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Alterations in the Expression of Transcription Factors and the Reduced Folate Carrier as a Novel Mechanism of Antifolate Resistance in Human Leukemia Cells*

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Lilah Rothem‡, Ami Aronheim§, and Yehuda G. Assaraf‡¶

From the Departments of ‡Biology and §Molecular Genetics, the Rappaport Institute for Research in the Medical Sciences and the B. Rappaport Faculty of Medicine, The Technion-Israel Institute of Technology, Haifa 31096, Israel

The human reduced folate carrier (hRFC) is the dominant transporter mediating the uptake of reduced folate cofactors and antifolate anticancer drugs. Defective antifolate uptake due to inactivating mutations in the hRFC gene is an established mechanism of drug resistance in various tumor cells. However, while antifolate transport is frequently impaired, either no or only a single hRFC allele is inactivated, suggesting that additional mechanism(s) of resistance are operative. Here we studied the relationship between the expression and function of transcription factors and antifolate resistance in transport-defective leukemia cells that poorly express or completely lack RFC mRNA. Stable transfection with a hRFC expression construct resulted in restoration of normal RFC mRNA expression and nearly wild type drug sensitivity in these antifolate-resistant cells. The loss of RFC gene expression prompted us to explore transcription factor binding to the hRFC promoter. The hRFC promoter contains an upstream GC-box and a downstream cAMP-response element (CRE)/AP-1-like element. Electrophoretic mobility shift assays and oligonucleotide competition revealed a substantial loss of nuclear factor binding to CRE and GC-box in these drug-resistant cell lines. Consistently, antibody-mediated supershift analysis showed a marked decrease in the binding of CRE-binding protein 1 (CREB-1) and specificity protein 1 (Sp1) to CRE and GC-box, respectively. Western blot analysis revealed undetectable expression of CREB-1, decreased ATF-1 levels, parental Sp1 levels, and increased levels of the short Sp3 isoforms, recently shown to repress hRFC gene expression. Transient transfections into these antifolate-resistant cells demonstrated a marked loss of GC-box-dependent, and CRE-driven reporter gene activities and introduction of CREB-1 or Sp1 expression constructs resulted in restoration of hRFC mRNA expression. These results establish a novel mechanism of antifolate resistance that is based on altered expression and function of transcription factors resulting in transcriptional silencing of the hRFC promoter.

Mammalian cells are devoid of reduced folate biosynthesis and therefore meet their folate growth requirement via uptake from exogenous sources (1). Reduced folates are absolutely essential cofactor vitamins involved in a host of one-carbon transfer reactions resulting in the biosynthesis of purines, deoxythymidylate, and methionine (1). Folate antagonists (*i.e.* antifolates) including methotrexate (MTX)[1] are potent inhibitors of purine and deoxythymidylate biosynthesis and thereby block DNA synthesis. Consequently, MTX is an integral component of chemotherapeutic regimens used in the treatment of various human malignancies (2). Folates and antifolates are hydrophilic divalent anions that cannot cross membranes via diffusion and are therefore taken up into mammalian cells by the reduced folate carrier (RFC) (3). Among the antifolates recognized as transport substrates by the RFC are the dihydrofolate reductase inhibitor, MTX (2), the novel thymidylate synthase inhibitors Tomudex (ZD1694, Raltitrexed) (4) and ZD9331 (5), as well as the glycinamide ribonucleotide transformylase inhibitor AG2034 (6). Various antifolate resistance phenomena pose a major obstacle toward curative cancer chemotherapy. For example, qualitative (*i.e.* inactivating mutations) (7–11) and quantitative alterations (*i.e.* decreased or abolished expression) in human RFC (hRFC) gene expression and/or function are documented mechanisms of MTX resistance in acute lymphoblastic leukemia (ALL) (12, 13) and osteogenic sarcoma patients (14). Although decreased expression of hRFC mRNA has been suggested as a mechanism of clinical resistance to MTX, the underlying molecular basis is still unknown (12–14).

It was recently shown that transcription of the hRFC gene is driven by at least two promoter elements: an upstream constitutive element consisting of a GC-box as well as a downstream inducible cAMP-response element (CRE)/AP-like element (CRE/AP-1) (15). Reporter gene assays revealed that the binding of Sp1 and the long Sp3 isoforms to the GC-box as well as that of CRE-binding protein (CREB-1) to the CRE/AP-1-like element result in activation of hRFC transcription (15). Using stepwise antifolate selection, the human CCRF-CEM leukemia cell lines $AG2034^{R2}$ and $ZD9331^{R1.5}$ were isolated, which displayed up to 2300-fold resistance to various antifolates due to abolished drug transport (11). This was a result of a marked decrease (or complete loss) of RFC mRNA expression. This major decrease in RFC mRNA expression was associated with alterations in the expression of transcription factors, resulting in a prominently decreased binding to both the constitutive and

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¶ To whom correspondence should be addressed: Dept. of Biology, The Technion-Israel Institute of Technology, Haifa 32000, Israel. Tel.: 972-4-829-3744; Fax: 972-4-822-5153; E-mail: assaraf@tx.technion.ac.il.

[1] The abbreviations used are: MTX, methotrexate; RFC, reduced folate carrier; hRFC, human RFC; CRE, cyclic AMP-response element; CREB-1, CRE-binding protein 1; Sp1, specificity protein 1; DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate synthetase; MRP1, multidrug resistance protein 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ALL, acute lymphoblastic leukemia; RT, reverse transcription; CAT, chloramphenicol acetyltransferase.

TABLE I
Oligonucleotides used for electrophoretic mobility shift assay
WT, wild type; M, mutant.

