAGTGTCTTGAATGAGTCACAGG	180
16	K L P H S V L L E I Q K E L L D Y K G V G I S V L E M S H R	
PSAT α	TCATCAGATTTTGCAGATTATTAACAATACAGAGAATCTTGTGCGGGAATTGCTAGCTGTCCAGACAACATAAGGTGATTTTCTG	258
PSAT β	TCATCAGATTTTGCAGATTATTAACAATACAGAGAATCTTGTGCGGGAATTGCTAGCTGTCCAGACAACATAAGGTGATTTTCTG	270
46	S S D F A K I I N N T E N L V R E L L A V P D N Y K V I F L	
PSAT α	CAAGGAGGTGGGTGCGGCCAGTTCAGTGTGTCCCTTAAACCTCATTGGCTTAAAGCAGGAAGGTGCGGACTATGTGGTACAGGA	348
PSAT β	CAAGGAGGTGGGTGCGGCCAGTTCAGTGTGTCCCTTAAACCTCATTGGCTTAAAGCAGGAAGGTGCGGACTATGTGGTACAGGA	360
76	Q G G G C G Q F S A V P L N L I G L K A G R C A D Y V V T G	
PSAT α	GCTTGGTCAGCTAAGCCGAGAAAGCCAAAGTTGGGACTATAAATATCGTTCACCCATAAAGTGGGAGTTATACAAAATTCCA	438
PSAT β	GCTTGGTCAGCTAAGCCGAGAAAGCCAAAGTTGGGACTATAAATATCGTTCACCCATAAAGTGGGAGTTATACAAAATTCCA	450
106	A W S A K A A E E A K K F G T I N I V H P K L G S Y T K I P	
PSAT α	GATCCAAGCACCTGGAACCTCAACCCAGATGCCTCCTACGTGATTATTGCGCAATGAGACGGTGCATGGTGTGGAGTTGACTTTATA	528
PSAT β	GATCCAAGCACCTGGAACCTCAACCCAGATGCCTCCTACGTGATTATTGCGCAATGAGACGGTGCATGGTGTGGAGTTGACTTTATA	540
136	D P S T W N L N P D A S Y V Y Y C A N E T V H G V E F D F I	
PSAT α	CCCGATGTCAAGGGAGCAGTACTGGTTTGTGACATGTCTCAAACCTCCTGTCCAAGCCAGTGGATGTTTCCAAGTTTGGTGTGATTTTT	618
PSAT β	CCCGATGTCAAGGGAGCAGTACTGGTTTGTGACATGTCTCAAACCTCCTGTCCAAGCCAGTGGATGTTTCCAAGTTTGGTGTGATTTTT	630
166	P D V K G A V L V C D M S S N F L S K P V D V S K F G V I F	
PSAT α	GCTGGTCCGAGAAAGTGTGGCTCTGCTGGGGTACCGTGGTATTGTCCGTGATGACCTGCTGGGGTTGCCCTCCGAGAGTGCCCC	708
PSAT β	GCTGGTCCGAGAAAGTGTGGCTCTGCTGGGGTACCGTGGTATTGTCCGTGATGACCTGCTGGGGTTGCCCTCCGAGAGTGCCCC	720
196	A G A Q K N V G S A G V T V V I V R D D L L G F A L R E C P	
PSAT α	TCGGTCTGGAATACAAGTGCAGGCTGGAACAGCTCCTTGTACAACACGCCTCCATGTTTCAGCATCTACGTATGGGCTTGGTCTG	798
PSAT β	TCGGTCTGGAATACAAGTGCAGGCTGGAACAGCTCCTTGTACAACACGCCTCCATGTTTCAGCATCTACGTATGGGCTTGGTCTG	810
226	S V L E Y K V Q A G N S S L Y N T P P C F S I Y V M G L V L	
PSAT α	GAGTGGATTAACAATGGAGGTGCCGCGCCATGGAGAAGCTTAGCTCCATCAAATCTCAAACAATTTATGAGATTATTGATAATTCT	888
PSAT β	GAGTGGATTAACAATGGAGGTGCCGCGCCATGGAGAAGCTTAGCTCCATCAAATCTCAAACAATTTATGAGATTATTGATAATTCT	900
256	E W I K N N G G A A A M E K L S S I K S Q T I Y E I I D N S	
PSAT α	CAAGGATTCTACGT-----	902
PSAT β	CAAGGATTCTACGTTTGTCCAGTGGAGCCCAAAATAGAAGCAAGATGAATATCCATTCCGATTGGCAATGCCAAAGGAGATGATGCT	990
286	Q G F Y V C P V E P Q N R S K M N I P F R I G N A K G D D A	
PSAT α	-----GTCTGTGGGAGGCATCCGGCCCTCTCTG	930
PSAT β	TTAGAAAAAGATTCTTGATAAAGCTCTTGAACCTCAATATGTTGTCTTGAAGGGCATAAGTCTGTGGGAGGCATCCGGCCCTCTCTG	1080
316	L E K R F L D K A L E L N M L S L K G H R S V G G I R A S L	
PSAT α	TATAATGCTGTCACAATTGAAGACGTTTCAAGAGCTGGCCGCTTCATGAAAAATTTTTGGAGATGCATCAGCTATGAACACATCCTAAC	1020
PSAT β	TATAATGCTGTCACAATTGAAGACGTTTCAAGAGCTGGCCGCTTCATGAAAAATTTTTGGAGATGCATCAGCTATGAACACATCCTAAC	1170
346	Y N A V T I E D V Q K L A A F M K K F L E M H Q L *	
PSAT α	CAGGATATACTCTGTTCTTGAACAACATACAAAGTTTAAAGTAAAC	1065
PSAT β	CAGGATATACTCTGTTCTTGAACAACATACAAAGTTTAAAGTAACTTGGGGATGGCTACAAAAAGTTAACACAGTATTTTCTCAAATGA	1260

