Purification and Properties of Human Cytosolic Folylpoly-γ-glutamate Synthetase and Organization, Localization, and Differential Splicing of Its Gene*

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Human cytosolic folylpolyglutamate synthetase (FPGS) was expressed in Escherichia coli and purified to homogeneity. Tetrahydrofolate and dihydrofolate were the most effective substrates, while 5-substituted folates were poor substrates. Most pteroyldiglutamates were better substrates than monoglutamates.

The human FPGS gene spans 12 kilobases and contains 15 exons and 14 introns. A single FPGS gene was located to chromosome region 9q34.1. Four exon 1 variants were identified, each of which was spliced to exon 2. The exon 1 variant corresponding to the isolated cDNA contains two ATG codons and multiple transcription start sites in this region generates mitochondrial and cytosolic FPGS (Freemantle, S. J., Taylor, S. M., Krystal, G., and Moran, R. G. (1995) J. Biol. Chem. 270, 9579–9584). Exons 1B and 1C, generated by alternate splicing in intron 1, and exon 1A, which is 5′ to exon 1 and may encode an additional mitochondrial isoform, are preceded by a number of potential promoter sites.

Chinese hamster ovary cell transfectants expressing FPGS activity in the mitochondria contained normal mitochondrial and low cytosolic folylpolyglutamate pools. Mitochondrial FPGS activity is required for mitochondrial folate accumulation, while cytosolic FPGS activity is needed for establishment of normal cytosolic folate pools. The reconstructed FPGS gene restored normal cytosolic and mitochondrial folate metabolism in hamster cells.

Folylpolyglutamate synthetase (FPGS); tetrahydrofolate

glutamate γ-ligase (ADP-forming), EC 6.3.2.17. activity is required for the synthesis and cellular retention of functional folate coenzymes and for the conversion of many antifolates to more active forms (1–6). FPGS activity is highest in proliferating tissues and activity, and mRNA levels increase after mitogen stimulation and decline during differentiation (7–10).

It has been proposed that a coordinate down-regulation of folate enzymes including FPGS, and a consequent limitation in macromolecule synthesis, may be an early programmed event in cell maturation (10). The ability of human leukemia blast cells to metabolize methotrexate to polyglutamate derivatives has been correlated with efficacy of methotrexate treatment (11), and a wide variation in FPGS activity and in FPGS mRNA levels has been found in leukemia blast cells (8, 12).

Increased expression of FPGS activity in model cells leads to increased sensitivity to antifolate drugs while decreased FPGS activity is a mechanism for resistance to many antifolates, both in model cell systems and in clinical samples (5, 13–16).

Mammalian FPGS is a low abundance protein which has hindered its isolation in sufficient quantities for detailed physical and biochemical analysis. Pig liver FPGS is the only mammalian enzyme to have been purified to homogeneity (17) although some characterizations of other crude or partially purified mammalian FPGS enzymes have been reported (18–22). In each case, only small amounts of protein have been obtained which has limited studies to kinetic analyses. The low abundance and instability of mammalian FPGS has complicated its purification in sufficient quantity to carry out mechanistic studies.

Mammalian cells possess both mitochondrial and cytosolic FPGS isozymes (23), and additional isoforms may be expressed in different tissues or in tumor cell (24). The Chinese hamster ovary (CHO) cell mutant AUXB1 lacks FPGS activity and as a result requires exogenous purines, thymidine, and glycine for growth (25–28). Studies using AUXB1 cells transfected with Escherichia coli folC gene (FPGS) constructs that target the expressed protein to the cytosol or mitochondria have demonstrated that cytosolic FPGS activity is required for cytosolic folate accumulation and purine and thymidylate biosynthesis, while mitochondrial FPGS activity is required for mitochondrial folate accumulation and glycine biosynthesis (23, 29).

Expression of E. coli FPGS solely in the mitochondria of AUXB1 cells restored normal folate accumulation and metabolism in the cytosol as well as in the mitochondria, demonstrating that pteroylglutamates synthesized in the mitochondria can be released into the cytosol, although these polyglutamate species cannot enter the mitochondria (14, 29).

We previously isolated a human FPGS cDNA (30) which encoded cytosolic FPGS and suggested that it might lack 5′

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) U24252 (upstream region to intron 4) and U24253 (intron 4 to intron 11).

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1 The abbreviations used are: FPGS, folypoly-γ-glutamate synthetase; PteGlu, pteroylglutamic acid; folic acid; H-PteGlu, tetrahydropteroylpoly-γ-glutamate; n, indicating the number of glutamate moieties; CHO, Chinese hamster ovary; 5′-RACE, rapid amplification of 5′-cDNA ends; RT-PCR, reverse transcription polymerase chain reaction; bp, base pair(s); kb, kilobases; PAGE, polyacrylamide gel electrophoresis; IPTG, isopropyl-β-D-galactopyranoside.

2 J.-C. Hsu, T. Garrow, and B. Shane, unpublished data.
sequences that could also encode the mitochondrial isozyme. Recently, Freemantle et al. (31) have described the organization of the 5' region of the human FPGS gene and have shown that the isolated cDNA lacks the 5' region of the mitochondrial leader sequence and that cytosolic and mitochondrial isozymes could be generated by alternate transcription start sites.

In this report, we describe the expression of human cytosolic FPGS in E. coli and the purification and properties of the enzyme. As a prelude to studying factors regulating the expression of FPGS in mammalian tissues and the role of the different isozymes, we have isolated and characterized the human gene.

In this report, we describe the organization of the complete human FPGS gene, its localization, and splicing variants, and present a rationale for the role and need for mitochondrial and cytosolic FPGS isozymes.

