A mutant Chinese hamster ovary cell line, glyB, that required exogenous glycine for survival and growth was reported previously (Kao, F., Chasin, L., and Puck, T. T. (1969) Proc. Natl. Acad. Sci. U. S. A. 64, 1284–1291). We now report that the defect in glyB cells causative of this phenotype is a point mutation in an inner mitochondrial membrane protein required for transport of folates into mitochondria. The CHO mitochondrial folate transporter (mft) was sequenced and compared with that from glyB cells. The hamster sequence was nearly identical to that of the recently reported human mitochondrial folate transporter. The corresponding cDNA from glyB cells contained a single nucleotide change that introduced a glutamate in place of the glycine in wild-type glyB cells. Transfection of the wild-type hamster mft cDNA into glyB cells allowed cell survival in the absence of glycine and the accumulation of folates in mitochondria. Transfection of the wild-type hamster cDNA into glyB cells allowed cell survival in the absence of glycine and the accumulation of folates in mitochondria, whereas transfection of the Glu-192 cDNA did not. Genomic sequence analysis and fluorescence in situ hybridization demonstrated a single mutated allele of the mft gene in glyB cells, whereas there were two alleles in CHO cells. We conclude that we have defined the cause of the glyB auxotrophy and that the glyB mft mutation identified a region of this mitochondrial folate carrier vital to its transport function.

The compartmentalization of folate cofactor interconversion and folate-dependent biosynthetic reactions between cytosol and mitochondria is essential for the proper function of mammalian folate metabolism (1, 2). In mitochondria, reduced folates are required for the initiation of mitochondrial protein synthesis, the synthesis of glycine from serine, and the glycine cleavage system, which allows the generation of 10-formyltetrahydrofolate (3, 4).

The transport of folates into the cytosol has been studied extensively (5, 6). Both the folate receptor (7) and the reduced folate carrier (8) have been identified as molecules in the passage of folates across the plasma membrane. In contrast, very little is known about the mechanism by which folates reach the mitochondrial matrix from the cytosol. Puck and co-workers (1, 9) isolated a CHO mutant cell line that was auxotrophic for glycine and that did not accumulate folate cofactors in their mitochondria (15). A recent study in our laboratory (10) led to the isolation of the human gene encoding a protein that complemented the glyB defect. We tentatively named this protein the human mitochondrial folate transporter (MFT) in response to the fact that the structure of this protein had the characteristics of members of the inner mitochondrial membrane carrier family. The human cDNA isolated in that experiment reinstituted folate uptake when transfected into glyB cells (10), strong evidence that the MFT was the endogenous protein responsible for the transport of folates into mammalian mitochondria. However, it remained possible that the complementation observed could have been through a compensatory or non-specific mechanism, and direct evidence that the CHO MFT was defective in glyB cells was needed to conclude that this protein is the endogenous mitochondrial transporter that facilitates the entry of folates into the mitochondrial matrix.

We now report the sequence of the hamster mft cDNA in wild-type CHO and mutant glyB cell lines and functional studies on these cDNAs. We conclude that we have identified the cause of the glyB auxotrophy, that the MFT is the mitochondrial folate transporter, and that we have found a region of this protein vital to its transport function.

**A Mutation Inactivating the Mitochondrial Inner Membrane Folate Transporter Creates a Glycine Requirement for Survival of Chinese Hamster Cells**

**Erin A. McCarthy‡, Steven A. Titus¶, Shirley M. Taylor§, Colleen Jackson-Cook¶, and Richard G. Moran¶¶***

From the Departments of ‡Pharmacology and Toxicology, §Pathology and Human Genetics, ¶Microbiology and Immunology, and the ¶¶Massey Cancer Center, Virginia Commonwealth University, Richmond, Virginia 23298

A mutant Chinese hamster ovary cell line, glyB, that required exogenous glycine for survival and growth was reported previously (Kao, F., Chasin, L., and Puck, T. T. (1969) Proc. Natl. Acad. Sci. U. S. A. 64, 1284–1291). We now report that the defect in glyB cells causative of this phenotype is a point mutation in an inner mitochondrial membrane protein required for transport of folates into mitochondria. The CHO mitochondrial folate transporter (mft) was sequenced and compared with that from glyB cells. The hamster sequence was nearly identical to that of the recently reported human mitochondrial folate transporter. The corresponding cDNA from glyB cells contained a single nucleotide change that introduced a glutamate in place of the glycine in wild-type glyB cells. Transfection of the wild-type hamster cDNA into glyB cells allowed cell survival in the absence of glycine and the accumulation of folates in mitochondria, whereas transfection of the Glu-192 cDNA did not. Genomic sequence analysis and fluorescence in situ hybridization demonstrated a single mutated allele of the mft gene in glyB cells, whereas there were two alleles in CHO cells. We conclude that we have defined the cause of the glyB auxotrophy and that the glyB mft mutation identified a region of this mitochondrial folate carrier vital to its transport function.

