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Pbx1 is a co-factor for Cdx-2 in regulating proglucagon gene expression in pancreatic A cells

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Abstract

A number of Hox and Hox-like homeodomain (HD) proteins have been previously shown to utilize members of the TALE HD protein family as co-factors in regulating gene expression. The caudal HD protein Cdx-2 is a transactivator for the proglucagon gene, expressed in pancreatic A cells and intestinal endocrine L cells. We demonstrate here that co-transfection of the TALE homeobox gene Pbx1 enhanced the activation of Cdx-2 on the proglucagon promoter in either the pancreatic A cell line InR1-G9 or BHK fibroblasts. The activation was observed for proglucagon promoter constructs with or without the binding motifs for Pbx1. Furthermore, mutating the penta-peptide motif (binding motif for TALE HD proteins) on Cdx-2 substantially attenuated its activation on proglucagon promoter, but not on the sucrase–isomaltase gene (SI) promoter, or its own (Cdx-2) promoter; suggesting that Cdx-2 utilizes Pbx1 as a co-factor for regulating the expression of selected target genes. Physical interaction between Cdx-2 and Pbx1 was demonstrated by co-immunoprecipitation as well as GST fusion protein pull-down. We suggest that this study reveals a novel function for Pbx1 in pancreatic islet physiology: regulating proglucagon expression by serving as a co-factor for Cdx-2.

Keywords: Proglucagon; Cdx-2; Pbx1; Homeobox gene; GST pull down

1. Introuction

The proglucagon gene is expressed in pancreatic islet A cells, intestinal endocrine L cells and selected endocrine neurons in the brain (Kieffer and Habener, 1999). It encodes three major peptide hormones: glucagon, glucagon like peptide 1 (GLP-1) and glucagon like peptide 2 (GLP-2). These hormones exert opposite or overlapping physiological functions (Drucker, 2003; Drucker et al., 1996; Meeran et al., 1999; Turton et al., 1996; Tang-Christensen et al., 2001).

It has been revealed that a number of transcription factors are able to interact with the first 300 bp proglucagon 5′ flanking (promoter) region (Knepel et al., 1990; Philippe et al., 1988). These interactions are considered to play sophisticated roles in regulating proglucagon expression in temporal and spatial manners. The transcription factors include the helix–loop–helix proteins E12/E47, Beta2/neuroD, members of the hepatocyte nuclear family (HNF3α, 3β and 3γ) and homeodomain (HD) proteins (Gauthier et al., 2002; Hussain et al., 1997; Jin and Drucker, 1996; Laser et al., 1996; Sander et al., 1997). We reported previously that the caudal HD protein Cdx-2 binds to two AT rich motifs within the G1 enhancer element of the proglucagon promoter and activates its transcription (Jin and Drucker, 1996). In addition, over-expressing Cdx-2 in the pancreatic InR1-G9 cell line was shown to stimulate endogenous proglucagon mRNA expression (Jin et al., 1997; Trinh et al., 1999).

Cdx-2 plays critical roles in embryo implantation and embryogenesis (Chawengsaksophak et al., 1997). In adult

Abbreviations: CRE, cAMP response element; EMSA, electrophoretic mobility shift assay; Exd, Extradenticle; GLP-1, glucagon like peptide 1; GLP-2, glucagon like peptide 2; glu, proglucagon; HD, homeodomain; LUC, luciferase; PDGP, proglucagon derived peptide; SI, sucrase–isomaltase; TALE, three amino acid loop extension

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animals, Cdx-2 is abundantly expressed in differentiated non-endocrine intestinal epithelia, and may have more than a dozen other potential target genes. Furthermore, Cdx-2 was shown to regulate the expression of its own promoter (da Costa et al., 1999; Xu et al., 1999). Cdx-2 was found to activate its own promoter only when the examination was conducted in Cdx-2 expressing cell lines, but not in Cdx-2 non-expressing fibroblasts (Xu et al., 1999). Taylor et al. also reported that Cdx-2 activates the sucrase–isomaltase (SI) gene promoter only in the Cdx-2 expressing intestinal Caco-2 cell line, but not in NIH-3T3 fibroblasts. In contrast, Cdx-2 was shown to activate the expression of proglucagon promoter in Cdx-2 expressing pancreatic and intestinal endocrine cell lines, as well as in BHK and NIH-3T3 cells (Jin and Drucker, 1996). These observations suggest that Cdx-2 may require cell type specific co-factors to regulate the expression of some of its downstream target genes, such as SI and Cdx-2 itself. For regulating the expression of proglucagon promoter, Cdx-2 may utilize a ubiquitously expressed co-factor. Because many Hox and Hox-like HD proteins, including another pancreatic cell Hox-like HD protein Pdx-1, were shown to utilize the three amino acid loop extension (TALE) HD protein Pbx1 as the co-factor, we propose that ubiquitously expressed Pbx1 also serves as a co-factor for Cdx-2 in regulating the expression of the proglucagon gene. We present here our experimental evidence that support this hypothesis.

2. Materials and methods

2.1. Materials

Cell culturing medium, fetal bovine serum (FBS), oligonucleotides and TRIzol reagent for RNA extraction, were purchased from Invitrogen Life Technology Inc. (Burlington, Ontario, Canada). Glutathione beads and reduced glutathione, as well as α-^{32}P labeled dCTP were obtained from Amersham Pharmacia Biotech (Baie d'Urfe, Quebec, Canada). Protein A sepharose was purchased from Upstate Biotechnology Inc. (UBI, Lake Placid, New York, USA). Restriction enzymes and DNA modification enzymes were molecular biology grade and were purchased from several sources. Other chemical reagents were purchased either from Bishop Canada Inc. (Burlington, Ontario, Canada) or Sigma–Aldrich Canada Ltd. (Oakville, Ontario, Canada).

