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Transcriptional Regulation of the Glucose-6-phosphatase Gene by cAMP/Vasoactive Intestinal Peptide in the Intestine

ROLE OF HNF4 α , CREM, HNF1 α , and C/EBP α *

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Gluconeogenesis is induced in both the liver and intestine by increased cAMP levels. However, hepatic and intestinal glucose production can have opposite effects on glucose homeostasis. Glucose release into the portal vein by the intestine increases glucose uptake and reduces food intake. In contrast, glucose production by the liver contributes to hyperglycemia in type II diabetes. Glucose-6-phosphatase (Glc6Pase) is the key enzyme of gluconeogenesis in both the liver and intestine. Here we specify the cAMP/protein kinase A regulation of the Glc6Pase gene in the intestine compared with the liver. Similarly to the liver, the molecular mechanism of cAMP/protein kinase A regulation involves cAMP-response element-binding protein, HNF4 α , CAAT/enhancer-binding protein, and HNF1. In contrast to the situation in the liver, we find that different isoforms of CAAT/enhancer-binding protein and HNF1 contribute to the specific regulation of the Glc6Pase gene in the intestine. Moreover, we show that cAMP-response element binding modulator specifically contributes to the regulation of the Glc6Pase gene in the intestine but not in the liver. These results allow us to identify intestine-specific regulators of the Glc6Pase gene and to improve the understanding of the differences in the regulation of gluconeogenesis in the intestine compared with the liver.

Endogenous glucose production results from glycogenolysis and gluconeogenesis. Gluconeogenesis is induced in insulinopenia states (*i.e.* fasting and diabetes) in the gluconeogenic organs: liver, kidney, and intestine (1–4). However, each gluconeogenic organ produces different amounts of glucose, and their production changes during fasting (5, 6). In postabsorptive and short fasting states, the liver is considered the major gluconeogenesis site. During fasting, glucose production decreases in the liver, whereas glucose production increases in the kidney and intestine (4–6). Increased hepatic glucose production is a major feature of type II diabetes (7, 8). In line with this, the expression of the key enzyme involved in glucose pro-

duction, glucose-6-phosphatase (Glc6Pase),² is increased in the liver in several animal models of diabetes (2, 9–11) and in diabetic humans (12). Accordingly, the induction of the Glc6Pase gene in hepatocytes increases glucose production and reduces glycogen synthesis (13), and overexpression of Glc6Pase in rat liver induces several of the abnormalities associated with early stage non-insulin-dependent diabetes mellitus (11). Whereas the induction of glucose production by the liver seems to exert a deleterious effect on glucose tolerance, glucose appearance in the portal vein may play a beneficial role in glucose homeostasis. For example, glucose release into the portal vein stimulates both peripheral and liver glucose uptake (14–16). Glucose release into the portal vein also proportionally inhibits hepatic glucose production (17, 18). Moreover, induction of intestinal glucose production by a protein-enriched diet has recently been shown to reduce food intake without altering endogenous glucose production (18). In contrast to hepatic glucose production, intestinal glucose production might have beneficial consequences on obesity and type II diabetes. Therefore, it is of general interest to specify the mechanism involved in the initiation of intestinal glucose production with regard to hepatic glucose production.

Intestinal gluconeogenic function depends on the expression of three key enzymes, glutaminase, phosphoenolpyruvate carboxykinase, and Glc6Pase (4, 10, 19), which is the most important to trigger glucose production (6, 20). Although regulation of the Glc6Pase gene in the liver has been extensively studied (21–30), little is known about the specific transcriptional control of the gene in the intestine (31, 32). In the intestine, Glc6Pase gene expression is strongly induced after birth and then rapidly decreases around the time of suckling-weaning transition (33). At a later stage, the Glc6Pase gene is expressed from duodenum to jejunum in normally fed rats and from duodenum to ileum in humans (10). Intestinal Glc6Pase mRNA is strongly induced in diabetic or fasted rats and is normalized upon insulin treatment or refeeding, respectively (10). Intracellular cAMP levels are increased by diabetes and fasting. Increased cAMP levels activate protein kinase A (PKA), which in turn phosphorylates cAMP-response element binding pro-

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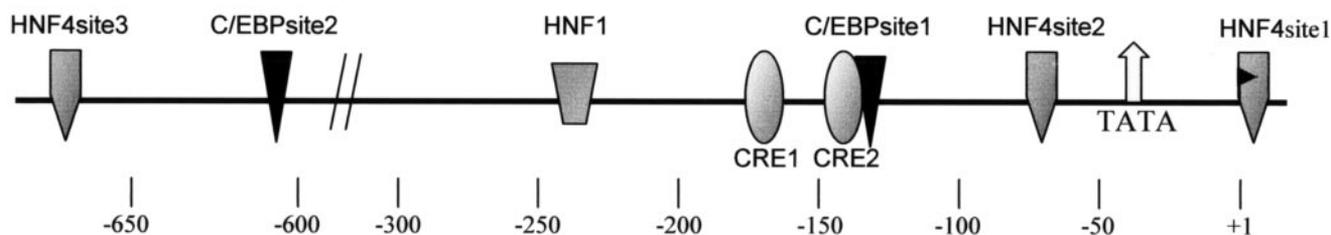
² The abbreviations used are: Glc6Pase, glucose-6-phosphatase; C/EBP, CAAT/enhancer-binding protein; CREB, cAMP-response element-binding protein; CREM, cAMP-response element binding modulator; PKA, protein kinase A; VIP, vasoactive intestinal peptide; PP, postprandial; CBP, CREB-binding protein.

TABLE 1

Mutated transcription factor binding sites

Mutated nucleotides are indicated in bold face type. The nucleotide positions of the binding sites are numbered relative to the transcription start site at +1.

HNF4 site 1	GATAGCAGA AATTCGT AGGGCCAGCCAGG (+2/+14) (23)
HNF4 site 2	TGTTTTTTT GCTTCAAGCTTGAC GGGCTAGGT (-79/-67) (39)
HNF4 site 3	TGGGTACGGAAAC GATATC CAGGTCTC (-668/-647) (23)
C/EBPsite1	ACATGTTTGCAT ATCGATA CTGAGATGCA (-135/-130) (27)
HNF1	TGTGCCCAAGTTAATA CGCGGTT CTGCCAAT (-214/-208) (34)
C/EBPsite2	GCCATCTCTCCA AGTCGAC GAAAAACATTT (-608/-603) (27)
CRE1	GTTTTGCTATTTT GAATTCAT CACCCTGAA (-165/-158) (27)
CRE2	CACCCTGAACATGTTT GAACTC ACCTACTG (-141/-134) (27)



teins (CREBs). PKA and CREB induce the transcription of the Glc6Pase gene in the liver and kidney (24, 25, 27, 28, 34). As a related point, CREB and PKA have also been shown to mediate the transcriptional effect of peptone hydrolysates on intestinal genes (cholecystokinin and proglucagon) (35, 36). The gluconeogenic genes phosphoenolpyruvate carboxykinase and Glc6Pase are endowed with CREB binding sites (24, 37) and are induced by a protein diet in the intestine but not in the liver (18). Thus, the study of the intestinal mechanism of cAMP regulation of the Glc6Pase gene might be useful to understand the mechanism of induction of intestinal glucose production as compared with hepatic glucose production.

In this study, we characterized the mechanism of cAMP regulation of the Glc6Pase gene in the intestine compared with the liver. In the liver, tissue-specific factors, such as HNF4 α (hepatocyte nuclear factor 4 α), HNF6, and CAAT/enhancer-binding protein (C/EBP) proteins have been involved in PKA regulation of the Glc6Pase gene (25, 27, 28, 38). In kidney cells, HNF1 has been suggested to play a special role in PKA regulation of the Glc6Pase gene (34). We have thus considered the contribution of these factors, which are all expressed in the small intestine, to the intestinal Glc6Pase gene transcription. Here we show that 1) Glc6Pase promoter activity is induced by cAMP and PKA in the intestine; 2) the molecular mechanism is similar to the liver but is slightly different; and 3) it involves the same families of transcription factors as in the liver, but different isoforms may contribute to the specific regulation in each tissue.