The compartmentalization of folate cofactor interconversion and folate-dependent biosynthetic reactions between cytosol and mitochondria is essential for the proper function of mammalian folate metabolism (1, 2). In mitochondria, reduced folates are required for the initiation of mitochondrial protein synthesis, the synthesis of glycine from serine, and the glycine cleavage system, which allows the generation of 10-formyltetrahydrofolate (3, 4).

The transport of folates into the cytosol has been studied extensively (5, 6). Both the folate receptor (7) and the reduced folate carrier (8) have been identified as molecules in the passage of folates across the plasma membrane. In contrast, very little is known about the mechanism by which folates reach the mitochondrial matrix from the cytosol. Puck and co-workers (1, 9) isolated a CHO mutant cell line that was auxotrophic for glycine and that did not accumulate folate cofactors in their mitochondria (15). A recent study in our laboratory (10) led to the isolation of the human gene encoding a protein that complemented the glyB defect. We tentatively named this protein the human mitochondrial folate transporter (MFT) in response to the fact that the structure of this protein had the characteristics of members of the inner mitochondrial membrane carrier family. The human cDNA isolated in that experiment reinstituted folate uptake when transfected into glyB cells (10), strong evidence that the MFT was the endogenous protein responsible for the transport of folates into mammalian mitochondria. However, it remained possible that the complementation observed could have been through a compensatory or non-specific mechanism, and direct evidence that the CHO MFT was defective in glyB cells was needed to conclude that this protein is the endogenous mitochondrial transporter that facilitates the entry of folates into the mitochondrial matrix.

We now report the sequence of the hamster mft cDNA in wild-type CHO and mutant glyB cell lines and functional studies on these cDNAs. We conclude that we have identified the cause of the glyB auxotrophy, that the MFT is the mitochondrial folate transporter, and that we have found a region of this protein vital to its transport function.

**EXPERIMENTAL PROCEDURES**

**Materials—**CHO-K1 cells were obtained from ATCC. glyB cells, a CHO-K1 derivative, were generously provided by Prof. Lawrence Chasin from Cornell University. V79 cells were obtained from Prof. Chia C. Chung from Michigan State University. All of the cell lines were negative for mycoplasma as detected by hybridization to a specific ribosomal RNA probe and were routinely grown in α-MEM supplemented with 10% fetal calf serum (FCS).

**Northern Blot—**RNA from CHO and glyB cells was isolated using the TRIsod reagent (Invitrogen). Poly(A)+ RNA was prepared using an Oligotex mRNA kit (Qiagen). Poly(A)+ mRNA (2 μg) was run in each lane of an agarose gel and blotted onto a nylon membrane (Biometra; ICB). A 726-nt PCR amplified region of CHO mft cDNA spanning a portion of the open reading frame was 32P-labeled by random priming and added to membranes in a Gilbert’s solution-based hybridization mixture at 1 × 106 cpm/ml. An overnight hybridization at 65 °C was followed by two washes in 2× SSC, 0.1% SDS at room temperature and at 50 °C, respectively.

**Sequence Analysis of Hamster mft—**A cDNA corresponding to a fragment of the hamster mft was amplified from both CHO and glyB mRNA using PCR primers predicted from human mft sequence. The forward primer was 5’-GCTGGAATGGTCAAGATCC-3’; and the reverse

*This work was supported in part by Grants CA-27605 and CA-104279 from the National Institutes of Health, DHHS. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AY611603.

**To whom correspondence should be addressed: Medical College of Virginia campus, Virginia Commonwealth University, 1101 E Marshall St., Richmond, VA 23298-0230. E-mail: rmoran@hsc.vcu.edu.**

**REFERENCES**

1. The abbreviations used are: CHO, Chinese hamster ovary; MFT, mitochondrial folate transporter; FISH, fluorescence in situ hybridization; FCS, fetal calf serum; MEM, minimum Eagle’s medium; nt, nucleotide; ES, energy transfer signature.
primary was 5'-CTAGCTTTACCTGGTTTACTGCGAAGG-3'. The PCR conditions were 94°C for 30 s, 58°C for 1.5 min, and 72°C for 1 min for a total of 35 cycles. The optimum PCR product was obtained when using the following conditions: 1.5 mM MgSO4, 200 mM 5' and 3' primers, 0.2 μM dNTPs, 1 μg of cDNA, 1× PCR enhancer (Invitrogen), 10× PCR amplification buffer, and Taq polymerase in a final volume of 25 μL. The 726-nt amplified fragment was subcloned into the pCRII-Topo vector (Invitrogen), and the insert from six colonies from two PCR reactions for each cell line was sequenced in both directions. The sequences were compared using the Gene Jockey program. The alignment of human, CHO, and glyB mft sequences were carried out in the MegAlign program. The genomic organization of human and mouse mfts was deduced by searching the mouse and human genome databases with the cDNA for human mft using the BLAST program.

