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Histone H3 methylation at lysine 4 on the *SLC2A5* gene in intestinal Caco-2 cells is involved in *SLC2A5* expression

Yuko Inamochi [a], Kazuki Mochizuki [a], Ayumi Osaki [a], Takeshi Ishii [b], Tsutomu Nakayama [b], Toshinao Goda [a,*]

[a] *Laboratory of Nutritional Physiology, The University of Shizuoka, Graduate School of Nutritional and Environmental Sciences and Global COE, 52-1 Yada, Shizuoka-shi, Shizuoka 422-8526, Japan*

[b] *Laboratory of Molecular Food Engineering, The University of Shizuoka, Graduate School of Nutritional and Environmental Sciences and Global COE, 52-1 Yada, Shizuoka-shi, Shizuoka 422-8526, Japan*

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ABSTRACT

Histone H3 methylation at lysine 4 (K4) is associated with euchromatic regions and is thought to be important for the transcriptional activation of genes during differentiation. In this study, we found that di- and tri-methylation of histone H3 at K4 and acetylation of histones H3 and H4 from the promoter/enhancer to the transcribed region close to the transcription initiation site of the solute carrier family 2, member 5 (*SLC2A5*) gene, and its expression, were induced by differentiation of intestine-like Caco-2 cells. These effects were accompanied by contact inhibition of cell growth of these cells. Furthermore, these modifications were induced by co-treatment with a synthetic glucocorticoid hormone dexamethasone and a p44/42 mitogen-activated protein kinase inhibitor PD89059. Our results suggest that methylation of histone H3 at K4 and acetylation of histones H3 and H4 are involved in *SLC2A5* gene induction associated with intestinal differentiation of Caco-2 cells.

Introduction

Intestinal absorptive cells rapidly mature during their transition from the crypt to the villus, and start to express many intestine-specific genes involved in digestion and absorption of nutrients. Previous studies, including our own, have shown that the expression of the hexose transporter, *SLC2A5*, often called glucose transporter 5 (GLUT5), a brush-border protein involved in fructose absorption from the lumen, is induced during the transition from the crypt to the villus [1]. In addition, several studies have documented that *SLC2A5* gene expression is coordinately enhanced by treatment with glucocorticoid or thyroid hormones and inhibitors of p44/42 mitogen-activated protein kinase (MAPK). These effects are mediated by glucocorticoid hormone receptor (GR) α and thyroid hormone receptor (TR) α-1, respectively [2,3]. It is known that p44/42 MAPK is inactivated during cell differentiation by contact inhibition of cell growth [4,5]. These results indicate that glucocorticoid hormone, thyroid hormone and p44/42 MAPK inhibition regulate the induction of *SLC2A5* gene expression in Caco-2 cells through GRα and TRα-1 during the differentiation to enterocyte-like cells.

Recent studies have shown that abrupt changes in gene expression, which frequently occur in differentiating cells, are accompanied by a major chromatin structural change that is triggered by modifications such as acetylation, methylation and phosphorylation of the histone tail [6,7]. Among several histone modifications that have been identified, acetylation of histone H3 at lysines (K) 9 and 14, of histone H4 at K5/8/12/16 and methylation of histone H3 at K4 are the most extensively studied, because regulation of these histone modifications is related to transactivation [8–10]. In particular, di- or tri-methylation at K4 is important for the initial activation of transcription because di-/tri-methylation of histone H3 at K4 is induced before the acetylation of histones and transactivation of genes. Indeed, methylation of histone H3 at K4 induces acetylation of histones [11,12]. Acetylation of histones leads to the recruitment of mRNA transcription complexes onto the promoter/enhancer region and mRNA elongation complexes to the transcribed region close to the transcription initiation site through binding of bromodomain-containing proteins to acetylated histones [13–15]. The molecular mechanisms involved in the activation of gene expression by methylation of histone H3 at K4 are still poorly understood. However, several studies have indicated that trimethylation also induces the recruitment of mRNA transcription and elongation complexes onto target genes. This occurs through the binding of proteins containing plant homeodomains (PHD), chromodomains, Tudor domains or WD40 repeat domains to the methylated histones [16,17]. Recently, we have demonstrated that acetylated histones H3 and H4 on the promoter/enhancer region

* Corresponding author. Fax: +81 54 264 5565.
E-mail address: gouda@u-shizuoka-ken.ac.jp (T. Goda).

