Diet-related variation in cellular retinol-binding protein type II gene expression in rat jejunum

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Cellular retinol-binding protein type II (CRBPII) is involved in the transport of vitamin A and its metabolism in the small intestine. In the present study, we demonstrated diet-related variations in CRBPII expression in rat jejunum. The CRBPII protein and mRNA levels increased in parallel after the start of feeding period regardless of whether the feeding period was restricted to the hours of darkness or of light. In addition, this variation was observed in the rats fed high-fat diet or low-fat diets, but not in those fed a fat-free diet or in fasted rats. A similar diet-induced variation was seen in the mRNA of liver-type fatty acid-binding protein in rat jejunum. In the transient transfection experiment, unsaturated fatty acid increased rat CRBPII gene promoter activity via the PPARα/retinoid X receptor-α heterodimer. Taken together, these results suggest that the diet-related variation in CRBPII expression in rat jejunum may be brought about by the transcriptional induction of CRBPII gene expression mainly triggered by dietary fatty acids.

CRBPII: L-FABP: small intestine: PPAR: dietary fat

It is well known that the capability to digest and absorb carbohydrates and proteins in the small intestine exhibits diurnal variations in the rat (Furuya *et al.* 1979). A number of reports have focused on the diurnal variations in carbohydrate-related proteins, including hexose transporters (Castello *et al.* 1995; Corpe & Burant, 1996) and disaccharidases (Saito *et al.* 1975, 1976). Although the precise mechanisms responsible for these diurnal variations are still unclear, it is evident that the rhythmic variations of these carbohydrate-related proteins are strictly dependent on feeding behaviour. Moreover, the expression of these carbohydrate-related proteins is also rapidly regulated by some dietary factors (Goda & Koldovsky, 1988; Kishi *et al.* 1999). Thus, carbohydrate-related proteins may be regulated by both diurnal rhythm and dietary factors. Little attention has, however, been given to the possible diurnal variations of digestive and absorptive capability for nutrients including fat and fat-soluble vitamins.

Cellular retinol-binding protein type II (CRBPII) is an abundant cytosolic protein found in the small intestinal enterocytes of many vertebrates (Ong, 1984; Ong & Page, 1987; Goda & Takase, 1989). CRBPII specifically binds retinol or retinal and may play an important role in the intestinal transport of vitamin A and its metabolism. Indeed, CRBPII–retinal and CRBPII–retinol complexes serve as substrates for the conversion of retinal into retinol, catalysed by retinal reductase (Kakkad & Ong, 1988), and for the conversion of retinol into retinyl esters, catalysed by lecithin:retinol acyltransferase (Ong *et al.* 1987). Thus, the alteration in CRBPII level in the small intestine may lead to changes in its capacity to absorb and transport vitamin A.

We and others have previously reported that intestinal CRBPII gene expression is altered in various physiological conditions: the level of CRBPII mRNA is, for example, abruptly elevated during the perinatal period in rats (Levin *et al.* 1987) and chicks (Suruga *et al.* 1997), and CRBPII gene expression can be induced by dietary fatty acids (Goda *et al.* 1994; Suruga *et al.* 1995; Takase *et al.* 1998). In the present study, we investigated whether diurnal variation or food consumption contributed to the expression of CRBPII in rat jejunum. We demonstrated that the variation in expression level of CRBPII in rat jejunum during the course of a day was triggered especially by the consumption of dietary fat but not by the diurnal rhythm of feeding.

Materials and methods

Animals and experimental diets

Three studies on variation in CRBPII gene expression were conducted. All the animals used in this study were 6-week-old male Wistar rats. All the rats were housed under a light cycle with 12 h darkness from 18.00 h to 06.00 h. The rats were killed by decapitation every 4 or 6 h as indicated.

In the first study, to investigate the diurnal pattern of jejunal CRBPII expression, the rats were separated into two groups; one group of animals were fed a standard laboratory chow diet (MF; Oriental Yeast Co., Tokyo, Japan) only during the dark period (from 18.00 h to 06.00 h) for 1 week. The other group of

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animals received the same diet during the 6 h period between 09.00 h and 15.00 h (a part of the light period) for 2 weeks.

