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The Reduced Folate Carrier Gene Is a Novel Selectable Marker for Recombinant Protein Overexpression

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ABSTRACT

Folate cofactors are one-carbon donors essential for the biosynthesis of purines and thymidylate. Mammalian cells are devoid of folate biosynthesis and are therefore folate auxotrophs that take up folate vitamins primarily via the reduced folate carrier (RFC). In this study, we showed that the human RFC (hRFC) gene can serve as a novel selectable marker for the overproduction of recombinant proteins. Toward this end, a hemagglutinin (HA) epitope tagged hRFC (hRFC-HA) was introduced into a bicistronic vector (pIRES2-EGFP), upstream of an enhanced green fluorescent protein (EGFP) reporter gene. Chinese hamster ovary cells deficient in RFC activity were isolated and transfected with this construct, followed by gradual deprivation of leucovorin, the sole folate source in the growth medium. Only cells with hRFC-HA overexpression were able to take up leucovorin and thereby survive these selective conditions. Western blot and immunofluorescence analyses confirmed that the hRFC-HA was overexpressed at extremely high levels, properly glycosylated and sorted out to the plasma membrane. This resulted in a ~450-fold increase in [^{3}H]methotrexate influx and ~100-fold increased sensitivity to methotrexate, relative to untransfected RFC-deficient cells. Flow cytometric analysis consistently revealed that EGFP was overexpressed ~100-fold above the autofluorescence level. Overproduction of hRFC-HA and EGFP was stably maintained for at least 2 months in a constant concentration of leucovorin. These results establish a novel RFC-based metabolic selection system for the efficient overexpression of recombinant proteins. Furthermore, the possible implications to subcellular transporter localization and restoration of MTX sensitivity in drug-resistant tumors by RFC-based gene therapy are discussed.

Folic acid and reduced folates are hydrophilic vitamins that are essential for the biosynthesis of purines, thymidylate, and certain amino acids in mammalian cells (Stockstad, 1990). Unlike prokaryotes and plants, mammalian cells cannot synthesize their own folate vitamins and therefore must rely on their uptake from exogenous sources to support RNA and DNA synthesis (Matherly and Goldman, 2003). Leucovorin (5-formyl-tetrahydrofolate) and related reduced folates, including 5-methyl-tetrahydrofolate, are precursors of one-carbon donors in the de novo biosynthesis of purines and thymidylate. The primary cellular transport system for the uptake of reduced folates and folic acid antagonists (i.e., antifolates) including methotrexate (MTX) is the reduced folate carrier (RFC) (Matherly and Goldman, 2003; Zhao and Goldman, 2003). The human RFC (hRFC) is an integral plasma membrane protein containing 591 amino acids, with 12 transmembrane domains, a short N terminus, and a long C terminus, both of which reside in the cytoplasm (Ferguson and Flintoff, 1999). The hRFC with a core molecular mass of ~65 kDa contains a single *N*-linked glycosylation site in the first extracellular loop but undergoes an extensive *N*-linked glycosylation, rendering it a broadly migrating transmembrane protein with an average molecular mass of ~85 kDa (Freisheim et al., 1992; Wong et al., 1998; Drori et al., 2000b).

RFC functions as a bidirectional anion exchanger (Goldman, 1971; Henderson and Zevely, 1981), taking up folate cofactors and exporting various organic anions, including thiamine pyrophosphate (Zhao et al., 2001a). RFC is a member of the major facilitator superfamily, a large group of carriers that transport diverse organic and inorganic compounds in both prokaryotes and eukaryotes (Pao et al., 1998). The central physiological role of the mammalian RFC has been demonstrated by targeted disruption studies of the murine *RFC* locus; knockout of both alleles of the murine *RFC* gene was found to be embryonic lethal (Zhao et al., 2001b). It

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ABBREVIATIONS: MTX, methotrexate; RFC, reduced folate carrier; hRFC, human reduced folate carrier; DHFR, dihydrofolate reductase; HA, hemagglutinin; LCV, leucovorin; F-MTX, fluorescein-MTX; TMQ, trimetrexate; CHO, Chinese hamster ovary; dFCS, dialyzed fetal calf serum; PBS, phosphate-buffered saline; HA, hemagglutinin; EGFP, enhanced green fluorescent protein; HBSS, HEPES-buffered saline solution; PCR, polymerase chain reaction; GS, glutamine synthetase.

was further shown that rescue of embryonic lethality in RFC knockout mice by maternal folic acid supplementation results in early neonatal failure of hematopoietic organs.

Folic acid antagonists (i.e., antifolates) exert their cytotoxic activity by inhibiting specific enzymes in the folic acid metabolic pathway. For example, MTX (which enters cells via the RFC, like many hydrophilic antifolates) potently inhibits dihydrofolate reductase (DHFR), the first and key enzyme in folate metabolism. Therefore, cells become folate-deficient and consequently fail to synthesize purines and thymidylate, thereby resulting in inhibition of DNA synthesis and cell death via apoptosis. We have shown recently that single-step and stepwise selection with MTX can lead to inactivation of the *hRFC* gene by different mechanisms, the vast majority of which lead to stable MTX-transport-deficient phenotypes (Jansen et al., 1998; Drori et al., 2000a; Rothem et al., 2002). Inactivating mutations in the *hRFC* coding region abolish its transport activity, thereby resulting in antifolate resistance (Jansen et al., 1998; Drori et al., 2000a; Rothem et al., 2002). We further showed that another central mechanism of impaired MTX transport is the loss of *hRFC* gene expression because of silencing of its promoter primarily resulting from the loss of expression and/or function of several transcription factors (Rothem et al., 2003, 2004a,b). In addition, *hRFC* promoter methylation, although rare (Rothem et al., 2004a), has also been documented as a mechanism of transcriptional silencing (Worm et al., 2001).

After single-step or stepwise selection with various hydrophilic antifolates, including MTX, we have shown that several clonal variants with a stable MTX transport-deficient phenotype could be isolated (Assaraf and Schimke, 1987). Furthermore, in previous studies, we have established that gradual deprivation of leucovorin from a folic acid-free growth medium resulted in a marked *RFC* gene amplification with a consequent 100-fold overproduction of MTX transport activity (Jansen et al., 1990; Drori et al., 2000b). Based on the above findings, we hypothesized that transfection of an *hRFC* gene as a potentially novel selectable marker into RFC-deficient cells followed by selection in gradually decreasing concentrations of leucovorin (LCV) as the sole folate source should result in overexpression of the *RFC* gene to allow for sufficient reduced folates to be transported into the folate-deprived cells. The co-overexpression of a gene of interest along with the selectable marker (i.e., hRFC) should be significantly enhanced in a bicstonic vector configuration. Indeed, we show here that the *hRFC* gene can be used as a selectable marker for the high-level overexpression of recombinant proteins. The possible implications to subcellular transporter localization and restoration of MTX sensitivity in drug-resistant tumors by RFC-based gene therapy are discussed.

