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Isolation and expression of an altered mouse dihydrofolate reductase cDNA

(gene transfer/eukaryotic expression vector/selectable marker)

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ABSTRACT **We have constructed a cDNA library from a murine cell line expressing high levels of a dihydrofolate reductase (tetrahydrofolate dehydrogenase; 5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) that displays an abnormally low affinity for methotrexate. From this library we have isolated a cDNA clone similar to, but distinguishable from, a cDNA clone previously demonstrated to encode the wild-type enzyme. Analysis of the nucleotide sequence of this cDNA clone allows us to predict that the altered dihydrofolate reductase differs from the wild-type enzyme at a single amino acid, reflecting the substitution of an arginine for a leucine residue in a region of the polypeptide thought to form a hydrophobic pocket essential for inhibitor binding. To confirm that this substitution was responsible for the altered properties of the enzyme, we genetically localized the region of the cDNA that specified resistance to methotrexate by *in vitro* recombination. These results reveal that a single nucleotide change in the codon specifying amino acid 22 of the enzyme was sufficient to alter the methotrexate sensitivity of the enzyme. We demonstrate that this altered gene can be employed as a dominant selectable marker in cultured cells expressing normal levels of wild-type dihydrofolate reductase.**

Dihydrofolate reductase (DHFR; tetrahydrofolate dehydrogenase, EC 1.5.1.3) catalyzes the NADPH-dependent reduction of folate to tetrahydrofolate, an essential cofactor in the synthesis of glycine, purines, and thymidine (1). The antineoplastic agent methotrexate (Mtx), a folate analogue, binds tightly to the catalytic site of the enzyme, resulting in a deficiency of thymidylate and subsequent cell death (2). The use of this inhibitor in cancer chemotherapy is hindered, however, by the emergence of resistant cells. Mutations that affect the transport of Mtx into cells have been described (3), as have cells that express increased levels of dihydrofolate reductase resulting from an amplification of DNA sequences that encode the enzyme (4). In addition, resistant cells can be found that express an altered dihydrofolate reductase exhibiting a decreased affinity for Mtx (5–10).

Recently, cDNA clones encoding wild-type DHFR have been obtained (11) that possess the ability to convert cells deficient in DHFR (12) to a DHFR$^+$ phenotype when appropriately engineered (13–15). However, attempts to utilize these constructions as a selectable marker in wild-type cells have not succeeded. Although wild-type cultured cells have been transformed with whole genomic DNA from cells expressing a DHFR with a reduced affinity for Mtx, transfer of the selected sequences is, as expected, inefficient (16–18). We reasoned that the isolation of a cDNA clone encoding an altered enzyme might provide a dominant, metabolically selectable marker that could efficiently facilitate the introduction of foreign genes into a wide variety of mammalian cells. We report here the characterization of a cDNA clone that encodes an altered DHFR and we demonstrate its utility as a dominant selectable marker in wild-type hamster and mouse cells.

MATERIALS AND METHODS

Nucleic Acid Manipulations. Cytoplasmic RNA was isolated from a clarified extract of 1×10^8 3T6-R400 mouse cells previously lysed by treatment with 20 ml of 0.15 M NaCl/1.5 mM $MgCl_2$/0.65% Triton X-100/10 mM Tris·HCl, pH 8.0, for 10 min at 4°C. Poly(A)$^+$ RNA was isolated by chromatography on oligo(dT)-cellulose (P-L Biochemicals). Construction and screening of a cDNA plasmid bank were performed by using standard techniques (19, 20). Plasmid DNA was prepared by using the method of Birnboim and Doly (21). Restriction and modification enzymes were purchased from either New England BioLabs or Bethesda Research Laboratories and used as directed by the supplier. Transformations were performed as described (19), using *Escherichia coli* strain 294 (22). DNA sequences were determined by the Maxam–Gilbert chemical procedure (23) and by the dideoxynucleotide chain termination method (24) after subcloning in phage M13 vectors (25).

