Expression of Escherichia coli Folypolyglutamate Synthetase in the Chinese Hamster Ovary Cell Mitochondrion

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Chinese hamster ovary (CHO) cell transfectants expressing Escherichia coli folypoly-γ-glutamate synthetase (FPGS) activity solely in their cytosol lack mitochondrial folypolyglutamates and are auxotrophic for glycine. Addition of a mammalian mitochondrial leader sequence targeted E. coli FPGS to the mitochondria of these cells. Mitochondrial expression of FPGS restored mitochondrial folypolyglutamate pools and overcame the glycine requirement. Pteroyltriglutamates functioned as effectively as the longer glutamate chain length folates found in wild type CHO cells in the metabolic cycle of glycine synthesis provided they were located in the mitochondria. Although folypolyglutamates cannot enter the mitochondria, mitochondrial folypolyglutamates can be released without prior hydrolysis and CHO transfectants expressing E. coli FPGS activity solely in the mitochondria possessed normal cytosolic folypolyglutamate pools. The proportion of cellular folate in the mitochondrion is governed by competition between mitochondrial and cytosolic FPGS activities.

Folate coenzymes act as donors and acceptors of one-carbon units in a series of interconnected metabolic cycles involving thymidylate and de novo purine biosynthesis, methionine biosynthesis and methyl group catabolism, serine and glycine interconversion and catabolism, and the catabolism of histidine and formate (1). The major forms of folate in tissues are polyγ-glutamate derivatives and intracellular metabolism of transported pteroylmonglutamates to polyglutamates is required for their cellular retention and for the formation of the active coenzymatic species. The major folate derivatives in wild type Chinese hamster ovary (CHO) cells are hexa- and heptaglutamate derivatives (2). AUXB1, a CHO mutant lacking folypolyglutamate synthetase (FPGS) activity, contains only pteroylmonglutamates, is defective in folate accumulation and is auxotrophic for products of one-carbon metabolism such as glycine, thymidine, and purines (3–6).

AUXB1 transfectants (AUX-coli) expressing the Escherichia coli FPGS gene (folIC) metabolize folates primarily to the triglutamate derivative (2). These triglutamates were retained by the cell as effectively as the longer polyglutamate derivatives normally found in CHO cells (2) and also appeared to function as effectively in supporting the metabolic cycles of thymidine and purine synthesis (6). However, AUX-coli remained auxotrophic for glycine while glycine synthesis was restored in AUXB1 transfectants expressing human FPGS (AUX-human) and containing folypolyglutamates of chain length ranging from four to eight (6). Further studies showed that WTT2 cells and AUX-human transfectants contained mitochondrial and cytosolic FPGS activity and that a significant proportion of cellular folate was located in the mitochondria of these cells, while AUX-coli, which only expresses a cytosolic FPGS activity, lacked mitochondrial folates despite possessing a normal cytosolic folate pool (7). The role of mitochondrial folate metabolism is poorly understood. Mammalian cells possess cytosolic and mitochondrial isozymes of serine hydroxymethyltransferase, the enzyme responsible for glycine synthesis, and loss of the mitochondrial activity results in a glycine auxotrophy (8, 9). The glycine-requiring phenotype of AUX-coli may have been due to a lack of mitochondrial folate rather than an inability of pteroyltriglutamate to support glycine synthesis.

The current study describes the development and characterization of model CHO cells for evaluating the role of mitochondrial FPGS activity and mitochondrial folate metabolism in the regulation of whole cell one-carbon metabolism. CHO cells expressing E. coli FPGS in the mitochondria were obtained by transfecting AUXB1 and AUX-coli cells with a modified folIC gene (mfolIC) encoding the mitochondrial leader sequence of human ornithine transcarbamoylase fused to the coding region of FPGS.

EXPERIMENTAL PROCEDURES

Materials

L-(U-14C)Glutamic acid (270 mCi/mmol) was obtained from Amer sham. [5,7,9-3H]Polic acid (PteGlu, 20 Ci/mmol). (6S)-[1H]Folic acid (5-formyl-H1[H]PteGlu, 40 Ci/mmol), and (6S)-[3H]-5-methyl-tetrahydrofolate (5-methyl-H[3H]PteGlu, 20 Ci/mmol) were obtained from Moravek Biochemicals. Dihydrofolate reductase (H2Pte), PteGlu-1, and PABAglu-1 were prepared as described previously (10, 11). PteGlu, amino acids, nucleosides, nucleotides, 4-chloro-1-naphthol, and vitamins were from Life Technologies, Inc, Vega, and Sigma. Restriction endonucleases, calf intestinal alkaline phosphatase, T4 DNA ligase, DNA polymerase I (Klenow fragment), hygromycin B (hyg), RNase (bovine pancreas, DNase-free), penicillin (pen), streptomycin (strept), trypsin, and soybean trypsin inhibitor were from Life Technologies, Inc and Boehringer Mannheim. Rabbit anti-E. coli FPGS antibody was prepared by Dr. Al Scott, Johns Hopkins University. Affinity-purified goat

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Fig. 1. Mammalian expression vectors used for transfection of CHO AUX-coli and AUXBl cells. Details are given under "Experimental Procedures.

anti-rabbit IgG (heavy and light chains specific, peroxidase-conjugated) was obtained from Jackson ImmunoResearch Laboratories. Peroxidase was obtained from Pharmacia LKB Biotechnology, Inc., and aliquots (40 ml) were stored in sterile polyethylene tubes (50 ml) at 4°C.

Cell Lines and Culture

Deficient medium (DMEM), a modification of a minimal essential medium lacking nucleosides, nucleotides, glycine, and folic acid, was supplied by JRH Biosciences. α-MEM normally contains glycine (600 µM) and folic acid (2.2 µM). DMEM contained methionine (100 µM), serine (240 µM), and vitamin B₁₂ (1 µM). Fetal bovine serum (FBS) was obtained from HyClone. Dialyzed FBS (dFBS) was prepared by dialysis at 4°C for 18 h against 6 × 10 volumes of sterile phosphate-buffered saline. DMEM was supplemented with 10% dialyzed serum (DMEM/dFBS). Media were also supplemented with combinations of glycine (G, 660 µM), hypoxanthine (H, 36 µM), thymidine (T, 36 µM), and/or PteGlu as indicated. Antibiotics (pen: 106 units/ml, strep: 0.1 µg/ml) were only used during transfection experiments when addition of nonsterile solutions to cultures was unavoidable, as indicated.

