Characterization of a Mutation in the Reduced Folate Carrier in a Transport Defective L1210 Murine Leukemia Cell Line

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This laboratory previously described an L1210 leukemia cell line (MTXrA) selected for resistance to methotrexate by virtue of impaired transport due to a functional defect in the translocation process. We now report on the sequence analysis of cDNAs encoding the reduced folate carrier from this line and identify a single mutation that results in the substitution of a proline for an alanine in a highly conserved transmembrane region of the protein. Transfection of the parental reduced folate carrier into MTXrA cells resulted in a cell line which exhibited a complete restoration of methotrexate uptake and an enhanced sensitivity to methotrexate. Northern analysis and specific [3H]MTX cell surface binding indicated that expression of the reduced folate carrier was elevated 5-fold in the transfectant compared to parental and MTXrA cells. The MTX influx properties of the transfectant cell line were identical to those of the well characterized reduced folate carrier from parental L1210 cells in terms of: 1) patterns of sensitivity to competing folates, 2) sensitivity to the organic anion sulfobromophthalein, 3) lack of energy dependence, and 4) capacity for trans-stimulation. We also provide new data which suggests that the nucleotide sequence 5' of the predicted ATG initiation codon may encode additional protein information in the form of a leader sequence. Finally, we demonstrate that the MTXrA line has both the mutant and the parental reduced folate carrier alleles but that expression appears to be restricted to the mutant allele. Thus, the methotrexate transport phenotype and resultant drug resistance in this cell line result from genetic/regulatory events at both alleles.

Resistance to methotrexate (MTX) is a major limiting factor in the clinical utility of this agent and can occur by a variety of mechanisms (1, 2). Of particular interest to this and other laboratories has been the resistance associated with impaired transport of the drug via the reduced folate carrier system. In murine L1210 leukemia cells, this carrier system mediates the rapid transport of reduced folates and the 4-amino analogs of folic acid and its kinetic and thermodynamic properties have been characterized in detail by several laboratories (3-5). Clones encoding a transmembrane carrier protein have recently been isolated from murine, hamster, and human cDNA libraries (6-9) and shown to restore folate transport in cell lines defective in carrier-mediated transport. Hydropathy plots of this ~60-kDa protein, designated RFC1 in the murine system, predict 12 α-helical transmembrane regions and suggest that it was likely a member of a superfamily of membrane spanning transporters. Based upon the relative molecular mass of membrane components which are labeled when murine cells are treated with various MTX-affinity analogs (M₀ = 43-48), the predicted M₀ of the cloned RFC1 protein is different from that predicted for the reduced folate carrier (10-12). This suggests that, in the murine system, there is either significant post-translational modification of RFC1 or that this protein is just one component of the reduced folate carrier system.

Several mechanisms of carrier-associated drug resistance have been characterized and shown to result from an increase in the influx Kᵢ, a decrease in the influx V_max, or changes in both parameters (1, 3). Changes in the latter parameter usually result from a decrease in the number of carrier sites. This laboratory previously described a MTX-resistant L1210 murine leukemia line (MTXrA) which exhibits a unique transport alteration due to an immobilization of the reduced folate carrier (10). In this cell line, the number of apparent carrier binding sites was slightly decreased, the affinity of these sites for MTX at the cell surface was unchanged, but the influx V_max for MTX was markedly decreased (10, 13). These observations were consistent with a highly specific loss of function, or mobility, of the reduced folate carrier. The MTXrA line exhibits multiple membrane protein alterations, but the functional change in transport is highly specific for the reduced folate carrier as transport of folic acid, amino acids, nucleosides, and sugars is not impaired (10).

In this paper, we describe the isolation and characterization of cDNAs encoding the RFC1 protein in the MTXrA line and identify a single mutation that appears to be responsible for the defect in MTX transport. We demonstrate that this MTX transport phenotype can be complemented by expression of the parental RFC1 in the MTXrA line and, further, that the restored transport has properties identical to those of the well characterized reduced folate carrier present in parental L1210 cells.

MATERIALS AND METHODS

Chemicals—[3, 5, 7-3H]MTX was obtained from Moravek Biochemicals and purified by high performance liquid chromatography prior to use.

Cell Culture—Parental L1210 cells were grown in RPMI 1640 medium (containing 2.2 mM folic acid) supplemented with 10% bovine calf serum (Hyclone), 2 mM glutamine, 20 mM 2-mercaptoethanol, penicillin (100 units/ml), and streptomycin (100 μg/ml).

