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REGULATION OF FOLATE-BINDING PROTEIN GENE EXPRESSION BY DNA METHYLATION IN METHOTREXATE-RESISTANT KB CELLS

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Abstract—Folate-binding protein (FBP) is responsible for the cellular transport of folate and methotrexate (MTX) in human KB (nasopharyngeal epidermoid carcinoma) cells. The levels of membrane-associated FBP and FBP mRNA are decreased 70–80% in an MTX-resistant KB subline (KB1BT) (Hsueh C-T and Dolnick BJ, *Oncol Res* **4**: 497–505, 1992). Southern blot analysis did not reveal any differences in FBP gene organization or copy number between KB1BT and KB cells. However, there was a 70% decrease in the FBP gene transcription rate and no change in FBP mRNA stability in KB1BT cells. Assessing genomic DNA methylation by *Msp*I and *Hpa*II restriction analysis suggested that the FBP gene in KB1BT cells was more methylated than in KB cells. These alterations in the expression, transcription rate and DNA methylation state of the FBP gene did not change when KB1BT cells were grown in the absence of MTX for 8 months (MTX-free KB1BT). When MTX-free KB1BT cells were exposed to 2.5 μM 5-aza-2′-deoxycytidine for 72 hr, the FBP gene became hypomethylated and the levels of membrane-associated FBP and FBP mRNA increased by 2- to 3-fold. These data indicate that decreased FBP gene expression in KB1BT cells results from increased DNA methylation.

Key words: folic acid; methotrexate; methylation; transcription; binding protein; drug resistance

FBP‡, a folate receptor expressed in certain normal and malignant cell lines and tissues [1, 2], is a high-affinity folate binder responsible for the cellular transport of folate and structurally related antifolates such as MTX in human KB (nasopharyngeal epidermoid carcinoma) cells [3–5]. Thus far, two forms of FBP, membrane-associated (M-FBP) and soluble, have been identified [6]. The M-FBP, anchored to the cell membrane via a glycosylphosphatidylinositol linkage [7], is involved in the receptor-mediated transport of folate, and recent evidence indicates that caveolae also participate in this transport process [8]. The function of soluble FBP remains unknown, but it is likely that soluble FBP is derived from protease or lipase digestion of M-FBP and may be involved in the storage, transport and uptake of folate [9, 10].

cDNAs for FBP mRNA have been cloned from KB cells [11, 12], human colon carcinoma cells [7], human ovarian carcinoma cells [13], human placenta [14, 15], and murine leukemia cells [16]. Expression of human FBP mRNA has been detected in choroid plexus, lung, thyroid, and kidney [1]. All the cDNAs derived from human cancer cells have the same protein coding sequences, but their 5′-untranslated regions vary and this may have functional significance [13]. There are at least three FBPs expressed in human placenta; one of these appears to be identical to KB FBP (putative adult form), while the other two (putative fetal forms) share approximately 70–80% identity with KB FBP [14, 15]. An FBP multi-gene family has been localized recently to chromosome 11q13, including an adult gene, a fetal gene, and one or more pseudogenes [17, 18].

MTX, a folate analog, has been used extensively in clinical cancer chemotherapy, including the treatment of head and neck cancer [19]. Defective MTX transport is a documented mechanism of MTX-resistance, both *in vitro* [20–22] and *in vivo* [23, 24]. Decreased FBP gene expression can mediate transport-defective MTX resistance in KB cells [25–27]. We showed previously that M-FBP and FBP mRNA levels are decreased by 70–80% in an MTX-resistant KB subline (KB1BT) when compared with the parental cells, and the decreased FBP gene expression leads to defective cellular MTX uptake [27]. The possible molecular mechanisms for the altered FBP gene expression in KB1BT cells were investigated in the present study. We observed an approximately 70% decrease in the transcription rate of FBP gene accompanied by FBP gene

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‡ Abbreviations: FBP, folate-binding protein; MTX, methotrexate; M-FBP, membrane-associated folate-binding protein; 5-aza-dC, 5-aza-2′-deoxycytidine; DHFR, dihydrofolate reductase; SSC, standard saline citrate (1 × SSC is 150 mM NaCl, 15 mM sodium citrate, pH 7.0); and ara-C, 1-β-D-arabinofuranosyl cytosine.

hypermethylation in KB1BT and KB1BT grown in the absence of MTX for 8 months (MTX-free KB1BT), as compared with KB cells. Moreover, cell exposure to a DNA methylation inhibitor induced FBP gene expression in MTX-free KB1BT cells. The implication of these findings is discussed.

MATERIALS AND METHODS

Materials. [α-^{32}P]dCTP (3000 Ci/mmol) and [α-^{32}P]UTP (3000 Ci/mmol) were obtained from DuPont/New England Nuclear (Boston, MA). [3′,5′,7,9-^{3}H]Folic acid (29 Ci/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA). Hybond N nylon membranes were obtained from Amersham (Arlington Heights, IL), and ultrapure cesium chloride, RPMI 1640 medium, horse serum and fetal bovine serum from GIBCO BRL (Gaithersburg, MD). MTX was obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute (Rockville, MD). Guanidine thiocyanate, actinomycin D and 5-aza-2′-deoxycytidine (5-aza-dC) were purchased from the Sigma Chemical Co. (St. Louis, MO), and restriction endonucleases from United States Biochemical (Cleveland, OH). All other reagents were of the highest quality available.

Cell lines and cultures. KB cells (ATCC CCL 17) and the clonally derived MTX-resistant KB1BT cell line were cultured in RPMI 1640 medium supplemented with 5% horse serum. KB1BT cells were maintained in the presence of 50 μM MTX unless otherwise specified. KB1BT exhibits DHFR gene amplification, and overexpresses DHFR and DHFR mRNA approximately 250-fold [28, 29]. Experiments involving 5-aza-dC treatment were performed 24 hr after cell passage, and cells (approximately 20–30% confluent) were exposed to the indicated concentration of 5-aza-dC. All other experiments were performed when cells were grown to confluence. Cell lines were examined every 3 months for Mycoplasma contamination and consistently tested negative.