Figure 1 Nucleotide and deduced amino acid sequences of HsPSAT α and HsPSAT β

The single-letter code-translated amino acid sequence is indicated below the nucleotide sequence from position 46 to 1158 bp (370 amino acid residues) in human PSAT β gene and from position 34 to 1008 bp (324 amino acid residues) in the PSAT α gene. The start codon is underlined and the termination codon is marked with an asterisk. The nucleotide numbering begins at the first C of PSAT α or at the first G of PSAT β , designated as +1, and is indicated on the right. Amino acid numbering of PSAT β is on the left.

(138 bp) among nine exons of the PSAT gene distributed over approx. 56 kb at 9q21.31 in the genomic DNA. Exon 8 was located between intron 7 (10274 bp) and intron 8 (793 bp), and consensus splice donor/acceptor sites were found at the exon–intron boundary (Figures 2B and 2C). These results indicate

that two forms of the human PSAT transcript were produced by alternative splicing of the primary transcript of the PSAT gene. Neither the polyadenylated [poly(A)] addition signal nor the poly(A) tail was detected in the 3'-untranslated region of both HsPSAT α and HsPSAT β .

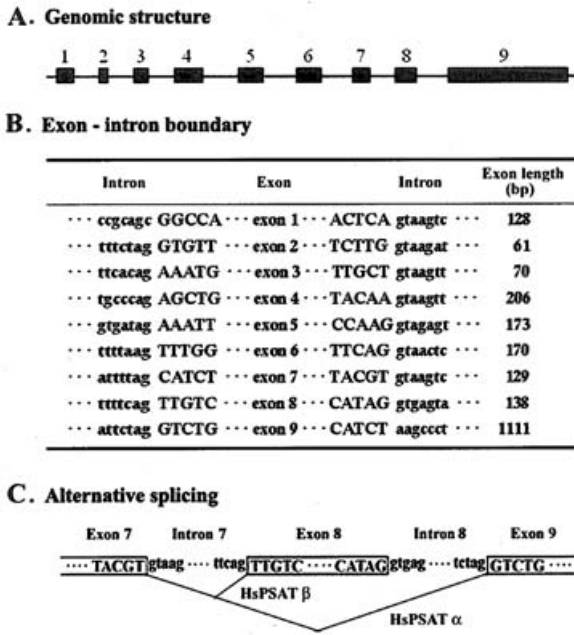


Figure 2 Genomic organization (A), exon-intron boundaries (B) and schematic representation of the alternative-splicing forms (C) of the human PSAT gene

Exons of PSAT gene are labelled 1–9 and are indicated by solid boxes. The exon–intron boundary and exon size in PSAT gene are shown. Two types of PSAT are shown, and the positioning of the PSAT β exon 8 between intron 7 (10 274 bp) and intron 8 (793 bp) is indicated. Exon sequences are indicated by upper-case letters and intron sequences are indicated by lower-case letters.

Comparison of the amino acid sequence of human PSAT α and PSAT β with related enzymes from other sources

As shown in Figure 3, the amino acid sequence of both PSAT α and PSAT β was aligned with nine PSAT sequences from other sources using the CLUSTALW program. The amino acid sequence of PSAT β appears to be more closely related to those from other sources because it possesses the 46-amino-acids insert, which is absent from PSAT α . The overall amino acid sequence of PSAT β shares 93.5% similarity with rabbit PSAT [15] and 92.4% similarity with mouse PSAT, indicating a high degree of similarity among mammalian PSAT enzymes. PSAT β shows 60.2% similarity with *Drosophila melanogaster* (GenBank® accession no. NP_652046.1), 51% similarity with *Caenorhabditis elegans* (GenBank® accession no. Z81516), 48.1% similarity with *Arabidopsis thaliana* [24] and 46.8% similarity with *Spinacia oleracea* (GenBank® accession no. D84061) counterparts. The sequence of PSAT β is 36.5% identical with the *S. cerevisiae* PSAT [25], and is 41.2 and 44.2% identical with individual PSATs from Gram-positive and Gram-negative bacteria, including *Bacillus subtilis* [26] and *E. coli* [17] respectively. These results indicate that mammalian PSAT enzymes show closer similarity with *D. melanogaster* when compared with other enzymes. Interestingly, the lower eukaryote yeast *S. cerevisiae* enzyme exhibits more similarity with the Gram-positive bacterial enzyme (*B. subtilis*) when compared with other eukaryotic enzymes as well as Gram-negative bacterial enzyme (*E. coli*). PSAT is known to be an enzyme that requires pyridoxal phosphate as the covalent cofactor and, thus, it is grouped into the class V subfamily of pyridoxal phosphate-dependent aminotransferases [27]. When the sequence of PSAT enzymes was multiply

