Allelic Variation in the Dihydrofolate Reductase Gene at Amino Acid Position 95 Contributes to Antifolate Resistance in Chinese Hamster Cells

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ABSTRACT

The Chinese hamster lung cell line DC-3F contains two polymorphic dihydrofolate reductase (DHFR) alleles that are defined by an Asp-Asn amino acid sequence difference at position 95 in the protein. Previously, we reported that the antifolate-resistant subline DC-3F/A3 overexpressed a Leu95 → Phe mutant of the Asp95 (21k) allele and that this was the basis of its resistance to methotrexate (MTX) and methasquin [P. W. Melera, J. P. Davide, C. A. Hession, and K. W. Scotto. Mol. Cell. Biol., 4:38-48, 1984]. We now show that another independently selected antifolate-resistant subline of DC-3F, DC-3F/AS5, in addition to being severely compromised in its ability to accumulate MTX, overexpresses a Leu95 → Phe mutant form of the Asn95 (20k) allele. Characterization of purified DHFR from these cells showed that the enzyme displayed a 6-fold higher K₉ for MTX (3.92 ± 0.17 psu) than the wild type (0.58 ± 0.10 psu), thus explaining its lowered sensitivity to drug. Unexpectedly, however, this value was 4-fold lower than that displayed by the DC-3F/A3 enzyme even though both contain the same (Leu95 → Phe) mutation and differ only at position 95. Indeed, we have also shown that the 21k and 20k wild type enzymes, both containing Leu at position 22, in fact differ by 3-fold (1.58 ± 0.08 and 0.58 ± 0.10 psu, respectively) in their K₉'s for MTX. This demonstrates that the amino acid at position 95 has an effect on the ability of DHFR to bind MTX. On the other hand, these allelic variants are indistinguishable from each other in their catalytic properties and in their respective K₉'s for dihydrofolate. Taken together, these characteristics are consistent with the observation that it is the wild type 21k allele which is preferentially overexpressed at a frequency of 3:1 in MTX-resistant Chinese hamster lung sublines derived by long-term selection in MTX.

The results of these studies are novel in that they establish a role for allelic variation in the DHFR gene as a contributor to antifolate resistance in mammalian cells. Moreover, they implicate amino acid position 95 in the maintenance of the structure of the MTX binding pocket.

INTRODUCTION

The folic acid analog MTX has been used clinically in the treatment of severe psoriasis, rheumatoid arthritis, and a variety of malignancies (1). The target of MTX is DHFR (5,6,7,8-tetrahydrofolate; NADPH oxidoreductase; EC 1.5.1.3) which catalyzes the NADPH-dependent reduction of H₂folate to H₄folate, and the H₄folate is a key intermediate in one-carbon metabolism. The clinical use of MTX, however, has been limited by cytotoxicity resulting from high doses and by the resistance of patients to the drug (2). Such clinical resistance manifests itself either as an intrinsic characteristic or as an acquired phenotype, and much effort has been focused upon understanding the biochemical and genetic basis of both (3).

A useful approach to the study of acquired resistance has been the study of cell lines selected for growth in the presence of MTX or its analogues. The results of these efforts have shown that antifolate resistance can be mediated by a variety of mechanisms including: (a) elevation of cellular DHFR activity as a result of DHFR gene amplification; (b) alteration of DHFR per se, resulting in decreased affinity for inhibitor; (c) alteration of drug transport into the cell; (d) reduction of MTX-polyglutamylation; (e) reduced thymidylate synthetase activity; and (f) enhancement of γ-glutamyl hydrolase activity. In some instances, more than one of these mechanisms has been found to be operative in a single cell line (4, 5).

We have shown that the Chinese hamster lung (CHL) cell line DC-3F is heterozygous at the DHFR locus (6, 7). The two polymorphic DHFR alleles, termed 20k and 21k because the DHFRs they encode display different mobilities upon SDS-PAGE with apparent molecular weights of 20,000 and 21,000, respectively (8), differ in their HindIII restriction profile (7), in the relative abundance of the different-sized polyadenylated transcripts they produce (7, 9), and in the enzymes they encode. The 20k and 21k alleles contain Asn and Asp at amino acid position 95, respectively (6). When subjected to step-wise increases in the concentration of MTX or its analogue methasquin (10, 11), either of these alleles can amplify, resulting in an overexpression of DHFR (12) and, therefore, resistance. Moreover, several of the drug-resistant sublines display resistance levels that are not proportional to their DHFR activity levels (13).

