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Impact of Extracellular Folate Levels on Global Gene Expression

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ABSTRACT

Methylation of DNA is associated with gene silencing. DNA methylation uses *S*-adenosylmethionine (SAM) as the methyl donor and the formation of SAM requires a continuous supply of folate from the extracellular milieu. Low extracellular folate levels are known to result in induction of expression of the human α folate receptor in nasopharyngeal epidermoid carcinoma cells. Low folate levels have been implicated in global activation of gene expression. We have investigated the impact of lowering the level of extracellular folate by performing cDNA microarray analysis of global gene expression in human nasopharyngeal carcinoma KB cells grown in folate-deplete and folate-replete medium. We found that expression of only eight genes reproducibly responded to variation of folate levels. Among those, three were up-regulated and five were down-regulated. Examination of one gene, H-cadherin, demonstrated down-regulation in response to folate depletion. Despite the low level of extracellular folate, there was hypermethylation of H-cadherin 5′ sequences. These data indicate that low extracellular folate positively and negatively influences the expression levels of a small cohort of genes. The data suggest that folate deficiency is associated with gene-specific methylation/demethylation, rather than global DNA demethylation and transcriptional activation.

Folates are essential vitamins. They participate in various biochemical reactions. The formation of purine and pyrimidine precursors for DNA and RNA synthesis, for example, requires folate cofactors (Wagner, 1985; Kane and Waxman, 1989; Henderson, 1990; Antony, 1996). Deficiency of folate can lead to serious clinical abnormalities. Megaloblastic anemia is one consequence of folate deficiency (Davis and Nicol, 1988). During pregnancy, the demand for folate increases, and folate deficiency in the mother before conception is thought to result in neural tube defects in the infant (Davis and Nicol, 1988). Deficiency in folate has also been implicated in cervical dysplasia (Butterworth et al., 1992; Glynn and Albanes, 1994). The biochemical processes affected by intracellular folate levels are currently being investigated, because knowledge of these mechanisms is important in understanding folate deficiency-related disease.

A multigene family of high-affinity folate binding proteins (FBP) designated the α, β, γ folate receptors are thought to be involved in intracellular folate transport (Wagner, 1985; Kane et al., 1988; Henderson, 1990; Antony, 1996). In cultured human nasopharyngeal carcinoma KB cells, expression of the human α folate receptor (αhFR) is inversely proportional to folate concentrations in the growth medium. When KB cells are continuously cultured in medium containing physiological folate concentrations (2–10 nM), the levels of receptor significantly increase compared with the receptor levels in cells maintained in standard Dulbecco's modified Eagle's medium (containing >2000 nM folic acid). This increase is accelerated when KB cells are passaged in very low folate medium containing <2 nM. Conversely, the addition of 100 nM 5-methyltetrahydrofolate (*N*-5 MTHF) to the growth medium prevents and reverses the increase in αhFR expression (Kane et al., 1988).

The mechanism(s) responsible for folate-mediated regulation of αhFR are unknown. No detectable differences are noted in the organization of the *αhFR* gene, or the size of its mRNA (Hsueh and Dolnick, 1993). However, αhFR protein levels (Kane et al., 1988) from folate-deficient or normal KB cells correlate well with mRNA levels (Sadasivan and Rothenberg, 1989). Regulation of *αhFR* gene expression by extracellular folate may therefore involve transcriptional and/or posttranscriptional controls. Altered methylation of *αhFR* gene sequences may represent one regulatory mechanism involved in folate-mediated regulation of αhFR. Methylation of DNA is known to be associated with gene silencing. Folates are directly involved in the formation of *S*-adenosylmethionine (SAM), the methyl donor of DNA methyltransferase (Chiang et al., 1996). Folate deficiency has been noted

ABBREVIATIONS: FBP, folate binding protein; αhFR, human α folate receptor; *N*-5 MTHF, 5-methyltetrahydrofolate; SAM, *S*-adenosylmethionine; KB-R, KB cells grown in standard media (containing >2000 nM folate); KB-D, KB cells grown in low folate media (containing 2–10 nM folate); PCR, polymerase chain reaction; SSII, Superscript II RT; RT, reverse transcription; bp, base pair(s); SAH, *S*-adenosylhomocysteine.

to decrease the levels of intracellular SAM in some systems (Balaghi and Wagner, 1993; Miller et al., 1994), suggesting that folate levels could influence DNA methylation and gene expression activities. Furthermore, depletion of intracellular SAM because of methyl deficiency has been demonstrated in vivo to decrease the overall methylation of DNA (Wainfan et al., 1989) and of specific genes including, *c-myc*, *c-fos*, and *Ha-ras* (Wu and Santi, 1987). It has been suggested that demethylation and enhanced expression of the specific genes examined may represent part of a process of general demethylation and transcriptional activation in response to methyl deficiency and lowered levels of intracellular SAM.

In the present study, we evaluated the impact of lowering extracellular folate levels on global gene expression in KB cells. We discovered the mRNA levels of only eight different genes were reproducibly affected by folate deficiency. Of particular interest was H-cadherin, a cell adhesion molecule whose gene expression is thought to be regulated by DNA methylation. H-cadherin mRNA levels were decreased by folate deficiency, and this effect was associated with increased methylation of H-cadherin 5′ gene sequences. Thus, a restricted number of genes respond to folate deprivation and the response is not limited to DNA demethylation and enhanced gene expression.

