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Feeding Rats Dietary Resistant Starch Reduces both the Binding of ChREBP and the Acetylation of Histones on the *Thrsp* Gene in the Jejunum

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S Supporting Information

ABSTRACT: We have previously reported that the thyroid hormone-responsive spot 14 protein (*Thrsp*) gene is expressed in rat jejunum. In this study, we found that jejunal mRNA and protein expressions of *Thrsp* were markedly reduced in rats fed a diet containing a high amount of resistant starch (RS), which is an indigestible starch, for 7 days, compared with those fed a regular starch diet. Furthermore, we found that the binding of carbohydrate response element binding protein (ChREBP), which is a key transcription factor for the *Thrsp* gene, and the acetylation of histones H3 and H4, which is one of the histone modifications for transactivation, on the *Thrsp* gene were reduced by feeding the RS diet. These results suggest that the reduction of jejunal *Thrsp* gene expression by feeding a diet rich in less-digestible starch is associated with decreases in the binding of ChREBP and the acetylation of histones on the gene.

KEYWORDS: Resistant starch, *Thrsp*, ChREBP, histone acetylation, small intestine

INTRODUCTION

Excessive carbohydrate intake is known to induce de novo lipogenesis in liver and adipose tissues and is tightly associated with the enhanced expression of genes coding lipogenic enzymes, such as fatty acid synthase (*Fas*) and acetyl CoA carboxylase (*Acc*).[1] It has been reported that the expression of these carbohydrate-responsive genes is regulated by carbohydrate response element binding protein (ChREBP), a transcriptional factor. The activated form of ChREBP is translocated to the nucleus via the dephosphorylation of serine residue 196 (Ser 196) by carbohydrate signals, whereas the inactivated form, which is phosphorylated at Ser 196, is located in the cytoplasm.[2] In addition, previous studies have presumed that thyroid hormone-responsive spot 14 protein (*Thrsp*), also known as *Spot 14*, is closely related to these carbohydrate-responsive genes, because *Thrsp* gene and protein expressions are also induced by carbohydrate intake and are paralleled by the expressions of these carbohydrate-responsive genes and proteins.[3−6] Recent studies have demonstrated that THRSP, located in the nucleus,[7] acts as a coactivator by interacting with nuclear transcriptional factors, such as thyroid hormone receptor (TR),[8,9] chicken ovalbumin upstream promoter-transcription factor 1 (COUP-TF1),[10] and the p53 coactivator ZAC1.[9] Furthermore, it has been shown that the cis-element for ChREBP, called carbohydrate response element (ChoRE), is located upstream of the *Thrsp* gene.[11] Therefore, it is speculated that carbohydrate signals are transmitted by THRSP as well as ChREBP. Recently, we have demonstrated that *Thrsp* is expressed in the rat upper small intestine (jejunum). We also showed that *Thrsp* gene expression is abruptly enhanced during the transient suckling−weaning period, the first 2−4 weeks after birth,[12] when the diet composition is gradually changed from one low in carbohydrate (milk) to one rich in carbohydrate (solid food).[13] This indicates that *Thrsp* gene expression in the small intestine is also closely associated with carbohydrate signals.

In several previous studies, we have demonstrated that carbohydrate signals in the small intestine regulate the acetylation of histones H3 and H4, which is a histone modification known to be important for transactivation,[14−16] on the carbohydrate-responsive genes related to carbohydrate digestion/absorption (e.g., sucrase-isomaltase (*Si*), maltase-glucoamylase (*Mgam*), and solute carrier family 5, member 1 (*Slc5a1*), also named SGLT1) as well as the expression of these genes.[17−20] Thus, it is very likely that the expression of the *Thrsp* gene is affected by carbohydrate intake, presumably regulated by the binding of ChREBP and the acetylation of histones.

In the present study, we hypothesized that the jejunal expression of the *Thrsp* gene is regulated by carbohydrate signals via the binding of ChREBP and the acetylation of histones H3 and H4, and we examined whether decreases in glucose inflow by feeding a diet rich in resistant starch (RS), which is abundant in amylose and less digestible than regular starch, alters the binding of ChREBP and the acetylation of histones H3 and H4 on the *Thrsp* gene as well as the gene expression.