2.2. Plasmids construction

The generation of Cdx-2/Luciferase (-374Cdx-LUC) and proglucagon-LUC (Glu-LUC) fusion gene constructs (−476 and −82) has been previously described (Jin and Drucker, 1995). The human sucrase–isomaltase gene promoter (−183–+54)/LUC fusion gene construct (SI-LUC) was kindly provided by Claude Asselin (Wu et al., 1992). Hamster Cdx-2 cDNA was originally provided by Michael German (German et al., 1992). For this study, the coding sequence of Cdx-2 was sub-cloned into the pcDNA3.1 vector (Invitrogene Life Technology Inc.) as the wild type Cdx-2 expression plasmid, and into the pGEX4T-2 vector (Amersham Pharmacia Biotech) for generating wild type Cdx-2-GST fusion protein (Xu et al., 1999). Human Pbx1, Pbx2 and Pbx3 cDNAs were gifts from Cornelis Murre (van Dijk et al., 1995). For this study, the cDNA fragments of these three genes were inserted into the pcDNA3.1 expression plasmid. Four additional Cdx-2 expression plasmids were generated. They carry site mutations on the Cdx-2 coding region, as illustrated in Fig. 3A. These constructs were generated by PCR using Vent polymerase, and sub-cloned into the pcDNA3.1 vector. Three additional Cdx-2-GST fusion protein constructs were generated. They carry site mutations or deletions on the Cdx-2 coding region, as illustrated in Fig. 7A. PCR products for sub-cloning were verified by DNA sequencing.

2.3. Cell culturing, plasmid transfection and LUC reporter gene analysis

The cultivation of pancreatic cell lines InR1-G9 and In111 has been described previously (Jin and Drucker, 1996). The human colon cancer cell line SW480 and two fibroblast cell lines, COS-7 and BHK, were obtained from American Type Culture Collection (ATCC). These cell lines were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (Chen et al., 2005; Jin and Drucker, 1996). For LUC reporter gene analysis, cell line transfection was conducted using the method of calcium phosphate precipitation. Sixteen to 18 h after the transfection, cells were harvested for LUC reporter gene analysis (Jin and Drucker, 1995). The same calcium phosphate precipitation method was used to generate Cdx-2 or Pbx1 protein expression or over-expression in BHK or COS-7 fibroblasts for verifying the expression level of the Cdx-2 expressing plasmids and for co-immunoprecipitation analysis, respectively.

2.4. Western blotting, co-immunoprecipitation and GST fusion protein pull-down assay

The methods for whole cell protein extraction, nuclear protein extraction, generation of Cdx-2 antibody and Western blotting have been previously described (Jin and Drucker, 1996; Trinh et al., 1999; Xu et al., 1999; Chen et al., 2005). The anti-β-actin monoclonal antibody was purchased from Sigma–Aldrich Canada Ltd. The anti-Pbx1 polyclonal antibody was purchased from UBI. The anti-GST polyclonal antibody was obtained from Amersham Pharmacia Biotech.

Whole cell lysates of the pancreatic InR1-G9 cells were prepared using radioimmunoprecipitation assay (RIPA) lysis buffer for co-immunoprecipitation (co-IP) (Branch and Mills, 1995). Following the determination of protein concentration, InR1-G9 whole cell lysates were diluted with the RIPA lysis buffer to a final concentration of 1 μg protein per μl. After a "pre-clear procedure" with protein A sepharose beads, the samples were incubated with the anti-Pbx1 antibody at 4 °C for 24 h with gentle rotation. The immune-complexes were then captured by protein A sepharose beads, followed by Western blotting.

GST fusion proteins were generated in the BL-21 strain of *Escherichia coli* with 0.2 μM IPTG as the inducer, purified with glutathione beads, and eluted using reduced glutathione, as previously described (Xu et al., 1999). To verify the generation of expected size GST and Cdx-2-GST fusion proteins for each plasmid construct, purified GST or Cdx-2-GST fusion proteins were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie blue staining. The method for the GST fusion protein pull-down assay has been previously described (Ma et al., 2003).

2.5. Densitometry analysis and statistics

All LUC reporter gene analyses in this study were performed at least in triplicate. Relative LUC activities were calculated in each set of experiments, normalized against total protein utilized in the LUC assay and the results of β-gal assay with the same sample. The results are presented as mean ± standard error of the mean (S.E.M.) ($n \geq 3$). Variations between two groups were analyzed by paired Student's t-test.

3. Results

3.1. Pbx1 enhances the activation on proglucagon promoter by Cdx-2

Cdx-2 was shown to bind to the G1 enhancer element (Jin and Drucker, 1996; Laser et al., 1996), while Pbx1 binds to G3, G5 and possibly the cAMP response element (CRE), located within the first 300 bp 5′ flanking region of the proglucagon gene (Fig. 1A; Herzig et al., 2000). The BHK fibroblasts, which do not express Cdx-2 or proglucagon (Jin and Drucker, 1996; Xu et al., 1999). We first asked the following two questions. (1) In BHK cells, whether Pbx1 on its own activate −476 GLU-LUC, which

