Hepatocyte nuclear factor-4α regulates human cellular retinol-binding protein type II gene expression in intestinal cells

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**ABSTRACT**

Cellular retinol-binding protein type II (CRBPII) is abundantly expressed in the small intestinal enterocytes of many vertebrates and plays important physiological roles in intestinal absorption, transport and metabolism of vitamin A. In the present study, we investigated regulation of human CRBPII gene expression using human intestinal Caco-2 BBe cells. We found that the human CRBPII gene contained a direct-repeat 1 (DR-1)-like nuclear receptor response element in the proximal promoter region, and that endogenous hepatocyte nuclear factor-4α (HNF-4α) was a major transcription factor binding to the DR-1-like element. Co-transfection of HNF-4α expression vector transactivated the human CRBPII gene promoter activity, whereas mutation of the DR-1-like element abolished the promoter activity. Stably transfected Caco-2 BBe cells overexpressing HNF-4α significantly increased endogenous CRBPII gene expression and retinyl ester synthesis. Reduction of HNF-4α protein levels by HNF-4α siRNA decreased CRBPII gene expression. Caco-2 BBe cells treated with phorbol 12-myristate 13-acetate, a protein kinase C activator, decreased nuclear HNF-4α protein level and its binding activity to the human CRBPII gene DR-1-like element, as well as CRBPII gene expression. Moreover, nuclear HNF-4α protein levels, its binding to human CRBPII DR-1-like elements, and CRBPII gene expression level were coordinately increased during Caco-2 BBe cell differentiation. These results suggest that HNF-4α is an important transcriptional factor that regulates human CRBPII gene expression.
and provide possibility for a novel function of HNF-4α in the regulation of human intestinal vitamin A absorption and metabolism.

**KEY WORDS**: Caco-2 BBc cells; vitamin A; direct repeat-1; gene regulation
INTRODUCTION

Vitamin A plays many important roles, including vision, organ development and cell differentiation, in different organisms (25, 26). In the small intestine, all-trans retinal or retinol binds to cellular retinol-binding protein type II (CRBPII), an abundant cytosolic protein, mainly found in the small intestinal enterocytes of many vertebrates (2, 21). The retinal or retinol-CRBPII complexes are substrate for conversion into retinoic acid, a biologically active form of vitamin A, by retinal reductase (1, 11) or into retinyl esters by lecithin:retinol acyltransferase for transport of vitamin A into liver and other tissues, respectively (6). In the study of human intestinal vitamin A absorption and metabolism, the human colonic adenocarcinoma cell line Caco-2 has been used as a suitable model (15, 17, 24). Human CRBPII expression increases in differentiating Caco-2 cells and the cells synthesize and secrete retinyl esters (15, 38). Thus, CRBPII plays a pivotal role in the intestinal vitamin A absorption and metabolism pathways in both human and rodent species.

It has been reported that various nuclear receptors, such as hepatocyte nuclear factor-4α (HNF-4α), peroxisome proliferator-activated receptor (PPAR), retinoic acid receptor (RAR), and retinoid X receptor (RXR), bind as homo- or heterodimers to the direct repeat-1 (DR-1)-type nuclear receptor response elements located in the 5′-flanking region of rat and mouse CRBPII genes, and increase their transcription activity in vitro (18, 19, 34, 35). In contrast to rat and mouse CRBPII genes, it has not been investigated whether these
nuclear receptors act on the human CRBPII gene. HNF-4α belongs to the nuclear receptor superfamily and is expressed in several endoderm-derived tissues including the liver, pancreas, kidney and intestine (28). Human small intestinal enterocyte also expresses HNF-4α protein as well as differentiated Caco-2 cells (10). HNF-4α regulates various genes that encode proteins involved in lipid, carbohydrate and drug metabolism in these tissues and cells via binding of HNF-4α homodimer to DR-1 elements (5, 12, 14, 20). The expression and DNA-binding activity of HNF-4α is regulated by various phosphorylation signals including the protein kinase C (PKC)/mitogen-activated protein kinase (MAPK) cascade, a signaling pathway involved in the control of fundamental cellular processes, including growth, differentiation, survival and metabolism (8, 13, 23, 33).

In the present study, we investigated whether human CRBPII gene promoter contains the unknown binding site for nuclear receptors using Caco-2 BBe cells, a microvillus-enriched subclone of Caco-2 cells. In differentiated Caco-2 BBe cells, CRBPII and β-carotene 15,15’-monooxygenase (BCMO1) are definitely expressed, compared with parental Caco-2 cells (3, 4, 41). We demonstrated the presence of a HNF-4α homodimer binding site on the DR-1-like element located in the human CRBPII gene proximal promoter region. Reporter analysis indicated that HNF-4α transactivated the human CRBPII gene DR-1-like element in Caco-2 BBe cells. Furthermore, stably transfected Caco-2 BBe
cells overexpressing HNF-4α increased endogenous CRBPII gene expression and retinyl ester synthesis. These novel findings suggest that HNF-4α is involved in vitamin A absorption and its metabolism in the human small intestine.

MATERIALS AND METHODS

Cell culture and treatment

Caco-2 BBe cells (CRL-2102) obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) were seeded onto type-I-collagen-coated plastic plates (Asahi Techno Glass, Tokyo, Japan) and cultured at 37°C in an atmosphere of 5% CO₂. The cells were maintained in DMEM (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% FBS (Biological Industries, Kibbutz Bet-Haemek, Israel), 1% non-essential amino acid solution (Invitrogen, Carlsbad, CA, USA), 20 mM HEPES (pH 6.5), 1% antibiotic-antimycotic mixed stock solution (Nacalai Tesque, Kyoto, Japan) and 10 μg/ml transferrin (Invitrogen). In some experiments, Caco-2 BBe cells at 14 days post-confluence were incubated for 24 h in serum-free medium supplemented with 0.1% DMSO, 1 μM phorbol 12-myristate 13-acetate (PMA; LC Laboratories, Woburn, MA, USA), with or without 4 μM GF109203X (Sigma-Aldrich), a PKC inhibitor, or 10 μM Rp-cAMP (BIOMOL International, LP, PA, USA), a PKA inhibitor, after pre-treatment with each inhibitor for 3 h. In the transfection study, Caco-2 BBe cells at 10 days post-confluence
were pre-treated with 1 μM PMA for 12 hr, and transfected as described below.