**EXPERIMENTAL PROCEDURES**

Materials—[2,3-14C]Glutamate (specific activity 270 mCi/mmol) was obtained from Amersham. (6S)-5-formyltetrahydroh[14]folate (folicinate, 5-formyl-H$_2$PteGlut, specific activity 20 Ci/mmol) was obtained from Moravek, and 3'-5'-32P-dCTP (3000 Ci/mmol) and 15-P32P-jATP were obtained from DuPont NEN. Folic acid, methotrexate, and aminopterin were obtained from Sigma and (Gambro-H$_2$PteGlut from Fluka. Reduced and oxidized folylpolyglutamates were synthesized as described previously (17, 32) or were obtained from Schirck Laboratories. Concentrations of folate derivatives were calculated from their absorbance spectra (33). Deficient α-minimal essential medium lacking purines, glucose, thymidine, and folate was obtained from J RH BiSciences. DNA restriction and modifying enzymes and RNAase A were obtained from Boehringer Mannheim, Promega, or New England Biolabs. AmpliTaq DNA polymerase was from Perkin-Elmer. Nitrocellulose was obtained from Schleicher and Schuell. Oligonucleotides were synthesized at the UC Berkeley Microchemical Facility. E. coli strain J M109(ΔDE3) was obtained from Promega. All other materials were obtained from commercial vendors.

Plasmid Construction for Expression of Human FPGS in E. coli—An NdeI restriction site was introduced at the first ATG in the human FPGS cDNA (pTZ18U-25) (30), accession number M98045) by mutagenesis using the procedure of Nakamaye and Ecksteine (34). The antisense primer 5'-CTGTTAATCCATCATGCTGGCTCTCTG-3' introduced an AT dinucleotide between bases 70 and 71 of the cDNA (Fig. 1). A 2117-bp NdeI-BamHI fragment from pTZ18U-25-NdeI, containing the entire open reading frame of human FPGS, was ligated into similarly treated pET3A (Novagen). Plasmid pET3A has the bacteriophage T7 gene 10 promoter and transcriptional termination sequences. This new construct, pET3A-25 was transformed into E. coli J M109(ΔDE3) for the production of unfused human FPGS.

To improve expression in E. coli, 12 of the first 27 codons in the human cDNA ORF were modified for optimal codon usage (35). The complementary oligonucleotides 5'-TATGGAATAACGAACGACTGTGACTGCTGA-ACACCTGCTGACAGAACCCTGCTTCTGGAGGAACGGTAAACGCC-AGGCGG-3' and 5'-32TATATTGGTCTGCTGAGACGGTGCTGACAGAACCCTGCTTCTGGAGGAACGGTAAACGCC-AGGCGG-3' were phosphorylated using T4 polynucleotide kinase, and the primers were annealed to each other by heating at 100°C for 3 min followed by slow cooling to room temperature in 60 mM Tris buffer, pH 7.5, containing 6 mM MgCl$_2$, 10 mM dithiothreitol, and 400 μM ATP. Five micrograms of pET3A-25 was digested with NdeI and BstEII and purified by agarose gel electrophoresis. Approximately 0.5 μg of this DNA was ligated with 10 μl of the annealed oligonucleotides in a total volume of 20 μl. Half of this reaction mixture was transformed into J M109(ΔDE3) and 4 transformants, whose inserts lacked an NdeI restriction site, were subjected to double-stranded sequencing (Sequenase, version 2) to verify the new primary structure at the 5' end of the cDNA (pET3A-25*).

Plasmid Construction for Expression of Human FPGS in CHO Cells—Two different constructs of the human FPGS cDNA in plasmid pSVK-3 (Pharamcia Biotech Inc.) were prepared as shown in Fig. 1. Plasmid pSVK-hFPGScyt was constructed by ligating the 2130-bp EcoRI fragment from pSE936-25 (30) into similarly treated pSVK-3. In some constructs, most of the 3'-untranslated region was deleted by digesting the plasmid with XbaI and religating (Fig. 1). Plasmid pSVK-hFPGScyt was made by ligating an 1860-bp XbaI fragment from pSE936-3 (30) into similarly treated pSVK-3. This construct adds an in-frame ATG upstream of the cDNA sequence (Fig. 1). Plasmids were purified by cesium chloride density centrifugation. These plasmid constructs were cotransfected into CHO-SV40-Δpoxanthine (AY1045) with 35S-labeled RNA encoding the human p53 tumor antigen splice site and a poly(A) signal. The inserts provide the 5'-promoter and transcriptional start region and upstream from a small tumor antigen splice site and a poly(A) signal. The inserts provide the 5'-promoter and transcriptional start region and upstream from a small

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**FIG. 1.** Plasmid constructs pSVK-hFPGScyt and pSVK-hFPGScit. The first 1815 bp of the human cDNA are represented. The 1833-bp EcoRI/XbaI fragment from pSE936-3 (21) was directionally subcloned into similarly treated pSVK-3 to create pSVK-hFPGScyt. In this construct, the first ATG (in bold at +71) originates from within the human cDNA and bypasses the partial mitochondrial leader sequence encoded by nucleotides -1 to +70 of the cDNA. The 1833-bp EcoRI/XbaI fragment from pSE936-3 was subcloned into similar pSVK-3 to create pSVK-hFPGScit. The orientation of the insert was checked by Smal digestion (unique Smal site in vector not shown). In this construct, the origin of the first ATG (at -26) is from the pSE936 vector and linker fusion. The subsequent 71% codons are encoded by the linker. The human cDNA begins at +1 and codes for the partial mitochondrial leader sequence. Further experimental details are described under "Experimental Procedures."
cells, the measurement of subcellular distributions of FPGS and marker enzyme activities, and the measurement of intracellular folate accumulation and folylpolyglutamate chain length distributions have been previously described (23, 29, 36).

**Folylpolyglutamate Synthetase Assay—** FPGS activity was monitored during enzyme purification procedures and in CHO cell extracts by the incorporation of \(^{14}C\)glutamate (250 \(\mu\)Ci) into folylpolyglutamate products using (Gamb-H\(_2\))PteGlu as the substrate, as described previously (23). Assays were normally conducted for 1 h at 37 °C. In kinetic studies with pure enzyme, the assay mixtures contained various concentrations of the substrate under investigation and fixed concentrations of (Gamb-H\(_2\))PteGlu (40 \(\mu\)M), ATP (1 mM), and d-glutamate (2 mM), as appropriate. The amount of FPGS was adjusted to ensure that less than 10% of the limiting substrate was converted to product at the lowest substrate concentration used.

**Enzyme Purification—** All buffer solutions were adjusted to the indicated pH at room temperature. Extracts were maintained at 0°C, and all other procedures were performed at 0–4°C.

