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Transcriptional Regulation of Cellular Retinol-Binding Protein, Type II Gene Expression in Small Intestine by Dietary Fat

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We have previously demonstrated that dietary fat, especially unsaturated fatty acids, induces cellular retinol-binding protein, type II (CRBPII) gene expression in rat jejunum. In the present study, we showed that feeding a high-fat diet caused parallel increases in jejunal CRBPII mRNA and CRBPII pre-mRNA levels. Nuclear run-on assay also revealed that this increase of CRBPII mRNA level by high-fat diet was, at least in part, triggered at a transcription level. Moreover, peroxisome proliferator-activated receptor alpha (PPARα) mRNA level was also increased in the jejunum by high-fat diet. Gel shift assay showed that the binding activity of rat jejunal nuclear protein to the nuclear receptor response elements located in the rat CRBPII gene (RXRE and RE3) was greater in rats fed high-fat diet than in those fed fat-free diet and were enhanced by addition of bacterially expressed PPARα protein. Also PPARα-retinoid X receptor alpha (RXRα) heterodimer was capable of binding to the CRBPII-RXRE and RE3 elements and these binding activities were enhanced by addition of some PPARα ligands in the gel shift assay. Taken together, these studies suggest that dietary fatty acids may lead to induction of CRBPII gene transcription through increases of PPARα as well as its ligand levels. © 1999 Academic Press

Key Words: **cellular retinol-binding protein, type II; dietary fat; peroxisome proliferator activated receptor; small intestine; gene transcription.**

Intestinal vitamin A uptake and metabolism are mediated by cellular retinol-binding protein, type II (CRBPII),[2] which is an abundant cytosolic protein found in the small intestinal enterocytes of many vertebrates (1–4). CRBPII binds specifically retinol or retinal. Indeed, CRBPII-retinal and -retinol complexes serve as substrate not only for the conversion of retinal into retinol catalyzed by retinal reductase (5), but also for the conversion of retinol into retinyl esters catalyzed by lecithin:retinol acyltransferase (6). Thus, the alteration of CRBPII levels in the small intestine may lead to changes in its capacity to uptake and transport vitamin A (7, 8).

We previously found that jejunum bypass operation led to marked increases in the amounts of CRBPII and apolipoprotein B in the residual jejunal segment (9), suggesting that CRBPII expression might be enhanced in the small intestinal segment where fat absorption was stimulated. Dietary fat enhanced the expression of CRBPII gene as well as its protein levels in rat jejunum (10). Moreover, this enhancement was brought about by unsaturated long-chain fatty acids (11). However, molecular mechanism mediating the effects of the fatty acids on CRBPII gene expression remains unknown.

Among the several potential mechanisms by which fatty acids may affect gene expression, the direct regulation by the *trans*-acting factors has been proposed. One of these candidates is peroxisome proliferator-activated receptors (PPARs)-mediated transcription

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[2] Abbreviations used: CRBPII, cellular retinol-binding protein, type II; L-FABP, liver-type fatty acid binding protein; PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; PPRE, peroxisome proliferator response element; RXRE, retinoid X receptor response element; HNF-4, hepatocyte nuclear factor-4; DR-1, direct repeat-1; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; DTT, dithiothreitol; PMSF, phenylmethyl-sulfonylfluoride.

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mechanism. PPARs are members of a superfamily of steroid/thyroid hormone receptors for retinoid, thyroid, vitamin D, and steroid hormones (12). PPARs are selectively activated by a variety of fibrate hypolipidemic drugs termed peroxisome proliferators, fatty acids and their metabolites, and others (13). Our recent study demonstrated that a potent PPARα activator clofibrate transcriptionally induced CRBPII gene expression in human small intestinal-like cell line, Caco-2 cells (14). PPARs bind to and activate transcription through peroxisome proliferator response element (PPRE) consisting of an imperfect direct repeat of two core motifs separated by 1 nucleotide (DR1) in a heterodimeric complex with other class of nuclear hormone receptors, i.e., retinoid X receptors (RXRs) (15–17). A few DR1-like sequences have been reported in the rat and mouse CRBPII gene's 5′-flanking regions, suggesting that CRBPII gene expression may be regulated by several ligand-activated or orphan nuclear receptor homo- and heterodimers through these DR1 sequences (18, 19). These observation led us to seek evidence in a whole animal that high-fat feeding-elicited CRBPII gene expression may be brought about by activating transcription through interaction of the heterodimer complex with the CRBPII gene response elements.

In the present study, we demonstrated that rat jejunal CRBPII gene expression was influenced by dietary fat at a transcription level and that this induction might be caused by dietary fat-induced enhancement of PPARα-RXR heterodimer binding to the nuclear receptor response element(s) located in the rat CRBPII gene.

MATERIALS AND METHODS

Animals. Six-week-old Wistar male rats were separated into two groups. One group of rats (fat-free diet group) were fed a low-fat diet containing 2.4% (w/w) corn oil (11) for 1 week and then fed a fat-free diet (11) for another week. The other group were fed a high-fat diet containing 24.7% (w/w) corn oil (10) for 2 weeks. The diets were isocaloric (305.8 kcal/100 g diet) and there was no difference in the amount of food-intake between the two dietary groups. Rats were killed by decapitation. The experimental procedures used in the present study met the guidelines of the animal usage committee of The University of Shizuoka.