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Table 1. Composition of Diets

	control		RS	
	g/100 g	% energy	g/100 g	% energy
cornstarch	55.0	50.4		
Hi-maize[a]			55.0	50.4
casein	20.0	18.3	20.0	18.3
lard	10.0	20.6	10.0	20.6
corn oil	5.0	10.3	5.0	10.3
cellulose	5.0		5.0	
AIN93 mineral mixture[b]	3.5		3.5	
AIN93 vitamin mixture[b]	1.0		1.0	
L-cystine	0.3	0.3	0.3	0.3
choline bitartrate	0.2		0.2	
total	100.0	100.0	100.0	100.0

[a] Hi-maize contains approximately 60% resistant starch. [b] Composition is given in the Supporting Information.

MATERIALS AND METHODS

Animals and Diets. Ten male Wistar rats (7 weeks old) were obtained from Japan SLC (Shizuoka, Japan) and were divided into two groups: control ($n = 5$) and RS ($n = 5$). Control animals received a diet rich in α-cornstarch (Oriental Yeast, Tokyo, Japan), which contains approximately 25% amylose. Rats in the RS group received a diet in which all α-cornstarch was replaced with the same amount of RS, which contains approximately 70% amylose and is supplied as Hi-maize (Hi-maize1043, Nippon NSC Ltd., Tokyo, Japan). The details of the diet compositions are shown in Table 1. Animals were allowed free access to the diets and water for 7 days. At day 7 after feeding, the rats were killed by decapitation and the entire small intestine was collected. The experimental procedures used in the present study conformed to the guidelines of the Animal Usage Committee of the University of Shizuoka.

Preparation of Intestinal Samples. The entire jejunoileum was removed and divided into three segments of equal length. The proximal one-third of the jejunoileum was flushed twice with ice-cold 0.9% NaCl solution. Two centimeter segments (200 mg each) were excised from the middle region of the jejunal loop, divided into two segments of equal length (100 mg each), and immediately stored at −80 °C until assayed by real-time reverse transcription (RT) polymerase chain reaction (PCR) and Western blotting. The remaining part of the jejunal loop was used for chromatin immunoprecipitation (ChIP) assays.

Real-Time RT-PCR. Total RNA was extracted by using the acidified guanidine thiocyanate method, as described by Chomczynski and Sacchi.[21] Total RNA samples were stored at −80 °C before use in real-time RT-PCR. The total RNA samples (2.5 μg) were converted into cDNA by RT using SuperScript III reverse transcriptase (Invitrogen, Tokyo, Japan) according to the manufacturer's instructions. To quantitatively estimate the mRNA levels of *Thrsp* and TATA-box binding protein (*Tbp*), PCR amplification was performed on a Light-Cycler 480 instrument system (Roche, Tokyo, Japan). Real-time RT-PCR reactions were carried out in a total volume of 10 μL containing 400 nM each of gene specific primers, cDNA, and SYBR Premix Ex Taq (Takara, Shiga, Japan). The cycle threshold (CT) values of *Thrsp* and *Tbp* detected by real-time RT-PCR were converted to signal intensities by using the ΔΔ method,[22] which calculates the signal intensities as twice the difference between the CT of the test gene (*Thrsp*) and that of a gene for normalization (*Tbp*). The formula is $2^{(\mathrm{CT}\ Thrsp - \mathrm{CT}\ Tbp)}$. The sequences of the PCR primer pair and the fragment size are shown in Table 2.

Western Blotting. Jejunal segments for protein extraction were homogenized in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, and protease inhibitors tablets (Complete Mini; Roche)) and centrifuged at 20000*g* for 30 min at 4 °C. The soluble supernatants, in which total protein concentrations were normalized using the Lowry method,[23] were collected and stored at −80 °C. The tissue extracts were separated by 14% SDS-PAGE and transferred to Immobilon paper (Millipore, Tokyo, Japan) at 80 V for 120 min in Tris/glycine/methanol transfer buffer. The membranes were blocked for 1 h in 10% skim milk in PBS with 0.05% Tween 20 and 0.5 M NaCl, pH 7.4 (PBS−Tween−NaCl) at room temperature. They were then incubated in 10% skim milk in PBS−Tween−NaCl with primary antibody at 4 °C for >12 h, using THRSP (Santa Cruz Biotechnology, Santa Cruz, CA) and α-tubulin (TUBA) (Cell Signaling, Beverly, MA) as first antibodies. After washing in PBS−Tween−NaCl, the membranes were incubated with anti-rabbit/mouse IgG conjugated to biotin (GE Healthcare, Tokyo, Japan) as second antibodies in 3% skim milk in PBS−Tween−NaCl. After washing in PBS−Tween−NaCl and incubating with horseradish peroxidase-conjugated antibiotin as a third antibody (Cell Signaling), signals were detected by chemiluminescence (ECL Plus; GE Healthcare), according to the manufacturer's instructions, and were quantified using a luminescent image analyzer LAS-3000 (Fujifilm, Tokyo, Japan). THRSP levels were normalized against TUBA.

