Single Nucleotide Polymorphisms in the Human Reduced Folate Carrier: Characterization of a High-Frequency G/A Variant at Position 80 and Transport Properties of the His[27] and Arg[27] Carriers[1]

Johnathan R. Whetstine, Andrew J. Gifford, Teah Witt, Xiang Y. Liu, Robin M. Flatley, Murray Norris, Michelle Haber, Jeffrey W. Taub, Y. Ravindranath, and Larry H. Matherly[2]

Departments of Pharmacology [J. R. W., L. H. M.] and Pediatrics [Y. R., J. W. T.], and the Experimental and Clinical Therapeutics Program [J. R. W., T. W., X. Y. L., R. M. F., J. W. T., Y. R., L. H. M.], Barbara Ann Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, Michigan 48201, Children's Hospital of Michigan, Detroit, Michigan 48201 [Y. R., J. W. T.], and Children's Cancer Institute Australia for Medical Research, Randwick, Sydney, New South Wales, Australia [A. J. G., M. N., M. H.]

ABSTRACT

The presence of sequence variants in the *human reduced folate carrier* (*hRFC*) was assessed in leukemia blasts from children with acute lymphoblastic leukemia (ALL) and in normal peripheral blood specimens. A CATG frame shift insertion at position 191 was detected in 10–60% of *hRFC* transcripts from 10 of 16 ALL specimens, by RFLP analysis and direct sequencing of *hRFC* cDNAs. In genomic DNAs prepared from 105 leukemia ($n = 54$) and non-leukemia ($n = 51$) specimens, PCR amplifications and direct sequencing of exon 3 identified a high-frequency G to A single nucleotide polymorphism at position 80 that resulted in a change of arginine-27 to histidine-27. The allelic frequencies of G/A80 were nearly identical for the non-leukemia (42.2% *CGC* and 57.8% *CAC*) and leukemia (40.7% *CGC* and 59.3% *CAC*) genomic DNAs. In cDNAs prepared from 10 of these ALL patients, identical allelic frequencies (40 and 60%, respectively) were recorded. In up to 62 genomic DNAs, *hRFC*-coding exons 4–7 were PCR-amplified and sequenced. A high-abundance C/T696 polymorphism was detected with nearly identical frequencies for both alleles, and a heterozygous C/A1242 sequence variant was identified in two ALL specimens. Both C/T696 and C/A1242 were phenotypically silent. In transport assays with [^{3}H]methotrexate and [^{3}H]5-formyl tetrahydrofolate, nearly identical uptake rates were measured for the arginine-27- and histidine-27-hRFC proteins expressed in transport-impaired K562 cells. Although there were no significant differences between the kinetic parameters for methotrexate transport for the hRFC forms, minor (~2-fold) differences were measured in the K_is for other substrates including Tomudex, 5,10-dideazatetrahydrofolate, GW1843U89, and 10-ethyl-10-deazaaminopterin and for 5-formyl tetrahydrofolate.

INTRODUCTION

The natural folates are members of the B-class of vitamins and are essential to cell proliferation (1). Because mammalian cells cannot synthesize these essential cofactors, folates must be actively transported into cells, whereupon they participate in one-carbon transfer biosynthetic reactions leading to purines, pyrimidines, serine, and methionine. The primary route for membrane transport of reduced folates into mammalian cells involves the RFC[3] (2, 3). Membrane transport via RFC is also an important determinant of antifolate therapeutics used in cancer chemotherapy (*e.g.*, MTX and Tomudex), and impaired uptake of antifolates by RFC is a frequent mode of drug resistance (4). Likewise, the loss of RFC function conceivably could contribute to disease states associated with decreased accumulations of folate cofactors (*e.g.*, fetal abnormalities, cardiovascular disease, and cancer; Refs. 5–7).

The *RFC* gene has been cloned and characterized from a number of sources (8–15). For the *hRFC*, an intriguing feature involves transcript heterogeneity. For instance, multiple cDNAs were described for *hRFC*, differing in their 5′-untranslated regions (13). Furthermore, variable deletions of *hRFC* open reading frame sequence have been reported, including a functional hRFC form encoded by a transcript with a 626-bp deletion (13) and a truncated hRFC resulting from a 987-bp deletion (15, 16). Point substitutions in the *hRFC* coding sequence have been described for *hRFC* cDNA clones isolated from different laboratories (see above), and point- and frame-shift mutations have

Received 7/25/01; revised 8/2/01; accepted 8/15/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Grant CA77641 from the National Cancer Institute, NIH, the Children's Leukemia Foundation of Michigan, the National Health and Medical Research Council (Australia), and the New South Wales Cancer Council (Australia). Mr. Gifford is the recipient of an Australian Postgraduate Award and an International Cancer Technology Transfer Fellowship (ICRETT) from the International Union Against Cancer.

[2] To whom requests for reprints should be addressed, at Experimental and Clinical Therapeutics Program, Karmanos Cancer Institute, 110 East Warren Avenue, Detroit, MI 48201. Phone: (313) 833-0715, extension 2407; Fax: (313) 832-7294; E-mail: matherly@kci.wayne.edu.

[3] The abbreviations used are: RFC, reduced folate carrier; MTX, methotrexate; hRFC, human RFC; SNP, single nucleotide polymorphism; Arg, arginine; His, histidine; ALL, acute lymphoblastic leukemia; gDNA, genomic DNA; RT-PCR, reverse transcription-PCR; 5-CHO-H_4PteGlu, 5-formyltetrahydro-folate.

been reported in transport-impaired cells selected *in vitro* for antifolate resistance (17–20). However, the relevance of these assorted sequence alterations to patient specimens is uncertain.

