Interaction between the homeodomain proteins Cdx2 and HNF1α mediates expression of the lactase-phlorizin hydrolase gene

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Lactase-phlorizin hydrolase is a brush-border enzyme which is specifically expressed in the small intestine where it hydrolyses lactose, the main carbohydrate found in milk. We have previously demonstrated in transgenic mice that the tissue-specific and developmental expression of lactase is controlled by a 1 kb upstream region of the pig lactase gene. Two homeodomain transcription factors, caudal-related homeodomain protein (Cdx2) and hepatic nuclear factor 1α (HNF1α), are known to bind to regulatory *cis* elements in the promoters for several intestine-specific genes, including lactase, and are present in mammalian intestinal epithelia from an early stage in development. In the present study, we examined whether Cdx2 and HNF1α physically interact and co-operatively activate transcription from the lactase-phlorizin hydrolase promoter. We show that the presence of both factors leads to a much higher level of transcription than the sum of the activation by either factor alone. The N-terminal activation domain of Cdx2 is required for maximal synergy with HNF1α. With the use of pull-down assays, we demonstrate a direct protein–protein interaction between Cdx2 and HNF1α. The interaction domain includes the homeodomain region of both proteins. This is the first demonstration of a functional interaction between two transcription factors involved in the activation of a number of intestine-specific genes. Synergistic interaction between tissue-restricted factors is likely to be an important mechanism for reinforcing developmental and tissue-specific gene expression within the intestine.

Key words: intestine, Caco-2, promoter, transcription factors, synergy.

INTRODUCTION

Lactase-phlorizin hydrolase (LPH) is a membrane-bound enzyme which hydrolyses milk lactose. LPH is expressed exclusively in the enterocytes of the small intestine and is highly expressed during the suckling period, but decreases after weaning in most mammals. This highly regulated expression pattern is controlled by *cis*-elements in the pig 5′ flanking 1 kb promoter region, which is sufficient to confer intestinal-specificity and post-weaning decline on the expression of a reporter gene [1]. Deletion and mutational analysis has identified multiple *cis*-acting sequence elements that bind transcription factors present in intestinal epithelia and in Caco-2 cells [2–4], an intestinal cell line which expresses LPH during differentiation [5]. The cell specificity of LPH expression is presumably a result of the interplay between these various proteins on DNA.

The proximal pig LPH promoter, from -227 to -17 relative to the transcriptional start site, is sufficient to activate a linked reporter gene in differentiated Caco-2 cells [2]. Therefore, we have further investigated the DNA-binding proteins which bind to the proximal LPH promoter. We have previously shown that caudal-related homeodomain protein (Cdx2) activates transcription from the LPH promoter through binding to a *cis* element, CE-LPH 1a, located close to the TATA box [6]. Mutation of this element does not completely abolish activation of the LPH promoter by Cdx2, probably owing to an additional Cdx2 binding site in the proximal promoter [6]. Furthermore, a binding site for hepatic nuclear factor 1α (HNF1α) is also present in the proximal LPH promoter [2]. The presence of both Cdx2 and HNF1α binding sites in the promoter is a shared feature of several intestinally expressed genes. These include LPH, sucrase-isomaltase and intestinal phospholipase A/lysophospholipase genes [2,7,8].

The homeodomain transcription factor Cdx2 is expressed specifically in the intestine [9]. The importance of Cdx2 for intestinal cell differentiation has been demonstrated by the conditional expression of Cdx2 in undifferentiated IEC-6 intestinal cells, which leads to a differentiated phenotype [10]. Furthermore, expression of Cdx2 is first detected in the gut of mouse embryos coincident with its earliest formation and is confined to the gut endoderm from embryonic day 12.5 [11]. Cdx2 is a potential tumour suppressor, since mice heterozygous for a null mutation in the Cdx2 gene have an increased propensity to develop colonic tumours [12].

HNF1α and HNF1β (or vHNF1) are related transcription factors which bind to DNA as homo- or hetero-dimers [13]. HNF1α and HNF1β have been known for some time to be important for liver-specific gene transcription, but are also expressed in other tissues, such as kidney and the intestinal epithelium [14,15]. Overexpression of either HNF1 results in transcriptional activation from the LPH promoter, although activation by HNF1α is 10-fold higher than by HNF1β [2]. Consistent with a role of HNF1α in the tissue-specific activation of LPH, expression of HNF1α protein in the mouse small intestine is detected from embryonic day 12.5 [16].

The role of co-operation between transcription factors in the regulation of intestine-specific gene expression is largely unexplored. We set out to investigate whether Cdx2 was able to synergize with HNF1α on the LPH promoter. In the present paper, we show that co-transfection of expression plasmids for both factors into Caco-2 cells leads to more than additive activation of a 210 bp pig LPH-promoter-driven reporter gene.

Abbreviations used: LPH, lactase-phlorizin hydrolase; Cdx2, caudal-related homeodomain protein; CE-LPH 1, *cis* element of lactase-phlorizin hydrolase; EMSA, electrophoretic-mobility-shift assay; SIF1, sucrase-isomaltase footprint 1; HNF, hepatic nuclear factor; GST, gluthathione S-transferase.

