Reduced Folate Carrier Gene Silencing in Multiple Antifolate-resistant Tumor Cell Lines Is Due to a Simultaneous Loss of Function of Multiple Transcription Factors but Not Promoter Methylation*

Received for publication, August 18, 2003, and in revised form, October 8, 2003
Published, JBC Papers in Press, October 9, 2003, DOI 10.1074/jbc.M309092200

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The human reduced folate carrier (hRFC) is the major uptake route for antifolates used in cancer chemotherapy. Here we explored the molecular basis for the decrease or loss of hRFC gene expression in seventeen tumor cell lines with resistance to multiple antifolates due to impaired antifolate transport. We studied the role of various cis-acting elements including CRE/AP-1-like element and GC-box in hRFC promoters A and B, respectively, as well as AP-2, Mzf-1 and E-box that are contained within or near four tandemly repeated sequences upstream of promoter A. Decreased or abolished binding to four \(^{32}\)P-labeled GC-box, Mzf-1, AP-1, E-box, or CRE oligonucleotides was detected in \(-50\%\)–\(-80\%\) of antifolate-resistant cell lines. Strikingly, \(-80\%\) of the cell lines displayed a simultaneously decreased binding to three or more of these hRFC promoter elements, whereas normal AP-2 binding was retained. The possible contribution of promoter methylation to hRFC gene silencing was also explored. None of the antifolate-resistant cell lines, except for MDA-MB-231 cells, showed hRFC promoter methylation; consistently, MDA-MB-231 was the only cell line that retained binding to all six cis-acting elements. Western blot analysis demonstrated decreased expression of transcriptional activators (pCREB-1, pATF-1, USF-1, c-Fos, c-Jun, Sp1, and Sp3) and/or increased expression of repressors (short Sp3 isoforms), whereas normal AP2α levels were retained. Transient expression of the relevant transcription factors restored, at least partially, both promoter binding and hRFC gene expression. This is the first report that transcriptional silencing of the hRFC gene in multiple tumor cell lines with resistance to various novel antifolates is a result of a simultaneous loss of function of multiple transcription factors but not promoter methylation.

Reduced folates are essential cofactors involved in various one-carbon transfer reactions resulting in the biosynthesis of nucleotides and amino acids essential for cell growth and DNA replication (1). Since mammalian cells lack the enzymatic capacity for the \textit{de novo} biosynthesis of tetrahydrofolate cofactors, these vitamins must be obtained from the diet (1). Folate analogues (i.e. antifolates) serve as anticancer drugs as they potently inhibit various enzymes involved in purine and deoxynucleotide biosynthesis resulting in inhibition of DNA synthesis and consequent cell death.

Reduced folates and antifolates are divalent anions that cannot traverse membranes by passive diffusion and are therefore transported into mammalian cells primarily by the reduced folate carrier (RFC) (2, 3). Among the antifolates that are recognized as transport substrates by the RFC are the dihydrofolate reductase inhibitors MTX, PT523 (4), and edatrexate (5), the novel thymidylate synthase inhibitors raltitrexed (Tomudex) (6) and ZD9331 (7), the glycinamide ribonucleotide transformylase inhibitor AG2034 (8) as well as the multitargeted antifolate (MTA), pemetrexed (9). In order for antifolates to exert their cytotoxic effect on tumor cells, high levels of RFC-dependent transport activity must be maintained (2, 3). Therefore, impaired drug transport results in decreased intracellular concentration of antifolates and consequent drug resistance; defective antifolate transport is a major mechanism of drug resistance in cultured tumor cells subjected to clinically relevant concentrations of various antifolates (10–14). Consistently, decreased hRFC gene expression and/or impaired antifolate transport are documented mechanisms of drug resistance in acute lymphoblastic leukemia (ALL) (15–17) and osteosarcoma patients that undergo MTX-containing chemotherapy (18–20). Using human osteosarcoma specimens at diagnosis we have shown recently that expression of low RFC protein levels at the time of initial biopsy correlates with a poor response to preoperative chemotherapy containing MTX (20).