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of *SLC2A5* gene are involved in *SLC2A5* gene induction in Caco-2 cells co-treated with a glucocorticoid hormone (dexamethasone, Dex) and a p44/42 MAPK inhibitor [3]. However, it is still unknown whether gene expression of *SLC2A5* during differentiation is accompanied by contact inhibition of cell growth in Caco-2 cells based on studies of histone acetylation, or whether methylation of histone H3 at K4 is involved in expression of intestinal gene, including *SLC2A5*, in intestinal cell lines or in the jejunum *in vivo*.

In the present study, we examined whether methylation of histone H3 at K4 and acetylation of histones H3 at K9/14 and H4 at K5/8/12/16 on the *SLC2A5* gene are involved in the induction of *SLC2A5* gene expression through contact inhibition of cell growth and by co-treatment with Dex and a p44/42 MAPK inhibitor.

Materials and methods

Cell culture. Caco-2 cells (American Type Culture Collection, Rockville, MD, USA) were seeded at a density of 0.6×10^4 cells/cm^2 on collagen-coated culture plates (Iwaki, Tokyo, Japan) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 1% non-essential amino acids (Invitrogen, Tokyo, Japan), 20 mM HEPES (pH 7.4), 1× antibiotic–antimycotic mixed stock solution (Nacalai Tesque, Kyoto, Japan) and 2 mM L-glutamate (Invitrogen) at 37 °C in a humidified atmosphere of 5% CO_2. Day 0 was regarded as the day when 100% confluence was reached, usually at 5 days after plating, and days 5 and 10 were regarded as 5 and 10 days, respectively, after reaching 100% confluence. For experiments involving treatment with Dex and a p44/42 MAPK inhibitor, Caco-2 cells were seeded at a density of 0.6×10^4 cells/cm^2 and cultured for 5 days in DMEM containing 10% FCS devoid of glucocorticoid hormone by treatment with charcoal/dextran. Then, cells were incubated with 50 μM PD98059 (PD), a p44/42 MAPK inhibitor, 1 μM Dex or vehicle (DMSO) alone for 48 h.

RNA analysis. Total RNA was extracted by the acidified guanidine thiocyanate method, as described by Chomczynski and Sacchi [18]. Total RNA was subjected to reverse transcription using Superscript III reverse transcriptase (Invitrogen). *SLC2A5* and *ACTB* (β-actin) cDNAs were amplified by real-time PCR using a LightCycler System (Roche Diagnostics, Tokyo, Japan) and SYBR Green I (Takara, Shiga, Japan). The cycle threshold (CT) values of *SLC2A5* and *ACTB* detected by real-time RT-PCR were converted into signal intensities by the delta–delta method [19], which calculates the signal intensity to be twice the difference between the cycle threshold of the test gene (*SLC2A5*) and that of a normalization gene (*ACTB*). The formula used was $[2^{(\mathrm{CT}\ SLC2A5 - \mathrm{CT}\ ACTB)}]$. The primer sequences used for real-time RT-PCR are shown in Table 1.

Chromatin immunoprecipitation (ChIP) assay. Cells were incubated in fixation solution (1% formaldehyde, 4.5 mM HEPES, pH 8.0, 9 mM NaCl, 0.09 mM EDTA and 0.04 mM EGTA) in 10% FBS/DMEM for 30 min at 37 °C. The reactions were terminated by the addition of glycine to a final concentration of 150 mM. After being washed in FACS solution (1× PBS, 2% bovine serum and 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 and 0.5 mM PMSF) to generate DNA fragments of size 200–500 bp. The ChIP assay used 1 μg of anti-acetyl histone H3 at K9/14 antibody (Millipore, Tokyo, Japan), anti-acetyl histone H4 at K5/8/12/16 antibody (Millipore), anti-mono-methyl-histone H3 at K4 antibody (Millipore), anti-di-methyl-histone H3 at K4 antibody (Millipore), anti-trimethyl-histone H3 at K4 antibody (Millipore) or normal rabbit IgG, and was performed as previously described [20]. The precipitated DNA was subjected to real-time PCR using primers corresponding to the indicated sites in the promoter/enhancer and transcription start sites. The CT values of the ChIP signals detected by real-time PCR were converted to a percentage of the ChIP signal for the input DNA, which was calculated by the delta–delta method [19] using the formula $100 \times [2^{(\mathrm{CT\ IP\ sample} - \mathrm{CT\ input})}]$. The primer sequences used in ChIP assays are indicated in Table 1.

