Summary It has been reported that the enzymic activity of fatty acid synthase (Fas) in rat liver increases during suckling-weaning transition. In this study, we investigated whether induction of the gene (Fasn) in the rat liver during the suckling-weaning transition is regulated by histone acetylation, and the nuclear transcription factors carbohydrate response element-binding protein (ChREBP) and sterol regulatory element-binding protein 1 (SREBP1). We observed that levels of Fas and Fasn increased during suckling-weaning transition. Binding levels of ChREBP and SREBP1 to upstream regions of Fasn increased during the suckling-weaning transition. Acetylation of histones H3 and H4 around Fasn increased during the transient period. Our results suggest that induction of liver Fasn during the suckling-weaning transition is closely associated with increased levels of ChREBP and SREBP1 binding, and acetylation of histones H3 and H4 around the gene.

Key Words FAS, ChREBP, SREBP1, histone acetylation, suckling-weaning transition

The suckling-weaning transition in laboratory rodents is accompanied by marked changes in dietary intake. During the suckling period, pups are fed milk, which is high in fat and low in carbohydrates. At the commencement of weaning (14–27 d post-partum) milk is progressively replaced by chow, which is high in carbohydrates and low in fat (1). This change in diet is followed by considerable hormonal modifications: an increase in plasma insulin concentration, and a decrease in plasma glucagon concentration (2). Carbohydrate intake and accompanying insulin secretion induce fatty acid synthesis in the liver and adipose tissues (3). It is supposed that dietary changes during the suckling-weaning transition would affect expression of genes related to fatty acid metabolism.

It is known that intake of a large amount of carbohydrates leads to enhanced activities for many liver enzymes necessary for converting glucose to fatty acids. Among the enzymes related to fatty acid synthesis, fatty acid synthase (Fas), which converts acetyl-coenzyme A to long-chain fatty acids, is a rate-controlling enzyme. Expression of Fas in the liver is higher in adult rats fed a high-carbohydrate diet compared with those fed a low-carbohydrate diet (3). Additionally, Fas expression in hepatocytes was induced by media high in glucose, and insulin treatment (4). These results suggest that expression of the Fas gene (Fasn) is regulated by carbohydrate flow and/or insulin action in the liver. Regarding postnatal development in rats, it has already been reported that Fasn mRNA levels and activity of Fas are very low in the liver (5). The mRNA and enzyme activity levels are induced with increasing amounts of carbohydrate in the diet during weaning. When weaning rats are fed a high-fat diet, increases in Fasn mRNA and Fas activity are abolished (5). Thus, carbohydrate and insulin signals seem to be important for the induction of Fasn in the liver of rat pups during the suckling-weaning transition as well as in adult rats.

Many recent studies have demonstrated that expression of genes related to fatty acid synthesis, including Fasn, are regulated by transcriptional factors such as carbohydrate response element-binding protein (ChREBP) and sterol regulatory element-binding protein 1 (SREBP1) (6). Glucose signaling enhances ChREBP binding to the carbohydrate response element (ChRE) upstream of target genes by inducing dephospho-
phorylation of the ChREBP. This results in increased transcription of the target genes (7). SREBP1 is synthesized in a precursor form, which is located in the endoplasmic reticulum and the nuclear membrane. After the precursor is cleaved into the active form in an insulin-dependent manner, the mature SREBP1 enters into the nucleus and activates transcription of target genes by binding to the sterol response element (SRE) (8). Cis-elements of ChREBP and SREBP1 are located in the promoter/enhancer region of Fasn (9, 10). It has already been reported that trans-activity of the upstream region of Fasn is enhanced by ChREBP (8) in hepatocytes, or by SREBP1 in adipocytes (11).