Wild type CHO cells (WT2) were obtained from Dr. Sharon Krag, Johns Hopkins University. Mutant CHO cells (AUXBl), which lack the ornithine transcarbamoylase mitochondrial leader coding sequence (3–6), were obtained from Dr. Victor Ling, Ontario Cancer Institute, Toronto. OxyB, a glycine-requiring CHO mutant (12), was obtained from Dr. L. Thompson, Lawrence Livermore National Laboratory. The generation of AUX-coli (D5–3A8) and D1–1A10, AUXBl transfectants expressing high and low E. coli FPGS activity solely in the cytosol and/or mitochondria, has been described previously (2). Cells were routinely cultured in DMEM/FBS + GHT under a water-saturated 5% CO₂ atmosphere in a 37°C incubator and were routinely passaged when they neared confluence as described previously (2, 6). Cell number was determined using a Coulter counter.

Mammalian Expression Vectors

pSV2 mammalian expression vectors, which were originally developed by Southern and Berg (13), were used in this study (Fig. 1). Their construction is briefly described below. Plasmids pSV2-β-globin (4.7 kb) (13) and pSV2-BamI-β-globin, a derivative of pSV2-β-globin with a BamHI linker inserted at the PvuII site, were obtained from Dr. Rebecca Dickstein, Johns Hopkins University. Plasmid pSVOTC2 (4.3 kb, amp) containing a human ornithine transcarbamoylase cDNA with a single BamHI site in the ornithine transcarbamoylase mitochondrial leader coding sequence (14, 15), was obtained from Dr. Arthur Horwich, Yale University. Plasmid pSP72 was from Promega. pLV99 (9.9 kb, a yeast β-globin cDNA plasmid containing a BamHI linker at the 3′ end of the cDNA) was obtained from Dr. Bill Sugden, McArdle Laboratory for Cancer Research, University of Wisconsin. pT719U, a derivative of pUC19 containing a fl phage replication origin, was obtained from Bio-Rad.

Large and small scale plasmid isolations from alkaline lysates were performed as described by Sambrook et al. (18). Restriction endonuclease, phosphatase, DNA polymerase I (Klenow fragment), RNase, and ligase treatments were carried out according to the manufacturers' instructions. Plasmids and restriction fragments were sized on agarose (0.8–1.5%) gels and visualized with ethidium bromide under ultraviolet light. Restriction fragments were isolated from agarose gel slices using GeneClean (Bio-101). Recombinant plasmids were isolated after transformation of E. coli strain RLM 569 (tr m’, obtained from Dr. Roger McMacken, Johns Hopkins University). Transformants were selected by antibiotic resistance. Oligonucleotide primers were synthesized by the Micro-Chemical Facility (University of California, Berkeley).

Construction of folC Gene with Mitochondrial Leader Sequence (mfolC)—A 1.4-kb HindIII-BamHI fragment from plasmid pAC5 (19), which contains the coding region of the E. coli folC gene (GenBank accession number J02808), was subcloned into HindIII-BamHI-treated pT719U and transformed into E. coli MV1101 (Bio-Rad) to generate pT719-folC (4.3 kb, amp). Single-stranded DNA was produced using helper phage M13K07, and an EcoRV cleavage site was generated between upstream nucleotides –3 and –4 (from start ATG, Fig. 2) by a C to T mutation at base –2 (20) to generate plasmid pT719-folCTR. DNA was sequenced by the method of Sanger et al. (21) using Sequenase 2 (U. S. Biochemical Corp.).

A HindIII-PvuII fragment (0.26 kb) of pSVOTC2, containing the cDNA for the leader sequence region of ornithine transcarbamoylase, was ligated into HindIII-EcoRI-treated pT719-folCTR (pT719-folCTR-4.4 kb) and isolated. This plasmid contains the folC gene with the ornithine transcarbamoylase leader peptide and sequence up to its amino-terminal sequence (folC, Fig. 2). A HindIII-EcoRI fragment of pT719-molC containing the molC gene was isolated and cloned into similarly treated pSP72 to generate pSP72-molC (3.99 kb), which contains the molC gene followed by a downstream BglII site.

Cloning of Hygromycin-resistance Gene in Mammalian Expression Vectors—A 0.95-kb fragment containing the E. coli hyg gene was obtained by BamHI digestion of pLV99. The β-globin gene was removed from pSV2-BamI-β-globin by HindIII-BglII digestion, and the remaining fragment (4.2 kb) and the hyg fragment were treated with phosphatase and blunt-ended with DNA polymerase I (Klenow fragment). Ligation of the two blunt-ended fragments resulted in plasmid pSV2-hyg (5.13 kb, Fig. 1) with elimination of the HindIII, BglII, and BamHI cloning sites. The orientation of the inserted hyg gene was checked by fragment sizes after digestion with EcoRI and BamHI.

Cloning of molC Gene with Mitochondrial Leader Sequence—pSV2–72 was constructed by ligation of HindIII-BglII-digested pSP72 (2.4 kb) to similarly treated pSV2-β-globin (4.2 kb). This removed the β-globin gene with its internal BamHI site from pSV2-BamI-β-globin and resulted in a plasmid with a single BamHI site which simplified further subcloning. A BamHI fragment (2.15 kb) of pSV2-hyg, containing all the elements required for expression of the hyg gene in mammalian cells (SV40 promoter, polyadenylation signal, and t-splice site) was ligated to BamHI and calf intestinal alkaline phosphatase-treated pSV2–72 to generate pSV2–72-hyg (8.75 kb).

The pSP72 stuffer fragment (2.4 kb) of pSV2–72-hyg was removed by HindIII-BglII digestion, and the remaining hyg-containing fragment (6.36 kb) was ligated to the molC fragment gene from HindIII-BglII digest of pSP72-molC to give pSV2-hyg-molC (7.92 kb, Fig. 1), which contains both the molC and hyg genes. A plasmid containing only the molC gene in a mammalian expression vector (pSV2-molC, 5.8 kb, Fig. 1) was made by ligation of HindIII-BglII-digested pSP72-molC to similarly treated pSV2-BamI-β-globin.

Transfection of CHO Cells

AUX-coli and AUXBl cells (2 × 10⁸) were plated on 60-mm dishes and cultured for 24 h in DMEM/FBS + GHT. Duplicate plates were then transfected with pSV2-hyg, pSV2-molC, or pSV2-hyg-molC (5 µg of DNA) or mock-transfected, by a modification (22) of the calcium phos-
plate precipitation method of Graham and van der Eb (23) as described previously (2). Following transfection and glycercol shock, the cells were rinsed once with 5 ml DMEM/dPBS + GHT + pen/strep and then incubated in the same medium (5 ml).