**J M109(DE3) harboring plasmid pET3A-25 was grown to midlog phase in Luria media containing 50 \(\mu\)g/ml ampicillin and 0.5% glycerol (15 liters). The cells were collected by centrifugation at 650 \(\times\) g for 10 min. The cell paste (114 g) was resuspended in 100 ml Tris-HCl buffer, pH 7.5 (300 ml), containing 2.5 mM EDTA, 50 mM mercaptoethanol, and 1 mM phenylmethylsulfonylfluoride, and the cells were disrupted using a Branson sonicator (power setting 8 using a 50% duty cycle for 3 min). The extract was then centrifuged at 12,000 \(\times\) g for 45 min, and the supernatant was decanted through a double layer of cheesecloth to give the crude extract (fraction 1).

The crude extract (325 ml) was applied to a hydroxyapatite (Bio-Rad) column (7.5 \(\times\) 7.5 cm) that had been equilibrated with 100 mM Tris-HCl buffer, pH 7.5, containing 2.5 mM EDTA, 50 mM mercaptoethanol, and 10% ethylene glycol (Buffer A). The column was washed with the equilibration buffer (1000 ml) and eluted with a linear gradient (1500 ml) of potassium phosphate buffer, pH 7.5 (0–150 mM) in the same buffer. Fractions containing FPGS activity were pooled (fraction 2).

Fraction 2 enzyme (450 ml) was applied to an Affi-Gel Blue (Bio-Rad) column (20 \(\times\) 1.5 cm) that had been equilibrated with Buffer A. The column was washed with Buffer A containing 150 mM KCl (300 ml) and a linear gradient (500 ml) of KCl (0.15–1 mM) in the same buffer. Fractions containing FPGS activity were pooled (fraction 3).

Fraction 3 enzyme (224 ml) was applied to a phenyl-agarose (BRL) column (20 \(\times\) 1 cm) that had been equilibrated with 100 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA, 600 mM KCl, and 50 mM mercaptoethanol. The column was washed with the equilibration buffer (180 ml), and enzyme activity was eluted with 100 mM Tris-HCl buffer, pH 8.2, containing 1 mM EDTA, 50 mM mercaptoethanol, and 20% (v/v) ethylene glycol. Active fractions were pooled and dialyzed against 100 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM EDTA and 50 mM mercaptoethanol (2 \(\times\) 12 liter).

The dialyzed enzyme (fraction 4; 213 ml) was applied to a heparin-agarose (BRL) column (20 \(\times\) 1 cm) equilibrated with 100 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and 50 mM mercaptoethanol. The column was washed with 150 ml of equilibration buffer and eluted with a linear gradient (300 ml) of KCl (0–500 mM) in the same buffer. Fractions containing FPGS activity were pooled and dialyzed against 2 \(\times\) 4 liters of 50 mM Tris-HCl buffer, pH 8.4, containing 0.5 mM EDTA, 50 mM mercaptoethanol, and 10% ethylene glycol (fraction 5).

Fraction 5 enzyme (20 ml) was applied to a DE52 (Whatman) column (20 \(\times\) 1 cm) equilibrated with 50 mM Tris-HCl buffer, pH 8.4, containing 0.5 mM EDTA, 10% (v/v) ethylene glycol, and 50 mM mercaptoethanol. The column was washed with equilibration buffer (150 ml) and eluted with a linear gradient (300 ml) of KCl (0–500 mM) in the same buffer. Fractions containing FPGS activity were pooled giving fraction 6 (11 ml).

Protein purity was analyzed by polyacrylamide electrophoresis in sodium dodecyl sulfate (SDS-PAGE) using a 4% stacking gel and a 12.5% separating gel (37). Protein bands were visualized by a silver staining procedure. Total protein was determined on pooled fractions by a modified Lowry procedure (38) using bovine serum albumin as the standard.

**Isolation of Genomic Clones—** A human lung fibroblast cell line W138 genomic library in the Lambda FIX II vector (obtained from Stratagene), was screened (10\(^6\) plaques) with \(^{32}P\)labeled primers generated using the Random Primed DNA Labeling Kit (Boehringer Mannheim). A human FPGS cDNA (30) as the template. Following plaque purification, 3 positive clones were obtained. The phage DNA from these clones were purified and characterized by restriction mapping and Southern hybridization using an EcoRI-BamHI 375-bp fragment of the 5' region and a 355-bp XbaI-EcoRI 3' region fragment of the human cDNA. XbaI fragments of the genomic clones, and the entire clones bordered by NotI sites, were subcloned into pBK5+ (Stratagene) for further analysis.

**DNA Sequencing and Intron Size Determination—** DNA was sequenced by the method of Sanger et al. (39) using Sequenase version 2.0 (United States Biochemical Corp.). Exon-intron junctions were determined by direct sequencing across the junctions using digoxigenin-labeled primers based on the cDNA sequence. Intron sizes were determined by sequencing through the region or by PCR using flanking primers. The FPGS gene sequence, with the exception of 4 introns, was determined and verified by sequencing both DNA strands.

**5'-RACE Analysis of FPGS cDNA Ends—** Total RNA was isolated from human HepG2 cells with guanidinium thiocyanate followed by cesium chloride centrifugation (40) or using Tri Reagent (Molecular Research Center) according to the manufacturer’s instructions. Primers used in this study are shown in Table I. cDNA corresponding to the 5' end of HepG2 mRNA was synthesized and amplified (41) using a Marathon cDNA Amplification Kit (Clontech) and following kit instructions with the following modifications. Antisense primer L5 was used, instead of oligo(dT), to synthesize the first strand cDNA. After the anchor primer (Clontech) ligation, the cDNA was first PCR-amplified with the anchor primer (Clontech) and an inner gene-specific antisense primer (L4). One \(\mu\)l of the resulting PCR product was reamplified with the anchor primer and another nested gene-specific antisense primer (L2). PCR was carried out at 94 °C for 5 min, and for 35 cycles at 94 °C for 1 min, 57 °C for 0.5 min, and 72 °C for 0.5 min, with a final extension at 72 °C for 5 min. PCR products were amplified on an agarose gel with \(6X\)Taq DNA polymerase (Promega) as size standards. Bands of interest were excised, and the DNA was purified using a QIAEX gel extraction kit (Qiagen) and cloned into a pGEM-T vector (Promega). In other studies, a 5'AmpliFINDER RACE kit (Clontech) was used. Primer L5 was used to initiate first strand cDNA synthesis. Anchor primer from the kit and primer L4 were used to amplify the cDNA with the same cycling profile as described above. The resulting PCR products were ethanol-precipitated and directly cloned into the pGEM-T vector. Ampicillin-resistant colonies were screened with a probe generated by