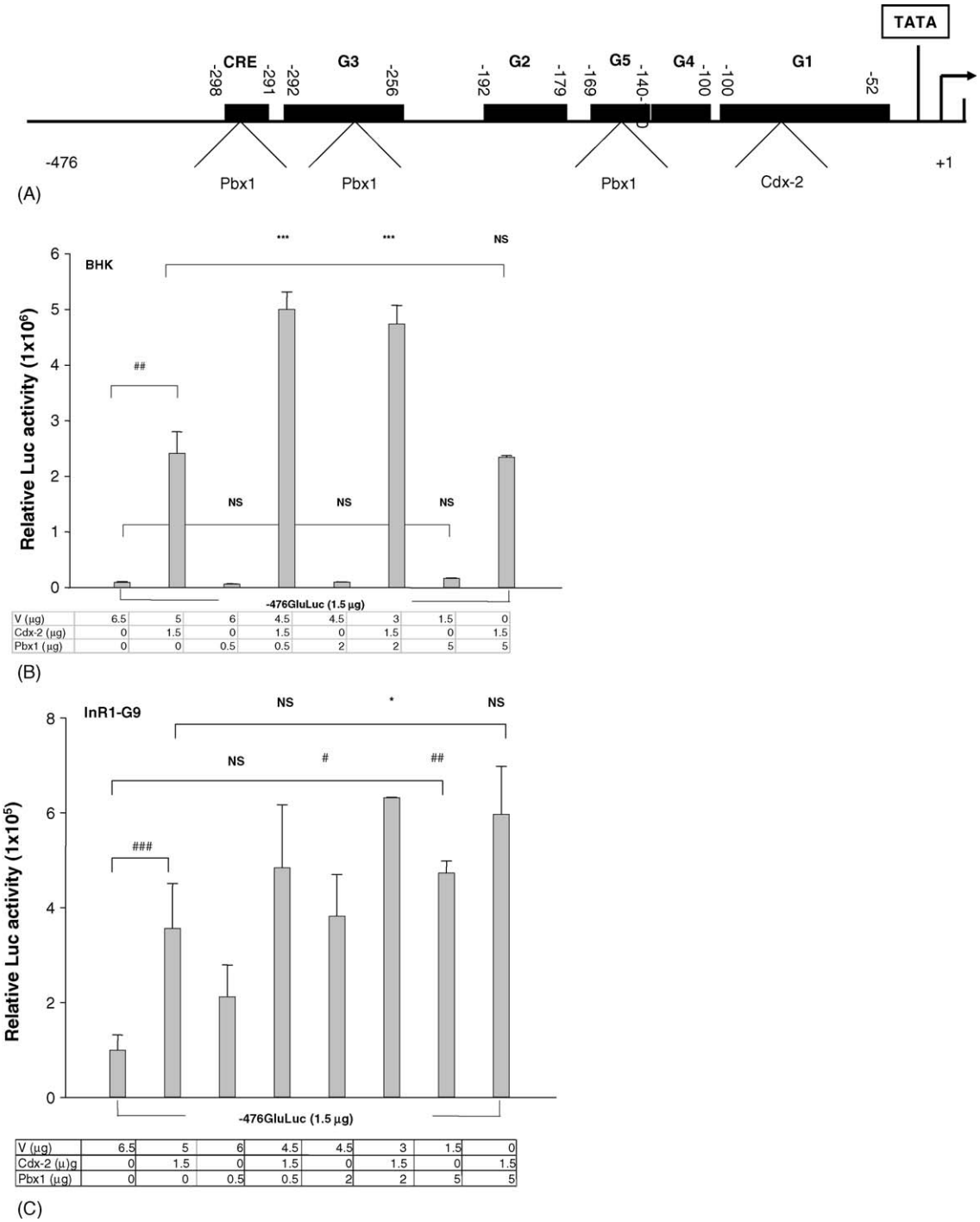

Fig. 1. Pbx1 enhances the activation on proglucagon promoter by Cdx-2. (A) Overall organization of the first 300 bp 5′ flanking region of the rat proglucagon promoter. CRE, cyclic AMP response element. G1–G5, proglucagon enhancer like elements. (B) Constant amount of −476 GLU-LUC (1.5 μg) and Cdx-2 expression plasmid (1.5 μg) were co-transfected into the BHK cells with indicated amount of Pbx1 expression plasmid and the empty vector pcDNA3.1 (V). Relative LUC activity was calculated in each set of experiments, normalized against total protein utilized in the LUC assay and the results of β-gal assay (mean ± S.E.M., $n \geq 3$). (C) Constant amount of −476 GLU-LUC (1.5 μg) and Cdx-2 expression plasmid (1.5 μg) were co-transfected into InR1-G9 cells with the indicated amount of Pbx1 expression plasmid and the empty vector pcDNA3.1 (V). NS, $P > 0.5$; # and *, $P < 0.5$; ##, $P < 0.01$; ### and ***, $P < 0.001$.

contains both Cdx-2 and Pbx1 binding sites. (2) In BHK cells, whether Pbx1 co-transfection would enhance the stimulatory effect of Cdx-2. For these purposes, a fixed amount of Cdx-2 expression plasmid and −476 GLU-LUC fusion plasmid (1.5 μg each) were co-transfected with increasing amounts of Pbx1. As shown in Fig. 1B, transfection of Pbx1 on its own (0.5, 2 and 5 μg) generated virtually no activation. In contrast, when co-transfected with Cdx-2, Pbx1 at concentrations of 0.5 and 2 μg, but not at 5 μg, substantially enhanced the activation of Cdx-2 on −476 GLU-LUC.

The same approach was then utilized in the InR1-G9 cell line. As shown in Fig. 1C, Pbx1 itself activated the expression of

−476 GLU-LUC in a dose dependent manner. Considering the lack of activation by Pbx1 on its own in the BHK fibroblasts, the activation observed here could be attributed to the existence of endogenous Cdx-2. Co-transfection of Cdx-2 with Pbx1 generated an additive effect. We have conducted this experiment nine times, with three different batches of plasmid DNA preparations. Each time, we observed an additive effect with two different Pbx1 concentrations. Fig. 1C shows that when we pool all the data together, the additive effect is confirmed only with 2.0 μg Pbx1.

3.2. Pbx1 enhances the activation by Cdx-2 on the proglucagon promoter construct without the Pbx1 binding motifs

To further verify the requirement of Cdx-2 for Pbx1 in activating proglucagon promoter, we conducted the examination in the InR1-G9 cells with the −82 GLU-LUC fusion gene construct, which contains Cdx-2 but not Pbx binding motifs. Two additional Pbx expression plasmids, Pbx2 and Pbx3 were also included in this experiment. In contrast with what we observed for the −476 GLU-LUC fusion gene, Pbx1, Pbx2, or Pbx3 co-transfection generated no appreciable activation on the expression of –82 GLU-LUC on its own (Fig. 2), suggesting biological significance of the three Pbx binding sites. When co-transfected with Cdx-2, Pbx1 or Pbx2, but not Pbx3, enhanced the activation on −82 GLU-LUC by Cdx-2 (Fig. 2).