Oligonucleotide[a]	Sequence of sense strand (5′ → 3′)
CREB WT	TCG ATT GGC TGA CGT CAG AGA G
CREB M	TGG ATT GGC **TCG** CGT C**C**G AGA G
SP1 WT	AGT CGA TCG GGG CGG GGC GAG
SP1 M	AGT CGA TCG G**TT** CGG GGC GAG
AP1 WT	AGC TAG CAT GAG TCA GAC

[a] For double-stranded oligonucleotides used in EMSA, only the sense strand is shown. Binding sites in the AP-1, CREB, and SP1 oligonucleotides are underlined with mutations in bold.

inducible elements of the hRFC promoter. This was associated with poor expression of CREB-1 and binding to the inducible CRE as well as substantially diminished Sp1 binding to the constitutive GC-box element in the hRFC promoter. The current study constitutes the first demonstration of a novel mechanism of antifolate resistance that is based on alterations in the expression of transcription factors and their binding to the hRFC promoter.

MATERIALS AND METHODS

Drugs, Biochemicals, and Radiochemicals—MTX was from Teva Pharmaceuticals Ltd. Novel antifolate drugs were generous gifts from the following sources: ZD9331 from Dr. A. L. Jackman, (Institute of Cancer Research, Sutton, United Kingdom), AG2034 from Dr. T. Boritzki (Agouron Pharmaceuticals, Inc.), and PT523 from Dr. W. T. McCulloch (Sparta Pharmaceuticals).

Tissue Culture and Antifolate Drug Selections—CCRF-CEM, a human T-cell leukemia line, and its antifolate-resistant sublines were maintained in RPMI 1640 medium containing 2.3 μM folic acid (Biological Industries, Beth-Haemek, Israel) supplemented with 10% fetal calf serum (Invitrogen), 2 mM glutamine, 100 units/ml penicillin G (Sigma), and 100 μg/ml streptomycin sulfate (Sigma). The cell lines $AG2034^{R2}$ and $ZD9331^{R1.5}$ were established by stepwise antifolate selection of parental CCRF-CEM cells in gradually increasing concentrations of AG2034 and ZD9331 as previously described (11). CCRF-CEM-7A cells with a high RFC overexpression were cultured in folic acid-free medium containing 10% dialyzed fetal calf serum and supplemented with 0.25 nM leucovorin as the sole folate source (16).

Antifolate Growth Inhibition—Parental cells and their antifolate-resistant sublines were first grown in antifolate-free growth medium for five to eight cell doublings. Thereafter, cells were seeded in 96-well plates (3×10^4/well) in growth medium (0.15 ml/well) containing various concentrations of different antifolates. After 3 days of incubation at 37 °C, viable cell numbers were determined by hemocytometer count using trypan blue exclusion. The percent inhibition of cell growth was calculated relative to untreated controls.

[^{3}H]MTX Transport—To examine the ability of the antifolate-resistant sublines to transport antifolates, we determined the initial rates of [^{3}H]MTX uptake relative to wild type CEM cells. Exponentially growing cells (2×10^7) were washed three times in transport buffer consisting of HEPES-buffered saline solution (11) and incubated at 37 °C for 3 min in same buffer (1-ml suspensions) containing 2 μM [^{3}H]MTX. Transport controls contained a 500-fold excess of unlabeled MTX (1 mM). Transport was terminated by the addition of 10 ml of ice-cold HEPES-buffered saline solution, after which cells were 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.

Electrophoretic Mobility Shift Assays—Nuclear extracts were prepared from exponentially growing cells (2×10^7 cells) as previously described (17). DNA-protein complexes were formed by incubating nuclear extract proteins (6 μg) with [γ-^{32}P]dCTP or [γ-^{32}P]dATP end-labeled CRE, AP-1, or GC-box double-stranded oligonucleotides (Table I) as detailed elsewhere (18). Oligonucleotide competition was performed with a 10–100-fold molar excess of unlabeled oligonucleotides (Table I). For supershift analysis, an aliquot of nuclear proteins (6 μg) was incubated for 1 h at 4 °C with anti-CREB-1, -ATF-1, -Sp1, and -Sp3 (Santa Cruz Biotechnology) antibodies (2–4 μg) in binding buffer according to the instructions of the manufacturer. Then, the radiolabeled oligonucleotide was added and DNA-protein immunocomplexes were allowed to form for 30 min at 4 °C. Complexes consisting of DNA, nuclear protein(s), and a specific antibody were resolved by electrophoresis on 6% non-denaturing polyacrylamide gels in 0.5× Tris borate-EDTA, pH 8.4, at 4 °C. The gels were then dried, and DNA-protein complexes were visualized by phosphorimaging. Protein concentration was determined by the colorimetric method of Bradford (19).

Semi-quantitative RT-PCR of hRFC and Various Housekeeping Genes—Exponentially growing cells (2×10^7) were harvested by centrifugation and washed with phosphate-buffered saline, and total RNA was isolated using the Tri-Reagent kit according to the instructions of the manufacturer (Sigma). A portion of total RNA (20 μg in a total volume of 20 μl) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (180 units, Promega) in a reaction buffer containing random hexamer primers, dNTPs, and ribonuclease inhibitor RNasin (Promega). Portions of cDNA (~50 ng) synthesized from parental CEM cells and their antifolate-resistant sublines were amplified using Expand polymerase (Roche Molecular Biochemicals) in a reaction buffer (total volume 25 μl) containing: 10 pmol of each primer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, and 2% Me_2SO according to the instructions of the manufacturer. The PCR reaction was performed as follows: initial melting at 95 °C for 5 min, followed by 35 cycles each of 1 min at 95 °C, annealing at 62 °C (56 °C for β-actin) for 45 s, elongation at 72 °C for 1 min, followed by a 10-min extension at 72 °C. Then, the PCR products were resolved on 2% agarose gels. The primers used for the semiquantitative RT-PCR of hRFC, DHFR, FPGS, MRP1, GAPDH, and β-actin are depicted in Table II.

