Regulation of lactase–phlorizin hydrolase gene expression by the caudal-related homoeodomain protein Cdx-2

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Lactase–phlorizin hydrolase is exclusively expressed in the small intestine and is often used as a marker for the differentiation of enterocytes. The *cis*-element CE-LPH1 found in the lactase–phlorizin hydrolase promoter has previously been shown to bind an intestinal-specific nuclear factor. By electrophoretic mobility-shift assay it was shown that the factor Cdx-2 (a homoeodomain-protein related to *caudal*) binds to a TTTAC sequence in the CE-LPH1. Furthermore it was demonstrated that Cdx-2 is able to activate reporter gene transcription by binding to CE-LPH1. A mutation in CE-LPH1, which does not affect Cdx-2 binding, results in a higher transcriptional activity, indicating that the CE-LPH1 site contains other binding site(s) in addition to the Cdx-2-binding site.

INTRODUCTION

Lactase–phlorizin hydrolase (LPH) is a membrane protein anchored by a short hydrophobic segment at its C-terminus [1]. It is expressed exclusively by the enterocytes of the mammalian small intestine [1,2]. During foetal development, LPH is also expressed at low levels in the colon [3]. Newborn mammals are dependent on high levels of lactase for hydrolysis of the lactose present in the milk. Most mammals lose LPH activity after weaning, a phenomenon often referred to as the post-weaning decline. However, two phenotypes exist in the human adult population: the lactose intolerant (adult-type hypolactasia) and the lactose tolerant (lactase persistence). Adult-type hypolactasia is the most common genetic condition in the human population and is characterized by an inability to hydrolyse lactose effectively because of lack of LPH activity after the age of 5–10 years. In contrast, lactase-persistent humans maintain high levels of lactase throughout their lifetime. This phenotype is the most common in Northern Europe.

Besides tissue-specific and developmental regulation, LPH is also regulated along the crypt–villus (vertical) axis and the duodenal–colonic (longitudinal) axis. Enterocytes are formed from stem cells in the crypt. The cells migrate from the crypt to the top of the villus and, during the migration, they differentiate and begin to express the microvillar hydrolases. LPH mRNA can first be detected at the transition zone between the crypt and the villus [4,5], a feature shared with e.g. sucrase–isomaltase and aminopeptidase N [6,7]. LPH is also regulated along the longitudinal axis. In the proximal jejunum, LPH levels are relatively low, rising to their highest values in the middle part of the small intestine, and then gradually declining towards the distal ileum [8–11].

It is generally agreed that LPH is mainly regulated at the transcriptional level [8–10,12–19]. However, additional post-transcriptional mechanisms modulate the final expression of LPH especially in the proximal jejunum [10,19]. A 1 kb section of the upstream region of the porcine LPH gene has been analysed for promoter activity in both transfection experiments [20] and transgenic mice [19]. This region contains the *cis* elements that are necessary for the post-weaning decline in LPH and the tissue-specific expression and the correct initiation of gene transcription along the crypt–villus axis. Along the longitudinal axis of the small intestine the transgene expression is also similar to the endogenous expression of LPH except in the proximal jejunum where high levels of transgene expression are detected in contrast with the relatively low endogenous LPH expression [19].

The 1 kb of the upstream region of the porcine LPH gene has been further analysed by deletion analysis, DNase I footprinting and electrophoretic mobility-shift assay (EMSA) (N. Spodsberg, J. T. Troelsen, P. Carlsson, H. Sjöström and O. Norén, unpublished work). Four regions with transcriptional importance have been identified: two regions from −894 to −798 and −227 to −142 which enhance transcription; one region from −299 to −227 which represses transcription; one region from −142 to −17 which is only able to drive a low level of transcription, but in a differentiation-dependent manner [20]. In the region from −142 to −17 one region (named CE-LPH1) from −40 to −54 has been shown to bind a nuclear factor (called NF-LPH1) [20]. Furthermore NF-LPH1 DNA-binding activity was only found in extracts of intestinal origin and was found to bind to a TTTAC-containing sequence. NF-LPH1-binding activity has been found to be high in newborn pigs [20] and rats [21], but relatively low in adult animals. The functional importance of this observation with regard to adult-type hypolactasia remains, however, to be established.