RNA analysis. Total RNA was extracted from rat jejunum by the acidified guanidine thiocyanate method as described by Chomczynski and Sacchi (20). Northern blot analysis was performed using ^{32}P-labeled cDNA probes as described previously (10). The cDNA probes used in the Northern blot analysis were as follows: rat CRBPII cDNA (10); rat liver-type fatty acid-binding protein (L-FABP) cDNA, a fragment corresponding to +40 to +423 (21), rat PPARα cDNA (11); rat 28S ribosomal RNA, a fragment corresponding to +3646 to +4205 (22). Ribonuclease protection assay was performed using ^{32}P-labeled antisense RNA probes. The cDNA templates for synthesis of antisense RNA probes were as follows: rat CRBPII intronic cDNA, a fragment corresponding to +230 to +516 (23); rat PPARα cDNA, a fragment corresponding to +378 to +523 (24); and rat RXRα cDNA, a fragment corresponding to +738 to +934 (16). These cDNAs were subcloned into pBluescript II SK+ vector (Stratagene, La Jolla, CA). Antisense RNA probes were synthesized with 0.5 μg each template using [α-^{32}P]UTP (800 Ci/mmol, ICN Biochemicals, Irvine, CA) and T3 or T7 RNA polymerase (Life Technologies, Gaithersburg, MD) according to the method of Sambrook *et al.* (25). Ribonuclease protection assay was performed as follows: typically 15 μg of rat jejunal total RNA was hybridized to a mixture of the above labeled probe (10,000 to 20,000 cpm) and 18 S rRNA as a control probe (300 cpm) (Ambion, Austin, TX) at 50°C for 16 h. For negative control of the ribonuclease protection assay, 15 μg yeast t-RNA was hybridized with mixture of antisense RNA probes instead of jejunal total RNA. Then, hybridized mixture was digested with 40 μg/ml ribonuclease A (Sigma, St. Louis, MO) and 1.1 U/ml ribonuclease T1 (Life Technologies, Gaithersburg, MD) at 30°C for 1 h. Protected fragments were separated on 6% denatured polyacrylamide gel. The gel was dried under vacuum and exposed to an image plate (Fuji Film, Tokyo, Japan) for 24 h. For quantification, specific mRNA signals from both Northern blot analysis and ribonuclease protection assay were scanned using a bioimage analyzer (BAS 3000, Fuji Film, Tokyo, Japan) and the signals were standardized using corresponding 28S or 18S rRNA signal, respectively.

Preparation of nuclei and nuclear extract. Rat jejunal nuclei were isolated according to the method of Krasinski *et al.* (26) with some modifications. Briefly, 1 g of rat jejunal mucosa was homogenized in 10 vol of ice-cold Lysis buffer [50 mM Tris–HCl (pH 7.5), 25 mM KCl, 5 mM $MgCl_2$, 0.32 M sucrose, 5 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonylfluoride (PMSF)] and centrifuged at 12,000*g* for 5 min at 4°C. The pellets were rehomogenized in 15 ml Lysis buffer containing 0.05% (v/v) Nonidet P-40 and the crude nuclei pellets were recovered by centrifugation at 1,500*g* for 10 min at 4°C. The recovered crude nuclei pellet was resuspended in 15 ml sucrose buffer [50 mM Tris–HCl (pH 7.5), 25 mM KCl, 1 mM $CaCl_2$, 2.4 M sucrose, 5 mM DTT, 0.1 mM PMSF] and layered over 5 ml sucrose buffer. Intact nuclei pellets were recovered by centrifugation at 75,000*g* for 30 min at 4°C. For the nuclear run-on assay, the purified nuclei pellet was resuspended in 100 μl nuclei storage buffer [40% glycerol, 50 mM Tris–HCl (pH 8.3), 5 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF]. The nuclei pellet was stored at −70°C until use. For the gel shift assay, the purified nuclei were resuspended in 50 μl nuclear protein extraction buffer [0.42 M NaCl, 20 mM Hepes (pH 7.9), 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 1 mM DTT, 25% glycerol, 1 μg/ml each of leupeptin, chymostatin, and pepstatin A], and extracted on ice for 30 min. The nuclear extract was centrifuged at 60,000*g* for 30 min and the supernatant was frozen in aliquots at −70°C. The nuclear protein concentration was determined using Coomassie protein assay reagent (Pierce, Rockford, IL).

Nuclear run-on assay. The nuclear run-on assay was performed essentially according to the method of Celano *et al.* (27). Briefly, about 2×10^7 nuclei per 100 μl were mixed with 100 μl reaction buffer composed of 10 mM Tris–HCl (pH 8.0), 5 mM $MgCl_2$, 0.3 M KCl, 2 mM DTT, 80 U ribonuclease inhibitor (Toyobo, Osaka, Japan), 1.5 mM each of ATP, GTP, TTP, 100 μCi [α-^{32}P]UTP (3000 Ci/mmol, ICN Biochemicals, Irvine, CA), and incubated for 30 min at 30°C. Nuclei were then digested with 20 U ribonuclease-free deoxyribonuclease I (Takara Shuzo, Kyoto, Japan) for 10 min at 30°C. The reaction was stopped by the addition of 24 μl stop buffer [100 mM Tris–HCl (pH 7.5), 50 mM EDTA, 5% SDS] and 4 μl of 10 mg/ml proteinase K (Wako Pure Chemicals, Osaka, Japan) and the mixture was further incubated at 50°C for 1 h. The elongated nascent RNA was purified according to the method of Chomczynski and Sacchi (20). The radiolabeled RNA (3×10^6 cpm) was hybridized with 2 μg cDNA probes (CRBPII and L-FABP cDNAs), which were immobilized on N+ nylon membrane for 72 h at 42°C in hybridization buffer as described previously (10). The membrane was washed in 2 × NaCl/sodium phosphate/EDTA buffer [1×; 0.18 M NaCl, 0.01 M sodium phosphate, 1 mM EDTA (pH 7.7)] containing 0.1% SDS for 15 min at 42°C twice, incubated in 2 × NaCl/sodium phosphate/EDTA buffer containing 10 μg/ml ribonuclease A for 30 min at 37°C, finally washed in 1 × NaCl/sodium phosphate/EDTA buffer containing 0.1%