Plasmids and probes. pG4ZF511, a gift from Dr. Stephen W. Lacey (University of Texas Southwestern Medical Center, Dallas, TX), contains a 991-bp *Eco*RI DNA fragment prepared from human Caco-2 (colon carcinoma) FBP mRNA [7]. The 544-bp human β-actin cDNA, spanning positions 317 to 860 of the mRNA sequence [30], was generated from KB RNA by using a β-actin mRNA sequence-specific complementary primer in a reverse transcription reaction and amplification of the resulting cDNA by the polymerase chain reaction [31]. For northern and Southern blot analyses, the *Eco*RI-restricted cDNA fragments from pG4ZF511 and pTZ18U-hFPGS, and β-actin polymerase chain reaction product were used in preparing ^{32}P-labeled probes by random priming [32]. p11/14 containing a chicken β-actin cDNA was a gift from Dr. Joseph T. Y. Lau (Roswell Park Cancer Institute, Buffalo, NY) [33]. pG4ZF511, p11/14 and pGEM-1 (Promega, Madison, WI) were used as DNA filter hybridization probes for nuclear run-off transcription assays.

Preparation and analysis of nucleic acids. RNA was isolated from cultured cells using guanidine thiocyanate according to the method of Chirgwin *et al.* [34]. Northern blot analysis and genomic DNA isolation were carried out as previously described [35]. Restriction enzyme digestion of DNA and Southern blot analysis were performed as described by Sambrook *et al.* [36]. To assess the equivalence of sample loading and gel transfer, RNA samples were treated with ethidium bromide prior to loading [37], and DNA samples were fractionated in agarose gels containing ethidium bromide. Samples were then visualized by UV illumination of the gels after electrophoresis and the nylon filters after capillary transfer. DNA and RNA blots were prehybridized at 42° for 12 hr, and then hybridized to the indicated ^{32}P-labeled DNA probe of specific activity greater than 10^9 cpm/μg at 42° for 12–16 hr. Unbound probe was removed by washing blots with two changes of a solution containing 2× SSC and 0.2% SDS for 30 min each at room temperature, and two changes for 30 min each of 0.2× SSC and 0.2% SDS at 60°. Blots were exposed to Kodak X-OMAT AR film with intensifying screens, and specific bands were quantitated with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Nuclear run-off transcription assay. Nuclei were isolated and further purified over a sucrose cushion by published methods [38, 39]. Nuclear run-off transcription assays with 5×10^7 nuclei and isolation of nuclear RNAs were performed based upon the method described by Celano *et al.* [40], except that 330 μCi [α-^{32}P]UTP and 0.6 mM ATP, GTP and CTP were used in the transcription reactions. The amount of [α-^{32}P]UTP incorporated into nuclear RNA was determined by the trichloroacetic acid precipitation method [36], and equal amounts of ^{32}P-labeled nuclear RNA from KB and KB1BT nuclei were used in filter hybridizations. Immobilization of the DNA probes onto nylon filters was performed according to the method of Kafatos *et al.* [41]. Five micrograms of denatured plasmid DNAs and 0.5 μg of genomic KB and KB1BT DNAs were dot-blotted to nylon filters using a 96-well filtration manifold with 3-mm wells. The filters were UV irradiated and prehybridized at 50° for 12–16 hr. ^{32}P-labeled RNAs from nuclear run-off reactions (usually more than 1×10^7 dpm) were incubated in 2 mL hybridization buffer at 80° for 10 min, then combined with filters containing the β-actin cDNA, FBP cDNA, pGEM-1 vector DNA and genomic DNA, and incubated at 50° for 72 hr. Filter-washing conditions, and buffer for prehybridization and hybridization were essentially the same as described by Flaspohler and Milcarek [42], except that 2 μg/mL RNase A was used in the washing solution. Autoradiography and Phosphorimager quantitation were performed as described above.

mRNA stability study. KB and KB1BT cells were exposed to 5 μg/mL actinomycin D as previously described [28]. Total cellular RNAs from drug-treated cells were isolated at various times, and analyzed by northern blotting with the ^{32}P-labeled FBP cDNA probe.

Membrane preparation and M-FBP quantitation. Membrane preparation and M-FBP quantitation were performed as previously described with some modifications [3]. Briefly, 2–4 × 10^6 cells from each

cell line were suspended in ice-cold homogenizing buffer (10 mM Tris–HCl, pH 7.5, and 2 mM dithiothreitol) at a concentration of 2×10^6 cells/mL. Cells were lysed by freeze-thawing and sonication [3]. The cell homogenates were centrifuged at 27,000 *g* for 30 min at 4°, then the pellets were resuspended in ice-cold homogenizing buffer, and protein estimation was performed by the method of Bradford [43]. M-FBP determination was performed by the [^{3}H]folic acid binding assay. The endogenously bound folates in 10 μg of membrane preparations were removed by treatment with 40 mM acetic acid at 4° for 10 min. After neutralization with sodium hydroxide, total M-FBP was determined by measuring binding in the presence of a large excess (100 nM) of [^{3}H]folic acid at 37° for 10 min. Subsequently the binding reactions were placed at 4°, and filtered through 0.45 μm Metricel membrane filters (Gelman Sciences, Inc., Ann Arbor, MI). The filters were washed with 20 mL of ice-cold homogenizing buffer, air-dried, and then counted in 5 mL of Ready Safe scintillation fluid (Beckman Instruments, Inc., Fullerton, CA). Counting efficiency was determined to be approximately 37%.

Statistical analysis. All experiments were performed at least twice with similar results, and the results of one representative experiment are reported. The curve-fitting analysis in the mRNA stability studies was performed by linear regression.

RESULTS

Southern blot analysis of the FBP gene. To determine whether an altered gene copy number or organization of the FBP gene could account for decreased FBP mRNA levels in KB1BT cells, genomic DNAs from KB and KB1BT cells were digested to completion by various restriction enzymes, and then subjected to Southern blot analysis with ^{32}P-labeled FBP cDNA as a probe. The *Apa*I, *Bam*HI, and *Pst*I-restriction patterns of the FBP gene were essentially the same between KB and KB1BT cells (Fig. 1), and additional restriction analysis by *Eco*RI and *Hind*III also yielded the same patterns in the two cell lines (data not shown). Moreover, there was no difference in the FBP gene copy number as determined by Phosphorimager analysis. Since there is a high degree of similarity between adult FBP cDNA, fetal FBP cDNAs and pseudogene(s) [14, 15, 17, 18], it is likely that the adult FBP cDNA probe used in this study hybridized to DNA fragments from more than one member of the FBP gene family. Nevertheless, identical restriction patterns indicate that both cell types are essentially identical with respect to gene organization and dosage.