Statistical analysis. Results are expressed as means ± SEM. Statistical significance was determined by Dunn's test based on the Kruskal–Wallis multiple range test.

Table 1
The sequences of oligonucleotide primers used for real-time PCR.

	Sequence
Target mRNA	
Human *SLC2A5* mRNA	5′-TGATCTTCCCGTTCATCCA-3′
	5′-GTCTCCGGGACAATCAAGAA-3′
Human *ACTB* mRNA	5′-CATGAAGTGTGACGTGGACAT-3′
	5′-TGATCTCCTTCTGCATCCTGT-3′
Region on the human SLC2A5 gene	
−5100	5′-GCTGAAAGCTGAGTCATGCAAGGA-3′
	5′-TCTGTCGCTGTCTGCTTAGGAACAT-3′
−2800	5′-TTCCTGGGCTCAAGCGATCC-3′
	5′-AGGGGAGTGGTCCTGGCAAA-3′
−2000	5′-CAGGGAGAAGTGGAAGATCTG-3′
	5′-GTCCAAAGTTTGTAGTCATCAGTC-3′
−1000	5′-TGGGACTTGTGAGACTTTGG-3′
	5′-TGTTTGGCACTCATTCAGCT-3′
−400	5′-CCACCAAGAGACACTTGACTG-3′
	5′-ACATGACACCAGGTAATCTGAG-3′
+0	5′-CAAAGTGCACCCAGAATGTC-3′
	5′-CATGCTCTGATCCTGTTGCT-3′
+1000	5′-GCTGAGCTGCATGAAAGCAAGA-3′
	5′-AAGGCAGGAAACCTACGTGACA-3′
+21,724 + 21,924	5′-ATCGTGCCTGCGATCTTAATGG-3′
	5′-TCAAATGATGTGGCGACTCTGC-3′
+30,900 + 31,100	5′-ACGTTCAGCTGATTTCCCAGT-3′
	5′-GAGAAAGCAGTTGGTTCACCT-3′
Over + 5900 + 6100	5′-AATGCTAAGTCCCTGTGATGG-3′
	5′-AAACGGGTCTGAACCTTCTTC-3′

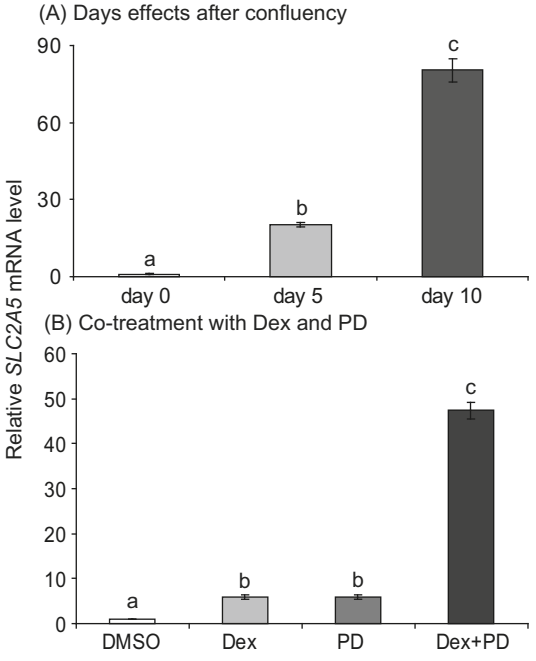

Fig. 1. Expression of *SLC2A5* mRNA during differentiation accompanied by contact inhibition of cell growth and by the treatment with Dex and PD in Caco-2 cells. (A) Cells were collected at days 0, 5 and 10 after reaching 100% confluency. (B) Cells were co-treated with Dex (1 μM) without/with PD (50 μM). Results of real-time RT-PCR for each mRNA were normalized to *ACTB* mRNA and are expressed as arbitrary units. Means ± SEM for six determinations are shown. Values not sharing a common superscript differ significantly ($P < 0.05$) from one another.

