Reduced Folate Carrier Gene Expression in Childhood Acute Lymphoblastic Leukemia: Relationship to Immunophenotype and Ploidy

Long Zhang, Jeffrey W. Taub, Michael Williamson, So C. Wong, Bharati Hukku, Jeanette Pullen, Y. Ravindranath, and Larry H. Matherly


ABSTRACT

Reduced folate carrier (RFC) transcripts in human leukemias were measured by a competitive PCR assay. Total RNAs were reverse transcribed and amplified in the presence of competitive templates for RFC and β-actin. RFC transcripts were normalized to transcripts for β-actin. In a series of K562 sublines, a ~30-fold range of RFC transcripts measured by PCR assay closely agreed with results of Northern analysis and varied in proportion to RFC protein on Western blots and [3H]methotrexate transport.

RFC transcripts varied over a 88-fold range in 49 specimens from 48 children with acute lymphoblastic leukemia (ALL). Median RFC transcripts were similar for 15 T-cell and 33 B-precursor ALL samples (RFC/β-actin = 6.13 × 10−3 and 7.92 × 10−3, respectively) and for 41 diagnostic (7.20 × 10−3) and 8 relapse (5.88 × 10−3) samples. Whereas PCR measurements of RFC transcripts approximated changes in methotrexate transport in B-precursor ALL blasts (n = 10), for T-ALL blasts (n = 12) there was no apparent relationship between these parameters. For hyperdiploid B-precursor blasts (n = 11) with greater than 52 chromosomes and three to five copies of chromosome 21, the median RFC transcript level was ~3-fold higher than that for diploid B-precursor blasts. RFC transcripts were also elevated for two of three B-precursor specimens with acquired trisomy 21.

Our results suggest that RFC gene expression is far more predictive of methotrexate uptake capacity in B-precursor than T-ALL and that increased copies of chromosome 21 in B-precursor ALL blasts are generally associated with increased RFC transcripts. Hence, the good prognosis for children with hyperdiploid B-precursor ALL treated with antimetabolite-based chemotherapy and the high levels of methotrexate and methotrexate polyglutamates accumulated may, in part, reflect elevated RFC gene expression and capacities for methotrexate transport.

INTRODUCTION

An active, receptor-mediated transport process for natural folates, termed the RFC, has been described in a wide array of human cells (1, 2). MTX is also transported by RFC (1, 2). Although impaired MTX transport has been described as a common mechanism of MTX resistance in cultured cells (1, 2) and in murine leukemia cells after MTX chemotherapy (4, 5), only limited success has been achieved in extending these findings to the clinic.

ALL is the prototype disease for studying MTX resistance in the clinic because MTX remains an important drug for this malignancy (4, 5) and leukemic blasts can be obtained in high purity from bone marrow or blood for laboratory studies. Our previous report used flow cytometry to assess the role of lineage-specific differences in DHFR and MTX transport as contributing factors in clinical response to MTX (6). Although compelling evidence was obtained that both inherent and acquired MTX resistance in childhood ALL involved changes in DHFR levels, MTX transport by indirect flow cytometry assay was largely invariant in over 90 T-cell and B-precursor ALL specimens from children at diagnosis or relapse (6). For the small subset of patients whose blasts appeared to exhibit defective MTX transport, the magnitude of this impairment was invariably low (6).

Our inability to detect impaired MTX transport at any appreciable frequency by flow cytometry in a large number of childhood leukemia patients was surprising given earlier results of in vitro and animal studies (1–3). Furthermore, impaired MTX transport was described as a mechanism of clinical resistance in human leukemias (7, 8). On this basis, we began to explore alternative means for evaluating MTX transport capac-

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3 The abbreviations used are: RFC, reduced folate carrier; MTX, methotrexate; ALL, acute lymphoblastic leukemia; DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate synthetase; RT-PCR, reverse transcription-PCR; DS, Down's syndrome.
ities in childhood ALL, with features including increased 
detection sensitivity and ease of quantitation, and a level of 
simplicity appropriate to routine analysis of patient specimens.