| Primer | Sequence (5' to 3') | Direction | Gene location | Exon |
|--------|---------------------|-----------|---------------|------|
| 2J     | GCGGATCCGCTCAGGAA | Antisense | 5             |
| L5     | CATAGCTCGAGGATACATT| Antisense | 1734 to 1755  |
| L4     | CCAAGCTGACGTGAGATTT| Antisense | 1596 to 1615  |
| L2     | ATGCTTTGCTCACTTGCTCACTTG | Antisense | 1438 to 1458  |
| L5A1   | TAACTCTGCTGAGGTA | Antisense | 116 to 135    | 1/1B|
| L5C1   | CCTTCTTTCACCTTCACCTTCACCTTT | Antisense | 461 to 480    |
| L5B1   | AGCCATCGCCGCACGCTTTC | Antisense | 180 to 161    | 1A  |
| L5B2   | CGACTTAAAAGCGGCCCCTTT | Antisense | 192 to 172    | 1A  |
| L51    | AGACGCGGCCTGCTCAGGAA | Antisense | 33 to 54     |
| L52    | GGCTGCTCGGCCCCGACATGAT | Antisense | 3 to 19     |
| PR1    | GGGCCACGCACCGCGCTCAAGCC | Antisense | 91 to 115    |
| PR2    | ACCTTCTTTCACCTTCACCTTCACCTTC | Antisense | 1392 to 1416 |
| T-1    | CAGACTCGGCTACGTGAACTC | Antisense | 430 to 453    |
| U2     | CAGGGGCCCAGtgAGCGATA | Sense | 116 to 134 |

* Numbered from the first base (+1) of the ATG codon of the mitochondrial leader sequence.

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amplification of the human FPGS cDNA using primers U2 and L2 labeled with [α-32P]CTP. Positive colonies were further analyzed by hybridization with 32P-labeled antisense oligonucleotides LS1A, LS1B, or LS1C which are specific for human FPGS exons 1 plus 1B, 1A, and 1C, respectively (Fig. 3). Positive colonies recognized by each probe were further characterized by restriction enzyme digestion, and clones with large size inserts were sequenced.

The 5' transcriptional ends of fetal liver FPGS mRNA was also assessed by RACE using human fetal liver 5'-RACE-Ready cDNA (Clontech), antisense primers 2', L5, L4, and L2 were used successively to PCR-amplify the 5' transcriptional end.

Primers Extension Analysis—Oligonucleotide primers complementary to the 5' end of the human FPGS cDNA sense strand sequence (PR1, PR2, LS1A, LS1L, LS1B, LS1B, T1, and T2) were labeled at the 5' end with γ-32P ATP (6000 Ci/mmol) using T4 polynucleotide kinase and purified on Sephadex G-25 Quick Spin columns (Boehringer Mannheim). Labeled probe (2 × 10^8 cpm) was hybridized to 50 μg of HepG2 total RNA in 1 × reverse transcriptase buffer (BRL) at 75°C for 10 min and 30°C for 60 min. Primer and RNA were coprecipitated with ethanol, and the pellet was resuspended in 50 μl of reaction solution containing 1 × reverse transcriptase buffer, avian myeloblastosis virus reverse transcriptase (40 units), 10 μM dithiothreitol, and 0.2 mM concentration of each dNTP with or without α-32P-dCTP. Positive colonies were further analyzed by hybridization with 32P-labeled antisense oligonucleotides L5A1, L5B1, L5C1 which are specific for human FPGS exons 1 plus 1B, 1A, and 1C, respectively (Fig. 3). Positive colonies recognized by each probe were further characterized by restriction enzyme digestion, and clones with large size inserts were sequenced.

Chromosomal Localization of FPGS Gene—A FPGS cDNA probe corresponding to the 5' end of the insert of this clone was used to probe a human chromosome 9 clone (Fig. 3) corresponding to the 5' end of the cDNA, which was subcloned into pBKS′ (Stratagene). The 2.7-kb fragment of the 5'-region of this insert, from the NotI site of the vector to the SacII site of the insert, was ligated to the SacII-NotI 3'-fragment (17 kb) of genomic clone 5 to construct the entire FPGS gene. The FPGS gene was subcloned into the NotI site of pBKS′.

Chromosomal Localization of FPGS Gene—A FPGS cDNA probe was labeled with biotin-11-dUTP by nick translation and hybridized to metaphase chromosomes prepared from normal male peripheral blood by the bromodeoxyuridine synchronization method (43). Chromatography on phenyl-agarose, heparin-agarose, and DEAE-cellulose, respectively. Purified enzyme was judged to be homogeneous by SDS-PAGE (Fig. 2). A single band was observed with an apparent Mr of about 61,000, which is consistent with the deduced amino acid sequence (455 amino acids, Mr = 60,128). This purification procedure has been repeated multiple times, starting with FPGS enrichments in crude extracts ranging from 0.1 to 1.2% and has resulted in homogeneous enzyme each time.

General Properties—Activity required a monovalent cation, K+ (20 mM) was most effective, followed by NH4+ and Rb+, while Na+, Li+, and Cs+ were ineffective. There was an absolute requirement for a reducing agent and the enzyme displayed a high pH optimum (pH 9.6). The Km values for L-glutamate and MgATP were 201 μM and 200 μM. Activity was stimulated by sodium bicarbonate (10 mM), which caused a 4-fold decrease in the Km for MgATP (54 μM) and a 20% increase in Vmax.

Folate and Analog Substrate Specificity—The kinetic constants for a variety of folates and folate analogs are shown in Table III. Enzyme concentrations and incubation times were adjusted to minimize the addition of more than one glutamate moiety to the folyl or anti folyl substrate and to ensure initial rate conditions. Marked substrate inhibition was observed with many of the substrates that displayed a high affinity for the enzyme. Vmax/Km values shown in Table III are the on rates for 3 A. Brenner, I. Atkinson, T. Garrow, and B. Shane, unpublished data.  

