Biochemical and Biophysical Research Communications 388 (2009) 79–85

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Functional elements in the minimal promoter of the human proton-coupled folate transporter

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ARTICLE INFO

*Article history:*
Received 22 July 2009
Available online 28 July 2009

*Keywords:*
Folates
Transport
Hereditary folate malabsorption
Promoter
Gene expression

ABSTRACT

The proton-coupled folate transporter (PCFT) is the dominant intestinal folate transporter, however, its promoter has yet to be revealed. Hence, we here cloned a 3.1 kb fragment upstream to the first ATG of the human PCFT gene and generated sequential deletion constructs evaluated in luciferase reporter assay. This analysis mapped the minimal promoter to 157 bp upstream to the first ATG. Crucial GC-box sites were identified within the minimal promoter and in its close vicinity which substantially contribute to promoter activity, as their disruption resulted in 94% loss of luciferase activity. We also identified upstream enhancer elements including YY1 and AP1 which, although distantly located, prominently transactivated the minimal promoter, as their inactivation resulted in 50% decrease in reporter activity. This is the first functional identification of the minimal PCFT promoter harboring crucial GC-box elements that markedly contribute to its transcriptional activation via putative interaction with distal YY1 and AP1 enhancer elements.

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## Introduction

Reduced folates serve as one-carbon donors in the *de novo* biosynthesis of purines and thymidylate and are therefore essential for DNA replication [1]. Folates are divalent anions under physiological pH and hence cannot traverse biological membranes [1,2], consequently, three transport systems can accommodate folate uptake [1,3] including the reduced folate carrier (RFC/SLC19A1), folate receptors (FRs) and the recently discovered proton-coupled folate transporter (PCFT/SLC46A1) [4]. PCFT displays optimal transport activity at acidic pH, and recognizes various folate and antifolate substrates with high affinities ($K_m$ = 0.3–5 μM) [5–7]. PCFT plays a seminal role in intestinal folate absorption and folate uptake across the choroid plexus-cerebrospinal fluid barrier [4,8]. Inactivating PCFT mutations were recently shown to underlie the autosomal recessive disease hereditary folate malabsorption (HFM) [4,9,10]. HFM is caused by impaired intestinal folate absorption with folate deficiency characterized by anemia, hypoimmunoglobulinemia and recurrent infections. However, although PCFT plays a central role in cellular folate physiology and pathological states associated with folate uptake deficiency [1,8], its promoter remains unidentified. Here we show that the minimal promoter of the human PCFT resides within a 157 bp region upstream to the first ATG which is transactivated by GC-box sites. We further identified remote enhancer elements including YY1 and AP1 which might interact with GC-box sites present in the minimal PCFT promoter, thereby resulting in transcriptional activation.

## Materials and methods

*Cell culture.* Human cervical carcinoma HeLa cells were maintained in RPMI-1640 medium (Invitrogen, GIBCO®) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin (Biological Industries) in a humidified atmosphere of 5% $CO_2$.

*Cloning of the 3.1 kb human PCFT promoter region and sequential deletion constructs.* Detailed in Supplemental data.

*Mutational analysis of specific nuclear factor binding elements.* Introduction of point mutations in the consensus sites of GC-box, AP2, YY1 and AP1 was performed with the QuikChange XL site directed mutagenesis kit according to the instructions of the manufacturer (Stratagene Inc.), using oligonucleotides described in Supplemental Table 1. Deletion mutations were introduced when mutating three adjacent sites at once. Due to the high complexity of the sequence surrounding the first ATG and the presence of a dense CpG island which makes it impossible to use the QuikChange kit, a 400 bp PCFT fragment containing point mutations in all five GC-box sites and one containing point mutations in all six AP2 sites (Fig. 1) were synthesized by GeneScript services (Piscataway, NJ, USA). Nucleotide sequences (Hy-labs services) of these constructs (termed construct C and E, respectively) are depicted in Supplemental data. To generate construct H, the pGL3-3.1 kb plasmid

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doi:10.1016/j.bbrc.2009.07.116