With the completion of the sequencing of the human genome (21, 22), there has been a surge of interest in the possible roles of gene variants in human disease, and potential opportunities for therapeutic intervention on this basis (23, 24). From the National Center for Biotechnology Information database, seven potential SNPs have been tentatively identified in *hRFC*, including three [positions 80 (G/A), 246 (C/G), and 696 (C/T)] in the *hRFC* coding sequence. Only the alteration at position 80 results in an amino acid substitution (*i.e.*, Arg/His^{27}). A recent study implied an effect of G/A80 in combination with C/T677 in 5,10-methylene tetrahydrofolate reductase on plasma folate levels and homocysteine pools (25); however, the effects of G/A80 on *hRFC* transport function were not addressed.

On these bases, we began a series of experiments to confirm the presence of putative *hRFC* sequence variants in leukemia blasts obtained from children and in normal (non-leukemia) specimens. For childhood ALL, we reasoned that the existence of such sequence variants might result in differences in the cellular uptake of MTX in ALL blasts, thus contributing to interpatient variations in response to chemotherapy with MTX. In normal tissues, differences in membrane transport of reduced folate cofactors could result in downstream effects on folate-dependent anabolic pathways so as to contribute to interpatient differences in susceptibilities to cardiovascular disease, fetal abnormalities, and/or cancer. In this report, we document the occurrence of high-frequency SNPs in leukemia and non-leukemia patient specimens and compare the functional properties of Arg^{27}-hRFC to His^{27}-hRFC resulting from a G to A SNP at position 80.

MATERIALS AND METHODS

Reagents. [3′, 5′, 7-^{3}H]MTX (20 Ci/mmol) and [3′, 5′, 7, 9-^{3}H] (6S) 5-CHO-H_4PteGlu (17 Ci/mmol) were purchased from Moravek Biochemicals. Unlabeled MTX and (6R,S) 5-CHO-H_4PteGlu were provided by the Drug Development Branch, National Cancer Institute, Bethesda, MD. Both labeled and unlabeled MTX were purified by HPLC before use (26). Several antifolate drugs were provided as follows: 10-ethyl-10-deazaaminopterin (CIBA-GEIGY Corp.; Ref. 27), GW184389 (GlaxoWellcome Pharmaceuticals; Ref. 28); Tomudex (Ann Jackman, Institute of Cancer Research, Surrey, England; Ref. 29), and (6R,S) dideaza-5,6,7,8-tetrahydrofolate (Lometrexol or DDATHF; Lily Research Laboratories; Ref. 30).

Restriction and modifying enzymes were obtained from Promega and New England Biolabs. Synthetic oligonucleotides were obtained from Genosys Biotechnologies, Inc., and Life Technologies, Inc. Tissue culture reagents and supplies were purchased from assorted vendors with the exception of fetal bovine serum and iron-supplemented calf serum, which were from Life Technologies, Inc., and Hyclone Laboratories, Inc., respectively.

Cell Culture. The wild-type K562 erythroleukemia cell line was obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in RPMI 1640 containing 10% heat-inactivated iron-supplemented calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere at 37°C in the presence of 5% CO_2/95% air. The transport-deficient K500E subline was selected from wild-type K562 cells by cloning in soft agar in the presence of 500 nM MTX (31). K500E cells were transfected with the wild-type Arg^{27}-*hRFC* cDNA (KS43) to generate the K43-6 subline, as described previously (31).

For *cytotoxicity* determinations with various antifolates (MTX, Tomudex, and GW1843U89), cells were cultured in 24-well dishes at 20,000 cells/ml in 2 ml of complete RPMI 1640 containing 10% dialyzed fetal bovine serum. Cells were counted with a hemacytometer after 120 h. The IC_{50}s were calculated as the concentrations of antifolate necessary to inhibit growth by 50% compared with control cells grown under identical conditions, except that the inhibitor was omitted. For assays of (6R,S) 5-CHO-H_4PteGlu growth requirements, cells were depleted of folates by culturing in folate-free RPMI 1640 (Life Technologies, Inc.) and 10% dialyzed serum containing adenosine (60 μM) and thymidine (10 μM), for 2 weeks. Growth was assayed after 120 h after culturing in complete folate-free medium without nucleosides but containing various concentrations of (6R,S) 5-CHO-H_4PteGlu (0–500 nM).

Isolation and Analysis of Total RNAs and gDNAs from Patient Specimens. The childhood acute leukemia specimens used for this study were obtained from the Children's Hospital of Michigan (Detroit, MI) and the Pediatric Oncology Group (Chicago, IL) and are those used in our previous studies of MTX response and resistance in pediatric ALL (32, 33). Patient samples were obtained at diagnosis after informed consent, and in accordance with protocols approved by the Committee on Investigation Involving Human Subjects at Wayne State University. Blasts were purified previously by standard Ficoll Hypaque density centrifugation. Total RNAs were prepared from 1–5 × 10^6 leukemia cells using the RNeasy mini-kit (Qiagen). cDNAs including the complete *hRFC* open reading frame (~1.8 kb) were prepared by reverse transcription with Superscript II (Life Technologies, Inc.) and oligo(dT) primer and PCR amplified with Taq polymerase (Promega) using PCR primers based on 5′- and 3′-untranslated region sequences (P8, 5′-CAGTGTCACCTTCGTCCCCT CCG-3′, and P10, 5′-CACCACCTC TTCCAGCAACAAA-3′). PCR conditions were 94°C for 1 min (1 cycle) and then 94°C for 30 s, 63°C for 45 s, and 72°C for 2 min (35 cycles) and ending with 72°C for 7 min (1 cycle). PCR products were subcloned into pGEM-T-Easy plasmid and five to seven separate clones were completely sequenced (see below) with a combination of universal and gene-specific primers. For amplifying the exon 3 sequence only, reverse transcription was performed with random hexamers and a RT-PCR kit from Perkin-Elmer, and the cDNAs were amplified with P8 and JW-RFC1 (5′-GGGTGATGAAGC TCTCCCCTGG-3′) primers [94°C for 5 min (1 cycle), 94°C for 15 s, 63°C for 45 s, and 72°C for 45 s (35 cycles), ending with 72°C for 7 min (1 cycle)]. PCR reactions were electrophoresed on a 2% LE agarose gel, and the amplicons were isolated from the gel with the Concert Rapid Gel Extraction kit (Life Technologies, Inc.). DNA sequences were obtained by automated DNA sequencing using the P8 primer and BigDye Terminator cycle sequencing reactions (Perkin-Elmer) at the DNA sequencing core of the Center of Molecular Medicine and Genetics (Wayne State University,