Materials and Methods

Drugs, Biochemicals, and Radiochemicals. LCV and MTX were purchased from Sigma-Aldrich Chemie BV (Zwijndrecht, the Netherlands). Fluorescein-MTX (F-MTX) was from Molecular Probes (Eugene, OR). [^{3}H]MTX (23.4 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA), purified before use by thin-layer chromatography (Jansen et al., 1990b), and stored at −80°C. Trimetrexate (TMQ) was a generous gift from Dr. D. Fry (Warner-Lambert/Parke-Davis, Detroit, MI). pIRES2-EGFP was purchased from BD Biosciences Clontech (Palo Alto, CA).

Isolation of MTX Transport-Deficient Cells. Chinese hamster ovary (CHO) AA8 cells were obtained from Dr. C. Hoy (originally from the lab of Dr. L.H. Thompson, Lawrence Livermore National Laboratory, Livermore, CA). Cells (5×10^5 cells/10 cm Petri dish; 20 ml of medium) growing under monolayer conditions were maintained in RPMI-1640 medium containing 2.3 μM folic acid (Biological Industries, Beth-Haemek, Israel) supplemented with 10% fetal calf serum (Invitrogen, Leek, The Netherlands), 2 mM glutamine, 100 units/ml penicillin G (Sigma) and 100 μg/ml streptomycin sulfate (Sigma) (Assaraf and Schimke, 1987). Single-step MTX selections were performed as follows: parental CHO AA8 cells were seeded (5×10^5 cells) in 10-cm Petri dishes in growth medium containing 10% dialyzed fetal calf serum (dFCS; Invitrogen) and 150 nM MTX (~10 times the 50% inhibitory concentration, IC_{50}). After 18 days of MTX exposure, several independent clones were picked (colony size, ~80–300 cells) using sterile cloning rings. Of these initial MTX-resistant clones, only one (i.e., C5 $MTX^{R0.15}$) was used for transfections.

Assay of F-MTX Staining and Competition with Hydrophilic and Lipophilic Antifolates. Cells were seeded in a 60-mm Petri dish and incubated in growth medium containing 2 μM F-MTX for 8 h at 37°C. Cells were then washed with PBS and subjected to competition with MTX and its lipophilic analog TMQ. After 3 h of antifolate competition, cells were detached by trypsinization and suspended in PBS containing 1% fetal calf serum; the residual green fluorescence per cell was determined with a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) with excitation set at 488 nm and emission at 525 nm. Autofluorescence of unstained cells was routinely recorded.

Construction of the pIRES2-EGFP/RFC-HA Vector. The human RFC-HA construct containing the human influenza virus hemagglutinin (HA) epitope tag (YPYDVPDYA) at the C terminus of the RFC was constructed as described previously (Ferguson and Flintoff, 1999; Liu and Matherly, 2002). The small HA epitope tag at the C-terminal end of the hRFC does not interfere with its folate/MTX transport activity (Ferguson and Flintoff, 1999; Liu and Matherly, 2002). This HA tag could be easily identified with commercially available monoclonal antibodies, thereby allowing for the facile follow-up after RFC-HA overexpression in the transfectants. This was essential to distinguish hRFC-HA expression from the endogenously inactivated hamster *RFC* gene product that may potentially undergo some reversion and re-expression. The hRFC cDNA was excised with XhoI and BamHI from the phRFC expression vector (Drori et al., 2000b), after which the HA epitope tag was inserted in the C terminus of the hRFC to yield the hRFC-HA as described previously (Ferguson and Flintoff, 1999; Liu and Matherly, 2002). For the construction of the hRFC-HA expression vector, we used a CMV-driven bicistronic vector termed pIRES2-EGFP (5.3 kb); the pIRES2-EGFP vector contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus between the multiple cloning site and the enhanced green fluorescent protein (*EGFP*) gene. This permits both the gene of interest (cloned into the MCS) and the *EGFP* gene to be translated from a single bicistronic mRNA. In brief, the hRFC-HA fragment (~ 2 kb) was ligated into the pIRES2-EGFP vector that was digested with BglII and SalI. The final ligation product (phRFC-HA-IRES2-EGFP) of ~7.3 kb is depicted in Fig. 3. An expression vector harboring the hRFC without an HA tag was constructed with the hRFC cDNA (Drori et al., 2000b). The hRFC in pcDNA3 was excised with XhoI and BamHI and directionally cloned upstream of the IRES element in the pIRES2-EGFP vector at the BglII and SalI site. The final ligation product (phRFC-IRES2-EGFP) of ~7.2 kb is depicted in Fig. 3.

Establishment of Stable RFC and RFC-HA Transfectants. Exponentially growing monolayer C5 $MTX^{R0.15}$ cells (2×10^7) were detached by standard trypsinization. Cells were then transfected by electroporation (1000 μF, 234 V) (Rothem et al., 2003) with 10 μg of the EGFP-based bicistronic expression vectors coding for the *hRFC* or *hRFC-HA* genes. After transfection, cells were transferred to folic acid-free RPMI-1640 medium supplemented with 10% dFCS and

only 2 nM LCV as the sole folate source and grown at 37°C for 48 h. Then, cells were transferred to folic acid-free RPMI-1640 medium containing 10% dFCS, either 450 or 600 μg/ml G-418 (Calbiochem, San Diego, CA) and 2 nM LCV as the sole folate source. Five weeks later, cells growing in 600 μg/ml G-418 and 2 nM LCV were subjected to a more stringent selection by simultaneously transferring them to a folic acid-free medium containing 1, 0.5, and 0.25 nM LCV as the sole folate source in the presence of a constant concentration of 600 μg/ml G-418. Cells were grown under these selective conditions for at least 1 month, at which time cells were further grown in the absence or presence of G-418.

Flow Cytometric Analysis of Cellular EGFP Expression. Monolayer cells growing in 25-mm tissue culture flasks were washed with PBS, detached by trypsinization, and suspended at 10^6 cells/ml in PBS containing 1% fetal calf serum. Cells were then analyzed for green fluorescence per cell using a flow cytometer at the above excitation and emission.

Inhibition of Cell Growth by Antifolates. For antifolate growth inhibition, C5 MTX$^{R0.15}$ and its RFC-HA transfectant cells (10^4/well) were seeded in 24-well plates in the respective growth medium containing various concentrations of MTX or its lipophilic antifolate TMQ for 3 days at 37°C. Thereafter, cells were detached by trypsinization and the number of viable cells was determined using trypan blue exclusion. IC_{50} is defined as the drug concentration at which cell growth was inhibited by 50% relative to untreated control cells.