Materials and Methods

Cell Culture. Wild-type human nasopharyngeal epidermoid carcinoma KB cells were obtained from the American Type Culture Collection (Manassas, VA). KB cells were maintained in Dulbecco's modified Eagle's medium with folic acid (folate replete, KB-R) or without folic acid (folate deplete, KB-D) containing L-glutamine, Earl's salts, and 10% fetal bovine serum (Biofluid Inc., Rockville, MD) at 37°C at 5% CO_2. Both cell lines were cultured in the same manner, asynchronous and continuously proliferating.

Northern Analysis. Samples containing 20 μg of total cellular RNA were resolved on a 1% agarose/0.66 M formaldehyde/0.023 3-(*N*-morpholino) propansulfonic acid gel. The RNAs were transferred to a nitrocellulose membrane (Portran; Schleicher and Schuell, Keene, NH) and then hybridized to a random ^{32}P-labeled αhFR cDNA probe (Promega, Madison, WI).

Western Analysis. Samples containing 100 μg of total cellular protein were electrophoresed on a 10% SDS-polyacrylamide gel and electroblotted onto a nitrocellulose membrane (Portran; Schleicher and Schuell, Keene, NH). Detection of αhFR was accomplished using polyclonal rabbit anti-αhFR antibody as described previously (Chung et al., 1993).

Nuclear Run On. Preparation of nuclei and nuclear run on reactions were performed according to standard procedures (Ausubel et al., 1997). Newly synthesized ^{32}P-labeled transcripts were hybridized to nitrocellulose filters containing 0.5, 1.0, or 2.5 μg of pGEM, β-actin and αhFR plasmid DNA. The abundance of each transcript was determined using Molecular Dynamics (Sunnyvale, CA) PhosphorImager analysis software.

Differential Display. Samples containing 0.25 μg of total RNA were reverse transcribed and PCR amplified with three sets of arbitrary 10 mers using the RNAmap kit (GenHunter Corporation, Brookline, MA). The amplified cDNAs were electrophoresed on 6% DNA sequencing gel and visualized by PhosphorImager.

Microarray Analysis. Human Oncochip cDNA arrays (National Cancer Institute Microarray Facility, Bethesda, MD) were manufactured as described by Eisen and Brown (1999). Arrays contained approximately 2200 elements, 2008 of which represented nonredundant named genes. A complete list of genes is available at http://nciarray.nci.nih.gov. Briefly, total RNA was combined with 1× Superscript II RT (SSII) reaction buffer (Invitrogen, Carlsbad, CA), 4 μg of oligo dT, 5 mM dNTP mix, 0.1 mM Cy3 or Cy5 dUTP, 10 mM dithiothreitol, and 20 to 40 units of RNAsin (Promega). The mixture was incubated at 65°C for 5 min and transferred to 42°C. The reaction was initiated by the addition of 400 units of SSII and incubated at 42°C for 25 min. Four hundred units of SSII were added a second time for 35 min at 42°C. The reaction was terminated by 50 mM EDTA. Residual RNA was hydrolyzed by 0.2 M NaOH at 65°C for 60 min. The reaction was cooled to room temperature and neutralized by one half volume of 1 M Tris-HCL, pH 7.5. Probes were cleaned using Microcon YM-30 spin columns (Amicon, Bedford, MA) and hybridized to Human Oncochip cDNA arrays at 65°C overnight. Arrays were washed repeatedly and analyzed using an Avalanche scanner (Molecular Dynamics) and ArraySuite Microarray Analysis software package (National Human Genome Research Institute, Bethesda, MD). Results of the microarray analysis represent a compilation of four independent experiments: 1) duplicates of Cy3-labeled KB-R and Cy5-labeled KB-D cDNA, and 2) reciprocal duplicates of Cy5-labeled KB-R and Cy3-labeled KB-D cDNA.

Real-Time Quantitative RT-PCR. First strand synthesis of cDNA was accomplished using the Taqman RT kit (Applied Biosystems, Foster City, CA). The reaction was inactivated by heating at 95°C for 5 min and diluted 5-fold. Primers for PCR amplification were designed using Primer Express software (Applied Biosystems). One tenth of the final RT reaction volume was combined with H-cadherin forward primers (H-cad1568F, 5′-GCTATGGAAACTTGGGAGTCA-3′; H-cad715F, 5′-TGATGACAGGTGCAGTTGTACATTTA-3′) and reverse primers (H-cad1624R, 5′-GCTCCTCAGCCTCTTCAGCTT-3′; H-cad789R, 5′-GTCCCGAATCCACAGTCGTACT-3′) and Sybr Green PCR master mix (Applied Biosystems). PCR amplification and detection of fluorescence incorporation was done using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) according to manufacturer's instructions. The C_T parameter is defined as the fractional cycle number at which the reporter fluorescence generated by binding of SYBR Green I dye onto double-stranded DNA passes a fixed threshold above the baseline. The C_T values were determined by Sequence Detection Analysis Software (Applied Biosystems). The relative change in gene expression was calculated ($2^{\Delta CT}$) and results were normalized to β-actin gene expression.

