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# Hypermethylation of the human proton-coupled folate transporter (SLC46A1) minimal trancriptional regulatory region in an antifolate-resistant HeLa cell line

Ndeye Khady Diop-Bove,[1] Julia Wu,[2] Rongbao Zhao,[1,2] Joseph Locker,[3] and I. David Goldman[1,2]

Departments of [1]Molecular Pharmacology, [2]Medicine, and [3]Pathology, Albert Einstein College of Medicine, Bronx, New York

## Abstract

**This laboratory recently identified a novel proton-coupled folate transporter (PCFT) that mediates intestinal folate absorption and transport of folates into the central nervous system. The present study focuses on the definition of the minimum transcriptional regulatory region of this gene in HeLa cells and the mechanism(s) underlying the loss of PCFT expression in the methotrexate-resistant HeLa R1-11 cell line. The *PCFT* transcriptional regulatory controls were localized between −42 and + 96 bases from the transcriptional start site using a luciferase-reporter gene system. The promoter is a G + C rich region of 139 nucleotides contained in a CpG island. HeLa R1-11 cells have no mutations in the *PCFT* open reading frame and its promoter; the transcription/translation machinery is intact because transient transfections in HeLa R1-11 and wild-type HeLa cells produced similar luciferase activities. Hypermethylation at CpG sites within the minimal transcriptional regulatory region was shown in HeLa R1-11 cells as compared with the parental PCFT-competent HeLa cells, using bisulfite conversion and sequence analysis. Treatment with 5-aza-2′-deoxycytidine resulted in a substantial restoration of transport and PCFT mRNA expression and small but significant decreases in methylation in the promoter region. *In vitro* methylation of the transfected reporter plasmid inhibited luciferase gene expression. Cytogenetics/fluorescence *in situ* hybridization indicated a loss of half the *PCFT* gene copies in HeLa R1-11 as compared with PCFT-competent HeLa cells. Taken together, promoter silencing through methylation and gene copy loss accounted for the loss of PCFT activity in antifolate-resistant HeLa R1-11 cells. [Mol Cancer Ther 2009;8(8):2424–31]**

## Introduction

Membrane transport of antifolates has been recognized as an important determinant of the activity of this class of agents. Traditionally, these studies have focused on methotrexate (MTX), until recently the only antifolate approved for the treatment of cancer in this country (1). Membrane transport of MTX into tumor cells is mediated almost exclusively by the reduced folate carrier (RFC), an anion exchanger and member of the superfamily of solute transporters (SLC19A1). When RFC is mutated or silenced, there is marked resistance to MTX (2). However, there had been evidence suggesting the presence of another pathway for MTX distinct from RFC in normal tissues and cancer cell lines, a folate/antifolate transport activity with a low-pH optimum (3–5). This RFC–independent mechanism was shown to have a low-pH optimum, unlike the neutral-pH optimum of RFC, and a very high affinity for pemetrexed (6).

Recently, this laboratory identified the molecular basis for this low-pH transport pathway with the cloning of the proton-coupled folate transporter (*PCFT*; SLC46A1), that reproduces all the properties of the low-pH folate transport activity recognized in mammalian cells (7, 8). From the physiologic perspective, PCFT plays a critical role in the transport of folates across the apical brush-border membrane of the proximal jejunum, wherein there is an acid microclimate (9), and in the transport of folates into the central nervous system. This was established when it was shown that there are loss-of-function mutations in this protein in the autosomal recessive disorder hereditary folate malabsorption (7, 10, 11), in which both processes are markedly impaired (12). Hence, PCFT is required for folate homeostasis and folate sufficiency in man. From an epidemiologic perspective and from studies in mouse models, folate deficiency is a risk factor for the development of colorectal and possibly other cancers (13–15). Conversely, there is evidence that folate excess may enhance tumor growth and progression once a malignancy is established (16).

Evidence is also emerging pointing to the role PCFT plays in the pharmacology of pemetrexed and potentially other antifolates. Transfection of *PCFT* in cells, to levels that approximate its constitutive expression in human tumors, potentiates the pharmacologic activity of pemetrexed (17). In a HeLa cell line (R5), in which RFC was deleted from the genome by chemical mutagenesis and MTX selective

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**Requests for reprints:** I. David Goldman, Departments of Medicine and Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461. Phone: 718-430-2302; Fax: 718-430-8550. E-mail: igoldman@aecom.yu.edu



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pressure, growth inhibition by pemetrexed was fully preserved because it was transport mediated by PCFT (5). However, these cells were resistant to MTX and highly resistant to ZD1694 and PT523. When, in addition, *PCFT* was silenced under a second round of MTX selective pressure, the HeLa R1 cell line emerged in which there was resistance to all these antifolates (18). The antitumor effects of PCFT are likely to be greater and have a broader spectrum of effectiveness against solid tumors *in vivo*, wherein the drug interacts with malignant cells in a hypoxic acidic environment (19, 20), in which the activity of PCFT is enhanced and the activity of RFC is diminished for all antifolates.