ChIP Assay. The mucosa removed from the jejunum was incubated with fixation solution (1% formaldehyde, 4.5 mM Hepes, pH 8.0, 9 mM NaCl, 0.09 mM EDTA, 0.04 mM ethylene glycol tetraacetic acid) in PBS for 30 min at 37 °C. The reaction was terminated by the addition of glycine to a final concentration of 150 mM. After being washed in FACS solution (1× PBS (−), 2% bovine serum, 0.05% NaN_3), the samples were sonicated in SDS lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 1% SDS, 0.5 mM phenylmethanesulfonyl fluoride) with protease inhibitors tablets (Complete Mini; Roche)/10 mL SDS lysis buffer when the DNA size of samples was 200−500 bp. The ChIP assay, using 1 μg of anti-ChREBP antibody (Santa Cruz Biotechnology), antiacetyl histone H3 at K9/14 antibody (Millipore, Tokyo, Japan), antiacetyl histone H4 at K5/8/12/16 antibody (Millipore), or normal rabbit IgG was performed as described previously.[17] The precipitated DNA was subjected to real-time PCR using primers that corresponded with the indicated sites in the promoter/enhancer and transcribed regions. The CT values of the ChIP signals detected by real-time PCR were converted to the percentage of the ChIP signal for the input DNA, which was calculated by the ΔΔ method,[22] using the formula $100 \times 2^{(\mathrm{CT\ IP\ sample} - \mathrm{CT\ input})}$. The sequences of the primers used in ChIP assays are indicated in Table 2.

Statistical Analysis. Each value is expressed as the mean ± SEM. Significance between two groups was evaluated by Student's *t* test. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Effects of an RS Diet on Jejunal mRNA and Protein Levels of *Thrsp* Gene. Jejunal mRNA (Figure 1A) and protein (Figure 1B) levels of *Thrsp* were 86 and 78% lower, respectively, in rats fed an RS diet compared with those fed a control diet ($P < 0.01$).

Effect of an RS Diet on the Binding of ChREBP and the Acetylation of Histones H3 and H4 in the Promoter/Enhancer and Transcribed Regions of the *Thrsp* Gene. To investigate whether the binding of ChREBP on the *Thrsp* gene is associated with a reduction in *Thrsp* gene expression by feeding rats an RS diet, we performed ChIP assays using antibodies for ChREBP. The average of ChIP signals for normal rabbit IgG was 0.0069 ± 0.0006%. The ChIP signals for ChREBP were relatively high in the promoter/enhancer (−2000 to −1 bp) and transcribed (500 bp) regions close to the transcription initiation site on the *Thrsp* gene in the control group, whereas the signals were

Table 2. Sequences of the Oligonucleotide Primers Used in This Study

	fragment size	sequence
Thrsp mRNA	118	5′-GAGAATGAGGCTGCTGAAACA-3′ 5′-TCAGGTGGGTAAGGATGTGAT-3′
Tbp mRNA	146	5′-CCCACAACTCTTCCATTCTCA-3′ 5′-TTTGGAGCTGTGGTACAATCC-3′

region on the *Thrsp* gene[a]		fragment size	sequence
Thrsp −2000	−2083 to −2064 −2013 to −1993	91	5′-ATGCAGCATGTAGCAACACC-3′ 5′-TGATAGACTGGGACCAGCATT-3′
Thrsp −1400	−1469 to −1449 −1387 to −1366	104	5′-GGAACACTCTGTTTGCCAGTT-3′ 5′-TGTAACTCCCTTTGAGTCCACA-3′
Thrsp −500	−542 to −522 −433 to −413	130	5′-AAGAGAACTGCTGGGACTTGA-3′ 5′-AGCTGTGAAACGAACAGAGGA-3′
Thrsp −1	−70 to −50 46−66	136	5′-GTCAATCTGCTGTCTGCTCAA-3′ 5′-AGTACCGATCCATGACCTTCA-3′
Thrsp 500	471−491 574−594	124	5′-ATGGAAGGTAATGGTGGTCGT-3′ 5′-TCCCAAGTGATAGTCCCCATA-3′
Thrsp 2000	1975−1995 2063−2083	109	5′-ACAGCAAGCAAACAGGTCACT-3′ 5′-CCATTGTCAAGACCAGCAGAT-3′

[a] The region is denoted relative to the transcription initiation site.