Cell Culture and Transfections. DHFR$^-$ Chinese hamster ovary (CHO) K1 DUX-B11 (12), wild-type CHO-K1 cells (12), and mouse Ltk$^-$ cells (26) were propagated as described. Transfections were performed by using the calcium phosphate coprecipitation method (27) as described (16), using a 20% (vol/vol) glycerol shock 3 hr after addition of DNA (28). Medium was replenished after the shock and the cells were allowed to grow for 2 more days in nonselective medium. Cells were then passaged into selective medium [F-12 medium lacking hypoxanthine, glycine, and thymidine (HGT)]. Medium was changed every 3–4 days; DHFR$^+$ clones were counted 10–14 days after selection by staining with crystal violet. Colonies from duplicate plates were subcloned with glass cylinder cloning rings (Bellco Glass). Mtx (Sigma) was added as appropriate when the cells were transferred to selective medium. Growth in Mtx was assayed by plating $0.5–1.0 \times 10^5$ cells in 60-mm dishes and adding selective medium. Cells were removed by trypsinization 72 hr later and counted.

RESULTS

Cloning of 3T6-R400 mRNA Sequences. To obtain cDNA clones specifying an altered DHFR we prepared a cDNA library from mRNA extracted from 3T6-R400 cells, which express a DHFR exhibiting a binding affinity for Mtx 1/270th of that of the wild-type enzyme (9). The cDNA library was constructed by using standard techniques (19) from oligo(dC)-tailed, oligo(dT)-primed cDNA prepared from poly(A)$^+$ cytoplasmic

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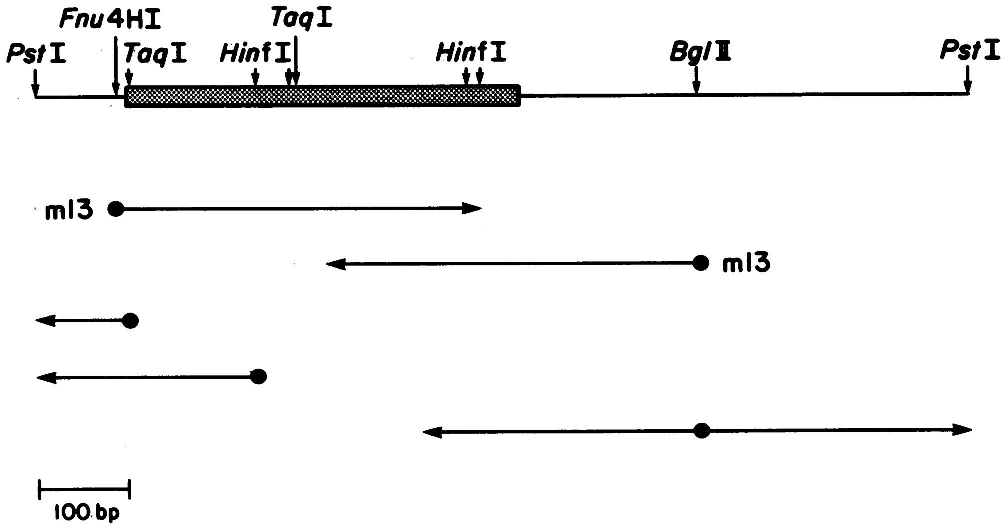
Abbreviations: DHFR, dihydrofolate reductase; Mtx, methotrexate; CHO, Chinese hamster ovary; bp, base pair(s); HGT, hypoxanthine/glycine/thymidine.

RNA. Approximately 2,500 colonies containing plasmids with inserts longer than 700 base pairs (bp) were grown on nitrocellulose filters and screened for the presence of DHFR-encoding sequences by *in situ* colony hybridization (20) with a ^{32}P-labeled nick-translated probe prepared from a wild-type murine DHFR cDNA [plasmid pDHFR-11 (11)]. Twelve colonies that hybridized strongly to the probe were isolated and plasmid DNA was prepared for further analysis (21). The 12 cDNA plasmids were digested with *Taq* I and electrophoresed on polyacrylamide gels; a 190-bp fragment characteristic of a full-length cDNA clone [based on the wild-type sequence (11)] was observed in 5 of the plasmids. One such clone (pR400-12) was chosen for further analysis.

