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Transcriptional regulation of PCFT by KLF4, HNF4α, CDX2 and C/EBPα: Implication in its site-specific expression in the small intestine

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ABSTRACT

Proton-coupled folate transporter (PCFT), which is responsible for the intestinal uptake of folates and analogs, is expressed only in the proximal region in the small intestine. The present study was to examine its transcriptional regulation, which may be involved in such a unique expression profile and potentially in its alteration, using dual-luciferase reporter assays in human embryonic kidney (HEK) 293 cells. The luciferase activity derived from the reporter construct containing the 5′-flanking sequence of −1695/+96 of the human PCFT gene was enhanced most extensively by the introduction of Krüppel-like factor 4 (KLF4). The KLF4-induced luciferase activity was further enhanced by hepatocyte nuclear factor 4α (HNF4α) synergistically. To the contrary, caudal-type homeobox transcription factor 2 (CDX2) and CCAAT/enhancer-binding protein α (C/EBPα) extensively suppressed the luciferase activity induced by KLF4 alone and also that induced by KLF4 and HNF4α. Western blot analysis using the rat small intestine indicated uniform expression of KLF4 along the intestinal tract, proximal-oriented expression of HNF4α, distal-oriented expression of CDX2 and C/EBPα. These results suggest that the activity of PCFT promoter is basically induced by KLF4 and the gradiented expression profile of PCFT may be at least in part accounted for by those of HNF4α, CDX2 and C/EBPα.

1. Introduction

Proton-coupled folate transporter (PCFT) has been recently identified as the transporter responsible for the intestinal uptake of folates and analogs that include antifolate drugs such as methotrexate [1]. Its characteristics, which feature specific affinity for reduced folates as well as folate and optimum operation driven by H^+ at acidic pH, are in agreement with those of the folate transport system that had long been hypothesized to be present in the small intestine [1–5]. Furthermore, the initial study on PCFT by Qiu et al. indicated that a loss of its function due to a homozygous mutation in its gene is responsible for hereditary folate malabsorption, underscoring its pivotal role in folate absorption [1]. It is also notable that PCFT is expressed abundantly in the upper and middle parts of the small intestine, but not at all in the lower part [3,4], and this unique expression profile is in excellent agreement with that of the activity of folate uptake, as we demonstrated in a study using rats [4]. Elucidating the mechanism regulating the unique expression profile of PCFT in the small intestine should be of interest and help understanding its potential alteration, which could have an impact on the absorption of antifolate drugs as well as folates.

Studies on PCFT have been expanding rapidly since its identification. They include those on its transcriptional regulation, which have identified Yin Yang 1 (YY1), activator protein 1 (AP1), AP2, nuclear respiratory factor 1 (NRF1) and vitamin D receptor (VDR) as transcription factors potentially involved in that [6–8]. However, all those studies are for transcriptional regulation in the cell lines of HeLa and Caco-2, and those identified transcription factors, which are present ubiquitously in various organs, are unlikely to be able to account for the specifically high expression of PCFT in the small intestine and its unique expression profile in that organ. We, therefore, attempted in the present study to identify transcription factors that could account for such a specific and unique expression profile of PCFT, assuming the involvement of those which are more specifically or abundantly expressed in the small intestine, and to elucidate the transcriptional regulation mechanism. We also examined the status of DNA methylation, because it has been reported to be an important epigenetic factor that determines the expression of PCFT in various cell lines [9,10].

2. Materials and methods

2.1. Materials

Rabbit polyclonal antibody for Krüppel-like factor 4 (KLF4), rabbit polyclonal antibody for CCAAT/enhancer-binding protein α (C/

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EBPα) and rabbit polyclonal antibody for caudal-type homeobox transcription factor 2 (CDX2) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), rabbit polyclonal antibody for hepatocyte nuclear factor 4α (HNF4α) was from Abnova (Taipei, ROC), mouse monoclonal anti-β-actin was from Sigma–Aldrich (St. Louis, MO, USA) and goat anti-rabbit IgG (Peroxidase AffiniPure Goat Anti-Rabbit IgG (H+L)) was from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). All other reagents were of analytical grade and commercially obtained.

2.2. Animals

Male Wistar rats, weighing about 300 g, were used without fasting in experiments to assess the expression of transcription factors and the status of DNA methylation in the small intestine. All the experiments were conducted with the approval of the Animal Ethics Committee of Nagoya City University Graduate School of Pharmaceutical Sciences.