3.3. Mutating the penta-peptide motif attenuates its activation on proglucagon promoter

Results from the above studies suggest that Pbx1, and possibly Pbx2, may serve as a co-factor for Cdx-2 in activating proglucagon expression. We then performed further LUC reporter gene analyses, addressing the question in a different way. Fig. 3A shows the overall organization of Cdx-2. A–C represent conserved regions among all members of the caudal HD proteins. The penta-peptide motif is located within

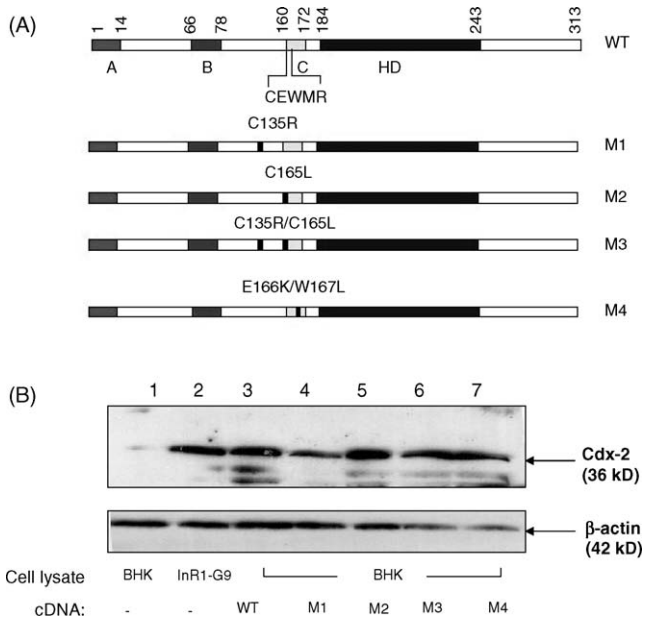

Fig. 3. Schematic representation of mutant Cdx-2 expression plasmids. (A) Schematic representation of the wild type and the mutant Cdx-2 expression plasmids. A–C are conserved regions among all members of the caudal HD protein family. CEWMR represents the penta-peptide motif of Cdx-2. Among these five amino acid residues, WM are conserved among the penta-peptide motifs of all Hox/Hox-like HD proteins (Peers et al., 1995). (B) Examination of Cdx-2 expression in BHK cells transiently tranfected with the wild type and the mutant Cdx-2 expression plasmids. Approximately 20 μg nuclear proteins from each sample was used for Western blotting with the anti-Cdx-2 polyclonal antibody. The same membrane was then stripped followed by re-probing with the anti-β-actin antibody (loading control).

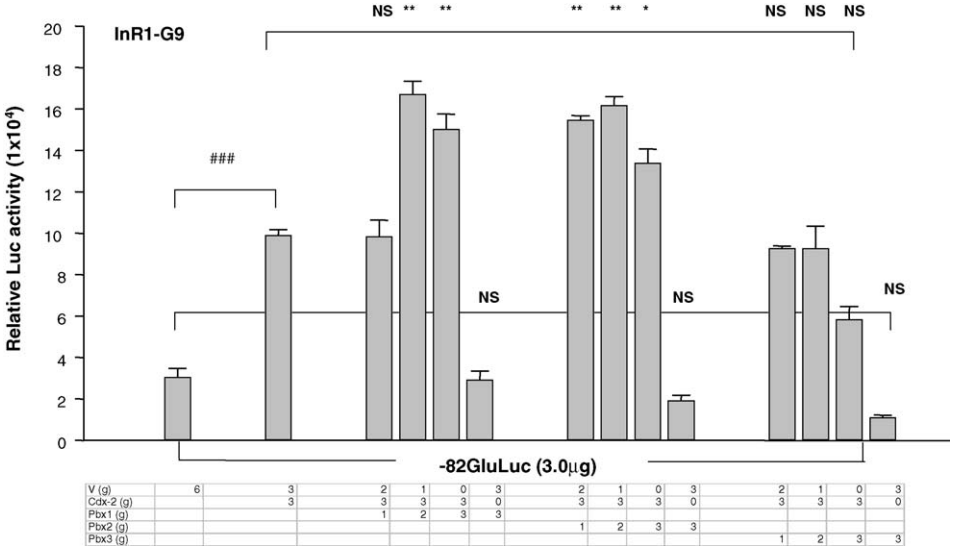

Fig. 2. Pbx1 enhances the activation on a Pbx-binding-site-deleted proglucagon promoter construct by Cdx-2. Constant amount of −82GLU-LUC (3.0 μg) and Cdx-2 expression plasmid (3.0 μg) were co-transfected into the InR1-G9 cells with the indicated amount of a given Pbx expression plasmid and the empty vector pcDNA3.1 (V). Relative LUC activity was calculated in each set of experiments, normalized against total protein utilized in the LUC assay and the results of β-gal assay (mean ± S.E.M., $n = 3$). NS, $P > 0.5$; *, $P < 0.05$; **, $P < 0.01$; ###, $P < 0.001$.

region C. It is generally accepted that Hox and Hox-like HD proteins utilize their penta-peptide motif to interact with Pbx proteins (Peers et al., 1995). If Cdx-2 recruits Pbx1 and such recruitment is important for proglucagon expression, mutating this motif will attenuate its stimulatory capability. Four mutant constructs were generated for this study. There are only two cysteine residues within the whole Cdx-2 molecule. Although these two cysteine residues are conserved among Cdx-2 homologues of human and rodent species, cysteine residues were not found among the penta-peptide motifs of other Hox/Hox-like HD proteins (Peers et al., 1995). Mutant 1 (M1) carries a site mutation on a cysteine residue outside of the penta-peptide motif, M2 carries the mutation on a cysteine residue within the penta-peptide motif and M3 carries mutations on both cysteine residues. M4, however, carries double mutations on glutamic acid and tryptophan residues of the penta-peptide motif. These two residues are well conserved among the penta-peptide motifs in different Hox/Hox-like HD proteins. These constructs, along with the wild type Cdx-2, were then utilized to examine whether these mutations would affect the ability of Cdx-2 to activate proglucagon, SI and Cdx-2 promoters. Fig. 3B shows that after transfection into BHK fibroblasts, these mutant constructs, as well as the wild type Cdx-2 construct, produced comparable amounts of nuclear Cdx-2, detected by Western blotting.

First, we examined the effect of these Cdx-2 mutations on activating proglucagon promoter in the InR1-G9 cell line. Compared with the wild type Cdx-2, all four mutant Cdx-2 constructs showed significant attenuation for activating −82 GLU-LUC (Fig. 4A). M3 showed the highest attenuation, approximately 80%; while M4 showed approximately 55% attenuation. Attenuated activation on proglucagon promoter by the M1–M3 mutants suggest that Cdx-2 dimerization is important in regulating proglucagon expression. Because the main purpose of this study is to examine the role of the penta-peptide motif, we then focused further examination on utilizing the M4 mutant. As shown in Fig. 4B, the activation of Cdx-2 M4 on the expression of −82 GLU-LUC in BHK cells was also significantly attenuated.

