Isolation and characterization of a variant dihydrofolate reductase cDNA from methotrexate-resistant murine L5178Y cells

R.Scott McIvor* and Christian C.Simonsen[1]

Institute of Human Genetics and Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55455 and [1]Genelabs, 505 Penebscot Drive, Redwood City, CA 94063, USA

ABSTRACT

Dihydrofolate reductase (DHFR) cDNA sequences were isolated from a methotrexate-resistant mouse L5178Y cell line previously shown to contain methotrexate-resistant dihydrofolate reductase enzyme activity. Specifically-primed reverse transcription products were amplified using the polymerase chain reaction and then cloned into a mammalian expression plasmid. Candidate clones were identified by restriction analysis and then functionally tested by transfection into mouse 3T3 fibroblasts, selecting for methotrexate-resistant colonies. Sequence analysis of the cDNA clones demonstrated the substitution of tryptophan (TGG) in place of the wild-type phenylalanine (TTC) at codon 31. Sequencing of PCR-amplified genomic DNA extracted from the drug-resistant L5178Y cells confirmed the tryptophan codon at position 31. Transfection of mammalian tissue culture cells with expression plasmids containing the trp31 DHFR sequence resulted in substantial methotrexate-resistant colony formation. Recombinant trp31 DHFR enzyme activity expressed in stably-transfected Chinese hamster ovary cells was approximately 20-fold less sensitive to methotrexate inhibition than wild-type mouse DHFR enzyme activity. We conclude that the cloned Trp31 DHFR sequence encodes an enzyme substantially resistant to methotrexate which confers a drug-resistance phenotype to cells in which it is expressed.

INTRODUCTION

Dihydrofolate reductase (DHFR; E.C. 1.5.1.3) catalyzes the reduction of folic acid to 7,8 dihydrofolate and of dihydrofolate to 5,6,7,8 tetrahydrofolate in mammalian cells (1). Methotrexate (Mtx, 4-amino-N[10]-methyl-folic acid) competitively inhibits DHFR and depletes dividing cells of tetrahydrofolate, necessary for the biosynthesis of precursors for protein and nucleic acid synthesis. The resultant anti-proliferative effect of methotrexate administration has made this drug an effective chemotherapeutic agent in the treatment of a number of different human malignancies (2, 3).

Resistance to Mtx in mammalian cells can arise as the result of increased expression of DHFR, commonly associated with amplification of the gene encoding DHFR (4), or expression of an altered DHFR protein having increased resistance to the drug (5−14). There have been several reports of DHFR activites with altered characteristics obtained from extracts of drug-resistant cells (5−14), and some of these have been molecularly defined (14−17). These studies have engendered insight into the relationship between DHFR structure and catalytic function and provided tools for molecular genetic studies as well. One variant murine DHFR in particular, isolated from murine 3T6 cells resistant to 400 μM Mtx and containing an arginine replacing leucine at codon position 22, has been used widely as a dominant selectable marker in mammalian systems (18).

A Mtx-resistant derivative of the mouse leukemia L5178Y cell line (L5178Y-R$_4$; 8−10) was recently reported to contain a DHFR enzyme extremely resistant to Mtx. To characterize the variant DHFR sequence expressed in these cells, we used the polymerase chain reaction to specifically amplify DHFR coding sequences and then clone the cDNA inserts. We report here that the single substitution of tryptophan for phenylalanine at codon position 31 was sufficient to allow for the methotrexate-resistant phenotype of these cells. Expression plasmids encoding the murine trp31 DHFR also conferred methotrexate resistance upon transfected mammalian cells, suggesting the usefulness of this gene as a selectable marker and mediator of drug-resistance.

MATERIALS AND METHODS

DNA Manipulations

Plasmids maintained in *Escherichia coli* K-12 strain 294 were extracted from chloramphenicol-amplified cultures and purified either by column chromatography on Biogel A50m or by equilibrium centrifugation in CsCl-ethidium bromide. Restriction enzymes were from New England Biolabs. T4 DNA ligase and MoMLV reverse transcriptase were obtained from BRL.

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* To whom correspondence should be addressed

Reagents and equipment (thermocycler) for the polymerase chain reaction were obtained from Perkin-Elmer/Cetus. Oligonucleotides (see Table 1) were synthesized on a Pharmacia Gene Assembler or obtained from the Microchemical Facility, Institute of Human Genetics, University of Minnesota.

cDNA Synthesis and Amplification

RNA was extracted from Mtx-resistant L5178Y-R_4 cells (8−10) by triton-X-100 lysis, removal of nuclei by centrifugation at 14,000×g (5 min.), phenol/chloroform extraction and ethanol precipitation (19). cDNA was synthesized (20) by reverse transcription of approximately 1 μg of total cellular RNA using 10 pmol antisense primer in 20 μl for 30 min. at 37°C. Completed reaction mixtures were then supplemented with 80 μl of 1 × Taq polymerase reaction buffer, 10 pmol sense primer and 2.5 U Taq DNA polymerase for PCR amplification (21)(1 min. 94°, 1 min. 45°, 1 min. 72°, 30 cycles).

DNA Sequencing

DHFR cDNA sequences were determined by using the dideoxy chain termination technique (22). A series of 8 synthetic 17-base oligonucleotides derived from the DHFR coding region were used to prime sequencing of double-stranded template (23). DHFR genomic sequences were determined by using the dideoxy chain termination technique (Sequenase kit 2.0, U.S. Biochem.) after sequential symmetric and asymmetric polymerase chain reactions (conditions described above) to generate single-stranded template (24).