Because of the physiologic and pharmacologic importance of *PCFT*, the basis for its regulation and the mechanisms by which it might be silenced in tumor cells is of considerable importance. The current study addresses the identification and characterization of the minimal transcriptional regulatory region of the *PCFT* gene and the mechanisms by which the expression of *PCFT* was silenced in the HeLa R1 antifolate-resistant cell line.

## Materials and Methods

### Materials

Tritiated MTX disodium salt, [3′,5′,7-$^3$H(N)]MTX ([$^3$H]MTX), was obtained from Moravek Biochemicals and purified by liquid chromatography (21).

### Cells and Culture Conditions

HeLa cells (cervical epitheloid carcinoma; RFC and PCFT competent), HeLa-derived R5 cells (RFC deleted; ref. 5), and HeLa R1-11 cells, a clonal derivative of the HeLa R1 cell line obtained from HeLa R5 cells by MTX selective pressure (RFC and PCFT deficient; refs. 17, 18), were maintained in monolayer culture at 37°C under 5% $CO_2$ in RPMI-1640 medium containing 2.3 μmol/L folic acid and supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. HeLa R1-11 cells have a stable phenotype and retain resistance to MTX after >3 mo in MTX-free medium (17).

### Transient Transfections and Luciferase Reporter Gene Activity

Human genomic DNA contained in the BAC Clone RP11-348E14 (Children's Hospital Oakland Research Institute BACPAC Resource Center) was used as a template to amplify nine fragments ($F_1$–$F_9$) of different lengths in the 5′ upstream region of PCFT. $F_1$ contains the segment −8/+96; $F_2$, −42/+96; $F_3$, −66/+96; $F_4$, −101/+96; $F_5$, −161/+96; $F_6$, −265/+96; $F_7$, −505/+96; $F_8$, −1,511/+96; and $F_9$, −2,005/+96. The numbering is relative to the transcriptional start site "A," which is based on database analysis (Fig. 2). The sequences for all the sense primers contain the *Kpn*I restriction site ($F_1$–$F_9$) and are as follows: 5′-cggggtaccaggcgcagacagcgcaagccc-3′, 5′-cggggtacccccgccggacatttaaggag-3′, 5′-cggggtaccggtggcctcaggtcacaggc-3′, 5′-cggggtacccacgcccagccaggtgcacc-3′, 5′-cggggtacctacgcacactttacaggtgag-3′, 5′-cggggtaccataccgtgcccagcacatagtaag-3′, 5′-cggggtaccatgccgaaggtagtggcagagcct-3′, 5′-cggggtacctcagctgctctgttctcaggaag-3′, and 5′-cggggtaccgagttagaaaagacctctaccctag-3′, respectively. Two antisense primers were used containing either *Xho*I or *Bgl*II restrictions sites: 5′-ccgctcgaggtgcgtgcgcggcggagctgtcg -3′ or 5′- ggaagatctgtgcgtgcgcggcggagctgtcg-3′. Amplimers were digested with *Kpn*I and *Xho*I or *Bgl*II, and subcloned into the multiple cloning sites of the promoterless pGL3- Basic vector (Promega), which contains the firefly luciferase gene. Clones were verified on an ABI 3730 DNA sequencer (Applied Biosystems) at the Albert Einstein Cancer Center Genomics Shared Resource using RVprimer3 and GLprimer2 (Promega). Reporter activity was assayed in HeLa cells by transient transfection using Lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol. The pGL3-Basic (promoterless) plasmid and the pGL3-SV40 promoter plasmid (Promega) were used as negative and positive controls, respectively. The phRG-B vector (Promega), which is identical to pGL3-Basic, except that it contains the Renilla luciferase gene, was modified in this laboratory by inserting the SV40 promoter using the same flanking restriction sites as in the pGL3-promoter plasmid (*Bgl*II and *Hin*dIII). For normalization, the phRG-B SV40 vector was used in cotransfection experiments. Sixteen to twenty-four hours postcotransfection, luciferase activities were measured using the Dual-Glo Luciferase kit (Promega). Firefly luciferase activities were normalized to Renilla luciferase. One-way ANOVA followed by the Tukey test were done to determine the statistical significance of the luciferase data in Fig. 1.