Results

SLC2A5 gene expression is induced during differentiation by contact inhibition of cell growth and by treatment with Dex and PD in Caco-2 cells

To examine whether *SLC2A5* expression was induced by enterocyte like differentiation in Caco-2 cells, cells were cultured for 0, 5 or 10 days after reaching confluence. As shown in Fig. 1, *SLC2A5* gene expression was lowest at 100% confluence (day 0). However, the expression of *SLC2A5* was clearly enhanced at days 5 and 10 after confluence, and the *SLC2A5* mRNA level was significantly higher on day 10 than on day 5 (Fig. 1A).

Next, to examine the effects of glucocorticoid hormone and inhibition of p44/42 MAPK on the *SLC2A5* gene expression, we performed real-time RT-PCR in Caco-2 cells treated with 1 μM Dex, with or without 50 μM of PD for 48 h. *SLC2A5* gene expression was significantly enhanced by treatment with Dex or PD alone, while co-treatment with Dex and PD synergistically enhanced gene expression (Fig. 1B).

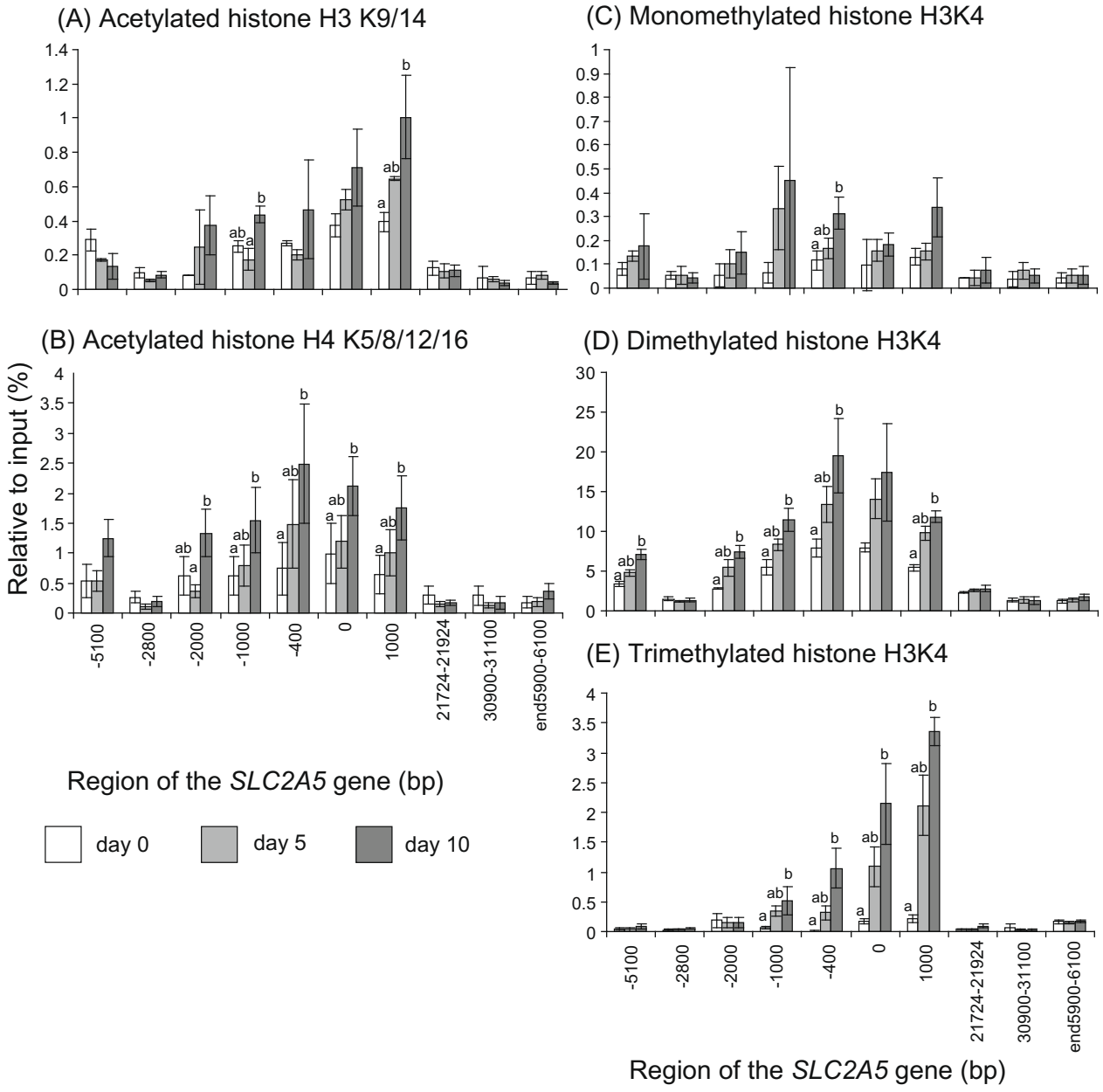

Fig. 2. Acetylated histone H3 at K9/14, acetylated histone H4 at K5/8/12/16 and methylated histone H3 at K4 on the *SLC2A5* gene in Caco-2 cells during differentiation accompanied by contact inhibition of cell growth. (A) Acetylated histone H3 at K9/14. (B) Acetylated histone H4 at K5/8/12/16. (C) Mono-methylated histone H3 at K4. (D) Di-methylated histone H3 at K4. (E) Tri-methylated histone H3 at K4. The samples were collected at days 0, 5 and 10 after reaching 100% confluence for ChIP assays. ChIP signals were detected by quantitative real-time RT-PCR following immunoprecipitation of the indicated nuclear proteins bound to the *SLC2A5* gene, and were normalized to input signals. The abscissa denotes the region on the *SLC2A5* gene relative to the transcription initiation site. Means ± SEM for six determinations are shown. Values not sharing a common superscript differ significantly ($P < 0.05$) from one another.

