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The Specific Expression Patterns of Lactase, Sucrase and Calbindin-D9k in Weaning Rats Are Regulated at the Transcriptional Level

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Summary During weaning, rat lactase-phlorizin hydrolase (LPH) expression decreased to the low levels found in adults, while sucrase-isomaltase (SI) sharply increased. Calbindin-D9k (CaBP) is specific to the intestine and expression peaked within a few days of weaning. The present study investigates whether these molecules are regulated at transcriptional or post-transcriptional levels and examines the effects of diet on regulation. At normal weaning on day 21, litters were separated from their dams and one group was fed with a standard laboratory diet (weaned (W) group). The other group received a diet containing lactose as the sole source of carbohydrate (lactose-fed (L) group). Mucosal cells were obtained from the proximal part of the rat small intestine and then the activity and concentration of LPH, SI and CaBP proteins and mRNAs were determined. Three parameters revealed the same changing patterns in LPH, SI and CaBP during development and there was significant ($p<0.001$) correlation between three parameters: LPH, $r=0.97$ for activity vs. protein, $r=0.99$ for activity vs. mRNA, $r=0.96$ for protein vs. mRNA, SI, $r=0.99$ for activity vs. protein, $r=0.98$ for activity vs. mRNA, $r=0.96$ for protein vs. mRNA, CaBP, $r=0.94$ for activity vs. protein, $r=0.97$ for activity vs. mRNA, $r=0.95$ for protein vs. mRNA. Expression of the three proteins did not differ between the L and W groups. Accordingly, it has been suggested that the expression of LPH, SI and CaBP during development is defined at the transcriptional level and dietary changes do not exert a primary effect on it.

Key Words lactase, sucrase, calbindin-D9k, weaning, rat

Absorptive enterocytes lining the intestinal villi are responsible for the terminal digestion and absorption of nutrients. To perform these specialized functions, they acquire the ability to regulate the expression of functionally relevant proteins such as digestive enzymes, receptors, transporters, and cytoplasmic carriers. Furthermore, regulation of these enterocyte-specific genes is time and region dependent. The expression of each gene sequentially changes during growth from the fetal period through suckling, weaning and adulthood. The diets of weaned mammals radically change from milk to a non-milk base. Different profiles of gene expression along the length of tract from duodenum to cecum result in regional differences in intestinal function.

We focused on the weaning period, because many biochemical activities must be involved during the dietary transition and they affect the regulation of the enterocyte-specific proteins; lactase-phlorizin hydrolase (LPH), sucrase-isomaltase (SI) and calbindin-D9k (CaBP). The expression profile of each of these is developmentally and regionally specific. The activity of LPH is elevated at the fetal stage, declines around day 21 of age (weaning) and remains thereafter in the adult at a level corresponding to 20% of that at birth (*1–3*). Along the longitudinal axis, the LPH gene is maximally expressed in the proximal small intestine and significantly declines in the distal segments of the intestine (*1*). The expression of SI is regulated in a contractive manner. Its activity is undetectable during suckling and increases to the adult level at day 16 or day 17 (*4*). A gradient from the proximal intestine to the colon of SI expression is established with the level being highest in the jejunum and undetectable in the colon (*2, 4*). The small intestine-specific calcium binding protein, CaBP, diffuses calcium ions across the cytoplasm of enterocytes from the brush border membrane to basolateral side (5). Its basal level is low in suckling and adult rats, but after weaning at day 21, it peaks and returns after several days to the basal level (*6*). The expression of CaBP is most abundant at the proximal region of the intestine (*7*).

Many investigators have reported the effects of dietary constituents on the expression of these genes. Dietary sucrose enhances SI and LPH gene expression at a transcription level in rats (*8, 9*). mRNA expression of CaBP fluctuates during 1,25-dihydroxyvitamin D_3 deficiency and repletion (5) and might be up- and

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down-regulated by low- and high-calcium diets, respectively, in rats (*10, 11*). The expression of CaBP is restricted to a very low level in VDR-ablated mice but recovered by a high-lactose, high Ca and high-phosphate diet to the level of non-ablated mice (*12*). The mechanisms underlying such regulation have not been fully defined.

The LPH, SI and CaBP genes provide valuable models with which to investigate the role of dietary constituents on gene expression. We prevented weaning by feeding a diet containing lactose to pups that were separated from their dams at the usual weaning age of day 21. Activity determination, Northern and Western blotting analyses of LPH, SI and CaBP were conducted using mucosal cells of proximal segment of intestine of weaning retarded rats.