Plasmid Construction

The plasmid pMH, used as a mammalian expression vector for cloning PCR-amplified DHFR sequences, was constructed as follows; pMAMD (25) was digested with BamHI to eliminate an adenosine deaminase coding sequence and then religated to isolate the plasmid pMMD. pMMD was then digested with SstII to eliminate a DHFR transcriptional unit between identical sequences derived from the hepatitis B virus surface antigen gene 3′ flanking region and then religated (see Results for functional characteristics of pMH).

pSV-DHFR plasmids were constructed by cloning the HindIII-NcoI DHFR fragment spanning the DHFR coding region into pFR400, replacing the arg22 DHFR coding sequence with the wild-type and trp31 sequences (15). Insertion of the appropriate

fragment was verified by double-stranded sequencing across the relevant region (23).

Mammalian Cell Culture and Gene Transfer

Mouse L5178Y-R_4 leukemia cells (8−10) were cultivated in Fishers medium containing 10% horse serum and 1 mM Mtx (amethopterin, Sigma). Mouse NIH 3T3 tk⁻ (lacking thymidine kinase), mouse L-tk⁻, and BHK-21 (baby hamster kidney) cells were cultivated in Dulbecco-modified Eagle medium (DMEM, Gibco)/10% newborn or fetal calf serum. Chinese hamster ovary (CHO) DUX-B11 (lacking DHFR) cells (26) were cultivated in Ham's F-12 (Gibco)/7% dialyzed fetal calf serum. All media were supplemented with penicillin and streptomycin.

DNA-calcium phosphate coprecipitate-mediated transfections were as previously described (27,28). Briefly, mouse NIH 3T3 cells or CHO DUX-B11 cells were subcultured one day prior to transfection and then exposed to coprecipitate (5 μg DNA) 3 hrs. (CHO) or overnight (3T3). The cells were then shocked with 15% glycerol (29) in phosphate-buffered saline (PBS) and, after a 2-day recovery period, subcultured into DMEM containing 0.1 μM Mtx (3T3) or F12 lacking glycine, hypoxanthine and thymidine and containing various levels of Mtx.

Lipofections (30) using DOTMA (BRL) were performed according to the manufacturer's recommendation. Briefly, plasmid DNA (5−10 μg) was mixed with 25 μg of DOTMA in a final volume of 100 μl and allowed to incubate at room temperature for 30 min. The DNA/DOTMA mixture was then added to freshly (24 hrs. before exposure) subcultured target cells (2×10⁵ cells). The cells were incubated with the mixture for 4 hrs. in serum-free medium and then supplemented with medium containing serum to a final concentration of 10%. The cells were then allowed to recover for 2 days prior to subculture into selective F-12 (CHO) or DMEM (L-tk⁻, BHK-21) media lacking glycine, hypoxanthine and thymidine and containing various levels of methotrexate.

DHFR Spectrophotometric Enzyme Assay

DHFR enzyme activity in cleared, sonicated cell extracts was determined at room temperature using a Beckman DU50 spectrophotometer as previously described (31,32, Vinh, D.B., and McIvor, R.S., manuscript submitted). One mL reactions contained 20 μM dihydrofolate (Sigma), 120 μM NADPH, 150 mM KCl, 10 mM β-mercaptoethanol and 50 mM Tris, pH 7.5.

Table 1. Oligonucleotide Sequences and Utilization.

No.	Orientation	Location[1]	Use[2]	Sequence[3]
1	Sense	−30 to −8	2,4,5	GGCTGGTA**A**GCTTTTATCCCCGC
3	Antisense	635 to 654	1	CTCATAGATCTAAAGCCAGC
5	Antisense	119 to 136	4	**CCGGAA**TTCCACTGAAGAGGTTG[4]
6	Antisense	57 to 78	5	AGGCCAGGGTCGGTCTCCGTT
7	Sense	intron 1[5]	5	CGGACTTGCACCTTTCGTCGC
11−20	Both	see Fig. 3	3	see Fig. 3

[1]Location; nucleotide numbers with respect to the translational start site (Fig. 3).
[2]Uses; 1- first strand cDNA synthesis, PCR amplification
    2- second strand cDNA synthesis, PCR amplification
    3- cDNA sequencing
    4- genomic DNA PCR amplification
    5- genomic DNA sequencing
[3] Sequences are given in the 5′ to 3′ direction. Deviations from the template sequence are in bold.
[4] Contains a 6-base 5′ tail to create an EcoRI site
[5] Nucleotides −50 to −70 upstream of the intron 1-exon 2 junction (ref 49). Exon 2 begins at nucleotide 87 in Figure 3.

Mtx, when included, was allowed to equilibrate with enzyme for 10 minutes before starting the reaction by addition of dihydrofolate. Continuous readings at 340 nm were transferred through an RS232 interface to an Apple Macintosh SE for determination of initial velocities using CricketGraph software ($\epsilon_{340} = 12$ mM^{-1}cm^{-1})

RESULTS

Cloning of PCR-amplified DHFR cDNA sequences

We used the polymerase chain reaction to specifically amplify DHFR cDNA sequences and facilitate the isolation of DHFR cDNA clones from a highly Mtx-resistant mouse L5178Y leukemia cell line (Fig. 1). A 21-base antisense oligonucleotide spanning a BglII site in the 3' untranslated region of the DHFR message was used to prime a first strand cDNA synthesis reaction (See Materials & Methods for reaction details). A 26-base sense oligomer from the 5' untranslated region was then added to the completed reverse transcription reaction mixture, which was then subjected to 30 cycles of PCR amplification. The upstream sense primer contained 2 mismatched bases which converted a 6 bp sequence to a unique HindIII site.