Assay of [^{3}H]MTX Transport. To determine the ability of the antifolate-resistant clone (i.e., C5 MTX$^{R0.15}$) and its hRFC- and hRFC-HA transfectants to take up antifolates, we measured the influx of [^{3}H]MTX relative to parental AA8 cells. Cells (2×10^7) from the mid-log phase of growth were washed three times in transport buffer consisting of HEPES-buffered saline solution (HBSS) (Rothem et al., 2002) and incubated at 37°C for 3 min in HBSS (1-ml suspensions) containing 2 μM [^{3}H]MTX (23.4 Ci/mmol). The specific activity of [^{3}H]MTX was 1320 dpm/pmol. Transport controls contained 1000-fold excess (2 mM) of nonradioactive MTX. [^{3}H]MTX transport was stopped by the addition of 10 ml of ice-cold HBSS. The cell suspension was then centrifuged at 500*g* for 5 min at 4° C, and the cell pellet was washed twice with 10 ml of ice-cold transport buffer. The final cell pellet was lysed in water and processed for scintillation counting.

Extraction of Membrane Proteins. Exponentially growing cells were detached by trypsinization, washed three times with PBS and harvested by centrifugation. Cells (1×10^6–3×10^6) were then incubated in a lysis buffer containing 50 mM Tris-HCl, pH 7.5, 50 mM 2-mercaptoethanol, 0.5% Triton X-100, and a Complete mini mixture of protease inhibitors (Roche, Mannheim, Germany) containing 10 μg/ml phenylmethyl sulfonylfluoride, 60 μg/ml aprotinin, 5 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM EGTA (pH 8), and 1 mM EDTA (pH 8) (Rothem et al., 2002). After 1 h of incubation on ice, the protein extract was centrifuged, and aliquots of the supernatant were stored at −80°C until analysis. Protein content was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Western Blot Analysis. hRFC-HA expression was determined by Western blot analysis as follows: microsomal proteins were resolved by electrophoresis on 10% polyacrylamide gels containing SDS and electroblotted onto nitrocellulose nylon membranes (Schleicher & Schuell, Riehen, Switzerland). The blots were then blocked for 1 h at room temperature in TBS buffer (150 mM NaCl and 0.5% Tween 20 and 10 mM Tris/Cl, pH 8.0) containing 1% skim milk. The blots were then reacted with the H12 monoclonal antibody (Covance Inc., Princeton, NJ) directed to the HA epitope (1:1000 dilution of the supernatant). Blots were then rinsed in the same buffer for 10 min in room temperature and reacted with a horseradish peroxidase-conjugated goat anti-mouse IgG (1:40,000 dilution; Jackson Immunoresearch Labs, West Grove, PA) for 1 h at room temperature. After three 10-min washes in TBS at room temperature, enhanced chemiluminescence detection was performed according to the manufacturer's instructions (Biological Industries).

Immunofluorescence Staining. Cells (10^4) were seeded in 24-well plates (1 ml of medium/well) on sterile glass coverslips and incubated for 3 days at 37°C. Then, the growth medium was removed and monolayer cells were washed twice with PBS and fixed with 4% formaldehyde in PBS for 10 min. The coverslips containing the fixed monolayer cells were washed twice with PBS and then incubated for 20 min in a solution of 80% methanol in double-distilled water. The coverslips were washed twice with PBS, blocked for 1 h at room temperature in PBS containing 1% skim milk, and reacted with anti-HA monoclonal antibody H12 (1:100 dilution; Covance Inc.). Then, a fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (1:100 dilution; Sigma) was added, after which two washes with PBS were performed. Cell nuclei were stained for 60 min at room temperature with 2 μg/ml of the DNA dye propidium iodide (Sigma). After four washes with 2 ml of PBS, the coverslips were mounted onto glass slides using fluoromount-G (Southern Biotech; Birmingham, AL) and the cells were then examined using a Bio-Rad confocal microscope.

Analysis of hRFC Gene Copy Number by Semiquantitative Genomic PCR. Genomic DNA isolated from hRFC-HA–transfected cells as well as from MCF-7 cells was diluted up to 3000-fold and then amplified using 10 pmol of each primer in 2× ReddyMix PCR master mix reaction buffer according to the instructions of the manufacturer (ABgene, Surrey, UK). The PCR reaction was performed as follows: initial melting at 95°C for 5 min, followed by 30 or 35 cycles each of 1 min at 95°C, annealing at 60°C for 45 s, elongation at 72°C for 1 min, followed by 10-min extension at 72°C. Then, the 276-base pair PCR product was resolved on 1% agarose gels containing ethidium bromide. The primers used for the semiquantitative genomic PCR were described previously (Rothem et al., 2002).

Results

Establishment of MTX Transport-Deficient Cells. We have shown previously that exposure of CHO AA8 cells to a single dose of 150 nM MTX (~10 times the IC_{50}) or other hydrophilic antifolates frequently results in drug resistance that is due to the loss of RFC transport activity (Assaraf and Schimke, 1987; Rothem et al., 2002). Identification of MTX transport-deficient clones was achieved using an assay in which F-MTX bound to intracellular DHFR was displaced by hydrophilic and lipophilic antifolates (Assaraf and Schimke, 1987). After the isolation of MTX-resistant CHO AA8 clones by a single-dose exposure to 150 nM MTX, characterization of the molecular basis of this antifolate resistance was undertaken. Thus, the F-MTX fluorescence of parental AA8 cells was completely displaced upon incubation with as low as 30 nM MTX (Fig. 1A), whereas 100-fold more MTX (i.e., 3 μM) was necessary to achieve a complete F-MTX displacement in C5 MTX$^{R0.15}$ cells, an MTX-resistant clone (Fig. 1B). TMQ (10 nM), a lipophilic antifolate that enters cells by diffusion (Fry and Besserer, 1988), consistently was sufficient to induce a complete loss of F-MTX staining in both parental AA8 (Fig. 1A) and C5 MTX$^{R0.15}$ cells (Fig. 1B). These results suggest that C5 MTX$^{R0.15}$ cells are markedly defective in MTX uptake.

Collateral Sensitivity of C5 MTX$^{R0.15}$ Cells to TMQ. Cells that are impaired in RFC transport activity, display a typical phenotype of hypersensitivity to the lipid-soluble antifolate TMQ in that they show marked shrinkage in the intracellular folate pools (Rothem et al., 2002). We therefore performed TMQ growth inhibition experiments with parental AA8 and C5 MTX$^{R0.15}$ cells; the latter cells were 7-fold

more sensitive to TMQ than their parental AA8 cells (IC_{50} = 38 ± 6 nM). These results provide further evidence to the loss of RFC transport activity in C5 $MTX^{R0.15}$ cells, thereby resulting in a markedly diminished intracellular folate pool and consequent collateral sensitivity to TMQ.

Loss of [^{3}H]MTX Transport in C5 $MTX^{R0.15}$ Cells. To provide direct evidence that C5 $MTX^{R0.15}$ cells are defective in MTX transport, we used the [^{3}H]MTX transport assay. Initial rates (3 min incubation at 37° C) of [^{3}H]MTX uptake were determined using an extracellular concentration of 2 μM. C5 $MTX^{R0.15}$ cells lost more than 98% of MTX transport present in parental AA8 cells (Fig. 2). Hence, C5 $MTX^{R0.15}$ cells were confirmed to be RFC transport-deficient by both the direct [^{3}H]MTX transport assay and the indirect F-MTX displacement assay.