Southern Blot Analysis. Samples containing 30 μg of genomic DNA were exhaustively digested with *Hpa*II or *Msp*I restriction endonucleases. Southern blot analysis was accomplished as described previously (Ausubel et al., 1997). DNAs transferred to nylon membranes were hybridized to a 1700-bp H-cadherin EST (American Type Culture Collection) labeled with [α-^{32}P]dCTP and [α-^{32}P]dGTP by the random primer method (Promega). *Hpa*II cleavage products (indicated by arrowheads in Fig. 5B) were quantitated using Scion Image Software (Scion Corp, Frederick, MD).

Sodium Bisulfite Sequencing. Modification of genomic DNA with sodium bisulfite was accomplished according to the methods of Kawakami et al. (Kawakami et al., 1999). The methyl-specific primers used for H-cadherin were designed to amplify fully modified DNA. (H-cad-MF, 5′-TTGGTTGGCGAGGTAGAGTTTT-3′; H-cad-MR: 5′-ACGCCCGACGACGTTTT-3′).

Measurement of SAM and *S*-Adenosyl Homocysteine. KB-R and KB-D cells were washed with cold phosphate-buffered saline, scraped from the dish, and pelleted. The pellets were suspended in 400 μl of cold 10% trichloroacetic acid and centrifuged. The trichloroacetic acid supernatants were used to measure SAM and *S*-adenosyl homocysteine (SAH) by a high-performance liquid chromatography method using two columns to resolve the respective peaks (Capdevila and Wagner, 1998).

Results

Folate-Mediated *αhFR* Gene Expression Is Regulated at the Level of mRNA Synthesis. KB cells were continuously cultured in either low folate (KB-D, containing

2–10 nM folate) or standard (KB-R, containing 2000 nM folate) growth media. In agreement with previous reports (McHugh and Cheng, 1979; Luhrs et al., 1986; Kane et al., 1988), we show that αhFR protein (Fig. 1A) and mRNA (Fig. 1B) levels were elevated by folate deprivation. Because protein and mRNA levels are correlated, the folate-mediated increase of *αhFR* gene expression may involve transcriptional and/or posttranscriptional regulation controls. To further investigate this issue, nuclear run-on assays were performed. Consistent with Western and Northern analyses, the rate of newly synthesized mRNA also increased approximately 5-fold in KB-D cells, relative to KB-R (Fig. 1C). These results strongly suggest that transcriptional mechanisms are involved in folate-mediated expression of αhFR.

Differential Display Analysis of the Influence of Extracellular Folate Levels on Gene Expression. The effect of folate concentrations on the expression of αhFR led us to investigate whether or not the expression of other genes is influenced by extracellular folate concentrations. Differential display analysis (Fig. 2A) detected increased and de novo expression of several PCR-amplified cDNA fragments (arrows) in KB-D compared with KB-R cells. The levels of folate may therefore influence regulatory mechanisms common to numerous genes in KB cells. Gene regulation by DNA methylation may represent one such mechanism. Folates are directly involved in the formation of SAM, the methyl donor of DNA methyltransferase. *N*-5 MTHF is the cofactor requirement for the methylation of homocysteine to generate methionine, the immediate precursor of SAM (Fig. 2B). The intracellular levels of SAM and its unmethylated counterpart SAH are thought to be important in regulating methyl transfer reactions. SAH is reported to be a competitive inhibitor of bacterial DNA methyltransferases, with respect to SAM (Wu and Santi, 1987). Therefore, the amounts of intracellular SAM and SAH could influence the overall levels of DNA methylation.

Folate Depletion Alters Intracellular Levels of SAM and SAH. To examine the effect of folate depletion on intracellular levels of SAM and SAH, we measured the amounts of SAM and SAH in KB-R and KB-D cells using a two-column high-performance liquid chromatography method as described under *Experimental Procedures*. As expected, Table 1 shows that SAH levels were almost 2-fold higher in KB-D compared with KB-R cells. The SAM levels increased by 1.7-fold. This resulted in a SAM/SAH ratio of 10.2 in KB-R versus 7.4 in KB-D cells.

The *αhFR* Gene Sequences Do Not Encompass a Classical CpG Island. In human cells, the CpG dinucleotide is normally under-represented. However, within the genome, there are CpG-rich clusters, called CpG islands, in which the density of CpG dinucleotides is similar to that of the GpCs, a non–under-represented dinucleotide (Tykocinski

