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SATURATION MUTAGENESIS AT DIHYDROFOLATE REDUCTASE CODONS 22 AND 31

A VARIETY OF AMINO ACID SUBSTITUTIONS CONFERRING METHOTREXATE RESISTANCE

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Abstract—Naturally occurring amino acid substitutions conferring resistance to methotrexate (MTX) have been reported previously at codon positions 22 (leu → arg, phe) and 31 (phe → ser, trp) of mammalian dihydrofolate reductases (DHFR). To explore the character of other substitutions, a polymerase chain reaction (PCR)-assisted saturation mutagenesis protocol was devised to introduce all possible codon sequences at positions 22 and 31 of the murine DHFR coding sequence in an expressible simian virus 40 (SV40)-regulated transcription unit. Nucleotide sequencing confirmed the presence of all four nucleotides at each of the three codon positions in the mutagenized material. Transfection of these "codon libraries" into DHFR-deficient Chinese hamster ovary cells resulted in an increased frequency of MTX-resistant colony formation in comparison with wild-type DHFR transfected cells. DHFR variants contained in different clones were characterized by PCR amplification and DNA sequencing, identifying six different amino acid substitutions at position 22 and seven substitutions at position 31. DHFR variants were extracted for determination of MTX inhibition character and catalytic activity, normalizing for the amount of DHFR protein by western blot analysis. A wide range of MTX sensitivities and catalytic activities were observed which is consistent with the role of these side chains in DHFR catalytic function. We observed that codon 22 variants were generally more resistant to MTX, but codon 31 variants retained substantially more catalytic activity (about 2.5-fold) at a given level of MTX resistance. This heterogeneity in catalytic and inhibition character has important implications for the function of different DHFR variants as mediators of drug resistance.

Key words: polymerase chain reaction; mammalian cells; gene transfer; gene therapy

MTX‡ (8-amino,10-methyl-pteryolglutamic acid) is a clinically important chemotherapeutic agent currently utilized in the treatment of many malignancies [1]. The primary site of MTX action is DHFR (EC 1.5.1.3), which catalyzes the reduction of folate to 7,8-dihydrofolate and further reduction to 5,6,7,8-tetrahydrofolate [2]. Derivatives of tetrahydrofolate are used subsequently for the synthesis of glycine, purines and thymidylate, essential precursors for macromolecular synthesis and cell proliferation. MTX is a folate analog that competitively inhibits DHFR. Therefore, when MTX is added to the environment of proliferating cells, the intracellular concentration of tetrahydrofolate is reduced, leading to aberrant DNA synthesis and subsequent cell death. This is thought to represent the antitumor effect of MTX.

Resistance to MTX has been a major problem in successful cancer chemotherapy. It is therefore essential to elucidate the multiple mechanisms by which resistance to MTX can arise. Under MTX selection, DHFR gene amplification can result in stoichiometrically increased production of the enzyme [3]. Cells can also become resistant to MTX by decreased cellular uptake of the drug [4]. Finally, several mutant DHFR forms that have arisen under MTX selection in mammalian cells have been reported to confer MTX resistance. The relevance of these variants to clinical resistance is not clear, but could prove to be of vast import. Substitutions of leucine at codon 22 by arginine [5] and phenylalanine [6, 7] have been reported for cDNA clones isolated from MTX-resistant mouse and hamster cells, respectively. At codon 31, substitutions of phenylalanine by serine [8] and by tryptophan [9] have been observed for MTX-resistant human and murine DHFRs, respectively. In addition to these naturally occurring drug-resistant DHFR variants, the function of specific active site amino acid side chains has been investigated by site-directed mutagenesis of human [10–12] and murine [13, 14] DHFR as well as the *Escherichia coli* enzyme [14, 15].

While the emergence of MTX-resistant variant

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‡ Abbreviations: CHO, Chinese hamster ovary; DHFR, dihydrofolate reductase; HBV, hepatitis B virus; IC_{50}, inhibitor concentration reducing enzyme activity to 50%; MTX, methotrexate; PCR, polymerase chain reaction; and SV40, simian virus 40.

DHFRs in tumor cells may pose a problem for effective cancer chemotherapy, such drug-resistant variants could be utilized to genetically render normal cells more resistant to MTX, thus providing a greater differential of sensitivity between tumor and normal cells for chemotherapeutic purposes [16–20]. Variant DHFRs, particularly the arg22 variant, have proven to be useful mediators of drug resistance for gene transfer studies in cultured mammalian cells [21–23] and in experimental animals [16–20]. Effectiveness of the arg22 variant is compromised, however, by reduced catalytic activity [24]. Other molecularly characterized DHFR enzyme variants, while retaining substantial catalytic activity, exhibit greater sensitivity to MTX. To characterize more completely the variety of DHFR genotypes potentially conferring MTX resistance upon mammalian cells, we adapted a polymerase chain reaction (PCR)-based overlap extension technique to conduct saturation mutagenesis experiments at nucleotides corresponding to codons 22 and 31 of the murine DHFR coding sequence. Furthermore, we used a functional DHFR transcription unit as a template for these reactions so that product sequences conferring drug resistance could be effectively screened and cloned by selection after transfection into cultured mammalian cells. This combination of saturation mutagenesis followed by selection in transfected mammalian cells proved to be a powerful approach for the establishment and characterization of a pharmacologically diverse array of variants.

MATERIALS AND METHODS

DNA manipulations. E. coli K-12 strain MM294 was used to maintain all plasmids. Plasmids were extracted from ampicillin-containing cultures by the alkaline lysis procedure and purified by cesium chloride-ethidium bromide gradient centrifugation [25]. Restriction enzymes and T4 DNA ligase were purchased from Gibco/BRL (Gaithersburg, MD) or New England Biolabs (Beverly, MA). Calf intestinal phosphatase was from Boehringer Mannheim (Indianapolis, IN). All oligonucleotides (see Table 1) were synthesized at the Institute of Human Genetics Microchemical Facility, University of Minnesota. The polymerase chain reaction was carried out using Taq polymerase from Boehringer Mannheim and a DNA thermal cycler from Perkin Elmer Cetus (Norwalk, CT).