Because of its unparalleled sensitivity, there has been 
growing interest in PCR-based approaches for studying tumor 
expression of genes relevant to chemotherapeutic response. If a 
protein can be identified whose level is critical to drug activity, 
PCR assays of gene expression can potentially be used to predict 
therapeutic response or acquired resistance accompanying 
chemotherapy. This strategy has been elegantly applied to a 
number of genes in patient tumors including thymidylate syn-
these (9), FPGS (10), and P-glycoprotein (11). Most recently, 
Gorlick et al. (8) described RT-PCR assays of RFC gene ex-
pression in assorted leukemic cells (ALL and acute myeloge-
nous leukemia) from both children and adults.

In this report, we adapt PCR-based methods to assays of 
RFC gene expression in cultured cells and leukemic blasts from 
children with ALL. RFC transcripts were found to correlate 
with levels of immunoreactive RFC proteins and/or direct measure-
ments of [$^{3}H$]MTX uptake in cultured cells and a majority of 
B-precursor ALL blasts; however, for T-ALL blasts, PCR as-
says were far less predictive of MTX uptake. Our finding of 
elevated RFC transcripts in hyperdiploid B-precursor blasts 
strongly suggests that increased levels of RFC gene expression 
and MTX transport may, in part, account for the high levels of 
MTX polyglutamates reported for these cells (12–14).

MATERIALS AND METHODS

Chemicals. (3',5',7-$^{3}H$)MTX (20 Ci/mmol) was pur-
bought from Moravek Biochemicals (Brea, CA). Unlabeled 
MTX was obtained from the Drug Development Branch, 
National Cancer Institute (Bethesda, MD). Both labeled and unla-
beled MTX were purified by reversed phase high-performance 
liquid chromatography as described previously (15). Tissue 
culture reagents and supplies were purchased from assorted 
vendors with the exception of iron-supplemented calf serum, 
which was obtained form the American Type Culture Collection 
(Rockville, MD). Cells were maintained in RPMI 1640 contain-
ing 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-impaired K500E and transport up-
regulated K562.4CF sublines were selected from wild-type 
K562 cells by cloning in soft agar and maintained as described 
previously (16).

Patient Specimens. Leukemia specimens (summarized 
in Table 1) were obtained from newly diagnosed or relapsed 
patients with ALL treated at the Children’s Hospital of Michi-
gan or Pediatric Oncology Group centers participating in the 
ongoing Pediatric Oncology Group 9400 ALL classification 
study. Three patients (sample numbers 22, 44, and 45) were 
treated at St. Jude Children’s Research Hospital (Memphis, TN). 
All patient samples were obtained after informed consent and in 
accordance with protocols approved by the Committees on

Investigation Involving Human Subjects at Wayne State Uni-
versity and St. Jude Children’s Research Hospital.

Blasts were separated from bone marrow by standard Ficoll 
Hypaque density centrifugation and were generally cryopre-
served in RPMI 1640/10% fetal bovine serum/10% DMSO in 
liquid nitrogen prior to experiment. Specimens were docu-
mented for patient age, sex, race, presenting WBC count, 
percentage of blasts in marrow, and immunophenotype; cytogeneric 
data were available for the B-precursor specimens.

The majority of patients in our analysis are still receiving 
chemotherapy. B-precursor patients were treated with assorted 
treatment protocols, generally including MTX during consoli-
dation (1 g/m²) and maintenance (20 mg/m²) phases. Chemother-
apy for T-ALL was typically more intensive than for B-
precursor ALL and frequently included anthracyclines and 
epidophyllotoxins, with or without intermediate dose i.v. 
MTX.

