Methylation-dependent Silencing of the Reduced Folate Carrier Gene in Inherently Methotrexate-resistant Human Breast Cancer Cells*

Jesper Worm‡, Alexei F. Kirkin‡, Karine N. Dhandzhugazyan‡, and Per Guldberg§

From the Institute of Cancer Biology, Danish Cancer Society, Strandboulevarden 49, DK-2100 Copenhagen, Denmark

The molecular basis of methotrexate resistance was studied in human MDA-MB-231 breast cancer cells, which are inherently defective in methotrexate uptake and lack expression of the reduced folate carrier (RFC). Transfection of MDA-MB-231 cells with RFC cDNA restored methotrexate uptake and increased methotrexate sensitivity by ~50-fold. A CpG island in the promoter region of RFC was found to be methylated in MDA-MB-231 cells, but was unmethylated in RFC expressing, methotrexate-sensitive MCF-7 breast cancer cells. Chromatin immunoprecipitation with antibodies against acetylated histones H3 and H4 showed that the RFC promoter was enriched for acetylated histones on expressed, unmethylated alleles only. Treatment of MDA-MB-231 cells with 5-aza-2′-deoxycytidine restored RFC expression but also led to increased methotrexate efflux and did not reverse methotrexate resistance. This suggests that 5-aza-2′-deoxycytidine up-regulates both methotrexate uptake and some methotrexate-resistance mechanism(s). Reverse transcription-polymerase chain reaction analysis showed increased expression levels of several ATP-dependent efflux pumps in response to 5-aza-2′-deoxycytidine treatment, including P-glycoprotein and members of the multidrug resistance-associated protein family. Up-regulation of P-glycoprotein in response to 5-aza-2′-deoxycytidine was associated with demethylation of a CpG island in the MDR1 promoter, whereas the mechanism(s) for 5-aza-2′-deoxycytidine-induced up-regulation of multidrug resistance-associated proteins is probably indirect. Dipyridamole inhibited methotrexate efflux and reversed methotrexate resistance in 5-aza-2′-deoxycytidine-treated MDA-MB-231 cells.

Methotrexate (MTX)1 is used as an immunosuppressive agent for treatment of autoimmune diseases and is included in combination chemotherapy regimens used in the treatment of various malignancies, including acute lymphoblastic leukemia, non-Hodgkin's lymphoma, osteosarcoma, and breast cancer (1–3). Like other 4-aminofolate analogues, MTX exerts its toxicity by competitively inhibiting dihydrofolate reductase, leading to intracellular depletion of tetrahydrofolate, impaired thymidylate, and de novo purine synthesis and inhibition of DNA replication (3, 4).

The major route for cellular uptake of MTX involves the reduced folate carrier (RFC), a bidirectional anion transporter with high affinity for reduced folates and antifolates but low affinity for folic acid (5). Once inside the cell, MTX is modified by the addition of glutamyl groups in a process catalyzed by folylpolyglutamate synthase (FPGS). Polyglutamylation causes retention of MTX within the cell and increases the cytotoxicity of this drug by sustaining maximal inhibition of its target enzyme. Hydrolysis of MTX polyglutamates is catalyzed by the lysosomal enzyme, γ-glutamyl hydrolase (γ-GH), and favors drug efflux by one of several possible mechanisms (4). MTX-efflux mechanisms seem to be ATP-dependent (6, 7) and may be mediated by members of the multidrug resistance-associated protein (MRP) family, including MRP1 (8), MRP2 (8), and MRP3 (9), or by as yet unknown proteins (10).

The development of resistance to MTX is a major problem in the use of this drug and may be a result of changes in the uptake or polyglutamylation of MTX, the hydrolysis of MTX polyglutamates, or the interaction of MTX with dihydrofolate reductase (4). Transport studies in in vitro models, including murine L1210 leukemia cells (11), Chinese hamster ovary cells (12), human CCRF-CEM leukemia cell lines (13), and human ZR-75-1 breast cancer cell lines (14, 15), have shown that down-regulation of RFC activity results in transport-mediated MTX resistance. Furthermore, transfection with RFC cDNA restored MTX sensitivity in RFC-deficient cells (14, 16–19). Recent studies in patients with acute lymphoblastic leukemia (20, 21) and osteosarcoma (22) have suggested that defective transport via the RFC may also be a common mechanism of clinical resistance to antifolates. However, while acquired dysfunction of the RFC in cell lines exposed to increasing MTX concentrations is associated with mutations in the RFC gene (11, 13, 23–31), no RFC mutations have been identified in clinical samples that could account for impaired MTX uptake in vivo.

To identify possible epigenetic mechanisms responsible for stable down-regulation of RFC function, we here investigated the cause of MTX resistance in human MDA-MB-231 breast cancer cells. This cell line was chosen as a model because it (i) is highly resistant to MTX (32), (ii) lacks expression of RFC (33), and (iii) was established from the malignant pleural effusion of a patient only 3 weeks after she had received MTX-
containing combination therapy (34), suggesting that the mechanism causing MTX resistance may have occurred in vivo. We report that a CpG island in the promoter region of RFC is aberrantly methylated in MDA-MB-231 cells and enriched for deacetylated histones. Treatment of MDA-MB-231 cells with the demethylating agent, 5-aza-2'-deoxycytidine (5-aza-CdR), induced re-expression of the RFC gene but did not in itself have a significant effect on MTX sensitivity, possibly due to the concomitant up-regulation of MTX-efflux mechanisms. Finally, we show that the effect of 5-aza-CdR on MTX uptake and MTX sensitivity of MDA-MB-231 cells can be modulated positively by dipyridamole (DFM) in a synergistic manner.

EXPERIMENTAL PROCEDURES

Materials—Unlabeled MTX, 5-aza-CdR, DFM, and probenecid (PBQD) were obtained from Sigma-Aldrich. (3′,5′)-7-32PMTX (97.3–99.6% purity (confirmed by thin-layer chromatography)) was from Amersham Pharmacia Biotech. Trichostatin A (TSA) was purchased from Wako. Gentamicin™ (G418-Sulfate), LipofectAMINETM, 99.6% purity (confirmed by thin-layer chromatography)) was purchased from Life Technologies, Inc. The RFC construct contains nucleotides 19–100997 of the human RFC cloned into the pcDNA3 expression vector (Invitrogen). The primers used for RFC promoter amplification were as described by Coffee et al. (38). The primers used for RFC promoter amplification were 5'-AGCTT-AATTGATTTTGGATTATTTGT-3', and 5'-TGCGGTTGTTGTGATGCG-3'.

