Dihydrofolate Reductase and Thymidylate Synthase Transgenes Resistant to Methotrexate Interact to Permit Novel Transgene Regulation*

Received for publication, June 10, 2015, and in revised form, August 2, 2015. Published, JBC Papers in Press, August 4, 2015, DOI 10.1074/jbc.C115.671123

David Rushworth,† Amber Mathews‡, Amir Alpert§, and Laurence J. N. Cooper‰

From the † Division of Pediatrics, Children’s Cancer Hospital, The University of Texas MD Anderson Cancer Center (MDACC), Houston, Texas 77030 and the ‡The University of Texas Graduate School of Biomedical Sciences at Houston, Houston, Texas 77030

Background: Methotrexate targets dihydrofolate reductase (DHFR) and thymidylate synthase (TYMS) to prevent cancer growth, and increases DHFR expression when applied.

Results: TYMS resistant to methotrexate inhibits the methotrexate induced increase in DHFR expression.

Conclusion: Thymidine synthesis regulates post-transcriptional expression of DHFR and TYMS.

Significance: Methotrexate-resistant DHFR and TYMS can be used to regulate cis and trans transgenes in primary T cells.

Methotrexate (MTX) is an anti-folate that inhibits de novo purine and thymidine nucleotide synthesis. MTX induces death in rapidly replicating cells and is used in the treatment of multiple cancers. MTX inhibits thymidine synthesis by targeting dihydrofolate reductase (DHFR) and thymidylate synthase (TYMS). The use of MTX to treat cancer also causes bone marrow suppression and inhibits the immune system. This has led to the development of an MTX-resistant DHFR, DHFR L22F, F31S (DHFR⁵⁶), to rescue healthy cells. 5-Fluourouracil-resistant TYMS T51S, G52S (TYMS⁵⁵) is resistant to MTX and improves MTX resistance of DHFR⁵⁶ in primary T cells. Here we find that a known mechanism of MTX-induced increase in DHFR expression persists with DHFR⁵⁶ and cis-expressed transgenes. We also find that TYMS⁵⁵ expression of cis-expressed transgenes is similarly decreased in an MTX-inducible manner. MTX-inducible changes in DHFR⁵⁶ and TYMS⁵⁵ expression changes are lost when both genes are expressed together. In fact, expression of the DHFR⁵⁶ and TYMS⁵⁵ cis-expressed transgenes becomes correlated. These findings provide the basis for an unrecognized post-transcriptional mechanism that functionally links expression of DHFR and TYMS. These findings were made in genetically modified primary human T cells and have a clear potential for use in clinical applications where gene expression needs to be regulated by drug or maintained at a specific expression level. We demonstrate a potential application of this system in the controlled expression of systemically toxic cytokine IL-12.

Antifolate drugs have been in use for seven decades in the treatment of cancer (1). MTX³ is a commonly used agent from this class and inhibits several folate-dependent enzymes including DHFR and TYMS. Inhibition of these proteins adversely affects the de novo synthesis of purine and thymidine nucleotides, which is vital for survival of rapidly replicating cells. MTX has proven valuable for treating rapidly replicating cancers such as leukemia, but the impact of MTX on healthy, replicating tissue leads to dose-limiting toxicities such as bone marrow suppression (2, 3). This disadvantage of MTX has led to intense study into mechanisms of resistance (4) and alternative anti-folates for the treatment of cancer (3).

Stemming from the discovery of DHFR mutants that conferred MTX resistance, a mutant that weakly binds MTX, DHFR⁵⁶, was developed (5). A strategy was formulated where bone marrow cells would be genetically modified ex vivo with DHFR mutants resistant to MTX (6). The use of DHFR⁵⁶ was later implemented in genetically modified T cells. T cells modified with DHFR⁵⁶ are desired following bone marrow transplant for relapsed leukemia to overcome immune suppression of MTX, allowing genetically modified T cells to survive and target cancer (7, 8). An advantage of DHFR⁵⁶ is that it can be used to select for transgenes useful for therapeutic efficacy such as tumor targeting proteins such as chimeric antigen receptors (8), suicide genes (9), or imaging genes (10). In an effort to broaden the application of DHFR⁵⁶, our group added a human TYMS mutant, discovered in a bacterial screen, resistant to 5-fluorouracil (5-FU) (11). Upon the addition of human mutant TYMS⁵⁵ with DHFR⁵⁶ in human T cells, we demonstrated