Reduced RFC mRNA levels can result from transcriptional silencing; the lack of hRFC transcripts in MTX-resistant MDA-MB-231 breast cancer cells with impaired MTX transport was due to promoter methylation (21). Furthermore, recent studies suggest a complex regulation of hRFC gene expression in normal tissues and tumor cell lines (22–24). Therefore, we recently initiated studies that focus on the elucidation of the molecular basis of the loss of hRFC gene expression in antifolate-resistant tumor cells. To this end, we recently found that alterations in the expression and binding of transcription factors to cAMP response element (CRE/AP-1-like element and GC-box in hRFC promoters A and B, respectively, underlie the loss of...
hRFC gene expression in two antifolate-resistant cell lines with impaired antifolate transport (25). Apart from the constitutive GC-box and inducible CRE/AP-1 element, additional promoter elements including AP-2, myeloid zinc finger 1 (Mzf-1), and E-box are contained within or near four tandemly repeated sequences upstream of promoter A (Fig. 1). Using electrophoretic mobility shift assay (EMSA) and antibody-mediated supershift analysis we screened 17 antifolate-resistant human leukemia and breast cancer cell lines displaying impaired antifolate transport associated with a marked decrease or complete loss of RFC mRNA expression. Strikingly, the vast majority of the cell lines displayed a simultaneous loss of binding to three or more cis-acting elements, whereas AP-2 binding was retained. The markedly decreased binding to these cis-acting elements was associated with decreased expression of various transactivators. This is the first demonstration that simultaneous alterations in the expression and function of multiple transcription factors that bind to various cis-acting elements are a major mechanism of loss of hRFC gene expression in tumor cell lines that display resistance to various novel antifolates due to impaired drug uptake. We further show that while hRFC promoter silencing via methylation is rare in antifolate-resistant cell lines, it appears to alleviate the stress that would otherwise lead to alterations in the expression and function of transcription factors.

MATERIALS AND METHODS

Drugs—MTX and aminopterin were from Sigma. Novel antifolate drugs were generous gifts from the following sources: AG2034, Dr. T. Boritzki (Aegouren Pharmaceuticals, Inc.); PT523, Dr. W. T. McCulloch (Sparta Pharmaceuticals); GW1843U89, Dr. G. Smith (Glaxo Welcome, Britain); Dicoumarol (Boritzki (Agouron Pharmaceuticals, Inc.); PT523, Dr. W. T. McCulloch (Sparta Pharmaceuticals); GW1843U89, Dr. G. Smith (Glaxo Welcome, Britain). Drugs—MTX and aminopterin were from Sigma. Novel antifolate drugs were generous gifts from the following sources: AG2034, Dr. T. Boritzki (Aegouren Pharmaceuticals, Inc.); PT523, Dr. W. T. McCulloch (Sparta Pharmaceuticals); GW1843U89, Dr. G. Smith (Glaxo Welcome, Britain); Dicoumarol (Boritzki (Agouron Pharmaceuticals, Inc.); PT523, Dr. W. T. McCulloch (Sparta Pharmaceuticals); GW1843U89, Dr. G. Smith (Glaxo Welcome, Britain). Drugs—MTX and aminopterin were from Sigma. Novel antifolate drugs were generous gifts from the following sources: AG2034, Dr. T. Boritzki (Aegouren Pharmaceuticals, Inc.); PT523, Dr. W. T. McCulloch (Sparta Pharmaceuticals); GW1843U89, Dr. G. Smith (Glaxo Welcome, Britain); Dicoumarol (Boritzki (Agouron Pharmaceuticals, Inc.); PT523, Dr. W. T. McCulloch (Sparta Pharmaceuticals); GW1843U89, Dr. G. Smith (Glaxo Welcome, Britain). Drugs—MTX and aminopterin were from Sigma. Novel antifolate drugs were generous gifts from the following sources: AG2034, Dr. T. Boritzki (Aegouren Pharmaceuticals, Inc.); PT523, Dr. W. T. McCulloch (Sparta Pharmaceuticals); GW1843U89, Dr. G. Smith (Glaxo Welcome, Britain); Dicoumarol (Boritzki (Agouron Pharmaceuticals, Inc.); PT523, Dr. W. T. McCulloch (Sparta Pharmaceuticals); GW1843U89, Dr. G. Smith (Glaxo Welcome, Britain). Drugs—MTX and aminopterin were from Sigma. Novel antifolate drugs were generous gifts from the following sources: AG2034, Dr. T. Boritzki (Aegouren Pharmaceuticals, Inc.); PT523, Dr. W. T. McCulloch (Sparta Pharmaceuticals); GW1843U89, Dr. G. Smith (Glaxo Welcome, Britain); Dicoumarol (Boritzki (Agouron Pharmaceuticals, Inc.); PT523, Dr. W. T. McCulloch (Sparta Pharmaceuticals); GW1843U89, Dr. G. Smith (Glaxo Welcome, Britain).

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**Fig. 1. Schematic representation of the hRFC promoter region.** Putative binding sites for various transcription factors are designated by geometrical shapes. The two dark gray boxes represent minimal promoters A and B (22), whereas the light gray boxes R1–R4 denote nearly identical tandem repeats upstream of promoter A. Nucleotide numbering was relative to translation initiation ATG which represents position +1 using accession number 7717445.