Transient Transfections with CRE- and GC-box-luciferase, CREB-1 and Sp1 Expression Constructs, and Reporter Activity—Logarithmically growing suspension cells (2×10^7) were harvested by centrifugation and transfected by electroporation (1000 microfarads, 234 V) with 10 μg of reporter constructs containing CRE-luciferase, GC-box-luciferase (pGL3-luciferase) and GC-box-chloramphenicol acetyltransferase (CAT) or with expression plasmids (10 μg) including pRSV-CREB-1 and pPacSp1 (kindly provided by Drs. M. E. Greenberg and G. Suske, respectively). Cells were then seeded at 2×10^6/ml in prewarmed growth medium. For reporter gene experiments, after 24 h of growth at 37 °C cells were harvested by centrifugation and washed with phosphate-buffered saline, cell lysates were prepared, and firefly luciferase activity was assayed using the dual luciferase kit (Promega) and a luminometer. Firefly luciferase activity was normalized with *Renilla* luciferase, following co-transfection with a *Renilla*-luciferase plasmid. For transient RFC mRNA expression, after 24 h of growth cells were harvested and total RNA was extracted. Results presented were obtained from at least three independent transfections performed in duplicate cultures and activity determinations.

Stable Transfections with a hRFC Expression Construct—Exponentially growing cells (2×10^7) were harvested by centrifugation and stably transfected by electroporation (1000 microfarads, 234 V) with 10 μg of an expression vector (pcDNA3-hRFC1) harboring the hRFC cDNA (10). Following 24 h of growth at 37 °C, cells were exposed to 600 μg/ml active G-418 (Calbiochem-Novabiochem, San Diego, CA). Stable transfectants expressing high levels of hRFC mRNA were obtained after at least 1 month of G-418 selection and were used for further analyses.

Western Blot Analysis—Nuclear extract proteins (20 μg) were resolved by electrophoresis on 10% polyacrylamide gels containing SDS, electroblotted onto a Protran cellulose nitrate membrane (Schleicher & Schuell), reacted with anti-CREB-1, -ATF-1, -Sp1, and -Sp3 (Santa Cruz Biotechnology Inc.) according to the instructions of the manufacturer. To examine RFC expression in parental and antifolate-resistant cells, microsomes were first isolated (20), and proteins were extracted in a buffer containing 0.5% Triton X-100 as previously described (11). Detergent-soluble proteins (12.5–150 μg) were resolved by electrophoresis, electroblotted onto a nylon membrane and blocked for 1 h at room temperature in TBS buffer (150 mM NaCl, 0.5% Tween 20, and 10 mM Tris/Cl at pH 8.0) containing 1% skim milk. The blots were then reacted with a polyclonal antiserum (1:700) prepared in mice against a C-terminal hRFC peptide (11), rinsed in the same buffer for 10 min in room temperature, and reacted with horseradish peroxidase-conjugated goat anti-mouse IgG (1:40,000 dilution, Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at room temperature. Following three washes (10 min each) in TBS at room temperature, enhanced chemiluminescence (ECL) detection was performed according to the manufacturer's instructions (Biological Industries, Beth Haemek, Israel). ECL was recorded on x-ray films using several exposure times, which were evaluated by scanning densitometry.

RESULTS

RFC Expression, Drug Transport, and Antifolate Resistance—We studied the molecular basis of antifolate resistance

TABLE II
Oligonucleotides used for RT-PCR of various housekeeping genes

Gene	Sense primer	Antisense primer
RFC	5′-CTGGCCTTCCTCACCTTCCA-3′	5′-CCGAGTTGAAGACCCACCAGA-3′
DHFR	5′-CTGCACAAATAGGGACGAGG-3′	5′-TAATGCCTTTCTCCTCCTGG-3′
FPGS	5′-CCGGCTGAACATCATCCA-3′	5′-CTTTCTGCCATGCGATCTTCT-3′
MRP1	5′-CAGGAGCAGGATGCAGAGGA-3′	5′-TGTAGTCCCAGTACACGGAAAGC-3′
β-Actin	5′-AAGAGAGGCATCCTCACCCT-3′	5′-TACATGCTGGGGTGTTGAA-3′
GAPDH	5′-AGGGGGGAGCCAAAAGGG-3′	5′-GAGGAGTGGGTGTCGCTGTTG-3′

in the human leukemia sublines ZD9331$^{R1.5}$ and AG2034^{R2}. These cell lines displayed up to 2300-fold resistance to various antifolates that use RFC as their primary route of uptake (Fig. 1*A*). This was associated with a marked decrease (95–97%) in the transport of [^{3}H]MTX (Fig. 1*B*). While detectable with 25 μg of microsomal proteins from parental CEM cells, RFC was not detectable even with 150 μg of proteins from AG2034^{R2} and ZD9331$^{R1.5}$ cells (Fig. 2*A*). Semiquantitative RT-PCR analysis showed that AG2034^{R2} cells expressed ~1/300th of parental RFC mRNA, whereas ZD9331$^{R1.5}$ cells had no detectable expression (Fig. 2*B*). In contrast, AG2034^{R2} and ZD9331$^{R1.5}$ cells expressed normal mRNA levels of various housekeeping genes including DHFR, FPGS, MRP1, and GAPDH (Fig. 2*C*), thereby suggesting that these cells did not suffer from a global transcriptional repression.

Restoration of Antifolate Sensitivity upon Transfection with a hRFC Expression Vector—To examine the impact of restoration of RFC expression on antifolate sensitivity, AG2034^{R2} and ZD9331$^{R1.5}$ cells were stably transfected with an expression vector harboring the wild type hRFC cDNA (Fig. 2*B*, *lane 1*). This resulted in restoration of normal RFC mRNA expression in these cell lines (Fig. 2*B*, *lane 1*). Consequently, AG2034^{R2} and ZD9331$^{R1.5}$ cells became up to 170-fold more sensitive to MTX, thereby approaching the sensitivity of wild type cells to this antifolate (Fig. 1*C*). These results establish that the loss of hRFC gene expression is a major underlying mechanism of antifolate drug resistance in these transport defective cell lines.