* This work was supported in part by National Institutes of Health Grants R01 (CA124782, CA120956, and CA141303) and P01 (CA148600); Burroughs Wellcome Fund; Cancer Prevention and Research Institute of Texas; Gillson Longenbaugh Foundation; Harry T. Mangurian, Jr., Fund for Leukemia Immunotherapy; Institute of Personalized Cancer Therapy; Leukemia and Lymphoma Society; Lymphoma Research Foundation; MDACC’s Sister Institution Network Fund; Miller Foundation; National Foundation for Cancer Research; Pediatric Cancer Research Foundation; and William Lawrence and Blanche Hughes Children’s Foundation. Some of the technology described was advanced through research conducted at the MDACC by Laurence J. N. Cooper, M.D., Ph.D. In January 2015, some technology generated by Dr. Cooper was licensed for commercial application to ZIOPHARM Oncology, Inc., and Intrexon Corporation in exchange for equity interests in each of these companies. Hence MDACC, Dr. Cooper, Dr. Rushworth, and Mr. Alpert have a financial interest in ZIOPHARM Oncology, Inc., and Intrexon Corporation. On May 7, 2015, Dr. Cooper was appointed as the Chief Executive Officer at ZIOPHARM. Dr. Cooper is now a Visiting Scientist at MDACC where he will continue to supervise the development of this technology.

1 Supported by a Floyd Haar, M. D., Memorial Endowed Research Award and a Pauline Altman-Goldstein Foundation Discovery Fellowship.
2 To whom correspondence should be addressed. Tel.: 713-792-9860; Fax: 713-792-9832; E-mail: ljncooper@mdanderson.org.

³ The abbreviations used are: MTX, methotrexate; DHFR, dihydrofolate reductase; TYMS, thymidylate synthase; 5-FU, 5-fluorouracil; PBMC, peripheral blood mononuclear cell; CoOq, codon-optimized; ic9, inducible caspase 9; ANOVA, analysis of variance; THF, tetrahydrofolate; 5,10-CH2THF, 5,10-methylenetetrahydrofolate; MFI, mean fluorescence intensity.
improved resistance of T cells to 5-FU and increased resistance to MTX beyond that of DHFR<sub>FS</sub> alone, in agreement with a series of experiments performed in murine bone marrow cells where linking of DHFR<sub>FS</sub> to a mutant of TYMS led to improved resistance to 5-FU and the antifolate pemetrexed (12, 13). We suggested that the addition of TYMS<sub>SS</sub> restores thymidine synthesis in MTX-treated T cells (9).

In working with DHFR and TYMS, it was noted that human DHFR is known to bind and inhibit translation of DHFR mRNA as a post-translational mechanism of regulation. Furthermore, MTX was shown to prevent this binding (14) and lead to an increase in DHFR protein expression. The nucleotide sequence to which DHFR protein binds DHFR mRNA within the coding domain is known (15, 16), and the specific amino acids that DHFR protein utilizes to bind DHFR mRNA have been elucidated (17). However, subsequent studies found that a mutation to mRNA binding amino acids of DHFR still resulted in increased DHFR protein expression in the presence of MTX (18). Conversely, mutations to amino acids unrelated to DHFR binding mRNA (17) prevented MTX-inducible expression of DHFR (18). This suggests that another mechanism of post-transcriptional regulation for DHFR is in effect, and findings detailed in this study lead us to propose that a mechanism independent of mRNA binding is affecting MTX-induced DHFR up-regulation. Notably, we demonstrate that this mechanism can be utilized for regulation of transgene expression when transgenes are located in a 3' cis arrangement with DHFR<sub>FS</sub>.