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**Table I**

Oligonucleotides used for electrophoretic mobility shift assay

| Oligonucleotide | Sequence of sense strand (5' → 3') |
|-----------------|-----------------------------------|
| CRE             | TCG ATT GCC TGA CCG AGA G         |
| GC-box          | AGT CCA TCG GGG CCG GCC GAG       |
| AP-1            | AGC TAG CAT GAG TCA GAC           |
| AP-2            | GAG TGA CCG CCC GGG GCC C         |
| Mzf-1           | GAG TAC CAG TCG GGA ACG C         |
| E-box           | GAG ACG GTC ACG TGG CTT A         |

For double-stranded oligonucleotides used in EMSA, only the sense strand is shown. The core consensus sequence is underlined.

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**Table II**

Transcription Factor Dysfunction and hRFC Gene Silencing

Transcription Factor Dysfunction and hRFC Gene Silencing

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**Semi-quantitative RT-PCR Analysis of hRFC Gene Expression**—Exponentially growing cells (1 × 10⁶) were harvested by centrifugation, 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 M-MLV (180 units, Promega) in a reaction buffer containing random hexamer primers, dNTPs, and a 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 Applied Science) in a reaction buffer (total volume 25 µl) containing: 10 pmol of each primer in 2xReddyMix PCR master mix reaction buffer according to the instructions of the manufacturer (ABgene, Surrey, UK). The PCR reaction was performed as follows: initial melting at 95 °C for 5 min, followed by 25–35 cycles each of 1 min at 95 °C, annealing at 62 °C for 45 s, elongation at 72 °C for 1 min, followed by 10 min extension at 72 °C. Then, the PCR products were resolved on 2% agarose gels containing ethidium bromide. The primers used for the semi-quantitative RT-PCR of hRFC and GAPDH were previously described (25).

**Transient Transfections with Various Expression Constructs**—Exponentially growing suspension cells (2 × 10⁶) were harvested by centrifugation and transfected by electroporation (1000 µF, 254 V) with 10 µg of the following expression plasmids: pcRed1 VP16 (kindly provided by Dr. M. E. Greenberg), pmc-c-fos, pHc-c-Jun and pPacSp1 (a gift from Dr. G. Suske). Cells were then seeded at 2 × 10⁶/ml in prewarmed growth medium. For transient RFC mRNA expression and EMSA, after...
TABLE II

Summary of the characteristics of the various cell lines

| Cell line          | Antifolate selection method | MTX transport (%) of control | Antifolate resistance range (fold) | RFC Mutation(s) | RFC protein expression | Ref. no. |
|--------------------|-----------------------------|------------------------------|-----------------------------------|-----------------|------------------------|----------|
| AG2034             | AG2034, Stepwise            | 3                            | 65–939                            | None            | 0‡                    | 14       |
| ZD9831             | ZD9831, Stepwise            | 6.5                          | 146–2272                          | E257Stop        | 0                      | 14       |
| PT C-3            | PT523, Single step          | 4.8                          | 107–2088                          | L203Stop        | 0                      | 14       |
| GW25              | GW1843, 24-h pulses         | 5                            | 36–1741                           | None            | 0.15                   | 26       |
| PT18-N            | PT523, Stepwise             | 1                            | 131–3461                          | L143P           | 0                      | Unpublished |

GW70

| Cell line          | Antifolate selection method | MTX transport (%) of control | Antifolate resistance range (fold) | RFC Mutation(s) | RFC protein expression | Ref. no. |
|--------------------|-----------------------------|------------------------------|-----------------------------------|-----------------|------------------------|----------|
| CEM/MTX           | MTX, Stepwise               | 1.1                          | 4–410                             | E45K            | 0.1                    | 11       |
| MTX15-N           | MTX, 24-h pulses            | 6                            | 11–66                             | None            | 0.1                    | 26       |
| PT C-7            | PT523, Single step          | 5.2                          | 53–695                            | G44R            | 0                      | 14       |
| EDX0.03           | EDX, Stepwise               | 3.5                          | 100–1284                          | E45K            | 0.15                   | 14       |
| MTX10.6           | MTX, Stepwise               | 5.6                          | 161–1451                          | S225Stop        | 0                      | 14       |
| AMT0.12           | AMT, Stepwise               | 5.9                          | 18–85                             | None            | 0                      | 14       |
| PT15-0.8          | PT523, Stepwise             | 5.7                          | 173–799                           | G239Stop        | 0                      | 14       |
| CEM-T             | MT, Stepwise                | 1.2                          | ND                                | G44R            | 0                      | Unpublished |
| CEM/MTX11         | MTX, Stepwise               | 7.5                          | ND                                | ND              | ND                     | Unpublished |
| MDA-MB-231        | None                        | 17.7                         | ~50                               | None            | 0                      | 21       |
| ZR-75-15MX        | MTX, Stepwise               | 3.15                         | ~200–2000                         | S361Stop        | 0                      | 30       |