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Although highest levels of activation by Cdx2 and HNF1α require intact binding sites for both factors in the promoter, HNF1α is apparently able to recruit Cdx2 into a transcriptionally-active complex when the Cdx2 binding sites are mutated. Furthermore, Cdx2 and HNF1α bind directly to each other *in vitro*. These findings demonstrate the importance of protein–protein interactions for high levels of expression of an intestine-specific target gene.

EXPERIMENTAL

Plasmid construction

The plasmid pBSK-Cdx2 contains the 1 kb cDNA coding for hamster Cdx2 (also called Cdx3) from pBAT-Cdx3 [17] inserted into the *Sac*I/*Eco*RI sites of pBluescript SK+ (Stratagene). PCR was carried out with the Advantage-GC cDNA PCR kit (ClonTech) on this template using 3′ primers annealing to the vector, and 5′ primers containing a *Hin*dIII restriction site (underlined) and an ATG translational start codon. The sequences of the 5′ primers used to create pCMV-Cdx2 WT, pCMV-Cdx2 (105–313) and pCMV-Cdx2 (165–313) are: 5′-GGGAAGCTTCACCATGTACGTGAGCTACCTC-3′, 5′-GGGAAGCTTCACCATGGGCTACAGCAG-3′, and 5′-GGGAAGCTTCACCATGCGGAAGCCCGCGCAG-3′, respectively. PCR products were inserted into the *Hin*dIII/*Apa*I sites of pRC/CMV (Invitrogen) and sequenced throughout to verify that no unintended mutations were present.

In order to obtain GST (glutathione S-transferase) fusion vectors, the full-length cDNA for hamster Cdx2 was subcloned from p86Cdx [4] into the *Sal*I/*Not*I sites of pGEX6P2 (Pharmacia). The *Hin*dIII-released inserts of pCMV-Cdx2 (105–313) and pCMV-Cdx2 (165–313) were inserted into a pGEX6P2 derivative which contains the *Sal*I/*Not*I polylinker from pBluescript, whereby the correct reading frame was retained. The plasmid pBSK-HNF1α contains the 2.4 kb cDNA coding for rat HNF1α from pRSV-HNF [18] inserted into the *Hin*dIII/*Bam*HI sites of pBluescript KS+. The C-terminal deletion constructs of pBSK-HNF1α were obtained by replacement of *Pst*I/partial *Nco*I fragments with a double-stranded oligonucleotide containing an in-frame stop codon. The pBSK-HNF1α Δ34–291 construct was created by deletion of a partial *Sma*I fragment from pBSK-HNF1α.

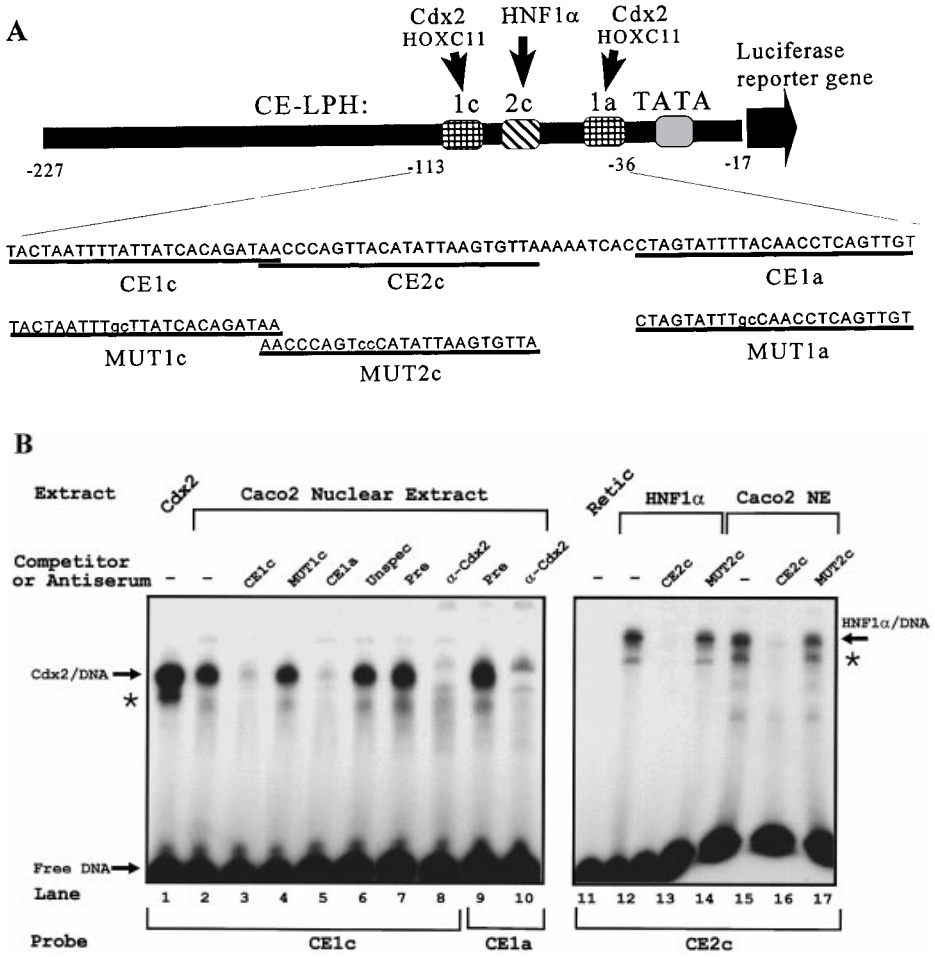
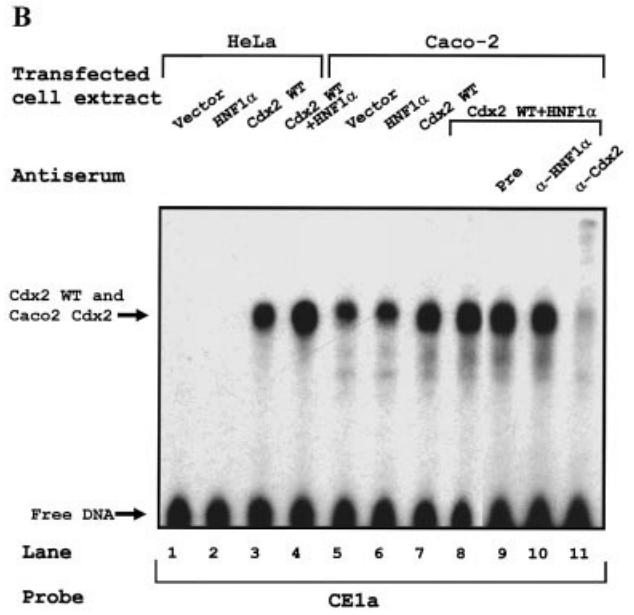