At 48 h after transfection, the remaining cells were transfected to duplicate 100-mm plates containing selective media (10 ml) consisting of DMEM/dPBS supplemented with GHT + hygromycin (0.4 mg/ml), HT + PteGlut (1 μM), or HT + PteGlut + hygromycin. After 8 days (with media changes at 4 days), one plate from each duplicate was fixed with methanol and stained with methylene blue, and the number of colonies were counted. Six distinct colonies from each remaining plate were transfected, using a cloning cylinder, to separate 35-mm dishes containing the same selective media, and the selection was continued for a further 8 days. Cells were then resuspended and diluted in selective media, and aliquots, equivalent to less than one cell per well, were transferred to 96-well microtiter plates. Colonies resulting from single cells were transferred after 7 days to selective media in 35-mm dishes. The transfectants were selectively cultured to near confluence in transition from 35-mm to 60-mm to 100-mm plates (approximately 1 month). Individual clones were then routinely passaged in DMEM/dPBS + GHT or stored at -80°C.

Cell Growth in Media Lacking Products of One-Carbon Metabolism

As an initial screen of the transfectants, near-confluent folate-depleted cells maintained in DMEM/dPBS + GHT were detached with trypsin and resuspended, and an aliquot was counted. Following centrifugation and resuspension in test media, duplicate aliquots of 5 x 10⁵ cells were plated on 6-well dishes in test media consisting of DMEM/dPBS supplemented with or without GHT, HT, or G. After 3 days incubation at 37°C, cells were detached with trypsin, resuspended in PBS + 0.02% trypsin inhibitor, and counted.

The folate requirement of transfectants cultured in media lacking products of one-carbon metabolism was assessed as described previously (6). Folate-depleted cells cultured in DMEM/dPBS + GHT medium were resuspended in various test media and replated at 1 x 10⁵ cells per 60-mm dish. The test media were DMEM/dPBS + GHT in which either G, H, or T was omitted and the media were supplemented with various concentrations of PteGlut (0–100 μM). Duplicate sets of plates were removed for cell counting at 24-h intervals, and media were replaced in the remaining plates at 48-h intervals. Cell doubling times were calculated by best fitting exponential growth curves to the data.

Assay of FPGS

FPGS activity in cell and subcellular extracts was measured in duplicate as described previously (2). Assay mixtures (0.5 ml) contained: 100 mM Tris/50 mM glycine buffer (pH 9.3), KCl (20 mM or 200 mM), diethiothreitol (5 mM), β-mercaptoethanol (10 mM); derived from the subcellular fraction, bovine serum albumin (50 μg), MacI (10 mM), ATP (5 mM), [methyl-3H]glutamate (500 μM; 1.25 μCi), H3Pte (50 μM) or (6-amino-13H)PteGlut (50 μM), extract (100 μl), and Triton X-100 (0.1%, mitochondrial fractions). The reaction mixtures were incubated at 37°C for 1 h. Reactions were stopped by the addition of 1.5 ml of ice-cold 30 mM β-mercaptoethanol. Labeled products were separated from glutamate on DEAE-cellulose columns. Unless indicated otherwise, mammalian FPGS activities reported in this paper were assayed using H3PteGlut and 200 mM KCl. In some experiments, 2 mM glutamate was used.

Cellular Uptake and Metabolism of Folate

Folate-depleted cells, cultured for several weeks in DMEM/dPBS + GHT medium, were replated in the same medium at 5 x 10⁶ cells per 100-mm dish or 5 x 10⁵ cells per 150-cm² flask (6 subcellular fractionation). After culturing for 24 h, the medium was replaced with the same medium containing various concentrations of (6H3)PteGlut, 5-formyl-H3PteGlut, or 5-methyl-H3PteGlut (2 mM-20 μM) with duplicate samples for each concentration. After the labeling period (usually 24 h), the cells were washed with ice-cold PBS (4 x 5 ml) and 0.1% trypsin in PBS (0.5 ml) was added (room temperature, 22°C). When the cells had detached from the plate (1-2 min), they were resuspended in cold PBS containing 0.01% soybean trypsin inhibitor (10 μl). Aliquots of the suspension were removed for counting cell number and total radioactivity. The remaining suspension was centrifuged at 200 x g for 5 min, and labeled folate in the cells extracted or subcellular fractions were prepared as described below. Polyglutamate chain length distributions were determined as described previously (2).

Subcellular FPGS and Folate Distributions

CHO cells were cultured as described previously in 150-cm² flasks (2, 6, 7). The monolayers were washed twice with ice-cold PBS (2 x 10 ml) and dislodged with 0.1% trypsin-EDTA. Cells were transferred with cold PBS containing 0.01% trypsin inhibitor (2 x 5 ml) to 50-ml plastic conical tubes. Aliquots were removed for cell counting, and the remaining cells were pelleted by centrifugation (500 x g, 10 min). Cell pellets were washed once with cold PBS (10 ml) and then resuspended in 10 ml of homogenization buffer (0.25% sucrose, 1% EDTA, pH 6.9), and aliquots were removed for assessing labeled folate accumulation. The remaining cells were homogenized, and crude subcellular fractions were prepared by differential centrifugation as described previously (7). Organelle marker enzymes were assayed as described previously (7).

Identification of the mfolC Gene Product by Western Blotting

Mitochondrial and cytosolic fractions of AUXB1 transfectants were prepared by differential centrifugation. The cytosolic fraction was concentrated using a microconcentrator (Centricron 10, Amicon). Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was carried out using a 5% stacking gel and a 10-15% gradient separating gel in a slab gel apparatus using the discontinuous buffer system of Laemmli (24). Proteins were transferred to nitrocellulose in a TransBlot Semi-Dry apparatus (Bio-Rad) at 1°C for 1 h at 15 V, according to the manufacturer's instructions.

Further manipulations were carried out at room temperature in a plastic dish on an oscillating rocker. The nitrocellulose was blocked for 1 h using PBS containing 3% non-fat milk. Anti-FPGS serum (16 μg/ml) was added to the blocking solution and allowed to adsorb for 12 h at 4°C. The blot was then washed four times with 25 ml of PBS + Tween 20 (0.05%) for 15 min each. Peroxidase-conjugated antibody was added at a 1:2000 dilution in blocking solution and allowed to adsorb for 2 h, and the blot was washed as before. The blot was then rinsed four times with 25 ml of PBS (no Tween), and 4-chloro-1-naphtol (0.6 mg/ml) in PBS containing 20% methanol and 0.02% H2O2 was added. Color development was stopped after 20 min by rinsing the blot with deionized water.