The transcriptional regulation of sucrase–isomaltase (SI), another gene exclusively expressed in the small intestine, has been analysed [22–25]. A DNA element consisting of a palindrome of the sequence TTTAT (named SIF1) has been shown to bind an intestinal-specific nuclear factor. We have shown that CE-LPH1 and SIF1 compete for binding to the same or closely related nuclear factors [26]. Recently it has been established that the major SIF1-binding activity in intestinal nuclear extracts is the homoeodomain protein Cdx-2 [27]. Cdx-3, the hamster homologue of Cdx-2, is also expressed in pancreatic cells [28,29]. For clarity we have used the name shCdx-2 (Syrian hamster Cdx-

Abbreviations used: LPH, lactase–phlorizin hydrolase; CE-LPH1, *cis*-element lactase–phlorizin hydrolase 1; NF-LPH1, nuclear factor lactase–phlorrhizin hydrolase 1; SIF1, sucrase–isomaltase footprint 1; shCdx-2, Syrian hamster Cdx-2; EMSA, electrophoretic mobility-shift assay.

The nucleotide sequence reported in this paper has been submitted to the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number Y08677.

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2) for Cdx-3 in the present work. The expression of Cdx-2 is much higher in the intestine than in the pancreas [27,29]. In the present work, we have established that Cdx-2 binds to CE-LPH1 and is of importance in the transcription of the LPH gene.

MATERIALS AND METHODS

Construction of LPH promoter–luciferase plasmids

A plasmid was generated containing an 877 bp upstream fragment of the pig LPH gene (−894 to −17) in front of a luciferase reporter gene by isolating a *Pst*I–*Sst*ILPH promoter gene fragment from pOVEC-LPH 894 (N. Spodsberg, J. T. Troelsen, P. Carlsson, H. Sjöström and O. Norén, unpublished work). The fragment was treated with Klenow enzyme to create a blunt end and cloned into pGL-2-Basic (Pharmacia, Uppsala, Sweden). This plasmid is named pGL2-LPH-894wt and was used to generate two plasmids with mutations in the *cis* element CE-LPH1 [26] (pGL2-LPH-894mut1 and pGL2-LPH-894mut2) by site-directed mutagenesis using the Chameleon kit (Stratagene). The sequence of the mutated CE-LPH1 in pGL2-LPH-894mut1 and pGL2-LPH-894mut2 is the same as the sequence of oligonucleotide mut1 and mut2 shown in Figure 1. The mutations were verified by sequencing the plasmid using a luciferase gene-specific primer.

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CE-LPH1-24:   5′ CTAGTAT TTTACAA C CTCAGT TGT    3′
              3′ GATCATA AAATGTT G GAGTCA ACA    5′

CE-LPH1-17:   5′   GATCT TTTACAA C CTCAGT TG     3′
              3′       A AAATGTT G GAGTCA ACCTAG 5′

mut1:         5′   GATCT TTgcCAA C CTCAGT TG     3′
              3′       A AAcgGTT G GAGTCA ACCTAG 5′

mut2:         5′   GATCT TTTACAA C CTgcGT TG     3′
              3′       A AAATGTT G GAcgCA ACCTAG 5′

mut3:         5′   GATCT TTTACAA g CTCAGT TG     3′
              3′       A AAATGTT c GAGTCA ACCTAG 5′

mut4:         5′   GATCT gggACAA C CTCAGT TG     3′
              3′       A cccTGTT G GAGTCA ACCTAG 5′

mut5:         5′   GATCT TTTcgcA C CTCAGT TG     3′
              3′       A AAAgcgT G GAGTCA ACCTAG 5′

mut6:         5′   GATCT TTTACAc g gTCAGT TG     3′
              3′       A AAATGTg c cAGTCA ACCTAG 5′

mut7:         5′   GATCT TTTACAA C CggcGT TG     3′
              3′       A AAATGTT G GccgCA ACCTAG 5′

mut8:         5′   GATCT TTTACAA C CTCAcg gG     3′
              3′       A AAATGTT G GAGTgc cCCTAG 5′

SIF1-33       5′ GGCTGGTGAGGGTGCAATAAAACTTTATGAGTA   3′
              3′ CCGACCACTCCCACGTTATTTTGAAATACTCAT   5′

SIF1-24       5′         GGGTGCA ATAAAACTTTATGAGTA   3′
              3′         GGGACGT TATTTTGAAATACTCAT   5′

Unspec        5′   GGTACTACTTCTAGCTTCGGAACT     3′
              3′   CCATGATGAAGATCGAAGCCTTGA     5′
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Figure 1 Sequence of the oligonucleotides used in EMSAs

The box indicates the regions protected in DNase I footprint analysis of CE-LPH1 [20]. Lowercase letters indicate the mutated residues in CE-LPH1 elements. The underlined letters are the Cdx-2-binding sites in SIF1 [27].