By codon-optimizing DHFR<sub>FS</sub> to remove the mRNA binding sequence previously identified (15, 16), we find that MTX continues to induce an increase in DHFR<sub>FS</sub> expression independent of the mRNA sequence and that the addition of TYMS<sub>SS</sub> unexpectedly blunts this increase. This finding suggests a novel method of post-transcriptional DHFR expression that is regulated by the enzymatic action of TYMS<sub>SS</sub>. In performing these experiments in primary human T cells, we also identified that the application of MTX decreases TYMS<sub>SS</sub> expression in an MTX-dependent manner, likely due to TYMS binding its own mRNA (15, 16). This codon-optimized (CoOp) DHFR<sub>FS</sub> otherwise had the same design as D<sup>F</sup>GiG and is known as FLAG-CoOp DHFR<sub>FS</sub>-2A-eGFP pSBSO (CoOp D<sup>F</sup>G). TYMS<sub>SS</sub> without codon optimization was co-expressed with a C terminus eGFP or RFP. The constructs are referred to as FLAG-TYMS<sub>SS</sub>-2A-GFP pSBSO (TSSS<i>G</i>) and FLAG-TYMS<sub>SS</sub>-2A-RFP pSBSO (TSSS<i>R</i>). A selection-free RFP plasmid NLS-mCherry pSBSO (RFP) was utilized as a control. The construct Myc-fLuc-NeoR pSBSO (NRF) was electroporated, and G418 (InvivoGen, Ant-<i>gn</i>-1) was used for selection as described below when alternative selection was desired. A construct to select for inducible caspase 9 (iC9) FLAG-DHFR<sub>FS</sub>-2A-iC9 pBSO (D<i>F</i>Si9) was also used. The design and synthesis of these constructs have been described elsewhere (9). The construct FLAG-TYMS<sub>SS</sub>-2A-IL-12p35-2A-IL-12p40 pBSO (TSSS<i>R</i>-IL-12) was synthesized from codon-optimized (GeneArt, Life Technologies) IL-12 p35 and IL-12 p40 transgenes and digested within the 2A regions to ligate IL-12 p35 and IL-12 p40 with a TYMS<sub>SS</sub> fragment also digested within the 2A region. TSSS<i>R</i> backbone digestion points 5' to the start site of TYMS<sub>SS</sub> and 3' to the IL-12p40 stop site ligated the three components into the TSSS<i>R</i> backbone in a four-part ligation. All constructs described integrate into the cellular genome with the aid of Sleeping Beauty transposase (20).

**Culture Conditions**—Both Jurkat cells and human PBMC were electroporated, maintained, and selected as described previously (9). Briefly, Jurkat cells were electroporated with transgene, and 48 h later, drug was applied for 2 weeks. Surviving cells were rested in complete media for another 3–5 weeks before testing. Human PBMC were electroporated, and the following day, T cells were propagated with activating and propagating cells at a 1:1 ratio including recombinant human IL-2. 48 h after co-culture was initiated, drug was applied to culture for a 2-week period. Propagation of PBMC with activating and propagating cells and IL-2 selectively expanded T cells and was repeated weekly. The T cells surviving drug treatment from days 2 to 14 were co-cultured without drug for another 3 weeks (9, 20, 21). On day 35 of propagation, T cells were stimulated with anti-CD3, anti-CD28, and IL-2 (9). MTX (Hospira, Lake Forest, IL), pemetrexed (Lilly, Indianapolis, IN), and 5-FU (APP Pharmaceuticals, Schaumburg, IL) were attained from the MDACC pharmacy, whereas raltitrexed (Abcam Biochemicals, Cambridge, MA, catalog number Ab142974) was not.

