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Effects of Antisense-based Folylpoly-γ-glutamate Synthetase Down-regulation on Reduced Folates and Cellular Proliferation in CCRF-CEM Cells

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ABSTRACT. The effect of down-regulation of folylpoly-γ-glutamate synthetase (FPGS) activity on intracellular reduced folate accumulation and cellular proliferation was examined, using an inducible antisense expression system in the human T-lymphoblastic leukemia cell line CCRF-CEM. FPGS catalyzes the addition of γ-glutamyl residues to natural folates and classical antifolates, which results in their enhanced cellular retention and increased cytotoxicity. As such, this enzyme has become a focus as a potential anticancer drug target. However, direct evidence to support this concept has been elusive. Hence, a study was undertaken using an antisense-based expression system to down-regulate FPGS activity. This inducible expression system was used to demonstrate that lower FPGS activity can lead to substantially lower intracellular folate content, which coincides with suppression of thymidylate synthesis and inhibition of cellular proliferation. BIOCHEM PHARMACOL **55**;12:2031–2037, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. folylpolyglutamate synthetase; antisense; reduced folates; cellular proliferation

FPGS§ (EC 6.3.2.17) catalyzes the magnesium- and ATP-dependent addition of γ-glutamyl residues to all natural folates and classical folate antagonists such as methotrexate [1–3]. Folates exist intracellularly as polyglutamate derivatives with 5–7 residues that enhance both their retention and substrate activity. Because FPGS is the only known enzyme that can catalyze polyglutamylation, it has been proposed that inhibition of FPGS activity could lead to folate depletion, with a corresponding impact on proliferation. Antifolates have played a prominent role in cancer treatment because of their ability to disrupt folate metabolism [4, 5], and FPGS inhibition could provide an additional target for that antagonism. However, direct demonstration that FPGS inhibition is antiproliferative has been elusive. Lowe and coworkers [6, 7] were able to show an association between FPGS level and folate content in FPGS-negative CHO cells (AUXB1) transfected with sheared human DNA to elevate FPGS, but only at higher medium folate levels, and the impact on proliferation was minimal. Another potential means to lower intracellular FPGS activity is exposure to inhibitors thereof, but many potent inhibitors are poorly taken up, and those that are adequately internalized could potentially target additional folate-metabolizing enzymes with the associated difficulty in interpretation of such results [8–10]. To more directly address the potential for this enzyme as a chemotherapeutic target, an inducible FPGS antisense expression system was developed, characterized, and used to examine the effects of down-regulation on reduced folate accumulation and cellular proliferation.

MATERIALS AND METHODS

Materials

[^{3}H]dATP (66 Ci/mmol), horseradish peroxidase conjugated to donkey antimouse-IgG, and an enhanced chemiluminescence system were purchased from Amersham. LV was obtained from Ben Venue Laboratories. DNA standards, *Eco*RI/*Not*I adapter, fetal bovine serum, Geneticin (G418), IPTG, MMLV reverse transcriptase, proteinase K, restriction endonucleases, RNase A, T4 DNA ligase, and trypsin were purchased from Life Technologies. [^{3}H]FdUMP (20 Ci/mmol) was obtained from Moravek Biochemicals. [^{3}H]dUrd (30 Ci/mmol) and [^{3}H]glutamic acid (50 Ci/mmol) was purchased from DuPont/NEN. *Taq* DNA polymerase was from Perkin Elmer Cetus. RNAguard (RNase inhibitor) and Sephadex G-25 and G-50 were

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§ *Abbreviations:* CH_2FH_4, methylenetetrahydrofolate; $5\text{-}CH_3FH_4$, 5-methyltetrahydrofolate; $10\text{-}CHOFH_4$, 10-formyltetrahydrofolate; $5\text{-}CHOFH_4$ or LV, 5-formyltetrahydrofolate or leucovorin; dUrd, deoxyuridine; FA, folic acid; FdUMP, 5-fluoro-2′-deoxyuridine-monophosphate; FH_2, dihydrofolate; FH_4, tetrahydrofolate; FPGS, folylpoly-γ-glutamate synthetase; IPTG, isopropylthio-β-D-galactoside; RT-PCR, reverse transcription-polymerase chain reaction; and TS, thymidylate synthase.