Protein Assay

Protein concentration was measured by a deoxycholate-trichloroacetic acid precipitation modification of the Lowry procedure (25) using bovine serum albumin as the standard.

RESULTS

Transfection of AUX-coli and AUXB1 Cells with the mfolC Gene—To investigate the role of mitochondrial FPGS activity on folate metabolism, AUX-coli, which express E. coli FPGS activity solely in the cytosol, and AUXB1 cells, which lack FPGS activity, were transfected with the E. coli mfolC gene containing a leader peptide ligated to its amino-terminus (mfolC). If the mfolC product is transported into the mitochondria and the ornithine transcarbamoylase leader sequence is processed normally, the mitochondria of the transformants should contain a modified E. coli FPGS protein with an additional five amino acids added at the amino terminus (Fig. 2). As it was not known whether the mfolC construct would be expressed in CHO cells or if the protein would be transported into mitochondria and expressed in an active form, nor whether the expected glutamate products would support mitochondrial glycin synthase, various plasmids (Fig. 1) were used to allow for selection by phenotypic complementation (+G + HT + PteGlut (1 μM) medium) and/or by antibiotic resistance (+hygromycin). G418 resistance could not be used as AUX-coli cells express the neo gene (2).

Transfection efficiencies were assessed after an 8-day culture under selective conditions. In AUX-coli and AUXB1 cells transfected with plasmids containing the hsg gene and selected in +GHT + hygromycin medium, colonies appeared at a rate of 0.1-1.3 x 10⁷ per transfected cell which is lower than efficiencies obtained previously with AUXB1 cells transfected under
similar conditions (2), and no colonies were observed in -glycine medium. Cells transfected with plasmids containing the mfolC gene and selected in -G + HT + PteGlu medium demonstrated similar transfection frequencies (0.2-0.4 x 10^5). The rate was slightly lower (0.1-0.2 x 10^5) when AUX-coli and AUXB1 cells transfected with pSV2-hyg-mfolC were subjected to double selection in -G + HT + PteGlu + hygromycin medium. The numerous colonies obtained in -glycine medium after transfection with plasmids carrying the mfolC gene suggested that the gene was expressed and active product might be present in the mitochondria. No colonies were observed with mock-transfected cells in -glycine (reversion frequency of less than 10^-5) or plus hygromycin medium.

**Growth of Transfectants**—Growth rates and nutritional requirements were initially assessed for 32 of the cloned transfectants. These transfectants were selected under -G or -G + hyg conditions and then cultured and cloned for several months under nonselective conditions. Ten out of fourteen AUXcoli-mcoli transfectants, AUX-coli cells transfected with pSV2-hyg-mfolC or pSV2-mfolC, retained the ability to grow in medium supplemented with PteGlu (1 µM) and lacking glycine with growth rates ranging from 14 to 125% that of WTT2. AUX-coli did not grow in this medium. As expected, all AUXcoli-mcoli transfectants grew in medium supplemented with glycine and folate but lacking purines and thymidine as did the parent AUX-coli line. All 18 AUX-mcoli transfectants, AUXB1 cells transfected with pSV2-hyg-mfolC or pSV2-mfolC, retained the ability to grow in medium lacking glycine with growth rates ranging from 13 to 84% of that of WTT2. Surprisingly, these transformants also grew in medium lacking purines and thymidine with growth rates ranging from 17 to 77% of that of WTT2 while AUXB1 was unable to grow under these conditions. All cells transfected with pSV2-hyg-mfolC were able to grow in the presence of hygromycin regardless of whether they were originally selected in -G or -G + hyg medium although the fastest growth rates were observed in transfected initially selected in -G + hyg medium.

**Subcellular Distribution of FPGS Activity in Transfectants**—With the exception of one cell line, all transfecteds expressed enzyme activity with characteristics of E. coli FPGS. H_Pte was a substrate (dihydrofolate synthetase activity), and enzyme activity was increased at 230 mM versus 20 mM KCI (2, 26). One cell line expressed FPGS activity with characteristics of mammalian enzyme and lacked dihydrofolate synthetase activity and was probably a revertant. Two transfectants of each class were chosen for more detailed study. The morphology of AUXcoli-mcoli-1 and -2 and AUX-mcoli-1 appeared identical with AUX-coli, and these cells appeared slightly larger than WTT2 cells. AUX-mcoli-2 was smaller than the other transfectants and grew at a faster rate.

FPGS activities are shown in Table 1. FPGS activity was higher in transfectants with H_Pte compared to H_PteGlu as a substrate, when assayed under standard conditions, and enzyme activity was significantly increased by increased KCI (200 mM) and glutamate (2 mM), which are characteristic properties of the E. coli protein (2, 26). WTT2 cells lacked activity with H_Pte as a substrate. As shown previously (7), enzyme activity was approximately equally distributed between the mitochondrial and cytosolic fractions in WTT2 cells, and the mitochondrial enzyme was of higher specific activity, while AUX-coli only contained cytosolic enzyme. All AUXcoli-mcoli transfectants expressed E. coli FPGS of very high specific activity in their mitochondria, and the mitochondrial fraction accounted for 50 to 70% of total cell enzyme activity. AUX-mcoli transfectants also expressed E. coli FPGS in their mitochondria but of lower specific activity than in the AUXcoli-mcoli transfectants. Some FPGS activity was found in the cytosolic fraction from AUX-mcoli transfectants, but the specific activity was very low and the distribution of enzyme activity was similar to that of the mitochondrial marker enzyme glutamate dehydrogenase. Similarly, the specific activity of FPGS was increased in the cytosolic fraction from AUXcoli-mcoli transfectants compared to AUX-coli, but the increase was consistent with mitochondrial enzyme contamination of this fraction. Although these data cannot eliminate the possibility of trace levels of expression of cytosolic FPGS from the mfolC construct, practically all, if not all, of the active FPGS resulting from expression of this gene in the transfectants was located in the mitochondria.

Western analyses of AUXcoli-mcoli transfectants using antibody to E. coli FPGS indicated the presence of two mitochondrial FPGS protein bands of similar intensity and differing in size by about 1 kDa (data not shown). The smaller size band appeared identical in size with the single bands observed with E. coli protein. The larger size band may have been the unprocessed precursor protein but its size suggested that it represented a processing intermediate (27). The two mitochondrial protein bands were also detected in AUX-mcoli transfectants, but no cytosolic protein was observed with these cells. These data also suggest little, if any, cytosolic FPGS in AUXcoli-mcoli transfectants and eliminate the possibility that accumulation of a partially active unprocessed precursor protein in the cytosol might result in some cytosolic FPGS.