Saturation mutagenesis. For the saturation mutagenesis experiments, 2.5 ng of *Sac*I or *Hind*III-digested pSV-DHFRwt plasmid [5, 9] were initially amplified between the 5′ end of the SV40 early promoter (primer #1) and a degenerative antisense primer at codon 22 (primer #2) or codon 31 (primer #5), and also between degenerative sense primers at codon 22 (primer #3) or codon 31 (primer #6) and the 3′ end of a hepatitis B virus (HBV) surface antigen gene sequence containing the polyadenylation signal (antisense primer #4), as depicted in Fig. 1. Reactions contained 100 pmol of SV40 or HBV primers and 40 pmol of degenerative primers. Reaction conditions were 30 sec 94°, 30 sec 55°, 2 min 72°, for 30 cycles. The initial upstream and downstream amplification products were purified from primers by using Millipore Ultrafree-MC filters, and a small portion (0.04%) of each was used for a subsequent PCR amplification using 50 pmol each of primers #1 and #4, 40 sec 94°, 40 sec 55°, and

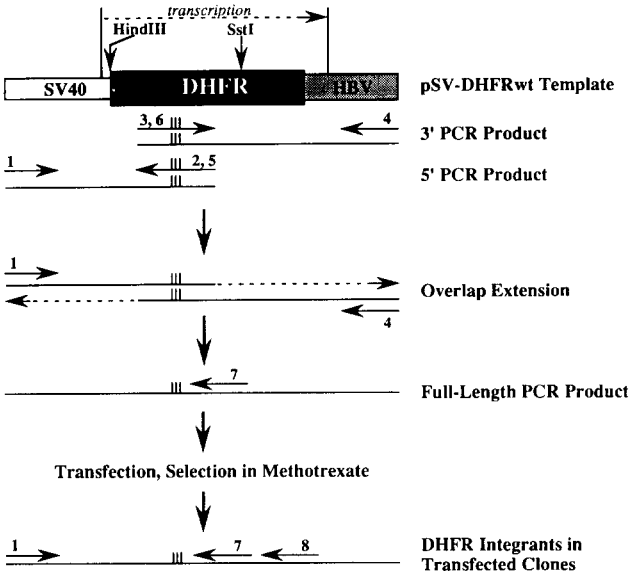

Fig. 1. Strategy for saturation mutagenesis of DHFR codons 22 and 31. Structure of the pSV-DHFRwt template is shown, including the DHFR coding sequence, SV40 early promoter, HBV polyadenylation signal, transcribed region, and restriction sites. Primers used for PCR and sequencing are indicated by the numbered arrows. PCR products are denoted by thin horizontal lines, with the location of the mutagenized codon (representing either codon 22 or 31) indicated by three short vertical lines.

3 min 72°, for 30 cycles. This reaction allowed initial extension of the overlap spanning codon 22 or 31 as well as amplification of the overlap extension product, a 1.8 kb mutagenized DHFR transcription unit. As controls for the electroporation experiment (see below), PCR-generated transcription units of wild-type DHFR, using pSV-DHFR as a template, and arg22 DHFR, using pFR400 [5] as a template, were also amplified using primers #1 and #4. Saturation mutagenesis products were prepared for sequencing (see below) by fragment isolation on agarose gels and extraction using Gene Clean II (Bio101).

Mammalian cell culture and gene transfer. Chinese hamster ovary (CHO) DUX-B11 cells (lacking DHFR) [26] were routinely cultivated in α-MEM (Sigma) containing 10% fetal or newborn calf serum (Intergen) and 10 μg/mL adenosine, deoxyadenosine, and thymidine (Sigma) as well as 50 U/mL penicillin, 50 μg/mL streptomycin, 0.125 μg/mL fungizone (Gibco-BRL) and 2 mM glutamine (Sigma). One day prior to electroporation, the cells were plated at less than 50% confluence to ensure that the target cells would be actively dividing. Cells were harvested with trypsin, washed twice with phosphate-buffered saline and then resuspended at 10^6 cells/mL in HEPES-buffered saline. Cells (10^6) were mixed with 0.3 μg of DNA in BRL disposable electroporation chambers and electroporated [27, 28] at 330 μF, 350 V and low resistance using a BRL Cell-Porator Electroporation System 1. Ten minutes following electroporation, the cells were plated in growth medium containing adenosine, deoxyadenosine and thymidine.

In an initial pilot experiment, cells were subcultured 2 days after electroporation into selective medium (α-MEM supplemented with 10% newborn calf serum, lacking adenosine, deoxyadenosine and thymidine, and containing 100 nM MTX; amethopterin, Sigma). Dense clonal outgrowth of these cells after 8 days necessitated their subsequent subculture at lower density in medium containing varying levels of MTX. To assure distinct clonal outgrowth of transfectants after the initial plating, a second experiment was performed in which the cells were subcultured at low density in selective medium 1 day after electroporation (5×10^4 cells per 25 cm dish at 0 and 100 nM MTX, 10^5 cells per 25 cm dish at 0.25 to 100 μM MTX). Ten days later, clones were scored, harvested and expanded in culture for further study.

DHFR expression plasmids were introduced into CHO DUX-B11 cells either by electroporation essentially as described above or by using the DNA-calcium phosphate co-precipitation technique as described elsewhere [9]. Two days post-transfection the cells were harvested and subcultured into selective medium containing various concentrations of MTX, collecting heterogeneous pools of clones after 2 weeks' selection for enzyme analysis.

DNA sequencing. The dideoxy chain termination technique [29] was used for all sequencing, employing the Sequenase 2.0 kit from United States Biochemicals. Template PCR products were prepared for sequencing by boiling for 5 min with primer and then snap cooling on powdered dry ice.

For determination of variant DHFR sequences in transfected clonal isolates, genomic DNA was extracted by using a nonionic detergent/proteinase K lysis protocol [30] and then a portion of the integrated DHFR sequence was amplified by PCR between primers #1 and #8 or #10. Reaction conditions were 40 sec 94°, 40 sec 55°, and 1 min 72°, for 40 cycles. Fifty-microliter reactions contained 25 μL of genomic DNA extract and 10 pmol of each primer. The PCR product was purified using Gene Clean II prior to sequencing. Antisense oligonucleotide #7, located just downstream of both codons 22 and 31 in the DHFR cDNA sequence, was used to prime reactions for sequencing PCR products amplified from transfectant cell DNA. Primers #7 and #8 were used to initiate sequencing reactions across codons 22 and 31, respectively, of saturation mutagenesis PCR products.

Variant DHFR recovery and plasmid construction. Variant DHFRs were retrieved from clones identified in the mutagenesis screen by PCR amplification between primers #9 and #10 (1 min 94°, 1 min 55°, and 1 min 72°, 40 cycles). PCR products were cloned into TA cloning vector PCR (Invitrogen) in a ligation reaction using 1 μL of PCR product under conditions suggested by the manufacturer. A 283 bp *Hin*dIII–*Sst*I fragment (5′ end of the DHFR coding sequence) was then isolated from these plasmids and subcloned into pFR400 (digested with *Hin*dIII and *Sst*I and dephosphorylated) in place of the arg22 DHFR sequence. Expression plasmids were purified by banding twice on CsCl/ethidium bromide density gradients [25] prior to transfection (described above).