Table I

| Gene (GenBank™ accession number) | Position | Sequence (5′–3′) | Product size | Annealing temperature |
|----------------------------------|----------|------------------|--------------|----------------------|
| RFC-U (U92868.1)                | 1336     | CCAATCCAATTATTTTGAATTAAAGAAA | 111 | 60 |
| RFC-M (U92868.1)                | 1446     | GCTTTGTAAATTTTGAATTAAAGAAA | 111 | 60 |
| FPGS-U (U24252.1)               | 856      | AAACAAACAAATTATTTTGAATTAAAGAAA | 143 | 55 |
| FPGS-M (U24252.1)               | 997      | TTAGGTTGTTGTTTGAATTAAAGAAA | 143 | 55 |
| MDR1-U (M29423.1)               | 809      | AGGTGGTTTTATGGGTATATATTTGT | 117 | 58 |
| MDR1-M (M29423.1)               | 925      | GTCCTAATACCGGAATCG | 115 | 60 |
| MRP3-U (AC004590)               | 100997   | TTTGTTTATATTGATATATTTGT | 170 | 55 |
| MRP3-M (AC004590)               | 101166   | AAAATTCAGATTCCCTGCTCCTAAAA | 170 | 60 |
| MRP3-M (AC004590)               | 101166   | AAAATTCAGATTCCCTGCTCCTAAAA | 170 | 60 |

RFC Promoter Methylation in MTX-resistant Breast Cancer Cells

Chromatin Immunoprecipitation—Confluent cells in T-75 flasks were incubated in phosphate-buffered saline (PBS) containing 1% formaldehyde for 10 min at 37 °C to cross-link DNA to chromatin-associated proteins, and then rinsed in ice-cold PBS, scraped, and collected by centrifugation at 4 °C. Chromatin immunoprecipitation using anti-acetyl-H3 and anti-acetyl-H4 antibodies (Upstate Biotechnology) was then carried out according to the manufacturer’s recommendations. The DNA-protein complexes were sonicated in a Branson Sonifier 250 under conditions that gave a range in DNA fragments from 200 to 1,000 base pairs, as determined by gel electrophoresis. The primers used for G6PD promoter amplification were as described by Coffee et al. (38). The primers used for RFC promoter amplification were 5'-GGCTTG-3', 5'-GCGGCAGAATGCGA-3', and 5'-GTACCAGATCTGCGGTTG-3', which amplify a region from positions 832 to 1005, according to GenBank™ accession number U92868.1. For G6PD amplification using a block thermocycler (GeneAmp PCR System 9600; PerkinElmer Life Sciences), reaction mixtures (25 μl) contained 10 pmol of each primer, 0.2 mM each dNTP, 1 unit of HotStarTag DNA polymerase (Qiagen), 1 μM PCR buffer (Qiagen), 0.2 mM c GST, 12% (v/v) sucrose, and bisulfite-modified DNA (∼50 ng). Reactions were initiated by “hot start,” followed by 40 cycles at 94 °C for 20 s, the appropriate annealing temperature for 20 s, and 72 °C for 20 s, followed by incubation at 72 °C for 15 min. The products were resolved on 2% agarose gels.

16 h. Modified DNA was recovered using glassmilk from the Geneclean II Kit (BIO 101, Inc.), according to the manufacturer’s protocol. The reaction was completed by incubating the recovered DNA in 0.3 N NaOH for 15 min at 37 °C, followed by ethanol precipitation. DNA was resuspended in DNA Hydration Solution (Gentra) and used immediately or stored at −80 °C until use. DNA treated in vitro with SssI methyltransferase (New England Biolabs) was used as a positive control for methylated RFC alleles. The primer sequences and annealing temperatures are given in Table I. PCR amplifications were performed in 25-μl reactions containing standard PCR buffer (PerkinElmer Life Sciences), 10 pmol of each primer, 0.2 mM each dNTP, 2 units of AmpliTaq (PerkinElmer Life Sciences), 0.2 mM c GST, 12% (w/v) sucrose, and bisulfite-modified DNA (∼50 ng). Reactions were initiated by “hot start,” followed by 40 cycles at 94 °C for 20 s, the appropriate annealing temperature for 20 s, and 72 °C for 20 s, followed by incubation at 72 °C for 15 min. The products were resolved on 2% agarose gels.

Chromatin Immunoprecipitation—Confluent cells in T-75 flasks were incubated in phosphate-buffered saline (PBS) containing 1% formaldehyde for 10 min at 37 °C to cross-link DNA to chromatin-associated proteins, and then rinsed in ice-cold PBS, scraped, and collected by centrifugation at 4 °C. Chromatin immunoprecipitation using anti-acetyl-H3 and anti-acetyl-H4 antibodies (Upstate Biotechnology) was then carried out according to the manufacturer’s recommendations. The DNA-protein complexes were sonicated in a Branson Sonifier 250 under conditions that gave a range in DNA fragments from 200 to 1,000 base pairs, as determined by gel electrophoresis. The primers used for G6PD promoter amplification were as described by Coffee et al. (38). The primers used for RFC promoter amplification were 5'-GGCTTG-3', 5'-GCGGCAGAATGCGA-3', and 5'-GTACCAGATCTGCGGTTG-3', which amplify a region from positions 832 to 1005, according to GenBank™ accession number U92868.1. For G6PD amplification using a block thermocycler (GeneAmp PCR System 9600; PerkinElmer Life Sciences), reaction mixtures (25 μl) contained 10 pmol of each primer, 0.2 mM each dNTP, 1 unit of HotStarTag DNA polymerase (Qiagen), 1 μM PCR buffer (Qiagen), 0.2 mM c GST, 12% (v/v) sucrose, and bisulfite-modified DNA. Reactions were started by initial denaturation at 95 °C for 15 min, followed by 37 cycles at 95 °C for 30 s, 67 °C for 30 s, and 72 °C for 40 s, followed by incubation at 72 °C for 15 min. For RFC amplification, the PCR conditions were as above, except that the reactions contained 1 × Q-solution (Qiagen), the number of cycles was 35, and the annealing temperature was 65 °C. PCR products were resolved on 2% agarose gels. For amplification on the LightCycler (Roche Molecular Biochemicals), reaction mixtures (20 μl) contained 10 pmol of each primer, 4 μl (for G6PD) or 5 μl (for RFC) MglCl, 5% dimethyl sulfoxide (for G6PD), or 1 × Q-solution (Qiagen) (for RFC), and 1 × FastStart DNA Master SYBR Green I (Roche Molecular Biochemicals). Reactions were started by initial denaturation at 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s, 70 °C for 15 s (for G6PD) or 72 °C for 15 s (for RFC) for 15 s, and 72 °C for 40 s.

Reverse Transcription-PCR (RT-PCR)—Total RNA was isolated from cultured cells using the Purescript RNA Isolation Kit (Gentra Systems, Inc.), according to the manufacturer’s instructions. cDNA was synthesized from total RNA (2 μg) using random hexamers, oligo(dT) primers, and Superscript II (Life Technologies, Inc.). The cDNA concentrations were normalized for β-actin gene message using the LightCycler sys-
gene by methylation-specific PCR, and for the presence of the Ki-ras 24-well plates. The coverslips were washed with folic acid-free RPMI to semiconfluency on sterile 15-mm round coverslips (Thermanox) in mine MTX binding to the cells (42), monolayers were incubated in the monolayers were washed four times with ice-cold PBS. To deter-
mined by transferring cells into ice-cold PBS. Values for nonspecific binding of [3H]MTX to coverslips preincubated in standard medium, incubated in MTX-loading medium and washed as above, were subtracted as background. When studying the effects of PBCD on efflux, PBCD (1 mM or 5 mM, with essentially the same result) was added to the MTX-loading medium during the last 10 min of the preloading period in order to provide access of PBCD in cis and trans orientation to the plasma membrane without compromising the loading procedure (7). Efflux was examined by placing MTX-loaded cell monolayers into preheated (37 °C) MTX-free medium. Efflux was ter-
minalized by transferring cells into ice-cold PBS. Values for nonspecific binding of [3H]MTX to coverslips preincubated in standard medium, incubated in MTX-loading medium and washed as above, were subtracted as background. When studying the effects of PBCD on efflux, PBCD (1 mM or 5 mM, with essentially the same result) was added to the MTX-loading medium during the last 10 min of the preloading period in order to provide access of PBCD in cis and trans orientation to the plasma membrane without compromising the loading procedure (7). Efflux was measured as above in medium containing 1 mM PBCD. when cells were plated directly in the wells of 24-well plates (not shown), but the use of coverslips resulted in a lower variation in the

continued...