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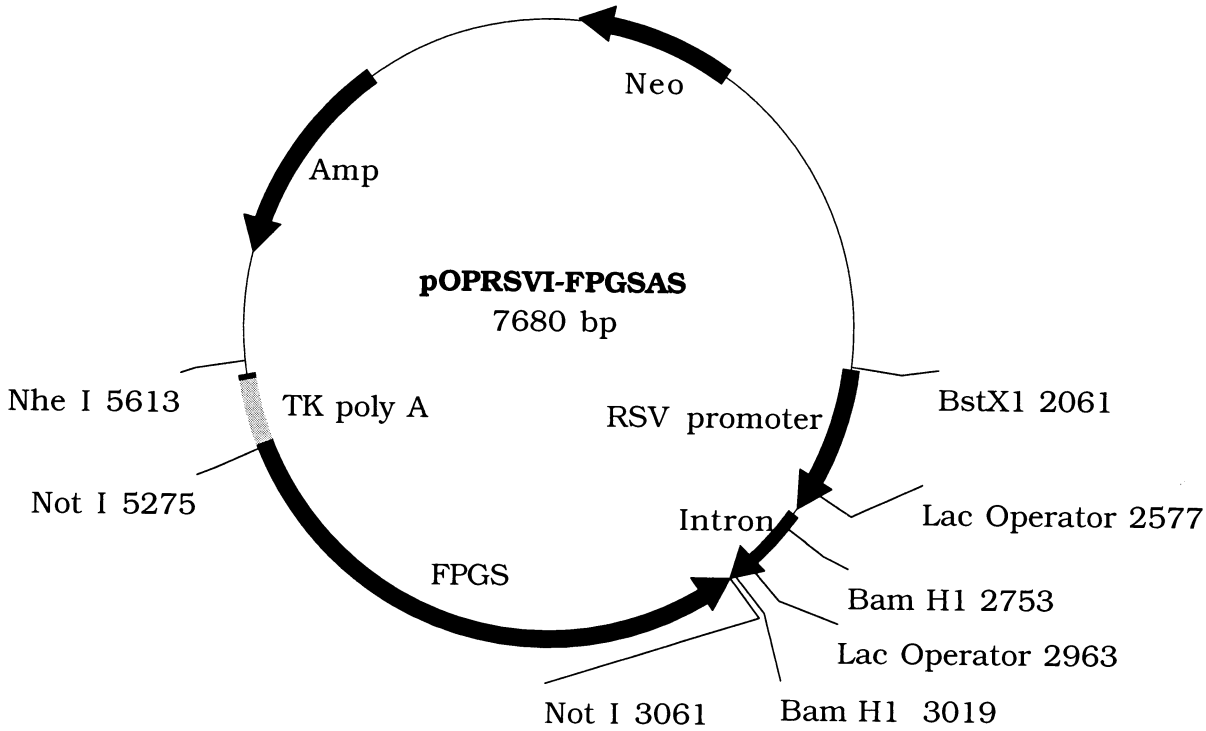

FIG. 1. Schematic representation of the FPGS antisense construct.

purchased from Pharmacia. The LacSwitch inducible mammalian expression vector system was purchased from Stratagene. TS (4 U/mg of protein) was purified from an *Escherichia coli* strain that overproduces *Lactobacillus casei* TS [11]. 5,10-Methylenetetrahydrofolate reductase (0.5 U/mg of protein) and 10-formyltetrahydrofolate dehydrogenase (0.2 U/mg of protein) were purified from pig liver as described previously [12, 13]. 5,10-Methenyltetrahydrofolate synthetase (0.1 U/mg of protein) was purified from rabbit liver [14]. Dihydrofolate reductase (1.25 U/mg of protein) was purified from *L. casei* [15]. FH4 was synthesized from FA via FH_2 by an enzymatic procedure described previously [16]. FdUMP, guanidine thiocyanate, RPMI-1640 medium, and other reagents were purchased from Sigma.

Cell Line

A human T-lymphoblastic leukemia cell line, CCRF-CEM, was purchased from the American Type Culture Collection and used for all studies [17]. Cells were maintained at 37° in 5% CO_2 in RPMI-1640 medium supplemented with 10% fetal bovine serum. Cells were passaged twice weekly, and exhibited a doubling time of 24 hr under these conditions.

Construction and Transfection of FPGS Antisense Expression Vector

Human FPGS cDNA (2.2 kb) was provided by Dr. B. Shane, University of California [18]. An *Eco*RI/*Not*I adapter was ligated to each end, digested with *Not*I, and cloned at the *Not*I site of the commercial mammalian expression vector pOPRSVI (Stratagene). This construct contains a neomycin-resistant gene for selection of transfected cells with G418 and two Lac operators that are located in the RSV promoter region (bp 2577–2613) and in the SV40 intron region (bp 2963–2999), that controls the expression of FPGS antisense (see Fig. 1). The Lac operator is repressed by LacI repressor protein, which is expressed by a second vector p3′SS (Stratagene). IPTG suppresses Lac repressor expression, which, in turn, induces expression of FPGS cDNA.

Antisense orientation of FPGS cDNA with respect to the Lac operator was established by predicted fragment size from multiple restriction enzyme digestions. Further confirmation was obtained from PCR product size using primers that predicted 320-bp fragments for antisense RNA versus a 7-kb fragment for sense RNA. Transfection of CCRF-CEM cells was accomplished with 20 μg each of the FPGS antisense expression vector, pOPRSVI-FPGSAS, and p3′SS, by electroporation. Two million cells in 200 μL of serum-free RPMI-1640 medium were electroporated at 110 V and 3000 microfarads using an Electro Cell Manipulator 600 (Biotechnologies & Experimental Research). Transfected cells were incubated with 800 μg/mL of G418 for 2 weeks for selection. Antibiotic-resistant single colonies were selected by limiting dilution in 96-well tissue culture plates.