In the second study, to investigate the effects of dietary fat on the variation in jejunal CRBPII gene expression, three types of diet containing different amounts of fat (Table 1) were given to three groups of rats. The first group of rats received access to a synthetic low-fat diet containing 2.4 % (w/w) corn oil only during the 6 h period between 09.00 h and 15.00 h for 2 weeks. The second group received access to a synthetic low-fat diet only during the 6 h period between 09.00 h and 15.00 h for 13 d and then received a synthetic fat-free diet only during the 6 h period between 09.00 h and 15.00 h on the fourteenth day. The third group was fed a high-fat diet containing 24 % (w/w) corn oil during the same 6 h period for 2 weeks.

In the third study, rats received access to a synthetic low-fat diet only during the 6 h period between 09.00 h and 15.00 h for 13 d and were then fasted during 6 h period only on the fourteenth day. The experimental procedures used in the present study met the guidelines of the animal usage committee of the University of Shizuoka.

Preparation of intestinal samples

The entire small intestine was removed, and the duodenum extending from the pylorus to the ligament of Treitz was discarded. The jejuno-ileum was divided into three segments of equal length. The proximal two-thirds (jejunum) of the jejuno-ileum were flushed with ice-cold 0.9 % NaCl solution. A 1.5 cm segment (100 mg) was excised from the middle region of the jejunal segment and immediately used for total RNA extraction. An adjacent 20 cm segment was saved at $-20°C$ for measurement of CRBPII content.

Quantification of jejunal CRBPII content

Mucosa was scraped from the jejunal segment with a glass microscope slide and homogenised in four volumes of ice-cold $0.2 \text{ M-K}_2\text{PO}_4$ buffer (pH 7.2). Following centrifugation at $10\,000\,g$ for 60 min at 4%, the cytosolic fraction was used for the measurement of CRBPII. Quantification of the jejunal CRBPII content was performed by sandwich-type ELISA using monospecific anti-rat CRBPII IgG as previously described (Takase et al. 1993).

RNA analysis

Total RNA was extracted from rat jejunum by the acidified guanidine thiocyanate method (Chomczynski & Sacchi, 1987).

Table 1. Composition of the experimental diets

Dietary components	Fat-free (g/kg diet)	Low-fat (g/kg diet)	High-fat (g/kg diet)
Vitamin-free casein	157.0	157.0	159.0
β-corn starch	580.0	526.0	36.0
Corn oil	–	24.0	247.0
Mineral mix, AIN-76	28.0	28.0	28.0
Vitamin mix, AIN-76	8.0	8.0	8.0
DL-Methionine	2.4	2.4	2.4
Choline bitartrate	1.6	1.6	1.6
2 % Agar solution	223.0	253.0	518.0

Northern blot analysis was performed using ^{32}P-labelled cDNA probes as previously described (Goda et al. 1994). The cDNA probes used in the Northern blot analysis were as follows: rat CRBPII cDNA (Goda et al. 1994), rat liver fatty acid-binding protein (L-FABP) cDNA (Suruga et al. 1999) and rat 28S ribosomal RNA (Suruga et al. 1999). For quantification of specific mRNA signals in the Northern blots, the density of radioactivity was scanned using a bio-image analyser (BAS 2000; Fujifilm, Tokyo, Japan), and the signals were normalised using the corresponding 28S rRNA level.

Plasmid construction, cell culture and transient transfection

Expression vectors for the mouse PPARα (pCMX-PPARα) and retinoid X receptor-α (RXRα; pCMX-RXRα) were generously provided from Dr RM Evans. Two types of rat CRBPII gene luciferase reporter vector were constructed as follows. First, the promoter region of the rat CRBPII gene (GenBank accession number M16400; 237-1037) was ligated into the PGV-B2 basic vector (TOYO Ink Co., Tokyo, Japan), and two tandem repeats of the RE3 region of the rat CRBPII gene (Suruga et al. 1999) were ligated into the PGV-P promoter vector (TOYO Ink Co.). These were designated prCRBPII pro-LUC and prCRBPII(RE3)2-SV40-LUC, respectively.