Cell culture, transfections and luciferase–β-galactosidase measurements

Cells were grown in minimum essential medium (Gibco–BRL Life Technologies) containing 10% calf serum. HeLa or Caco-2 cells at 80% confluence were treated with trypsin and plated at 2×10^6 cells per 35 mm Petri dish the day before transfection. Medium was changed 4 h before transfection. DNA was precipitated using the calcium phosphate method: 219 μl of DNA solution was mixed with 31 μl of 2 M $CaCl_2$. Then 250 μl of 2 × HBS (1 × HBS is 280 mM NaCl, 10 mM KCl, 1.5 mM Na_2HPO_4, 50 mM Hepes), pH 7.12, was added slowly to the DNA during gentle shaking. The precipitate was incubated for 15 min at room temperature and subsequently distributed to three 35 mm dishes containing the cells. The cells were incubated overnight with the precipitated DNA, and this was followed by a medium change. At 48 h after transfection, the cells were harvested and analysed using the protocol of the Dual Light System (Tropix).

PCH110 [a β-galactosidase expression plasmid (Pharmacia); 1 μg] was included in all transfections. β-Galactosidase activity was used as an internal standard for transfection efficiency. pGL2-LPH-894wt, pGL2-LPH-894mut1 or pGL2-LPH-894mut2 (10 μg in each case) was used in the transfection experiments. In some experiments 1.25 μg of pBAT-Cdx-3 (a shCdx-2 expression plasmid kindly provided by Dr. Michael Germany [28]) was co-transfected. The total amount of DNA per transfection was adjusted to 13.5 μg with the plasmid pRC-CMV (Invitrogen).

HeLa cells were transfected with pBAT-Cdx-3 in order to prepare cell extracts containing shCdx-2. pBAT-Cdx-3 (10 μg) was calcium phosphate precipitated as previously described and distributed over HeLa cells seeded in a 100 mm Petri dish at a density of 2×10^6 the day before transfection. Whole cell extracts were prepared from the HeLa cells 48 h after the transfection using the protocol of Schöler et al. [30].

Electrophoretic mobility-shift and supershift assays

Nuclear extracts from differentiated Caco-2 cells were prepared as previously described [31]. EMSA was performed by mixing 5 μl of nuclear extracts from Caco-2 cells (5–10 μg) or from whole cell extracts from shCdx-2-transfected or non-transfected HeLa cells (2 μg) with 20 μl of gel shift buffer (25 mM Tris/HCl, pH 7.8, 5 mM $MgCl_2$, 6 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol, 0.5 mM PMSF, 5% Ficoll, 2.5% glycerol, 50 ng/μl dIdC). In some experiments, unlabelled double-stranded oligonucleotides were added (Figure 3). The samples were incubated for 10 min on ice, and then 3 fmol (Figure 2A) or 25 fmol (Figure 3) of ^{32}P-end-labelled double-stranded oligonucleotide (probe) was added and the mixture was incubated for 15 min on ice. Gel shift loading buffer [0.2% Bromophenol Blue, 10% glycerol, 0.5 × TBE (1 × TBE is 45 mM Tris/borate, pH 8.3, 1 mM EDTA); 3 μl] was added and the samples were run on a 5% non-denaturing polyacrylamide gel. DNA–protein complexes were visualized by autoradiography.

Supershift analyses were performed by mixing nuclear extracts, gel shift buffer and dIdC as described for EMSA. The samples were incubated for 10 min on ice. The probe was added and the samples were incubated for 15 min at room temperature. Antiserum against the C-terminal portion of shCdx-2 (a gift from Dr. Michael German) or preimmune serum was added and the samples were incubated for 10 min at room temperature. Finally gel shift loading buffer was added and the samples were run on a 5% non-denaturing polyacrylamide gel. DNA–protein complexes were visualized by autoradiography.

Table 1 Activation of reporter gene transcription by Cdx-2 through CE-LPH1 of the LPH promoter

Caco-2 and HeLa cells were transfected with three different LPH promoter–luciferase constructs with or without shCdx-2-expression plasmid (pBAT-Cdx-3): pGL2-LPH-894wt containing the wild-type LPH promoter (from position −17 to −894) in front of the luciferase gene; pGL2-LPH-894mut1 containing the LPH promoter with a mut1 mutation in the CE-LPH1 site (−40 to −54); pGL2-LPH-894mut2 containing the LPH promoter with a mut2 mutation in the CE-LPH1 site (−40 to −54). The mut1 and 2 mutations are as shown in Figure 1, oligonucleotide mut1 and mut2 respectively. The resulting luciferase activities were corrected for variation in transfection efficiency and normalized to the value of co-transfection with pGL2-LPH-894wt and shCdx-2, expressed as percentages. Means ± S.D. were calculated from three experiments.