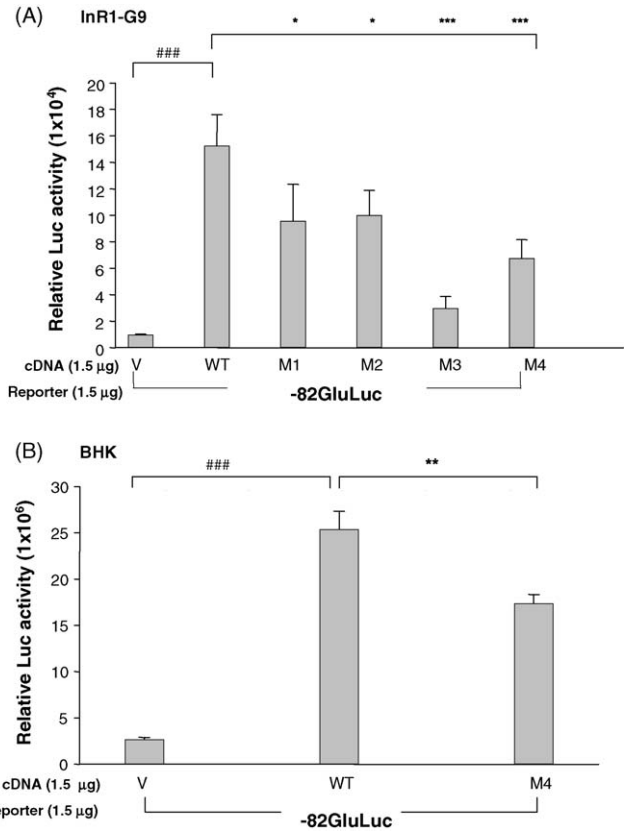

Fig. 4. Mutating the penta-peptide motif of Cdx-2 attenuates its activation on proglucagon promoter. (A) InR1-G9 cells were co-transfected with 1.5 μg of the indicated Cdx-2 expression plasmid, or pcDNA3.1 (V), along with 1.5 μg −82 GLU-LUC. Relative LUC activity was calculated in each set of experiments, normalized against total protein utilized in the LUC assay and the results of β-gal assay (mean ± S.E.M., $n = 3$). (B) BHK fibroblasts were co-transfected with 1.5 μg wild type (WT), or M4 mutant Cdx-2 expression plasmid, or pcDNA3.1 (V), along with 1.5 μg −82 GLU-LUC. *, $P < 0.05$; **, $P < 0.01$; ### and ***, $P < 0.001$.

3.4. Mutating the penta-peptide motif does not inhibit the activation on SI and Cdx-2 promoters

The effect of the penta-peptide motif mutation on the activation of Cdx-2 and SI promoters was then examined. We found that in all cell lines examined, all of the mutant Cdx-2 expression plasmids activated these two promoters either comparable to that activated by the wild type Cdx-2, or much stronger than the wild type Cdx-2. Fig. 5A shows that when examined in the pancreatic InR1-G9 cells, all the mutant Cdx-2 expression plasmids generated stronger activation on the Cdx-2 promoter, compared with the activation generated by the wild type Cdx-2. Fig. 5B shows that when co-transfected into the non-endocrine colon cancer cell line SW480, M1 and M4 generated comparable activation on the Cdx-2 gene promoter, compared with the wild type Cdx-2; while M2 and M3 mutants generated stronger activation on the Cdx-2 promoter. Fig. 5C shows that when co-transfected into the SW480 cell line, M1 generated comparable activation on the SI promoter with that generated by the wild type Cdx-2 transfection, while three other mutants generated much stronger activation.

3.5. Detection of physical interaction between Cdx-2 and Pbx1

To investigate the physical interaction between Cdx-2 and Pbx1, we first examined and demonstrated Pbx1 expression in the cell lines utilized in this study (Fig. 6A, lanes 1–5). Lane 6 in Fig. 6A shows the detection of increased expression of Pbx1 in COS-7 fibroblasts that were transiently transfected with Pbx1 expression plasmid.

Co-immunoprecipitation (Co-IP) was then employed to detect the interaction between Cdx-2 and Pbx1 in untreated InR1-G9 cells. Anti-Pbx1 and anti-Cdx-2 antibodies were utilized as IP antibody and Western blotting antibody, respectively (Fig. 6B). Lane 1 shows the detection of the 36 kD Cdx-2 protein in InR1-G9 cell lysate. Lane 3 shows that anti-Pbx1 antibody precipitated Cdx-2. Lane 4 shows that the anti-β-actin antibody