2.3. Cell cultures

Human embryonic kidney (HEK) 293 cells were maintained at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

2.4. Cloning of the 5′-flanking region of the PCFT gene and preparation of deletion reporter constructs

The region spanning positions −1695 to +96 (relative to the transcription start site), which encompasses the 5′-flanking region of the human PCFT gene was cloned from the genomic DNA of HEK293 cells and incorporated into pGL4.10(luc2) vector containing the firefly luciferase reporter gene (Promega, Madison, WI, USA), as detailed in Supplementary data. Deletion constructs (−1036/+96, −526/+96, −243/+96, −185/+96, −94/+96, −24/+96) were prepared from −1695/+96 construct, as also detailed in Supplementary data.

2.5. Cloning of transcription factors

HNF1α, HNF4α, HNF4γ, GATA binding protein 4 (GATA4), CDX2, C/EBPα, KLF4, KLF5 and KLF13 were cloned, as detailed in Supplementary data.

2.6. Luciferase assay

HEK293 cells were seeded on 96-well plates coated with poly-L-lysine (4.0×10^4 cells/100 μl/well), transfected with one of the reporter plasmids and one or a few plasmids carrying the cDNA of a transcription factor by lipofection using HilyMax (Dojindo Laboratories, Kumamoto, Japan) as a transfection reagent, according to the manufacturer's instructions, and cultured for 48 h for transient expression before luciferase assays. The cells were transfected with 200 ng/well of total plasmids of a reporter construct and transcription factors at a constant ratio in each set of experiments. The total amount of plasmids was kept constant by the addition of an appropriate amount of the empty pCI-neo vector when fewer transcription factors were introduced into the cells. The cells were also cotransfected with 20 ng/well of pGL4.74 (hRluc/TK) vector (Promega) for the expression of *Renilla* luciferase as an internal control of transfection efficiency.

Firefly and *Renilla* luciferase activities were determined using the Dual-Glo Luciferase Assay System (Promega), according to the manufacturer's protocol, and an ARVO MX plate reader (PerkinElmer Life Sciences, Boston, MA, USA) for the detection of luminescence. The firefly luciferase activity was normalized to the *Renilla* luciferase activity.

Because HEK293 cells have little PCFT expressed [2], we assumed that they are transcriptionally inactive for PCFT in practice and can be used for the investigation of its transcriptional regulation, using exogenously introduced PCFT promoter and transcription factors. To assess the effect of transcription factors on the promoter activity, the firefly luciferase activity, which was normalized to the *Renilla* luciferase activity, was evaluated in terms of the relative one further normalized to the basal activity in the absence of exogenously introduced transcription factors.

2.7. Western blot analysis

The 5 or 10 cm sections of duodenum, jejunum, midgut and ileum were isolated from male Wistar rats and the expression profiles of KLF4, HNF4α, CDX2, and C/EBPα proteins were examined by Western blotting, as detailed in Supplementary data.

2.8. ChIP assay

Chromatin immunoprecipitation (ChIP) assay was performed to examine the binding of KLF4 to the region between −209 and −18, which was suggested to include a promoter element for KLF4 binding, of the human PCFT promoter, as detailed in Supplementary data.

2.9. DNA methylation analysis

Genomic DNA (10 μg) was extracted from the jejunum and ileum of the male Wistar rats and DNA methylation was assessed by a bisulfite DNA sequencing method, as detailed in Supplementary data.

2.10. Data analysis

Experimental data are presented as the means ± SE, and statistical analysis was performed using two-tailed, unpaired Student's *t*-test or, when multiple comparisons were needed, analysis of variance (ANOVA) followed by Dunnett's test, with $p < 0.05$ considered significant.

3. Results

3.1. Activation of PCFT promoter by KLF4

As the first step to identify transcription factors involved in the transcriptional regulation of the expression of human PCFT in the small intestine, luciferase reporter assays were performed with the reporter construct containing −1695/+96 segment of the 5′-flanking region of the human PCFT gene in HEK293 cells, assessing changes in luciferase activity induced by cotransfection of plasmids for the expression of several transcription factors, which are known to be specifically present in the small intestine. Those tested were HNF1α, HNF4α, HNF4γ, GATA4, CDX2, C/EBPα, KLF4, KLF5 and KLF13. As shown in Fig. 1A, luciferase activity was enhanced extensively by a factor of about 16 by KLF4, compared with the basal activity in the absence of any exogenously introduced transcription factor (no factor). HNF4α also enhanced the luciferase activity, though only modestly by a factor of about 2. All the other transcription factors were, however, found not to alter the luciferase activity. These results suggest that KLF4 could be the principal activator of the PCFT promoter.