	Luciferase activity (%)	
	−shCdx	+shCdx-2
Transfection of Caco-2 cells		
pGL2-LPH-894wt	3.6 ± 0.8	100 ± 7.0
pGL2-LPH-894mut1	2.4 ± 0.3	22.8 ± 2.2
pGL2-LPH-894mut2	4.9 ± 0.2	148.4 ± 11.0
Transfection of HeLa cells		
pGL2-LPH-894wt	1.4 ± 0.9	100 ± 7.2
pGL2-LPH-894mut1	1.1 ± 0.1	63.6 ± 2.3
pGL2-LPH-894mut2	1.7 ± 1.2	217.0 ± 17.6

Western-blot analysis

Nuclear Caco-2 extract (25 μg of protein) and whole cell extracts from shCdx-2-transfected or non-transfected HeLa cells (20 μg of protein) were analysed by SDS/PAGE together with appropriate molecular-mass markers and were electrotransferred to an ECL membrane (Amersham). The blot was incubated with antiserum against shCdx-2 (diluted 1:2000) for 1 h at room temperature. Antibody binding was detected using chemiluminescence (ECL system; Amersham). Washes, incubations and development were performed according to the instructions of the manufacturer.

RESULTS

Cdx-2 activates transcription through the LPH promoter

The only cell line reported to express LPH is Caco-2 cells [32]. However, the level of LPH expression is very low in these cells even after differentiation. Caco-2 cells were transfected with a reporter plasmid containing 878 bp of the LPH promoter in front of a luciferase reporter gene (pGL2-LPH-894wt) in order to test whether undifferentiated Caco-2 cells are able to drive the reporter gene transcription from this LPH promoter construct by its endogenous transcription factors (Table 1). Transfection of pGL2-LPH-894wt resulted in a very low luciferase activity (five times the background level), indicating the lack of (an) essential transcription factor(s) for LPH transcription in the undifferentiated Caco-2 cells. However, co-transfection of pGL2-LPH-894wt and pBAT-Cdx-3 (shCdx-2 expression plasmid) resulted in a 28-fold activation of the reporter gene expression.

Two DNA plasmids containing two different point mutations in the CE-LPH1 element of pGL2-LPH-894wt were generated by site-directed mutagenesis (pGL2-LPH-894mut1 and pGL2-LPH-894mut2). In EMSA experiments the mut1 mutation was previously shown to abolish protein binding, whereas the mut2 mutation had no effect on binding [26]. The reporter gene activity in Caco-2 cells transfected with pGL2-LPH-894mut1 and pGL2-