EXPERIMENTAL PROCEDURES

Reporter Plasmids, Expression Vectors, and Antibodies—The rat Glc6Pase promoter constructs containing regions up to nucleotide -1,640 relative to the transcription start site and cloned into the “pGL2 basic” vector (indicated by “B” in construct names; Promega) upstream of a luciferase reporter

gene and its mutant forms were previously described (see Table 1) (23, 27, 39). The pCR3-HNF4 α expression vector encodes the HNF4 α protein, the PKA expression vector encodes the catalytic subunit of protein kinase A (generous gifts from M. Raymondjean and B. Viollet) (40), and the recombinant expression vector pDGT.23-1 encodes a dominant negative form of HNF4 α protein (generous gift from T. Leff) (41). Plasmids used for transfection were purified using the Plasmid maxikit (Jet Star; Genomed).

The following commercially available antibodies were used for chromatin immunoprecipitation or Western blot assays: CBP (sc-369), HNF4 α (sc-8987), C/EBP α (sc-9315), C/EBP β (sc-150), β -tubulin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and CREB (New England Biolabs). The antibody against cAMP-response element binding modulator (CREM) was previously described (42), and the antibodies against HNF1 α and HNF1 β were generous gifts from M. Pontoglio (43).

Animals and Tissue Sampling—Male Sprague-Dawley rats (260–280 g) were housed as previously described (6). Fasted rats were deprived of food for 48 h with free access to water. About 1 g of liver and 2 cm of intestine (jejunum) were sampled as previously described (6). This protocol was performed according to the rules of our local ethics committee for animal experimentation. Each tissue sample was cut in small pieces, immersed in 10 ml of 1% formaldehyde in phosphate-buffered saline, and fixed at room temperature for 10 min. Cross-linking was stopped by adding 1 M glycine directly to the medium at a final concentration of 125 mM for 10 min, and samples were rinsed twice with ice-cold phosphate-buffered saline and then frozen in liquid nitrogen.

Cell Culture and Transfection—HepG2 human hepatoma cells and CaCo2 human colonic cells were grown in Dulbecco's

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modified Eagle's medium supplemented with 6% (HepG2) or 15% (CaCo2) fetal bovine serum, 5 mM glutamine, streptomycin (1 μ g/ml), and penicillin (1 unit/ml) at 37 °C in a humidified 5% CO₂, 95% air atmosphere. For transient transfection, 1 day (for HepG2) or 2 days (for CaCo2) before transfection, 200,000 cells were plated out in 35-mm wells in 6-well cell culture plates. Complete medium was refreshed 1 h prior to transfection. HepG2 and CaCo2 cells were transfected using ExGen500 (Euromedex) as recommended by the supplier (23), with 1 μ g of *Glc6Pase*-LUC plasmid, 1 ng of pCMV-RL (Promega) to correct for transfection efficiency, and 100 ng of expression vectors, except when differently indicated in the figure. The total amount of DNA (2 μ g) was kept constant by the addition of pBluescript SK+ plasmid. The cells were harvested 48 h after transfection and then washed three times with phosphate-buffered saline and lysed with passive lysis buffer (Promega). *Renilla* luciferase and firefly luciferase activities were determined with a BCLBook luminometer (Promega) using the Dual-Luciferase kit assay reagent (Promega). The levels of firefly luciferase activities were normalized by means of the *Renilla* luciferase activities. For stable transfection, CaCo2 cells were transfected as described above with the -1640/+60B construct containing a gene conferring resistance to puromycin. Two days after transfection, transfected clones were selected by treatment with 2 μ g/ml puromycin and were named *Glc6Pase*-CaCo2 cells. For the differentiation process, 400,000 *Glc6Pase*-CaCo2 cells were plated out in 35-mm wells in 6-well cell culture plates, and cultured for 15 days. Firefly luciferase activity was determined as described above. Protein concentration in the lysates was determined with the Bradford method. The levels of firefly luciferase activities were normalized to the protein concentrations. Cells were treated with forskolin, vasoactive intestinal peptide (VIP), or glucagon (Sigma) in serum-free Dulbecco's modified Eagle's medium supplemented with glutamine and antibiotics, as indicated in the figures. Measure of cAMP level was performed on differentiated and undifferentiated CaCo2 cells with the cAMP-¹²⁵I direct biotrack assay (Amersham Biosciences) as described by the supplier. Statistical analyses were performed using Student's *t* test for unpaired data.

Western Blot—30 μ g of whole cell extracts from HepG2, CaCo2, differentiated CaCo2, and HNF4 α -transfected CaCo2 cells, were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to Immobilon membrane (Millipore Corp.). Immunoblotting was performed using anti-HNF4 (1:1000), anti-CREM (1:1000), or anti-CREB (1:1000) antibodies and visualized by chemiluminescence. Membranes were stripped with Reblot plus strong solution (Euromedex) and analyzed for β -tubulin expression as a control.

Chromatin Immunoprecipitation Assay—About 500 mg of tissue was reduced to powder in liquid nitrogen, lysed in 20 ml of cell lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, pH 8) for 20 min at 4 °C and then homogenized in a Dounce potter. After centrifugation (2,500 \times g, 8 min, 4 °C), nuclei in the pellet were lysed in 20 ml of nuclei lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 0.5 M NaCl, pH 8, 1% Triton, 0.5% sodium deoxycholate, 0.5% Sarcosyl, 1 mM phen-

ylmethylsulfonyl fluoride, 1 μ g/ml aprotinin, 1 μ g/ml leupeptin) for 20 min at 4 °C. Chromatin obtained was then sheared with the enzymatic shearing kit (Active Motif) as recommended by the supplier, yielding chromatin fragments of 200–500 bp in size. Samples were centrifuged at 14,000 \times g for 4 min at 4 °C to remove debris, and the supernatant was collected. 50 μ l of the supernatant was kept as a positive control (input). Each immunoprecipitation was performed with about 50 μ g of chromatin in radioimmune precipitation buffer (140 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, pH 8). To reduce nonspecific background, each chromatin sample was precleared with 60 μ l of a solution of protein A-Sepharose (100 mg/ml radioimmune precipitation buffer), supplemented with 300 μ g/ml sonicated salmon sperm DNA (Fermentas) and 1 mg/ml bovine serum albumin, for 1 h at 4 °C on a rotating wheel. Chromatin complexes were immunoprecipitated for 16–18 h at 4 °C while rotating with 10 μ g of primary antibody or without antibody (mock) as a negative control. Immune complexes were collected with 40 μ l of a solution of protein A-Sepharose (100 mg/ml), supplemented with 300 μ g/ml sonicated salmon sperm DNA (Fermentas) and 1 mg/ml bovine serum albumin, for 3 h at 4 °C on a rotating wheel, followed by centrifugation at 14,000 \times g for 30 s at 4 °C. The beads were washed five times for 10 min at 4 °C with radioimmune precipitation buffer, once with LiCl washing buffer (10 mM Tris-HCl, pH 8, 250 mM LiCl, 1 mM EDTA, pH 8, 0.5% Nonidet P-40, 0.5% sodium deoxycholate), and twice with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8). To digest RNA and proteins present in the samples, RNase (50 mg/ml), SDS (1%), and proteinase K (400 μ g/ml) were added, and the samples were incubated overnight at 37 °C. DNA was purified by phenol/chloroform extraction and precipitated in the presence of 100 μ g of glycogen. PCR amplification was performed using primers specific for the -326/+150 bp region of the *Glc6Pase* gene (forward, 5'-CAGGAGCCACACAGTTGAAACAGA-3'; reverse, 5'-AGGGT-GATTTACGTAAAATAGCAAA-3') or the -174/+44 bp region of the *Glc6Pase* gene (forward, 5'-TTTGCTATTT-TACGTAAATCACCCCT-3'; reverse, 5'-GTACCTCAGGAA-GCTGCCA-3').