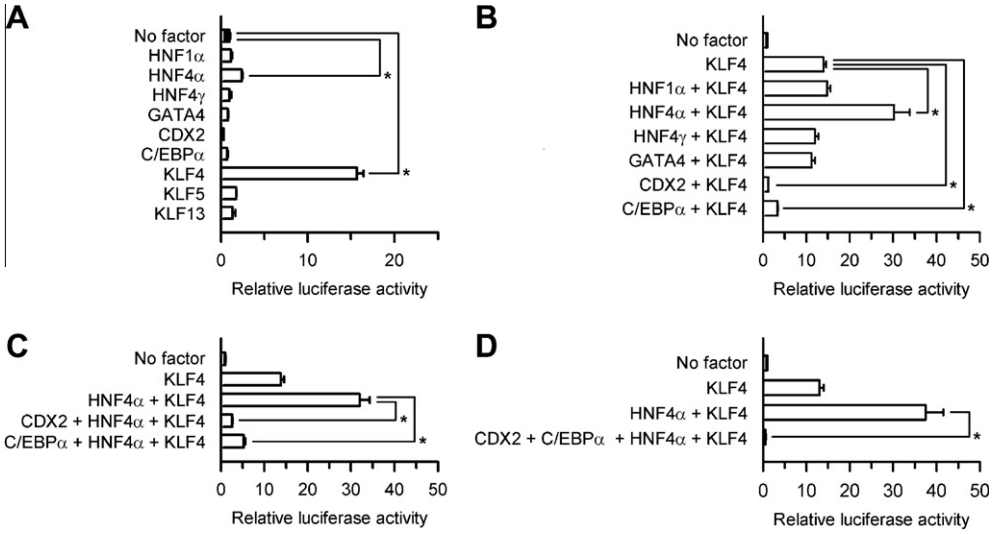

Fig. 1. (A) Effect of various transcription factors on the activity of the human PCFT promoter. HEK293 cells were cotransfected with the reporter construct (−1695/+96) and a plasmid for each transcription factor with 1:1 ratio (200 ng of total plasmid DNA). (B) Effect of various transcription factors on the KLF4-induced activity of the human PCFT promoter. HEK293 cells were cotransfected with the reporter construct (−1695/+96), a plasmid for KLF4 and a plasmid for each transcription factor with 1:1:1 ratio (200 ng of total plasmid DNA). (C) Suppression by CDX2 and C/EBPα of the synergistic activation of the PCFT promoter by KLF4 and HNF4α. HEK293 cells were cotransfected with the reporter construct (−1695/+96) and plasmids for KLF4, HNF4α and CDX2, or C/EBPα, with 1:1:1:1 ratio (200 ng of total plasmid DNA). (D) Combined suppressing effect of CDX2 and C/EBPα on the synergistic activation of the PCFT promoter by KLF4 and HNF4α. HEK293 cells were cotransfected with the reporter construct (−1695/+96), KLF4, HNF4α, CDX2 and C/EBPα with 1:1:1:1:1 ratio (200 ng of total plasmid DNA). In every set of experiments, empty pCI-neo vector was substituted for plasmids for nuclear factors when they were not used. The relative luciferase activities are presented as the means ± SE ($n = 4$). *Significantly different at $p < 0.05$.

3.2. Effect of transcription factors on the KLF4-induced activation of the PCFT promoter

Since it has been reported that various transcription factors can interact with KLF4 and modulate its activity, we examined the effect of HNF1α, HNF4α, HNF4γ, GATA4, CDX2 and C/EBPα on the KLF4-induced luciferase activity with the reporter construct of −1695/+96. As shown in Fig. 1B, the KLF4-induced luciferase activity was found to be doubled to be about 30 as the relative value from about 15 by HNF4α, indicating its synergistic cooperation with KLF4. To the contrary, CDX2 and C/EBPα were found to suppress that activity extensively to be close to the basal level, indicating their role as factors to suppress the KLF4-induced PCFT promoter activation. It is also notable that CDX2 was a little more potent in the suppression than C/EBPα. HNF1α, HNF4γ and GATA4 were, on the other hand, found not to alter that activity.

