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Leptin Transcriptionally Enhances Peptide Transporter (hPepT1) Expression and Activity via the cAMP-response Element-binding Protein and Cdx2 Transcription Factors*

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PepT1 is an intestinal epithelial apical membrane transporter that is expressed in the small intestine, with little or no expression in the normal colon. However, we previously demonstrated that colonic PepT1 may be expressed during chronic inflammation. To begin elucidating inflammatory hPepT1 signaling, we herein investigated the long term leptin treatments, on PepT1 expression and activity in Caco2-BBE cells, and began to reveal the involved signaling pathways. We successfully cloned the 723-bp hPepT1 promoter region and identified the human transcription initiation site 86 bp upstream from the translation start site. Leptin treatment dose- and time-dependently increased hPepT1 promoter and *transport activities* in Caco2-BBE cells, with maximal activity observed in cells treated with 100 nM leptin for 8 h. Under these conditions, we observed 2-fold increases in hPepT1 mRNA and *protein expression*, as well as increased transport activity. Our molecular analyses of possible signal-transduction pathways revealed that leptin treatment enhanced the intracellular levels of cAMP and phosphorylated cAMP-response element-binding protein (CREB) protein in Caco2-BBE cells, whereas our deletion, mutation, and CDX2 overexpression analyses demonstrated that interaction of the Cdx2 and phosphorylated CREB transcription factors was essential for leptin-induced hPepT1 transcription in Caco2-BBE cells. Our results indicate that leptin, which is increased in inflamed colonic mucosa, triggers colonic expression of hPepT1 via the CREB and Cdx2 transcription factors. These findings provide important new insights into the mechanisms of intestinal inflammation and may suggest new therapeutic modalities in the future.

Gut epithelial cells absorb small dietary peptides via the action of apical membrane peptide transporters. A cDNA encoding an apical membrane protein accounting for this pep-

tide transport capability has been cloned from humans and has been designated hPepT1 (1–7). hPepT1 is an integral membrane-spanning protein expressed predominantly in the small intestine (8, 9), with additional expression recently reported in immune cells (10). hPepT1 is generally not expressed in the esophagus, stomach, or normal colon (8, 9, 11); however, colonic hPepT1 expression has been observed in inflamed colon and inflammatory bowel disease (11). It has been suggested that expression of hPepT1 in the inflamed colon could initiate uptake of small di-tri bacterial peptides, such as formyl-Met-Leu-Phe or muramyl dipeptide, which are abundantly present in the colonic lumen and have been associated with inflammation (11–13). The induction of hPepT1 expression in chronic colitis and in patients with short-bowel syndrome may indicate that specific inflammation-associated signaling pathways are activated (11, 14). However, no previous study has directly investigated the activation of transcriptional activity of hPepT1.

Leptin, which is encoded by the *ob* gene (15), is an adipocyte-secreted hormone involved in central regulation of body weight (16–18) via specific receptors in the hypothalamus (19). Nonadipose tissues, such as skeletal muscle (20), pituitary gland (18), and stomach (22), may also produce luminal leptin (22–24). We recently reported that the normal colon lumen contains low (nanomolar) concentrations of leptin. Because no leptin staining was detected in epithelial cells from normal small and large intestines, we suggest that this leptin arises from the gastric gland (25). Interestingly, we detected strong leptin staining in colonic epithelial cells from inflamed tissues, and the luminal leptin concentration was up to >15-fold higher than normal during inflammation (25). The intestinal luminal leptin and the leptin in colonic epithelial cells provide an additional local pool of leptin. The latter observation suggests that colonic epithelial cells may be in contact with high leptin concentration and may play important physiopathological roles in the colon during inflammation.

Various leptin receptor isoforms, including the functional long isoform (Ob-Rb), have been detected in rat intestines from the duodenum to the colon and in the Caco2 model intestinal cell line (26–30). Several studies have investigated the possible role of leptin in digestive absorption and/or secretion. For example, our group demonstrated that luminal leptin can regulate the activity of transporters such as monocarboxylate transporter 1 (31), whereas another study showed that intravenous leptin infusion attenuated the increased synthesis and secretion of apoAIV induced by intraduodenal infusion of lipids

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) DQ370174.

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² Recipient of a research fellowship award from the Crohn's and Colitis Foundation of America.

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in rats (28). Leptin administered to the basolateral side of Caco2 cells was found to inhibit the secretion of triglycerides, the biosynthesis of apoB-100 and apoB-48, and the output of chylomicron and low density lipoproteins (29). Furthermore, we previously demonstrated that short term leptin treatment increased hPepT1 activity *in vivo* and *in vitro* (26). The 5'-flanking region of the hPepT1 gene has been cloned, and investigations into the regulation of its transcription have indicated that the Sp1 transcription factor functions in basal transcriptional regulation of hPepT1 (32). However, no previous study has investigated transcriptional regulation of hPepT1 in response to hormones, particularly during intestinal inflammation. In the present study, we investigated the molecular mechanisms underlying the leptin-induced transcriptional activation of hPepT1 in the intestinal epithelia.

MATERIALS AND METHODS

Cell Culture—Caco2-BBE cells (passages 45 and 75) were grown in high glucose Dulbecco's Vogt-modified Eagle's medium (Invitrogen) supplemented with 14 mM NaHCO₃, 10% fetal bovine serum, and penicillin/streptomycin (100 units/100 μg/ml, Invitrogen). Transfected cells were maintained in the same medium containing 1.2 mg/ml G418 (Invitrogen). Cells were kept at 37 °C in a 5% CO₂ atmosphere and 90% humidity, and the medium was changed daily. Monolayers were subcultured every 7 days with 0.25% trypsin (Invitrogen). Transport experiments were performed with confluent monolayers plated on permeable supports (area = 1 cm²; pore size, 0.4 μm; Transwell-Clear polyester membranes from Costar VWR, Suwanee, GA) and examined 15 days post-plating. For extraction of proteins, membranes, or RNA, Caco2-BBE cells were plated in 6-well plates (Costar VWR) at a density of 10⁴ cells/cm² and prepared 15 days post-plating.

Isolation of the 5'-Flanking Region of the hPepT1 Gene—Genomic DNA was extracted from Caco2-BBE cells using the REDExtract-N-Amp Tissue PCR kit (Sigma). The promoter of the hPepT1 gene was amplified using PCR primers specific to the 5'-flanking region of the human PepT1 gene (sense, 5'-AAA GAA AGG ATG ATT CTT AAA GAA AGG AAA TG-3'; and antisense, 5'-CAT GGC GGC GGC TCC CAG GGC TCC TGC-3'). The PCR conditions consisted of 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 4 min, with a final extension at 72 °C for 10 min. The PCR product was gel-purified, subcloned into the pCR 2.1-TOPO vector (Invitrogen), and confirmed by sequence analysis. For promoter activity, the hPepT1 promoter pCR 2.1-TOPO was digested with SacI and XhoI and subcloned into the firefly luciferase reporter vector, pGL3-Basic (Promega, Madison, WI).

5'-Rapid Amplification of cDNA Ends—RACE⁴ was performed with total RNA isolated from Caco2-BBE cells and the GeneRacer Kit (Invitrogen), according to the manufacturer's

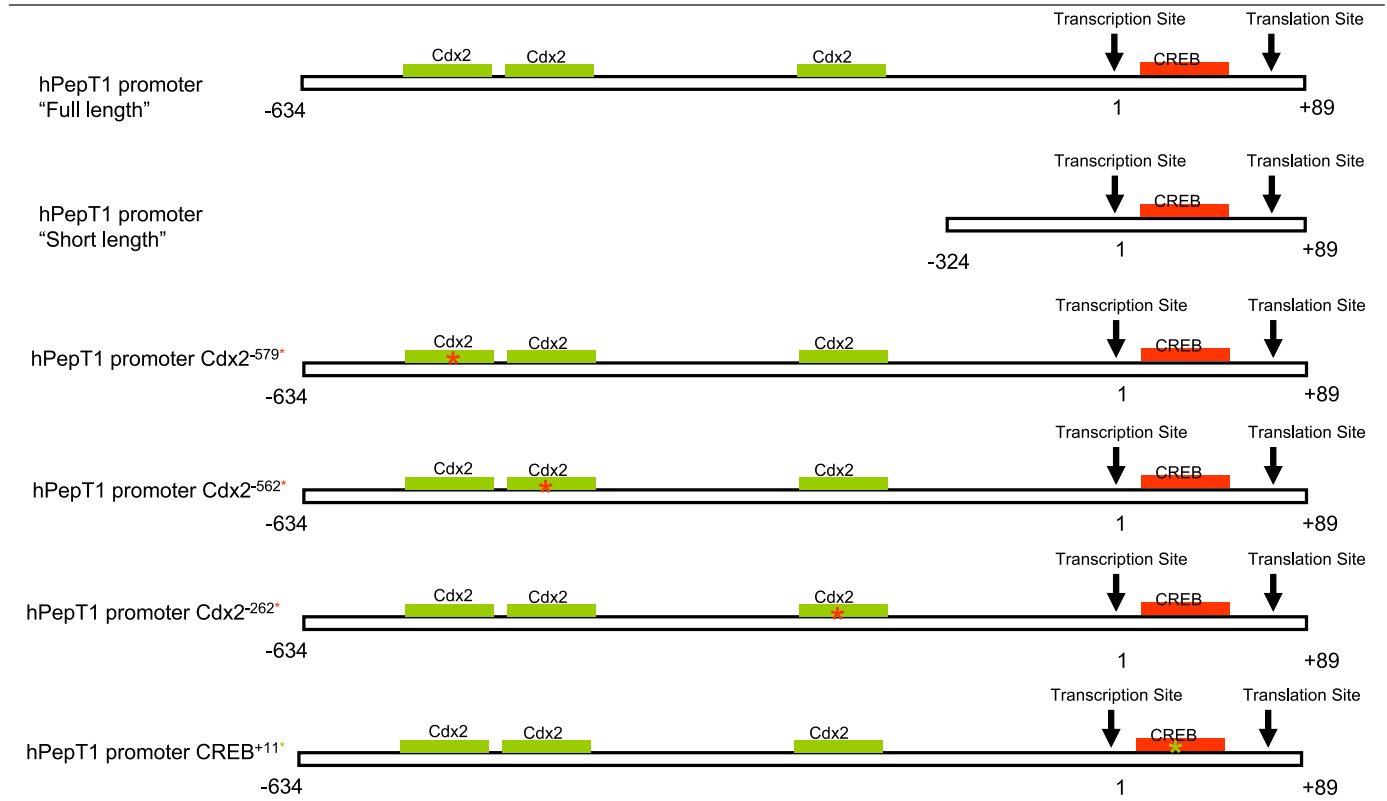
instructions. Briefly, total RNA was dephosphorylated, decapped, and ligated to the provided GeneRacer RNA oligonucleotide. The resulting RNA was reverse-transcribed using a human PepT1-specific primer (hPepT1 5'-RACE400, 5'-AAG GAC AGC ACC ACG TGC ACA GGA A-3') and the provided Superscript III reverse transcriptase. The 5'-end of PepT1 was amplified from this cDNA using the hPepT1 5'-RACE400 primer and the provided GeneRacer 5' Primer. The resulting product was further amplified with the nested primers hPepT1 5'-RACEnest (5'-GAT GGC GGT GGA CAG GTT ATC ATC C-3') and the provided GeneRacer 5' nested primer. The resulting RACE product was subcloned into the pCR-4Blunt-TOPO vector (Invitrogen) and sequenced.

Preparation of the Reporter Constructs—The -634/+89 hPepT1 promoter, contained in the pCR 2.1-TOPO vector (Invitrogen), was digested with SacI and XhoI, and the product was subcloned into the pGL3-Basic firefly luciferase reporter vector (Promega). Site-directed mutations in the putative CREB and Cdx2 binding sites of the -634/+89 construct were introduced using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA), with the following primers: hPepT1 promoter with mutated CREB, sense (5'-CAA CGG GGC CGG GCC TGG AAT TCA GGT CGG AGG AGT AG-3') and antisense (5'-CTA CTC CTC CGA CCT GAA TTC CAG GCC CGG CCC CGT TG-3'); hPepT1 promoter with the first Cdx2 site mutated, sense (5'-GAA ATG TAG AAT CCC CCA GAG ATG CTT TCA AAG GTT-3') and antisense (5'-AAC CTT TGA AAG CAT CTC TGG GGG ATT CTA CAT TTC-3'); hPepT1 promoter with the second Cdx2 site mutated, sense (5'-GGT TGA ATC TCA AAA TGA AGC CAC ACA CAC ACT CT-5') and antisense (5'-AGA GTG TGT GTG TGG CTT CAT TTT GAG ATT CAA CC-3'); hPepT1 promoter with the third Cdx2 site mutated, sense (5'-AAC CTC CCC TTA GAC TTC TTC GAA ATG CAC ATT CTG G-3') and antisense (5'-CCA GAA TGT GCA TTT CGA AGA AGT CTA AGG GGA GGT T-3'). The PCR conditions consisted of 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 4 min, with a final extension at 72 °C for 10 min. All mutants were confirmed by DNA sequencing. The truncated promoter construct (Table 1), containing deletions of all three Cdx2 sites (-324/+89) was amplified using specific primers (sense, 5'-AGC CCC GAC CTC CTG AGT CAG-3'; and antisense, 5'-CAT GGC GGC GGC TCC CAG GGC TCC TGC-3') and cycling conditions of 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 4 min, with a final extension at 72 °C for 10 min. The resulting PCR product (324 bp) was gel-purified and subcloned into the pCR 2.1-TOPO vector (Invitrogen) for confirmation by sequence analysis. This was subsequently digested with BamHI and subcloned into the pGL3-Basic firefly luciferase reporter vector (Promega).