Association between acetylation of histone H3 at K9/14 and histone H4 at K5/8/12/16 or methylation of histone H3 at K4 with SLC2A5 gene induction during differentiation accompanied by contact inhibition of cell growth in Caco-2 cells

To investigate whether acetylation of histone H3 at K9/14, acetylation of histone H4 at K5/8/12/16 and methylation of histone H3 at K4 are associated with the induction of *SLC2A5* during differentiation and contact inhibition of cell growth in Caco-2 cells, we performed ChIP assays using antibodies for acetylated histone H3 at K9/14, acetylated histone H4 at K5/8/12/16 and mono-/di-/trimethylated histone H3 at K4. The ChIP signals for normal rabbit IgG were <0.04% per input. The ChIP signals for acetylated histone H3 at K9/14 tended to be higher on day 10 than on day 0 on the regions ranging from −1000 bp to 1000 bp, and significant differences were observed between day 0 and day 10 at 1000 bp (Fig. 2A). The ChIP signals for acetylated histone H4 at K5/8/12/16 were significantly higher on day 10 than on day 0 on the regions ranging from −1000 bp to 1000 bp (Fig. 2B). Regarding the methylation of histone H3 at K4, the ChIP signals for mono-methylated histone H3 at K4 were significantly higher on day 10 than on day 0 at −400 bp (Fig. 2C). The signals for di-methylated histone H3 at K4 tended to be higher on day 10 than on day 0 on the regions ranging from −2000 bp to 1000 bp and at −5100 bp. Significant