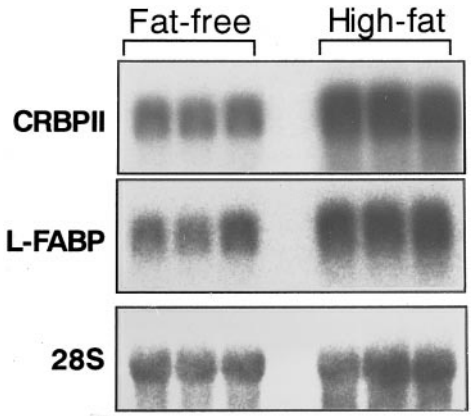

FIG. 1. Effect of high-fat diet on CRBPII and L-FABP mRNA levels in rat jejunum. Rats (N = 3/group) were fed a fat-free or a high-fat diet for 2 weeks as described under Materials and Methods. Jejunal total RNAs (10 μg) prepared from three different rats were analyzed for each mRNA level by Northern blot hybridization as described in Materials and Methods. The 28 S rRNA signals were for loading control.

SDS for 15 min at 42°C twice. The washed membranes were exposed to an image plate for 72 h. Quantification of the signals was performed by densitometric scanning of the spots using bioimage analyzer and signals were standardized for the corresponding 28S ribosomal RNA signals.

Productions of RXRα and PPARα protein. Both rat RXRα (16) and PPARα (24) full-length cDNAs were generated by reverse transcribed-PCR methods. The rat RXRα and PPARα cDNAs were subcloned into the *Eco*RI and *Bam*HI site of pcDNA1.1/Amp expression vector (Invitrogen, Carlsbad, CA), respectively, and RXRα and PPARα proteins were synthesized using TNT T7 Quick coupled transcription/translation system (Promega, Madison, WI), according to the protocol of the manufacturer. The production of *in vitro* translated proteins were analyzed by ^{35}S incorporation and direct visualization on SDS–PAGE. Rat PPARα cDNA was also subcloned into the *Bam*HI site of pET-15b vector (Novagen, Madison, WI) and the expression construct was transformed into *Escherichia coli* BL21(DE3). Cultures were grown at 37°C in Luria–Bertani medium containing 200 μg/ml ampicillin. The culture was induced at an optical density (at 595 nm) of 0.6 using 1 mM isopropyl β-D-thiogalactopyranoside and grown further for 24 h at 18°C before harvesting by centrifugation. The cell pellet was resuspended in Buffer A [50 mM Tris–HCl (pH 7.9), 2 mM EDTA, 100 mM NaCl, 1 mM PMSF, 1 mM DTT] and sonicated with a microtip to lyse the cells. The cell pellet was collected by centrifugation at 10,000g for 30 min at 4°C and then resuspended in Buffer A containing 0.1% Triton X-100. The solution was mixed by a vortex and the soluble protein fraction containing PPARα was collected by centrifugation at 10,000g for 30 min at 4°C and then dialyzed against Ni^{2+} starting buffer [20 mM Tris–HCl (pH 7.9), 5 mM imidazole, 0.5 M NaCl]. The dialyzed solution was purified using HiTrap chelating column (Pharmacia Biotech., Tokyo, Japan). The protein was eluted from the column in the elution buffer [20 mM Tris–HCl (pH 7.9), 1 mM imidazole, 0.5 M NaCl]. The eluted PPARα was subjected to thrombin cleavage according to the protocol of the manufacturer (Invitrogen) and the final PPARα preparation was stored in aliquots at −70°C.