The results of DHFR cDNA PCR amplification are shown in figure 2, where 10 μL (out of 100 μL) of each reaction was loaded onto a 0.7% agarose gel stained with ethidium bromide. A 600 bp fragment was observed in lane 1, containing the complete reaction, but not if the RNA (lane 2) or reverse transcriptase (lane 5) were left out, verifying that the 600 bp PCR product was derived from an RNA source.

The PCR product of the complete reaction (fig. 2, lane 1) was digested with HindIII and BglII, which cut within the primer

regions at the 5' and 3' ends, respectively. These sequences were then directionally cloned between HindIII and BamHI sites in a mammalian expression plasmid, pMH (pMH construction detailed in Materials & Methods). pMH contains a single HindIII site flanked upstream by a 1834 bp mouse metallothionein promoter sequence (33), and a single BamHI site flanked downstream by a 585 bp sequence from the hepatitis B-virus surface antigen gene spanning the polyadenylation signal (19). 5' to 3' directional inserts of coding sequences between HindIII and BamHI should thus provide functional transcription units. Bacterial transformants were screened by restriction mapping of extracted plasmid DNA for those containing DHFR inserts (approximately 1/3). A total of 7 DHFR cDNA clones were thus identified (nos. 15, 21, 23, 25, 30, 31, and 36). Three of these clones (nos. 15, 21, and 23) were also tested and found to generate Mtx-resistant colonies after transfection into mouse 3T3 cells. Extracts of cells expanded from one of these clones (#21) were found to contain DHFR activity substantially resistant to Mtx (data not shown), providing evidence for the presence of a Mtx^r-DHFR coding sequence on these plasmids.

cDNA sequence analysis

A composite DHFR cDNA sequence compiled from cDNA sequencing results is shown in Figure 3. The entire DHFR insert sequence of cDNA clone #21 was determined using the dideoxy chain termination technique. The sequence was identical to the established murine DHFR cDNA sequence (15,34) with the following exceptions: (i) A two base-pair change at codon 31 (TTC to TGG) which would cause a phenylalanine to tryptophan substitution; and (ii) an A to G transition at position 1 of codon 13, which would result in the substitution of aspartic acid for the wild-type asparagine at this residue.

Since multiple changes were observed in the DHFR sequence of clone #21, possibly due to alterations introduced by reverse transcription (35,36) or PCR amplification (37,38), we felt it necessary to confirm these results by using several different methods. First, five other cDNA clones (Nos. 15, 23, 30, 31, and 36) were sequenced across codons 13 and 31. All five contained the trp31 (TGG) double nucleotide change, and all five
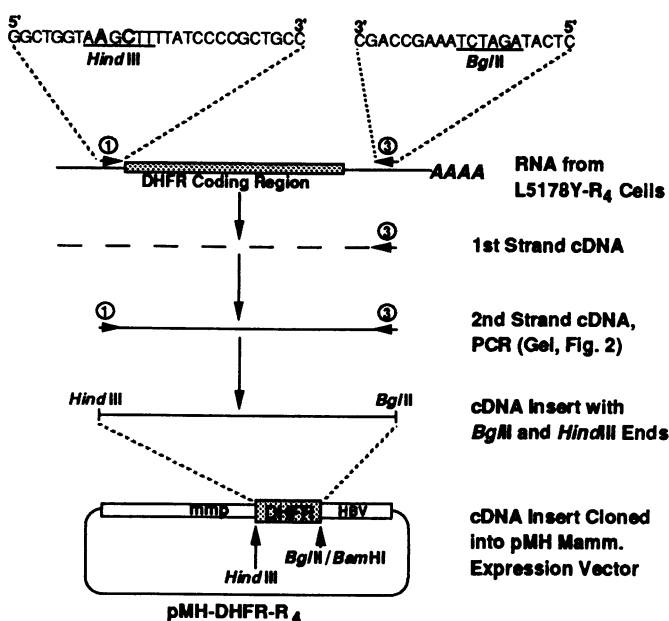

Figure 1. Strategy for cDNA synthesis, amplification and cloning of the murine DHFR coding sequence into pMH, a mammalian expression vector. The primer sequences are given, with *Bgl*II and *Hind*III sites underlined in the downstream (no. 3) and upstream (no. 1) primers, respectively. Base mismatches in the upstream primer, included to provide a unique *Hind*III site, are in outline type. Stippled boxes; DHFR coding sequence. Additional open boxes in pMH-DHFR-R₄; a 1.8 kb sequence containing the promoter region from the mouse metallothionein I gene (mp; 33), and a 585 bp sequence from the hepatitis B virus surface antigen gene including the polyadenylation signal (HBV;19).
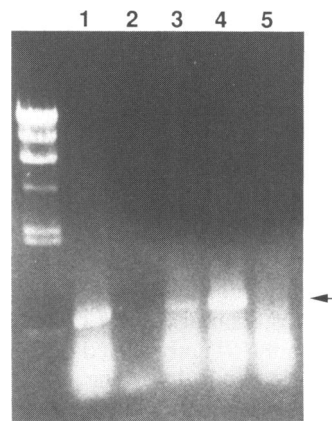