DNA Sequence Analysis. The sequence of the entire cDNA insert of plasmid pR400-12 was determined by using a combination of the Maxam–Gilbert (23) and dideoxy chain termi-

a



b

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             A                                                 G                                       Fnu4HI
GGGGCGGGGCCTTAGCTGCGCAAGTGGTACACAGCTCAGGGCTGCGATTTCGCGCCAAACTTCACGGCAATCCTAGCGTGAAGGCTGGTAGGATTTTATCCCCGCTGCCATC   ATG

                                                                                   leu
    TaqI                           10                                      20       T                                30
val arg pro leu asn cys ile val ala val ser gln asn met gly ile gly lys asn gly asp arg pro trp pro pro leu arg asn glu
GTT CGA CCA TTG AAC TGC ATC GTC GCC GTG TCC CAA AAT ATG GGG ATT GGC AAG AAC GGA GAC CGA CCC TGG CCT CCG CTC AGG AAC GAG

                                   40                          HinfI       50                                        60
phe lys tyr phe gln arg met thr thr thr ser ser val glu gly lys gln asn leu val ile met gly arg lys thr trp phe ser ile
TTC AAG TAC TTC CAA AGA ATG ACC ACA ACC TCT TCA GTG GAA GGT AAA CAG AAT CTG GTG ATT ATG GGT AGG AAA ACC TGG TTC TCC ATT

              TaqI                 70                                      80                                        90
pro glu lys asn arg pro leu lys asp arg ile asn ile val leu ser arg glu leu lys glu pro pro arg gly ala his phe leu ala
CCT GAG AAG AAT CGA CCT TTA AAG GAC AGA ATT AAT ATA GTT CTC AGT AGA GAA CTC AAA GAA CCA CCA CGA GGA GCT CAT TTT CTT GCC

                                   100                                     110                                       120
lys ser leu asp asp ala leu arg leu ile glu gln pro glu leu ala ser lys val asp met val trp ile val gly gly ser ser val
AAA AGT TTG GAT GAT GCC TTA AGA CTT ATT GAA CAA CCG GAA TTG GCA AGT AAA GTA GAC ATG GTT TGG ATA GTC GGA GGC AGT TCT GTT

                                   130                                     140                                       150
tyr gln glu ala met asn gln pro gly his leu arg leu phe val thr arg ile met gln glu phe glu ser asp thr phe phe pro glu
TAC CAG GAA GCC ATG AAT CAA CCA GGC CAC CTC AGA CTC TTT GTG ACA AGG ATC ATG CAG GAA TTT GAA AGT GAC ACG TTT TTC CCA GAA

                                   160                                     170                                       180
ile asp leu gly lys tyr lys leu leu pro glu tyr pro gly val leu ser glu val gln glu glu lys gly ile lys tyr lys phe glu
ATT GAT TTG GGG AAA TAT AAA CTT CTC CCA GAA TAC CCA GGC GTC CTC TCT GAG GTC CAG GAG GAA AAA GGC ATC AAG TAT AAG TTT GAA

                    186
val tyr glu lys lys asp OC                                                                                      BglII
GTC TAC GAG AAG AAA GAC TAA CAGGAAGATGCTTTCAAGTTCTCTGCTCCCCTCCTAAAGCTATGCATTTTTATAAGACCATGGGACTTTTGCTGGCTTTAGATCTATGAGTAA

                               G   CC  CCC    C C                                                                (T)
TTATTTCTTTAGGGAGGGGTAGTTGGAAGAATTGTTTGTTTTGTGATCTTGGGGATGGAACCTAAGACCCAGTGCGTGCTGAGCAAATGCTATACTGCTGAGCCACCCCAACCCTAGCCC

                               T  C   CT                      T C-   TT      TC    -    -       (T)          (GA)
CTATATAATTCTAAACAATATGTTGTCATTTCCCAGTAATCTAACAAGGTATAGTAAAAGTGCCTTAAGAAATGTCACTTGCTATAAAGGTCTCAGTGCCCCTCCCATGAGACCTCAAGT

CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
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FIG. 1. Nucleotide sequence of 3T6-R400 DHFR cDNA. (*a*) Strategy used to determine the nucleotide sequence of the 3T6-R400 cDNA. A 660-bp coding fragment resulting from digestion with *Fnu*4HI and *Bgl* II was introduced into phage M13mp8 and mp9 vectors and its sequence was determined by the dideoxynucleotide chain termination method (24). The sequence of the 1,014-bp cDNA insert was also determined by the chemical modification method of Maxam and Gilbert (23) from the *Taq* I, *Hin*fI, and *Bgl* II sites. (*b*) Complete nucleotide sequence of the DHFR cDNA isolated from 3T6-R400 cells, along with the inferred protein sequence. The stippled bar in *a* represents the inferred DHFR-encoding sequence. The nucleotides that differ from the wild-type DHFR sequence obtained from mouse S-180 cells (11, 29) are indicated as follows: the nucleotides from the wild-type cDNA are shown above, deletions are marked with hyphens, and insertions are shown in parentheses. The wild-type sequence reflects a silent T-to-C change from the published sequence (11) in the third position of the histidine codon at position 130 to conform with our sequencing results. Amino acids are numbered according to Stone *et al.* (30). OC, ocher termination.