In the present study, we have analyzed the antifolate-resistant phenotype of DC-3F/A55, a CHL cell line known to amplify the DHFR gene and to overexpress an altered form (13) of the 20k enzyme (8). Our interest in this subline was based upon its high level of resistance to MTX, about 4,500-fold (13) relative to its modest DHFR overexpression (13), characteristics that allow this mutant form of DHFR to act with high efficiency as a dominant selectable marker in gene transfection experiments (15) and allow amplification of cotransfected genes as well (16).

In the present study, we have analyzed the antifolate-resistant phenotype of DC-3F/A55, a CHL cell line known to amplify the DHFR gene and to overexpress an altered form (13) of the 20k enzyme (8). Our interest in this subline was based upon its high level of resistance to MTX, about 4,500-fold (13) relative to its modest DHFR overexpression level, i.e., approximately 4-fold (17), and the fact that mutations of the 20k allelic form of Chinese hamster DHFR had not been previously characterized at the molecular level. In addition to confirming that DC-3F/A55 cells contain an altered form of DHFR (13), the results of this work show that the altered form is a Leu95 → Phe mutant of the 20k allele and that resistance in this subline is also mediated by a dramatic decrease in MTX transport. Moreover, we provide evidence to show that amino acid residue 95, the site of allelic variation, contributes to the differential affinity of Chinese hamster DHFR for MTX and that the influence of position 95 on MTX binding is enhanced by the Leu to Phe substitution at amino acid residue 22. Hence, allelic variation at amino acid position 95 in the DHFR gene contributes to antifolate resistance in CHL cells.

MATERIALS AND METHODS

Materials. Minimum essential media, F-12, fetal calf serum, Dulbecco's phosphate-buffered saline, restriction endonucleases, T₅ DNA ligase, and isopropyl β-D-thiogalactopyranoside were purchased from BRL. The expression

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3 The abbreviations used are: MTX, methotrexate; DHFR, dihydrofolate reductase; H₂folate, 7,8-dihydrofolate; H₄folate, 5,6,7,8-tetrahydrofolate; CHL, Chinese hamster lung; CHO, Chinese hamster ovary; cDNA, complementary DNA; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PFGS, polyfolypolyglutamate synthetase.
vector pKK223-3, oligodeoxynucleoside acid_{12-18} and Sephade G-100 were obtained from Pharmacia LKB Biotechnology, Inc. G-50 quick-spin columns and hydroxyapatite were supplied by Boehringer Mannheim and Bio-Rad, respectively. RNAsin and AMV reverse transcriptase were purchased from Promega, and Sequenase 2.0 in kit form was purchased from United States Biochemicals Corp. Taq DNA polymerase was obtained from Perkin Elmer/Cetus, and [α-32P]dATP was purchased from New England Nuclear, Inc. [3H]MTX (specific activity, 10–20 Ci/mmol) and [3H]H2folate (specific activity, 29 Ci/mmol) were supplied by Moravek Biochemicals and were purified prior to use by thin layer chromatography as described previously (18). MTX and (d5)-folinic acid were a gift from Lederle Laboratories and SAFEC S.A. Fine Chemicals, Barbengo, Switzerland. NADPH, NADP+, folic acid, and leupeptin were purchased from Sigma. H2folate was prepared from folic acid by dihydrofolate reductase (19). Oligodeoxynucleosides used for polymerase chain reaction and sequencing were synthesized by phosphoramidite chemistry (20) using an Applied Biosystems model 391 DNA synthesizer and purified via oligonucleotide purification cartridges according to Applied Biosystems.

Cell Lines and Cultures. The antifolate-sensitive CHL cell line DC-3F and the MTX-resistant subline DC-3F/A55 were grown as described by Biedler et al. (10). The folinic-acid selected CHL cell line, DC-3F/FA14, was grown in minimal essential media-F-12 special media (without hypoxanthine, folic acid, and thymidine) plus 10% dialyzed fetal calf serum and 15 μg (6S)-folinic acid (21).