FPGS Activity

FPGS activity was determined by the [^{3}H]glutamic acid incorporation microassay previously reported [19]. Cells

were disrupted in a lysis buffer using an Ultrasonic W-380 Sonicator (Heat Systems Ultrasonics) and centrifuged. These supernatants were incubated with [^{3}H]glutamic acid and FH_4 at 37° for 30 min, a time point demonstrated to be in the linear region of product accumulation [19]. *L. casei* TS and FdUMP were added and allowed to react at 37° for 30 min to form a ternary complex. Reaction mixtures were then passed through Sephadex G-50 columns to remove free radioactive ligand. Bound radioactivity was determined by scintillation counting, and used to establish FPGS activity.

Estimation of FPGS mRNA by RT-PCR

Quantitative RT-PCR techniques were used to measure FPGS mRNA levels in antisense transfected cells as previously described [20]. Briefly, cells were incubated with 5 mM of IPTG for 72 hr. Total cellular RNA was isolated by the guanidinium thiocyanate extraction method [21], and the concentration was determined spectrophotometrically at 260 nm. Total RNA was reverse transcribed with MMLV reverse transcriptase using random oligo primers. PCR amplification of incremental amounts of resultant cDNA was carried out with specific gene primer pairs for FPGS and 0.5 μCi of [^{3}H]dATP using *Taq* DNA polymerase. PCR cycles were: 1 min at 94°, 1 min at 55°, and 1 min at 72° for 30 cycles. Following PCR, the product was centrifuged through Sephadex G-50 columns to separate free labeled nucleotide from nucleic acid incorporated [^{3}H]dATP, and radioactivity was determined by scintillation counting. The linear amplification regions were determined by plotting the amount of PCR product versus the amount of starting cDNA. The slopes of the linear amplification regions under each condition were used to calculate the expression ratio (transfected cells/control cells). The FPGS primers were 5′-GTCTTCAGCTGCATTTCACATGCCTTGCAAT GGA-3′ and 5′-CTACTGGGACAGTGCGGGCTCCA GCAGCTT-3′, which encompass bases 1489–1708 of the FPGS cDNA sequence [18]. They were prepared with a 380B DNA synthesizer (Applied Biosystems). Automated fluorescence sequencing confirmed that the sequence of this PCR product was identical to the published FPGS cDNA.

Estimation of Folate Polyglutamate Chain Length

The polyglutamate chain length of the intracellular CH_2FH_4 + FH_4 pool was determined by a modification of the previously described ternary complex method [22]. Briefly, cells were washed with cold PBS, and lysed by freeze/thaw in the presence of 100 mU of TS and 250 nM of [^{3}H]FdUMP to immediately entrap cellular folate polyglutamates. This approach prevented hydrolysis by folylpolyglutamate hydrolase, which was present in lysates [23]. Electrophoresis on 7% polyacrylamide was used to separate complexes based on the number of charged glutamate residues present. After transfer to Immobilon-P membranes, *L. casei* TS antibody, developed in rabbits as described below, and horseradish peroxidase conjugated to donkey anti-rabbit IgG were introduced. Then the ECL system was used to visualize bands, and their relative intensity was quantitated by scanning densitometry. This approach to detection of ternary complexes was compared with fluorography of [^{3}H]FdUMP-labeled complexes [22] and found to provide greater sensitivity with the same relative detection of individual polyglutamate species.

Antibody Preparation

Polyclonal antibodies raised against *L. casei* TS were produced in female New Zealand white rabbits. Approximately 100 μg of purified TS mixed with Freund's complete adjuvant was injected intradermally. Two weeks later an additional 100 μg of protein mixed in Freund's incomplete adjuvant was administered intradermally. Four weeks after the first immunization, a booster dose of 50 μg of protein in Freund's incomplete adjuvant was injected intradermally. Rabbits were bled 2 weeks after the last injection to collect antisera.

Estimation of Reduced Folates

The ternary complex assay described previously was used to quantitate reduced folates [22]. This assay is based upon enzymatic cycling of reduced folates to CH_2FH_4 followed by entrapment into a stable ternary complex with excess *L. casei* TS and [^{3}H]FdUMP [24]. Briefly, cells were washed twice with cold PBS, lysed by sonication, and centrifuged at 10,000 *g* for 5 min at 4° to remove cellular debris. Resultant supernatants were immediately boiled for 3 min and recentrifuged to remove precipitated protein. Aliquots of supernatant were incubated at 25° for 30 min in a 50 mM of Tris–HCl buffer (pH 7.4) with 20 mU of *L. casei* TS, 125 nM of [^{3}H]FdUMP, 50 mM of sodium ascorbate, and 1 mM of EDTA in a total volume of 200 μL. Additional enzymes and cofactors were added as necessary to convert each reduced folate to the CH_2FH_4 form [25, 26]. After stopping reactions by boiling for 10 min in 1% SDS, aliquots were applied to Sephadex G-25 mini-columns and eluted by centrifugation to separate tritiated complexes from free [^{3}H]FdUMP. Radioactivity was determined by scintillation counting and used to estimate reduced folate content.

[^{3}H]dUrd Incorporation Assay

Incorporation of tritium from [^{3}H]dUrd into DNA requires the synthesis of thymidylate and, hence, can be used to determine the effect of FPGS down-regulation on intracellular TS activity [27]. Briefly, after treatment cells were incubated with 2 μCi [^{3}H]dUrd for 30 min, washed twice with cold PBS, and lysed, and DNA was isolated as previously reported [20]. DNA-incorporated radioactivity was measured by scintillation counting.