**Uptake and Subcellular Distribution of Folate by Transfectant Cells**—Cells were cultured in medium containing a physiological level of [3H]folinate (7 nM) for 24 h, and postnuclear supernatants from the CHO cells were fractionated on a self-forming Percoll density gradient (Fig. 3). Labeled folate from WTT2 cells (not shown), AUXcoli-mcoli, and AUX-mcoli cells was associated with heavy and light mitochondrial fractions and with the cytosolic fraction, while no organellar peaks were observed with AUXcoli extracts (7). All transfecteds metabolized folinate primarily to the triglutamate, the characteristic major end product of E. coli FPGS, and expression of E. coli FPGS in the mitochondria of AUXcoli-mcoli and AUX-mcoli restored the ability of the cells to accumulate mito-
glutamate, and 20 mM KCl, except where noted. Values are shown for two separate experiments and are the means of duplicate assays. As described under "Experimental Procedures." FPGS and glutamate dehydrogenase (GDH) activity in mitochondrial (Mito) and cytoplasmic (Cyto) fractions were measured as described under "Experimental Procedures." FPGS assay mixtures contained 50 μM H4PteGlu or H2Pte, 500 μM glutamate, and 20 mM KCl, except where noted. Values are shown for two separate experiments and are the means of duplicate assays.

**Fig. 4.** The concentrating ability of the cells is expressed as the concentration of 1 mmol/l h/mg. CHO cells were cultured in +GHT medium, and subcellular fractions were prepared by nitrogen bomb cavitation and differential centrifugation as described under "Experimental Procedures." FPGS and glutamate dehydrogenase (GDH) activity in mitochondrial (Mito) and cytoplasmic (Cyto) fractions were measured as described under "Experimental Procedures." FPGS assay mixtures contained 2 mM glutamate and 200 mM KCl.

**Fig. 3.** Percoll density gradient distribution of folate in postnuclear supernatant of CHO cell transfectants. AUXcoli-mcoli-1 (O—O) and AUX-mcoli-1 cells (—●—●) were incubated in DMEM/dFBS + GHT medium containing [3H]folinic acid (7 nM) for 24 h. Subcellular fractionation, Percoll density gradient centrifugation, and enzyme assays were carried out as described under "Experimental Procedures." M indicates the heavy and light mitochondrial fractions, as indicated by glutamate dehydrogenase activity.

**Table I.** Subcellular distribution of folypolyglutamate synthetase activity in CHO cell transfectants

| Cell        | Fraction | FPGS specific activity | Enzyme distribution | FPGS | Enzyme distribution |
|-------------|----------|------------------------|---------------------|------|---------------------|
|             |          | H4PteGlu | H2Pte | % | pmol/h/mg | % | pmol/h/mg |
| WTT2        | Mito     | 529      | 0     | 46 | 674      | 0 | 89 |
|             | Cyto     | 155      | 0     | 54 | 266      | 0 | 67 |
| AUX-coli    | Mito     | 5        | 79    | 98 | 27       | 2,241 | 97 | 71 |
|             | Cyto     | 52       | 415   | 60 | 68       | 22,870 | 52 | 87 |
| AUXcoli-mcoli-1 | Mito     | 643      | 10,020| 40 | 32       | 3,068 | 48 | 13 |
|             | Cyto     | 237      | 968   | 63 | 77       | 10,240 | 72 | 85 |
| AUXcoli-mcoli-2 | Mito     | 426      | 6,570 | 37 | 23       | 575 | 28 | 15 |
| AUX-mcoli-1 | Mito     | 198      | 958   | 74 | 73       | 1,230 | 80 | 90 |
|             | Cyto     | 18       | 47    | 26 | 27       | 44 | 20 | 16 |
| AUX-mcoli-2 | Mito     | 145      | 1,581 | 76 | 74       | 4,140 | 86 | 84 |
|             | Cyto     | 45       | 77    | 24 | 26       | 103 | 14 | 16 |

*Assay mixtures contained 2 mM glutamate and 200 mM KCl.

Folate (2 to 48%), WTT2 cells and the transfectants accumulated similar levels of labeled vitamin. As the medium folate increased to pharmacological levels (up to 20 μM), the ability of the various transfectants to accumulate folate was similar and all accumulated very high levels of folate (about 600 μM) while the ability of WTT2 to accumulate folate was more limited. Under physiological conditions, folate accumulation by WTT2 and AUX-coli is limited by transport (6). At physiological levels (20 μM), accumulation by WTT2 is limited by FPGS activity while accumulation by AUX-coli cells, which express high levels of FPGS, remains limited by influx rates (6). The ability of AUX-mcoli to accumulate high levels of folate as effectively as AUX-coli and AUXcoli-mcoli demonstrates that transport of folate into the mitochondria in AUX-mcoli is not limiting under these conditions. Similar studies with labeled folinic acid, which is transported, and consequently concentrated, less effectively than folinate by CHO cells, showed qualitatively similar effects (data not shown).

The effect of medium folate on intracellular pteroylpolyglutamate distributions is shown in Table III. There was little difference in the type of folypolyglutamate that accumulated in the various transfectants over a wide range of medium folate or folinate concentrations. Extending the culture time from 24 to 72 h, the proportion of tetraglutamate that accumulated, but triglutamate was still the major intracellular derivative. Increases in medium folate cause a shortening of intracellular folate glutamate chain lengths in WTT2 cells and complete loss of very long chain length derivatives (6).

**Effect of Medium Folate Concentration on Subcellular Folate Accumulation and Metabolism—**The ability of WTT2 cells and transfectants to accumulate labeled medium folinate is shown in Fig. 4. The concentrating ability of the cells is expressed as the ratio of intracellular to extracellular folate concentrations and assumes 1 pmol/10^6 cells is equivalent to an intracellular concentration of 1 μM (2). Intracellular folate concentrations for the transfectants may be slightly overestimated due to the slightly larger size of these cells. At physiological levels of medium folinate (2 to 48%), WTT2 cells and the transfectants accumulated similar levels of labeled vitamin. As the medium folate increased to pharmacological levels (up to 20 μM), the ability of the various transfectants to accumulate folate was similar and all accumulated very high levels of folate (about 600 μM) while the ability of WTT2 to accumulate folate was more limited. Under physiological conditions, folate accumulation by WTT2 and AUX-coli is limited by transport (6). At physiological levels (20 μM), accumulation by WTT2 is limited by FPGS activity while accumulation by AUX-coli cells, which express high levels of FPGS, remains limited by influx rates (6). The ability of AUX-mcoli to accumulate high levels of folate as effectively as AUX-coli and AUXcoli-mcoli demonstrates that transport of folate into the mitochondria in AUX-mcoli is not limiting under these conditions. Similar studies with labeled folinic acid, which is transported, and consequently concentrated, less effectively than folinate by CHO cells, showed qualitatively similar effects (data not shown).