Nuclear Factor Binding to [^{32}P]-labeled CRE, AP-1, and GC-box Oligonucleotides—To explore the basis for the loss of RFC mRNA expression, nuclear proteins from parental CEM and antifolate-resistant cells were isolated and examined by an electrophoretic mobility shift assay. This electrophoretic mobility shift assay assessed the binding of nuclear factors to [^{32}P]-labeled CRE, GC-box, and AP-1 double-stranded oligonucleotides (Table I). A 3-band binding pattern to [^{32}P]-labeled CRE (Fig. 3*A*) and [^{32}P]-labeled GC-box (Fig. 3*B*) was observed in parental CEM cells, whereas AG2034^{R2} and ZD9331$^{R1.5}$ cells had a markedly decreased binding to both oligonucleotides (Fig. 3, *A* and *B*) but retained normal binding to [^{32}P]-labeled AP-1 (Fig. 3*C*). To examine the specificity of nuclear factor binding to these consensus binding sites, two independent parameters were used: (a) competition of binding to CRE and GC-box with unlabeled oligonucleotides and (b) binding to mutant CRE and GC-box oligonucleotides (Table I). The binding of nuclear proteins from parental cells to [^{32}P]-labeled CRE, and GC-box was competed in a dose-dependent manner, with a 10–100-fold molar excess of the unlabeled oligonucleotides (data not shown). In contrast, the residual binding of nuclear proteins from AG2034^{R2} and ZD9331$^{R1.5}$ cells to CRE and GC-box could not be competed even with a 100-fold molar excess of the unlabeled oligonucleotides. Furthermore, nuclear proteins from both parental and antifolate-resistant sublines failed to bind to the mutant [^{32}P]-labeled CRE and [^{32}P]-labeled GC-box oligonucleotides and displayed a similar nonspecific basal binding (data not shown). These results suggest that the residual binding to CRE and GC-box in these antifolate-resistant sublines was largely nonspecific.

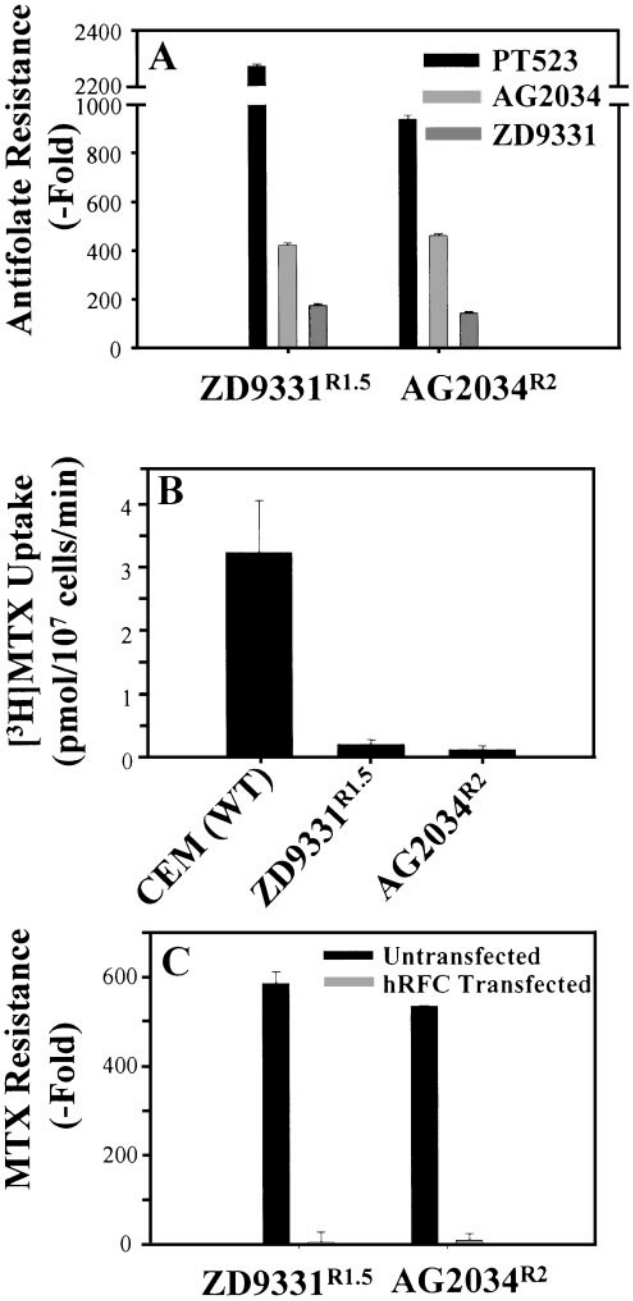

FIG. 1. **Antifolate resistance and [^{3}H]MTX transport in parental CEM cells and their antifolate-resistant sublines.** Levels of antifolate resistance in AG2034^{R2} and ZD9331$^{R1.5}$ cells prior to (*A*) or after stable transfection with a hRFC cDNA (*C*). Initial rates of [^{3}H]MTX transport (*B*) were determined as described under "Materials and Methods." The influx of [^{3}H]MTX in parental CEM cells was: 4.0 ± 0.6 pmol [^{3}H]MTX/min × 10^7 cells. Results shown are the means of four independent experiments ± S.D.

