The caudal-related homeobox gene Cdx-2/3 is a critical “master” control gene in embryogenesis. Mice heterozygous for a null mutation in Cdx-2/3 exhibit multiple malfunctions including tail abnormalities, stunted growth, a homeotic shift in vertebrae, and the development of multiple intestinal adenomatous polyps, indicating that Cdx-2/3 is haplo-insufficient. In vitro studies have identified more than a half-dozen downstream target genes expressed in pancreatic and intestinal cells for this transcription factor. We have examined the transcriptional properties of the mouse Cdx-2/3 promoter. This promoter could be autoregulated in pancreatic and intestinal cells that express endogenous Cdx-2/3. In contrast, Cdx-2/3 transfection represses the Cdx-2/3 promoter in fibroblasts, which do not express endogenous Cdx-2/3. Since Cdx-2/3 activates proglucagon gene promoter in both pancreatic and intestinal cells and in fibroblasts, we suggest that some, yet to be identified, cell type-specific components are required for activating selected target gene promoters of Cdx-2/3, including the Cdx-2/3 promoter itself. Cdx-2/3 binds to the TATA box and another AT-rich motif, designated as DBS, within an evolutionarily conserved proximal element of the Cdx-2/3 promoter. The DBS motif is critical for the autoregulation, whereas the TATA box may act as an attenuating element for the autoregulatory loop. Finally, overexpression of Cdx-2/3 in a pancreatic cell line activated the expression of the endogenous Cdx-2/3. Taken together, our results indicate that the dose-dependent phenotype of Cdx-2/3 expression on its downstream targets in vivo could be regulated initially via a transcriptional network involving cell type-specific autoregulation of the Cdx-2/3 promoter.

Homeodomain (HD) proteins are tissue-restricted transcription factors involved in fetal development, organogenesis, and cell type determination. HD proteins regulate the expression of genes involved in morphogenesis, cell growth, differentiation, and apoptosis. Alterations in HD protein expression may result in aberrant organ development, inherited diseases, and the formation of tumors (1–9). Cdx-2/3 is one of the three caudal-related homeobox genes identified in mammals. Cdx-2/3 maternal transcripts are expressed at pre-implantation stages and at the time of implantation in trophoectodermal cells. The embryonic expression is first seen at 8.5 days post-coitum (d.p.c.), and its expression increases in the endoderm just before the endoderm intestinal epithelial transition (~15 d.p.c.). In adults, Cdx-2/3 is only expressed in differentiated intestinal epithelial cells, endocrine (L) cells, and in pancreatic A and B cells (10–16). We and others (13–16) have demonstrated that Cdx-2/3 is a critical transcriptional activator for proglucagon gene in pancreatic A cells and intestinal L cells. Cdx-2/3 homozygous null mutant mice die between 3.5 and 5.5 d.p.c., whereas heterozygotic mutants show tail abnormalities, stunted growth, and a homeotic shift in vertebrae (9). In addition, 90% of the heterozygotic mutants develop multiple intestinal adenomatous polyps (9). These extraordinary multiple malfunctions observed in the heterozygotic mutant mice indicate that Cdx-2/3 transcriptional activity must be strongly dosage-dependent since a normal cellular phenotype appears to require expression of both alleles (i.e. Cdx-2/3 exhibits haplo-insufficiency).

In this paper, we have isolated the mouse Cdx-2/3 promoter and studied the transcriptional properties of this promoter. We found that although Cdx-2/3 is able to activate proglucagon gene promoter in both the Cdx-2/3-expressing pancreatic and intestinal cell lines, and the non-expressing fibroblasts, it activates its own promoter in a cell type-specific manner. Cdx-2/3 was shown to be able to bind to its TATA box and another AT-rich motif designated as DBS (downstream binding site) within an evolutionarily conserved proximal element of the Cdx-2/3 promoter. The DBS motif was found to be critical for Cdx-2/3 autoactivation, whereas the TATA box may act as an attenuating element for the autoregulatory loop.

EXPERIMENTAL PROCEDURES

Materials—Radioisotopes were obtained from Amersham Pharmacia Biotech. Oligonucleotides were provided by AGCT Co. (Toronto, Canada). Restriction enzymes and other DNA modification enzymes were molecular biology grade and were purchased from several sources.