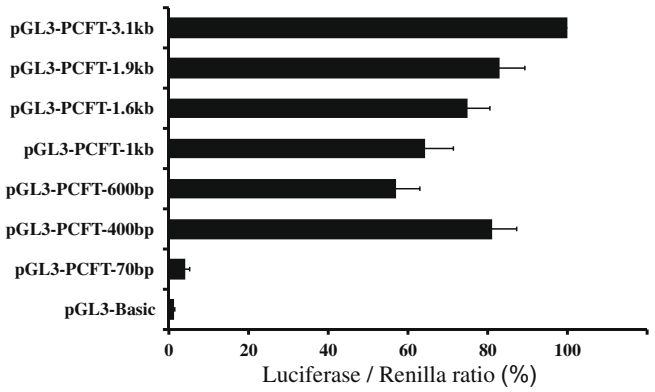


**Fig. 1.** Sequential deletion analysis of the human PCFT promoter. Sequential deletion constructs of the pGL3-PCFT-3.1 kb vector were generated and tested for their ability to drive luciferase activity. Results are presented as a firefly luciferase/*Renilla* luciferase ratio (%), normalized to the pGL3-PCFT-3.1 kb vector set as 100%. Results in the entire paper are the means of at least three independent experiments performed in duplicates ± SD.

was digested with NheI and MluI to allow the removal of 343 bp, following which the plasmid was self-ligated. Constructs F, G and I were generated by replacing the 400 bp fragment in the pGL3-3.1 kb plasmid located between NheI and NcoI by the same fragment from either construct C, E or A, respectively. All mutations were verified by DNA sequencing.

*Transient transfections with pGL3-PCFT expression vectors and luciferase reporter gene assay.* Cells were grown in 24-mm dishes for 24 h, following which they were transiently co-transfected with the above pGL3-PCFT constructs along with pRL-O control plasmid (*Renilla* luciferase) using the jetPEI transfection reagent (Polyplus-transfection) according to the instructions of the manufacturer. Cells were harvested 24 h after transfection and processed for luciferase and *Renilla* activities using the Dual-Luciferase Reporter Assay System (Promega) as described by the manufacturer. The results in the entire paper are the means of at least three independent experiments performed in duplicates ± SD.

*Electrophoretic mobility shift assay and antibody-mediated supershift analysis.* Nuclear extracts were prepared as previously described [11]. DNA–protein complexes were formed as detailed elsewhere [12] using the oligonucleotides described in Supplemental Table 2. Competition experiments were performed with a 100-fold molar excess of non-radiolabeled oligonucleotides. For supershift analysis, an aliquot of nuclear proteins (6 μg) was incubated for 20 min on ice with 4 μg of either anti-YY1 or anti-IgG antibody (Santa Cruz Biotechnology) prior to the formation of protein–DNA complexes. Complexes were resolved by electrophoresis on 6% non-denaturing polyacrylamide gels in Tris borate–EDTA, pH 8.4, at 4 °C. Gels were then dried and DNA–protein complexes were visualized by phosphorimaging. EMSA gels shown in the entire paper are representative of at least three independent experiments.

## Results and discussion

### *Deletion analysis and identification of the core PCFT promoter*

Despite the central role PCFT plays in intestinal folate absorption, its promoter has not been revealed. As a first step towards this end, we first used bioinformatics programs designed to identify promoter regions including "Genomatix-MatInspector" and "Web promoter scan service"; these programs predicted the human PCFT promoter to be either 564 or 250 bp upstream to the first ATG, respectively. We therefore cloned a large 3.1 kb fragment, upstream to the translation initiation site, into a pGL3-Basic vector (Nucleotide sequence of the 3.1 kb fragment is shown in Supplemental materials and methods). Sequential deletion analysis was undertaken (Fig. 1) using HeLa cells as recipient cells for transfections based on their high level expression of PCFT mRNA [4,13]. HeLa cells were transiently co-transfected with various promoter constructs (described in Supplemental materials and methods) along with the control *Renilla* plasmid followed by luciferase reporter assay. The 70 bp construct displayed only marginal luciferase activity (~4%), that was comparable to the background activity obtained with the empty pGL3-Basic vector (Fig. 1). In contrast, the 400 bp construct, predicted by both bioinformatics programs to contain the PCFT promoter region, displayed 80% of the luciferase activity obtained with the 3.1 kb PCFT construct. Addition of a 200 bp fragment as present in the 600 bp construct resulted in 30% decrease in luciferase activity, relative to the 400 bp fragment, hence suggesting the presence of transcriptional repressors in this 200 bp fragment. Interestingly, however, further gradual elongation of the 600 bp construct to 1, 1.6 and 1.9 kb resulted in a stepwise augmentation in reporter activity such that the latter construct displayed luciferase activity that was comparable to that obtained with the 400 bp construct (Fig. 1). Hence, this incremental increase in luciferase activity suggests the gradual addition of stimulatory binding sites. Consistent with the bioinformatics predictions, this initial deletion analysis suggests that the minimal promoter region is contained within a 343 bp fragment located between nucleotides −70 and −400, relative to the first ATG.