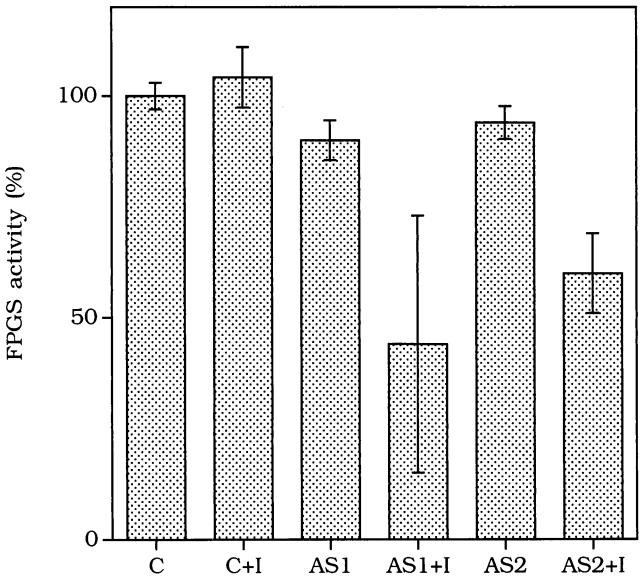

FIG. 2. Evaluation of FPGS activity from antisense transfected cells. Ten million CEM (C) and two FPGS antisense transfected cell lines (AS1 and AS2) were treated with 5 mM of IPTG (I) for 72 hr. FPGS activity was determined by [^{3}H]glutamic acid incorporation microassay [16] and presented as a percentage where 100% represents 240 pmol/hr/mg of protein. Values represent the means ± SEM from 3 separate experiments.

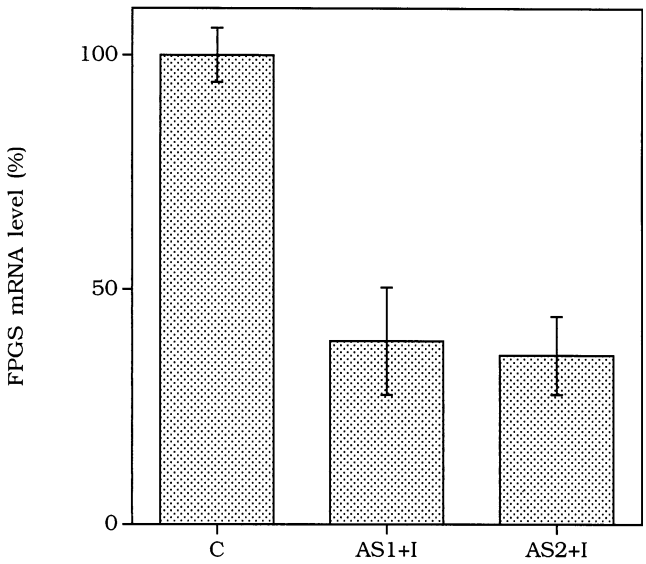

FIG. 3. FPGS mRNA levels in transfected CEM cells induced to express FPGS antisense RNA. Ten million CEM (C) and two FPGS antisense transfected cell lines (AS1 and AS2) were treated with 5 mM of IPTG (I) for 72 hr. Total cellular RNA was isolated and reverse transcribed. PCR amplification was performed using primer pairs specific for FPGS RNA. Incorporation of [^{3}H]dATP was estimated by passage of PCR products over Sephadex G-50 columns followed by scintillation counting. Values are the means ± SEM from 3 separate experiments.

RESULTS

A two-part inducible expression system for FPGS antisense was constructed with the vector shown in Fig. 1 and a second vector that causes IPTG sensitivity. This dual vector system was transfected into CCRF-CEM cells by electroporation. Two antisense transfected clones, AS1 and AS2, were selected with antibiotic and used for further study. FPGS activity was determined by the [^{3}H]glutamic acid incorporation microassay in lysates from these clones [19], and results are shown in Fig. 2. It can be seen that activity is suppressed 40–50% compared with control cells. There was essentially no suppression of FPGS activity in untransfected control cells treated with IPTG and only a modest loss of activity in uninduced FPGS antisense transfected cells. In addition, there was no suppression of FPGS activity in cells transfected with the vector system without the FPGS antisense insert (results not shown). To support the concept that this FPGS activity down-regulation is antisense based, the level of FPGS mRNA was evaluated by RT-PCR in IPTG-induced clones. The results shown in Fig. 3 indicate a loss of 50–60% of FPGS mRNA.

To evaluate the impact of antisense down-regulation of FPGS activity on intracellular polyglutamylation, folate-depleted cells were exposed to high levels of LV for 4 hr, and accumulation of folate polyglutamates was evaluated by the ternary complex approach [22]. Table 1 shows that IPTG induction of antisense expression in both of the transfected cell lines resulted in suppressed formation of longer chain length polyglutamates compared with controls. The distribution of polyglutamate species associated with the CH_2FH_4 + FH_4 pool is shown as a percentage of the total, but absolute concentrations of each can be calculated from the total cellular concentration of this pool shown in Table 2. Hence, antisense transfection of this cell line resulted in both lower cell-free extract FPGS activity and impaired intracellular polyglutamylation of reduced folates.