Plasmids—The plasmid pBAT7.Cdx-2/3, which contains the hamster Cdx-2/3 cDNA, and the control expression vector pBAT7 were provided by Dr. Michael German (11). Construction of pBAT7.Cdx-2/3.AS, which contains the Cdx-2/3 cDNA in the antisense orientation, was described previously (13). HOX11 cDNA was provided by Dr. Robert Hawley (17). It was subcloned into the expression vector pBAT7. Other plasmids were either generated from this study or previously described (13, 15, 16).

GST-Cdx-2/3 Fusion Protein—The full-length hamster Cdx-2/3 cDNA was inserted into a pGEX plasmid (pGEX4T-2, Amersham Pharmacia Biotech). Following transforming a host Escherichia coli strain, the expression of GST-Cdx-2/3 fusion protein was induced with isopropyl-1-thio-β-D-galactopyranoside. The protein was purified with a glutathione-Sepharose 4B column. The purity of the protein was verified by Coomassie Blue staining to be a single band and confirmed as Cdx-2/3 protein by Western blot analysis.

Cell Culture, Transient Transfection, and RNA Analysis—All the cell lines were grown in Dulbecco’s modified Eagle’s medium supplemented
with serum as described previously (13) or based on ATCC instruction. BHK, Rat-1, NIH3T3, InR1-G9, and Caco-2 cell lines were transfected by the calcium phosphate precipitation method (13). STC-1 cells were transfected using LipofectAMINE. The cells were harvested for the LUC reporter gene analysis 14-16 h after the transfection. Methods for RNA extraction and Northern blot analysis were described previously (13).

Nuclear Protein Extraction and EMSA—Nuclear proteins from the cultivated cell lines were prepared as described by Schreiber et al. (18). Electrophoretic mobility shift assay (EMSA) was performed as described previously (13).

Western Blot Analysis—The rabbit polyclonal antibody against a carboxyl-terminal portion of hamster Cdx-2/3 (amino acids 260-313) was prepared previously (16). For Western blot analysis, 5-10 mg of nuclear proteins from cultivated cell lines were size-fractionated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Potran, Schleicher & Schuell). Cdx-2/3 immunoreactive protein was detected with an ECL Western blot analysis system (Amersham Pharmacia Biotech) with the peroxidase-linked anti-rabbit immunoglobulin as the second antibody.

RESULTS

The Structure of the Mouse Cdx-2/3 Promoter—We isolated the mouse Cdx-2/3 gene by screening a mouse λ phage genomic library using the hamster Cdx-2/3 cDNA as the probe (11). A 12-kb fragment carrying the mouse Cdx-2/3 gene including three exons, two introns, and the 5'- and 3'-flanking sequences was obtained. It contains five BamHI fragments. Results from partial DNA sequencing analyses and physical mapping indicate that the three internal BamHI fragments, measuring 7.2 kb, correspond to the mouse Cdx-2/3 gene (10). Fig. 1 shows the overall structure of the mouse Cdx-2/3 promoter and the DNA probes utilized for EMSA in this study.