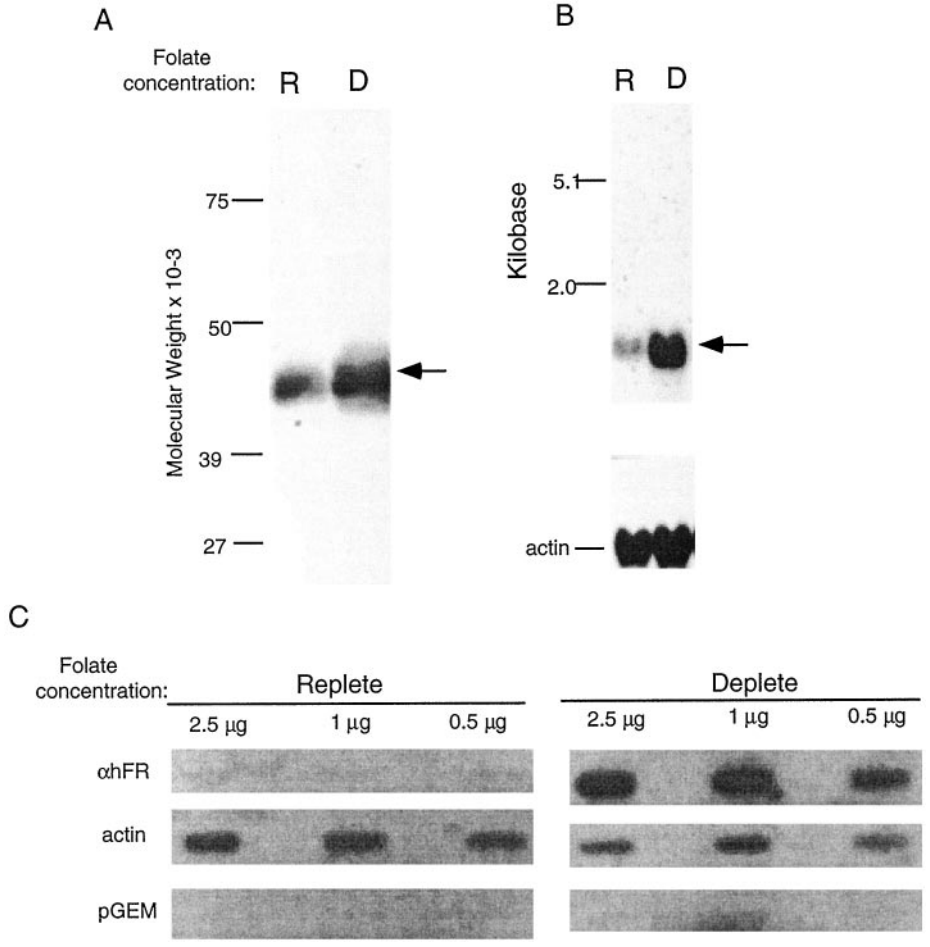

Fig. 1. Western (A), Northern (B), and nuclear run-on analyses (C) of KB-R and KB-D cells was accomplished as described under *Materials and Methods*. Arrows indicate increased levels of αhFR protein and mRNA in KB-D cells.

and Max, 1984). Expression of genes containing CpG islands within their 5′ region is known to be decreased or completely silenced by methylation of their CpG island (Bird, 1986). To test whether or not DNA methylation plays a role in folate-mediated gene expression, we decided to investigate the methylation status of folate-regulated genes that contain CpG islands. We determined the frequency of CpG dinucleotides within the *αhFR* gene sequences. A CpG/GpC map of the *αhFR* complete gene sequence indicates no CpG island (Fig. 3). These results suggest that the mechanism by which folate regulates αhFR expression may not involve CpG methylation directly. Perhaps the gene products of other factors that are regulated by folate/DNA methylation influence the expression levels of αhFR.