Cloning and Expression of DC-3F/A55 Dihydrofolate Reductase cDNA in Escherichia coli. Total RNA isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method from midlog phase DC-3F/A55 cells (22) was reverse transcribed using oligodeoxynucleoside acid_{12-18} or the DHFR-specific 3' primer (PR1, 5'-GGGCTTGTAGCCTTCTTCTC-3') as described by Ausubel et al. (23). Full length dihydrofolate reductase coding sequences were then amplified from cDNA/RNA hybridomers by the polymerase chain reaction using two dihydrofolate reductase specific primers, PR2 (5'-CCGGATCATGGTTCGACCGCTG-3') and PR1 (5'-GGGCTTGTAGCCTTCTTCT-3'), according to the GeneAmp DNA Amplification kit protocol supplied by Perkin Elmer/Cetus. The amplification was performed in a programmable heat block (DNA Thermal Cycler; Perkin Elmer/Cetus Instruments) by denaturing the templates at 94°C for 1 min, annealing the template-primers at 53°C for 1 min, and extending them at 72°C for 1 min over 35 cycles. The resulting 572-base pair DHFR double-stranded cDNAs were ligated into Smal digested pKK223-3 (24) after purification from excess primers by gel electrophoresis following elution from the gel. The recombinant DNA was then transformed into JM 109 competent cells which had been prepared using the CaCl2 method. The bacteria containing CHL DHFR were identified by selection in 100 μg/ml of trimethoprim and 100 μg/ml of ampicillin on minimal agar plates. Bacteria that do not express mammalian DHFR are sensitive to trimethoprim and will not grow under these conditions. The nucleotide sequences of the wild type DHFR cDNA were obtained from Melera et al. (30). Six of the cDNAs sequenced were used products from three independent PCR reactions utilizing different primer combinations. The cloned DC-3F/A55 DHFR cDNAs from 59 bacterial colonies resistant to trimethoprim were then sequenced. Six (10%) of these contained C at nucleotide position 67 and A at position 286, and thus represent transcripts from the wild type 20k DHFR allele (Table 1). The remaining 53 colonies, representing 90% of the cloned cDNA's, also contained A at position 286 indicating that, they, too, were derived from the 20k allele. However, they contained T at position 67, which changes the amino acid at residue 22 from Leu to Phe. This amino acid sequence alteration is known to be the basis for the mutant form of DHFR overexpressed by DC-3F/A3 cells (14). In that case, however, it is the 21k allele and not the 20k allele that contains the mutation. Thus, DC-3F/A3 and DC-3F/A55 express the bulk of their DHFR from different alleles, but both contain the same Leu22 → Phe mutation. Since the 59 DHFR cDNAs analyzed were obtained from multiple, independent cloning experiments, it is unlikely that the six representatives of the wild type 20k allele represent cloning artifacts. Rather, their presence suggests that a functional wild type 20k allele persists in the DC-3F/A55 genome and that the DHFR cDNA of DC-3F/A55 cells was cloned into the expression vector pKK223-3 and sequenced as described in "Materials and Methods." The sequence of DC-3F/A55 DHFR is compared at amino acid positions 22 and 95 to the wild and mutant forms of CHL DHFRs published previously (Refs. 6 and 14). Six of the cDNAs sequenced represent the wild type (WT) of the 20k enzyme and the remaining 53 cDNAs contained T at the first nucleotide of the codon for amino acid position 22, thereby representing a mutant form (Met(Val)(Leu22 → Phe) of the 20k enzyme.

Table 1. Nucleotide sequence comparison of Chinese hamster lung DHFR genes at amino acid positions 22 and 95

<table>
<thead>
<tr>
<th>Codon</th>
<th>Amino acid position 22</th>
<th>Amino acid position 95</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC-3F/A75 (WT, 21k)</td>
<td>CTT(Leu)</td>
<td>GAT(Asp)</td>
</tr>
<tr>
<td>DC-3F/A3 (Mut, 21k)</td>
<td>CTT(Leu)</td>
<td>GAT(Asp)</td>
</tr>
<tr>
<td>DC-3F/MQ19 (WT, 20k)</td>
<td>CTT(Leu)</td>
<td>AAT(Asn)</td>
</tr>
<tr>
<td>DC-3F/A55 (Mut, 20k)</td>
<td>CTT(Leu)</td>
<td>AAT(Asn)</td>
</tr>
</tbody>
</table>

* The nucleotide sequences of DC-3F/A75 DHFR gene were obtained from Melera et al. (6).
* The nucleotide sequences of DC-3F/A3 and DC-3F/MQ19 DHFR genes were obtained from Melera et al. (14).