Dual-luciferase Reporter Assay—Caco2-BBE cells (50,000 cells/well) were cultured in 12-well plates for 1 day. At 50–70% confluence, the cells were rinsed and then incubated with 1000 μl of Opti-MEM (Invitrogen), 5 ng of a construct encoding *Renilla* luciferase (phRL-CMV), 2 μg of the relevant hPepT1 promoter construct, and 10 μg/ml Lipofectin (Invitrogen). In each experiment, cells were incu-

⁴ The abbreviations used are: RACE, rapid amplification of cDNA ends; CREB, cAMP-response element-binding protein; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; MES, 4-morpholineethanesulfonic acid; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; RLA, relative luciferase activity; PKA, protein kinase A; pCREB, phosphorylated CREB; CMV, cytomegalovirus; Sar, sarcosine.

TABLE 1
PepT1 promoter constructs



bated with the same total amount of plasmid DNA. After 24 h, the cells were washed twice with phosphate-buffered saline and lysed with the provided buffer (Luciferase Assay Kit, Promega). Subsequently, the Caco2-BBE cells were stimulated with the indicated concentrations of leptin or vehicle for 2, 4, 8, 12, 24, or 48 h. For the luciferase activity assay, 20 μ l of cell extract was mixed with 100 μ l of luciferase assay substrate (Promega), and the resulting luminescence was measured for 10 s in a luminometer (Luminoskan, Thermal Labsystems, Needham Heights, MA). Each luciferase activity was normalized with regards to that of the control *Renilla* luciferase. Extracts were analyzed in duplicate, and at least two independent experiments were performed for each experiment. The observed luciferase activity reported in each figure is expressed as the percentage of the activity obtained with regards to the most active construct used in that figure. The DNAs and transfected cell lines are as indicated in the individual experiments.

Reverse Transcription-PCR Analysis of Cdx2 Expression—Caco2-BBE cells were cultured to confluence on plastic supports (area, 9.4 cm²) for 14 days, and total RNA was isolated using a Micro Fast Track™ kit (Invitrogen). RNA yield was determined by ultraviolet spectrophotometry, and 3 μ g of each total RNA was primed with oligo(dT) and reverse transcribed with avian myeloblastosis virus reverse transcriptase, using the cDNA cycle kit (Invitrogen). The reverse-transcribed cDNA was PCR-amplified with primers specific for Cdx2 (sense, 5'-AGC CAA GTG AAA ACC AGG AC-3'; and antisense, 5'-AGC CAA GTG AAA ACC AGG AC-3'; yielding a

722-bp product) or glyceraldehyde-3-phosphate dehydrogenase (internal control; sense, 5'-ACC ACA GTC CAT GCC ATC AC-3'; and antisense, 5'-TCC ACC ACC CTG TTG CTG TA-3'). The cycling conditions consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 2 min, and 72 °C for 3 min, with a final extension step at 72 °C for 7 min. The resulting PCR products were separated by 1% agarose gel electrophoresis and visualized by ethidium bromide. The desired fragment (722 bp) was gel-extracted using the QIAquick gel extraction kit (Qiagen, Valencia, CA), ligated into the Topo Mammalian Expression Vector system (Promega), grown and purified using the Mini-Prep plasmid kit (Qiagen), and confirmed by sequencing (Lark Sequencing, Houston, TX). Subconfluent Caco2-BBE cells were transfected with Cdx2-Topo or empty vector, using the Trojane transfection reagent (Avanti, Alabaster, AL) according to the manufacturer's instructions, for 3–4 h in Opti-MEM I medium (Invitrogen). Transfectants were selected in medium containing 1.2 mg/ml G418 (Invitrogen).

cAMP Analysis—Caco2-BBE cells were stimulated apically and basolaterally with 100 nM leptin or 10 μ M forskolin for the indicated times, and cAMP levels were measured from whole cell lysates using a competitive cAMP immunoassay kit (Tropix, Bedford, MA). Luminescence was read with a luminoscan (Ascent Thermo Labsystems, Needham Heights, MA).

Membrane Extractions—Caco2-BBE cells were grown at confluency, pelleted, homogenized in 5 mM HEPES containing protease inhibitors (Roche Diagnostics, Mannheim, Germany),

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incubated for 30 min at 4 °C, and then centrifuged at 13,000 × *g* for 30 min at 4 °C. The resulting pellet was suspended in phosphate-buffered saline by repeated passage through an 18-gauge needle, and the resulting protein solution was boiled for 5 min at 100 °C in Laemmli buffer supplemented with 0.5% β-mercaptoethanol.

Immunoprecipitation—Cells were washed with ice-cold phosphate-buffered saline and then lysed on ice in 1 ml of radioimmune precipitation assay lysis buffer (150 mM NaCl, 0.5% sodium deoxycholate, 50 mM Tris·HCl (pH 8.0), 0.1% SDS, 0.1% Nonidet P-40) supplemented with protease inhibitors (Roche Diagnostics). The lysates were centrifuged at 10,000 × *g* for 15 min at 4 °C, and the resulting supernatants were subjected to immunoprecipitation and immunoblot analysis. Immunoprecipitation was performed using the Catch and Release Reversible Immunoprecipitation Kit (Upstate Biotech, Lake Placid, NY), according to the manufacturer's instructions under non-denaturing conditions. Briefly, 500 μg of cell lysate, 4 μg of anti-phospho-CREB antibody (Upstate Biotech), 10 μl of antibody capture affinity ligand, and washing buffer were mixed, applied to a spin column, and incubated at room temperature for 30 min. The spin column was then placed in a capture tube and centrifuged at 5,000 rpm for 30 s to collect the flow-through. The spin column was washed three times (5,000 rpm, 30 s) using the provided wash solution, and the target protein was eluted in its native form with 70 μl of non-denaturing elution buffer and centrifugation at 5,000 rpm for 30 s. For immunoblot analysis, the eluate was resolved by SDS-PAGE and transferred onto nitrocellulose membranes overnight at 4 °C. The blots were blocked for 1 h with 5% nonfat dry milk in blocking buffer, washed with blocking buffer, and incubated for 1 h at room temperature with the appropriate antibody. The blots were then incubated for 30 min at room temperature with the appropriate secondary antibody, and probed using an ECL system (Amersham Biosciences).

Western Blot Analysis—Cells were lysed for 30 min at 4 °C in radioimmune precipitation assay buffer (150 mM NaCl, 0.5% sodium deoxycholate, 50 mM Tris·HCl (pH 8.0), 0.1% SDS, 0.1% Nonidet P-40) supplemented with protease inhibitors (Roche Diagnostics). The homogenates were centrifuged at 13,000 × *g* for 30 min at 4 °C, and the supernatants were collected for Western blot analysis. Protein concentrations were determined using the Folin method (DC Protein Assay kit, Bio-Rad), and equal amounts of protein were mixed in Tricine sample buffer (Bio-Rad), boiled for 5 min, resolved on 7.5% (Bio-Rad) or 8% polyacrylamide gels (VWR, Westchester, PA), and then transferred to nitrocellulose membranes. Membranes were blocked overnight at 4 °C or for 1 h at room temperature with 5% nonfat milk in blocking buffer, and then incubated for 1 h at room temperature, along with rabbit anti-hPepT1 antibody (1:1000 dilution (11) or mouse anti-V5 antibody (1:5000 dilution, Invitrogen). The membranes were washed three times for 15 min each in blocking buffer and then incubated for 1 h at room temperature with an anti-rabbit horseradish peroxidase-conjugated antibody (1:1000, Amersham Biosciences). The membranes were washed, and immunoreactive proteins were detected with an ECL kit (Amersham Biosciences). The band

intensities were quantitated using a gel documentation system (Alpha Innotech, San Leandro, CA).

Northern Blot Analysis—The 2.6-kb hPepT1 cDNA (11) was used as probe for Northern blot analysis. Caco2-BBE cells were incubated with or without 100 nM leptin for 8 h, and total RNA was isolated using the Tri-Reagent (Molecular Research, Cincinnati, OH). Total RNA (20 μg) was denatured for 15 min at 65 °C in 20 mM HEPES (pH 7.2), 1 mM EDTA, 50% formamide and 6% formaldehyde, and then resolved by electrophoresis on a 1% agarose gel containing 2% formaldehyde. The separated RNAs were transferred to a nylon membrane (PerkinElmer Life Sciences), and covalently cross-linked by exposure to UV light. The hPepT1 cDNA probe was labeled with [α -³²P]CTP using the Rediprime II random prime labeling system (Amersham Biosciences) and hybridized to the membrane in 7% SDS, 1% bovine serum albumin, 10% polyethylene glycol 8000, 250 mM NaCl, 1.25 mM EDTA, 125 mM NaPO₄, and 1 mg of DNA salmon sperm (Ambion, Austin, TX). A mouse glyceraldehyde-3-phosphate dehydrogenase probe was used as the control (Ambion), and visualization was made by autoradiography. The band intensities were quantitated using a gel-documentation system (Alpha Innotech).

Uptake Experiments—Cells were grown on filters for 15 days, washed twice with Hanks' balanced salt solution (Sigma-Aldrich), and placed in containing 10 mM HEPES (pH 7.4) in the basolateral compartment and 10 mM MES (pH 6.2) in the apical compartment. The monolayers were allowed to stabilize in these buffers for 15 min at 37 °C. The apical compartment was then loaded with Hanks' balanced salt solution-10 mM MES (pH 6.2) containing 20 μM [¹⁴C]Gly-Sar with or without 20 mM Gly-Leu, or the basolateral compartment was loaded with Hanks' balanced salt solution-10 mM MES (pH 7.2) without [¹⁴C]Gly-Sar or Gly-Leu. After samples were incubated for 15 min at room temperature, the supernatants were removed, the cells were washed twice, the filters were cut, and cell-associated radioactivity was determined by liquid scintillation counting in a β-counter. The results are expressed as specific uptake of [¹⁴C]Gly-Sar mediated by hPepT1, calculated as follows: (uptake of [¹⁴C]Gly-Sar) – (uptake of [¹⁴C]Gly-Sar in the presence of Gly-Leu).

Electrophoretic Mobility Shift Assays—Probes for the electrophoretic mobility shift assays (EMSAs) were prepared using a standard protocol. Forward Cdx2 (GAATCTCAAATAAAGCCACACA) and Reverse Cdx2 (TGTGTGGCTTTATTTTGAGATTC) are complementary oligonucleotides for the second Cdx2⁻⁵⁶² binding site, Forward* Cdx2 (TAAGTAACTCAAGCCACACAAAA) and Reverse* Cdx2 (TTTTGTGTGGCTTGAGTTACTTA) are complementary oligonucleotides for the second Cdx2⁻⁵⁶² binding site mutant. The probes were synthesized with high-performance liquid chromatography purification, represent the transcript factor binding sites, and were first end-labeled with a Biotin 3' End DNA Labeling Kit (Pierce). Briefly, 0.2 unit/μl of terminal deoxynucleotidyltransferase, 0.5 μM biotin-11-UTP, and 100 nM of each single-stranded oligonucleotide were incubated in 1× terminal deoxynucleotidyltransferase reaction buffer for 30 min at 37 °C. EDTA (2.5 μl of 0.2 M) was added to stop the reaction, and 50 μl of chloroform: isoamyl alcohol was added to extract the labeled oligonucleo-

tides. For annealing, concentrated complementary oligonucleotides were mixed together at a 1:1 molar ratio and incubated at 95 °C for 5 min. The heat was then gradually reduced over 3 hours until the oligonucleotides reached room temperature. Standard EMSAs were performed using the LightShift Chemiluminescent EMSA Kit (Pierce). Briefly, 20 fmol of each biotin end-labeled target oligonucleotide pair was incubated in EMSA binding buffer (100 mM Tris, 500 mM KCl, 10 mM dithiothreitol, pH 7.5) containing 2.5% glycerol, 5 mM MgCl₂, 50 ng/μl poly(dI-dC), 0.05% Nonidet P-40, and 5 μg of Caco2-BBE nuclear proteins for 15 min at room temperature. For the competition EMSA, we added 200-fold (8 pmol) of unlabeled paired oligonucleotides. For the supershift EMSA, we added 2 μg of related Cdx2 antibody (Zymed Laboratories Inc. laboratories, San Francisco, CA) and anti-CREB or pCREB antibody (Upstate, Charlottesville, VA). Complexes were resolved by electrophoresis on native 5% TBE Criterion Precast Gels (Bio-Rad) in 0.5× TBE tris borate-EDTA buffer at 110 V/gel. Gels were semi-dry transferred to Biodyne B Pre-cut Modified Nylon Membranes (0.45 μm, Pierce), using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). The membranes were then cross-linked (UVC-508 Ultraviolet Cross-linker, Ultra LUM, Claremont, CA), and visualized using the Chemiluminescent Nucleic Acid Detection System (Pierce).