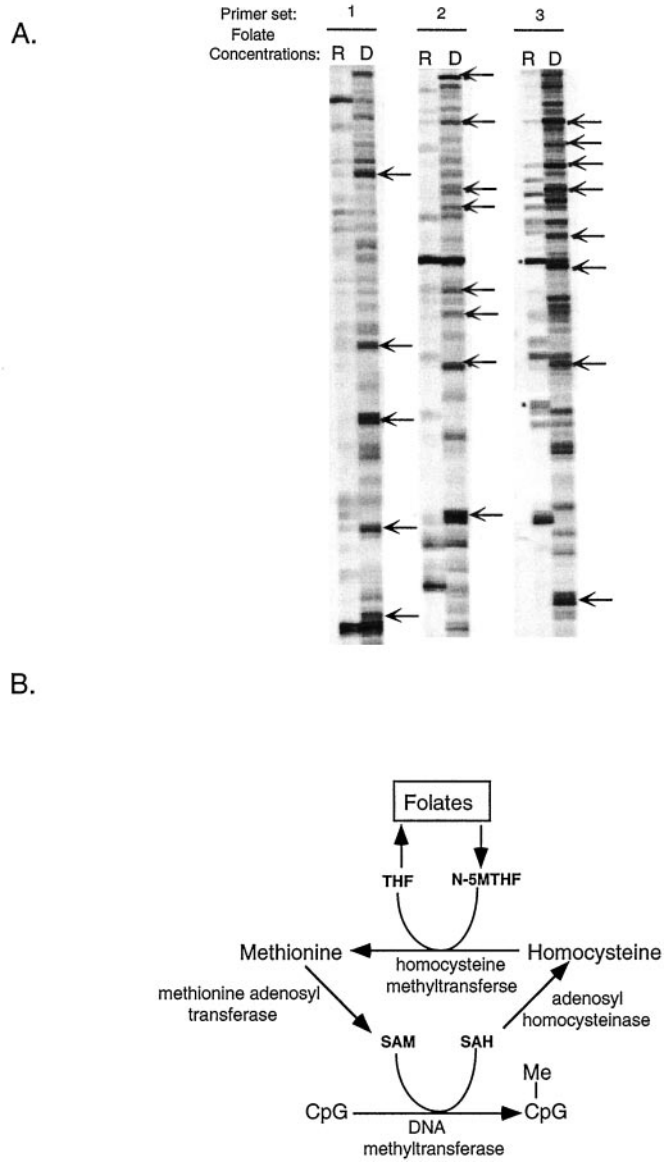

Fig. 2. A, differential display analysis of KB-R and KB-D cells. Differential display, using three unique amplimer sets (1, 2, 3), was accomplished using the GenHunter kit (Brookline, MA), according to manufacturer's instructions. Arrows indicate increased and unique mRNAs in KB-D cells. B, DNA methylation utilizes SAM, a metabolic product of folate metabolism. *N*-5 MTHF is the cofactor requirement for the methylation of homocysteine to generate methionine, the immediate precursor of SAM.

TABLE 1

Measurement of intracellular SAM and SAH

KB-R and KB-D cells were analyzed for intracellular SAM and SAH levels using two-column high-performance liquid chromatography as described under *Materials and Methods.*

Cells	SAM	SAH	SAM/SAH
	pmol/10^6 cells		
KB-R	896.9	87.7	10.2
KB-D	1150.0	154.8	7.4

Low Extracellular Folate Both Positively and Negatively Influence the Levels of Genes Expressed. The differential display analysis suggested that extracellular folate levels may have a general influence on gene expression, including those factors that regulate αhFR. To test for a global impact of extracellular folate on gene transcription and to identify the genes regulated, a microarray analysis was performed using RNA isolated from KB-D and KB-R cells. The mRNA levels of only eight different genes were altered in a highly reproducible manner by folate depletion (Table 2). Three genes were up-regulated and five were down-regulated. We were interested in H-cadherin, a protein related to the superfamily of cell adhesion molecules; its expression has been shown to be significantly reduced in human breast carcinoma cell lines and breast cancer specimens (Lee, 1996; Lee et al., 1998). Furthermore, in human lung cancer cell lines, loss of H-cadherin expression was accompanied by hypermethylation at the 5′ region of this gene (Sato et al., 1998). In contrast to the effect of folate levels on *αhFR* gene expression, microarray analysis detected a decrease in H-cadherin mRNA levels by approximately 2.5-fold in KB-D versus KB-R cells. This result was confirmed by real time quantitative RT-PCR (Fig. 4) using primer sets targeted to two different locations (715/789 bp and 1568/1624 bp) within the H-cadherin open reading frame.

H-Cadherin Contains a 5′ CpG Island That Is Differentially Methylated in KB-D and KB-R Cells. Because H-cadherin is known to be down-regulated by DNA methylation in other systems (Sato et al., 1998), we wondered whether decreased H-cadherin expression by low extracellular folate involved hypermethylation of H-cadherin gene sequences in KB cells. To investigate this issue, we determined whether methylation of CpG dinucleotides within the 5′ region H-cadherin was increased in KB-D versus KB-R cells. A CpG/GpC map of the H-cadherin gene indicates the presence of a CpG island that encompasses the first exon and its immediate 5′ sequences (Fig. 5A). Differential methylation of H-cadherin CpG dinucleotides was examined by the *Hpa*II methyl-sensitive restriction enzyme, which selectively hydrolyses nonmethylated DNA sequences, and its isoschizomer *Msp*I, which cleaves DNA regardless of methylation status. Compared with KB-R cells, Fig. 5B shows decreased cleavage of H-cadherin sequences by *Hpa*II in KB-D cells (indicated by the arrowhead). The intensity of the cleavage product decreased by about 7.4-fold as determined by Scion Image software.

Quantitative Analysis of Methylated CpGs within the H-Cadherin CpG Island. To quantitatively access the levels of CpG methylation of H-cadherin in KB-R and KB-D cells, we performed real-time PCR using methyl-specific primers and sodium bisulfite-treated genomic DNA. As

shown in Fig. 6A, sodium bisulfite treatment of genomic DNA selectively converts nonmethylated cytosines to uracil residues, whereas methylated cytosines remain nonreactive (Rein et al., 1998). Figure 6B indicates a 40% increase of H-cadherin CpG methylation in KB-D compared with KB-R cells. Taken together, these results strongly suggest that decreased expression of H-cadherin by intracellular folate deficiency is associated with hypermethylation of the CpG island.

Discussion

Regulation of αhFR has been studied extensively in the KB cell line. KB cells express high levels of the αhFR relative to human tissue. Previous reports indicate that the concentration of extracellular folate contributes to the levels of αhFR in these cells. Expression of the αhFR increases (about 5- to 10-fold) when KB cells are maintained in media containing low folate (<10 nM) compared with cells cultured in standard media (containing 2000 nM

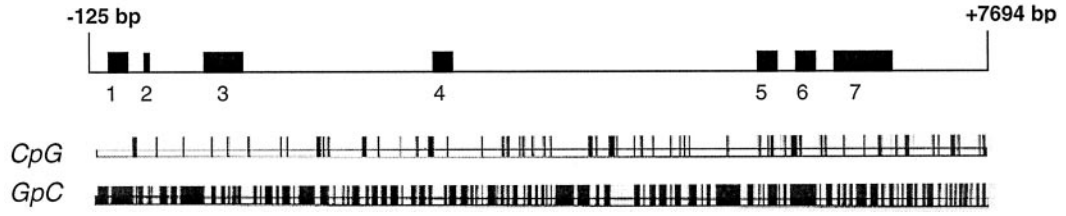

Fig. 3. A CpG island can be defined as a 5′ CG-rich region in which the frequencies of CpG and GpC sequences are approximately equal. A CpG/GpC map of *αhFR* gene sequences indicates no CpG island. The solid boxes in the αhFR gene structure diagram represent exons 1 to 7.