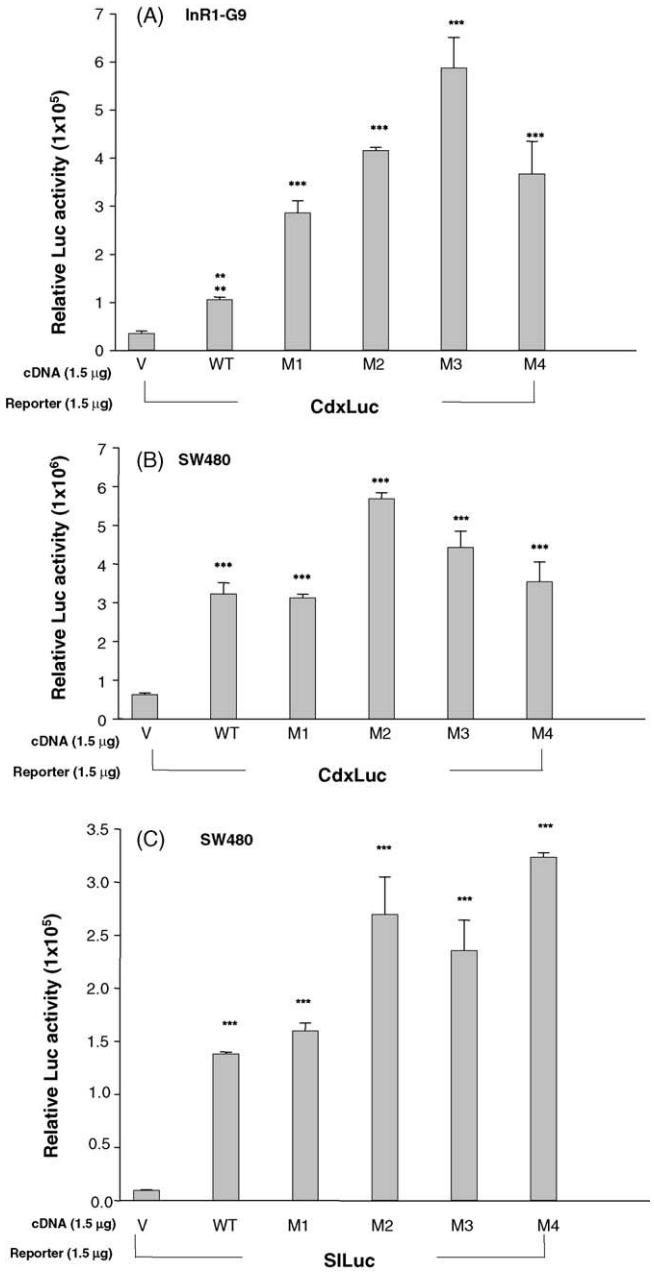

Fig. 5. Mutating the penta-peptide motif of Cdx-2 does not attenuate its activation on Cdx-2 and SI promoters. (A) InR1-G9 cells were co-transfected with 1.5 μg indicated Cdx-2 expression plasmid, or pcDNA3.1 (V), along with 1.5 μg −374Cdx-LUC. (B and C) SW480 cells were co-transfected with 1.5 μg of the indicated Cdx-2 expression plasmid, or pcDNA3.1 (V), along with 1.5 μg −374Cdx-LUC (B) or SI-LUC (C). Relative LUC activity was calculated in each set of experiments, normalized against total protein utilized in the LUC assay and the results of β-gal assay (mean ± S.E.M., $n = 3$). **, $P < 0.01$; ***, $P < 0.001$.

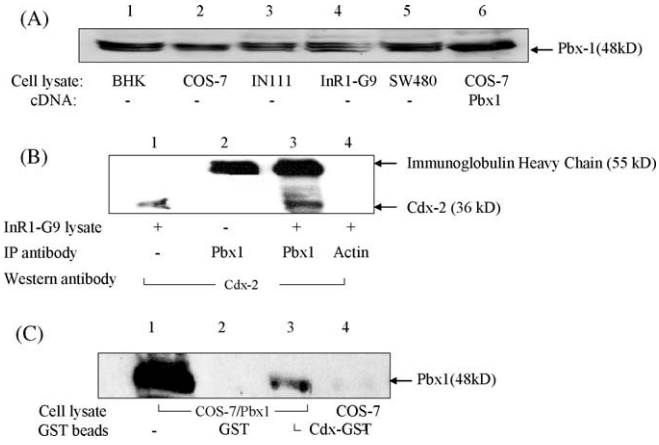

Fig. 6. Detection of physical interaction between Cdx-2 and Pbx1. (A) Approximately 20 μg total protein from indicated untreated cell lines (lane 1–5) and Pbx1 transfected COS-7 cells (lane 6) were used for Western blotting with the anti-Pbx1 antibody. (B) Pbx1 co-precipitates with Cdx-2 in untreated InR1-G9 cells. Anti-Pbx1 (rabbit polyclonal, lanes 2 and 3) and anti-β-actin (mouse monoclonal, lane 4) antibodies were utilized in IP against untreated InR1-G9 cell lysates (lanes 3 and 4) or no cell lysate negative control (lane 2). The presence of Cdx-2 in the immunocomplex was detected by Western blotting. Lane 1 contains 10 μg total protein from untreated InR1-G9 cells. (C) Cdx-2-GST pulls down Pbx1 in COS-7 cell lysates. Approximately 20 μg total cell protein from Pbx1 transfected COS-7 cells was incubated with 20 μg GST alone (lane 2) or Cdx-2-GST (lane 3). Following the pull-down procedure, the presence of Pbx1 was detected by the anti-Pbx1 antibody. Lane 1 contains Pbx1 transfected COS-7 cell lysate (approximately 20 μg total protein) as positive control for Western blotting. Lane 4 shows that a trace amount of Pbx1 in untransfected COS-7 cells was also pulled down by Cdx-2-GST.

could not precipitate Cdx-2. Lane 2 is a no cell lysate negative control, showing the position of the immunoglobulin heavy chain of the immunoprecipitation antibody.

To further confirm the physical interaction, GST fusion protein pull-down assays were conducted. Although we were able to pull-down Pbx1 from untreated COS-7 cell lysate, and from cell lysates of other cell lines with the wild type Cdx-2-GST fusion protein, the signal was very weak (Fig. 6C, lane 4, data not shown). We then transiently transfected the Pbx1 expression plasmid into the COS-7 cell line. Lane 3 in Fig. 6C shows an abundant amount of Pbx1 in Pbx1 transfected COS-7 cell lysate was pulled-down by Cdx-2-GST. Pbx1 in the same Pbx1 transfected COS-7 cell lysate, however, could not be pulled down by GST alone (lane 2).

We next performed further GST pull-down to ask the question whether the penta-peptide motif of Cdx-2 is the sole determinant in its interacting with Pbx1. In a preliminary experiment, we utilized the Cdx-2(M)-GST. This mutant is identical with the Cdx-2 expression plasmid M4 (Fig. 3A), except for that the mutant Cdx-2 cDNA was inserted into the pGEX4T-2 plasmid for the generation of a GST-fusion protein (Fig. 7A). We have repeatedly observed that this mutant Cdx-2 is still able to interact with Pbx1 in Pbx1 transfected COS-7 cells. We then made two additional Cdx-2-GST fusion proteins. Cdx-2(D)-GST bears a deletion from animo acid residue 163 to amino acid residue 174. Cdx-2(T)-GST contains only the N-terminal portion of the Cdx-2 molecule (Fig. 7A). Fig. 7B shows our verification of correspondent GST fusion proteins by Coomassie blue staining, after SDS-PAGE. Fig. 7C shows that both the wild type Cdx-2-GST and Cdx-2(M)-GST were able to pull-down Pbx1 from Pbx1 transfected COS-7 cell lysate (lanes 2 and 3). Deleting the penta-peptide motif [Cdx-2(D)-GST] was shown to substantially attenuate, but not abolish the interaction (lane 4). GST alone or Cdx-2(T)-GST, however, could not pull-down the Pbx1 protein in this experiment (lanes 5 and 6).