3.3. Suppression by CDX2 and C/EBPα of the synergistic activation of the PCFT promoter by KLF4 and HNF4α

It should be of interest to examine if CDX2 and C/EBPα, which suppressed the KLF4-induced activation of the PCFT promoter, could suppress its synergistic activation by KLF4 and HNF4α. As shown in Fig. 1C, each of CDX2 and C/EBPα was found to indeed suppress the synergistic induction of luciferase activity extensively to be close to the basal level, as observed in the suppression of KLF4-induced one (Fig. 1B). The combined effect of CDX2 and C/EBPα was even greater, resulting in a complete suppression of the synergistic induction of luciferase activity (Fig. 1D).

3.4. Sequential deletion analysis of the PCFT promoter for activation by KLF4 and its synergistic enhancement by HNF4α

Focusing on KLF4 as the suggested principal activator and HNF4α as a cooperating enhancer, we attempted to identify the segments of the PCFT promoter involved in its activation by these transcription factors by sequential deletion analysis. In this analysis, luciferase reporter assays were performed with a series of reporter constructs containing sequentially deleted segments of the 5′-flanking region of the PCFT gene (−1695/+96, −1036/+96, −526/+96, −243/+96, −185/+96, −94/+96, −24/+96), assessing changes in luciferase activity induced by cotransfected KLF4 alone, or with HNF4α. As shown in Fig. 2, the luciferase activity induced by KLF4 was unchanged at about 13 as the relative value when the incorporated 5′-franking region spanning to −1695 was deleted sequentially to be spanning to −1036, −526, −243, and −185. The luciferase activity was then reduced when the incorporated 5′-flanking region was further deleted to be spanning to −94, and was reduced some more to be close to the basal level when it was shortened to be spanning to −24. Therefore, it is most likely that the region of −185 to −95 contains a promoter element for

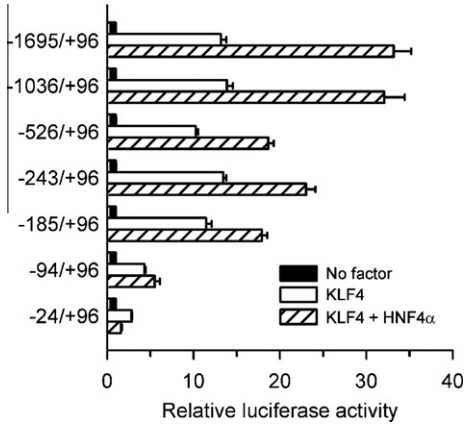

Fig. 2. Sequential deletion analysis of the human PCFT promoter for activation by KLF4 and its synergistic enhancement by HNF4α. HEK293 cells were cotransfected with one of the deleted reporter constructs and plasmids for KLF4 and HNF4α with 1:1:1 ratio (200 ng of total plasmid DNA). Empty pCI-neo vector was substituted for plasmids for nuclear factors when they were not used. The relative luciferase activities are presented as the means ± SE ($n = 4$). *Significantly different at $p < 0.05$.

KLF4 and the region of −94 to −25 may also play a role in KLF4-induced promoter activation. The synergistic enhancement of KLF4-induced luciferase activity by HNF4α was greatest (about 150% increase) for the segments of −1695/+96 and −1036/+96. It was weaker (about 70% increase) for the segments in the subsequent range (−526/+96, −243/+96, −185/+96) and was not observed for the shortest ones of −94/+96 and −24/+96. Therefore, it is most likely that the region of −1036 to −527 and −185 to −95 may include elements for HNF4α action.

3.5. Binding of KLF4 to the PCFT promoter

To further focus on the role of KLF4 as the principal activator and confirm its binding to the PCFT promoter, we performed ChIP assay. The −209/−18 segment of the PCFT gene, which includes the region suggested to contain the promoter element for KLF4 (−185 to −95), was successfully detected by amplification by polymerase chain reaction (PCR) after precipitation by antibody directed against KLF4 (Fig. 3A). The segment was also detected after precipitation by anti-histone H3 antibody (positive control), but only negligibly after precipitation by normal IgG (negative control). This result indicates the specific binding of KLF4 to an element within the designated segment of the PCFT promoter.