* MTX transport V_max in ZR-75 (WT) was 10.8 ± 0.8 pmol/mg protein/min.
* Initial rate of MTX transport in parental CEM (WT) cells was 4.11 ± 0.56 pmol/10⁵ cells/min.
* RFC protein expression relative to CEM (WT) or ZR-75 (WT).
* RFC below the level of detection on Western blot.
* ND, not determined.
* Patient-derived.

24 h of incubation at 37 °C, cells were harvested and total RNA and nuclear proteins were extracted.

Western Blot Analysis—Nuclear proteins (30 μg) were resolved by electrophoresis on 10% polyacrylamide gels containing SDS, electroblotted onto Protran cellulose nitrate membranes (Schleicher & Schuell), reacted with anti-pCREB-1/pATF-1 (New England Biolabs), c-Jun, and AP-2 (abcam, Cambridge, UK), Sp1 (Serotec, Oxford, UK), USF-1, Sp3, c-Fos (abcam, Cambridge, UK), corresponding 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.

Determination of hRFC Promoter Methylation—We have devised two independent assays in order to explore hRFC promoter methylation in the various antifolate-resistant cell lines.

A) Bisulfite DNA Sequencing Assay—The first assay is based on bisulfite modification of genomic DNA followed by genomic PCR and DNA sequencing. Bisulfite treatment of genomic DNA was carried out using CpGenome DNA modification kit according to the instructions of the manufacturer (Intergen) under conditions that allowed the complete conversion of all cytosine residues (but not 5-methylcytosine) to uracil. DNA treated in vitro with SssI methyltransferase (New England Biolabs) was used as a positive control for methylated hRFC alleles. Following bisulfite modification, DNA was PCR-amplified using Redymix (ABgene) and 10 pmol of each primer (5’-ATCCCAAACCCCAAAA-3’ and 5’-GGATTTTAGGGTTAGTTT-3’), corresponding to a promoter region of hRFC where methylation was previously found to result in transcriptional silencing (21) (see Fig. 9). All positions refer to ATG of the translation initiation as +1 using accession number 7717445. Reaction mixtures were incubated at 95°C for 3 min, followed by 35 cycles of 1 min at 95 °C, 2 min at 53 °C, and 3 min extension at 72 °C; a final extension at 72°C for 10 min was performed to complete the genomic amplification process. Products were then resolved by electrophoresis on a 1.5% agarose gel, purified (Qiagen), and sequenced.

B) Southern Blot-based Assay—Genomic DNA from the various cell lines was co-digested with NciI (Fermentas) and MmuI along with either SmalI (methylation-sensitive restriction enzyme) or Xmal (a methylation insensitive isozyme of Smal) according to the instructions of the manufacturer (New England Biolabs). Following digestion, DNA was fractionated by electrophoresis (2.2 V/cm on 0.8% agarose gels containing 0.2 μg/ml ethidium bromide. DNA in the gel was then...
depurinated with 0.25 M HCl, denatured with 1.5 M NaCl, 0.5 M NaOH, neutralized with 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.5, transferred to a ZetaProbe-GT nylon membrane (Bio-Rad), and UV cross-linked. Fragments were analyzed by Southern blot analysis using a 537-bp 32P-labeled genomic hRFC probe (see Fig. 9). Following an overnight hybridization at 65°C, blots were washed under high stringency conditions with a final wash in a solution of 0.1 M SSC, 0.1% SDS at 65°C for 30 min, and visualized by a phosphorimaging device.

RESULTS

Loss of [3H]MTX Transport and Antifolate Resistance—In the present study we used a large panel of seventeen antifolate-resistant leukemia and breast cancer cell lines, the features of which are summarized in Table II; these previously published characteristics also include initial rates of [3H]MTX transport. The up to 99% loss of [3H]MTX uptake in these antifolate-resistant cell lines was associated with a marked decrease in RFC protein expression. Furthermore, in approximately two thirds of the cell lines, heterozygous nonsense mutations and/or missense mutations were present. Consequently, these cell lines displayed up to 3,500-fold cross-resistance to various hydrophilic antifolates.

hRFC mRNA Expression—The decreased RFC protein levels suggested a consistent loss of hRFC gene expression. We therefore used semi-quantitative RT-PCR analysis in order to determine hRFC mRNA levels (normalized to GAPDH) in the various antifolate-resistant cell lines. Whereas readily detectable in parental CEM cells after 25 cycles of RT-PCR, hRFC cDNA was observed in neither of the antifolate transport-defective cell lines (Fig. 2A); consistently, after 30 cycles of RT-PCR, decreased RFC cDNA levels could be detected in eight of the seventeen antifolate-resistant cell lines, whereas no detectable expression could be observed in the remainder (Fig. 2B).