Southern hybridization analysis. DNA was extracted as previously described [31] and then digested with restriction endonuclease and RNase overnight. The digested DNA was electrophoresed on 1.0% agarose and blotted onto Nytran according to the manufacturer's instructions (Schleicher & Schuell). Prehybridizations and hybridizations were conducted as previously described [31]. The blots were probed with a PCR product generated from the HBV 3′ flanking sequence (primer #11 to primer #4, 55° 1 min, 72° for 1 min, 94° for 1 min for 40 cycles, radiolabeled using the Oligolabelling kit from Pharmacia), washed twice in 0.1 × SSPE (SSPE = 0.18 M NaCl, 5 mM Na_2HPO_4, 5 mM NaH_2PO_4, 1 mM disodium EDTA, pH 7.0) for 30 min and then autoradiographed using an intensifying screen.

DHFR spectrophotometric enzyme assay. Cell extracts were prepared by sonication and assayed for DHFR activity spectrophotometrically at 340 nm on a Beckman DU50 as previously described [32, 33]. Reaction mixtures (1 mL) contained 100 mM Tris/Cl^-, pH 7.5, 150 mM KCl, 10 mM β-mercaptoethanol, 30 μM dihydrofolate, 120 μM NADPH (all components from Sigma) and extract at room temperature. MTX, when included, was added to otherwise complete reaction mixtures 10 min prior to initiation of the reaction by addition of dihydrofolate. Control reactions lacking dihydrofolate were carried out for each extract to determine the level of background NADPH oxidation, which was subtracted from the DHFR signal. Progress curves were transferred through an RS232 interface

Table 1. Oligonucleotide sequences and utilization

No.	Orientation	Location*	Use†	Sequence‡
1	Sense	−482 to −463	1, 2, 3	**GGCATATGAAAGACCCC**CAGTCCATGACCTACGAACC
2	Antisense	55 to 81	1	CGGAGGCCAGGGNNNGTCTCCGTTCTT
3	Sense	55 to 81	1	AAGAACGGAGACNNNCCCTGGCCTCCG
4	Antisense	1206 to 1225	1, 2, 5	**GGCATATGAAAGAACCC**GACGGAAGGAAAGAAGTCAG
5	Antisense	82 to 108	2	TTGGAAGTACTTNNNCTCGTTCCTGAG
6	Sense	82 to 108	2	CTCAGGAACGAGNNNAAGTACTTCCAA
7	Antisense	119 to 135	4	**CCGGAA**TTCCACTGAAGAGGTTG
8	Antisense	170 to 194	3, 4	TTCTTCTCAGGAATGGAGAACCAGG
9	Sense	−75 to −52	3	GCTATTCCAGAAGTAGTGAGGAGG
10	Antisense	336 to 356	3	CTGCCTCCGACTATCCAAACC
11	Sense	924 to 945	5	ATGTCAACGACCGACCTTGAGG

* Location: nucleotide numbers with respect to the DHFR translational start site of pSV-DHFRwt.
† Uses: 1—saturation mutagenesis, codon 22
2—saturation mutagenesis, codon 31
3—PCR amplification of integrated DHFR sequences from genomic DNA
4—sequencing of DHFR DNA amplified from genomic DNA
5—PCR amplification to generate a probe for Southern analysis
‡ Sequences are given in the 5′ to 3′ direction and nucleotides different from pSV-DHFRwt are in bold.
N indicates positions where all four nucleotides were inserted.

to an Apple Macintosh SE computer for initial velocity determinations using Cricket Graph Software ($\varepsilon_{340} = 12\ mM^{-1}\ cm^{-1}$). Extract-to-extract coefficient of variation was less than 10% [33]. Protein concentration was determined by using the Bradford assay [34] as formulated by Bio-Rad.

Western blot analysis. Cell extracts prepared for enzyme assays (above) were electrophoresed under reducing conditions [35] on 15% polyacrylamide gels containing 0.1% SDS [36]. Proteins were electroblotted onto Immobilon-P membranes (Millipore) [37] and blocked with 1% normal goat serum and 5% BLOTTO overnight at 4° according to Faassen *et al.* [38]. The filters were then probed with a 0.5% dilution of a polyclonal anti-murine DHFR rabbit antibody (provided by Dr. Bruce Dolnick) followed by washing with a goat anti-rabbit IgG peroxidase conjugate and stained as previously described [38]. DHFR protein signals were then quantitated by videodensitometry [39].

RESULTS

Saturation mutagenesis at codons 22 and 31 of the murine DHFR coding sequence. To introduce all possible nucleotide triplets at DHFR codon positions 22 and 31, we employed an overlap extension protocol in conjunction with PCR and sets of complementary mutagenic primers (Fig. 1). A wild-type DHFR cDNA expression plasmid (pSV-DHFRwt [5, 9]), containing an SV40 early promoter and a polyadenylation signal from HBV surface antigen gene, was used as template. Two sets of complementary oligonucleotide pools were synthesized, corresponding to the DHFR coding sequence around codon 22 (primers #2 and #3) and codon 31 (primers #5 and #6), except that all four nucleotides were inserted at each of the three nucleotide positions for these two codons (Table 1). For each of the two mutagenesis experiments, two PCR reactions were conducted to amplify the 5′ and 3′ portions of the transcription unit (Fig. 1). A sense primer (#1) from the SV40 promotor was used in conjunction with a degenerative antisense primer (#2 or #5) to amplify the 5′ end of the transcription unit. A separate PCR reaction was used to amplify the 3′ region of the transcription unit using a sense degenerative primer (#3 or #6) and an antisense primer (#4) downstream of the HBV polyadenylation signal. Following ultrafiltration to remove the primers, the two PCR products were denatured and mixed, allowing the overlap spanning codons 22 or 31 to be annealed and extended to form complete transcription units. Primers #1 and #4 were then used to amplify these transcription units in a final PCR reaction. The pSV-DHFRwt plasmid DNA used as template for initial amplification of upstream and downstream regions was first digested with *Sst*I or *Hin*dIII, respectively, to avoid regeneration of the starting template sequence in the final PCR step.

The final PCR products were characterized by using a portion of each as template in a sequencing reaction, priming from a downstream site and sequencing across codons 22 and 31 (using primers #7 and #8, respectively; Fig. 1). The sequencing results demonstrated the presence of all four nucleotides at each of the three nucleotide positions within codons 22 and 31 of the PCR-mutagenized products (Fig. 2). This PCR-mediated saturation mutagenesis process thus resulted in the formation of a heterogeneous pool of PCR products representing a "codon library", with complete murine DHFR cDNA transcription units encoding different amino acids at codons 22 and 31.