### *Multiple GC-box elements markedly contribute to the transcriptional activity of the minimal promoter*

Bioinformatics analyses suggested the presence of five consensus GC-box elements and six consensus AP2 motifs in the putative promoter (Fig. 2A). To determine the functionality of these motifs, EMSA was performed using the authentic promoter sequences of these *cis*-acting elements along with nuclear extracts from HeLa cells (Fig. 2B); EMSA with GC-box sequences 1 and 3–5 revealed specific nuclear protein–oligonucleotide complexes (designated **a** and **b**) which were eliminated upon competition with excess of the corresponding nonradioactive oligonucleotides (Fig. 2A and B, compare lanes 1 and 9 to 2 and 10, respectively); whereas, GC-box 2 showed barely detectable binding (Fig. 2A and B, lane 5). Furthermore, competition with excess consensus GC-box oligonucleotide completely eliminated protein–oligonucleotide complexes **a** and **b** in GC-box 1 and 3–5 (Fig. 2B, compare lanes 1 and 9 to lanes 3 and 11, respectively), whereas the mutant GC-box oligonucleotide failed to do so (Fig. 2B, compare lanes 1 and 9 to lanes 4 and 12). No binding or competition was obtained in EMSA experiments with the six AP2 sites (data not shown). Hence GC-box sites 1 and 3–5 appear to be functional elements. To determine whether these *cis*-acting elements can actually drive reporter gene expression, we mutated or deleted individual or all consensus GC-box and AP2 sites either in the context of the 400 bp or the 3.1 kb constructs and determined their luciferase reporter activity (Fig. 2C). Relative to the luciferase activity of the 400 bp construct, a 10 bp deletion eliminating the cluster of three GC-box sites (i.e., 3–5) and the sixth AP2 site (construct A) resulted in a ~30% decrease in luciferase activity. A further inactivating point mutation in GC-box site 1 (construct B) resulted in an additional ~30% decrease in reporter activity, thereby amounting to a total decrease of 60%, relative to the 400 bp construct. Whereas, mutational inactivation of all five GC-box sites (construct C) had no additional deleterious effect, hence exhibiting 53% of control luciferase activity. These results are consistent with the EMSA experiments hence indicating that GC-box sites 1 and 3–5 have both binding and transcriptional capacity, whereas GC-box site 2 lacks these capabilities.