Assay of [$^{3}H$]MTX Uptake in Patient Blasts. RFC-
mediated MTX uptake was assayed in patient blasts using a 
aviation of our earlier published methods (16, 17). Incubations 
were with 1 µM high radiospecific activity (5.6 mCi/µmol) 
[$^{3}H$]MTX for 30 min at 37°C in 0.5 ml of complete culture 
medium plus 10% serum (while shaking). Drug uptake was 
quenched with ice-cold Dulbecco’s PBS; cells were washed 
(three times) with ice-cold PBS and solubilized in 0.5 N NaOH 
for assays of radioactivity and protein content. Net drug uptake 
under these conditions was expressed as pmol [$^{3}H$]MTX per mg 
of total cell protein. Because net MTX uptake after 30-min drug 
uptake exceeded MTX bound to DHFR by >10-fold, even in 
specimens with elevated DHFR, this parameter provided a 
good approximation of RFC-mediated membrane transport. 
Furthermore, for a small number of samples in which they were 
directly measured, MTX polyglutamates comprised <20% of 
the intracellular drug forms under conditions of our uptake assay 
(data not shown). RFC involvement was directly confirmed in a 
number of ALL specimens by specifically blocking [$^{3}H$]MTX 
uptake with GW18438U9 (Glaxo-Wellcome Co.), a tight binding 
RFC substrate (18).

All of the drug uptake measurements were performed with 
previously cryopreserved ALL specimens and were repeated 
two to three times with nearly identical results. The data re-
ported are mean values from replicate experiments. In prelimi-
nary experiments, we found no difference in capacities for 
[$^{3}H$]MTX uptake for identical fresh (never frozen) and cryopre-
served ALL specimens, as long as the following conditions were 
mets: (a) after thawing, frozen blasts were preincubated for 
20–24 h at 37°C in complete culture medium plus 10% serum 
prior to assay; and (b) cell viabilities, measured at the time of 
experiment by trypan blue exclusion, were 80% or greater 
typically >90%).

Western Analysis of Immunoreactive RFC Protein.
Polyclonal antibodies to RFC (anti-RFC/ps) were prepared in 
New Zealand White rabbits by Research Genetics (Huntsville, 
AL), using a synthetic RFC peptide [PEDSLGAVG-
PNLQRQS, corresponding to amino acids 504–521 (17)] 

4 Unpublished data.
### Table 1: Characteristics of ALL specimens

<table>
<thead>
<tr>
<th>Specimen Type*</th>
<th>Specimen</th>
<th>Age (yr)*</th>
<th>Sex</th>
<th>Race*</th>
<th>WBC(^{a,c})</th>
<th>% blasts</th>
<th>RFC-β-Actin ((&lt;10^{9}))</th>
<th>PT430(^{d})</th>
<th>Cytogenetics</th>
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</table>

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* AA, African American; AI, American Indian; Aq21, acquired trisomy 21; H, Hispanic; HD, hyperdiploid; ND, not determined; W, white.

* At diagnosis.

* Units for WBCs are cells/μl.

* Relative PT430 fluorescence upon treatment with 0.5 μM MTX to 0.5 μM trimetrexate. As described previously (6), increased fluorescence ratios measured by flow cytometry correspond to impaired RFC-mediated MTX transport. Specimens 1, 5, and 22 contained dual populations with differing capacities for PT430 displacement by MTX. The percentages of each subpopulation are: 1, 78/22; 5, 86/14; 22, 90/10.

* Archival karyotype.

* Paired relapse/diagnostic specimen.
conjugated to keyhole limpet hemocyanin (19). Preparation of plasma membranes and gel electrophoresis conditions were described previously (16, 19). Immunoblot detection on electrophoresed plasma membrane proteins was performed using protein A-agarose (20) purified RFC/ps and an enhanced chemiluminescence kit (Boehringer Mannheim) according to the manufacturer’s recommendations. Light emission was recorded on X-ray film, and the intensities of the signals were quantitated by densitometry.