Growth Inhibition Studies—0.2-ml cultures (8.0 × 10⁶ cells/ml) in 96-well plates were continuously exposed to the appropriate concentra-
tion of MTX in replete medium for 72 h following which cell numbers were determined by hemocytometer count and viability determined by trypan blue exclusion.

Influx of MTX—Cells were harvested, washed, with 0°C HBS buffer (20 mM HEPEs, 140 mM NaCl, 5 mM KCl, 2 mM MgCl2, 5 mM glucose, pH 7.4), and resuspended in HBS to 1 × 10⁷ cells/ml. Uptake was initiated by the addition of radiolabeled MTX and samples taken over 4 min. For [³H]MTX transport inhibition studies, folic acid, and 5-formyltetrahydrofolate were added simultaneously with the drug. To assess the effects of sulfobromophthalain and azide, cells were incubated 10 min at 37 °C in the presence of these compounds (100 µM and 10 mM, respectively) prior to the addition of [³H]MTX. In studies utilizing azide, glucose was absent from the transport buffer. For transport stimulation studies, cells were incubated with 60 µM di-5-formyltetrahydrofolate for 30 min at 37 °C, washed two times with ice-cold transport buffer, and resuspended in transport buffer at 37 °C containing [³H]MTX. For all of the studies, uptake was terminated by injecting 1.0 ml of the cell suspensions into 9.0 ml 0°C acid HBS (pH 4.5). Cells were collected by centrifugation, washed twice in 0°C HBS (pH 7.4), and processed for determination of intracellular tritium as described previously (14).

Analysis of Specific MTX Binding to the Reduced Folate Carrier at the Cell Surface—Cells were harvested, washed, resuspended in 0°C HEPEs buffer (160 mM HEPEs, 2 mM MgCl2, pH 7.4) to 1 × 10⁷ cells/ml and 250-µl aliquots incubated with 10 µM [³H]MTX for 10 min at 0 °C. Bound and free ligand were separated by centrifugation at 9400 g for 10 min at 4 °C. Aliquots of the reaction mixture were used for dry weight determinations of cell pellets as described previously (14). Specific binding was determined as the difference between 1.0 ml aliquots incubated with 100 nM [³H]MTX and the same volume of MTX buffer in place of the radiolabeled ligand. Bound and free ligand were separated by centrifugation as above.

Northern Analyses—RNA was isolated using the RNAzol B (Biotech) procedure, fractionated by electrophoresis on 1.0% formaldehyde-agarose gels, fixed to nylon membranes (Schleicher and Schuell), and hybridized with nick-translated probes prepared using the random primer procedure (15). Quantitation of specific transcripts was performed by direct analysis of radioactive bands using a Molecular Dynamics 445 SI Phosphoimager and transcript levels normalized to glyceraldehyde-3-phosphate dehydrogenase.

Cloning of the Reduced Folate Carrier—Poly(A)⁺ mRNA was isolated from 1 × 10⁷ cells using QuickPrep (Pharmacia Biotech Inc.) and resuspended in 5 µl of sterile water. Using 0.5 µl of the poly(A)⁺ RNA, first strand synthesis was performed with oligo(dT) in a final volume of 20 µl using a reverse transcription kit (Life Technologies, Inc./BRL).

RESULTS

Cloning of the Reduced Folate Carrier—We previously described an L1210 cell line, designated MTXrA, which exhibited a 100-fold increased resistance to MTX due to impaired transport associated with a loss of function of the reduced folate carrier (10). Recently, the reduced folate carrier, or a component (designated RFC1) of this carrier system, was cloned by several groups (6–9). Therefore, studies were undertaken to determine whether a mutation in this transport protein could be the basis for the MTX transport defect and resultant MTX resistance exhibited by the MTXrA cell line. Using primers based on the published RFCl murine sequence, a cDNA encoding the translated region of RFC1 was isolated from parental cells by RT-PCR and used to probe a cDNA library generated from the L1210 and CHO cells. Several clones were identified and complete sequence analysis performed on the one (accession number U32469) having the longest insert (2283 bp). As the RFCl transcript identified upon Northern analysis is ~2300 nt (Fig. 1), this clone appears to encode a nearly full-length cDNA. Translation of the 123 nucleotides upstream of the predicted ATG start codon results in an additional 41-amino acid residues.