**Flow Cytometry**—T cells were prepared for flow cytometry by washing once in FACs buffer, and surface-stained for anti-human CD3-APC (BD Pharmingen, 340661, 1:33 dilution), as described previously (21). Intracellular staining for Myc tag Alexa Fluor 488 (MBL International, M047-A48, 1:33 dilution) and anti-human IL-12 p40/p70-PE (BD Pharmingen, 554575,
1:20 dilution) also proceeded as described previously (21). Transgene expression was analyzed on either a BD LSRFortessa or a BD FACSCalibur (BD Biosciences), with the same flow cytometer used consistently within a given experiment. FlowJo v 10.0.5 (TreeStar Inc., Ashland, OR) was used to analyze flow cytometry data.

**A** No MTX

![Graph showing MFI for different conditions without MTX]

**B** Δ MTX

![Graph showing MFI for different conditions with MTX]

**C** No MTX

![Graph showing MFI for different conditions without MTX]

**D** Δ MTX

![Graph showing MFI for different conditions with MTX]

**E**

- No DNA Jurkat No Trx
- ΔG Jurkat No Trx
- [ΔG & TS55R] Jurkat No Trx

**F**

- No DNA T cell day 21
- [ΔG & RFP] T cell day 21
- [ΔG & TS55R] T cell day 21

**G** [ΔFSG & TS55R] Jurkat

\[ R^2 = 0.90 \]

slope = 1.3

**Legend**

- DΔG: DHFRΔG-2A-eGFP
- TS55G: TYMS55-2A-eGFP
- TSΔR: TYMSΔR-2A-RFP
- NRF: fLuc-2A-NeoR
- RFP: NLS-mCherry
**Statistical Analysis**—Statistical analysis and graphical representation were performed as before (9). Briefly, non-Gaussian distributions of a multivariate nature were analyzed by one-way ANOVA Kruskal–Wallis test followed by Dunn’s multiple comparison test. Univariate tests (experimental versus control) utilized t tests. Two-way ANOVA was performed when appropriate and followed by Sidák’s multiple comparison test. The analysis is denoted in context. Pearson’s correlation and linear regression analysis were performed where denoted. Statistical significance was designated as \( \alpha < 0.05 \). Means with standard deviations are depicted within each figure.

**Results and Discussion**

Mayer-Kuckuk et al. (22) demonstrated that DHFR linked to a transgene of interest can be used to induce an increased expression of that transgene in vitro and in vivo when MTX is administered. We attempted to recapitulate an increase in the expression of MTX-resistant DHFR\( ^{FS} \) after the administration of MTX within human T cells, which Skacel et al. (18) has shown in a hamster cell line. As a control for our studies, DHFR\( ^{FS} \) transgene was codon-optimized to remove any of the mRNA sequence or structure that contributed to mRNA binding (16). It was hypothesized that this modification would increase DHFR\( ^{FS} \) expression by removing DHFR protein binding to DHFR\( ^{FS} \) mRNA and prevent the increase of DHFR\( ^{FS} \) expression previously noted in the presence of MTX. The expression of DHFR\( ^{FS} \) and CoOp DHFR\( ^{FS} \) selected for uniform expression in Jurkat T cell line is shown in Fig. 1A. CoOp DHFR\( ^{FS} \) did not contribute to a significantly higher expression of DHFR\( ^{FS} \) as indicated by a cis-expressed eGFP, nor did it prevent MTX-induced increases in transgene expression as noted in Fig. 1B. This was unexpected. However, the loss of MTX-induced increase in DHFR\( ^{FS} \) expression was noted when TYMSS\( ^{S} \) was co-expressed with DHFR\( ^{FS} \), as seen in Fig. 1, A and B (9). The addition of TYMSS\( ^{S} \) led to an insignificant reduction in the expression of un-optimized DHFR\( ^{FS} \) in the absence of MTX. The addition of MTX was unable to induce the same increase in DHFR\( ^{FS} \) expression seen during the sole expression of either DHFR\( ^{FS} \) version. Thus, TYMSS\( ^{S} \) is playing a role in the MTX-inducible increase of DHFR\( ^{FS} \), and likely through the restoration of thymidine synthesis.