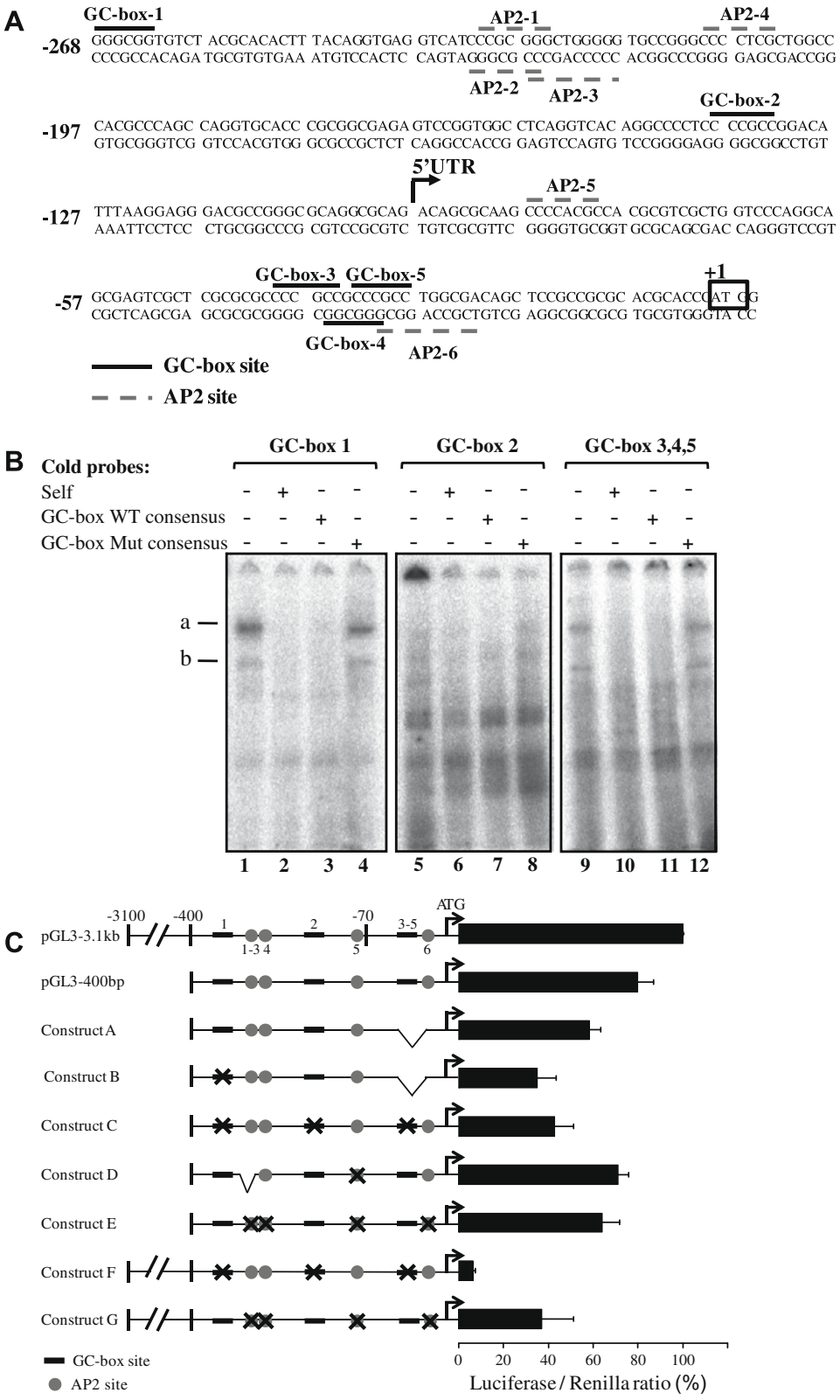


**Fig. 2.** Functional analysis of putative transcription factor binding sites in the basal promoter. (A) Localization of potential GC-box and AP2 binding sites within the 5′-flanking region of the PCFT gene, as predicted by bioinformatics analyses. (B) EMSA using nuclear extract from HeLa cells and oligonucleotides containing the authentic sequence of GC-box sites from the proximal promoter region. DNA–protein complexes (**a** and **b**) were resolved by electrophoresis and examined by a Phosphorimager. Molar excess competitions (100-fold) were performed using nonradioactive probes. (C) Luciferase reporter gene assay in HeLa cells following co-transfection with pRL-O *Renilla* plasmid and various PCFT promoter constructs; pGL3-3.1 kb or pGL3-400 bp harboring point mutations (indicated by "X") and/or deletion (indicated by a triangular gap) of the indicated GC-box (black rectangles) or AP2 sites (grey circles). Results are presented as a luciferase/*Renilla* ratio (%), normalized to the pGL3-3.1 kb vector assigned as 100%.

We next explored the contribution of the AP2 sites to the transactivation of the PCFT promoter (Fig. 2C); a construct containing a 10 bp deletion of the cluster of three AP2 sites 1–3 and an additional point mutation in AP2 site 5 (construct D) displayed only a modest decrease (11%) in luciferase activity. Consistently, inactivating point mutations eliminating all six AP2 sites (construct E) resulted in a relatively small, yet statistically significant, cumulative loss of 20% in luciferase activity ($P = 0.0007$), relative to the 400 bp construct. Both the luciferase reporter assays as well as the EMSA experiments demonstrate a modest contribution of the AP2 elements to promoter activity.