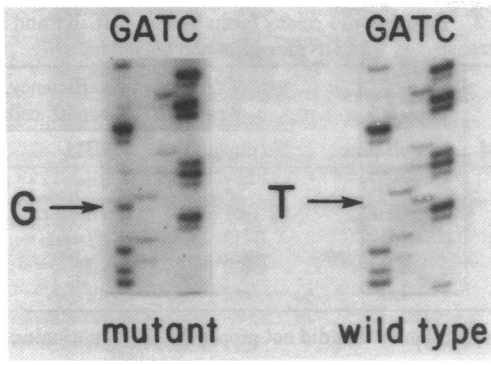

FIG. 2. Comparison of mutant and wild-type DHFR cDNA sequence autoradiographs. Plasmids pDHFR-11 (11) and pR400-12 were digested with *Fnu*4HI and *Bgl* II and the sequence of the fragment spanning the DHFR coding region was determined as described in Fig. 1. The region of the sequencing gel illustrating the T-to-G nucleotide substitution at position +68 is shown.

nation methods (24). Fig. 1*a* illustrates the strategy employed to determine the sequence. The complete sequence of the cDNA insert is shown in Fig. 1*b* and is compared to the published sequence of the cDNA encoding the wild-type enzyme (11, 29, 31). It is evident that few nucleotide differences distinguish the 3T6-R400 cDNA from the wild-type cDNA sequence; the majority of these occur in the 3′ nontranslated region and generally reflect single base substitutions, insertions, and deletions. Significantly, only a single nucleotide difference exists between the wild type and mutant cDNAs in the coding region.

To directly confirm this change, the sequences of the DHFR cDNA derived from 3T6-R400 cells and the wild-type DHFR cDNA clone pDHFR-11 were analyzed in parallel. A portion of the sequencing autoradiogram that demonstrates this nucleotide substitution is presented in Fig. 2, revealing that where a thymine residue is present at position +68 in the wild-type DHFR cDNA (11), a guanine residue is present in the corresponding cDNA from cells expressing the altered enzyme.

Genetic Mapping of the Functionally Altered Region of the DHFR cDNA. The nucleotide sequence of the DHFR cDNA clone pR400-12 was consistent with the interpretation that it encoded an altered DHFR (see *Discussion*); it was possible, however, that the clone represented an allelic or polymorphic variant of the wild-type gene. To confirm that the cDNA clone pR400-12 isolated from 3T6-R400 cells encoded an altered enzyme we sought to express the gene product in heterologous cells and demonstrate its resistance to Mtx. Accordingly, we constructed fusions between the simian virus 40 early promoter and the cDNA clone pR400-12, using the plasmid DNA vector pCV342E, which permits the expression of inserted heterologous genes after introduction of these vectors into recipient cells (32). To unequivocally establish that the nucleotide substitution at position +68 of the DHFR cDNA was responsible for the altered properties of the enzyme, we prepared plasmid expression vectors based on either the wild-type or mutant gene that were identical except for the substitution at this single position. This was accomplished by digesting both pDHFR-11 and pR400-12 with restriction endonucleases *Fnu*4HI and *Bgl* II, isolating the respective 660-bp fragments that encompassed the encoding region, and separately introducing them into the plasmid expression vector to generate plasmids pFD11 (based on wild-type DHFR) and pFR400 (based on 3T6-R400 DHFR) (Fig. 3).

To correlate the altered nucleotide with a functional difference in the properties of the enzyme, we separately transfected cultures of DHFR$^-$ cells with either the pFD11 or the pFR400 plasmid constructions and selected colonies in the absence of HGT. Colonies arising from each transfection were subcloned, grown, and then plated in the presence of various levels of Mtx.