Analysis of a Defective Reduced Folate Carrier

The parent expression vector pTK-PGK used in the transfection studies was constructed as follows: plasmid pcDNA3 (13) was restricted with BamHI and XhoI to remove all FR-α coding sequences, blunt-ended with Klenow, and religated. The resultant plasmid was linearized with EcoRI (5′ of the PGK promoter) and the pmC1NeoPoly(A) (neo⁺) cassette (derived from pmC1NeoPoly(A) in which the single mutation present in the neo⁺ cassette was corrected) was inserted. To generate the parental RFCl expression vector pPGK-RFCl, the pTK-PGK vector was linearized with Eco1316I and DNA encoding the RFCl protein from the L1210 parental cells (nt 38–1584 based on the published sequence by Dixon et al. (6).) was inserted.

MTXrA cells (1 × 10⁵ cells) were electroporated (250 V, 200 microfarads) with 40 µg of BglII-linearized pPGK-RFCl in a final volume of 800 µl of RPMI 1640 folate-free medium without serum. Cells were brought to 10 ml with RPMI 1640 medium and allowed to recover 36 h following which they were adjusted to 1 × 10⁷ cells/ml in RPMI 1640 medium containing 418 (75 µg of active drug/ml) and distributed into 96-well plates at approximately 20,000 cells per well. Positive (G418 resistant) wells developed within 8–10 days and these cells were expanded further for further study.

Genomic Amplification and Restriction Analysis—Amplification of a genomic RFCl-specific 520-bp fragment was performed using the following primers which flanked the mutation identified in the MTXrA RFCl: 5'-CTGGCAGTACACGACCAGCTC-3' and 5'-TTCAC-CAGTTCACCTTGAACATG-3'. Genomic DNA was isolated using DNAzol (Molecular Research Center, Inc.) and 300 ng used for the amplification reaction under the following conditions: 10 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM (NH₄)₂SO₄, 3 mM MgSO₄, 0.1% Triton X-100, 300 µM of each deoxynucleotide, 0.4 µM each primer, and 1.0 unit of VENT polymerase (New England Biolabs) in a 50 µl reaction volume. Following an initial melt for 2 min at 96 °C, amplification was carried out for 31 cycles of 45 s at 96 °C, 40 s at 62 °C, and 1 min at 72 °C. The 520-bp fragment was gel purified, restricted, and fractionated on a 6% TBE polyacrylamide gel.

RESULTS

Cloning of the Reduced Folate Carrier—We previously described an L1210 cell line, designated MTXrA, which exhibited a 100-fold increased resistance to MTX due to impaired transport associated with a loss of function of the reduced folate carrier (10). Recently, the reduced folate carrier, or a component (designated RFC1) of this carrier system, was cloned by several groups (6–9). Therefore, studies were undertaken to determine whether a mutation in this transport protein could be the basis for the MTX transport defect and resultant MTX resistance exhibited by the MTXrA cell line. Using primers based on the published RFCl murine sequence, a cDNA encoding the translated region of RFC1 was isolated from parental cells by RT-PCR and used to probe a cDNA library generated from the MTXrA cell line. Several clones were identified and complete sequence analysis performed on the one (accession number U32469) having the longest insert (2283 bp). As the RFCl transcript identified upon Northern analysis is ~2300 nt (Fig. 1), this clone appears to encode a nearly full-length cDNA. Translation of the 123 nucleotides upstream of the predicted ATG start codon results in an additional 41-amino acid residues.
which no longer contains the in-frame TGA termination codon predicted by Dixon et al. (6). The two closed reading frames each contain at least one termination codon. Interestingly, these same upstream nucleotide sequences from both human and CHO cells encode an additional 31 and 33 amino acid residues, respectively, and neither encodes a termination codon (7–9). As shown in Fig. 1, 13 of the initial 16 amino acid residues (and 20 of 33 overall) predicted for the L1210 sequence are identical to those predicted for this upstream sequence in CHO cells. These data raise the possibility that the nucleotide sequence in this 5′-region may actually encode additional protein information, perhaps in the form of a signal sequence which directs the RFC1 protein (or one of its potential splice forms, see below) to a target membrane. Accordingly, the predicted stretch of 41 amino acid residues is well within the size range for mitochondrial signal sequences and it contains a high proportion (39%) of those residues (Ala, Arg, Leu, and Ser) commonly found in such targeting sequences and a low proportion (7%) of those (Asp, Glu, Ile, and Lys) which are rarely found (17, 18). Likewise, the predicted human and CHO protein sequences encode, respectively, 58 and 52% of the four common residues and only 0 and 9% of the four uncommon residues.