Chromatin Immunoprecipitation Assay—CREB and Cdx2 chromatin immunoprecipitation (ChIP) assays were performed using the ChIP assay kit (Upstate Cell Signaling Solutions, Lake Placid, NY), according to the manufacturer's instructions, with some modifications. Briefly, Caco2-BBE cells were grown to confluence, and then treated with or without leptin (100 nM) for 8 h. The cells were then fixed with 1% formaldehyde for 10 min at 37 °C (to initiate cross-linking), scraped off the plate, washed with ice-cold phosphate-buffered saline, and resuspended in 200 μl of SDS lysis buffer for 10 min on ice. The cells were then sonicated with 3 sets of 12-s pulses at 35% power, to shear the DNA into 200- to 1000-bp fragments. The samples were centrifuged, and the supernatant was diluted in ChIP dilution buffer and pre-cleared with a protein A-agarose/salmon sperm DNA slurry to reduce the nonspecific background. The samples were then immunoprecipitated with anti-p-CREB or CREB antibody overnight at 4 °C. The complexes were collected in a protein A-agarose/salmon sperm DNA slurry for 1 h at 4 °C, and then washed once each with the provided low salt, high salt, and LiCl wash buffers, and twice in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The immunoprecipitated chromatin was eluted from the protein A using freshly prepared elution buffer (100 mM NaHCO₃, 1% SDS), and the protein-DNA cross-links were reversed by treatment with NaCl (200 mM) at 65 °C for 4 h. The DNA was purified by incubation with proteinase K at 45 °C for 1 h, followed by phenol/chloroform extraction and ethanol precipitation with glycogen. The Cdx2 (1, 2) and Cdx2 (3) promoter elements in the immunoprecipitates were detected by PCR using specific primers (Cdx1chipfor, 5'-TCT TAA AGA AAG GAA ATG TAG AAT CC-3' and Cdx1chiprev, 5'-TGT GTG TGT GAA TGA GGA TTG A-3'; and Cdx3chipfor, 5'-CCC ACA GTG GTT TCC AAA GT-3' and Cdx3chiprev, 5'-AGC CAG TCT AAA CGC GGA TT-3').

Statistical Analysis—All evaluations were performed using the Excel (Microsoft), SigmaPlot (SPSS, Chicago, IL), and InStat version 3.06 (GraphPad, San Diego, CA) programs, with data reported as means ± S.D. Multiple groups were compared by analysis of variance, using Tukey's post-hoc test.

RESULTS

Molecular Cloning and Analysis of the 5'-Flanking Region of the hPepT1 Gene—The putative proximal promoter region of the human *PepT1* gene (722 bp) was obtained by PCR amplification using primers specific to the 5'-flanking region of the *PepT1* gene sequence. GRAILEXP (compbio.ornl.gov/grailxp/) analysis revealed that this region was 60% G+C and contained a predicted CpG island. The isolated 722-bp sequence confirmed the previous finding (32) that the region proximal to the transcription start site lacks a canonical TATA or CAAT box (Fig. 1A). RACE was used to identify the transcription start site in polyadenylated RNA from human epithelial cells (Caco2-BBE). As shown in Fig. 1B, our results show for the first time that the *hPepT1* gene possesses one major transcription start site 86 bp upstream from the translation start site. Analysis with the TFSEARCH prediction program (www.cbrc.jp/) revealed that the cloned promoter region contained multiple putative binding sites for heat shock factor, located at -523 and -379 in the sense direction and at -636, -654, -547, and -507 in the antisense direction. Binding sites for the alcohol dehydrogenase gene regulator were located at -284, -171, and -128 in the sense direction and at -455, -181, -203, and -143 in the antisense direction. Binding sites for Cdx-2, a caudal-related homeodomain transcription factor important for intestinal gene transcription and epithelial cell differentiation (33, 34), were identified at -675 and -586 in the sense direction and at -299 in the antisense direction.⁵ Binding sites for the stimulating protein 1 were identified at -238 and -44 in the sense direction, whereas a binding site for cAMP responsive element-binding protein (CREB) was located downstream of the transcription start site at +7 in the antisense directions (Fig. 1A).

Leptin Enhances hPepT1 Promoter Activity—To test the response of the hPepT1 promoter to leptin, hPepT1 promoter-encoding reporter constructs were transfected into Caco2-BBE cells, a model system known to express endogenous hPepT1 and leptin receptors. Transfected Caco2-BBE cells were left untreated (controls) or were preincubated with 100 nM leptin for 0–48 h, and promoter activity was assessed in terms of luciferase activity. Our results revealed that leptin treatment of Caco2-BBE monolayers dose- and time-dependently increased hPepT1 promoter activity, with a maximal increase of ~1.6-fold seen after 8-h exposure to 100 nM leptin (Fig. 2, A and B).

Leptin Increases hPepT1-mediated Di-peptide Uptake in Caco2-BBE Monolayers—As shown in Fig. 3, addition of 100 nM to the apical and basolateral of Caco2-BBE monolayers significantly increased hPepT1-mediated Gly-Sar uptake after 8 and 12 h but not at earlier or later time periods of leptin treatment (145.76 pmol/filter/h ± 1.5256 with 100 nM leptin for 8 h and 89.630 pmol/filter/h ± 6.8848 without leptin, 126.3600 pmol/

⁵ A. Driss, V. Nduati, and D. Merlin, GenBank™ accession number: DQ370174.

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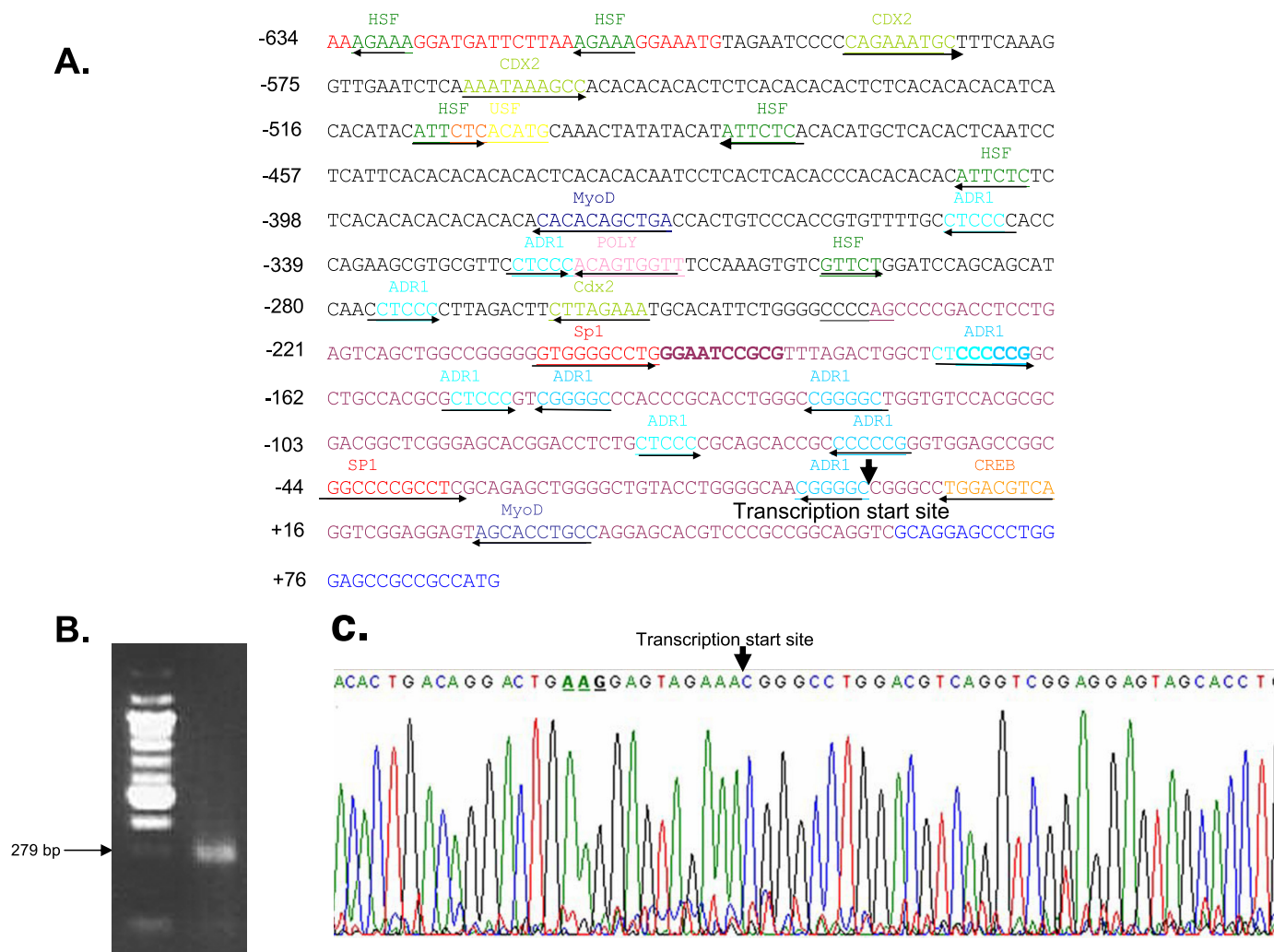


FIGURE 1. Characterization of the cloned 5'-flanking region of the human *PepT1* gene. *A*, sequence of the cloned 5'-flanking region of the human CD98 gene. The TFSEARCH prediction program (www.cbrc.jp/) was used to search the cloned hPepT1 5'-flanking region (722 bp) for putative transcription factor binding sites, as shown on the sequence (arrows indicate the direction). *B*, localizing the transcription initiation start site. The transcription initiation site was identified by RACE analysis of total RNA from Caco2-BBE cells. Lane 1, 100-bp DNA ladder (molecular size marker); lane 2, 5'-RACE PCR product. *C*, mapping the transcription initiation site. Gel-purified 5'-RACE PCR products were cloned into PCR2.1 and sequenced. The Chromas software package was used for sequence analysis.

filter/h \pm 7.8900 with 100 nM leptin for 12 h and 90.5600 pmol/filter/h \pm 9.5600) without leptin. Fig. 4 shows that treatment with leptin for 8 h is dose-dependent with a maximal increase of hPepT1 activity at 100 nM leptin (104.4283 pmol/filter/h \pm 2.3983 with 100 nM leptin *versus* 42.635 pmol/filter/h \pm 8.963 without leptin).

As shown in Fig. 5 (*A* and *B*), addition of leptin to the apical or basolateral compartments significantly increased hPepT1-mediated Gly-Sar uptake by 58 and 33%, respectively (apical, 82.87 pmol/filter/h \pm 10 without leptin *versus* 138.09 pmol/filter/h \pm 1.2 with 100 nM leptin; basolateral, 72.39 pmol/filter/h \pm 23 without leptin *versus* 111.32 \pm 5.6 pmol/filter/h with 100 nM leptin). In addition, we observed that the levels of hPepT1 immunoreactive proteins (\sim 100 kDa) found in the membrane fractions were 26 and 60% higher in monolayers exposed to 8 h of leptin treatment on the basolateral and apical sides, respectively (Fig. 5, *C–E*). Together, these results demonstrate that, under our experimental conditions, leptin treatment led to increased membrane hPepT1 protein expression and enhancement of hPepT1-mediated transport events.

Leptin Increases hPepT1 mRNA and Protein Expression—The mRNA expression levels of hPepT1 were analyzed by Northern blotting of total RNA from Caco2-BBE monolayers that were left untreated (control) or were treated with 100 nM luminal leptin for 8 h. As shown in Fig. 6 (*A* and *B*), the 3.2-kb band representing the hPepT1 mRNA was significantly increased (\sim 1.5-fold) in leptin-treated cells *versus* controls, indicating that leptin treatment increases hPepT1 expression at the transcriptional level.