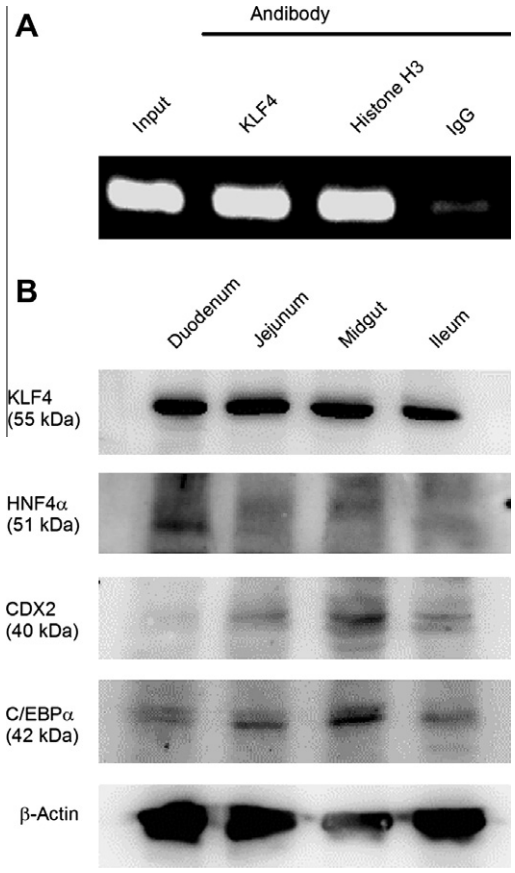

Fig. 3. (A) Specific binding of KLF4 to the human PCFT promoter. ChIP assays were performed to assess the binding of KLF4 to the human PCFT promoter (−209/−18). Cross-linked DNA–protein complexes from HEK293 cells transfected with the plasmid for −1695/+96 segment of the human PCFT gene and that for KLF4 were immunoprecipitated with anti-KLF4 antibody, anti-histone H3 antibody (positive control) and normal IgG (negative control), decross-linked and PCR-amplified with specific primers to detect the 192 bp segment. Input represents the control sample without immunoprecipitation. (B) Western blots for transcription factors in the rat small intestine. Bands for β-actin are also shown as references.

3.6. Distribution of transcription factors along the intestinal tract

To clarify the possible roles of those transcription factors that were found to regulate the transcription of the PCFT gene in the unique distribution profile of PCFT along the intestinal tract, we examined their distribution in the small intestine by Western blotting, using rat as a model animal, in which the profile of the intestinal distribution of PCFT is similar to that in human. As shown in Fig. 3B, KLF4, the principal activator, was expressed almost uniformly along the entire small intestine. The expression of HNF4α, a cooperative factor that can synergistically enhance KLF4-induced PCFT promoter activation, was highest in the duodenum and declined along the intestinal tract toward the ileum, where it was almost negligible. The expression of CDX2, a factor to suppress KLF4-induced PCFT promoter activation, was highest in the midgut, detectable at similarly lower levels in the jejunum and ileum, but not detectable in the duodenum. The expression profile of C/EBPα, another suppressing factor, was similar to that of CDX2, although it was expressed modestly also in the duodenum. Based on these results, it may be possible to assume that PCFT expression is lowest in the ileum due to the combined effect of the lack of synergistic enhancement of KLF4-induced transcription by HNF4α and of the suppression of the transcription by CDX2 and C/EBPα. It may be also that PCFT expression is high in the duodenum and jejunum due to the prevailing effect of HNF4α to enhance KLF4-induced transcription.

3.7. DNA methylation status of the PCFT promoter in the small intestine

DNA methylation of promoters at position 5 of cytosine in CpG dinucleotides is an epigenetic mechanism of gene silencing [11,12]. It has been reported for the human PCFT gene that such DNA methylation in a CpG-rich region within a CpG island of −200 to +100 plays a major role in suppressing its transcription in cell lines expressing PCFT only poorly [9]. We, therefore, assessed in the rat small intestine the status of methylation of the PCFT gene in the region of −194 to +291, which includes the corresponding major part of the CpG island involved in the regulation of the human PCFT gene, to examine if the status of DNA methylation might be involved in the expression profile of PCFT. However, DNA methylation analysis by a bisulfite DNA sequencing method revealed that the CpG-rich region has not been methylated at all practically, with only sparse and unreproducible observation of methylation signals, not only in the jejunum but also in the ileum (Fig. 4). The result indicates that DNA methylation is not involved in the unique expression profile of PCFT in the small intestine.