Construction of expression vector and luciferase reporter vector

The pcDNA3.1-HNF-4α plasmid was generated by subcloning of the rat full-length HNF-4α2 cDNA fragment, which corresponded to nucleotides 1-1446 (GenBank Acc. no., NM_022180) (7), into pcDNA3.1 vector that contained neomycin resistance gene (Invitrogen). The phCRBPII-(DR1)2-tk-LUC luciferase reporter vector contained two copies of the DR-1-like element of human CRBPII gene in front of the herpes virus thymidine kinase promoter of ptkLUC+ vector (RIKEN BioResource Center, Tsukuba, Japan). The oligonucleotide sequence of human CRBPII DR-1-like element (underlined) was as follows:

5′-TGCTTCTGCCCTTTGAACCTCTTTATTGCTTCTGCCCTTTGAACCTCTTTATTGCCCTTTGCTTCTGCCCTTTGAACCTCTTTATTGC-3′. The human CRBPII gene promoter fragment (1016 bp) spanning nucleotides 1826-2841 (GeneBank Acc. no. AF338345) was amplified by the polymerase chain reaction (PCR) from Caco-2 BBe cells genomic DNA using a forward primer,

5′-ACATCTTGGAATAGAGTGTC-3′ and a reverse primer,

5′-CGGTGAGGGTTTGTGGTG-3′. The PCR product was ligated into pGL3-basic vector (Promega, Madison, WI, USA) to construct phCRBPIIpro-LUC vector, which contained a DR-1-like element and TATA box. Additionally, PCR-based mutagenesis was performed to introduce mutations into the DR-1-like element of phCRBPIIpro-LUC vector using
PrimeSTAR Mutagenesis Kit (TAKARA Bio, Ohtsu, Shiga, Japan). The mutated human CRBPII gene DR-1-like element primers consisted of a forward primer, 5′-TCTACTACGTCTTCCTCTTATCTTCAGAGTC-3′ and a reverse primer, 5′-GGAAGACGTAGTAGAAGCACAAACTCAAAG-3′. The mutated luciferase reporter vector was designated phCRBPIIpro(DR-1mut)-LUC.

Establishment of stably transfected cells

Caco-2 BBc cells were plated at a density of 2.4 × 10^6 cells per plate of a 10-cm type-I-collagen-coated plate in DMEM supplemented with 10% FBS. The cells were grown overnight, and transfected using FuGENE HD Transfection Reagent (Roche Diagnostics, Indianapolis, IN, USA) with 20 μg pcDNA3.1-HNF-4α or pcDNA3.1 mock vector. Forty-eight hours after transfection, the cells were trypsinized and seeded in DMEM supplemented with 10% FBS containing 1.2 mg/ml Geneticin (G418). Starting at 4 weeks post-plating, G418-resistant Caco-2 BBc cell foci were isolated using cloning rings, detached with trypsin-EDTA, and transferred to 24-well collagen-coated plates. The Caco-2 BBc cell clones were expanded, and stored at −140°C until use. To examine the effects of stable overexpression of HNF-4α on endogenous CRBPII mRNA levels, the cloned Caco-2 BBc cells were maintained in medium that contained 0.6 mg/ml G418.
**Transient transfection and dual luciferase reporter assay**

Caco-2 BBe cells were plated at a density of $1 \times 10^4$ cells per well in a 96-well type-I-collagen-coated plate in DMEM supplemented with 10% FBS. The cells were grown overnight, and transient transfection was performed using FuGENE HD Transfection Reagent with 100 ng pcDNA3.1-HNF-4α or pcDNA3.1 mock vector, 100 ng phCRBPII-(DR1)$_2$-tk-LUC, phCRBPIIpro-LUC or phCRBPIIpro(DR-1mut)-LUC vector and 2 ng phRG-tk-LUC vector (Promega) as internal *renilla* luciferase control vector, in Opti-MEM medium (Invitrogen). The medium was replaced with DMEM supplemented with 10% FBS 15 min after transfection, and the cells were harvested 48 h after transfection. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega).

**siRNA transfection**

Caco-2 BBe cells were trypsinized and plated at a density of $2.0 \times 10^5$ cells per well in a six-well type-I-collagen-coated plate in DMEM supplemented with 10% FBS. The cells were grown overnight and siRNA transfection was performed using Lipofectamine RNAiMAX (Invitrogen) with 30 pmol HNF-4α siRNA or control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The cells were harvested 48 h after transfection, and nuclear HNF-4α protein and CRBPII mRNA levels were analyzed by Western blotting and
real-time RT-PCR, respectively.

Preparation of nuclear extracts

Caco-2 BBe cells were washed twice with ice-cold PBS, scraped with a rubber policeman, and transferred into 15-ml tubes. Caco-2 BBe cells were pelleted, resuspended in 1.0 ml ice-cold cell lysis buffer [10 mM Tris-HCl (pH7.4), 10 mM NaCl, 3 mM MgCl₂, 0.5% Nonidet P40 (NP40), 1 mM DTT and 0.5 mM PMSF], and stored on ice for 5 min. The lysate was homogenized and centrifuged at 3,000 × g for 5 min at 4°C. The nuclear pellets were resuspended in 700 μl ice-cold cell lysis buffer and centrifuged at 3,000 × g for 5 min at 4°C. The pellets were resuspended twice in 500 μl ice-cold nuclei storage buffer [40% glycerol, 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT and 0.5 mM PMSF], and centrifuged at 3,000 × g for 5 min at 4°C. The pellets were resuspended in 100 μl nuclear protein extraction buffer [25% glycerol, 0.42M NaCl, 20 mM HEPES (pH7.9), 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 0.5 mM PMSF], incubated on ice for 30 min, and centrifuged at 105,000 × g for 30 min at 4°C. The supernatant containing nuclear extracts was stored at −80°C until use. Protein concentration was determined using the Coomassie Protein Assay Reagent (Pierce, Rockford, IL, USA).