Gel shift assay. The specific DNA-binding of nuclear proteins from rat jejunum and the binding of *in vitro*-translated PPARα and/or RXRα was monitored by a gel shift assay using ^{32}P-fill-in-labeled double-stranded RXRE and Regardless oligonucleotides located in the rat CRBPII gene (19). The sequence of labeled probes are as follows: RXRE (from −640 to −599; Ref. 23), 5′-CTGCTGTCACAGGTCACAGGTCACAGGTCACAGTTCATTTTC-3′; and RE3 (from −76 to −52; Ref. 23), 5′-GCTTATGACCTTTGACTCTGTTAT-3 (underlines indicate direct repeat). Rat jejunal nuclear extracts, *in vitro*-translated RXRα, and/or PPARα were incubated at room temperature for 15 min with 1 μg polydeoxyiosinic-deoxycytidylic acid (Pharmacia Biotech., Tokyo, Japan) in 10 μl of binding buffer containing 10 mM Tris–HCl (pH 8.0), 150 mM KCl, 0.05% Nonidet P-40, 6% glycerol, 1 mg/ml bovine serum albumin, 0.5 mM DTT, and 0.1 mM PMSF. Each labeled probe (about 2×10^4 cpm/5 fmol) was then added and the mixture was incubated for an additional 20 min at room temperature. For competition analysis, the gel shift assay was performed using 100-fold molar excess of unlabeled RXRE, RE3, and PPRE of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) synthase gene (5′-TCTGAGACCTTTGGCCCAGTTTTTCTGAGG-3′ (28); underlines indicate direct repeat) or unrelated sequence for ubiquitous nuclear factor Sp1 (5′-GATCGGGGCGGGGCGAG-3′). Ligands for nuclear receptors were dissolved in Me_2SO and were added directly to the incubation mixture prior to the addition of labeled probes. Final concentrations of Me_2SO in the reaction mixture were adjusted to 0.4%. The oligonucleotide probe–protein complexes were separated from the unbound probe on 5% polyacrylamide gels in the presence of 0.5 × Tris-borate-EDTA buffer [1×; 90 mM Tris, 90 mM boric acid, and 2 mM EDTA, pH 8.0] at 4°C at 180 V for 90 min. After electrophoresis, the gel was dried and analyzed using bioimage analyzer as described above.

Statistical analysis. Results were expressed as mean ± SEM. The significance of differences between groups was determined by Student's t test.

RESULTS

Dietary fat induces CRBPII gene expression at transcription level. Effect of dietary fat intake on the CRBPII mRNA levels in rat jejunum was studied. In this study, no significant differences in the body-weight gain and the daily food consumption were observed between the fat-free diet group and the high-fat diet group (data not shown). As shown in Fig. 1 and Table I, feeding a high-fat diet produced a 3.3-fold increase in rat jejunal CRBPII mRNA level compared with fat-free diet group. This increase was parallel with the increase in its pre-mRNA levels which were determined by highly-sensitive ribonuclease protection assay using

TABLE I

Summary of the Results from Figs. 1–3

Gene	mRNA level[a]	pre-mRNA level[a]	Transcription Rate[a]
CRBPII	3.30 ± 0.42*	4.21 ± 0.81*	1.63 ± 0.12*
L-FABP	3.70 ± 0.35*	—[b]	2.66 ± 0.34*
PPARα	1.79 ± 0.16*	—[b]	—[b]
RXRα	1.06 ± 0.06	—[b]	—[b]

[a] Data obtained from the results of Figs. 1–3 were normalized by using the 18S or 28S rRNA signals and expressed as relative values of high-fat diet group representing the mean value of the corresponding fat-free diet group (N = 3) as 1.0. Values are mean ± SEM for N = 3/group.

[b] Not measured.

* Significantly different from the corresponding fat-free diet group (Student's t test, $P < 0.05$).

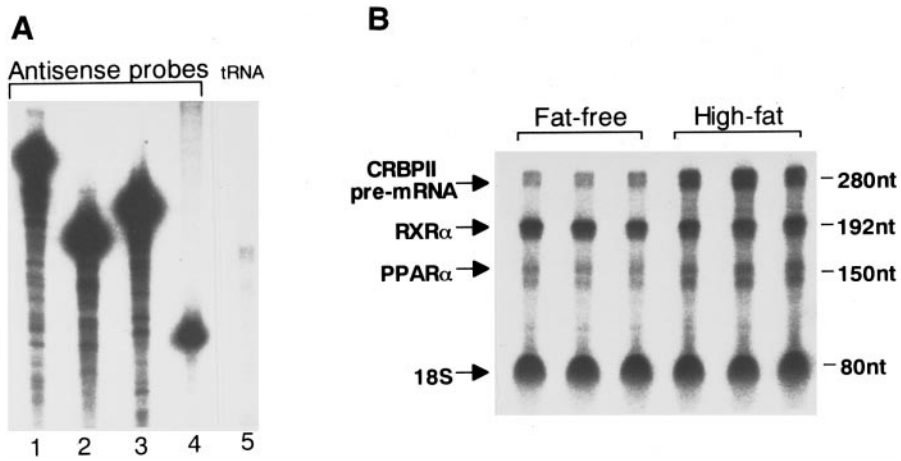

FIG. 2. Ribonuclease protection assay of jejunal CRBPII pre-mRNA, and PPARα and RXRα mRNA levels. (A) Synthesized antisense RNA probes (lane 1: CRBPII gene intron sequence; lane 2: PPARα; lane 3: RXRα; and lane 4: 18 S rRNA) were shown. Fifteen micrograms yeast tRNA was hybridized with mixture of each antisense RNA probe and was treated with ribonuclease A and T1 as a control (lane 5) as described in Materials and Methods. (B) Rats were the same as described in the legend of Fig. 1. Jejunal total RNAs (15 μg) prepared from three different rats were analyzed for CRBPII pre-mRNA, PPARα, and RXRα mRNA levels by ribonuclease protection assay as described in Materials and Methods. The protected size of each transcript was shown as a nucleotide (nt). The 18 S rRNA signals were used for normalization of each mRNA level.

rat CRBPII intron cDNA as a probe (4.2-fold, Fig. 2 and Table I). Furthermore, nuclear run-on assay revealed that the transcription rate of the CRBPII gene was significantly elevated in rats fed a high-fat diet (1.6-fold, Fig. 3 and Table I). In addition, jejunal L-FABP mRNA levels (3.7-fold, Fig. 1 and Table I) and its transcription rate (2.7-fold, Fig. 3 and Table I) were also elevated in rats fed a high-fat diet compared with those of a fat-free diet.