TABLE II

| Gene (GenBank™ accession number) | Position | Sequence (5'→3') | Product size | Number of PCR cycles | Annealing temperature °C |
|---------------------------------|----------|------------------|--------------|----------------------|--------------------------|
| RFC (S78996) | 1077 | GAGCAGACTTGGCTGTGGCT | 188 | 34 | 63 |
| FPGS (XM_005598) | 1264 | GCCAGCTCGAGAATAGAAGGT | 290 | 28 | 60 |
| γ-GH (NM_003878) | 790 | CATCCGAGAGCTAGAGAGCA | 300 | 25 | 60 |
| γ-GH (NM_003878) | 649 | GGAGCCCTCGCTGAGAATT | 300 | 25 | 60 |
| DHFR (XM_003991) | 948 | TATGCTTCTCTTCTGATAT | 360 | 25 | 63 |
| MDR1 (M14758) | 1050 | CACTGGTGGTAACTACAO | 317 | 34 | 60 |
| MRP1 (XM_007791) | 2572 | CAGACAGAGTACCAAG | 280 | 30 | 66 |
| MRP2 (XM_005809) | 2851 | CAGTCAGGAGAGG | 220 | 30 | 66 |
| MRP3 (XM_008420) | 3798 | CAGTGGAGGCTGGTGAGTATT | 331 | 30 | 66 |
| GAPDH (XM_006959) | 413 | AGGGGGGCGCAAGAG | 514 | 25 | 60 |
| 926 | GAGGAGTTGGTCCGCGTTG | 514 | 25 | 60 |

RFC cDNA Transfection—MDA-MB-231 cells were seeded in 6-well plates and transfected with 1 μg of plasmid DNA complexed with LipofectAMINE, according to the manufacturer’s procedure. G418-re-
sistant transfectant clones isolated by limiting dilution were evaluated.

MTX Uptake—Cells grown to semi-confluency in 24-well plates were added to the wells of 1640 medium containing 10 μM MTX, and cells were kept at 37 °C for 3 min at 120 Ci/n. Then plates were placed on ice, and cells were fixed by guest on July 24, 2018http://www.jbc.org/Downloaded from

MTX Cytotoxicity—Cells were plated in triplicate in 96-well microti-

plates at a density of 1000 cells/well in 0.1 ml of standard medium. Next day, serial dilutions of MTX from 1 nM to 30 μM, final concentra-
tions) were added to the wells in 0.1 ml of standard medium in the presence or absence of 10 μM DFM. After another 5 days, the amount of cells was evaluated by using the sulforhodamine B (SRB) colorimetric cytotoxicity assay (44) with the following modifications: 150 μl of the medium were aspirated from each well, and plates were centrifuged for 3 min at 120 x g. Then plates were placed on ice, and cells were fixed by adding 150 μl of ice-cold 20% (v/v) trichloroacetic acid and incubating on ice for 1 h. The cells were then washed, dried, colored, and destained according to the original procedure (44). Bound SRB-dye was substituted for measuring radioactivity by scintillation counting. Protein concentrations were determined by the biuret method (BCA) protein assay, according to the procedure given by the manufacturer (Pierce). Bovine serum albumin was used as a standard. MTX uptake was determined as the difference between the radioactivity of samples incubated at 37 and 4 °C, and was expressed as picomole of MTX/mg of total cellular protein.

MTX Efflux—Cells, grown to semi-confluency on coverslips in 24-well plates, were washed with folate acid-free RPMI 1640 medium containing 10 μM HEPES, pH 7.3, and incubated at 37 °C for 40 min in the same medium supplemented with 1 μM [3H]MTX. When the coverslips were transferred into ice-cold PBS and washed by three successive changes of the buffer. Efflux was examined by placing MTX-loaded cell monolayers into preheated (37 °C) MTX-free medium. Efflux was termi-
nalized by transferring cells into ice-cold PBS. Values for nonspecific binding of [3H]MTX to coverslips preincubated in standard medium, incubated in MTX-loading medium and washed as above, were subtracted as background. When studying the effects of PBCD on efflux, PBCD (1 mM or 5 mM, with essentially the same result) was added to the MTX-loading medium during the last 10 min of the preloading period in order to provide access of PBCD in cis and trans orientation to the plasma membrane without compromising the loading procedure (7). Efflux was measured as above in medium containing 1 mM PBCD. Protein concentrations were determined as described for MTX uptake. The initial MTX efflux rate was obtained as the slope from linear regression analysis of the efflux data and was expressed in femtomole of MTX/s/mg of protein. Quantitatively similar results were obtained whether the cells were plated directly in the wells of 24-well plates (not shown), but the use of coverslips resulted in a lower variation in the experimental data at short efflux intervals. For efflux rate constant determinations, the fraction of MTX remaining within the cells after incubation for 30 min at 37 °C was subtracted from the corresponding efflux data, and the logarithm of the percentage of the initial “free” MTX level was plotted as a function of time (43).
RFC Promoter Methylation in MTX-resistant Breast Cancer Cells

Fig. 1. Effects of RFC cDNA transfection on MTX sensitivity (A and C) and MTX uptake (B and D) of MDA-MB-231 cells. Data are shown for MCF-7 and MDA-MB-231 cells (A and B), and MDA-MB-231 cells stably transfected with the p5′RFC construct (2 clones), the p5′ΔRFC-EGFP construct (2 clones), or an empty pcDNA3 expression vector (1 clone) (C and D). Panels A and C, cells were seeded in 96-well plates and grown for 24 h in 0.1 ml of medium without MTX, and then 0.1 ml of medium containing MTX at different concentrations was added. Cells were continuously exposed to MTX for 5 days, and cell growth was determined relative to untreated cells, using the SRB colorimetric assay. The results represent the means of at least three independent determinations performed in triplicate. Panels B and D, cell monolayers were incubated in medium containing 1 μM [3H]MTX at 37°C for the indicated times. MTX uptake was determined relative to cells incubated in the same medium at 4°C for 30 s. Data on MTX uptake during the first 2 min (p5′RFC transfectants) or 3 min (p5′ΔRFC-EGFP and empty vector transfected) of incubation of cells with MTX were used for calculating initial MTX-influx rates. The results represent the means of three experiments performed in duplicate. Symbols in B and D are the same as in A and C, respectively.

However, pretreatment of cells with 1 μM 5-aza-CdR resulted in a ~2-fold lower SRB absorbance at day 6. Therefore, in preliminary experiments, 1 μM 5-aza-CdR-treated cells were also plated at a higher initial density (2 × 10^5 cells/well), but this did not change the results on MTX cytotoxicity.