However, it is still unclear whether ChREBP and SREBP1 bind to the promoter/enhancer region of Fasn in vivo, and whether their binding is associated with induction of the gene during suckling-weaning transition. This could be examined using chromatin immunoprecipitation (ChiP) assays. Recent studies have demonstrated that an abrupt change in gene expression, which accompanies maturation and differentiation of cells, is regulated by histone modifications such as acetylation, methylation, and phosphorylation, as well as by transcriptional factors (12). In particular, the acetylation of histones H3 and H4 is thought to play a central role in the regulation of transcription. Hyperacetylation of histones H3 and H4 is associated with the euchromatin region of the genome (13). The subsequent acetylation of histones H3 and H4 is accompanied by binding of nuclear transcriptional factors, including ChREBP and SREBP1, to the promoter/enhancer region of genes. Such histone modifications have been shown to induce the binding of transcriptional machinery including co-activators, the SWI/SNF complex, transcriptional factors, and RNA polymerase II to their target genes (14, 15). Therefore, it is very likely that abrupt induction of liver Fasn during the suckling-weaning transition in rats is regulated by histone modifications and bindings of transcription factors such as ChREBP and SREBP1.

In this study, we examined whether the induction of the Fasn gene during the suckling-weaning transition in rats is regulated by a coordinated increase in binding of ChREBP and SREBP1, along with acetylation of histones H3 and H4.

MATERIALS AND METHODS

Animals. Pregnant Sprague-Dawley rats carrying fetuses at 10 d after gestation were obtained from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan). After the pups were born, they were kept with their mothers. Both mothers and pups were given free access to a standard laboratory chow diet consisting of 54.4% carbohydrates, 23.6% crude protein, and 5.3% crude lipids (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) throughout the experimental period. Rat pups were killed at 13, 20 and 27 d after birth. The experimental procedures used in the present study met the guidelines of the Animal Usage Committee of the University of Shizuoka.

Preparation of hepatic samples. The liver was washed with ice-cold 0.9% NaCl solution and then divided into three parts for RNA extraction, immunoblotting, and ChIP assays. The tissues were immediately frozen in liquid nitrogen except for those for ChIP assays, and stored at −80°C.

Blood biochemical parameters. Serum in developing rats was collected by decapitation. Serum glucose and triacylglycerol concentrations were measured using commercial kits (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Serum insulin concentration was measured using a Rat Insulin ELISA Kit (AKRIN-010; Shibayagi, Gunma, Japan).

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). The hepatic tissue was homogenized in a solution consisting of 4 mM guanidine thiocyanate, 25 mM sodium citrate (pH 7.5), 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol. Homogenates were immediately frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted by the acidified guanidine thiocyanate method, as described by Chomczynski and Sacchi (16). The RNA samples (2.5 μg) were reverse transcribed into cDNA using Super Script III reverse transcriptase™ (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. PCR amplification was performed on a Light-Cycler 480 system (Roche Diagnostics, Mannheim, Germany). Reactions were carried out in a total volume of 10 μL and contained gene-specific primers, cDNA, and SYBER Green Master I (Roche Diagnostics). The cycle threshold (CT) values for each gene detected by qRT-PCR were converted into signal intensities by the delta-delta CT method (17).

We chose 18S rRNA as the control gene. Specific primers were used to determine mRNA expression levels of genes encoding the following proteins: Fasn (5′-GGA TGT CAA CAA GCC CAA GTA-3′ and 5′-TTA CAG AGG AGA AGG CCA CAA-3′); ChREBP (5′-AAT CCC AGC CCC TAC ACC-3′ and 5′-CTG GGA GGA GCC AAT GTG-3′); SREBP1 (5′-ACA AGA TTG TGG AGC TCA AG-3′ and 5′-TGG GCA GAG CAG ATT TA-3′); and 18S rRNA (5′-GCT TGG CCG TTC TTA-3′ and 5′-TCC TTC GTC ATT GCA ATT AAC C-3′).