Expression of these transgenes in primary T cells was evaluated to recapitulate the findings of MTX-inducible increases in DHFR\( ^{FS} \) expression that were prevented by TYMSS\( ^{S} \). Expression of DHFR\( ^{FS} \), TYMSS\( ^{S} \), or [DHFR\( ^{FS} \) & TYMSS\( ^{S} \)] was achieved with stability and purity by selecting from days 2–14 of propagation with the respective drugs MTX, 5-FU, and G418 when the selection vector containing neomycin resistance was included (9). The expression of DHFR\( ^{FS} \)-linked eGFP and TYMSS\( ^{S} \)-linked RFP can be noted in Fig. 1C for DHFR\( ^{FS} \), TYMSS\( ^{S} \) or [DHFR\( ^{FS} \) & TYMSS\( ^{S} \)]. Again it is noted that DHFR\( ^{FS} \) expression is increased in the presence of MTX (Fig. 1D), but this increase is blunted and no longer significant when TYMSS\( ^{S} \) is co-expressed with DHFR\( ^{FS} \), as in Jurkat cells. Of note, expression of TYMSS\( ^{S} \) without DHFR\( ^{FS} \) was successfully achieved in primary T cells by selection with 5-FU and a trans neomycin resistance gene selected by G418 (9). When TYMSS\( ^{S} \) was tested for inducible changes in the presence of high doses of MTX (Fig. 1D), it was found that TYMSS\( ^{S} \)-linked RFP decreased significantly. MTX induced a reduction in the expression of TYMSS\( ^{S} \) that MTX-resistant DHFR\( ^{FS} \) restored. Although not definitive, these findings could indicate that TYMSS\( ^{S} \) is being repressed by a lack of 5,10-methylenetetrahydrofolate (5,10-CH\( ^{2} \)THF). This is consistent with the findings of Chu et al. (19) that 5,10-CH\( ^{2} \)THF prevents TYMS-mediated repression of TYMS mRNA translation. Those findings indicate that in a healthy cell where DHFR activity is uninhibited, TYMS is not significantly bound to its mRNA. However, we propose that MTX, which leads to a drop in 5,10-CH\( ^{2} \)THF, is causing TYMS protein to bind TYMS and TYMSS\( ^{S} \) mRNA, preventing expression (23). It should be noted that TYMSS\( ^{S} \) is equivalent to the native sequence with the exception of the point mutations (9).