Figure 2. Amplification of DHFR RNA sequences by using the polymerase chain reaction. 1 μg of total RNA was reverse transcribed and then subjected to 30 cycles of PCR amplification as described in Materials and Methods. 10 μl samples (of 100 μl total) were loaded onto a 0.7% agarose gel. Lane 1, Complete reaction; Lane 2, RNA omitted; Lane 3, Reverse transcriptase added *after* 37° C incubation; Lane 4, Reaction supplemented with pDHFR11 cDNA clone (34); Lane 5, Reverse transcriptase omitted. Arrow; 600 bp DHFR fragment.
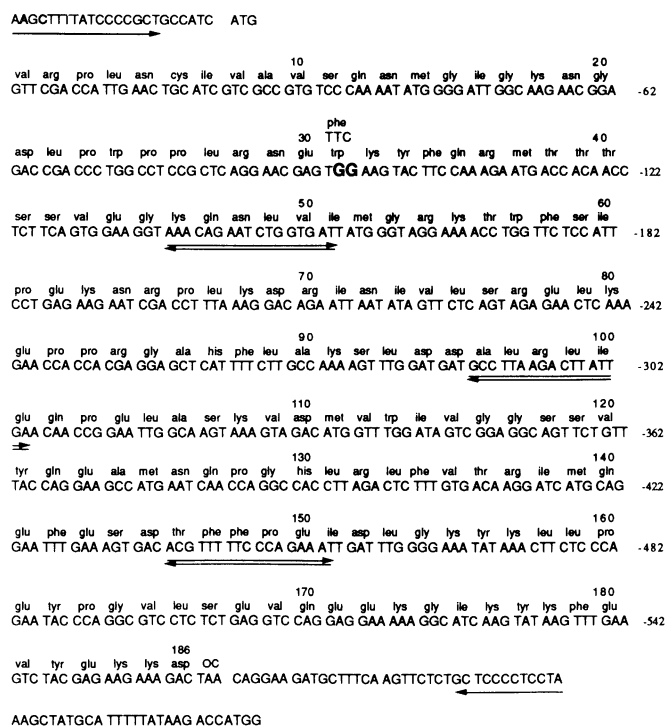
AAGCTTTATCCCCGCTGCCATC ATG

               10   20

val arg pro leu asn cys ile val ala val ser gln asn met gly ile gly lys asn gly
GTT CGA CCA TTG AAC TGC ATC GTC GCC GTG TCC CAA AAT ATG GGG ATT GGC AAG AAC GGA -62

                           phe
             30 TTC   40
asp leu pro trp pro pro leu arg asn glu trp lys tyr phe gln arg met thr thr thr
GAC CGA CCC TGG CCT CCG CTC AGG AAC GAG TGG AAG TAC TTC CAA AGA ATG ACC ACA ACC -122

                   50   60
ser ser val glu gly lys gln asn leu val ile met gly arg lys thr trp phe ser ile
TCT TCA GTG GAA GGT AAA CAG AAT CTG GTG ATT ATG GGT AGG AAA ACC TGG TTC TCC ATT -182

                  70   80
pro glu lys asn arg pro leu lys asp arg ile asn ile val leu ser arg glu leu lys
CCT GAG AAG AAT CGA CCT TTA AAG GAC AGA ATT AAT ATA GTT CTC AGT AGA GAA CTC AAA -242

                  90   100
glu pro pro arg gly ala his phe leu ala lys ser leu asp asp ala leu arg leu ile
GAA CCA CCA CGA GGA GCT CAT TTT CTT GCC AAA AGT TTG GAT GAT GCC TTA AGA CTT ATT -302

                  110   120
glu gln pro glu leu ala ser lys val asp met val trp ile val gly gly ser ser val
GAA CAA CCG GAA TTG GCA AGT AAA GTA GAC ATG GTT TGG ATA GTC GGA GGC AGT TCT GTT -362

                  130   140
tyr gln glu ala met asn gln pro gly his leu arg leu phe val thr arg ile met gln
TAC CAG GAA GCC ATG AAT CAA CCA GGC CAC CTT AGA CTC TTT GTG ACA AGG ATC ATG CAG -422

                  150   160
glu phe glu ser asp thr phe phe pro glu ile asp leu glu gly lys tyr lys leu leu pro
GAA TTT GAA AGT GAC ACG TTT TTC CCA GAA ATT GAT TTG GGG AAA TAT AAA CTT CTC CCA -482

                  170   180
glu tyr pro gly val leu ser glu val gln glu glu lys gly ile lys tyr lys phe glu
GAA TAC CCA GGC GTC CTC TCT GAG GTC CAG GAG GAA AAA GGC ATC AAG TAT AAG TTT GAA -542

                  186
val tyr glu lys lys asp OC
GTC TAC GAG AAG AAA GAC TAA CAGGAA GATGCTTTCA AGTTCTCTGC TCCCCTCCTA

AAGCTATGCA TTTTTATAAG ACCATGG

Figure 3. Composite cDNA sequence of DHFR RNA expressed in Mtx-resistant L5178Y-R$_4$ cells (determined as described in Materials and Methods). The sequence is shown from the PCR-engineered *Hind*III site (altered nucleotides in outline type) through the unique *Nco*I site at nucleotide position 627. Nucleotides are numbered (on the right) from the start codon. Oligonucleotides used for priming sequencing reactions are indicated by arrows underneath the specific priming sequence. Amino acids are numbered (on top) starting with valine, one residue beyond the methionine start codon. Altered bases (at codon 31) are indicated in bold with the normal, wild-type murine DHFR sequence indicated above the variant sequence.

contained the wild-type sequence (AAT) at codon 13, indicating that the GAT at codon 13 found in clone #21 was either an artifact or reflected a rare message type in the Mtx-resistant L5178Y-R$_4$ cells (hence a wild-type AAT codon is indicated in Fig. 3 for the composite coding sequence). The results of transfection studies and sequencing amplified genomic material also supported this conclusion (see below).