**Competitive RT-PCR Assay of RFC Expression.** Total RNA was isolated from 1–10 X 10⁶ lymphoblasts using a RNeasy mini kit (Qiagen). First-strand cDNAs were synthesized from 1 μg of total RNA in 20 μl of reaction mix containing 50 pmol of random hexa DNA oligonucleotide primers, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 1 mM dNTPs, and 50 units of Moloney murine leukemia virus reverse transcriptase. After 1 h of incubation at 42°C, the reaction was stopped by heating at 99°C for 5 min. Aliquots of the cDNA mixtures were amplified by PCR in the presence of serially-diluted, 276-bp RFC competitive mimic template (prepared by deleting a 50-bp AccI-AccI fragment from a 326-bp wild-type RFC cDNA fragment). The PCR reaction (50 μl) contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM MgCl₂, 0.2 mM dNTPs, 0.5% DMSO, 1.25 units Taq polymerase (Promega), and 120 nm primers [P1: 5’-CGCACGCTTCTTCTCAACCGC-3’ (nucleotides 622 to 643 as described in Ref. 17) and P2: 5’-ATCGCGTGGGAGGCGACATCT-3’ (nucleotides 927 to 948)]. PCR was performed for 35 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C, followed by 7 min at 72°C. To normalize levels of RFC expression, parallel tubes were prepared with competitive template and commercial primers for β-actin (Clontech), and PCR was performed for 35 cycles of 30 s at 94°C, 45 s at 60°C, and 1 min at 72°C, followed by an additional 7 min at 72°C. Aliquots (10 μl) were analyzed on agarose gels (2% for RFC, 1.5% for β-actin) and stained (1–2 h) with SYBR green I (FMC Bioproducts), and the relative levels of fluorescent PCR products were quantitated with a Molecular Dynamics Storm 860 fluorescence and radioactivity imaging system and ImageQuant software. The logarithm of the ratio of endogenous target RFC (or β-actin) to RFC (β-actin) competitor were plotted against the log attomoles of competitor template added to the amplification reaction (21); attomoles of endogenous target were calculated using the equivalence point of the target and competitor templates (21). No PCR products were detected in negative controls, including cDNA reaction mixtures prepared without RNA and mock cDNA reactions prepared without reverse transcriptase.

For all samples, PCR measurements were repeated two to four times on separate days. Overall, the mean variation between replicate measurements on the same patient specimen was 26.61% (±20.02%; SD) for RFC (n = 49); for β-actin, the variation averaged 23.13% (±13.43%; n = 49). These values are well within the range of variation reported previously (9, 22) for RT-PCR measurements of transcripts for other human genes. The data reported are mean values of replicate PCR measurements. For comparison of relative levels of RFC transcripts between samples, ratios of mean RFCs to mean β-actins are reported.

**Northern Hybridizations.** Total RNAs (20 μg) were separated by formamide-agarose electrophoresis, blotted onto nylon filters, and hybridized with 32P-labeled RFC cDNA (K543) (17). Equal RNA loading was confirmed by stripping and reprobing with 32P-labeled β-actin cDNA. Relative intensities of the signals were determined with a Molecular Dynamics densitometer and ImageQuant software.

**RESULTS**

**Quantitation of RFC Transcripts in K562 Sublines by Competitive RT-PCR.** Competitive PCR reactions were performed with constant cDNAs (prepared from transport-impaired K500E cells) and increasing concentrations of RFC competitor. The levels of 326-bp product derived from the endogenous RFC transcript and 276-bp RFC competitor product were quantitated, and the logarithms of this ratio were plotted against the log attomoles of competitor template added to the amplification reaction (21); attomoles of endogenous target were calculated from the equivalence point of the target and competitor templates (Fig. 1B). By this approach, correlation coefficients consistently exceeded 0.95.

To correct for differences in RNA levels or quality, or differences in the efficiencies of reverse transcription between samples, RFC transcript levels were normalized to levels of β-actin transcripts, measured in separate tubes with a commercial competitive β-actin template. Following amplification, the 838-bp β-actin target and 619-bp competitor template products were analyzed, as described above.