RESULTS

Nucleic Acid Sequence of DC-3F8/A55 DHFR cDNA. To determine the nature of the putative alteration present in the DC-3F8/A55 DHFR gene, the 572-base pair DHFR coding region was amplified by reverse transcription-polymerase chain reaction from total RNA and cloned into the expression vector pKK223-3 which had been digested with the restriction enzyme Smal. Multiple clonings were performed using products from three independent PCR reactions utilizing different primer combinations. The cloned DC-3F8/A55 DHFR cDNAs from 59 bacterial colonies resistant to trimethoprim were then sequenced. Six (10%) of these contained C at nucleotide position 67 and A at position 286, and thus represent transcripts from the wild type 20k DHFR allele (Table 1). The remaining 53 colonies, representing 90% of the cloned cDNA's, also contained A at position 286 indicating that, they, too, were derived from the 20k allele. However, they contained T at position 67, which changes the amino acid at residue 22 from Leu to Phe. This amino acid sequence alteration is known to be the basis for the mutant form of DHFR overexpressed by DC-3F/A3 cells (14). In that case, however, it is the 21k allele and not the 20k allele that contains the mutation. Thus, DC-3F/A3 and DC-3F/A55 express the bulk of their DHFR from different alleles, but both contain the same Leu22 → Phe mutation. Since the 59 DHFR cDNAs analyzed were obtained from multiple, independent cloning experiments, it is unlikely that the six representatives of the wild type 20k allele represent cloning artifacts. Rather, their presence suggests that a functional wild type 20k allele persists in the DC-3F/A55 genome and that the

Dihydrofolate Reductase Purification. DHFR purification from monolayer CHL cells was performed using G-100 gel filtration and hydroxyapatite column chromatography as described by Schweitzer et al. (27) except that the crude extracts were subjected to 25% ammonium sulfate saturation instead of 40% saturation, and the soluble fraction was applied to a G-100 gel filtration column chromatography. The purified enzyme was adjusted with glycerol to a final concentration of 10% and stored at −80°C until needed. Purity was analyzed by SDS-PAGE followed by Coomassie blue staining. Protein concentra
mutation responsible for the majority of the cDNAs analyzed emerged after or during the first round of gene amplification that occurred in these cells.

Characterization of Purified DHFRs. Purified enzyme from DC-3F8/A55 cells was assayed for sensitivity to MTX, and the resulting inhibition curve was compared to those for DHFRs isolated from DC-3F8/A75, DC-3F/MQ19, and DC-3F/A3 cells. The latter three cell lines overexpress the 21k wild type, 20k wild type, and 21k mutant forms of CHL DHFR, respectively (6, 14). As shown in Fig. 1, the inhibition curve for the DC-3F8/A55 enzyme differed from both the wild type enzymes and from the 21k mutant form as well. Calculation of inhibiting concentrations indicated that the DC-3F8/A55 enzyme had a 6-fold higher value (15.8 ns) than the two wild type enzymes (2.4 ns and 2.6 ns for the M, 21,000 and M, 20,000 enzymes, respectively) but a 4-fold lower value than the DC-3F/A3 enzyme (56.7 ns). These results confirmed the mutant nature of the DC-3F8/A55 enzyme and suggested that its affinity for MTX had been reduced. In addition, the DC-3F8/A55 and DC-3F/A3 DHFRs exhibit differential sensitivity to MTX which reflects the Asn-Asp difference present at the site of allelic variation.

Since the presence of Phe at position 22 clearly alters the sensitivity of both the DC-3F8/A55 and DC-3F/A3 enzymes to MTX as compared to their respective wild type allelic forms, we sought to determine what effect, if any, this change might have on the functional characteristics of the enzymes. The steady state kinetic properties of the wild type and mutant DHFRs from both alleles were, therefore, determined. The enzymes used for this analysis were judged to be more than 90% pure by SDS-PAGE analysis (data not shown). K_m’s for H2folate and NADPH, as well as k_cat’s for all four enzymes, are given in Table 2.