To examine the impact of down-regulation of FPGS activity on intracellular reduced folate content, total folate levels in IPTG-induced cells were examined using the ternary complex assay [22, 25, 26]. It can be seen in Fig. 4 that the total reduced folate content, determined from separate estimation of the CH_2FH_4 + FH_4, 5-CH_3FH_4, 10-$CHOFH_4$, 5-$CHOFH_4$, and FH_2 + FA pools, was suppressed 30–35%. In addition to steady-state folate content, the effect of down-regulation of FPGS activity on the

TABLE 1. Folate polyglutamate formation in FPGS down-regulated CEM cells after incubation with LV

	Polyglutamate distribution (% of total)						
Cell line	**glu1**	**glu2**	**glu3**	**glu4**	**glu5**	**glu6**	**glu7**
CEM + I	10	12	15	15	20	19	9
AS1 + I	20	22	25	20	7	4	2
AS2 + I	40	22	20	10	4	3	1

CEM and two FPGS antisense transfected cell lines (AS) were grown in 10 nM LV for 72 hr in the presence of 5 mM of IPTG (I) before introduction of 10 μM of LV for 4 hr. After incubation for 4 hr, polyglutamate chain length distribution was estimated densitometrically from immunoblots of electrophoretically separated ternary complexes.

TABLE 2 Effects of down-regulation of FPGS activity on folate pools in CEM cells

Cell line	Reduced folates (pmol/mg protein)					
	CH_2FH_4 + FH_4	5-CH_3FH_4	10-$CHOFH_4$	5-$CHOFH_4$	FH_2 + FA	Total
CEM	61.9 ± 9.0 (7)	18.1 ± 9.9 (2)	667.9 ± 72.5 (73)	146.6 ± 48.5 (16)	17.1 ± 15.0 (2)	911.6 ± 32.1
AS1 + I	44.1 ± 6.3 (11)	3.3 ± 2.0 (1)	279.7 ± 52.9 (71)	63.1 ± 12.0 (16)	2.3 ± 1.9 (1)	392.5 ± 62.3
AS2 + I	39.0 ± 5.9 (7)	12.3 ± 3.9 (2)	417.7 ± 64.6 (70)	114.1 ± 18.0 (19)	10.3 ± 2.9 (2)	593.4 ± 31.9

CEM and FPGS antisense transfected (AS) cells (1×10^7) were grown in 10 nM of LV medium for 72 hr in the presence of 5 mM of IPTG (I) before introduction of 10 μM of LV. After a 4-hr incubation, each reduced folate pool was determined by the ternary assay. Values represent the means ± SEM of duplicate analyses from 3 separate experiments. Numbers in parentheses indicate percent of total for each folate.

uptake and retention of folates was also examined. CCRF-CEM cells were first depleted of folate for 72 hr followed by exposure to high LV (10 μM). Intracellular reduced folate accumulation was monitored over an 8-hr period. It can be seen in Fig. 5 that total folate content had plateaued by 4 hr in both control and the two antisense-induced cell lines. However, FPGS down-regulated cells acquired only approximately 50% as much total folate as control cells. The effect of down-regulation of FPGS activity on the distribution among intracellular folate pools was also evaluated, and results are shown in Table 2. It can be seen that each individual reduced folate pool was influenced to essentially the same extent as the total pool. Therefore, while there was a substantial impact of down-regulation of FPGS activity on total steady-state folate content, there was little impact on folate pool distribution.

Because TS requires CH_2FH_4 as a substrate, and it has been shown that polyglutamylation of CH_2FH_4 enhances TS activity [28, 29], the impact of down-regulation of FPGS activity on intracellular thymidylate synthesis was determined using the incorporation of [^{3}H]dUrd into DNA [27]. It can be seen in Fig. 6 that there was about 30–40% suppression of [^{3}H]dUrd incorporation in FPGS down-regulated cells compared with control or uninduced transfected cells. Because impaired polyglutamylation could inhibit growth, the impact of down-regulation of FPGS activity on proliferation was examined. FPGS antisense transfected and control cells were maintained in the presence of IPTG, and cell growth was monitored for 6 days. It can be seen in Fig. 7 that the antisense-induced clones grew more slowly and the confluence level achieved was well below that of uninduced clones or controls.