### Bisulfite Conversion

Differences in methylation patterns in the HeLa R5 and HeLa R1-11 cell lines that express moderate or no PCFT, respectively, were studied using bisulfite conversion, followed by PCR and sequencing. Genomic DNA was isolated from HeLa R5, HeLa R1-11, and HeLa R1-11 treated with 5-aza-2′-deoxycytidine (5-aza-CdR) using the DNeasy Tissue kit

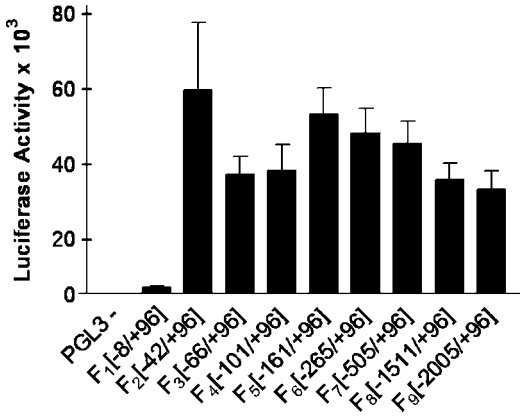


**Figure 1.** Assessment of human *PCFT* promoter activity by luciferase gene expression reporter assays. Promoter regions of *PCFT* designated $F_1$ to $F_9$ were subcloned into the pGL3-Basic vector (-). Using Lipofectamine (0.75 μL per well), HeLa cells (4,000 cells seeded in a 96-well plate) were transiently cotransfected 2-d postseeding with the pGL3 constructs (200 ng) and the phRG-B SV40 plasmid (15 ng), which contains the Renilla luciferase gene. At 16 to 24 h after transfection, luciferase activities were measured using the Dual-Glo Luciferase kit (Promega) and a POLARstar OPTIMA microplate reader (BMG LABTECH). Firefly luciferase activities were normalized with Renilla luciferase. Data are the mean ± SE from three experiments, each done in triplicate.

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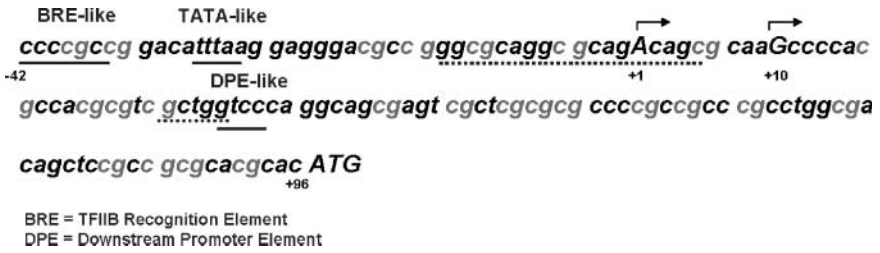


**Figure 2.** Schematic representation of the upstream structure of the *PCFT* gene. The sequence (−42/+96) is shown, including a predicted promoter sequence (www.fruitfly.org/seq_tools/promoter.html), TFIIB recognition element, and TATA-like sequences (*underlined*), reported initiation sites (*capitalized bps with arrows*; NM_080669, BC010691) arbitrarily positioned at +1 and +10. *Dots*, conserved human and mouse nucleotides; a downstream promoter element-like sequence is *underlined*. The GC-rich nature of the human *PCFT* minimal transcriptional regulatory region is shown by a high G + C content (77%) and the presence of 24 CpG dinucleotides (*grey*).

(Qiagen Sciences). The bisulfite reaction was done as described previously (22) with slight modifications. Genomic DNA (~1 μg in 10 μL) was sheared five times using a 27-gauge syringe then denatured with 0.34 mol/L NaOH for 20 min at room temperature. Sixty-eight microliters of freshly prepared bisulfite solution (2 mol/L sodium bisulfite; 6 mol/L urea; 0.6 mmol/L hydroquinone, pH 5.0) was added, following which PCR was done under the following conditions: 2 min at 95°C × 1, 2 h at 75°C followed by 1 min at 95°C × 3. The bisulfite adduct was then removed using Qiagen columns from the EpiTect Bisulfite Kit. The human *PCFT* minimal promoter region was amplified using the following sense and antisense primers, 5′-TAG GGT TTT TTA TTT GTT AGG TTT TT-3′ and 5′-CAC ACT TTA CAA ATA AAA TCA TCC C-3′, respectively. Amplified products were gel purified and sequenced using two degenerate inner forward and reverse primers: 5′-ATT AGY GGT TTT ATY GGG TTT YGG-3′ and 5′-CTC CCY GCY GAA CAT TTA AAA A-3′, respectively, with Y representing a C or a T. The fraction of methylated C was calculated by measuring the height of each C and T and then dividing C by the sum of the heights of C + T. Figure 4 represents the degree of methylation on the Y-axis at each CpG site plotted on the X-axis. The numbering is based on the transcriptional start site. Student paired *t* tests were used to assess the significance of methylation changes between untreated and 5-aza-CdR–treated HeLa R1-11 cells.

### *In vitro* Methylation

Construct $F_2$ (−42/+96) was methylated *in vitro* by treatment with the CpG methyltransferase M.Sss1 (New England BioLabs, Inc.), according to the manufacturer's protocol. Methylation was confirmed by resistance to the methylation-sensitive restriction enzyme BstUI.