### TABLE II

| Fraction | Volume | Activity | Protein | Specific Activity | Purification | Yield |
|----------|--------|----------|---------|------------------|--------------|-------|
| Crude    | 325 ml | 2,560 mg | 30.6 mg | 82.5 μL/mg | 1 | 100    |
| Hydroxylapatite | 450 | 1,540 | 8.90 | 173 | 2 | 83    |
| Affi-Gel blue | 224 | 2,660 | 1.39 | 1,910 | 23 | 71    |
| Phenyl-agarose | 213 | 1,180 | 0.156 | 7,580 | 92 | 30    |
| Heparin-agarose | 20 | 7,920 | 0.671 | 11,800 | 143 | 19    |
| DEAE-cellulose | 11 | 12,900 | 0.701 | 18,400 | 223 | 17 |

* Nanomoles of glutamate incorporated into folate product per h. Assay mixtures contained 5 mM ATP, 40 μM H4PteGlu, and 250 μM glutamate.
The folate analogs 5-deazaazaaydotetrahydrofolate, an inhibitor of transformylases involved in purine synthesis (47), and 2-methyl-5,8-dideazaalosolate, an inhibitor of thymidylate synthase (48), were very effective substrates of human FPGS (Table III). Polyglutamates of these compounds are much more potent inhibitors of their target enzymes than the monoglutamate derivatives. Aminopterin was a very good substrate for the enzyme with the 4-amino substitution decreasing Km values by about 15-fold compared to PteGlu, while methotrexate was less effective due to an increased Km. Extension of the glutamate chain of methotrexate decreased substrate effectiveness primarily due to a drop in Vmax values, and the triglutamate derivative was an extremely poor substrate.

Organization of the Human FPGS Gene—A Lambda Fix II library was screened as described under “Experimental Procedures,” and three clones were obtained, two of which were found to be identical by restriction enzyme mapping and Southern analysis. One clone (C5, Fig. 3) lacked the 5′ region of the FPGS cDNA. The second clone (C9) overlapped clone C5 and contained an additional 10 kb of 5′ region, but lacked the region corresponding to the 3′ end of the cDNA (Fig. 3). Xbal fragments were subcloned into pBKS+ and intron/exon junctions were sequenced using primers to various regions of the cDNA (Table IV). All intron/exon splice junctions follow the GT-AG rule (49). The XbaI fragments were sequenced in both directions, and the sequence of the gene, with the exception of parts of four intronic regions, was obtained. The human FPGS gene spans about 12 kb and consists of 15 exons and 14 introns (Fig. 3, Table IV). Two sequences, corresponding to the 5′ region of the gene to the 5′ end of intron 4 (accession number U24252) and the 3′ region of intron 4 to the 5′ region of intron 11 (accession number U24253), have been deposited in the GenBank™ data base. The coding sequence of the gene was in agreement with our previously published cDNA sequence (30).

Freemantle et al. (31, U14939) recently reported the sequence of the 5′ genomic region of human FPGS and demonstrated that the originally isolated cDNA lacked 56 bp of coding region, including an upstream ATG which was preceded by Sp1 sites, and that multiple transcription start sites in this region can result in cytosolic and mitochondrial forms of FPGS. Our sequence is in agreement except for some differences in intron 1. These include additional C residues at +160, +167, and +337 (numbered from the ATG codon of the mitochondrial leader sequence) and GC transpositions at +215/216 and +350/351, and G instead of A at +982. An additional 170-bp BamHI fragment (residues +396 to +566) is not present in U14939. This additional region is not a coding artifact as it contains part of an alternate exon 1 identified by 5′-RACE of human mRNA (see below). The C5 clone starts at a Sau3Al (BamHI) site at residue +566.

Differential Splicing and Alternate Exon Usage—5′-RACE analysis of RNA from HepG2 cells using nested antisense primers to exons 3 and exon 2 regions resulted in multiple species of different length. The products were cloned and sequenced, and the longest form obtained for the exon 1 region started at +43 (Fig. 3), which agrees with the transcription start site suggested by Freemantle et al. (22). The sequences of the RACE products were identical with the gene and cDNA sequences except HepG2 mRNA had a G at position +64 instead of an A. Additional alternate exon 1 regions (Fig. 3, Table IV), that were

![Figure 2. Sodium dodecyl sulfate gel electrophoresis of DE52 purified human folylpolyglutamate synthetase. Experimental details are described under “Experimental Procedures.” M₉ (× 10⁻⁹) of protein standards are shown.](image-url)

**Table III**

| Substrate          | Kₘ (µM) | Vₘₐₓ (µmol/h/mg) | Vₘₐₓ/Kₘ (rel) |
|--------------------|---------|------------------|---------------|
| H₂PteGlu           | 59.6    | 66               | 4.9           |
| H₂PteGlu₂          | 16.3    | 83               | 12.3          |
| H₂PteGlu₃          | 20.5    | 56               | 12.3          |
| PteGlu₄            | 12.3    | 34               | 13.3          |
| PteGlu₅            | 64.1    | 1                | 0.07          |
| H₄PteGlu           | 0.81    | 85               | 460           |
| H₄PteGlu₂          | 47.6    | 96               | 8.3           |
| (6G)H₄PteGlu       | 1.6     | 83               | 23.3          |
| (6S)H₄PteGlu       | 4.4     | 100              | 100           |
| (6S)H₄PteGl₂       | 3.3     | 82               | 109           |
| (6S)H₄PteGl₃       | 1.4     | 19               | 60            |
| (6S)H₄PteGl₄       | 1.6     | 10               | 29            |
| (6S)H₄PteGl₅       | 1.4     | 0.6              | 1.9           |
| (6R)-10-formyl-H₄PteGl₂ | 3.7     | 23               | 49            |
| (6R)-10-formyl-H₄PteGl₃ | 2.7     | 30               | 49            |
| (6S)-5-formyl-H₄PteGl₂ | 105     | 85               | 3.6           |
| (6S)-5-formyl-H₄PteGl₃ | 13.6    | 68               | 23            |
| (6S)-5-methyl-H₄PteGl₂ | 48.1    | 83               | 7.6           |
| Aminopterin        | 4.4     | 118              | 118           |
| Methotrexate (Glu-1)| 71.9    | 94               | 5.8           |
| Methotrexate (Glu-2)| 50.6    | 16               | 1.4           |
| Methotrexate (Glu-3)| 148.6   | 3.2              | 0.086         |
| 5-Deazaacyclotetrahydrofolate | 5.3     | 107              | 89            |
| 2-Methyl-5,8-dideazaflolate | 2.8     | 101              | 158           |