Establishment of a Novel RFC-Based System for Target Gene (e.g., EGFP) Overexpression. C5 $MTX^{R0.15}$ cells shown to be MTX transport-deficient were used as the host for the transfection of a bicistronic hRFC construct that contained a downstream *EGFP* reporter gene (Fig. 3). To discern between the endogenous and the transfected hRFC, an HA tag was inserted in the C terminus of hRFC (i.e., hRFC-HA).

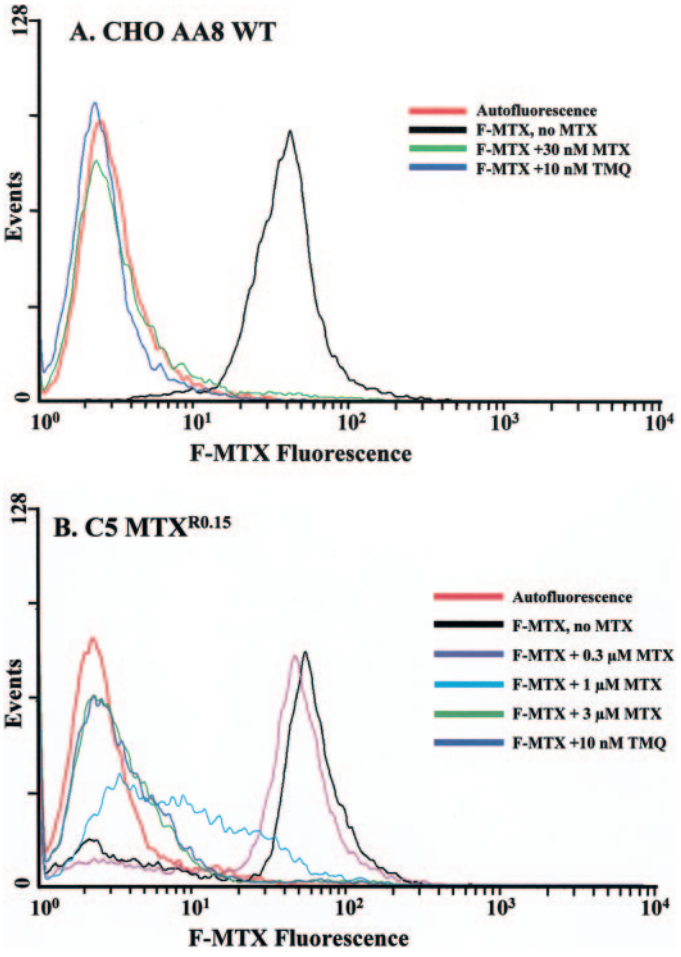

Fig. 1. Antifolate displacement of F-MTX–labeled cells. Wild-type CHO AA8 cells (A) and their MTX-resistant clone C5 $MTX^{R0.15}$ cells (B) were stained with F-MTX and subjected to a competition with various concentrations of MTX or TMQ. Mean fluorescence per cell was then determined by a flow cytometer. Displacement of F-MTX with MTX indicates functionally active RFC, whereas retention of F-MTX fluorescence after competition with high MTX concentrations is indicative of MTX transport defect (i.e., loss of RFC function).

Twenty-four hours after transfection of the hRFC and hRFC-HA expression constructs into the clonal C5 $MTX^{R0.15}$ subline, cells were incubated in a folic acid-free medium containing nanomolar concentrations of the reduced folate LCV as the sole folate source. Then, a gradual deprivation of LCV was undertaken in the presence of a constant concentration of G-418 over the subsequent weeks. Flow cytometric analysis of viable cells after several weeks of LCV deprivation (i.e., 2 nM through 0.25 nM) revealed a 10- to 100-fold increase in EGFP fluorescence in both the untagged (Fig. 4A) and the HA-tagged hRFC-HA transfectants (Fig. 4B), relative to the autofluorescence of untransfected cells. Because these results pointed to the impact of LCV deprivation on the

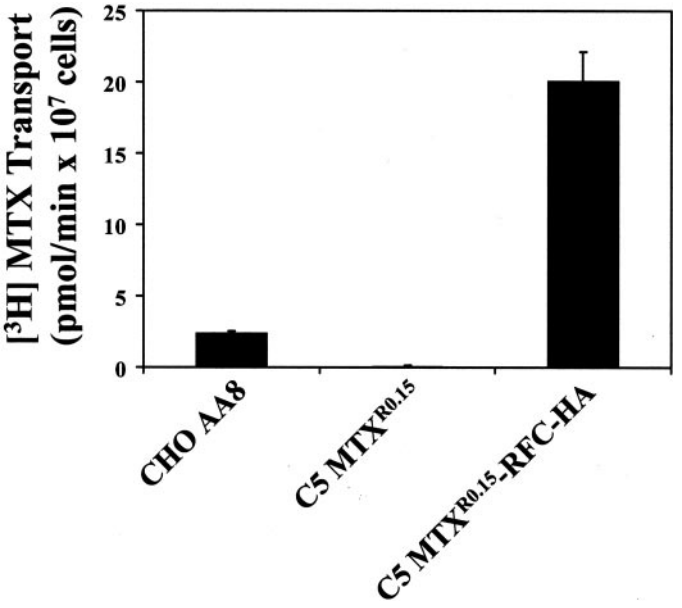

Fig. 2. [^{3}H]MTX transport in parental AA8, C5 $MTX^{R0.15}$, and RFC-HA transfectant cells. Initial rates of [^{3}H]MTX transport were determined in parental AA8, MTX-resistant cells as well as their RFC-HA transfectants as described under *Materials and Methods*. Results shown are the means of three independent experiments ± S.D.

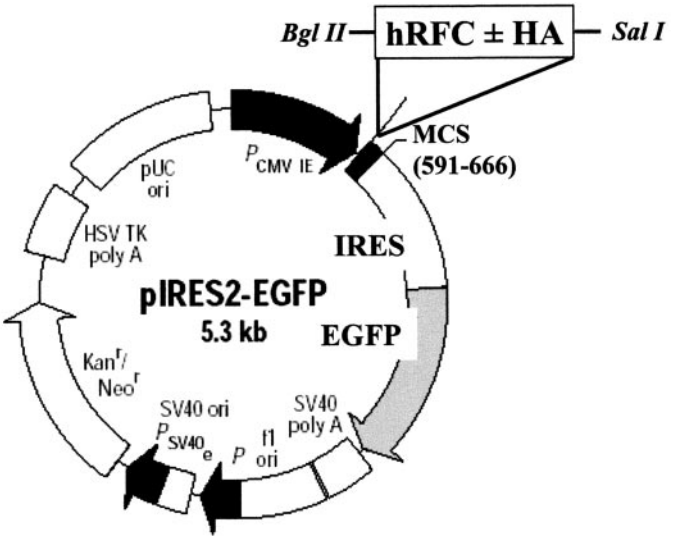

Fig. 3. Bicistronic vector harboring the HA-tagged or untagged hRFC and the *EGFP* reporter gene. RFC and RFC tagged with hemagglutinin in the C-terminal fragments (length of ~2 kilobase pairs) were cloned into pIRES2-EGFP vector. EGFP served as a reporter gene of interest.