MTX resistance after electroporation into cultured mammalian cells. Because the saturation mutagenesis protocol generated complete transcription units, it was possible to express these DHFR sequences in mammalian cells without further rearrangement to investigate the variety of DHFR genotypes

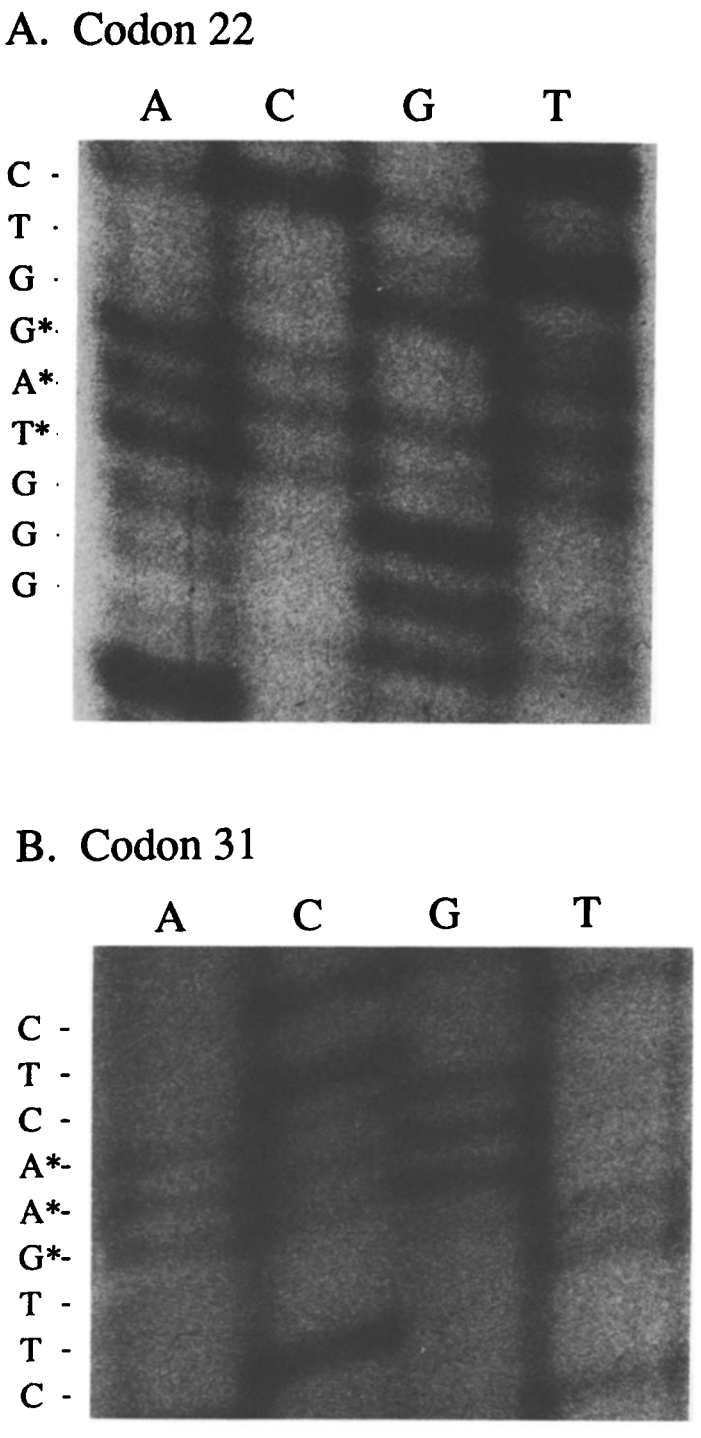

Fig. 2. Nucleotide sequence analysis of saturation mutagenized PCR products. Sequencing reactions were initiated using primer #7 or primer #8 (Fig. 1) to extend across codon 22 (A) or codon 31 (B). The wild-type DHFR sequence spanning codon 22 (A) or codon 31 (B) is indicated, with an asterisk indicating those positions at which the wild-type nucleotide has been replaced by all four nucleotides.

potentially conferring MTX resistance. DHFR-deficient CHO cells (CHO DUX-B11; [26]) were electroporated with 0.3 μg of each PCR-generated DHFR "codon library" and subcultured after 24 hr into selective medium. CHO DUX-B11 cells require an exogenous source of purines and thymidine, so the outgrowth of clones in selective medium lacking adenosine, deoxyadenosine, and thymidine required

Table 2. Transfection efficiencies at various concentrations of MTX

Sample	[MTX]					
	0	0.1 μM	0.25 μM	1 μM	10 μM	100 μM
Pos. 22*	5.6†	1.2	0.75	0.2	0	0
Pos. 31*	11.8	2.6	1.7	0.7	0	0
wt DHFR	7.7	0.7	0.15	0	0	0
arg22 DHFR	5.4	4.9	3.7	1.8	0.85	0.05
H_2O	0	0	0	0	0	0

DHFR-deficient CHO cells were electroporated with the indicated samples and then plated into selective medium containing the indicated concentration of MTX.

* pSV-DHFR, saturation mutagenized at position 22 or 31 as described in the text.

† Transfection efficiencies × 10^4.

the acquisition of a functional DHFR (Table 2). To determine if the saturation mutagenesis protocol introduced into the DHFR coding sequence mutations conferring MTX resistance, we assessed the frequency of colony formation in selective medium containing various concentrations of MTX (0, 0.1, 0.25, 1.0, 10, and 100 μM). Transfection with a control wt DHFR sequence resulted in no colony formation at 1 μM MTX. In contrast, saturation mutagenesis at codons 22 or 31 resulted in the isolation of several drug-resistant clones at 1 μM MTX and a marked increase in the frequency of drug-resistant colony formation at 0.25 and 0.1 μM MTX in comparison with wt DHFR transfected cells (Table 2). The saturation mutagenesis procedure thus increased the frequency of drug-resistant colony formation, indicating the expression of MTX-resistant DHFR variants after transfecting into CHO cells.

DHFR sequences in MTX-resistant clones. MTX-resistant clones were expanded in culture, and genomic DNA was extracted to determine the codon sequences at positions 22 or 31 that resulted in the drug-resistant phenotype. A portion of the integrated DHFR transcription unit was amplified by PCR between the SV40 sense primer (#1) and a downstream antisense primer from the DHFR coding sequence (#8; Fig. 1). This PCR product was purified from its primers and then used as template for a sequencing reaction initiated from an internally nested antisense primer downstream of codon 31 (#7; Fig. 1).

A total of fifty-four clones were studied, thirty-five of which were difficult to interpret due to multiple DHFR integrants identifiable on sequencing gels. Eleven codon-22 variants and eight codon-31 variants were identified in clones containing single integrants, resulting in six different codon-22 substitutions and seven different codon-31 substitutions (Table 3). This included a variety of both conservative and non-conservative amino acid substitutions at both codon positions. While several of the variants recapitulated substitutions that have been associated previously with MTX resistance (arg22 [5], phe22 [6, 7], and ser31 [8]), most of the observed substitutions have not been described previously. Multiple clones for several of the substitutions were observed (trp22, tyr22, arg22, ser31, his31), sometimes including more than one codon sequence, supporting the role of these specific substitutions in the expression of a drug-resistance phenotype.