Due to the functional role of the GC-box sites, and to a lesser extent the AP2 sites in the minimal promoter, we explored their impact in the context of the 3.1 kb construct (Fig. 2C). Inactivation of all five GC-box sites in the 3.1 kb construct (construct F) revealed a dramatic loss of 94% in luciferase activity. Moreover, mutating all six AP2 sites in the context of the 3.1 kb construct (construct G) decreased luciferase activity by 63%; these marked decreases in reporter activity exceeded our predictions as mutating the GC-box or AP2 sites in the 400 bp context showed only ~50% and 20% decrease, respectively. These results may be indicative of the presence of (a) transcriptional suppressor elements upstream to the 400 bp fragment (as suggested above for the 600 bp construct) and/or (b) Sp1 or AP2 family members which bind to their proximal binding sites and are crucial for the transactivation capacity of factors that bind to upstream enhancer elements.

### *The minimal PCFT promoter is a 157 bp fragment upstream to the translation initiation site*

To provide further evidence that the 343 bp fragment residing between nucleotides −400 and −70 defined in the deletion analysis, contains the minimal PCFT promoter, a pGL3-3.1 kb construct lacking this region was generated (construct H) and tested for its ability to drive luciferase reporter activity (Fig. 3). As would be expected from a construct lacking the putative core promoter region, only background (4.5%) luciferase activity was observed, hence being comparable to that observed with the 70 bp construct (Fig. 1). This result suggests that the remaining ~2.7 kb fragment upstream to the core promoter consists of possible enhancer/repressor elements that have no autonomous transcriptional capacity but in the same time may readily interact with proximal *cis*-acting elements in the minimal promoter region. Since the cluster of GC-box sites 3–5 (Fig. 2A), in the 70 bp fragment, displayed significant transcriptional activity in the 400 bp construct (Fig. 2C, construct A) and although by itself has diminutive luciferase activity (Fig. 1), we examined its contribution to the luciferase activity of the 3.1 kb fragment; a 3.1 kb construct harboring a deletion of this cluster (construct I) showed an 88% drop in reporter activity (Fig. 3), hence recapitulating the deleterious effect achieved by the 343 bp deletion. Since this GC-box cluster has a crucial role in driving luciferase reporter activity, we expanded the 343 bp fragment to contain this cluster, hence becoming 400 bp long.

After verifying the contribution of the GC-box and AP2 sites to the promoter activity, we pin-pointed the minimal promoter in this 400 bp region. Sequential deletions were introduced based on the positions of the GC-box and AP2 sites, the first of which is located 271 bp upstream to the first ATG (Fig. 2A); three small constructs designated pGL3-271, pGL3-257 and pGL3-157 were generated (Fig. 3). pGL3-271, harboring all five GC-box sites and six AP2 sites, exhibited a 36% increase in luciferase activity relative to the 400 bp construct ($P = 0.00000001$, Fig. 3), thus being transcriptionally comparable to the entire 3.1 kb construct; this increase in transcriptional activity suggests the presence of repressor elements in the 130 bp fragment that was deleted. An additional deletion of only 14 bp, containing GC-box site 1, resulted in a 40% decrease in reporter activity, relative to the 271 bp construct (pGL3-257; Fig. 3), hence corroborating the functionality of this site. Deletion of an additional 100 bp, therefore eliminating AP2 sites 1–4, had no further affect (Fig. 3, pGL3-157), thus demonstrating the negligible autonomous effect of the AP2 sites on promoter activity. The 70 bp fragment is devoid of autonomous activity (~4% reporter activity, Figs. 1 and 3), however, it is crucial for promoter activity in the 3.1 kb fragment (construct I, Fig. 3). Collectively, these results establish that the PCFT minimal promoter is contained within 157 bp exhibiting 64% luciferase activity of the entire 3.1 kb fragment.