Restoration of RFC mRNA Expression upon Transient Transfection with CREB-1 and Sp1 Expression Constructs—The putative loss of function of transcription factors that are involved

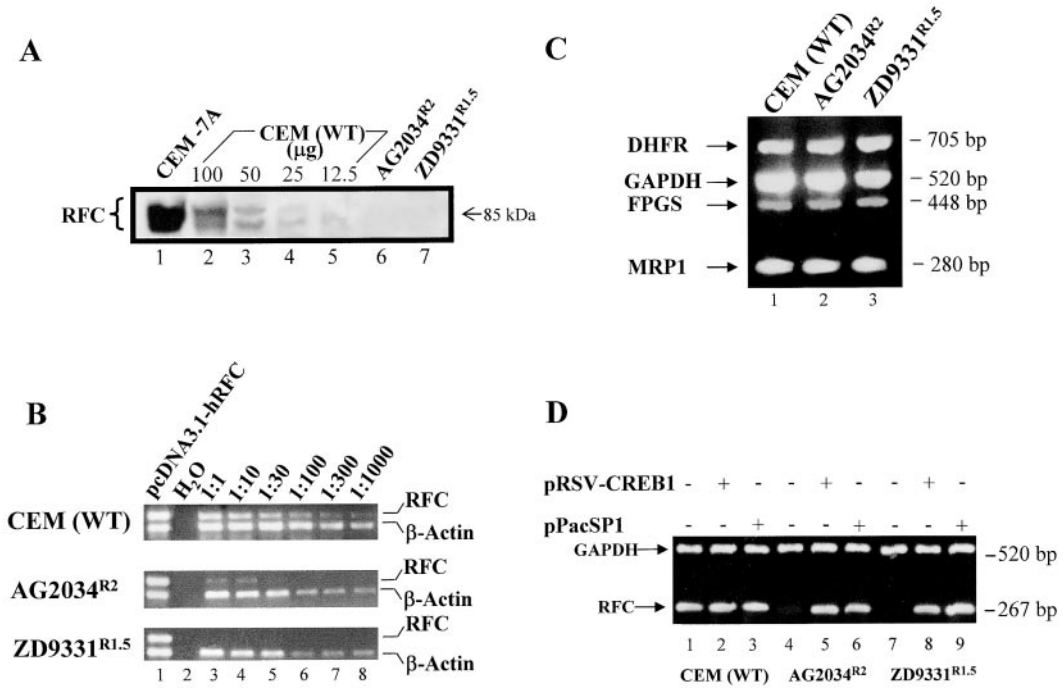

FIG. 2. **Expression of RFC and various housekeeping genes in wild type and antifolate-resistant cells.** Western blot analysis with microsomal proteins from RFC-overexpressing CEM-7A cells (50 μg, *lane 1*), wild type CEM cells (100, 50, 25, and 12.5 μg, *lanes 2–5*, respectively), as well as AG2034^{R2} and ZD9331$^{R1.5}$ cells (150 μg, *lanes 6* and *7*, respectively) was performed with anti-hRFC antibodies as detailed under "Materials and Methods." Messenger RNA levels of RFC (*B*), DHFR, GAPDH, FPGS, and MRP1 (*C*) were determined by semiquantitative RT-PCR as detailed under "Materials and Methods." RFC mRNA expression determined by various dilutions of total cDNA prior to or after transient transfection of parental and antifolate-resistant cells with the expression vectors pRSV-CREB-1 and pPacSp1 (*D*) was normalized to β-actin (*B*) or GAPDH (*D*). RFC mRNA expression in wild type and antifolate-resistant cells stably transfected with an expression vector harboring the hRFC cDNA is also shown (*B*, *lane 1*). The H_2O group represents a negative PCR control in which no cDNA was present (*B*, *lane 2*).

in the binding to CRE and GC-box prompted us to explore the impact of the introduction of CREB-1 and Sp1 on RFC mRNA expression (Fig. 2*D*). Transfection of AG2034^{R2} and ZD9331$^{R1.5}$ cells with expression constructs harboring CREB-1 (pRSV-CREB1) or Sp1 (pPacSp1) resulted in restoration of normal RFC mRNA expression (Fig. 2*D*).

Loss of CRE- and GC-box-driven Reporter Gene Activity—To assess CRE- and GC-box-mediated transcription, parental cells and their antifolate-resistant sublines were transiently transfected with CRE-luciferase (Fig. 4*A*), GC-box luciferase (Fig. 4*B*), and GC-box-CAT (Fig. 4*C*), and reporter gene activity was determined. The activities of firefly luciferase and CAT were normalized to that of a promoterless *Renilla* construct. Antifolate-resistant cells had a substantial decrease in luciferase activity driven by the CRE-containing construct, relative to parental cell transfectants (Fig. 4*A*). Reporter gene activity driven by two independent constructs containing GC-box was markedly reduced in these antifolate-resistant cells (Fig. 4, *B* and *C*). Thus, these drug-resistant cell lines had a major loss in GC-box- and CRE-dependent transcription, resulting in a marked or complete loss of hRFC mRNA expression.

Antibody-mediated Supershift Analysis—It was recently shown that CREB-1 and Sp1 bind to CRE and GC-box in the hRFC promoter, respectively (15). To identify alterations in the binding of specific transcription factors to CRE (Fig. 5*A*) and GC-box (Fig. 5*B*), antibody-mediated supershift analysis was used. Preincubation of nuclear proteins either with antibodies to CREB-1 and ATF-1 or a combination of the two followed by [^{32}P]-labeled CRE-protein-antibody complex formation revealed supershifts in parental CEM cells (Fig. 5*A*, *complexes A–C* in *lanes 2–4*, respectively; note the elimination of *bands 1* and/or *2*). Likewise, [^{32}P]-labeled GC-box supershifts in parental CEM cells were obtained with anti-Sp1 (Fig. 5*B*, *complex A* in *lane 2*; note the elimination of band 2) and anti-Sp3 antibodies (Fig. 5*B*; note the elimination of *bands 1* and *3* in *lane 3*). Furthermore, the combination of anti-Sp1 and -Sp3 antibodies eliminated all three [^{32}P]-labeled GC-box bands and formed a high molecular weight complex (Fig. 5*B*, *complex B* in *lane 4*). In contrast, nuclear proteins from AG2034^{R2} (*lanes 5–8*) and ZD9331$^{R1.5}$ cells (*lanes 9–12*) neither showed [^{32}P]-labeled CRE supershifts with anti-CREB-1 or ATF-1 antibodies (Fig. 5*A*) or [^{32}P]-labeled GC-box supershifts with antibodies to Sp1 (Fig. 5*B*). However, anti-Sp3 antibodies eliminated *band 3* of [^{32}P]-labeled GC-box in both AG2034^{R2} and ZD9331$^{R1.5}$ cells (Fig. 5*B*, *lanes 7* and *11*, respectively).