RESULTS

MTX Resistance in MDA-MB-231 Cells Is Due to Defective RFC-mediated MTX Uptake—It was shown previously that MDA-MB-231 cells exhibit a low sensitivity to MTX (32). Indeed, in cytotoxicity studies, MDA-MB-231 cells were ~50-fold less sensitive to MTX than MCF-7 cells (Fig. 1A). These data are consistent with the data presented in Fig. 1B, showing that MCF-7 cells accumulate almost 5 times more [3H]MTX than MDA-MB-231 cells during a 1-h incubation period. RT-PCR analysis using primers that amplify all known RFC transcript types (45) confirmed the data of Moscow et al. (33) that MDA-MB-231 cells lack expression of RFC mRNA (not shown), suggesting that impaired transport via the RFC is the main mechanism for MTX resistance in these cells. Accordingly, restoration of RFC expression should confer sensitivity to MTX. MDA-MB-231 cells were transfected with either of the p5′RFC and p5′ΔRFC-EGFP constructs (35) or the empty pcDNA3 expression vector. Green fluorescence was seen clearly at the periphery of the p5′ΔRFC-EGFP-transfected cells, confirming expression of the protein and correct targeting of the product to the plasma membrane (not shown). As shown in Fig. 1C, stable clones that expressed either p5′ΔRFC or the p5′ΔRFC-EGFP fusion protein showed a sensitivity to MTX (average IC₅₀ of 21.0 ± 3.3 and 13.5 ± 4.0 nm, respectively) similar to that of MCF-7 cells (IC₅₀ of 18.0 ± 2.7 nm), while vector-transfected clones remained as insensitive to MTX as the parent MDA-MB-231 cell line. The initial rate of MTX influx, measured also in cell monolayers, was 0.17 ± 0.03 pmol of MTX/min/mg of protein in control cells and increased to 0.90 ± 0.10 and 0.96 ± 0.06 pmol of MTX/min/mg in the two p5′RFC-EGFP transfecteds, and to 3.70 ± 0.24 and 4.10 ± 0.21 pmol of MTX/min/mg in the two p5′ΔRFC transfectants (Fig. 1D). Transfection with either of the constructs increased the net uptake of MTX to a level similar to that observed in MCF-7 cells, while transfection with the empty vector was without any effect on MTX uptake (Fig. 1D). In p5′RFC transfectants, the MTX uptake was generally ~2-fold higher than in p5′ΔRFC-EGFP-transfected cells (Fig. 1D). Furthermore, MTX sensitivity in cytotoxicity assays correlated broadly with efficiency of MTX uptake. Collectively, these transfection data support previous indirect evidence (33) that MTX resistance in the MDA-MB-231 cell is caused by lack of RFC-mediated transport.

A CpG Island at the RFC Gene Promoter Is Methylated in MDA-MB-231 Cells—Transcriptional silencing of tumor suppressor genes in neoplastic cells has often been associated with de novo methylation of promoter CpG islands (46). The RFC promoter contains a ~1400-base pair region that meets the criteria for a CpG island (47) in having a GC content of 0.77 and a CpG:GpC ratio of 0.91. To examine the methylation status of this CpG island, SssI-methylated DNA and DNA from MDA-MB-231 cells, MCF-7 cells, and normal peripheral blood lymphocytes was treated with sodium bisulfite and amplified with...
RFC Promoter Methylation in MTX-resistant Breast Cancer Cells

Fig. 2. Aberrant methylation of the RFC promoter CpG island in MDA-MB-231 cells. Genomic DNA from the MDA-MB-231 and MCF-7 cell lines and peripheral blood lymphocytes (PBL) and universally SssI-methylated DNA was treated with sodium bisulfite and PCR amplified with primer pairs specific for methylated (M) and unmethylated (U) RFC alleles. SssI-methylated DNA provided a positive control for methylated RFC alleles.

primer pairs specific for methylated and unmethylated RFC alleles. MDA-MB-231 contained only methylated RFC alleles, whereas MCF-7 cells and peripheral blood lymphocytes contained only unmethylated alleles (Fig. 2). Thus, methylation of the RFC promoter correlated with transcriptional silencing, suggesting that changes in the methylation status is a mechanism for regulating RFC expression.

RFC Promoter Methylation Is Associated with Histone Deacetylation—Since transcriptional regulation by DNA methylation has been suggested to involve histone deacetylation (38), it was explored whether differences in histone acetylation exist between the RFC alleles of MDA-MB-231 and MCF-7 cells by using a chromatin immunoprecipitation assay with antibodies against acetylated histones H3 and H4. Analysis of immunoprecipitated DNA by PCR indicated that RFC DNA levels were reduced relative to G6PD DNA levels in immunoprecipitates from MDA-MB-231 cells compared with MCF-7 cells, using either anti-acetyl-H3 or anti-acetyl-H4 antibodies (Fig. 3). Quantitative real-time PCR analysis using the LightCyder System showed that the ratio of RFC to G6PD DNA was >45-fold higher in MCF-7 cells than in MDA-MB-231 cells when using anti-acetyl-H3 antibodies, and >20-fold higher when using anti-acetyl-H4 antibodies. These results demonstrate a direct correlation between RFC promoter methylation, histone deacetylation, and loss of RFC expression.

Effects of DNA Demethylation and Histone Deacetylase Inhibition on RFC Expression—Cells treated with 5-aza-CdR generally show reduced levels of DNA methylation and increased expression of genes silenced by DNA methylation (46), whereas treatment with TSA increases the levels of histone acetylation and expression of at least some target genes (48, 49). Recent studies have suggested a synergistic effect of demethylation and histone deacetylase inhibition in re-expression of genes silenced by de novo methylation (50, 51). To further investigate the roles of DNA methylation and histone acetylation in the control of RFC transcription, MDA-MB-231 cells were treated with 5-aza-CdR and/or TSA. 5-Aza-CdR reactivated RFC expression and increased the levels of transcription in a concentration-dependent manner, as determined by RT-PCR analysis (Fig. 4). By contrast, TSA failed to reactivate this gene in MDA-MB-231 cells and did not potentiate the effect of 5-aza-CdR (Fig. 4), even though it induced expression of the estrogen receptor α gene in the same cells (not shown).

Reactivation of RFC Expression by 5-Aza-CdR Does Not Reverse MTX Resistance—Recently, it was shown that treatment with 5-aza-CdR can reverse resistance to γ-interferon of cells with death-associated protein kinase promoter methylation (52), and sensitize drug-resistant ovarian and colon human tumor xenografts with hMLH1 promoter methylation to a number of cytotoxic drugs (53). Having shown that RFC silencing in MDA-MB-231 cells is associated with RFC methylation and that transcription can be activated by treatment with 5-aza-CdR, we next investigated whether 5-aza-CdR could reverse resistance to MTX. Contrary to what was expected, treatment with 5-aza-CdR did not significantly influence the sensitivity of MDA-MB-231 cells to MTX. Only a small, statistically insignificant, albeit reproducible, increase in MTX sensitivity was observed after treatment with 5-aza-CdR at a concentration of 0.2 μM or lower, and even a modest decrease in MTX sensitivity was observed at higher concentrations of 5-aza-CdR, e.g. 0.5 and 1 μM (Fig. 5), so that the MTX IC50 was 0.84 μM for control cells and varied from 0.72 μM for cells pretreated with 0.2 μM 5-aza-CdR to 1.03 μM for cells pretreated with 1 μM 5-aza-CdR. The effect of higher concentrations of 5-aza-CdR could, at least in part, be a result of slower cell proliferation (see “Experimental Procedures”). The lack of significant changes in MTX sensitivity despite up-regulation of RFC expression pointed to the possibility that increased uptake of MTX could be compensated by a 5-aza-CdR-mediated increase in the efflux of this drug, leading to no changes in the net accumulation of MTX.