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but appears to be due to the high capacity of cytosolic FPGS in these cells to polyglutamate folate and thus prevent its transport into mitochondria. Mitochondrial folate accumulation by AUX-mcoli-1 cells remained high at both low and high medium folinate, but the proportion did decrease between 5 and 24 h. Although total cellular folate accumulation by AUX-mcoli-1 cells under low and high medium folinate conditions increased about 5-fold between 5 and 24 h, cytosolic folate accumulation increased 10- and 8-fold, respectively. These data again suggest that the cytosolic folate pool in these cells is derived from the efflux of mitochondrial folate.

Folate accumulation by AUX-coli is limited by transport rather than by FPGS activity because these cells express high levels of FPGS (6), and folate accumulation only becomes proportional to FPGS levels in AUX-coli transfectants that express less than 30% of the FPGS activity of the AUX-coli transfectant used in this study (2). If the AUX-mcoli transformants express any cytosolic FPGS activity, the maximum level, after allowing for mitochondrial contamination of the cytosolic extract (Table 1), can be calculated to be less than 1% of that of AUX-coli and 10% of that of D1-IA10. However, under low and high medium folinate conditions, AUX-mcoli transfectants accumulated 70 to 80% as much cytosolic folate as AUX-coli and higher levels of cytosolic folate than were found in D1-IA10 (Tables II and IV), again suggesting that the cytosolic folate pool in these cells was derived from mitochondrial polyglutamates. GlyB, a glycine-requiring CHO mutant (12) that is defective in mitochondrial folate transport, accumulated pharmacological levels of folate as effectively as WTT2, despite the lack of mitochondrial folate metabolism (Table IV). However, FPGS levels in the mitochondria and cytosol of GlyB are about 2-fold elevated compared to WTT2 cells.

**Folatepolyglutamate Chain Length Specificity of One-carbon Metabolism**—The ability of pteroyltetraglutamates to support metabolic cycles of one-carbon metabolism was assessed by cultivating cells in medium lacking various products of one-carbon metabolism and determining the intracellular folate concentration that supported half-maximal growth rates. For most of the transfectants, the maximal growth rates obtained in the different media were lower than for WTT2 but similar to the parent AUXB1 mutant (Table V). Maximal growth rates of AUX-mcoli-2, which was smaller than the other transfectants, were similar to WTT2.

Table V shows medium PteGlu concentrations that supported half-maximal growth rates for the different cell lines under the different nutritional conditions. The data are typical of that found in two or three separate trials. The high folate requirement of AUX-coli in medium lacking glycine was reduced for the AUX-mcoli transfectants and was reduced further in AUX-coli transfectants. All transfectants grew well in medium lacking thymidine or purines. As shown previously, AUXB1 cells did not grow in medium lacking glycine or purines, and growth in the absence of thymidine required very high medium PteGlu (Table V).

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Subcellular folate concentrations that supported half-maximal growth rates for the different cell lines under the different nutritional conditions are shown in Table VI. The data were derived from growth requirement (Table V) and cellular labeling experiments with PteGlu (similar to data shown in Fig. 4), and from subcellular distribution studies (Tables II and IV). The very high intracellular folate level required by AUX-coli to
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TABLE III
Effect of medium folate concentration on folylpolyglutamate chain length in CHO transfectants

Folate-depleted cells were cultured for 24 h in DMEM/dFBS + GHT medium containing the indicated [3H]PteGlu or (6S)-5-formyl-[3H]PteGlu concentration, and intracellular folylpolyglutamates were identified as described under "Experimental Procedures."

| Cell       | Medium folate | Polyglutamate chain length distribution |
|------------|---------------|----------------------------------------|
|            |               | 1 | 2 | 3 | 4 | 5 | 6 |
| AUXcoli    | 20 μM PteGlu  | 2 | 4 | 80| 14| 0 | 0 |
| AUXcoli    | 6 nm 5-formyl-H4PteGlu | 1 | 4 | 85| 10| 0 | 0 |
| AUXcoli    | 2 μM 5-formyl-H4PteGlu | 2 | 2 | 78| 17| 1 | 0 |
| AUXcoli-mcoli-1 | 20 μM PteGlu | 9 | 2 | 75| 13| 1 | 0 |
| AUXcoli-mcoli-1 | 6 nm 5-formyl-H4PteGlu | 1 | 1 | 82| 15| 1 | 0 |
| AUXcoli-mcoli-1 | 20 μM 5-formyl-H4PteGlu | 11 | 3 | 78| 8 | 0 | 0 |
| AUXcoli-mcoli-2 | 6 nm 5-formyl-H4PteGlu | 2 | 2 | 86| 8 | 0 | 0 |
| AUXcoli-mcoli-2 | 6 nm 5-formyl-H4PteGlu | 1 | 1 | 79| 16 | 4 | 1 |
| AUXcoli-mcoli-1 | 20 μM PteGlu | 12 | 3 | 82 | 3 | 0 | 338 | 0 |
| AUXcoli-mcoli-1 | 6 nm 5-formyl-H4PteGlu | 0 | 1 | 92 | 6 | 0 | 0 |
| AUXcoli-mcoli-2 | 20 μM 5-formyl-H4PteGlu | 5 | 5 | 89 | 1 | 0 | 0 |
| AUXcoli-mcoli-2 | 6 nm 5-formyl-H4PteGlu | 1 | 1 | 87 | 11 | 1 | 0 |

* Cultured for 72 h.