RESULTS

Induction of the *Glc6Pase* Promoter Activity by PKA: Synergistic Effect of HNF4 α in CaCo2 Cells—HNF4 α plays a key in the transcription of *Glc6Pase* in the liver (32). Moreover, the *Glc6Pase* promoter exhibits a basal activity in transiently transfected CaCo2 cells, albeit lower than in HepG2 cells (32). Since the *Glc6Pase* gene was induced during the differentiation process of CaCo2 cells (10), we analyzed the amount of HNF4 α in these cells by Western blot. Consistent with a role of HNF4 α in this process, the amount of HNF4 α protein was much higher in differentiated CaCo2 cells than in undifferentiated cells. In undifferentiated cells, the amount of HNF4 α protein was similar to the level obtained in differentiated cells or in HepG2 cells when 50 ng of HNF4 α expression vector was co-transfected (Fig. 1A). Moreover, overexpression of HNF4 α increased *Glc6Pase* promoter activity by about 4–5-fold (Fig. 1B, *white*

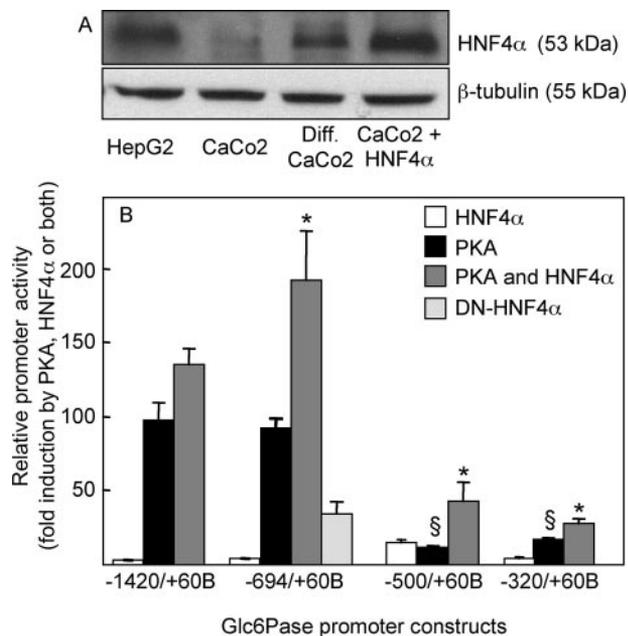


FIGURE 1. Regulation of the Glc6Pase promoter activity by PKA and HNF4 α . *A*, HNF4 α expression was analyzed by Western blotting on whole cell extracts from HepG2, CaCo2, differentiated CaCo2, and undifferentiated CaCo2 transfected with HNF4 α . Total protein content was assessed by β -tubulin expression. *B*, undifferentiated CaCo2 cells were transiently transfected with Glc6Pase promoter constructs in the presence of HNF4 α (white bars), PKA (black bars), PKA and HNF4 α (dark gray bars), or DN-HNF4 α (light gray bar). Results are expressed as -fold induction over basal promoter activity and are expressed as a mean \pm S.E. of six independent experiments. *, value significantly different from induction of promoter activity by PKA ($p < 0.05$). §, value significantly different from induction of promoter activity by PKA of $-694/+60B$ and $-1420/+60B$ constructs ($p < 0.01$).

bars). These data suggested a key role for HNF4 α in the induction of the transcription of the Glc6Pase gene in the intestine.

In the liver, PKA has been shown to induce Glc6Pase promoter activity by a mechanism involving HNF4 α (27). To characterize an interaction between PKA and HNF4 α in the regulation of the Glc6Pase gene in the intestine, serial 5'-deleted promoter fragments (from -1420 to -320 bp) were transiently transfected in CaCo2 cells in the presence of the catalytic subunit of PKA. The activity of all promoter fragments was markedly increased by PKA (Fig. 1*B*, black bars). As previously described in HepG2 cells (27), deletion of the promoter sequence from -694 to -500 bp resulted in a strong decrease of PKA stimulation, from about 100-fold for the $-1420/+60B$ and the $-694/+60B$ constructs to about 20-fold for the $-500/+60B$ and the $-320/+60B$ constructs (Fig. 1*B*, black bars). Interestingly, the promoter activity was much higher in the presence of both HNF4 α and PKA than in the presence of PKA or HNF4 α alone. For all constructs (except the $-1480/+60B$ construct), the effect was synergistic and not only additive (Fig. 1*B*). Consistent with these results, the induction of the promoter activity of the $-694/+60$ construct by PKA was strongly inhibited (60%) in the presence of a dominant negative form of HNF4 α (Fig. 1*B*). These results suggested that HNF4 α and PKA synergistically induce Glc6Pase transcription.

Relative Contribution of the Different HNF4 α Binding Sites to the Synergistic Induction of the Glc6Pase Activity—Three HNF4 α binding sites have been shown to contribute to the regulation of the Glc6Pase promoter activity by PKA in the

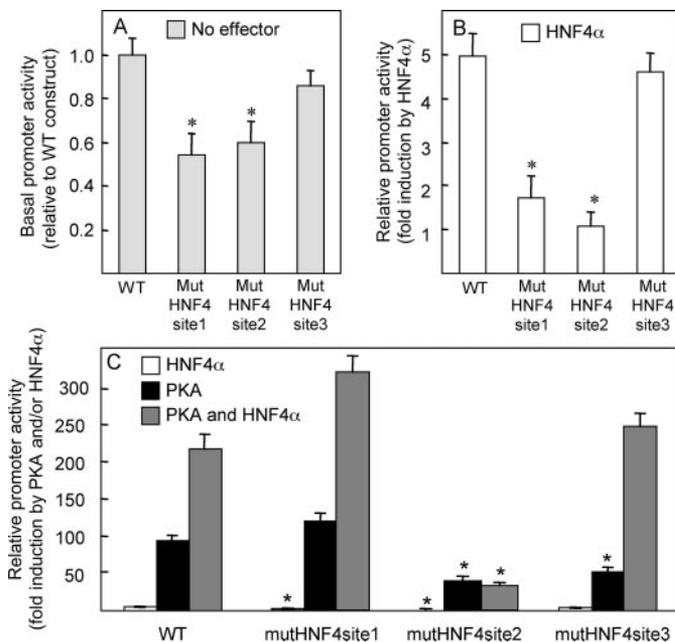


FIGURE 2. Contribution of HNF4 α binding sites to the regulation of the Glc6Pase gene by PKA. *A*, CaCo2 cells were transfected with the wild type $-694/+60B$ construct (WT) or the mutated constructs as indicated. Results are expressed relative to the activity of the $-694/+60B$ construct. *B* and *C*, CaCo2 cells were transfected with the wild type $-694/+60B$ construct or the mutated constructs as indicated in the presence of HNF4 α (white bar), PKA (black bar), or PKA and HNF4 α (dark gray bar). Results are expressed as -fold induction over the promoter activity in the absence of PKA and/or HNF4 α and are expressed as a mean \pm S.E. of six independent experiments. *, value significantly different from wild type construct ($p < 0.01$).

liver, whereas they had no individual role in the transcriptional activity (27). To further delineate the mechanism of the synergistic stimulation of the Glc6Pase promoter by PKA and HNF4 α in the intestine, we studied the relative importance of each of the three HNF4 binding sites. Regarding basal promoter activity, mutation of HNF4 site 1 ($+2/+14$ bp) resulted in a 47% decrease of the basal activity, and mutation of HNF4 site 2 ($-79/-67$) resulted in a 40% decrease of the basal activity, whereas mutation of HNF4 site 3 ($-668/-647$) had no significant effect (Fig. 2*A*). Moreover, mutations of HNF4 site 1 and HNF4 site 2 resulted in a 60 and 75% decrease of the HNF4 α -induced promoter activity, respectively, whereas deletion of HNF4 site 3 had no effect (Fig. 2*B*). Thus, HNF4 α (via sites 1 and 2) seems to have a crucial role in the basal activity of the Glc6Pase promoter in intestinal cells, but not in hepatic cells (27).