Relative levels of RFC transcripts, measured by RT-PCR, were in close agreement with transcript levels measured by Northern analysis for wild-type K562, K562.4CF, and K500E cells (Refs. 17 and 19; Table 2). However, the ability of the PCR method to accurately resolve differences between low levels of gene expression (i.e., less than for wild-type cells) was greater. In Fig. 2, data are shown from a mixing experiment in which theoretical amounts of RFC transcripts from K500E and wild-type total RNAs (based on Northern analyses) were reverse-transcribed. For each mixture, RFC and β-actin templates were separately amplified in the presence of competitor templates with respective primers, and relative amounts of target templates were calculated, as described above. The close agreement between relative theoretical and actual RFC to β-actin ratios over a ~10-fold range aptly demonstrates the ability of the competitive RT-PCR assay to resolve small differences in relative RFC transcripts with high accuracy. Furthermore, the 30-fold differences in levels of RFC gene expression among wild-type K562, K562.4CF, and K500E cells paralleled changes in the levels of immunoreactive RFC protein on Western blots (19), and, likewise, relative capacities for [3H]MTX uptake (Ref. 19; Table 2).
Fig. 1  Analysis of RFC transcripts by RT-PCR. A, 1 µg of total K500E RNA was reverse transcribed, and identical cDNA aliquots were amplified by PCR over 35 cycles in the presence of 3.52 × 10^{-4}, 1.49 × 10^{-4}, 7.45 × 10^{-5}, 3.72 × 10^{-5}, 1.86 × 10^{-5}, or 9.31 × 10^{-6} attomoles/µL of RFC competitor (Lanes 1–6). DNA size standards are shown in Lane M. For the gel shown, samples (10 µL each) were analyzed on a 2% agarose gel, stained with SYBR green I, and photographed on a transilluminator. B, the logarithms of the ratios of PCR products derived from the endogenous RFC template and RFC competitor (RFCc) were plotted against the log attomoles/µL of competitor template added to the amplification reaction. A Pearson's correlation of 0.998 was calculated. As depicted, the level of endogenous cDNA target (7.4 × 10^{-5} attomoles/µL) was extrapolated from the equivalence point of target and competitor PCR products.

Table 2  Correlations between MTX transport, RFC protein, and RFC transcripts in K562 sublines

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Transporta</th>
<th>Immunoblotb,c</th>
<th>Northernsb</th>
<th>RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.00</td>
</tr>
<tr>
<td>K500E</td>
<td>0.17</td>
<td>ND</td>
<td>0.10</td>
<td>0.12</td>
</tr>
<tr>
<td>K562.4CF</td>
<td>6.09</td>
<td>7.2</td>
<td>3.46</td>
<td>2.73</td>
</tr>
</tbody>
</table>

a Relative MTX transport data were from Matherly et al. (16).
b Data are from Wong et al. (19).
c Calculated from densitometric analysis as described in “Materials and Methods.”
d Normalized RFC transcript levels are expressed as RFC:β-actin ratios, as described in the text.

d ND, not detected.

Measurements of RFC Transcripts in Leukemic Blasts from Children with ALL. Experiments were performed to extend our RT-PCR methods to measurements of RFC transcripts in 49 leukemic blast specimens from children with ALL at diagnosis (28 B-precursor and 13 T-cell) and relapse (5 B-precursor and 3 T-cell). Patient characteristics are summarized in Table 1.

In striking contrast to our earlier inability to detect significant differences in MTX transport in leukemic blasts by flow cytometry (6, 23), 88-fold variations in relative RFC transcripts were detected by RT-PCR (Table 1; Fig. 3). For 23 specimens, both RT-PCR and flow cytometry assays were performed with notably different results (Table 1). RFC to β-actin ratios for ALL blasts were typically 3–10-fold lower than those for wild-type K562 cells. This level approached the limit of detection for our Northern blotting assays. Similarly, immunoreactive RFCs could not be detected in blast cells by standard Western blotting with RFC-specific antiserum (data not shown).

RFC to β-actin ratios were compared between B-precursor and T-ALL specimens (Fig. 3A). Although the median (6.13 × 10^{-3} and 7.92 × 10^{-3}, respectively) values for RFC to β-actin were not significantly different for 15 T-cell and 33 B-precursor
ALL samples ($P = 0.16$ by the Mann-Whitney $U$ test), marked differences were measured in the range of RFC values $(0.88 \times 10^{-3}$ to $11.81 \times 10^{-3}$ and $0.54 \times 10^{-3}$ to $47.18 \times 10^{-3}$, respectively). There were no significant differences in the median RFC values for specimens obtained at diagnosis $(7.20 \times 10^{-3}; n = 41)$ and relapse $(5.58 \times 10^{-3}; n = 8; P = 0.76)$. 