**CHO λ cDNA Library Screen—** A CHO λ-Zap CMV-XR library (Stratagene) was plated at 50,000 plaque-forming units/plate on 20 150-mm NZY agar plates after incubation with XLI-Blue MRF’ cells. A 313-nt PCR-amplified fragment from the 5’ end of CHO mft cDNA was 32P-labeled by random priming and added to the membranes of duplicate nitrocellulose lifts in a hybridization mixture at 1× 106 cpm/mL. This PCR product was cloned and sequenced prior to use, and its identity as an upstream fragment of the hamster mft was deduced from homology with the human mft. After overnight incubation at 65°C, membranes were washed sequentially to a final stringency of 0.5× SSC, 0.1% SDS at 50°C.agar. Agar plugs containing positive plaques were excised, and secondary and tertiary screening was performed to isolate a single plaque.

**Transfection Studies—** glyB cells actively growing in α-MEM (Invitrogen) supplemented with 10% FCS were plated at 2× 104 cells on each of the 24 100-mm dishes. After 24 h, 5 μg of plasmid DNA combined with 2 μl CaCl2 and 2× HEBS (HEPES, NaCl, KCl, Na2PO4, and glucose) was overlaid on the cells in a total volume of 1 ml (11). After 30 min at room temperature, 9 ml of complete medium was added to each plate and the plates were incubated at 37°C overnight. The following day, a brief Me2SO shock was performed and the medium was changed.

**Subcellular Distribution of Cellular Folates—** Cell cultures (6×108/175 cm²) were grown in α-MEM, which contains glycine, supplemented with dialyzed FCS and 0.3 μM H11032/H11032-αKTN-NT (Moravek) 48 h. Cells were detached with trypsin and pelleted by centrifugation. The pellets were resuspended in 10 ml of PBS (+5% FCS), counted, and re-pelleted. The pellets were resuspended in 2 ml of homogenization solution (0.25M sucrose and 1 mM EDTA), and the various cellular fractions were isolated using a Dounce homogenization procedure described previously (10). Aliquots of fractions were prepared for scintillation counting under uniform ionic conditions, and radioactivity was converted to folate content by counting an aliquot of the culture medium under identical conditions.

**Fluorescence In Situ Hybridization (FISH) Analysis—** For FISH, metaphase spreads from V79, CHO, and glyB cells were prepared as described previously (12). Two fragments of CHO genomic DNA were PCR-amplified and directly labeled with the Spectrum Orange fluorochrome using a nick translation kit (Vysis). These fragments corresponded to the regions of the hamster mft gene spanning predicted exons 2–4 including the two intervening introns (5.7 kb) and the region spanning predicted exons 6 and 7 including the intervening intron (1.3 kb). These two PCR products were cloned and partially sequenced to confirm the identity with the mft gene. Representative images were documented using a CytoVision image analysis system (Applied Imaging).

**RESULTS**

**The Mitochondrial Folate Transporter Is Expressed in glyB Cells—** Earlier studies by Kao et al. (1) and Chasin et al. (14) establish four complementation groups among CHO mutants that were auxotrophic for glycine. A cell line defining one of these complementation groups, designated glyB, was found to have a defect in its ability to accumulate folates in the mitochondria (10, 15), a process required for the activity of mammalian mitochondrial serine hydroxymethyltransferase (16).

To understand what was causing this mutant phenotype, we examined the expression of the hamster homolog of a human gene (mft) previously identified (16) to stimulate folate influx into glyB mitochondria. Initially, the human mft cDNA sequence was used to design primers for PCR amplification of the corresponding hamster cDNA. The primers were designed in regions that retained the highest homology among other inner mitochondrial membrane proteins such as the energy sequence motifs and the putative membrane-spanning domains (17–20).

With one set of primers, a single 726-nt fragment was amplified using cDNA from both CHO and glyB cells. The identity of this cDNA fragment as the hamster mft was clear from the homology with the human gene (see below). Thus, it was used subsequently to probe poly(A)+ RNA from CHO and glyB cells on a Northern blot (Fig. 1). A diffusely migrating mRNA band was seen at 2.3 kb in both cell lines. With longer exposures, a faint band at 1.6 kb also could be detected with longer exposures. The blot was stripped and re-probed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA for normalization.

**FIG. 1.** Northern blot of mRNA from CHO and glyB cells. Poly(A)+ mRNA (2 μg/lane) from the two cell lines were run on a 1% gel, blotted onto a nylon membrane, and probed with a 726-nt portion of the CHO mft cDNA. A major band at 2.3 kb was detected in both cell lines, and a faint band at 1.6 kb also could be detected with longer exposures. The blot was stripped and re-probed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA for normalization.