Transcription rate of the FBP gene. The transcription rates of the FBP gene between KB and KB1BT cells were compared by a nuclei run-off assay. Nuclei from KB and KB1BT cells were isolated, and the amounts of RNA transcription were measured for β-actin, FBP and genomic DNAs by filter hybridization (Fig. 2A). No significant difference was observed in the transcription rate of the β-actin gene between KB and KB1BT nuclei; however, there was an approximately 70% decrease

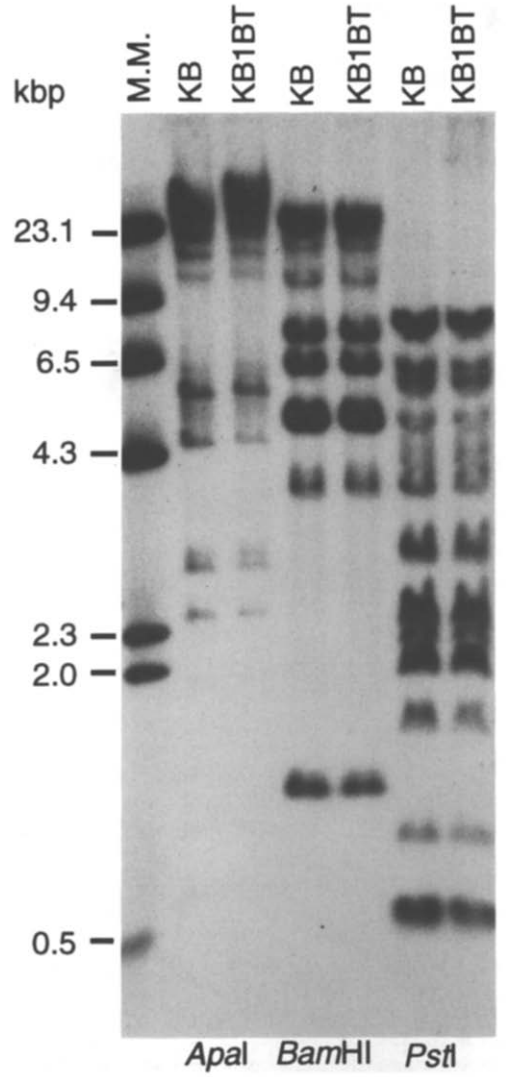

Fig. 1. Southern blot analysis of the FBP gene in KB and KB1BT cells. KB and KB1BT genomic DNAs (15 μg) were digested with various restriction endonucleases (as indicated), fractionated by electrophoresis through 0.6% agarose gels, blotted to nylon filters, then hybridized with a ^{32}P-labeled FBP cDNA probe. M.M. represents the *Hin*dIII-digested λ phage DNA molecular weight markers, and the sizes of the markers are indicated to the left in kbp.

in the transcription rate of the FBP gene in KB1BT cells, compared with KB cells (Fig. 2B).

FBP mRNA stability. In a previous report, we observed that increased FBP mRNA stability is partly responsible for increased FBP gene expression in KB cells grown under folate-deficient conditions [44]. Thus, FBP mRNA stability was explored to determine whether altered mRNA stability could account for the decreased FBP mRNA levels in KB1BT cells. KB and KB1BT cells were exposed to 5 μg/mL of the transcription inhibitor actinomycin D [45]. At various times RNAs were isolated and

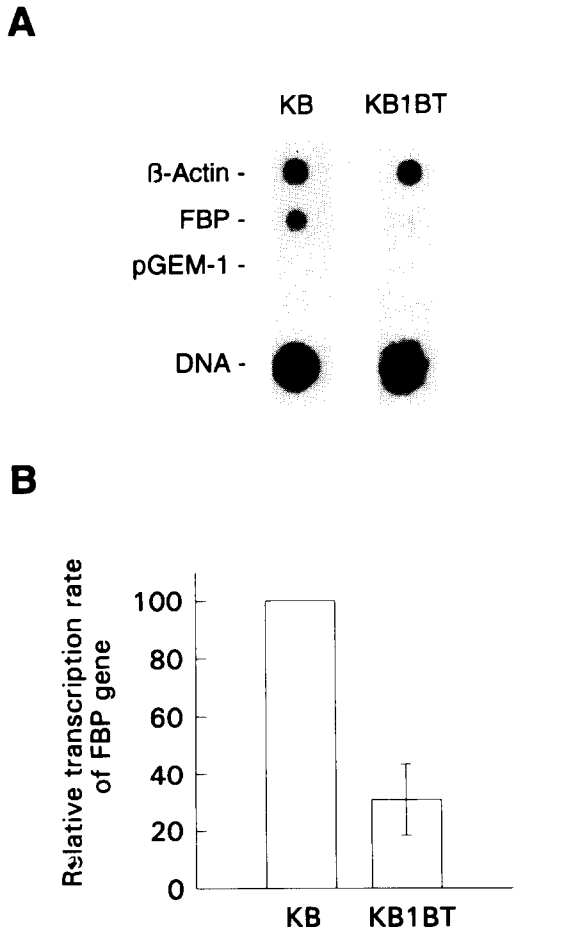

Fig. 2. Transcription rate of the FBP gene in KB and KB1BT cells. (A) Nuclear run-off transcription analysis. Nuclei (5×10^7) from KB and KB1BT cells were used in nuclear run-off transcription assays as described under Materials and Methods. Equal amounts of ^{32}P-labeled nuclear RNA were hybridized to filters blotted with 5 μg of denatured plasmid DNAs containing chicken β-actin cDNA, human FBP cDNA and pGEM-1 vector, and 0.5 μg KB or KB1BT genomic DNA. (B) Relative transcription rate of the FBP gene. The radioactivity of each dot of the filters was quantitated by a Phosphorimager. Background hybridization to pGEM-1 was subtracted from values for β-actin cDNA, FBP cDNA and genomic DNAs. The transcription rate of the FBP gene was normalized to that of the β-actin gene, and presented as the percent change from the relative transcription rate of KB cells. The data shown are the means ± SEM of three experiments. Similar results were observed when the transcription rate of the FBP gene was normalized to that of respective genomic DNA.