Alterations in the Expression and Function of Transcription Factors—As alterations in the function of transcription factors could also result from changes in their expression, we determined CREB-1, ATF-1 (Fig. 6*A*), Sp1 (Fig. 6*B*), and Sp3 levels (Fig. 6*C*) by Western blot analysis. Parental CEM cells expressed substantial levels of CREB-1 and ATF-1 (Fig. 6*A*, *lane 1*), whereas AG2034^{R2} and ZD9331$^{R1.5}$ cells did not contain detectable levels of CREB-1 (Fig. 6*A*, *lanes 2* and *3*, respectively). In addition, these cells had decreased ATF-1 levels (Fig. 6*A*, *lanes 2* and *3*, respectively). Reprobing the Western blot with anti-Sp1 antibodies revealed similar Sp1 levels in parental cells and their antifolate-resistant sublines (Fig. 6*B*, *lanes 1–3*). Parental CEM cells expressed both the long and short isoforms of Sp3 (Fig. 6*C*, *lane 1*), whereas AG2034^{R2} and ZD9331$^{R1.5}$ cells showed increased levels of the short Sp3 isoforms (Fig. 6*C*, *lanes 2* and *3*, respectively). As the short Sp3 isoforms were recently shown to act as inhibitors of hRFC transcription (15), we examined the possibility that a repressor activity was present in these antifolate-resistant cells that could interfere with GC-box binding. A nuclear protein mixture (vol:vol) derived from parental and ZD9331$^{R1.5}$ cells showed a normal binding to [^{32}P]-labeled CRE (Fig. 3*D*, compare *lane 3* with *1*) but a poor binding (<15% of wild type) to the [^{32}P]-labeled GC-box (Fig.

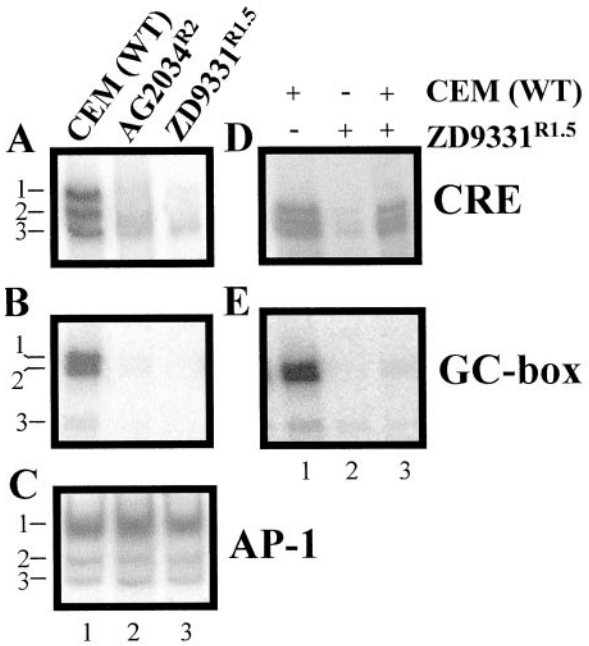

FIG. 3. **Electrophoretic mobility shift assay of [^{32}P]-labeled CRE, GC-box, and AP-1 oligonucleotides.** Nuclear proteins (6 μg) from parental CEM, AG2034^{R2}, and ZD9331$^{R1.5}$ cells were first incubated with [^{32}P]-labeled CRE (*A*), GC-box (*B*), and AP-1 (*C*) oligonucleotides, resolved by electrophoresis on polyacrylamide gels, and viewed by phosphorimaging. A portion of nuclear proteins (6 μg total) from parental CEM cells was mixed with an equal amount of nuclear proteins from ZD9331$^{R1.5}$ cells, and the binding to [^{32}P]-labeled CRE (*D*) and [^{32}P]-labeled GC-box (*E*) oligonucleotides was examined. The nuclear protein-CRE/GC-box/AP-1 complexes are denoted on the *left* by *1*, *2*, and *3*.

3*E*, compare *lane 3* with *1*). These results suggest that an inhibitory component was present in the nuclear extract from ZD9331$^{R1.5}$ cells that appeared to disrupt GC-box binding.

DISCUSSION

The aim of the current study was to identify the molecular basis underlying the loss of RFC mRNA expression in antifolate-resistant human leukemia cell lines displaying impaired MTX transport. We find substantial alterations in the expression of CREB-1, ATF-1, and the short isoforms of Sp3. These alterations were associated with a prominent decrease in the binding of transcription factors to the inducible (CRE) and constitutive (GC-box) elements in the hRFC promoter, presumably leading to a marked decrease in RFC mRNA expression. Consistently, introduction of CREB-1 and Sp1 into these antifolate-resistant cells resulted in restoration of RFC mRNA expression. Importantly, stable transfection of a hRFC cDNA driven by a potent cytomegalovirus promoter, resulted in restoration of normal RFC mRNA expression and nearly wild type sensitivity to MTX. These results provide the first demonstration of a novel mechanism of antifolate drug resistance that is based upon altered expression and function of transcription factors resulting in transcriptional silencing of the hRFC promoter.