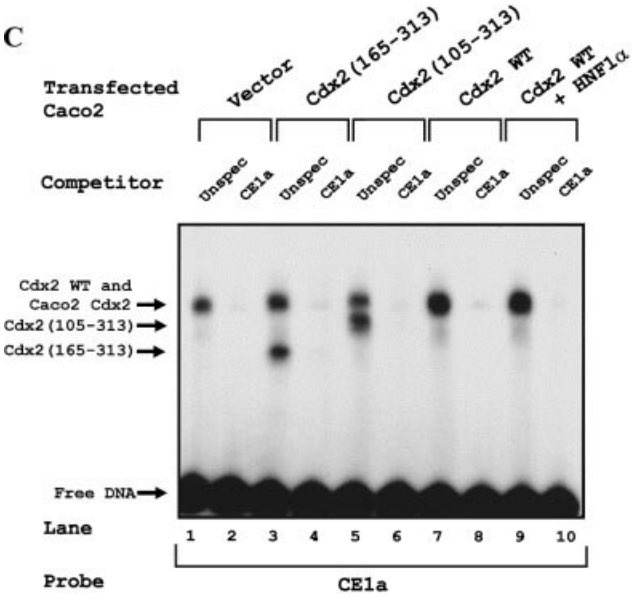
Figure 1 Binding sites for Cdx2 and HNF1α in the proximal LPH promoter

The location of the binding sites CE-LPH 1c, CE-LPH 2c and CE-LPH 1a, and the factors which interact with these sites within the proximal LPH promoter, are indicated (**A**). The sequence of the LPH-113/-36 probe, numbered relative to the start of transcription in the LPH gene, is shown. The sequence corresponding to the double-stranded probes used in EMSAs are underlined, and the bases which differ from the wild-type sequence are written in lower-case. (**B**) EMSA analysis with the indicated double-stranded probes was carried out with *in vitro*-translated Cdx2 (lane 1), HNF1α (lanes 12–14) or unprogrammed reticulocyte lysate (lane 11). Caco-2 nuclear extracts were assayed in lanes 2–10 and 15–17. Competitor DNA (100-fold excess), preimmune serum (Pre) or antiserum against hamster Cdx2 (α-Cdx2) were included as indicated. The main protein–DNA complexes formed are indicated with arrows. Minor complexes are marked by asterisks.

A

	LPH227WT - HNF1α	LPH227WT + HNF1α
Transfection of HeLa cells		
Vector	1.0 ± 0.9	9.4 ± 6.6
Cdx2 WT	81.2 ± 7.4	246.8 ± 41.0
Transfection of Caco2 cells		
Vector	1.0 ± 0.2	4.8 ± 0.4
Cdx2 WT	77.3 ± 4.1	695.0 ± 13.8
Cdx2 (105-313)	3.4 ± 0.6	70.3 ± 2.2
Cdx2 (165-313)	1.3 ± 0.3	19.2 ± 3.6





Construction of the LPH227WT and mutated derivatives in the pGL3 vector (Promega) has previously been described [4]. The sequence of each mutated element is shown in Figure 1(A) (mutated bases are in lower-case letters).

In vitro transcription/translation and GST pull-down assay

In vitro coupled transcription/translation using rabbit reticulocyte lysate (Promega) was carried out with pBSK-Cdx2 or pBSK-HNF1α plasmid templates in the absence or presence of [^{35}S]methionine as appropriate, according to the manufacturer's protocol. GST fusion proteins were expressed in *Escherichia coli* strain BL21 and bound to GSH–Sepharose beads using standard protocols (Pharmacia). Proteins were > 90 % pure as judged by Coomassie Blue-stained protein gel. Beads with bound GST or GST fusion protein (1 μg) were incubated *in vitro* with 5 μl of ^{35}S-labelled protein in 40 μl of binding buffer [20 mM Hepes (pH 7.4)/100 mM KCl/5 mM EDTA/5 mM EGTA/10 % (v/v) glycerol/0.5 % (w/v) BSA/ 0.4 % (v/v) Nonidet P40/1 mM dithiothreitol]. Binding reactions and washing steps were carried out as described [19]. Bound proteins were released by boiling in SDS-sample buffer [20] and resolved on 10 % (w/v) NuPAGE gels (Novex, San Diego, CA, U.S.A.).