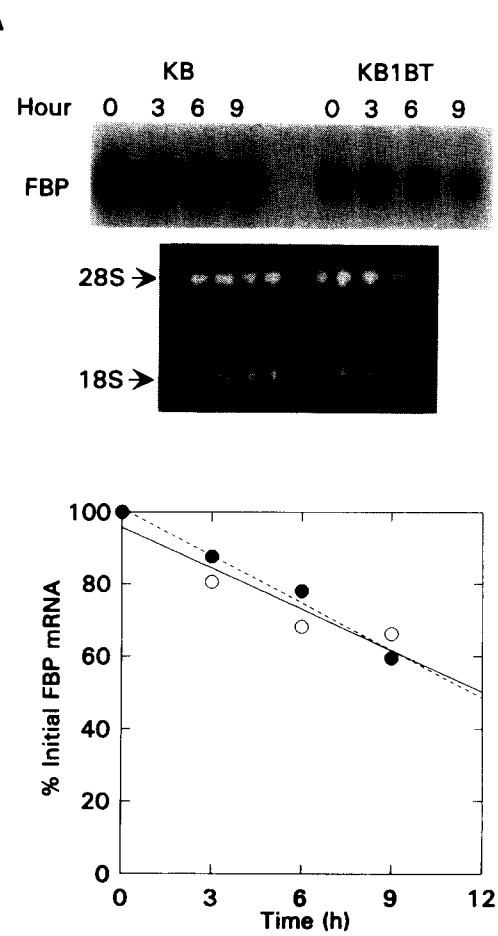

Fig. 3. FBP mRNA stability in KB and KB1BT cells. (A) Northern blot analysis. KB and KB1BT cells were exposed to actinomycin D (5 μg/mL) for the indicated times. Total cellular RNAs (10 μg) at each time point were subjected to northern blot analysis with ^{32}P-labeled FBP cDNA as a probe, as described under Materials and Methods. The photograph of the nylon filter exhibiting ethidium bromide fluorescence is displayed in the lower panel, demonstrating equal loading and even transfer of the gel. (B) Half-life determination of FBP mRNA. Specific FBP mRNA bands for KB (○) and KB1BT cells (●) in the RNA blots were quantitated by a Phosphorimager. The lines represent the results of best fit analysis determined by linear regression.

subjected to northern blot analysis with ^{32}P-labeled FBP cDNA as a probe (Fig. 3A). There was no difference between KB and KB1BT cells in FBP mRNA stability within the time frame studied (Fig. 3B) The FBP mRNA half-life in KB cells grown in RPMI 1640 medium supplemented with 5% horse serum was observed to be approximately 12 hr in the present study, while the FBP mRNA half-life in KB cells cultured in minimum essential medium supplemented with 10% fetal bovine serum was found to be 5.7 hr in a previous report [44]. The difference in the FBP mRNA stability under those two cell culture conditions was also reflected in the FBP mRNA levels.* Since serum and medium conditions were not variables in the current study, this phenomenon was not investigated further.

Methylation pattern of the FBP gene. Methylation of cytosine at specific CpG dinucleotides has been implicated in transcriptional control of gene expression [46–49]. To compare the methylation

* Hsueh C-T and Dolnick BJ, unpublished observation.

patterns of the FBP gene between KB and KB1BT cells, *Msp*I-and *Hpa*II-restricted genomic DNAs were subjected to Southern blot analysis and probed with ^{32}P-labeled FBP cDNA. *Msp*I and *Hpa*II are isoschizomer restriction-enzymes that recognize the sequence CCGG. *Msp*I does not cleave DNA when the external cytosine is methylated and its activity is unaffected by internal cytosine methylation, whereas *Hpa*II does not digest DNA if the internal cytosine is methylated [50]. Identical restriction patterns generated from *Msp*I and *Hpa*II digestions are indicative of unmethylated DNA, whereas DNA methylation is revealed by the presence of higher molecular weight DNA fragments after *Hpa*II digestion. While the *Msp*I-restriction patterns of the FBP gene from KB and KB1BT cells were identical (Fig. 4A), the *Hpa*II-restriction patterns were not (Fig. 4B). The *Hpa*II-restricted FBP gene in KB1BT cells exhibited a novel fragment of approximately 12 kbp, and diminished intensity in several fragments of approximately 10.4 to 9.4, 7.2, 5.6 and 4.5 kbp, when compared with KB cells. These results indicate CpG methylation is increased at some *Hpa*II/*Msp*I sites of one (or more) FBP gene in KB1BT cells.

Effects of MTX and 5-aza-dC on the expression and DNA methylation state of the FBP gene in KB1BT cells. KB1BT cells are not stably amplified for DHFR [28]. When KB1BT cells were grown in the absence of MTX for 8 months (approximately 65–70 generations), the DHFR mRNA levels decreased by 95–99% (data not shown), but there were no significant changes in the levels of M-FBP or FBP mRNA (Fig. 5A, lane 3), the FBP gene transcription rate (data not shown), or the FBP gene methylation state (Fig. 5B, lane 3). These data indicate that the methylation state of the FBP gene in KB1BT cells is stably inherited, even when the selection pressure of MTX exposure is removed.

It is very likely that DNA hypermethylation suppresses FBP gene expression in KB1BT cells through decreased transcriptional activity, even in the absence of MTX. To test further this hypothesis, KB1BT cells grown in the absence of MTX for 8 months (MTX-free KB1BT cells) were exposed for 72 hr to 2.5 μM 5-aza-dC, a DNA methylation inhibitor [46]. The 72-hr exposure time corresponds to approximately three cell generations for KB1BT cells grown in the absence of MTX for 8 months, and the concentration of 5-aza-dC used is within the effective concentration range which induces significant DNA hypomethylation without severe cytotoxicity [51, 52]. The levels of FBP mRNA and M-FBP, and FBP gene methylation in the treated cells were compared with those of untreated cells. After exposure to 5-aza-dC, MTX-free KB1BT cells exhibited decreased FBP gene methylation (Fig. 5B, lane 4) and a 2- to 3-fold increase in the levels of FBP mRNA (Fig. 5A, lane 4) and M-FBP (determined by M-FBP quantitation, data not shown). There was no induction in FBP gene expression when KB cells were exposed to 2.5 μM 5-aza-dC for 72 hr (data not shown).

