In the present study, therefore, we examined the effects of testosterone on the promoter activities of rOCTs to understand the role of this hormone in the gender differences of rOCT2 expression.

MATERIALS AND METHODS

Materials

Restriction enzymes were obtained from New England BioLabs (Beverly, MA, USA). T4 kinase and T4 DNA ligase were purchased from TaKaRa (Ootu, Japan). [α-^{32}P] CTP was obtained from Amersham Biosciences, Inc. (Buckinghamshire, UK). Testosterone was purchased from Nacalai Tesque (Kyoto, Japan). Nilutamide was obtained from Sigma (St. Louis, MO, USA).

Determination of Putative Transcriptional Start Sites

The putative transcriptional start sites for rOCT1-3 were determined by 5′-rapid amplification of cDNA ends (5′-RACE) using the rat Marathon-Ready cDNA kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions. The rOCT2 gene-specific primers for the 5′-RACE were designed and synthesized based on the genomic sequence. The 5′-RACE was performed with adapter primer 1 that came with the kit and a gene-specific primer of rOCT1 (accession number NM_012697), 5′-CACAACCAGGGAGCCCAGGAAGAAG CCC-3′ (538 to 511). Nested polymerase chain reaction (PCR) was performed with adapter primer 2 and a nested gene-specific primer of rOCT1, 5′-CAGGAAGGCTTGTTTCTGGAAC CAGCCA-3′ (106 to 79). The rOCT2 gene-specific primers are as follows: a gene-specific primer of rOCT2 (accession number NM_031584), 5′-CCTTCATAAGAGGTTGTTAA GCCTGCCACTGGA-3′ (605 to 577); a nested gene-specific primer of rOCT2, 5′-GGAGGCACCAGACAGCAGGCT AAGAGG-3′ (187 to 160). The rOCT3 gene-specific primers are as follows: a gene-specific primer of rOCT3 (accession number NM_019230), 5′-GCCCAGGAAGACCACACCAA CGAAGAG-3′ (520 to 493); a nested gene-specific primer of rOCT3, 5′-GTCAGGCACAGCAGCAGGAACACGCG CC-3′ (474 to 450).

Genomic Cloning of rOCT1, rOCT2, and rOCT3 Promoters

rOCT1 and rOCT3 promoters were isolated from the rat genome (Clontech) by a PCR-based method using the following primers designed based on the rat genomic DNA (accession number AC114389): rOCT1 sense 5′-GGACGCGTCCA TGCTCTGCGAACTGAGGT-3′ and antisense 5′-GGCTC GAGGACTGCCACCAGGGGTTCAT-3′; rOCT3 sense 5′-GGACGCGTCCCTTCGAAGCAGAGGGAAAA-3′ and antisense 5′-GGAGATCTTGCAGGAATAGCCTCCAGT GC-3′. On the other hand, the rOCT2 promoter was isolated from the rat genomic library (Clontech) with a conventional plaque hybridization method. The probe was prepared by PCR using rat genomic DNA (Clontech) as a template. The primers, designed based on the rat genomic DNA (accession number AC114389), were as follows: 5′-GGCTTGGGAGAT GGCTAAGTA-3′ and 5′-TCACAGCCATGTGGGACA TGT-3′. Phage DNA with a long rOCT2 promoter was prepared with a QIAGEN lambda midi kit (Qiagen, Hilden, Germany) and partially sequenced. The transcription factor-binding sites were predicted with TRANSFAC 5.0 software (http://www.gene-regulation.com/cgi-bin/pub/programs/match/bin/match.cgi?), with a core similarity of 0.95 and a matrix similarity of 0.90.

Construction of Reporter Gene and Rat Androgen Receptor Expression Plasmid

Approximately 3-kb fragments corresponding to the 5′-flanking regions of the rOCT genes were subcloned into a pGL3-Basic luciferase gene vector (Promega, Madison, WI, USA) to yield rOCT1 (−3025/+23), rOCT2 (−3036/+242), and rOCT3 (−3001/+31). The deletion constructs rOCT2 (−1895/+242), rOCT2 (−819/+242), rOCT3 (−1095/+31), and rOCT3 (−515/+31) were prepared with the restriction enzymes. The mouse mammary tumor virus (MMTV) gene excised from pMSG (Amersham Biosciences) was subcloned into pGL3 to yield MMTV-pGL3.

ARE mutants were constructed using QuikChange® II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Primer sequences are listed in Table I.

Table I. Sequences of Mutation Primers

Primer	Sequence
ARE-1 S	5′-GGCCTCTGTGGTAGAGGA**GCC**ACTTAATCTTGCTGC-3′
ARE-1 AS	5′-GCAGCAAGATTAAGTGGCTCCTCTACCACAGAGGCC-3′
ARE-2 S	5′-CCTATGAGGACCAAGC**GCC**ACTCTCATGTCCTTCCTG-3′
ARE-2 AS	5′-CAGGAAGGACATGAGAGTGGCGCTTGGTCCTCATAGG-3′
ARE-3 S	5′-CCTTGGCACAGGA**GCC**TCTCCTTGACTCTCACCTG-3′
ARE-3 AS	5′-CAGGTGAGAGTCAAGGAGAGGCTCCTGTGCCAAGG-3′
ARE-4 S	5′-GCGTCCTGATACAGAC**GCC**ACCCATGAGTCAGTCAC-3′
ARE-4 AS	5′-GTGACTGACTCATGGGTGGCGTCTGTATCAGGACGC-3′
ARE-5 S	5′-CAGCAGGAAAGAGAGACTACC**GCC**TTCCCTGGCATTTGG-3′
ARE-5 AS	5′-CCAAATGCCAGGGAAGGCGGTAGTCTCTCTTTCCTGCTG-3′

Underlined sequences are putative ARE sequences, and bold characters indicate positions of the ARE mutation.
ARE = androgen response element.

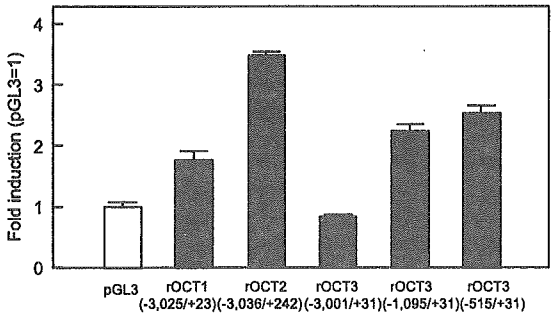

Fig. 1. Promoter activities of rat organic cation transporter (rOCT) genes. rOCT1–3 promoter constructs were transfected into LLC-PK_1 cells for luciferase assays. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Each column represents the mean ± SE of three independent experiments.

cDNA for rat AR (rAR: accession number, NM_012502) was isolated from rat kidney cDNA by a PCR-based method using the following primers: sense 5′-GGGATCCAGGATG GAGGTGCAGTTAGGG-3′ (991 to 1011) and antisense 5′-GGCTCGAGTTTCCAAATCTTCACTGTGTG-3′ (3713 to 3693). PCR was performed using Pfu polymerase (Stratagene) as follows: 95°C for 3 min; 35 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 8 min; and a final extension at 72°C for 10 min. The PCR product was subcloned into the expression vector pBK-CMV (Stratagene).

