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Title Page

SPECIES SPECIFIC DIFFERENCES IN TRANSLATIONAL REGULATION OF DIHYDROFOLATE REDUCTASE

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Running Title Page

Running Title: Translational regulation of dihydrofolate reductase

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Abbreviations: DHFR, dihydrofolate reductase; MTX, methotrexate; TMTX, trimetrexate; RTX, raltitrexed; PTX, pemetrexed; NADPH, reduced nicotinamide adenine dinucleotide phosphate, EGFP, enhanced green fluorescent protein; CHO, Chinese hamster ovary; SDS, Sodium dodecyl sulfate; PMSF, phenyl methyl sulfonyl fluoride; PBS, phosphate buffered saline.

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Abstract

We have observed that rodent cell lines (mouse, hamster) contain approximately 10 times the levels of dihydrofolate reductase as human cell lines, yet the sensitivity to methotrexate (ED50), the folate antagonist that targets this enzyme, is similar. Our previous studies showed that dihydrofolate reductase protein levels increased after methotrexate exposure; and we proposed that this increase was due to the relief of feedback inhibition of translation as a consequence of methotrexate binding to dihydrofolate reductase. In the current report we show that unlike what was observed in human cells, DHFR levels do not increase in hamster cells following methotrexate exposure. We provide evidence to show that although there are differences in the putative mRNA structure between hamster and human mRNA in the dihydrofolate reductase binding region previously identified, " hamsterization" of this region in human dihydrofolate reductase mRNA did not change the level of the enzyme or its induction by methotrexate. Further experiments showed that human dihydrofolate reductase is a promiscuous enzyme, and that it is the difference between the hamster and human dihydrofolate reductase protein, rather than the DHFR mRNA, that determines the response to methotrexate exposure. We also present evidence to suggest that the translational upregulation of dihydrofolate reductase by MTX in tumor cells is an adaptive mechanism that decreases sensitivity to this drug.

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Introduction

Dihydrofolate reductase (DHFR, 5, 6, 7, 8-tetrahydrofolate: NADP+ oxidoreductase, EC 1.51.3) catalyzes the reduction of dihydrofolate to tetrahydrofolate, utilizing NADPH as a cofactor. Tetrahydrofolate and its one–carbon adducts are essential cofactors in the synthesis of thymidylate, purines and some amino acids (Blakley, 1984). Inhibition of DHFR results in a depletion of the reduced folate pools, inhibition of DNA synthesis, and cell death. MTX, a tight binding inhibitor of DHFR, is used for the treatment of acute lymphocytic leukemia, non-Hodgkins lymphoma, osteosarcoma, choriocarcinoma, breast, and head and neck cancer, as well as a variety of other nonmalignant diseases (Bertino, 1997; Chu, 1996).

Within days following exposure to MTX, DHFR activity increases approximately six fold in blast cells of leukemic patients (Bertino et al., 1962). Following this initial report, several studies of human cells *in vivo* and *in vitro* showed that DHFR protein levels rapidly increase, or are "induced", in response to MTX treatment (Bertino et al., 1962; Bertino et al., 1963; Hillcoat and Bertino, 1969; Hillcoat et al., 1967a). This rapid adaptive response differs from the increase in DHFR due to gene amplification in cells after long term MTX selection (Alt et al., 1978). The rapid increase in DHFR protein levels seen upon exposure to MTX was unaffected by the transcriptional inhibitor actinomycin D, but was blocked by the translational inhibitor cycloheximide (Hillcoat et al., 1967a; Hillcoat et al., 1967b) ruling out an increase in transcription as the mechanism. As the increase in DHFR is associated with MTX bound to the protein, it was suggested that MTX protected DHFR from degradation (Bertino et al., 1970). Later studies showed that the half-life of DHFR protein was unaltered in the presence of MTX (Cowan et al., 1986; Domin et al., 1982), indicating that protection from degradation was not the explanation for the increase.

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The first suggestion that translational regulation may play a role in DHFR induction came from studies showing that DHFR mRNA levels remain the same in the presence or absence of MTX (Bastow et al., 1984; Cowan et al., 1986). Based on studies demonstrating an interaction between DHFR protein and its cognate mRNA, a model was proposed to account for increases in DHFR protein levels in response to MTX exposure, namely that the binding of human DHFR protein to its cognate mRNA results in decreased translation, and that addition of MTX disrupts this auto-regulation (Chu et al., 1993; Ercikan et al., 1993). Using an *in vitro* translation assay our laboratory (Ercikan et al., 1993) and Chu et al (Chu et al., 1993) demonstrated that addition of exogenous DHFR protein in a rabbit reticulocyte system inhibited translation of DHFR mRNA. Utilizing both RNA gel shift assays and UV cross-linking competition studies, the DHFR binding site was localized to a 100 base pair region in the coding region between nucleotides 380-480 (Ercikan-Abali et al., 1997). Computer modeling of mRNA folding in this region revealed two possible stem-loop structures suitable for DHFR binding between nucleotides 407-470. We postulated that DHFR binds to its own mRNA within this coding region resulting in repression of translation. The addition of inhibitor, substrate, or co-factor alters DHFR conformation, resulting in disruption of this RNA-protein complex and subsequent resumption of translation (Ercikan-Abali et al., 1997). Tai et al. (Tai et al., 2004a) using gel shift and nitrocellulose filter binding assays subsequently localized the binding site to an 82-nucleotide sequence corresponding to nucleotides 401-482, consistent with our initial findings.

In this report we show that DHFR induction by MTX differs between human and rodent cells. While there is an increase in DHFR protein levels in human cell lines in response to MTX treatment, there is no change in DHFR protein levels in either mouse or hamster cells upon MTX treatment. We attribute this difference to differences in the protein structure of DHFR between

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rodents and human rather than differences in mRNA structure. Importantly, we demonstrate that translational up regulation of human DHFR decreases sensitivity of cells to this drug. Finally, we propose that DHFR protein may be a moonlighting enzyme acting as an RNA-binding protein.

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Materials and Methods

Materials. CMRL 1066 media, RPMI 1640, fetal bovine serum (FBS), dialyzed FBS (dFBS), G418 sulfate (Geneticin), penicillin, streptomycin, and trypsin were purchased from Gibco BRL (Gaithersburg, MD). The pEGFPN3 vector was from Clontech (Palo Alto, CA) and the pcDNA3 vector was from Invitrogen (Carlsbad, CA). The QuikChange Site-Directed Mutagenesis Kit was purchased from Stratagene (La Jolla, CA). Oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) or Biosource International (Camarillo, CA). ECL reagents were supplied by Amersham Pharmacia Biotech (Piscataway, NJ). The antibody to green fluorescent protein was from Roche Molecular Biochemicals (Indianapolis, IN). The antibodies used as loading control, anti β-tubulin antibody and anti GAPDH were obtained by Sigma (St Louis, MO) and Chemicon (Temecula, CA). The rabbit polyclonal antibody to human DHFR was custom produced by Research Genetics/Invitrogen (Carlsbad, CA) and the monoclonal antibody to DHFR was from BD Biosciences (San Diego, CA). Secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Enyzmes were from NEB (Beverly, MA). Aprotinin, PMSF, and sodium orthovanadate were obtained from Sigma (St Louis MO).