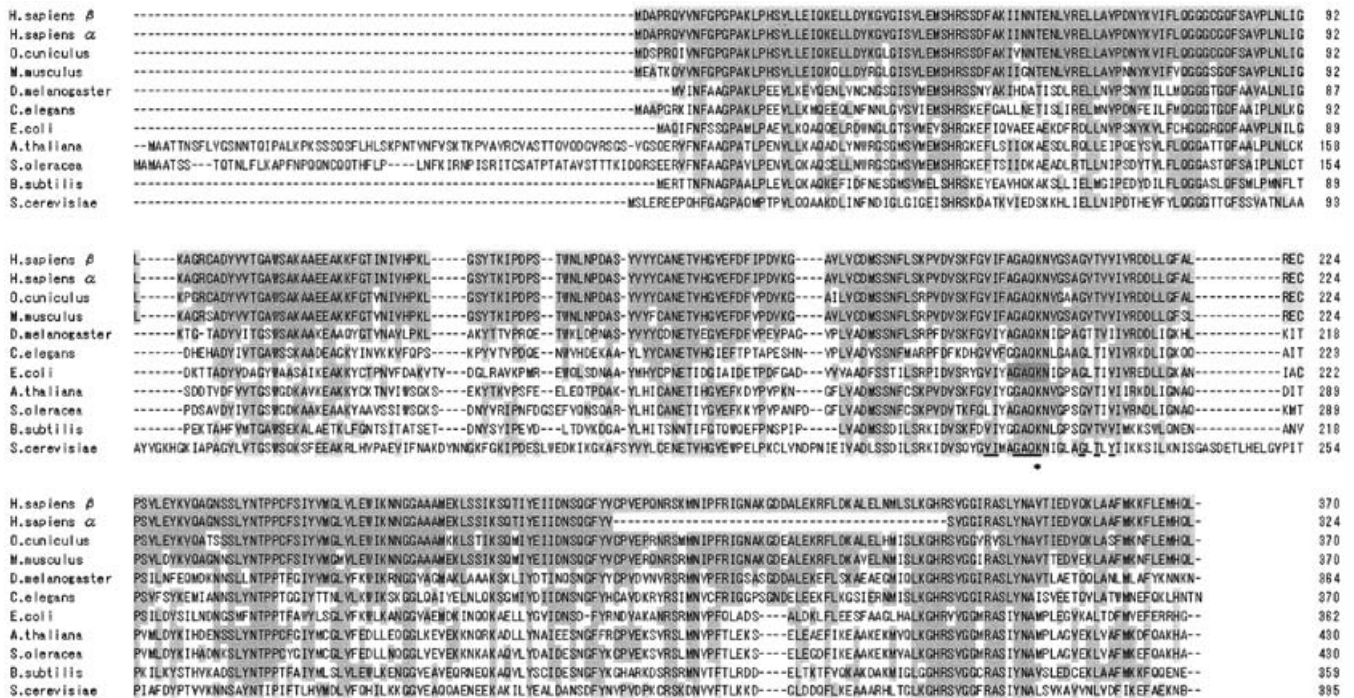


Figure 3 Comparison of amino acid sequences of PSAT α and PSAT β from human Jurkat T-cells, and PSAT β from *Oryctolagus cuniculus*, *Mus musculus*, *D. melanogaster*, *C. elegans*, *E. coli*, *A. thaliana*, *S. oleracea*, *B. subtilis* and *S. cerevisiae*

Amino acids are displayed in single-letter abbreviation after alignment for maximal identity using the CLUSTALW program. The conserved sequence (LIVMFYA)(2)-X(2)-(GSTAC)(2)-(HQR)-K-X(4,6)-G-X-(GSAT)-X-LIVMFYS involved in binding with the required cofactor pyridoxal phosphate (vitamin B₆) is underlined, and the lysine residue which is known to attach covalently with vitamin B₆ is indicated by an asterisk.

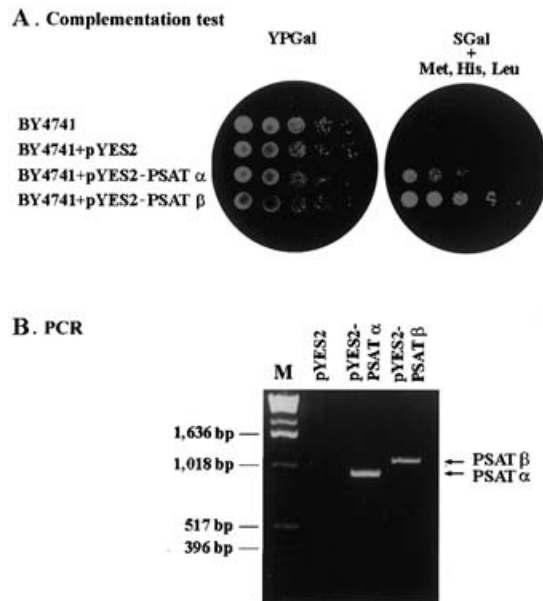


Figure 4 Functional complementation of *S. cerevisiae* serine auxotroph BY4741 by the human PSAT α and PSAT β (A), and identification of both PSATs in the individual transformants by PCR (B)

The *S. cerevisiae* BY4741 cells were transformed with pYES2-PSAT α , pYES2-PSAT β or the empty vector pYES2, as indicated. Complementation was assayed by monitoring the growth of serially diluted yeast strains on YPGal (complete medium) plate and comparing it with the growth on SGal (minimal medium) plate.

aligned, the binding domain for the required cofactor pyridoxal 5'-phosphate (vitamin B₆) appeared to share a significant identity. This domain contains a consensus sequence consisting of (LIVMFYA)(2)-X(2)-(GSTAC)(2)-(HQR)-K-X(4,6)-G-X-(GSAT)-X-LIVMFYS, which includes the lysine residue known to be covalently bound to this vitamin [27,28].