Cell culture and transfections

Caco-2 cells were grown in minimum essential medium (Life Technologies) containing 10 % calf serum. Transient transfections were carried out using the calcium phosphate method as previously described [6]. All transfections contained 1 μg of a β-galactosidase expression plasmid as an internal standard for transfection efficiency (pCH110; Pharmacia) and 2.5 μg of wild-type (LPH227WT) or mutated (LPH227MUT) constructs. In addition, 1.25 μg of pCMV-Cdx2 WT, pCMV-Cdx2 (105–313) or pCMV-Cdx2 (165–313), and/or 0.63 μg of pRSV-HNF [18], made up to 2.5 μg with pRC-CMV, were co-transfected. Cells were harvested 48 h after transfection and analysed using the Dual Light System (Tropix, Bedford, MA, U.S.A.).

Electrophoretic-mobility-shift assays (EMSAs)

The LPH-113/-36 probe was generated by annealing and extending two overlapping oligonucleotides from −113 to −68 (end-labelled with [γ-^{32}P]ATP) and from −36 to −91 in the LPH promoter, using Advantage KlenTaq polymerase (ClonTech), followed by gel purification of the probe. Double-stranded oligonucleotide probes corresponding to the sequences shown in Figure 1(A) were end-labelled with [γ-^{32}P]ATP, gel-purified and used in EMSAs as described [6]. EMSA reactions contained 1–2 μl of *in vitro* transcription/translation products (Promega), 3.5 μg of Caco-2 nuclear extract [21] or 1 μg of whole cell lysate from transfected Caco-2 cells. In some experiments a 100-fold excess of unlabelled double-stranded oligonucleotide (competi-

Figure 2 Transcriptional synergy by Cdx2 deletion proteins and HNF1α

The LPH227WT reporter construct was co-transfected into HeLa and Caco-2 cells with or without an HNF1α expression plasmid (**A**). The effect of co-transfecting the indicated expression plasmids for wild-type (WT) or N-terminally deleted Cdx2 was tested. Luciferase activity was first corrected for transfection efficiency, and then expressed as fold-activation relative to LPH227WT alone. The means ± S.D. were obtained from three experiments. HD, homeodomain. (**B**) and (**C**) EMSA analysis with the CE1a double-stranded probe was carried out with extracts prepared from HeLa or Caco-2 cells transfected as in (**A**). Preimmune serum (Pre) or antibodies against HNF1α (α-HNF1α) or hamster Cdx2 (α-Cdx2) or competitor DNA (100-fold excess) were included as indicated. The main protein–DNA complexes formed are indicated with arrows.

tor) was included in the reaction (Figure 1A). The sequence of the unspecific competitor is given in [6]. Supershift analysis with antiserum against the C-terminal portion of hamster Cdx2 [17], antiserum specific for HNF1α [22] or preimmune serum was carried out as described in [6].

RESULTS

Binding of Cdx2 and HNF1α to the proximal LPH promoter

We have previously shown that Cdx2 binds to the CE-LPH 1a *cis* element in the proximal LPH promoter [6]. Since Cdx2 was still able to activate transcription from the LPH promoter when CE-LPH 1a was mutated, we suggested that Cdx2 might bind to an additional *cis* element in the LPH promoter which contains a TTTA(T/C) consensus sequence for Cdx2 binding [6]. A probe corresponding to the proposed CE-LPH 1c element was therefore tested in EMSA (Figure 1). *In vitro*-translated Cdx2 binds to the CE1c probe, with a similar mobility as a factor present in nuclear extracts from differentiated Caco-2 cells (Figure 1B, lanes 1 and 2). The complex formed between CE1c and the Caco-2 nuclear factor is specific, as shown by competition with unlabelled CE1c or CE1a, but not by an unrelated unspecific oligonucleotide. MUT1c contains a mutation of two bases within the TTTAT core sequence, and no longer competes for binding. Antibodies directed against hamster Cdx2 block formation of the complex between CE1a and a Caco-2 nuclear factor, without producing a supershifted complex, as previously demonstrated [6]. Formation of the complex with CE1c is also blocked by Cdx2 antibodies, but not by preimmune serum (Figure 1B), suggesting that Cdx2 present in Caco-2 nuclear extracts binds to both CE-LPH 1a and CE-LPH 1c elements.

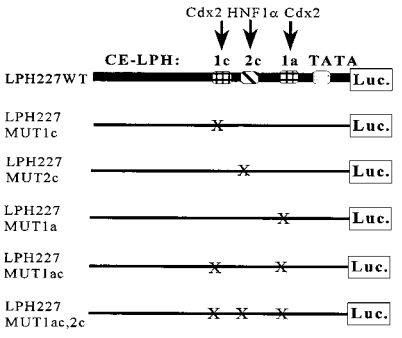
Caco-2 nuclear extracts contain HNF1α, which binds to the *cis* element CE-LPH 2c [2]. As demonstrated in Figure 1(B), *in vitro* translated HNF1α is also able to bind to the CE2c probe, and an oligonucleotide containing a 2 bp mutation within CE2c (MUT2c) does not compete for HNF1α binding.