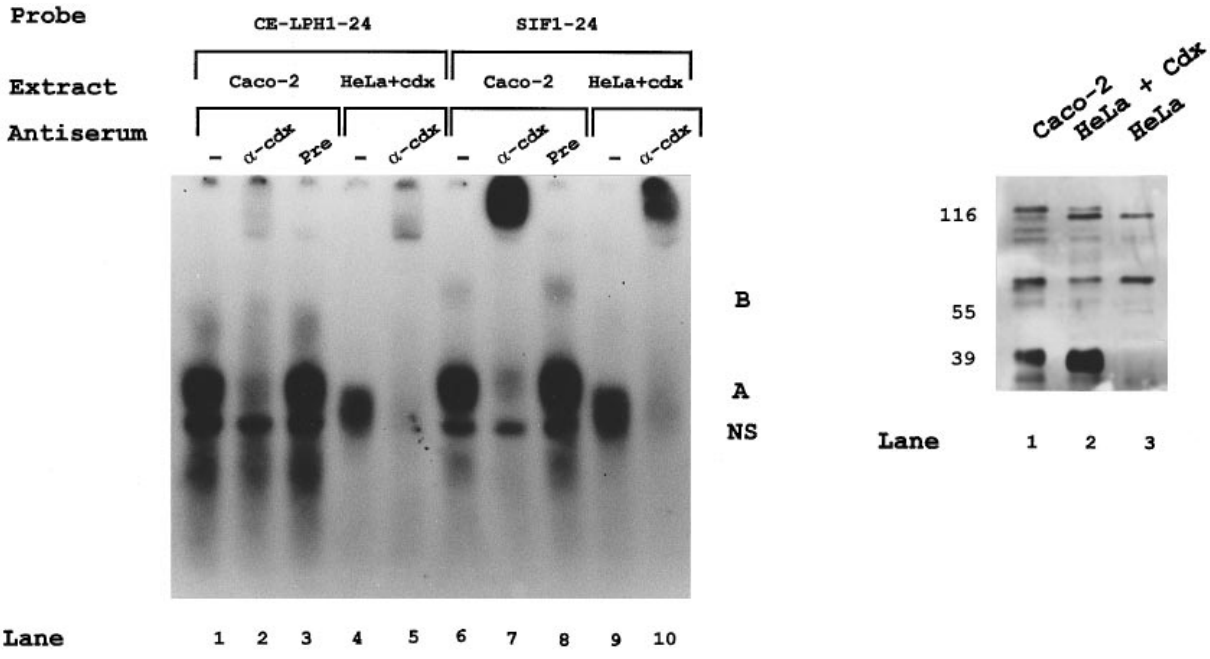

Figure 2 Supershift assay of SIF1 and CE-LPH1 using Caco-2 extract and extracts from shCdx-2-transfected HeLa cells

Left, Caco-2 extracts were assayed using CE-LPH1-24 (lanes 1–3) or SIF1-24 (lanes 6–8) oligonucleotides as probe. Antiserum against shCdx-2 (α-cdx) was added in lanes 2 and 7. In lanes 3 and 8 preimmune serum (Pre) was added. Extracts from shCdx-2-transfected HeLa cells were assayed using CE-LPH1-24 (lanes 4 and 5) or SIF1-24 (lanes 9 and 10) oligonucleotides as probe. In lanes 5 and 10 antiserum against shCdx-2 was added (α-cdx). The monomer complex (A) of Cdx-2 and SIF1 and the dimer complex (B) are indicated. NS marks a band generated by a non-specific binding activity in the Caco-2 extract. The probe was run out of the gel. Right, Western-blot analysis using antiserum against shCdx-2. Caco-2 nuclear extract (lane 1), extract from shCdx-2-transfected HeLa cells (lane 2) and extract from untransfected HeLa cells (lane 3) were separated by SDS/PAGE using a 10% polyacrylamide gel. The molecular masses (kDa) of size markers are indicated.