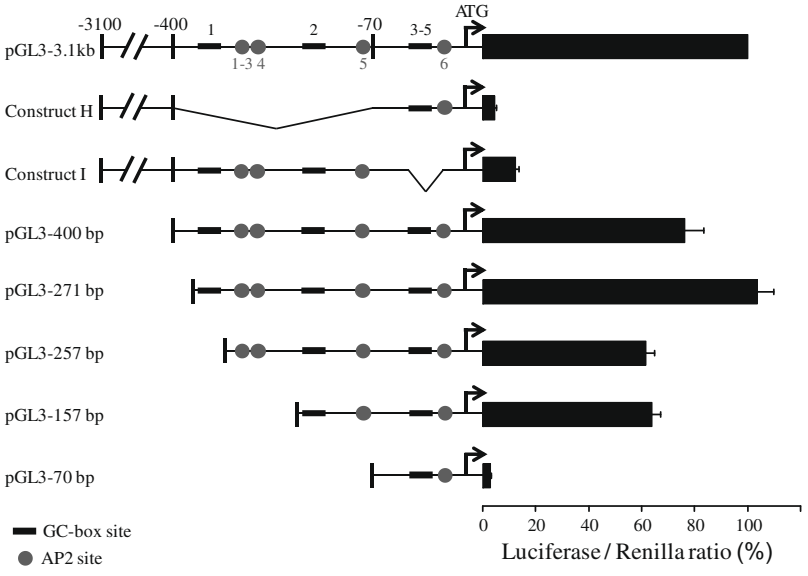


**Fig. 3.** Identification of the minimal PCFT promoter. Luciferase reporter gene assay in HeLa cells following transient co-transfection with pRL-O plasmid and various PCFT promoter constructs; pGL3-3.1 kb harboring deletions (indicated by a triangular gap) in the basal promoter or sequential deletions of the pGL3-400 bp. GC-box sites are represented by black rectangles, whereas AP2 sites are represented by grey circles. The results are presented as a luciferase/*Renilla* ratio (%), normalized to the pGL3-PCFT-3.1 kb vector assigned as 100%.