Synergistic activation of the LPH proximal promoter by Cdx2 and HNF1α

In order to find out if Cdx2 and HNF1α synergize on the LPH proximal promoter, Caco-2 and HeLa cells were transfected with the LPH227WT construct, containing the proximal promoter from -227 to -17 in front of the luciferase reporter gene. Although subconfluent Caco-2 cells contain endogenous Cdx2 and HNF1α [2,23], the activity of the reporter construct alone is low (Figure 2A). This activity is increased 5-fold by co-transfection of the expression plasmid for HNF1α, and 77-fold by Cdx2 alone. Co-transfection of HNF1α and Cdx2 expression plasmids leads to a 700-fold activation of the LPH227WT construct, which greatly exceeds the sum of the activation obtained by each activator alone. For comparison, HeLa cells were co-transfected with the same constructs, since these cells have no endogenous expression of Cdx2, HNF1α or LPH [4,5,16]. Synergy between Cdx2 and HNF1α was also observed in HeLa cells (Figure 2A), but to a lower extent than in Caco-2 cells.

In order to investigate the effect of overexpressing HNF1α on Cdx2 DNA-binding activity, the CE1a binding site was used as a probe in EMSA, using extracts from transfected cells. The complex observed with extracts from Caco-2 cells co-transfected with expression constructs for Cdx2 and HNF1α co-migrates with that obtained using HeLa cell extracts in which there is no endogenous Cdx2 or HNF1α (Figure 2B, compare lanes 4 and 8). Formation of this complex is inhibited by antibodies specific for Cdx2 and not by anti-HNF1α antibodies, suggesting that Cdx2 is the only protein in transfected Caco-2 cells present in the complex with the CE1a probe. In Caco-2 cells, co-transfection of the HNF1α expression plasmid together with Cdx2 does not enhance the DNA-binding activity of Cdx2 as shown by EMSA (Figure 2B, compare lanes 7 and 8) or lead to increased Cdx2 protein levels as shown by Western-blot analysis (results not shown).

It has previously been demonstrated that the activation domain of Cdx2 lies in the N-terminal 180 amino acids [24,25]. In order to determine if the N-terminal region of Cdx2 is also important for transcriptional synergy with HNF1α, various deletion constructs of Cdx2 were tested in co-transfection assays using Caco-2 cells. EMSA analysis of cell extracts from transfected Caco-2 cells confirmed that each Cdx2 deletion construct was expressed, and retained specific DNA-binding activity (Figure 2C). Removal of the first 104 amino acids in the Cdx2 (105–313) mutant decreases the transcriptional activity of Cdx2 alone by 96% in Caco-2 cells co-transfected with the LPH227WT reporter construct (Figure 2A). However, synergy is still observed between Cdx2 (105–313) and HNF1α. The Cdx2 (165–313) mutant, in which amino acids 1–164 were removed, is no longer able to activate LPH227WT alone in Caco-2 cells (Figure 2). However,



	Vector	HNF1α	Cdx2	Cdx2+ HNF1α
LPH227WT	0.04 ± 0.00	0.27 ± 0.07	11.2 ± 1.4	100 ± 10.9
LPH227 MUT1c	0.05 ± 0.03	0.40 ± 0.05	3.7 ± 0.5	68.9 ± 9.7
LPH227 MUT2c	0.00 ± 0.01	0.00 ± 0.01	10.0 ± 0.5	40.1 ± 3.6
LPH227 MUT1a	0.01 ± 0.01	0.29 ± 0.09	1.0 ± 0.3	43.8 ± 1.1
LPH227 MUT1ac	0.00 ± 0.03	0.37 ± 0.00	0.7 ± 0.2	25.9 ± 2.7
LPH227 MUT1ac,2c	0.00 ± 0.03	0.04 ± 0.02	0.6 ± 0.4	5.1 ± 2.3

Figure 3 DNA binding is required for synergistic activation of the LPH promoter by Cdx2 and HNF1α

The indicated reporter constructs were co-transfected into Caco-2 cells with and without the expression vectors for Cdx2 and HNF1α as indicated. The location of point mutations (Figure 1A) in the LPH promoter are shown schematically with crosses. Luciferase activity was first corrected for transfection efficiency. After subtraction of the background activity of the pGL3-Basic plasmid (devoid of LPH promoter sequence), reporter activation was expressed relative to LPH227WT plus Cdx2 and HNF1α (arbitrarily set at 100). The means $\pm$ S.D. were obtained from three experiments.