DISCUSSION

We reported previously that the levels of M-FBP and FBP mRNA in KB1BT cells are decreased by 70–80% when compared with parental KB cells, and that the decreased FBP gene expression leads to defective cellular uptake of MTX [27]. Since the levels of M-FBP and FBP mRNA are decreased to approximately the same degree in KB1BT cells, the altered FBP gene expression is probably not translationally regulated. In the current study, genomic organization and gene copy number of the FBP gene were compared between KB and KB1BT cells, and no differences were found. Saikawa *et al.* [26] have reported similar findings in MTX-resistant KB cells grown under folate-deficient conditions (less than 10 nM folate vs 2.3 μM folate in standard medium) with decreased FBP gene expression. These findings suggest that chromosomal deletions, additions or translocations of the FBP gene are not involved in the down-regulation of FBP gene expression in KB cells grown either under folate-replete or folate-deficient conditions.

An approximately 70% decrease in the transcription rate of the FBP gene was observed in KB1BT cells compared with KB cells. Since the degrees of decrease in FBP gene transcription rate and expression levels are approximately the same in KB1BT cells, FBP gene expression is likely transcriptionally down-regulated in KB1BT cells. An increase in the FBP gene methylation state was found in KB1BT cells compared with KB cells. The alterations in the DNA methylation state, transcription and expression of the FBP gene were stably inherited from passage to passage, even when KB1BT cells were grown in the absence of MTX for up to 8 months. Further exposure of those MTX-free KB1BT cells to a DNA methylation inhibitor resulted in FBP gene hypomethylation and a 2- to 3-fold induction of FBP gene expression. These data suggest that DNA hypermethylation suppresses FBP gene expression in KB1BT cells through decreased transcriptional activity. Many lines of evidence indicate that DNA methylation is somatically inherited and is mediated by a specific DNA methyltransferase [53, 54]. An inverse correlation between the extent of DNA methylation and the level of gene expression has been demonstrated for many genes [55–57]. Several methyl-CpG binding proteins have been identified recently [48, 58]. It is speculated that these proteins could complex with methylated CpG dinucleotides in the promoter regions of certain genes, and suppress gene expression by interfering with the access of transcription factors.

We also observed hypermethylation of the folylpolyglutamate synthetase gene in KB1BT compared with KB, but there was no difference in the folylpolyglutamate synthetase mRNA levels between those two cell lines as determined by Northern blot analysis [59]. This suggests that increased DNA methylation in KB1BT cells is not restricted to the FBP gene, but that only the expression of some genes such as FBP correlate inversely with DNA methylation. The sensitivity of gene expression to DNA methylation may depend upon whether altered methylation sites are critical for transcription. Since the complete genomic sequence of the FBP multi-gene family is not

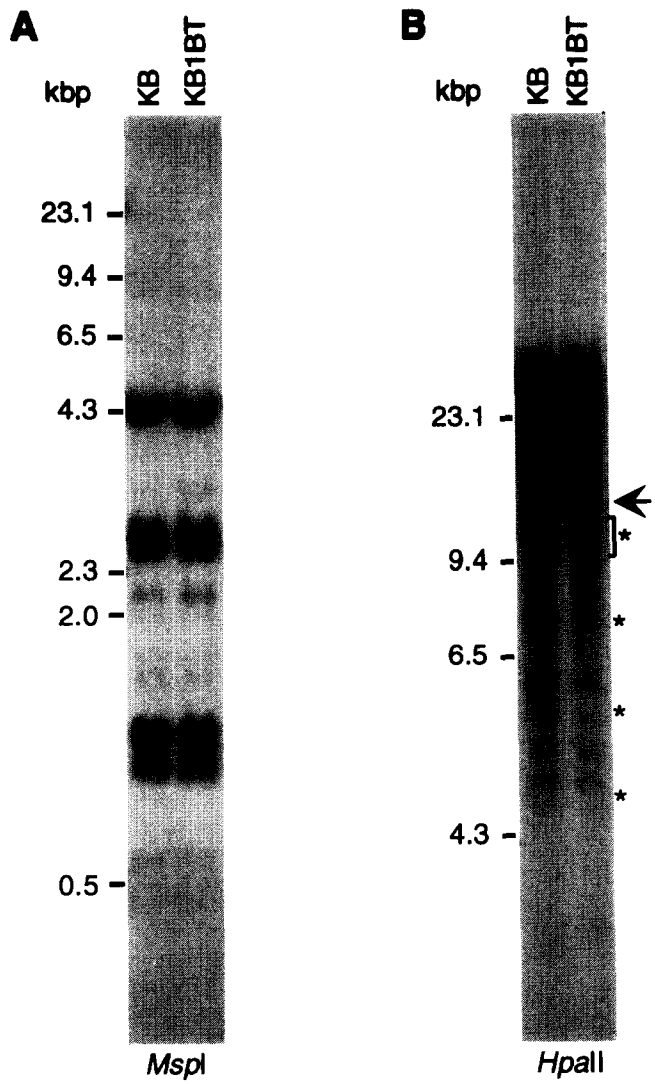

Fig. 4. DNA methylation state of the FBP gene in KB and KB1BT cells. KB and KB1BT genomic DNAs (15 μg) were restricted by *Msp*I and *Hpa*II, fractionated by electrophoresis through 0.6% agarose gels, blotted to nylon filters, and then probed with ^{32}P-labeled FBP cDNA. The sizes of *Hin*dIII-digested λ phage DNA molecular weight markers (not shown) are indicated to the left in kbp. (A) *Msp*I-restriction patterns of the FBP gene in KB and KB1BT cells. (B) *Hpa*II-restriction patterns of the FBP gene in KB and KB1BT cells. The arrow to the right indicates the position of one *Hpa*II-restriction fragment of the FBP gene that was present in KB1BT cells, but not evident in KB cells. Asterisks indicate those *Hpa*II-restriction fragments of the FBP gene that were less apparent in KB1BT, compared with KB cells.

currently available, we were unable to identify which *Hpa*II/*Msp*I sites of the KB1BT FBP gene harbor the methylation changes. CpG islands in the gene promoters are regions of interest for the transcriptional regulation of CpG methylation, though other sites of methylation changes can also affect gene expression [60–62]. It is possible there are other gene products modulating FBP gene expression, and that their expression is controlled by DNA methylation.