Table 1 Primers for PCR of gDNAs

Exon	Sense primer	Antisense primer
3	5′-CTGACTCCACCCCTCCTTCCAGGCACAGTG-3′	5′-CCACATGCCTGCTCCCGCGTGAACTTCT-3′
4/5	5′-CGAACGAGATCACGCCGGTGCTGTC-3′[a]	5′-TGGCGATGGGCACGAGGAACTGGT-3′
4	5′-CGAACGAGATCACGCCGGTGCTGTC-3′[a]	5′-AGCAGCGTGGAGGCAGCATCT-3′
5	5′-CCCCTCTCAGGCGCCATCACGTCCTTC-3′[a]	5′-GTGGAGGGCCTGGGGGAGCAGCAA-3′
6	5′-GCTTTTCCCTGTGGCTGCTGCTTATT-3′	5′-ACAATGTCCCCACAAGTAGCTTCCATCC-3′
7	5′-TACTCCGTGTACTTCCTGATCCTGTCCA-3′	5′-ACCCACCTCTTCCAGCAACAAAGC-3′

[a] Two primer sets were used for amplifying exons 4 and 5 in separate experiments.

Detroit, MI) and the Western Kentucky University Biotechnology Center (Bowling Green, KY).

gDNAs were isolated from 3–5 $\times$ 10^6 ALL blasts using the DNA Isolation Kit from Puregene (Gentra Systems, Inc.). gDNAs from non-leukemic adult individuals were prepared from peripheral blood samples and provided by the Children's Cancer Institute Australia for Medical Research. Exons 3–7 were amplified from gDNAs (200 ng) with Taq polymerase (Promega) over 35 cycles [94°C for 5 min (1 cycle), 94°C for 15 s, 61–63°C for 45 s, and 72°C for 45 s (35 cycles), ending with 72°C for 7 min (1 cycle)]. The primers are summarized in Table 1. GC-rich reagent (Roche) was included in the amplifications from gDNAs. The amplicons were isolated and directly sequenced with the upstream primers by automated DNA sequencing.

RT-PCR/RFLP Analysis of CATG-191 in *hRFC* Transcripts from ALL Blasts. For PCR-RFLP screening for the presence of a CATG insertion at position 191 in *hRFC* transcripts, RNAs were reverse transcribed and PCR amplified with P8 and P7 (5′-GCCAGCGAGATGTAGTTGAGCGT-3′) primers. The PCR products were subcloned into pGem-T-Easy plasmid (Promega). For each patient, 10–11 bacterial colonies were PCR amplified and digested with Nsp1, and products were electrophoresed on a 2% LE agarose gel with ethidium bromide staining. By this approach, Nsp1 digestion of wild-type sequence (*i.e.,* no CATG) would generate 215- and 403-bp fragments, whereas the presence of CATG-191 would result in 240-, 167-, and 215-bp restriction fragments.

Preparation of the His^{27} *hRFC* Expression Construct and Transfection of Transport-impaired K562 Cells. "SOE" PCR (34) was used generate the His^{27}- (A80) *hRFC* cDNA, using Arg^{27}- (G80) *hRFC* cDNA (KS43) in pCDNA3 as template (13). The primary PCR step was designed to amplify two separate products for the secondary PCR step and used two primer sets: (*a*) R27H, 5′-CTCCGGTCCTGGCGGCACCTC-GTGTGCTACCTT-3′ and reHA-7, 5′-CTCGTTCCACAGGA-TGTGCAC-3′; and (*b*) T7 and reR27H, 5′-AAGGTAGCA-CACGAGGTGCCGCCAGGACCGGAG-3′. The R27H/reR27H primers were completely complementary and included the desired A80 mutation. PCR conditions for the primary amplification were 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min (30 cycles). Secondary PCR was carried out under identical conditions with the T7/reHA-7 primer set and the primary PCR products as template. The amplicon was isolated, digested with BbrPI and *Not*I, and ligated into BbrPI and *Not*I-digested KS43 *hRFC* cDNA in pCDNA3. The presence of A80 in the His^{27}-*hRFC* cDNA construct was confirmed by automated DNA sequencing.

His^{27}-*hRFC* cDNA in pCDNA3 was transfected into transport-impaired K500E cells with lipofectin, as described previously (31). G418-resistant clones were isolated, expanded and screened for immunoreactive hRFC protein on Western blots and for [^{3}H]MTX uptake.

Preparation of Plasma Membranes and Western Analyses. Plasma membranes were prepared by differential centrifugation as described earlier (35). Membrane proteins were electrophoresed on 7.5% gels in the presence of SDS (36) and electroblotted onto polyvinylidene difluoride membranes (37) for detection with protein A-purified hRFC antibody (20) and enhanced chemiluminescence (Roche). Densitometry was performed on a Kodak Digital Science Image Station 440CF.

Transport of [^{3}H]MTX and [^{3}H](6S) 5-CHO-H_4PteGlu. Initial [^{3}H]MTX or [^{3}H](6S) 5-CHO-H_4PteGlu uptake was measured over 180 s at 1 μM, as described previously (13, 20, 31). Levels of intracellular radioactivity were expressed as pmol/mg protein, calculated from direct measurements of radioactivity and protein contents of the cell homogenates. Protein assays were by the method of Lowry *et al.* (38). Kinetic constants (K_t, V_{max}, K_i) were calculated from Lineweaver-Burk and Dixon plots as in our previous reports (13, 20, 31).