**A Point Mutation in the mft Gene in glyB Cells—** To obtain the full hamster mft sequence, a CHO λ cDNA library was screened with a 313-nt PCR product amplified from the 5’ region of the hamster cDNA. Three of the longest clones were sequenced. An open reading frame of 954 nt was identified in these cDNAs that encoded a protein of 317 residues with a predicted molecular weight of 35,125 daltons. The predicted hamster protein was 89% identical to the reported human MFT (Fig. 2). The longest 5’-untranslated region found was 203 nt, and the 3’-untranslated region appeared to be 1189 nt. The sequence can be retrieved from GenBank™ under accession number AY611603. As with the human MPT, hydrophathy consider-
of both PCR-amplified CHO cDNAs and from an analysis of
mft frame of hamster transfer motif (see subgroup of mitochondrial inner membrane transport proteins
with the human gene, the hamster 3rd, and 5th predicted transmembrane domains (Fig. 2). As
proteins were identified, which were placed precisely after the 1st,
sequence characteristic of mitochondrial inner membrane pro-
terns identified six putative transmembrane domains in the
transmembrane domains are
dicted by homology to other family members (23). The six predicted
boxed sequences
top clones. The additional amino acids shown below the hamster sequence
position corresponding to the glyB
tation that only an A residue was found in glyB genomic DNA at the

top right

Sequence obtained from this 626-nt genomic fragment revealed
a larger genomic DNA marked by the presence of intron 5.
6 were used to PCR-amplify the region of the glyB mutation on

genic locus. Hence, the primers from predicted exons 5 and
was predicted that codon 192 would be in exon 5 of the hamster
B
codon 192 (Fig. 3A). As-
the recently completed mouse genome sequence (Fig. 3
resented the mouse mft
gene, the region of genomic DNA
mft mutation and that only
A FISH analysis was used to determine the
presence of only one mutated allele and a second wild-type but epigenetically silenced allele in glyB
cells. However, by such sequencing alone, we could not differ-
entiate the presence of only one copy of the mft in the CHO
gene or a mutation in one allele followed by a gene conver-
sion event, which fixed the same mutation in each of two copies of
the gene, or the loss of an allele during the selection of the

glyB cells. In
CHO Cells Have Two Alleles of the mft Gene, but glyB Cells Have Only One—A FISH analysis was used to determine the
number of signals present for the mft gene in the metaphase
spreads of CHO, V79, and glyB cell lines. The modal numbers
of 21, 22, and 19 chromosomes, respectively, were noted in the
metaphase spreads from these cell lines. In the CHO metaphase
spreads, two small chromosomes showed bright hybridization
signals with the probes for the hamster mft gene (Fig. 4A). Based on the information gained from the FISH and addi-
tional GTG-banding studies (data not shown), these signals
appeared to localize toward the ends of a Z-12 chromosome and
a chromosome 11 (21). The same number of FISH signals was
seen on metaphase chromosomes from the hamster V79 lung
fibroblast cell line (data not shown), leading to the conclusion
that CHO and V79 cells lines had two copies of the mft gene. In

![Fig. 2. Predicted amino acid sequence of the open reading frame of hamster mft. The sequence shown was obtained from translation
of both PCR-amplified CHO cDNAs and from an analysis of λ clones. The additional amino acids shown below the hamster sequence (top)
represent residues different in the human MFT sequence. The boxed sequences indicate the positions of the three energy motifs predicted
by homology to other family members (23). The six predicted transmembrane domains are overscored.](Image 1)

![Fig. 3. Position of mutation in glyB cDNA and genomic DNA. A, the mft open reading frames from CHO and glyB cDNAs were isolated by PCR and multiple PCR products were cloned and sequenced. A G to A transition was detected at nucleotide 575 of all sequenced cDNAs from glyB cells as shown in the electropherograms. B, similarity of intron-exon structure of the human and mouse mft genes. BLAST (www.ncbi.nlm.nih.gov/BLAST) searches of the human and mouse genes with the human and mouse cDNAs resulted in the indicated intron and exon placement for the mft gene that were positioned on human chromosome 8 and mouse chromosome 15. C, the sequence of genomic DNA in glyB cells showed only a single allele at nt 575. Primers were selected on sequence predicted to be on exons 5 and 6 based on homology with the human and mouse genes (above), and the 626-bp PCR fragment was sequenced.](Image 2)
The open reading frame of mft homologs were subcloned into pcDNA3.1 and stably transfected into glyB cells. In each experiment, triplicate plates were selected in medium containing 1 mg/ml G418 and three more plates were selected in glycine-deficient medium containing G418. Colonies were fixed, stained, and counted after 10 days of selection. Percent rescue values were derived from the number of colonies under double selection divided by the number of colonies under single selection.

### Table 1

| Construct    | G418 + glycine | G418 – glycine | % Rescue | n |
|--------------|----------------|----------------|----------|---|
| pcDNA3.1(−)  | 72             | 0.3            | 0.4*     | 6 |
| CHO          | 80             | 70             | 87       | 7 |
| glyB         | 65             | 4              | 66*       | 7 |
| Mouse        | 101            | 114            | 113*      | 2 |
| Zebrafish    | 104            | 46             | 44       | 2 |
| Human        | 96             | 56             | 59       | 2 |

* Colonies found were typically very small.