### 5-aza-CdR Treatment of HeLa R1-11 and HeLa R5 Cells and Assessment of [$^3$H]MTX Influx

HeLa R1-11 and HeLa R5 cells ($2.5 \times 10^5$) were seeded in glass vials (Research Product International Corp.), and after a day, fresh medium containing 1 μmol/L 5-aza-CdR (Sigma-Aldrich) was added and changed daily. After a 3-d exposure to 1 μmol/L 5-aza-CdR, cells were trypsinized, counted, and reseeded ($2.5 \times 10^5$) in drug-free medium (23). Forty-eight hours later, [$^3$H]MTX influx was assessed over 1 min at a concentration of 0.5 μmol/L in MES buffer saline (MBS; 20 mmol/L 4-morpholinepropane-sulfonic acid, 140 mmol/L NaCl, 5 mmol/L KCl, 2 mmol/L $MgCl_2$, and 5 mmol/L glucose; pH 5.5), as described previously (17). HEPES buffered saline buffer (0°C) was added to stop uptake, following which cells were washed twice, each for 5 min in this buffer, then lysed in 500 μL 0.2 mol/L NaOH at 65°C for 45 min. A portion (400 μL) of lysate was assayed

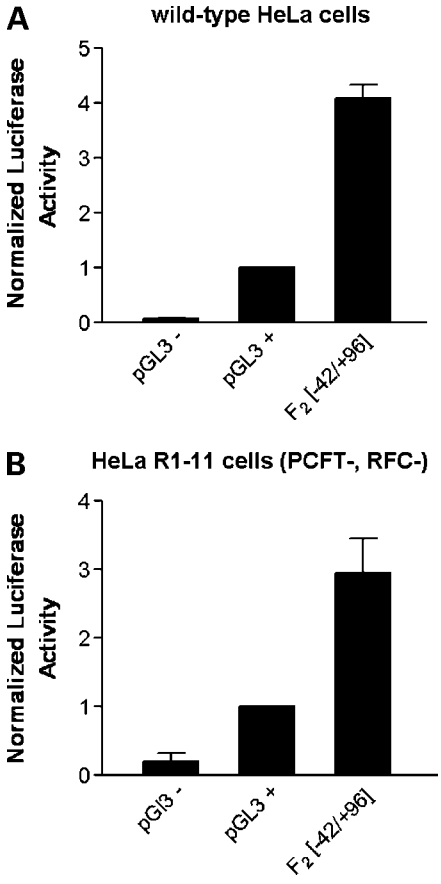


**Figure 3.** Assessment of the human PCFT core promoter activity ($F_2$, −42/+96) in HeLa cells. **A,** wild-type HeLa cells. **B,** PCFT-deficient, RFC–deficient HeLa R1-11 cells. The pGL3-SV40 promoter plasmid (pGL3+) was assigned the value of 1. Data are the mean ± SE from three experiments, each done in triplicate.

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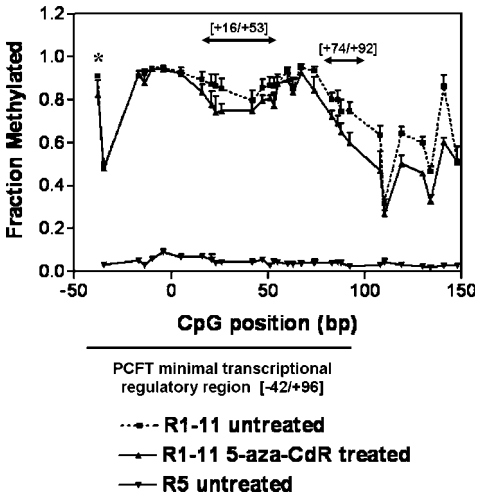


**Figure 4.** Assessment of DNA methylation in the *PCFT* minimal transcriptional regulatory region (−42 to +96) by bisulfite conversion and sequence analysis among HeLa R5, HeLa R1-11, and HeLa R1-11 cells treated with 5-aza-CdR. The fraction of methylated C was calculated by measuring the height of each C and T and then dividing C by the sum of the height of C + T shown on the Y-axis. The location of each CpG dinucleotide is relative to the transcriptional start site (*X-axis*). The CpG sites within the minimal transcriptional regulatory region are indicated along with CpG sites within the first exon at positions beyond +100. Data are the mean ± SE from three independent experiments. The data were analyzed using the Student's *t* test. *Asterisk*, statistical significance at the −38 CpG site. The regions between +16 to +53 and between +74 to +92 are also demarcated, and each showed statistically significant demethylation (see text).

for radioactivity and 10 to 20 μL was assayed for protein determination using the bicinchoninic acid protein assay kit (PIERCE). Cellular uptake is expressed in picomoles per milligram protein per minute.

### Quantitation of Human PCFT mRNA Levels by Real-time PCR

Total RNA was isolated from untreated and 5-aza-CdR–treated HeLa R5 and HeLa R1-11 cells using TRIzol Reagent (Invitrogen), according to the manufacturer's instructions. cDNA was synthesized from total RNA (5 μg) using oligo $(dT)_{12\text{-}18}$ primers and Superscript II reverse transcriptase (Invitrogen). *PCFT* mRNA was quantitated by real-time PCR using Power SYBR Green PCR Master Mix (Applied Biosystems). The primers used for the *PCFT* gene and the housekeeping gene (β-actin) were reported previously (7). All assays were carried out in triplicate.