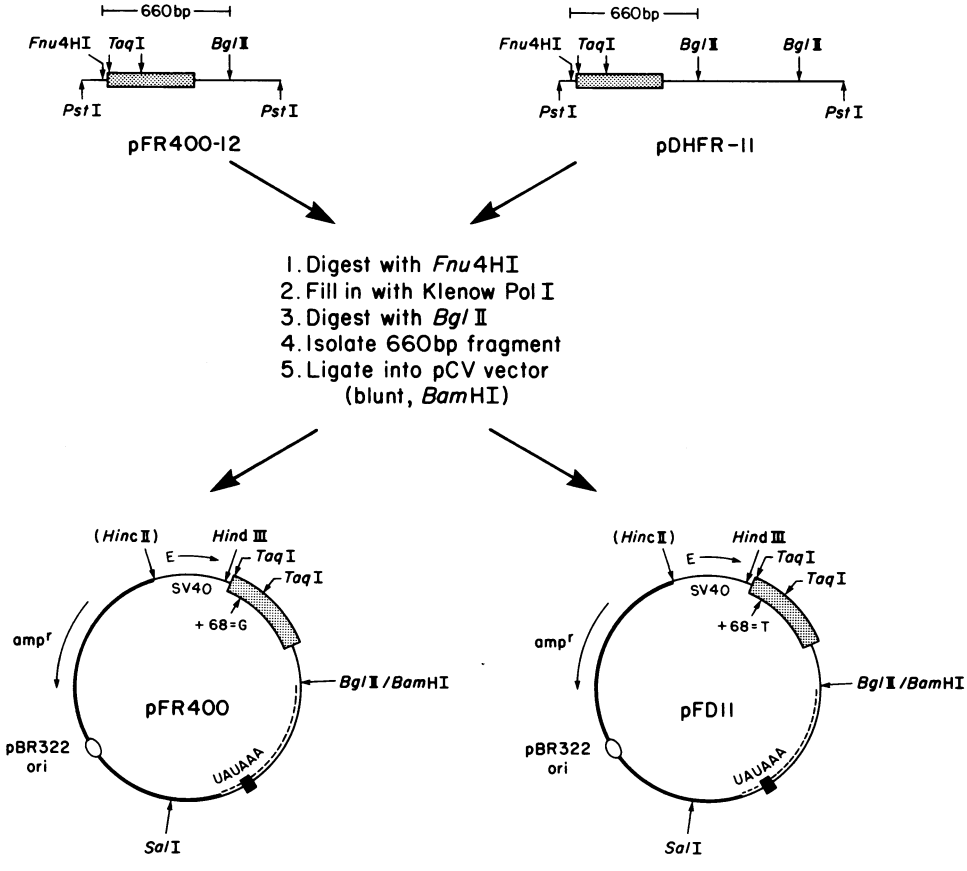

FIG. 3. Construction of DHFR expression plasmids. The DHFR cDNA plasmids pDHFR-11 and pR400-12 were digested with *Fnu*4HI and the single-stranded fragment ends were filled in by *E. coli* DNA polymerase I large fragment. After cleavage with *Bgl* II, the 660-bp fragment was isolated by electroelution from polyacrylamide gels and inserted into the expression plasmid pCV342E. This plasmid is a derivative of p342E [containing the early promoter of simian virus 40 (SV40), and the 548-bp *Bam*HI/*Bgl* II fragment containing the polyadenylylation site of the surface antigen gene from hepatitis B virus] (32). Thick line, pBR322-derived sequences (ori, origin; amp^r, ampicillin resistance); parallel broken and solid lines, hepatitis B virus-derived sequences; solid line, SV40 or DHFR sequences (E, direction of transcription of SV40 early mRNA); stippled bar, DHFR protein-encoding sequence.

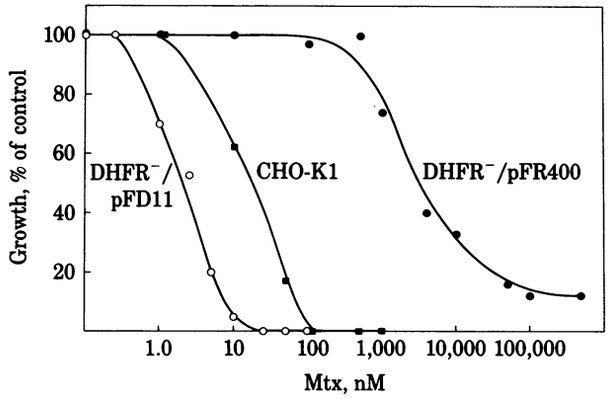

FIG. 4. Inhibition of growth of HGT-independent cells by Mtx. DHFR⁻ cells were separately transfected with pFD11 and pFR400 DNA, and DHFR⁺ colonies were selected in medium lacking HGT. After subcloning, individual colonies were passaged into larger culture dishes. The cells were treated with trypsin and plated into 60-mm dishes at approximately 1×10^5 cells per dish. Mtx was added to the growth medium at the concentrations indicated. The cells were incubated for 3 days and counted. The data from a typical subclone after transfection with pFD11 and pFR400 DNA are presented, along with the Mtx sensitivity of the CHO wild-type parent. The data are expressed as the percentage of cells growing at the indicated level of Mtx relative to growth in the absence of Mtx.