### The G192E Mutation Results in Glycine Auxotrophy

To establish whether the mutation identified in the glyB mft gene was causative of the mutant phenotype of this cell line, we stably transfected the mft cDNA from CHO and glyB cells back into glyB recipient cells and determined whether these cDNAs complemented the auxotrophy of these cells. The region of cDNA encoding the open reading frame from both CHO and glyB were re-cloned into the mammalian expression vector pcDNA3.1(−) and transfected into glyB cells using a calcium phosphate-mediated procedure. After 3 days, replicate plates were placed under single selection with G418 as a transfection frequency control or under double selection using both G418-containing and glycine-deficient media. After 10 days of selection, the plates were stained for visualization of colonies. Transfection of the wild-type CHO cDNA into glyB cells resulted in a similar rate of colony formation under single or double selection (Table 1), indicating that the CHO mft cDNA could complement the auxotrophy of the glyB cells efficiently. The glyB cells transfected with the mutant construct and placed under double selection were unable to form colonies, but these transfections survived under single selection (Fig. 5A). In every experiment, a few small colonies (<20 cells/colony) were found on plates transfected with glyB cDNA, but these adherent cell clusters were incapable of continuous growth when transferred in selective medium in 24-well dishes. As seen in previous experiments (10), the human MFT was able to complement the glycine auxotrophy but to a somewhat lesser extent than we observed with the CHO construct. glyB cells transfected with the vector alone had formed colonies only on single selection dishes. Hence, these transfection experiments indicated that the single base pair alteration located at residue 192 in the glyB mft gene is capable of producing the mutant phenotype of glyB cells.

### Wild-type MFT Reinstates Folate Levels in Mitochondria of glyB Cells

Colonies of glyB cells transfected with either CHO or glyB mft were selected for clonal expansion. glyB cells expressing the wild-type CHO construct could be maintained in the absence of glycine, but glyB cells transfected with glyB mft cDNA could only survive in medium supplemented with exogenous glycine. To establish a cell line expressing the glyB mft under the control of the cytomegalovirus promoter, glyB cells were transfected with the glyB mft cDNA and exposed to double selection for 10 days. The few weakly adherent small cell colonies were selected for clonal expansion. glyB cells expressing the wild-type CHO construct were unable to form colonies, but these transfections survived under single selection (Fig. 5A).
The Hamster Mitochondrial Folate Transporter

Fig. 5. A, complementation of glycine auxotrophy in glyB cells by transfection of CHO mft cDNA. Triplicate plates of glyB cells were transfected with CHO and glyB mfts, human mft, and vector (pcDNA3.1(−)) alone. Plates then were fed with media containing G418 in the presence or absence of glycine. After 10 days, colonies were stained with Giemsa. B, mouse and zebrafish mft cDNAs also complement the glyB deficiency. glyB cells were transfected with cDNAs identified by data base searching as in A (see “Experimental Procedures” for details).

Identification of the Mouse and Zebrafish mfts—Several mft candidate homologs from other species were identified easily from the NCBI data base that had high levels of identity to the candidate homologs from other species. We compare the sequences of an unidentified mouse cDNA of mouse origin with cDNAs from zebrafish and monkey (GenBank™ accession number BC048057), all of which appear to represent mft genes from these species. However, we have not detected such an effect (Tables I and III), because the glyB cell acts like a null function cellular background. As such, the glyB promises to be a valuable tool for study of the mechanism of the process of transport of folates into the mitochondrial matrix since this function can be selected for in this mammalian cell line.

The high level of sequence identity between human and hamster proteins (Fig. 2) prompted us to search the EST and completed genome databases for probable mft sequences from other organisms. We compare the sequences of an unidentified cDNA of mouse origin with cDNAs from zebrafish and monkey (GenBank™ accession number AB060253), all of which appear to represent mft genes from these species based on homology (Fig. 6) and function (Fig. 5). Also compared are the yeast gene for a mitochondrial inner membrane transporter for flavin (GenBank™ accession number NP012132) and the human ATP/ADP transporter (GenBank™ accession number Q09737). We compared the sequence similarities of the folate-specific carriers and also compared them with related family members.

We had isolated a human gene previously that could complement the defect of a cell line rendered auxotrophic for glycine and incapable of accumulating folates into their mitochondria. Herein, we report that the homologous hamster protein is mutated in glyB cells and that the single point mutation distinguishing the hamster MFT proteins in CHO and glyB cells explains the mitochondrial folate transport defect in glyB cells. We conclude that the MFT protein can be identified definitively as the endogenous mitochondrial folate transporter.