CV-1 cells were purchased from American Type Culture Collection (Rockville, MD, USA) and cultured at 37°C in a humidified atmosphere (5 % CO$_2$, 95 % air) in Dulbecco's modified eagle's medium (Sigma, St Louis, MO, USA) supplemented with 10 % (v/v) fetal calf serum (Biological Industries, Kibbutz Bet-Haemek, Israel), 2 mM-glutamine (Sigma, St Louis, MO, USA), 20 mM-HEPES and 1 % (v/v) antibiotic–antimycotic mixed solution (Nacalai Tesque, Kyoto, Japan). For the transfection, cells were plated at a density of 3.0×10^4 cells per well in a ninety-six-well cell culture plate (Iwaki glass, Tokyo, Japan). Twenty-four hours after plating, the culture medium was removed and the cells washed once with Opti-MEM (Invitrogen, Tokyo, Japan).

Transfection was performed using Tfx-10 reagent (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. Briefly, each well was transfected with 100 ng constructed luciferase reporter vector, 50 ng each of pCMX-RXRα and pCMX-PPARα expression vectors (or 100 ng pCMX mock vector) and 2 ng Renilla luciferase control vector phRG-TK (Promega Corporation), which were complexed with 0.6 μl Tfx-10 reagent in 50 μl Opti-MEM. One hour after transfection, the DNA–Tfx-10 complex was removed and cells were incubated for 48 h in a serum-free culture medium containing 2 mg/ml bovine serum albumin (BSA) with or without 100 μM fatty acids. Luciferase activity was determined by the dual-luciferase reporter assay system (Promega Corporation) and expressed as fold-activation relative to control (pCMX mock vector only, no fatty acids).

Statistical analysis

All results were subjected to one-way ANOVA. Differences in mean values between groups were tested using Ryan's multiple range test, or differences in mean values between the two groups were analysed by Student's t test (Stat-Light, Yukmus Co., Tokyo, Japan). Both differences were considered statistically significantly different at $P < 0.05$.

Results

Feeding-related variation in jejunal CRBPII protein and mRNA levels

When rats were fed a standard laboratory diet during the dark period (18.00 h to 06.00 h), both CRBPII protein and mRNA levels were elevated during the feeding period; the lowest levels were seen at 16.00 h, and both levels gradually increased during the dark period (Fig. 1(A)). To examine whether these variations in CRBPII expression were related to the feeding period or were synchronised to the light–dark cycle, the feeding period was shifted to the time between 09.00 h and 15.00 h (i.e. the light period). As a result, the pattern of variation in jejunal CRBPII mRNA levels shifted, and remarkable increases in CRBPII mRNA levels were observed after the start of the feeding period in spite of the light (Fig. 1(B)).
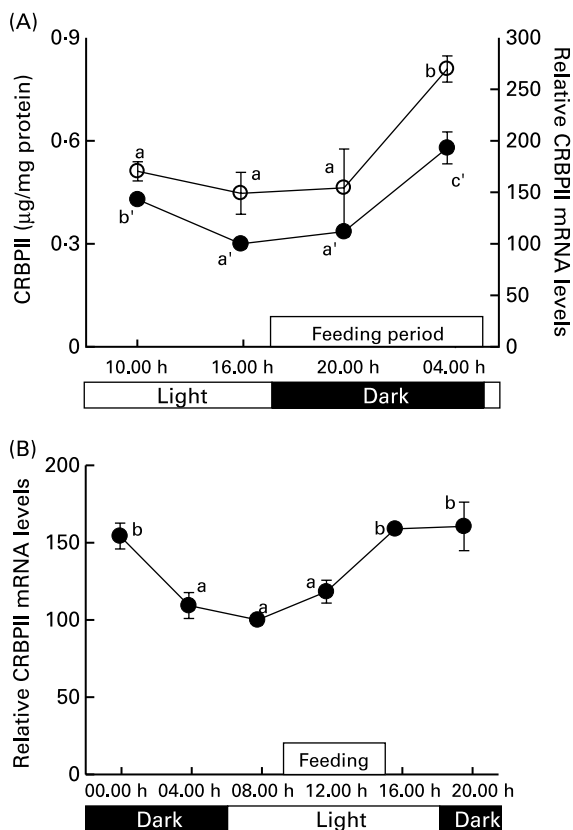