Fig. 4 shows the results for 10 B-precursor ALL specimens (Fig. 4A) and 12 T-ALL specimens (Fig. 4B), illustrating the relationships between RFC to $\beta$-actin ratios measured by RT-PCR assay and [H]MTX ($1 \mu$M) uptake after 30 min. For the 10 B-precursor specimens, a Spearman’s correlation (rho) of 0.455 ($P = 0.17$) was calculated, which changed to a highly significant value ($r = 0.783$ and $P = 0.05$) when the two outlying values were excluded. Although the variations in [H]MTX uptake closely correlated with relative levels of RFC transcripts over a $\sim$6-fold range of uptake for these eight B-precursor samples, the range of relative RFC transcripts exceeded that for relative drug uptake by $\sim$4-fold. With T-cell ALLs ($n = 12$), [H]MTX uptake was generally lower than for B-precursor ALLs, and there was no apparent relation to RFC expression ($r = 0.002$ and $P = 0.995$; Fig. 4B).

For B-precursor ALL, differences in the relative levels of RFC transcripts between groups of patients were reflective of chromosomal ploidy (Fig. 3B). Indeed, an unusually high level of RFC gene expression was detected for the 11 B-precursor patients characterized by hyperdiploid karyotypes (54–59 chromosomes for samples 20–30 in Table 1; Fig. 3B). Although two of these samples (nos. 29 and 30) were obtained at relapse, there were no significant differences between the karyotypes at diagnosis and relapse. Five hyperdiploid samples exhibited RFC to $\beta$-actin ratios exceeding $2.5 \times 10^{-3}$ and included the highest values recorded in our patient cohort. Median RFC to $\beta$-actin ratios were significantly increased for hyperdiploid B-precursor blasts over diploid B-precursor blasts ($1.13 \times 10^{-2}$ versus $4.26 \times 10^{-3}; P = 0.0097$ by Mann-Whitney $U$ test). Interestingly, RFC expression was also slightly elevated in two (samples 18 and 19 in Table 1) of three non-DS B-precursor patients with 47 chromosomes and acquired trisomy 21 (1.28 $\times 10^{-2}$ and 1.31 $\times 10^{-2}$, respectively). However, this pattern was not observed for two B-precursor specimens (samples 15 and 16) from patients with DS.

DISCUSSION

Studies aimed at establishing the role of RFC in clinical MTX response have long been hampered by the lack of a uniformly sensitive and quantitative method of analysis. RFC expression in patient specimens is frequently far lower than for cultured cells and often approaches the limit of detection for standard drug uptake or blotting assays. Although we (6, 23) and others (7, 8) previously used a fluorescent MTX analogue and flow cytometry for indirectly evaluating RFC function in human leukemia cells, our more recent experience suggested that this method may not be sufficiently sensitive to reliably measure MTX transport capacities of leukemic blasts from children with ALL. Moreover, with uncharacterized leukemia samples from patients, data interpretation is often subjective, and it is frequently difficult to establish suitable criteria for classifying a specimen as transport-impaired versus competent.

Notably, our experience differs from that of Gorlick et al. (8), who successfully used flow cytometry to detect impaired MTX transport in assorted leukemic samples (ALL and acute myelogenous leukemia) from both children and adults. This discrepancy may reflect differences in patient or disease characteristics, assay methodologies, or data interpretation, as noted above.
assays of RFC (8, 26), lies in the fact that it is not necessary to measure the PCR products exclusively during the exponential phase of amplification. This allows useful data to be obtained, even at plateau phase. Because the extent of RNA degradation or efficiency of reverse transcription, combined with sampling errors, can profoundly influence the yield of PCR products obtained, we separately amplified β-actin competitor and target templates and calculated empirical RFC to β-actin ratios as measures of relative RFC gene expression. Other genes could also be used for normalizing our RFC results because when glyceraldehyde-3-phosphate dehydrogenase transcripts were used as a basis for normalizing RFC expression in a small number of samples (with significantly differing RFC:β-actin ratios), qualitatively similar results were obtained (not shown).