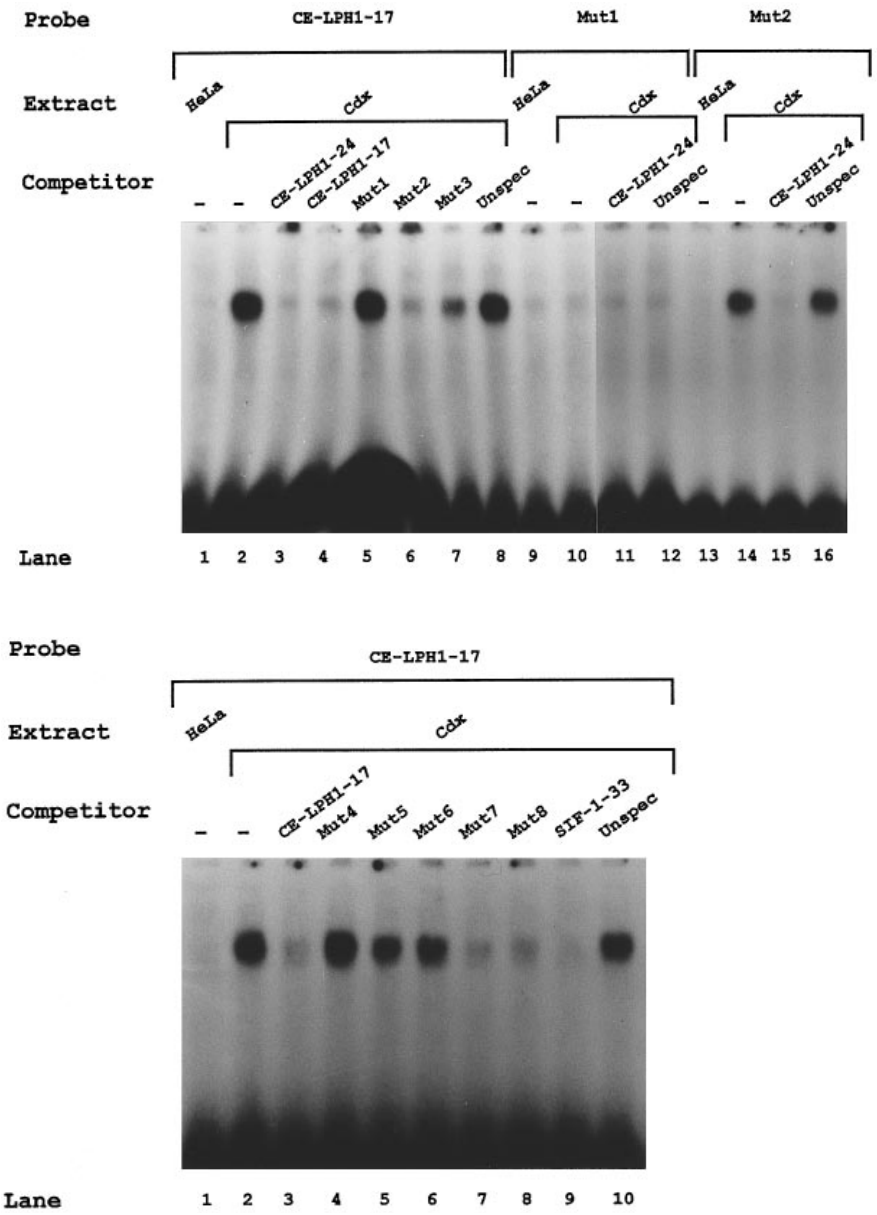

Figure 3 Effect of mutations in CE-LPH1

Top, Whole-cell extracts from untransfected HeLa cells (HeLa) and HeLa cells transfected with shCdx-2 (Cdx) were used in EMSAs. The probes used in the EMSAs were CE-LPH1-17 (lanes 1–8), mut1 (lanes 9–12) and mut2 (lanes 13–16) as indicated. Competitors were added as indicated (competitor). Bottom, Whole-cell extracts from untransfected HeLa cells (HeLa) and HeLa cells transfected with shCdx-2 (Cdx) were used in EMSAs with CE-LPH1-17 as probe. Competitors were added as indicated.

LPH-894mut2 alone was again very low, but co-transfection with the shCdx-2 expression plasmid (pBAT-Cdx-3) and pGL2-LPH-894mut1 resulted in a reporter gene expression at 23 % of the level using the wild-type promoter (pGL2-LPH-894wt) and pBAT-Cdx-3. However, the reporter gene level was 48 % higher using co-transfection of pGL2-LPH-894mut2 and pBAT-Cdx3 in comparison with the results from co-transfecting pGL2-LPH-894wt and pBAT-Cdx3 (Table 1).

A set of transfections using the same combinations of plasmids as in the experiments with Caco-2 cells was performed in HeLA cells and they gave similar results (Table 1). shCdx-2 activates the reporter gene transcription from pGL2-LPH-894wt by a factor of 73. However, co-transfecting pBAT-Cdx-3 and pGL2-LPH-894mut1 leads to a reporter gene activity 63 % of the level using pGL2-LPH-894wt and pBAT-Cdx-3. Co-transfection of pBAT-Cdx-3 and pGL2-LPH-894mut2 increased the luciferase activity by more than 2-fold compared with pGL2-LPH-894wt and pBAT-Cdx-3.

Cdx-2 binds to CE-LPH1

Nuclear extracts were prepared and used in EMSAs in order to characterize the expression of Cdx-2 in Caco-2 cells. The SIF1 element from the sucrase–isomaltase promoter containing two Cdx-2-binding sites binds specifically a factor in Caco-2 nuclear extracts [24–27]. With SIF1-24 as probe, a prominent DNA–

protein complex is formed (Figure 2, left, lane 6). Furthermore an antibody directed against the C-terminal part of the shCdx-2 supershifts the protein–DNA complex (Figure 2, left, lane 7), whereas the addition of preimmune serum did not result in the formation of an SIF1–protein–antibody complex (Figure 2, left, lane 8).

Previous results have indicated that the SIF1 element and CE-LPH1 element bind related or identical proteins [26]. An EMSA analysis, with CE-LPH1-24 as a probe, demonstrated that Caco-2 cells contain a factor that binds the CE-LPH1 element (Figure 2, left, lane 1) [26]. The intensity of the CE-LPH1–protein band is weakened by the addition of antiserum against shCxd-2. Only a weak supershift is seen, suggesting that the formation of the CE-LPH1–protein complex is inhibited by the antiserum against shCxd-2 (Figure 2, left A, lane 2). The inhibition is specific, as the addition of a preimmune serum does not influence the formation of the CE-LPH1–protein complex (Figure 2, left, lane 3).