RESULTS AND DISCUSSION

Identification of *hRFC* Transcript Variants in B-Precursor ALL Blasts. By sequencing multiple (five to seven) full-length *hRFC* cDNA clones amplified from each of four B-precursor ALL specimens, we identified three distinct and reproducible sequence alterations. (*a*) A CATG insertion was detected at position 191 (relative to the ATG translation start at position 1) in one or two clones from all four ALL specimens. This 4-bp insertion is identical to that previously described in MTX-resistant CCRF-CEM cells (CEM/MTX-1; Ref. 20) and occurs at the donor splice junction for exon 3, suggesting its origin by alternative splicing. The result of the frame shift is the synthesis of a M_r ~48,000 protein with mostly nonsense sequence (20). (*b*) For all four lymphoblast specimens, cDNAs were identified with both a G (CGC) and an A (CAC) at position 80 (*i.e.,* codon 27). (*c*) A C to T transition at position 696 (codon 232) was also identified in these cDNAs. The former would result in a substitution of His for Arg^{27}, whereas the latter would be phenotypically silent (*i.e.,* Pro→Pro).

RT-PCR/RFLP Analysis of CATG-191 in *hRFC* Transcripts from ALL Blasts. An RT-PCR/RFLP strategy was developed for screening total RNAs for the CATG insertion at position 191, using RNAs obtained from 16 ALL specimens (10

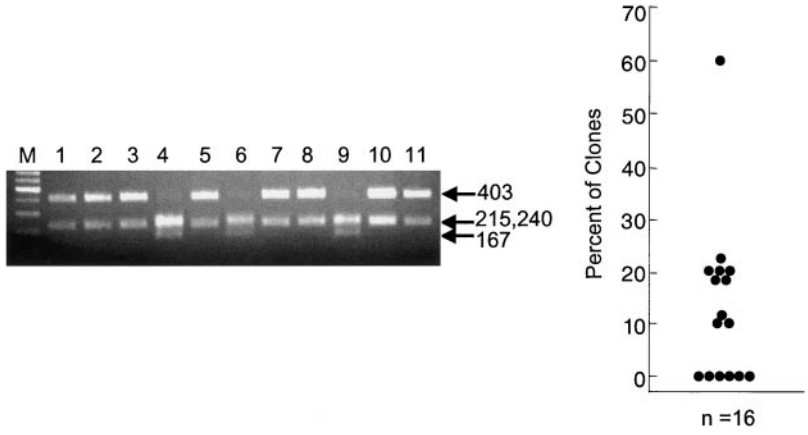

Fig. 1 PCR-RFLP analysis of CATG-191 in *hRFC* transcripts. *Left*, an ethidium bromide-stained agarose gel of the Nsp1-digested PCR products for a single patient, exhibiting both wild-type cDNA sequence (403- and 215-bp products; *Lanes 1, 2, 3, 5, 7, 8, 10,* and *11*) and that including CATG-191 (240-, 167-, and 215-bp products; *Lanes 4, 6,* and *9*). *Right*, the distribution of transcripts containing CATG-191 among 16 ALL specimens, as described in the text.

Table 2 Frequencies of G/A80 SNP in ALL and non-ALL specimens

gDNAs from ALL and non-ALL specimens were PCR amplified with exon 3-specific primers (see "Materials and Methods") and the amplicons directly sequenced for identification of the homozygous (*CGC/CGC* or *CAC/CAC*) or heterozygous (*CGC/CAC*) phenotypes at codon 27.

Type	*n*	Homozygous *CGC/CGC* Arg/Arg	Heterozygous *CGC/CAC* Arg/His	Homozygous *CAC/CAC* His/His	Allelic frequencies (%) *CGC*	*CAC*
ALL	54	10 (0.19)	24 (0.44)	20 (0.37)	40.7	59.3
Non-ALL	51	9 (0.18)	25 (0.49)	17 (0.33)	42.2	57.8
Total	105	19 (0.18)	49 (0.49)	37 (0.33)	41.4	58.6

B-precursor and 6 T-ALL). After the initial RT-PCR, the amplicons were subcloned and multiple (10 or 11) clones for each sample were screened for the presence of a novel Nsp1 restriction site generated by the CATG-191 insertion, resulting in a unique pattern of restriction fragments (215 and 403 bp in wild type or 240, 167, and 215 bp with CATG insertion; Fig. 1*A*). Of the 16 ALL specimens tested, 10 ALL specimens expressed transcripts with CATG-191 at frequencies ranging from 10 to 60% (Fig. 1*B*). Thus, the CATG-191 phenotype, previously described for CEM/MTX-1 cells *in vitro* (20), is indeed clinically relevant. At high frequencies, the presence of CATG-191 could result in significantly decreased levels of functional hRFC protein and levels of MTX transport despite high levels of *hRFC* transcripts. Although we were unable to establish any direct correlation between levels of MTX uptake and frequency of CATG-191 among our patient specimens (not shown), for the B-precursor ALL patient with the highest frequency of CATG-191 (60%), there was a strikingly disproportionate increase in the level of *hRFC* transcripts relative to MTX uptake (33).

Confirmation of a High-Frequency G to A Transition at Position 80. To assess the frequency of a G/A80 transition in the *hRFC* coding sequence, we screened 54 gDNAs prepared from ALL specimens (34 BP-ALL and 20 T-ALL). gDNAs were PCR amplified with specific primers for exon 3 (includes the ATG translation start site), and the 194 bp amplicons were sequenced by automated sequencing. For comparison, 51 gDNAs from normal (non-leukemia) individuals were screened for G/A80, as well. Among these 105 leukemia and non-leukemia amplifications of exon 3, the only alteration detected was G/A80. The allelic frequencies for G/A80, based on the Hardy-Weinberg equation, are summarized in Table 2 and are virtually identical for the non-leukemia (42.2% CGC and 57.8% CAC) and leukemia gDNAs (40.7% CGC and 59.3% CAC). These results confirm that G/A80 represents a high-frequency SNP in the *hRFC* coding sequence.