### Fluorescence *In situ* Hybridization

BAC DNA (RP11-348E14) was labeled by nick translation using biotin-16-dUTP (Roche Diagnostics), hybridized to metaphase chromosomes, and detected by Alexa Fluor 647–conjugated streptavidin antibody (Invitrogen). A probe for human chromosome 17 (Albert Einstein Cancer Center Genome Imaging Facility) was cohybridized. Metaphase chromosomes were derived from HeLa wild-type, R5, and R1-11 cell lines, as previously described.[4]

[4] http://www.riedlab.nci.nih.gov/protocols.asp

## Results

### Definition of the *PCFT* Minimum Transcriptional Regulatory Region

The minimal promoter of the human *PCFT* gene was identified using a luciferase-reporter transfection system. Figure 1 shows the luciferase activities of constructs that map the upstream *PCFT* sequence. The data suggest that the −42/+96 fragment with respect to the transcriptional start site, determined by database analysis, is sufficient for maximal *PCFT* promoter activity. When an ANOVA was done, there were no significant differences among and between the $F_2$ and $F_9$ constructs (Fig. 1). Hence, no additional enhancer or repressor elements could be identified over the region between −2,005 and +96. Thus, transcriptional regulation is confined to a region that contains the core promoter and a more proximal segment (Fig. 2).

### Analysis of the Basis for Loss of PCFT Activity in HeLa R1-11 Cells

HeLa R1-11 cells were derived from HeLa R5 cells, which lack genomic RFC but retain a moderate level of *PCFT* expression and activity. In contrast, HeLa R1-11 cells have essentially no folate transport activity, and *PCFT* mRNA is not detected (7). Sequence analysis indicated that there are no mutations in the upstream (−1,288/+96) or coding regions (data not shown). However, transient transfection of the *PCFT* promoter–luciferase construct [$F_2$(−42/+96)] in HeLa R1-11 cells showed expression levels similar to those observed after transfection into wild-type HeLa cells, indicative of intact transcriptional/translational mechanisms (Fig. 3A and B). Hence, the loss of PCFT activity in HeLa R1-11 cells is consistent with a regulatory change raising the possibility of an alteration at the epigenetic level.

### Methylation Status of the *PCFT* Minimal Transcriptional Regulatory Region

Studies were undertaken to explore the possibility that *PCFT* silencing in HeLa R1-11 cells is due to altered DNA methylation in the promoter region. The human *PCFT* minimal transcriptional regulatory region is G + C rich and contained in a CpG island that comprises 584 nucleotides and includes exon 1 and 168 bp in the upstream region. The UCSC genome browser gives a CpG count of 64, a G + C percentage of 73.5%, and a ratio of observed to expected CpG of 0.81 (24). Among the 139 nucleotides in the minimal regulatory region, there are 60 C and 46 G, thus a G + C content of 77% and 24 CpGs. Methylation analysis at those CpGs showed a difference between the two cell lines (Fig. 4). HeLa R5 cells showed virtually no methylation; in contrast, the *PCFT* promoter region was highly methylated in HeLa R1-11 cells.

### Impact of the Methylation of the Putative PCFT Promoter on Transcriptional Activity

To directly assess the impact of methylation on transcriptional activity, the −42/+96 human *PCFT* construct was treated with Sss1 methyltransferase. Methylation was confirmed by the observation that this abolished cleavage of the *PCFT* DNA by BstUI (Fig. 5A). When the methylated *PCFT* promoter–luciferase construct was transiently

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expressed in HeLa cells, luciferase activity similar to a negative control plasmid was observed (Fig. 5B). Hence, methylation of the *PCFT* promoter abolishes its activity.

**Reactivation of *PCFT* Gene Expression in HeLa R1-11 Cells Treated with 5-aza-CdR**

When HeLa R1-11 cells were treated for 72 hours with the DNA methyltransferase inhibitor, 5-aza-CdR, there was a 2.6-fold increase in PCFT-mediated [$^3$H]MTX influx compared with untreated cells (Fig. 5C). Treatment of HeLa R5 cells, in which the PCFT −42/+96 region was in a demethylated state, resulted in ~42% decrease in transport mediated by this carrier. This was associated with 70% ± 9.7% inhibition of cell growth based upon cell count, consistent with the inhibitory effect of this agent on DNA synthesis (25). Likewise, there was comparable inhibition of cell growth in HeLa R1-11 cells treated with this drug (61 ± 3.2%). Despite this growth inhibition, the level of transport activity in 5-aza-CdR–treated HeLa R1-11 cells increased to half that of the 5-aza-CdR-treated HeLa R5 cells (Fig. 5C). These results were correlated with the effects on *PCFT* mRNA levels, which increased ~5-fold in HeLa R1-11 cells treated with 5-aza-CdR compared with untreated cells (Fig. 5D). There was no difference in *PCFT* mRNA levels in 5-aza-CdR–treated and untreated HeLa R5 cells; hence, inhibition of transport by 5-aza-CdR was likely related to a nonspecific toxic effect of this agent associated with the inhibition of cell growth. When the level of methylation was assessed in HeLa R1-11 cells treated with 5-aza-CdR and compared with untreated cells, there was a small (7%) global ($P < 0.002$) decrease in methylation when all the CpG sites within the minimal transcriptional regulatory region were analyzed (−38 to +92; $P < 0.006$; Fig. 4). Specifically, the decrease in the most upstream CpG site (−38) was significant at $P = 0.03$. Two other parts of the minimal transcriptional regulatory region, from +16 to +53 and +74 to +92, showed more substantial demethylation ($P < 0.0001$). These represent