Complementation of *S. cerevisiae* SER1-deletion mutation by HsPSAT α and HsPSAT β

To investigate whether both PSAT α and PSAT β are catalytically active enzymes, we found that HsPSAT α or HsPSAT β can rescue the SER1-deletion mutation of *S. cerevisiae* by successful complementation. When *S. cerevisiae* serine auxotroph BY4741 (*his3*, *leu2*, *met15*, *ura3*, Δ *ser1::Neo^R*) was transformed with the expression vector pYES2 containing the coding region for PSAT α (pYES2-PSAT α) or PSAT β (pYES2-PSAT β), in which the expression of each protein was regulated by the GAL1 promoter, the transformants were able to grow on a SGal minimal medium plate in the absence of L-serine (Figure 4A). Under these conditions, the recipient *S. cerevisiae* BY4741 and the transformant with the empty vector pYES2 failed to grow without supplementation of L-serine. To confirm whether the transformants grown on a SGal minimal medium possess each expression vector construct of PSAT α or PSAT β , PCR was performed. As shown in Figure 4(B), the transformants appeared to contain pYES2-PSAT α and pYES2-PSAT β respectively. Although both PSAT α and PSAT β proteins could functionally rescue the SER1-deletion mutation of *S. cerevisiae*, the efficiency of the complementation by PSAT β appeared to be higher than that by PSAT α . These results suggest that PSAT β is catalytically more active than PSAT α in *S. cerevisiae*.

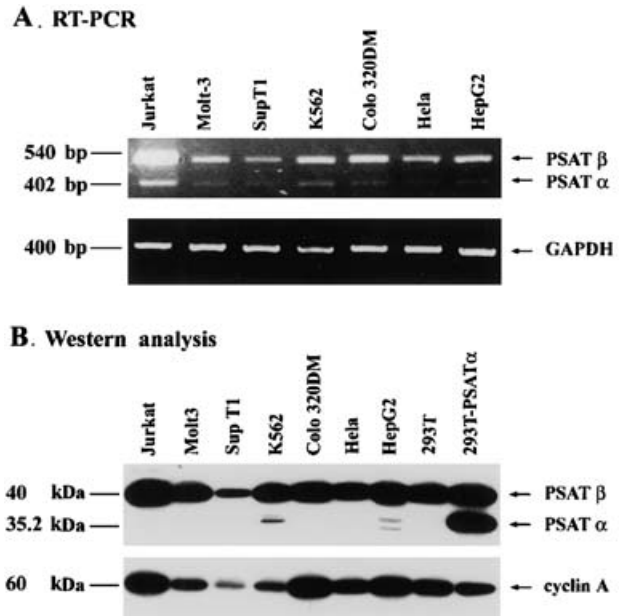


Figure 5 RT-PCR analysis of individual transcripts (A) and Western-blot analysis of individual proteins (B) of PSAT α and PSAT β in various human tumour cell lines

Total RNA isolated from each human tumour cell line was used as template for RT-PCR using the specific primers as described in the Experimental section. GAPDH was used as the housekeeping control gene. Each cell lysate (20 μ g) was electrophoresed on a 4–12% NuPAGE gradient gel and electrotransferred to Immobilon-P membrane. The PSAT α , PSAT β and cyclin A proteins were detected as described in the Results section. Human-transformed primary embryonic kidney cell line 293T was transfected with pCAGGS-PSAT α and its cell lysate was used as the control to detect PSAT α protein.

Expression of PSAT α and PSAT β in human cell lines

Since our results suggested that both PSAT α and PSAT β play a role as functional enzymes, and that PSAT β might be catalytically more active than PSAT α , the abundance of PSAT α and PSAT β transcripts in different cell types was analysed by RT-PCR. As shown in Figure 5(A), the presence of PSAT α and PSAT β transcripts in individual cells was detected by RT-PCR as 402 and 540 bp products respectively. Although two different transcripts corresponding to PSAT α and PSAT β were detected in all cell lines tested, the expression level of PSAT β mRNA was more abundant than that of PSAT α mRNA, which appeared as a weaker band where the PSAT β mRNA was expressed. The dominant PSAT β transcript was expressed at high levels in human leukaemia Jurkat, colon adenocarcinoma COLO 320DM and hepatocellular carcinoma HepG2, and expressed weakly in leukaemia MOLT-3. The differential level of PSAT α and PSAT β proteins in these cell lines was also analysed by Western blotting using a rabbit polyclonal antibody that could recognize both PSAT α and PSAT β proteins. The level of cyclin A, which is required for the progression through the S-phase of the cell cycle [29], was also investigated as the control. As shown in Figure 5(B), PSAT β (40 kDa) protein was detectable in proportion to the amount of mRNA in the cell lines tested, whereas PSAT α (35.2 kDa) protein was detectable only as a very faint band in HepG2 and human chronic myelogenous leukaemia K562. There is an additional PSAT α (34 kDa) protein detected as a faint band in HepG2. It is noteworthy that human-transformed primary embryonic kidney cell line 293T appeared to express only PSAT β protein unless the cells were transfected with the pCAGGS-PSAT α construct to overexpress PSAT α protein. These results indicate that PSAT β