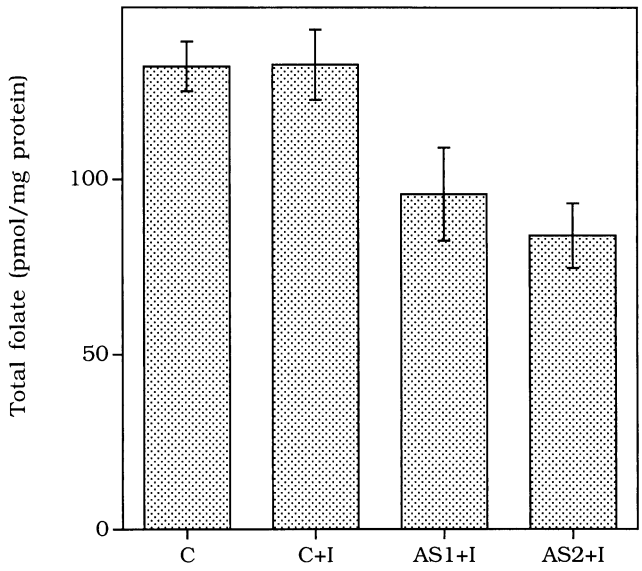

FIG. 4. Effect of FPGS antisense expression on total folate content of CEM cells. Ten million CEM (C) and two FPGS antisense transfected cell lines (AS1 and AS2) were treated with 5 mM of IPTG (I) in standard RPMI-1640 medium for 72 hr. Individual folate pools were measured by the ternary complex assay, and summed to obtain total folate. Values represent the means ± SEM of duplicate analyses from 3 separate experiments.

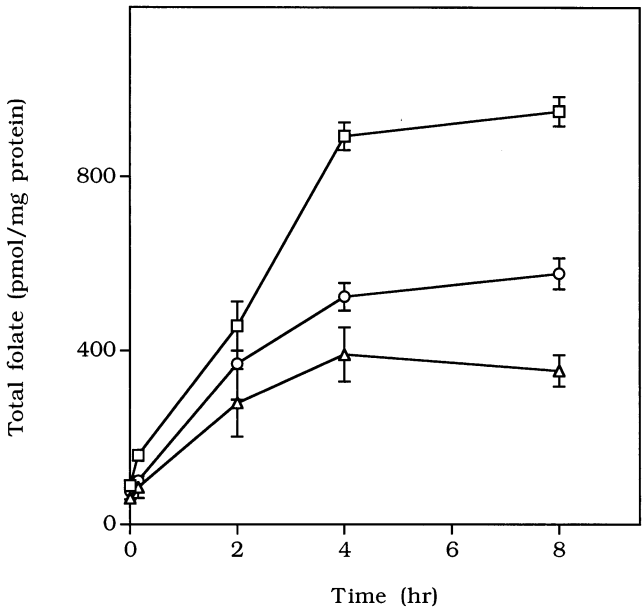

FIG. 5. Time dependence of total folate accumulation by FPGS down-regulated CEM cells after introduction of 10 μM of LV. Ten million control (□) and FPGS antisense transfected AS1 (△) and AS2 (○) cells were treated with 5 mM of IPTG in a medium containing 10 nM of LV for 72 hr. After the introduction of 10 μM of LV, reduced folates were monitored by the ternary complex assay and summed to obtain total folate. Values represent the means ± SEM of duplicate analyses from 3 separate experiments.

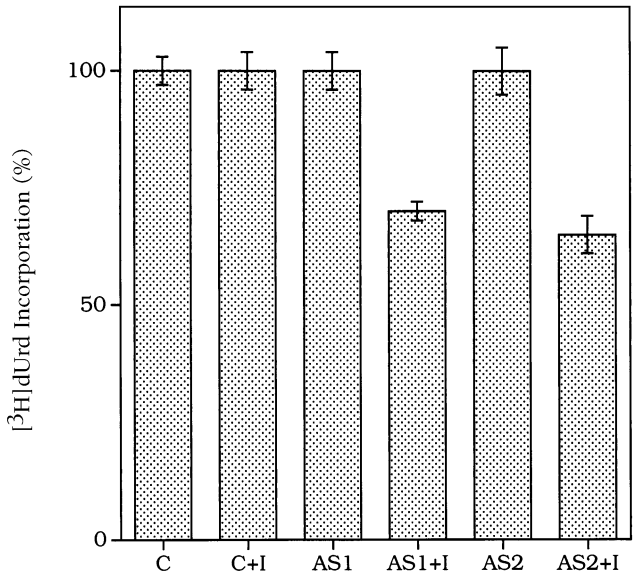

FIG. 6. Effects of FPGS down-regulation on intracellular TS activity. Ten million CEM (C) and two FPGS antisense transfected cell lines (AS1 and AS2) were treated with 5 mM of IPTG (I) for 72 hr. Cells were then exposed to [^{3}H]dUrd to determine intracellular TS activity through incorporation of radioactivity into DNA. Results are presented in terms of percentages where 100% represents 29,040 cpm/μg of DNA. Values represent the means ± SEM from 3 separate experiments.

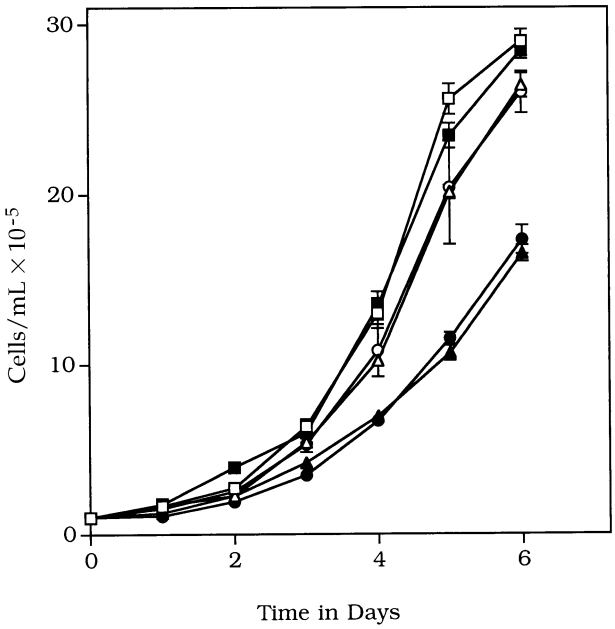

FIG. 7. Effects of FPGS antisense expression on the growth of CEM cells. Ten million IPTG treated (■) and untreated (□) sets of control cells and two IPTG-treated (▲, ●) and -untreated (△, ○) sets of transfected cells were grown for 6 days in standard RPMI-1640 medium with repleting every 72 hr. Values represent the means ± SEM from 3 separate experiments.