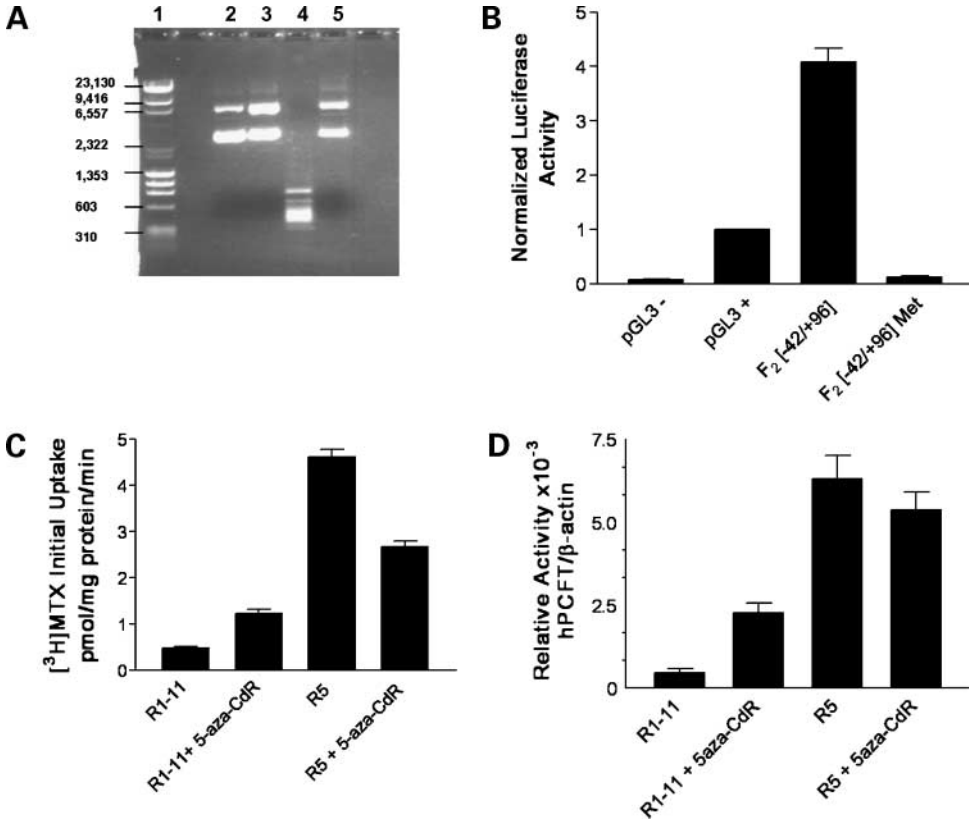


**Figure 5.** Impact of methylation on promoter activity and 5-aza-CdR treatment in HeLa R1-11 and HeLa R5 cells **A,** DNA methylation and its effect on the activity of the *PCFT* core promoter. Confirmation of *in vitro* methylation of the *PCFT* promoter–luciferase construct $F_2$ (−42/+96) by protection from BstUI digestion. $F_2$ was methylated by Sss1 methylase for 4 h and then treated with BstUI. *Lane 1*, λ DNA/*Hin*dIII and Phi X174 RF/*Hae*III molecular weight markers; sizes are shown on the left in bp; *lane 2*, unmethylated $F_2$; *lane 3*, $F_2$ methylated by Sss1; *lane 4*, unmethylated $F_2$ treated with BstUI; and *lane 5*, $F_2$ methylated by Sss1 and treated with BstUI. *Lanes 2* to *5*, 500 ng DNA. **B,** normalized luciferase activities of unmethylated and methylated $F_2$ (−42/+96) in wild-type HeLa cells. Data are the mean ± SE from three experiments, each done in triplicate. **C,** reactivation of PCFT function in HeLa R1-11 and HeLa R5 cells ($2.5 \times 10^5$ cells seeded in transport vials) treated with 1 μmol/L 5-aza-CdR for 72 h. Following treatment, the same number of cells was reseeded in drug-free media for 48 h, following which [$^3$H]MTX influx was assessed over 1 min (pH 5.5) and a concentration of 0.5 μmol/L. Data are the mean ± SE from four experiments, each done in triplicate. **D,** quantitation of *PCFT* mRNA levels by real-time PCR in HeLa R1-11 and HeLa R5 cells treated with and without 5-aza-CdR. Y-axis, the ratio of the relative expression levels of human *PCFT* to β-actin mRNAs. Data are the mean ± SE from an experiment using two sets of *PCFT* primers and done in triplicate.