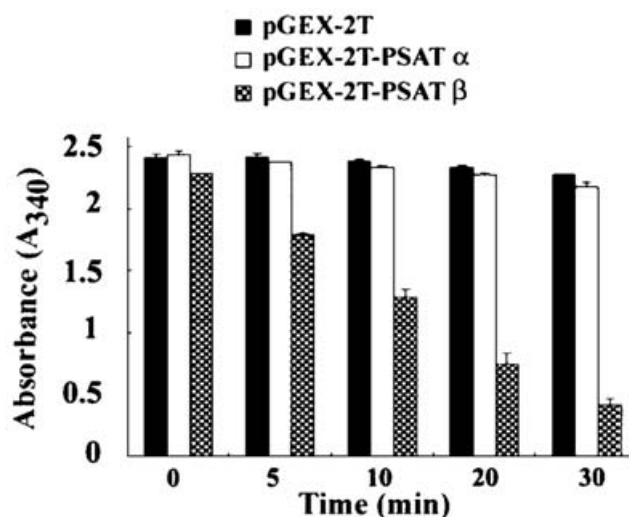


Figure 6 Enzymic activity of GST-PSAT α and GST-PSAT β

Relative enzyme activities of GST-PSAT α and GST-PSAT β were detected by employing equivalent amounts of individual cell lysates, and the cell lysate of *E. coli* transformed with empty vector was used as the control.

rather than PSAT α is the physiologically functional enzyme and plays a major role in the human cells.

Comparison of the enzyme activity of PSAT α and PSAT β

To compare the catalytic enzyme activity of these two forms of human PSAT, individual PSAT proteins were expressed in *E. coli* system as GST fusion proteins, and the enzymic activities of the GST-PSAT α and GST-PSAT β were determined. There was no PSAT protein detectable by anti-human PSAT α antibody in the cell lysate of the *E. coli* transformant harbouring pGEX2T vector, whereas the cell lysates of the transformants harbouring pGEX-2T-HsPSAT α or pGEX-2T-HsPSAT β appeared to express individual GST-PSAT α (61.2 kDa) and GST-PSAT β (66 kDa) proteins (results not shown). The relative enzyme activities that were measured by employing individual cell lysates showed that GST-PSAT β has 6.8 times higher enzymic activity when compared with GST-PSAT α (Figure 6). These results demonstrate that PSAT β is the physiologically functional form of human PSAT responsible for serine biosynthesis in human cells, whereas PSAT α , the short form of PSAT protein, is a hypoactive enzyme and thus not effective *in vivo*.

Tissue distribution of PSAT-specific mRNA

When the tissue distribution of the human PSAT-specific mRNA was analysed with 32 P-labelled 1065 bp HsPSAT α cDNA probe using a human multiple tissue Northern blot, an approx. 2.2 kb mRNA specific for PSAT was detected (Figure 7). The size estimate is only approximate on these blots, since the blot length was only 7 cm. The 2.2 kb mRNA was expressed at high levels in the brain, liver, kidney and pancreas, but very weakly in the thymus, prostate, testis and colon (mucosal lining). However, it was not detectable in the spleen, ovary, small intestine, peripheral blood mononuclear cells, heart, placenta, lung and skeletal muscle. On the contrary, the 2.1 kb mRNA specific for PHGDH that catalyses the first step in the phosphorylated pathway by oxidizing 3-phosphoglycerate to 3-phosphohydroxypyruvate was expressed at high levels in prostate, testis, ovary, brain, liver,

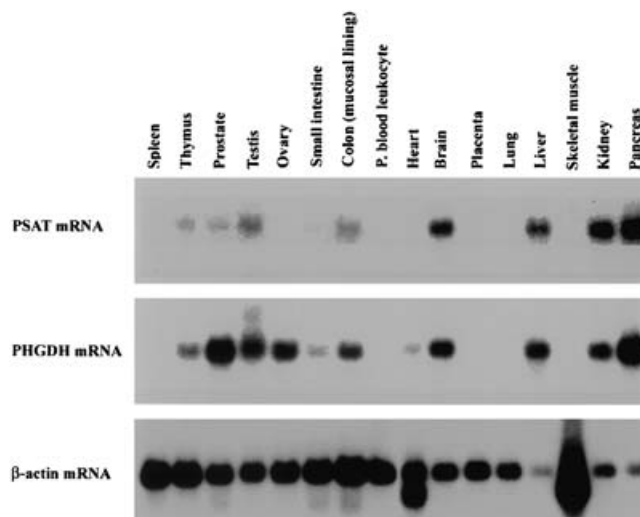


Figure 7 Northern-blot analysis of tissue distribution of PSAT-specific mRNA

Multiple-tissue Northern-blot membranes (MTNTM I and II; ClonTech Laboratories), each containing 2 μ g of poly(A)⁺ RNA/lane, were hybridized with 32 P-labelled PSAT α , PHGDH and β -actin cDNA probe.

kidney and pancreas, and weakly expressed in thymus, colon and heart. As compared with the PHGDH gene, the PSAT gene has a more restricted tissue-specific expression. In particular, both PSAT and PHGDH were expressed abundantly in the brain. These results suggest that the expression of PSAT is tissue-specific, but is not restricted to tissues with a high proliferative capacity.