Regulation of PPARα gene expression in rat jejunum by dietary fat. We next studied the effects of dietary fat on the gene expression of PPARα because PPARα was shown to be activated by their ligands, e.g., fatty acids (29, 30), and they were the most possible candidate for the nuclear factor involved in the dietary fat-mediated regulation of CRBPII gene expression. The PPARα mRNA level in the jejunum was significantly elevated in rats fed the high-fat diet (1.8-fold increase compared with the fat-free group, Fig. 2 and Table I). On the other hand, the mRNA level of RXRα, which dimerizes with PPARs, was unaffected by the dietary manipulation in rat jejunum (Fig. 2 and Table I).

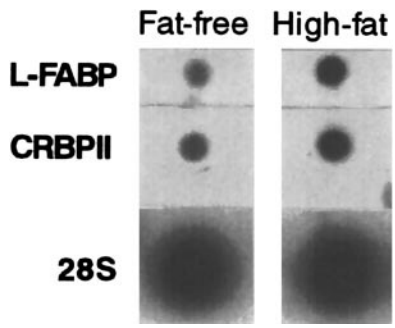

FIG. 3. High-fat diet induces CRBPII and L-FABP gene expressions in rat jejunum at the transcription level. Rats were the same as in Fig. 1. Nuclear run-on assays were performed using isolated rat jejunal nuclei. [α-^{32}P]UTP-labeled transcripts were hybridized with denatured cDNA probes bound to nylon membrane as described in Materials and Methods. The 28S rRNA cDNA was used for normalization of each transcription level. The figure is a representative of three independent experiments.

Properties of jejunal nuclear proteins-binding to the nuclear receptors response elements on CRBPII gene. The formation of DNA–protein complexes that consist of the nuclear receptor response elements (RXRE and RE3) reported in the rat CRBPII gene (19) and rat jejunal nuclear proteins were examined by gel shift assay. Some complexes of jejunum nuclear proteins bound to each of ^{32}P-labeled DNA oligonucleotide were observed (Fig. 4). In order to estimate the binding specificities of these protein–DNA complexes, the competitive gel shift analysis was performed using unlabeled 100-fold molar excess own sequences (RXRE and RE3), well-known HMG-CoA synthase gene's PPRE, or unrelated sequence (Sp-1) as competitors. As shown in Fig. 4, we recognized totally three prominent specific bindings of protein–DNA as indicated by alphabets. Both bindings of complexes B on the RXRE element and C on the RE3 element were also competed by HMG-CoA synthase PPRE sequence (Fig. 4, compare lanes 2 vs 5, and lanes 7 vs 10). The signal densities of complexes B and C were greater in rats fed the high-fat diet than those fed the fat-free diet (Fig. 5, compare lanes 2 vs 3, and 6 vs 7, respectively) and were en-

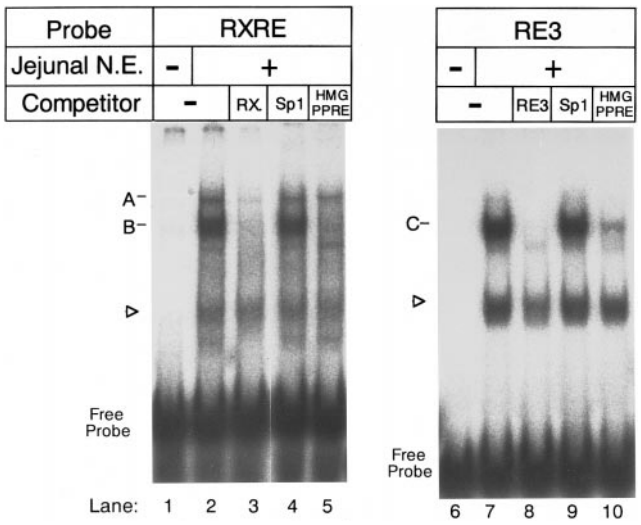

FIG. 4. Analysis of binding properties of rat jejunal nuclear proteins to RXRE and RE3 elements located in rat CRBPII gene. Gel shift assay was performed with rat jejunal nuclear proteins (4 μg) and ^{32}P-labeled RXRE and RE3 probes as described under Materials and Methods. Assays were conducted in the absence (−) or presence of 100-fold molar excess of unlabeled respective oligonucleotides as competitors. The signals A–C represent specific DNA–protein complexes. The competitors used in this study: RX, RXRE; RE3; HMG-PPRE, HMG-CoA synthase gene's PPRE; and Sp-1 oligonucleotides.

hanced by the addition of bacterially expressed PPARα protein (Fig. 5, compare lanes 3 vs 4 and lanes 7 vs 8).