This possibility was tested in the experiments shown in Fig. 6. Semiconfluent monolayers of untreated MDA-MB-231 cells and cells pretreated with 0.2 or 1 μM 5-aza-CdR were rinsed with folate-free medium and incubated in the presence of 1 μM [3H]MTX, and the accumulation of MTX in the cells was measured as a function of time. 5-Aza-CdR-treated cells accumulated MTX more rapidly than untreated cells during the first 20 min of incubation, but they reached an earlier steady-state level, so that the net uptake after 1 h of incubation was 3.6 pmol of MTX/mg of protein for untreated cells versus 3.0 and 2.7 pmol of MTX/mg of protein for cells treated with 0.2 and 1 μM 5-aza-CdR, respectively (Fig. 6A). To study the effect of 5-aza-CdR on MTX efflux, monolayers of control and 5-aza-CdR-treated cells were incubated for 40 min in folate-free medium containing 1 μM [3H]MTX and subsequently transferred to MTX- and folate-free medium. As shown in Fig. 6, B and C, MTX efflux was more rapid from 5-aza-CdR-treated cells than from control cells, with initial efflux rates of 0.046 ± 0.005, 0.085 ± 0.010, and 0.098 ± 0.012 pmol of MTX/mg of protein for control cells and cells treated with 0.2 μM and 1 μM 5-aza-CdR, respectively. The corresponding efflux rate constants were 0.61 ± 0.05, 1.00 ± 0.08, and 1.46 ± 0.11 min−1 (Fig. 6C). Collectively, these data suggest that 5-aza-CdR induces up-regulation of one or more MTX-efflux mechanisms.

Expression Levels of Various Efflux Pumps Are Increased by 5-Aza-CdR Treatment—To identify mechanisms responsible for increased MTX efflux in 5-aza-CdR-treated MDA-MB-231 cells, RT-PCR assays were performed to assess the expression levels of proteins implicated in MTX transport and/or resistance. 5-Aza-CdR had no effect on the expression levels of γ-GH and led to an increase in FPGS expression (Fig. 7), which would exclude a decrease in MTX polyglutamylation as a cause of increased efflux. On the other hand, 5-aza-CdR led to activation or increased expression levels of several members of the ATP-binding cassette family of transporter proteins. Untreated MDA-MB-231 cells had undetectable levels of MDR1 P-glycoprotein, whereas expression was activated by 5-aza-CdR at a concentration of 0.1 μM and further increased at a concentration of 0.2 μM (Fig. 7). In untreated cells, weak PCR signals were obtained for three members of the MRP family, MRP1, MRP2, and MRP3, which have all been implicated in transport of MTX (8, 9), and the levels were markedly higher in cells treated with 5-aza-CdR (Fig. 7). Thus, many efflux pumps associated with drug resistance, including resistance to MTX,
seem to be controlled by DNA methylation and are activated or up-regulated at the transcriptional level by 5-aza-CdR in a concentration-dependent manner.

To investigate whether the effects of 5-aza-CdR on the expression of FPGS, P-glycoprotein, MRP1, MRP2, and MRP3 are direct or indirect, the methylation status of the corresponding genes was examined. The genes encoding FPGS, P-glycoprotein, and MRP3 all contain CpG islands in their promoter regions, which could be targets for methylation. Methylation-specific PCR analysis revealed only unmethylated alleles of the FPGS and MRP3 genes in MDA-MB-231 cells (Fig. 8A), suggesting that demethylation in cis is not responsible for 5-aza-CdR-induced up-regulation of these genes. By contrast, MDA-MB-231 cells contained only methylated alleles of the MDR1 gene, and unmethylated alleles appeared upon treatment with 5-aza-CdR (Fig. 8B). Hence, up-regulation of P-glycoprotein in these cells is associated with demethylation of the MDR1 promoter, as has previously been shown for other cell types (54). The genes encoding MRP1 and MRP2 do not contain CpG islands in their promoters, suggesting that up-regulation of these genes by 5-aza-CdR is probably indirect, although it cannot be excluded that methylation of single CpG sites in these genes may play a role in transcriptional regulation. The lack of FPGS, MRP1, MRP2, and MRP3 promoter CpG island methylation is consistent with the incomplete shutdown of expression of these genes in MDA-MB-231 cells (Fig. 7).

Synergistic Effect of 5-Aza-CdR and DPM on MTX Resistance—In order to further characterize the MTX transport sys-
tem(s) that is up-regulated in MDA-MB-231 cells in response to treatment with 5-aza-CdR, the effects of two modulators of multidrug resistance, PBCD and DPM, were examined. PBCD is an inhibitor of MRP activity (55) and inhibits both inward and outward flux of MTX (7), although efflux is considerably more sensitive to inhibition than is influx, as determined in murine L1210 cells (56) and human CCRF-CEM cells (57). DPM potentiates the cytotoxic action of a broader range of drugs and exhibits a more complex mechanism of drug-resistance suppression (58). In control cells, i.e. MDA-MB-231 cells grown in the absence of 5-aza-CdR, both PBCD and DPM exhibited a minor, statistically insignificant inhibition of MTX efflux (Fig. 9). Treatment of MDA-MB-231 cells with 0.2 \mu M 5-aza-CdR led to a 2-fold increase in the initial efflux rate, and this efflux could not be significantly suppressed by 1 mM PBCD. However, the presence of 10 mM DPM reduced the initial MTX efflux rate from 84 to 40 fmol of MTX/s/mg of protein, i.e. to the level observed in cells grown in the absence of 5-aza-CdR (Fig. 9). The corresponding efflux rate constants were 0.61 min\(^{-1}\) for control cells, and 0.58 and 1.04 min\(^{-1}\) for 5-aza-CdR-pretreated cells in the presence and absence of DPM, respectively (not shown).

Fig. 10A shows the effects of DPM and PBCD on MTX net uptake in control and 5-aza-CdR-pretreated MDA-MB-231 cells. In the presence of 10 mM DPM, the net uptake during a 30-min incubation period was increased by 12% in control cells, compared with cells in the absence of this inhibitor, and by 36 and 48% in cells pretreated with 0.2 and 1 mM 5-aza-CdR, respectively. The values were even higher, 15, 45, and 60%, respectively, when only free, exchangeable MTX within the cells was considered. In the presence of 0.5 mM PBCD, the net

**FIG. 6.** MTX uptake (A) and efflux (B) in 5-aza-CdR-treated and control MDA-MB-231 cells. Panel A, MTX uptake by cells in semiconfluent monolayers was measured as described in the legend to Fig. 1. Panel B, semiconfluent cell monolayers on coverslips were preloaded with MTX by incubation in folic acid-free medium containing 1 \mu M [\(^{3}\)H]MTX at 37 °C for 40 min and then incubated in the same medium without MTX at 37 °C for the times indicated. Efflux was terminated by washing cells with ice-cold PBS, and the remaining [\(^{3}\)H]MTX radioactivity was counted. Results are mean values of three independent experiments performed in duplicate ± S.D. Panel C, semilogarithmic analysis of the data shown in B. The free intracellular MTX level was determined by subtracting the drug fraction remaining in the cells after incubation at 37 °C for 30 min, and the logarithm of the percentage of the initial free MTX level was plotted as a function of time. Efflux rate constants were determined as the slopes of the lines. Symbols in B and C are the same as in A.