Immunoblotting. Frozen tissues were homogenized in radio-immunoprecipitation assay (RIPA) buffer (1% Nonidet P-40, 0.1% SDS, 20 mM Tris-HCl pH 8.0, 5 mM EDTA, 150 mM NaCl) that contained phosphatase inhibitors (1 mM NaMoO₄, 50 mM NaF, and 1 mM NaVO₄) and protease inhibitors (1 tablet of complete Mini/10 mL; Roche Diagnostics). Lysates were centrifuged (14,800 × g, 30 min, 4°C) and total protein concentration in the supernatant was determined with the Lowry method (18). Hepatic extracts were separated by 10% SDS-PAGE and proteins in the gels were transferred to Immobilon membranes (Millipore, Billerica, MA). Membranes were blocked for 2 h in skim milk (3–10%) made up in PBS (pH 7.4) supplemented with 0.05% Tween-20 (PBS-T) at room temperature. Membranes were then incubated at 4°C for at least 12 h with the following primary antibodies diluted in PBS-T containing 3–10% skim milk: anti-FAS (Cell Signaling Technology, Inc., Danvers, MA), anti-ChREBP (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-SREBP1.
Morishita S et al.

(F ACS) solution (PBS, 2% bovine serum, 0.05% NaN_3), at 4˚C. After washing in fluorescence activated cell sorter
for 15 min. Lysates were centrifuged at 1,200
for 10 min. Samples were sonicated in SDS lysis buffer containing
AT-3
and 5
AGC AAC G-3
′
500 bp (5
-ATC ACC CTA TTG CCT
2
AAG GTT GGT CTT-3
and 5
-GGA CGG GAG ATG ATG
′
AGT GAT CTG C-3
2
′
ACT GCC CAT TGG TTT TGT C-3′ and 5′-CCC AGT AAC CAT GTG TTT TGT C-3′).

Chromatin immunoprecipitation (ChIP) assays. Fresh liver was homogenized in 4.5 mM HEPES buffer (pH 8.0) containing 1% formaldehyde, 9 mM NaCl, 0.09 mM EDTA, and 0.04 mM ethylene glycol tetraacetic acid, and incubated at 37˚C for 30 min. The reaction was terminated by treatment with 150 mM glycine at 37˚C for 15 min. Lysates were centrifuged at 1,200 × g for 15 min at 4˚C. After washing in fluorescence activated cell sorter (FACS) solution (PBS, 2% bovine serum, 0.05% NaN_3), samples were sonicated in SDS lysis buffer containing 10 mM EDTA, 1% SDS, and protease inhibitors (1 tablet of complete Mini/10 mL; Roche Diagnostics) in 50 mM Tris-HCl, pH 8.0, to obtain 200–500 bp DNA fragments. The ChIP assay was performed as described previously (19), with the following antibodies: anti-ChREBP (Santa Cruz Biotechnology); anti-SREBP1 (Santa Cruz Biotechnology); anti-acetyl-histone H3 (Millipore); anti-acetyl-histone H4 (Millipore); and control rabbit IgG. Precipitated DNA was analyzed by real-time PCR with primers complementary to sites in the promoter/enhancer and transcribed regions of the Fasn gene. ChIP results are expressed as a percent of the PCR signal for input DNA, transcribed regions of the Fasn gene. ChIP results are expressed as a percent of the PCR signal for input DNA.

RESULTS

Changes in blood parameters during suckling-weaning

Serum glucose levels were maximal at 20 d after birth, and decreased to a lower level at 27 d after birth. Serum insulin concentrations were higher at 20 and 27 d after birth than at 13 d after birth. Serum triacylglycerol concentration gradually decreased during weaning, and its level was significantly lower at 27 d after birth than at 13 and 20 d after birth (Table 1).

Table 1. Biochemical parameters in blood samples.