Regarding the induction of the promoter activity by PKA, mutation of HNF4 site 1 had no effect either on PKA induction of the Glc6Pase promoter activity or on the synergistic induction in the presence of HNF4 α (Fig. 2*C*, black bars and dark gray bars). In contrast, mutation of HNF4 site 2 led to a 70% decrease of the induction of the promoter activity by PKA (Fig. 2*C*, black bars) and completely abolished the synergistic induction of the promoter activity by PKA and HNF4 α (Fig. 2*C*, dark gray bars). Mutation of HNF4 site 3 resulted in a 40% decrease of the stimulation by PKA (Fig. 2*C*, black bars) but had no effect on the synergistic stimulation by HNF4 α and PKA (Fig. 2*C*, dark gray bars). To further delineate the role of HNF4 α binding sites, we performed double mutations of sites 1 and 3 and mutations of all three HNF4 binding sites on the $-694/+60B$ con-

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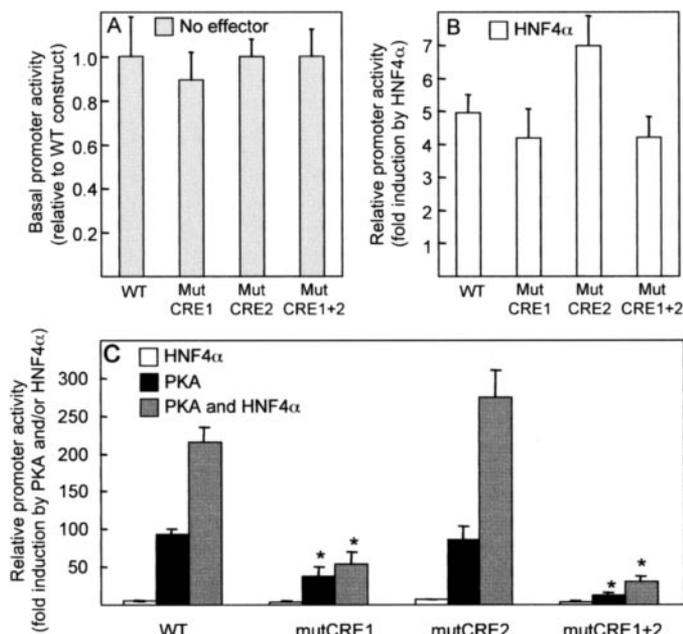


FIGURE 3. Contribution of CREB binding sites to the regulation of the *Glc6Pase* gene by PKA. *A*, CaCo2 cells were transfected with the wild type $-694/+60B$ construct or the mutated constructs as indicated. Results are expressed relative to the activity of the $-694/+60B$ construct. *B* and *C*, CaCo2 cells were transfected with the wild type $-694/+60B$ construct or the mutated constructs as indicated in the presence of HNF4 α (white bar), PKA (black bar), or PKA and HNF4 α (dark gray bar). Results are expressed as -fold induction over the promoter activity in the absence of PKA and/or HNF4 α and are expressed as a mean \pm S.E. of six independent experiments. *, value significantly different from wild type construct ($p < 0.01$).

struct. There were no additional effects on the synergy when both HNF4 sites 1 and 3 were mutated compared with the construct HNF4 site 3 (data not shown). The mutation of all HNF4 α binding sites resulted in a total loss of basal activity of the promoter construct (data not shown). The effect of this triple mutation was not studied further.

In summary, 1) HNF4 α site 1 and site 2, but not site 3, are crucial for basal *Glc6Pase* promoter activity; 2) HNF4 α site 2 and site 3, but not site 1, are involved in the PKA response of the *Glc6Pase* promoter; and 3) only HNF4 α site 2 is determinant for the synergistic induction of the *Glc6Pase* gene transcription by PKA and HNF4 α . HNF4 site 2 has been shown to recruit PGC-1 (peroxisome proliferator-activated receptor- γ co-activator-1) (39). However, we did not find any significant increase of the PKA stimulation of the *Glc6Pase* promoter activity by overexpression of PGC1 (data not shown).

Specific Involvement of CREM Protein in the PKA Regulation of the *Glc6Pase* Gene in the Intestine—PKA regulated transcription via the phosphorylation of the members of the CREB/CREM/activating transcription factor family. These factors bind a consensus palindromic 5'-TGACGTCA-3' sequence as homo- or heterodimers (see Refs. 44–46 for a review). Thus, we studied the contribution of CREB/CREM family proteins in the synergistic activation of the *Glc6Pase* gene by PKA and HNF4 α . CREB has been involved in the cAMP regulation of the *Glc6Pase* gene in the liver via two binding sites ($-165/-158$ bp named CRE1, and $-141/-134$ bp named CRE2) (24, 28). In CaCo2 cells, mutation of both CRE binding sites had no effect on either basal (Fig. 3A) or HNF4 α -induced promoter activities

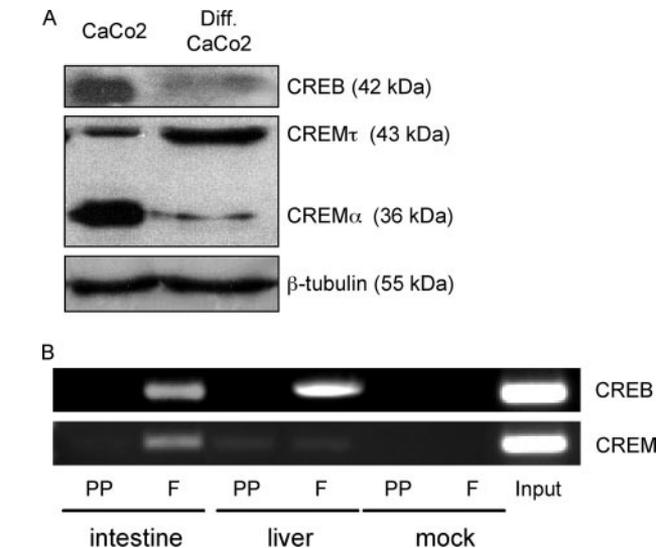


FIGURE 4. CREM contributes to the specific transcriptional regulation of the *Glc6Pase* gene in the intestine. *A*, protein expression was analyzed by Western blotting on whole cell extracts from CaCo2 and differentiated CaCo2 cells. Proteins were detected with anti-CREB (top) or anti-CREM (bottom). Total protein content was assessed by β -tubulin expression. *B*, CREM and CREB binding to the *Glc6Pase* promoter was analyzed by immunoprecipitation of chromatin from intestine or liver of postprandial (PP) or 48 h-fasted rats (F) with anti-CREM or anti-CREB antibodies or without antibody as a control (mock). The PCR amplifications of immunoprecipitated fragments of the $-174/+44$ bp region of the *Glc6Pase* gene are shown. The mock lane shows the results of samples precipitated without antibody. The input lane shows the result of samples not subjected to immunoprecipitation. The data shown are representative of at least two experiments.

(Fig. 3B). Mutation of the CRE1 site resulted in a 60% decrease of the stimulation of promoter activity by PKA (Fig. 3C, black bars) and in a strong decrease of the synergistic stimulation by PKA and HNF4 α (Fig. 3C, dark gray bars). Mutation of the CRE2 site had no effect (Fig. 3C). Interestingly, mutation of both sites almost completely abolished the stimulation by PKA either in the presence or the absence of HNF4 α (Fig. 3C). These results were consistent with results obtained in liver and kidney cells, where only CRE1 appears as a *bona fide* CRE, whereas CRE2 is an accessory site for the regulation by PKA (34). The synergistic induction of the promoter activity of the $-694/+60B$ construct by HNF4 α and PKA thus also appeared to depend on CREB binding sites.