For 10 of the ALL patients, including the 4 originally screened for *hRFC* mutations, total RNAs were reverse transcribed and PCR amplified for sequencing. In this analysis, the allelic distributions for codon 27 were in near-perfect agreement with those obtained by genomic sequencing and included 1 of 10 *CGC/CGC* (Arg^{27}), 6 of 10 *CGC/CAC* (Arg^{27}/His^{27}), and 3 of 10 *CAC/CAC* (His^{27}). The allelic frequencies for the cDNAs are 40% *CGC* and 60% *CAC*. Thus, both the *CGC* and *CAC* alleles are transcribed equally well.

The allelic frequencies obtained by direct sequencing of 105 gDNA specimens are distinctly different from those previously reported by PCR-RFLP analysis with CfoI (25) and in our own experience using *Dra*III or *Hae*II digestions to distinguish the *CGC* allele (*Dra*III-insensitive and *Hae*II-sensitive) from the *CAC* allele (*Dra*III sensitive and *Hae*II insensitive) in gDNAs from normal and leukemia patients (39), including many of those characterized by sequencing. The allelic frequencies by

PCR amplification-*Dra*III digestions were 60% *CGC* and 40% *CAC* (not shown), whereas those by *Hae*II digestion were 56.6% *CGC* and 43.4% *CAC*, a striking contrast from those obtained by direct sequencing. These data suggest that the use of RFLP analysis to identify and quantitate this *hRFC* polymorphism can lead to an appreciable level of inaccuracy.

Screening for Other High-Frequency *hRFC* Polymorphisms. The demonstration of a frequent G/A variant at codon 27 of the *hRFC* coding sequence among 105 individuals led us to expand the scope of our search for high frequency polymorphisms that may occur in exons 4 through 7. Coding exons 4–7 were PCR amplified from 53 gDNAs (47 ALL and 6 non-ALL) and directly sequenced. The sequences for each exon were then compared with wild-type *hRFC* coding sequence (GenBank accession no. U19720). For an additional three specimens (two ALL and one non-ALL) exons 3, 4, 5, and 7 (*i.e.*, not exon 6) were also sequenced; for an additional 6 non-ALL specimens, sequence data were obtained for exons 3–6 (*i.e.*, not exon 7). Similar to our cDNA data (see above), C/T696 was identified as a frequent polymorphism that had an allelic frequency nearly equal among the 62 gDNAs (30 *CCT* and 32 *CCC*) sequenced. G/A80 and C/T696 did not cosegregate in our patients, indicating there is no linkage between these two SNPs. The only other alteration detected in 62 gDNAs was a heterozygous C/A transversion at position 1242 in two ALL specimens, resulting in a silent mutation (*i.e.*, both *ATC* and *ATA* at codon 414 encode isoleucine).

Characterization of the His^{27} hRFC Protein Resulting from G/A80. From topology models for hRFC, Arg^{27} is predicted to lie in the first transmembrane domain, a region implicated as important to carrier function (17, 19, 40–42). To directly compare the transport properties of His^{27}-hRFC with Arg^{27}-hRFC, we transfected the His^{27}-*hRFC* cDNA into hRFC-null K500E cells, exactly as in our earlier study with the Arg^{27}-hRFC-expressing K43-6 cells (31). Two stable clones (designated R27H2-4 and R27H2-8) that expressed moderate levels of His^{27}-hRFC protein on Western blots (only slightly lower than for the K43-6 cells) were isolated and characterized (Fig. 2*A*).

In transport assays of [^{3}H]MTX (1 μM) and [^{3}H](6S) 5-CHO-H$_4$PteGlu (1 μM), uptake rates over 180 s in K43-6 (Arg^{27}-hRFC) exceeded that for the His^{27}-hRFC-expressing R27H2-4/R27H2-8 sublines by ~39 and 31%, respectively (Fig. 2*B*).

Relative uptake rates for the KS43-6 cells and the two His^{27}-hRFC-expressing lines were identical when uptake data were normalized for hRFC protein expression (Fig. 2*C*). However, these normalized values may be slightly biased, because hRFC expression and transport are not always proportional in transfected cells (31).

Transport kinetic parameters (*i.e.*, V_{max} and K_t) for MTX were, likewise, nearly identical between K43-6 cells, and the R27H2-4 and -8 sublines (Table 3). Furthermore, there were, at most, only minor (~2-fold) differences in the K_is for folate/antifolate transport substrates between the Arg^{27}-hRFC- and His^{27}-hRFC-expressing cells (Table 3). Finally, there were no significant differences between K43-6 and R27H2-4 cells in their sensitivities to growth inhibition by MTX (IC_{50}s of 54 and 56 nM, respectively), Tomudex (IC_{50}s of 4.1 nM for both lines),