Western blot analysis
Nuclear extracts (20 μg) separated by SDS-PAGE (10%) were transferred onto PVDF membrane (GE Health Care, Buckinghamshire, UK) using wet blotting apparatus. After blocking of the membrane by 5% ECL Blocking Reagent (GE Health Care) for 1 h at room temperature, the blots were incubated for 1 h at room temperature with anti-rabbit HNF-4α antibody (sc-8987x; Santa Cruz Biotechnology), which was diluted to 1:2,000 in PBS-Tween. Following three washes of 5 min in PBS-Tween, the secondary antibody (peroxidase-linked anti-rabbit IgG) diluted to 1:2,000 in PBS-Tween was added and incubated for 1 h at room temperature. The blots were developed with the ECL Western Blotting Detection Reagent (GE Health Care) and analyzed using LAS-1000plus (Fuji Film, Tokyo, Japan).

Electrophoresis mobility shift assay (EMSA)

Complementary oligonucleotides that contained sequences from human CRBP1 DR1-like element were annealed and radio-labeled using exo-free Klenow fragment in the presence of [α-32P]dCTP. The following oligonucleotide was used as a DNA probe for human CRBP1 gene DR-1-like element (underlined):

5′-GATCTGTGCTTCTGCCCTTTGAACCTCTTATCT-3′. EMSA reaction mixtures (10 μl final volumes) included 5 μg nuclear extract protein, 5× EMSA buffer [50 mM Tris-HCl (pH 8.0), 750 mM KCl, 0.25% NP-40 and 30% glycerol], and 1 μl of 1 mg/ml poly (dl-dC).
After incubation for 15 min at room temperature, [α-32P]dCTP labeled probe (20,000 cpm) was added, and the reaction was incubated for a further 20 min at room temperature. Protein/DNA complexes were separated on a 5% polyacrylamide gel in 0.5× Tris-borate-EDTA (TBE) buffer. For supershift analysis, 1 μl rabbit polyclonal antibody against human HNF-4α (sc-8987x) or RXRα (sc-553x; Santa Cruz Biotechnology) was pre-incubated with EMSA reaction mixtures for 15 min at room temperature before the probe was added. For analysis of binding activity of in vitro synthesized nuclear receptor, human HNF-4α, RXRα and PPARα proteins were synthesized by TNT T7 Quick Coupled Transcription/Translation System (Promega), as described previously (36). For competition analysis, the nuclear extracts were incubated with the labeled probe, and 50-fold excess unlabeled human CRBPII DR1-like element, rat CRBPII gene DR-1 element (RE3) (36), or sucrose-isomaltase footprint (SIF)-1 element (32) as a nonspecific competitor. After electrophoresis, the gel was dried and analyzed using an FLA-3000G image analyzer (Fuji Film).

Quantitative real-time RT-PCR analysis

Total RNA was extracted from Caco-2 BBe cells using TriPure Isolation Reagent (Roche Diagnostics). The extracted total RNA was treated with DNase I using a Turbo DNA-free kit (Ambion, Austin, TX, USA) before the reverse transcription reaction. First
strand cDNA was synthesized from individual samples of total RNA (1 μg) using ExScript RT Reagent Kit (TAKARA Bio) according to manufacturer’s instructions. All primers for PCR analysis were designed using Primer Express Software ver. 3.0 (Applied Biosystems, Foster City, CA, USA), and were tested for specificity using a BLAST search (Table 1). The PCR was performed on the synthesized cDNA product (1 μl) with gene-specific primer pair using SYBR Premix Ex Taq (TAKARA Bio), according to the manufacturer’s protocol. The amplification conditions were as follows: denaturation at 95°C for 10 s, and annealing/extension at 60°C for 34 s for 40 cycles. The PCR products were analyzed using ABI PRISM 7300 Real-Time PCR System (Applied Biosystems). Each mRNA level was normalized to the corresponding ribosomal protein, large, P0 (RPLP0) mRNA levels as an internal control.