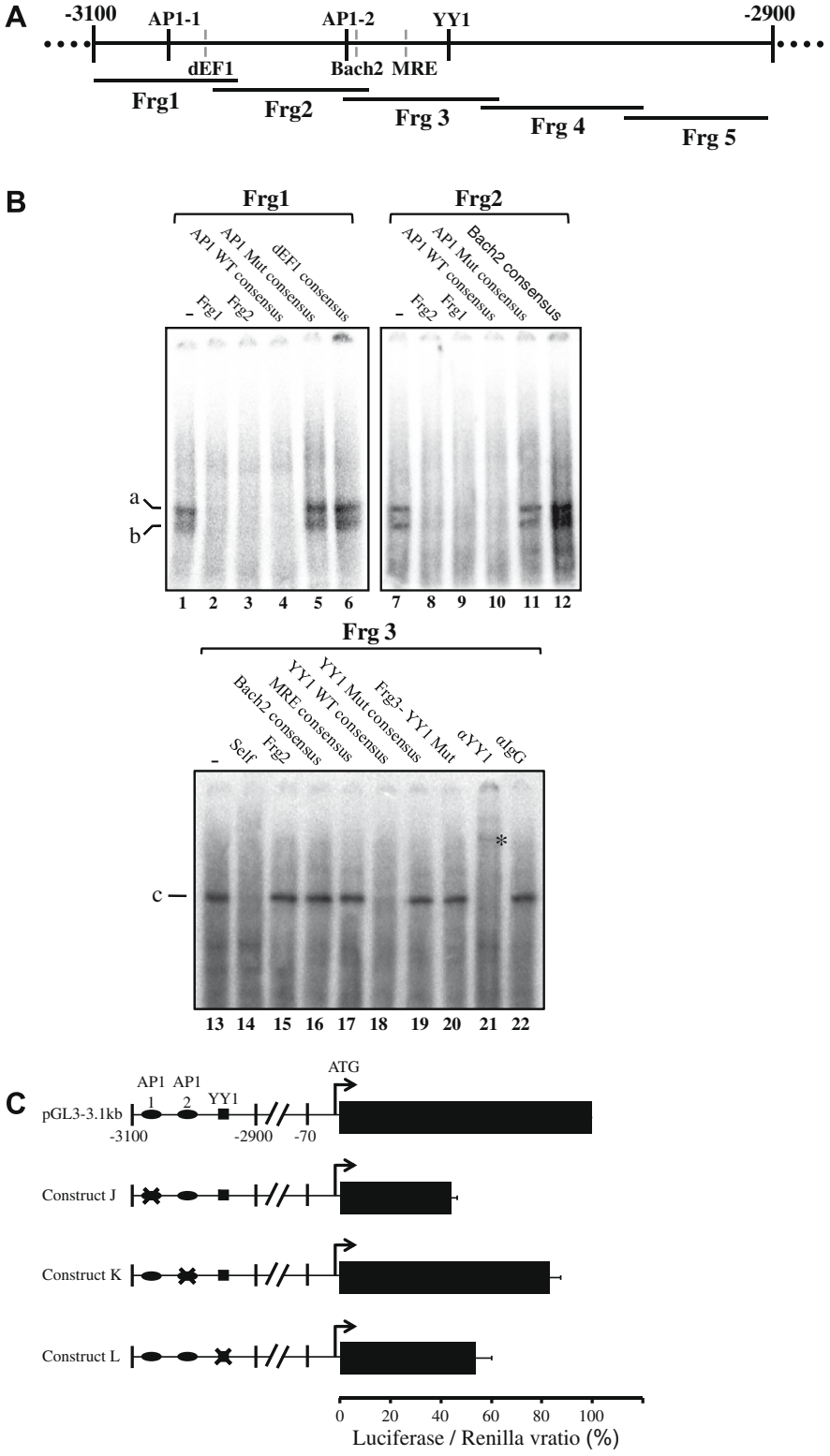


**Fig. 4.** Functional analysis of upstream YY1- and AP1-binding sites. (A) A scheme of the most upstream 207 bp fragment from the PCFT promoter with the predicted positions of potential nuclear factor binding sites. This fragment was divided into five partially overlapping sub-fragments termed fragments 1–5. (B) EMSA performed with HeLa nuclear extracts and the authentic sub-fragments 1–3. Specific complexes (**a**–**c**) and the high molecular weight complex formed using antibody-dependent supershift analysis (denoted by an asterisk) were resolved by electrophoresis and examined by a Phosphorimager. Molar excess competitions (100-fold) were performed using the denoted nonradioactive probes. (C) Luciferase reporter assay following co-transfection of HeLa cells with pRL-O plasmid and various promoter constructs; pGL3-3.1 kb harboring inactivating point mutations (indicated by "X") in either YY1 (indicated by solid square) or AP1 (indicated by black ellipses) sites. Results are presented as a luciferase/*Renilla* ratio (%), normalized to the pGL3-PCFT-3.1 kb vector, assigned as 100%.

Previously we have shown that human leukemia cells, with silenced PCFT gene harbor dense CpG island methylation particularly at nucleotides −200 through +100 [13] – a region coinciding with our presently identified minimal promoter; 5′-deoxy-2′-azacytidine-dependent demethylation restored PCFT gene expression [13]. Hence, hypermethylation-dependent silencing of this promoter region recapitulates its functionality as a putative core promoter region crucial for gene expression.

While evaluating transcription factors associated with transactivation of genes involved in folate uptake and metabolism including RFC (SLC19A1) [14], FRα [15], folylpoly-γ-glutamate synthetase [16] and thymidylate synthase [17], one notes that these promoters along with the PCFT promoter, are GC-rich, TATA-less and GC-box-regulated. Deletion of GC-box sites from the RFC promoter resulted in 90% repression of gene expression [14]; these findings are consistent with our minimal PCFT promoter results. Hence, the common basal regulatory elements shared by these folate transporters and folate-dependent enzymes may be explained by their ubiquitous expression, thereby reflecting the absolute requirement for these housekeeping gene transcripts essential for DNA replication and cell proliferation.