Fig. 1. Diet-related variation in cellular retinol-binding protein (CRBP) II protein and CRBPII mRNA levels in rat jejunum. (A) Rats had access to a standard laboratory diet only during the dark period (18.00 h to 06.00 h) for 1 week and were killed by decapitation at 6 h intervals. Total RNA was isolated from the jejunum, and the CRBPII mRNA levels (●) were quantified by Northern blot hybridisation using 28S rRNA signals for normalisation. Jejunal cytosolic CRBPII protein (○) was quantified by ELISA and expressed as μg/mg jejunal mucosal protein. (B) Rats were fed a standard laboratory diet only during the time indicated during the light hours (from 09.00 h to 15.00 h) for 2 weeks and were killed by decapitation at 4 h intervals. Total RNA was isolated from the jejunum, and the CRBPII mRNA levels were quantified by Northern blot hybridisation using 28S rRNA signals for normalisation. Values represent means with their standard errors for three rats for each time point. [a,b,c]Values with unlike superscript letters on the same curve are significantly different at $P < 0.05$ by Ryan's multiple range test.

Dietary fat-induced variation in jejunal CRBPII and L-FABP mRNA levels

We previously reported that dietary fat, especially unsaturated fatty acids, enhanced CRBPII mRNA levels in rat jejunum (Goda *et al.* 1994; Suruga *et al.* 1995; Takase *et al.* 1998). Therefore, we next investigated whether dietary fat affected the variation in jejunal CRBPII mRNA level. Rats were meal-fed either an isoenergic low-fat or high-fat diet during the 6 h period (from 09.00 h to 15.00 h) each day for 2 weeks. In both dietary groups, CRBPII mRNA levels were similarly increased after the start of feeding (Fig. 2). Overall mRNA levels were higher in the animals fed the high-fat diet than in animals fed the low-fat diet at all times investigated over the 24 h period (Fig. 2). CRBPII mRNA levels were significantly higher ($P < 0.05$) at 20.00 h than at 08.00 h in both dietary groups (2·9-fold in animals fed the low-fat diet and 2·3-fold in those fed the high-fat diet). However, no increase in CRBPII mRNA level after the start of feeding was seen in the rats fed a fat-free diet on the last day (Fig. 2). In addition, when the rats were fasted on the last day, CRBPII mRNA levels did not change between 08.00 h and 20.00 h (Fig. 2). L-FABP, which is considered to be involved in intestinal and liver fatty acid transport, showed a similar variation in mRNA level in rat jejunum to that of CRBPII (Fig. 2). The animals fed the high-fat diet also exhibited greater L-FABP, as well as CRBPII, mRNA levels than those fed the low-fat diet at any time of day (Fig. 2).

Rat CRBPII gene promoter transactivation by fatty acids via PPARα/RXRα heterodimer

In order to clarify the mechanism of dietary-fat induced CRBPII gene expression, we performed transient transfection assay. Rat CRBPII gene promoter activity increased in the CV-1 cells transiently expressing PPARα and RXRα (Fig. 3(a)) in the absence of fatty acids. Furthermore, linoleic acid and arachidonic acid further induced the PPARα/RXRα heterodimer-mediated transactivity. Similar results were observed in the reporter vector which was ligated only rat CRBPII gene RE3 element (Fig. 3(b)).