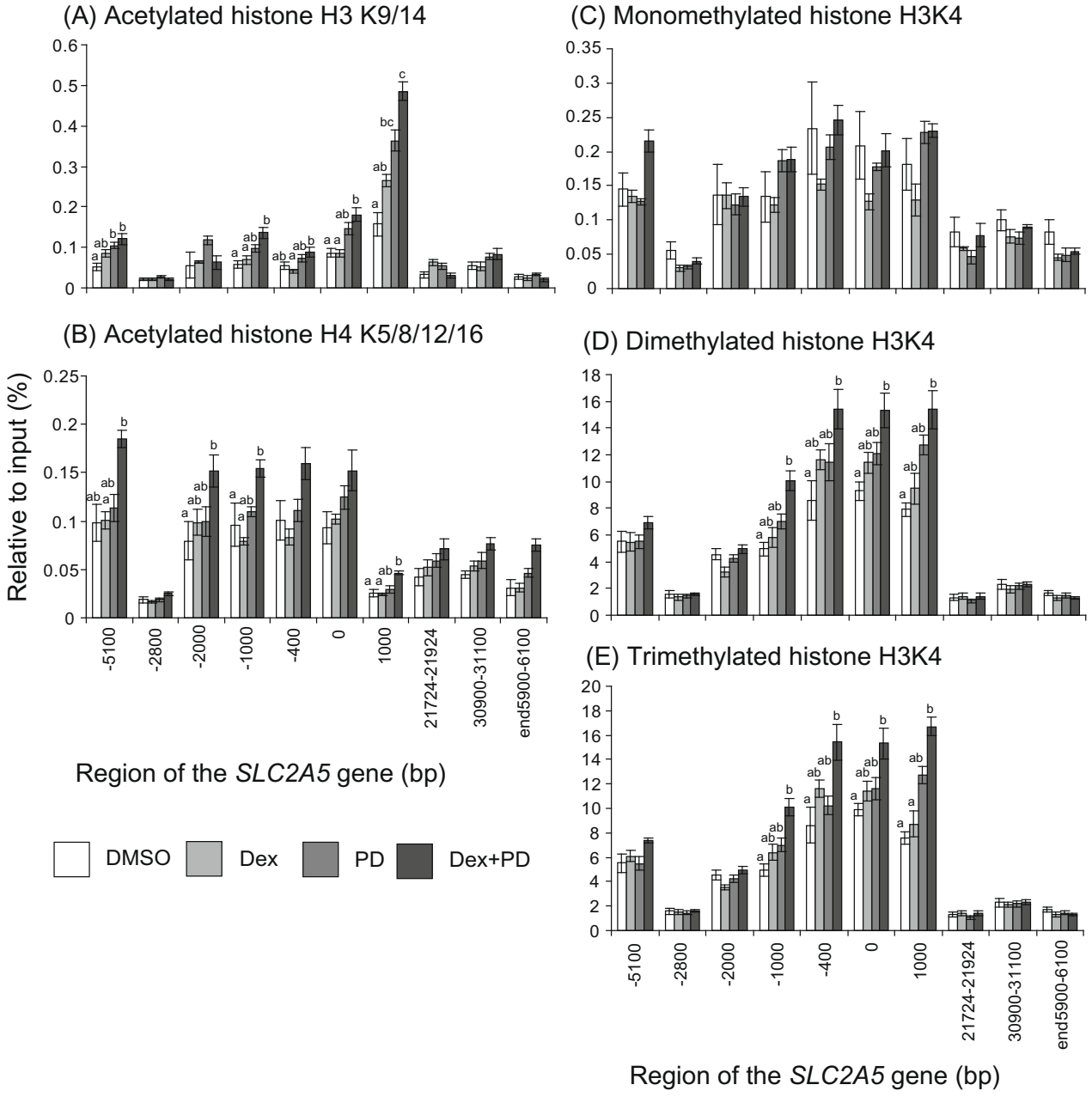

Fig. 3. Acetylation of histone H3 at K9/14 and histone H4 at K5/8/12/16, and methylation of histone H3 at K4 on the *SLC2A5* gene induced by treatment with Dex and PD in Caco-2 cells. (A) Acetylated histone H3 at K9/14. (B) Acetylated histone H4 at K5/8/12/16. (C) Mono-methylated histone H3 at K4. (D) Di-methylated histone H3 at K4. (E) Trimethylated histone H3 at K4. Caco-2 cells were treated with Dex and PD and collected for ChIP assays. ChIP signals were detected by quantitative real-time RT-PCR following immunoprecipitation of the indicated nuclear proteins bound to the *SLC2A5* gene, and were normalized to input signals. Means ± SEM for six determinations are shown. The abscissa denotes the region on the *SLC2A5* gene relative to the transcription initiation site. Values not sharing a common superscript differ significantly ($P < 0.05$) from one another.

differences between days 0 and 10 were observed at −5100 bp, −2000 bp, −1000 bp, −400 bp and +1000 bp (Fig. 2E). The ChIP signals for tri-methylated histone H3 at K4 were significantly higher on day 10 than on day 0 on the regions ranging from −1000 bp to 1000 bp (Fig. 2E).