Testing the glyB mft cDNA for a Dominant Negative Effect—Because previous literature (22) indicates that other inner mitochondrial transporters function as homodimers, we had expected that the co-expression of a functional MFT and the glyB MFT in the same cell line would result in an impaired activity of the functional MFT, i.e. that the glyB mft cDNA would have a dominant negative effect. The transfection of either CHO or mouse mft into a glyB cell background (Table I) did not support this expectation. To more formally test this hypothesis, the glyB mft cDNA under the strong cytomagelivirus promoter was transfected into CHO cells and the rate of colony formation was compared in the presence and absence of glycine. Surprisingly, the data from this experiment (Table III) demonstrated that transfection of the glyB mft cDNA was no different from the transfection of the pcDNA control alone, indicating no detectable dominant negative effect and leading us to the conclusion that the glyB MFT was not forming a dimer with co-existing functional MFT species.

DISCUSSION

The point mutation identified in the glyB mft cDNA resulted in the substitution of a glycine residue for a glutamic acid within the fourth predicted transmembrane domain, an almost entirely hydrophobic region of this protein. The placement of a negatively charged amino acid within a hydrophobic membrane-spanning domain probably either disrupts the insertion of the protein into the hydrophobic environment of the inner mitochondrial membrane or interferes with proper folding and results in the targeting of the mutant MFT for proteolytic degradation. Either of these effects would explain the loss of MFT function in the glyB cells. It is thought that the inner mitochondrial membrane transporters function only after assembly as a 12-transmembrane domain homodimer (22). Hence, we had expected that the inactivated glyB MFT transporter might have a dominant negative effect on the function of transfected mft species. However, we have not detected such an effect (Tables I and III), because the glyB cell acts like a null function cellular background. As such, the glyB promises to be a valuable tool for study of the mechanism of the process of transport of folates into the mitochondrial matrix since this function can be selected for in this mammalian cell line.

The point mutation identified in the glyB mft cDNA resulted in the substitution of a glycine residue for a glutamic acid within the fourth predicted transmembrane domain, an almost entirely hydrophobic region of this protein. The placement of a negatively charged amino acid within a hydrophobic membrane-spanning domain probably either disrupts the insertion of the protein into the hydrophobic environment of the inner mitochondrial membrane or interferes with proper folding and results in the targeting of the mutant MFT for proteolytic degradation. Either of these effects would explain the loss of MFT function in the glyB cells. It is thought that the inner mitochondrial membrane transporters function only after assembly as a 12-transmembrane domain homodimer (22). Hence, we had expected that the inactivated glyB MFT transporter might have a dominant negative effect on the function of transfected mft species. However, we have not detected such an effect (Tables I and III), because the glyB cell acts like a null function cellular background. As such, the glyB promises to be a valuable tool for study of the mechanism of the process of transport of folates into the mitochondrial matrix since this function can be selected for in this mammalian cell line.
in an attempt to deduce structures needed specifically for folate transport. The structural divergence/similarities in several regions of these proteins are noteworthy and suggestive of the roles of these peptides in MFT function as described below.

Energy Transfer Signature Motif—In the hamster MFT (Fig. 2), as with the other mammalian MFTs (Fig. 6), there are three peptides located immediately after the 1st, 3rd, and 5th transmembrane domains that fit the energy transfer signature (ES) consensus (23–26): P\(\times\)D/E/X/L/I/V/A/T\(\times\)R/K\(\times\)L/R/H/L/I/V/ M/F/Y/\(\times\)Q/G/A/I/V/M). The ES peptides for some yeast mitochondrial transporters have been shown to be involved in targeting proteins to the inner mitochondrial membrane (27, 28).

Cell lines were grown in a -MEM supplemented with 0.3 \(\mu\)Ci/ml \(\text{[3H]}\)folate acid for 48 h. The cells were harvested, homogenized, and centrifuged differentially to isolate mitochondria (see “Experimental Procedures”). Each fractionation was performed on triplicate cultures, and data were combined from two experiments. The percentage of cellular folates in the mitochondrial compartment (in parenthesis) was derived by comparing mitochondrial folates with the sum of folate levels in the cytosol plus mitochondria.

| Cell line/transfected DNA | CHO/none | glyB/none | glyB/CHO-mft | glyB/glyB-mft |
|---------------------------|----------|----------|-------------|--------------|
| Cellular compartment      | pmol folate/10\(^6\) cells |
| Cytosol                   | 5.5 ± 0.62 | 3.6 ± 0.49 | 6.1 ± 0.92 | 2.2 ± 0.45 |
| Mitochondria              | 0.66 ± 0.12 (11%) | 0.033 ± 0.009 (0.96%) | 0.60 ± 0.12 (9.0%) | 0.036 ± 0.009 (1.6%) |
| Unbroken cells/nucleus    | 1.8 ± 0.70 | 0.63 ± 0.13 | 2.6 ± 0.58 | 0.75 ± 0.23 |
| Sum of compartments       | 8.0 ± 0.94 | 4.3 ± 0.51 | 9.3 ± 1.1 | 3.0 ± 0.51 |