Analysis of genomic sequences PCR-amplified from Mtx-resistant L5178Y cell DNA

To determine whether the asp13 or trp31 substitutions were encoded by DHFR gene sequences in Mtx^r-L5178Y-R$_4$ cells, a PCR strategy was devised to specifically amplify a 500 bp region of the DHFR gene for sequencing across both codons 13 and 31 (Fig. 4A). The upstream primer (#1) was the same 26-base oligonucleotide from the 5' untranslated region which was used for DHFR cDNA amplification. The downstream antisense primer was a 23-base oligonucleotide (#5) from the 3' end of exon 2. Mtx^r-L5178Y-R$_4$ cell DNA (1 μg) and pMg3 DNA (1 ng)(39) were subjected to sequential symmetric and then asymmetric (both strands, separately) PCR amplification (24). To sequence across codon position 13, sense and antisense templates were primed (Fig. 4A) with antisense oligonucleotide #6 and sense oligonucleotide #1, respectively. Mtx^r-L5178Y-R$_4$ and Mg3 (wild-type) sequences were identical in this region,

including those located at codon position 13 (sense, AAT; antisense, TTA; Fig. 4B).

To sequence across codon 31, antisense template was primed (Fig. 4A) with oligonucleotide #7. As observed in Fig. 4C, there was no indication that the wild-type phe codon (TTC) was present in the L5178Y-R$_4$ cell DNA. The trp substitution (TGG) was observed for L5178Y-R$_4$ cell DNA, replacing the wild-type phe codon (TTC) observed for pMg3 at this position. Close inspection of the sequencing autoradiograph suggested that the L5178Y-R$_4$ cells also contained a weaker, but definitive, T signal at position 2 of codon 31, resulting in a leucine codon (TTG). Whether the predicted leu31 codon represents a second mutation or whether it preceded the trp mutation cannot be ascertained by the sequence shown in Fig. 4. We can only note that an earlier-existing leu alteration could have arisen by a single mutation within codon 31 (TTC to TTG), whereupon a single additional change (TTG to TGG) could account for the trp variant.

Analysis of drug-resistance function by transfection in cultured mammalian cells

To characterize the utility of the isolated Mtx^r-DHFR cDNA as a drug-resistance marker, gene transfer experiments were conducted using several different mammalian cell lines as target cell populations. The trp31 DHFR coding sequence was inserted into a mammalian expression vector containing an SV40 early promoter to regulate initiation of transcription (see ref. 15, Fig. 3 for map of a comparable arg22 DHFR expression plasmid). To directly compare the trp31 coding sequence with the wild-type and arg22 sequences in their ability to render mammalian cells resistant to methotrexate, comparable mammalian expression plasmids were transfected into various cells by using the DOTMA lipofection system (see Materials and Methods). Two days post-transfection the cells were subcultured into selective medium (lacking glycine, hypoxanthine and thymidine; GHT) containing different levels of methotrexate. Clones were scored after 10−21 days (Figure 5).

CHO cells deficient in DHFR activity (Figure 5A) cannot survive in the absence of GHT. Therefore, colony-formation in the absence of GHT provides a nutritional assessment of a transfecting plasmid's ability to provide sufficient DHFR activity for cell survival. After transfection of CHO-DHFR$^-$ cells, the wild-type DHFR sequence provided the greatest number of transfectant colonies (Fig. 5A). DHFR$^+$ colony formation was slightly reduced for trp31, and severely reduced for arg22. This relationship between absolute DHFR$^+$ colony-formation and altered DHFR sequence types reflects the relative activities of these different DHFR enzymes (18). Wild-type DHFR$^+$ transfectant colony-formation was almost completely inhibited by 100 nM Mtx in the selective medium, and was completely absent at 500 nM. Trp31 DHFR$^+$ colony-formation, however, was substantially resistant to 100 and 500 nM Mtx (43% and 11%, respectively). Arg22 DHFR transfectants generated a low level of colony-formation which was relatively unaffected by Mtx. Transfection with the trp31 DHFR sequence thus resulted in DHFR$^+$ colony-formation which was less resistant to Mtx than arg22 DHFR transfection, but provided a much higher level of colony-formation.