Southern analysis to determine DHFR integrant copy number. To verify the DHFR integrant copy number in candidate clones identified by DNA sequencing, DNA was extracted and digested either with *Sst*I, which cuts the DHFR transcription unit once, or with *Eco*RI, which does not cut at all. Under these conditions, different integrants will generate differently sized fragments identifiable on Southern blots hybridized with a probe for the transfected sequences (in this case, HBV sequences hybridizing to the 3′ end of the transcription unit). For most of the clones depicted in Fig. 3, a single band was observed after digestion with either *Sst*I or *Eco*RI, indicating that these clones contained a single variant DHFR integrant. Ser31-2, which was isolated after consecutive platings at 100 nM and 100 μM MTX, appeared to contain amplified copies of a single integrant apparent in both *Eco*RI and *Sst*I digests, consistent with the high level of activity identical in character to ser31-1 extracts (see below). In contrast to the clones containing single integrants, several clones contained multiple integrants, including trp22-2, arg22-2, and gly,leu-31 (see below).

DHFR catalytic activity and MTX inhibition character. To assess the enzymatic and MTX inhibition character of the variant DHFRs identified by nucleotide sequencing, cell extracts were prepared from at least one clonal cell population for each of the different amino acid substitutions at codons 22 and 31. DHFR activities were assessed at a range of MTX concentrations (Fig. 4) to determine the IC_{50} (that MTX concentration reducing enzyme activity to 50%) for each of the variants (recorded in Table 3).

There was a marked heterogeneity in MTX inhibition character among the different DHFR variants identified (Table 3). Codon 22 substitutions varied between a clone expressing ala22, with a relatively low MTX sensitivity, to the previously described and highly drug-resistant arginine variant [5, 24]. Substitution of leucine at position 22 by more bulky or more hydrophilic amino acid side chains

Table 3. Substitutions associated with MTX resistance after saturation mutagenesis

Substitution	Codon	IC_{50}*	Activity†	DHFR/μg‡	pmol/min/DHFR§	%wt‖
(A) Pos. 22						
leu (wt)¶	CUA	4.5 nM	23	1.8	13	100
ala	GCU	50 nM	6.3	2.8	2.2	18
phe	UUC	200 nM	4.4	1.4	3.2	26
asn	AAC	400 nM	1.2	0.71	1.6	13
trp-1	UGG	1.3 μM	4.3			
trp-2	UGG	1.4 μM	4.5	2.0	2.2	18
tyr-1	UAU	1.9 μM	1.5			
tyr-2	UAC	1.7 μM	1.1			
tyr-3	UAC	2.0 μM	1.2	0.55	2.2	17
tyr-4	UAC					
arg-1**	CGC	9 μM	0.53	3.3	0.16	1.3
arg-2	AGG		0.16	0.65	0.25	2.0
(B) Pos. 31						
phe (wt)¶	UUC	4.5 nM	23	0.98	23	100
arg	CGC	10 nM	7.9			
ser-1††	AGC	20 nM	13	0.14	87	380
ser-2**	UCA	30 nM	1170	13	93	400
gln	CAG	75 nM	36	2.6	14	59
glu††	GAA	80 nM	9.3	0.30	31	140
trp‡‡	UGG	100 nM	2.2	0.63	3.5	15
his-1	CAU	180 nM	26	1.8	15	66
his-2	CAU	280 nM	3.0	0.21	14	61
asn	AAC	375 nM	6.3	0.61	10	45

* The IC_{50} values were determined from results depicted in Fig. 4.

† DHFR specific activity in crude cell extracts, nmol/min/mg protein.

‡ DHFR videodensitometric signal/amount of protein loaded on a western blot, Fig. 5.

§ DHFR specific activity divided by DHFR protein/μg.

‖ Percentage of activity (pmol/min/DHFR) compared with that of wild type activity.

¶ Wild-type DHFR clone was established by transfection with pSV-DHFRwt constructs rather than by saturation mutagenesis.

** Clones isolated from initial pilot experiment; arg22-1 and ser31-2 were selected at 100 μM MTX.

†† Clones isolated from initial pilot experiment; glu31 and ser31-1 were selected at 100 nM MTX.

‡‡ The trp31 clone was established by transfection with Tg-trp31 expression construct, which contains the trp31 mutation inserted into the DHFR gene transcriptionally regulated by the natural DHFR promoter, the first two introns, and the HBV polyadenylation signal (Morris JA and McIvor RS, unpublished observation).

(asparagine, tryptophan, tyrosine, arginine) resulted in a 10- to 2000-fold reduction in MTX sensitivity. Substitution at position 31 resulted in a less extreme (2- to 80-fold) reduction in MTX sensitivity than at position 22 and included both smaller and more bulky hydrophilic side chains.

Specific DHFR activity for each of the extracts was also determined in the absence of MTX. However, clonal variation in specific activity reflects not only differences in the catalytic character of the particular DHFR variant but also differences in the level of DHFR protein being expressed. We therefore conducted western blot analysis to determine the comparative levels of DHFR protein being expressed in the different transfectant cell lines (Fig. 5). The amount of DHFR protein contained in each sample was quantitated by videodensitometry and then used to standardize the DHFR specific activity assessed for each extract. This allowed quantitation of the catalytic activity associated with specific DHFR protein (pmol/min/DHFR; Table 3). The reliability of this computation was demonstrated by its reproducibility for several pairs of clones expressing the same DHFR variant at different levels, i.e. his31, ser31 and arg22, the latter two pairs being generated from different triplet sequences.

For codon 22 variants, the IC_{50} ranged from 50 nM to 9 μM, and an intermediate to very reduced catalytic activity (26 to 1.3%) was observed for each of the variants. Arg22 activity was reduced 50- to 100-fold in comparison with wt DHFR, consistent with the 20-fold reduction in V_{max} previously reported for the enzyme purified from the MTX-resistant mouse 3T6-R400 cell line [24]. The arg22-2 cell line did contain two integrants identifiable on Southern blots (Fig. 3). However, when examining its enzymatic activity, arg22-1 and arg22-2 had very similar characteristics. This would suggest that the other integrated sequence in arg22-2 is nonfunctional. Similarly, trp22-2 extracts had similar enzymatic characteristics as trp22-1, suggesting a nonfunctional, second DHFR sequence integrated in the trp22-2 cell line as well.