TABLE 2

Microarray analysis

Total RNA derived from KB-D and KB-R cells was labeled with Cy3 or Cy5 fluorescent nucleotide analogs and then hybridized to Human Oncochip cDNA array (National Cancer Institute, Bethesda, MD), as described under *Materials and Methods.* Results are a compilation of four independent experiments: duplicates of Cy3-labeled KB-R and Cy5-labeled KB-D cDNA, and their reciprocal duplicates. Arrows indicate the detected change in mRNA levels in KB-D cells relative to KB-R cells.

Identified Gene	Description	Average Fold δ Expression
Brain-expressed HHCPA78 homolog	A human 1α,25-dihydroxy-vitamin D3 hydroxylase	↑ 4.3 (± 0.83)
Keratin type 1 cytoskeleton 14	Cytoskeleton protein	↓ 5.1 (± 0.54)
Calmegin	Putative testis-specific chaperon.	↑ 2.5 (± 0.32)
Cyclin D3	Promotes progression through the G_1 phase of the cell cycle.	↓ 2.5 (± 0.19)
H-cadherin	Cell adhesion molecule	↓ 2.5 (± 0.40)
Gravin	A kinase scaffold protein	↓ 3.0 (± 1.28)
Insulin-induced protein 1	Expressed in proliferating liver during regeneration and development.	↑ 2.5 (± 0.11)
Interferon-inducible protein 1-8U	Laminin receptor homolog	↓ 2.3 (± 0.18)

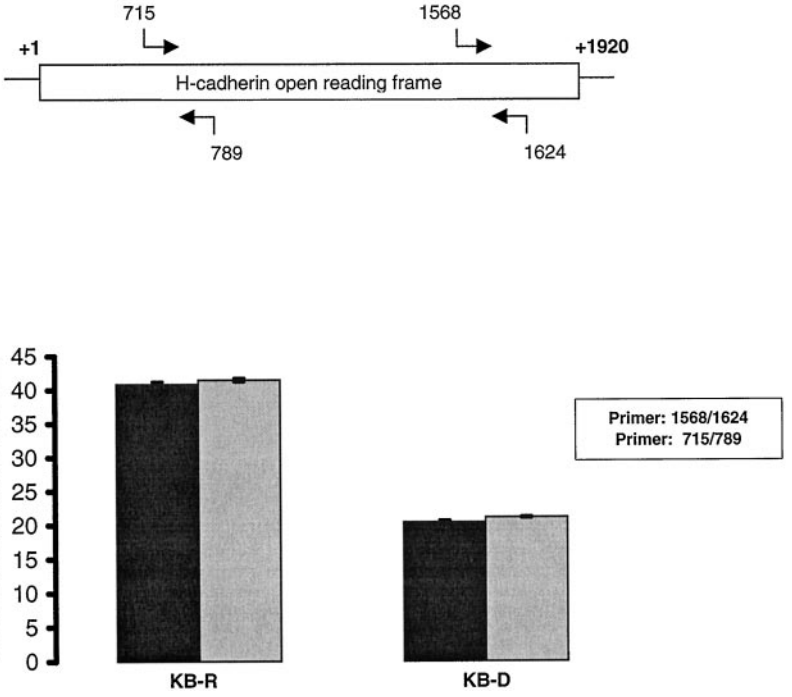

Fig. 4. Relative amounts of H-cadherin mRNA levels in KB-R and KB-D cells, as estimated by real time quantitative RT-PCR. A 2.5-fold decrease of H-cadherin mRNA in KB-D compared with KB-R cells was noted using two primer sets (primers 1568/1624, dark shade; primers 715/789, light shade) targeted to separate regions of H-cadherin open reading frame sequences.

folate) (McHugh and Cheng, 1979; Luhrs et al., 1986; Kane et al., 1988; Sadasivan and Rothenberg, 1989). Our results agree. We showed an increase in αhFR protein and mRNA levels in KB-D relative to KB-R cells (Fig. 1). Furthermore, the rate of αhFR mRNA synthesis also increased in KB-D cells by approximately 5-fold (Fig. 1C). However, this observation contrasts with that of Hsueh and Dolnick (1993), where no differences in the rate of αhFR mRNA synthesis were found. Instead, they reported that increased mRNA stability of αhFR mRNA contributes to increased αhFR levels in folate deficient KB cells. They showed that the half-life of αhFR mRNA under low folate conditions was increased by approximately 2.5-fold, whereas the steady-state mRNA levels increased by approximately 5- to 10-fold. Although differences in mRNA stability may account for a portion of the αhFR increase in folate-depleted KB cells, other regulatory mechanisms must be involved to explain fully the folate-mediated induction of αhFR in KB cells.