Cell Culture and Luciferase Assay

The porcine kidney epithelial cell line LLC-PK_1 was obtained from American Type Culture Collection (ATCC CRL-1392; Rockville, MD) and cultured as described previously (16). For the luciferase assay, the cells were seeded at 1.5×10^5 cells into 24-well plates in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% charcoal-stripped fetal bovine serum (FBS). Cells were transfected by 5-h exposure to LipofectAMINEplus (Invitrogen Japan KK, Tokyo, Japan), with each well containing 0.6 μg of the rOCT2 (−3036/+242) or equimolar amount of other reporter constructs, 0.1 μg of the rAR expression vector, and 30 ng of an internal control vector for transfection efficiency, namely, the *Renilla* luciferase (pRL-TK) reporter plasmid (Promega) in serum-free DMEM. The medium was changed to DMEM supplemented with 10% charcoal-stripped FBS, containing testosterone, nilutamide, or the vehicle control, dimethyl sulfoxide. After 43-h incubation, the cells were harvested and lysed, and luciferase activity was determined using a dual luciferase assay kit (Promega) and a LB940 luminometer (Berthold, Bad Wildbad, Germany). Each reporter construct was assayed in triplicate wells, and each experiment was repeated three times.

Statistical Analysis

The data were expressed as the mean ± SE. The significance of differences between the vehicle-treated and testosterone-treated groups was analyzed using Dunnet's *post hoc* analysis. Other analyses were conducted with Student's *t* test. Significance was set at $p < 0.05$.

RESULTS

Determination of the Transcriptional Start Site(s) for rOCTs in Rat Kidney Using 5′-RACE

The transcriptional start site(s) for rOCTs in the rat kidney were identified using 5′-RACE. The putative transcriptional start sites were determined using the longest RACE product. Sequencing of the amplified bands revealed that the terminal position of rOCT1 cDNA with the longest 5′-untranslated region was located 63 nucleotides above the start codon, which is 26 bp upstream of the 5′-end of rOCT1 cDNA reported previously (4). The terminal position of rOCT2 cDNA was located 306 nucleotides above the start codon, which is 266 bp upstream of the 5′-end of rOCT2 cDNA (5). The terminal position of rOCT3 cDNA is 35 nucleotides above the start codon, which is 359 bp downstream of the 5′-end of rOCT3 cDNA (8). Therefore, the terminal position of

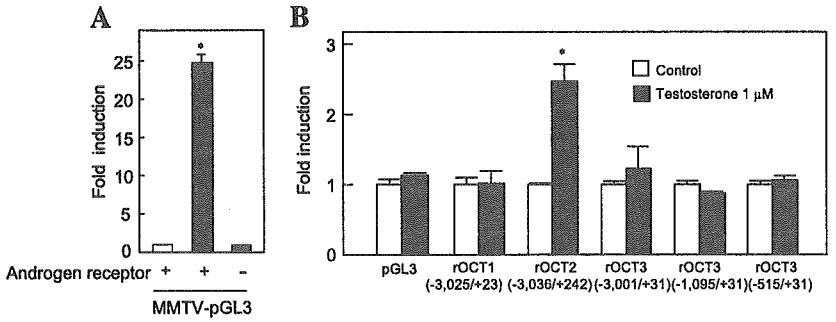

Fig. 2. (A) *Trans*-activation of the mouse mammary tumor virus (MMTV) promoter by rat androgen receptor (rAR) in the presence of testosterone. (B) *Trans*-activation of rOCT1–3 promoters by rAR in the presence of testosterone. Constructs were transiently transfected into LLC-PK_1 cells with rAR and pRL-TK. The cells were cultured for 43 h with vehicle or 1 μM testosterone, and luciferase activity was measured. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Each column represents the mean ± SE of three independent experiments. *$p < 0.05$, significantly different from control.

rOCT cDNA with the longest 5′-untranslated region was numbered with +1 as the transcription start site in this study.

Isolation and Analysis of 5′-Flanking Region of rOCT Genes

Based on the transcriptional start site, we then isolated the promoter region (about 3 kb) of each transporter and prepared reporter constructs. For luciferase assay, we used LLC-PK_1 cells because LLC-PK_1 cells possessed organic cation transport activities (16,17) and pig organic cation transporter OCT2p (18). Figure 1 shows the basal promoter activities of each transporter in LLC-PK_1 cells. Reporter constructs for rOCT1 and rOCT2 showed significant promoter activity. A reporter construct for rOCT3 (−3001/+31) did not have promoter activity, but those for rOCT3 (−1095/+31) and rOCT3 (−505/+31) did, suggesting that a repressive region is located in the rOCT3 promoter region −3001 to −1095. These findings suggest that all promoter constructs function appropriately.

Using these constructs, the effects of testosterone on the promoter activities were assessed. The functional activity of rAR was confirmed by a reporter assay using MMTV reporter construct in the presence of 1 μM testosterone (Fig. 2A). AR has been shown to *trans*-activate MMTV promoter using testosterone (19). In the absence of rAR, MMTV promoter activity was not enhanced by testosterone; native AR was not expressed in LLC-PK_1 cells. As shown in Fig. 2B, the activity of the rOCT2 promoter was significantly enhanced by testosterone, but that of the rOCT1 and rOCT3 promoters was not. These results were consistent with our previous results of Northern blotting (11). We therefore further characterized the transcriptional mechanisms by which the rOCT2 promoter is stimulated by testosterone.

Figure 3 shows the nucleotide sequence for 1000 bp upstream of the translation start site of the rOCT2 gene. Putative binding sites for many transcription factors were identified by TRANSFAC with a core similarity of 0.95 and a matrix similarity of 0.90, including activating protein (AP)-1, octamer-binding factor (Oct)-1, HNF-3/Fkh Homolog (HFH)-3, and a CCAAT box.

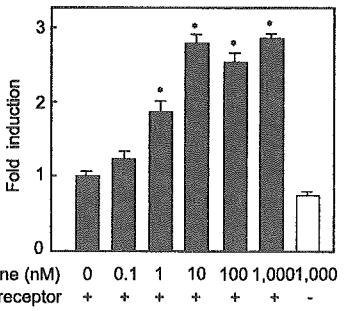

Fig. 4. *Trans*-activation of the rOCT2 promoter (−3036/+242) by rAR in the presence of testosterone. Constructs were transiently transfected into LLC-PK_1 cells with rAR and pRL-TK. The cells were cultured in the presence or absence of testosterone for 43 h. The vector pBK-CMV was used instead of rAR, and luciferase activity was measured. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Each column represents the mean ± SE of three independent experiments. $*p < 0.05$, significantly different from 0 nM testosterone.

Region of 5′-Flanking Sequence Required for Response to Testosterone

As shown in Fig. 4, a reporter construct for rOCT2 (−3036/+242) was significantly activated by testosterone in a concentration-dependent manner, and about a 3-fold increase was observed with 10 nM testosterone. Testosterone did not activate the rOCT2 promoter construct in the absence of the rAR expression vector. Nilutamide, an antiandrogen drug, acts as a competitive inhibitor of the androgen receptor (20). Nilutamide blocked the activation of the rOCT2 promoter by testosterone in a dose-dependent manner (Fig. 5), but nilutamide is a partial agonist of androgen receptor; rOCT2 activity is not completely suppressed by nilutamide. These findings suggest that rOCT2 promoter activity is stimulated by AR. Therefore, we tried to identify ARE(s) that work to stimulate rOCT2 promoter activity.