DISCUSSION

Because FPGS regulates polyglutamylation and thus retention of intracellular folates, it has been proposed as an anticancer drug target [1–3]. However, this concept has been difficult to directly demonstrate in a quantitative manner with FPGS inhibitors because their intracellular specificity for FPGS versus other folate-metabolizing enzymes cannot be assured [8–10]. And, a FPGS negative CHO cell system transfected with sheared human DNA to elevate FPGS expression did not cause profoundly diminished proliferation [7]. In this report, we address the concept through use of an antisense expression system to directly suppress intracellular FPGS activity.

The antisense expression system developed for these studies has allowed several questions related to proliferation to be addressed. First, the extent to which intracellular reduced folate content depends upon FPGS activity has not been clear. Longer chain length polyglutamates have been shown to be preferentially retained [1–3], but whether modest changes in FPGS activity can influence folate pool levels has been somewhat uncertain. In a sheared human DNA transfection system with AUXB1 cells, Shane and coworkers were only able to demonstrate an effect of FPGS level on intracellular folate content at high medium folate levels [7]. In this study, we have demonstrated that the folate content of CCRF-CEM transfectants was responsive to the level of FPGS activity at both low and high medium folate levels. In medium that contained 2.3 μM of FA (standard RPMI-1640), and in folate-depleted medium, steady-state folate content in antisense-induced cells was only approximately half that in control cells. In both instances, folate content was almost directly proportional to FPGS activity. These results strongly support the concept that FPGS-mediated polyglutamylation is a powerful determinant of homeostasis between intra- and extracellular folate pools. An additional outcome of this study is demonstration that there is little influence of FPGS on intracellular folate pool distribution, at least over the limited suppression of folate content examined in this system. That is, as total folate content falls in response to lower FPGS levels, each pool declines to nearly an equivalent extent so that the relationship between individual pools is not altered.

It has been shown that TS displays a distinct preference for longer chain length polyglutamates of CH_2FH_4 [28, 29]. Consistent with this, the results in this study show that intracellular TS activity in FPGS antisense transfected cells was about 70% that in control cells. However, because CH_2FH_4 levels were also lower, it is not possible to assign an unequivocal role to polyglutamate status versus cofactor level.

In summary, FPGS was examined as a potential anticancer drug target. Inducible antisense transfection permitted down-regulation, which allowed demonstration that lowering FPGS activity by about 50% causes significantly slower intracellular polyglutamylation of newly introduced folate, lower steady-state folate content, diminished thymidylate synthesis, and cellular proliferation. Thus, based on these studies, FPGS appears to be a suitable target for the development of anticancer agents.