Regulation of folate-induced gene expression by DNA methylation is one plausible explanation. Folates are directly involved in the synthesis of SAM, the methyl donor of DNA methyltransferase (Fig. 2B). Others have demonstrated that extracellular folate concentrations can influence the levels of intracellular SAM in some systems (Miller et al., 1994) and that depletion of SAM by methyl deficiency, which includes choline, folic acid, methionine, and vitamin B_{12}, decreases the overall methylation of DNA (Wainfan et al., 1989) and of specific genes (Wu and Santi, 1987) in vivo. In the current study, folate deficiency in KB cells was achieved by continuous culture in low folate medium. Unlike previous reports of methyl-deficiency, the intracellular levels of SAM and SAH increased in KB-D compared with KB-R model cell lines (Table 1).

Folates are needed for the methylation of homocysteine to generate methionine, the immediate precursor of SAM. A deficiency in folate would increase the levels of homocysteine and reverse the SAH hydrolase reaction, leading to elevated levels of SAH. SAH has been reported to be a competitive inhibitor of bacterial DNA methyltransferase, with respect to SAM (Wu and Santi, 1987). A decrease of DNA methylation reactions caused by elevated SAH would result in increased levels of free intracellular SAM. Furthermore, compared with the ratio of SAM to SAH, the absolute levels of SAH are thought to be a more important indicator of whether methylation reactions are inhibited, (Capdevila et al., 1997). Table 1 shows a small change in the SAM/SAH ratio in KB-D (7.4) verses KB-R (10.2) cells. However, the increase in SAH in KB-D compared with KB-R cells was approximately 2-fold. For these reasons, an increase in the levels of both intracellular SAM and SAH would be an expected consequence of physiologic folate deficiency.

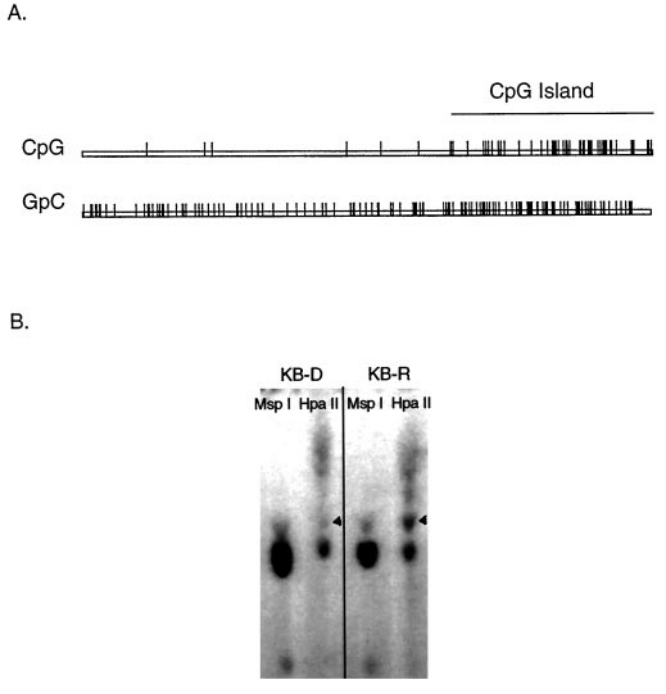

Fig. 5. A, a CpG/GpC map of H-cadherin 5′ sequences reveals a CpG island encompassing the first exon and intron and proximal 5′ sequences. B, methyl-sensitive restriction enzyme analysis of H-cadherin genomic sequences in KB-R and KB-D cells. Genomic DNA derived from KB-R and KB-D cells was cleaved exhaustively with methyl-sensitive *Hpa*II and its methyl-insensitive isoschizomer *Msp*I. Cleavage products were detected using a ^{32}P-labeled H-cadherin EST (American Type Culture Collection), as described under *Materials and Methods*. The arrowheads indicate decreased *Hpa*II cleavage in KB-D compared with KB-R cells.