overexpression of the downstream EGFP reporter, we examined whether the selectable hRFC-HA marker placed upstream of the EGFP reporter gene was also overexpressed. Thus, hRFC-HA expression was determined in transfectant cells by Western blot analysis using monoclonal antibodies to the HA epitope (Fig. 5). C5 MTX$^{R0.15}$ RFC-HA transfectant cells growing in a selective medium containing 1 nM LCV and 600 μg/ml G-418 expressed extremely high levels of the hRFC-HA, even when exposing the film for a few seconds after the enhanced chemiluminescence reaction (Fig. 5A, lanes 2 and 5). Consistent with our previous results, this transporter protein appeared as a broadly migrating, highly glycosylated protein with an average molecular mass of ~85 kDa (Wong et al., 1998; Drori et al., 2000b; Rothem et al., 2002), compared with its unglycosylated core molecular mass of ~65 kDa (Wong et al., 1998; Drori et al., 2000b). As expected, no anti-HA signal could be detected with the untransfected C5 MTX$^{R0.15}$ cells (Fig. 5, lanes 1 and 4). Consistently high levels of hRFC-HA were also observed in the transfectants that were gradually deprived of LCV from 2 nM through 0.25 nM (Fig. 5B, lanes 2–5).

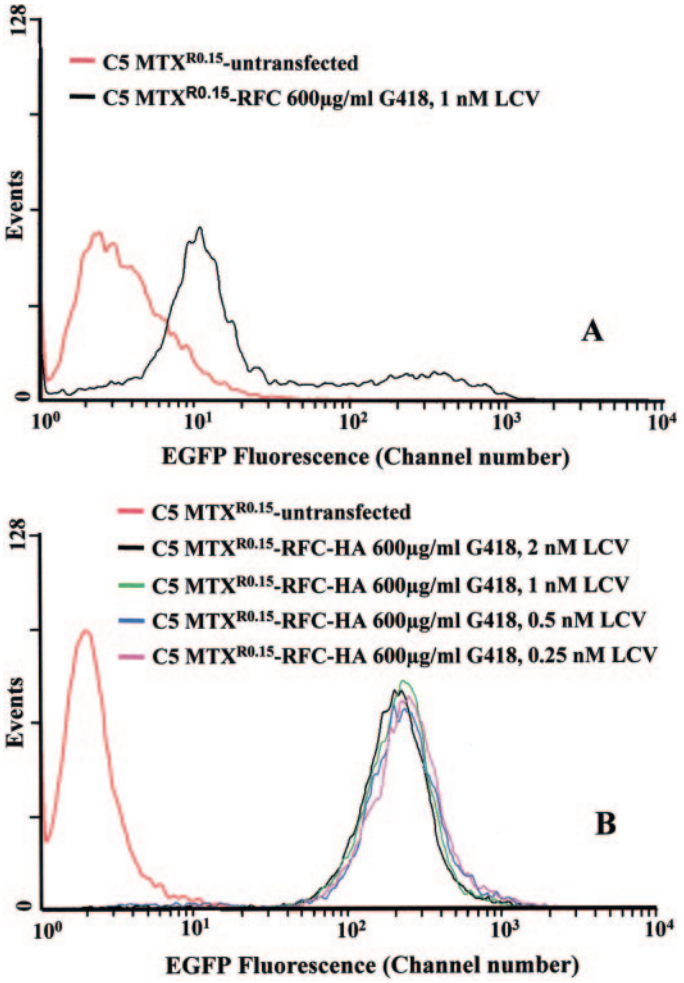

Fig. 4. Flow cytometric analysis of EGFP expression in C5 MTX$^{R0.15}$ cells transfected with pIRES2-EGFP/RFC ± HA vector. Untransfected C5 MTX$^{R0.15}$ cells (A and B) as well as their RFC (A) and RFC-HA transfectants (B) doubly selected for 6 weeks in folic acid-free medium supplemented with 0.25 to 2 nM LCV and 600 μg/ml G418 were analyzed by flow cytometry for mean linear green fluorescence per cell.

Subcellular Localization of hRFC-HA. To ascertain that the glycosylated hRFC-HA was properly targeted to the plasma membrane, we used confocal laser microscopy after immunofluorescent staining of paraformaldehyde-fixed cells with an anti-HA monoclonal antibody (Fig. 6). The intense green fluorescence corresponding to the highly overexpressed hRFC-HA was confined to the plasma membrane of the hRFC-HA transfectant (Fig. 6A), whereas no staining was observed when no anti-HA antibody was added (Fig. 6B). In the latter group, however, a weak, whole-cell green fluorescence of the overexpressed EGFP was observed only upon signal intensification. In contrast, in the untransfected cells, no fluorescent signal was apparent when the anti-HA antibody was added (Fig. 6C). Counterstaining with the red DNA dye propidium iodide served to localize nuclei and their borders (Fig. 6, D–F); merging the hRFC-HA green fluorescence and the red nuclear fluorescence in the transfectant cells clearly established that hRFC-HA was highly confined to the plasma membrane (Fig. 6G). In contrast, only red nuclear fluorescence could be detected in transfectants to which no HA antibody was added (Fig. 6H) or in untransfected cells incubated with an anti-HA antibody (Fig. 6I).

Functionality of the Overexpressed hRFC-HA. To confirm that the overexpressed hRFC-HA was a functional transporter, [^{3}H]MTX transport and MTX growth inhibition assays were carried out. Indeed, the high level overexpression of hRFC-HA was associated with a dramatic increase in [^{3}H]MTX transport (Fig. 2); the hRFC-HA transfectant growing in 1 nM LCV displayed a MTX influx activity of 20.1 ± 2.0 relative to 0.045 ± 0.015 in untransfected C5 MTX$^{R0.15}$ cells (Fig. 2). Thus, the hRFC-HA transfectant exhibited increases