Tissue culture and transfections. The Chinese hamster ovary cell line DG44, deleted for both alleles of DHFR, was a kind gift of Dr. L. Chasin (Columbia University, New York, NY). Parental DG44 cells were maintained in CMRL 1066 media supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. Human (C85), hamster (CHO) and mouse (3T6) cells were grown in RPMI 1640 supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. All transfections were performed using DOTAP transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN). Parental cells were seeded at a density of 2.5 X 10^4

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cells/10-cm dish. When cells reached 10-15% confluency they were transfected with 10 μg of plasmid DNA plus 70 μl of DOTAP reagent as per manufacturer's protocol. Fresh media was supplied 24 h post transfection. Selection in 750 μg/ml of G418 sulfate for plasmids containing the neomycin resistance gene began 48 h later. A parallel mock transfection, which contained everything but plasmid DNA, was performed concurrently with each transfection. Approximately 14 days later, individual single clones could be isolated by ring cylinders and expanded into stable resistant cell lines. At the same time pooled batches were harvested and expanded into stable lines. Neither mock-transfected (only DOTAP reagent) nor parental cells survived the G418 selection.

Preparation of Cell lysates and Western Blotting. *Preparation of cell lysates.* Cells were harvested by 2 min trypsinization and collected by centrifugation (800 x g) for 5 min. Cell pellets were washed twice in 1X PBS, and resuspended in ice-cold RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing 100 μg/ml PMSF, 30 μl/ml Aprotinin (Sigma, St Louis, MO), and 1 mM sodium orthovanadate. After 30 min incubation on ice, cells were sonicated 3 times at 10 second bursts, using a VibraCell Sonicator (30% output, Vibracell Danbury CT). Following sonication 100μg/ml PMSF was added and cells were incubated on ice for 30minutes. The lysate was centrifuged at 15,000 X g for 20 min at 4^{o}C. The supernatant was collected and used immediately or stored at –70^{o}C.

Western Blotting. Protein samples were resolved on 10% SDS-PAGE gels. Transfer was performed by standard electroblotting protocols onto nitrocellulose membranes. Equal loading was determined by using Ponceau S and anti-β tubulin antibody. Immunoblots were incubated with anti-EGFP, anti-DHFR, anti-GAPDH or anti-β-tubulin antibodies at room

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temperature for 1 h using PBS-T buffer containing 0.1% Tween 20 and 5% nonfat dry milk powder. Blots were washed at room temperature and incubated for 1hour with goat anti-mouse IgG as per standard protocols. Chemiluminescence was used to visualize bands by following manufacturer's instruction (ECL kit, Amersham Pharmacia Biotech, Piscataway, NJ). Blots were stripped and reprobed according to protocol included with the ECL kit.

Site-directed mutagenesis. The DHFR-EGFP fusion vector generated as described above served as the template in subsequent Quikchange^{TM} site-directed mutagenesis reactions to create the majority of variants used in this study. Mutagenesis was performed as per manufacturer's protocol included in the QuikChange^{TM} kit (Stratagene, La Jolla, CA). Briefly, each 50 µl reaction contained 10 ng of double-stranded DNA template (pEGPN3-DHFR), 125 ng of sense mutagenic primer, 125 ng of antisense mutagenic primer, 1 µl of dNTP mix, and 5 µl of 10X manufacturer's reaction buffer to which 1 µl of *PfuTurbo* DNA polymerase (2.5 U/µl) was added. Thermocycling was performed in the GeneAmp 9600 (Perkin-Elmer/Cetus, Norwalk, CT) thermocycler according to the following parameters: T_1, 95^0C, 30 sec, (1 cycle) followed by T_1, 95^0C, 30 sec; T_2, 55^0C, 1 min; T_3, 68^0C 12 min. (16 cycles). Following cycling, the reaction was cooled on ice. To digest the parental (nonmutated) template strain 1 µl of *Dpn* I restriction enzyme (10 U/µl) was incubated with the reaction for 1 h at 37^0C. One microliter of the digested reaction mix containing the mutated plasmid was transformed into XL-1 Blue Supercompetent cells as per standard protocols. Clones were isolated, and each variant was sequenced by UMDNJ-RWJMS DNA Core Facility in Piscataway, NJ to confirm the presence of the specific mutation. The sequences of the primers used for mutagenesis are shown in Table 1.

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Cytotoxicity Assay. Cytotoxicity to antifolates was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay according to CellTiter 96 Aqueous One Solution protocol (Promega, Madison, WI). Four thousand cells were seeded in 96 well plates in RPMI 1640 media supplemented with 10% dialyzed FBS, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. The wells in the first row of the plate were used as blank and contained only media but no cells. The wells in the second row of the plate were used as control and contained transfected DG44 cells in the media but no drug. Following incubation of the cells with MTX for 96 h, MTS assay was performed. The absorbance at 490 nm was measured using a plate reader. The percentile cell survival was determined by Softmax Pro software and sigmoidal dose-response curve fit of the graphs drawn by GraphPad Prism 4 software was used to determine EC_{50} values. All experimental points were set up in replicate 8 wells, and all experiments were repeated 7 times.

In-vitro transcription and translation assay. DHFR-EGFP constructs were generated with PCR using a primer at the 5'end that the T7 promoter. After phenol chloroform extraction and precipitation of the PCR products using 90% ethanol, in vitro transcription was performed using T7 RNA polymerase according to the manufacturer's instruction (Promega, Madison, WI). The integrity and the size of the RNA were verified using gel electrophoresis, and the concentration and the purity of RNA samples were determined by reading the absorbance at λ=260 nm and taking the ratio of λ=260 nm to λ=280 nm respectively.

The nuclease treated rabbit reticulocyte lysate system (Promega, Madison, WI) was used to translate the in-vitro transcribed RNA samples. 7.5 pmol of each transcript, 23 μL rabbit reticulocyte lysate, 40 U rRNAsin inhibitor, 1mM amino acid mixture which lacked methionine, 5 μL [^{35}S]-methionine and an aliquot of nuclease free water to a final volume of 55 μL were

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added and the reaction mixture was incubated for 30 min at 37°C. The cell free translation products were run on a 12% SDS-PAGE gel and autoradiographed for visualization.

Flow cytometry. Expression of EGFP after 1 μM MTX treatment for 48 h was determined using flow cytometry. After trypsinization of the DHFR-EGFP transfected DG44 cells, the cells were washed and suspended in media. Cytometric analysis was performed using FACSCalibur (BD Biosciences, San Jose, CA) equipped with CellQuest software. EGFP fluorescence was detected using a 525/50 band pass filter. The induction efficiency of variants of DHFR is expressed as a percentage (the number of cells expressing EGFP over the total number of cells).

Measurement of DHFR activity. The specific activity of DHFR from lysates of hamster and the variants of human DHFR-EGFP was determined as described by Ercikan-Abali (1996).

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Results

Rodent DHFR levels do not increase in the presence of antifolates. DHFR protein levels are up regulated in response to MTX in all human cell lines tested (Bastow et al., 1984; Bertino et al., 1962; Chu et al., 1993; Cowan et al., 1986; Ercikan-Abali et al., 1997). In contrast, there was no detectable increase in DHFR levels in hamster (CHO) and mouse (3T6) cell lines following exposure to 100 nM MTX for 24 or 48 h, while DHFR protein levels were increased in a human colon cancer cell line, C85 (Figure 1a).