Autoregulation of the Mouse Cdx-2/3 Promoter—A 906-base pair BamHI/SmaI fragment containing the DNA sequence from -769 to +137 of the mouse Cdx-2/3 gene was inserted into a promoterless plasmid pBlue (13), immediately adjacent to the coding sequence of the firefly luciferase (LUC) reporter gene. We first examined the transcriptional property of this promoter in the BHK fibroblasts (Fig. 2A), Rat1, and NIH3T3 (data not shown). The mouse Cdx-2/3 promoter is very moderately active in fibroblasts, activating the LUC reporter gene only 6-9-fold. Co-transfecting this promoter with the Cdx-2/3 cDNA resulted in an activation of the reporter gene expression (Fig. 2A). The activation is dose-dependent at lower dosages (0.5-2.5 µg) of Cdx-2/3 transfection but is slightly dropped when 5 µg of Cdx-2/3 was utilized. However, co-transfecting the Cdx-2/3 promoter with either an empty expression vector (pBAT7) or a Cdx-2/3 cDNA in the antisense orientation (AS) (13) shows no substantial effect on the reporter gene activity. These results would suggest that the expression of Cdx-2/3 is positively autoregulated in cells that express the endogenous Cdx-2/3. One may argue that the increased activity of Cdx-2/3 promoter in InR1-G9 and Caco-2 cells compared with that in the BHK fibroblasts could be due to a higher background activity of the pBluc, utilized in this study as a control, in the BHK cells. However, we have previously found that Cdx-2/3 is able to activate proglucagon gene promoter in both pancreatic and intestinal cells and in the BHK fibroblasts (13, 15). To examine further the phenomenon of cell type-specific autoregulation, we repeated the transfection assay in both the InR1-G9 cells and the BHK fibroblasts against the proglucagon gene promoter and the Cdx-2/3 promoter. For each cell line, these two promoters were transfected in combination with a wider range of dosages of Cdx-2/3 cDNA. Because these two promoters were examined in the same batch experiment, only one set of pBluc served as the control for both promoters. Our results demonstrate that, at all the dosages utilized (0.05-10 µg), Cdx-2/3 activates both the Cdx-2/3 and the proglucagon gene promoters in the InR1-G9 cells (data not shown). However, at all the dosages utilized, Cdx-2/3 represses Cdx-2/3 promoter but activates the proglucagon gene promoter in the BHK fibroblasts (Fig. 2B). About 4-fold activation was observed when 0.05 µg of Cdx-2/3 cDNA was utilized, and the highest activation was achieved with 0.5 µg of Cdx-2/3 cDNA (~8-fold). When 5 and 10 µg of Cdx-2/3 cDNA were used, the activation dropped to about 6- and 5-fold, respectively. Western blot analysis shows that comparable amounts of Cdx-2/3 were expressed in the BHK cells transfected with a given dosage of Cdx-2/3 in combination with either Cdx-2/3 or proglucagon promoters (data not shown). Therefore, we suggest that some as yet to be determined cell type-specific component(s) is/are required for activating selected target gene promoters of Cdx-2/3, including the Cdx-2/3 promoter itself.

To locate the cis elements critical for Cdx-2/3 autoregulation, a series of 5' deletion Cdx-2/3/LUC fusion genes were generated. Deletion of the DNA sequence from -769 to -51 base pairs had no substantial effect on Cdx-2/3 activation (Fig. 3A), indicating that the cis elements critical for the autoregulation are located mainly within the proximal region of the promoter. Fig. 3B shows that transfection of another homeobox gene, HOX11, into a fibroblast, a pancreatic, and an intestinal cell line had no appreciable activation or repression on the Cdx-2/3 promoter.

| Name | Nucleotide sequence (5' to 3') |
|------|--------------------------------|
| TATA box | DBS |
| PE | CRE | Cdx-2/3 | OCT | TATA | DBS |
| USE | OCTACTC| Cdx-2/3Cdx-2/3 | OCTACTC | TATA | OCTACTC |
| M1 | OCTACTC | Cdx-2/3Cdx-2/3 | OCTACTC | TATA | OCTACTC |
| M2 | OCTACTC | Cdx-2/3Cdx-3 | OCTACTC | TATA | OCTACTC |
| M3 | OCTACTC | Cdx-2/3Cdx-2/3 | OCTACTC | TATA | OCTACTC |
| DSE | OCTACTC | Cdx-2/3Cdx-2/3 | OCTACTC | TATA | OCTACTC |
| DSE.1 | OCTACTC | Cdx-2/3Cdx-2/3 | OCTACTC | TATA | OCTACTC |
| DSE.2 | OCTACTC | Cdx-2/3Cdx-2/3 | OCTACTC | TATA | OCTACTC |
| DSE.3 | OCTACTC | Cdx-2/3Cdx-2/3 | OCTACTC | TATA | OCTACTC |
| M4 | OCTACTC | Cdx-2/3Cdx-2/3 | OCTACTC | TATA | OCTACTC |
| M5 | OCTACTC | Cdx-2/3Cdx-2/3 | OCTACTC | TATA | OCTACTC |