To assess the utility of the trp31 DHFR sequence as a dominant selectable marker, the same three pSV-DHFR expression plasmids (WT, trp31, and arg22) were transfected into cells containing endogenous DHFR activity (CHO-K1, Fig. 5B,
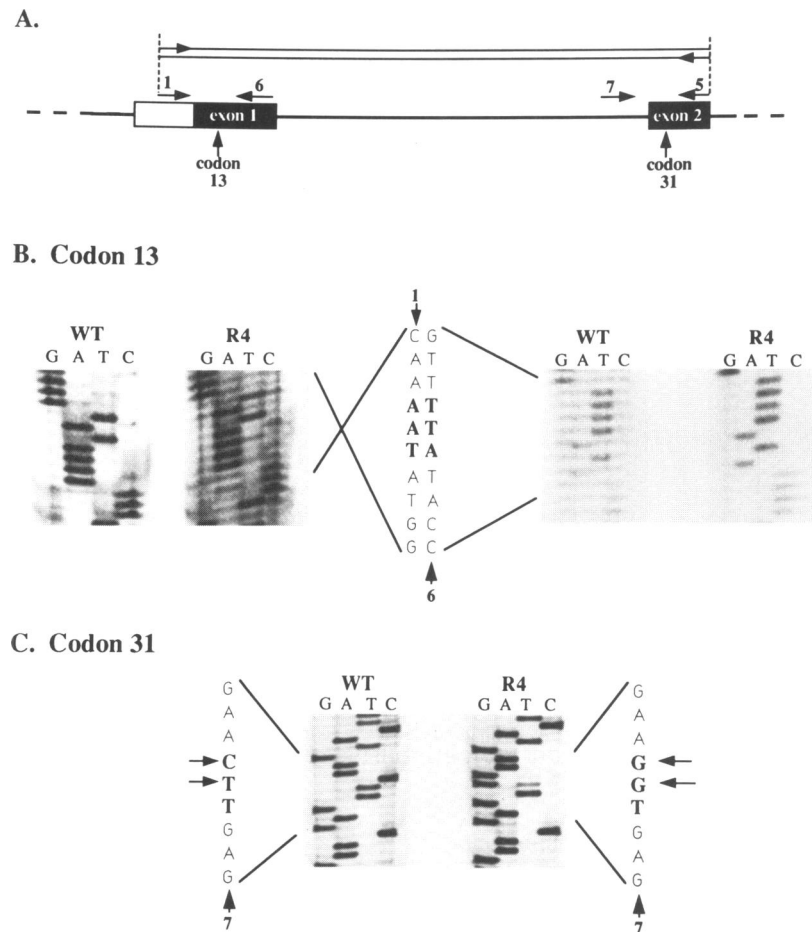
Figure 4. PCR amplification and sequencing of DHFR gene sequences contained in L5178Y-R₄ cells. A. Strategy. A map of the relevant region of the murine DHFR gene encompassing both codons 13 and 31 is shown along with the locations of oligonucleotide primers in sense (nos. 1 and 7) and antisense (nos. 5 and 6) orientations. Symmetric and asymmetric PCR products were generated with primers 1 and 5 as indicated at the top. Oligonucleotides were then used to prime sequencing of complementary, asymmetrically amplified single-stranded template. B and C. Autoradiograms showing sequence across codons 13 and 31 (in bold type) for L5178Y-R₄ DNA and also for pMg3 (a cloned murine DHFR minigene construct; 39) as a wild-type control. Codon 13 was sequenced in both directions and codon 31 was sequenced in the sense direction. The primers used for specific sequencing reactions shown in the figure are indicated in the direction the sequence was generated.

parental line to the DHFR-deficient DUX-B11 line; BHK-21, Fig. 5C; and mouse L-tk⁻, Fig. 5D), selecting for drug-resistant colonies. As observed in DHFR⁻ CHO cells, transfection with the trp31 DHFR sequence in general resulted in higher absolute colony numbers than transfection with the arg22 DHFR sequence. Transfection with the wild-type DHFR did not support Mtx-resistant colony-formation. L-cell drug-resistant colony-formation was also highest for trp31 DHFR transfectants, although significant colony-formation was observed only at low levels (100 nM) of Mtx, perhaps owing to low expression levels using the SV40 early promoter in mouse cells (40). These results demonstrated the utility of the trp31 DHFR sequence as a selectable marker which is dominant-acting in target cell populations that contain endogenous DHFR activity as well as in DHFR-deficient cells.

Mtx growth inhibition of stably transfected Chinese hamster ovary cells

To determine the effect of methotrexate on the growth characteristics of transfectants stably expressing different DHFR's and adapted to selective growth conditions, CHO-DHFR⁻ cells were transfected with pSV-DHFR plasmids encoding the wild-type, trp31, or arg22 enzyme. DHFR⁺ colonies were isolated in selective medium (see Materials and Methods) and expanded in culture. These stable transfectants were then plated into selective medium containing different levels of methotrexate and cell populations quantitated after 3 days in culture (Figure 6). Growth of wild-type DHFR transfectants was inhibited by 50% at 10 nM Mtx, while 50% reduction in the growth of trp31 DHFR transfectants required Mtx in excess of 100 nM. Growth of arg22 DHFR transfectants was substantially affected only at Mtx concentrations exceeding 1 uM. Stable transfection with a trp31 DHFR expression plasmid thus resulted in resistance to Mtx which was intermediate between that of wild-type murine DHFR and arg22 DHFR.