The protein expression levels of hPepT1 were analyzed by Western blot analysis using rabbit anti-hPepT1 antibody on cytosol and membrane protein lysate from Caco2-BBE monolayers that were untreated or treated with 100 nM leptin for 8 h. The cytosol and membrane amount of hPepT1-immunoreactive protein (\sim 100 kDa), was significant higher by 30 and 23%, respectively, after 8-h leptin treatment when compared with untreated cells (Fig. 6, *C* and *D*). Together these results demonstrate that 100 nM leptin for 8-h treatment induces increased of hPepT1 expression and translocation to the membranes. Treatment with 100 nM leptin for 8 h is optimal to increase hPepT1

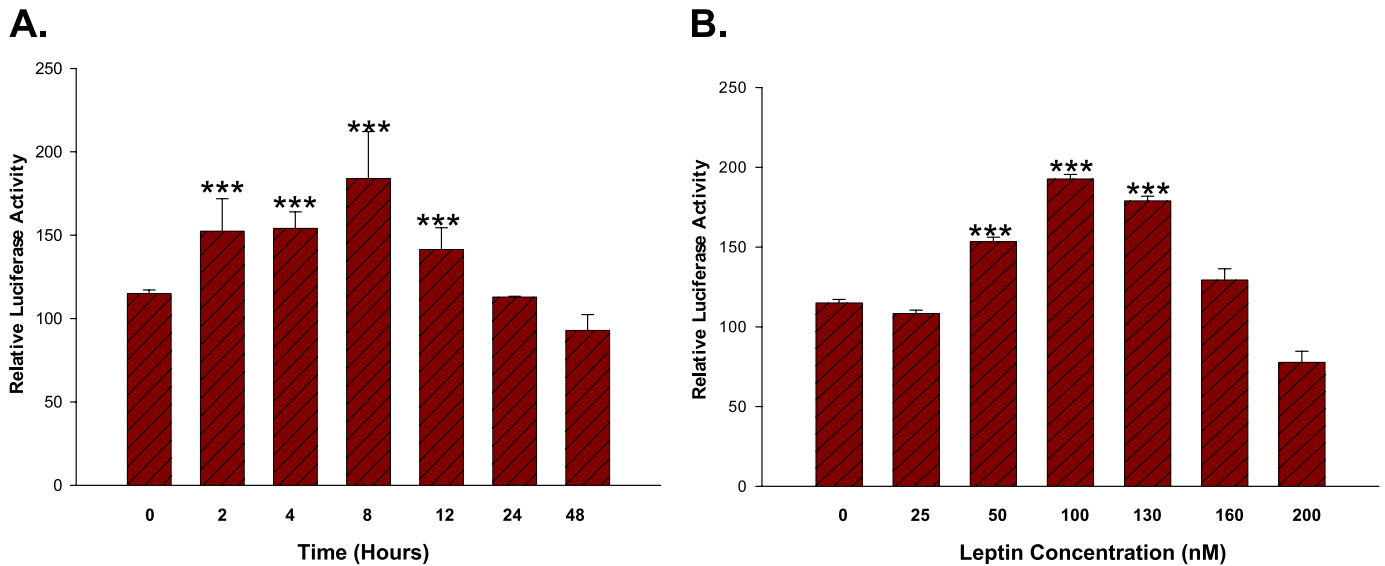


FIGURE 2. Leptin increases hPepT1 promoter activity in Caco2-BBE cells. The cloned hPepT1 promoter region was inserted upstream of the luciferase coding region in the pGL3-basic plasmid. *A*, functional activities of the hPepT1 promoter construct in Caco2-BBE cells stimulated with 100 nM leptin for 0, 2, 4, 8, 12, 24, and 48 h. *B*, functional activities of the hPepT1 promoter construct in Caco2-BBE cells stimulated with 25, 50, 100, 130, 160, and 200 nM leptin for 8 h. Luciferase activities were normalized to *Renilla* luciferase activities driven by the phRL-CMV control vector. Activities are expressed as -fold induction over that seen in cells transfected with empty pGL3-basic vector. Each value represents the mean \pm S.D. of at least three independent sets of transfection experiments performed in triplicate. ***, $p < 0.001$.

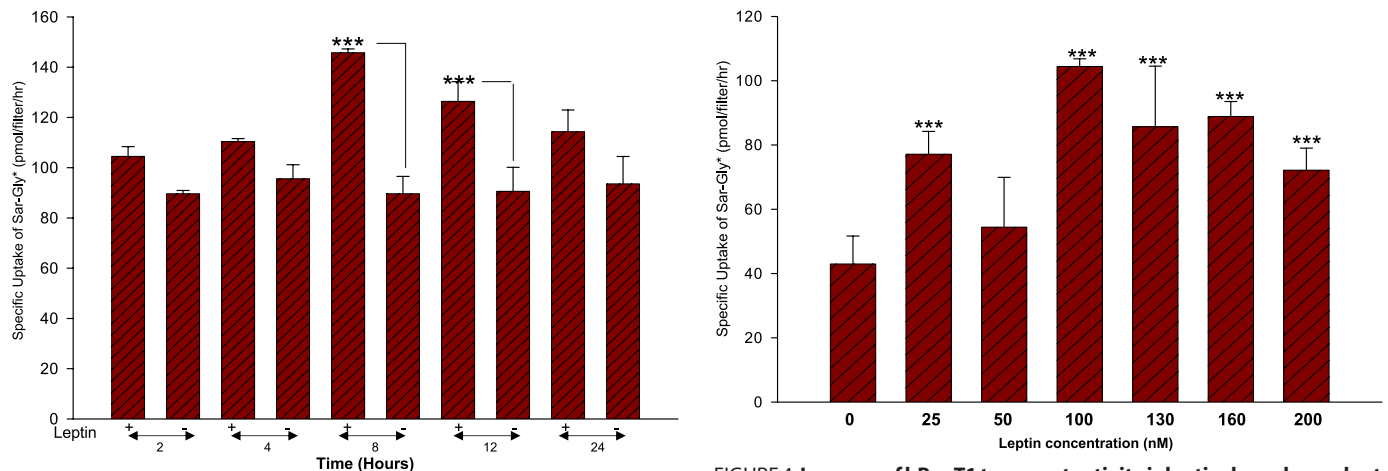


FIGURE 3. Increase of hPepT1 transport activity is leptin time-dependent. Caco2-BBE monolayers grown on filters were left untreated (–) or were treated apically and basolaterally with 100 nM leptin (+) for 2, 4, 8, 12, or 24 h. Uptake experiments were performed with 20 μ M [14 C]Gly-Sar \pm 20 mM Gly-Leu at an apical pH of 6.2, whereas the pH of the basolateral side was maintained at pH 7.2. The filters were cut and the cells were lysed, and the remaining intracellular [14 C]Gly-Sar was measured. The results are expressed as specific uptake of [14 C]Gly-Sar. Values represent means \pm S.D. of three determinations. ***, $p < 0.001$.

promoter activity, hPepT1 mRNA and protein expressions, and hPepT1 transport activity.

The CREB Binding Site Is Necessary for Leptin-induced hPepT1 Promoter Activity—We then sought to examine possible signal transduction pathways responsible for leptin-induced hPepT1 responses. We demonstrated that cAMP levels transiently increased following treatment of Caco2-BBE cells with 100 nM leptin, peaking 30 min post-treatment (relative light units, 0.3 relative light unit \pm 0.02 without leptin versus 5.7 relative light units \pm 0.5 with leptin; Fig. 7). Similarly, the positive control (10 μ M forskolin) transiently increased intracellular cAMP levels, with the response peaking 30 min post-treat-

FIGURE 4. Increase of hPepT1 transport activity is leptin dose-dependent. Caco2-BBE monolayers grown on filters were left untreated (0) or were treated apically and basolaterally with 25, 50, 100, 130, 160, and 200 nM leptin for 8 h. Uptake experiments were performed with 20 μ M [14 C]Gly-Sar \pm 20 mM Gly-Leu at an apical pH of 6.2, whereas the pH of the basolateral side was maintained at pH 7.2. The filters were cut, and the cells were lysed, and the remaining intracellular [14 C]Gly-Sar was measured. The results are expressed as specific uptake of [14 C]Gly-Sar. Values represent means \pm S.D. of three determinations. ***, $p < 0.001$; **, $p < 0.01$.

ment (Fig. 7). Immunoblot analysis revealed that CREB and phospho-CREB were both present in Caco2-BBE cells and that phospho-CREB levels increased following 30-min leptin stimulation (Fig. 8, *A* and *B*). We then used deletion analysis to test whether the leptin-mediated increase of intracellular cAMP might activate hPepT1 promoter activity via the CREB binding site. Our results revealed that point mutations (CG replace by AT) in the CREB binding transcription site completely inhibited leptin-induced hPepT1 transcription activity; specifically, the wild-type promoter yielded (RLA: relative luciferase activity) 42 RLA \pm 7 in the absence of leptin and 82 RLA \pm 4 in the presence of 100 nM leptin, whereas the mutated hPepT1 pro-

Leptin Increases hPepT1 Transcription Activity

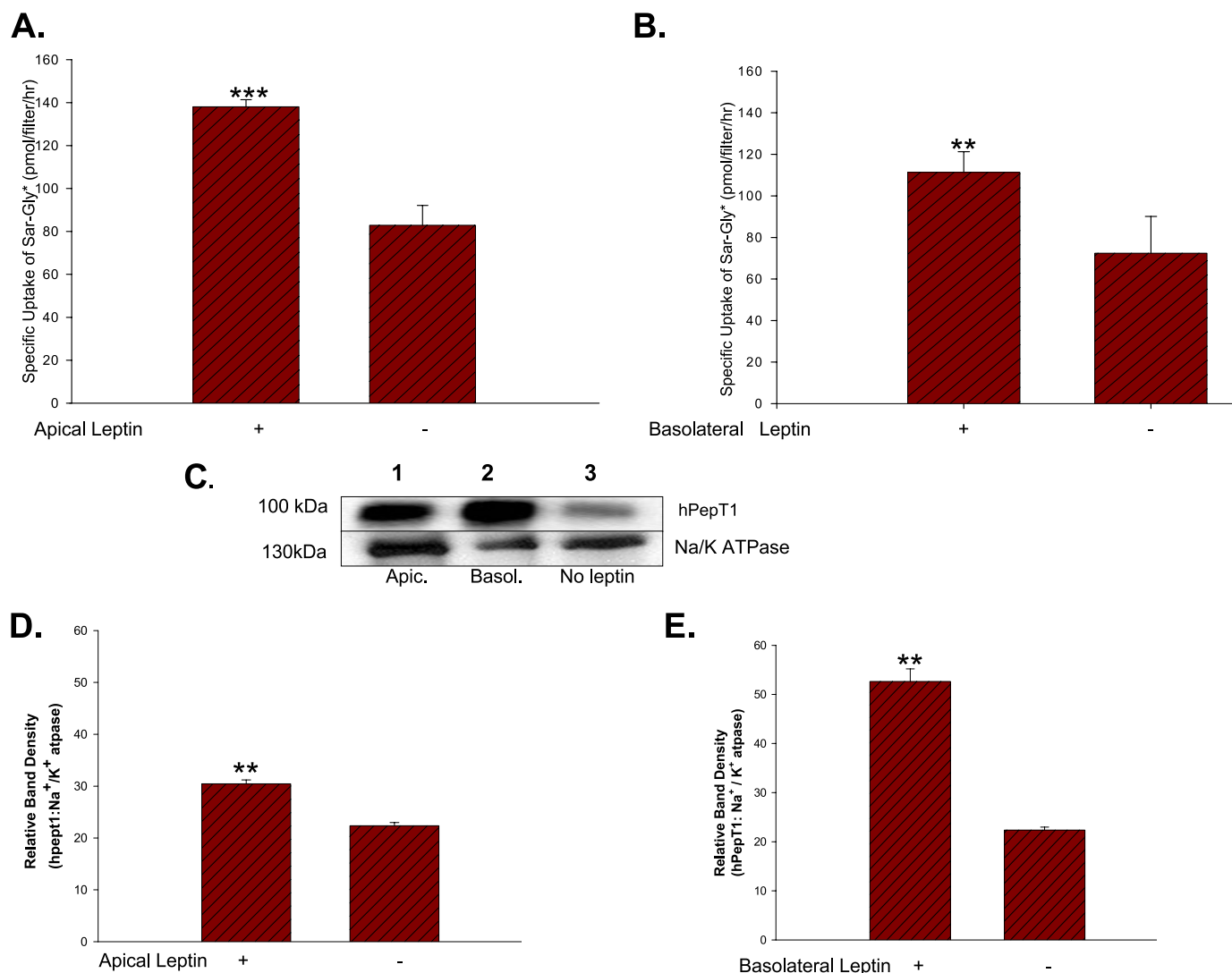


FIGURE 5. Leptin increases hPepT1 transport activity and hPepT1 protein expression. Caco2-BBE monolayers grown on filters were left untreated (–) or were treated apically (A, +) or basolaterally (B, +) with 100 nM leptin for 8 h. Uptake experiments were performed with 20 μ M [14 C]Gly-Sar \pm 20 mM Gly-Leu at an apical pH of 6.2, whereas the pH of the basolateral side was maintained at pH 7.2. The filters were cut and the cells were lysed, and the remaining intracellular [14 C]Gly-Sar was measured. The results are expressed as specific uptake of [14 C]Gly-Sar. Values represent means \pm S.D. of three determinations. ***, $p < 0.001$; **, $p < 0.01$; NS, not significant. C, Caco2-BBE cell monolayers were preincubated with 100 nM apical or basolateral leptin or vehicle for 8 h. Membrane proteins were extracted, and 40 μ g/lane was subjected to 4–20% SDS-PAGE followed by transfer to a nitrocellulose membrane. The blot was immunostained with a rabbit anti-hPepT1 antibody (lane 1, monolayers treated with apical 100 nM leptin for 8 h; lane 2, monolayers treated with basolateral 100 nM leptin for 8 h; lane 3, monolayers treated with vehicle for 8 h). D and E, scanning densitometry of hPepT1 membrane protein expression. Densitometric analysis of hPepT1 membrane protein immunoblots from monolayers treated with 100 nM apical (D) or basolateral (E) leptin for 8 h. The changes are expressed as mean \pm S.D. of three analyses. The level of hPepT1 was calculated in relation to the vehicle-treated cells, and the value of each time control was arbitrarily set to 1. **, $p < 0.01$ versus vehicle.