Fig. 6. Sequence comparison of eukaryotic MFTs with related mitochondrial carrier family proteins. MFT candidates from various species have been aligned along with the yeast flavin mitochondrial transporter and the human ATP/ADP carrier. The mammalian MFT homologs retain nearly identical sequence within conserved regions (transmembrane domains and energy sequences). The zebrafish MFT identified functionally herein had a 68% identity with human MFT and showed high homology across the energy sequence motifs and transmembrane domains. The flavin and ATP/ADP carriers, which are known to shuttle their substrates across the inner mitochondrial membrane, share similarities within the energy sequences (hamster residues 42–50, 140–148, and 244–252), but across other regions of the protein, there is very limited homology.
in sequence among the three ES motifs. However, each of the energy signal motifs, taken one at a time, is nearly identical across the several mammalian MFT homologs currently identified. The variation in sequence among the three ES peptides of a single protein, yet near identity at each of the three ES signatures across proteins, is highly suggestive that each ES peptide has a somewhat different but conserved function. It is also very interesting that the ES sequences of MFT are remarkably divergent from those of other inner membrane proteins (e.g. compare the human MFT and human ATP/ADP transporter (Fig. 6) at any of the three ES repeats) (Fig. 6) or even comparing the mammalian sequences with zebrafish within a single ES motif. This would not be expected if the only function of the ES peptides were to bind to a common set of proteins responsible for the trafficking of all of the mitochondrial transporters to their site of insertion in the inner membrane. This seems to imply either that there are substantial differences in binding partners for trafficking of individual inner membrane transporters or that these peptides have other functions involved in the transport of substrates. Others (26) have noted that there is a subgroup of the 75 mitochondrial transport proteins predicted from the yeast genome sequence distinguishable by the fact that they bear a PIW or PLW sequence in the second ES signature. All of the mammalian sequences we identify as mft homologs have the PIW tripeptide with the exception of the zebrafish, which instead has a PVW at this position, placing the MFT transporters in this small subgroup, which includes the yeast flavin transporter \textit{flx1}.

\textbf{Inner Membrane Loop Sequences}—By homology with other more thoroughly characterized mitochondrial transporters (26, 31, 32), it is predicted that the MFT proteins are inserted with the N- and C-terminal peptides facing the intermembrane space (Fig. 7). With this type of distribution in the inner membrane, three loops located directly after transmembrane domains 1, 3, and 5 are predicted to extrude into the matrix side of the membrane. The peptides that comprise these loop regions retain profound homology among the predicted mammalian MFT homologs. This does not hold true when one compares across mitochondrial metabolite carriers for other substrates compared with the MFT homologs (compare the features of the mammalian or zebrafish MFT inner loop sequences with those of the human ATP/ADP transporter or yeast \textit{flx1} carriers) (Fig. 6). It is also noted that several charged residues are universally present on all of the mammalian folate carriers and are placed identically in these loops. The presence of multiple charged residues in these predicted solvent-exposed loop peptides is to be expected from previous literature (26, 33). However, some of these charged residues seem to be conserved only in the predicted MFT homologs, suggesting a role specific to the transport of folates.

\textbf{Outer Membrane Loop Sequences}—Two loop sequences are predicted in the membrane-inserted MFT that project into the intramembrane space after the 2nd and 4th transmembrane domains in addition to the N- and C-terminal peptides. The 1st

\begin{table}
\begin{tabular}{llll}
Construct & Average colony count/plate & \% Rescue  \\
& G418 + glycine & G418 – glycine &  \\
pcDNA3.1(\textsuperscript{+}) & 227 \textpm 73 & 310 \textpm 42 & 137 \textpm 48  \\
GlyB & 140 \textpm 12 & 165 \textpm 27 & 118 \textpm 22  \\
\end{tabular}
\end{table}

\textbf{Transfection of glyB mft cDNA into CHO cells}

The cDNA for the mutant mft found in glyB cells was cloned into pcDNA3.1 and transfected into CHO cells. After 10 days of selection on medium containing glycine and G418 or glycine-deficient medium containing G418, the colonies found on triplicate plates were fixed and stained. Colony sizes were equivalent in all of the groups.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{hamster_mft_structure.png}
\caption{\textbf{Predicted structure of the hamster mitochondrial folate transporter.} The projected orientation of the MFT protein in the inner mitochondrial membrane is shown as predicted by hydropathy plot analysis and homology to other mitochondrial inner membrane proteins. The model predicts that both the N and C termini face the intermembrane space, that there are six membrane-spanning regions, and that there three energy motifs are located immediately after membrane-spanning regions 1, 3, and 5. The G to A transition in glyB cells at residue 192 (yellow) would be predicted to be in the fourth membrane-spanning region.}
\end{figure}
and 2nd intramembrane loops of the identified MFTs are highly homologous across species as is the C-terminal peptide, in distinct contrast to the sequences of the N-terminal peptides that differ significantly among even the mammalian MFT proteins (Fig. 6). When compared with peptides of other metabolite carriers in the inner mitochondrial membrane, there are several invariant charged residues spaced at intervals of approximately three residues apart. Currently, the role of these residues in recognition and transport of folates is being studied.