Mtx inhibition characteristics of the trp31 DHFR expressed in DHFR-deficient Chinese hamster ovary cells

To more directly characterize the gene product of the cloned trp31 DHFR cDNA sequence as a drug-resistant enzyme, stable transfectants of CHO DHFR⁻ cells were extracted by sonication and then DHFR activity was determined at different concentrations of methotrexate (Figure 7). Methotrexate inhibition kinetics for trp31 extracts were displaced to an inhibitor
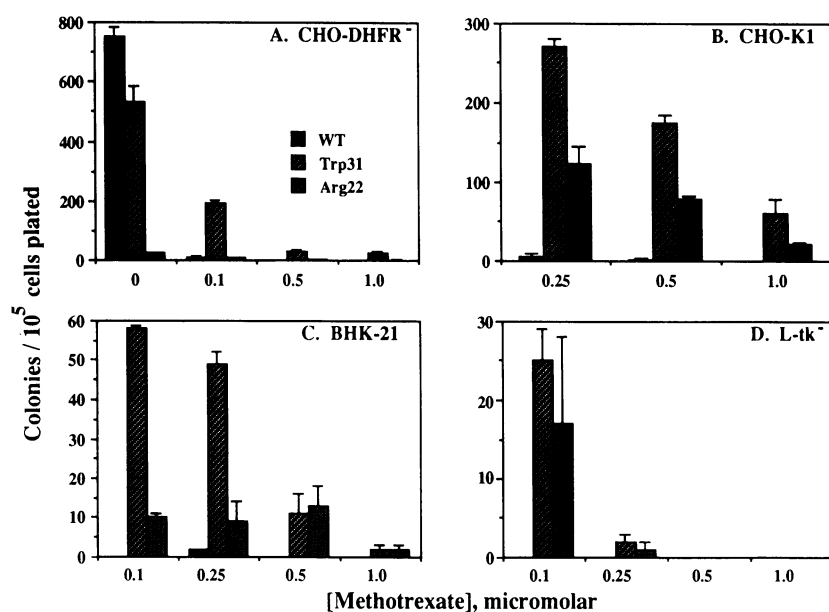
Figure 5. Methotrexate-resistant colony formation after transfection with different DHFR expression plasmids. Cells were transfected with pSV-DHFR plasmids containing the wild-type, trp31 or arg22 DHFR sequences (as described in Materials and Methods) and then plated in duplicate into medium containing the indicated level of methotrexate, 5×10^4 cells per plate. Colonies containing greater than 50 cells were scored after 10−21 days. Data are expressed as the mean (with standard error, bar) of at least two replicate experiments (n) as follows: A. CHO-DUX-B11 (DHFR⁻) cells, n = 6; B. CHO-K1, n = 2; C. BHK-21, n = 2; D. L-tk⁻, n=2.

concentration approximately 20-fold higher (50% inhibition at greater than 100 nM) in comparison with wild-type murine DHFR activity (50% inhibition at around 5 nM). A low level of arg22 DHFR was extremely resistant to Mtx, maintaining greater than 50% of the uninhibited level at an inhibitor concentration of 3 μM. Inhibition of enzyme activity thus closely paralleled the inhibition of colony-formation by Mtx observed for transfectants of these three murine DHFR isoforms (Fig. 5) and growth inhibition of stable transfectants (Fig. 6). These results directly associate the trp31 mutation with a catalytically altered DHFR gene product which is less sensitive to methotrexate, the expression of which is capable of rendering cells drug-resistant.

DISCUSSION

cDNA clones containing sequences encoding a murine methotrexate-resistant dihydrofolate reductase were isolated and characterized by sequencing and expression in transfected mammalian cells. A sequence change at codon 31 (phe to trp substitution), observed by cDNA and genomic DHFR gene sequence analysis, was associated with drug-resistance in transfection and enzyme inhibition experiments. One practical consequence of sequence variations at the DHFR locus rendering this enzyme resistant to methotrexate is that this phenotype itself can be used for the purpose of screening DHFR inserts for those encoding such a drug-resistant enzyme (when cloned into an appropriate mammalian expression plasmid, Fig. 1). Candidate clones can thus easily be tested directly for the presence of a drug-resistance function by transfection in mammalian cells and assay for the outgrowth of drug-resistant mammalian cell colonies.

We used PCR to specifically enrich DHFR sequences and facilitate cDNA cloning. However, in addition to the trp31 substitution, we also observed a single base change at codon 13 (AAT to GAT) in one of six cDNA clones. This variant may
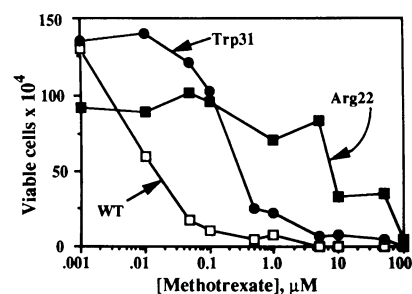

Figure 6. Methotrexate growth inhibition of stable transfectants of CHO-DHFR⁻ cells expressing different murine DHFR's. 2.5×10^5 cells stably transfected with pSV-DHFR plasmids containing the wild-type, trp31, or arg22 DHFR sequence were plated in duplicate into medium containing different levels of Mtx and then cell number quantitated after 3 days growth. wt, pSV-DHFR transfectant; trp31, pSV-DHFR-trp31 transfectant; arg22, pFR400 (pSV-DHFR-arg22) transfectant.
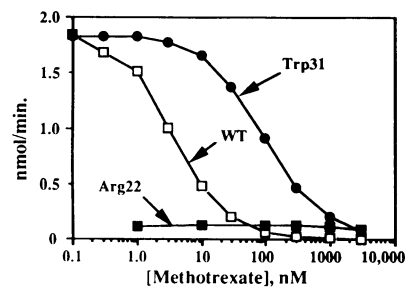