motor showed activities of 42 RLA \pm 9 in the absence of leptin and only 48 RLA \pm 6 in the presence of leptin (Fig. 9). In addition, Fig. 10 shows that the selective PKA inhibitor H89 abrogated the increased of hPepT1 promoter activity stimulated by leptin in a dose-dependent manner (10 μ M H89 158.8200 RLA \pm 2.2700 versus 40 μ M H89 75.4600 RLA \pm 8.6900; controls 93.6200 RLA \pm 4.3600 in the absence of leptin and presence of 40 μ M H89; 100.0000 RLA \pm 0.63 in the absence of both leptin and H89). Together, these results suggest the hypothesis that leptin treatment increases intracellular cAMP levels, leading to activation of PKA, which forms the molecular connection between cAMP and CREB. The catalytic subunit of PKA then likely translocates into the nucleus and phosphorylates CREB, leading to the stimulation of *hPepT1* gene transcription.

The Cdx2 Binding Sites Are Necessary for Leptin-induced hPepT1 Promoter Activity—hPepT1 promoter contains three Cdx2 binding sites (Fig. 1A), we next tested whether Cdx2 binding is required for leptin-induced hPepT1 promoter activity. We used deletion experiments similar to those described above to examine whether the Cdx2 binding sites were required for leptin-induced hPepT1 promoter activity. Our results revealed that deletion of the Cdx2 binding sites abrogated leptin-induced increases in hPepT1 promoter activity (Fig. 11A), suggesting that Cdx2 might play a role in leptin-stimulated hPepT1 transcription (wild-type promoter, 41 RLA \pm 7 without leptin, 184 RLA \pm 2 with 100 nM luminal leptin; Cdx2-deleted promoter, 60 RLA \pm 7 without leptin, 48 RLA \pm 5 with 1 μ M luminal leptin; Fig. 11A). We then used point mutation of the

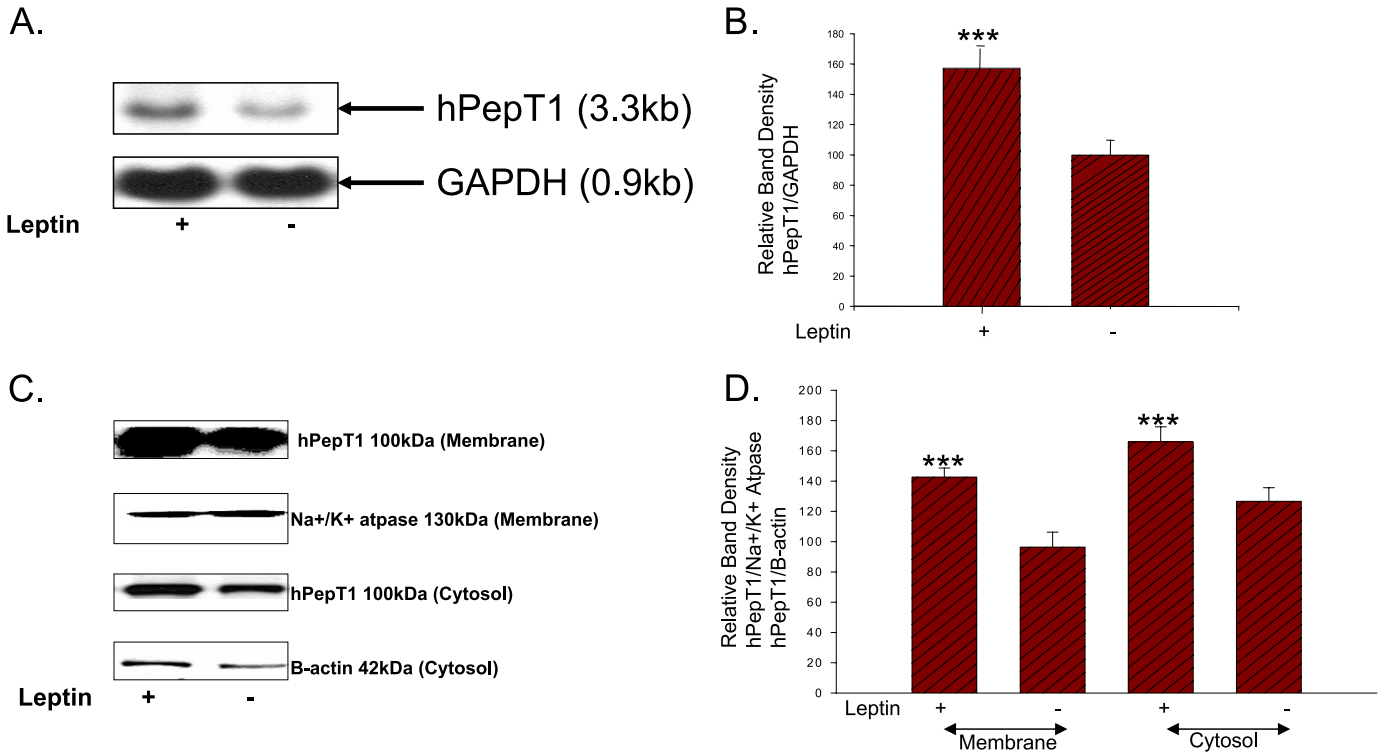


FIGURE 6. Leptin increases hPepT1 mRNA and protein expression. *A*, Caco2-BBE cell monolayers were preincubated with 100 nM luminal leptin or vehicle for 8 h, and total RNA (20 μ g/lane) was subjected to Northern blot analysis with a probe specific to the hPepT1 transcript (2.6 kb). The same blot was stripped and re-probed with the glyceraldehyde-3-phosphate dehydrogenase cDNA (control). *B*, densitometric analysis of the Northern blot results. The changes are expressed as mean \pm S.D. of three analyses. The levels of hPepT1 mRNA were calculated *versus* those found in the vehicle-treated cells at each time, with the value of each time control arbitrarily set to 1. *******, $p < 0.001$ *versus* vehicle. *C*, Caco2-BBE cell monolayers were preincubated with 100 nM leptin (+) or vehicle (-) for 8 h before those membrane and cytosolic proteins were extracted. Membrane (25 μ g/lane) and cytosolic (25 μ g/lane) proteins was subjected to 8% SDS-PAGE followed by transfer to nitrocellulose membrane. The blot was immunostained with a rabbit anti-hPepT1, mouse anti-Na/K ATPase, mouse anti- β -actin. *D*, densitometric analysis of immunoblots of hPepT1 membrane and cytosol proteins. The changes are expressed as mean \pm S.D. of three analyses. The level of hPepT1 was calculated in relation to the vehicle-treated cells, and the value of each time control was arbitrarily set to 1. *******, $p < 0.001$ *versus* vehicle.

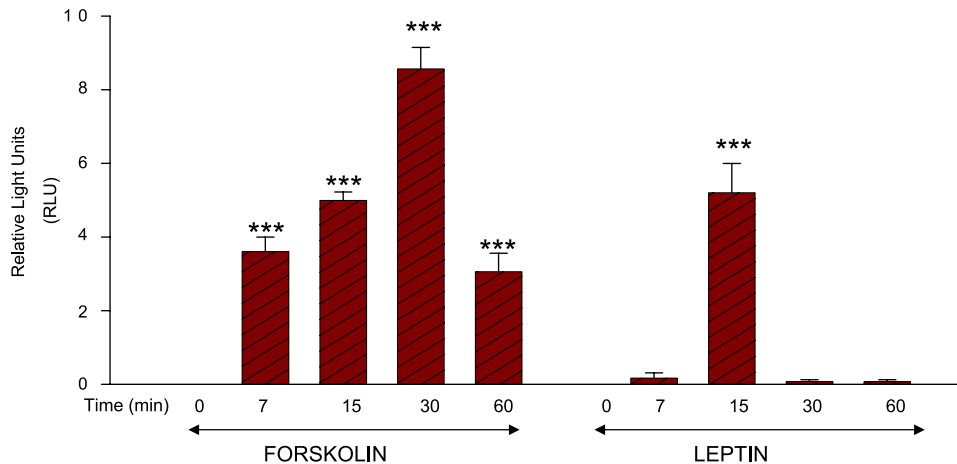


FIGURE 7. Leptin increases intracellular cAMP in the colonic epithelial cells, leading to the subsequent activation of CREB. Leptin increases intracellular cAMP in Caco2-BBE cells. Caco2-BBE monolayers were stimulated with 10 μ M forskolin or 100 nM leptin for 7, 15, 30, and 60 min, and cAMP levels were measured from cell lysates. The data represent values obtained from three independent experiments with three samples per condition; cAMP levels are given in relative light units (RLU) \pm S.D. ($n = 3$). *******, $p < 0.001$.

individual Cdx2 binding sites to assess which is/are involved in the leptin-stimulated hPepT1 promoter response. As shown in Fig. 11B, mutation of the Cdx2 binding sites located at -579 or -564 prevented the leptin-induced increase of hPepT1 promoter activity (RLA 53.96 \pm 5.72 *versus* 42.36 \pm 6.36 and RLA 42.36 \pm 3.59 *versus* 39.76 \pm 4.56, respectively), whereas muta-

tion of the Cdx2 binding site located at -262 had no such effect (RLA 95.63 \pm 9.6 for the mutant *versus* 39.76 \pm 7.86 in the control). To confirm the involvement of Cdx2, we cloned Cdx2 (Fig. 12A) from Caco2-BBE cells, generated a Cdx2-overexpressing Caco2-BBE cell line (Fig. 12B), and examined whether leptin treatment increased hPepT1 promoter activity to a greater degree in the Cdx2-overexpressing cell line. Our results revealed that Caco2-BBE cells overexpressing Cdx2 transfected with reporter constructs driven by the wild-type hPepT1 promoter did indeed have an enhanced hPepT1 response to leptin when compared with controls harboring empty reporter vectors

(Cdx2-overexpressing cells harboring wild-type hPepT1 reporter constructs, 41 RLA \pm 6 without leptin, 92 RLA \pm 6 with 100 nM leptin; controls, 42 RLA \pm 6 without leptin, 48 RLA \pm 7 with 100 nM leptin; Fig. 13A), whereas those transfected with reporter constructs driven with Cdx2 binding site-deleted hPepT1 promoter sequences did not (Cdx2-overex-