Decreased Binding of Transcription Factors to Various hRFC Promoter Elements in Antifolate-resistant Cell Lines—These RT-PCR data established that RFC gene expression was profoundly suppressed in various cell lines. To explore potential alterations in the binding of transcription factors to the various cis-acting elements in the hRFC promoter region, we used electrophoretic mobility shift assay (EMSA). Nuclear factor binding to consensus 32P-labeled oligonucleotides including CRE, E-box, AP-1, Mzf-1, and GC-box was markedly decreased (Fig. 3, A–F, respectively), whereas normal AP-2 binding was retained in all antifolate-resistant cell lines (Fig. 3F). Fig. 4A summarizes the percentage of cell lines with decreased oligonucleotide binding; 47–76% of the cell lines had decreased transcription factor binding either to GC-box, Mzf-1, AP1, E-box, or CRE. Strikingly, ~76% of the cell lines displayed a simultaneous decrease in the binding to three or more hRFC promoter elements, whereas, only ~18% of the cell lines had one or two hRFC elements altered in binding (Fig. 4B). In contrast, MDA-MB-231 was the only cell line that retained normal binding to all six cis-acting elements.

Altering the Expression of Various Transcription Factors—Recently we have shown that decreased CRE- and GC-box binding in the hRFC promoter is due to alterations in the expression of the relevant transcription factors (25). We therefore used Western blot analysis in order to survey the levels of pCREB-1, pATF-1, USF-1, c-Jun, c-Fos, Sp1, Sp3 isoforms, and AP-2a (Fig. 5).

pCREB and pATF-1—All cell lines with decreased CRE binding either had prominently diminished or undetectable levels of phosphorylated CREB-1 (pCREB), the transcriptionally active form of CREB (Fig. 5A). Consistently, the vast majority of cell lines with decreased pCREB levels retained near normal levels of pATF-1 (Fig. 5A), whereas only a few cell lines had decreased levels of both pCREB-1 and pATF-1 (Fig. 5A, compare lanes 3 and 11 with 1). A ~35-kDa pATF/pCREB family member that was barely detectable in parental cells was overexpressed in almost all cell lines with decreased CRE binding (Fig. 5A).
USF-1—The majority of cell lines with decreased E-box binding had substantially diminished levels of USF-1 (Fig. 5B). Consistently, PT[32P]CRE protein-antibody complex formation, revealed supershifts in parental CEM cells (Fig. 6A, left panel), complexes A and B in lanes 2 and 3, 4, respectively; note the elimination of band 1 with anti-CREB-1 and band 2 with anti-ATF-1 antibodies, respectively). In contrast, CEM/MTX cells with a markedly decreased CRE binding had only residual ATF-1 complex formation (Fig. 6A, right panel). These results are consistent with the retention of pATF-1 expression (Fig. 5A).

E-box—Antibodies against USF-1 supershifted band 1 and formed a high molecular weight complex A (Fig. 6B, compare lane 2 with 1). In contrast, CEM/MTX cells that lost band 1 but not band 2 of E-box binding (Fig. 3B) did not display any supershift (Fig. 6B, right panel).

AP-1—We used antibodies against AP-1 activators (c-Jun and c-Fos) and repressors (Jun B) (Fig. 6C). Anti-c-Jun antibodies revealed a supershifted complex B (Fig. 6C, left panel). Whereas anti-c-Fos antibodies eliminated bands 2 and 3, anti-Jun B antibodies eliminated band 2 and formed complex A (Fig. 6C, left panel). Consistently, co-treatment with both anti-c-Jun, c-Fos, and Jun B antibodies eliminated bands 2 and 3, reduced the intensity of band 1 and formed complexes A and B (Fig. 6C, left panel). In contrast, CEM/MTX with an abolished AP-1 binding (Fig. 3C) failed to show any detectable supershift (Fig. 6C, right panel).