TABLE IV
Whole-cell and subcellular folinate accumulation by CHO cell transfectants

Folate-depleted cells were cultured in +GHT medium containing (6S)-5-formyl-[3H]PteGlu (7 nm or 2 μM) for 5 or 24 h, as indicated, and crude subcellular fractions were prepared by differential centrifugation as described under "Experimental Procedures."

| Cell       | Fraction | 7 nm | 2 μM |
|------------|----------|------|------|
|            |          | 5 h  | 6 h  | 5 h  | 6 h  | 5 h  | 6 h  |
|            |          | pmol/10^6 cells (% total) | pmol/10^6 cells (% total) | pmol/10^6 cells (% total) | pmol/10^6 cells (% total) |
| WTT2       | Total    | 0.45 | 1.9  | 6.1  | 21   |
|            | Mito     | 0.21 (47) | 0.72 (38) | 0.61 (10) | 1.8 (9) |
|            | Cyto     | 0.24 | 1.2  | 5.4  | 18.5 |
| AUXcoli    | Total    | —    | 3.0  | —    | 363  |
|            | Mito     | —    | 0.03 (1) | —    | 3.6 (1) |
|            | Cyto     | —    | 3.0  | —    | 359  |
| AUXcoli-mcoli-1 | Total | 0.65 | 3.0  | 88   | 295  |
|            | Mito     | 0.04 (6) | 0.18 (6) | 2.6 (3) | 8.9 (3) |
|            | Cyto     | 0.61 | 2.8  | 86   | 286  |
| AUXcoli-mcoli-1 | Total | 0.76 | 4.7  | 71   | 338  |
|            | Mito     | 0.50 (66) | 2.1 (45) | 36 (51) | 71 (21) |
|            | Cyto     | 0.26 | 2.6  | 35   | 267  |
| AUXcoli-mcoli-2 | Total | —    | 3.0  | —    | 274  |
|            | Total    | 42   | 1.4  | —    | 233  |
| AUXcoli-mcoli-2 | Gly B* | —    | 2.1  | —    | 37   |

Same as AUXcoli except expressing approximately 10-fold less E. coli FPGS activity.

WTT2 mutant defective in mitochondrial folate transport. (R.-F. Huang and B. Shane, unpublished data.)

TABLE V
Medium folate concentrations supporting half-maximal growth rates and cell doubling times in media lacking products of one-carbon metabolism

Experimental conditions are described under "Experimental Procedures." Cells were cultured for 4 days in modified complete medium (DMEM/dFBS + GHT) supplemented with various concentrations of folinic acid (0-100 μM) and lacking H or G or T as indicated, and growth was measured at 24-h intervals. Data are representative of two or three separate experiments.

| Cell line | Medium PteGlu at half-maximal growth rate | Cell doubling time* |
|-----------|------------------------------------------|---------------------|
|           | -Glycine | -Purines | -Thymidine | +GHT | -G | -H | -T |
| WTT2      | 275      | 160      | 30         | 30   | 12 | 12 | 12 |
| AUXB1     | 400      | 150      | 30         | 30   | 15 | 15 | 28 |
| AUXcoli   | 2,000    | 100      | 40         | 40   | 15 | 18 | 16 |
| AUXcoli-mcoli-1 | 1,200 | 140      | 20         | 20   | 16 | 14 | 15 |
| AUXcoli-mcoli-2 | 400    | 250      | 50         | 50   | 17 | 17 | 17 |
| AUXcoli-mcoli-1 | 275    | 150      | 30         | 30   | 13 | 12 | 12 |

* Cell doubling time in indicated media supplemented with excess PteGlu.

No growth at 100 μM medium PteGlu.

Doubling time at 100 μM medium PteGlu.

Support growth in medium lacking glycine was reduced in the AUXcoli-mcoli transfectants and was reduced further in AUXcoli transfectants to levels that were similar to WTT2 cells. However, mitochondrial folate concentrations that supported half-maximal growth rates under these conditions were similar in all the transfectants and in WTT2 cells, indicating that pteroylglutamates function as effectively as the longer chain length folylpolyglutamates in WTT2 cells in the metabolic cycles of glycine synthesis, provided the folate is in the mitochondria. Similarly, no significant differences were observed in...
the cytosolic folate levels that supported thymidylate and purine synthesis between the various transfectants and WTT2 cells. AUX-mcoli-2 consistently required lower intracellular folate levels than AUX-mcoli-1 to support these metabolic cycles. However, the folate concentration in the cytosol and mitochondria of AUX-mcoli-2 would be very similar to AUX-mcoli-1 due to the smaller size of AUX-mcoli-2.

**DISCUSSION**

Although it has been known for some time that mammalian cells and tissues contain mitochondrial and cytosolic folypolyglutamate pools (7, 12, 28–30), the role of mitochondrial folate metabolism in cellular one-carbon metabolism is poorly understood. In the current study, we describe model CHO cells which were developed for assessing the metabolic role of mitochondrial folate metabolism. The presence of mitochondrial FPGS activity in the transfectants demonstrated that the E. coli FPGS protein, targeted by the human ornithine transcarbamoylase leader sequence, could be successfully transported into the CHO cell mitochondria and be expressed as an active enzyme. In the construct used, there are 5 additional amino acid residues between the normal cleavage site of the ornithine transcarbamoylase leader sequence and the start methionine of the mature FPGS protein. Although we did not test whether proteolytic processing occurred at the normal proOTC cleavage site, Western analysis demonstrated a mitochondrial band indistinguishable in size from mature FPGS as well as what appeared to be a processing intermediate. proOTC is normally processed via a two-step proteolytic cleavage (27). The active FPGS in mitochondria implies that the extra amino-terminal residues on the FPGS protein did not significantly affect enzyme activity, and that this prokaryotic protein seems to refold correctly after unfolding and translocation across the mitochondrial membrane. These transfectants, expressing E. coli FPGS activity with different ratios of mitochondrial to cytosolic enzyme activity, provide a potentially useful model for studying the role of mitochondrial folate metabolism in one-carbon metabolism.

It has been suggested, based on *in vitro* studies, that folate-mediated reactions in the mitochondria may generate one-carbon units for utilization in cytosolic processes, and a novel pathway has been proposed for a one-carbon shuttle between the two compartments using serine and formate (31). GlyA, a CHO mutant which lacks mitochondrial serine hydroxymethyltransferase activity, is auxotrophic for glycine (8) although it is not clear why the cytosolic hydroxymethyltransferase, which normally accounts for about 25% of the total cell enzyme in CHO cells, cannot meet its glycine requirement. Our results also directly demonstrate this requirement for mitochondrial glycine synthesis in the CHO cell. AUX-coli cells, which lack mitochondrial folate activity but possessing normal cytosolic folate pools, were auxotrophic for glycine when cultured in medium containing physiological levels of folate, while transfectants expressing mitochondrial FPGS activity and containing mitochondrial folate pools were prototrophic for glycine.