Discussion

A number of studies have reported the presence of diurnal variations or circadian rhythms in intestinal digestive and absorptive capability. Investigations into the diurnal variations in disaccharidase and glucose transporters in the rodent's small intestine have revealed that these diurnal variations were closely related to food intake, but that the increase in their protein and/or mRNA levels occurred before the onset of feeding (Saito *et al.* 1975, 1976; Furuya *et al.* 1979; Castello *et al.* 1995; Corpe & Burant, 1996). Although the precise mechanism leading to the diurnal variations in these enterocyte-specific proteins is unknown, several possibilities have been suggested. One is related to diurnal variation in the enterocyte cell populations. The number of absorptive cells and the villus height in rat jejunum were reported to be elevated before the start of feeding (Stevenson *et al.* 1979), which appeared to correlate with diurnal variations in sucrase activity (Saito *et al.* 1975, 1976) and glucose transporter mRNA levels (Corpe & Burant, 1996). Furthermore, it has recently been reported that the diurnal variations in some disaccharidase and glucose transporters in the rat small intestine might be
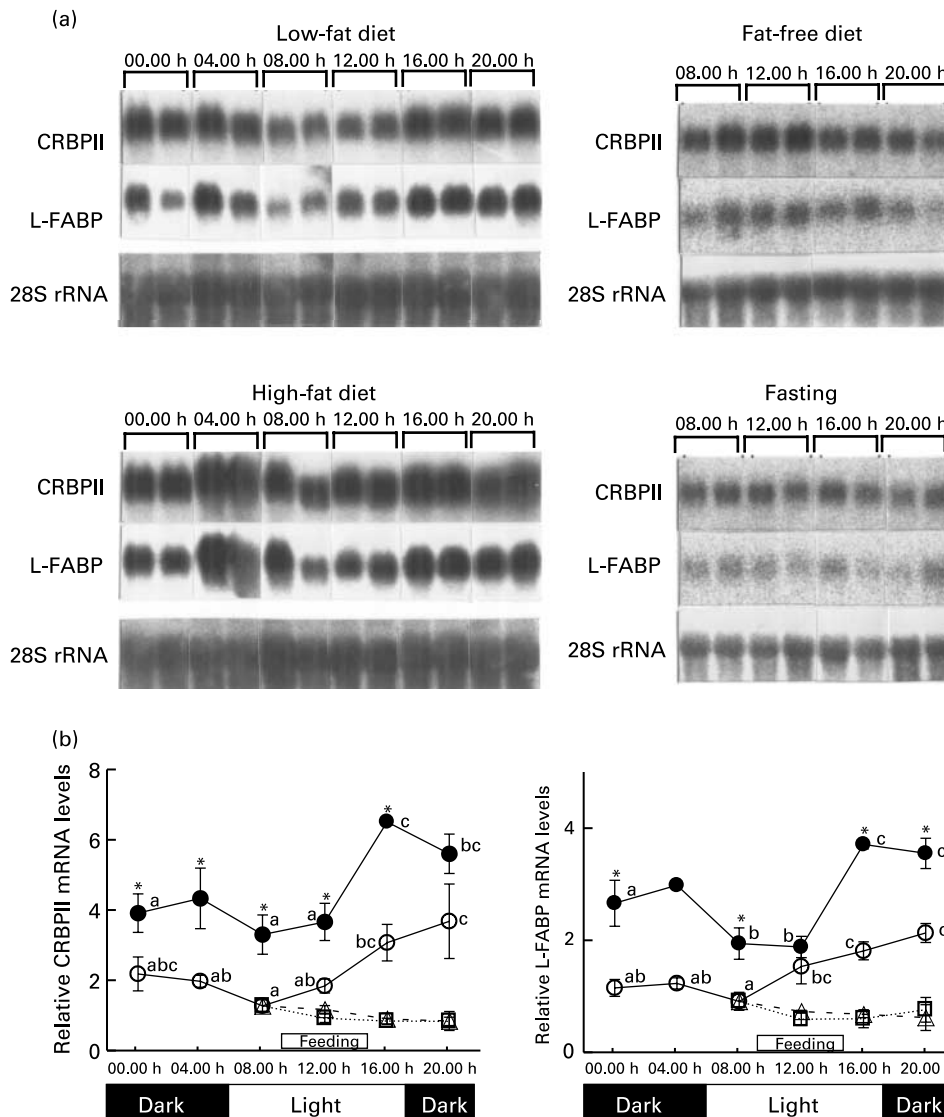
Fig. 2. Effects of dietary fat on the variations of cellular retinol-binding protein II (CRBPII) and liver fatty acid-binding protein (L-FABP) mRNA levels in rat jejunum. Rats had restricted access to either a low-fat diet or a high-fat diet during the time indicated (between 09.00 h and 15.00 h) for 13 d, and on the last day they were fed various diets or fasted. For further details, see pp. 885–886. The rats were killed at 4 h intervals. (a) Results of Northern blot analysis of the jejunal CRBPII, L-FABP and 28S rRNA transcripts. Two representative blots at each time are shown for comparison. (b) Graphical representation of relative jejunal CRBPII and L-FABP mRNA levels normalised for corresponding 28S rRNA levels and expressed in arbitrary units. Values represent means and their standard errors for at least three rats for each time point except for the L-FABP mRNA levels of rats fed a high-fat diet, at 04.00 h (n 2). [a,b,c,d]Values among the same diet group with unlike superscript letters on the same curve are significantly different at $P < 0.05$ by Ryan's multiple range test. *Significant difference at $P < 0.05$ compared with the respective mRNA levels of rats fed the low-fat diet at the corresponding time point (Student's t test). (–●–) High-fat diet; (–○–) low-fat diet; (–△–) fat-free diet; (–□–) fasting.