Expression of PSAT during differentiation and retrodifferentiation of U937 cells

The human monoblastoid cell line U937 stops proliferating and differentiates into monocytes in the presence of PMA. PMA-induced monocytic differentiation of U937 cells is believed to be reversible because a long-term culture in the absence of PMA restores the proliferating state by a retrodifferentiation process [18]. Since L-serine is known to serve as a precursor for the formation of nucleotides that are directly associated with cellular replication, and since it has been shown previously [12] that the expression level of the PHGDH mRNA is regulated in U937 cells during induced differentiation and retrodifferentiation, it seems probable that the expression level of the PSAT mRNA may also be regulated by PMA.

To test this prediction, the expression of 2.2 kb PSAT mRNA in U937 cells was examined during induced differentiation and retrodifferentiation. After U937 cells were treated with 32 nM PMA for 36 h, the cells no longer incorporated [3 H]TdR (Figure 8A). Under the same conditions, the expression of the 2.2 kb PSAT-specific mRNA, which is the only transcript-detectable in U937 cells, decreased to a barely detectable level 6 h after the treatment with PMA (Figure 8B). In accordance with the decrease in the level of PSAT-specific mRNA, the protein level of 40 kDa PSAT β was significantly down-regulated 6 h after PMA treatment and became undetectable within 24 h (Figure 8C). When U937 cells treated with 32 nM PMA for 48 h were cultured in the absence of PMA, [3 H]TdR incorporation began to increase from day 11 and reached a remarkable level in 13 days (Figure 9A). Under these conditions, the 2.2 kb PSAT mRNA appeared to be detectable on day 5 and its remarkable

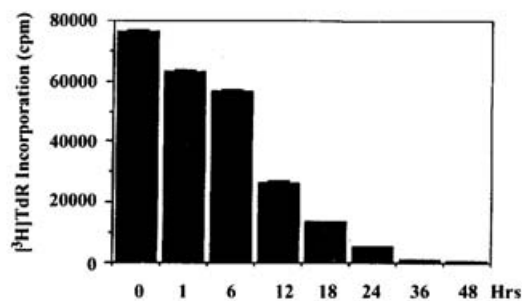
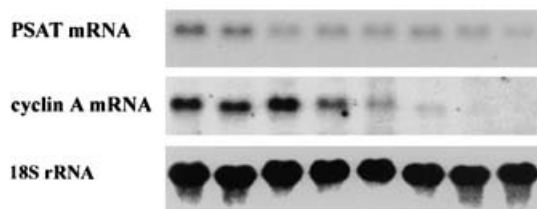
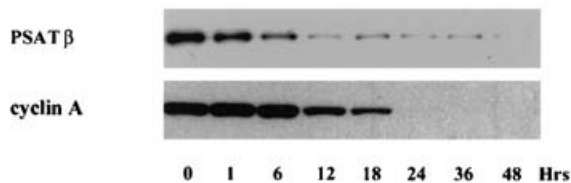
A. Thymidine incorporation**B. Northern analysis****C. Western analysis**

Figure 8 Kinetic analysis of [³H]TdR incorporation (A), and expression of Hs PSAT-specific mRNA (B) and protein (C) during PMA-induced monocytic differentiation of U937 cells

For proliferation assay, U937 cells (10^5 /well) were treated with 32 nM PMA in 96-well plates and pulsed for 4 h with 1 μ Ci of [³H]TdR at the times indicated. Equivalent cultures were incubated for the indicated time periods and prepared for RNA extraction and cell lysates. Northern- and Western-blot analyses were performed as described in the Experimental section.

accumulation occurred on day 11, whereas PSAT β protein was first detected on day 9 and increased up to day 11 (Figures 9B and 9C). Under these differentiation and retrodifferentiation conditions, PSAT α protein was not detected in U937 cells. The expression pattern of PSAT β in U937 cells during induced differentiation and retrodifferentiation appears to be similar to that of human cyclin A, which is known to accumulate in the S-phase and to support cellular replication [29]. These results suggest that the expression level of PSAT mRNA can be regulated depending on the cellular proliferative status and may be up-regulated in the S-phase to promote the phosphorylated pathway of serine synthesis.

Expression of PSAT during cell-cycle progression

To confirm whether the expression level of PSAT mRNA is up-regulated in the S-phase of the cell cycle, the change in the level of PSAT-specific mRNA during cell-cycle progression of Jurkat T-cells was investigated by Northern-blot analysis. As shown in Figure 10, treatment of Jurkat T cells with hydroxyurea or nocadazole for 20 h blocked the cells in late G₁ or the G₂/M