In a previous report, we observed a decrease in FBP gene methylation but no change in the FBP gene transcription rate in KB cells grown in folate-deficient versus folate-replete medium [44]. Hypomethylation of the FBP gene induced by exposure to DNA methylation inhibitors also fails to increase FBP gene expression in KB cells grown in folate-replete medium. Moreover in KB cells, increased FBP mRNA stability is partly responsible for the induction of FBP gene expression under folate-deficient growth conditions. Based upon those results and the current findings, we speculate that a

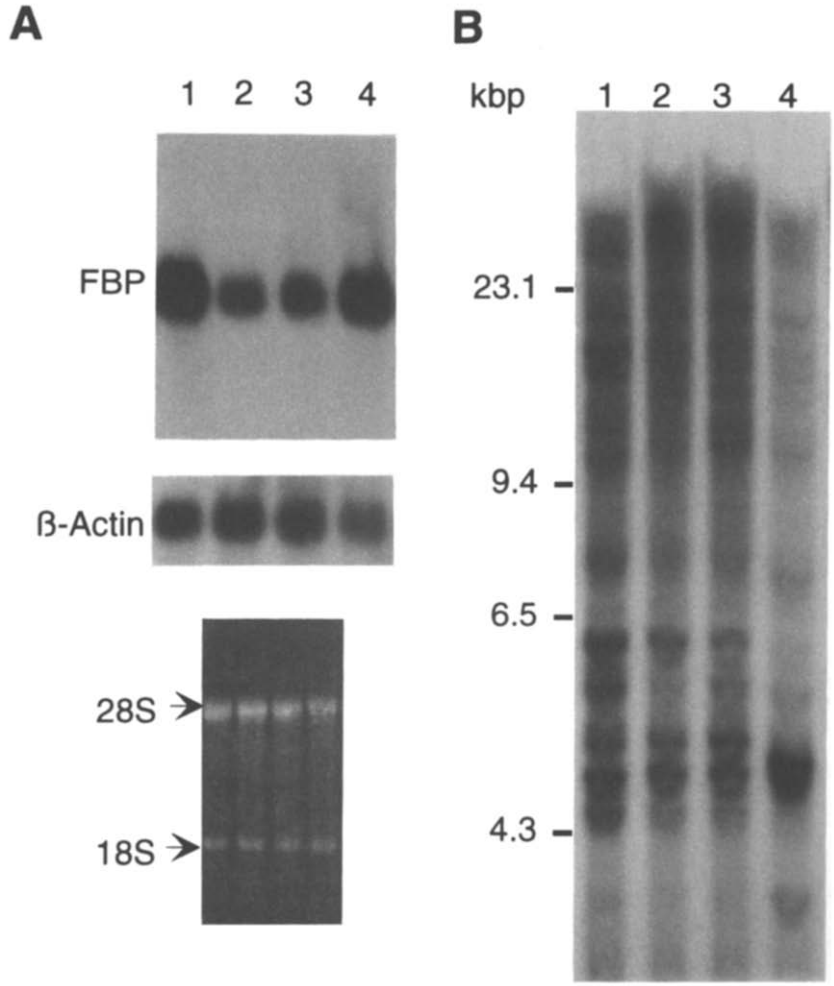

Fig. 5. Effects of MTX and 5-aza-dC on the FBP mRNA levels and FBP gene methylation in KB1BT cells. (A) Northern blot analysis. Northern blot analysis was performed as described, and the nylon filters were sequentially hybridized to ^{32}P-labeled FBP and β-actin cDNAs. The photograph of the nylon filter exhibiting ethidium bromide fluorescence is shown in the lower panel, demonstrating equal loading and even transfer of the gel. (B) *Hpa*II-restriction patterns of the FBP gene. Southern blot analysis was performed as previously described. The positions of *Hin*dIII-digested λ phage DNA molecular weight markers (not shown) are indicated to the left in kbp. Lane 1, KB cells. Lane 2, KB1BT cells. Lane 3, KB1BT grown in the absence of MTX for 8 months. Lane 4, KB1BT cells grown in the absence of MTX for 8 months but treated with 2.5 μM 5-aza-dC for 72 hr.

certain degree of FBP gene hypomethylation is required for basal-level gene expression, but that under folate-deficient conditions further increase in FBP gene expression may rely on mechanisms besides DNA methylation.

Drug-induced DNA hypermethylation has been documented as one component of the response of human tumor cells to toxic concentrations of commonly used cancer chemotherapy agents including MTX [63]. It has also been reported that ara-C is able to induce DNA hypermethylation in mammalian cells [46, 64]. It has been shown that human tumor cells resistant to ara-C, presumably due to the presence of an inactive deoxycytidine kinase gene, could be reactivated for deoxycytidine kinase enzyme expression by a DNA methylation inhibitor with concomitant resensitization toward ara-C [65, 66]. It is possible that hypermethylation induced by chemotherapeutic agents could selectively suppress expression of certain genes and lead to drug resistance in tumor cells. If indeed, the increased DNA methylation of the FBP gene leads to decreased FBP gene expression in certain cancer cells and tissues, the clinical combination of DNA methylation inhibitors and MTX may be beneficial in preventing and reversing MTX resistance resulting from decreased FBP gene expression, or in increasing cellular uptake of MTX by turning on FBP gene expression in some MTX-unresponsive tumors. However, further mechanistic studies regarding FBP gene expression and a demonstration of clinical MTX resistance resulting from decreased FBP gene

expression are needed before justification would exist for such treatment regimens.