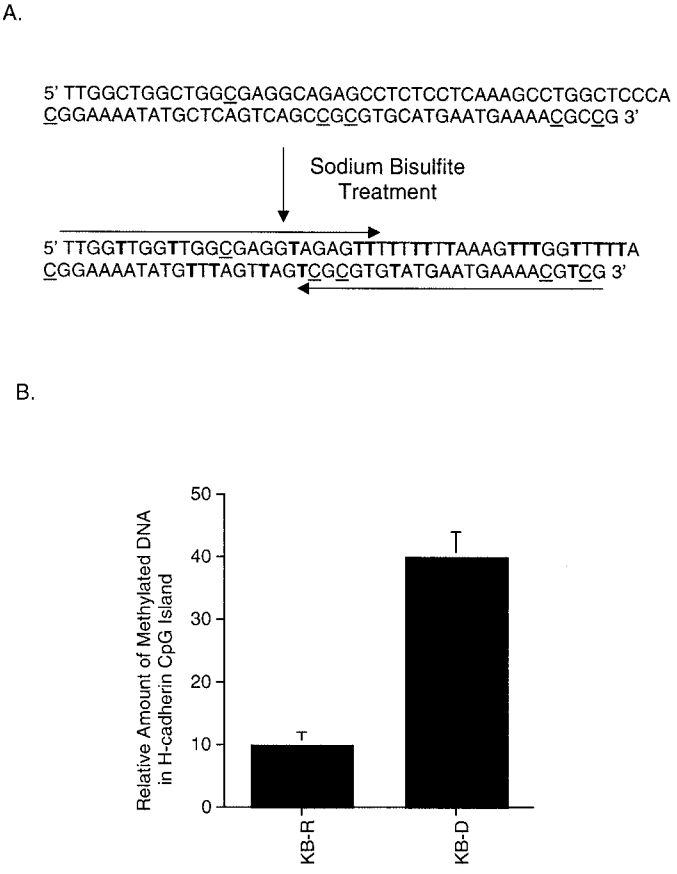

Fig. 6. Measurement of methylated CpGs in KB-R and KB-D cells using real-time quantitative PCR. Sodium bisulfite treatment of genomic DNA selectively converts nonmethylated cytosines to uracil residues, whereas methylated cytosines are not converted. A, a region within the H-cadherin CpG island was amplified using methyl-CpG-specific PCR primers (arrow) and sodium bisulfite-treated genomic DNA from KB-R and KB-D cells. The methylated Cs are underlined and the converted Cs are depicted as Ts in bold type. B, quantitative real-time PCR was used to determine the level of methylated CpG sequences in KB-R and KB-D cells. Results are a compilation of three independent RT-PCR reactions and have been normalized to β-actin gene expression.

The differential display analysis in the present study supports the hypothesis that folate deficiency induces global DNA hypomethylation, thereby increasing the transcriptional activity of genes whose expression is normally suppressed by DNA methylation. We show increased and de novo expression of numerous PCR-amplified cDNA fragments using three different amplimer sets (Fig. 2A). It is possible that these transcripts represent demethylated genes, including genes for which expression is not normally present in these cells or required for cell survival in culture.

We questioned whether or not αhFR induction by folate deficiency involved DNA methylation. In human cells, methylation of DNA normally occurs at the 5 position of cytosines that reside 5′ to guanines. Expression of genes containing 5′ CG-rich clusters, called CpG islands, is known to be decreased or completely silenced by CpG methylation (Tykocinski and Max, 1984; Bird, 1986). By constructing a CpG map, we determined that the *αhFR* gene sequences do not encompasses a CpG island (Fig. 3). Folate regulation of αhFR may not involve CpG methylation directly; however, extracellular folate levels and CpG methylation may influence the gene products of factors that regulate αhFR. Alternatively, mechanisms that do not involve DNA methylation may play a role in folate-mediated regulation of αhFR. These may include mechanisms that are involved in end-product feedback regulation of folate homeostasis. Such a system has been noted in the cholesterol-induced activation of sterol regulatory element-binding proteins (Nohturfft et al., 2000).

The present study examined the impact of extracellular folate levels on global gene expression by microarray analysis. We identified only eight genes for which mRNA levels were reproducibly different in KB-R and KB-D cells. Three genes were up-regulated and five were down-regulated (Table 2). It is unexpected that deficiency in the extracellular levels of folate would effect the expression of only a small cohort of genes. Folates serve as cofactors for numerous biochemical processes, including DNA synthesis and methyl transfer reactions. In the present study, we have sampled the expression of only 2008 genes and have used a cell line with its own unique genetic characteristics. A more accurate reflection of the effect of extracellular folate levels on gene expression might entail higher density cDNA chips and examination of additional cell types.

We determined that H-cadherin mRNA levels decreased by approximately 2.5-fold in KB-D, compared with KB-R cells (Fig. 4). We examined whether this was associated with increased methylation, because previous reports suggest that loss of H-cadherin activity may be caused by hypermethylation of its 5′ GC-rich sequences (Sato et al., 1998). We discovered that folate deficiency produced the same effect. Southern blot analysis showed decrease cleavage of genomic DNA from KB-D, compared with KB-R cells, by *Hpa*II, a methyl-sensitive restriction enzyme. Additionally, quantitative real-time PCR analysis using methyl-specific primers and sodium bisulfite-treated genomic DNA showed a significant increase (40%) in CpG methylation of H-cadherin sequences in KB-D cells (Fig. 6B).

It is paradoxical that a decrease in folate, the cofactor required for the synthesis of SAM, would induce DNA hypermethylation. The data demonstrate the gene-specific nature of response to low extracellular folate. H-cadherin is one member of a large family of transmembrane glycoproteins that mediates cell-cell adhesion and maintains normal tissue architecture (Nagafuchi and Takeichi, 1989; Takeichi, 1991,1993; Stappert and Kembler, 1993). It is now clear that cadherin dysfunction is implicated in tumor development (Nagafuchi and Takeichi, 1989, 1993; Takeichi, 1991; Stappert and Kembler, 1993). Repression of H-cadherin under folate-deficient conditions may be an effector of the malignant phenotype that has been associated with low folate. Future studies are required to determine whether regulation of the transcription program by folate plays a role in malignant transformation.

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