**Chromatin immunoprecipitation (ChIP) assay**

Caco-2 BBc cells were fixed with 1% formaldehyde for 5 min at room temperature. The fixation was stopped by 0.125 M glycine for 5 min at room temperature. The cells were washed twice with ice-cold PBS, scraped with a rubber policeman, and transferred into 15-ml tubes. Caco-2 BBc cells were pelleted, resuspended in 200 μl lysis buffer [50 mM Tris-HCl (pH8.0), 1% SDS, 10 mM EDTA, 1.0 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml leupeptin], and stored on ice for 10 min. The cells were sonicated to achieve an average
genomic DNA size of 500-1,000 bp using Bioruptor (Cosmo Bio, Tokyo, Japan), a device for cell disruption, in cold water (4°C). The sonicated mixtures were centrifuged at 14,000 × g for 15 min at 4°C. The supernatants containing chromatin were diluted in 1,800 μl ChIP dilution buffer [50 mM Tris-HCl (pH8.0), 167 mM NaCl, 1.1% Triton X-100, 0.11% sodium deoxycholate, 1.0 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml leupeptin]. Aliquots of 50 μl of the dilutions were kept as a positive control (input). Chromatin complexes were pre-incubated with 50% Salmon Sperm DNA/Protein G Agarose Slurry (Millipore, Temecula, CA, USA) for 2 h at 4°C, and were immunoprecipitated for 18 h at 4°C, with 4 μg rabbit polyclonal antibodies against human HNF-4α (sc-8987x), or with normal goat IgG (sc-2028; Santa Cruz Biotechnology). Immune complexes were collected with 50% Salmon Sperm DNA/Protein G Agarose Slurry for 2 h incubation at 4°C, followed by centrifugation at 10,000 × g for 10 s at 4°C. The agarose beads were washed once for 5 min at 4°C with 1× RIPA/150 mM NaCl [50 mM Tris-HCl (pH8.0), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate/150 mM NaCl], 1× RIPA/500 mM NaCl, LiCl washing buffer [10 mM Tris-HCl (pH8.0), 250 mM LiCl, 1 mM EDTA, 0.5% NP-40, 0.5% sodium deoxycholate], and twice with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The beads that contained DNA fragments were eluted by resuspending in 200 μl elution buffer [10 mM Tris-HCl (pH8.0), 300 mM NaCl, 5 mM EDTA, and 0.5% SDS], followed by decrosslinking for 6 h at 65°C. To digest RNA and proteins present in the samples, RNase A
and proteinase K were added, and the samples were incubated for 30 min at 37°C and 1 h at 55°C, respectively. DNA was purified by phenol/chloroform extraction and ethanol precipitation. PCR amplification was performed using specific primers that are located around human CRBPII gene DR-1-like element (GeneBank Acc. no. AF338345, -131 to +108; 5′-CCTATAGTCTCGCTGAACTTTACAA -3′; reverse, 5′-AAAGTTTTTCATTACTCTCCATCTC-3′) or unrelated primer that are located at far upstream region of human CRBPII gene (GeneBank Acc. no. AF338345, -1944 to -1799; forward, TTTGGCATGGCTTTATCACACA; reverse, TCTGCTCTGACCTTGGTTTGG). The amplification conditions were as follows: denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s for 30 cycles. To quantification of in vivo association of endogeneous HNF-4α with human DR-1-like element by ChIP assay quantitative real-time PCR was performed as described above, and the ChIP signals were normalized to input signals.

Extraction of retinyl esters and HPLC analysis

The stably transfected Caco-2 BBc cells were grown on a six-well type-I-collagen-coated plate until 7 days post-confluence, and were incubated in serum-free medium supplemented with 1 μM all-trans retinol (Sigma-Aldrich), 1% insulin/transferrin/selenium (ITS) mix (Sigma-Aldrich), 6 mM sodium taurocholate (WAKO
Pure Chemical, Osaka, Japan) and 0.6 mg/ml G418 for 3-24 h. The cells were washed twice with ice-cold PBS, scraped with a rubber policeman, and transferred into 1.5-ml tubes. The pellets were resuspended in 500 μl homogenate buffer (50 mM Tris-HCl, pH 7.6, containing 0.25 M sucrose, 16 mM EDTA, 20 mM ascorbic acid, 25 mM KCl and 5 mM MgCl₂) and 500 μl ethanol. The samples were then extracted with 5 ml n-hexane containing 100 μg/ml butylated hydroxytoluene. After centrifugation at 400 × g for 5 min at 4°C, the hexane extracts were evaporated to dryness under nitrogen. The residue was dissolved in 50 μl methanol. HPLC was performed using a Shimadzu LC-10AD system fitted with a μ−Bondapak C18 column (3.9 × 300 mm; Waters, Milford, MA, USA), with 100% methanol as the mobile phase, at flow rate of 1.5 ml/min.

Statistical analysis

Data were subjected to one-way ANOVA. The significance in mean values among different groups was analyzed using Duncan’s multiple-range test or Student’s t test. P < 0.05 was used to indicate statistical significance.

RESULTS

Binding of nuclear receptors to the human CRBPII gene DR-1-like element

We studied the binding site of transcriptional factors within the 5′-flanking region of
the human CRBPII gene using the TFSEARCH program (http://mbs.cbrc.jp/research/db/TFSEACHJ.html). We found the DR-1-like sequence ‘TGCCCTtTGAACC’, which was located at position –80 to –68 of the human CRBPII gene promoter (GeneBank Acc. no. AF338345) (Fig. 1). It has previously been reported that various nuclear receptors, including HNF-4α, RXRα and PPARα, bind to DR-1 elements as homo- or heterodimers (18, 19). Therefore, we examined binding of these nuclear receptors to the human CRBPII DR-1-like element by EMSA, to further characterize the relationship between human CRBPII and HNF-4α, RXRα and PPARα. EMSA clearly showed a signal for binding of Caco-2 BBe cell nuclear extract to the human CRBPII DR-1-like element (Fig. 2A, lane 2). The binding signal was specifically supershifted in the presence of HNF-4α specific antibody (Fig. 2A, lane 3), but not RXRα antibody (Fig. 2A, lane 4).

Moreover, EMSA was also performed using in vitro synthesized HNF-4α, RXRα and PPARα. In vitro synthesized HNF-4α strongly bound to the human CRBPII DR-1-like element as a homodimer (Fig. 2B, lane 4). RXRα homodimer bound to the same element very weakly in the presence of RXR ligand 9-cis retinoic acid (Fig. 2B, lane 2), whereas RXRα/PPARα heterodimer did not bind to the element in the presence of PPARα agonist WY 14643 (Fig. 2B, lane 3). We confirmed that the addition of excess unlabeled human CRBPII gene DR-1-like element, as well as rat CRBPII gene DR-1 element (RE3) abolished the binding signal (Fig. 2C, lane 2 and 4). On the other hand, the addition of excess
unlabeled SIF-1 element, which is the binding site for the intestine-specific homeodomain transcription factor CDX2 but not HNF-4α (32), did not affect the binding signal (Fig. 2C, lane 5). To further investigate whether endogenous HNF-4α protein bound to the human CRBPII DR-1-like element in the nucleus of Caco-2 BBe cells in vivo, we performed a ChIP assay. As shown in Fig. 2D, endogenous nuclear HNF-4α protein bound to the human CRBPII DR-1-like element (lane 1). Immunoprecipitation with control IgG, or without antibody as a negative control, was undetected by PCR amplification (lanes 2 and 3). We confirmed that the ChIP signal was undetected by PCR amplification using unrelated primers located at far upstream human CRBPII promoter region (–1944 to –1799) (Fig. 2D).