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REFERENCES

1. Weitman SD, Lark RH, Coney LR, Fort DW, Frasca V, Zurawski VR and Kamen BA, Distribution of the folate receptor GP38 in normal and malignant cell lines and tissues. *Cancer Res* **52**: 3396–3401, 1992.
2. Weitman SD, Weinberg AG, Coney LR, Zurawski VR, Jennings DS and Kamen BA, Cellular localization of the folate receptor: Potential role in drug toxicity and folate homeostasis. *Cancer Res* **52**: 6708–6711, 1992.
3. McHugh M and Cheng Y-C, Demonstration of a high affinity folate binder in human cell membranes and its characterization in cultured human KB cells. *J Biol Chem* **254**: 11312–11318, 1979.
4. Antony AC, Kane MA, Portillo RM, Elwood PC and Kolhouse JF, Studies of the role of a particulate folate-binding protein in the uptake of 5-methyl-tetrahydrofolate by cultured human KB cells. *J Biol Chem* **260**: 14911–14917, 1985.
5. Deutsch JC, Elwood PC, Portillo RM, Macey MG and Kolhouse JF, Role of the membrane-associated folate binding protein (folate receptor) in methotrexate transport by human KB cells. *Arch Biochem Biophys* **274**: 327–337, 1989.
6. Elwood PC, Kane MA, Portillo RM and Kolhouse JF, The isolation, characterization, and comparison of the membrane-associated and soluble folate-binding proteins from human KB cells. *J Biol Chem* **261**: 15416–15423, 1986.
7. Lacey SW, Sanders JM, Rothberg KG, Anderson RGW and Kamen BA, Complementary DNA for the folate binding protein correctly predicts anchoring to the membrane by glycosyl-phosphatidylinositol. *J Clin Invest* **84**: 715–720, 1989.
8. Anderson RGW, Kamen BA, Rothberg KG and Lacey SW, Potocytosis: Sequestration and transport of small molecules by caveolae. *Science* **255**: 410–411, 1992.
9. Elwood PC, Deutsch JC and Kolhouse JF, The conversion of the human membrane-associated folate binding protein (folate receptor) to the soluble folate binding protein by a membrane-associated metalloprotease. *J Biol Chem* **266**: 2346–2353, 1991.
10. Verma RS, Gullapalli S and Antony AC, Evidence that the hydrophobicity of isolated, *in situ*, and *de novo*-synthesized native human placental folate receptors is a function of glycosyl-phosphatidylinositol anchoring to membranes. *J Biol Chem* **267**: 4119–4127, 1992.
11. Sadasivan E and Rothenberg SP, The complete amino acid sequence of a human folate binding protein from KB cells determined from the cDNA. *J Biol Chem* **264**: 5806–5811, 1989.
12. Elwood PC, Molecular cloning and characterization of the human folate-binding protein cDNA from placenta and malignant tissue culture (KB) cells. *J Biol Chem* **264**: 14893–14901, 1989.
13. Coney LR, Tomassetti A, Carayannopoulos L, Frasca V, Kamen BA, Colnaghi MI and Zurawski VR, Cloning of a tumor-associated antigen: MOv18 and MOv19 antibodies recognize a folate-binding protein. *Cancer Res* **51**: 6125–6132, 1991.
14. Ratnam M, Marquardt H, Duhring JL and Freisheim JH, Homologous membrane folate binding proteins in human placenta: Cloning and sequence of a cDNA. *Biochemistry* **28**: 8249–8254, 1989.
15. Page ST, Owen WC, Price K and Elwood PC, Expression of the human placental folate receptor transcript is regulated in human tissues: Organization and full nucleotide sequence of the gene. *J Mol Biol* **229**: 1175–1183, 1993.
16. Brigle KE, Westin EH, Houghton MT and Goldman ID, Characterization of two cDNAs encoding folate-binding proteins from L1210 murine leukemia cells. *J Biol Chem* **266**: 17243–17249, 1991.
17. Ragoussis J, Senger G, Trowsdale J and Campbell IG, Genomic organization of the human folate receptor genes on chromosome 11q13. *Genomics* **14**: 423–430, 1992.
18. Sadasivan E, Cedeno M and Rothenberg SP, Genomic organization of the gene and a related pseudogene for a human folate binding protein. *Biochim Biophys Acta* **1131**: 91–94, 1992.
19. Jolivet J, Cowan KH, Curt GA, Clendeninn NJ and Chabner BA, The pharmacology and clinical use of methotrexate. *N Engl J Med* **309**: 1094–1104, 1983.
20. Sirotnak FM, Moccio DM, Kelleher LE and Goutas LJ, Relative frequency and kinetic properties of transport-defective phenotypes among methotrexate-resistant L1210 clonal cell lines derived *in vivo*. *Cancer Res* **41**: 4447–4452, 1981.
21. Frei E III, Rosowsky A, Wright JE, Cucchi CA, Lippke JA, Ervin TJ, Jolivet J and Haseltine WA, Development of methotrexate resistance in a human squamous cell carcinoma of the head and neck in culture. *Proc Natl Acad Sci USA* **81**: 2873–2877, 1984.
22. Rosowsky A, Wright JE, Cucchi CA, Lippke JA, Tantravahi R, Ervin TJ and Frei E III, Phenotypic heterogeneity in cultured human head and neck squamous cell carcinoma lines with low-level methotrexate resistance. *Cancer Res* **45**: 6205–6212, 1985.
23. Rodenhuis S, McGuire JJ, Narayanan R and Bertino JR, Development of an assay system for the detection and classification of methotrexate resistance in fresh human leukemic cells. *Cancer Res* **46**: 6513–6519, 1986.
24. Norris MD, Haber M, Kavallaris M, Brian MJ, Lutze LH, White L and Stewart BW, Reduced drug accumulation as the mechanism of extreme clinical resistance to methotrexate in the human T-cell leukemia xenograft, LALW-2. *Cancer* **68**: 981–987, 1991.
25. Knight CB, Chabner BA and Elwood PC, The human folate receptor in KB cells is altered in transport mediated, acquired methotrexate resistance. *Blood* **74**: 244a, 1989.
26. Saikawa Y, Knight CB, Saikawa T, Page ST, Chabner BA and Elwood PC, Decreased expression of the human folate receptor mediates transport-defective methotrexate resistance in KB cells. *J Biol Chem* **268**: 5293–5301, 1993.
27. Hsueh C-T and Dolnick BJ, Folate-binding protein mRNA is decreased in methotrexate-resistant KB cells. *Oncol Res* **4**: 497–505, 1992.
28. Will CL and Dolnick BJ, 5-Fluorouracil inhibits dihydrofolate reductase precursor mRNA processing and/or nuclear mRNA stability in methotrexate-resistant KB cells. *J Biol Chem* **264**: 21413–21421, 1989.
29. Dolnick BJ, Zhang Z-G, Hines JD and Rustum YM, Quantitation of dihydrofolate reductase and thymidylate synthase mRNAs *in vivo* and *in vitro* by polymerase chain reaction. *Oncol Res* **4**: 65–72, 1992.
30. Nakajima-Iijima S, Hamada H, Reddy P and Kakunaga T, Molecular structure of the human cytoplasmic β-actin gene: Interspecies homology of sequences in the introns. *Proc Natl Acad Sci USA* **82**: 6133–6137, 1985.
31. Maley LM, *The Establishment of a Quantitative*