Table II shows sequences, positions, and homology to the consensus sequence of ARE within the 3000 bp of rOCT2

```
-694  GGAAGGGGGAAAGAGAGAGGAAGGCGGGGGACACACAGAAGACAGACAGATTTACATTCCAGTTTTATAATGCAGGTTTGTCAGCAGGCTACCCCCGTCT  -595
                                                                  Oct-1                                HFH-3
-594  TTGTTTTGCTTGGCAGCATCAGTATGCTCTGGGATCCTTCTGTCCAGCAGGAAAGAGAGACTACCTGTTTCCCTGGCATTTGGATACCCTTAGCAGCATC  -495
                                Hand1/E47
-494  CTGAAGGAGCATCCCTAAGTTAGATGGAACAACCTGGAACAACGGAATGACAGTAGAACAAATACACCCATGTCCTGCGTTTGCACTTGACCTATATGTA  -395

-394  AGGCAAATGTGGTACTCTTTCCTCACAAGGGGGTGAAGAGGAAGGACACATCAGGGTTGAGAGGTAAGAATGCTAAACTGGAATGAACCCTTGGAAACGT  -295
                                                                                                  RFX-1
-294  CAGACTGATTCAATCTGAGAGAAATCAAAGCACGCCGCCACAGCATGTCGCTCAAGTGCATCGCTTTCAGGGGGATTCAGGAGCTTGGATCCAGGACCCC  -195
           AP-1                                          Nkx2-5
-194  CCAGACCCTGATGCAAATTGCACTGGGTGTGTATAACCAAGTGCGCGTGTGTCTGTGTCACACCTACAAGAGAGGCTGGCTGTGTAGGTAACAAGGATAT  -95
           Oct-1                                 N-Myc
-94   ATTAATTTTCTGGCCACTTAATGTGCTAGGGAAAGATTAACCAGCTGTGATATGTTAAGAGACTCAGATTTCTCCCCGTGGGATACAGAGAAGTGATTGT  +6
           CCAAT box                                                                                  ▲
+7    TTGTAGTGCTGCTGTGAGGCAGAGACAAACTCACAGAGAATGAGGTTACAATAAACACATCACACTCCAGGGCCTGCTCTGGGCATTTCCGGGCAGCAAG  +106
                                                                                                NF-κB
+107  ATCTTCCCCCATTGGAGAGAGGTGCGATTGGAAGTTGGCCTGTCCGAGGGACGTCTGACCCAAAGGCTGGTCCCTCTCTAAATAAGCTAATGCTCACCCT  +206
                                                                                  AP-1
+207  TTGACTCTTGGTCAAGAGTCATCCTGAGCTGAGCGCGCTGCCTTCAGCAGCATTTGGAGCCCTGTGGGTGAAGCCTCAGGAACAGCTGCCTCCAGGGACC  +306
                          △
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Fig. 3. Transcriptional elements of the rOCT2 promoter. A 1000-base genomic DNA sequence immediately upstream of the start codon site is listed. An open triangle indicates the putative transcriptional start position, and a closed triangle indicates the 5′-end of rOCT2 cDNA published so far (NM_031584).

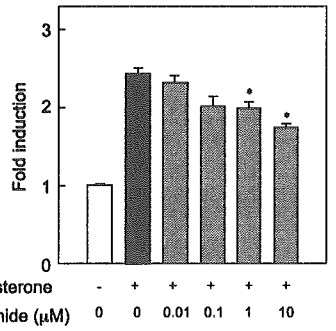

Fig. 5. The effect of nilutamide on *trans*-activation of the rOCT2 promoter (−3036/+242) by rAR in the presence of testosterone. Constructs were transiently transfected into LLC-PK_1 cells with rAR and pRL-TK. The cells were cultured for 43 h with vehicle and 10 nM testosterone in the absence or presence of various concentrations of nilutamide, and luciferase activity was measured. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Each column represents the mean ± SE of three independent experiments. *p < 0.05, significantly different from testosterone (+) in the absence of nilutamide.

promoter region. The ARE located furthest from the transcriptional start site was designated ARE-1 and that located closest was designated ARE-5. To determine which region(s) in the 5′-flanking region of rOCT2 gene is involved in expressional regulation by testosterone, constructs with deletions of the 5′-flanking region of rOCT2 gene were prepared and their luciferase activities were measured (Fig. 6). On deletion upstream to position −819, there was no induction by testosterone, suggesting that ARE-5 is not involved in the testosterone induction. But, on deletion up to position −1895, testosterone produced a 2-fold increase in activity, indicating that ARE-3 and/or -4 may work as response element(s). Furthermore, as the full-length promoter showed a 3-fold increase in activity, ARE-1 and/or -2 may also function as response elements.

To identify the functional sites for AR's activation, each ARE was mutated. As AR binds to specific response elements organized as an imperfect palindrome sequence (GGTACA nnnTGTTCT), we decided that the 3 bp at position 10–12 were changed to GCC in this sequence because left half-site of ARE is important for androgen receptor and ARE interaction (21). The promoter activity of rOCT2 with a mutation in each ARE revealed that mutated constructs of ARE-1 and ARE-3 were not affected by testosterone (Fig. 7). The other mutated constructs exhibited promoter activity to various extents in response to testosterone. These findings suggested that ARE-1 and ARE-3 play important roles in the activation of the rOCT2 promoter by testosterone.

DISCUSSION

Previously, we and others reported that the expression of rOCT2 mRNA in the kidney differed with gender, but neither rOCT1 nor rOCT3 (11,22), and that exogenous testosterone significantly stimulated only rOCT2 expression in the kidney of both male and female rats (12,23). Serum levels of testosterone were increased to about 1 μM in testosterone-administered rats. In the present study, we demonstrated that rOCT2 promoter activity was stimulated by 1 μM testosterone, whereas rOCT1 and rOCT3 promoters were not (Fig. 2B). These results are consistent with previous *in vivo* findings. 5′-Flanking region about 3000 bp of rOCT2 gene contained five putative AREs. The reporter assay using a series of deletion constructs and mutant constructs for each ARE revealed that ARE-1 (−2975 to −2960) and ARE-3 (−1340 to −1325), which have more similarity to the ARE consensus sequence than any other region, were responsible for the stimulation of rOCT2 promoter activity by testosterone (Figs. 6–7). As the promoter regions of rOCT1 and rOCT3 used in the present study do not have sequences highly homologous to ARE, the absence of an effect by testosterone on these two promoters is reasonable.

It was reported that there was no gender difference in rOCT2 mRNA expression in organs such as the liver and cerebellum (22). This may be a result of the low basal levels of rOCT2 mRNA or of the weak expression of AR in these organs compared to the kidney (24). We previously demonstrated that rOCT2 is predominantly expressed in the kidney, suggesting that some unidentified kidney-specific transcription factors cooperate to stimulate the rOCT2 promoter activity in the presence of testosterone. HEK293 cells (a human embryo kidney cell line) did not show any organic transporter activity (25). When HEK293 cells were used to measure rOCT2 promoter activity in the presence of AR and testosterone, there was no stimulative effect by testosterone (data not shown). It is probable that LLC-PK_1 cells express some transcription factor(s) necessary to express organic cation transporters. Further studies are needed to identify the kidney-specific transcription factors required for rOCT2 expression.