**FIG. 7.** Up-regulation of efflux pumps by 5-aza-CdR. Reverse-transcribed RNA was produced from untreated MCF-7 and MDA-MB-231 cells and MDA-MB-231 cells treated with 0.1 or 0.2 \mu M 5-aza-CdR for 3 days, and amplified with primer pairs for six different genes implicated in MTX transport and/or resistance. GAPDH RT-PCR provided a control for initial RNA amounts (bottom panel).
uptake was increased by 4, 11, and 15% in control cells and cells pretreated with 0.2 and 1 μM 5-aza-CdR, respectively (Fig. 10A).

The presence of DPM in itself did not affect the growth of control or 5-aza-CdR-pretreated MDA-MB-231 cells (see “Experimental Procedures”), in agreement with previous data on MDA-MB-436 cells (59), and did not increase the sensitivity of untreated cells to MTX (Fig. 10B), consistent with the efflux (Fig. 9) and net uptake (Fig. 10A) data. Nevertheless, when the cells were pretreated with 0.2 μM 5-aza-CdR, they became nearly 4-fold more sensitive to MTX in the presence of DPM (MTX IC50 0.68 versus 0.18 μM). Furthermore, in cells pretreated with 1 μM 5-aza-CdR, DPM induced an even larger (>6-fold) increase in sensitivity to MTX (MTX IC50 1.02 μM versus 0.16 μM) (Fig. 10B). Since there were observed no cytotoxic effects of 10 μM DPM itself on control or 5-aza-CdR-treated cells, these data suggest that the extracellular nucleoside salvage pathway does not play a significant role in the growth of both control and 5-aza-CdR-treated MDA-MB-231 cells and is not critical for the survival of untreated cells in the presence of MTX (Fig. 10B). Accordingly, our data suggest that inhibition of MTX efflux in 5-aza-CdR-treated cells by DPM is responsible for, or at least significantly contributes to, the potentiation of the cytotoxic effects of MTX.
Qualitative and/or quantitative defects in RFC-mediated transport have been recognized as a common mechanistic basis of antifolate resistance. This study describes the first case of an epigenetic alteration responsible for stable down-regulation of RFC expression in the absence of a selective pressure. Human MDA-MB-231 breast cancer cells grown in medium containing normal folate concentrations exhibit negligible levels of RFC mRNA expression due to DNA methylation at the RFC promoter, probably through the coupled removal of acetyl groups from the associated histone proteins. These data conform to a current model in which methyl-CpG-binding domain proteins and complexes, including MeCP1 and MeCP2, mediate transcriptional repression through the recruitment of histone deacetylases (60–62).

Consistent with an important role of DNA methylation in RFC silencing, incubation of MDA-MB-231 cells with 5-aza-CdR resulted in readily detectable levels of RFC mRNA in a concentration-dependent manner. On the other hand, although the methylated RFC promoter was enriched for deacetylated core histones H3 and H4, treatment of MDA-MB-231 cells with TSA did not induce expression of RFC at concentrations that induced expression of the estrogen receptor α gene in the same cells (63). Furthermore, there was no synergistic effect of TSA and 5-aza-CdR on RFC expression, in contrast to what has been recently reported for some other genes silenced by promoter methylation (38, 50). These results may suggest that inhibition of histone deacetylation is not sufficient to “unlock” the RFC gene from the silenced chromatin state induced by DNA methylation, and, accordingly, that DNA methylation is dominant over histone deacetylase activity. Alternatively, methylation-dependent transcriptional repression of RFC may not depend on a TSA-sensitive histone deacetylase. Recent studies in yeast have revealed six different histone deacetylases, of which at least two are insensitive to TSA (64). That mammalian histone deacetylases may also exhibit differential sensitivity to TSA was suggested by a recent study in which TSA failed to reactivate the human HPRT gene on the inactive X chromosome in human/hamster somatic cell hybrids, even when the HPRT promoter had been partially demethylated (65).

Exposure to 5-aza-CdR did not significantly influence the sensitivity of MDA-MB-231 cells to MTX despite up-regulation of RFC mRNA expression. It is possible that this apparent lack of response can be explained by a very low fraction of cells expressing RFC upon 5-aza-CdR treatment. Nevertheless, a more likely possibility is that 5-aza-CdR is able to modulate the steady-state accumulation of MTX by shifting the balance between MTX influx and efflux. Indeed, 5-aza-CdR treatment of MDA-MB-231 cells was shown to increase MTX efflux in a concentration-dependent manner. Total free intracellular antifolate after incubation of cells with MTX is comprised of the monoglutamate and its polyglutamate derivatives. A change in either of these two components can alter the total level of pharmacologically active drug in the cell. The level of MTX polyglutamate derivatives is decreased by a decrease in FPGS activity (66) or an increase in γ-GH activity (67). A decrease in MTX influx via RFC or an increase in efflux via this carrier (43) or another exporter (4) will decrease the free MTX level, that is the substrate for FPGS, and result in a lower level of accumulation of the polyglutamate derivatives. Treatment of MDA-MB-231 cells with 5-aza-CdR did not influence the level of γ-GH mRNA expression but caused an increase in FPGS expression. On the other hand, 5-aza-CdR caused a transcriptional up-regulation of several efflux pumps including MRP1, MRP2, and MRP3. Up-regulation of MRP expression by 5-aza-CdR was not associated with demethylation in cis and may instead involve the modulation of one or more transactivating regulators. MRP2 was recently shown to be involved in excretion of MTX into bile (68), and ovarian carcinoma 2008 cells transfected with MRP1, MRP2, or MRP3 are highly resistant to MTX in short-term (4 h) drug exposure experiments (8, 9). Furthermore, the addition of PBCD, an inhibitor of MRP activity and MTX influx and efflux (7–9), reversed the MTX-resistance phenotype of these cells. Nevertheless, the role of MRPs in conferring long-term MTX resistance is unclear; in 7-day drug exposure experiments, the sensitivity of MRP-transfected 2008 cells was not different from untransfected cells (8, 9). Incubation of 5-aza-CdR-treated MDA-MB-231 cells with PBCD neither reversed the enhanced MTX efflux nor markedly increased the net accumulation of MTX. Thus, although the expression of several MRPs seems to be controlled indirectly by DNA methylation and is up-regulated by 5-aza-CdR treatment, our data do not support a major role of these proteins in counteracting the effect of increased RFC expression in 5-aza-CdR-treated MDA-MB-231 cell monolayers.