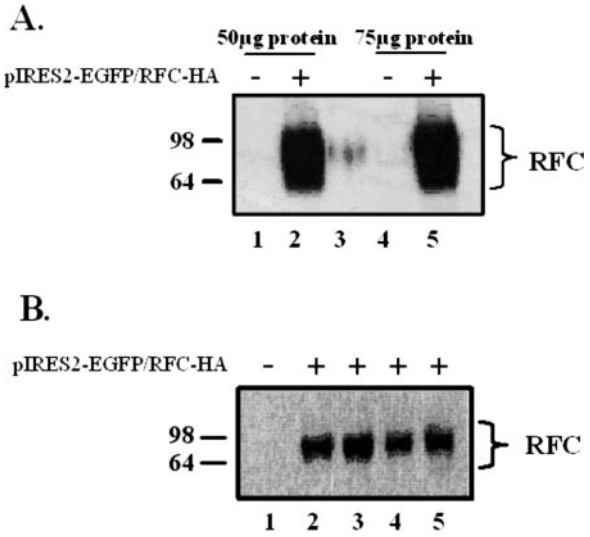

Fig. 5. Western blot analysis of RFC-HA expression in C5 MTX$^{R0.15}$ cells. A, Triton X-100–soluble proteins were first extracted from untransfectant CHO C5 MTX$^{R0.15}$ cells (lanes 1 and 4), and their RFC-HA transfectants were grown for 4 weeks in folic acid-free medium containing 600 μg/ml G-418 and 1 nM LCV (lanes 2 and 5). Proteins (50 μg, lanes 1 and 2; 75 μg, lanes 4 and 5; no proteins were loaded in lane 3) were then resolved by 10% SDS polyacrylamide gel electrophoresis, transferred to a nylon membrane, and reacted with a monoclonal antibody to the HA tag. The membranes were developed using a standard enhanced chemiluminescence procedure. B, in addition, Western blot analysis showed that all transfectants that expressed high levels of RFC grew with 600 μg/ml G418 and in a range of LCV concentrations (lanes 2–5). Lane 1, untransfected C5 MTX$^{R0.15}$ cells (negative control); Lanes 2, 3, 4, and 5, C5 MTX$^{R0.15}$-RFC-HA transfectants grown with 2, 1, 0.5, and 0.25 nM LCV, respectively.

in MTX transport activity as high as ~450-fold relative to their C5 MTX$^{R0.15}$ counterpart.

RFC-deficient cells overexpressing a functional transporter are expected to be much more sensitive to MTX than their MTX-resistant C5 MTX$^{R0.15}$ cells. Thus, untransfected C5 MTX$^{R0.15}$ cells displayed a very high MTX IC_{50} value of ~3 μM (Fig. 7), as would be expected from MTX transport-defective cells (Assaraf and Schimke, 1987; Rothem et al., 2002, 2003). In contrast, the hRFC- and hRFC-HA transfectants displayed IC_{50} values of ~27 and ~29 nM, respectively, making them 2 orders of magnitude more sensitive to MTX than the untransfected C5 MTX$^{R0.15}$ cells (Fig. 7). Hence, the hRFC- and hRFC-HA transfectants expressed 100- to 450-fold more functional hRFC and hRFC-HA transporters than their untransfected C5 MTX$^{R0.15}$ counterparts. These results establish that C-terminal HA tagging of the hRFC did not interfere with its transport function, in that transfected cells containing or lacking the HA tag showed an equal increase in MTX sensitivity. This is consistent with previous studies showing that HA-tagged hRFC was as functional as the untagged RFC (Wong et al., 1998; Ferguson and Flintoff, 1999; Liu and Matherly, 2002).

Stability of EGFP Expression under Constant LCV Concentrations. To examine the stability of reporter gene (i.e., EGFP) overexpression under constant metabolic selection conditions, EGFP levels were determined in a constant concentration of 1 to 2 nM LCV in the presence or absence of G-418 for up to 2 months. Flow cytometric analysis revealed that EGFP expression was higher in transfectants growing in a constant concentration of LCV in the absence of G-418 than in its presence (Fig. 8). These results suggest that a high level of EGFP overexpression was stably maintained in the hRFC-HA transfectants at nanomolar concentrations of LCV for at least 2 months in the absence of G-418.

Molecular Basis of hRFC-HA and EGFP Overexpression. We showed recently that gradual deprivation of LCV from the growth medium of human leukemia cells with intact RFC function results in *RFC* gene amplification (Drori et al., 2000b) and consequent overexpression of this transporter (Jansen et al., 1990; Drori et al., 2000b). Thus, genomic DNA was isolated from the hRFC-HA transfectant and from MCF-7 human breast cancer cells (a control cell line with a single *hRFC* copy per haploid), after which semiquantitative genomic PCR was undertaken using up to 3000-fold genomic DNA dilutions (Fig. 9). The intensity of the 276-base pair hRFC product after 30 and 35 cycles of genomic PCR amplification in both the hRFC-HA transfectant and MCF-7 cells was identical. These results suggest that the high level of hRFC-HA overexpression was not due to gene amplification.

Discussion

Several lines of evidence establish that the novel bicistronic RFC-based metabolic selection system devised here is an efficient and thus attractive approach for the overproduction of recombinant proteins. First, flow cytometric analysis of reporter gene (EGFP) overexpression in transfectant cells established that gradual deprivation of LCV from the growth medium resulted in a ~100-fold increase in EGFP fluorescence, relative to the autofluorescence of untransfected cells. Western blot and immunofluorescence analyses consistently confirmed the high-level overexpression of the selectable marker (i.e., hRFC) that, under physiological conditions, is ubiquitously expressed in various tissues and cell lines, albeit at relatively low levels. Second, both the reporter and the selectable marker retained high level overexpression even after 2 months of growth in a moderately selective medium

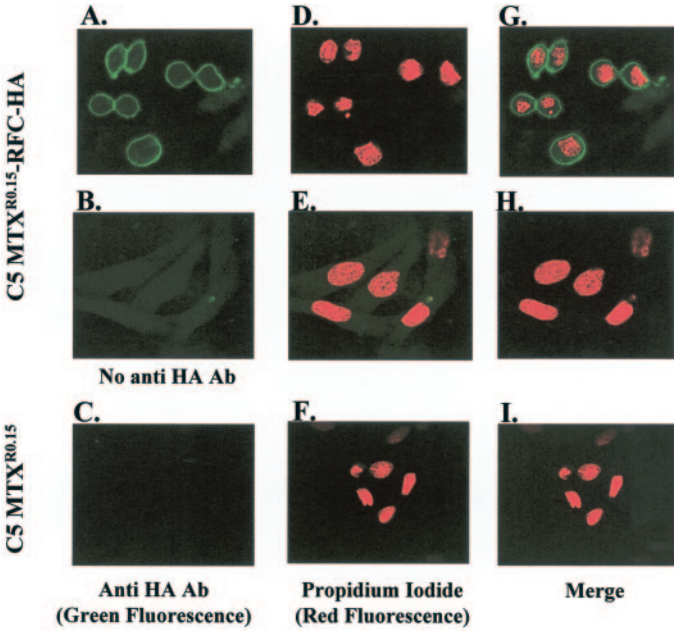

Fig. 6. Immunofluorescence analysis of RFC-HA expression and subcellular localization. RFC-HA transfectants cultured in 1 nM LCV and 600 and μg/ml G418 (A, B, D, E, G, and H) and their parental MTX$^{R0.15}$ cells (C, F, and I) were fixed with 4% formaldehyde, treated with lysis buffer, and stained with an HA-specific monoclonal antibody (A and C) followed by secondary fluorescein-conjugated antibodies (green fluorescence). Cells were then counterstained with the red fluorescence DNA dye propidium iodide (D–F). Merging the green and red fluorescence (G–I) facilitated the identification of nuclei and the plasma membrane localization of the overexpressed RFC-HA (G). A negative control of RFC-HA transfectants to which no anti-HA antibody was added was also included (B).