GC-box—Anti-Sp1 antibody eliminated band 2 and formed complex A, whereas anti-Sp3 antibody eliminated bands 1 and 3, and formed complexes B and C in nuclear extracts from parental CEM cells (Fig. 6D, left panel). Consistently, treatment with both antibodies eliminated bands 1, 2, and 3 and formed complexes A, B, and C (Fig. 6D, left panel). In contrast, CEM/MTX cells (Fig. 3E), which had a marked loss of GC-box binding, showed only residual supershifts with both anti-Sp1 and -Sp3 antibodies (Fig. 6D, right panel).

Restoration of cis-Element Binding after Transient Expression of Various Transcription Factors—The loss of expression and/or binding of various transcription factors in the multiple
FIG. 5. Western blot analysis of transcription factor expression in parental and antifolate-resistant cell lines. Nuclear proteins (30 μg) from parental and antifolate-resistant sublines were resolved by polyacrylamide gels containing SDS, transferred to a cellulose nitrate Protran membrane, and reacted with antibodies against human pCREB-1/pATF-1 (A), USF-1 (B), c-Jun (C), c-Fos (D), Sp1 (E), and Sp3 (F) as detailed under “Materials and Methods.” The blots were then reprobed with antibodies to AP-2α (G).
cell lines prompted us to explore the impact of their transient expression on restoration of: (a) transcription factor binding to the various hRFC promoter elements, and (b) RFC mRNA expression. Transfection of representative cell lines with expression constructs harboring CREB-1, c-Fos, and c-Jun, or Sp1 resulted in a partial or complete restoration of binding to CRE (Fig. 7A), AP-1 (Fig. 7B), and GC-box (Fig. 7C), respectively. This resulted in at least a partial restoration of hRFC mRNA expression relative to parental cells (Fig. 8).

Assessment of hRFC Promoter Methylation in Antifolate-Resistant Cell Lines—Recently it was shown that methylation of hRFC promoter A occurs in MTX-resistant MDA-MB-231 cells resulting in transcriptional silencing and impaired drug transport (21). Thus, to explore the frequency of hRFC promoter methylation in the various antifolate-resistant cell lines, we devised two hRFC promoter methylation assays. The first involves the bisulfite DNA sequencing technique that is based on the treatment of genomic DNA with bisulfite thereby converting only unmethylated cytosines to uracils. Examination of a region upstream to promoter A that contains 13 CpGs (Fig. 9A, see dotted line) that was shown (21) to be fully methylated in MDA-MB-231 cells (Fig. 9B), revealed that none of the parental and antifolate-resistant cell lines showed CpG island methylation (Fig. 9B). In the second approach we devised a Southern blot assay that is based on genomic DNA digestion with methylation-sensitive (SmaI) and -insensitive restriction isoenzymes (XmaI); this assay was designed to analyze the methylation status of a more downstream region of the hRFC promoter A. Blots were then hybridized with a 32P-labeled 537 bp genomic hRFC probe (Fig. 9A, see light gray box). The 600-bp band obtained after SmaI digestion was diagnostic for unmethylated DNA and was observed in all cell lines except for MDA-MB-231 cells that contained methylated CpG islands and therefore could not undergo digestion with SmaI (Fig. 9C). Consistently, XmaI-digested DNA yielded a 600-bp fragment in all cell lines including MDA-MB-231 cells. These results dem-

Fig. 6. Antibody-mediated supershift analysis. Nuclear proteins (6 μg) from parental cells and a representative antifolate-resistant cell line, CEM-MTXR1 with loss of binding to multiple transcription factor recognition sites, were first incubated with antibodies (4 μg) against pCREB-1/pATF-1 (A), USF-1 (B), c-Jun, c-Fos, and JunB (C), as well as Sp1 and Sp3 (D) for 24 h at 4 °C. Then, binding to 32P-labeled CRE (A), E-box (B), AP-1 (C), and GC-box (D) oligonucleotides was determined by a phosphorimager. The supershifted antibody-nuclear protein(s)-oligonucleotide complexes are denoted on the left by A, B, and C.
onstrate that regions upstream and downstream of hRFC promoter A are not methylated in any of the antifolate-resistant cell lines examined here except for MDA-MB-231 cells. Strikingly, the only MTX-transport defective cell line that retained intact binding to all hRFC promoter elements was MDA-MB-231 (Fig. 3, A–F). In contrast, no promoter methylation was found in ZR-75-MTX R, another MTX-resistant breast cancer cell line with impaired antifolate transport, decreased Mzf-1 binding (Fig. 3D) and a heterozygous premature translation termination mutation in the hRFC (Table II). Thus, the loss of hRFC mRNA expression in MDA-MB-231 cells that did not harbor any RFC mutation (Table II) could be solely attributed to hRFC promoter methylation. These results suggest that alterations in the binding of transacting factors and promoter methylation appear to be mutually exclusive mechanisms of transcriptional silencing of the hRFC gene.