The CaCo2 cell line is a known model of intestinal cells differentiation (47). Interestingly, CREM is a control gene for the differentiation process of spermatogenesis (48). Both activator (τ) and repressor (α , β , γ) isoforms are produced from the CREM gene (49). Since *Glc6Pase* gene expression increases during differentiation of CaCo2 cells, we characterized the presence of CREM and CREB proteins in this cell model. Western blot analyses showed that CREB is reduced during CaCo2 cell differentiation (Fig. 4A, upper panel). Conversely, we observed a switch in the expression of the repressor CREM α to the activator CREM τ during differentiation of CaCo2 cells (Fig. 4A, lower panel). This result suggests that CREM τ might be the principal activating protein of the CREB/CREM family controlling *Glc6Pase* gene expression in differentiating CaCo2 cells. To further document these data in the intestine, we studied the binding of CREB and CREM to the *Glc6Pase* promoter by chromatin immunoprecipitation. We used chromatin from liver

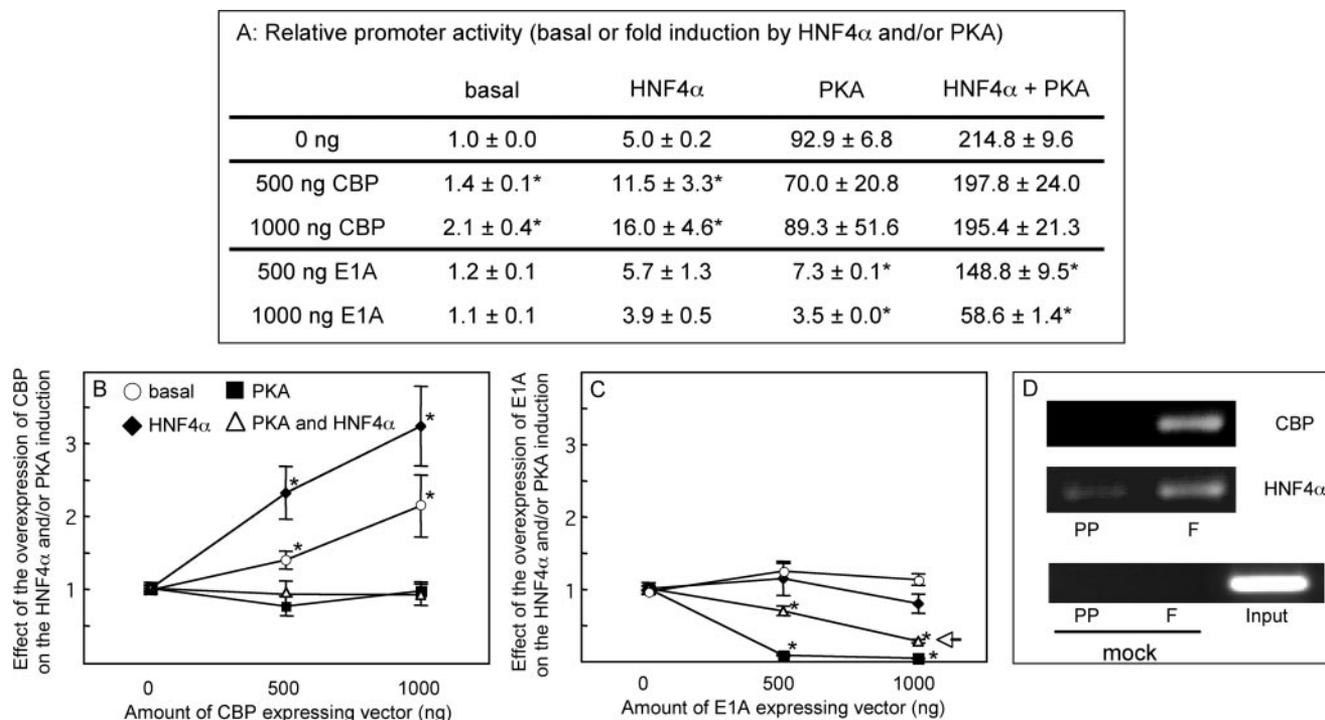


FIGURE 5. Effect of CBP on the stimulation of the Glc6Pase promoter activity. CaCo2 cells were transfected with the wild type $-694/+60B$ construct in the presence of increasing amounts of expression vectors of CBP or E1A. Promoter activity was analyzed in basal conditions (*white circles*) or in the presence of HNF4 α (*black diamonds*), PKA (*white triangles*), or PKA and HNF4 α (*black squares*). *A*, results are expressed as -fold induction over the basal promoter activity. *B* and *C*, graphics represent the effect of CBP (*B*) or E1A (*C*) on the -fold induction of the promoter activity by PKA and/or HNF4 α . Results are expressed as a mean \pm S.E. of six independent experiments. *, value significantly different from induction of promoter activity by CBP or E1A ($p < 0.01$). *D*, CBP and HNF4 α binding to the Glc6Pase promoter were analyzed by immunoprecipitation of chromatin from the intestine of postprandial (PP) or 48 h-fasted rats (F) with anti-CBP or anti-HNF4 α antibodies. The PCR amplifications of immunoprecipitated fragments of the $-174/+44$ bp region of the Glc6Pase gene are shown. The *mock lane* shows the results of samples precipitated without antibody. The *input lane* shows the result of samples not subjected to immunoprecipitation. The data shown are representative of at least two experiments.

and intestine of fed postprandial (PP) and 48 h-fasted rats. Fig. 4*B* shows that CREB and CREM were not bound to the intestinal Glc6Pase promoter in the fed PP state and that both bound to the promoter after fasting. In contrast, only CREB bound to the liver Glc6Pase promoter in fasting state (Fig. 4*B*), similar to the regulation of the Glc6Pase gene in the liver (27). Moreover, these data clearly suggest an important contribution of CREM to Glc6Pase gene expression specifically in the intestine.

CBP Is Involved in the PKA Regulation of the Glc6Pase Gene in CaCo2 Cells—Both HNF4 α and CREB/CREM can recruit CBP as co-activator (50, 51). Phosphorylation of CREB or CREM by PKA allows them to recruit CBP and induce transcription (45). CBP is present at limiting concentrations in many cell types (52), so its recruitment to one given promoter is a crucial event for the activation of transcription. To test if synergistic effect on the transactivation of the Glc6Pase promoter by PKA and HNF4 α could derive from a more efficient recruitment of CBP to the Glc6Pase promoter, we performed co-transfection experiments. We transfected increasing amounts of an expression vector of CBP to trigger a high level recruitment to the promoter. In this type of experiment, if CBP is efficiently recruited to the promoter without additional transfection, increasing the amount of CBP protein by transfection should not have any effect on the promoter activity. On the contrary, if CBP concentration is limiting for its recruitment, then increasing the amount of CBP protein by transfection should increase promoter activity. In CaCo2 cells, overexpression of CBP

increased basal (Fig. 5*B*, *white circles*) and HNF4 α -induced promoter activities (Fig. 5*B*, *black diamonds*), whereas it had no effect on the promoter activities stimulated by PKA (irrespective of the presence of HNF4 α) (Fig. 5*B*, *white triangles* and *black squares*, respectively). These results suggested that HNF4 α alone was not sufficient to recruit CBP to the Glc6Pase promoter and that CBP was efficiently recruited to the promoter in the presence of PKA. Immunoprecipitation of chromatin confirmed that, in the intestine, CBP was recruited on the Glc6Pase promoter after a 48-h fasting, whereas HNF4 α was already bound to the Glc6Pase promoter in the PP state (Fig. 5*D*). To further understand the synergistic activation of the Glc6Pase promoter activity by PKA in the presence of HNF4 α , we used the capacity of E1A to inhibit CBP acetyltransferase activity (53). Overexpression of E1A decreased the promoter activities induced by PKA (irrespective of the presence of HNF4 α) (Fig. 5*C*, *white triangles* and *black squares*, respectively), whereas it had no effect on either basal (Fig. 5*C*, *white circles*) or HNF4 α -induced promoter activities (Fig. 5*C*, *black diamonds*). These results confirmed the contribution of CBP in the transactivation of the Glc6Pase promoter by PKA. Moreover, a higher amount of E1A, which reflected a higher inhibition of CBP acetyltransferase activity, was needed to induce the same decrease in the stimulation of the promoter activity by both PKA and HNF4 α than by PKA alone (Fig. 5*C*, *arrow*). In summary, these results suggest that after PKA stimulation, CBP exhibits a higher transactivation activity in the presence of