Other transporters have gender differences. For example, the level of rat organic anion transporter 2 (rOAT2) mRNA in the kidney and liver is higher in female than male rats (26,27), and the level of rOAT3 mRNA in male kidney is higher than

Table II. Comparison of the AREs in the Rat OCT2 Promoter Region

Position	Sequence	Homology (%)
ARE consensus	5′-**GGTACAnnnTGTTCT**-3′	–
ARE-1 (−2975 to −2960)	5′-**GGTAGAggaAGCTCT**-3′	80
ARE-3 (−1340 to −1325)	5′-**GGCACAggaTGCTCT**-3′	87
ARE-4 (−1268 to −1253)	5′-**GATACAgacTGTACC**-3′	80
ARE-5 (−548 to −533)	5′-**GAGACTaccTGTTTC**-3′	73

OCT = organic cation transporter.

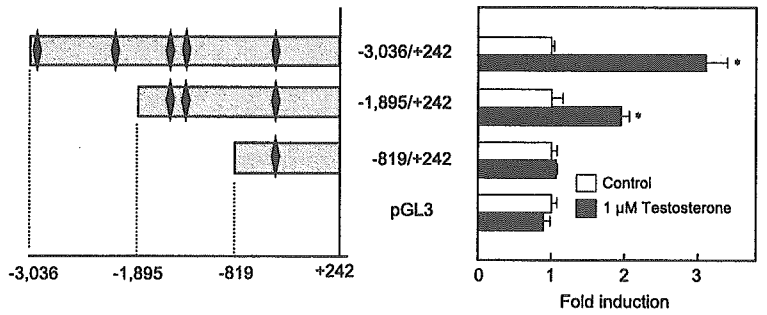

Fig. 6. *Trans*-activation of serial deletions of the rOCT2 promoter by rAR in the presence of testosterone. Various deletion constructs [equimolar amounts of the −3036/+242 construct (0.6 μg)] were transiently transfected into LLC-PK$_1$ cells with rAR and pRL-TK. The cells were cultured for 43 h with vehicle or 1 μM testosterone, and luciferase activity was measured. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Black diamonds indicate AREs. Each column represents the mean ± SE of three independent experiments. *$p < 0.05$, significantly different from control.

in females (27). Also, gender differences in rat renal cortical OAT1 and OAT3 levels (male > female) are caused by a stimulatory effect of androgens (28). Recently, Ohtsuki *et al.* (29) demonstrated that the expression of OAT3 in rat brain capillary endothelial cells was regulated by testosterone. Although Ohtsuki *et al.* (29) did not carry out reporter assay, it is conceivable that the expressions of the rOAT1 and rOAT3 genes are mediated by the interaction of AR and ARE on these promoters. The present study will be helpful to clarify transcriptional mechanisms of the induction of promoter activities of rOAT1 and rOAT3 by testosterone.

In humans, an evidence is the accumulation of gender differences in the efficacy and toxicity of drugs, and it is thought that physiological factors including body weight, plasma volume, and gastric emptying time are responsible for the variation in drug sensitivity between men and women (30). Recent studies have revealed that other differences, such as cytochrome P450 (CYP), cause gender-related variations in the pharmacokinetics of drugs. It is known that erythromycin (31), nifedipine (32), and verapamil (33), which are metabolized by CYP3A, have greater clearance in women than men. A sex-based difference in the expression of CYP3A4 was detected in the liver (34), but not in the intestine (35). In the renal clearance, amantadine and pramipexole, which are transported by OCT2 (36,37), also exhibit a gender difference (38,39). The renal clearance of amantadine was greater in men than women and was significantly reduced by quinine and quinidine only in men (40). In contrast, the pharmacokinetic parameters of cimetidine and procainamide do not differ between the sexes (41). It is noted that because the luminal efflux may be a rate-limiting step for renal secretion of organic cations (42), large differences in expression of a basolateral human (h)OCT2 may not result in similar large differences in renal clearance. There are some putative AREs in the promoter region of hOCT2, but the positions and sequences are different from those of rOCT2. Further studies are needed to clarify whether the expression of hOCT2 differs with gender, and whether the hOCT2 promoter interacts with the human androgen receptor.

In conclusion, a physiological concentration of testosterone (~10 nM) specifically enhanced transcription of rOCT2 gene, but not of rOCT1 or rOCT3 genes. ARE-1 (−2975 to

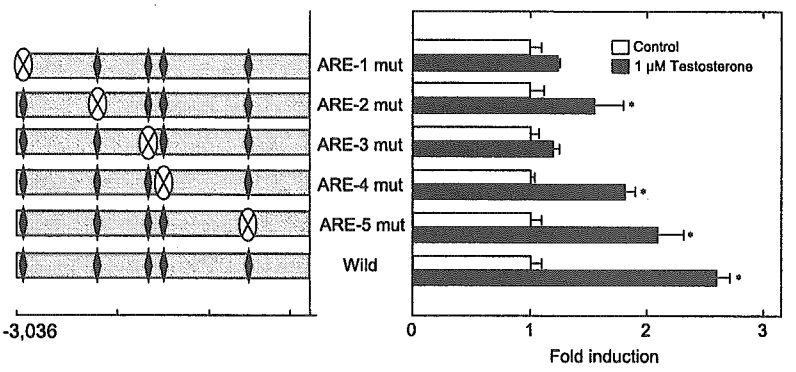

Fig. 7. *Trans*-activation of ARE-mutated rOCT2 promoters by rAR in the presence of testosterone. Constructs were transiently transfected into LLC-PK$_1$ cells with rAR and pRL-TK. The cells were cultured for 43 h with vehicle or 1 μM testosterone, and luciferase activity was measured. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Black diamonds indicated AREs. Each column represents the mean ± SE of three independent experiments. *$p < 0.05$, significantly different from control.

−2960) and ARE-3 (−1340 to −1325) in the rOCT2 promoter region would play important roles for the enhanced transcription of rOCT2 gene. These findings would account for the transcriptional mechanisms underlying the gender difference in the renal expression of rOCT2 and provide useful information to understand the renal handling of organic cations.

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The transcription factor Cdx2 regulates the intestine-specific expression of human peptide transporter 1 through functional interaction with Sp1

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Abbreviations:
Cdx2, caudal-related homeobox protein 2
ChIP, chromatin immunoprecipitation
GAPDH, glyceraldehydes-3-phosphate dehydrogenase
HNF, hepatocyte nuclear factor
PEPT1, H+/peptide cotransporter 1
TBS, Tris-buffered saline

ABSTRACT

H^+/peptide cotransporter 1 (PEPT1, SLC15A1) localized at the brush-border membranes of intestinal epithelial cells plays important roles in the intestinal absorption of small peptides and a variety of peptidemimetic drugs. We previously demonstrated that transcription factor Sp1 functions as a basal transcriptional regulator of human PEPT1. However, the factor responsible for the intestine-specific expression of PEPT1 remains unknown. In the present study, we investigated the effect of the intestinal transcription factors on the transcription of the PEPT1 gene and found that only Cdx2 markedly trans-activated the PEPT1 promoter. However, the promoter region responsible for this effect lacked a typical Cdx2-binding sequence, but instead, possessed some Sp1-binding sites. In vitro experiments using Caco-2 cells showed that (1) mutation of the Sp1-binding site diminished the effect of Cdx2, (2) co-expression of Cdx2 and Sp1 synergistically trans-activated the PEPT1 promoter and (3) Sp1 protein was immunoprecipitated with Cdx2 protein. These results raise the possibility that Cdx2 modulates the PEPT1 promoter by interaction with Sp1. The significance of Cdx2 in vivo for PEPT1 regulation was shown by the determination of mRNA levels of Cdx2 and PEPT1 in human tissue. In gastric samples, some with intestinal metaplasia, the levels of PEPT1 and Cdx2 mRNA were highly correlated. Taken together, the present study suggests that Cdx2 plays a key role in the transcriptional regulation of the intestine-specific expression of PEPT1, possibly through interaction with Sp1.