While the nucleotide sequence of the translated region of RFC1 from parental cells was identical to the published murine sequence, a single mutation (nt G429 → C429; numbering is based on the published (6) murine L1210 sequence) was identified in all of the RFC1 clones derived from the MTX′A′ line. This mutation resulted in the substitution of proline for alanine at amino acid residue 130. This residue is also an alanine in the RFC1 protein isolated from both human and CHO cells and is located in a highly conserved region in the middle of the predicted fourth transmembrane domain of the protein.

Transfection Studies—To verify that the alanine → proline mutation identified in RFC1 was responsible for the defective MTX transport phenotype observed in the MTX′A′ cell line, an expression vector encoding the parental RFC1 was transfected into this line in an effort to complement the mutation and restore transport function. MTX′A′ cells were electroporated with the expression vector pPGK-RFC1 ("Materials and Methods") and G418 resistant clonal lines were isolated. A typical transfectant, designated MTX′A′-R16, was chosen for the remaining experiments described below.

As shown in Fig. 2, the transcript encoding the endogenous RFC1 (~2300 nt) was present in all three (L1210 parent, MTX′A′, and MTX′A′-R16) cell lines. In contrast, the RFC1-encoding transcript produced by the pPGK-RFC1 expression vector was diagnostically shorter than the endogenous message and was present only in the transfected cell line. Phosphoimage analysis of multiple samples indicated that the steady state level of total RFC1 message present in the MTX′A′-R16 line was ~5-fold higher than in the parental line. Conversely, there was a ~30% decrease in the total level of RFC1 message expressed in the MTX′A′ line compared with the parental line. The level of reduced folate carrier expressed on the surface of these three cell lines was determined by measuring specific cell surface binding of [3H]MTX at 0°C. As shown in Table I, the level of specific [3H]MTX binding roughly correlated with the total level of RFC1 message present upon Northern analysis.

MTX Influx Studies—As shown in Fig. 3, the 5-fold increase in the level of RFC1 expression in the MTX′A′-R16 transfectant resulted not only in a restoration of MTX uptake, but in a nearly 10-fold increase in MTX influx compared with L1210 parental cells. In fact, influx of 0.1 μM [3H]MTX in MTX′A′-R16 cells was slightly greater than the influx of 1 μM [3H]MTX in the parental line. The recovery of MTX transport in the MTX′A′-R16 cell line was reflected in its restored sensitivity to MTX in growth inhibition studies. This transfectant exhibited an IC50 of ~4.0 nm, a value which is 200- and 2.4-fold less than that of MTX′A′ and L1210 parental cells, respectively. Taken together, these results strongly suggest that the mutation identified in RFC1 in the MTX′A′ cell line is responsible for the observed MTX transport defect.

As there is virtually no uptake of MTX in the MTX′A′ cell line, transport in MTX′A′-R16 cells must be mediated entirely by the transfected RFC1 and should exhibit properties essentially identical to those of the well characterized L1210 parental cell line. In parental cells, MTX influx mediated by the classical reduced folate carrier has the general characteristics of a carrier mediated process (19), is unaffected by metabolic poisons (13, 20, 21), exhibits sensitivity to structurally unrelated organic and inorganic ions (22–24), and shows preference for MTX and reduced folates (kM=1–5 μM) compared with folic acid (kM=100–200 μM) (5, 25).

As shown in Fig. 4, [3H]MTX influx in the transfectant was relatively insensitive to the presence of 5 μM folic acid but was inhibited nearly 60% by 5 μM 5-formyltetrahydrofolate. As in parental L1210 cells, the addition of the inorganic anion sulfobromophthalain (100 μM) nearly abolished the influx of [3H]MTX in the transfectant. In contrast, the presence of 10 mM azide had essentially no effect on [3H]MTX influx. Finally, transport by the classical reduced folate carrier in parental L1210 cells shows the characteristic of influx stimulation by the presence within the intracellular compartment of a similar substrate which shares this carrier. Accordingly, there was a 1.13-fold trans-stimulation of [3H]MTX influx when the transfectant was preloaded with dl-5-formyltetrahydrofolate. These results demonstrate that the restored (and augmented) MTX influx mediated by the transfected RFC1 has properties of the well characterized reduced folate carrier and that RFC1 indeed encodes this classical murine folate transporter.