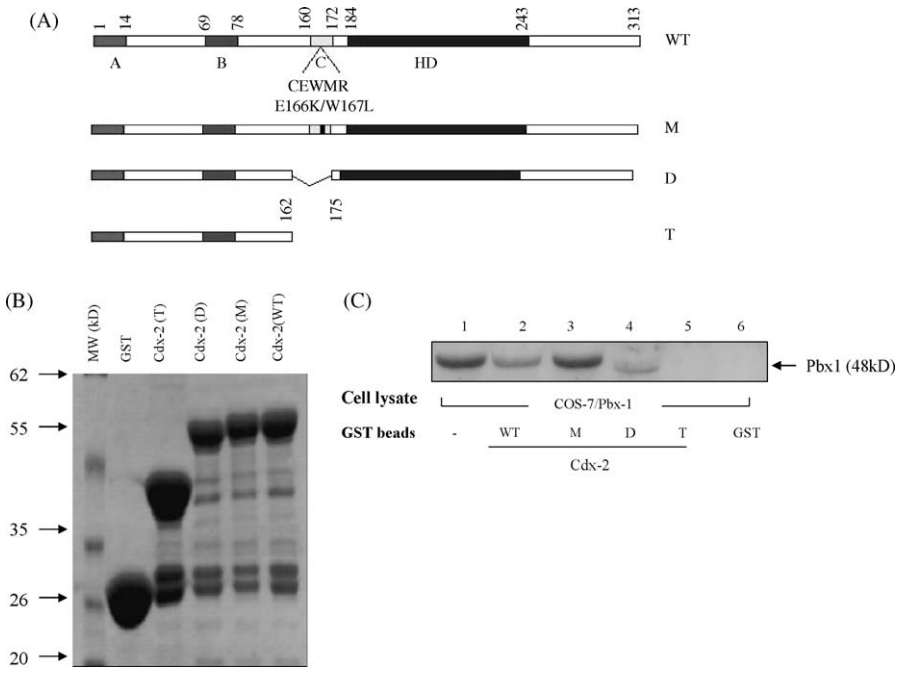

Fig. 7. Mutating the penta-peptide motif of Cdx-2 does not abolish its interaction with Pbx1. (A) Schematic representation of deletion and mutation constructs of Cdx-2-GST. Cdx-2(M)-GST is identical with Cdx-2 (M4) expression plasmid in its Cdx-2 coding region. (B) Coomassie blue staining shows the GST protein alone (lane 2) and Cdx-2-GST fusion proteins (lanes 3–6). (C) Approximately 20 μg total cell protein from Pbx1 transfected COS-7 cells were incubated with 20 μg GST alone (lane 5) or the indicated Cdx-2-GST fusion proteins (lanes 2, 3, 4 and 6). Following the pull-down procedure, the presence of Pbx1 was detected by anti-Pbx1 polyclonal antibody. Lane 1 contains Pbx1 transfected COS-7 cell lysate (approximately 20 μg total protein) as positive control for Western blotting.

4. Discussion

The generation and analyses of *Cdx*-2$^{-/-}$ mice indicated that this caudal gene has multiple biological functions (Chawengsaksophak et al., 1997). Consistently, numerous in vitro studies have identified more than two dozen potential target genes of Cdx-2 (Uesaka et al., 2004). To regulate the expression of different target genes in temporal and spatial manners, Cdx-2 may utilize different co-factors. Indeed, Cdx-2 was shown to interact with Pax6 and this interaction is important in regulating proglucagon expression (Hussain and Habener, 1999). In addition, Cdx-2 may interact with HNF1α and this interaction appears to regulate the expression of LPH and claudin-2 (Boudreau et al., 2002; Mitchelmore et al., 2000; Sakaguchi et al., 2002). Our current study, however, is focused on investigating whether Pbx1 serves as a co-factor for Cdx-2 in regulating proglucagon expression. We raised this hypothesis based on the following facts. First, many Hox and Hox-like proteins were shown to utilize Pbx proteins as co-factors (Peers et al., 1995). Second, the penta-peptide motif for interacting with Pbx proteins is present in all caudal protein members. Third, *Pbx*1$^{-/-}$ mice die at E15 and E16 with hypoplasia or aplasia of many organs, including the pancreas (Kim et al., 2002). All these observations suggest an important role of Pbx1 in normal pancreatic development and physiology. Finally, in contrast with cell type specific activation of its own promoter and SI promoter, Cdx-2 activated the proglucagon promoter in BHK cells (Taylor et al., 1997; Xu et al., 1999), indicating that a co-factor utilized in this activation, if any, is ubiquitously expressed. Members of the Pbx HD proteins fall into this category. Through cooperative DNA binding, Pbx proteins enhance the binding specificity and affinity of Hox/Hox-like HD proteins on their target promoter (Neuteboom and Murre, 1997; Phelan and Featherstone, 1997). Furthermore, the transcriptional activity of some preformed Hox/Pbx complexes could be further modulated by additional closely related HD proteins including Meis, HTH and Prep1 (Ferretti et al., 2000; Herzig et al., 2000; Schnabel et al., 2000; Shanmugam et al., 1999). We show here that Pbx1 on its own did not activate the expression of −476 GLU-LUC in BHK fibroblasts, although the construct contains the Pbx1 binding sites. Pbx1, however, did activate −476 GLU-LUC in the Cdx-2 expressing InR1-G9 cells. These results, in combination with our observation that Pbx1 co-transfection with Cdx-2 enhanced the activation on proglucagon promoter constructs with or without the Pbx1 binding sites, collectively suggest that Pbx1 serves as a co-factor for Cdx-2 in regulating proglucagon expression. We then demonstrated that mutating the penta-peptide of Cdx-2 attenuated its stimulatory effect on proglucagon promoter, but not on Cdx-2 and SI promoters. We suggest that Pbx proteins serve as co-factor of Cdx-2 in regulating the expression of its selected target genes.