**FIGURE 1.** DHFR\( ^{FS} \) cis-transgene expression increases in the presence of MTX independent of mRNA sequence, and the increase is suppressed by restoration of thymidine synthesis. A, Jurkat cells were genetically modified to express FLAG-DHFR\( ^{FS} \), 2A-eGFP pSBSO (D\( ^{G} \)) with resistance to MTX (n = 4), CoOp D\( ^{G} \), with known mRNA binding elements D\( ^{G} \) removed (n = 5), and [D\( ^{G} \) & FLAG-TYMS\( ^{S} \)-2A-RFP pSBSO (TS\( ^{S} \))]\( ^{R} \), with enhanced resistance to MTX beyond D\( ^{G} \) alone through the addition of MTX-resistant TYMSS\( ^{S} \) (n = 7). Genetically modified Jurkat cells were selected for 2 weeks in 1 \( \mu \)M MTX before culturing without MTX for 3–5 weeks. In A, the stable fluorescent protein expression, in the absence of MTX, is depicted by MFI. B, panel I, Jurkat cells were treated for 72 h with 0.5 \( \mu \)M MTX or no treatment. The MFI difference (\( \Delta \) = eGFP MFI MTX-treated − eGFP MFI-untreated) is depicted. B, panel II, a histogram representative of the data used in panel I demonstrates the MTX-induced change in eGFP MFI for DHFR\( ^{FS} \) and CoOp DHFR\( ^{R} \) in Jurkat cells. No Trx, no treatment. C, in primary T cells, transgenes DHFR\( ^{FS} \), TYMSS\( ^{S} \), or the combination were selected with FLuc-Neo- pSBSO (NRP) for 2 weeks in the presence of cytotoxic drug and then propagated without selection for 3 weeks (see “Experimental Procedures”). On day 35, T cells were stimulated with anti-CD3, anti-CD28 antibodies, and 50 IU/ml IL-2 in the presence or absence of MTX for 72 h. The fluorescent protein MFI of untreated cells is shown. D, panel I, depicts the MFI after 72 h of treatment with 0.5 \( \mu \)M MTX in comparison to no treatment. In D, panel II, a representative histogram demonstrates the observed shift in eGFP fluorescence for DHFR\( ^{FS} \) T cells in the presence or absence of MTX (n = 5). E, a trans regulatory pattern of DHFR- and TYMSS-linked proteins was observed. A representative flow plot from the 1 \( \mu \)M DHFR\( ^{FS} \)-selected Jurkat cells left untreated, as in A, demonstrates that in untreated Jurkat cells of either unselected mock-electroporated (No DNA) or D\( ^{G} \), there is a globular appearance in the RFP channel. However, co-expression of DHFR\( ^{FS} \) with TYMSS\( ^{S} \) in [D\( ^{G} \) & TS\( ^{S} \)] \( ^{R} \) Jurkat cells leads to a linear clustering. F, primary T cells were electroporated with DHFR\( ^{FS} \) and co-transformed with either RFP control or FLAG-TYMS\( ^{S} \)-2A-RFP pSBSO (TS\( ^{S} \)) before propagation, as in C. A representative flow plot of primary human T cells from the same donor where [D\( ^{G} \) & RFP], [D\( ^{G} \) & TS\( ^{S} \)]\( ^{R} \) and untransformed T cells were electroporated Jurkat cells in antifolates MTX (0, 0.01, 0.1, 0.5, 1, 5 \( \mu \)) \( \mu \), pemetrexed (0, 10, 50, 100 \( \mu \)), and raltitrexed (0, 1, 5, 10 \( \mu \)). The MFI of D\( ^{G} \) and TS\( ^{S} \) for each expression pattern was plotted after days 2–14 in selection. The values were plotted, and a linear fit was performed with the R\(^{2}\) from the Pearson’s correlation and the slope of the linear regression provided on the graph. These data are assembled from four technical replicates. H, a model of post-transcriptional regulation of DHFR and TYMS mRNA into the respective proteins is depicted with colored arrows indicating increase (green) or decrease (red) in specific small molecules. Black pointed arrows indicate promotion, while black bars indicate inhibition of protein translation. All experiments other than G were independently repeated twice. Kruskal-Wallis test was used to determine significant differences with multivariate analyses. Unmarked statistically significant results refer to comparison between experimental cells and the “No DNA” untreated control cells of equivalent measure. ns = not significant; * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001. TMP, thymidine monophosphate; UMP, uridine monophosphate; DHF, dihydrofolate.
Based on findings in Fig. 1, A and B, we propose that DHFR\textsuperscript{FS} expression is also regulated by the synthesis of thymidine. Likewise, based on findings in Fig. 1, C and D, we propose that TYMS\textsuperscript{SS} expression is regulated by the synthesis of tetrahydrofolate (THF). As a derivative of THF is used to make thymidine, a logical conclusion was made that DHFR\textsuperscript{FS} regulates the expression of TYMS\textsuperscript{SS} and TYMS\textsuperscript{SS} regulates the expression of DHFR\textsuperscript{FS}. If this is the case, a correlated expression of DHFR\textsuperscript{FS} and TYMS\textsuperscript{SS} should be noted within individual cells. A correlated expression of DHFR\textsuperscript{FS} and TYMS\textsuperscript{SS} was indeed observed in flow cytometry plots of Jurkat cells in Fig. 1E and primary T cells in Fig. 1F. A control RFP vector co-expressed with DHFR\textsuperscript{FS} but not modulated by cis expression with TYMS\textsuperscript{SS} did not appear to have the same co-expression pattern (Fig. 1F). To quantify this observation, Jurkat cells expressing [DHFR\textsuperscript{FS} & TYMS\textsuperscript{SS}] were treated with antifolates MTX, pemetrexed, and raltitrexed at varying concentrations for 2 weeks. DHFR\textsuperscript{FS}-linked eGFP mean fluorescence intensity (MFI) and TYMS\textsuperscript{SS}-linked RFP MFI for each separate well were then plotted and compared. The linked expression between DHFR\textsuperscript{FS} and TYMS\textsuperscript{SS} was significant and fit a linear regression (Fig. 1G). These findings support a general mechanism for regulation of DHFR and TYMS, which leads to a linear co-expression of DHFR\textsuperscript{FS} and TYMS\textsuperscript{SS} independent of MTX. This model is shown in Fig. 1H.