It was recently found that the hRFC promoter is driven by an inducible CRE/AP-1-like element and a constitutive GC-box element (15). This study showed that CREB-1 and c-Jun in human fibrosarcoma HT1080 cells, as well as CREB-1 and ATF-1 in human hepatocellular carcinoma HepG2 cells, were the cell-specific transcription factors involved in CRE binding (the inducible element) in the hRFC promoter. We consistently find here that CREB-1 and ATF-1 were expressed at substantial levels in wild type human leukemia CEM cells and displayed a functional binding to CRE. The marked decrease or

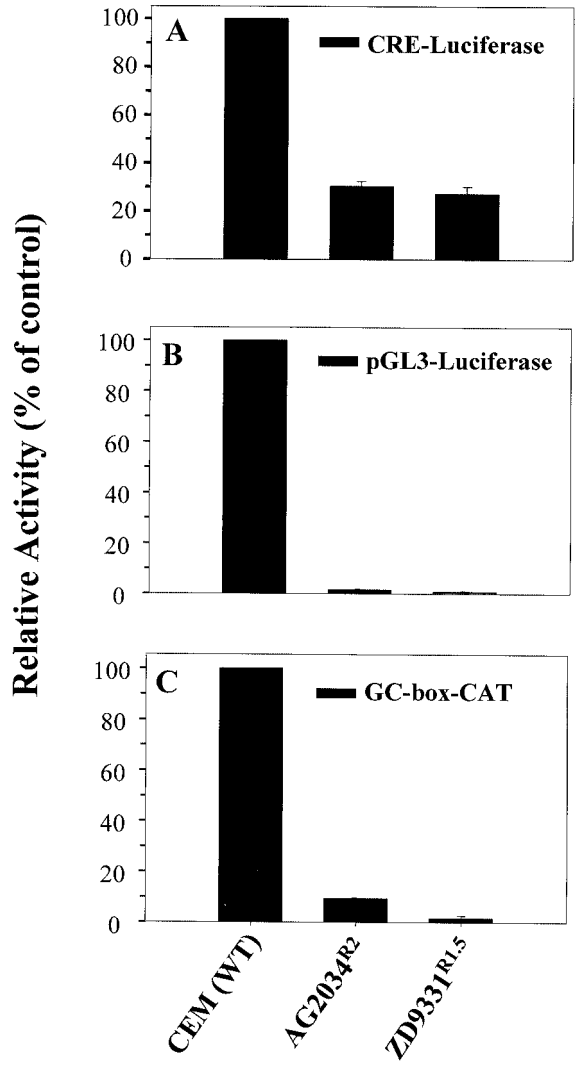

FIG. 4. **CRE and GC-box reporter gene activities following transient transfection into parental and antifolate-resistant cells.** CRE-luciferase (*A*), GC-box-luciferase (*B*), GC-box-CAT (*C*), and control *Renilla* constructs were transfected by electroporation into parental CEM and antifolate-resistant cells. Cells were then lysed and reporter gene activities were normalized to *Renilla* luciferase. Results presented are averages ± S.D. derived from three experiments. The 100% control activity was obtained from parental CEM cells.

loss of RFC mRNA expression in AG2034^{R2} and ZD9331$^{R1.5}$ cells, respectively, was associated with a decreased expression (or lack) of CREB-1 and ATF-1. These results strongly suggest that CREB-1 and ATF-1 are essential components for the transcriptional activation of the inducible element in the hRFC promoter in leukemia cells. However, antifolate-resistant cancer cell lines and malignant tumors of different cell lineages may potentially express alternative, cell-specific transacting factors of the basic region-leucine zipper (bZip) family, which may also contribute to hRFC transcription.

Transient transfections of either Sp1 or the long Sp3 isoform resulted in a potent activation of the constitutive GC-box element in the hRFC promoter (15). In contrast, introduction of the short Sp3 isoform resulted in a potent repression of the Sp1-dependent activation of the hRFC promoter. Consistently, ZD9331$^{R1.5}$ and AG2034^{R2} cells showed elevated levels of the short Sp3 isoforms; the latter is likely to act as a repressor of the Sp1-mediated activation of the constitutive GC-box element in the hRFC promoter. This suggestion could gain support from the following results and considerations. First, the