We considered several possible explanations for the difference in response of hamster versus human cells following exposure to MTX. Although DHFR levels are regulated at both the transcriptional and translational level, we had previously shown that steady state levels of DHFR mRNA are not altered by MTX in either hamster or human cells, suggesting that differential regulation occurs downstream of RNA synthesis/degradation, perhaps at the level of translation. Translation of DHFR mRNA is controlled by sequences within the coding region and the 3'UTR (Mishra et al., 2007). In order to determine whether the differential response to MTX was regulated by differences in the coding region, chimeric constructs of either human or hamster DHFR coding region fused to EGFP and under the control of the constitutive CMV promoter were constructed. Using a functional rapid fluorescence assay, we tested the relative increase in EGFP fluorescence of the two DHFR-EGFP fusion proteins; EGFP alone was used as a negative control to set the baseline fluorescence. As shown in Figure 1b, there was a specific increase (>2-fold) in the human DHFR-EGFP fusion protein levels in response to MTX treatment. However, when hamster DHFR-EGFP was transfected into the DG44 cell line, there was no detectable increase in fusion protein levels in response to MTX treatment (Figure 1b and c). Moreover, the three mutants of human DHFR (S118A, L22R and E30A) that are not

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upregulated by MTX and other antifolates (Skacel, 2004) were also not increased in response to MTX (Figure 1b). We had previously reported that a mutant DHFR, with its sole cysteine residue changed to either serine or glycine, increased after treatment with MTX and trimetrexate (an antifolate with similar affinity towards DHFR as MTX), but not after treatment with raltitrexed (with thymidylate synthase as its major target (K_i = 1 nM), and DHFR as a secondary target (K_i = 92 nM) (29) or pemetrexed (pemetrexed-polyglutamates potently inhibit both thymidylate synthase (K_i = 1.3 nM) and DHFR (K_i = 7.2 nM) and to a lesser extent, 5-phosphoribosylaminoimidazolecarboxamide transformylase (GARFT) (K_i = 65 nM))(Skacel et al., 2005). We therefore determined whether DHFR from hamster cells would also show differences in upregulation to various antifolates. Hamster DHFR-EGFP transfected cells exposed to MTX, trimetrexate, raltitrexed and pemetrexed did not show an increase in DHFR , similar to what had been observed for the DHFR mutants that lack negative feedback regulation of DHFR translation (Figure 1d).

Lack of upregulation of rodent DHFR by antifolates is due to failure of hamster DHFR protein to bind to its cognate mRNA. The current model for translational regulation of DHFR by its cognate mRNA is that DHFR protein interacts through its NADPH binding site within the coding region of its own mRNA (nucleotides 380-480). We proposed that DHFR exists in at least two conformers, one of which has higher affinity for NADPH and the other for DHFR mRNA. Binding of MTX to the folate binding site of DHFR would result in a change in the conformation of DHFR protein and the release of DHFR mRNA from the DHFR-mRNA complex, hence overcoming translational detainment of the enzyme by its own mRNA.(Skacel et al., 2005) The three mutants of human DHFR identified that have no changes within the putative mRNA binding region and yet are not upregulated by MTX, indicates that the lack of

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upregulation by these mutants may be due to a change in their protein structure rather than s difference in their mRNAs. As both the protein and mRNA sequence of hamster DHFR differs from human DHFR, we investigated the role of each in the divergent regulation of human and rodent DHFR. To accomplish this, we initially compared the cis-acting regulatory elements on hamster DHFR mRNA with those of human wt and non-RNA binding mutant DHFR mRNAs.

Our earlier work and studies by Tai et al (Tai et al., 2004b) demonstrated that the DHFR binding site was localized to a 100 base pair sequence in the coding region between nucleotides 380–480. Analysis of human DHFR mRNA in this region using the mfold program (Zuker and Stiegler, 1981) revealed two potential stem-loop structures which could form stable secondary structures suitable for DHFR protein binding ($\Delta G = -12.3$ kcal/mol). The difference between hamster and human DHFR in the predicted stem-loop structure is only four nucleotides with 89% homology. Although the hamster DHFR RNA also formed two similar stem-loop structures, the stability of these two stem loops was lower than the stability of the human stem loops (ΔG=-6.1 vs ΔG=-12.3 kcal/mol). Therefore, we hypothesized that the difference in RNA folding due to four nucleotides differences between human and hamster within this binding region may be responsible for the lack of translational up-regulation of hamster DHFR by antifolates (Figure 2 a and b). In order to test this hypothesis, we "hamsterized" the human DHFR-EGFP fusion construct within the region that forms the putative two stem-loop structures by changing the four nucleotides in the human DHFR to the hamster nucleotides. The hamsterized human DHFR-EGFP was transfected into the DG44 cell line and clones, as well as batches, were selected by resistance to G418 and stable cell lines were generated. Transfected cells were exposed to l µM of MTX or media without MTX for 24 h and 48 h and the fusion protein levels were detected using an anti-EGFP antibody. As shown in Figure 2 c and d, individual clones and as well as the

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batch of transfected cells with hamsterized human DHFR were induced in response to MTX, indicating that four nucleotide difference between hamster and human DHFR mRNA could not explain the difference in response to MTX between the species.

Earlier studies indicated that the putative DHFR mRNA binding site is localized to a larger stretch of nucleotides (100 bp) than just the nucleotides that formed the stem loop structures (Ercikan-Abali et al., 1997; Tai et al., 2004a). Therefore, we also examined the role of the nucleotides that are not part of the stem and loop structures but still lie within the l00bp-binding region. Figure 3a shows the sequence of the entire putative binding site that includes nucleotides 380-480 within the coding region compared to the hamster DHFR sequence within the same region. There are four-nucleotide differences upstream of the stem and loop structures and one nucleotide difference downstream. We denoted the "hamsterized" part of the binding region as the core binding region and using this construct, we performed additional rounds of site-directed mutagenesis using the hamsterized human DHFR-EGFP as the template, generating three additional chimeric constructs. One construct, a "half-mutated" binding region (BR1) had the core 4 nucleotides as well as the four additional upstream nucleotides "hamsterized". A second construct, "half-mutated" binding region (BR2) had the four core nucleotides hamsterized, as well as the downstream nucleotide that was not conserved between human and hamster (G to C). The third construct, a fully mutated binding region (FMBR) DHFR-EGFP, had all nine nucleotides "hamsterized" within the binding region of human DHFR mRNA. After constructing these vectors, we transfected them into DG44 cells and derived stable clones and cell lines. Treatment of the cells with 1 μM MTX for 24 h and 48 h, resulted in induction of DHFR-EGFP levels in all cell lines (Figure 3b). Thus, the difference in primary sequence within the putative DHFR binding regions of the human and hamster DHFRs did not account for

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the species difference in MTX-induced DHFR increases. Therefore we considered that the difference might lie within the DHFR protein itself, in that the human protein might have characteristics lacking in the hamster DHFR protein that allows interaction with its cognate mRNA.