regulated by the nuclear transcription factor hepatocyte nuclear factor-1 (Rhoads *et al.* 1998).

The results in the present study indicated that both CRBPII protein and CRBPII mRNA exhibited a diet-related variation in rat jejunum. It should be noted that the pattern of diet-related variation in CRBPII content in the jejunum (Fig. 1) was distinct from that of disaccharidase activities, which were reported to start increasing prior to the feeding period (Saito *et al.* 1976). Since it was apparent that the significant increase in CRBPII mRNA occurred several hours after the start of the feeding period (Fig.1), we considered it possible that some component(s) in the diet might be responsible for the feeding-dependent variation at the gene expression level.

We previously demonstrated that jejunal CRBPII and L-FABP mRNA levels were increased by feeding a diet rich in long-chain triacylglycerols (Goda *et al.* 1994; Suruga *et al.* 1999; Mochizuki *et al.* 2001) and that the increase was triggered specifically by dietary unsaturated fatty acids derived from long-chain triacylglycerols (Suruga *et al.* 1995). Therefore, in this study we examined whether the diet-related variation in jejunal CRBPII and L-FABP mRNA levels would be influenced by the fat content of the diet. As we expected, the CRBPII and L-FABP mRNA levels of animals fed the high-fat diet were higher than those of rats fed the low-fat diet at any time investigated throughout the 24 h period (Fig. 2). In addition, mRNA levels increased after the onset of feeding in the groups of rats fed a low-fat diet or a high-fat
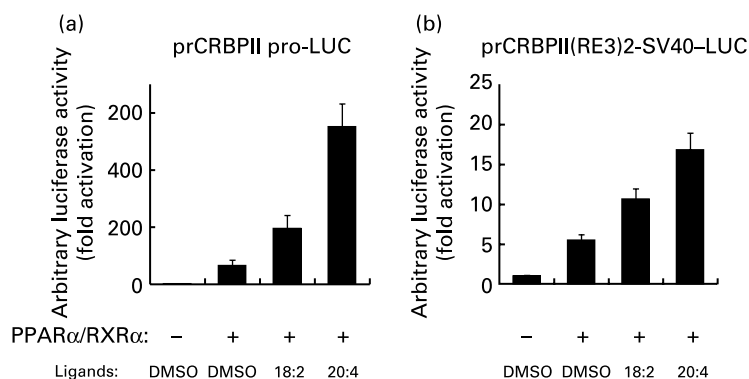
Fig. 3. Fatty acids increase the rat cellular retinol-binding protein II (CRBPII) gene promoter activity via the PPARα/retinoid X receptor-α (RXRα) heterodimer. CV-1 cells plated on ninety-six-well plate were co-transfected with pCMX mock vector (−) or both pCMX-RXRα and pCMX-PPARα expression vectors (+), phRG-TK R*enilla* control luciferase vector and prCRBPII-pro-LUC vector (a) or prCRBPII(RE3)$_2$-SV40-LUC vector (b) using Tfx-10 transfection reagent. For further details, see pp. 885–886. After the transfection, cells were incubated for 48 h in the serum-free medium containing vehicle alone (0·1 % dimethyl sulfoxide (DMSO)) or fatty acid (100 μM-linoleic acid (18:2) or 100 μM-arachidonic acid (20:4)). Cell lysates were harvested and dual-luciferase activities were determined. Values (means and their standard errors, *n* 6) were expressed as fold induction over pCMX mock vector.