Herzig et al. (2000) have examined the function of Pbx proteins with another TALE HD protein, Prep1, in regulating proglucagon expression. Most of their experiments were conducted using the JEG-3 choriocarcinoma cell line. Their results showed that Pbx/Prep1 repressed proglucagon promoter transcription in this non-proglucagon producing cell line, and suggested that this inhibitory effect could be involved in the establishment of islet cell-specific proglucagon expression. Herzig et al. (2000) performed electrophoretic mobility shift assays

(EMSA), showing that Pbx1 and Prep1 formed heterodimers and that the heterodimers were capable of binding to the G3 and G5 enhancer elements, as well as the CRE motif. The binding was dependent on the existence of both the Pbx protein (including Pbx1a, Pbx1b, and Pbx2, but not Pbx3) and the Prep1 protein. Similarly, we found that both Pbx1 and Pbx2, but not Pbx3, enhanced the stimulatory effect of Cdx-2 on the expression of proglucagon gene promoter. However, unlike Herzig et al., we did not observe the repression on the proglucagon promoter by Pbx1 transfection in our BHK cell system. This could be due to the use of different non-proglucagon producing cell lines (BHK versus JEG-3), different GLU-LUC reporter gene constructs, and/or the lack of using the Prep1 cDNA in our transfection experiment. Furthermore, we noticed that, at a higher dosage (5 μg), Pbx1 cDNA co-transfection attenuated the activation by Cdx-2 in the BHK cells (Fig. 1B). Thus, Pbx1 may be able to work in concert with Cdx-2 to either activate or repress proglucagon expression, depending on its expression level.

We found that mutating the penta-peptide motif of Cdx-2 generated no attenuation on its activation of the Cdx-2 and SI promoters. This observation is of interest, as it provides an example of cell type specific and promoter specific nature of the co-factors of HD proteins. Indeed the discovery of Pbx as well as Meis proteins as co-factor for Hox and Hox-like HD proteins has advanced the view that interactions with these co-factors are critical to target promoter selectivity. However, much less is known regarding to what extent each of the Hox or Hox-like proteins could regulate the expression of its target genes without the participation of these ubiquitously expressed co-factors. A few recent studies have shed some light in answering this challenging question. Pbx1 was also shown to interact with Pdx-1, a counterpart of Cdx-2, expressed in pancreatic B cells and somatostatin producing δ cells (Kim et al., 2002; Peers et al., 1995). Although Pdx-1 utilizes Pbx1 in regulating somatostatin expression in pancreatic δ cells and ELA1 expression in pancreatic acinar cells, it regulates insulin expression in a Pbx1 independent manner (Liu et al., 2001). In addition, although HOXB6 may use Pbx proteins as co-factors in exerting many other biological functions, it does not need Pbx1 in repressing globulin gene expression in K562 leukemia cells (Shen et al., 2004). Furthermore, the penta-peptide motif in HOXB6 is not required for this function (Shen et al., 2004). Other studies have also shown that a given HD protein may utilize a given co-factor for its selected functions, but not for the other functions (Galant et al., 2002; Strubin et al., 1995; Shore et al., 2002).

We found that Cdx-2 bearing the mutations on its penta-peptide motif exerted enhanced activation on SI and Cdx-2 promoters. Similar observations have been made by Chan et al. (1996) in studying the mouse Hoxb-1 gene and its *Drosophila* homologue labial (LAB), which indicates an additional role of the penta-peptide motif. Their results suggested that the penta-peptide motif in LAB HD protein plays a role in preventing the interaction between its HD and its target DNA sequences. LAB carrying a mutation within the penta-peptide motif was shown to have increased ability to activate transcription in vivo. Based these observations, Chan et al. (1996) suggested that the interaction between LAB/Hox and Exd/Pbx may release this inhibitory effect of the penta-peptide motif. Although the importance of the penta-peptide motif for interacting with Pbx proteins by Hox and Hox-like proteins has been interpreted in many circumstances (Peers et al., 1995), the functions of other domains are not dispensable. First, HD of Hox proteins was suggested to involve the protein–protein interactions (Johnson et al., 1995; Peers et al., 1995). The residues flanking the penta-peptide motif to HD also contribute to the interaction (Shanmugam et al., 1997). *Drosophila* HD protein Ubx was demonstrated to utilize Exd as a co-factor in the specification of the body plan (Chan et al., 1994). Although the penta-peptide motif in Ubx is important for the interaction with Exd, Ubx without the penta-peptide motif still retained its associations with Exd (Johnson et al., 1995). LAB cannot bind to its target DNA sequence without Exd. However, in the presence of Exd, the complex comprising Exd and LAB without the penta-peptide motif on their target DNA sequence was still detected, suggesting that the interaction could occur without this motif (Chan et al., 1996). HoxA9 was shown to utilize Pbx1 as the co-factor, and these two HD proteins were suggested to be involved in the development and progression of acute myeloid leukemia (Thorsteinsdottir et al., 1999). Although the tryptophan residue within the penta-peptide motif of HoxA9 is essential for its interaction with Pbx1, this residue is dispensable for the role of HoxA9 in immortalizing myeloid progenitor cells and for the cooperative transactivational activity by HoxA9 and Pbx1 (Calvo et al., 2000). Considering these findings in other Hox and Hox-like HD proteins and our results, we suggest that Cdx-2 may interact with Pbx1 through both the penta-peptide motif and another as yet to be defined motifs.

In conclusion, this study reveals an additional molecular mechanism for Pbx1 in pancreatic islet physiology: regulating proglucagon expression by serving as a co-factor of Cdx-2. Our study also indicated that Cdx-2 may only require Pbx proteins as co-factors in regulating the expression of its selected, but not all, target genes. Considering recent findings in studying other Hox and Hox-like HD proteins, one may suggest that Hox/Hox-like proteins and Pbx/Meis proteins exert overlapping but not identical biological functions. Although Hox/Hox-like proteins may utilize co-factors other than Pbx/Meis for exerting their selected functions, Pbx/Meix proteins may exert certain biological functions that are not directly related to Hox/Hox-like proteins. Indeed, a recent study has revealed that Pbx/Meis protein may serve as co-factors for MyoD in regulating the expression of MyoD dependent promoters including that of myogenin (Berkes et al., 2004). Since Cdx-2 exerts multiple and important functions via regulating the expression of more than two dozen potential target genes in temporal and spatial manners, effort should be made to address the following two questions: (1) What other Cdx-2 downstream target genes could be regulated with Pbx proteins as co-factors? (2) What are the co-factors for Cdx-2 in regulating the expression of its target genes when Pbx proteins are not directly involved?

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