We began to test this new hypothesis by analyzing a number of human DHFR mutants that alter the amino acid sequence. Tai and colleagues showed that a mutation in nucleotide 419 (U to C) dramatically reduced the binding of wt human DHFR to its cognate mRNA (Tai et al., 2004b). However it was not clear whether the loss of binding between DHFR protein with its cognate mRNA correlated with the loss of MTX-induced translational regulation. Therefore we created the same mutation and tested its ability to be induced by MTX. As shown in Figure 4, DHFR 419 U→C, (DHFR M140T mutant) was still up regulated by MTX. We then examined the human DHFR S118A mutant that has similar characteristics as hamster DHFR, i.e. both are not up regulated by MTX (Figure 5a). DG44 cells that were already transfected with the mutant S118A-EGFP were transfected with a flag-tagged wt DHFR protein expression vector. This allowed us to observe the changes in both human wt and mutant DHFR protein (Figure 5b). Five individual cell lines established from stable transfectants containing both flag-tagged DHFR and the mutant S118A-EGFP demonstrated increased fusion protein levels upon exposure to 1μM MTX for 24 h. The levels of S118A-EGFP fusion protein increased in response to MTX comparable with flag-tagged wt DHFR in the doubly transfected cell lines. Moreover, the basal level of S118A protein levels in doubly transfected cell lines were significantly reduced as compared to the singly transfected cell line indicating the restoration of negative feedback regulation of the human DHFR S118A mutant by expression of wt human DHFR in these cells.

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Hence, introduction of wt human DHFR into the S118A transfected cells re-established the ability of this mutant to be induced by MTX (Figure 5a and b).

In a second experiment, we used a metastatic human colon cancer cell line (C85) in which the endogenous DHFR levels are up regulated by MTX (Figure 1a). These cells were transfected with either wt human or hamster DHFR-EGFP and batches resistant to G418 were expanded. Polyclonal DHFR antibody was used to detect endogenous human DHFR and anti-EGFP antibody was used to detect the fusion protein of wt hamster DHFR-EGFP. When these cells were exposed to 0.1 or 1 μM MTX for 48 h there was a marked increase in hamster DHFR-EGFP protein levels in response to MTX treatment (Figure 5c). Thus, in the presence of human DHFR protein, hamster DHFR protein was now induced by MTX, indicating that human DHFR protein is somewhat promiscuous, i.e. it also binds to DHFR hamster DHFR mRNA and inhibits its synthesis, similar to the results obtained with the human DHFR S118A variant that is not responsive to MTX-induced upregulation (Figure 5a). Therefore wt human DHFR protein by binding to both wt hamster DHFR and the human DHFR S118A variant mRNA regulated the translation of these mRNAs and DHFR levels were increased in the presence of MTX.

Translational regulation of human DHFR by MTX is an intrinsic mechanism of resistance. Cells transfected with the three human DHFR mutants that lacked translational feedback regulation had a higher cellular baseline level of DHFR than cells transfected with wt DHFR (Skacel, 2004), As hamster DHFR baseline levels are also high, we hypothesized that the reason for the increased level of DHFR in hamster cells as compared to human cells was a difference in translation efficiency. Therefore we compared the translational efficiency of hamster and human mutant DHFR mRNAs. When equal concentrations of *in vitro* transcribed DHFRs (human wt, S118A, L22R, E30A and hamster DHFR transcripts) were prepared and

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translated using a rabbit reticulocyte assay, the human mutant and hamster wt DHFR transcripts were translated to a greater extent than wt human DHFR mRNA (Figure 6a). The half-life of the mutant and the wt human and hamster DHFRs were compared. After treating the transfected DG44 cells with cycloheximide to inhibit protein synthesis, cell lysates were collected at different time points and DHFR levels were analyzed using Western blotting. There was no significant difference in half-lives among the mutants of human DHFR that are not induced by MTX, wt human and hamster DHFR (data not shown). Therefore, the higher basal levels of the variants of human DHFR and hamster DHFR may be attributed to the fact that these proteins do not bind to their own mRNA to inhibit translation. Next, we determined the basal levels and specific activity of DHFR in DG44 cells transfected with either wt human DHFR-EGFP or wt hamster DHFR-EGFP. The basal levels were established by titrating wt hamster DHFR levels and by comparing 30 μg of total lysate of wt human DHFR with wt hamster DHFR (Figure 6b). The basal level of wt hamster DHFR was 10-fold higher than that of wt human DHFR transfectants. In addition, DHFR protein levels were comparable to the specific activity obtained for human and hamster DHFR (Figure 6c). The kinetic properties of human and hamster DHFR are similar (the Km of dihydrofolate for human and hamster DHFR are 0.02-0.37 μM and 0.42 μM, respectively and the Kms for NADPH for human and hamster are 0.16-1.44 μM and 0.64 μM, respectively (Ercikan-Abali et al., 1996; Lewis et al., 1995; Schweitzer et al., 1989; Wu et al., 1997)). In addition, there was no statistical difference ($p=0.26$) in the ED50 values for MTX between DG44 transfectants of human and hamster DHFR when one-tailed paired t-test was used to analyze the data using Graph Prism 4 (Figure 6d). The observation that hamster DHFR is expressed at ~10 times the level of human DHFR, but human DHFR is induced ~ 10-fold by MTX indicates that human cells respond to MTX by translationally upregulating DHFR levels

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and that this mechanism is an intrinsic mechanism of resistance to antifolates. Hence, the upregulation of DHFR protein in the presence of MTX leads to decreased sensitivity to this drug.

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Discussion

Earlier, we observed that DHFR in rodent (mouse and hamster) cells did not increase following MTX treatment as compared to DHFR levels in human, monkey and dog cells (Skacel, 2004). Herein, we demonstrate that this is due to a critical difference in the translational regulation of human and rodent DHFR. Based on the aforementioned model, we considered two explanations for the lack of MTX-induced translational regulation of hamster DHFR: i) a difference between the cis-acting regulatory elements on hamster versus human DHFR mRNA or ii) a difference in the trans-acting regulatory domain of hamster DHFR versus human DHFR.

To test the first possibility, we compared the human DHFR mRNA cis element to that of the hamster. Target elements for RNA binding proteins are generally found in loops, bulges or interior loops, which offer conformations for recognition and binding (reviewed in Frankel, 2000). Modeling studies using Zuker's RNA m-fold program identified two putative stem and loop structures within this region for both species. However, these structures had significant species-specific differences in their predicted stability, with the human structures approximately twice as stable as the comparable structures in rodent DHFR mRNA structures. The bulges within these structures were also dissimilar. However, "hamsterizing" the human protein by replacing the human sequence with the respective hamster nucleotides had no affect on the ability of MTX to upregulated the hamsterized DHFR, strongly suggesting that the observed difference in DHFR mRNA structure was not the explanation for the lack of upregulation of hamster DHFR.

Next we addressed the second possibility i.e. that the divergent translational regulation between hamster and human DHFR is due to the differences in the trans-acting regulatory (i.e. protein) domains between human and hamster DHFR. Previously, we identified a variant of

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human DHFR (S118A) residing in the NADPH binding pocket was similar to wt human DHFR in its enzymatic properties and yet was not upregulated by MTX (Skacel et al., 2005). We hypothesized that this human DHFR mutant might mimic the hamster DHFR, perhaps in a shared inability to bind to DHFR mRNA, thereby effecting translational control. If this were the case, the wild type human DHFR protein should be able to compensate for the hamster and S118A defects, bind to their respective mRNAs and regulate their translation. Using co-transfection experiments, we indeed showed that both hamster and S118A DHFR protein levels were elevated after MTX administration in the presence of wt human DHFR, strongly implicating differences in DHFR protein/conformation in the lack of a translational autoregulatory mechanism for the mutant and hamster DHFR is sequence. By extrapolation, human DHFR protein is an RNA-binding protein, while hamster is not. These findings also may explain the increased basal levels of hamster DHFR protein and variants of human DHFR that are not upregulated by MTX (Skacel et al., 2005). There is growing evidence that many enzymes have additional regulatory functions that are not related to their enzymatic activities. These enzymes have been referred to as “moonlighting” enzymes (Jeffery, 1999). Thus the ability of DHFR to regulate its translation would indicate that DHFR may also be a moonlighting enzyme.