Treatment of MDA-MB-231 cells with 5-aza-CdR also induced expression of the MDR1 gene product, P-glycoprotein, a transmembrane efflux pump that has broad substrate specificity and is associated with the multidrug resistance (MDR) phenotype over a wide range of human cancers (69). P-glycoprotein has primarily been implicated in outward transport of lipophilic compounds that enter the cell by passive diffusion across the plasma membrane, and MTX resistance is not a general characteristic of cells with MDR1 overexpression (70). Nevertheless, a number of studies have shown a correlation between P-glycoprotein expression and MTX resistance in MTX-transport defective cell lines (71–74). In one of these studies (74), MTX-resistant human T-cell leukemia cells carrying a truncating RFC mutation were selected for growth in vincristine. In these cells, the increased expression levels of P-glycoprotein correlated not only with a decrease in the sensitivity to vincristine, but also to a further decrease in the sensitivity to MTX. In contrast, the parental, MTX-sensitive cell line carrying a wild-type RFC gene did not show any decrease in MTX sensitivity after selection in vincristine. Collectively, these previous data suggest that P-glycoprotein may contribute to multifactorial MTX resistance in cells with impaired uptake of MTX via the RFC.

DPM and its derivatives may potentiate the cytotoxicity of a wide spectrum of drugs by (i) reacting directly with drug-transporting efflux systems (58, 75, 76), (ii) affecting the intracellular pool of nucleotides via inhibition of the nucleoside salvage pathway (77), and (iii) increasing the intracellular amount of long-chain polyglutamyl MTX derivatives (59, 77). The first effect is evident already in short-term assays (minutes) (75), whereas the two latter require much longer times (hours) of incubation with DPM (59, 77). In the present study, DPM alone had no effect on MTX transport or sensitivity in MDA-MB-231 cells. Nevertheless, DPM acted synergistically with 5-aza-CdR in MDA-MB-231 cells in inhibiting MTX efflux and increasing MTX net uptake and sensitivity. Suppression of 5-aza-CdR-induced MTX efflux did not require preincubation with DPM, which is consistent with a direct effect on the transport machinery, rather than an indirect effect on nucleotide pools. DPM has been reported to sensitize MDR cells to MDR-associated drugs by inhibiting the efflux of these drugs. Specifically, DPM has been shown to prevent photoaffinity labeling of P-glycoprotein by [3H]azidopine in membrane vesicles (58), which suggests a direct effect of DPM. Furthermore, BIBW222, a potent phenylteridine analogue of DPM, was shown to interfere with P-glycoprotein and be highly effective in reversing the MDR phenotype (76). Thus, our data would be
consistent with a role for P-glycoprotein in mediating increased MTX efflux in 5-aza-CdR-treated MDA-MB-231 cells. Nevertheless, this evidence is only indirect and other mechanisms must be considered. Recently, by excluding all known mechanisms of MTX resistance, Volk et al. (10) provided evidence that MTX cross-resistance in mitoxantrone-selected MCF-7 cells is due to up-regulation of an ATP-dependent transporter for MTX. This as yet unidentified efflux pump may also be a candidate for mediating enhanced MTX efflux in 5-aza-CdR-treated MDA-MB-231 cells.

With the cloning of the human (14, 18, 19), mouse (16), and hamster (17) RFC genes, an understanding of the molecular basis for transport-related MTX resistance has become possible. In all human and rodent cells selected in medium containing high concentrations of MTX, impaired MTX uptake has been associated with mutations in RFC. Some initial reports identified RFC mutations that resulted in markedly impaired MTX transport, whereas the transport capacity for reduced folate and folic acid was retained or even increased (11, 13, 26–28, 31). These findings suggested that in vitro selection to MTX resistance may result in the production of highly selective transport changes that minimally affect folate cofactor uptake. However, other reports have identified a broader range of RFC mutations in MTX-selected human and rodent cells, including several causing protein truncation (25, 29, 30, 79). These latter changes are predicted to result in an RFC-null phenotype similar to that mediated by transcriptional RFC silencing in MDA-MB-231 cells, suggesting that complete impairment of RFC function is fully compatible with growth of some cells. Indeed, Pinard et al. (32) showed that MDA-MB-231 cells express high levels of the membrane folate receptor (MFR-α) when grown in medium with normal concentrations of folate and high levels of both MFR-α and -β when adapted to growth in low-folate medium, which could account for sufficient internalization of folates in these cells in the absence of RFC-mediated uptake.

Recently, Ma et al. (15) showed that short-term exposure of folate-depleted ZR-75-1 breast cancer cells to low doses of MTX resulted in transcriptional down-regulation of RFC expression. Co-incubation of these cells with MTX and 5-aza-CdR at a concentration of 1 μM resulted in a net decrease in MTX accumulation, leading these authors to exclude DNA methylation at the RFC promoter as a cause of acute down-regulation of RFC in response to MTX. Recent studies have shown that MTX can up-regulate the p53 tumor suppressor (80), which in turn causes RFC down-regulation (78), which would provide a plausible basis for an acute response to MTX. Nevertheless, on the basis of the 5-aza-CdR data presented here, a role of DNA methylation in mediating RFC down-regulation after short-term exposure to MTX cannot yet be entirely excluded.

Acknowledgments—We thank Dr. Wayne F. Flitoff for providing plasmid constructs and Dr. Pieter Spee for assistance with the chromatim immunoprecipitation assay.