Codon 31 variants were not nearly as impaired catalytically. Indeed, the assessed catalytic activity was increased 1.4-fold for glu31 and 4-fold for ser31, a difference not previously observed for the ser31 human [10] and murine [13] DHFR variants

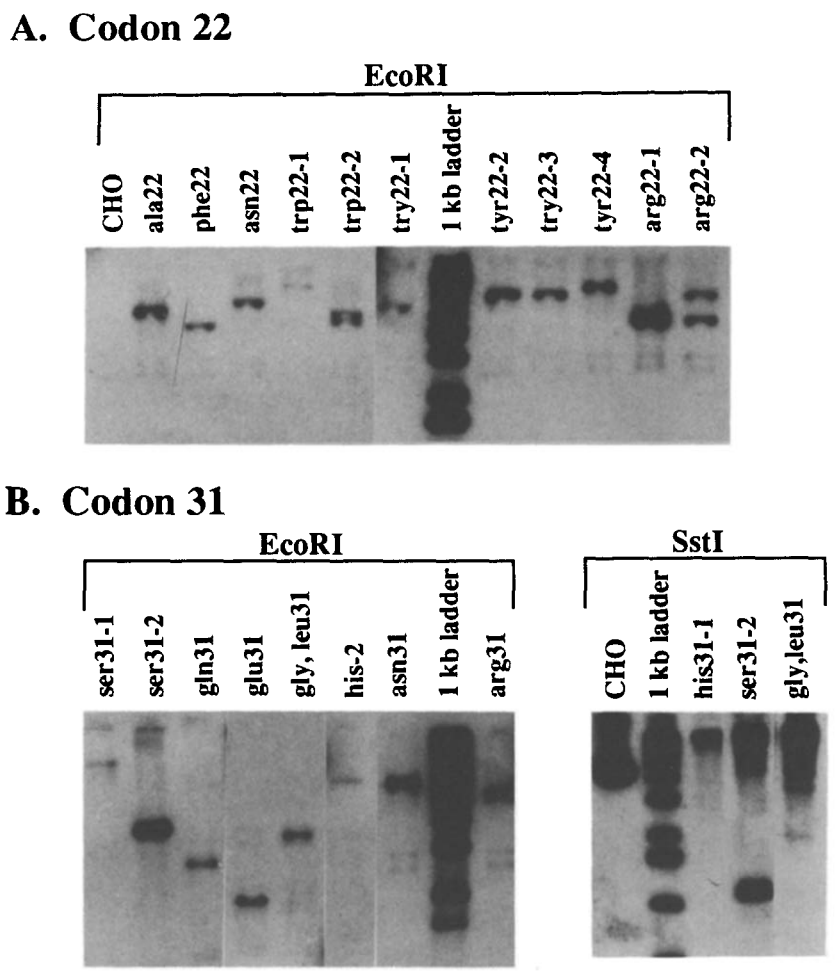

Fig. 3. Southern blot of variant DHFR clones digested with *Sst*I (Fig. 1) or *Eco*RI, probed for HBV sequences located at the 3′ end of transfected DHFR transcription units (Fig. 1). Samples were extracted for DNA, digested, blotted and probed, as described in Materials and Methods. Lanes loaded with samples or with 1 kb ladder marker are indicated.

expressed in *E. coli*. Other codon 31 variants retained between 59 and 66% of wt DHFR catalytic activity, substantially higher (about 2.5-fold) than codon 22 variants exhibiting similar levels of drug resistance (compare his31 and asn31 with phe22 and asn22).

Retrieval and transfection of variant DHFR sequences. To verify their effectiveness as mediators of drug resistance, several of the DHFR variants were recovered from clones transfected with saturation mutagenized material and used to construct SV40-regulated mammalian expression plasmids for transfection experiments. Genomic DNA (extracted from clones trp22-2, tyr22-3, ser31-2, gly,leu-31, and his-31) was used as a template to amplify a 283 bp sequence between primers #9 and #10, cloning the PCR product into the TA cloning vector pCR. The region spanning codons 22 and 31 was recovered from the TA clones by digestion with *Hin*dIII and *Sst*I (see Fig. 1) and inserted in place of the arg22 sequence in pFR400 [5]. The nucleotide sequence between *Hin*dIII and *Sst*I was determined for each plasmid. Only the engineered sequence changes at position 22 or position 31 were observed, except for the recovered leu31 sequence which sustained additional substitutions at codon 16 (isoleucine to phenylalanine) and at codon 63 (lysine to arginine). This sequence recovery process also served to isolate distinct DHFR variants from clones containing more than one DHFR integrant (e.g. leu31 and gly31).

CHO DUX-B11 cells were transfected with SV-DHFR plasmids containing the recovered variant DHFR sequences as well as wild-type and arg22 controls, and then subcultured into medium containing different concentrations of MTX (as described in Materials and Methods). Fourteen days later, colonies were harvested as heterogeneous transfectant cultures for further enzymological analyses. DHFR variants trp22, tyr22, ser31, gly31 and his31 all conferred substantial MTX resistance as gauged by colony formation in 0.25 μM and 1.0 μM MTX.

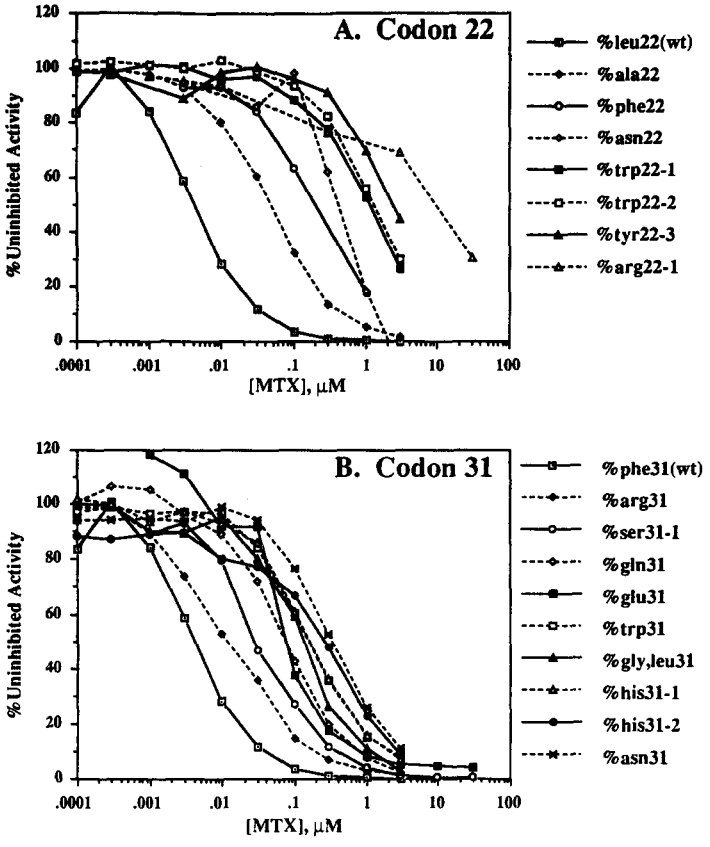

Fig. 4. MTX concentration–response of variant DHFR enzyme activities. Cell extracts were prepared and assayed for DHFR activity at increasing concentrations of MTX, as described in Materials and Methods. Specific activities are expressed as a percentage of that observed for an uninhibited control reaction (see Table 3). The IC_{50} values were read directly from the concentration–response curve for each variant (Table 3).