*HNF-4α enhances transcriptional activity of the human CRBPII gene promoter*

The effect of HNF-4α on human CRBPII DR-1-like-element-mediated transcription activity was examined. A transient co-transfection assay was performed using two luciferase reporter constructs (wild-type phCRBPIIpro-LUC vector and mutated phCRBPIIpro(DR-1mut)-LUC vector) (Fig. 3A). When the HNF-4α expression vector was co-transfected into Caco-2 BBe cells with the wild-type phCRBPIIpro-LUC vector, luciferase activity was significantly increased 2.0-fold as compared with that of the mock control (Fig. 3B). However, mutations in the human CRBPII DR-1-like element using phCRBPIIpro(DR-1mut)-LUC vector resulted in significant reduction of basal luciferase
activity, which was ~50% of that of the wild-type (Fig. 3B). Moreover, co-transfection of
the HNF-4α expression vector did not increase the luciferase activity in
phCRBPIIpro(DR-1mut)-LUC vector (Fig. 3B).

Effect of nuclear HNF-4α protein expression level on endogenous CRBPII gene
expression in C2BBe cells

In order to investigate whether nuclear HNF-4α expression was affected by
endogenous CRBPII gene expression in Caco-2 BBe cells, we performed two independent
experiments. First, we established a Caco-2 BBe clone that was stably transfected with
HNF-4α expression vector, and confirmed the overexpression of nuclear HNF-4α protein
level. As shown in Fig. 4A, two signals that corresponded to proteins of different molecular
weight were seen. In the cells that were stably transfected with HNF-4α expression vector,
the higher molecular weight signal increased markedly. The luciferase activity derived from
phCRBPII-(DR-1)x-tk-LUC vector was significantly increased approximately nine-fold in
the cells that overexpressed HNF-4α, as compared with the mock vector-transfected cells
(Fig. 4B). Moreover, overexpression of HNF-4α significantly increased endogenous
CRBPII mRNA level. ALPI mRNA level, which is known as a HNF-4α target gene in
human small intestine (20), also increased, but not significantly (p =0.267), in the cells that
overexpressed HNF-4α (Fig. 4C). Second, we performed siRNA experiment to inhibit
endogenous HNF-4α expression in Caco-2 BBe cells. We confirmed that nuclear HNF-4α protein levels were dramatically decreased in cells transfected with HNF-4α siRNA, as compared with control siRNA (Fig. 5A). The HNF-4α siRNA-transfected Caco-2 BBe cells also showed significantly lower levels of endogenous CRBPII and ALPI mRNA (Fig. 5B).

Effects of PMA-induced decrease in nuclear HNF-4α expression on CRBPII gene expression in Caco-2 BBe cells

HNF-4α expression level and its DNA-binding activity have been shown to decrease under treatment with PMA, a PKC activator, in human hepatoma HepG2 cells (8). We also showed a significant decrease in endogenous nuclear HNF-4α protein level, especially the higher molecular weight one, in PMA-treated Caco-2 BBe cells, as compared with DMSO-treated Caco-2 BBe cells (Fig. 6A). GF109203X, a PKC inhibitor, at 4 μM prevented the PMA-induced decrease in nuclear HNF-4α protein levels (Fig. 6A). However, since high concentration (> 2 μM) of GF109203X is also known to inhibit not only PKC but also PKA (39), we additionally confirmed the possible involvement of PKA on PMA-induced decrease in nuclear HNF-4α protein levels in Caco-2 BBe cells using Rp-cAMP (a specific PKA inhibitor). The treatment of Rp-cAMP did not restore PMA-induced decrease in HNF-4α protein expression (Fig. 6A). CRBPII mRNA level was also significantly decreased in PMA-treated Caco-2 BBe cells (Fig. 6B). Although CRBPII
mRNA level was not changed by GF109203X or Rp-cAMP alone, GF109203X but not Rp-cAMP significantly prevented PMA-induced decrease in CRBPII mRNA level (Fig. 6B). We confirmed that the HNF-4α homodimer binding activity to the human CRBPII DR-1-like element in PMA-treated Caco-2 BBe cells was weaker than that with DMSO-treatment, whereas PMA-treatment together with GF109203X restored the binding activity (Fig. 6C). The luciferase activity expressed from wild-type phCRBPIIpro-LUC vector was significant lower in PMA-treated Caco-2 BBe cells than that of DMSO-treated cells (Fig. 6D). In contrast, the luciferase activity expressed from phCRBPIIpro(DR-1mut)-LUC vector did not changed in PMA-treated Caco-2 BBe cells (Fig. 6D).

Coordinate changes in CRBPII gene expression, nuclear HNF-4α expression and the binding activity to DR-1-like element during Caco-2 BBe cell differentiation

We examined the changes in nuclear HNF-4α protein levels during Caco-2 BBe cell differentiation. The nuclear HNF-4α protein levels significantly increased during proliferation and early differentiation (days –2 to 7), and then remained constant during the late stage of differentiation (days 7 to 14) (Fig. 7A). Similarly, the mRNA levels of CRBPII and ALPI, which is known as an HNF-4α target gene and a differentiation marker in crypt–villus enterocytes (20), were also low during the proliferation stage (days –2 to 0),
and then significantly increased during the early stage of differentiation (days 0 to 7). The higher mRNA levels of CRBPII and ALPI remained constant during the late stages of differentiation (days 7 to 14). The changes in the binding activity of HNF-4α homodimer to the human CRBPII DR-1-like element examined by EMSA were similar to those of nuclear HNF-4α protein levels during Caco-2 BBe cell differentiation (Fig. 7C). We performed the quantification of \textit{in vivo} nuclear HNF-4α-binding activity to the human CRBPII DR-1-like element by ChIP assay and real time PCR. The changes in the \textit{in vivo} nuclear HNF-4α-binding activity increased, but not significantly (p = 0.258, one-way ANOVA), during days –2 to 7, and then remained constant during days 7 to 14 (Fig. 7D).