**DISCUSSION**

We have recently shown that loss of hRFC gene expression in two drug-resistant cell lines with impaired antifolate transport is associated with alterations in the expression and binding of transcription factors to the inducible CRE/AP-1 and constitutive GC-box in hRFC promoters A and B, respectively (25). The aim of the current study was to explore whether multiple antifolate-resistant cell lines with decreased (or abolished) hRFC mRNA expression display altered binding of transcription factors to additional cis-acting elements that are contained within or near four tandemly repeated sequences upstream of promoter A including E-box, AP-2 and Mzf-1. Extending on our recent findings (25), we also examined the status of transcription factor binding to CRE/AP-1 and GC-box in this large panel of cell lines that are resistant to various antifolates. We strikingly found that the majority of these cell lines displayed loss of binding to E-box, AP-1, and Mzf-1, cis-acting elements that were not previously associated with decreased hRFC gene expression. Furthermore, as much as ~80% of the cell lines displayed a simultaneous loss of binding to three or more hRFC promoter elements; this was consistently associated with decreased expression of the relevant transcription factors that act as transcriptional activators including USF-1, c-Fos, c-Jun, pCREB-1, and pATF-1. In contrast, although binding to AP-2 was previously shown to promote hRFC gene expression (23), all the seventeen antifolate-resistant cell lines retained normal AP-2 binding and wild-type AP2α expression. Transient transfection of individual transcription factors restored, at least in

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**Fig. 7. Restoration of promoter element binding after transient expression of transcription factors in antifolate-resistant cell lines lacking CRE, AP-1, and GC-box binding.** Drug-resistant cell lines with decreased (or loss) of binding to cis-acting elements due to specific alterations in transcription factor expression, were transiently transfected with expression vectors harboring specific transcription factors. Then, the binding to 32P-labeled CRE, AP-1, and GC-box (panels A, B, and C, respectively) was determined.
part, both binding to the cis-acting elements and hRFC gene expression. Using two experimental approaches, we found that none of the antifolate-resistant cell lines contained hRFC promoter methylation, except for MDA-MB-231. Consistently, the latter cells retained normal binding to all hRFC promoter elements. We conclude that the simultaneous decrease in the expression and/or function of multiple transcription factors involved in activation of hRFC gene expression is a frequent mechanism of resistance to multiple novel antifolates in malignant tumor cell lines with impaired antifolate transport. Our results further suggest that decreased binding of transactivators to sequences upstream to promoter A including four nearly identical tandem repeats containing Mzf-1 and GC-box as well as a 5'-neighbor E-box appear to play an important role in the silencing of the hRFC gene in antifolate-resistant cell lines.

Of the seventeen antifolate-resistant cell lines, only MDA-MB-231 cells retained normal binding to all six hRFC promoter elements that were examined here. This inherently MTX-resistant breast cancer cell line was recently found to completely lack hRFC transcripts (30) due to silencing of the hRFC gene via promoter methylation (21). In contrast, the MTX-resistant breast cancer cell line ZR-75-8MTK8 with barely detectable hRFC mRNA levels displayed a markedly decreased Mzf-1 binding. Furthermore, neither ZR-75-8MTK8 cells nor any of the other antifolate-resistant cell lines had hRFC promoter methylation. These results strongly suggest that hRFC promoter methylation and alterations in the binding of various transcription factors to different cis-acting elements in the hRFC promoter region are presumably mutually exclusive mechanisms of gene silencing. The following evidences support the conclusion that the presence of CpG island methylation in the hRFC promoter may alleviate the biochemical stress imposed by antifolates that would otherwise lead to decreased binding of transcription factors to various hRFC promoter elements. (a) Methylated CpG islands can directly interfere with the binding of transcription factors to their cognate recognition sites (31). Most mammalian transcription factors have GC-rich binding sites and contain many CpGs in their DNA recognition elements. Binding by several of these factors is impeded or abolished by CpG methylation. (b) DNA methylation can directly influence the translational positioning of a nucleosome at specific DNA sequences in vitro and could lead to chromatin remodeling and consequent masking of essential regulatory elements by nucleosomes (32). (c) In addition to these direct modalities of physical masking of cis-acting elements, evidence exists for mechanisms of indirect repression mediated by proteins that specifically bind to methylated DNA. These include methyl-CpG-binding protein 1 (MeCP1) (33) and MeCP2 (34), as well as a family of novel mammalian proteins known as methyl-CpG binding domain 1, 2, and 4 (MBD1, 2 and 4) (35–37). Strikingly, MeCP2 and MBD2 that harbor a transcriptional repression domain (TRD) are thought to repress transcription by recruiting a histone deacetylase complex that modifies chromatin structure (37, 38). Indeed, Worm et al. (21) have shown histone deacetylase complex recruitment in the hRFC gene in MDA-MB-231 cells with promoter methylation. Hence, these evidences emphasize the high efficiency of the direct and indirect mechanisms of transcriptional repression mediated by promoter methylation thereby eliminating the biochemical stress that would otherwise lead to loss of function of various transactivators.