Although the cloning data suggested the coexistence of wild type and mutant forms of 20k allelic DHFR in DC-3F8/A55 cells, the great majority (≥90%) of the cDNAs analyzed and presumably, the enzyme present as well, was the mutant form of the 20k allele. Yet no significant difference was observed in any of the enzymatic properties between the mutant 20k enzyme from DC-3F8/A55 and its wild type 20k enzyme isolated from DC-3F/MQ19 cells. In addition, the catalytic properties of the 20k enzymes were not different from those of the 21k enzymes, although there was a 1.4-fold difference in K_m for NADPH between the mutant DC-3F8/A55 and DC-3F/A3 enzymes. Hence, while the mutant forms of DHFR clearly display differences in their affinities for MTX, they are catalytically similar to each other and to their wild type counterparts.

Binding of [3H]-labeled Ligands. Since the data of Fig. 1 and Table 1 suggest that the amino acid at position 95 in hamster DHFR impacts upon the affinity of the enzyme for MTX, we determined by equilibrium dialysis the dissociation constant (K_d) of MTX for both wild type and mutant DHFRs in the presence of NADPH. As shown in Table 3, the K_d for MTX binding to DC-3F8/A55 enzyme was increased 6.7-fold compared to the wild type enzyme of the same allele (DC-3F/MQ19). The DC-3F/A3 enzyme showed a 9.9-fold increase in K_d compared to its wild type counterpart (DC-3F/A75). Comparison of the two mutant DHFRs indicates that K_d of the mutant form of the M, 21,000 enzyme (DC-3F/A3) is 4-fold higher than that of the M, 20,000 mutant enzyme (DC-3F/A55). This is consistent with the lower affinity of the 20k mutant enzyme for drug. Of further interest however, was the finding that K_d for MTX binding to wild type 21k and 20k enzymes (DC-3F/A75 and DC-3F/MQ19) was approximately 3-fold different (1.58 ± 0.08 and 0.58 ± 0.10 pm, respectively), although inhibition of their activity by MTX was indistinguishable. This indicates that while the Leu to Phe mutation at amino acid residue 22 dramatically increases the K_d for each allele, a more subtle difference in MTX affinity, not readily detectable by enzyme activity inhibition studies, exists between the wild type enzymes per se. Next, the dissociation constant for H2folate (the normal substrate for DHFR) was determined in the presence of NADP+ for both wild type DHFRs. The results (Table 3) showed that K_d’s of DC-3F/MQ19 and DC-3F/A75 DHFR for [3H]H2folate were 71.8 ± 9.1 and 80.6 ± 5.4 nm, respectively. Hence, the wild type alleles do not differ in their K_d’s for normal substrate but do differ by 3-fold in their K_d’s for MTX.

Table 2 Steady state properties of wild and mutant CHL dihydrofolate reductase

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>NADPH constants (μM)</th>
<th>H2folate constants (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC-3F/MQ19</td>
<td>12.36 ± 0.72*</td>
<td>5.76 ± 0.54</td>
</tr>
<tr>
<td>DC-3F/A75</td>
<td>12.36 ± 1.02</td>
<td>5.39 ± 1.02</td>
</tr>
<tr>
<td>DC-3F/A55</td>
<td>14.20 ± 0.86</td>
<td>5.92 ± 1.24</td>
</tr>
<tr>
<td>DC-3F/A3</td>
<td>10.39 ± 0.53</td>
<td>5.37 ± 0.98</td>
</tr>
</tbody>
</table>

* Mean, ± SE (n = 3)

Table 3 Dissociation constants for MTX and H2folate binding to the wild and mutant CHL dihydrofolate reductase

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>MTX (μM)</th>
<th>H2folate (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DC-3F/MQ19</td>
<td>0.58 ± 0.10*</td>
<td>71.8 ± 9.1</td>
</tr>
<tr>
<td>DC-3F/A75</td>
<td>1.58 ± 0.08</td>
<td>80.6 ± 5.4</td>
</tr>
<tr>
<td>DC-3F/A55</td>
<td>3.92 ± 0.17</td>
<td>ND</td>
</tr>
<tr>
<td>DC-3F/A3</td>
<td>15.70 ± 1.20</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Mean, ± SE (n = 3)