MATERIALS AND METHODS

Animals and experimental design. Sixteen pregnant female Sprague-Dawley rats (15 wk old) of similar body weight purchased from Saitama Experimental Animal Supply (Saitama, Japan) were individually housed in temperature and humidity-controlled rooms (25±1°C, 65±5%, respectively) with lights on from 0800 to 2000 h. A standard laboratory chow diet (MF, Oriental Yeast Co., Tokyo, Japan) and water were continuously available. The composition of MF (g/kg) was as follows: carbohydrate 523, protein 246, lipids 56 and minerals and vitamins that were sufficient to maintain growth (Table 1). On the day after parturition, rats were considered to be 1 d old; dams and all pups were weighed every day beginning on day 5. At day 21, litters were separated into two groups of four pups each. The weaned (W) group was fed with mashed standard laboratory chow and the other (L) group received a diet containing lactose as the sole source of carbohydrates (Table 1).

The Animal Care Advisory Committee of Kagawa Nutrition University approved the study design and protocol.

Preparation of intestinal samples. Suckling and weanling pups were sacrificed by decapitation at 1000 h, and the small intestine extending from the ligament of Treitz to the cecum was removed. Mucosal cells were scraped from the proximal intestine (first half) with a glass slide and homogenized in four volumes of 10 mmol/L potassium phosphate buffer (pH 6.0) containing 1.5 mmol/L NaN_3 and 0.1 mmol/L phenylmethylsulfonyl fluoride. This homogenate was used for the activity determination and Western blot analysis of LPH and SI. For calcium binding activity assay, the scraped sample was homogenized in four volumes of 13.7 mmol/L Tris-HCl buffer (pH 7.4) containing 0.12 mol/L NaCl and 4.74 mmol/L KCl. The supernatant fraction after centrifugation at 39,000×*g* for 20 min was heated at 60°C for 10 min, and heat-denatured protein was discarded after centrifugation at 10,000×*g* for 30 min. The supernatant was used for Western blot analysis.

Activity assays. The activities of lactase and sucrase were determined for LPH and SI, respectively, according to the method described by Dahlqvist (*13*), using 28 mmol/L each of lactose and sucrose as respective substrates.

Table 1. Composition of experimental diets.

	Lactose diet	MF[4]
	g/kg diet	
Ingredient		
Lactose[1]	360	
Casein[2]	250	
Corn oil[3]	120	
L-Methionine[1]	4	
Cellulose powder[4]	20	
Vitamin mixture[5]	10	
Mineral mixture[6]	100	
H_2O	136	
Energy (kcal/kg diet)	349	360
Protein (g/kg diet)	216	246
Fat (g/kg diet)	120	56
Carbohydrate (g/kg diet)	360	523
Dietary fiber (g/kg diet)	20	31

[1] Wako Pure Chemical Industries, Ltd., Osaka, Japan.
[2] Vitamin-free casein from milk, Oriental Yeast, Co., Ltd., Japan.
[3] Commercially available.
[4] Oriental Yeast, Co., Ltd.
[5] Harper's vitamin mixture (*22*), Oriental Yeast, Co., Ltd.
[6] Harper's mineral mixture (*22*), Oriental Yeast, Co., Ltd.

The activity of CaBP was determined for calcium binding activity using the method of Wasserman and Taylor (*14*) with some modifications. Chelex-100 resin (Bio-Rad Laboratories, Inc., Richmond, CA, USA) was washed and equilibrated with the same buffer as that used for tissue homogenization so that 0.2 mL of resin suspension contained 0.1 mL of packed resin. The supernatant fraction (625 μL) containing 300 μg protein was mixed with 625 μL of 18.5 kBq ^{45}Ca (Amersham Biosciences, Uppsala, Sweden), and then CaBP was allowed to bind ^{45}Ca for 5 min with vigorous shaking. A resin suspension (50 μL) was added to this mixture and the solution was shaken for another 5 min. The mixture was then separated by centrifugation at 15,000×*g* for 2 min and 50 μL aliquots of the supernatant were placed in glass liquid scintillation vials containing Bray's solution (*15*). Levels of ^{45}Ca radioactivity were measured using a Packard 1600TR liquid scintillation detector (Packard Instrument Company, Meriden, CT, USA).

Protein was measured by the method of Lowry et al. (*16*) with bovine serum albumin as the standard. Activity is expressed per milligram of protein.