Acknowledgment—We thank Prof. Larry Chasin (Columbia University) for generously providing the glyB cells.

REFERENCES
1. Kao, F., Chasin, L., and Puck, T. T. (1969) Proc. Natl. Acad. Sci. U. S. A. 64, 1284–1291
2. Appling, D. R. (1991) FASEB J. 5, 2645–2651
3. Wang, F. K., Koch, J., and Stokstad, E. L. (1967) Biochem. Zeit. 263, 13602–13609
4. Goldman, I. D., Lichtenstein, N. S., and Oliverio, V. T. (1968) J. Biol. Chem. 243, 5007–5017
5. Dixon, K. H., Lanphier, B. C., Chiu, J., Kelley, K., and Cowan, K. H. (1994) J. Biol. Chem. 269, 17–20
6. Bringle, K. E., Spinella, M. J., Westin, E. H., and Goldman, I. D. (1994) Biochem. Pharmacol. 47, 357–345
7. Kao, F. T., and Puck, T. (1975) Genetics 79, (suppl.) 343–52
8. Titus, S. A., and Moran, R. G. (2000) J. Biol. Chem. 275, 36811–36817
9. Graham, F. L., and van der Eb, A. J. (1973) Virolology 52, 456–467
10. Jackson-Cook, C., Bae, V., Edelman, W., Brothman, A., and Ware, J. (1996) Cancer Genet. Cytogenet. 87, 14–23
11. Landegent, J. K., Jansen in de Wal, N., Dirks, R. W., Baan, F., and van der Ploeg, M. (1987) Hum. Genet. 77, 366–370
12. Chasin, L. A., Feldman, A., Knustam, M., and Urlaub, G. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 718–722
13. Taylor, R. T., and Hanna, M. L. (1982) Arch. Biochem. Biophys. 217, 609–623
14. Schirch, L., and Peterson, D. (1980) J. Biol. Chem. 255, 7801–7806
15. Saraste, M., and Walker, J. E. (1982) FEBS Lett. 144, 250–254
16. Aquila, H., Link, T. A., and Klingenberg, M. (1985) EMBO J. 4, 2369–2376
17. Runswick, M. J., Powell, S. J., Nyren, P., and Walker, J. E. (1987) EMBO J. 6, 1367–1373
18. Runswick, M. J., Walker, J. E., Bisaccia, F., Iacobazzi, V., and Palmieri, F. (1990) Biochemistry 29, 11033–11040
19. Worton, R. G., Ho, C. C., and Duff, C. (1977) Somatic Cell Genet. 3, 27–45
20. Runswick, M. J., Walker, J. E., Bisaccia, F., Iacobazzi, V., and Palmieri, F. (1990) Biochemistry 29, 11033–11040
21. Nelson, D. R., Felix, C. M., and Swanson, J. M. (1998) J. Biol. Chem. 273, 14269–14276
22. Palmieri, F. (1994) FEBS Lett. 346, 48–54
23. Kuan, J., and Saier, M. H., Jr. (1993) Crit. Rev. Biochem. Mol. Biol. 28, 209–233
24. Barouch, A. (1992) Nucleic Acids Res. 20, (suppl.) 2015–8
25. Schroers, A., Burkovski, A., Wohlrab, H., and Kramer, R. (1998) J. Biol. Chem. 273, 14269–14276
26. Schirch, L., and Peterson, D. (1980) J. Biol. Chem. 255, 7801–7806
27. Saraste, M., and Walker, J. E. (1982) FEBS Lett. 144, 250–254
28. Endres, M., Neupert, W., and Brunner, M. (1999) EMBO J. 18, 3214–3221
29. Koehler, C. M., Jarosch, E., Tokatlidis, K., Schmid, K., Schweyen, R. J., and Schatz, G. (1998) Science 279, 369–373
30. Koehler, C. M., Merchant, S., Ophliger, W., Schmid, K., Jarosch, E., Delфин, I., Junne, T., Schatz, G., and Tokatlidis, K. (1998) EMBO J. 17, 6477–6486
31. Capobianco, L., Bisaccia, F., Michel, A., Sluse, F. E., and Palmieri, F. (1995) FEBS Lett. 357, 297–300
32. Brandolin, G., Le Saux, A., Trezequet, V., Lautquin, G. J., and Vignais, P. V. (1993) J. Bioenerg. Biomembr. 25, 459–472
A Mutation Inactivating the Mitochondrial Inner Membrane Folate Transporter Creates a Glycine Requirement for Survival of Chinese Hamster Cells
Erin A. McCarthy, Steven A. Titus, Shirley M. Taylor, Colleen Jackson-Cook and Richard G. Moran

J. Biol. Chem. 2004, 279:33829-33836.
doi: 10.1074/jbc.M403677200 originally published online May 12, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M403677200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 33 references, 12 of which can be accessed free at
http://www.jbc.org/content/279/32/33829.full.html#ref-list-1