*Values are relative to that obtained with (6S)-H₄PteGlu (equals 100). Vₘₐₓ 59 µmol/h/mg, kₘₐₓ 0.99 s⁻¹; kₘₐₓ/Kₘ 0.225 × 10⁻⁶ M⁻¹·s⁻¹.*
distinct from exon 1 and from any previously isolated cDNA sequence, were also detected and, in each case, the 5' sequence of the product obtained (Fig. 3) was identical with a region of the gene and the junction of the 5' region with exon 2 matched consensus splice junctions (Table IV). Several of these species were due to alternate splicing in intron 1 at positions 139. If exon 1B arose by extension of exon 1, and the translation product started at the exon 1 cytosolic ATG (+127), the resulting mRNA would be out of frame with codons in the exon 2 region. However, translation of this variant could start at an alternate ATG at the beginning of exon 2 at position +1373 (Table IV) equivalent to amino acid residue 9 in the cytosolic form of the protein, although this unlikely (see below).

None of the 5'-RACE products obtained for exon 1C, which varied in size, extended as far upstream as exon 1B and the longest form obtained started at +285 (Fig. 3). Primer extension analysis, using primers specific to the exon 1C region (L5C1 and T-1), indicated multiple species with major starts at +330 and +373 and failed to indicate longer species that extended into exon 1B. This region is preceded by potential Sp1 binding sites (+264 to +269 and +356 to +361) and an E2A site (+331 to +338). Exon 1C lacks an in-frame start ATG although translation of this variant could also start at the alternate ATG at the beginning of exon 2 (Table IV).

An additional splice variant encoded an upstream exon (exon 1A, Table IV, Fig. 3) spliced to exon 2. Again, 5'-RACE products of various lengths, with different 5' ends, were obtained, the longest starting at position -213 (Fig. 3). All 5' sequences terminated at residue -160, a consensus splice junction (Table IV). Primer extension analysis using exon 1A specific probes (L5B1, L5B2, Table I) indicated several starts equivalent to positions -288 and -226, assuming no additional splicing in this region (see below).

Exon 1A precedes all the Sp1 binding sites but is preceded by a computer identified Adh1 promoter site (-256 to -251), imperfect TATA sites (-240 to -235, -434 to -429), an APRT promoter site (-469 to -462), and an E-box insulin-responsive element (-456 to -449) (50). Exon 1A does not contain a start ATG, although translation of the longest 5'-RACE product for exon 1A would give a peptide (SPGWCTRKGRLFSGARGL) with characteristics of a mitochondrial leader sequence (51).

In initial 5'-RACE studies, the major transcripts obtained from HepG2 mRNA encoded the exon 1A variant while exon 1 and exon 1C variants predominated in the human fetal liver library. Further 5'-RACE studies indicated all four variants in HepG2 mRNA. Differences in distributions between experiments probably reflected PCR variations. To further investigate the distribution of these variants and to check for other variants, 5'-RACE was performed on HepG2 mRNA using an exon 4 primer (L5) for first strand cDNA synthesis and PCR was performed with an exon 3 antisense primer (L4), but no nested primer was used. The products obtained were cloned into a pGEM-T vector and...
1000 individual colonies of E. coli transformants expressing this vector were screened with a labeled probe composed of the most 3’ 23 bp of exon 1 and the most 5’ 98 bp of exon 2. 548 clones gave a positive signal. The positive clones were then screened with labeled primers L5A1, L5B1, or L5C1 (Table I) which are specific for exons 1 and 1B, 1A, and 1C, respectively. The exon 1/1B probe hybridized with 287 colonies (52%). Nine of these clones were sequenced and seven encoded the exon 1C variant specific primers. Five of these were sequenced. Three extended only 5bp into exon1, while 2 were unspliced at the exon 1 variant. Seventeen percent (92) of the clones recognized by the exon 1/1B probe were negative when probed with the exon 1A variant. Seventeen percent (92) of the clones recognized by the exon 1C probe hybridized to 87 colonies (16%). However, only 3 of 11 clones sequenced encoded the exon 1C variant. The exon 1C probe hybridized with 87 colonies (16%).

Role of Mitochondrial and Cytosolic FPGS Isozymes—FPGS is located in the mitochondria and cytosol of eukaryotic cells and mitochondrial FPGS activity is required for mitochondrial 1-carbon metabolism (23, 29) and for a normal 1-carbon flux in the cytosol.4 In our initial report on the human FPGS cDNA (30), expression of the complete cDNA containing exons 2 to 15, but lacking all exon 1 regions, under the control of an SV40 promoter, also failed to restore growth under these conditions. As synthesis of a functional FPGS from the exon 1B and 1C variants would require translation from an ATG at the start of exon 2 (Table IV), it is unlikely that exon 1B and 1C mRNA variants are translated to produce a functional FPGS.

4 R.-F. Huang and B. Shane, unpublished data.
Subcellular distribution of FPGS activity and folylpolyglutamates in CHO cells

Folate-depleted cells were cultured in deficient α-minimal essential medium containing glycine, thymidine, hypoxanthine, and (6S,5S)-5-formyl-H$_2$[(6H)]pterGlu (10 nM) for 24 h, and subcellular fractions were prepared as described under “Experimental Procedures.”

| Cell   | Folate activity | GDH activity | Polyglutamate chain length distribution |
|--------|-----------------|--------------|----------------------------------------|
|        | pmol/h/mg       | %            | %                                      |
| CHO WTT2a | Cyto 155        | 54           | 26                                     |
|         | Mito 529        | 46           | 74                                     |
| AUX-hFPGScyt1 | Cyto 723       | 96           | 25                                     |
| AUX-hFPGScyt2 | Cyto 3582      | 98           | 99                                     |
| AUX-hFPGSmit  | Cyto 44        | 25           | 27                                     |
|         | Mito 388        | 75           | 73                                     |

a Data for wild type CHO cell from Ref. 29.

were evaluated. Signals were clearly seen on two chromosomes at least one chromosome band 9q34.1 in 40% of cells and at no other sites in greater than one cell.