<table>
<thead>
<tr>
<th>Days after birth</th>
<th>Day 13</th>
<th>Day 20</th>
<th>Day 27</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose (mg/dL)</td>
<td>208±7\textsuperscript{a}b</td>
<td>260±9\textsuperscript{a}</td>
<td>187±10\textsuperscript{b}</td>
</tr>
<tr>
<td>Insulin (ng/dL)</td>
<td>0.15±0.01\textsuperscript{a}</td>
<td>0.24±0.02\textsuperscript{b}</td>
<td>0.22±0.02\textsuperscript{b}</td>
</tr>
<tr>
<td>Triacylglycerol (mg/dL)</td>
<td>146±19\textsuperscript{a}</td>
<td>121±16\textsuperscript{a}</td>
<td>66±4\textsuperscript{b}</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SE; n=6–8. Values not sharing a common letter are significantly different from one another (p<0.05, Tukey’s multiple range test).

Statistical analysis. Group differences were analyzed by analysis of variance (ANOVA) followed by Tukey’s multiple range test. A p-value less than 0.05 was considered significant.

Hepatic changes in Fasn mRNA and Fas protein levels

The mRNA level for Fasn in the liver was low at 13 d after birth, and it gradually increased during the weaning period; Fas protein levels at 27 d after birth were 6.6-fold higher than those at 13 d after birth (Fig. 1A). Similarly, the Fas protein level in the liver increased during the weaning period; Fas protein levels at 27 d after birth were 3.1-fold higher than those at 13 d after birth.

Fig. 1. Hepatic changes in Fasn mRNA (A) and Fas protein (B) levels during the suckling-weaning transition in rats. The mRNA levels were normalized to 18S rRNA expression (A). The protein levels were normalized to TFIIB (B). Means±SE for 6–8 animals are shown. Values not sharing a common letter are significantly different from one another (p<0.05, Tukey’s multiple range test).

TTC-3′ and 5′-AAT CCC TTC CAG ACA CAT GAC-3′; FAS+2.000 bp (5′-TGA AGG ATC TGT CCA AGT TCG-3′ and 5′-CTC GTG CCA AAA TAG CTT CAT-3′) and FAS+5.000 bp (5′-CCC AGT AAC CAT GTG TTT TGT C-3′ and 5′-CCC AGT AAC CAT GTG TTT TGT C-3′).

(Santa Cruz Biotechnology), and anti-TFIIB (Santa Cruz Biotechnology). After washing in PBS-T, membranes were incubated at 4˚C for at least 12 h with anti-rabbit IgG conjugated to biotin (GE Healthcare, Little Chalfont, UK). Membranes were washed in PBS-T and incubated with horseradish peroxidase conjugated with anti-biotin (Cell Signaling, Tokyo, Japan). Positive signals were then detected by chemiluminescence (ECL Plus; GE Healthcare, Tokyo, Japan) according to the manufacturer’s instructions. Relative signal intensities were evaluated with Multi Gauge (version 3.0; Fuji Film).

GROUP DIFFERENCES WERE ANALYZED BY ANALYSIS OF VARIANCE (ANOVA) FOLLOWED BY TUKEY’S MULTIPLE RANGE TEST. A P-VALUE LESS THAN 0.05 WAS CONSIDERED SIGNIFICANT.
Regulation of Fasn Expression

Hepatic changes in protein levels and in vivo binding of ChREBP and SREBP1 around Fasn gene

The ChREBP protein (93 kDa) levels were near constant during the suckling-weaning transition, with no significant differences observed among the levels at 13, 20 or 27 d after birth. The SREBP1 precursor protein (125 kDa) levels were significantly higher at 13 d after birth than those at 20 and 27 d after birth, whereas mature SREBP1 protein (68 kDa) levels tended to be higher at 20 and 27 d after birth than those at 13 d after birth (Fig. 2A).

Next, we determined binding levels of ChREBP and SREBP1 around the Fasn gene using ChIP assays. The ChIP signals detected for control IgG were less than 0.03% per input for all regions of the Fasn gene (data not shown). The binding levels of ChREBP on the Fasn gene were significantly increased during the weaning period at the promoter/enhancer (−7,000, −2,000, −1,000 and −500 bp) and transcribed (+1,000 bp) regions. The binding levels of SREBP1 on the Fasn gene were significantly increased during the weaning period at an enhancer region (−7,000 bp; Fig. 2B).