Figure 7. Methotrexate inhibition of DHFR activities expressed in CHO-DHFR⁻ cells. Extracts were prepared and assayed for DHFR activity at different concentrations of methotrexate as described in Materials and Methods. Assayed enzyme activity is plotted vs. methotrexate concentration. wt, .34 mg pSV-DHFR transfectant; trp31, .29 mg pSV-DHFR-trp31 transfectant; arg22, .33 mg pFR400 (pSV-DHFR-arg22) transfectant.

have resulted from an error introduced during the processes of reverse transcription or PCR amplification. Although reported error frequencies vary widely, an inherent infidelity is generally recognized for both of these enzymes (35−38). It is not known which (if either) of these two enzymes was responsible for this sequence anomaly, but our observations underscore the potential hazard of their use in molecular cloning experiments. Analysis of PCR-amplified DHFR gene sequences demonstrated the presence of only wild type sequence at codon 13 and the trp codon (TGG) at position 31 in the Mtx^r-L5178Y cells (Fig. 4). There was also a reduced T signal at position 2 of codon 31, perhaps representative of a very minor portion of DHFR gene sequences, which are amplified in these cells (9, and data not shown). This leucine codon (TTG) may have existed as an intermediate in the generation of the trp codon (TGG) from wild-type phe (TTC) in these cells. However, none of the 6 cDNA clones sequenced across codon 31 contained the TTG (leu) sequence at this position, suggesting that the leu31 sequence represents at best a minor portion of the DHFR transcripts in these cells.

Substitution of phenylalanine by tryptophan at position 31 is of structural significance since phe31 is one of few amino acids which are conserved in both prokaryotic and eukaryotic DHFR's (41). Crystallographic studies have indicated hydrophobic interactions with substrate and inhibitor at this position for the murine (42), chicken (43,44), *E. coli* (44−46) and *Lactobacillus casei* (46) enzymes. Reduced affinity for methotrexate has previously been reported for a naturally-occuring phe31 to serine substitution in the human enzyme (14) and also for an engineered phe31 to arginine substitution in the murine enzyme (47). Other mammalian DHFR sequence changes reported to render the wild-type enzyme less sensitive to inhibition by methotrexate include a glutamine to proline substitution at codon 35 of the murine DHFR sequence, isolated using a selective system in *Bacillus subtilis* (16). Substitution at codon 22 was originally reported for a murine cDNA sequence (leu22 to arginine; 15) isolated from Mtx-resistant 3T6 cells, and has subsequently been reported for Chinese hamster lung cells (leu22 to phenylalanine; 13) and for Chinese hamster ovary cells (also leu22 to phenylalanine; 17). The genetic observations implicating these side chains in DHFR function are consistent with structural studies and may be of use in the design of antifolates as chemotherapeutic agents (48).

CHO DHFR⁻ cells transfected with the trp31 variant expressed DHFR activity which was approximately 20-fold more resistant to methotrexate than murine wild-type DHFR activity in enzyme assays containing saturating levels of substrates. These results clearly contrast with those reported (8) for enzyme partially purified (as the fall-through from Mtx affinity columns) from the same Mtx-resistant L5178Y-R_4 cells, where much higher levels of Mtx (100 μM) were required for inhibition. This difference in Mtx inhibition character could reflect isozymic variance, perhaps the result of post-translational modification in the Mtx-resistant L5178Y-R_4 cells. We have not yet kinetically characterized the recombinant trp31 enzyme, but our results from transfection studies provide some insight into the nature of this variant in comparison with the arg22 variant and the wild-type enzyme. Overall expression of DHFR activity was manifested in these experiments as the total number of colonies observed when transfected CHO-DHFR⁻ cells were plated into selective medium lacking GHT and containing no methotrexate (Fig. 5A). Since the only difference between transfected plasmids was in the few base changes in the DHFR coding region (aside from some minor differences in linker sequences in the 5′ untranslated region of pFR400, the arg22 expression plasmid; 15), resulting

differences in DHFR+ colony-formation were most likely associated with variation in DHFR activity among the different DHFR's expressed from these plasmids. For example, the arg22 variant enzyme has a V_{max} which is reduced 20-fold from that of the wild type (11), and transfection of DHFR⁻ cells with an arg22 DHFR expression plasmid (pFR400) resulted in DHFR+ colony-formation which was substantially reduced in comparison with wild-type DHFR transfection (Fig. 5A). Transfection with a trp31 DHFR expression plasmid resulted in DHFR+ colony-formation which was reduced only slightly in comparison with wild-type DHFR transfection, indicating the likelihood that the trp31 DHFR enzyme has an intrinsic activity which is intermediate between the wild-type enzyme and the arg22 variant.

These transfection experiments demonstrated the usefulness of the trp31 DHFR sequence as a selectable marker in DHFR-deficient CHO cells (Fig. 5A) and as a dominant-acting selectable marker in mouse NIH 3T3 (used to initially screen clones by expression), CHO-K1, BHK21 (baby hamster kidney) and mouse Ltk⁻ cells (Fig. 5B-D). Although the drug-resistance conferred by trp31 DHFR expression does not extend to methotrexate concentrations nearly as high as that conferred by arg22 DHFR expression, the greater activity of the trp31 DHFR may make this enzyme superior for the purpose of rendering mammalian cells and tissues resistant to relatively moderate (under 1 uM) levels of methotrexate. Finally, the trp31 DHFR may be superior to the widely-used arg22 DHFR as an amplifiable marker since it is more sensitive to methotrexate and should amplify to a greater degree when selected in high levels of methotrexate, a possibility which we are currently investigating.

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