Polymerase Chain Reaction for Human β-Actin mRNA. M.S. Thesis, State University of New York at Buffalo, Buffalo, 1991.
32. Feinberg AP and Vogelstein B, A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal Biochem* **132**: 6–13, 1983.
33. Wang X, O'Hanlon TP and Lau JTY, Regulation of β-galactoside α2,6-sialylytransferase gene expression by dexamethasone. *J Biol Chem* **264**: 1854–1859, 1989.
34. Chirgwin JM, Przybyla AE, MacDonald RJ and Rutter WJ, Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**: 5294–5299, 1979.
35. Will CL and Dolnick BJ, 5-Fluorouracil augmentation of dihydrofolate reductase gene transcripts containing intervening sequences in methotrexate-resistant KB cells. *Mol Pharmacol* **29**: 643–648, 1986.
36. Sambrook J, Fritsch EF and Maniatis T, *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989.
37. Rosen KM and Villa-Komaroff L, An alternative method for visualization of RNA in formaldehyde agarose gels. *Focus* **12**: 23–24, 1990.
38. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA and Struhl K, *Current Protocols in Molecular Biology*. Greene Publishing Associates and Wiley-Interscience, New York, 1987.
39. O'Conner JL and Wade MF, Determination of coexisting nuclear transcription rates and cytoplasmic mRNA levels for gonadotropin subunit genes in rat anterior pituitary. *Biotechniques* **12**: 238–243, 1992.
40. Celano P, Berchtold C and Casero RA Jr, A simplification of the nuclear run-off transcription assay. *Biotechniques* **7**: 942–944, 1989.
41. Kafatos FC, Jones CW and Efstratiadis A, Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. *Nucleic Acids Res* **7**: 1541–1552, 1979.
42. Flaspohler JA and Milcarek C, Use of *in vitro*-transcribed RNAs as immobilized targets during RNA:RNA hybridization. *Biotechniques* **13**: 68–72, 1992.
43. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
44. Hsueh C-T and Dolnick BJ, Altered folate-binding protein mRNA stability in KB cells grown in folate-deficient medium. *Biochem Pharmacol* **45**: 2537–2545, 1993.
45. Reich E and Goldberg IH, Actinomycin and nucleic acid function. *Prog Nucleic Acid Res Mol Biol* **3**: 183–234, 1964.
46. Jones PA and Taylor SM, Cellular differentiation, cytidine analogs and DNA methylation. *Cell* **20**: 85–93, 1980.
47. Cedar H, DNA methylation and gene activity. *Cell* **53**: 3–4, 1988.
48. Bird A, The essentials of DNA methylation. *Cell* **70**: 5–8, 1992.
49. Strobl JS, A role for DNA methylation in vertebrate gene expression? *Mol Endocrinol* **4**: 181–183, 1990.
50. Nelson M and McClelland M, Effects of site-specific methylation on DNA modification methyltransferases and restriction endonucleases. *Nucleic Acids Res* **17** (Suppl): R389–R415, 1989.
51. Momparler RL, Bouchard J and Samson J, Induction of differentiation and inhibition of DNA methylation in HL-60 myeloid leukemic cells by 5-aza-2′-deoxycytidine. *Leuk Res* **9**: 1361–1366, 1985.
52. Nyce J, Gene silencing in mammalian cells by direct incorporation of electroporated 5-methyl-2′-deoxycytidine 5′-triphosphate. *Somat Cell Mol Genet* **17**: 543–550, 1991.
53. Bolden AH, Nalin CM, Ward CA, Poonian MS and Weissbach A, Primary DNA sequence determines sites of maintenance and *de novo* methylation by mammalian DNA methyltransferase. *Mol Cell Biol* **6**: 1135–1140, 1986.
54. Holliday R, The inheritance of epigenetic defects. *Science* **238**: 163–170, 1987.
55. Riggs AD and Jones PA, 5-Methylcytosine, gene regulation, and cancer. *Adv Cancer Res* **40**: 1–30, 1983.
56. Compere SJ and Palmiter RD, DNA methylation controls the inducibility of the mouse metallothionein-I gene in lymphoid cells. *Cell* **25**: 233–240, 1981.
57. Gloudemans T, Pospiech I, Van Der Ven LTM, Lips CJM, Schneid H, Den Otter W and Sussenbach JS, Expression and CpG methylation of the insulin-like growth factor II gene in human smooth muscle tumors. *Cancer Res* **52**: 6516–6521, 1992.
58. Boyes J and Bird A, DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. *Cell* **64**: 1123–1134, 1991.
59. Hsueh C-T, *Human Folate Receptor: Regulation of Gene Expression and Its Role in Mechanisms of Methotrexate Resistance*. Ph.D. Thesis, State University of New York at Buffalo, Buffalo, 1993.
60. Keshet I, Yisraeli J and Cedar H, Effect of regional DNA methylation on gene expression. *Proc Natl Acad Sci USA* **82**: 2560–2564, 1985.
61. Zhang Z-X, Kumar V, Rivera RT, Pasion SG, Chisholm J and Biswas DK, Suppression of prolactin gene expression in GH cells correlates with site-specific DNA methylation. *DNA* **8**: 605–613, 1989.
62. Klages S, Mollers B and Renkawitz R, The involvement of demethylation in the myeloid-specific function of the mouse M lysozyme gene downstream enhancer. *Nucleic Acids Res* **20**: 1925–1932, 1992.
63. Nyce J, Drug-induced DNA hypermethylation and drug resistance in human tumors. *Cancer Res* **49**: 5829–5836, 1989.
64. Nyce J, Liu L and Jones PA, Variable effects of DNA-synthesis inhibitors upon DNA methylation in mammalian cells. *Nucleic Acids Res* **14**: 4353–4367, 1986.
65. Antonsson BE, Avramis VI, Nyce J and Holcenberg JS, Effect of 5-azacytidine and congeners on DNA methylation and expression of deoxycytidine kinase in the human lymphoid cell lines CCRF/CEM/0 and CCRF/CEM/dCk^{-1}. *Cancer Res* **47**: 3672–3678, 1987.
66. Avramis VI, Mecum RA, Nyce J, Steele DA and Holcenberg JS, Pharmacodynamic and DNA methylation studies of high-dose 1-β-D-arabinofuranosyl cytosine before and after *in vivo* 5-azacytidine treatment in pediatric patients with refractory acute lymphocytic leukemia. *Cancer Chemother Pharmacol* **24**: 203–210, 1989.