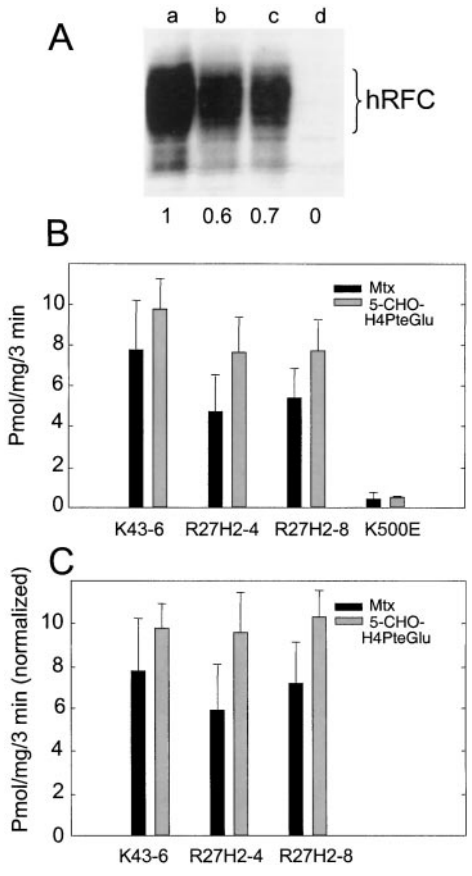

Fig. 2 His^{27}-hRFC expression and function in K562 transfectants. *A*, Western blot of membrane proteins from K43-6 (Arg^{27}-hRFC; *Lane a*), hRFC-null K500E (*Lane d*), and the His^{27}-hRFC-expressing R27H2-4 and R27H2-8 cells (*Lanes b* and *c*, respectively). Proteins (20 μg) were fractionated on a 7.5% gel in the presence of SDS and electroblotted onto a polyvinylidene difluoride membrane. Detection was with an anti-hRFC antibody and an enhanced chemiluminescence kit. The relative levels of hRFC protein by densitometry are noted. *B* and *C*, the relative uptakes of [^{3}H]MTX and [^{3}H](6S) 5-CHO-H$_4$PteGlu over 180 s at 37°C. *B*, uptake rates without normalization for hRFC expression; *C*, the normalized transport data using the relative hRFC expression levels from the Western blot. All transport results are the mean values of three to four separate experiments (±SD).

or GW1843U89 (IC_{50}s of 1.1 and 1.2 nM, respectively), nor were there differences in their (6R,S) 5-CHO-H$_4$PteGlu growth requirements (50% maximal growth at 1.9 and 1.6 nM, respectively). Collectively, these results demonstrate that the His^{27}-hRFC protein exhibits, at most, only minor functional differences from Arg^{27}-hRFC in terms of substrate affinities and/or transport efficiencies.

Conclusion. A major goal of human genetics is to identify the roles of common genetic variants that result in altered susceptibilities to disease. This requires identifying gene variations in human populations and assembling an extensive catalogue of SNPs and characterizing their functional significance and associations with particular diseases. The identification of SNPs that result in altered proteins can also provide molecular insights into the bases for interpatient variability in therapeutic

Table 3 Kinetic constants for Arg^{27}-hRFC and His^{27}-hRFC-expressing cells

Kinetic constants were determined from Lineweaver-Burk and Dixon plots for the K43-6 and R27H2-4 and R27H2-8 transfectants, expressing the Arg^{27}-hRFC and His^{27}-hRFC, respectively, using [^{3}H]MTX. The data shown are the mean values from multiple experiments (shown in parentheses) ± SD. V_{max} (normalized) is the calculated V_{max}, normalized to levels of hRFC protein on Western blots (*i.e.*, Fig. 2).

Parameter	Substrate	K43-6 (Arg^{27}-hRFC)	R27H2-4 (His^{27}-hRFC)	R27H2-8 (His^{27}-hRFC)
K_t (μM)	MTX	2.38 ± 1.36 (n = 5)	3.04 ± 2.74 (n = 4)	1.62 ± 0.43 (n = 5)
V_{max} (pmol/mg/3 min)	MTX	29.06 ± 8.48 (n = 5)	17.07 ± 5.58 (n = 4)	18.44 ± 6.43 (n = 5)
V_{max} (normalized)	MTX	29.06	28.45	26.34
K_i (μM)	ZD1694	3.00 ± 1.63 (n = 4)	1.65 ± 0.82 (n = 4)	1.93 ± 0.29 (n = 3)
K_i (μM)	GW1843U89	1.44 ± 0.65 (n = 3)	0.55 ± 0.13 (n = 3)	0.89 ± 0.48 (n = 3)
K_i (μM)	DDATHF	2.16 ± 0.50 (n = 2)	1.62 ± 0.28 (n = 2)	1.68 ± 0.31 (n = 2)
K_i (μM)	10-EDAM	2.17 ± 0.72 (n = 3)	1.28 ± 0.14 (n = 3)	1.12 ± 0.19 (n = 3)
K_i (μM)	(6S) CHO-H_4PteGlu	6.74 ± 2.22 (n = 5)	3.86 ± 1.54 (n = 5)	4.03 ± 1.45 (n = 5)

responses and toxicities to drugs (23, 24). Given the narrow therapeutic window for most cancer therapies, this is particularly important for the clinical management of cancer (43). By identifying specific gene polymorphisms involved in altered drug metabolism or transport, pharmacogenetic screens can be developed to determine whether particular groups of patients may be predisposed to unacceptable drug toxicities and/or poor antitumor responses for specific classes of anticancer drugs. This potentially could lead to more individualized therapies for cancer.

From the critical role of hRFC in membrane transport of folates for cellular proliferation and tissue regeneration and of antifolates for cancer therapy, interest in *hRFC* gene variants is particularly acute. In this report, we have identified four sequence alterations in *hRFC* in patient specimens. These include a four bp (CATG) frameshift mutation at position 191, apparently resulting from aberrant mRNA splicing, a silent C to T alteration at position 696 in exon 4, a silent C to A transversion at position 1242 in exon 6, and a high-frequency (G to A) SNP at position 80 in exon 3 that results in a substitution of Arg^{27} by His^{27}.

The high frequency CATG insertion would result in a nonfunctional carrier and could possibly contribute to low levels of hRFC-protein and -MTX transport in ALL lymphoblasts from patients. The allelic frequencies of *CAC* and *CGC* at codon 27 were ~60 and 40%, respectively, among the 105 patients in our analysis. The change of a strongly basic amino acid (Arg) to a weak base (His) in a region of the carrier documented to influence folate substrate binding and rates of uptake (19, 40–42) might be expected to alter hRFC transport properties. However, by directly comparing the transport properties of Arg^{27}-hRFC to His^{27}-hRFC in stable K562 transfectants, no significant differences in the uptake rates of either MTX or 5-CHO-H_4PteGlu were observed, and only minor differences were calculated in the relative affinities for an assortment of transport substrates. Collectively, these data strongly argue for a lack of major functional differences between the Arg^{27}- and His^{27}-hRFCs for reduced folate cofactors and for various antifolates used in cancer chemotherapy.