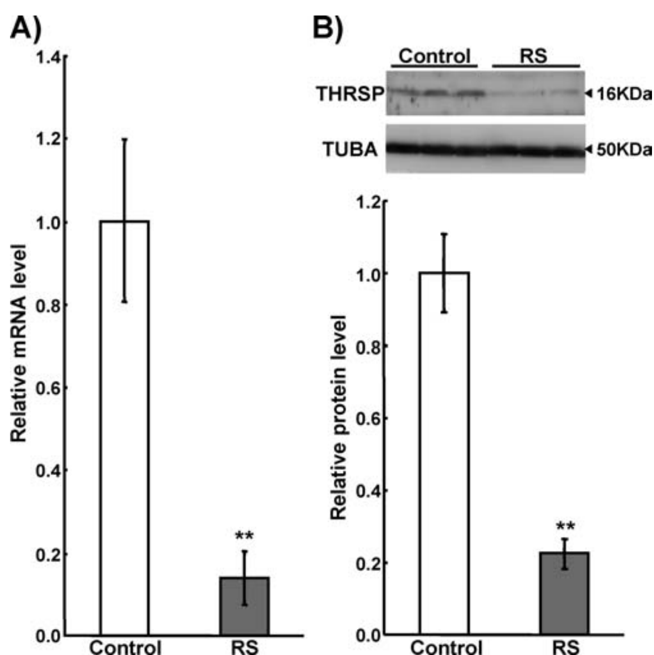

Figure 1. mRNA and protein levels of the *Thrsp* gene in the jejunum of rats fed a control diet or an RS diet for 7 days: (A) *Thrsp* mRNA level; (B) THRSP protein level. Mean ± SEM of five animals is shown. **,$P < 0.01$, RS diet versus control diet (Student's *t* test).

significantly reduced in the promoter/enhancer (−1400 and −500 bp, $P < 0.05$; −1 bp, $P < 0.01$) regions in the RS group (Figure 2B).

Next, to investigate whether the acetylation of histones H3 and H4 on the *Thrsp* gene is associated with a reduction in *Thrsp* gene expression by feeding rats an RS diet, we performed ChIP assays using two antibodies for acetylated histone H3 at K9/14 and acetylated histone H4 at K5/8/12/16. We chose the antibodies against the specific acetylated lysine residues, K9 and K14, of histone H3 and against the specific acetylated lysine residues, K5, K8, K12, and K16, of histone H4 because the acetylated parts of both histones are closely associated with the euchromatin region and the acetylated lysine residues induce transcription through recruiting transcriptional complexes.[24] The ChIP signals for acetylated histone H3 at K9/14 were relatively high in the promoter/enhancer (−2000 to −1 bp) regions on the *Thrsp* gene in the control group, whereas the signals were significantly reduced in both the promoter/enhancer (−2000, −1400, −500, and −1 bp, $P < 0.05$) and transcribed (500 bp, $P < 0.05$) regions in the RS group (Figure 2C). The ChIP signals for acetylated histone H4 at K5/8/12/16 were relatively high in the promoter/enhancer (−2000 to −1 bp) regions on the *Thrsp* gene in the control group, whereas the signals were significantly reduced in promoter/enhancer (−2000 bp, $P < 0.01$; −1400 and −500 bp, $P < 0.05$) regions in the RS group (Figure 2D).

DISCUSSION

In this study, we found that the jejunal mRNA of the *Thrsp* gene was reduced by feeding rats an RS diet (Figure1A). In a preliminary study, we found that the *Thrsp* mRNA levels were inversely associated with the amount of dietary RS (0% RS (control diet), 27.5% RS, and 55% RS (RS diet), (Shimada et al unpublished results). In addition, we demonstrated that the