Leptin Increases hPepT1 Transcription Activity

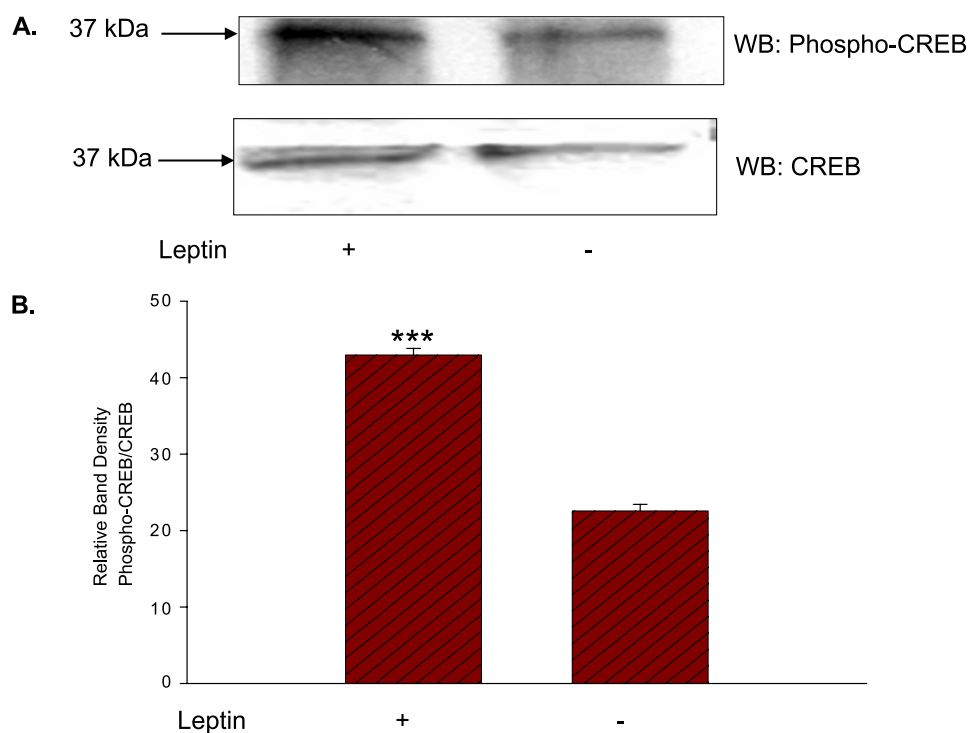


FIGURE 8. Leptin induces pCREB in Caco2-BBE cells. Caco2-BBE cells were pretreated with (lane 1, +) or without (lane 2, -) 100 nM leptin for 30 min. *A*, equal amounts (20 μ g) of protein from whole cell lysates were resolved on 10% acrylamide gels and subjected to Western blot analysis of pCREB (Phospho-CREB) and CREB. *B*, densitometric analysis of pCREB immunoblots from cells treated with or without 100 nM leptin. The changes are expressed as mean \pm S.D. of three analyses. The level of pCREB was calculated in relation to the vehicle-treated cells, and the value of each time control was arbitrarily set to 1. ***, $p < 0.001$ versus vehicle.

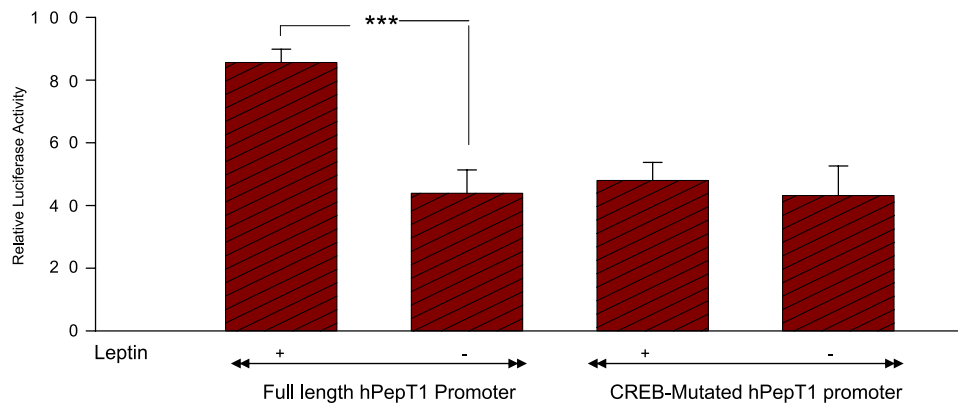


FIGURE 9. CREB is required for leptin-induced hPepT1 promoter activity. Caco2-BBE cells were cotransfected with reporter plasmids containing the 722-bp cloned hPepT1 5'-flanking region (full-length hPepT1 promoter), the cloned promoter with mutations in the CREB binding site (CREB-mutated) or the *Renilla* luciferase control plasmid. Transfectants were treated with vehicle (-) or with 100 nM leptin (+) for 8 h, and the luciferase activity was determined. The data represent fluorescence ratios relative to unstimulated transfected cells, plotted as mean \pm S.D. from duplicate determination of two samples per group ($n = 3$). ***, $p < 0.01$ versus vehicle.

pressing cells harboring mutant hPepT1 reporter constructs, 45 RLA \pm 8 without leptin, 52 RLA \pm 8 with 100 nM leptin; controls, 46 RLA \pm 3 without leptin, 47 RLA \pm 5 with 100 nM leptin; Fig. 13*B*). These results collectively demonstrate that Cdx2 is necessary for leptin-stimulated hPepT1 promoter activity. We then sought to confirm the site-specific results in Cdx2-overexpressing Caco2-BBE cells. As shown in Fig. 14, mutation of the Cdx2 binding sites located at -579 or -564 blocked leptin-induced hPepT1 promoter activity in Cdx2-overexpressing Caco2-BBE cells, but mutation of the Cdx2 site at -262 had no such effect.

Together, these results suggest that the -579 and -564 Cdx2 binding sites are crucial for leptin-induced increases of hPepT1 promoter activity.

Cdx2 and Phospho-CREB Interact in Leptin-stimulated Caco2-BBE Cells—We then used co-immunoprecipitation assays to examine the possible interaction between Cdx2 and phosphorylated CREB (pCREB). When lysates from leptin-stimulated Caco2-BBE cells overexpressing V5-tagged Cdx2 were immunoprecipitated with anti-phospho CREB antibody, and the immunoprecipitates were probed with an anti-V5 antibody, the anti-V5 antibody recognized the expected 42-kDa band representing Cdx2 (Fig. 15*A*, lane 1). In contrast, no such co-immunoprecipitation of pCREB and Cdx2 was observed in unstimulated Caco2-BBE cells overexpressing V5-tagged Cdx2 (Fig. 15*A*, lane 2) or in stimulated Caco2-BBE transfected with the vector alone (Fig. 15*A*, lane 3). These results suggest that leptin treatment induces phosphorylation of CREB, leading to physical interaction of pCREB and Cdx2 and subsequent up-regulation of hPepT1 gene transcription. To further confirm the importance of Cdx2 and pCREB for activation of the hPepT1 gene stimulated by leptin we used gel shift and supershift to characterize their binding to Cdx2 (Fig. 15*B*). Gel shifts were carried out with nuclear extracts as a source of protein from unstimulated or leptin-treated Caco2-BBE, along with biotin-labeled double-stranded oligonucleotides containing the respective consensus binding sites, mixed with a series of unlabeled double-stranded oligonucleotide competitors or relevant Cdx2, CREB, and pCREB antibodies for the supershift analysis. DNA-protein complexes were detected in unstimulated and leptin-treated nuclear extracts (Fig. 15*B*, lanes 2 and 9). The unlabeled wild-type and labeled mutant oligonucleotides markedly diminished formation of all retardation complexes in nuclear complex from unstimulated and leptin-stimulated Caco2-BBE cells (Fig. 15*B*, lanes 3 and 4, and 10 and 11). Finally, gel-shift analysis revealed that incubation of the DNA-protein complexes from unstimulated and leptin-treated nuclear proteins with Cdx2 antibody shifted the migrating

bands in an upward direction (Fig. 15B, lanes 5 and 12). Similar results were found when using anti-CREB antibody (Fig. 15B, lanes 7 and 14). In contrast, incubation of the DNA-protein complexes from leptin-treated but not for unstimulated nuclear proteins with pCREB antibody shifted the migrating bands in an upward direction (Fig. 15B, lanes 6 and 13). These results suggest that Cdx2 and pCREB likely interact with Cdx2 binding sites and participate to the activation of hPepT1 promoter activity under leptin stimulation. To confirm this hypothesis, we performed chromatin immunoprecipitation analysis. Leptin-stimulated or unstimulated Caco2-BBE were cross-linked and immunoprecipitated with anti-pCREB or anti

CREB antibodies. The precipitates were then PCR-amplified with primers specific to CREB, or each of the three Cdx2 binding sites. Our results revealed that, in unstimulated cells, CREB but not pCREB binds to the Cdx2 binding sites (Fig. 15C), whereas, in leptin-stimulated cells, CREB and pCREB bound to all Cdx2 binding sites (Fig. 15C). These results collectively suggest that the interaction between pCREB and Cdx2 is important for leptin-induced hPepT1 promoter activity.

DISCUSSION

We previously reported that hPepT1 is expressed in the inflamed colon tissues of inflammatory bowel disease patients (11). It is believed that colonic hPepT1 expression may initiate the inflammatory cascade by transporting bacterial products such as formyl-Met-Leu-Phe and muramyl dipeptide (12, 13). However, no previous work has sought to elucidate the mechanisms governing hPepT1 expression in the colon. In an effort to improve our understanding of hPepT1 expression in colonic mucosa and inflammatory bowel disease, we herein sought to identify and characterize the regulatory elements controlling leptin-induced hPepT1 gene expression.

Leptin has been shown to rapidly enhance hPepT1-mediated di-peptide transport in rats and in Caco2-BBE cells *in vitro*, at least in part by triggering the translocation of

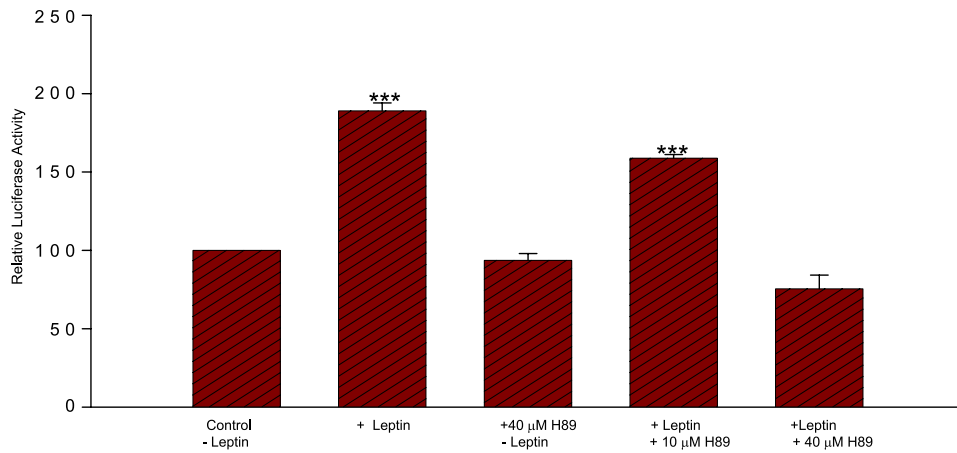


FIGURE 10. PKA is required for leptin-induced hPepT1 promoter activity. Caco2-BBE cells were co-transfected with reporter plasmids containing the 722-bp cloned hPepT1 5'-flanking region (full-length hPepT1 promoter) or the *Renilla* luciferase control plasmid. Transfectants were treated with vehicle (-) or with 100 nM leptin (+) in the presence or absence of the PKA inhibitor H89 at 10 and 40 μM concentrations for 8 h, and luciferase activity was determined. The data represent fluorescence ratios relative to unstimulated transfected cells, plotted as mean ± S.D. from duplicate determination of two samples per group (n = 3). ***, p < 0.01 versus vehicle.