diet, but not in the rats fed a fat-free diet (Fig. 2). These results suggest that dietary fat may be a dominant factor in the diet-related variation in small intestinal CRBPII and L-FABP gene expression.

It has been reported that rat CRBPII gene expression might be transcriptionally regulated by several nuclear receptor superfamilies via nuclear receptor response elements located in the CRBPII gene 5′-flanking region (Mangelsdorf *et al.* 1991; Nakshatri & Chambon, 1994; Nakshatri & Bhat-Nakshatri, 1998). A similar nuclear receptor response element also existed in the rat L-FABP gene 5′-flanking region (Issemann *et al.* 1992). Among various types of nuclear receptor, PPAR and hepatocyte nuclear factor-4 were activated by fatty acids and/or their metabolites as their ligands (Forman *et al.* 1997; Hertz *et al.* 1998). We and others also reported that the PPAR/RXR heterodimer and hepatocyte nuclear factor-4 homodimer bound to the rat CRBPII and L-FABP gene's nuclear receptor response elements (Nakshatri & Chambon, 1994; Poirier *et al.* 1997; Nakshatri & Bhat-Nakshatri, 1998; Suruga *et al.* 1999; Mochizuki *et al.* 2001). We further demonstrated in this study that unsaturated fatty acids such as linoleic acid and arachidonic acid, a major dietary fatty acid component and potent natural PPAR ligand (Forman *et al.* 1997), increased the promoter activity of rat CRBPII gene via the PPARα/RXRα heterodimer on the RE3 element located in the proximal 5′-flanking region of the rat CRBPII gene (Fig. 3). Schachtrup *et al.* (2004) also recently reported that PPAR/RXR heterodimer could transactivate the murine L-FABP nuclear receptor response element. It was therefore suggested that other PPAR/RXR heterodimer-dependent genes in the small intestine might show a similar diet-related variation induced by feeding of dietary fat.

Whereas CRBPII is the major cytosolic retinol-binding protein in the small intestine (Ong, 1984), other types of retinol-binding proteins, such as CRBP and retinol-binding protein, are abundantly expressed in the liver. We observed that hepatic retinol-binding protein and CRBP mRNA levels did not show a diet-related variation (K. Suruga, S. Takase and T. Goda, unpublished results). Although L-FABP mRNA levels in the jejunum exhibited a diet-related variation similar to that of CRBPII mRNA (Fig. 2), hepatic L-FABP mRNA levels were unchanged during the 24 h period (K. Suruga, S. Takase and T. Goda, unpublished results). These results

suggested that the diet-related variations in the expression of the genes encoding for the binding proteins of vitamin A and fatty acids might be prominent only in the small intestine.

In conclusion, the present study demonstrated that jejunal gene expression related to vitamin A and fatty acid metabolism showed a diet-related variation that was induced by feeding dietary fat at transcriptional level but not by a diurnal rhythm of feeding.

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