Potential Splice Forms—Additional sequence analysis of random RT-PCR-generated clones identified a potential RFC1 splice variant which was present in a small percentage of the clones. This specific variant exhibited a 119-bp deletion (loss of nt T601 → nt G979) and is considered to result from alternative splicing because: (a) it is present in clones isolated from both

| Parental L1210 | MTX′A′ | MTX′A′-R16 |
|---------------|-------|-----------|
| Total RFC1 transcript | 1.00  | 0.72  | 4.93  |
| Specific [3H]MTX binding | 1.00  | 0.44  | 4.25  |
and Methods." Data represent the mean ± S.E. of four experiments.

**FIG. 3.** Transport of [3H]MTX in parental L1210, MTX/A, and MTX'A-R16 cell lines. Cells were harvested, washed, and resuspended in HEPES-buffered saline, and at time 0, exposed to 1 μM [3H]MTX (closed symbols) or 0.1 μM [3H]MTX (open symbol). The graph is representative of four experiments.

**FIG. 4.** Effect of various treatments on initial rates of [3H]MTX uptake in MTX'A-R16 cells. MTX'A-R16 cells were harvested, washed, and resuspended in HEPES-buffered saline, and at time 0, simultaneously exposed to 0.1 μM [3H]MTX and either 5 μM folic acid or 5 μM 5-formyltetrahydrofolate. Details of the sulfobromophthalein (BSP), azide, and trans-stimulation treatments are described under "Materials and Methods." Data represent the mean ± S.E. of four experiments.

TABLE II

| Restriction enzyme | Expected restriction fragment sizes |
|--------------------|-----------------------------------|
| BsaHI (+)          | 520                               |
|                     | 390a                              |
|                     | 130a                              |
| Sau96I (-)          | 115                               |
|                     | 220a                              |
| NlaIV (+)           | 360                               |
|                     | 230a                              |
|                     | 130a                              |

*Novel restriction fragments generated by the gain (+) or loss (−) of a restriction site in the mutated RFC1 allele.*

the parental and mutant cell lines, (b) it arose in several independent RT-PCR reactions, and (c) the 3' junction of this 119-bp deletion maps to the exact 3' site of an internal deletion found by Williams et al. (7) in an RFC1 cDNA isolated from a CHO cDNA library (nt 940 based on the CHO sequence). While this 119-bp deletion alters the normal RFC1 reading frame, the resulting novel polypeptide continues for an additional 72 amino acid residues before reaching a termination codon. In its entirety, the predicted protein that would result from translation of this splice form is one-third shorter than the full-length RFC1 and has a novel hydrophilic COOH terminus which comprises 20% of the protein. Interestingly, this predicted polypeptide essentially bisects the full-length RFC1, having only the first six hydrophobic transmembrane domains which are present in the full-length RFC1.

Identification of RFC1 Alleles—The original MTX'A donal line was isolated by a single step selection in 50 nM MTX and has essentially no MTX transport capacity. Accordingly, in the present study, no cDNA clones encoding a functional RFC1 were isolated from these cells by either RT-PCR or from the cDNA library. This data suggests that either the MTX'A line does not have an allele which encodes a functional RFC1 protein or alternatively, that this cell line has two distinct RFC1 alleles (functional and mutated) but that it expresses only the mutated allele. Fortuitously, the mutation present in the MTX'A line generates two new restriction sites (BsaHI and NlaIV) and results in the loss of another (Sau96I). Thus, it was possible to distinguish between the two RFC1 alleles utilizing genomic PCR and subsequent restriction analysis of the amplified fragment. As detailed in Table II and shown in Fig. 5, the amplified fragment from parental L1210 cells generates a restriction pattern that is characteristic of the functional RFC1 allele only. In the MTX'A line, however, the amplified fragment generates a restriction pattern which is indicative of both the functional and mutated alleles. While the inability of the MTX'A cell line to express a functional RFC1 could be due to an inactivating gene rearrangement at the RFC1 gene locus, no such event was evident by Southern analysis (data not shown).

**DISCUSSION**

We previously described an L1210 cell line, designated MTX'A, which exhibited a 100-fold increased resistance to MTX by virtue of a novel, functional defect in the reduced folate carrier (10, 13). A cDNA (RFC1) thought to encode the reduced folate carrier has recently been isolated and, in the present study, we cloned and characterized cDNAs encoding this protein from the MTX'A cell line and identified a single mutation in the RFC1 protein that results in the substitution of a proline residue for an alanine. When the parental RFC1 was transfected into MTX'A cells, MTX sensitivity was regained and MTX transport restored. The transport properties of the restored MTX uptake in the transfectant were identical to those of the well characterized classical reduced folate carrier present in parental L1210 cells. This confirms that RFC1 does indeed encode this transport protein and that, in the MTX'A cell line, the mutation identified in this protein is responsible for the defective MTX transport phenotype.