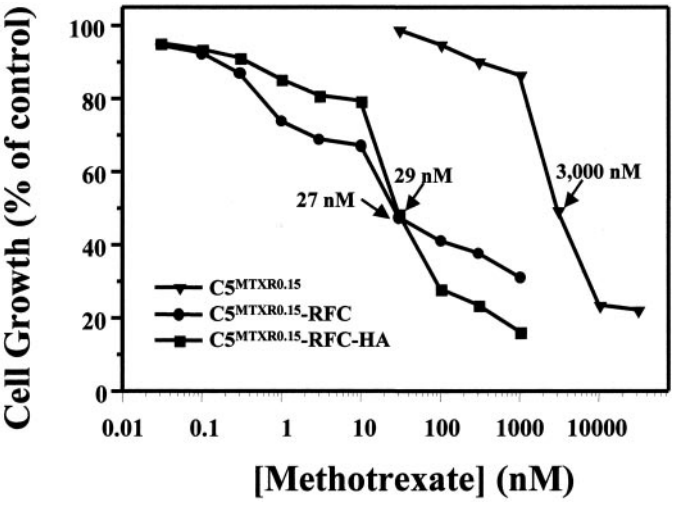

Fig. 7. MTX growth inhibition of C5 MTX$^{R0.15}$ cells and their RFC and RFC-HA transfectants. C5 MTX$^{R0.15}$ cells (▼) and their RFC (●) and RFC-HA (■) transfectants were incubated for 3 days in various concentrations of MTX. Then, cell survival was determined by trypan blue exclusion, and the IC_{50} values were determined as detailed under *Materials and Methods* (see arrows). The 100-fold increase in MTX sensitivity in both transfectants establishes that these cells overexpressed a functional RFC that is capable of transporting MTX.

lacking G-418. Third, the metabolic selection based upon gradual folate deprivation did not interfere with vital cellular functions, including:

1. *N*-linked glycosylation of the selectable marker, hRFC. The latter has a core molecular mass of ~65 kDa, but upon extensive *N*-linked glycosylation, it becomes a broadly migrating ~85-kDa glycoprotein (Wong et al., 1998; Drori et al., 2000b). Indeed, transfected cells that were gradually deprived of LCV highly overexpressed hRFC-HA with an average molecular mass of ~85 kDa.
2. Sorting of proteins to their target destination was intact; immunofluorescence and confocal laser microscopy revealed that hRFC-HA was properly sorted out to the plasma membrane.

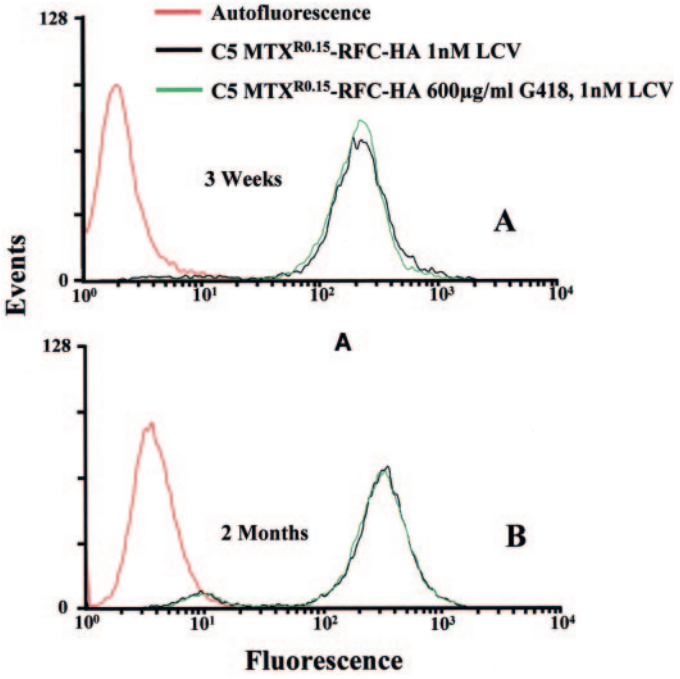

Fig. 8. Stability of EGFP expression under conditions of constant LCV concentration. Flow cytometric analysis of EGFP expression in RFC-HA–transfected C5 MTX$^{R0.15}$ cells. RFC-HA transfectants were grown in selective medium containing dFCS and 1 or 2 nM LCV in the presence of 600 μg/ml G-418 or after G-418 removal. RFC-deficient C5 MTX$^{R0.15}$ cells (negative control), which could not grow in the selective medium, were grown in nonselective medium containing 2.3 μM folic acid and supplemented with 10% fetal calf serum.

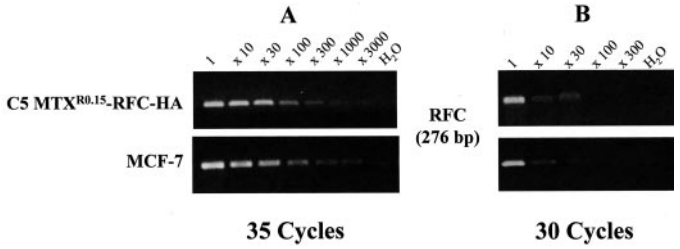

Fig. 9. Analysis of *hRFC* gene copy number in hRFC-HA transfectants by semiquantitative genomic PCR. To assess whether the transfected *RFC* gene underwent gene amplification during the LCV deprivation, semiquantitative genomic PCR was performed for 35 (A) or 30 cycles (B) using up to 3000-fold dilutions of the genomic DNA as detailed under *Materials and Methods*. DNA isolated from MCF-7 cells was used as a control human DNA that contains a single copy *hRFC* gene per haploid. The endogenous hamster *RFC* gene was not amplified under these PCR conditions.

3. hRFC-HA transfectant cells showed no significant prolongation in the growth rate relative to their C5 MTX$^{R0.15}$ counterpart; these cell lines displayed a similar doubling time of ~28 h.

The potency of the hRFC-HA gene as a functional selectable marker is exemplified by the following findings. First, the transfectants exhibited high-level overexpression of the hRFC-HA protein, as revealed by both immunoblotting and immunofluorescence. Second, these transfectant cells displayed a dramatic increase (450-fold) in the initial uptake rates of MTX, a bona fide transport substrate of the RFC (Goldman, 1971; Matherly and Goldman, 2003; Zhao and Goldman, 2003). Third, the introduction of the hRFC-HA into MTX-resistant C5 MTX$^{R0.15}$ cells rendered them ~100-fold more sensitive to MTX than their untransfected counterparts. This further confirmed that the epitope-tagged hRFC-HA was fully functional when compared with the untagged hRFC. This finding of the functionality of the C-terminally epitope tagged hRFC is in accord with previous reports (Wong et al., 1998; Ferguson and Flintoff, 1999; Liu and Matherly, 2002). It is reasonable to propose that future transfection of one of the mutant hRFC transporters that we have recently identified with a poor folate/MTX transport V_{max} would further facilitate hRFC overexpression to meet the high cellular requirement for reduced folates (Jansen et al., 1998; Drori et al., 2000a; Rothem et al., 2002).