The involvement of the DHFR NADPH binding site in autoregulation of DHFR through RNA binding is notable, but not unprecedented. A number of dehydrogenases have been shown to interact with RNA through their NAD/NADPH binding sites (Hentze, 1994; Nagy et al., 2000). One of the most noteworthy, glyceraldehyde-3-phosphate dehydrogenase, binds tRNA, AU-rich sequences at the 3’untranslated region of mRNA, hammerhead ribozyme and viral cis-acting regulatory elements (Evguenieva-Hackenberg et al., 2002). The possibility that human DHFR may bind to other mRNA species is currently being explored.

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From a clinical standpoint, the demonstrated ability of MTX to upregulate human wt DHFR could result in increased resistance of human tumor cells to MTX treatment. Higher DHFR levels are correlated with increased resistance to MTX; therefore it would be reasonable to expect that the DG44 cells transfected with hamster DHFR would be more resistant to MTX than the DG44 cells transfected with human DHFR. However, despite the divergent basal levels (~ 10-fold) of DHFR, IC50 values for MTX in both cell lines were statistically similar, due to translational upregulation of human DHFR, but not hamster DHFR, in the presence of MTX. Therefore, in contrast to the genetic change (gene amplification) which results in resistance following multiple exposures to MTX, translational upregulation of human DHFR by antifolates is a rapid adaptive mechanism of resistance. While this mechanism may protect normal renewal tissues from the toxicity of MTX, it may also decrease the sensitivity of tumor cells to therapy. This adaptive mechanism of human DHFR was also shown with pyrimethamine, an antifolate that is used in the treatment of malaria. The malaria parasite, Plasmodium falciparum, has a bifunctional DHFR-thymidylate synthase enzyme synthesized from the same mRNA transcript. This transcript binds to the bifunctional enzyme, but administration of antifolates for the treatment of malaria does not release DHFR-TS mRNA from binding to its protein Therefore, while the parasite is killed by pyrimethamine, the ability of the human host to upregulate DHFR allows the host to overcome drug toxicity by increasing human DHFR protein levels (Zhang and Rathod, 2002).

We conclude that divergent translational regulation of human and hamster DHFR is due to the differences in their protein properties rather than differences in their mRNAs (Figure 7). Not only does human DHFR bind to its cognate mRNA, but also it binds to hamster DHFR mRNA. Translational regulation of human DHFR allows cells to rapidly respond to cytotoxic

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effects of MTX; hence this is an adaptive mechanism. We are currently testing novel antifolates that do not share MTX's ability to upregulate DHFR levels, and thus may be more effective anticancer agents.

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Footnotes

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Y.C. H. and N.E.S have contributed equally to the studies presented in this manuscript and should be considered co-first authors.

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Figure Legends

Figure 1. a) Western blot analysis of total cell lysates from human and rodent cell lines after MTX exposure. Human (C85), Hamster (CHO) and Mouse (3T6) cells were exposed to l μM MTX or media without MTX for 24 and 48 h. b) Increase in EGFP fluorescence of various transfectants after 1 μM MTX treatment for 24 h. EGFP and wt human DHFR-EGFP transfectants is negative and positive control. c) Human and hamster DHFR-EGFP transfectants of DG44 cells were exposed to either 1 or 10 μM MTX in the presence of hypoxanthine and thymidine in order to prevent cell death due to MTX treatment for 24 and 48 h. d) DHFR-EGFP transfectants were exposed to 1 μM MTX, trimetrexate (TMTX), raltitrexed (RTX) and pemetrexed (PTX) respectively. a-c) 50 μg of total cell lysate was loaded and probed with either an anti-DHFR antibody (a) or EGFP antibody (b and c). Equal loading was determined by either α-tubulin or GAPDH respectively.

Figure 2. a) Comparison of the primary sequence of binding region (BR) of human DHFR to hamster DHFR. b) Secondary structures of human and hamster DHFR within the binding region are obtained using m-fold program. c) and d) "Hamsterized" human DHFR-EGFP protein levels increase upon exposure to MTX. (c) Batches and (d) four individual clones from DG44 cells transfected with hamsterized DHFR-EGFP. Cells were exposed to 1 μM MTX or media without MTX for 24 h and 48 h. 50 μg of total cell lysate was loaded and probed with an anti-EGFP antibody. Equal loading was determined by Ponceau S staining (not shown).

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Figure 3. (a) Experimental design for fully mutated binding region (FBMR) and half mutated binding regions (BR1 and BR2). Human DHFR-EGFP with FMBR and human DHFR-EGFP with a half mutated binding regions demonstrate increases in fusion protein levels upon exposure to MTX. (b) Batches from DG44 cells transfected with a FMBR, BR1 and BR2 human were exposed to 1 μM MTX or media without MTX for 24 h and 48 h. 50μg of total cell lysate was loaded and probed with an anti-EGFP antibody. Equal loading was determined by Ponceau S staining (data not shown).

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Figure 4. The only ATG within the binding region was mutated to ACG which resulted in M140T. Following exposure to 0.1 and 1 μM MTX for 24 and 48 h, human DHFR variant M140I resulted in increased levels of DHFR-EGFP fusion protein. Western blots were performed on total cell lysates from DG44 cells transfected with M140T. Cells were exposed to MTX or media without MTX in the presence of hypoxanthine and thymidine in order to prevent cell death due to MTX treatment. 50 μg of total cell lysate was loaded and probed with an anti-EGFP antibody. Equal loading was determined by GAPDH antibody.

Figure 5. Wt human DHFR restores the lack of feedback regulation in hamster and human DHFR S118A variant both of which are not responsive to MTX-induced upregulation. a) DG44 cells stably transfected with DHFR S118A-EGFP were exposed to 1μM MTX for 24 h demonstrating the lack of upregulation of DHFR by MTX. DHFR S118A-EGFP levels were detected using antibody against EGFP. Equal loading was determined with GAPDH antibody. b) DG44 cells transfected with DHFR S118A-EGFP were transfected once more with flag-tagged

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wt human DHFR. While flag-tagged wt human DHFR protein levels were detected with anti-flag antibody, mutant DHFR S118A-EGFP levels were detected with anti-EGFP antibody. Five stable clones of doubly transfected cell lines were exposed to 1μM MTX for 48 h and DHFR protein were detected using Western blotting. Graph is depicting the quantitative analysis of the data in (b). The intensity of DHFR S118A-EGFP protein bands was normalized to the intensity of GAPDH. c) C85 cells transfected with hamster DHFR-EGFP were exposed to 0.l and 1μM MTX for 48 h. 50 μg of total cell lysate was loaded and probed with either DHFR antibody for endogenous levels of wt human DHFR and EGFP antibody for wt hamster-EGFP fusion protein. To control for equal loading, blots were stripped and reprobed with an α-tubulin antibody.