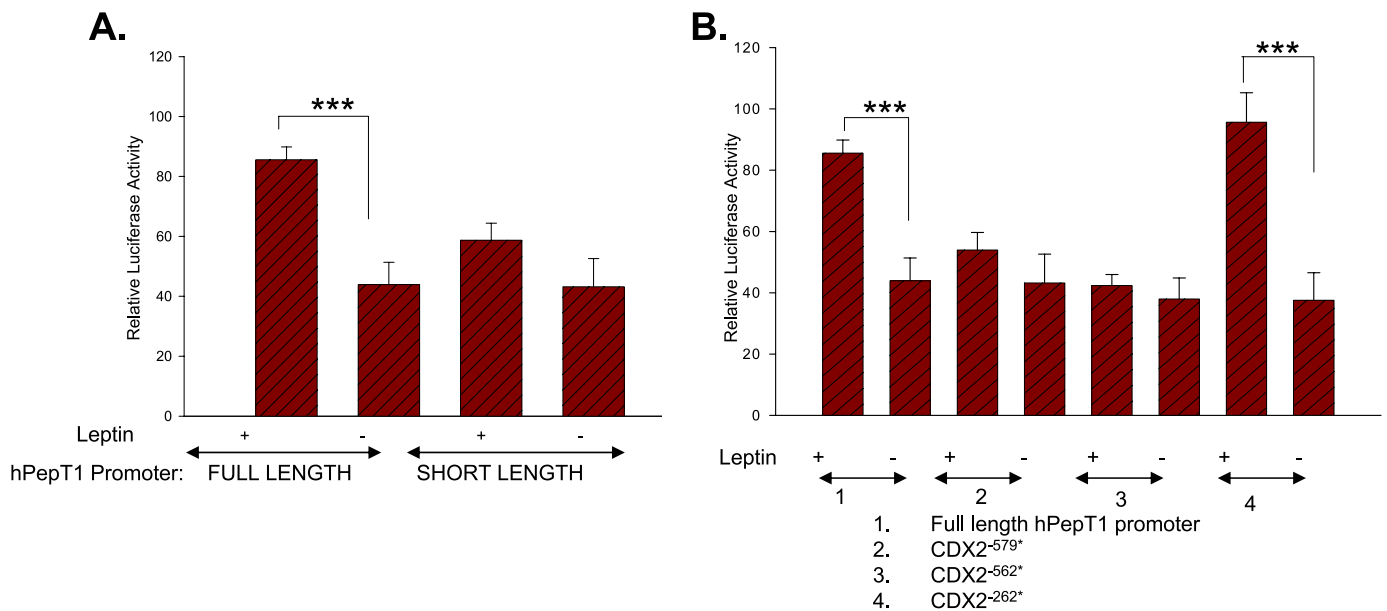


FIGURE 11. Cdx2 is crucial for leptin-induced hPepT1 promoter activity. Caco2-BBE cells were cotransfected with *Renilla* luciferase control plasmids and reporter plasmids containing the full-length hPepT1 promoter or 324 bp of the hPepT1 promoter lacking the three Cdx2 binding sites (short length) (A), or the full-length wild-type hPepT1 promoter or full-length sequences containing mutations of the Cdx2 binding sites at -579 (Cdx2^{-579*}), -562 (Cdx2^{-562*}), or -262 (Cdx2^{-262*}) (B). Transfectants were treated with 100 nM leptin (+) or with vehicle (-) for 8 h, and luciferase activity was determined. The data represent fluorescence ratios relative to unstimulated transfected cells ± S.D. observed in two separate experiments plotted as mean ± S.D. from duplicate determinations of two samples per group (n = 3). ***, p < 0.001.

CDX2 protein domains

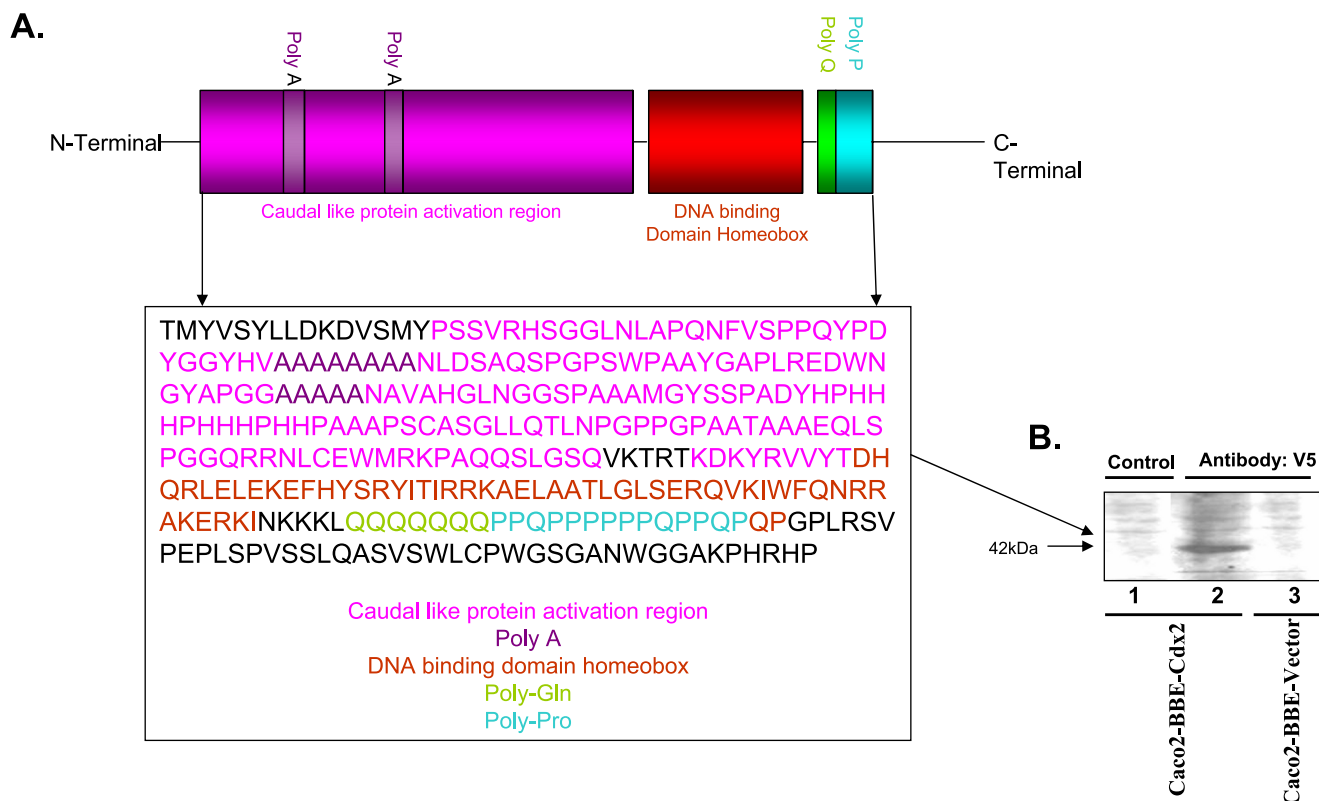


FIGURE 12. **Overexpression of Cdx2 in Caco2-BBE.** *A*, Cdx2 is expressed in confluent Caco2-BBE cells and is composed of caudal-like protein activation and a DNA binding domain homeobox regions. *B*, we cloned Cdx2 from Caco2-BBE cells and generated a Caco2-BBE cell line overexpressing Cdx2. Caco2-BBE cells stably transfected with Topo vector containing V5-tagged Cdx2 (lanes 1 and 2) or empty Topo vector (lane 3) were lysed, total cell proteins (20 μ g/lane) were resolved with 4–20% SDS-PAGE and transferred to a nitrocellulose membrane, and the blot was immunostained with mouse IgG (lane 1) and a mouse anti-V5 antibody (lanes 2 and 3).

hPepT1 from intracellular pools to the apical plasma membrane (26). This effect of leptin was also observed in the small intestine, where PepT1 is normally expressed in the apical membrane of absorptive cells (26). However, the present findings suggest that leptin may also up-regulate transcription of the gene encoding hPepT1.

In the present study, we demonstrated that long term leptin treatment increased hPepT1 protein expression and the associated hPepT1 transport activity in Caco2-BBE cells. The effect of leptin on the hPepT1 promoter and transport activities were both abrogated when cells were treated for 24 h with leptin. We could speculate that some negative signaling feedback on hPepT1 expression could occur under these conditions. This effect was observed regardless of whether leptin was added to the apical or basolateral membrane of the Caco2-BBE monolayers, which contrasts with the previous observation that short term leptin treatment at the apical but not basolateral side enhanced hPepT1 transport events in Caco2-BBE monolayers (26). Together, these observations suggest that short and long term leptin treatments enhance hPepT1 activity via different mechanisms in Caco2-BBE cells. Our present results indicate that the long term enhancement is due at least in part to leptin-induced transcription of the gene encoding hPepT1. The exogenous leptin used in our study is ~10 times its endogenous plasma levels. However, additional pool of leptin in inflamed

colonic epithelial cells (25) could provide high local concentration of leptin and affect colonic cellular functions. Indeed, it has previously been shown by pharmacokinetic studies with exposure with exogenous 125 I-leptin that the gastrointestinal tract has the highest local concentration and uptake of leptin, followed by the stomach and liver (35). Therefore, the presence of its receptors strongly suggests a local action of leptin. Furthermore, the long term leptin treatments with leptin concentration of 100 nM, similar to that used in our study, have been shown to have functional effects such as significantly increased bromodeoxyuridine-uptake in adherent clonal cells derived from human gastric adenocarcinoma cells and to inhibit insulin-stimulated glucose uptake in L6 rat skeletal muscle cells (36, 37). Although other factors could contribute to the increased of hPepT1 transcription associated with intestinal inflammation *in vivo*, our results highlight a potentially important direct effect of leptin for increasing hPepT1 transport activity in colonic epithelial cells in intestinal inflammation.

In an effort to elucidate the signaling pathways underlying this transcriptional activation, we cloned and characterized the 5'-flanking region of the *hPepT1* gene in Caco2-BBE cells. The identified promoter region was 100% homologous to the one previously cloned from human genomic DNA (32) and did not harbor a TATA or CAAT box. We used RACE to identify the

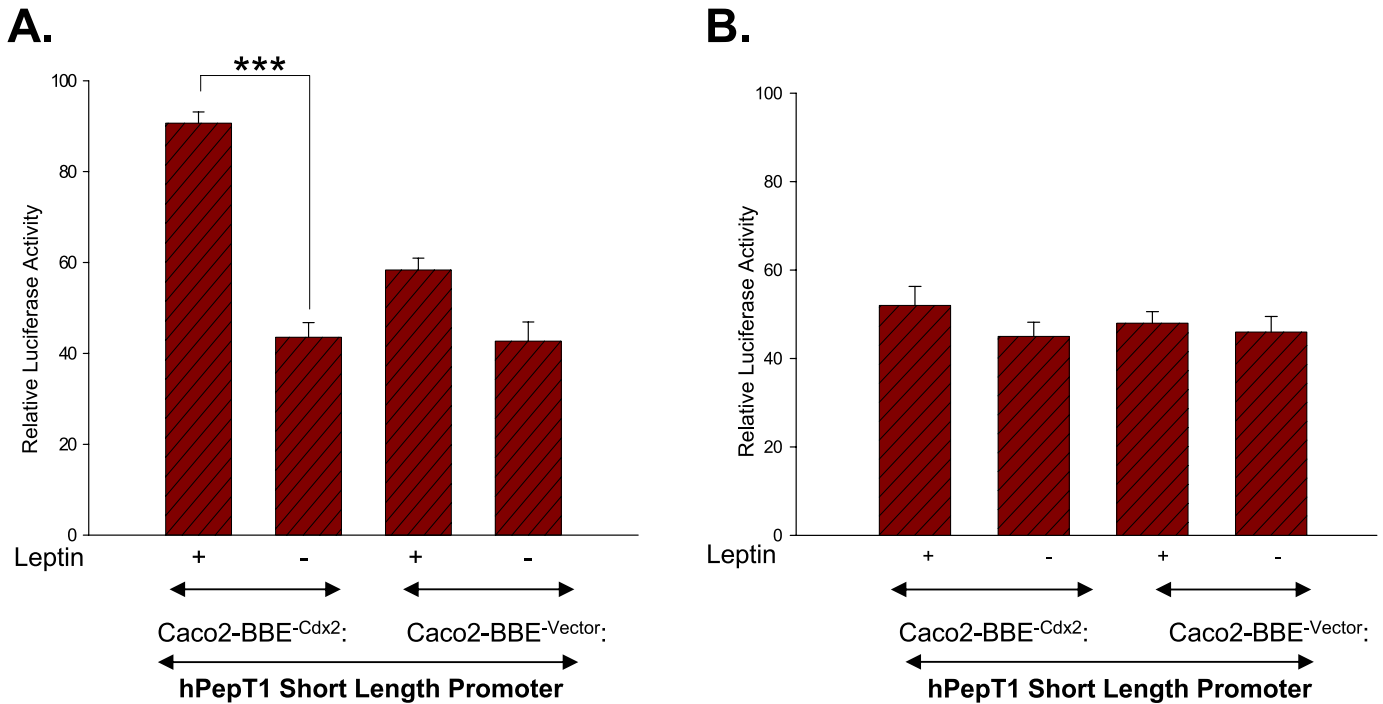


FIGURE 13. Cdx2-overexpressing Caco2-BBE cells enhance *hPepT1* promoter activity stimulated by leptin. Cdx2-overexpressing Caco2-BBE cells (Caco2-BBE^{-Cdx2}) or empty vector controls (Caco2-BBE^{-Vector}) were cotransfected with *Renilla* luciferase control plasmids and reporter plasmids, including the full-length *hPepT1* promoter (A) and the short-length construct described above (B). Transfectants were treated with 100 nM leptin or with vehicle (-) for 8 h, and luciferase activity was determined. The data represent fluorescence ratios relative to unstimulated transfected cells ± S.D. observed in two separate experiments plotted as mean ± S.D. from duplicate determinations of two samples per group (n = 3). ***, p < 0.001 versus vehicle.