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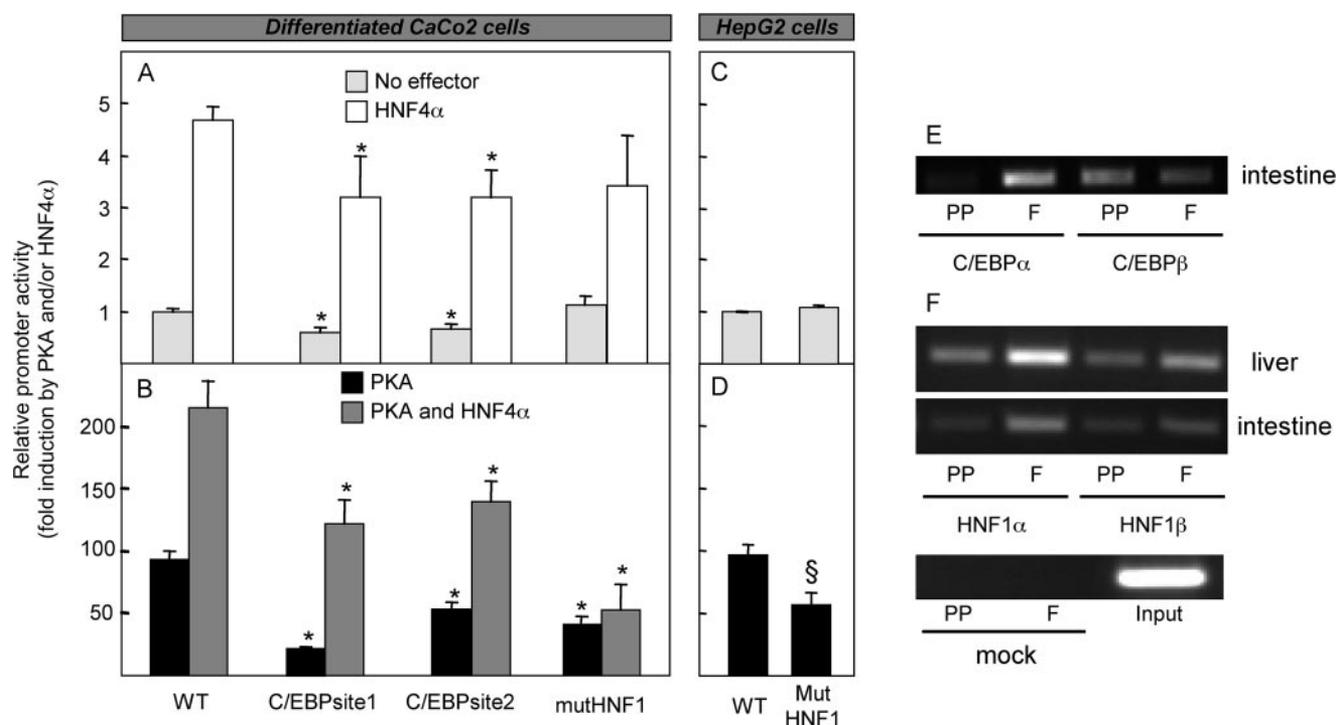


FIGURE 6. Contribution of C/EBP and HNF1 proteins to the PKA regulation of the Glc6Pase gene. *A* and *B*, CaCo2 cells were transfected with the wild type $-694/+60B$ construct or the mutated constructs as indicated either alone (light gray bar) or in the presence of HNF4 α (white bar), PKA (black bar), or PKA and HNF4 α (dark gray bar). *C* and *D*, HepG2 cells were transfected with the wild type $-694/+60B$ construct or the mutated constructs as indicated either in the absence (light gray bar) or in the presence of PKA (black bar). Results are expressed as -fold induction over the promoter activity of construct transfected alone and are expressed as a mean \pm S.E. of six independent experiments. * and §, value significantly different from wild type construct ($p < 0.01$). *E*, C/EBP binding to the Glc6Pase promoter were analyzed by immunoprecipitation of chromatin from intestine or liver of postprandial (PP) or 48 h-fasted rats (F) with anti-C/EBP α or anti-C/EBP β antibodies. The PCR amplifications of immunoprecipitated fragments of the $-174/+44$ bp region of the Glc6Pase gene are shown. *F*, HNF1 binding to the Glc6Pase promoter was analyzed by immunoprecipitation of chromatin from intestine or liver of postprandial or 48 h-fasted rats with anti-HNF1 α or anti-HNF1 β antibodies. The figure represents the PCR amplification of immunoprecipitated fragments of the $-326/-150$ bp region of the Glc6Pase gene. The data shown are representative of at least two experiments.

HNF4 α and indicate that, after PKA stimulation, CBP is better recruited to the promoter in the presence of HNF4 α .

C/EBP and HNF1 Contribute to the Potentiated PKA Induction of the Glc6Pase Promoter in an Intestine-specific Way—C/EBP and HNF1 proteins are involved in PKA regulation of the Glc6Pase gene in the liver (27) and in the kidney (34), respectively. Since these proteins are also expressed in the intestine, we assessed their involvement in PKA regulation of the Glc6Pase gene. Mutations of C/EBP site 1 ($-135/-130$ bp) or C/EBP site 2 ($-608/-603$) resulted in a 40% decrease of the basal promoter activity (Fig. 6A, gray bars) and in a 30% decrease of the induction by HNF4 α of the promoter activity (Fig. 6A, white bars). These results suggested that C/EBP proteins were involved in the promoter activity of the Glc6Pase gene in the intestine. Regarding PKA regulation, the mutation of the C/EBP site 1 resulted in a 60% decrease of the stimulation of the Glc6Pase promoter activity by PKA and the mutation of C/EBP site 2 in a 40% decrease of the stimulation by PKA (Fig. 6B, black bars). Moreover, mutations of either C/EBP site 1 or C/EBP site 2 resulted in a 40% decrease of the synergistic stimulation of the promoter activity by PKA and HNF4 α (Fig. 6B, dark gray bars). These results were confirmed by immunoprecipitation of intestine chromatin. The analysis of Fig. 6E shows that both C/EBP isoforms were bound to the intestinal Glc6Pase promoter upon fasting. Interestingly, only C/EBP α binding seems to be regulated by fasting, since its binding was increased in 48 h-fasted rats whereas C/EBP β binding

did not change. This suggests a more important role of the C/EBP α isoform than the C/EBP β isoform in cAMP regulation of the Glc6Pase gene in the intestine.