Similarly, HeLa cells were analysed for Cdx-2-binding activity by EMSA. No specific binding was detected (Figure 3, top, lane 1). EMSAs with extracts from HeLa cells transfected with pBAT-Cdx-3 resulted in the generation of a specific binding activity to both the SIF1 and the CE-LPH1 probes (Figure 2, left, lanes 4 and 9). However, the shCdx-2–DNA complex from transfected HeLa cells seems to have slightly higher mobility than the complexes in EMSAs using Caco-2 extracts probably because of species differences between human Cdx-2 and hamster Cdx-2. A supershift analysis of the shCdx-2 binding to CE-LPH1 and SIF1 using shCdx-2-transfected HeLa-cell extract gave similar results to those using Caco-2-cell extracts. Thus anti-shCdx-2 is able to supershift the SIF1–protein complex (Figure 2, left, lane 10), whereas the formation of the CE-LPH1–protein complex is inhibited by anti-shCdx-2 (Figure 2, left, lane 5).

The specificity of the antiserum against shCdx-2 was analysed by Western-blot analysis (Figure 2, right). A major Cdx-2 immunoreactive band of approx. 37 kDa was detected in Caco-2 cells and HeLa cells transfected with shCdx-2 expression plasmid (lanes 1 and 2). In contrast, no 37 kDa immunoreactive band could be detected in the non-transfected HeLa cells (lane 3). This result is in accordance with the results from a similar Western-blot analysis using the same shCdx-2 antiserum against a BHK-cell extract and shCdx-2-transfected BHK cells [29]. Some minor bands at various concentrations in both Caco-2 and shCdx-2-transfected HeLa-cell extracts were also recognized by the anti-shCdx-2 serum. However, these weak bands cannot represent proteins that bind CE-LPH1 as the same bands were also present in the non-transfected HeLa-cell extract without CE-LPH1-binding activity (Figure 3). This eliminates the possibility that the anti-shCdx-2 antibody recognizes CE-LPH1-binding proteins other than Cdx-2 in Caco-2.

Cdx-2 has been shown to bind SIF1 both as monomer and dimer [27], which results in two bands in the EMSA. The monomer is called the A-complex and the dimer is called the B-complex (Figure 2, left) [27].

As seen in Figure 2 (left) the CE-LPH1 complex has a similar mobility to the monomeric SIF1–Cdx-2 band (A-complex). As oligonucleotides of the same length (24 bp) containing CE-LPH1 (CE-LPH1-24) or SIF1 (SIF1-24) sites were used in the supershift assay (Figure 2, left), it can be speculated that only one Cdx-2 molecule binds to CE-LPH1. No band corresponding to the SIF1–Cdx-2 band dimer (B-complex) is seen. However, a weak band with a lower mobility than the prominent band is present. As the mobility of this minor CE-LPH1 complex is higher than that of the B-complex, it is probably not a Cdx-2 dimer.

In order to characterize the nucleotide residues that are important in the Cdx-2–CE-LPH1 interaction, a series of oligonucleotides containing mutations in the CE-LPH1 site were synthesized (Figure 1) and analysed for their binding ability. mut1 and mut2 each contain a 2 bp mutation. mut3 contains a 1 bp mutation. mut4, 5, 6, 7 and 8 all contain systematic 3 bp mutations in the CE-LPH1 site progressing from the 5′ to the 3′ end. EMSAs were carried out using the mutated CE-LPH1 oligonucleotides and an extract from shCdx-2-transfected HeLa cells (Figure 3). mut1 is not able to form a complex with Cdx-2, whereas mut2 binds Cdx-2. The mutation scan of the protein-binding activity of CE-LPH1 demonstrates that mut4 to mut6 do not compete for Cdx-2 binding, whereas mut7 and 8 do. Similar results were obtained using Caco-2 nuclear extracts (results not shown).