PPARα-RXRα-heterodimer binds to CRBPII gene-RXRE and RE3 elements. To test the binding activities of PPAR-RXR heterodimer to the RXRE and RE3 elements, gel shift assay was performed using *in vitro*-synthesized PPARα and RXRα. As shown in Fig. 6, *in vitro*-synthesized PPARα alone did not bind specifically to RXRE and RE3 elements (lanes 3 and 9). On the other hand, RXRα alone could bind to RE3 element as homodimer (Fig. 6, lane 8), but not to RXRE (Fig. 6, lane 2) in this gel shift assay. When these two receptors were combined, a clearly distinct band was seen for RXRE and RE3 elements (Fig. 6, lanes 4 and 10, respectively). The heterodimer-mediated binding was competed with the unlabeled own sequence (Fig. 6, lanes 5 and 11), but not with the unrelated Sp1 sequence (Fig. 6, lanes 6 and 12). The binding activity of PPARα-RXRα heterodimer for the RE3 elements were enhanced by addition of various long-chain fatty acids (Fig. 7A, compare lanes 1 vs 2–7), but not by medium-chain fatty acid (caprylic acid) and 9-*cis* retinoic acid (Fig. 7B, compare lanes 1 vs 2–4 and 11–13, respectively). These PPARα ligand-induced binding activity of PPARα-RXRα heterodimer depended on the added ligand concentration (Fig. 7B, lanes 5–7 and 8–10). Control experiments indicated that the PPARα ligands (palmitic acid and arachidonic acid) did not enhance, but rather repressed the DNA binding activity of the RXRα homodimer (Fig. 7C, lanes 5–10), which was inducible by RXRs ligand, i.e., 9-*cis* retinoic acid (Fig. 7C, lanes 11–13). Similar results were also observed for the RXRE elements (data not shown).

DISCUSSION

Many studies have previously reported that dietary fat (especially polyunsaturated fatty acids) regulates positively or negatively many gene expressions related to lipid and carbohydrate metabolisms through certain types of transcriptional regulation [see reviews of Ref. 31). Our previous studies showed that a diet rich in long-chain triacylglycerol increased the CRBPII mRNA level as well as CRBPII protein level (10). This increase in CRBPII mRNA levels was brought about especially by unsaturated long-chain fatty acids in long-chain triacylglycerol diet within a few hours (11). Conversely, the rat jejunal CRBPII expression was decreased at protein and mRNA levels after the intake of a fat-free diet (32). Thus, dietary fatty acids are considered to be one of the modulator of CRBPII gene expression in rat small intestine.

In the present study, the induction mechanism for the fat-induced CRBPII gene expression was explored by the two independent measures of transcriptional rate, i.e., the ribonuclease protection assays of pre-

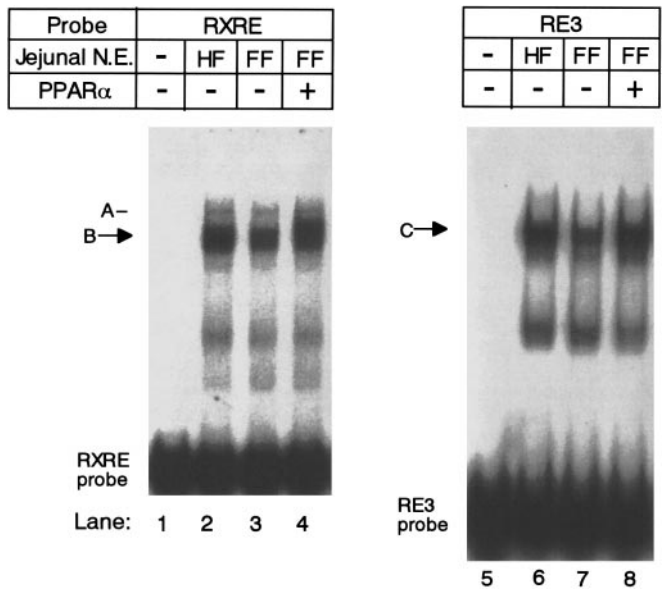

FIG. 5. Enhancement of binding activity of jejunal nuclear extract to RXRE and RE3 elements in the rats fed a high-fat diet. The nuclear extracts used in this figure were extracted from the combined nuclei derived from each three rat fed a fat-free or high-fat diet. Gel shift assay was performed using jejunal nuclear proteins (4 μg) of rats fed the fat-free (FF) or high-fat (HF) diet for 2 weeks with or without bacterially synthesized PPARα (100ng). Rats were the same as in Fig. 1.

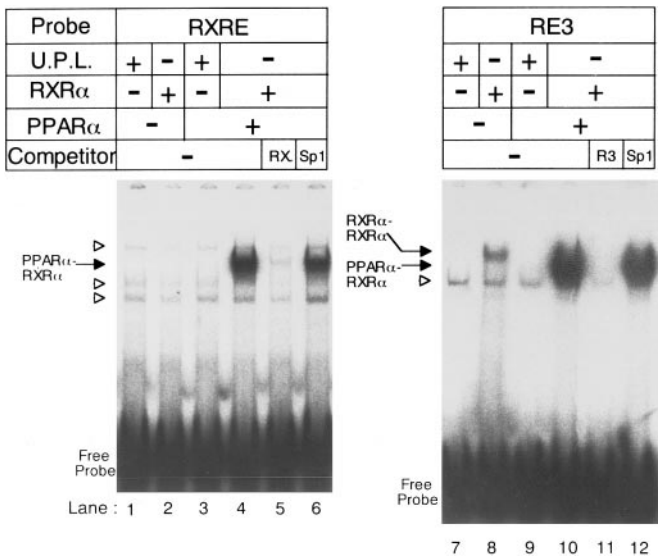

FIG. 6. PPARα-RXRα heterodimer binds to the RXRE and RE3 elements. The gel shift assay was performed using labeled RXRE and RE3 as probes, and unprogrammed lysate (U.P.L.), *in vitro*-translated RXRα (1 μl), and/or PPARα (3 μl). The total amount of reticulocyte lysate was maintained constant in each reaction (4 μl) through the addition of U.P.L. The positions of PPARα-RXRα heterodimer and RXRα homodimer were indicated by the arrows. Assays were also conducted in the absence (−) or presence of 100-fold molar excess of unlabeled respective oligonucleotides as competitors. The competitors used in this study: RX, RXRE; R3, RE3; and Sp-1 oligonucleotides. Open triangles indicate nonspecific bindings probably derived from reticulocyte lysate.