HNF1 has been involved in PKA regulation of the Glc6Pase gene in the kidney (34) and plays an important role in the regulation of intestinal gene expression (see Ref. 54 for a review). In CaCo2 cells, mutation of the single HNF1 binding site ($-214/-207$) resulted in a 40% decrease of the induction of the promoter activity by PKA (Fig. 6B, black bars) and in a 65% decrease of the synergistic induction by PKA and HNF4 α (Fig. 6B, dark gray bars) but did not decrease basal promoter activity or HNF4 α -induced activity (Fig. 6A, gray and white bars). HNF1 has not been involved in the PKA regulation of the Glc6Pase gene in the liver (34). To clarify the role of HNF1 in the regulation of the Glc6Pase gene, we studied the effect of the mutation of HNF1 binding site on the Glc6Pase promoter activity in liver cells. In our hands, the mutation of the HNF1 binding site also resulted in a 40% decrease of the induction of promoter activity by PKA in HepG2 cells (Fig. 6D, black bars), whereas it had no effect on basal promoter activity in either cells (Fig. 6, A and C, gray bars). To further document these results, we performed immunoprecipitation of chromatin from liver and intestine of fed postprandial and 48 h-fasted rats. Fig. 6F shows that, in both tissues, HNF1 α and β were bound to the promoter in the fed state and might increase their binding after

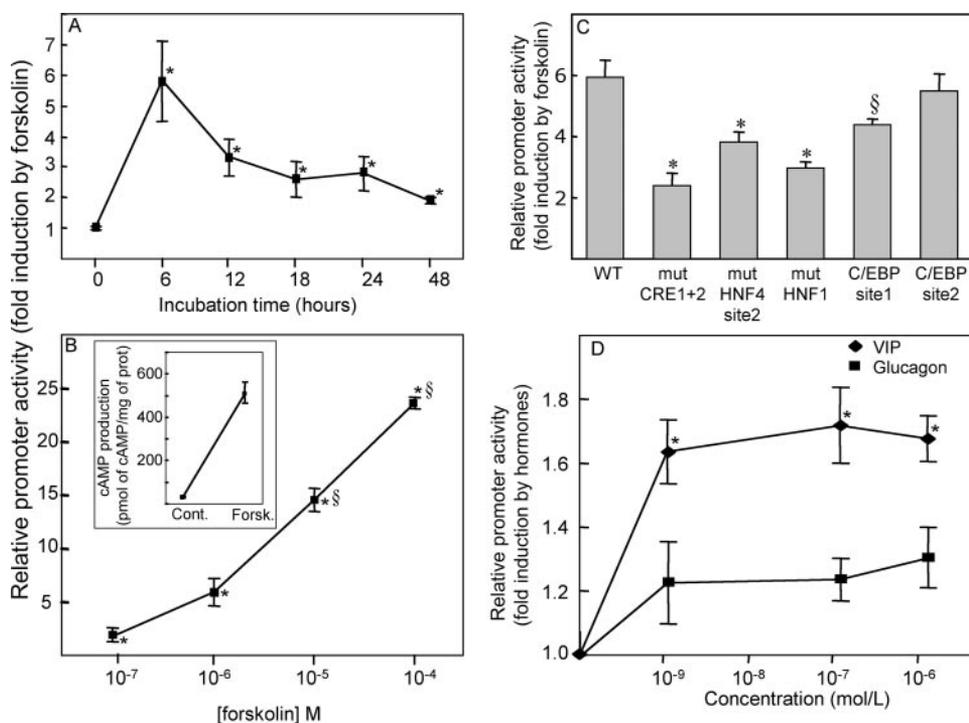


FIGURE 7. Induction of the Glc6Pase promoter activity by forskolin and VIP. A and B, differentiated Glc6Pase-CaCo2 cells ($-1420/+60B$ construct) were treated with 10^{-6} M forskolin at different incubation times (A) or for 6 h with different concentrations of forskolin (B). Relative promoter activities are expressed as -fold induction over promoter activity in untreated cells and are expressed as a mean \pm S.E. of six independent experiments. *, value significantly different from untreated cells ($p < 0.01$). Inset in B, cAMP production as measured in untreated differentiated CaCo2 cells (Cont.) or treated with 10^{-4} M forskolin for 6 h (Forsk.). The cAMP level is expressed as pmol of cAMP/mg of proteins and represents mean \pm S.E. of three independent experiments. C, CaCo2 cells were transfected with the wild type $-694/+60B$ construct or the mutated constructs as indicated in the presence of 50 ng of HNF4 α expression vector and treated for 6 h with 10^{-5} M forskolin. Relative promoter activities are expressed as -fold induction over promoter activity in untreated cells and are expressed as a mean \pm S.E. of three independent experiments. *, value significantly different from untreated cells ($p < 0.01$). §, value significantly different from untreated cells ($p < 0.05$). D, differentiated Glc6Pase-CaCo2 cells were treated for 6 h with different concentrations of VIP (black diamonds) or glucagon (black squares). Results are expressed as -fold induction over the promoter activity of untreated cells and are expressed as a mean \pm S.E. of three independent experiments. *, value significantly different from untreated cells ($p < 0.01$).

fasting. Thus, HNF1 α and - β are involved in the PKA regulation of the Glc6Pase gene in both the liver and intestine.

Forskolin and VIP Stimulated the Glc6Pase Gene in Enterocyte-like Cells at a Transcriptional Level—CaCo2 cells spontaneously differentiate in enterocyte-like cells after confluence (47) with concomitant induction of the endogenous Glc6Pase expression (47). To confirm that the Glc6Pase promoter is induced by cAMP in an intestinal context closer to the *in vivo* situation, we used differentiated CaCo2 cells. CaCo2 cells were stably transfected with the longest Glc6Pase promoter construct, $-1420/+60B$ and named Glc6Pase-CaCo2. Differentiated Glc6Pase-CaCo2 cells were treated with 10^{-6} M of forskolin (an activator of adenylate cyclase) for 6–48 h (Fig. 7A). Glc6Pase promoter activity was significantly increased up to a maximum after 6 h of forskolin treatment and then remained sustained up to 48 h (Fig. 7A). Fig. 7B shows that the stimulation of Glc6Pase promoter activity by forskolin was dependent on the dose. Maximal induction was obtained with 10^{-4} M forskolin in differentiated CaCo2 (Fig. 7B). In line with the latter, the intracellular cAMP level was induced by 16-fold after 6 h of treatment by 10^{-4} M forskolin (Fig. 7B, inset). These results

suggest that the Glc6Pase gene is transcriptionally induced by cAMP in the intestinal context.

PKA is the main effector of cAMP regulation. We thus aimed to check whether molecular mechanisms of induction of the Glc6Pase promoter by PKA studied above were involved in the transcriptional induction initiated by increased cAMP levels. CaCo2 cells were transfected either with wild type and mutated constructs of the $-694/+60$ promoter construct and treated with 10^{-5} M forskolin for 6 h to induce an intracellular increase of cAMP level. Glc6Pase constructs were transfected in the presence of an expression vector of HNF4 α to obtain a substantial induction by forskolin. Forskolin treatment resulted in a 6-fold increase of the Glc6Pase promoter activity (Fig. 7C). Mutation of CREB binding sites (CRE1 and -2) resulted in a 60% decrease of the induction by forskolin and HNF4 α , consistent with the crucial role of CREB in the synergistic regulation of the Glc6Pase promoter activity by PKA and HNF4 α . Mutation of HNF4 α binding site 2 resulted in a 35% decrease of the induction of the promoter activity by forskolin and HNF4 α and mutation of HNF1 binding site resulted in a 45% decrease of the induction by forskolin and HNF4 α (Fig. 7C). However,

the mutation of C/EBP binding site 1 induced a small decrease of the induction of the Glc6Pase promoter activity by forskolin and HNF4 α , and the mutation of C/EBP site 2 had no significant effect on the induction of the promoter activity (Fig. 7C). These results are consistent with the rather weak inhibitory effect of mutations of C/EBP binding site 1 or 2 on the induction of the Glc6Pase promoter activity by PKA and HNF4 α (see Fig. 6B). In summary, the binding sites, which are the most important for the synergistic regulation of the Glc6Pase promoter activity by PKA and HNF4 α (e.g. CRE1 and CRE2, HNF4 site 2 and HNF1) are also crucial in the induction of the promoter activity initiated by increased cAMP levels. Finally, we study the effect of hormones well known to specifically induce cAMP levels in hepatocytes (glucagon) and in enterocytes (VIP) (55). To study the specific sensitivity of the Glc6Pase promoter activity to these hormones in enterocytes, we treated differentiated Glc6Pase-CaCo2 cells with increased concentration of glucagon and VIP for 6 h. Fig. 7D shows that the promoter activity was significantly induced in differentiated Glc6Pase-CaCo2 cells after a 6-h treatment with 10^{-9} to 10^{-6} M VIP, whereas glucagon had no significant effect. These results are in agree-

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ment with previous works that showed high adenylate cyclase sensitivity to VIP in differentiated CaCo2 cells as well as in isolated intestinal epithelial cells from rat (55, 56) (probably due to the same amount of VIP receptors (57)).