Hepatic changes in acetylation of histones H3 and H4 around Fasn gene

The acetylated histone H3 levels gradually increased during the weaning period on the transcribed regions (+1,000, +2,000 and +5,000 bp); the levels at 27 d were significantly higher than those at 13 d after birth (Fig. 3). The acetylated histone H4 levels gradually increased during the weaning period on the promoter/enhancer (−7,000 and −2,000 bp) and transcribed regions (+1,000, +2,000 and +5,000 bp) regions; the levels at 27 d were significantly higher than those at 13 and 20 d after birth (Fig. 3).

DISCUSSION

We have demonstrated in this study that both Fas protein and Fasn mRNA levels are increased in parallel in the liver during the suckling-weaning transition in rats, and that the rise in the Fasn mRNA levels are accompanied by increases in the bindings of ChREBP and SREBP1 to the upstream regions of the Fasn gene and the acetylation of histones H3 and H4 around the Fasn gene. The results presented here are consistent with those from previous studies which demonstrated that the expression of the Fasn gene is regulated by ChREBP.
and SREBP1c, two major transcription factors which transduct carbohydrate and insulin signals, respectively, to carbohydrate- and/or insulin-dependent genes in the liver (6), and further suggested that induction of the Fasn gene during the suckling-weaning transition is regulated at the transcriptional level.

Previous studies showed that hepatic Fasn mRNA levels remained extremely low in ChREBP knockout mice fed a high-starch diet (60% starch) compared with wild-type mice fed the same diet, despite increased glucose and insulin levels in the blood (20). Over-expression of SREBP1c, an isoform of SREBP1, was shown to induce the expression of the Fasn gene in hepatocytes (21). A previous in vitro study demonstrated, using gel shift assays, that ChREBP in the nuclear extract of rat liver bound to sequences of ChRE located in the promoter of Fasn (9). Luciferase assays have shown that a SREBP1 binding site in the Fasn promoter region is associated with its transactivation in the hepatic cell line HepG2 (10).

In this study, we have demonstrated using ChIP assays that the bindings of ChREBP and SREBP1 to the hepatic Fasn promoter/enhancer regions are elevated during the suckling-weaning transition. This is the first study to demonstrate that the bindings of these transcription factors to the promoter/enhancer region of the Fasn gene are modulated in vivo. Two isoforms of SREBP1 are known to be present (SREBP1a and SREBP1c), and the antibody we used in this study was able to detect both SREBP isoforms. Because previous studies have demonstrated that SREBP1c is predominantly expressed in the liver (22), we believe that SREBP1c is the dominant form that bound to the upstream region of the Fasn gene in this study.

It should be noted that ChREBP and active SREBP1 protein levels were not significantly changed during the suckling-weaning transition. It remains unknown which factors regulate the binding activity of these transcription factors. A recent study has reported that protein kinase A is involved in phosphorylation of SREBP1 in cultured hepatoma cells, and that the phosphorylation led to decreased binding of SRE (23). Another study has reported that SREBPs are negatively regulated by small ubiquitin-related modifier-1 (24). In addition, it has been reported that Ser196 of ChREBP is dephosphorylated by protein phosphatase 2A, which is activated by the glucose metabolite, xylulose-5-phosphate. The decrease in glucose concentration induces activation of cAMP-activated protein kinase, and it leads to enhanced phosphorylation of ChREBP at Ser196, which in turn results in reduced ChREBP trans-activity (25). Thus, it is very likely that posttranslational modifications of these transcription factors are altered during the suckling-weaning transition. Further studies should examine whether the posttranslational modifications of SREBP1 and ChREBP are related to the regulation of the Fasn gene in the liver during the suckling-weaning transition in rats.