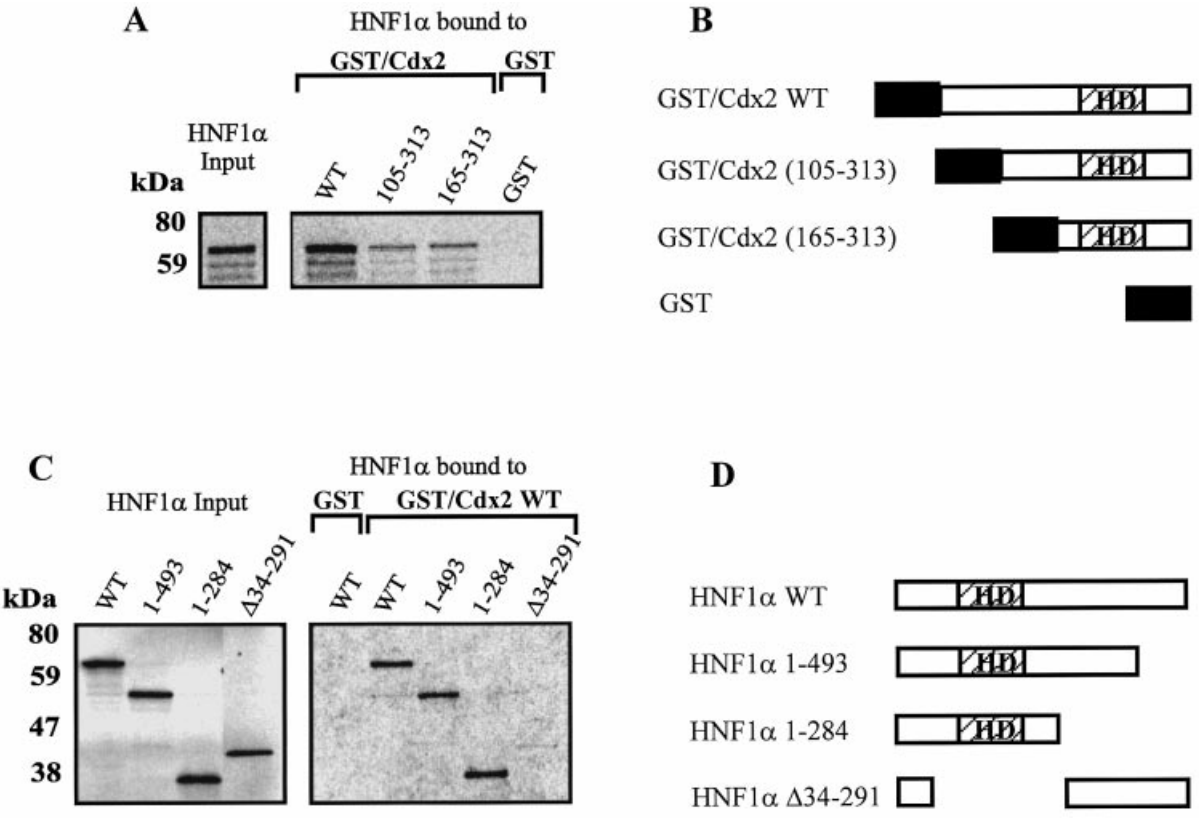


Figure 4 Association between Cdx2 and HNF1α

(**A**) *In vitro* [^{35}S]methionine-labelled HNF1α was incubated with the indicated GST fusion proteins bound to GSH–Sepharose beads, and the bound HNF1α was eluted and analysed on a 10% (w/v) NuPAGE gel. About 20% of the input was also analysed (HNF1α Input). (**B**) Structure of the GST fusion proteins. The black box represents the GST portion. (**C**) NuPAGE gel of the *in vitro* [^{35}S]methionine-labelled HNF1α wild-type (WT) and deleted proteins used in the GST-pull down assay (HNF1α Input). The indicated *in vitro* [^{35}S]methionine-labelled HNF1α protein was incubated with GST or GST–Cdx2 WT bound to GSH–Sepharose beads, and the bound HNF1α was eluted and analysed on a 10% (w/v) NuPAGE gel. (**D**) Structure of the HNF1α WT and deletion constructs. HD, homeodomain.

there is a small stimulation of HNF1α-dependent activation by the Cdx2 (165–313) mutant, probably due to interactions with HNF1α, since Cdx2 (165–313) and HNF1α associate *in vitro* (Figure 4A).

Transcriptional synergy by Cdx2 and HNF1α is likely to occur through the simultaneous binding of both factors to the LPH promoter. In order to test the requirement of Cdx2 and HNF1α binding sites in the proximal LPH promoter for synergistic activation, various mutated promoter constructs were analysed in Caco-2 cells (Figure 3). The activity of each LPH promoter construct is given relative to LPH227WT co-transfected with Cdx2 and HNF1α expression plasmids, after subtraction of the corresponding background activity of the promoterless pGL3-Basic. The basal activity of the LPH promoter in Caco-2 cells that do not overexpress Cdx2 or HNF1α is very low. Co-transfection of the HNF1α expression plasmid alone results in slight stimulation of reporter gene activity from the wild-type LPH promoter, whereas mutations in the CE-LPH 2c site (LPH227MUT2c and LPH227MUT1ac2c) decrease the activity to the background levels observed with pGL3-Basic. Activation by Cdx2 alone is decreased 11-fold by a mutation in the CE-LPH 1a site (LPH227MUT1a), which blocks binding to this site [5], whereas a mutation in the CE-LPH 1c site (LPH227MUT1c) decreases the activation by Cdx2 alone by 3-fold. However, synergistic stimulation by HNF1α in the presence of co-expressed Cdx2 is observed in all the constructs containing mutations in Cdx2 binding sites (LPH227MUT1c, LPH227MUT1a and LPH227MUT1ac). This demonstrates that Cdx2 binding to CE-LPH 1a and CE-LPH 1c is important for activation of the pig proximal LPH promoter by Cdx2 alone, in agreement with previous results [6], but is not required for transcriptional synergy with HNF1α. In contrast with these results, mutation of the HNF1α binding site in the LPH227MUT2c construct decreases the fold-stimulation by HNF1α on Cdx2-mediated activation to below that observed for the wild-type template (LPH227WT). This suggests that binding of HNF1α to the promoter is required for synergy with Cdx2. The low level of stimulation by HNF1α on templates containing mutated CE-LPH 2c sites might be due to residual binding of HNF1α to the mutated element. Mutation of the CE-LPH 1a, CE-LPH 1c and CE-LPH 2c sites in the LPH227MUT1ac,2c construct leads to approximately background levels of stimulation by Cdx2 and HNF1α, similar to that observed for pGL3-Basic, which lacks any promoter sequence.