ND, not determined.
Allelic Variation in DHFR and Antifolate Resistance

Albrecht et al. (13) originally reported that the antifolate-resistant subline of DC-3F, DC-3F/A55, contains an altered form of DHFR. The results presented in this report extend that observation to include studies of several vertebrate DHFRs and NMR spectroscopic analysis of human DHFR have shown that Leu22 is located in the folate-binding pocket and forms hydrophobic contacts with bound inhibitors (32, 33). Disruption of these hydrophobic contacts alters the affinity of the enzyme for drug and, in the case of the Leu22 → Arg mutation reported in the mouse enzyme (34), increases $K_d$ for MTX by 210-fold while reducing the catalytic activity by 95%. Substitution of Phe, with its bulky nonpolar side chain, for Leu22 is much less disruptive than the positively charged Arg as indicated by the observation that the Phe mutation does not alter catalytic activity and increases $K_d$ for MTX by only 6-fold. That this mutation does not effect catalytic activity—(Tables 2 and 3) is consistent with previous results obtained from studies of the Leu22 → Phe mutant found in the 21k form of DHFR present in the CHO cell line Pro-3MTXR (31).

Of interest is the observation that the Leu22 → Phe mutant increases $K_d$ for MTX of the 20k enzyme by 6.7-fold while the same mutation in the 21k enzyme increases $K_d$ by 9.8-fold (Table 3). Since the two mutant enzymes differ only at position 95 where the 20k enzyme contains Asn and the 21k enzyme contains Asp (Table 1), it is apparent that this position effects the interaction of enzyme with drug. It is also clear that this effect is enhanced when Phe occupies position 22, although it is also indicated by the 3-fold difference in $K_d$'s for MTX displayed by the wild type allelic forms that contain Leu22 (Table 3). Further evidence for the effect of amino acid position 95 on DHFR structure has come from site-directed mutagenesis studies showing that the thermal stability of the enzyme can be greatly altered by substituting different amino acid residues at this location. It is clear, therefore, that even though amino acid position 95 is not located near the drug-binding site of DHFR but instead is found on the αE helix, the amino acid residue at that location does have an impact on drug binding. Interestingly, however, the wild type allelic forms do not differ in their respective $K_d$'s for the normal substrate, dihydrofolate, or in the other kinetic properties measured (Tables 2 and 3). This suggests that their structural differences are subtle and specifically affect drug binding rather than normal enzyme function. Such differences might be expected for allelic variants of enzymes that are retained in a population and are neither selected for or against under normal growth conditions but manifest themselves only in the presence of drug, at which time one may provide a selective advantage over the other. Such an occurrence may be relevant to the DHFR alleles discussed here, because it is the 21k allele which displays the lowest affinity to MTX and is most frequently overexpressed in DC-3F cell cultures selected over long periods for growth in MTX (8, 35).

Moreover, preliminary results from transfection experiments in which DHFR-CHO cells have been transformed to DHFR+ via expression of either the wild type 20k or the 21k form of enzyme show that a survival advantage is afforded cells that express the 21k form during selection for growth in MTX (36).

In addition to sustaining a mutation in the 20k DHFR allele, DC-3F/A55 cells have also undergone an alteration in their capacity to accumulate MTX (Fig. 2). The inability to retain drug is an effective mechanism of resistance (3, 37, 38) and can be mediated in a number of different ways including: (a) a decrease in the formation of polyglutamylated MTX caused by a loss of FPGS activity; (b) enhanced formation of short chain di- and monoglutamates of MTX that are known to efflux rapidly as compared to triglutamate and longer species (39); (c) increased catabolism of polyglutamates by elevated levels of γ-glutamyl hydrolase (40); (d) a mutation in FPGS; (e) a mutation in the reduced folate transporter that is also responsible for the uptake of MTX (37, 41); or (f) decreased expression of folate receptors (42). Of these, only loss of FPGS activity per se can be ruled out.

Fig. 2. Accumulation of $[^{3}H]$MTX at 37°C by Chinese hamster lung cells. Transport of $[^{3}H]$MTX by DC-3F/A55 (Δ), DC-3F/FA14 (●), and parental DC-3F (○) cells were determined in the presence of 1.5 μM of $[^{3}H]$MTX as described by Jansen et al. (26). Each time point indicates the average of duplicate measurements. Bars, upper and lower intervals. The absence of bars indicates that, for those points, very similar values were obtained; therefore, the bars cannot be visualized.

**DISCUSSION**

R. O. Moran, personal communication.


6 McKissick and Melera, unpublished observations.

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out as a possible mechanism since it has been shown that no difference in FPGS activity can be demonstrated between DC-3F8/A55 and parental DC-3F cells.4 The remaining possibilities are under investigation.

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