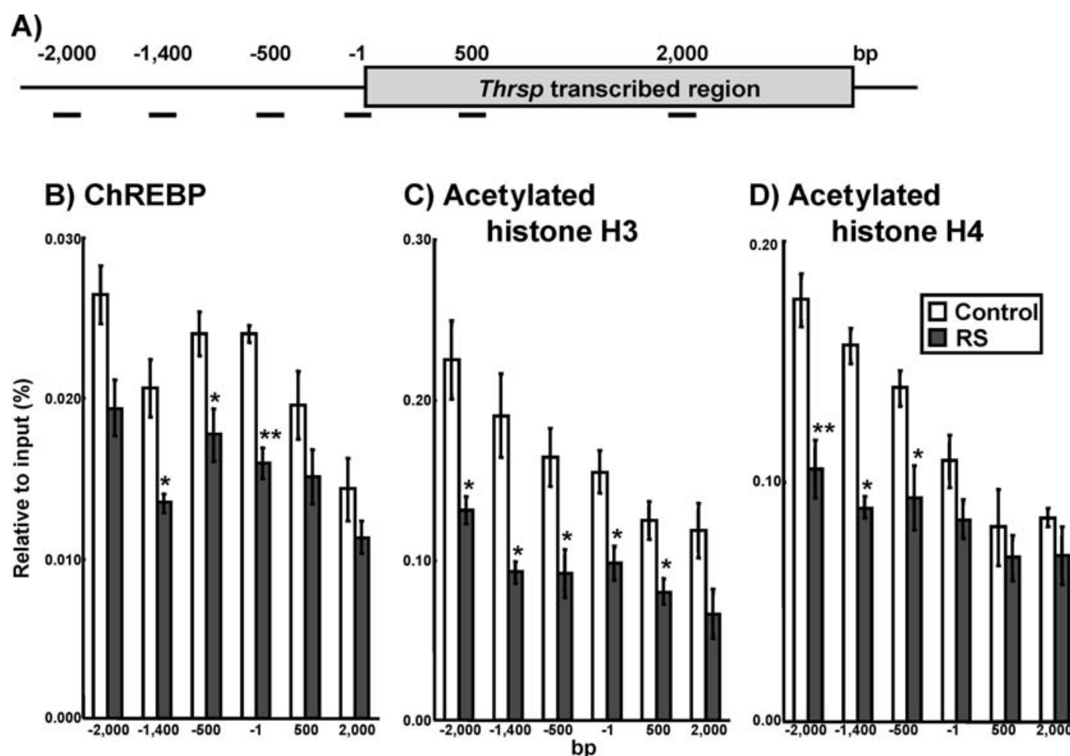

Figure 2. Binding of ChREBP and acetylation of histones H3 and H4 on the *Thrsp* gene in the jejunum of rats fed a control diet or an RS diet for 7 days: (A) primer pairs used for PCR in ChIP assays; (B) ChREBP; (C) acetylated histone H3 at K9/14; (D) acetylated histone H4 at K5/8/12/16. Mean ± SEM of five animals is shown. ∗, $P < 0.05$, ∗∗,$P < 0.01$, RS diet versus control diet (Student *t* test).

extent of the reduction in mRNA levels was similar to that of the reduction in protein levels. Many studies have demonstrated that the *Thrsp* gene and protein respond to carbohydrate signals in the liver.[3−6] Our results suggest that carbohydrate signals induce *Thrsp* gene and protein expressions in the small intestine. Furthermore, we found that ChREBP was strongly bound to the promoter/enhancer (−2000 to −1 bp) regions on the *Thrsp* gene in rats fed a control diet. It has been shown that ChoRE, the consensus sequence for ChREBP, is composed of two 6 bp motifs known as E boxes and located on −1448 to −1422 bp from the translation start site of rat *Thrsp* gene.[11] However, in vivo binding to the elements and/or promoter/enhancer regions of the gene was still unknown. This study is the first to demonstrate that ChREBP is directly bound to the promoter/enhancer regions of the *Thrsp* gene. It should be examined further by DNAase footprinting assay whether the ChREBP is bound to the putative ChoRE (−1448 to −1422 bp) on the *Thrsp* gene. Interestingly, we found that the ChREBP binding to the promoter/enhancer (−1400 to −1 bp) regions was significantly reduced by feeding rats the RS diet. These results suggest that the reduction of the ChREBP binding on the *Thrsp* gene by the dietary RS is closely associated with the reduction in the gene expression.