ACKNOWLEDGMENTS

We gratefully acknowledge the contributions of Kenneth Bark and Dawn Clifford in certain of the formative experiments that led to this study. We thank the Pediatric Oncology Group for providing childhood ALL specimens for our ongoing studies of MTX response and resistance in pediatric ALL.

REFERENCES

1. Stokstad, E. L. R. Historical perspective on key advances in the biochemistry and physiology of folates. *In:* M. F. Picciano, E. L. R. Stokstad, and J. F. Gregory (eds.), Folic Acid Metabolism in Health and Disease, pp. 1–21. New York: Wiley-Liss, Inc., 1990.

2. Goldman, I. D., and Matherly, L. H. The cellular pharmacology of methotrexate. Pharmacol. Ther., *28:* 77–102, 1985.

3. Sirotnak, F. M., and Tolner, B. Carrier-mediated membrane transport of folates in mammalian cells. Annu. Rev. Nutr., *19:* 91–122, 1999.

4. Jansen, G. Receptor- and carrier-mediated transport systems for folates and antifolates: exploitation for folate-based chemotherapy and immunotherapy. *In:* A. L. Jackman (ed.), Antifolate Drugs in Cancer Therapy, pp. 293–322. Totowa, NJ: Humana Press, Inc., 1999.

5. Butterworth, C. E., and Bendich, A. Folic acid and the prevention of birth defects. Annu. Rev. Nutr., *16:* 73–97, 1996.

6. Mason, J. B., and Levesque, T. Folate: effects on carcinogenesis and the potential for cancer chemoprevention. Oncology (Huntingt.), *10:* 1727–1736, 1996.

7. Refsum, H., Ueland, P. M., Nygard, O., and Vollset, S. E. Homocysteine and cardiovascular disease. Ann. Rev. Med., *49:* 31–62, 1998.

8. Dixon, K. H., Lampher, B. C., Chiu, J., Kelley, K., and Cowan, K. H. A novel cDNA restores reduced folate carrier activity and methotrexate sensitivity to transport-deficient cells. J. Biol. Chem., *269:* 17–20, 1994.

9. Williams, F. M. R., Murray, R. C., Underhill, T. M., and Flintoff, W. F. Isolation of a hamster cDNA clone coding for a function involved in methotrexate uptake. J. Biol. Chem., *269:* 5810–5816, 1994.

10. Moscow, J. A., Gong, M., He, R., Sgagias, M. K., Dixon, K. H., Anzick, S. L., Mettzer, P. S., and Cowan, K. H. Isolation of a gene encoding a human reduced folate carrier (RFC1) and analysis of its expression in transport-deficient, methotrexate-resistant human breast cancer cells. Cancer Res., *55:* 3790–3794, 1995.

11. Prasad, P. D., Ramamoorthy, S., Leibach, F. H., and Ganapathy, V. Molecular cloning of the human placental folate transporter. Biochem. Biophys. Res. Comm., *206:* 681–687, 1995.

12. Williams, F. M. R., and Flintoff, W. F. Isolation of a human cDNA that complements a mutant hamster cell defective in methotrexate uptake. J. Biol. Chem., *270:* 2987–2992, 1995.

13. Wong, S. C., Proefke, S. A., Bhushan, A., and Matherly, L. H. Isolation of human cDNAs that restore methotrexate sensitivity and reduced folate carrier activity in methotrexate transport-defective Chinese hamster ovary cells. J. Biol. Chem., *270:* 17468–17475, 1995.

14. Tolner, B., Roy, K., and Sirotnak, F. M. Structural analysis of the human *RFC-1* gene encoding a folate transporter reveals multiple promoters and alternatively spliced transcripts with 5′ end heterogeneity. Gene, *211:* 331–341, 1998.

15. Zhang, L., Wong, S. C., and Matherly, L. H. Structure and organization of the *human reduced folate carrier* gene. Biochim. Biophys. Acta, *1442:* 389–393, 1998.

16. Drori, S., Sprecher, H., Shemer, G., Jansen, G., Goldman, I. D., and Assaraf, Y. G. Characterization of a human alternatively spliced truncated reduced folate carrier increasing folate accumulation in parental cells. Eur. J. Biochem., *267:* 690–701, 2000.

17. Drori, S., Jansen, G., Mauritz, R., Peters, G. J., and Assaraf, Y. G. Clustering of mutations in the first transmembrane domain of the human reduced folate carrier in GW1843U89-resistant leukemia cells with impaired antifolate transport and augmented folate uptake. J. Biol. Chem., *275:* 30855–30863, 2000.

18. Gong, M., Yess, J., Connolly, T., Ivy, S. P., Ohnuma, T., Cowan, K. H., and Moscow, J. A. Molecular mechanism of antifolate transport deficiency in a methotrexate resistant MOLT-3 human leukemia cell line. Blood, *89:* 2494–2499, 1997.

19. Jansen, G., Mauritz, R., Drori, S., Sprecher, H., Kathmann, I., Bunni, M., Priest, D. G., Noordhuis, P., Schornagel, J. H., Pinedo, H. M., Peters, G. J., and Assaraf, Y. G. A structurally altered human reduced folate carrier with increased folic acid transport mediates a novel mechanism of antifolate resistance. J. Biol. Chem., *273:* 30189–30198, 1998.

20. Wong, S. C., Zhang, L., Witt, T. L., Proefke, S. A., Bhushan, A., and Matherly, L. H. Impaired membrane transport in methotrexate-resistant CCRF-CEM cells involves early translation termination and increased turnover of a mutant reduced folate carrier. J. Biol. Chem., *274:* 10388–10394, 1999.