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References

1. McGuire JJ and Coward JK, Pteroylpolyglutamates: Biosynthesis, degradation, and function. In: *Folates and Pterins* (Eds. Blakeley RL and Benkovic SJ), Vol. 1, pp. 135–190. John Wiley, New York, 1984.
2. Shane B, Folylpolyglutamate synthesis and role in the regulation of one-carbon metabolism. *Vitam Horm* **45:** 263–335, 1989.
3. Cichowicz DJ and Shane B, Mammalian folylpoly-γ-glutamate synthetase. 1. Purification and general properties of the hog liver enzyme. *Biochemistry* **26:** 504–512, 1987.
4. Priest DG and Bunni MA, Folates and folate antagonists in cancer chemotherapy. In: *Folate in Health and Disease* (Ed. Bailey LB), pp. 379–403. Marcel Deckker, New York, 1995.
5. Allegra CJ, Antifolates. In: *Cancer Chemotherapy: Principles and Practice* (Eds. Chabner BA and Collins JM), pp. 110–153. J.B. Lippincott Co., Philadelphia, 1990.
6. Osborne CB, Lowe KE, and Shane B, Regulation of folate and one-carbon metabolism in mammalian cells. I. Folate metabolism in Chinese hamster ovary cells expressing *Escherichia coli* a human folypoly-γ-glutamate synthetase activity. *J Biol Chem* **268:** 21657–21664, 1993.
7. Lowe KE, Osborne CB, Lin B-F, Kim J-S, Hsu J-C and Shane B, Regulation of folate and one-carbon metabolism in mammalian cells. II. Effect of folylpoly-γ-glutamate synthetase substrate specificity and level on folate metabolism and folylpoly-γ-glutamate specificity of metabolic cycles of one-carbon metabolism. *J Biol Chem* **268:** 21665–21673, 1993.
8. McGuire JJ, Hsieh P, Franco CT and Piper JR, Folylpolyglutamate synthetase inhibition and cytotoxic effects of methotrexate analogs containing 2,ω-diaminoalkanoic acids. *Biochem Pharmacol* **35:** 2607–2613, 1986.
9. Rosowsky A, Freisheim JH, Moran RG, Solan VC, Bader H, Wright JE and Radike-Smith M, Methotrexate analogues. 26. Inhibition of dihydrofolate reductase and folylpolyglutamate synthetase activity and *in vitro* tumor cell growth by methotrexate and aminopterin analogues containing a basic amino acid side chain. *J Med Chem* **29:** 655–660, 1986.
10. Sanghani PC, Jackman A, Evans VR, Thornton T, Hughes L, Calvert AH and Moran RG, A strategy for the design of membrane-permeable folylpoly-γ-glutamate synthetase inhibitors: "Bay-region"-substituted 2-desamino-2-methyl-5,8-dideazafolate analogs. *Mol Pharmacol* **45:** 341–351, 1994.
11. Pinter K, Davisson VJ and Santi DV, Cloning, sequencing, and expression of the *Lactobacillus casei* thymidylate synthase gene. *DNA* **7:** 235–241, 1988.
12. Mathews RG, Methylenetetrahydrofolate reductase from pig liver. *Methods Enzymol* **122:** 372–381, 1986.
13. Rios-Orlandi EM, Zarkadas CG and MacKenzie RE, Formyltetrahydrofolate dehydrogenase-hydrolase from pig liver: Simultaneous assay of the activities. *Biochim Biophys Acta* **871:** 24–35, 1986.
14. Hopkins S and Schirch V, 5,10-Methenyltetrahydrofolate synthetase. Purification and properties of the enzyme from rabbit liver. *J Biol Chem* **259:** 5618–5622, 1984.
15. Dunlap RB, Harding NGL and Huennekens FM, Thymidylate synthetase from amethopterin-resistant *Lactobacillus casei*. *Biochemistry* **10:** 88–97, 1971.
16. Moran RG, Spears CP and Heidelberger C, Biochemical determinants of tumor sensitivity to 5-fluorouracil: Ultrasensitive methods for the determination of 5-fluoro-2′-deoxyuridylate, 2′-deoxyuridylate, and thymidylate synthetase. *Proc Natl Acad Sci USA* **76:** 1456–1460, 1979.
17. Foley GF, Lazurus H, Farber S, Uzman BG, Boone BA and McCarthy RE, Continuous culture of lymphoblasts from peripheral blood of a child with acute leukemia. *Cancer Res* **18:** 522–529, 1965.
18. Garrow TA, Admon A and Shane B, Expression cloning of a human cDNA encoding folylpoly(γ-glutamate) synthetase and determination of its primary structure. *Proc Natl Acad Sci USA* **89:** 9151–9155, 1992.
19. Antonsson B, Barredo J and Moran RG, A microassay for mammalian folylpolyglutamate synthetase. *Anal Biochem* **186:** 8–13, 1990.
20. Liu Y, Bhalla K, Hill C and Priest DG, Evidence for involvement of tyrosine phosphorylation in taxol-induced apoptosis in a human ovarian tumor cell line. *Biochem Pharmacol* **48:** 1265–1272, 1994.
21. Chomczynski P and Sacchi N, Single-step of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162:** 156–159, 1987.
22. Priest DG and Doig MT, Tissue folate polyglutamate chain length determination by electrophoresis as thymidylate synthase–fluorodeoxyuridylate ternary complexes. *Methods Enzymol* **122:** 313–319, 1986.
23. Romanini A, Lin JT, Niedzwiecki D, Bunni MA, Priest DG and Bertino JR, Role of folylpolyglutamates in biochemical modulation of fluoropyrimidines by leucovorin. *Cancer Res* **51:** 789–793, 1991.
24. Santi DV, McHenry CS and Perriard ER, A filter assay for thymidylate synthetase using 5-fluoro-2′-deoxyuridylate as an active site titrant. *Biochemistry* **13:** 467–470, 1974.
25. Bunni M, Doig MT, Donato H, Kesavan V and Priest DG, Role of methylenetetrahydrofolate depletion in methotrexate-mediated intracellular thymidylate synthesis inhibition in cultured LI210 cells. *Cancer Res* **48:** 3398–3404, 1988.
26. Schmitz JC, Grindey GB, Schultz RM and Priest DG, Impact of dietary folic acid on reduced folates in mouse plasma and tissues. Relationship to dideazatetrahydrofolate sensitivity. *Biochem Pharmacol* **48:** 319–325, 1994.
27. Priest DG, Bunni M and Sirotnak FM, Relationship of reduced folate changes to inhibition of DNA synthesis induced by methotrexate in L1210 cells *in vivo*. *Cancer Res* **49:** 4204–4209, 1989.
28. Kisliuk RL, Gaumont Y, Lafer E, Baugh CM and Montgomery JA, Polyglutamyl derivatives of tetrahydrofolate as substrates for *Lactobacillus casei* thymidylate synthase. *Biochemistry* **20:** 929–934, 1981.
29. Kisliuk RL, Pteroylpolyglutamates. *Mol Cell Biochem* **39:** 331–345, 1981.