Based on the above model in Fig. 1H, it appears that TYMS\textsuperscript{SS} expression will be stabilized by DHFR\textsuperscript{FS} from strong expression changes in the presence of MTX. This was tested in Fig. 2A with primary T cells expressing DHFR\textsuperscript{FS} along with either RFP or TYMS\textsuperscript{SS} linked to RFP by applying increasing doses of MTX. As expected, DHFR\textsuperscript{FS}-linked eGFP was increased by rising concentrations of MTX, and this increase was blunted by the presence of TYMS\textsuperscript{SS} (Fig. 2A, panel I). This conserves the model in Fig. 1H where restoration of thymidine synthesis prevents the MTX-induced increase in DHFR\textsuperscript{FS}. Further conserving the model, RFP linked to TYMS\textsuperscript{SS} did not significantly increase...
over any concentration of MTX used (Fig. 2A, panel II). When DHFR<sup>FS</sup>-linked eGFP increased, so too did the control RFP, and a rise in the expression of RFP alone was not expected. A possible explanation is that this increase was noted above 0.5 μM MTX, and DHFR<sup>FS</sup> alone is only resistant to 0.5 μM MTX (9). This suggests that higher doses of MTX begin to select for cells with higher gene content of DHFR<sup>FS</sup> and associated transgenes. This is supported by Kacherovsky et al. (10) where greater DHFR<sup>FS</sup> expression was selected by the addition of MTX. Notably, DHFR<sup>FS</sup> co-expressed with TYMS<sup>SS</sup> is resistant to concentrations of up to 1 μM MTX. This further supports the use of TYMS<sup>SS</sup> to modulate transgene expression and prevent unwanted selection toward higher gene expression levels of genes expressed in cis or trans with DHFR<sup>FS</sup>.

Our group previously described a construct of DHFR<sup>FS</sup> cis expressing an inducible suicide gene, iC9 (9). This construct, called D<sup>F</sup>|iC9, selects for T cells expressing D<sup>F</sup>|iC9 in the presence of MTX and ablates D<sup>F</sup>|iC9<sup>+</sup> T cells in the presence of drug that activates iC9 to induce apoptosis (24). Based on the above findings, the DHFR<sup>FS</sup> in D<sup>F</sup>|iC9 could be used to modulate and potentially ablate the expression of a transgene of interest that is otherwise too toxic to express without regulation. IL-12 is such a transgene. IL-12 is a cytokine capable of inducing a strong immune response against tumors of specific T cells (25). However, systemic IL-12 is highly toxic and hence of low efficacy (26). Groups seeking to apply IL-12 to a specific T cells (25). However, systemic IL-12 is highly toxic and hence of low efficacy (26). Groups seeking to apply IL-12 to a clinical setting have sought inducible systems to overcome the toxicity related to this cytokine where T cells express IL-12 after activation (27). Presented here is an alternative approach where IL-12 is expressed cis to TYMS<sup>SS</sup> to decrease and stabilize the expression level of IL-12. In Fig. 2B, a flow plot demonstrates the expression of IL-12 cis-expressed with TYMS<sup>SS</sup> and iC9 cis-expressed with DHFR<sup>FS</sup> expression. The primary T cells were either left untreated or treated with high doses of MTX for 7 days. This expression pattern appears to indicate that IL-12 can be stably expressed even in toxic doses of MTX. A further analysis of similarly manipulated donors (Fig. 2C) demonstrates the potential of TYMS<sup>SS</sup> when co-expressed with DHFR<sup>FS</sup> to stabilize the expression of the potentially toxic transgenen of interest (here IL-12).