Fig. 4 illustrates that the growth of a representative colony arising after transfection with pFD11 DNA was inhibited 50% by approximately 2 nM Mtx, whereas a representative colony arising after transfection with pFR400 DNA was not similarly inhibited until concentrations of Mtx in excess of 2,500 nM were employed. The wild-type parent CHO-K1 cells exhibited a resistance to Mtx intermediate in value to the transfected subclones. To confirm that this result was not peculiar to the limited number of cell clones analyzed, we determined the Mtx resistance of mass cultures transfected with either pFD11 or pFR400. Cells were separately transfected with these plasmids and various levels of Mtx were added to the selective medium (−HGT) at the initial time of selection. As illustrated in Table 1, only cultures transfected with plasmid pFR400 produced colonies in concentrations of Mtx higher than 25 nM. Colonies were observed even at Mtx concentrations as high as 100 μM, although at a reduced number. It was thus apparent that DHFR⁺ cells derived from transfections utilizing plasmid constructions containing DHFR cDNA from 3T6-R400 cells were able to propagate in much higher levels of Mtx than DHFR⁺ cells derived from transfections with similar plasmids containing DHFR

Table 1. Frequency of colony formation by DHFR⁻ cells transfected with mutant and wild-type DHFR expression plasmids

	Transfection efficiency, colonies/μg per 10^6 cells		
Mtx, nM	No plasmid	pFD11	pFR400
0	<0.3	$>10^3$	330
1	—	330	310
5	<0.3	260	230
10	—	140	240
25	—	30	250
50	<0.3	<0.3	240
100	<0.3	<0.3	200
250	—	<0.3	170
500	—	<0.3	85
1,000	—	<0.3	75
10,000	—	—	45
100,000	—	—	10

Table 2. Frequency of colony formation by CHO-K1 and Ltk⁻ cells transfected with DHFR expression plasmids

		Transfection efficiency, colonies/μg per 10^6 cells		
Mtx, nM	Cell line	No plasmid	pFD11	pFR400
250	CHO	<1	5*	270
500	CHO	<1	5*	90
100	Ltk⁻	<2	<2	80
250	Ltk⁻	<2	<2	44
500	Ltk⁻	<2	<2	14

* Nonviable colonies that did not propagate upon subcloning.

cDNA from wild-type cells, thereby localizing the genetic basis for the altered gene function to nucleotide +68.

Evidence That the Altered DHFR cDNA Functions in Wild-Type Cells. The above data clearly indicate that, after transfection, pFR400 DNA can confer a high level of Mtx resistance to DHFR⁻ CHO cells. In view of the fact that Mtx inhibits the growth of many wild-type (DHFR⁺) cell lines at concentrations greater than 250 nM (16, 18, 33), we tested whether we could employ the altered cDNA as a dominant selectable marker in cells expressing normal levels of unaltered enzyme. We utilized as recipients both the wild-type progenitor of the DHFR⁻ cell line (CHO-K1) and mouse Ltk⁻ cells, the latter being frequently used in gene transfer experiments utilizing the thymidine kinase gene (25, 34). Cells were transfected with either pFD11 or pFR400 DNA, and colonies able to grow in the presence of various concentrations of Mtx were selected. As expected (16, 18, 33), no viable colonies appeared in control cultures or in cultures transfected with pFD11 DNA at any level of Mtx tested (Table 2). However, colonies arose with a frequency of $4–25 \times 10^{-5}$ in cultures transfected with pFR400 DNA after selection with 250–500 nM Mtx. Such colonies survived repeated subclonings and could be propagated in the continued presence of high levels of Mtx (unpublished data), indicating that introduction and expression of the altered gene had occurred.