**DISCUSSION**

Mammalian FPGS enzymes are low abundance and unstable proteins which has hampered their purification or isolation in significant quantities. Overexpression of a FPGS cDNA has allowed the purification of the human enzyme to homogeneity and characterization of its properties. The only other mammalian FPGS enzyme to have been purified, the pig liver protein, has similar general properties to other mammalian FPGS enzymes. The human chromosome 9 ideogram shows the location of the FPGS gene.

![Fig. 4. Fluorescence in situ localization of FPGS gene to human chromosomal region 9q34.1–34.2.](http://www.jbc.org/content/doi/10.1074/jbc.M104.024473)

The enzyme has a pH optimum of approximately 9.4, which is similar to the pH optima of bacterial (55–57), yeast, and pig liver (17) enzymes. Other studies have reported a pH optima of about 8.3 (18, 21). This discrepancy is most likely due to the use of higher concentrations of glutamate in previous assays and
possibly decreased stability of the enzyme in crude extracts at the higher pH. Studies with the pig liver enzyme showed that \( V_{\text{max}} / K_{\text{m}} \) values for glutamate demonstrated a steep pH profile with an optimum at pH 9.4 (17), similar to the pK of the amino group of glutamate, and that the enzyme functioned well at physiological pH provided higher levels of glutamate were provided. The \( K_{\text{m}} \) for glutamate reported here is lower than that reported for extracts from human leukemia cells (54) or liver (21) (0.4 to 1.2 m\( \text{M} \)), which presumably reflects the lower pH values of the assays used in the other studies.

The folate substrate specificity of the human enzyme was qualitatively similar to that reported for the purified pig liver enzyme (32) although some differences were noted from that reported for crude preparations from leukemia cells (54) and human liver (21). This may reflect that the specific activity of the enzyme used in the current study was about 50,000-fold higher than that used in the previous studies (21). The maximum catalytic rate of human FPGS (1 s\(^{-1}\)) is about 40% of that of the pig liver enzyme (32). The unsubstituted reduced folates, H\(_4\)PteGlu and H\(_2\)PteGlu, and aminopterin were the preferred substrates for the enzyme while PteGlu bound less effectively. 10-Formyl-H\(_2\)PteGlu was also a good substrate although with a reduced \( V_{\text{max}} \) while 5-substitution of reduced folate caused a large elevation in \( K_{\text{m}} \). Previous studies have suggested that (65)- and (6ambo)-5-formyl-H\(_2\)PteGlu are very good substrates (\( K_{\text{m}} \) about 5 \( \mu \text{M} \)) for human FPGS while the 6R isomer is a poor substrate (\( K_{\text{m}} \) about 100 \( \mu \text{M} \)) (21, 46). However, the high \( K_{\text{m}} \) obtained for (65)-5-formyl-H\(_{2}\)PteGlu in the current study (105 \( \mu \text{M} \)) is consistent with values obtained for other folates substituted at the 5-position, and also with the value obtained with homogenous pig liver enzyme (32). The lower values obtained by other investigators may have been due to metabolism of (65)-5-formyl-H\(_{2}\)PteGlu to 10-formyl-H\(_{2}\)PteGlu. This conversion is catalyzed by 5,10-methenyltetrahydrofolate synthetase and 5,10-methylene tetrahydrofolate cyclohydrolase, enzymes that would be present in crude tissue extracts. The FPGS reaction mixture contains all the substrates necessary for this interconversion, and this would also explain the poor substrate activity of (6R)-5-formyl-H\(_{2}\)PteGlu.

Studies with model systems have demonstrated that cellular accumulation of folate, which requires its conversion to polyglutamate derivatives of chain length at least three, is dependent on FPGS activity levels with physiological levels of poor substrates for the enzyme and becomes highly dependent on FPGS activity levels with pharmacological levels of all folates (28, 36). Pharmacological doses of 5-formyl-H\(_{2}\)PteGlu are used in combination with thymidylate synthase inhibitors in some chemotherapeutic regimens to elevate tissue folate levels (58). The poor substrate activity of 5-formyl-H\(_{2}\)PteGlu for human FPGS suggests that the ability of tissues to accumulate 5-formyl-H\(_{2}\)PteGlu will be highly dependent on the level of FPGS activity. In addition, 5-formyl-H\(_{2}\)PteGlu accumulation may also be dependent on the level of 5,10-methenyltetrahydrofolate synthetase activity, which metabolizes this folate to 5-formyl-H\(_{2}\)PteGlu, a more effective substrate for FPGS. This enzyme has a \( K_{\text{m}} \) of about 1 \( \mu \text{M} \) for 5-formyl-H\(_{2}\)PteGlu (59) and would be operating under \( V_{\text{max}} \) conditions when pharmacological levels of 5-formyl-H\(_{2}\)PteGlu are provided.

The specificity for folypolyglutamate substrates is qualitatively similar to that reported for the pig liver enzyme (32) except that diglutamate derivatives, with the exception of H\(_{2}\)PteGlu\(_{2}\), compared to their respective pteroylmonoglutamates, tended to be somewhat better substrates. This was most pronounced for 5-formyl-H\(_{2}\)PteGlu\(_{2}\), which exhibited a 7-fold decrease in \( K_{\text{m}} \) compared to the monoglutamate. \( V_{\text{max}} \) values fell beyond the diglutamate and H\(_{2}\)PteGlu\(_{2}\), derivatives were the most effective polyglutamate substrates.

The 4-amino substitution of pteroylmonoglutamate increases its substrate effectiveness for mammalian FPGS but greatly impairs substrate activity with di- and longer polyglutamate derivatives (60–62). Methotrexate is a fairly poor substrate for mammalian FPGS enzymes and activity drops significantly with extension of the polyglutamate chain. The major drop in activity for the pig liver and CHO enzymes occurs at the diglutamate. However, activity falls off less sharply with the human enzyme and the large fall off in activity occurs at the triglutamate.