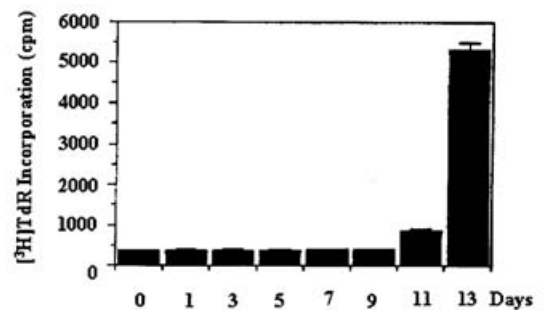
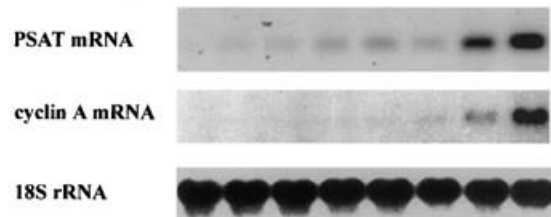
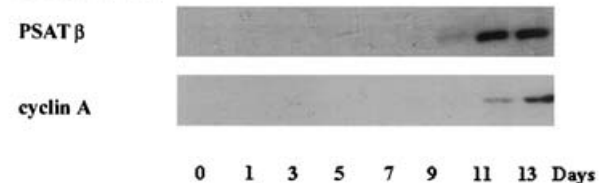
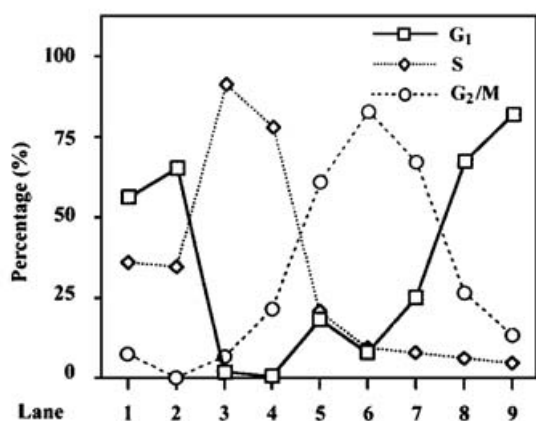
A. Thymidine incorporation**B. Northern analysis****C. Western analysis**

Figure 9 Kinetic analysis of [³H]TdR incorporation (A), and expression of PSAT-specific mRNA (B) and protein (C) during retrodifferentiation of U937 cells

U937 cells, treated with 32 nM PMA for 48 h, were washed three times with 1 \times HBSS containing 2% FBS, and cultured in the absence of PMA at a density of 10^5 cells/well in 96-well plates, and pulsed for 4 h with 1 μ Ci of [³H]TdR at the times indicated. Equivalent cultures were incubated for the indicated time periods, and prepared for RNA extraction and cell lysates. Northern- and Western-blot analyses were performed as described in the Experimental section.

boundary efficiently; approx. 70% of the cells remained at G₁ and 90% of the cells at the G₂/M phase respectively. Since both hydroxyurea [19] or nocadazole [20] are known to be reversible blocking agents of the cell-cycle progression, reversal of the blocking was easily accomplished by washing the cells and then resuspending them in the reagent-free medium at 37 °C. The blocked cells could continue the cell-cycle progression homogeneously at least until 7 h after releasing them from the blocking. To obtain Jurkat T cells at the individual specific stages of the cell cycle, the cells were released from the blocking points for 2, 4 or 7 h. The cells were processed sequentially for RNA extraction. Northern-blot analysis revealed that the expression level of 2.2 kb PSAT mRNA reaches a maximum in the S-phase and decreases to the basal level as the cells move to the G₂/M boundary (Figure 10B). The basal level of the PSAT mRNA expression is sustained until the cells traverse through the M phase and enter the G₁ phase. The expression pattern of the PSAT mRNA during cell-cycle progression appeared to be essentially the same as that for PHGDH and cyclin A. These results demonstrate that the expression level of PSAT mRNA along with PHGDH mRNA is regulated during cell-cycle progression, and reaches a maximum

A. Cell cycle progression



B. Northern analysis

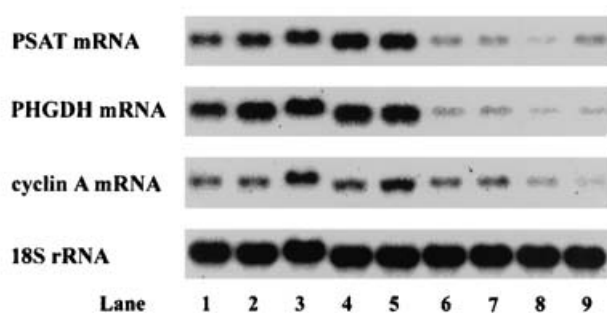


Figure 10 Expression of PSAT-specific mRNA during cell-cycle progression

Jurkat T-cells blocked at late G₁ or the G₂/M boundary by the treatment of hydroxyurea or nocadazole were induced to continue cell-cycle progression for the indicated time periods after releasing from the blocking. The cell cycle was also analysed by flow cytometry after staining the cellular DNA with propidium iodide (A). Expressions of PSAT-, PHGDH- and cyclin A-specific mRNA during cell-cycle progression were analysed by Northern blotting (B). Total RNA (15 µg) extracted from Jurkat T-cells untreated (lane 1), cells blocked with hydroxyurea (lane 2), and released from the blocking for 2 h (lane 3), 4 h (lane 4) and 7 h (lane 5) or cells blocked with nocadazole (lane 6), and released from the blocking for 2 h (lane 7), 4 h (lane 8) and 7 h (lane 9), were electrophoresed, transferred and probed with ³²P-labelled PSAT α , PHGDH, cyclin A and 18 S rRNA cDNA.

in the S-phase. These results also suggest that the capacity of the phosphorylated pathway for L-serine synthesis is enhanced in replicating cells in which there is a high L-serine requirement for the nucleotide precursor formation that is coupled with DNA replication.