mRNA using intron probe and the nuclear run-on assays. These results indicate that feeding of high-fat diet induces CRBPII gene expression at a transcription level. We also demonstrated that not only CRBPII but also L-FABP gene was up-regulated at the transcription level by a high-fat diet in rat jejunum (Fig. 3 and Table I). This result was consistent with the previous report which showed that L-FABP gene expression was induced in the small intestine (ileum) at transcription level by oral administration of fat in mice (33). We have recently observed that CRBPII and L-FABP genes are regulated in parallel by long chain-fatty acids or its analogue in rat jejunum and in human intestine-like cell line, Caco-2 cells as well (Suruga *et al.*, unpublished work). These results suggest that there is a common transcriptional induction mechanism between intestinal CRBPII and L-FABP in which fatty acids may serve as a natural transcriptional inducer.

Various fatty acids, especially long-chain fatty acids, were reported to be able to activate PPARs, serving as their ligands (29, 30). These reports raised a possibility that the fatty acid-induced CRBPII and L-FABP gene expression may be caused through PPAR-mediated pathway. Both PPARα transcript and their partner RXRα transcript were coexpressed in the rat jejunum (Fig. 3, Table I, and Ref. 11). Interestingly, jejunal PPARα mRNA level was increased by intake of the high-fat diet for 2 weeks (Fig. 2 and Table I) and this increase was dependent on the amounts of fat in the diet (data not shown). Our previous study showed that jejunal PPARα mRNA level was not influenced by force-feeding of the high-fat diet at 6 h after the feeding when jejunal CRBPII mRNA level was already elevated (11). Although these two results appear to contradict each other, we consider that there may be two independent steps in increasing jejunal CRBPII gene expression through PPAR-mediated mechanism as follows: (1) in a short period after the fat intake (in our previous study Ref. 11), jejunal CRBPII mRNA level may be increased mainly by the increase in the amounts of fatty acids or its metabolites derived from them which are served as PPARα ligands; (2) after a long-term intake of fat (in this study), both PPARα level and its ligand levels in the small intestine would increase and they may result in more marked increase in CRBPII mRNA level.

The results from gel shift assay showed that small intestinal nuclear extracts in the rats fed a high-fat diet contained greater amounts of proteins which bound to the RXRE and RE3 than those in the rats fed a fat-free diet (Fig. 5) and that the densities of these binding complexes were enhanced by the addition of PPARα protein (Fig. 5). These results suggest that an increase in the jejunal PPARα expression level by intake of high-fat diet may result in increase of binding of small intestinal nuclear extract to the RXRE and RE3 elements.

We also showed that PPARα-RXRα heterodimer was capable of binding to RXRE and RE3 elements (Fig. 6). This result suggests that PPARα-RXRα heterodimer may be one of the transcriptional regulators of rat CRBPII gene. Very recently, it was reported that the promoter activity of the 5′-flanking region of mouse CRBPII gene containing RE3 element was enhanced by a potent PPARα ligand, Wy-14,643 in the cells which were overexpressed with PPARα and RXRα (34). Our present studies further demonstrated that various long-chain fatty acid could specifically induce the binding activity of PPARα-RXRα heterodimer to the RXRE and RE3 elements (Fig. 7). These results suggest that dietary fat-induced increases of PPARα ligand concentration may result in the induction of PPARα-RXRα heterodimer binding activity to RXRE and RE3 elements in rat jejunal nuclei. This result is in good agreement with the previous report of Forman *et al.*, who demonstrated the ligand-induced increase in the binding of PPAR-RXR heterodimer to the PPRE element of acyl-CoA oxidase gene (29). On the other hand, the PPARα ligands (palmitic acid and arachidonic acid), but not caprylic acid, had repressive effects at higher concentration (i.e., 20 and 40 μM) (Fig. 7C). Although it was reported that fatty acids including arachidonic