Recently we have demonstrated that carbohydrate signals induce histone acetylation on carbohydrate-responsive genes related to carbohydrate digestion or absorption (e.g., *Si*, *Mgam*, and *Slc5a1*) as well as the expression of these genes in the small intestine.[17−20] This indicates that the histone acetylation on these carbohydrate-responsive genes and the gene expression are associated with the inflow of available carbohydrate, namely, glucose, in the small intestine. In this study, we showed that a decrease in glucose inflow into the jejunum by feeding the RS diet led to a reduction in the acetylation of histone H3 in both the promoter/enhancer (−2000 to −1 bp) and transcribed (−1 to 500 bp) regions and led to a reduction in the acetylation of histone H4 in the promoter/enhancer (−2000 to −500 bp) regions of the *Thrsp* gene. It is reported that histone acetylation in the promoter/enhancer region leads to recruitment of mRNA transcriptional complexes, whereas histone acetylation in the transcribed region leads to recruitment of mRNA elongation complexes.[25−27] Therefore, it is likely that the repression of histone H3 acetylation by the intake of RS is involved in the repression of both mRNA transcription initiation and mRNA elongation and that the repression of histone H4 acetylation by the intake of RS is involved in the repression of mRNA transcription initiation of the *Thrsp* gene. In addition, this result suggests the possibility that the reduction in acetylation is associated with the reduction in ChREBP binding on the *Thrsp* gene. It has been reported that nuclear transcriptional factors including ChREBP induce histone acetylation in the promoter/enhancer regions through the binding of histone acetyl-transferases (HATs) and that the histone acetylation recruits mRNA transcriptional complexes.[25−27] Thus, it is most likely that inflow of glucose into the jejunum induces histone acetylation as well as ChREBP binding on the *Thrsp* gene and that the reduction of *Thrsp* gene expression by dietary RS is involved in the repression of histone acetylation triggered by a decrease in the amount of glucose derived from dietary RS in the jejunum. Further studies are needed to determine whether the binding of HATs and transcriptional complexes on the *Thrsp* gene are reduced by feeding the RS diet. However, it is still unclear if the histone acetylation is specific to the *Thrsp* gene, because the expression of many genes is regulated by histone acetylation. Histone acetylation is notably induced in the cellular differentiation processes.[14] Intestinal cells are known to transit and differentiate from stem

cells in the crypt to absorptive cells in the villus, where many genes related to digestion/absorption of nutrients are expressed. The transition of differentiation from the crypt to the villus in rodent small intestine takes only 2−3 days, and during the transition the genes participating in digestion/absorption are expressed.[28] Using cryostat sectioning to cut frozen jejunum into several fractions from the top of the villus to the bottom of the crypt, we have recently demonstrated that histone acetylation on the *Si* gene and its expression are induced during the transition of differentiation from the crypt to the villus.[29] In addition, our previous study has shown that carbohydrate signals induce *Si* gene expression at the transitional period of differentiation.[30] These studies indicate that expression of the *Si* gene and the acetylation of histones on the gene are associated with differentiation to the villus and that the event is characteristic of the small intestine. Thus, the reductions in *Thrsp* gene expression and in histone acetylation on the gene by dietary RS may also be related to a decrease in the transition of differentiation from the crypt to the villus. Whether jejunal *Thrsp* mRNA and histone acetylation on the gene along the crypt−villus axis are altered by the intake of RS diet should be further investigated by cryostat sectioning technique.

Because expression of THRSP is closely associated with expression of lipogenic enzymes such as FAS and ACC in the liver,[1] it is speculated that THRSP is a regulator for lipogenic enzyme expression in the liver. Although the function of THRSP in the small intestine remains unclear, THRSP reportedly acts as a coactivator by interacting with nuclear transcriptional factors, such as TR, COUP-TF1, and ZAC1.[8−10] Particularly, several studies, including our own, indicate that TRα-1 regulates carbohydrate-responsive genes such as *Si*, *Slc5a1*, solute carrier family 2, member 5 (*Slc2a5*), also named GLUT5, and solute carrier family 2, member 2 (*Slc2a2*), also named GLUT2, in the small intestine.[31,32] In addition, expression of these carbohydrate digestion/absorption-related genes is regulated by several transcriptional factors, such as caudal type homeobox 2 (CDX2), hepatocyte nuclear factor 1 (HNF1), and cAMP responsive element binding protein (CREB).[33−36] Taken together, jejunal THRSP may act as a nuclear transcriptional factor and bind to the promoter/enhancer regions of genes related to carbohydrate digestion/absorption by coactivating the nuclear transcriptional factors. Therefore, reduced expression of jejunal THRSP might lead to suppression of postprandial hyperglycemia, hyperinsulinemia, and hyperlipidemia through reduced expression of genes related to the digestion and absorption of carbohydrates.

In conclusion, we have demonstrated for the first time that the reduction of *Thrsp* gene expression by an RS diet in rats is associated with decreases both in the binding of ChREBP and in the acetylation of histones H3 and H4 on the gene.

ASSOCIATED CONTENT

Supporting Information. Additional tables showing the compositions of the AIN93 mineral and vitamin mixtures. This material is available free of charge via the Internet at http://pubs.acs.org.

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