Although different levels of medium folate supported half-maximal growth rates of the AUXcoli-mcoli and AUX-mcoli transfectants and WTT2 cells, and intracellular folate levels also varied under these conditions, mitochondrial folate concentrations in all these cells were essentially the same reflecting that this pool supplied folate cofactor for glycine synthesis and that pteroyltriglutamates were as effective as longer polyglutamate derivatives in glycine synthesis. The glycine auxotrophy of AUX-coli cells, but not AUXB1 cells, could be overcome by very high medium folate concentrations. AUX-coli cells accumulate very high cytosolic pteroyltriglutamate levels under these conditions, and it is possible that this allows sufficient glycine synthesis to occur in the cytosol. However, although pteroyltriglutamates do not enter the mitochondria, pteroylmonoo- and possibly diglutamates would be expected to be transported but would not be concentrated by AUX-coli mitochondria due to the lack of FPGS activity. We have previously shown that AUX-coli mitochondria contain trace amounts of pteroyltetra- and pteroylhexaglutamates, reflecting that a small proportion of the cytosolic folates in this cell line are also mono- and diglutamate derivatives (7). Under conditions of very high cytosolic folate levels, sufficient pteroyldiglutamate may be present in the mitochondria of AUX-coli cells to allow glycine synthesis. Half-maximal growth rates of AUX-coli cells in the absence of glycine required about 100 pmol of folate/10⁶ cells, and pteroyldiglutamate accounted for about 4 pmol/10⁶ cells. If 25% of this was in the mitochondria, this would be similar to the mitochondrial folate concentrations that supported half-maximal growth rates in the other transfectants. Very high levels of medium folate do not support growth of GlyA cells under these conditions, which would also suggest that elevated cytosolic folate is not sufficient to allow glycine synthesis.

The ability of AUX-mcoli transfectants to support the cytosolic cycles of thymidylate and purine synthesis was unexpected as it was anticipated that these cells would lack cytosolic folates. However, these transfectants contained cytosolic folylpolyglutamates. Cytosolic folate concentrations that supported similar rates of thymidylate or purine synthesis were similar for all the transfectants and for WTT2 cells and, as has been previously reported (6), these metabolic cycles also appear to function effectively with pteroyltriglutamates.

We have previously shown that folate accumulation by AUX-coli, which expresses high levels of *E. coli* FPGS, is limited primarily by folate influx rather than by FPGS activity (6). AUXcoli-mcoli transfectants were selected by their ability to grow in the absence of glycine, and all of these transfectants, including the two which were characterized in detail in this report, expressed extremely high levels of mitochondrial FPGS.

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**Table VI**

| Cell line       | Glycine | Purines | Thymidine |
|-----------------|---------|---------|-----------|
|                 | Cyto    | Mito    | Cyto      | Mito     | Cyto    | Mito     |
| WTT2            | 0.81    | 0.49    | 0.43      | 0.27     | 0.12    | 0.08     |
| AUXB1           | ~0.5    |         | ~0.60     | 0.03     | ~0.20   | ~0.01    |
| AUXcoli         | ~100    | 0.5     | 0.47      | 0.10     | 0.17    | 0.03     |
| AUXcoli-mcoli-1 | 9.6     | 0.66    | 0.60      | 0.68     | 0.22    | 0.18     |
| AUXcoli-mcoli-2 | 1.4     | 1.1     | 0.83      | 0.68     | 0.22    | 0.18     |
| AUXmcoli-1      | 1.1     | 0.45    | 0.56      | 0.24     | 0.14    | 0.06     |
| AUXmcoli-2      | 0.47    | 0.20    | 0.19      | 0.03     |         |          |

*1 pmol/10⁶ cells is equivalent to an intracellular concentration of approximately 1 μM.

*No growth or mitochondrial pool negligible or absent.*
activity although the proportion of cellular folate in the mitochondria of these cells was relatively low. This could not be due to a limitation in mitochondrial folate transport as, under similar culture conditions, AUX-mcoli transfectants accumulated higher absolute levels of mitochondrial folate despite possessing lower mitochondrial FPGS activities. Subcellular folate accumulation in AUX-mcoli transfectants was governed by competition between cytosolic and mitochondrial FPGS, and very high levels of mitochondrial FPGS were required to enable mitochondrial folate accumulation and to overcome the ability of the cytosolic enzyme in these cells to trap all entering folate. Although FPGS is a very low abundance protein in cultured mammalian cells, these levels are normally sufficient to enable retention of all transported folate under physiological conditions (6). One metabolic reason for FPGS to be present at such low levels would be to allow mitochondrial accumulation of folate. Overexpression of human FPGS in the cytosol of mammalian cells results in an inability to accumulate mitochondrial folate.

AUX-mcoli transfectants appeared to lack cytosolic FPGS activity although the possibility of trace levels of cytosolic enzyme activity could not be excluded. No accumulation of a precursor protein was detected in the cytosol, and the half-life of most mitochondrial precursor proteins is very short (32). Although a large proportion, in some cases the majority, of cellular folate was located in the mitochondria of these cells, they also contained a normal cytosolic folylpolyglutamate pool. Various lines of evidence indicated that the cytosolic folate pool was derived from mitochondrial efflux of folylpolyglutamates. The proportion of mitochondrial folate decreased with culture time, and the size of the cytosolic folate pool increased in a cooperative fashion rather than linearly with time. Even if AUX-mcoli cells did express some cytosolic FPGS activity, the calculated maximum level was clearly insufficient to account for cytosolic folate accumulation. Studies described in the accompanying report (33) also indicate that efflux of mitochondrial folate occurs without prior hydrolysis of folylpolyglutamates and that this efflux is not limited to short chain folylpolyglutamates but also occurs with the longer polyglutamate derivatives typically found in mammalian cells. Preliminary studies have also indicated that expression of human FPGS in the mitochondria of AUXR1 cells results in normal cytosolic folate pools.3 It should be noted that although mitochondrial folate efflux may be a significant factor in expanding the cytosolic folate pool, the actual rate of efflux would be insignificant compared with the rate of one-carbon fluxes in the various metabolic cycles of one-carbon metabolism in the CHO cell. Consequently, folate efflux could not play a metabolic role as a carrier of one-carbon moieties to connect mitochondrial and cytosolic one-carbon metabolism.

The mechanism of folylpolyglutamate efflux from mitochondria has not been established but clearly differs from mitochondrial folate influx. The mitochondrial folate transporter is specific for reduced folates and will not transport pteroyltriglutamates (34, 35). It differs from the plasma membrane transporter in CHO cells in that methotrexate is not transported (34).

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