As for the endogenous regulatory mechanism proposed in Fig. 1H, there is evidence to support a natural regulatory pathway leading to linked expression of TYMS and DHFR. Regulation of TYMS expression by TYMS binding its own mRNA (19) and regulation of DHFR expression by DHFR binding its own mRNA (14) are both translationally regulated by the presence of folates. Our findings indicate that a DHFR mRNA-independent translational or post-translational mechanism is also involved in directing the co-expression of DHFR and TYMS. In this study, DHFR<sup>FS</sup> and TYMS<sup>SS</sup> expression was tracked by fluorescent proteins linked by a 2A ribosomal “slip” site. These linkages express two independent proteins due to slippage of the completed N terminus peptide at the 2A sequence. In this study, the N terminus peptide was DHFR<sup>FS</sup> and TYMS<sup>SS</sup> (28). The above findings of MTX-inducible changes suggest that if the fluorescent protein is regulated by either DHFR<sup>FS</sup> or TYMS<sup>SS</sup>, then this regulation is occurring before or during translation rather than after the proteins separate post-translationally. Thus, our findings add complexity to the translational regulation of DHFR and TYMS to meet an unknown biological need for combined expression of DHFR and TYMS. Findings by Anderson et al. (29) demonstrated that DHFR and TYMS co-localize as a multi-enzyme complex within the nucleus during replication of DNA. Stabilization and construction of this multi-enzyme complex within the cell may explain the need for tight translational control of DHFR and TYMS.

In conclusion, we have demonstrated an endogenous mechanism of post-transcriptional regulation for DHFR and TYMS that can be co-opted with either DHFR<sup>FS</sup> or TYMS<sup>SS</sup> to regulate expression of cis- or trans-expressed transgenes. The purpose of this study is to describe a novel system for transgene regulation in gene therapy. This system is of significant clinical interest as the genes described are human transgenes of low immunogenicity that utilize MTX to regulate transgene expression. MTX is readily available for use in both hospital and clinical settings. As these studies demonstrate that this phenomenon occurs in primary human T cells, this system can readily be used in the field of transgenic T cell immunotherapy to improve the safety and efficacy of T cell therapies used to treat cancer (30). What remains to be determined is how an improved understanding of the regulatory mechanism surrounding DHFR and TYMS expression may lead to a better understanding of developmental disorders and improved chemotherapeutics.

Author Contributions—D. R. designed, acquired, analyzed, and interpreted experiments and wrote the manuscript. A. M. designed experiments, acquired data, and was involved in critical revisions of the manuscript. A. A. acquired data and was involved in critical revisions of the manuscript. L. J. N. C. designed experiments and approved the final version of the manuscript for publication. All authors reviewed the results and approved the final version of the manuscript.

Acknowledgments—We extend gratitude to the dedicated work of Helen Huls, Tiejuan Mi, and Drs. Kumar, Belousova, and Singh for laboratory support. We thank Drs. Dat Tran, Dean Lee, and George McNamara for assistance in manuscript preparation, and Dr. David Spencer for assistance in developing and utilizing iC9. We thank the MDACC South Campus Flow Cytometry Facility, supported by the NCI Cancer Center Support Grant P30CA16672 through the National Institutes of Health and Cancer Center Core Grant (CA16672), for use of their facilities.

References
directed double mutants of dihydrofolate reductase. Cancer Res. 56, 4142–4145
Dihydrofolate Reductase and Thymidylate Synthase Transgenes Resistant to Methotrexate Interact to Permit Novel Transgene Regulation
David Rushworth, Amber Mathews, Amir Alpert and Laurence J. N. Cooper

doi: 10.1074/jbc.C115.671123 originally published online August 4, 2015

Access the most updated version of this article at doi: 10.1074/jbc.C115.671123

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 11 of which can be accessed free at http://www.jbc.org/content/290/38/22970.full.html#ref-list-1