DISCUSSION

We have described the isolation, characterization, and utility of a cDNA clone (pR400-12) encoding a DHFR that exhibits a reduced affinity for the folate analog Mtx. This clone was obtained from mRNA extracted from 3T6-R400 cells, previously demonstrated to express high levels of a DHFR displaying a Mtx binding affinity 1/270th that of the wild type (9). DNA sequence analysis revealed that only a single nucleotide substitution distinguished the wild-type cDNA from the altered cDNA in the coding region, corresponding to a T-to-G transversion 68 nucleotides downstream of the translational initiation codon. This change, in the second nucleotide of the codon specifying amino acid 22 of the enzyme, would result in the incorporation of an arginine residue at this position of the protein, rather than a leucine residue as found in the wild-type protein. It is unclear how this substitution alters the enzyme's affinity for Mtx, but the nature and location of this change is provocative. First, Leu-22 has been found from x-ray crystallographic data to be one of several residues that form a small binding pocket for, and is in hydrophobic contact with, the triazine ring of 2,4-diamino-5,6-dihydro-6,6-dimethyl-5-(4'-methoxyphenol)-s-triazine, a chemical and functional analogue of Mtx (35). The replacement of the hydrophobic side chain of a leucine residue with that bearing the positively charged guanidinium group of an arginine residue could perturb this interaction. Second, an identical leucine-to-arginine substitution at position 28 (30, 36) has been identified in a bacterial DHFR exhibiting a reduced affinity for trimethoprim (an analog of Mtx) (36). Finally, it provides an explanation of data

obtained by Haber *et al.* who demonstrated that the altered enzyme exhibits a significant basic shift in its migration on a pH gradient relative to the wild-type enzyme (9).

To directly implicate this particular nucleotide substitution in the alteration of gene function, we supplemented these results with an independent series of experiments designed to genetically localize the region of the cDNA clone that specified this functional alteration. Constructing DHFR expression vectors that differed at only the single nucleotide representing the alteration occurring at position +68 of the DHFR cDNA, we found that although both vectors were capable of converting $DHFR^-$ cells to a $DHFR^+$ phenotype (Table 1), only the transfection of the 3T6-R400-based vector resulted in cell growth when assayed in the presence of Mtx (Table 1, Fig. 4). No significant decrease in the frequency of colony formation was detected until concentrations of Mtx in excess of 1,000 nM were employed, well beyond the concentration required to inhibit the enzymatic activity of the wild-type gene product. The number of copies of the DHFR gene we observe in our initial transformants is low [between one and five (unpublished data)], consistent with previous studies suggesting that a few copies of a mutant DHFR gene are capable of rendering cells resistant to high concentrations (>1,000 nM) of Mtx (6, 18).

In view of the ability of pFR400 DNA to convert $DHFR^-$ cells to a $DHFR^+$ phenotype capable of growing in Mtx concentrations sufficient to suppress the endogenous enzyme activity of many wild-type cells, we have explored the use of the mutant cDNA as a dominant selectable marker in cells producing normal levels of an unaltered DHFR. After transfection of both wild-type CHO cells and mouse Ltk^- cells with pFR400 DNA, viable colonies resistant to high levels of Mtx arise with a frequency of $4–25 \times 10^{-5}$. This frequency is at least 100-fold above that observed with the wild-type DHFR vector and is comparably above the background rate of appearance of resistant colonies that result from amplification of the endogenous DHFR genes (16, 32). Because this selection protocol is sufficient to inactivate the endogenous enzyme activity of a wide variety of other cells as well (unpublished data), the utility of this approach may be limited only by the ability of cells to acquire and stably incorporate foreign DNA. This would provide a distinct advantage over other selectable markers such as thymidine kinase (26, 34), adenine phosphoribosyltransferase (37), and hypoxanthine phosphoribosyltransferase (38), all of which require recipient cells of unusual phenotypes.

The ability to transfer specific genes into cultured cells affords a unique opportunity to study the function of exogenous genes in a new environment. Other dominant selectable markers, such as provided by the bacterial genes encoding xanthine–guanine phosphoribosyltransferase (39) and aminoglycoside 3′-phosphotransferase (neomycin resistance) (40, 41) have been reported that have facilitated these studies. The use of an altered DHFR cDNA as the basis of selection should allow the introduction (16, 37) and expression (17, 42) of coselected genes as well. In addition, because cotransfected sequences can be amplified by using DHFR vectors (13–15, 42), the altered cDNA may provide a means by which the effects of gene dosage on foreign gene function can be assessed in a wide variety of cell types.

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