Western blot analysis. For the preparation of LPH and SI antiserum, these proteins were purified from the proximal intestine by published methods (*17, 18*). For the CaBP antiserum, a bacterially expressed fusion protein, glutathione *S*-transferase and CaBP, was used after purification by a glutathione-Sepharose column. Corresponding antibodies were raised in rabbits using these

proteins and applied for Western blot analysis as previously described (*17*). The final chemiluminescence signals on the membranes were detected on Fuji X-ray film (Fuji Photo Film Co., Ltd., Tokyo, Japan), and density was quantified using an ATTO Densitograph AE-6920-M-SS (ATTO Co., Tokyo, Japan).

RNA isolation and Northern blot analysis. Total RNA and poly $(A)^+$ RNA were prepared using Wako ISOGEN (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and Oligotex-dT30 Super (Takara Shuzo Co. Ltd., Kyoto, Japan), respectively, in accordance with the manufacturers' manuals. As probes for hybridization, cDNA encoding rat LPH was prepared by amplification of rat intestinal poly $(A)^+$ RNA using reverse transcription polymerase chain reaction, as previously described (*17*). The rat SI (*19*) and CaBP (*20*) cDNA clones were gifts from Dr. S. J. Henning (Baylor College of Medicine, Houston, TX, USA) and Dr. H. F. DeLuca (University of Wisconsin-Madison, Wisconsin, WI, USA). Northern blot analysis was performed as described (*17*).

Statistical analysis. All results are expressed as means±SE. We tested the daily difference in mean values of body weight between the L and W groups using Student's *t* test. Growth rate was compared between those two groups, combining all values from day 21 to 27 by one-way ANOVA. Differences between the L and W groups at day 24 and 27 were tested with Student's *t* test. Correlation coefficient analysis was performed using SPSS 11.5 software (SPSS Inc., Chicago, IL, USA). Differences were considered significant at $p<0.05$.

RESULTS

Expression of LPH, SI and CaBP is regulated at transcriptional level

Figure 1 shows the developmental changes of LPH, SI and CaBP in terms of activity, mRNA and protein concentrations. We determined the lactase and sucrase activities of LPH and SI, respectively, as well as the calcium binding activity of CaBP. All values are indicated as a ratio (%) of the value at day 21. Figure 1A shows that the lactase activity of LPH, amounts of LPH protein and mRNA were maximal at day 7 and declined with development. The three parameters significantly correlated ($p<0.001$): $r=0.97$ for activity vs. protein, $r=0.99$ for activity vs. mRNA and $r=0.96$ for protein vs. mRNA. These findings indicated that the transcription of LPH is regulated during the developmental process. Figure 1B shows undetectable levels of SI activity, protein and mRNA at day 7 and very high levels at day 21. There was significant ($p<0.001$) correlation between protein and mRNA concentrations and activity during development: $r=0.99$ for activity vs. protein, $r=0.98$ for activity vs. mRNA and $r=0.96$ for protein vs. mRNA. These findings indicated that SI expression is regulated at the transcriptional level during development. In Fig. 1C, the calcium binding activity of CaBP, concentrations of CaBP protein and mRNA show low basal levels at day 7 and in adult rats at day 105. However, after weaning by day 24, the three parameters peaked for several days. The significant correlation