\textit{Increase of retinyl ester synthesis in Caco-2 BBe cells overexpressing HNF-4α}

The stably transfected Caco-2 cells overexpressing CRBPII increased uptake and esterification of all-trans retinol as shown previously (15, 17). We aimed to establish whether the HNF-4α-induced increase in CRBPII gene expression increased retinyl ester (retinyl palmitate) synthesis. As shown in Fig. 8, retinyl ester formation was significantly higher in the HNF-4α-overexpressing Caco-2 BBe cells than in mock vector-transfected cells at 3-24 h after addition of all-trans retinol.
DISCUSSION

We found that 5’-flanking region of the human CRBPII gene contained a DR-1-type nuclear receptor response element within its proximal promoter region. We demonstrated that endogenous HNF-4α homodimer specifically bound to the human CRBPII DR-1-like element. The nuclear HNF-4α protein expression levels were correlated with those of endogenous CRBPII in Caco-2 BBe cells. Overexpression of HNF-4α enhanced CRBPII gene expression level and retinyl ester synthesis in Caco-2 BBe cells.

Some nuclear receptors such as HNF-4α and RXRα homodimer, and RXRα/PPARα heterodimer bind to the DR-1 element (RE3; TGACCTtTGACTC) located in the proximal promoter region of the rat and mouse CRBPII gene (18, 19). We showed by EMSA and ChIP assay that nuclear HNF-4α homodimer specifically bound to the human CRBPII DR-1-like element in vitro and in vivo. Mutation of this element significantly reduced the basal promoter activity and abolished HNF-4α-induced increase in the promoter activity of human CRBPII gene in Caco-2 BBe cells, which indicates that HNF-4α might be involved in basal transcription of human CRBPII gene in the small intestine. Unlike rat and mouse CRBPII gene DR-1 elements (18, 19, 35), EMSA showed that the human CRBPII gene DR-1-like element bound very weakly to RXRα homodimer in the presence of 9-cis retinoic acid, but did not bind to RXRα/PPARα heterodimer in the presence of WY 14643 (Fig. 2). It has been reported that all-trans and 9-cis retinoic acid increase human CRBPII gene
expression in Caco-2 cells (16, 36) and Caco-2 BBe cells (S. Miyamoto, unpublished observation). Zhang et al. have demonstrated that the human CRBPII gene promoter sequence within 2.8 kb upstream of the 5′-flanking region is activated by 9-cis retinoic acid in differentiated but not undifferentiated Caco-2 cells (42). Our preliminary study showed that 9-cis retinoic acid significantly increased luciferase reporter activity derived from phCRBPII-(DR-1)2-tk-LUC vector in the differentiated Caco-2 BBe cells and non-intestinal CV-1 and HEK-293 cells, but not undifferentiated Caco-2 BBe cells (S. Miyamoto, unpublished observation). Therefore, the weak binding of RXRα homodimer to the human CRBPII DR-1-like element in the presence of 9-cis retinoic acid (Fig. 2) was likely to have contributed in part to the 9-cis retinoic acid-induced increase in CRBPII gene expression in differentiated Caco-2 BBe cells. We have also reported that some fatty acids and PPARα agonists such as ETYA, clofibrate and WY 14643 increase CRBPII gene expression in rat jejunum and Caco-2 cells (36–38). However, we did not find the response element for PPARα/RXRα heterodimer in the human CRBPII gene 5′-flanking region, including its DR-1-like element, although the rat CRBPII gene DR-1 elements (RXRE and RE3) are responsive to PPARα/RXRα heterodimer (34). Thus, the functional properties of CRBPII gene DR-1 elements located in the proximal promoter region seem to differ between humans and rodents.

It has been reported that several phosphorylation signals modulate HNF-4α
expression levels or its binding activity as a transcription factor (9, 29, 33, 40). In the present study, endogenous nuclear HNF-4α expression level in Caco-2 BBe cells and its binding activity to the human CRBPII gene DR-1-like element decreased in PMA-treated Caco-2 BBe cells, which led to decreased CRBPII promoter activity and gene expression. Their PMA-induced decreases in both CRBPII gene expression level and nuclear HNF-4α protein levels were restored by GF109203 but not Rp-cAMP, which indicates that the PKC-cascade-mediated phosphorylation signal is involved in regulation of human intestinal CRBPII gene expression, through modulation of HNF-4α levels and its binding activity. The intestinal epithelium is continuously exposed to various stimuli such as dietary components, bacteria, endotoxin, luminal growth factors, and inflammatory mediators. These stimuli activate various intracellular signaling cascades including PKC (22). Further studies are needed to investigate the effect of PKC-related physiological stimuli on regulation of CRBPII-mediated vitamin A absorption and metabolism.

In the present study, we also showed a significant increase in HNF-4α nuclear protein levels and binding activity of endogenous HNF-4α homodimer to the human CRBPII DR-1-like element, as well as CRBPII gene expression levels during Caco-2 BBe cell differentiation. Previous studies have reported the importance of HNF-4α as a transcription factor for villus-specific gene expression during enterocyte differentiation (30, 31). Moreover, Zhang et al. have demonstrated that human CRBPII gene minimal promoter
region (–103 to +58), which contains DR-1-like element, is activated during Caco-2 cell
differentiation (42). Therefore, we speculate on the possibility that HNF-4α is a regulator of
human CRBPII gene expression during Caco-2 BBe cell differentiation.

Stably transfected Caco-2 cells that overexpress CRBPII show increased retinol
uptake and esterification (15, 17). Therefore, we aimed to confirm whether enhanced
CRBPII gene expression in stably transfected Caco-2 BBe cells, which overexpressed
HNF-4α, increased retinyl ester synthesis. HNF-4α also enhances the transcriptional
activity of human microsomal triglyceride transfer protein (MTP) gene, which is an
important carrier protein involved in intestinal retinyl ester incorporation into chylomicron
(27). Thus, HNF-4α might be an important regulatory transcription factor in vitamin A
absorption and its metabolism in human small intestine.