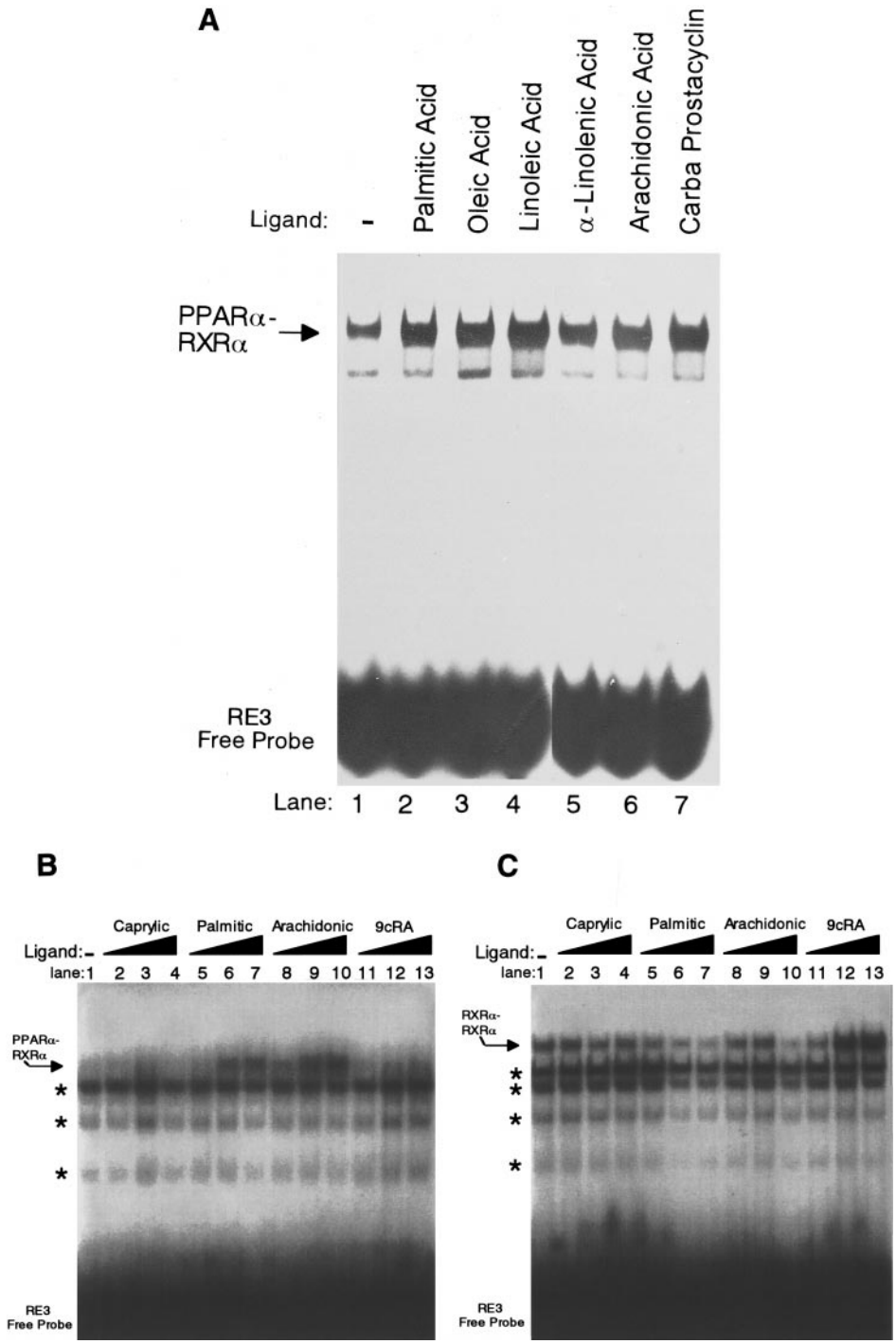

FIG. 7. Effects of fatty acids for the binding activity of a PPARα-RXRα heterodimer to RE3 element. (A) The gel shift assay was performed using *in vitro*-translated RXRα (0.15 μl) and PPARα (0.45 μl) in the absence or presence of various fatty acids (40 μM each of palmitic acid, oleic acid, linoleic acid, α-linolenic acid, or arachidonic acid, or 10 μM carba-prostacyclin). (B) The gel shift assay was performed using *in vitro*-translated RXRα (0.15 μl) and PPARα (0.45 μl) in the absence or presence of various amounts of fatty acids (5, 20, or 40 μM each of caprylic acid, palmitic acid, and arachidonic acid) or 9-*cis* retinoic acid (9cRA; 0.1, 1, or 5 μM). Asterisks indicate nonspecific binding complexes derived from reticulocyte lysate. (C) The same assay as described in B was performed using RXRα alone (1 μl). Asterisks indicate nonspecific binding complexes derived from reticulocyte lysate.

acid were not able to bind to RXRα as ligand (35), we observed that the repressive effect on the binding of RXRα homodimer were specific for the long-chain fatty acids. It remains unclear at present about the cause of these repressive effects of long-chain fatty acids on the DNA binding.

Another type of nuclear receptor superfamily, hepatocyte nuclear factor-4 (HNF-4)-α, which is expressed abundantly in the small intestine (36), was shown to bind to CRBPII-RXRE and RE3 elements as homodimer and activate CRBPII gene promoter activity (19, 34). Very recently, Hertz *et al.* demonstrated that HNF-4-α was activated by fatty acyl-CoA thioesters as ligand (37). However, it should be pointed out that some acyl-CoA esters of long-chain fatty acids (oleic, linoleic, and α-linolenic acids, which were able to induce CRBPII mRNA levels (11) were inhibitors of HNF-4-α (37). Thus, it is unlikely that HNF-4 may be involved in the induction of CRBPII gene expression by dietary fatty acids.

In conclusion, a strong stimulation of CRBPII gene expression is elicited by fatty acids through a transcriptional mechanism probably mediated by PPARα-RXRα heterodimer bound to CRBPII-RXRE and RE3 elements with increases of both PPARα levels and its ligand levels.

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