In this study, we showed that ChREBP was bound around −7,000 bp of the rat liver Fasn gene and the binding was enhanced during the suckling-weaning transition. The result is consistent with a previous report showing that ChRE is located around the enhancer region (from −7,214 bp to −7,190 bp) of the rat Fasn gene (26). The binding of SREBP1 at −500 bp of the rat Fasn gene observed in this study is likely associated with the binding of SREBP1 on the SRE, which was reported to be located around the promoter region (from −151 bp to −142 bp) close to the CpG island and TATA box of the rat Fasn gene (27). In addition, we demonstrated in this study that the binding of SREBP1 at −7,000 bp was significantly increased from 20 d to 27 d after birth. It is still unclear whether SRE is located around −7,000 bp of the rat Fasn gene, because we were unable to find consensus sequences for the SRE (ATCACCCCA) in these regions. Further studies should examine whether SRE is located around −7,000 bp of the rat Fasn gene by DNase I footprinting and/or luciferase reporter assays, and whether ChREBP and/or SREBP1 play a crucial role in enhancing promoter activity of the Fasn gene as a component of transcription initiation factors in rat liver during the suckling-weaning transition period.

We found in this study that acetylation of histones H3 and H4 on the promoter/enhancer and/or transcribed regions of the Fasn gene were increased markedly from 13 d to 27 d after birth. This result suggests that acetylation of histones H3 and H4 is associated with increased Fasn gene expression in the liver of rats during development. SREBP1 binds specifically to the CREB-binding protein (CBP) and p300, both of which have histone acetyltransferase activity (28). CBP has
been shown to enhance SREBP1-dependent transactivation in HeLa cells (28). ChREBP assembles the transcriptional complex containing CBP onto the prototypical glucose responsive L-type pyruvate kinase gene (29). Acetylation of histone N-terminal tail regions catalyzed by histone acetyltransferases leads to target gene induction, by controlling the accessibility of transcriptional machinery to chromatin (30). Thus, it is likely that not only binding of transcription factors, but also acetylation of histones, is involved in formation of transcriptional machinery onto the promoter region of the rat Fasn gene during the suckling-weaning transition. It should be investigated in future studies whether binding of transcriptional machinery to the promoter region of the rat Fasn gene is altered during the suckling-weaning transition period.

It should be noted that serum glucose levels were elevated at 20 d, but decreased to a lower level at 27 d after birth, whereas serum insulin levels increased to higher levels at 20 d and 27 d after birth, and serum triacylglycerol levels were gradually decreased during the suckling-weaning transition period (Table 1). These changes in blood parameters were not apparently associated with the rises in mRNA levels of the Fasn gene and associated bindings of ChREBP, SREBP1 and acetylated histones around the Fasn gene. We speculate that the discrepancy is attributable to a change in the functional capability of the liver and adipose tissue during the suckling-weaning transition period (31), which plays a pivotal role in the uptake and utilization of glucose in an insulin-dependent manner. During the late-weaning period, i.e., between 20 and 27 d after birth, the reduction of serum glucose levels is likely caused by increased uptake of glucose in the liver as well as in the muscle and adipose tissue, along with maturation of insulin signal transduction system in these tissues.

In conclusion, we have demonstrated in this study in vivo that induction of the rat liver Fasn gene during the suckling-weaning transition period is associated with enhanced binding of ChREBP and SREBP1, and acetylation of histones H3 and H4 around the gene.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research (20590233) and for Young Scientists (22680054) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, the Global COE program, the Center of Excellence for Innovation in Human Health Sciences of the Ministry of Education, Science, Sports and Culture of Japan, and a grant from the Mishima Kaiau Memorial Foundation.

REFERENCES

19) Homma K, Mochizuki K, Goda T. 2007. Carbohydrate/fat ratio in the diet alters histone acetylation on the sucrose-isomaltase gene and its expression in mouse small intest-


29) Burke SJ, Collier JJ, Scott DK. 2009. cAMP opposes the glucose-mediated induction of the L-PK gene by preventing the recruitment of a complex containing ChREBP, HNF4alpha, and CBP. *FASEB J* **23**: 2855–2865.