DISCUSSION

Glc6Pase appears to be the most important enzyme in the triggering of intestinal gluconeogenesis (6, 18). To delineate the specific regulation of gluconeogenesis in the intestine compared with the liver, we have studied the transcriptional regulation of this critical gene in the intestine with increased cAMP levels. We have shown that, in the intestine as in the liver, the Glc6Pase gene is transcriptionally induced by cAMP. The transcription factors that are involved in the PKA regulation of the Glc6Pase gene in the liver, such as CREB, HNF4 α , and C/EBP (27), also contribute to the regulation of this gene in the intestine. In addition, we have demonstrated the specific contribution of CREM in PKA regulation of the Glc6Pase gene in the intestine. Most interestingly, we have here highlighted that the specific role of each factor in the regulation of the Glc6Pase gene is different in the liver and in the small intestine. This may have an impact on the differential regulation observed *in vivo*.

Here we elucidate the mechanism of the expression of the Glc6Pase gene in the intestine, and, for the first time, we identify tissue-specific factors also expressed in the liver that are important for the basal activity of the gene in the intestine. A high expression of HNF4 α protein seems to be crucial to produce a high basal activity of the Glc6Pase promoter in CaCo2 cells. Moreover, in these cells, HNF4 α expression is correlated with Glc6Pase gene expression (*i.e.* increase during differentiation), and mutations of HNF4 binding sites result in a decrease of basal promoter activity. The importance of HNF4 α in the regulation of the Glc6Pase gene expression is confirmed *in vivo* by chromatin immunoprecipitation experiments, which show that HNF4 α is bound to the rat promoter. In hepatocytes, HNF4 α knockdown or mutation of the Glc6Pase-HNF4 α binding sites has no effect on the basal activity of the Glc6Pase promoter (27, 58). In contrast with hepatocytes, in enterocyte-like cells, HNF4 α is not dispensable regarding the induction of the basal activity of the Glc6Pase promoter. In line with our results, such a crucial role of HNF4 α in the specific expression of other intestinal genes (*e.g.* alipoprotein A IV (58), intestinal alkaline phosphatase gene (59), and guanylyl cyclase C (60)) has previously been shown. Both C/EBP α and - β proteins are involved in the regulation of Glc6Pase gene expression in the liver, where they appear to equally contribute to basal and cAMP-induced promoter activities (27, 61). Conversely, our work here suggests that, in the intestine, only C/EBP β contributes to the basal promoter activity of the Glc6Pase gene (Fig. 6). Our results also show that C/EBP is involved in the activation of the Glc6Pase gene by HNF4 α . These results suggest that a multifactor cooperativity is a critical determinant of intestinal Glc6Pase gene activation by HNF4 α . In line with our previous results in relation to the regulation of the Glc6Pase gene by CDX1 (32), this work further confirms that the Glc6Pase promoter appears to be representative of specific intestinal genes (62, 63).

The Glc6Pase promoter activity is regulated in both tissues

by PKA. In CaCo2 cells, we further show a specific synergistic effect of PKA and HNF4 α on the Glc6Pase promoter activity. This synergistic activation does not depend on the distal promoter region (-694/-500 bp), which is involved in a potentiated PKA responsiveness in hepatocytes (27). Nevertheless, in the intestine, this region also contributes to a higher induction of the promoter activity by PKA (Fig. 1). Our mutagenesis experiments show that the synergistic effect depends on the HNF4 α site 2 present in the proximal promoter region and particularly involves the binding sites CRE1, HNF1, and C/EBP1 and -2 in a mechanism linked to CBP recruitment. HNF1 and HNF4 have already been shown to cooperate in the regulation of the intestinal fatty acid binding protein (64). Both HNF4 α and HNF1 α are able to induce the acetyltransferase activity of CBP (65). Moreover, CBP increases the nuclear translocation of HNF4 α by acetylation on a lysine residue, which in turn induces an increase in the interaction between HNF4 α and CBP (66). C/EBP proteins are also known to interact with CBP (67). We thus propose that, on the Glc6Pase promoter, tissue-specific factors (HNF4 α , C/EBP, and HNF1) provide an initial platform for the selective recruitment and further assembly of a multicomponent coactivator complex (including CREB proteins and CBP) on the promoter.

Regarding the regulation of the Glc6Pase gene by PKA, we highlight several differences between liver and intestinal transcription complexes. Indeed, whereas HNF4 α and C/EBP α and - β are already bound to the promoter in the basal state in the liver (27), C/EBP α protein is recruited to the Glc6Pase promoter only upon fasting in the intestine (Fig. 6). Furthermore, we show that both HNF1 isoforms seem to be equally involved in the regulation of the Glc6Pase gene in the liver and the intestine. On the contrary, a specific regulation of intestinal genes by the HNF1 α isoform regarding HNF1 β has been observed with the intestine-specific genes, such as sucrase isomaltase and the dipeptidylpeptidase IV (68, 69). In a similar manner, O'Brien and co-workers (34) have suggested that, in the kidney, only the HNF1 α isoform is able to induce Glc6Pase promoter activity. In this regard, Glc6Pase thus seems to retain a hepatic feature, even in the intestinal context, since both HNF1 isoforms have been suggested to be equivalent in liver gene expression (70). Most interestingly, we emphasize in this work the importance of CREB/CREM proteins in the regulation of the Glc6Pase promoter activity in the intestine. Although the modulation of CREB activity by specific transduction pathways has been extensively studied, little is known about the selectivity code by which the different proteins of the CREB family regulate the tissue-specific expression of genes. CREB and CREM are derived from multiexonic genes, the alternative splicing of which generates a complex array of isoforms that can act either as activators or repressors of transcription (for reviews, see Refs. 45 and 49). The relative amount of CREB and different isoforms of CREM appears to be particularly important in various processes, as in the orchestration of spermatogenesis (71). In the latter, there is a switch in the expression of CREM α isoform to CREM τ isoform at puberty (72). We here report for the first time that CREM seems to specifically contribute to the PKA regulation of the Glc6Pase gene in the intestine. Interestingly, we also observed a switch in the expression of specific

CREM isoforms from CREM α isoform (repressor) to CREM τ (activator isoform) during the differentiation process of CaCo2 cells, which correlates with the expression of the Glc6Pase gene. In relation to the role of CREB, the antibody raised against this protein used in this study might also recognize CREM isoforms, according to the manufacturer. We thus cannot ensure whether only CREM or both CREB and CREM may bind to the Glc6Pase promoter *in vivo*.

In conclusion, we have here delineated the specific transcriptional regulation of the Glc6Pase gene in the intestine. We have shown that 1) HNF4 α is crucial for the transcription of the Glc6Pase gene in the intestine, and 2) the basal promoter activity of the Glc6Pase gene depends on the cooperation between HNF4 α and C/EBP β . Moreover, we point out the specificity of intestinal regulation with regard to the liver by delineating the molecular mechanism of the transcriptional regulation of this gene by cAMP. 1) We show the synergistic action of different transcription factors isoforms (HNF1, HNF4, C/EBP, and CREB/CREM) in the regulation of the Glc6Pase promoter activity by PKA; 2) we highlight for the first time the involvement of CREM protein in the specific regulation of an intestinal gene by PKA. Since PKA and CREB/CREM proteins are also involved in the transcriptional regulation of specific intestinal genes by peptones (35, 36), the mechanism described here could also contribute to the regulation of the Glc6Pase gene by protein-enriched diet (18). Thus, these results provide a better understanding of the differences in the regulation of expression of a key gluconeogenesis gene in the intestine compared with the liver.

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cAMP/Vasoactive Intestinal Peptide in the Intestine: ROLE OF HNF4 α , CREM,
HNF1 α , and C/EBP α**

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