1. Introduction

Di- and tripeptides are taken up into the intestinal and renal epithelial cells by H^+-coupled peptide transporters (PEPT1/SLC15A1 and PEPT2/SLC15A2). Many functional studies using heterologous expression systems have demonstrated molecular natures in their transport characteristics. For example, despite having similar substrate specificity, PEPT1 and PEPT2

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were characterized as low- and high-affinity type transporters, respectively [1,2]. In addition, the two transporters differ in their tissue distribution and play distinct physiological roles. PEPT1 is expressed predominantly in the small intestine and slightly in the kidney [1,2]. On the other hand, PEPT2 is expressed mainly in the kidney, but it is also expressed in various tissues such as lung [3], choroid plexus [4] and mammary gland [5], and plays tissue-specific roles. As PEPT1 has broad substrate specificity, the intestinal absorption of several pharmacologically active drugs such as oral β-lactam antibiotics and the anti-viral agent valacyclovir are mediated by this transporter, and therefore, PEPT1 also plays important roles not only as a nutrient transporter but also as a drug transporter [2].

Previously, we isolated the promoter region of PEPT1 and demonstrated that the transcription factor Sp1 plays an important role in the basal transcriptional regulation of PEPT1 [6]. But, as Sp1 is expressed ubiquitously, the intestine-specific expression of PEPT1 cannot be controlled only by Sp1; thus, an intestine-restricted transcription factor is assumed to be involved.

The transcription factor Cdx2 is a member of the caudal-related homeobox gene family and expressed mainly in the intestine [7]. Cdx2 plays important roles in the early differentiation, proliferation and maintenance of intestinal epithelial cells [7,8], and in the transcription of intestinal genes, such as the sucrase–isomaltase [9], lactase–phlorizin hydrolase (LPH) [10], claudin-2 [11] and UDP glucuronosyl-transferases genes (UGTs) [12] through binding to a TTTAT/C consensus sequence. Over-expression of Cdx2 in undifferentiated rat IEC-6 intestinal epithelial cells leads to the development of a differentiated phenotype [8]. Furthermore, in humans, CDX2 has been reported to be associated with intestinal metaplasia in the stomach [13] in which ectopic expression of CDX2 is speculated to cause the gastric epithelial cells to trans-differentiate and take the intestinal phenotype. In our recent study, PEPT1 was also found to be expressed in the stomach, induced by intestinal metaplasia [14].

Considering the functions of Cdx2 mentioned above and overlapping of its expression with PEPT1, it is possible to suggest a link between these two genes. In the present study, we investigated the role of Cdx2 in the transcriptional regulation of PEPT1 using the human intestinal cell line Caco-2 cells. In addition, the correlation between PEPT1 and CDX2 mRNA expression levels in human gastric tissue samples developing intestinal metaplasia was also assessed.

2. Materials and methods

2.1. Materials

The anti-CDX2 monoclonal antibody was purchased from BioGenex (San Ramon, CA). The polyclonal antibody recognizing human Sp1 was from Upstate (Charlottesville, VA). The anti-FLAG M2 monoclonal antibody and anti-FLAG M2 monoclonal antibody conjugated to agarose gel (anti-FLAG M2 affinity gel) were obtained from Sigma (St. Louis, MO). The mouse Cdx2 expression vector (pRc/CMV-Cdx2) was a gift from Dr. Eun Ran Suh (University of Pennsylvania). The human HNF-1α and HNF-1β expression vectors were kindly supplied by Dr. Marco Pontoglio (Institute Pasteur, Paris, France). The CMV-Sp1 expression vector was kindly provided by Dr. Robert Tjian (University of California, Berkeley). The FLAG-Cdx2 expression plasmid was constructed by cloning the *Hin*dIII fragment of pRc/CMV-Cdx2 into pFLAG-CMV-6a (Sigma) at the *Hin*dIII restriction site. All other chemicals used were of the highest purity available.

2.2. Cloning of the 5′-regulatory region of PEPT1 gene and preparation of deletion reporter constructs

Cloning of the 5′-regulatory region of the PEPT1 gene and preparation of various reporter constructs were carried out as previously described [6]. Briefly, the 2940-bp flanking region upstream of the transcription start site was subcloned into the firefly luciferase reporter vector, pGL3-Basic (Promega, Madison, WI). This full-length reporter plasmid is hereafter referred to as −2940/+60. The 5′-deleted (−1111/+60, −960/+60, −401/+60, −247/+60, −172/+60 and −21/+60) constructs were generated by digestion of the −2940/+60 construct with the restriction enzymes. The −35/+60 construct was generated by PCR. Site-directed mutations in putative Sp1-binding sites were introduced into the −172/+60 construct with a Quik Change XL site-directed mutagenesis kit (Stratagene, La Jolla, CA).

2.3. Cell culture, transfection and reporter gene assay

Caco-2 cells were obtained from the American Type Culture Collection (ATCC CRL-1392) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% non-essential amino acids. Caco-2 cells were plated into 24-well plates (3×10^5 cells/well) and transfected the following day with the reporter constructs, the expression plasmid for the transcription factor and 2.5 ng of the *Renilla reniformis* vector, pRL-TK (Promega), using Lipofectamine 2000 (Invitrogen Japan KK, Tokyo, Japan) according to the manufacturer's recommendations. The medium was changed after 24 h. The firefly and *Renilla* activities were determined 48 h after the transfection using a dual luciferase assay kit (Promega) and a LB940 luminometer (Berthold, Bad Wildbad, Germany). For the immunoprecipitation and chromatin immunoprecipitation experiments, Caco-2 cells were plated into 60-mm dishes (1.2×10^6 cells/dish) and transfected the following day with the expression plasmid for FLAG-Cdx2 using Lipofectamine 2000.

2.4. Immunoprecipitation and Western blotting

Caco-2 cells expressing FLAG-Cdx2 were washed with PBS twice, scraped off, and suspended in lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.5 mM PMSF and 1% protease inhibitor cocktail (Nacalai tesque, Kyoto, Japan)). After an incubation at 4 °C for 15 min, the cells were disrupted by vigorous vortexing and repeated passages through a 24-gauge needle. The homogenate was centrifuged at 4 °C and 20,000 ×*g* for 10 min, and the supernatant was recovered. Immunoprecipitation of FLAG-Cdx2 was performed using anti-FLAG affinity gel at 4 °C overnight. The gel was washed with the lysis buffer five times. The immunoprecipitated FLAG-Cdx2 proteins were solubilized