The rationale behind the choice of the *RFC* gene as a selectable marker is based upon the following considerations:

1. Unlike bacteria and plants, mammalian cells are folate auxotrophs, in that they are devoid of folate biosynthesis. Hence, mammalian cells rely on an obligatory uptake system (i.e., the RFC) that can transport these anionic vitamins into proliferating cells. The central role that this transporter plays has recently been demonstrated by targeted disruption of the murine *RFC* gene, resulting in embryonic lethality (Zhao et al., 2001b).
2. Mammalian cells grown in medium that is very low in folate cofactors cannot support purine and thymidylate biosynthesis, thereby resulting in inhibition of DNA synthesis and cell death.
3. Provision of nanomolar concentrations of oxidized folates such as folic acid or reduced folates (e.g., LCV) is sufficient to rescue folate-deprived cells from cell death (Jansen et al., 1990, 1998; Drori et al., 2000a, 2000b; Rothem et al., 2002). This is strongly supported by in vivo data with *RFC* knockout mice, in which maternal folic acid supplementation rescued RFC-null embryos from the lethal effect of the loss of folate uptake (Zhao et al., 2001b).
4. We have shown recently that gradual deprivation of LCV from the growth medium of human leukemia cells results in *RFC* gene amplification (Drori et al., 2000b), 100-fold overexpression of the RFC protein, and a parallel increase in MTX transport activity (Jansen et al., 1990; Drori et al., 2000b). It is remarkable that this *RFC* amplicon retained the original telomeric position of the *RFC* gene in chromosome 21 (Drori et al., 2000b). Hence, the *hRFC* gene can be readily amplified and/or highly overexpressed in response to folate deprivation.

The *hRFC* gene is a unique example of gene amplification achieved in the absence of a cytotoxic drug selection (Schimke, 1988). However, in the current study, we found that the overexpression of the hRFC-HA was not due to gene amplification. Hence, we are currently characterizing the mechanisms that stably augment the transcription and/or translation of the *hRFC-HA* gene, resulting in overproduction of the hRFC-HA protein and the EGFP reporter.

Recombinant expression systems that are based on metabolic selection have been described previously. First, CHO or other mammalian cells lacking the ammonia breakdown enzyme glutamine synthetase (GS) were transfected with the GS gene followed by selective growth in medium lacking L-glutamine in the presence or absence of increasing concentrations of the cytotoxic GS inhibitor L-methionine-*S*-sulfoximine (Bebbington et al., 1992; Johnstone and Harfst, 1992; Pu et al., 1998; Zou and Sun, 2004). This led to the overexpression of various proteins, including a soluble form of the human interleukin-4 receptor α chain (Pu et al., 1998), transforming growth factor-β1 (Zou and Sun, 2004), and integral membrane proteins, such as thyrotropin receptor, because of 3- to 50-fold amplification of the thyrotropin receptor gene (Johnstone and Harfst, 1992). Second, two additional metabolic selective systems have been developed that employ the *Escherichia coli* tryptophan synthase B2 (*TrpB*) gene as well as the histidinol dehydrogenase (*HisD*) gene (Hartman and Mulligan, 1988) in an attempt to result in recombinant gene transfer. Thus, the TrpB gene product catalyzes the conversion of indole + L-serine to L-tryptophan, whereas histidinol dehydrogenase, the HisD gene product, catalyzes the two-step NAD^+-dependent oxidation of L-histidinol to L-histidine. Again, the use of millimolar concentrations of the cytotoxic L-histidinol was the major selective force in the HisD system. However, despite the initially successful functional complementation of mammalian cells' auxotrophy, these systems are not suited for target protein overexpression; in fact, since their introduction, there has been very little use of these metabolic selective systems.

The human RFC-based bicistronic metabolic selection for target protein overexpression is well suited for bioreactor scaling-up purposes. First, rather than using an expensive (e.g., hygromycin, geneticin, MTX) and/or highly toxic selective agent (e.g., L-methionine-*S*-sulfoximine or L-histidinol) our system uses nanomolar concentrations of a reduced folate vitamin, LCV. The latter is inexpensive, stable in culture medium, rapidly and efficiently taken up by the RFC into mammalian cells; once within cells, it is rapidly converted to the one-carbon donor forms necessary for purine and thymidylate biosynthesis. Furthermore, we used here RFC-deficient CHO recipient cells that can readily grow both under monolayer and suspension culture conditions. This is an important advantage when large-scale growth is considered.

Our current results shed light on the subcellular localization of the hRFC. Using polyclonal antiserum to a small peptide corresponding to a predicted intracellular loop linking the 6th and 7th transmembrane domains of the hRFC, it was found that apart from its plasma membrane localization, this transporter is also present in the mitochondrial membrane (Trippett et al., 2001). It was therefore suggested by Trippett et al. (2001) that this transporter might be active in folate transport into mitochondria. In contrast, however, our current study re-examined this mitochondrial subcellular localization by tagging the hRFC with a small C-terminal HA tag that interferes with neither *N*-linked glycosylation nor its MTX transport activity. Our results do not support a mitochondrial localization for the hRFC, because upon immunofluorescence with anti-HA antibodies, this hRFC-HA was present in the plasma membrane but not in mitochondria. In support of our current data is the recent cloning of the human mitochondrial folate transporter, which is completely distinct of the hRFC and is localized in the mitochondrial inner membrane (Titus and Moran, 2000). Mutational inactivation of this mitochondrial folate transporter consistently resulted in glycine auxotrophy (McCarthy et al., 2004). Furthermore, our present results are in accord with the plasma membrane localization of the murine RFC in various tissues (Wang et al., 2001).

The present study suggests that this efficient hRFC-based bicistronic overexpression system is readily capable of restoring MTX uptake and consequent antifolate sensitivity upon transport-deficient cells that originally display high-level antifolate resistance. Hence, this system could potentially be regarded as a prototypic hRFC-based gene therapy approach for restoration of MTX sensitivity in MTX-resistant tumors that have lost RFC function as a result of loss of transporter expression and/or mutational inactivation. This may be particularly feasible in that tumor-bearing individuals may become progressively folate-deficient. It was shown recently that loss of hRFC expression and/or MTX transport function is an important mechanism of MTX resistance in osteosarcoma (Guo et al., 1999; Ifergan et al., 2003; Yang et al., 2003) and childhood acute lymphoblastic leukemia (Gorlick et al., 1996, 1997; Kaufman et al., 2004). This was consistent with studies using antifolate-resistant cell lines in which frequent mutational inactivation of the *hRFC* gene (Jansen et al., 1998; Drori et al., 2000a; Rothem et al., 2002) and loss of gene expression were demonstrated (Rothem et al., 2003, 2004a,b).

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