DISCUSSION

This is the first investigation of the molecular cloning and characterization of two alternatively spliced forms (HsPSAT α and HsPSAT β) of human PSAT cDNA that encodes the PSAT, catalysing the conversion of 3-hydroxyypyruvate into 3-phosphoserine for the phosphorylated pathway. HsPSAT α possesses an ORF comprising 324 amino acids, encoding a 35.2 kDa protein (PSAT α), and HsPSAT β consists of 370 amino acids with a molecular mass of 40 kDa (PSAT β). The difference between PSAT α and PSAT β is mainly due to the 46-amino-acid peptide insert between Val²⁹⁰ and Ser³³⁷ of PSAT β , which does not exist in PSAT α . This 46-amino-acid insert appears to

be produced by differential splicing of the PSAT transcript at exons 7 and 9. Multiple alignment of PSAT α and PSAT β with those from other sources using the CLUSTALW program revealed that the short form, PSAT α , is not present in the other organisms reported, indicating that the primary transcript of the PSAT gene does undergo the alternative splicing event only in the human system. It would be of interest to investigate further the molecular mechanism associated with this species-selective alternative splicing. It is noteworthy that mammalian PSATs are closer to the Gram-negative bacterial enzyme than to the lower eukaryote yeast *S. cerevisiae* enzyme. The Gram-positive bacterial PSAT (*B. subtilis*) exhibits more similarity with the yeast enzyme than to other Gram-negative bacterial enzymes. Similarly, it has been reported that *B. subtilis* PHGDH is closer to eukaryotic enzymes than to other bacterial PHGDH enzymes [12,30,31]. These results suggest that the ancestral gene of PSAT as well as PHGDH involved in the phosphorylated pathway began diverging before the separation between eukaryotes and prokaryotes occurred. To characterize the biological properties of PSAT α and PSAT β , we performed complementation of SER1-deletion mutation of *S. cerevisiae* by the expression plasmid pYES2 containing HsPSAT α or HsPSAT β , and demonstrated that PSAT β was catalytically more active than PSAT α because the efficiency of the complementation by PSAT β was much higher than PSAT α . In our studies, the expression of PSAT β mRNA, when measured by RT-PCR, was significantly more abundant than PSAT α mRNA in all human cells tested. At the same time, Western-blot analysis demonstrated that the dominant PSAT β was easily detectable in proportion to the mRNA level, but PSAT α was not detectable in most human cell lines tested except in K562 and HepG2, which appeared to express PSAT α at a barely detectable level. These results indicated that PSAT β rather than PSAT α has a physiological role in the human system. This was further supported by our finding that GST-PSAT α fusion protein possessed a barely detectable level of enzyme activity, and the relative enzyme activity of GST-PSAT β was 6.8 times higher than that of GST-PSAT α .

When we performed Northern-blot analysis, a single approx. 2.2 kb transcript corresponding to PSAT mRNA was detected in healthy human tissues. This indicated that the 138 bp difference in size between the coding region of PSAT α and PSAT β was not detectable by Northern-blot analysis. The 2.2 kb mRNA is expressed at high levels in the brain, liver, kidney and pancreas, and at low levels in the thymus, prostate, testis and colon (mucosal lining), suggesting that the expression of PSAT is tissue-specific. Compared with the PHGDH gene, the PSAT gene has a more restricted tissue-specific expression. Only in four tissues, those of brain, liver, kidney and pancreas, is there an abundant expression of both PHGDH mRNA and PSAT mRNA, suggesting that these tissues are the major sites for the endogenous L-serine biosynthesis via the phosphorylated pathway. In particular, the abundant expression of both PHGDH [12] and PSAT in the brain, along with the previous result showing an inefficient transport of L-serine via the blood-brain barrier to the CNS [6,7], apparently supports the necessity for *de novo* biosynthesis of serine by the phosphorylated pathway in the CNS. The importance of L-serine in cellular replication and thus the highest capacity of the phosphorylated pathway in the S-phase of the cell cycle are corroborated by the results from Northern-blot analysis, which demonstrate that the mRNA specific for both PSAT and PHGDH [12], abruptly down-regulated in accordance with growth arrest of U937 cells on PMA-induced monocytic differentiation, can be recovered when the PMA-treated cells restore cell growth by a retrodifferentiation process. Although PSAT α was not detected under these conditions, the change in the level of PSAT β protein

was essentially the same as that of its transcript. The fact that the accumulation of PSAT mRNA reaches a maximum in the S-phase of the cell cycle is more obvious when Northern-blot analysis was performed using the total RNA extracted from Jurkat cells at the specific stages of the cell cycle. Since the protein level of PSAT β was well reflected by the level of the mRNA (results not shown), these results suggest that the capacity of the phosphorylated pathway for L-serine biosynthesis fluctuates during cell-cycle progression and reaches a maximum in the S-phase to support cell proliferation.

Taken together, these results demonstrate that two types of PSAT transcript (HsPSAT α and HsPSAT β) expressed in the human system result from alternative splicing, and that PSAT β protein contains a 46-amino-acid insert between Val²⁹⁰ and Ser³³⁷, which does not exist in PSAT α . These results further demonstrate that PSAT β rather than PSAT α is the physiologically functional enzyme required for the phosphorylated pathway and that the accumulation of PSAT mRNA is regulated depending on tissue specificity as well as cellular proliferation status with a maximum level in the S-phase. These findings will be useful for creating a new concept or target for the diagnosis and therapy of human serine metabolism-related disorders and cancer.

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