in SDS sample buffer, separated on a 10% polyacrylamide gel at room temperature, and transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA) by semidry electroblotting. Blots were blocked with 5% nonfat dry milk in Tris-buffered saline (TBS; 20 mM Tris, 137 mM NaCl, pH 7.5) with 0.1% Tween 20 (TBS-T) for 3 h at room temperature. The blots were washed in TBS-T and then incubated with the anti-FLAG M2 monoclonal antibody (10 μg/ml, 1 h at room temperature) or anti-Sp1 polyclonal antibody (1 μg/ml, overnight at 4 °C). Blots were washed three times with TBS-T, and the bound antibody was detected on X-ray film by enhanced chemiluminescence with a horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG antibody (Amersham Pharmacia Biotech, Piscataway, NJ).

2.5. *Chromatin immunoprecipitation (ChIP)*

Caco-2 cells expressing FLAG-Cdx2 were cross-linked with 1% formaldehyde at room temperature for 10 min. Cells then were rinsed with ice-cold PBS twice, scraped off, and suspended in lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.5 mM PMSF and 1% protease inhibitor cocktail). Cells were sonicated three times for 15 s each time at 40% of the maximal setting (VP-5S, TAITEC, Koshigaya, Japan) and centrifuged at 4 °C and 20,000 ×*g* for 10 min. After the supernatants were collected and diluted in lysis buffer, immunoprecipitation was performed overnight at 4 °C with anti-FLAG affinity gel. The gel was then washed five times with lysis buffer and extracted with 1.5% SDS, followed by 0.5% SDS. Eluates were pooled and heated at 68 °C for 6 h to reverse the formaldehyde cross-linking. Chromatin-associated proteins were digested with proteinase K at 55 °C. The DNA was recovered by phenol–chloroform extraction and ethanol precipitation. Pellets were dissolved in 20 μl of TE buffer and used as a template for PCR. Primers used for amplifying the PEPT1 promoter were 5′-GACTGGCTCTCCCCCGGCCTGCCACG-3′ and 5′-CCCGGCCCCGTTGCCCCAGGTACAGC-3′ (−209 to −26 upstream of the transcriptional start site). PCR was performed using Advantage GC Genomic Polymerase Mix (BD Biosciences, Franklin Lakes, NJ) and cycling conditions were as follows: 1 min of denaturation at 95 °C, followed by 30 cycles of 30 s of denaturation at 94 °C, 3 min of primer annealing and extension at 68 °C, and 3 min of final extension at 68 °C.

2.6. *Human gastric tissue sample*

The gastric mucosal samples from normal stomachs were obtained from cancer patients ($n = 30$) during surgery at the First Department of Surgery, Kyoto University Hospital. Normal mucosal samples were resected at the site most distant from the affected portions. Three samples from different portions were resected in some patients. Pathologists diagnosed intestinal metaplasia in some patients. No patients underwent preoperative chemotherapy and/or radiation therapy. The samples were frozen in liquid nitrogen and stored at −80 °C until RNA extraction. This study was conducted in accordance with the Declaration of Helsinki and its amendments, and was approved by the Ethics Committee of Kyoto University (G-39). Written informed consent was obtained from all patients for surgery and the use of their resected samples.

2.7. *Real-time PCR*

Isolation of total RNA from the human stomach samples and real-time PCR were carried out as described previously [14]. The primer–probe set used for CDX2 was pre-developed TaqMan Assay Reagents (Applied Biosystems, Foster, CA). Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) mRNA was also measured as an internal control with GAPDH Control Reagent (Applied Biosystems).

2.8. *Data analysis*

The results were expressed relative to the result obtained with the pGL3-Basic vector set as 1 and represent the means ± S.E. ($n = 3$). Two or three experiments were conducted, and representative results were shown. In the mutational experiment, statistical analysis was performed with the one-way ANOVA followed by Scheffé *F* post hoc testing.

3. Results

3.1. *Cdx2 activates transcription of the PEPT1 promoter–reporter construct*

To investigate whether Cdx2 activates the PEPT1 promoter, the −2940/+60 reporter construct was transiently transfected into Caco-2 cells simultaneously with Cdx2 expression plasmids. Besides Cdx2, the transcription factor hepatocyte nuclear factor (HNF)-1α is also expressed in the intestine and involved in the expression of some intestinal genes although it was first discovered in the liver [15,16]. In the regulation of LPH expression, Cdx2 is reported to directly interact with HNF-1α [10]. Thus, the effect of HNF-1α and a related transcription factor, HNF-1β, on the PEPT1 promoter was also assessed. Cdx2 over-expression resulted in a four-fold increase in promoter activity (Fig. 1). However, HNF-1α could neither activate the PEPT1 promoter nor enhance its activity driven by Cdx2. HNF-1β could activate it only a little as compared to Cdx2. Thus, we focused on Cdx2 as a possible regulator of PEPT1 expression and further investigated the Cdx2-responsive region in the PEPT1 promoter.

3.2. *Cdx2-responsive region located near the Sp1-binding sites*

To determine the elements contributing to the expression of PEPT1, we carried out a promoter 5′-deletion analysis (Fig. 2). The promoter activity in the absence of Cdx2 was strongest with the −401/+60 construct and gradually decreased by the deletion between −401 and −35, consistent with our previous result [6]. The promoter activity in the presence of Cdx2 increased three- to four-fold as compared to that in the absence of Cdx2 with the −2940 to −172 constructs whereas it was completely diminished with the −35/+60 construct, suggesting that the Cdx2-responsive region is located between −172 and −35. Unexpectedly, this region lacked a consensus Cdx2-binding site but contained multiple Sp1-binding sites as reported previously [6]. We could not find any transcription factor-binding sites which are likely to be responsible for the

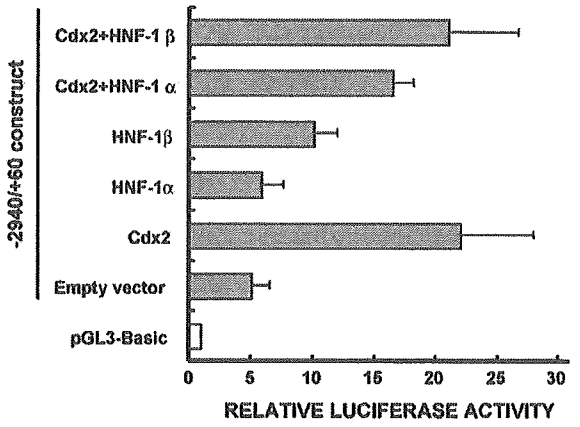

Fig. 1 – Effects of Cdx2, HNF-1α and HNF-1β over-expression on the PEPT1 promoter activity. Caco-2 cells were transiently transfected with 250 ng of the −2940/+60 construct and 250 ng of the expression vector for Cdx2, HNF-1α or HNF-1β. The total amount of transfected DNA (750 ng) was kept constant by adding empty vector. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Data are reported as the relative fold-increase compared with pGL3-Basic and represent the mean ± S.E. ($n = 3$).