Figure 6. a) Synthesis of wt human, hamster and variants of human DHFR-EGFP using an *in vitro* reticulocyte translation assay. b) DHFR-EGFP protein levels of hamster is higher than the wt human DHFR-EGFP. Serial dilutions of hamster DHFR-EGFP lysates were compared to 30 μg of wt human DHFR-EGFP transfected cells. Westerns were probed with an anti-EGFP antibody. Loading was controlled using GAPDH antibody. c) DHFR activity of hamster and human DHFR variants that are not up-regulated by MTX are higher than the wt DHFR-EGFP. d) Cytotoxicity of MTX to DG44 cells transfected with wt human and hamster DHFR-EGFP.

Figure 7. Model for divergent translational regulation of rodent and human DHFR. Wt human DHFR mRNA translation is feedback-regulated through the interaction of DHFR protein with its cognate mRNA. The cis-acting regulatory elements on human DHFR mRNA were localized within the coding region, and the trans-acting regulatory domain was suggested to be within the NADPH binding site of DHFR. Wt human DHFR protein has at least two conformations, one of

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which preferentially is bound to NADPH, and the other is bound to DHFR mRNA. These two conformers are in equilibrium and can interconvert. Binding of MTX to the binary complex of DHFR-NADPH shifts the equilibrium towards the NADPH bound conformer, releasing DHFR mRNA to be translated. However, three variants of human DHFR protein (S118A, L22R and E30A) and hamster are unable to bind their own cognate mRNA which leads to increased synthesis of these proteins. Therefore, while binding of MTX to the wt human DHFR-mRNA complex leads to resumption of DHFR synthesis, hamster and three variants of human DHFR proteins are not induced by MTX.

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Table 1. Primers used in the constructions of variants of human DHFR

Primer		
DHFR	sense	5' GGCGGCAAGCTTATGGTTGGTTCGCTAAACTGC 3'
	antisense	3' GCCGCCGGATCCATCATTCTTCTCATACTTC 3'
BR1	sense	5' GGAAGCCATGAATCAGCCAGGCCATCTCAGACTCTTTGTGACAAGGATCATGC 3'
	antisense	5' GCATGATCCTTGTCACAAAGAGTCTGAGATGGCCTGGCTGATTCATGGCTTCC 3'
BR2	sense	5' CCAGAAATTGATTTGGAGAAATATAAACTTCTCCCAGAATACCCAGGTG 3'
	antisense	5' CACCTGGGTATTCTGGGAGAAGTTTATATTTCTCCAAATCAATTTCTGG 3'
T419C	sense	5' CTTAAACTATTTGTGACAAGGATCACGCAAGACTTTGAAAGTGAC 3'
	antisense	5' GTCACTTTCAAAGTCTTGCGTGATCCTTGTCACAAATAGTTTAAG 3'

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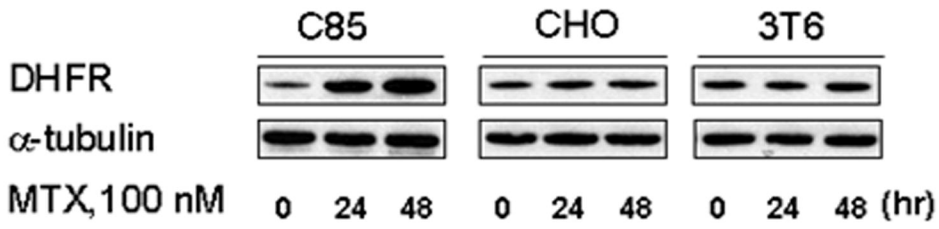
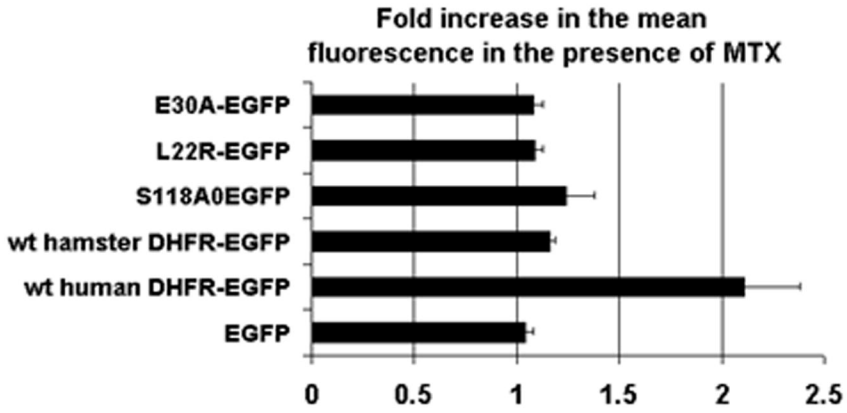
Figure 1

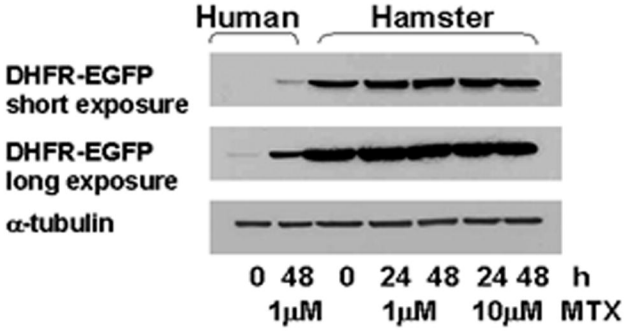
a)



b)



c)



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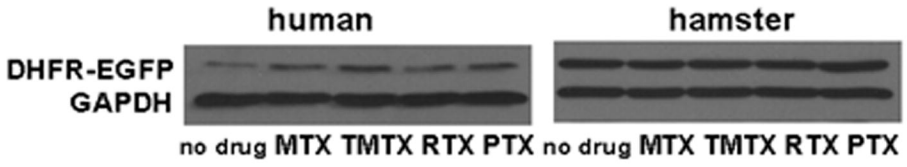