In conclusion, we demonstrated that HNF-4α specifically regulated gene expression
of human CRBPII, which plays a pivotal role in vitamin A absorption and metabolism in
Caco-2 BBe cells. Therefore, we propose a novel physiological role for HNF-4α on vitamin
A absorption and its metabolism in human small intestine.

GRANTS

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FIGURE LEGENDS

Fig. 1. Human CRBPII gene promoter sequence.

The sequence of proximal promoter region of human CRBPII gene was submitted to the TFSEARCH program. The DR-1-type nuclear response element, TATA box and initiation codon are boxed. The transcription start site is position +1.

Fig. 2. Binding properties of nuclear receptors to the human CRBPII DR-1-like element.

(A) EMSA was performed using the $^{32}$P-labeled human CRBPII gene DR-1-like element as a probe. Assay was conducted in the absence (–) or presence (+) of nuclear extract (NE; 5 μg) from 14 days post-confluent Caco-2 BBe cells, anti-HNF-4α antibody (H; 1 μl) or anti-RXR antibody (R; 1 μl). (B) EMSA was conducted in the absence (–) or presence (+) of in-vitro-translated RXRα, PPARα and HNF-4α. R: RXRα (5 μl) plus 1 μM 9-cis retinoic acid; RP: RXRα (3 μl) plus PPARα (2 μl) + 10 μM WY 14643; H: HNF-4 (1 μl). The position of HNF-4α and RXRα homodimer complexes are indicated by arrows. (C) Competition analysis was performed using 50-fold excess amounts of the unlabeled human CRBPII gene DR-1-like element, rat RE3 or human SIF-1 probe. (D) In ChIP assays, soluble chromatin was immunoprecipitated with anti-HNF-4α antibody (HNF-4α), with goat IgG (IgG) and without antibody (NA) as a negative control. Immunoprecipitated
fractions were analyzed by PCR with specific or unrelated primers for amplification of human CRBPII promoter gene containing DR-1-like elements (–80 to –68) or approximately 2.0 kbp upstream (–1944 to –1799), respectively. PCR was also performed with total chromatin content as an input control (Input).

Fig. 3. HNF-4α-mediated transactivation of wild-type and mutated human CRBPII gene DR-1-like element.

(A) Wild-type (wt) and mutated (mut) human CRBPII gene promoter vector constructs are shown. Underlined nucleotides on the DR-1-like element were mutagenized. (B) Caco-2 BBc cells seeded onto a 96-well plate were co-transfected with 100 ng each of phCRBPIIpro-LUC or phCRBPIIpro(DR-1mut)-LUC, 100 ng pcDNA3.1-HNF-4α or pcDNA3.1 vector, and 2 ng phRG-tk-LUC. After transfection, the cells were incubated with DMEM supplemented with 10% FBS for 24 h, and dual luciferase assays were performed. The luciferase activity of the cells transfected with pcDNA3.1 as a mock control was equivalent to 100%. Values represent means ± SEM (n=4 each group). Values not sharing common superscript letters were significantly different at P < 0.05 (Duncan’s test).

Fig. 4. Increase in endogenous CRBPII gene expression in the stably transfected Caco-2 BBc cells overexpressing HNF-4α.
(A) Representative Western blot analysis of nuclear HNF-4α protein from Caco-2 BBe cells stably transfected with pcDNA-HNF-4α expression vector (HNF-4α) or mock vector (mock). (B) Stably transfected Caco-2 BBe cells seeded onto a 96-well plate were transfected with 100 ng phCRBPII-(DR1)2-tk-LUC reporter vector. After 48 h transfection, dual-luciferase assays were performed. (C) CRBPII and ALPI mRNA levels were quantified by real-time RT-PCR. The graph represents the relative mRNA levels of each gene normalized to RPLP0 mRNA abundance, expressed in arbitrary units. Luciferase activity and mRNA levels of the cells stably transfected with pcDNA3.1 as a mock control were equivalent to 1.0. Values represent means ± SEM (n=4 each group). *Significant difference from the value for the mock control at P < 0.05. NS: not significant (Student’s t test).

Fig. 5. Effect of CRBPII gene expression by HNF-4α siRNA transfection in Caco-2 BBe cells.

(A) Representative Western blot analysis of nuclear HNF-4α protein from Caco-2 BBe cells transfected with HNF-4α or control siRNA. (B) CRBPII and ALPI mRNA levels cells were quantified by real-time RT-PCR. The graph represents the relative mRNA levels of each gene normalized to RPLP0 mRNA abundance, expressed in arbitrary units. mRNA levels in cells transfected with control siRNA were equivalent to 1.0. *Significant difference from the value for the control siRNA at P < 0.05 (Student’s t test).
Fig. 6. Effects of PMA on CRBPII promoter activity and gene expression, nuclear HNF-4α protein expression and binding activity in Caco-2 BBe cells.

(A)-(C) Caco-2 BBe cells at 14 days post-confluence were treated for 24 h in serum-free medium supplemented with 0.1% DMSO or 1 μM PMA, with or without 4 μM GF109203X (GF) or 10 μM Rp-cAMP after pre-treatment with GF or Rp-cAMP for 3 h. (A) Representative Western blot analysis of nuclear HNF-4α protein from Caco-2 BBe cells. (B) CRBPII mRNA levels were quantified by real-time RT-PCR. The graph represents the relative mRNA levels of each gene normalized to RPLP0 mRNA abundance, expressed in arbitrary units. mRNA levels of the cells treated with DMSO only were equivalent to 1.0. Values represent means ± SEM (n=4 each group). Values not sharing common superscript letters were significantly different at P < 0.05 (Duncan’s test). (C) EMSA was performed using the 32P-labeled DR-1-like element of human CRBPII gene as a probe and the extracted Caco-2 BBe cell nuclear proteins (5 μg). (D) Caco-2 BBe cells at 10 days post-confluence in a 96-well plate were treated with 1 μM PMA for 12 hr. The transfection and luciferase activity assay performed as described under “Materials and Methods”. The luciferase activity of the cells transfected with phCRBPIIpro-LUC was equivalent to 100%. Values represent means ± SEM (n=6 each group). *Significant difference from the value for the control at P < 0.05 (Student’s t-test). NS: not significant (Student’s t-test).
Fig. 7. Changes in nuclear HNF-4α protein levels and binding activity during Caco-2 BBe cell differentiation.