effect of Cdx2 except for Sp1 in this region. We next carried out a mutational analysis to determine whether the effect of Cdx2 was mediated through these Sp1-binding sites. The promoter activity in the absence of Cdx2 was reduced with the mutation of Sp-A, Sp-B or Sp-C sites, consistent with our prior study [6]. Trans-activation by Cdx2 was markedly decreased with the construct possessing the mutation of Sp-A or Sp-C sites (Fig. 3). These deletion and mutational analyses collectively suggest that Cdx2 may functions via interaction with Sp1 on the PEPT1 promoter.

3.3. *PEPT1 promoter was synergistically activated by Cdx2 and Sp1*

Sp1 has been shown to trans-activate the PEPT1 promoter [6]. We therefore determined whether Cdx2 enhances the promoter activity in cooperation with Sp1. Cdx2 or Sp1 alone caused a 1.5–2-fold increase of the promoter activity, whereas co-expression of Cdx2 and Sp1 resulted in a four-fold increase in the promoter activity (Fig. 4), suggesting a synergistic effect.

3.4. *Protein–protein interaction of Cdx2 and Sp1*

The synergistic effect of Cdx2 and Sp1 observed in the co-expression experiment, together with the observations from the mutational analysis, raise the possibility that these two proteins interact physically to regulate PEPT1 expression. We therefore investigated the interaction of Cdx2 and Sp1 within the cell using co-immunoprecipitation. Whole-cell extracts of Caco-2 cells transfected with the expression vector for FLAG-Cdx2 or empty vector were subjected to immunoprecipitation followed by Western blotting. Detection with anti-FLAG antibody confirmed that FLAG-Cdx2 protein was expressed in the cells transfected with FLAG-Cdx2 and appropriately immunoprecipitated (Fig. 5, upper panel). In the input samples, as expected, the band of Sp1 protein was detected both in the cells transfected with FLAG-Cdx2 and empty vector, while in the immunoprecipitated sample, it was detected only in the FLAG-Cdx2 transfected cells (Fig. 5, lower panel). These findings show that endogenous Sp1 protein was co-immunoprecipitated with FLAG-Cdx2 and suggest that Cdx2 and Sp1 were associated in a protein complex in Caco-2 cells.

3.5. *Cdx2 associates with the PEPT1 promoter*

As mentioned above, the Cdx2-responsive region lacked a consensus Cdx2-binding site, and an electrophoretic mobility shift assay failed to demonstrate the binding of Cdx2 with the PEPT1 promoter (data not shown). Thus we adopted an

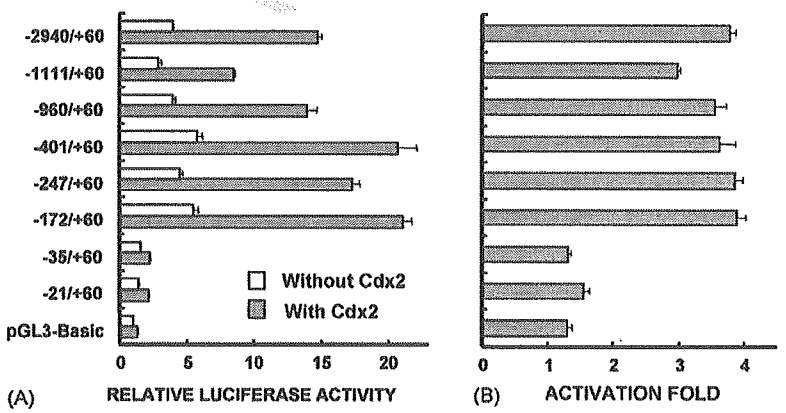

Fig. 2 – Identification of the Cdx2-responsive region in the PEPT1 promoter. A series of deleted promoter constructs (equimolar amounts of the −2940/+60 construct (500 ng)) and 500 ng of the Cdx2 expression vector or empty vector were transiently transfected into Caco-2 cells for luciferase assays. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Data are reported as the relative fold-increase compared with the pGL3-Basic vector (A) or as the ratio of Cdx2-expressing vector to empty vector (B) and represent the mean ± S.E. ($n = 3$).

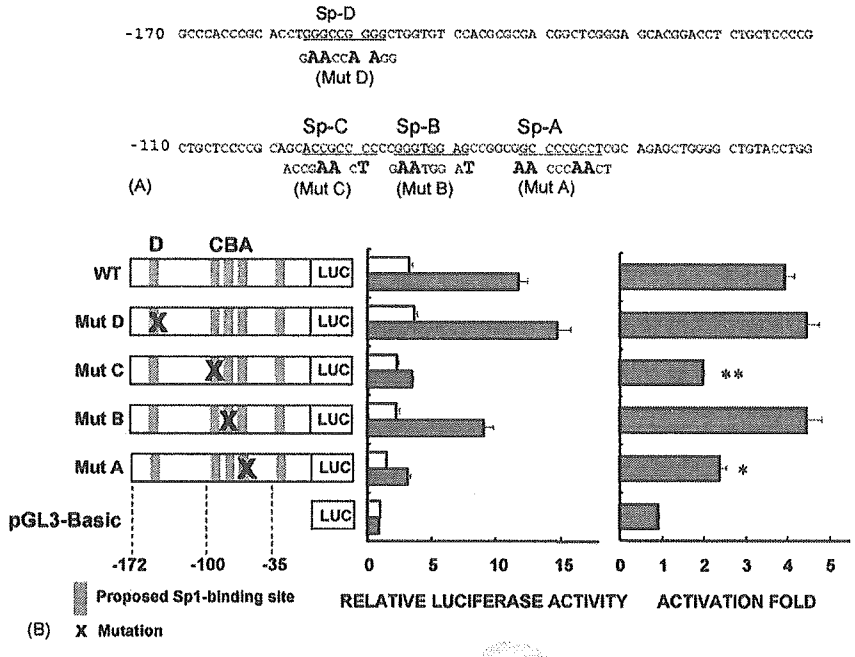

Fig. 3 – Mutational analysis of Sp1-binding sites in the Cdx2-responsive region in the PEPT1 promoter. (A) The nucleotide sequence of the promoter region from −170 to −41 is shown with the putative Sp1-binding elements (Sp-A, Sp-B, Sp-C, Sp-D, underlined). Site-directed mutations that destroy Sp1-binding elements were introduced individually and designated mut A, mut B, mut C and mut D. The nucleotides altered for mutational analysis are shown in bold letters under the wild-type sequence. (B) The mutated −172/+60 constructs (500 ng) and 500 ng of the Cdx2 expression vector or empty vector were transiently expressed in Caco-2 cells for luciferase assays. Firefly luciferase activity was normalized to *Renilla* luciferase activity. Data are reported as the relative fold-increase compared with the pGL3-Basic vector and as the ratio of Cdx2-expressing vector to empty vector, and represent the mean ± S.E. ($n = 3$). (* and **) Significantly different from wild type (WT), $^{*}p < 0.05$, $^{**}p < 0.01$.

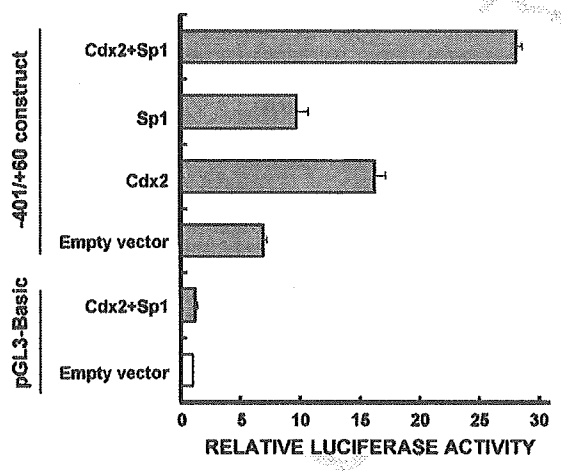

Fig. 4 – Synergistic activation of the PEPT1 promoter by Cdx2 and Sp1. Caco-2 cells were transiently transfected with 150 ng of the −401/+60 construct and the expression vector for Cdx2 (500 ng) or Sp1 (1000 ng). The total amount of transfected DNA (1650 ng) was kept constant by adding empty vectors. Data are reported as the relative fold-increase compared with pGL3-Basic and represent the mean ± S.E. ($n = 3$).

alternative methodology, the ChIP assay, to investigate the association of Cdx2 with the PEPT1 promoter. An approximately 200-bp fragment of the PEPT1 promoter covering the Cdx2-responsive region, which had the Sp1-binding sites, was recovered by immunoprecipitation of FLAG-Cdx2 from the transfected cells, whereas only a trace amount of the fragment was recovered from the mock-transfected cells (Fig. 6).

3.6. *The level of PEPT1 mRNA is correlated with that of CDX2 in the gastric samples with the intestinal metaplasia*

mRNA levels of PEPT1 and CDX2 in the human gastric samples, some with intestinal metaplasia, were determined using quantitative real-time PCR (Fig. 7). PEPT1 and CDX2 mRNA levels differed by more than 100-fold between the samples. The mRNA level of PEPT1 was highly correlated with that of CDX2. Furthermore, both PEPT1 and CDX2 were expressed at apparently higher levels in the samples diagnosed pathologically with intestinal metaplasia as compared to the normal tissue.

4. Discussion

The molecular mechanisms responsible for the intestine-specific expression of PEPT1 are largely unknown. In the

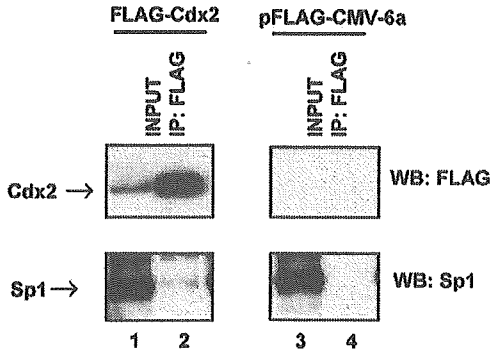

Fig. 5 – Physical interaction of Cdx2 with Sp1 protein. Whole-cell extracts of Caco-2 cells transfected with FLAG-Cdx2 or pFLAG-CMV-6a (empty vector) were subjected to immunoprecipitation followed by Western blotting. Proteins were immunoprecipitated with anti-FLAG M2 affinity gel (lanes 2 and 4, indicated as IP:FLAG). Whole-cell extracts before immunoprecipitation were also analyzed (lanes 1 and 3, indicated as INPUT). FLAG-Cdx2 and Sp1 protein were detected with anti-FLAG M2 monoclonal antibody (upper panels) and anti-Sp1 polyclonal antibody (lower panels), respectively.

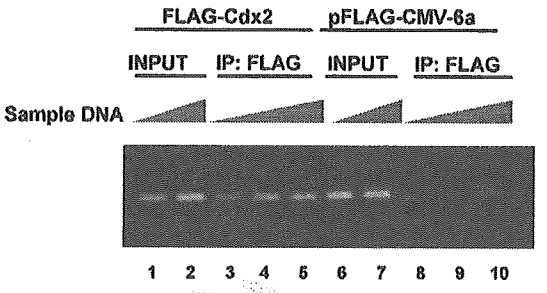