Based on hydrophobicity plots, the RFC1 protein is predicted to have 12 α-helical transmembrane spanning domains (6). The alanine → proline substitution identified in the MTX'A line occurs in one such domain in a highly conserved region of the protein. Transmembrane α-helices of integral membrane transport proteins, such as the family of glucose transporters, are believed to form channels through which substrates pass (26,
While proline residues frequently occur in such transmembrane helices (serving both structural and dynamic roles), this imino acid generally has significant α-helix destabilizing features (28). Random insertion of a proline residue into a protein would be expected to have adverse structural consequences due to the induction of backbone kinks. In the present case, such a structural alteration in a transmembrane region of RFC1 could result in a functional change that readily prevents substrate passage into the cell (i.e. 100-fold decrease in V_{max}) without having an effect on substrate binding (i.e. unchanged K_m).

The MTX’A cell line is essentially unable to transport MTX and all of the RFC1-encoding cDNAs isolated from this line contained the alanine→proline mutation. Therefore, it was apparent that the MTX’A cell line either did not have a parental (i.e. functional) RFC1 allele or that, if one was present, it was not expressed. Southern analyses and diagnostic restrictions of an amplified genomic RFC1 fragment unequivocally demonstrated that a structurally unaltered parental RFC1 allele was present in the MTX’A cell line. As such, it appears that the initial selection of the donor MTX’A cell line in 50 nM MTX was the result of two events: 1) a G→C conversion mutation which led to the substitution of a proline for an alanine in one RFC1 allele; and 2) either a second mutation or epigenetic mechanism which led to a silencing of the functional RFC1 allele. While a second mutational event may have occurred, it would likely present in the 5’-regulatory region of the RFC1 gene and would not have been detected in this study. Alternatively, drug-induced DNA hypermethylation and subsequent inactivation of the functional RFC1 allele is also quite possible. DNA hypermethylation has been shown to occur as a normal cellular response to drugs such as MTX which inhibit DNA synthesis (29, 30). Furthermore, as DNA methylation is often associated with gene silencing, Nye (29) has suggested that hypermethylation may aid in the development of drug resistance by inactivating genes whose products are required for drug toxicity. In fact, a previous study by Hsueh and Dolnick (31) has shown that decreased expression of a folate receptor due to hypermethylation of the encoding gene is, in large part, responsible for the MTX resistance which developed in a human KB cell line following selection for growth on the drug.

Williams et al. (7) identified several RFC1 clones from a CHO cDNA library that contained internal deletions and were suggestive of alternative splicing. In the present study, a potential alternative RFC1 splice form was identified in a number of RT-PCR generated clones isolated from these L1210 cell lines. While multiple RFC1 transcripts were not visible on Northern analysis, the size of this potential splice form may not differ significantly from the major RFC1 transcript or its steady state level of expression may simply be too low to visualize. The nucleotide sequence of this splice form predicts a protein which contains the first six transmembrane domains of the RFC1 protein followed by a novel hydrophilic carboxyl terminus of 72 amino acid residues. While there is no evidence that this transcript is ultimately translated into a functional protein, it should be noted that other investigators have demonstrated the presence of alternative MTX transport systems with properties distinct from the classical reduced folate carrier (32–36). Additionally, Horne et al. (37) have characterized a transporter that mediates the translocation of reduced folates into mitochondria. The potential presence of additional protein information (42 amino acid residues, see Fig. 1) encoded upstream of the putative ATG start codon raises the possibility that putative RFC1 splice forms could be targeted to different membrane systems. Together, these observations raise the possibility that alternative splice forms of the RFC1 gene transcript may give rise to transmembrane carriers which have distinct structural, biochemical, and thermodynamic properties.

Finally, the homologous murine expression system resulting from a combination of the MTX’A cell line and the pTK-PGK expression vector developed in this study will be a very useful model for expression and characterization of RFC1 transport proteins that contain specifically engineered mutations. Such structure-function studies may aid in the development of additional pharmacological agents which utilize this carrier for transport into the cell.

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