Several lines of evidence indicate that loss of RFC function typically associated with a ~95% decrease in MTX transport activity in multiple antifolate-resistant cell lines is based upon the coexistence of several modalities of drug resistance: (a) Two-thirds of the seventeen antifolate-resistant cell lines studied here harbored antifolate transport inactivating mutations (missense and/or nonsense). The large proportion of premature translation termination mutations in these antifolate-resistant cell lines could clearly contribute to the loss of hRFC mRNA expression via nonsense-mediated mRNA decay (NMD) (39, 40). Recent studies have indicated that an mRNA species harboring a premature translation termination mutation (i.e. stop codon) may be rapidly degraded via a quality control mechanism known as NMD. However, since these nonsense mutations were all heterozygous and as human leukemia cells frequently contain three RFC alleles, one or more normal RFC allele(s) were retained, thereby subjecting them for additional modalities of loss of function. (b) Indeed, all the antifolate-resistant cell lines examined here either had a markedly decreased or a complete loss of hRFC gene expression. This was associated with the simultaneous loss of binding of multiple transcription factors to their hRFC promoter elements. (c) Using Southern blot analysis we recently found that the majority of these antifolate-resistant cell lines displayed hRFC allele loss.2 This finding is consistent with previous studies that also identified hRFC deletions in antifolate-resistant human erythroleukemia cells exhibiting a 90% loss of hRFC mRNA expression (41).

Loss of binding to CRE, E-box, and AP-1 in the multiple antifolate-cell lines could be explained by decreased levels of the relevant activating transcription factors including pCREB-1 and pATF-1, USF-1, as well as c-Jun and c-Fos, respectively. However, the levels of Mzf-1 could not be exam-

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2 Y. Kaufman, I. Ifergan, L. Rothem, G. Jansen, and Y. G. Assaraf, submitted for publication.
ined due to the lack of commercially available antibodies (23). In contrast, the loss of binding to GC-box could not be attributed to decreased Sp1 or Sp3 levels in various antifolate-transport-defective cell lines, except for CEM-MTXR1 and PTR0.5/N cells, which displayed markedly decreased Sp1 and Sp3 levels, respectively. One viable possibility that may be consistent with these results is that Sp1 and Sp3 undergo post-translational modifications that compromise their transactivator capabilities. Members of the Sp/Krüppel-like family of transcription factors undergo several post-translational modifications that modulate their transactivating potentiability including O-linked glycosylation, serine and/or threonine phosphorylation and acetylation (42). It was specifically shown that the introduction of an O-linkage of N-acetylglucosamine to the activation domain of Sp1 inhibits its transcriptional capability in vitro (43) and in vivo (44). Furthermore, the corepressor mSin3A was found to recruit O-linked N-acetylglucosamine transferase to promoters resulting in O-linked glycosylation of transcription factors like Sp1 yielding transcriptional repression (42). Interestingly, O-linked glycosylated proteins are also phosphoproteins and these two types of modifications may be reciprocally regulated in Sp1 (45). Threonine phosphorylation of the zinc-finger domain of Sp1 by casein kinase II results in a reduced affinity of Sp1 for its consensus binding site (46, 47). In the present study we found that two cell lines (AG2034R2 and EDXR0.03) with loss of GC-box binding had normal Sp1 levels but markedly increased levels of both the long and short forms of Sp3. These results may be consistent with the current literature that the short forms of Sp3 act as transcriptional repressors. Furthermore, the long form of Sp3 that, as opposed to all members of the Sp family, contains an inhibitory domain that may inhibit transcription; this activity is apparently regulated...
by acetylation of a critical lysine residue in the inhibitory domain (48). Further studies are underway to explore whether post-translational modifications of Sp1 and Sp3 including O-linked glycosylation, phosphorylation, and acetylation occur in antifolate-resistant cells displaying loss of GC-box binding while retaining normal Sp1 levels.

Acknowledgments—We thank Yaffa Both for technical assistance, Dr. Michael E. Greenberg for the pRSV-CREB-1 expression vector, and Dr. Guntram Suske for the pPacSp1 construct.

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