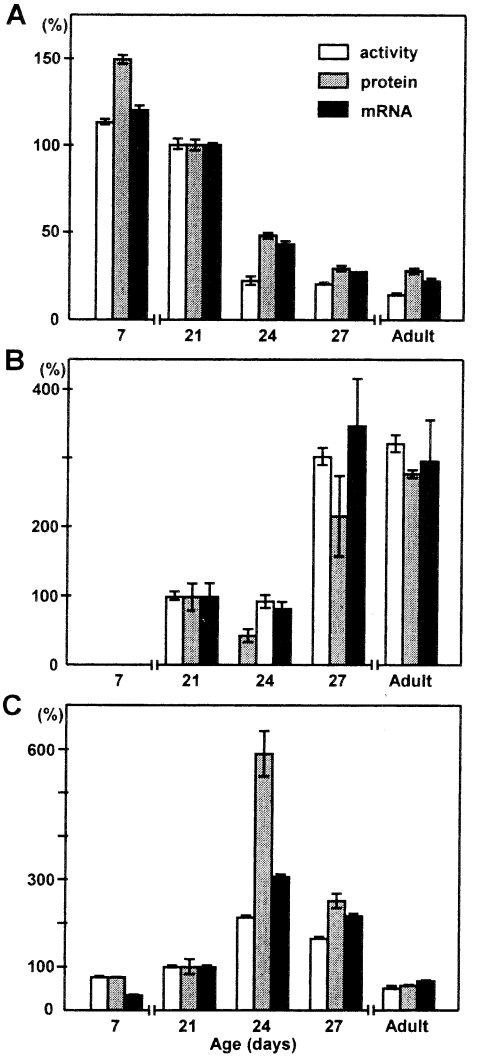

Fig. 1. Developmental changes of activities, protein and mRNA concentrations of LPH (A), SI (B) and CaBP (C). Activities of LPH and SI were determined as enzyme activities of lactase for LPH and sucrase for SI and calcium binding activity for CaBP. Protein and mRNA concentrations were determined by Western and Northern blot analyses, respectively. All data were calculated as a percentage of the value of day 21 and are expressed as means±SE, $n=4$. The profile of LPH (A) shows that peak level at suckling sharply decreased at weaning to low adult levels with high correlation ($p<0.001$) as follows: $r=0.97$, activity vs. protein; $r=0.99$, activity vs. mRNA; $r=0.96$, protein vs. mRNA. Values for SI (B) were closely correlated, being very low level before weaning and then increasing to high adult levels. Significant ($p<0.001$) correlations were as follows: $r=0.99$, activity vs. protein; $r=0.98$, activity vs. mRNA; $r=0.96$, protein vs. mRNA. Expression of CaBP differed from that of LPH or SI, as it peaked after weaning (C). Significant ($p<0.001$) correlation was as follows: $r=0.94$, activity vs. protein; $r=0.97$, activity vs. mRNA; $r=0.95$, protein vs. mRNA.

($p<0.001$) between activity vs. protein ($r=0.94$), activity vs. mRNA ($r=0.97$) and protein vs. mRNA ($r=0.95$) also indicated that CaBP is regulated at the transcriptional level during development.

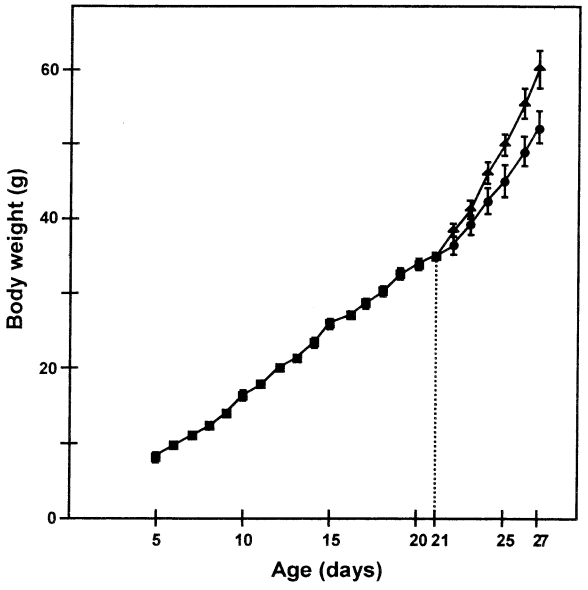

Fig. 2. Body weights of suckling (closed squares), lactose-fed (closed circles) and weanling rats (closed triangles) compared during development from day 5 to 27. At day 21, litters were separated from dams and divided into weanling (W) and lactose-fed (L) groups. The L group received lactose as the sole carbohydrate source as shown in Table 1. Data are expressed as means±SE, $n=8$ before day 21 and $n=4$ for the W and L groups. No significant difference ($p<0.05$) in body weight on each day between the W and L groups from day 21 to 27.

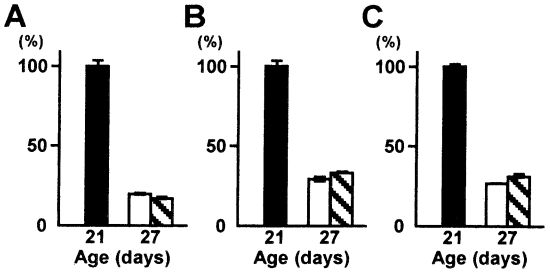

Fig. 3. Activity (A), protein (B) and mRNA (C) concentrations of LPH in weanling (W, open bar) and lactose-fed (L, hatched bar) groups at day 27. Data were calculated as ratios (%) of value at day 21 (closed bar) and are expressed as means±SE, $n=4$. Levels of all three parameters decreased with no significant difference ($p<0.05$, Student's t-test) between the W and L groups at day 27.