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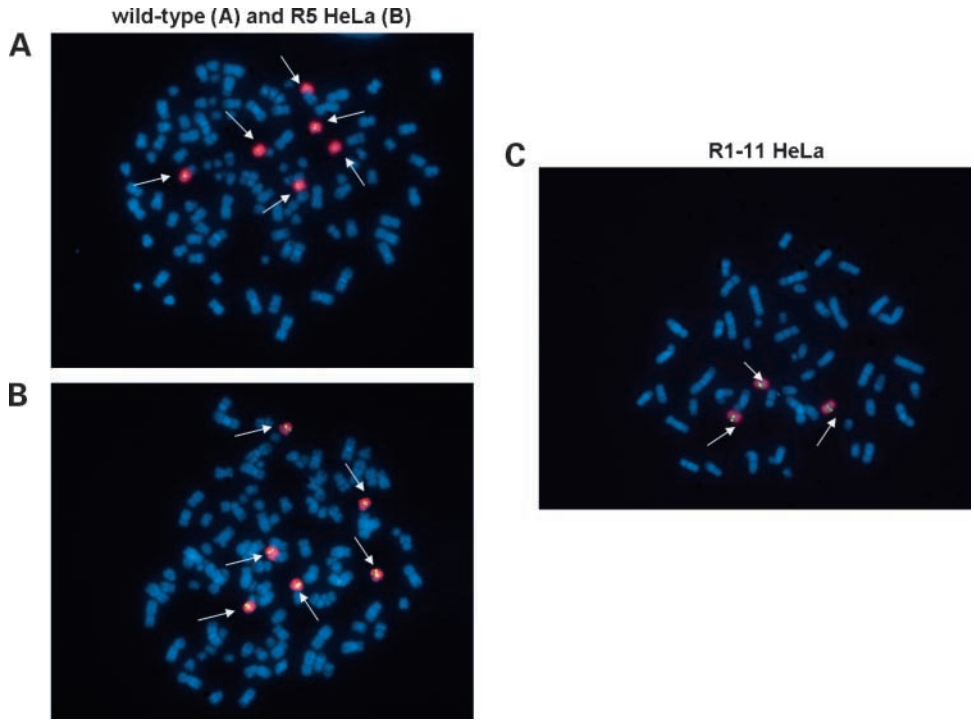


**Figure 6.** Determination of *PCFT* gene copy number in HeLa wild-type, R5, and R1-11 cell lines by fluorescence *in situ* hybridization analysis. A PCFT probe (*green*) and a chromosome 17 painted probe (*red*) were used to analyze metaphase spreads from wild-type HeLa cells (**A**), HeLa R5 cells (**B**), and HeLa R1-11 cells (**B**). Arrows, both signals.

potential sites for significant regulatory actions. There was, in addition, significant ($P < 0.003$) demethylation within the coding region (+108 to +148).

### Cytogenetic Analysis

To determine whether an altered gene copy number contributed to the loss of PCFT expression and function in HeLa R1-11 cells, cytogenetic studies were undertaken using probes for chromosome 17 and the *PCFT* gene. As indicated in Fig. 6A and B, wild-type and HeLa R5 cells have six copies of chromosome 17 and six copies of *PCFT*; HeLa R1-11 cells have three copies of chromosome 17 and three copies of *PCFT* (Fig. 6C). Hence, HeLa R1-11 cells have lost half their *PCFT* DNA copies. This is consistent with the observation that treatment of HeLa R1-11 cells with 5-aza-CdR restores PCFT transport activity but only to a level approximately half that of HeLa R5–treated cells.

## Discussion

These studies provide insight into the regulation of the human *PCFT* gene and the mechanism by which the HeLa R1-11 cell line lost PCFT activity under MTX selective pressure. The data localized all transcriptional regulation of the *PCFT* gene to the interval of −42 to +96 bases, which contains the minimum transcriptional regulatory region. To explore the physiologic relevance of this finding, a BLAST search was done and the databases of all expressed sequenced tags were aligned with the first 600 nucleotides of the *PCFT* gene (NM_080669). These clones indicated two alternative transcription start sites at +1 and +10. It is therefore possible that the entire regulatory region is contained within the interval of −42 to +96 bases upstream from the transcriptional start site. This segment includes the core promoter but is large enough to include other *cis*-regulatory elements. Real-time PCR showed that PCFT expression is ~1/100th that of β-actin (Fig. 5D), consistent with the moderate level of PCFT protein expression in HeLa cells.