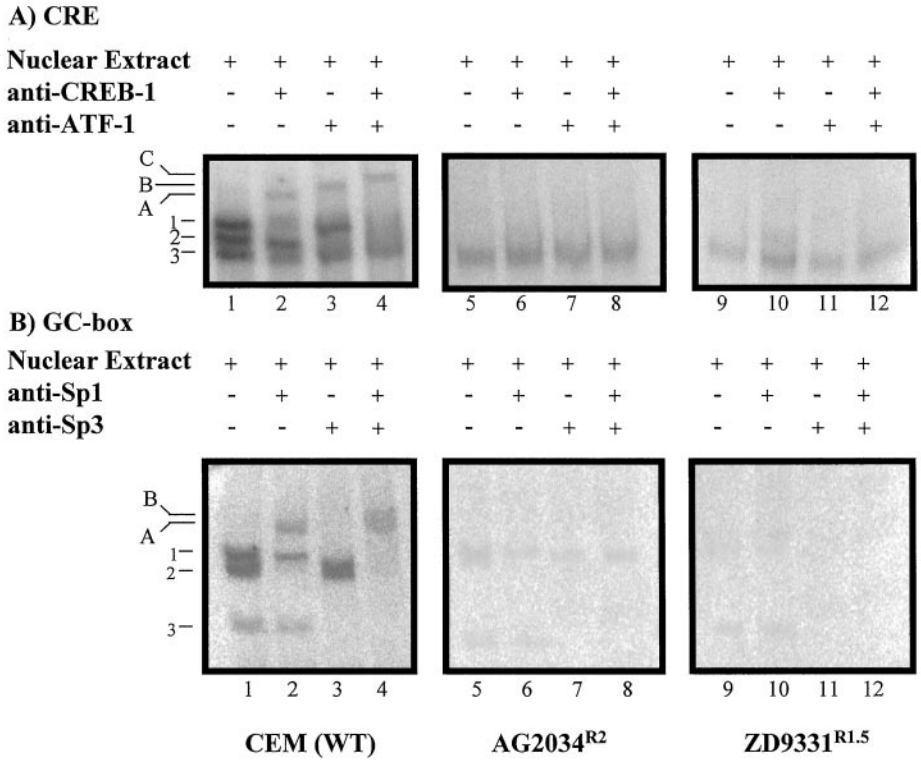

FIG. 5. **Antibody-mediated supershift analysis with [^{32}P]-labeled CRE and [^{32}P]-labeled GC-box oligonucleotides.** Nuclear proteins (6 μg) from parental CEM and antifolate-resistant cell lines were first incubated with antibodies (2–4 μg) to CREB-1, ATF-1, Sp1, and Sp3 for 1 h at 4 °C. Then, the binding to [^{32}P]-labeled CRE (*A*) or [^{32}P]-labeled GC-box (*B*) oligonucleotides was examined by phosphorimaging. The antibody-nuclear protein(s)-oligonucleotide complexes are denoted on the *left* by *A*, *B*, and *C*.

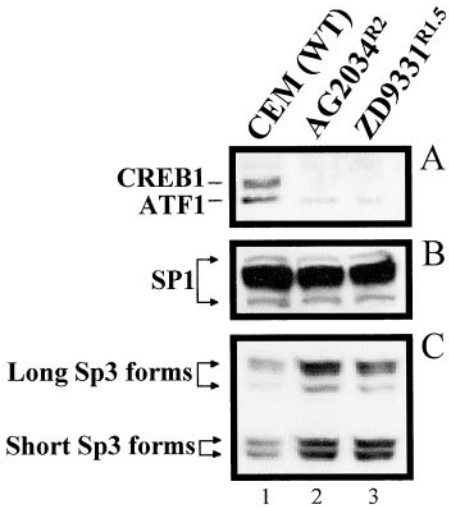

FIG. 6. **Western blot analysis of transcription factor expression in parental and antifolate-resistant cell lines.** Nuclear proteins (20 μg) from parental CEM and antifolate-resistant sublines were resolved by polyacrylamide gels containing SDS, transferred to a cellulose nitrate Protran membrane, and reacted with antibodies to human CREB-1/ATF-1 (A) as detailed under "Materials and Methods." The blots were then reprobed with antibodies to Sp1 (*B*) and Sp3 (*C*).

1:1 mixing of nuclear proteins from parental and ZD9331$^{R1.5}$ cells markedly blocked GC-box binding but did not interfere with CRE binding. Second, it is likely that transfection of ZD9331$^{R1.5}$ and AG2034^{R2} cells with an Sp1 expression construct should result in Sp1 levels that exceed those of the short Sp3 isoforms, resulting in restoration of RFC mRNA expression. Thus, the short Sp3 isoforms that are expressed at elevated levels in these antifolate-resistant cells appear to competitively block Sp1 binding to the GC-box, thereby repressing transcriptional activation of the hRFC gene.

We have recently shown that a predominant mechanism of resistance to MTX and various novel hydrophilic antifolates is the loss of RFC-mediated drug transport in multiple human leukemia sublines (8, 10, 11). These transport-defective sublines harbored various inactivating mutations that primarily clustered in the first transmembrane domain of the hRFC (8, 10, 11). In contrast, AG2034^{R2} cells were devoid of RFC mutations (11), had an extremely poor expression of RFC mRNA, and had no detectable levels of RFC protein. These cells consequently displayed an abolished antifolate transport. Thus, the loss of RFC expression in AG2034^{R2} cells constitutes the first mechanism of antifolate-resistance that appears to rely solely on the loss of transcriptional activation of the hRFC promoter. In contrast, ZD9331$^{R1.5}$ cells harbored a heterozygous premature translation termination mutation (Glu257Stop) but retained a wild type hRFC allele (11). Although this wild type allele could have been substantially transcribed, ZD9331$^{R1.5}$ cells were devoid of RFC mRNA expression. These results suggest that although mutations are frequently observed in the human (7–11) and the murine (21) RFC genes, the mutational inactivation of a single allele is insufficient and may be accompanied by transcriptional silencing of both alleles via alterations in the expression and function of certain transcription factors. A strong support to this suggestion derives from the fact that various antifolate-resistant human (7–11) and mouse (21) leukemia sublines harbored both RFC-inactivating mutations and prominently decreased RFC mRNA levels.

The present findings have potentially important implications for the development of clinical resistance to MTX and novel antifolates, including ZD1694 (Raltitrexed, Tomudex) (4) and multitargeted antifolate (Pemetrexed) (22), which use RFC as their primary route of entry. Several studies with specimens derived from ALL (12, 13) and osteosarcoma (14) have shown that defective drug transport via the RFC may be a mechanism of resistance to MTX. Consistently, decreased RFC mRNA expression has also been documented in specimens from ALL (12, 13) and osteosarcoma patients (14). Surprisingly however, while inactivating mutations in the RFC gene were a frequent mechanism of antifolate resistance in human (7–11) and animal tumor cell lines (21), the frequency of RFC mutations in

ALL specimens was extremely low.[2] These results suggest that transcriptional silencing of the hRFC gene either by altered expression and function of transcription factors or alternatively by hRFC promoter methylation (23) may prove as factors that contribute to antifolate resistance. Importantly, the collateral sensitivity of various transport-defective phenotypes to lipophilic antifolates including trimetrexate (11) may be exploited to eradicate antifolate-resistant tumors (24).

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