Body weight in both groups spontaneously increased

We examined the growth rates of eight pups from one dam. At day 21 (usual weaning date) eight pups were separated from their dam and divided into two groups of four each. The L group was fed with the purified diet containing lactose as described in Table 1, and the W group was fed with mashed regular chow. Figure 2 shows that the body weights of both groups steadily increased from day 21 to 27. Although the growth rate of the L group was slower than that of the W group, there was no significant difference in either body weight

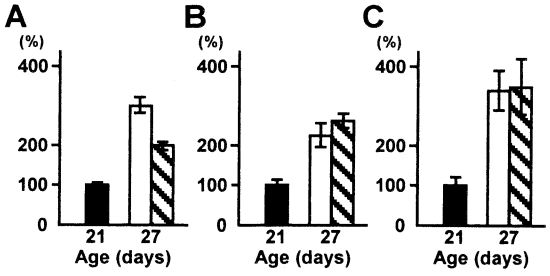

Fig. 4. Activity (A), protein (B) and mRNA (C) concentrations of SI in weanling (open bar) and lactose-fed (hatched bar) groups at day 27. Data were calculated as a percentage of the value of day 21 (closed bar) and expressed as means±SE, $n=4$. Levels of all three parameters in both groups sharply increased with no significant difference ($p<0.05$, Student's t-test) between those two groups at day 27.

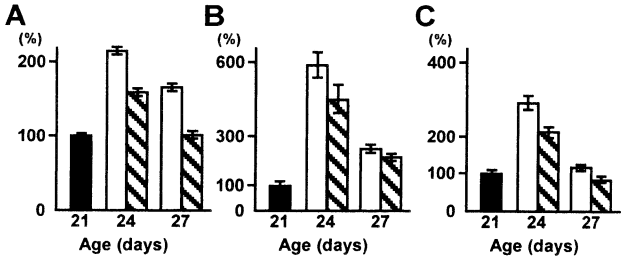

Fig. 5. Calcium binding activity (A), protein (B) and mRNA (C) concentrations of CaBP in weanling (open bar) and lactose-fed (hatched bar) groups at day 24 and 27. Data were calculated as ratios (%) of value at day 21 (closed bar) and were expressed as means±SE, $n=4$. Levels of all three parameters peaked at weaning with no significant difference ($p<0.05$, Student's t-test) between those two groups at day 27.

or daily change of body weight between the L and W groups during the period from day 21 to 27.

Changes of LPH, SI and CaBP at weaning are not affected by diet

Figure 3 compares LPH between the L and W groups at day 27. Lactase activity (A), as well as protein (B) and mRNA (C) concentrations were analyzed by Western and Northern blot analyses, respectively. The density of each band on the membranes was quantified and data from the W and L groups at day 27 were shown as ratios (%) of the value at day 21. The three parameters remarkably decreased to similar levels at day 27 with no significant differences between the W and L groups. As suggested by Fig. 1A, the expression of LPH during development was regulated at the transcriptional level, and moreover, dietary changes such as terminating lactose ingestion did not affect the specific decrease at weaning. Figure 4 shows the results of SI, and panels A, B and C show the determination of activity, Western and Northern blotting analyses, respectively. In agreement with Fig. 1B, the expression of SI showed a remarkable increase at day 27 and remained high during adulthood. In the L group, the enzyme activity, SI protein and mRNA were induced to similar levels as the

W group. These findings suggest that the increased SI expression at weaning is developmentally determined and independent of sucrose. Changes in levels of CaBP differed considerably at weaning from LPH and SI (Fig. 5). While the levels in young sucklings and adult rats were low, they apparently peaked at weaning (Fig. 1C). This characteristic peak appeared in both groups and the difference was not significant.

DISCUSSION

We analyzed the regulation of the intestine-specific proteins, LPH, SI and CaBP. We examined correlations among the activity, protein and mRNA expression of these proteins to define whether they are regulated at the transcriptional, post-transcriptional, translational or post-translational levels. Although the weaning period includes many changes; from milk to a non-milk diet, from liquid to solid diet, from lactose to sucrose as a carbohydrate source, we fed rats with a diet containing lactose even after weaning (L group) to define the effect of weaning on the expression of these three proteins.