DISCUSSION

The CE-LPH1 element was previously identified by DNase I footprinting [20]. Two subregions in CE-LPH1 were protected in the footprint analysis: one region covering the sequence TTACAA and a second region with sequence CTCAGT (Figure 1). A hypersensitive site was detected between these protected regions [20]. The first 130 bp of the LPH promoter is conserved between species (human, pig, rat and rabbit [20,33–36]). However, further upstream sequences show only a small degree of homology. The porcine CE-LPH1 element (-40 to -54) is 100 % conserved with respect to man, 79 % with respect to rat and 85 % with respect to rabbit. The conservation of CE-LPH1 between species and its position close to the TATA box indicates its importance in transcription initiation of the LPH gene. Previous analysis of the LPH promoter in Caco-2 cells has shown that reporter gene transcription is up-regulated from a 126 bp fragment containing the CE-LPH1 element and the TATA box during differentiation of the cells [20]. In the present study, undifferentiated and subconfluent Caco-2 cells with very low LPH expression were used to analyse LPH promoter–luciferase DNA constructs. In accordance with our previous results [20], undifferentiated Caco-2 cells are not able to activate transcription from a reporter plasmid containing the LPH promoter (Table 1). The presence of Cdx-2 clearly activates its transcription. However, mutation of the Cdx-2-binding site in CE-LPH1 (mut1) does not completely eliminate this activation, but reduces it to 23 %, even though this mutation abolishes binding of Cdx-2 to CE-LPH1 in EMSA (Figure 3, top). This indicates that an alternative Cdx-2 site(s) is present in the LPH promoter. Analysis of the LPH promoter sequence reveals several other putative Cdx-2-binding sites. Of special note is a TTTAT sequence present at position -102 to -106 in the porcine promoter and a region at position -353 to -339 which is 86 % identical with CE-LPH1 (N. Spodsberg, J. T. Troelsen, P. Carlsson, H. Sjöström and O. Norén, unpublished work). The TTTAT sequence is identical with the Cdx-2-binding site in the sucrase–isomaltase promoter (Figure 1) [27]. Mutation of the other protected region in the CE-LPH1 element (mut2) results in a higher transcriptional activation in the presence of Cdx-2 than the wild-type CE-LPH1. It can be speculated that this is due to more efficient binding of Cdx-2 to mut2 than to the wild-type CE-LPH1. However, mut2 competes with approximately the same efficiency as CE-LPH1 for binding to Cdx-2 in EMSA (compare lanes 4 and 6 in Figure 3, top), indicating that Cdx-2 binds with a similar affinity. The existence of a binding site for a transcriptional repressor present in Caco-2 cells corresponding to the downstream-protected region in the footprint [20] (Figure 1) therefore seems more plausible. The results of the transfection experiments using HeLa cells (Table 1) support this hypothesis, as the mutation in pGL2-LPH-894mut2 increases the reporter gene expression 2-fold

compared with pGL2-LPH-894wt. The sucrase–isomaltase gene has also been suggested to be regulated by a repressor mechanism operating in non-enterocytic cells in the intestine [37]. The existence of a repressor for Cdx-2 activation in non-enterocytic cells could also explain why LPH and sucrase–isomaltase are not expressed in the colon in spite of the high level of Cdx-2 expression [27,38,39]. We have not yet been able to demonstrate specific binding to the putative repressor site of CE-LPH1 by EMSAs, which might be because of the conditions used in EMSAs.

Recently Cdx-2 has been shown to be involved in the expression of carbonic anhydrase 1 gene in the colon [40]. Cdx-2 from Caco-2 cells binds to two DNA elements in the colon promoter of the carbonic anhydrase 1 gene. One element is 100 % identical with the Cdx-2 site in CE-LPH1. It is interesting that carbonic anhydrase is not expressed in Caco-2 cells even though Cdx-2 is expressed.

The importance of Cdx-2 in enterocytic differentiation has been investigated by generating stable clones of IEC-6 cells with inducible Cdx-2 expression [41]. IEC-6 is an undifferentiated rat intestinal cell line that does not exhibit endogenous expression of Cdx-2. These clones differentiate into both goblet-like and enterocyte-like cells after induction of Cdx-2 expression, and furthermore they express sucrase–isomaltase.

shCdx-2 was originally cloned from an expression cDNA library from an insulin-producing hamster cell line by its ability to bind to the FLAT element of the insulin promoter [28]. In both hamster and human the expression of Cdx-2 in the pancreas is very low and cannot be detected by Northern blot and RNase mapping [27,28]. However, the pancreatic hamster cell line InR1-G9 produces Cdx-2 in measurable amounts [29]. Cdx-2 activates the transcription of the proglucagon gene in these cells and other endocrine cell lines from both the small and the large intestine [29]. Thus Cdx-2 appears to be involved in gene expression and probably also differentiation into endocrine cells, goblet cells and enterocytes [27,29]. Cell type allocation by the small intestinal stem cells cannot, however, be dependent on Cdx-2 expression alone as three of the cell types in the small intestinal epithelium express this protein [27,29].

Cdx-2 is probably not the only factor in Caco-2 cells able to bind CE-LPH1, as screening of a cDNA library of differentiated Caco-2 cells for factors binding to CE-LPH1 by the one-hybrid screening system in yeast [42] resulted in cloning of HOX C11, a member of the Hox-family of homoeodomain factors (C. Mitchelmore, J. T. Troelsen, H. Sjöström and O. Norén, unpublished work). HOXC11 is the human homologue of the murine factor Hoxc-11. HOXC11 also binds specifically to the TTTAC element in the CE-LPH1 element. Thus the differentiated Caco-2 cells express at least two factors that are able to bind CE-LPH1. Further experiments are required to reveal the functional importance of the two factors that are able to bind to CE-LPH1.

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