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Figure 2

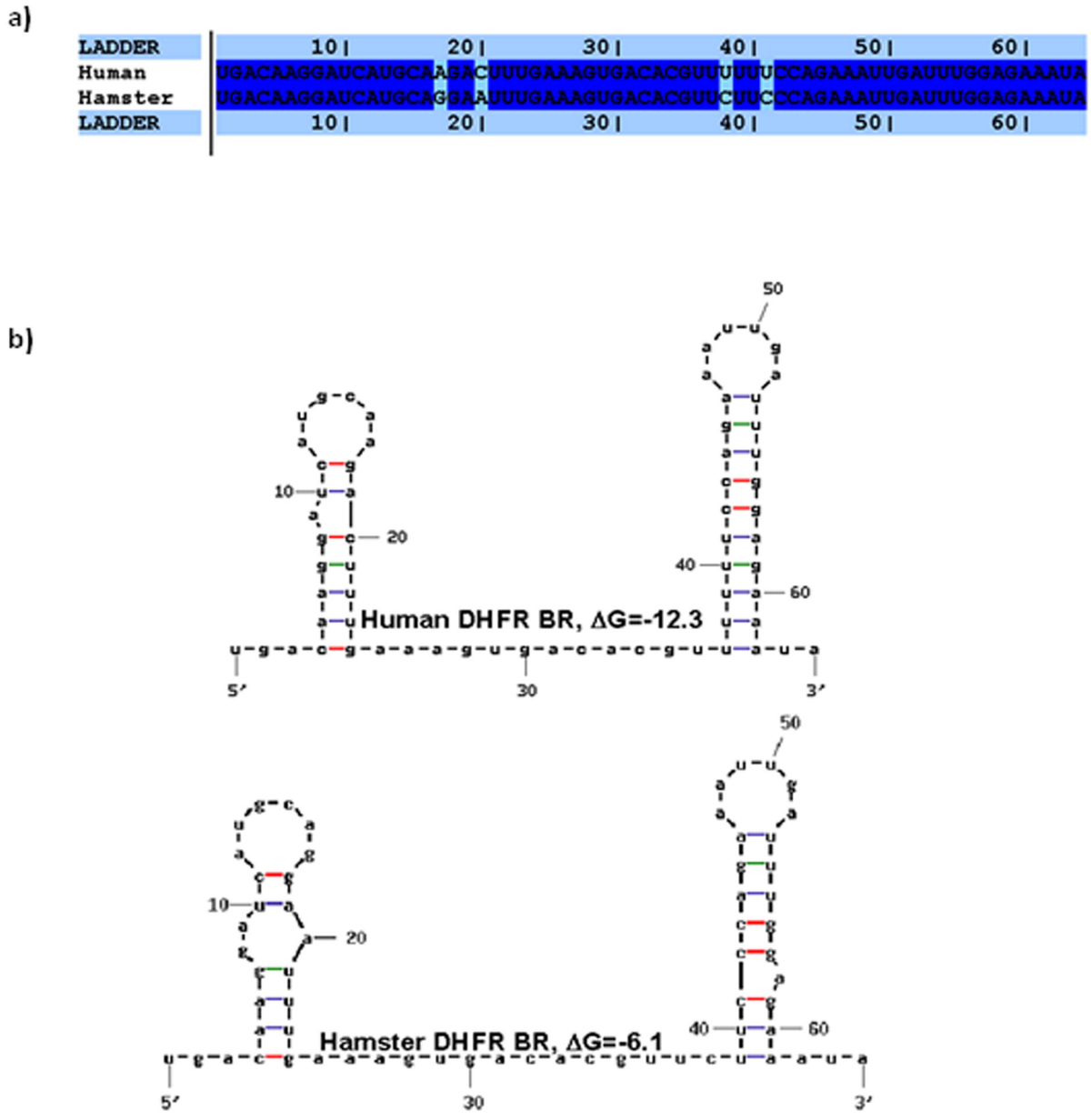

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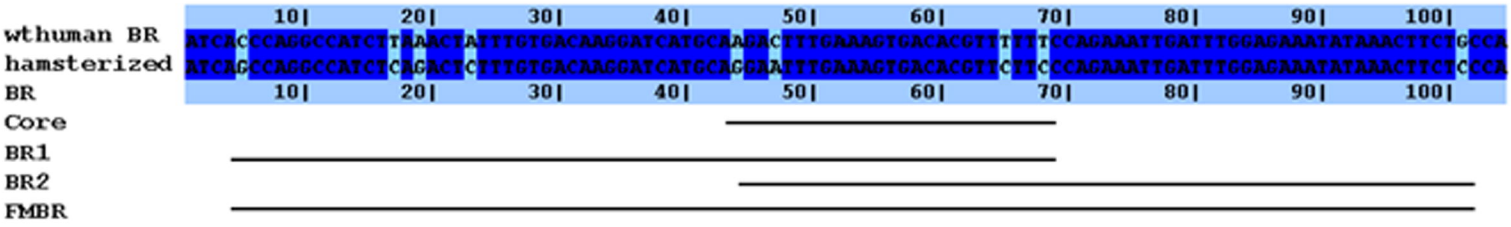
Figure 3

a)



b)

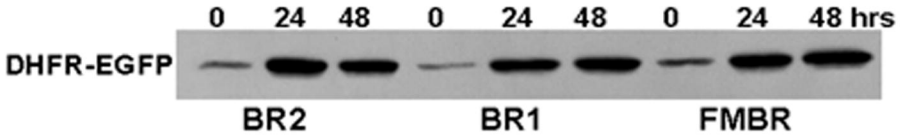


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Figure 4

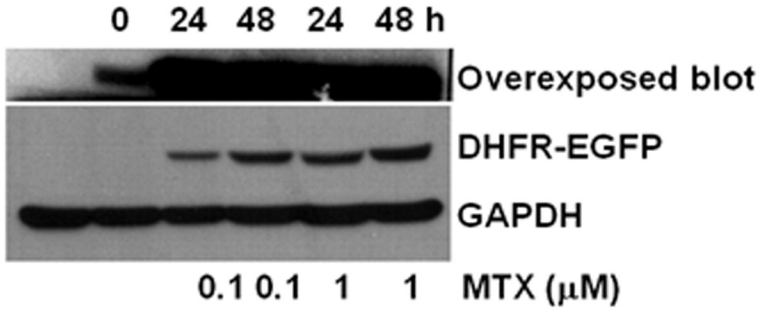

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Figure 5

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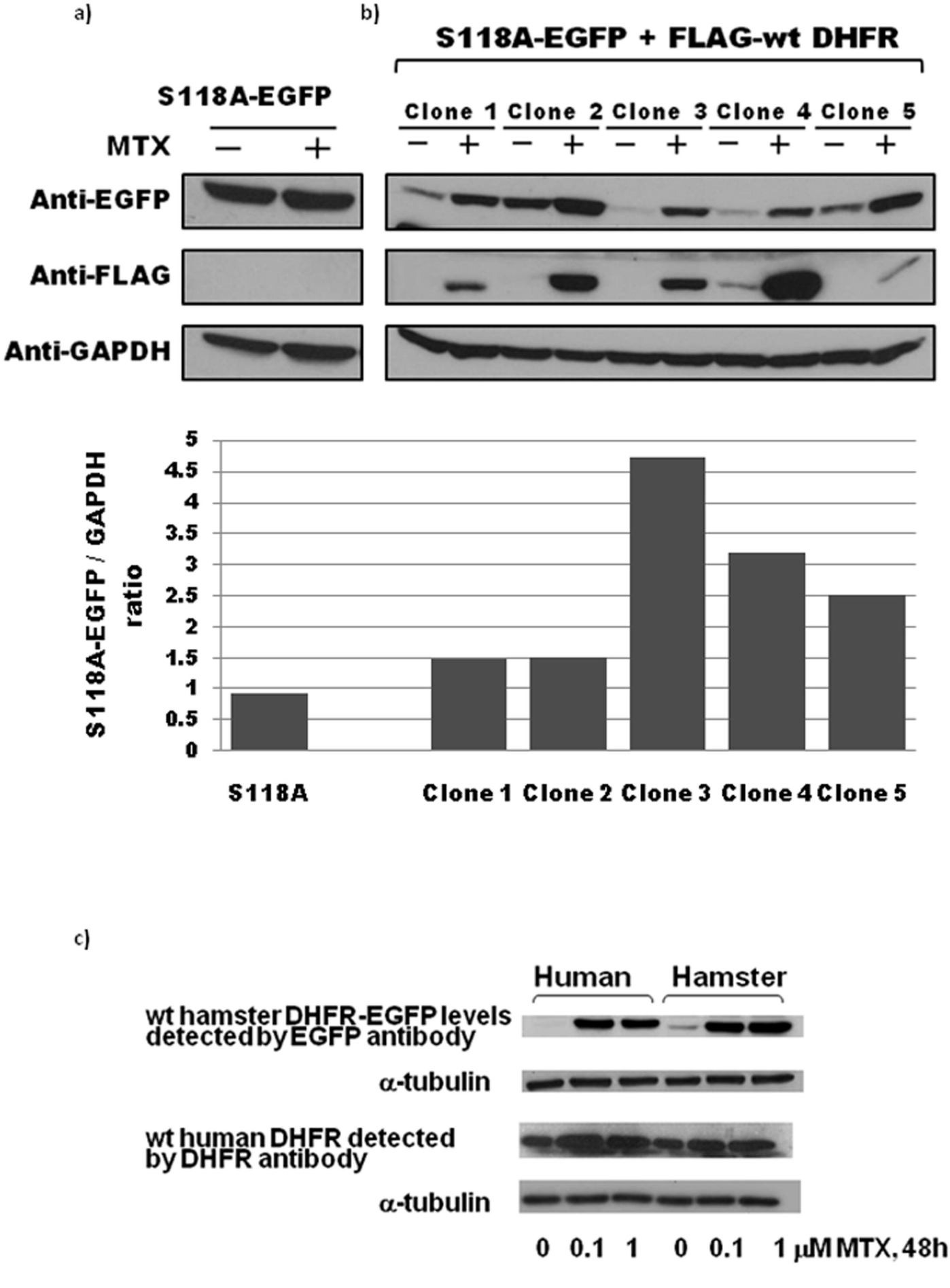