In vitro association between Cdx2 and HNF1α

In order to understand the molecular mechanisms behind the observed synergy, we investigated whether HNF1α and Cdx2 interact with each other *in vitro*, using GST pull-down assays. *E. coli*-expressed GST, or fusion proteins of GST with Cdx2, were

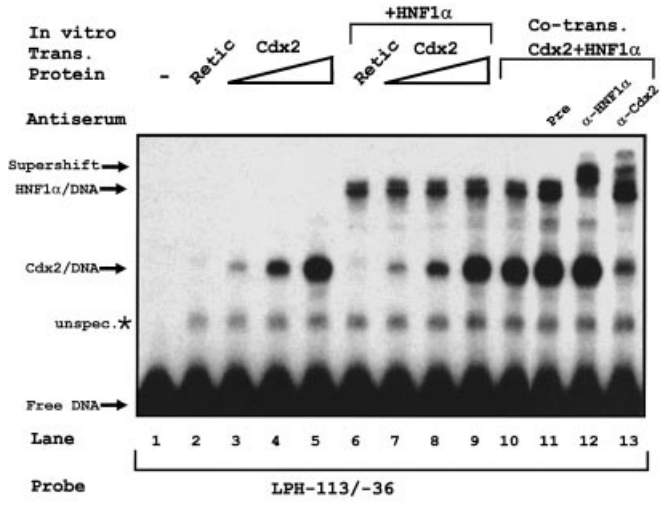


Figure 5 Binding of *in vitro*-translated Cdx2 and HNF1α to the LPH-113/-36 probe

EMSA analysis with the LPH-113/-36 probe (Figure 1) was carried out with increasing amounts of *in vitro*-translated Cdx2 (0.1 μl in lanes 3 and 6, 0.5 μl in lanes 4 and 8, and 2 μl in lanes 5 and 9) or unprogrammed reticulocyte lysate (lanes 2 and 6). *In vitro*-translated HNF1α was included in lanes 6–9. In lanes 10–13, co-translated Cdx2 + HNF1α was used. The total volume of lysate added in lanes 2–13 was kept constant. Preimmune serum (Pre) or antibodies against HNF1α (α-HNF1α) or hamster Cdx2 (α-Cdx2) were included as indicated (lanes 11–13). The main protein–DNA complexes formed are indicated with arrows. The asterisk represents an unspecific complex formed by the lysate.

immobilized on GSH–Sepharose beads, and used to demonstrate that *in vitro*-translated HNF1α is pulled down by GST/Cdx2 WT protein, but not by GST alone (Figure 4A). The GST fusion proteins with Cdx2 (105–313) and Cdx2 (165–313) are also able to pull down labelled HNF1α protein, albeit to a lesser extent than full-length Cdx2 (Figure 4A). Thus the C-terminal half of Cdx2, which includes the homeodomain region, contains an interaction domain with HNF1α. This interaction is probably stabilized by the N-terminal region of Cdx2, as suggested by the stronger interaction of HNF1α with full length Cdx2 (Figure 4A).

The region of HNF1α that is required for interaction with Cdx2 was similarly tested (Figure 4C). Constructs with a progressive deletion of the C-terminal activation domain of HNF1α retain the ability to bind Cdx2 in the *in vitro* assay. However, deletion of the region encompassing the HNF1α homeodomain (HNF1α Δ34–291) leads to a marked reduction in this association (Figure 4C). Thus the homeodomain of HNF1α is most likely to be the region of interaction with Cdx2, although the possibility exists that deletion of the homeodomain region leads to a conformational change outside the homeodomain that disturbs the HNF1α–Cdx2 interaction. In order to test the specificity of this interaction, the ability of another homeodomain protein, HOXC11, to associate with GST–Cdx2 WT was tested and was shown to also bind (results not shown).

Protein–protein interactions between Cdx2 and HNF1α might lead to the observed synergistic activation of LPH through an induced conformational change resulting in increased transcriptional activity of either factor, and/or via co-operative DNA binding. In order to test this, the region between −113 and −36 was tested in EMSA with *in vitro*-translated proteins (Figure 5). We were unable to demonstrate facilitated binding of the factors to the DNA probe, even when Cdx2 and HNF1α were co-translated in the same reticulate lysate (lane 10). The identity of the proteins in each complex was confirmed by supershift (lanes 11–13). Formation of a ternary complex was probably not observed, owing to the low ratio of bound to free probe, and to the EMSA conditions which disrupt labile complexes.