21. International Human Genome Sequencing Consortium. Initial sequencing and analysis of the human genome. Nature (Lond.), *409:* 860–921, 2001.

22. Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. D., Yandell, M., Evans, C. A., and Holt, R. A. The sequence of the human genome. Science (Wash. DC), *291:* 1304–1351, 2001.

23. McLeod, H. L., and Evans, W. E. Pharmacogenomics: unlocking the human genome for better drug therapy. Ann. Rev. Pharmacol. Toxicol., *41:* 101–121, 2001.

24. Peltonen, L., and McKusick, V. A. Dissecting human disease in the postgenomic era. Science (Wash. DC), *291:* 1224–1229, 2001.

25. Chango, A., Emery-Fillon, N., de Courcy, G. P., Lambert, D., Pfister, M., Rosenblatt, D. S., and Nicolas, J. P. A polymorphism (80G→A) in the *reduced folate carrie*r gene and its associations with folate status and homocysteinemia. Mol. Genet. Metab., *70:* 310–315, 2000.

26. Fry, D. W., Yalowich, J. C., and Goldman, I. D. Rapid formation of poly-γ-glutamyl derivatives of methotrexate and their association with dihydrofolate reductase as assessed by high pressure liquid chromatography in the Ehrlich ascites tumor cell *in vitro*. J. Biol. Chem., *257:* 1890–1896, 1982.

27. Grant, S. C., Kris, M. G., Young, C. W., and Sirotnak, F. M. Edatrexate, an antifolate with antitumor activity: a review. Cancer Investig., *11*: 36–45, 1993.

28. Smith, G. K., Bigley, J. W., Dev, I. K., Duch, D. S., Ferone, R., and Pendergast, W. GW1843: a potent, noncompetitive thymidylate synthase inhibitor—preclinical and preliminary clinical studies. *In:* A. L. Jackman (ed.), Anticancer Development Guide: Antifolate Drugs in Cancer Therapy, pp. 203–227. Totowa, NJ: Humana Press, Inc., 1999.

29. Hughes, L. R., Stephens, T. C., Boyle, F. T., and Jackman, A. L. Raltitrexed, a polyglutamatable antifolate thymidylate synthase inhibitor: Design and preclinical activity. *In:* Jackman A. L. (ed.), Anticancer Development Guide: Antifolate Drugs in Cancer Therapy, pp. 147–165. Totowa, NJ: Humana Press, Inc., 1999.

30. Mendelsohn, L. G., Worzalla, J. F., and Walling, J. M. Preclinical and clinical evaluation of the glycinamide ribonucleotide formyltransferase inhibitors Lometrexol and LY309887. *In:* A. L. Jackman (ed.), Anticancer Development Guide: Antifolate Drugs in Cancer Therapy, pp. 261–280. Totowa, NJ: Humana Press, Inc., 1999.

31. Wong, S. C., McQuade, R., Proefke, S. A., Bhushan, A., and Matherly, L. H. Human K562 transfectants expressing high levels of reduced folate carrier but exhibiting low transport activity. Biochem. Pharmacol., *53:* 199–206, 1997.

32. Matherly, L. H., Taub, J. W., Wong, S. C., Simpson, P. M., Ekizian, R., Williamson, M., Buck, S., Amylon, M., Pullen, J., Camitta, B., and Ravindranath, Y. Increased frequency of expression of elevated dihydrofolate reductase in T-cell *versus* B-precursor acute lymphoblastic leukemia in children. Blood, *90:* 578–579, 1997.

33. Zhang, L., Taub, J. W., Williamson, M., Wong, S. C., Hukku, B., Pullen, J., Ravindranath, Y., and Matherly, L. H. *Reduced folate carrier* gene expression in childhood acute lymphoblastic leukemia: relationship to immunophenotype and ploidy. Clin. Cancer Res., *4:* 2169–2177, 1998.

34. Horton, R. M., Cai, Z. L., Ho, S. N., and Pease, L. R. Gene splicing by overlap extension: tailor-made genes using the polymerase chain reaction. Biotechniques, *8:* 528–535, 1990.

35. Matherly, L. H., Czajkowski, C. A., and Angeles, S. M. Identification of a highly glycosylated methotrexate membrane carrier in K562 human erythroleukemia cells up-regulated for tetrahydrofolate cofactor and methotrexate transport. Cancer Res., *51:* 3420–3426, 1991.

36. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.), *227:* 680–685, 1970.

37. Matsudaira, P. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem., *262:* 10035–10038, 1987.

38. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem., *193:* 265–275, 1951.

39. Gifford, A. J., Whetstine, J. R., Bark, K. S., Kulkarni, J. S., Taub, J. W., Haber, M., Norris, M. D., and Matherly, L. H. Mutations in the *reduced folate carrier* gene (*RFC*) in childhood acute lymphoblastic leukemia (ALL). Proc. Am. Assoc. Cancer Res., *41:* 758, 2000.

40. Zhao, R., Assaraf, Y. G., and Goldman, I. D. A mutated reduced folate carrier (*RFC1*) with increased affinity for folic acid, decreased affinity for methotrexate, and an obligatory anion requirement for transport function. J. Biol. Chem., *273:* 19065–19071, 1998.

41. Zhao, R., Assaraf, Y. G., and Goldman, I. D. A reduced folate carrier mutation produces substrate-dependent alterations in carrier mobility in murine leukemia cells and methotrexate resistance with conservation of growth in 5-formyltetrahydrofolate. J. Biol. Chem., *273:* 7873–7879, 1998.

42. Tse, A., Brigle, K., Taylor, S. M., and Moran, R. G. Mutations in the *reduced folate carrier* gene which confer dominant resistance to 5,10-dideazatetrahydrofolate. J. Biol. Chem., *273:* 25953–25960, 1998.

43. Evans, W. E., and Relling, M. V. Pharmacogenomics: translating functional genomics into rational therapeutics. Science (Wash. DC), *286:* 487–491, 1999.