(A) Representative Western blot analysis of nuclear HNF-4α protein from Caco-2 BBe cells during differentiation. (B) CRBPII and ALPI mRNA levels were quantified by real-time RT-PCR. The graph represents the relative mRNA levels of various genes normalized to RPLP0 mRNA abundance, and expressed in arbitrary units. mRNA levels in Caco-2 BBe cells at 2 days before confluence (−2 days) were equivalent to 1.0. Values represent means ± SEM (n=6 each day). Values not sharing common superscript letters were significantly different at P < 0.05 (Duncan’s test). (C) EMSA was performed using the 32P-labeled DR-1-like element of human CRBPII gene as a probe and the extracted Caco-2 BBe cells nuclear proteins (5 μg). (D) In vivo HNF-4α homodimer binding activity to the human CRBPII gene DR-1-like element was analyzed by ChIP assay and quantified by real-time PCR. The values were normalized to those of the input signals. Values represent means ± SEM (n=3-4 each day). Values not sharing common superscript letters were significantly different at P < 0.05 (Duncan’s test).

Fig. 8. Retinyl ester synthesis in stably transfected Caco-2 BBe cells overexpressing HNF-4α.
Caco-2 BBe cells stably transfected with pcDNA/HNF-4α expression vector (HNF-4α) or mock vector (mock) were grown on six-well plates until 7 days post-confluence. Cells were treated with 1 μM retinol in serum-free medium containing 1% ITS, 6 mM sodium taurocholate, and 0.6 mg/ml G418 for 3-24 h. At the indicated time, these cells were analyzed for contents of retinyl ester (retinyl palmitate) in cell extracts. Values represent means ± SEM (n=4 each group). *Significant difference from the value for the mock control at P < 0.05 (Student’s t test).
Table 1 Oligonucleotide primers for real-time RT-PCR

<table>
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<tr>
<th>Gene</th>
<th>Accession Number</th>
<th>Forward primer 5’→3’</th>
<th>Reverse primer 5’→3’</th>
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<tbody>
<tr>
<td>CRBPII</td>
<td>NM_004164</td>
<td>AGCACATTCGCAACTATGATG</td>
<td>CCAGGTGACCAGTGCCTTAAC</td>
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<tr>
<td>ALPI</td>
<td>NM_001631</td>
<td>AGCAAGCAGGAAAGTCAGTGG</td>
<td>CCAAGTGCGGTCACTGTGT</td>
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<tr>
<td>RPLP0</td>
<td>NM_053275</td>
<td>GGCTACCCAACGTTGGGATCA</td>
<td>GCCCTGACCTTTTACGAGTG</td>
</tr>
</tbody>
</table>

RPLP0: ribosomal protein, large, P0.
-144 TGTTTCGTGGGTACCTATAGTCTCGCTGAACCTTACAAAAGAAAAGTTCTTT

DR-1

-94 TTGAGTTGTGCTTCTGCTTTTGAACCCTTTATCTTCAAGGCACTTACAAAACC

TATA box

-44 TGGCCCACTAGGTGCTTTTGAACCCTTTATCTTCAAGGCACTTACAAAACC

Initiation codon

57 CATGACAAGGGACCAGAATGGGAACCTGGGAGATGGAGAGTAATGAAAAACT

107 TTGAGGGCTACATGAAGGCCCTGGGTAAGGAGGAGACTGGGGATGGGGAG

Figure 1
Figure 2

(A) and (B) show gel shift assays with probes and antibodies. The probes are labeled as + or - depending on their presence or absence. The nuclear receptor and antibody are marked with H, R, and P. The supershift indicates the presence of antibody. RXRα homodimer, HNF-4α homodimer, and non-specific bands are also shown. Lane numbers are 1 to 4.

(C) and (D) show additional gel shift assays. N.E. and 50x competitor are marked as + or -. HNF-4α homodimer and free probe are indicated. Lane numbers are 1 to 5 or 1 to 4 for different gels.

Figure 2
Figure 3

(A)

DR-1-like element

TATA BOX

wt: tc TGCCCT t TGAACC ttatatcttt----ggeTATAaaa

mut: tc TACTAC g TCTT CC tc----

hCRBPII gene 5'-flanking region

Luciferase

(B)

Relative luciferase activity (% of wild type mock)

0 50 100 150 200 250

mock HNF-4α mock HNF-4α

Wild type Mutation

Figure 3
Figure 4

(A) HNF-4α protein

(B) Relative luciferase activity

(C) CRBPII

Relative mRNA levels

mock HNF-4α

ALPI

Relative mRNA levels

mock HNF-4α

NS

mock HNF-4α

mock HNF-4α
Figure 5

(A) HNF-4α protein

(B) CRBPII and ALPI mRNA levels after control siRNA and HNF-4α siRNA treatment.
Figure 6
Figure 7

(A) HNF-4α protein

(B) CRBPII and ALPI mRNA levels over time:
- CRBPII: Relative mRNA level increases significantly from -2d to 0d, peaking at 7d and decreasing slightly at 14d.
- ALPI: Relative mRNA level increases from -2d to 0d, with a slight increase at 7d.

(C) HNF-4α homodimer activity

(D) In vivo HNF-4α binding activity:
- Activity increases from -2d to 14d, showing significant differences at each time point.
Figure 8