*Contribution of upstream YY1 and AP1 sites to promoter activity*

Bioinformatics analysis suggested that a 207 bp fragment, located between −3.1 kb and −2.9 kb, is rich in regulatory elements (Fig. 4A); hence, this remote fragment was divided into five overlapping sub-fragments (termed fragments 1 through 5), each of ~50 bp (Fig. 4A, see Supplemental Table 2). Upon EMSA, only fragments 1, 2 and 3 displayed discrete complexes which were eliminated upon competition with excess of their respective unlabeled oligonucleotide (Fig. 4B, compare lanes 1, 7, 13 to 2, 8, 14, respectively; note complexes **a**–**c**). As fragments 1 and 2 displayed identical EMSA patterns (Fig. 4B, compare lanes 1 and 7) and since they both contain potential AP1 sites (Fig. 4A), we determined by EMSA whether these sites are functional. Fragments 1 and 2 exhibited identical, two doublet bands (i.e., bands **a** and **b**) that were eliminated upon cross-competition with nonradioactive fragments (Fig. 4B, compare lanes 1 and 7 to lanes 3 and 9), thereby indicating that they efficiently compete with each other; furthermore, AP1 consensus but not an established mutant oligonucleotide, eliminated the identical **a** and **b** complexes (Fig. 4B, lanes 4, 10 vs. lanes 5, 11). Furthermore, neither dEF1 nor Bach2 oligonucleotides (predicted to reside within fragments 1 and 2, respectively) competed out these complexes (Fig. 4B, lanes 6, 12). These results establish a direct binding of nuclear factors to the AP1 sites in fragments 1 and 2 in an upstream promoter region. EMSA experiments with fragment 3 exhibited a distinct complex which was competed with its unlabeled oligonucleotide (Fig. 4B, complex **c**, compare lanes 13 and 14), but not with unlabeled fragment 2 (Fig. 4B, compare lanes 13 and 15) or with fragments 1, 4 and 5 (data not shown). As bioinformatics analysis suggested that fragment 3 contains binding sites for Bach2, MRE and YY1 (Fig. 4A), competitions with nonradioactive consensus sites were performed; Bach2 and MRE oligonucleotides failed to eliminate complex **c** (Fig. 4B, compare lanes 13 to 16 and 17, respectively), whereas YY1 consensus oligonucleotide eliminated complex **c** (Fig. 4B, compare lanes 13 and 18). Furthermore, competition either with a mutant YY1 consensus oligonucleotide or fragment 3 harboring a mutant YY1 site failed to eliminate complex **c** (Fig. 4B, compare lane 13 to lanes 19 and 20, respectively). YY1-specific antibody, but not a control anti-IgG antibody, targeted complex **c** in a supershift assay, hence mediating a supershift to a higher molecular weight form (Fig. 4B, compare lanes 13 and 21, note the asterisk on the right side of the supershifted band). These findings establish that YY1 binds to the PCFT promoter at an upstream YY1 consensus.

Since YY1- and AP1-binding factors are known to act either as transcriptional repressors or transactivators [18–21], we used luciferase reporter assay to assess their activity in this upstream PCFT promoter region, by generating pGL3-3.1 kb-based constructs harboring inactivating point mutations either in the YY1 or one of the two AP1 sites (Fig. 4A and C). Disruption of the first or second AP1 sites (i.e., AP1-1 and AP1-2) resulted in a 56% or 20% decrease in luciferase activity, respectively (Fig. 4C, constructs J and K), relative to the wt 3.1 kb construct. Hence, AP1-1 and to a lesser extent AP1-2 site contribute to promoter activity. Moreover, mutation of the YY1 site resulted in a ~50% reduction in luciferase activity (Fig. 4C, construct L), thereby suggesting its functionality as a transcriptional activator. The collaboration between YY1 and AP1-binding proteins including FosB, JunB, and JunD has been experimentally demonstrated [18,20]. Moreover, since these point mutations lead to a lower luciferase activity than the one obtained with the 271 bp construct, one may conclude that some repressor element(s) reside along the upstream 2.7 kb sequence.

In summary, the current paper constitutes the first identification and initial characterization of the minimal PCFT promoter residing in a 157 bp proximal fragment in which crucial GC-box elements markedly contribute to transcriptional activation also via a possible interaction with distal YY1 and AP1 enhancer elements, thereby resulting in transcriptional activation of the PCFT promoter.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.07.116.

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