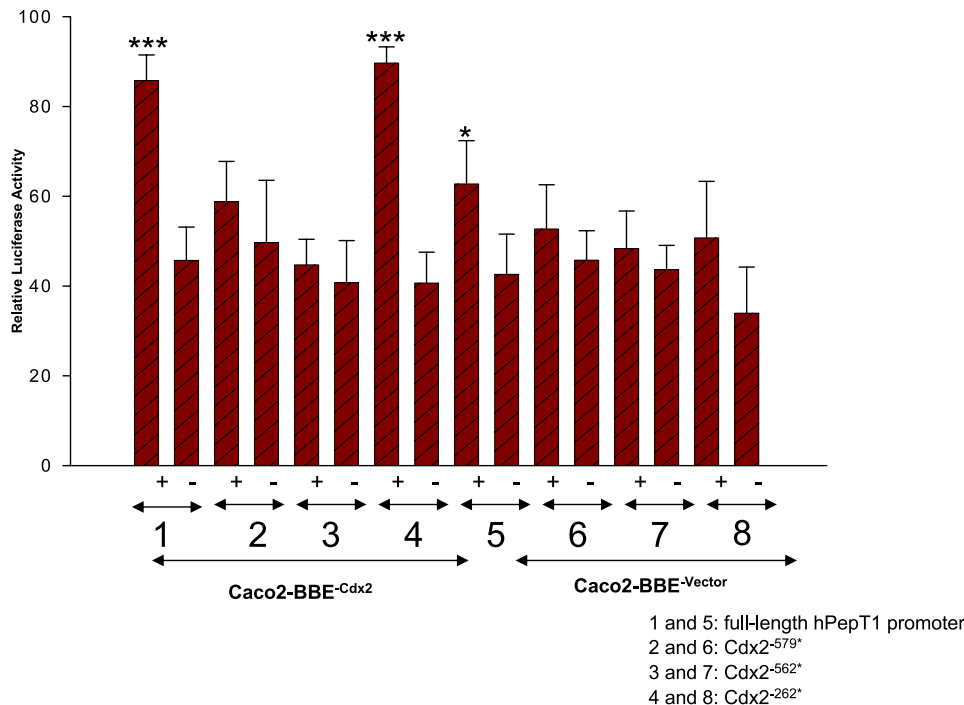


FIGURE 14. Leptin treatment triggers an association between Cdx2 and pCREB. Cdx2-overexpressing Caco2-BBE cells (Caco2-BBE^{-Cdx2}) or empty vector controls (Caco2-BBE^{-Vector}) were co-transfected with *Renilla* luciferase control plasmids and reporter plasmids, including the full-length wild-type *hPepT1* promoter (1 and 5) or full-length sequences containing mutations of the Cdx2 binding sites at -579 (Cdx2^{-579*}) (2 and 6), -562 (Cdx2^{-562*}) (3 and 7), or -262 (Cdx2^{-262*}) (4 and 8). Transfectants were treated with 100 nM leptin (+) or with vehicle (-) for 8 h, and luciferase activity was determined. The data represent fluorescence ratios relative to unstimulated transfected cells ± S.D. observed in two separate experiments plotted as mean ± S.D. from duplicate determinations of two samples per group (n = 3). ***, p < 0.001 versus vehicle.

transcription initiation site of the human *hPepT1* gene for the first time and identified a single major transcription initiation site located 86 bp upstream from the first ATG codon. The transcription start sites of the rat and mouse *PepT1* genes have been located at 31 and 80 bp upstream of the start ATG, respectively (38, 39), indicating that the transcriptional start sites (and possibly promoter activities) differ by species.

Further analysis of the *hPepT1* promoter region identified a CREB binding site downstream from the transcription start site, suggesting that cAMP and/or CREB may be involved in regulation of *hPepT1* transcription. Previous studies have shown that leptin treatment can elevate intracellular cAMP levels in different cell models. For example, leptin induces lipolysis and inhibits insulin secretion by enhancing adenylate cyclase and cAMP in pancreatic B cells (40). In the present work, we showed that leptin treatment enhanced the activity of a luciferase reporter construct con-

Leptin Increases *hPepT1* Transcription Activity

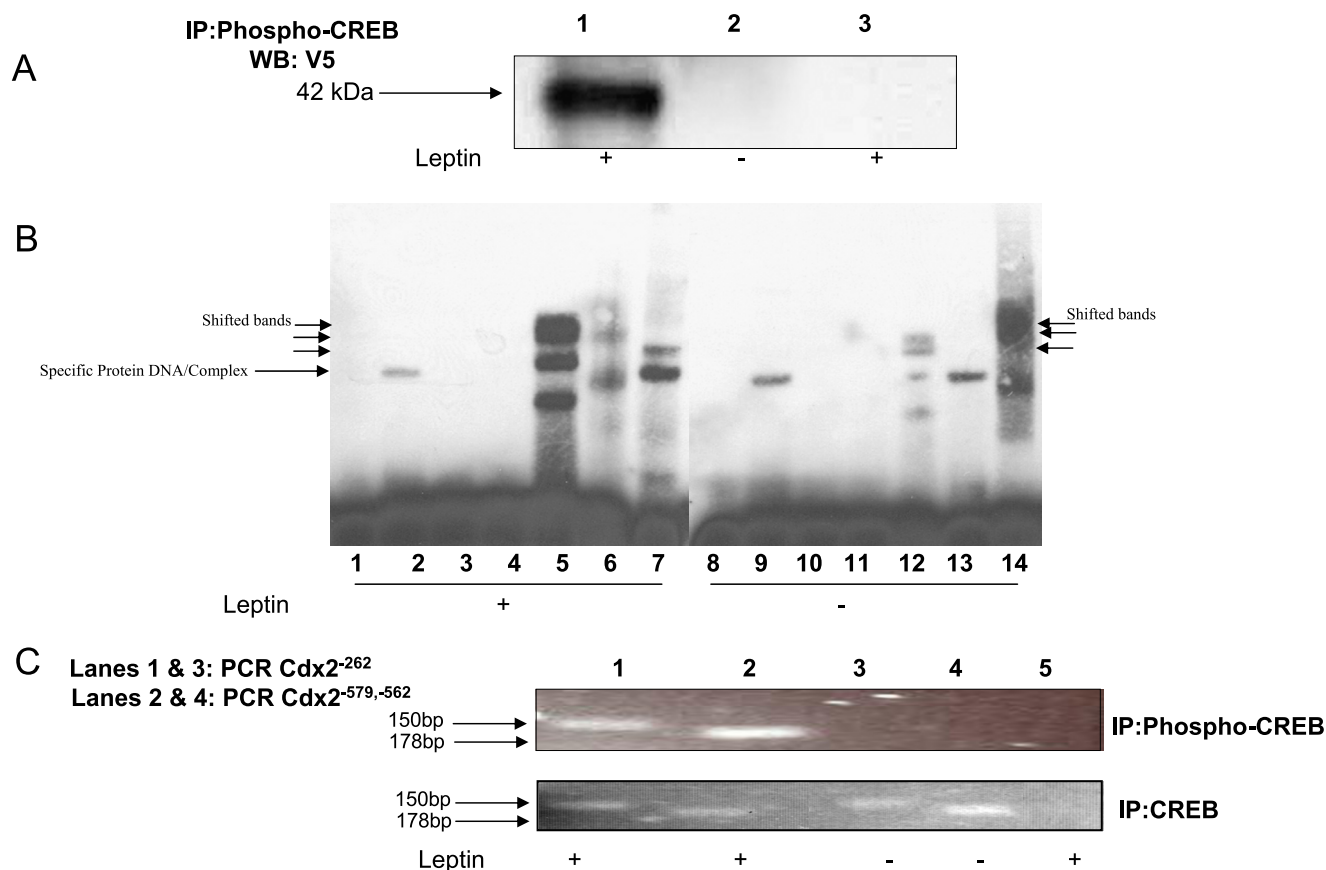


FIGURE 15. Leptin induces association between pCREB and Cdx2 and binding to the *hPepT1* promoter. *A*, Caco2-BBE cells overexpressing Cdx2 (lanes 1 and 2) or transfected with vector alone (lane 3) were treated with 100 nM leptin for 30 min (lanes 1 and 3) or un-treated (lane 2). Cells were lysed, and the lysates were immunoprecipitated anti-pCREB (lanes 1–3). The immunoprecipitates were subject to 8% SDS-PAGE and transferred to nitrocellulose membranes, and the blot was immunostained with an anti-V5 antibody. *B*, nuclear extracts (5 μ g) from 30-min leptin treated (lanes 2–7) or un-treated Caco2-BBE (lanes 9–14) were incubated with biotin-labeled oligonucleotides (lanes 2 and 9), with biotin-labeled oligonucleotides in the presence of a 200-fold excess of cold competitor oligonucleotide (lanes 3 and 10), with mutated biotin-labeled oligonucleotides (lanes 4 and 11), Cdx2 oligonucleotide with antibodies against CDX2 (lanes 5 and 12) or pCREB (lanes 6 and 13) and CREB (lanes 7 and 14) for electrophoretic mobility and supershift assays. Lanes 1 and 2 represent biotin-labeled oligonucleotide alone. *C*, soluble chromatin was prepared from Caco2-BBE cells treated with 100 nM Leptin or vehicle for 30 min. Protein-bound DNA complexes were immunoprecipitated with antibody against p-CREB or against CREB. After cross-link reversal, purified DNA was amplified with primers specific for the Cdx2⁻²⁶² (lanes 1 and 3) or Cdx2^{-579,-562} (lanes 2 and 4). PCR products were amplified using diluted chromatin that was not immunoprecipitated (lane 5). Gel shown of PCR products is representative of two separate experiments.

taining the wild-type CREB sequence from the 5'-flanking region of the *hPepT1* gene, but not that of a construct containing mutations at the CREB site, indicating that an intact CREB site is necessary for leptin-induced *hPepT1* promoter activity. In contrast, our results revealed that the presence or absence of the CREB site did not appear to affect the basal transcriptional activity of *hPepT1* in Caco2-BBE cells, suggesting that CREB binding may be related to leptin-mediated (*i.e.* inflammatory) signaling.

In a given cell type, only a small portion of the possible CREB target genes are induced by cAMP under each condition (41, 42). This flexibility requires the involvement of additional CREB regulatory partners for promoter activation. For example, CREB-binding protein CBP/p300 acts as a cofactor for transcriptional activation by CREB (43). The Cdx proteins are involved in the regulation of intestine-specific genes, such as sucrase-isomaltase, intestinal phospholipase A/lysophospholipase, lactase-phlorizin hydrolase, p21, claudin-2, and the iron transport protein hephaestin (2, 7, 26, 32, 43). Cdx2 itself appears to play critical roles in gut differentiation, proliferation, and neoplasia (44), and it was recently demonstrated that the

gastric mucosa of transgenic Cdx2-overexpressing mice are morphologically changed to intestinal metaplastic mucosa, including microvilli, and *PepT1* expression (44). Notably, our promoter sequence analysis identified three putative Cdx2 binding sites, causing us to speculate that Cdx2 might also be involved in regulating leptin-induced *hPepT1* gene transcription. Further experiments revealed that overexpression of Cdx2 in Caco2-BBE monolayers enhanced leptin-induced *hPepT1* promoter activity but that this effect was abrogated in *hPepT1* promoter constructs lacking the Cdx2 binding sites. More specifically, the two distal Cdx2 binding sites, located at -579 and at -562, were critical to leptin-induced *hPepT1* promoter activity, whereas the Cdx2 site located at -262, proximal to the CREB binding site, did not affect leptin-stimulated *hPepT1* promoter activity.

We then assessed whether CREB and Cdx2 appeared to interact with one another during leptin-induced transcription of *hPepT1*. Our results revealed that pCREB protein levels increased following leptin stimulation, and pCREB and Cdx2 could be co-immunoprecipitated from leptin-stimulated Caco2-BBE cells but not from resting Caco2-BBE cells. Con-

sistent with the latter result, gel-shift and chromatin immunoprecipitation analyses revealed that pCREB interacts with the Cdx2 binding sites after leptin stimulation. Based on our collective findings, we herein propose that the presence of leptin in inflamed colonic mucosa (25) could result to a local high concentration of leptin in contact with colonocytes. Our results suggest that the presence of high leptin concentrations result in increased intracellular cAMP in the colonic epithelial cells, triggering intracellular protein kinase signaling cascades, such as the second messenger system, leading to the subsequent phosphorylation/activation of CREB and Cdx2, which is followed by increased expression of hPepT1 and enhanced uptake of the small di-tri bacterial products that perpetuate intestinal inflammation (21). These findings provide important new insights into intestinal inflammation and may lead to new therapeutic modalities in the future.

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