Extracts from the heterogeneous pools of transfectant clones were assayed for DHFR activity at different concentrations of MTX (Fig. 6) for comparison with clones transfected immediately after saturation mutagenesis. As observed in the originally established transfectant clones, codon 22 variants were much more reduced in specific DHFR activity in comparison with codon 31 variants (Table 4). The IC_{50} values observed for trp22, tyr22, ser31, and his31 transfectants (Table 4) were close to those values observed in extracts from the corresponding clones isolated in the mutagenesis screen (Table 3), verifying the biochemical character of these variants expressed in CHO cells after transfecting with plasmids in which the precise sequence of the DHFR coding region had been determined. In spite of additional substitutions at positions 16 (ile to phe) and 63 (lys to arg), the IC_{50} for leu31 was close to that of wild-type DHFR (phe31), corroborating recently published enzymological results on this variant generated by site-directed mutagenesis [14]. The newly isolated gly31 variant was quite resistant to MTX, with an IC_{50} of about 0.3 μM in this assay. Enzymatic analysis of these DHFRs expressed after transfection of isolated plasmids thus supported the character of these variants initially established in clones transfected with saturation mutagenized material.

DISCUSSION

We conducted saturation mutagenesis experiments at DHFR codon positions 22 and 31 to investigate the effect of different amino acid substitutions on MTX resistance and catalytic activity. DHFR sequence variants conferring drug resistance were selected by isolation of MTX-resistant clones after transfection of mutagenized DHFR transcription units into DHFR-deficient CHO cells. A variety of different substitutions at DHFR codon positions 22 and 31 were found to confer MTX resistance upon the enzyme.

PCR-assisted saturation mutagenesis was an effective means for generating mutations at codons 22 and 31 of the murine DHFR coding sequence. Our data indicate that all four nucleotides were introduced at each of the three codon positions. We cannot be sure that the PCR/overlap extension protocol did not bias the amplification of certain sequences for which the conditions used might have

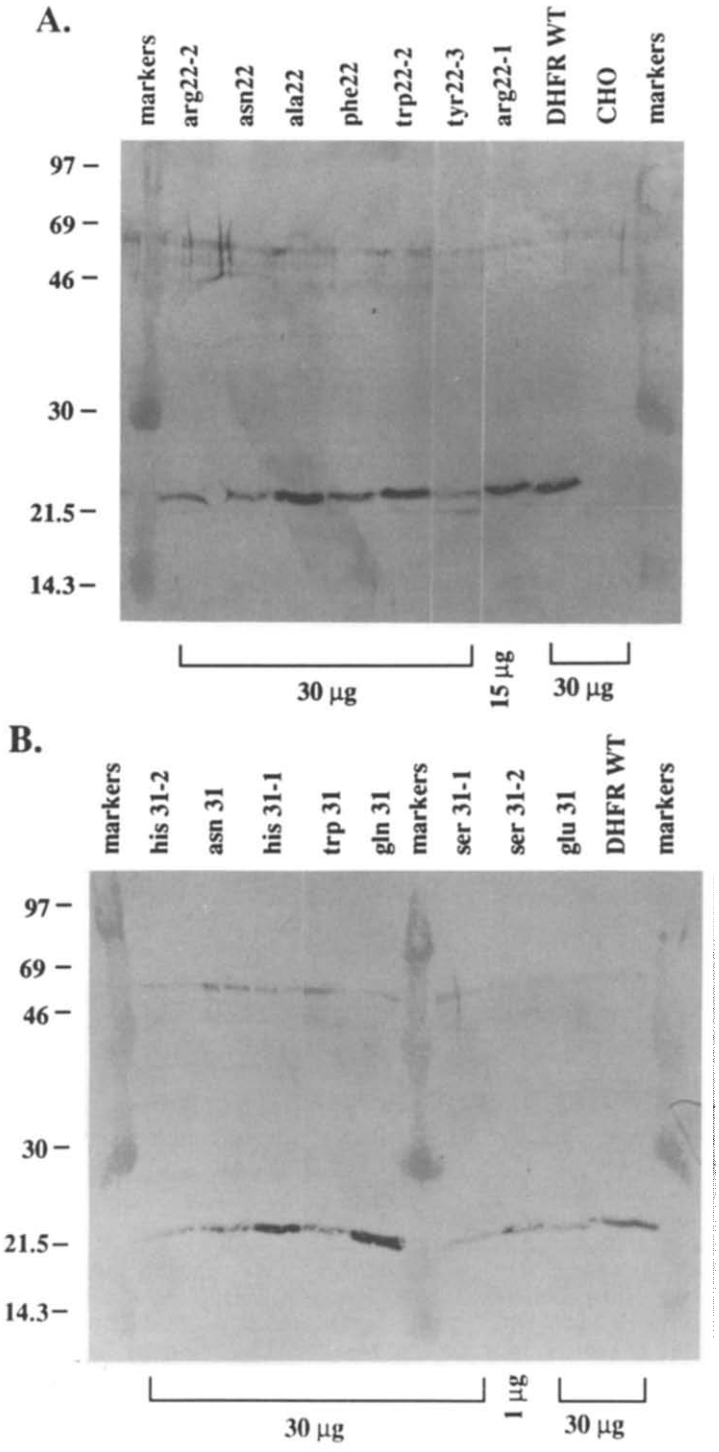

Fig. 5. Western blot of extracts from MTX-resistant CHO clones probed with an anti-murine DHFR antibody. Each sample, indicated on the top, was loaded using the amount of protein indicated on the bottom. The location of molecular weight markers (in kDa) is indicated on the left. DHFR signals (24 kDa) were quantitated by videodensitometry [39], and the results are summarized in Table 2. (A) Codon 22 variants. (B) Codon 31 variants.