REFERENCES

1. Jolivet, J., Cowan, K. H., Curt, G. A., Clendeninn, N. J., and Chabner, B. A. (1983) N. Engl. J. Med. 309, 1094–1010
2. Moscow, J. A., Connolly, T., Myers, T. G., Cheng, C. C., Paull, K., and Cowan, K. H. (1997) Int. J. Cancer 74, 184–190
3. Jansen, G. (1993) Annu. Rev. Nutr. 13, 71–89
4. Gorlick, R., Goker, E., Trippett, T., Steinherz, P., Elisseyeff, Y., Mazumdar, M., Flitoff, W. F., and Bertino, J. R. (1999) Blood 93, 2126–2301
5. Plumb, J. A., Strathdee, G., Sludden, J., Kaye, S. B., and Brown, R. (2000) Cancer Cell 7, 1107–1112
6. Sirotnak, F. M., and Tolner, B. (1999) Cancer Res. 59, 14746–14752
7. Sokol, O., Shrestha, K., Evans, S., Parkin, J. D., and Zalcberg, J. R. (1997) Blood 89, 21207–21212
8. Henderson, G. B., and Zevely, E. M. (1985) Int. J. Cancer 37, 674–679
9. Gorlick, R., Goker, E., Trippett, T., Steinherz, P., Eliseeyev, Y., Mazumdar, M., Flitoff, W. F., and Bertino, J. R. (1999) Blood 93, 2126–2301
10. Wong, S. C., Proefke, S. A., Bhushan, A., and Matthery, L. H. (1995) J. Biol. Chem. 270, 14746–14747
11. Jansen, G., Mauritz, R., Drori, S., Sprecher, H., Kathmann, I., Bunni, M., Priest, D. G., Noordhuis, P., Schornagel, J. H., Pinedo, H. M., Peters, G. J., and Assaraf, Y. G. (1998) J. Biol. Chem. 273, 30189–30196
12. Guo, W., Healey, J. H., Meyers, P. A., Ladanly, M., Huvos, A. G., Bertino, J. R., and Gorlick, R. (1999) Clin. Cancer Res. 5, 621–627
13. Jansen, G., Mauritz, R., Drori, S., Sprecher, H., Kathmann, I., Bunni, M., Priest, D. G., Noordhuis, P., Schornagel, J. H., Pinedo, H. M., Peters, G. J., and Assaraf, Y. G. (1999) Cancer Res. 59, 14752–14757
14. Sirotnak, F. M., and Tolner, B. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6914–6919
15. Underhill, T. M., and Flintoff, W. F. (1989) Somat. Cell Mol. Genet. 15, 141–148
16. Jolivet, J., Cowan, K. H., Curt, G. A., Clendeninn, N. J., and Chabner, B. A. (1983) N. Engl. J. Med. 309, 1094–1010
17. Williams, F. M., Murray, R. C., Underhill, T. M., and Flitoff, W. F. (1994) J. Biol. Chem. 269, 5811–5816
18. Schlemmer, S., and Flitoff, W. F. (1999) J. Biol. Chem. 274, 16269–16278
19. Dicker, A. P., and Kawakami, K. (1999) Cancer Res. 59, 5810–5814
20. Sirotnak, F. M., Moccio, D. M., and Young, C. W. (1981) J. Biol. Chem. 256, 1107–1112
21. Gorlick, R., Goker, E., Trippett, T., Steinherz, P., Eliseeyev, Y., Mazumdar, M., Flitoff, W. F., and Bertino, J. R. (1999) Blood 93, 2126–2301
22. Guo, W., Healey, J. H., Meyers, P. A., Ladanly, M., Huvos, A. G., Bertino, J. R., and Gorlick, R. (1999) Clin. Cancer Res. 5, 621–627
23. Brige, K. E., Spinella, M. J., Sierra, E. E., and Goldman, I. D. (1995) J. Biol. Chem. 270, 2274–2279
24. Kool, M., van der, L. M., de Haas, M., Schefer, G. L., de Vre, J. M., Smith, A. J., Jansen, G., Peters, G. J., Ponne, N., Schefer, R. J., Ellerink, R. P., Baas, F., and Borst, P. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6914–6919
25. Jolivet, J., Cowan, K. H., Curt, G. A., Clendeninn, N. J., and Chabner, B. A. (1983) N. Engl. J. Med. 309, 1094–1010
26. Hooijberg, J. H., Broxterman, H. J., Kool, M., Assaraf, Y. G., and Goldman, I. D. (1999) Mol. Pharmacol. 56, 68–76
27. Tse, A., Brige, K., Taylor, S. M., and Moran, R. G. (1998) J. Biol. Chem. 273, 25953–25960
28. Saucy, M., and Flitoff, W. F. (1999) J. Biol. Chem. 274, 26703–26707
29. Brige, K. E., Spinella, M. J., Sierra, E. E., and Goldman, I. D. (1997) J. Biol. Chem. 272, 19056–19071
30. Annu. Rev. Nutr. 5, 71–89
31. Katzenellenbogen, R. A., Baylison, S. B., and Herman, J. G. (1999) Adv. Cancer Res. 72, 141–196
32. Zeelenberg, P., el Osta, A., deSilva, M., Wall, D. M., Hu, X. F., Slater, A., Henderson, G. B., and Zevely, E. M. (1985) Int. J. Cancer 35, 1–12
33. Sirotnak, F. M., Moccio, D. M., and Young, C. W. (1981) J. Biol. Chem. 256, 1107–1112
RFC Promoter Methylation in MTX-resistant Breast Cancer Cells

59. Kennedy, D. G., Van den Berg, H. W., Clarke, R., and Murphy, R. F. (1986) *Biochem. Pharmacol.* 35, 3053–3056
60. Jones, P. L., Veenstra, G. J., Wade, P. A., Vermaak, D., Kass, S. U., Landsberger, N., Strouboulis, J., and Wolff, A. P. (1998) *Nat. Genet.* 19, 187–191
61. Nan, X., Ng, H. H., Johnson, C. A., Laherty, C. D., Turner, B. M., Eisenman, R. N., and Bird, A. (1998) *Nature* 393, 386–389
62. Ng, H. H., Zhang, Y., Hendrich, B., Johnson, C. A., Turner, B. M., Erdjument-Bromage, H., Tempst, P., Reinberg, D., and Bird, A. (1999) *Nat. Genet.* 23, 55–61
63. Yang, X., Fergusson, A. T., Nass, S. J., Phillips, D. L., Butash, K. A., Wang, S. M., Herman, J. G., and Davidson, N. E. (2000) *Cancer Res.* 60, 6890–6894
64. Carmen, A. A., Griffin, P. R., Calaycay, J. R., Rundlett, S. L., Suka, Y., and Grunstein, M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* 96, 12356–12361
65. Chen, C., Yang, M. C., and Yang, T. P. (2001) *J. Biol. Chem.* 276, 320–328
66. Roy, K., Mitsuji, K., and Siretnik, F. M. (1997) *J. Biol. Chem.* 272, 5587–5593
67. Rhee, M. S., Wang, Y., Nair, M. G., and Galivan, J. (1993) *Cancer Res.* 53, 2227–2230
68. Masuda, M., Hiruka, Y., Yamauchi, M., Nishigaki, R., Kato, Y., Nishimura, K., Suzuki, H., and Suzuki, Y. (1997) *Cancer Res.* 57, 3506–3510
69. Gottesman, M. M., and Pastan, I. (1993) *Annu. Rev. Biochem.* 62, 385–427
70. Bosch, I., and Croop, J. (1996) *Biochim. Biophys. Acta* 1288, F37–F54
71. de Graaf, D., Sharma, R. C., Mechetner, E. B., Schimke, R. T., and Roninson, I. B. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 1238–1242
72. Norris, M. D., de Graaf, D., Haber, M., Kavallaris, M., Madaﬁfiglio, J., Gilbert, J., Kwan, E., Stewart, B. W., Mechetner, E. B., Gutkov, A. V., and Roninson, I. B. (1996) *Int. J. Cancer* 65, 613–619
73. van Triest, B., Pinedo, H. M., Telleman, F., van der Wilt, C. L., Jansen, G., and Peters, G. J. (1997) *Biochem. Pharmacol.* 53, 1855–1866
74. Gifford, A. J., Kavallaris, M., Madaﬁfiglio, J., Matherly, L. H., Stewart, B. W., Haber, M., and Norris, M. D. (1998) *Int. J. Cancer* 78, 176–181
75. Cabral, S., Leis, S., Bover, L., Nembrot, M., and Mordoh, J. (1984) *Proc. Natl. Acad. Sci. U. S. A.* 81, 3200–3203
76. Chen, H. X., Bamberger, U., Heckel, A., Guo, X., and Cheng, Y. C. (1993) *Cancer Res.* 53, 1974–1977
77. Goel, R., and Howell, S. B. (1992) in *New Drugs, Concepts and Results in Cancer Chemotherapy* (Muggia F. M., ed) pp. 19–44, Kluwer Academic, Boston, MA
78. Ding, B. C., Whetstone, J. R., Witt, T. L., Schuetz, J. D., and Matherly, L. H. (2001) *J. Biol. Chem.* 276, 8713–8719
79. Sadliah, H., Murray, R. C., Williams, F. M., and Flintoff, W. F. (2000) *Biochem. J.* 346, 509–518
80. Krause, K., Wasner, M., Reinhard, W., Haugwitz, U., Dohna, C. L., Mossner, J., and Engelhardt, K. (2000) *Nucleic Acids Res.* 28, 4410–4418