For a series of K562 sublines with a ~30-fold range of RFC transcripts, relative transcripts by PCR assay were nearly identical to those measured on Northern blots. Furthermore, differences in RFC transcript levels in cultured cells were paralleled by changes in amounts of immunoreactive RFC protein or initial rates of [3H]MTX transport.

As with earlier studies of RFC expression in leukemic blasts (8), we found a wide range (88-fold) of RFC transcript levels in blasts from children with ALL at diagnosis and relapse. Although RFC transcripts were frequently low in relapsed ALL, there were no overall differences in PCR measurements of RFC expression between diagnostic and relapse samples or between B-precursor and T-cell ALL blasts. We were able to demonstrate a remarkably close correlation between our PCR results and [3H]MTX uptake for 8 B-precursor ALL specimens. However, two additional B-precursor specimens exhibited disproportionate levels of RFC expression, and for 12 T-ALL specimens there was no obvious relationship between RFC gene expression and low levels of [3H]MTX uptake. Hence, RT-PCR assay of RFC gene expression appears to be predictive of MTX uptake capacity only for the majority of B-precursor ALLs.

The bases for lack of correlation between uptake and expression for a subset of B-precursor ALLs and for the majority of T-ALLs are presently under investigation. Likely possibilities include the inefficient translation of RFC transcripts to mature carrier, or the existence of mutated or otherwise nonfunctional RFC proteins, analogous to RFC alterations described in vitro (27–29).

Of particular interest was the finding that RFC levels were dramatically increased in a disproportionate number of hyperdiploid B-precursor patients over diploid patients. Hyperdiploidy (>50 chromosomes or a DNA index >1.16) has been documented to confer a favorable prognosis in B-precursor ALL patients treated with MTX-based chemotherapy (30) and has been associated with substantially higher levels of MTX polyglutamates over diploid ALL (12–14). Because no significant differences were noted previously in the levels of FPGS activity between hyperdiploid (n = 5) and nonhyperdiploid (n = 26) ALL blasts [905 pmol/h/mg versus 764 pmol/h/mg, respectively (12)], it would appear that elements separate from FPGS must determine the relative levels of MTX polyglutamates accumulated. The localization of the RFC gene to chromosome 21 [q22.2–22.3 (31)] and the demonstration that increased copies of chromosome 21 occur in ~97% of hyperdiploid B-precursor ALL patients (32), strongly implies that elevated MTX polyglu-
tamylation must, at least in part, be secondary to increased RFC gene copy and expression and, consequently, increased MTX transport in these patients.

Although the elevated RFC transcripts in our hyperdiploid specimens may result from increased RFC gene dosage and extra copies of chromosome 21 in this fashion, we were unable to establish a strict correlation between total copies of chromosome 21 and RFC transcripts. Moreover, there were no obvious relationships between RFC gene expression and increased copies of other chromosomes or additional cytogenetic abnormalities (i.e., chromosomal translocations); however, because of the limited numbers of hyperdiploid patients available for analysis, these possibilities cannot entirely be discounted. Whereas the slightly increased RFC to β-actin ratios in two of three non-DS patients with 47 chromosomes and acquired trisomy 21 as the sole cytogenetic alteration further suggests that a gene dosage effect may, in some instances, still come into play, the relationship between the well-progression associated with this chromosomal abnormality (33) and MTX polyglutamate synthesis is uncertain. A similar explanation was proposed (4) to account for the well-established sensitivity of DS patients to MTX (34); however, increased RFC expression was not seen for the two DS patients in our cohort.

In conclusion, our results expand upon earlier studies (8) and document the potential predictive value of PCR assays of RFC gene expression in assessing clinical response of B-precursor ALL to chemotherapy with MTX. Although RT-PCR assays were poorly predictive of RFC function in T-ALL blasts, for cultured cells and the majority of B-precursor blasts, RFC transcripts correlated with other measures of MTX uptake capacity, including levels of immunoreactive RFC protein and/or direct assays of 

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