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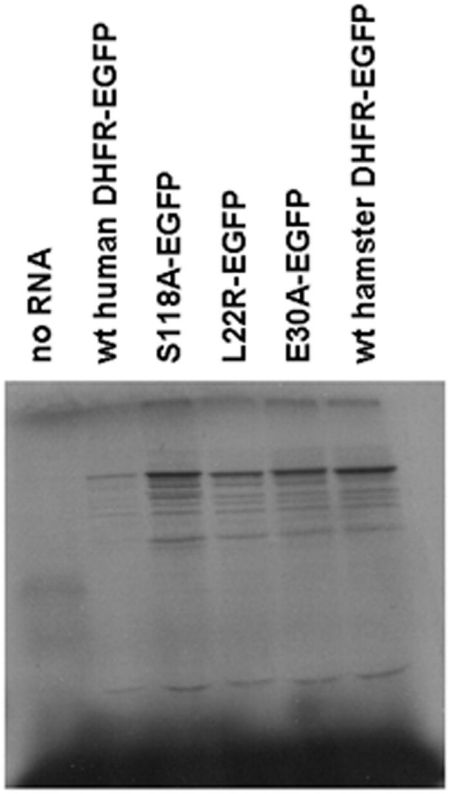
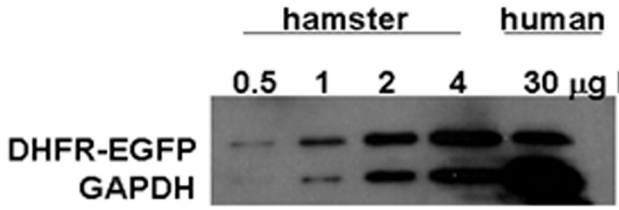
Figure 6

a)



b)



c)

DHFR-EGFP	Specific activity (mU/mg)	fold increase
human wt DHFR-EGFP	0.53	1
hamster wt DHFR-EGFP	8.7	16

d)

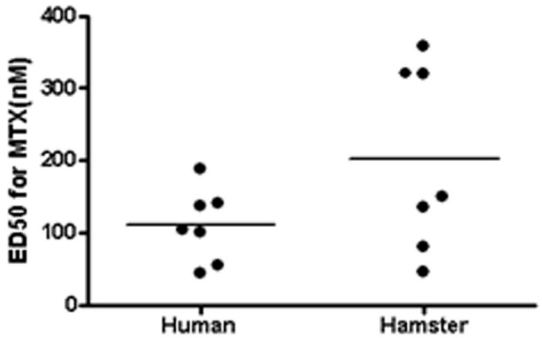


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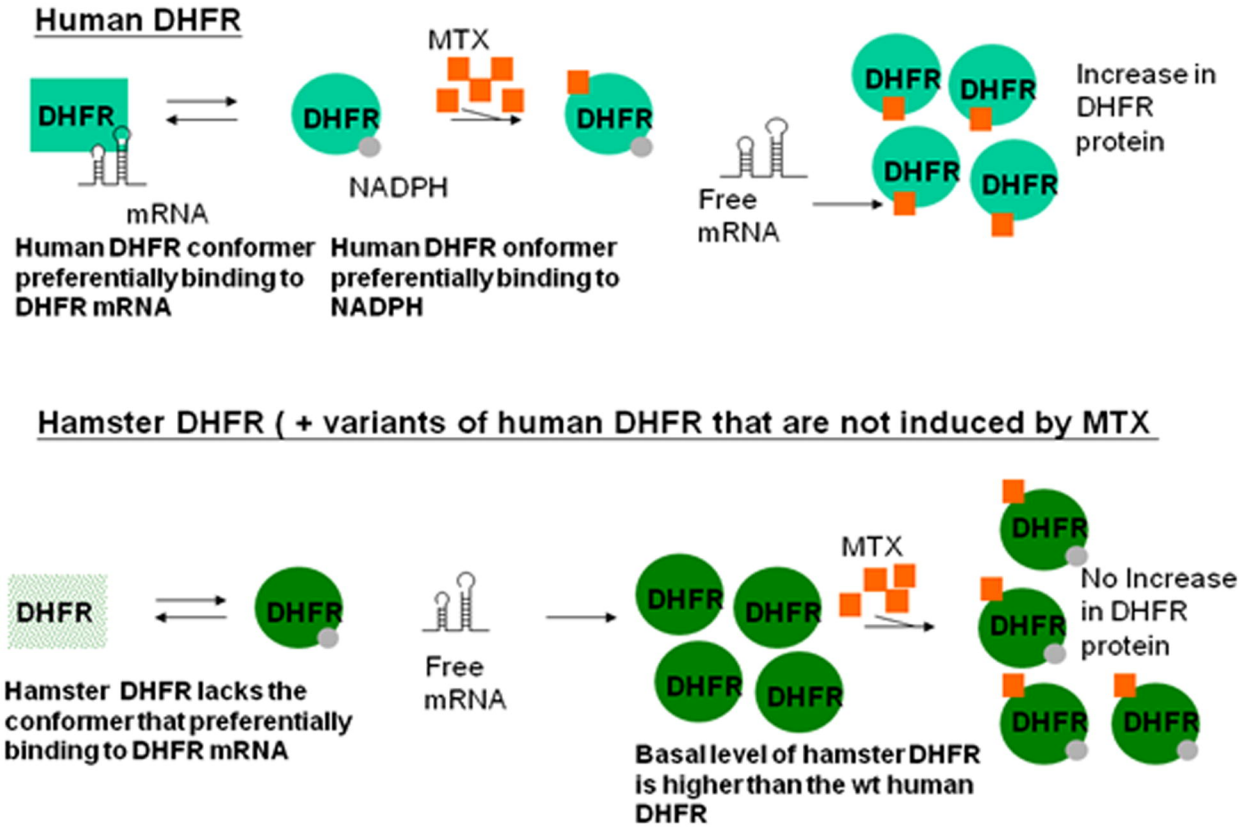
Figure 7



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