The *PCFT* gene was only very recently identified, so there is little information on factors that alter its expression. However, a marked increase in *PCFT*, RFC, and folate receptor mRNA expression in small intestine was observed in mice fed a folate-deficient, as compared with a folate-replete, diet (26, 27). *In vitro* studies showed a much smaller increase in PCFT expression with folate deprivation (2.5-fold increase in *PCFT* mRNA levels and 1.6-fold increase in PCFT-mediated [$^3$H]folic acid uptake) in Caco-2 cells grown with 0.25 μmol/L, as compared to 100 μmol/L, folic acid (28). The present article suggests one possible mechanism by which dietary folate availability might alter the expression of PCFT: through its effects on DNA methylation. Folate depletion has been implicated in global hypomethylation (29), which might enhance PCFT transcription resulting in enhanced intestinal folate absorption and folate repletion. Likewise, folate excess might result in *PCFT* hypermethylation suppressing transcriptional activity of this gene. Such a regulatory feedback mechanism could be an important factor in the maintenance of folate homeostasis. Although folate status and its effects on methylation affects the expression of the other folate transporters (27), *PCFT* alone is capable of altering the level of folate absorption in the proximal small intestine, thus modulating net organismal folate levels.

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The HeLa cell lines used in the current studies played a crucial role in the identification of PCFT (5, 7). The present study explored the basis for the loss of PCFT expression in the HeLa R1-11 clonal derivative of the RFC–deficient, *PCFT*-deficient HeLa R1 line. No mutation could be detected in the coding or 5′ upstream region (−1,288/+96) of this gene. Furthermore, when the *PCFT* promoter–luciferase construct $F_2$ (−42/+96) was transiently transfected in the MTX-resistant HeLa R1-11 and wild-type HeLa cells, the same fold increase in activity was observed with respect to the pGL3-SV40 positive control plasmid (Fig. 3), consistent with intact transcription/translation machinery. The defect in HeLa R1-11 cells turned out to be due to profound hypermethylation of the minimal transcriptional regulatory region in comparison to the HeLa R5 line as established by bisulfite conversion and sequence analysis (Fig. 4). *PCFT* mRNA expression and PCFT function in the R1-11 cells was substantially increased by treatment with 5-aza-CdR (Fig. 5C and D). This was accompanied by small but significant changes in methylation (Fig. 4). This degree of demethylation may have produced a modification in chromatin structure that was sufficient to restore a level of transcriptional activity that could account for these findings. A low level of 5-aza-CdR demethylation under these conditions is, in fact, expected because this agent only acts on newly synthesized DNA and HeLa R1-11 cells underwent less than one division over the 3 days of exposure to the drug. Although a longer exposure to 5-aza-CdR would likely have resulted in a greater degree of demethylation, this would have been accompanied by a greater degree of cytotoxicity. It is also possible that the changes in PCFT mRNA and folate transport observed were related to a small fraction of the cell population in which there was a greater level of demethylation. These results are consistent with a recent report in which cells from patients with myelodysplastic syndrome that were treated with several cycles of low-dose 5-aza-CdR resulted in restoration of p15 protein expression after the first cycle, whereas demethylation of the gene occurred much more slowly over several subsequent cycles of drug treatment (30). Hence, the return of activity preceded gross demethylation in the cell population. The failure to achieve complete restoration of PCFT function is attributed to the loss of half the *PCFT* gene copies in HeLa R1-11 as compared with HeLa R5 cells, as assessed by fluorescence *in situ* hybridization (Fig. 6), and a nonspecific toxic effect of 5-aza-CdR on transport unrelated to PCFT expression.

HeLa R1-11 cells were selected in the presence of MTX with folic acid as the growth source. With the loss of PCFT, there was a marked contraction of folate cofactor pools in these cells (5). However, despite the deficiency of cellular folates, the *PCFT* promoter was methylated and PCFT activity diminished. This suggests that the toxic effects of the antifolate produced a greater selective pressure than folate depletion.

Methylation-associated suppression of the expression of other folate transporters was reported for RFC in MTX-resistant MDA-MB-231 breast cancer cells (31) and folate receptor in nasopharyngeal epidermoid cancer cells, KB1BT (32). When these cells were treated with 5-aza-CdR, the RFC and folate receptor genes were re-expressed; however, the levels of expression and function in KB1BT-treated cells were far less than in wild-type cells.

In summary, the human *PCFT* minimum transcriptional regulatory region in HeLa cells has been defined and hypermethylation of the promoter region, and gene copy loss were shown to be the basis for the silencing of the *PCFT* gene in a MTX-resistant cell line derived from HeLa R5. Recently, hypermethylation of a larger upstream region of this gene was shown to be associated with the low level of PCFT expression and low-pH folate transport activity in two leukemia cell lines. However, function was not assessed before or after treatment with 5-aza-CdR (33). Understanding the mechanisms of *PCFT* regulation offers the possibility of identifying approaches to modulate expression of this gene that could be used to diminish the impact of folate-deficiency states and enhance the delivery of antifolates to cancer cells.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Ndeye Khady Diop-Bove, Julia Wu, Rongbao Zhao, et al.

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