The improved substrate activity of the diglutamate makes human cells particularly sensitive to methotrexate. Methotrexate accumulation by mammalian cells, which involves metabolism to at least the triglutamate, and its cytotoxic efficacy are very sensitive to the level of FPGS activity (13). CHO cell transfectants expressing human FPGS activity accumulate methotrexate more effectively than wild type CHO cells expressing the same levels of CHO FPGS activity and are more sensitive to the antifolate (13).

AUX-hFPGS\(_{\text{cot}}\) transfectants, which expressed human FPGS activity solely in the cytoplasm, were unable to accumulate folate in the mitochondria and remained glycine auxotrophs. These data support our earlier findings that mitochondrial folate accumulation is dependent on mitochondrial FPGS activity and that the defect in glycine biosynthesis was due to a lack of mitochondrial folate despite the presence of cytoplasmic folates (23, 29). AUX-hFPGS\(_{\text{mit}}\) transfectants, which appeared to express FPGS activity solely in the mitochondria, also contained long chain cytosolic folypolyglutamates and were prototrophic for thymidine and purines, which are synthesized in the cytosol, as well as glycine. These results mirrored our observations with transfectants expressing E. coli FPGS in the mitochondria of AUXB1 cells (AUX-mcoli) (14, 29).

Pulse-chase studies with AUX-mcoli and wild type CHO cells indicated a slow release of folypolyglutamates from the mitochondria to the cytosol of these cells, and this was more pronounced in the AUX-mcoli transfectants, which synthesize shorter polyglutamate species (predominantly triglutamate) than wild type cells. Although the possibility that AUX-hFPGS\(_{\text{mit}}\) transfectants express trace levels of human FPGS in the cytosol can not be totally eliminated, previous studies suggest that cytosolic metabolism of folate to polyglutamates cannot account for the cytosolic folate pool in AUX-hFPGS\(_{\text{mit}}\) cells. AUXB1 cells transfected with human genomic DNA and expressing human FPGS at the trace levels that could be present in AUX-hFPGS\(_{\text{mit}}\) cells (36) contain folypolyglutamates that are of shorter chain length than was found in this study.

The proportion of cellular folate in the cytosol of AUX-hFPGS\(_{\text{mit}}\) transfectants was much lower than in AUX-mcoli transfectants, ranging from about 4 to 8% of total cell folate after adjustment for mitochondrial folate contamination (as judged by glutamate dehydrogenase distributions), and was of shorter glutamate length than mitochondrial folate, suggesting that efflux of polyglutamates from the mitochondria shows a preference for the shorter chain length species. About 60% of folate in wild type CHO cells is cytosolic. Although expression of mammalian FPGS in the mitochondria is sufficient to generate a cytosolic folypolyglutamate pool and to allow cytosolic 1-carbon metabolism, expression of a cytosolic FPGS is required for the generation of a normal folate pool in the cytosol.

The FPGS gene was mapped to chromosome region 9q34.1 and no additional chromosomal signals, suggestive of a second closely related gene, were observed, and the reconstructed FPGS gene restored AUXB1 cells to the wild type phenotype. Reversion frequencies of the AUXB1 mutant are also consistent with a single genetic mutation causing the multiple auxotrophy
The organization of the 5' region of the human gene is in general agreement with that described recently by Freemantle et al. (31). The exon 1 variant contains two ATG codons and alternate transcription start sites in this region generate mRNAs encoding mitochondrial and cytosolic FPGS isoforms (31). However, we have identified two additional splice variants (exons 1B and 1C) that arise by alternate splicing in the intron 1 region and an additional variant (exon 1A) that is transcribed by a promoter upstream of the exon 1 promoter region. All variants are spliced to exon 2.

The exon 1C variant is not an extended form of exon 1, and primer extension and RACE analyses suggested multiple transcription start sites in the intron 1 region. This region contains a number of potential promoter sites including Sp1 binding sites. Preliminary studies suggest that exon 1B and 1C variants are unlikely to generate functional FPGS. Exon 1A is preceded by a number of potential promoter sites including a binding site for CTF/NF-1 (CCAAT-binding transcription factors, nuclear factor 1) with the consensus sequence GCCAAT (52) and an E-box, generally represented by the sequence CAnNTG. We have not yet identified the transcription start site(s) of exon 1A, and the possibility of further upstream introns cannot be excluded. The deduced sequence of the peptide encoded by exon 1A is characteristic of a mitochondrial leader sequence and exon 1A may encode an additional mitochondrial isoform of FPGS.

In our original cloning of a human FPGS cDNA from an Epstein-Barr virus-transformed lymphobocyte library, we obtained four clones, all of which encoded the exon 1 variant (30). As the cDNAs were cloned by functional complementation of an E. coli FPGS mutant, this may suggest that the other splice variants may not encode functional protein or may reflect the absence of these variants in the library used. However, it should be noted that we did not obtain any exon 1 variants with a complete mitochondrial leader sequence and, if any of the variants encode additional mitochondrial isoforms, it is probable that they would not be isolated by the complementation cloning procedure used. A single 2.5-kb band was observed in Northern analyses of HepG2 and MCF-7 cell mRNA using the human FPGS cDNA as a probe. However, the other exon 1 variants would be of similar expected size (within 0.1 kb) and would not be distinguished by Northern analyses. We have shown, using exon 1 variant specific probes, that the exon 1C variant is a major species (2.4 kb) in MCF-7 cells.

Decreased FPGS activity has been identified as a mechanism for cellular resistance to antifolates (5, 13–16, 64). However, the decreased FPGS activity is not always accompanied by decreases in FPGS mRNA levels (65). Although this may reflect modulation of translation rates or the presence of a mutation in the mRNA, this could also be due to down-regulation of a functional mRNA species being masked by the presence of variant mRNAs which do not encode active FPGS. Future studies on the regulation of FPGS activity should quantitate individual mRNA variants before conclusions on the absence of transcriptional control are reached. If some of the different mRNA species encode functional FPGS, this may explain the report of differences in substrate specificity for enzyme from different tissues of the same animal (24). We are currently investigating whether the different splice variants observed in this study are translated and whether they encode functional FPGS.

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Purification and Properties of Human Cytosolic Folylpoly-γ-glutamate Synthetase and Organization, Localization, and Differential Splicing of Its Gene
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