We retarded weaning by feeding the diet containing lactose to the L group thereafter. The diet was adjusted to that of standard laboratory chow containing the same amount of calories but with lactose as the sole source of carbohydrate and corn oil for lipids. Because the AIN-93 purified diet (*21*) contains sucrose as the medium for mineral and vitamin mixtures, we excluded this SI substrate by using Harper's mineral and vitamin mixtures (*22*) for the L group diet. To avoid any deleterious effects of this diet, we weighed the animals each day to confirm that growth rates were similar to those rats in the W group (Fig. 2). However after day 27, probably because of malnutrition, the L group could not maintain the same growth rate as the W group, so we terminated the experiment at this time.

Based on our finding of a correlation between the developmental profiles of activity, protein and mRNA expression, we concluded that LPH, SI and CaBP were transcriptionally regulated during weaning. Moreover, the three parameters did not differ between the L and W groups. Thus, dietary changes induced by weaning do not significantly impact transcriptional regulation at weaning. Although the L group was fed with a diet containing lactose, LPH expression declined in the same manner as in the W group. The expression of SI was significantly induced in the L group at weaning without a sucrose substrate. The expression of CaBP in the L group after weaning also peaked. The sequential gene expression of LPH, SI and CaBP during development was very firmly programmed at the transcriptional level.

All three genes were isolated and the 5′ regulatory regions were analyzed in detail. In vitro binding studies were conducted to identify a specific nuclear protein and its *cis*-element. The colon carcinoma cell line, Caco-2, was used for transfection to determine interaction between transcription factors and the *cis*-element that was identified by the binding assays in vitro. This small intestine-like cell line can differentiate to express LPH, SI and CaBP. Moreover, transgenic mice were the most appropriate model with which to determine the effect of a specific promoter sequence in vivo. In vitro binding studies have indicated that the *cis*-element, CE-LPH1, specifically interacts with the intestinal nuclear protein NF-LPH1 in suckling pigs or rats (*23, 24*). This protein was important in identifying the tissue specificity of LPH and its specific expression during development (*25*). *Cis*-elements that are necessary for the SI expression have also been identified in Caco-2 cells and are named SIF1, 2 and 3 (*26*). The homeodomain protein, Cdx-2 has been isolated as an SIF1-specific binding protein (*27*) and is also specific to CE-LPH1 (*28*). The intestine-specific transcription factors, GATA 4,5,6, HNF-1 and Cdx-2, increase the transcription of LPH and SI in Caco-2 cells (*29–32*). Each factor is uniquely distributed in the gradient from the proximal to the distal parts of the intestine and in a developmental manner. These factors are expressed only in intestinal enterocytes (*33–35*) and their binding sites in the LPH, SI and CaBP genes are adjacent to the TATA box and to each other (*29–32*). Thus, one hypothesis states that these transcription factors are all important regulators of LPH, SI and CaBP gene expression. Co-transfection studies of Caco-2 cells and several transgenic mice with various combinations of transcription factors have revealed a complex pattern of effectiveness that differs from the sum of the activation of any of these factors alone (*32*). These results imply a correlation among these transcription factors for the time- and position-dependent regulation of LPH and SI (*32*). However which factor is responsible for the decline of LPH mRNA at weaning remains unanswered. Transgenic mice carrying several promoter regions of pig (*36*) and rat LPH (*37*) have been established and these regions contain *cis*-elements that direct the down regulation of LPH transcription at weaning. However the mechanisms that underlie the process are poorly understood and evidence regarding the transcription factors and the consensus sequences that are responsible for the signals derived from dietary stimuli are scarce.

Only CaBP directly interacts with a nutrient, an active vitamin D metabolite, and this induces CaBP gene expression (*38, 39*). Several candidate consensus sequences for the vitamin D receptor (VDR) have been suggested in CaBP 5′ promoter region (*40, 41*), but their interaction remains speculative. The 4,580 base pairs of the 5′ regulatory region of CaBP in transgenic mice can target reporter transgene expression in the intestine and cause this transgene to respond to the active vitamin D metabolite (*42*). Moreover, response elements to Cdx-2 and VDR have been determined (*41, 42*). How interaction between these transcription factors and sequences is involved in the time- and position-dependent regulation of CaBP during the transition from suckling to weaning remains obscure.

Not all of the transcription factors and *cis*-elements that are involved in developmental regulation have been identified, and additional factors and sequences

remain to be determined. Therefore, the investigation concerning the relevant role of nutritional constituents has hereafter to be developed in terms of nutrition using the strategy of molecular biology.

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