4. Discussion

The present study has indicated that KLF4 can be a major transcription factor that activates the promoter of the human PCFT gene. KLF4, which belongs to the family of zinc-finger transcription factors, is also known as gut-enriched Krüppel-like factor (GKLF) and highly expressed in terminally differentiated epithelial cells at the villi of the small intestine [13]. It has also been reported that KLF4 is involved as a transcriptional activator in the intestine-specific expression of various proteins, such as intestinal alkaline phosphatase [14] and sodium-dependent multivitamin transporter [15]. It is likely that PCFT is one of such proteins. Two other KLF proteins, KLF5 and KLF13, were tested for their ability to activate the PCFT promoter, but they could not, indicating that the activator activity is a specific one for KLF4 (Fig. 1A). The analysis using the sequentially deleted reporter constructs of the PCFT promoter indicated that the minimal promoter is contained within the region of

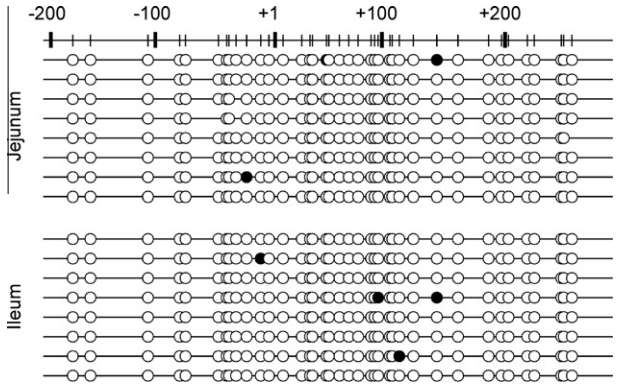

Fig. 4. Methylation status of the PCFT promoter. Genomic DNA extracted from rat jejunum and ileum was bisulfite-treated, amplified by PCR, and then introduced into pCR2.1-TOPO vector for sequencing to determine methylation status. Each line represents a different clone, and each row represents a different CpG site. Eight independent clones were analyzed in each intestinal site. Open circles and closed circles represent the dinucleotides of unmethylated CpG (TpG) and methylated CpG, respectively.

185 bp upstream to the transcription initiation site, with a putative element for KLF4 binding in the region of −185 to −95. The direct binding of KLF4 to a segment (−209/−18) containing that region was confirmed by ChIP assay, although the specific element to which KLF4 binds has not been identified yet. We identified a consensus sequence for KLF4 binding [16] in the segment of −124 to −118 (RRGGYGY, where R = A/G and Y = C/T), and attempted to examine their role in the promoter activity by disrupting it by introduction of point mutations. However, it was unsuccessful, being unable to observe any significant mutation-induced alteration in the promoter activity (data not shown).

HNF4α is known to play a role in transcriptional regulation of various genes in the small intestine [17,18], although it was initially identified as a transcription factor required for the expression of liver-specific proteins involved in the metabolism of sugars and lipids [19,20]. Moreover, HNF4α is reportedly known to regulate type 1 iodothyronine deiodinase (DIO1) with GATA4 and KLF9, a KLF protein [21]. HNF4α, which activated the PCFT promoter only modestly, was found to interestingly enhance the KLF4-induced activation extensively in a synergistic manner. The elements involved in the synergistic enhancement by HNF4α were suggested to be present in the regions of −1036 to −527 and −185 to −95 by sequential deletion analysis (Fig. 2). However, analysis using TRANSFAC (http://www.gene-regulation.com/pub/databases.html), a sequence analysis program, indicated a consensus sequence for HNF4α in the region of −612 to −600 in the former (G/AGGNCAAAGG/TTCA/G) [22], but not in the latter. The synergistic enhancement may be caused by interaction of HNF4α with KLF4, or may require some other elements for HNF4α binding.

CDX2 and C/EBPα were found to potently suppress the KLF4-induced activation of the PCFT promoter in the presence of HNF4α as its synergistic enhancer, as well as in its absence. Although these transcription factors are well known for their role as transcriptional activators to various genes, they are also known to act as factors to suppress transcription to some genes [23,24]. Because KLF4 as the principal activator of the PCFT promoter is uniformly present all along the intestinal tract, the role of CDX2 and C/EBPα seem to be important in the lack of PCFT expression in the ileum. In addition, the gradiented expression of HNF4α, being higher in the upper small intestine and absent in the ileum, seems to be important in realizing PCFT expression in the upper small intestine. It is also notable that DNA methylation was found not to be involved in the suppression of expression in the ileum. Thus, it is likely that KLF4, HNF4α, CDX2 and C/EBPα are at least in part involved in the transcriptional regulation of the expression of PCFT in the small intestine and in determining its unique expression profile. Although the mechanism of the transcriptional regulation of PCFT is not fully clear yet, information obtained in the present study should be useful for further exploring factors and mechanisms that may alter PCFT expression in the small intestine and the absorption of folates and antifolate drugs.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.004.

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