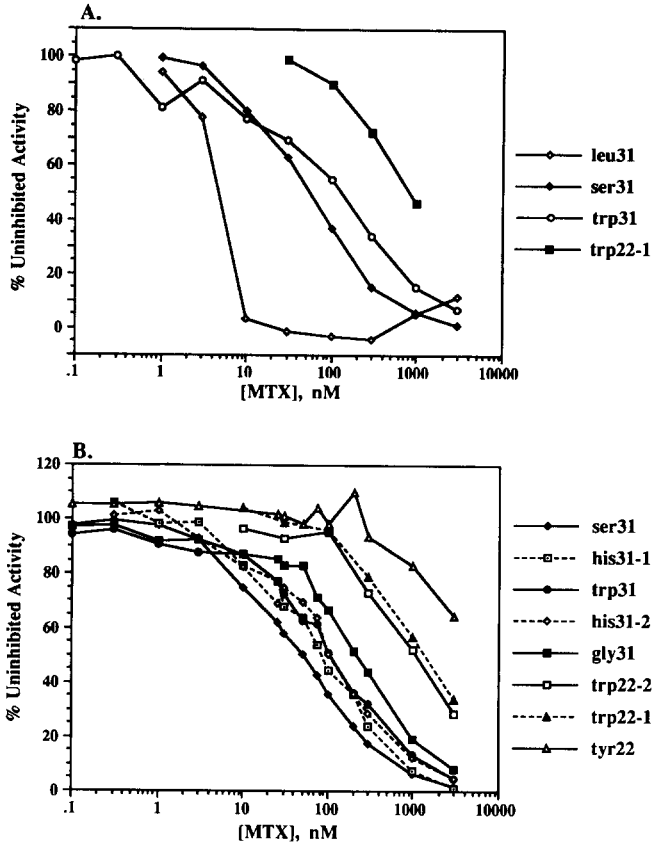

Fig. 6. MTX concentration–response of variant DHFRs expressed in heterogeneous transfectant cultures. Cultures were established, extracted and assayed for DHFR activity at various MTX concentrations, as described in Materials and Methods. Specific activities are expressed as a percentage of that observed for an uninhibited control reaction (see Table 4). The IC_{50} values were read directly from the concentration–response curve for each variant (Table 4). (A) Transfectant populations selected at 0 MTX. (B) Transfectant populations selected at 250 nM MTX.

Table 4. Retrieval and transfection of variant DHFR sequences

		Cells selected at 0 MTX		Cells selected at 250 nM MTX	
Substitution	Codon	IC_{50}*	Activity†	IC_{50}*	Activity†
(A) Pos. 22					
trp-1	UGG	850 nM	0.5	1.5 μM	0.8
trp-2	UGG			1.3 μM	0.7
tyr	UAC			6.0 μM	2.3
(B) Pos. 31					
leu	CUA	4.5 nM	1.6		
ser	UCC	55 nM	2.8	50 nM	6.9
his-1	CAU			90 nM	11.9
his-2	CAU			100 nM	16.1
trp‡	UGG	120 nM	6.8	100 nM	4.5
gly	GGC			225 nM	9.1

* The IC_{50} values were determined from results depicted in Fig. 6.
† DHFR specific activity in crude cell extracts, nmol/min/mg protein.
‡ The trp31 transfectants were established by gene transfer with pSV-DHFR-trp31.

been more favorable for hybridization and priming of DNA synthesis by Taq polymerase. However, out of a total of twenty-two DHFR integrants characterized, fifteen different codon sequences were observed, indicating a diversity of sequences introduced by the mutagenesis procedure. It is also likely that additional sequence alterations occurred in some of the DHFR transcription units expressed in the transfected CHO clones due to errors introduced by Taq polymerase during saturation mutagenesis. However, several DHFR variants (trp22, tyr22, arg22, ser31 and his31) were reproducibly identified and characterized, implicating these substitutions as contributing primarily to the observed differences from wild-type enzyme in catalytic and MTX inhibition character. In addition, several DHFR variants (trp22, tyr22, his31, ser31, gly31) were recovered in order to verify the absence of sequence changes other than those engineered at positions 22 or 31, and the character of DHFR variant enzyme expressed in cells transfected with these recovered sequences was indistinguishable from that expressed in the originally established clones.

In vitro, site-directed mutagenesis has been used previously to explore the role of specific amino acid side chains in DHFR-mediated catalysis [10–15]. In this study, we chose to combine *in vitro* saturation mutagenesis of a complete DHFR transcription unit with selection for MTX resistance after transfection into DHFR-deficient CHO cells, allowing the selective procedure itself to screen for those substitutions conferring drug resistance. The surprising result was that a variety of conservative and non-conservative substitutions at codon positions 22 and 31 conferred MTX resistance upon the expressed DHFR enzyme. Furthermore, there are other substitutions conferring drug resistance at these positions which were not identified in the selection screen but which have been characterized after *in vitro* mutagenesis, i.e. lys22 and glu22 [21], or after cloning, such as trp31 [9]. Assessment of the different substitutions conferring drug resistance was most likely limited, therefore, by the total number of colonies isolated and characterized. Thus, it is also likely that other substitutions at these positions would confer drug resistance, consistent with the variability in character of those substitutions which have been observed, and implying that indeed *most* substitutions at these positions confer MTX resistance. The existence of such a variety of substitutions which are functionally tolerated and associated with MTX resistance strengthens the possibility that emergence of such mutant DHFR forms may contribute to drug resistance of tumor cells *in vivo*.

The catalytic and MTX inhibition character of the observed DHFR codon 22 and codon 31 variants is consistent with the role of these amino acid side chains in DHFR function as demonstrated by enzymological and crystallographic studies. Leucine-22 is located in a hydrophobic pocket known to be responsible for the binding of substrate and inhibitor [40]. We observed that replacement of leucine by alanine resulted in a 10-fold increase in IC_{50}, indicating the importance of *any* deviation in side chain size at this position. The bulkier substitutions at position 22 had a more profound effect on MTX sensitivity, presumably due to steric interactions. Phenylalanine-31 is believed to play an active role in substrate and inhibitor binding by stabilizing interactions with both pteridine and *p*-aminobenzoyl moieties [41]. Replacement of phenylalanine-31 by both smaller (leucine, serine, glutamine, glutamic acid, glycine, asparagine) or larger (histidine, tryptophan [9]) amino acid side chains may disrupt these interactions. That such diverse active site structures continue to function catalytically attests to the adaptability of the enzyme active site surface.

MTX-resistant DHFRs have provided useful tools as dominant selectable markers for mammalian gene transfer and may be applicable to the protection of normal cells and tissues from MTX toxicity during chemotherapy *in vivo*. The arg22 variant has been most extensively utilized as a mediator of MTX resistance in cultured cells and in animals [16–23]. However, the extreme resistance of arg22 to MTX is compromised by its low level of catalytic activity (see Table 3, and Ref. 24), resulting in reduced frequencies of colony formation (in comparison with trp31 [9]) after gene transfer into cultured mammalian cells. It is inherent from the screening process that was used for isolation, i.e. selection in MTX, that any of the array of DHFR variants identified in this study would function as a dominant selectable marker. Furthermore, these variants provide a variety of MTX-resistant DHFRs with different catalytic and MTX inhibition characteristics that can be tested for their effectiveness in mediating drug resistance after gene transfer in mammalian systems. The use of variants more intermediate in character than arg22, such as trp22, tyr22, gly31, his31 or asn31, enzymes which are substantially resistant to MTX (IC_{50} increased 30- to 400-fold over wt DHFR; Table 3) but which retain 17–66% of wt DHFR catalytic activity, may provide more optimized combinations of MTX sensitivity and catalytic character for mediating drug resistance in mammalian cells and tissues.

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