DISCUSSION

Functional synergy between Cdx2 and HNF1α in transcription of LPH

Using mutagenesis, co-transfections and EMSA analyses, we have identified two binding sites for Cdx2 and one binding site for HNF1α in the pig proximal promoter of the intestinal specific gene, LPH. We demonstrate here that Cdx2 synergizes with HNF1α in transcriptional activation of the LPH gene promoter. In agreement with recent experiments which demonstrated that the N-terminal 180 amino acids of mouse and hamster Cdx2 encode a transcriptional activation domain [24,25], our deletion analysis of Cdx2 shows that activation of the LPH promoter-driven reporter gene by the Cdx2 construct lacking the N-terminal 104 amino acids, Cdx2 (105–313), is only 4% of that activated by full-length Cdx2. However, we show that Cdx2 (105–313) is still capable of synergistic stimulation with HNF1α, although the total level of transcriptional activation by Cdx2 (105–313) and HNF1α is about 10-fold lower than for wild-type Cdx2 and HNF1α. Even the Cdx2 construct which is transcriptionally inactive when co-transfected alone with the reporter plasmid, Cdx2 (165–313), stimulates HNF1α-dependent activity, though to a lesser extent. This N-terminally deleted Cdx2 protein retains DNA-binding activity and the ability to interact *in vitro* with HNF1α, suggesting that it may interact with HNF1α bound to the promoter.

Recent studies have shown the direct interaction of Cdx2 with the paired homeodomain protein Pax-6, the homeodomain protein Pdx-1 and the transcriptional co-activator CBP [23,26,27]. The region in Cdx2 important for interaction with CBP was localized to the homeodomain [23]. Our *in vitro* and transfection studies implicate the C-terminal half of Cdx2, containing the homeodomain, to be involved in the physical interaction with HNF1α. Since deletion of the homeodomain of HNF1α disrupts the *in vitro* interaction with Cdx2, the homeodomain of HNF1α is most likely to be the region of interaction with Cdx2. Involvement of the homeodomain in both DNA-binding and protein–protein interaction has previously been observed, for example in the Pit-1–Oct-1 interaction [28].

Synergistic activation of the LPH promoter occurs even when the Cdx2 binding sites in the proximal promoter are mutated. This suggests that DNA-bound HNF1α is able to tether Cdx2 to the promoter via protein–protein interactions. We were unable to demonstrate co-operative binding of the factors to DNA by EMSA analysis. This might be due to the EMSA conditions, or to the absence of modifications or cofactors which may stabilize the *in vivo* interaction of DNA-bound Cdx2 and HNF1α. Protein–protein interactions between Cdx2 and HNF1α may induce a conformational change in either protein leading to increased transcriptional activity. In this regard, it has been reported that the transcriptional activity of a LexA:Cdx2 chimaera containing the first 180 amino acids of hamster Cdx2 is at least 4-fold higher than that of a chimaera containing full-length Cdx2 [25].

Implications for intestinal gene expression

Our data show the synergistic activation of a pig LPH promoter-driven construct by overexpression of Cdx2 and HNF1α in the

intestinal Caco-2 cell line. Synergy was also observed in transient transfections in HeLa cells, which do not contain endogenous Cdx2 or HNF1α. Since the observed synergy was higher in Caco-2 cells, it is suggested that cell-specific factors are involved, in a similar way to that proposed for the activation by Cdx2 from enhancers [24].

Cdx2 and HNF1α are both expressed in epithelial cells in the small intestine from an early stage in development, consistent with an important role in intestinal development [11]. Binding sites for Cdx2 and HNF1α are present in the proximal promoters of several intestine-specific genes, including LPH, sucrase–isomaltase and intestinal phospholipase A/lysophospholipase [2,7,8]. Mutagenesis and deletion studies have demonstrated the importance of the binding sites for Cdx2 and HNF1α in the expression of LPH and sucrase-isomaltase by endogenous transcription factors present in Caco-2 cells [2,3,29].

Why might the synergy between Cdx2 and HNF1α be of importance for small-intestinal gene expression? One possibility is that much higher levels of tissue-specific expression would result from the simultaneous presence of both transcription factors. Thus LPH expression is low in tissues where only HNF1α is expressed, such as the liver and kidney, owing to the absence of Cdx2. However, this cannot be the full explanation for tissue-specificity, since LPH is not expressed in the adult colon or in undifferentiated Caco-2 cells, even though both Cdx2 and HNF1α are present [2,23]. Another possibility is that the factors have different functions during crypt–villus differentiation in the small intestine. For example, HNF1α has been implicated in chromatin remodelling of target genes [30]. Finally, the synergistic activation of an intestine-specific gene by two factors allows for the fine tuning of developmental and spatial expression by targeting of either factor.

From these findings, we suggest that the intestine-specific expression pattern of LPH may be due, at least in part, to the simultaneous presence of HNF1α and Cdx2. Furthermore, we infer that the frequent occurrence of binding sites for these factors in the *cis* elements of intestine-specific genes is of functional importance. Thus it will be of interest to determine whether HNF1α and Cdx2 are also able to synergize in the activation of other intestinal genes.

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