Effects of Dex and PD on histone H3 acetylation at K9/14, histone H4 acetylation at K5/8/12/16 and methylation of histone H3 at K4 on the SLC2A5 gene in Caco-2 cells

To investigate whether acetylation of histone H3 at K9/14, acetylation of histone H4 at K5/8/12/16 and methylation of histone H3 at K4 are associated with the *SLC2A5* gene expression in Caco-2 cells treated with Dex and PD, we performed ChIP assays using antibodies for acetylated histone H3 at K9/14, acetylated histone H4 at K5/8/12/16 and mono-/di-/tri-methylated histone H3 at K4. The ChIP signals for normal rabbit IgG were <0.04% per input. The ChIP signals for acetylated histone H3 at K9/14 on the upstream/transcriptional regions of the *SLC2A5* gene (at −5100 bp, from −1000 bp to 1000 bp) were markedly enhanced by co-treatment with Dex and PD (Fig. 3A). The ChIP signals for acetylated histone H4 at K5/8/12/16 tended to be enhanced by co-treatment with Dex and PD throughout the upstream/transcriptional regions of the *SLC2A5* gene, with significant differences observed at −5100 bp, −2000 bp, −1000 bp and at 1000 bp (Fig. 3B). Treatment with Dex and PD remarkably enhanced the di-methylation levels of histone H3 at K4 from the promoter/enhancer region to transcribed region close to transcription initiation site of the *SLC2A5* gene (from −1000 bp to 1000 bp) (Fig. 3D). Tri-methylation of histone H3 from the promoter/enhancer to the region close to the transcription initiation site of the *SLC2A5* gene (from −1000 bp to +1000 bp) was also markedly enhanced by co-treatment with Dex and PD (Fig. 3E).

Discussion

In this study, we have demonstrated that *SLC2A5* gene expression increases during differentiation, which is accompanied by contact inhibition of cell growth, and in response to co-treatment with Dex and PD in Caco-2 cells (Fig. 1). This result is consistent with the findings of previous studies [21–23]. In this study, we showed that acetylation of histone H3 at K9/14 and acetylation of histone H4 at K5/8/12/16 on the *SLC2A5* gene were progressively increased after the onset of differentiation on broad regions ranging from promoter/enhancer region to the transcribed region close to the transcription initiation site. Our previous study demonstrated that co-treatment with Dex and a p44/42 MAPK inhibitor PD induced acetylation of histones H3 and H4 in the promoter/enhancer region of *SLC2A5* gene [2]. The current study has shown that the enhancement of acetylation of histone H3, which was induced either by differentiation/contact inhibition of cell growth, or by treatment with Dex and p44/42 MAPK inhibitor, occurred at the transcribed region close to the transcription initiation site of the *SLC2A5* gene, whereas the basal level as well as the induced levels of acetylation of histone H4 was observed primarily on the promoter/enhancer region of *SLC2A5* gene. It is known that histone acetylation in the transcribed region leads to recruitment of mRNA elongation complexes, whereas histone acetylation in the promoter/enhancer region is associated with enhanced recruitment of mRNA transcriptional complexes [13–15]. Therefore, it is likely that histone H3 acetylation is involved in the regulation of mRNA elongation and histone H4 acetylation is involved in the regulation of mRNA transcription initiation of the *SLC2A5* gene.

Several recent studies have demonstrated that methylation of histone H3 at K4 is important for the initial activation of transcription, because di-/tri-methylation of histone H3 at K4 is induced before acetylation and transactivation of genes, and methylation of histone H3 at K4 precedes histone acetylation [11,12]. However, it has not been tested whether methylation of histone H3 at K4 affects the expression of genes such as *SLC2A5* in the small intestine *in vivo* or in cell lines. As shown in Fig. 2E, tri-methylation of histone H3 at K4 was induced during the differentiation from the promoter/enhancer region to the transcribed region close to transcription initiation site of the *SLC2A5* gene. In addition, we showed that treatment with Dex and a p44/42 MAPK inhibitor induced tri-methylation of histone H3 at K4 from the promoter/enhancer region to the transcribed region close to transcription initiation site of the gene. Thus, our study is consistent with previous studies which have demonstrated that histone H3 at K4 is highly tri-methylated in the transcription initiation site and plays a role in recruiting Pol II and enhancing mRNA elongation [24,25]. In addition, we found that di-methylation of histone H3 at K4 was highly enhanced during differentiation from the promoter/enhancer to the transcribed region close to the transcription initiation site. Considering our present results and those of previous reports together, it is likely that tri-methylation and di-methylation of histone H3 at K4 are involved in mRNA elongation as well as in mRNA transcription initiation, but this hypothesis should be investigated by further studies.

In conclusion, we have demonstrated that histone H3 methylation at K4 is involved in *SLC2A5* gene induction in Caco-2 cells during differentiation, as well as by co-treatment with a glucocorticoid hormone and a p44/42 MAPK inhibitor.

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