Fig. 6 – Association of Cdx2 with the PEPT1 promoter. Chromatin immunoprecipitation analysis of the endogenous PEPT1 promoter in Caco-2 cells transfected with FLAG-Cdx2 or pFLAG-CMV-6a (empty vector) was performed. The immunoprecipitated DNA fragments were purified and amplified by PCR with primers spanning the Cdx2-responsive region, and subjected to agarose gel electrophoresis. Serially diluted samples of DNA were used for PCR amplification. Lanes 1, 2 and 6, 7 indicate INPUT DNA. Lanes 3–5 and 8–10 show immunoprecipitated DNA.

present study, we provide the first evidence that Cdx2 regulates the transcription of PEPT1 using Caco-2 cells. Unlike other intestinal genes, such as the genes for LPH [10], claudin-2 [11] and UGTs [12], neither HNF-1α nor HNF-1β could trans-activate the PEPT1 promoter, although Cdx2 markedly enhanced the activity of the PEPT1 promoter. Deletion analysis revealed that the Cdx2-responsive region was located between bases −172 and −35 relative to the transcription start site. Computational analysis showed the lack of a canonical Cdx2-binding site in this region, but the presence of several GC-boxes which we previously identified as Sp1-binding sites. Sp1 has been reported to interact with various transcription factors or co-factors, such as estrogen receptor [17], p300/CREB-binding protein [18] and homeobox protein, Hox proteins [19]. Thus we tried to elucidate whether Cdx2 interacts with Sp1 to regulate the PEPT1 promoter.

Introducing mutations into Sp1-binding sites reduced the effect of Cdx2. Furthermore, co-expression of Cdx2 and Sp1 synergistically activated the PEPT promoter. These results suggest that the trans-activating effect of Cdx2 might be mediated via a Sp1-dependent mechanism. Among Sp1-binding sites located in Cdx2-responsive region, Sp-A, Sp-B and Sp-C were involved in the basal promoter activity, whereas only Sp-A and Sp-C appeared to be critical for Cdx2 effect, indicating that the regulatory effect of Cdx2 is site-dependent.

Co-immunoprecipitation of FLAG-tagged Cdx2 precipitated the endogenous Sp1 protein, suggesting the formation of a transcriptional complex involving Cdx2 and Sp1. In addition, ChIP assays indicated that Cdx2 protein was present on the

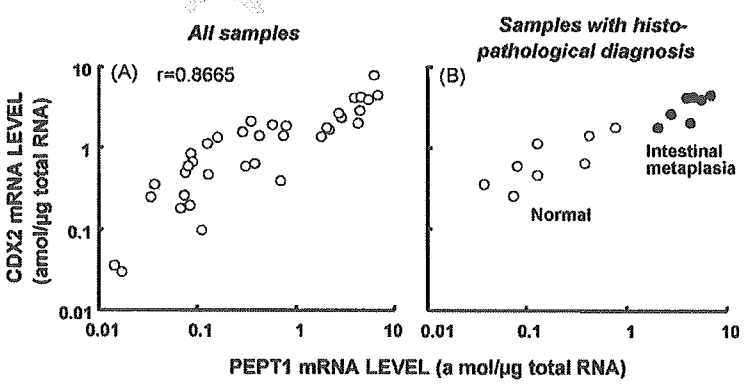

Fig. 7 – Correlation between PEPT1 and CDX2 mRNA levels in the human gastric tissue samples. The mRNA levels of PEPT1 and CDX2 were quantified with real-time PCR analysis in the gastric mucosal samples. Some of these tissue samples were diagnosed by a pathologist and proved to be intestinal metaplasia. (A) All samples were plotted. (B) The samples of patients diagnosed by a pathologist were plotted. Open and closed symbols indicate the normal samples and the samples proved to be intestinal metaplasia, respectively.

PEPT1 promoter at the whole cell level. Considering the lack of Cdx2-binding site in the Cdx2-responsive region and the functional and physical interaction between Cdx2 and Sp1 mentioned above, one explanation for the regulatory mechanism of Cdx2 may be that Cdx2 protein associates with the PEPT1 promoter via the complex formation with Sp1. Although Cdx2 has been reported to interact with the transcription factors such as HNF-1α [10–12] and GATA proteins [20], in all cases, Cdx2 directly binds to its cognate binding site on the promoter of target genes. By contrast, Cdx2 is speculated to exert its effect without direct binding to its cognate binding sequence on the PEPT1 promoter. The nature of the physical interaction between Cdx2 and Sp1 has yet to be determined. One possibility is that Cdx2 directly binds to Sp1 protein. Another possibility is indirect binding mediated by a common cofactor or some adaptor proteins. In both cases, to our knowledge, this is a novel mechanism of transcriptional regulation by Cdx2. Further studies will be needed to obtain the additional proof for supporting and fully characterizing this proposed mechanism.

In order to demonstrate the significance of Cdx2 for PEPT1 expression in vivo, we next investigated the expression profile of Cdx2 and PEPT1 mRNA using human tissue samples. In the gastric samples, some of which had intestinal metaplasia, PEPT1 and Cdx2 mRNA levels were highly correlated. The fact that ectopic expression of Cdx2 accompanied the expression of PEPT1 in human tissues strongly supports the role of Cdx2 demonstrated by in vitro reporter experiments. In addition, a recent study showed that expression of PEPT1 was induced in the gastric epithelium in a transgenic mouse expressing Cdx2 exclusively in the gastric epithelium [21].

The similarities between PEPT1 and Cdx2 in their expression profile are observed not only at the tissue level but also at the cellular level. PEPT1 is localized to brush-border membranes of the absorptive epithelial cells of the small intestine, and this protein is abundant at the tip of the villus and scarce at the crypt base [22]. Cdx2 also has a gradient of expression in the crypt–villus axis being primarily expressed in the villus [7].

It has been reported that intestinal PEPT1 is regulated by various factors [23], such as thyroid hormone [24], dietary conditions [25,26], diurnal rhythm [27] and a selective σ-ligand, pentazocine [28]. In addition, the ectopic induction of PEPT1 expression in the colon, where PEPT1 was not expressed under normal conditions, was observed in cases of functional deficiency of the small intestine such as ulcerative colitis, Crohn's disease and short-bowel syndrome [23]. It is not clear at present whether Cdx2 plays some parts in these regulatory functions. However, Cdx2 exerts physiological roles in the differentiation of intestinal epithelial cells and maintenance of intestinal phenotype. It is possible that Cdx2 helps to regulate colonic PEPT1 expression under such pathological conditions.

In conclusion, we demonstrated that Cdx2 regulated the PEPT1 promoter activity in Caco-2 cells using reporter assays, and confirmed the significance of Cdx2 in vivo in a correlation analysis of mRNA expression in human tissue samples. In addition, it may be possible that Cdx2 physically and functionally interacts with Sp1, and associates with the PEPT1 promoter although no cognate Cdx2-binding site is evident. These results collectively indicate that Cdx2 plays a key role in the transcriptional regulation for the intestine-specific expression of PEPT1, and have implications as a basis for future investigations of efficient enteral nutrition and drug therapy.

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