FIG. 1. Overall structure of the mouse Cdx-2/3 promoter and the DNA probes utilized for EMSA. A, CRE, cAMP response element; OCT, octamer binding motif. Two Cdx-2/3-binding sites, TATA box and DBS, identified in this study are underlined. B, PE, the proximal element of the mouse Cdx-2/3 promoter; USE, upstream sub-element of PE, DSE, downstream sub-element of PE, DBS, downstream binding site.
Cdx-2/3 Expressed by Pancreatic and Intestinal Cells Binds to the Proximal Element of the Cdx-2/3 Promoter—To examine if Cdx-2/3 binds to its own promoter, EMSA was applied using the nucleotide probes listed in Fig. 1B. We first examined if Cdx-2/3 expressed in pancreatic and intestinal cell lines binds to the proximal element of the Cdx-2/3 promoter. Nuclear extracts from InR1-G9 cells (Fig. 4) and Caco-2 cells (data not shown) were examined using a probe designated as PE (Proximal Element, Fig. 1B), which contains the DNA sequence from +53 to +18 of the Cdx-2/3 promoter. We were unable to obtain an autoradiograph with well separated complexes. Furthermore, the addition of either preimmune serum or anti-Cdx-2/3 serum caused increased overall binding activity (lanes 6–11). This is not unexpected because many different transcription factors may bind to the TATA box and the adjacent regions. However, when the anti-Cdx-2/3 antibody was added, supershifted complexes were observed (lanes 7 and 8, defined as Cdx/Ab), which are comparable to the supershifted complexes obtained using the GC sub-element of G1 enhancer in the rat proglucagon gene as the probe (lanes 12 and 13; see Ref. 13). Cdx/Ab complexes were not observed when a preimmune serum was used (lanes 9–11), and they were not observed when nuclear extract from BHK fibroblasts was utilized (data not shown). Taken together, we suggest that Cdx-2/3 expressed by parent pancreatic and intestinal cell lines binds to the proximal element of the Cdx-2/3 promoter.

Fig. 3. Localization of the Cdx-2/3 autoactivation domain. A, 5 μg of Cdx-2/3-Luc fusion gene was transfected into InR1-G9 cells with 2.5 μg of pBAT7.Cdx-2/3 (+Cdx) or pBAT7.Cdx-2/3.AS (+AS). Relative luciferase activity was calculated as fold (average of three individual assays) against the luciferase (LUC) activity obtained by transfecting 5 μg of pBluc and 2.5 μg of pBAT7.Cdx-2/3.AS into the InR1-G9 cells. B, Hox11 (TCL3, see Ref. 17) does not activate Cdx-2/3 promoter in the pancreatic and intestinal cell lines examined. Five μg of Cdx-2/3-Luc reporter gene (−50/+137) was transfected into indicated cell line with 2.5 μg of indicated cDNA. The data are expressed as mean relative luciferase activity (n = 3) ± S.E. normalized to the activity obtained after transfection of pBluc in the same experiment.
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pancreatic and intestinal cell lines binds to the proximal region of its own promoter, although a specific complex that contains Cdx-2/3 cannot be defined.

Cdx-2/3 binds to the TATA box of the Cdx-2/3 promoter—Cdx-2/3 is able to bind to the TATA box of the calbindin-D9k gene (19). To examine if Cdx-2/3 is also able to bind to its own TATA box, a probe designated as USE (Upstream sub-element of PE) was designed. Again, we were unable to obtain an autoradiograph with well separated complexes when this shorter probe was examined using the nuclear extracts from pancreatic and intestinal cell lines (data not shown). To circumvent this problem, a GST-Cdx-2/3 fusion protein was generated and used in the following assays.

GST-Cdx-2/3 forms a complex with USE (Fig. 5, lane 2). This complex could be supershifted by anti-Cdx-2/3 antibody (lane 3), competed by unlabeled USE (lanes 4–6), but not by a unrelated probe (lanes 7–9). Within USE, except for the TATA box, there is an ATT motif 5’ of the TATA box (Fig. 1B). Mutating this ATT motif (M1) or a 3’ motif (M3) had no effect on the complex formation (lanes 11, 12, and 14). However, when the TATA box was mutated (M2), the formation of the complex was abrogated (lane 13), indicating that Cdx-2/3 binds to its own TATA box.

Cdx-2/3 binds to another AT-rich motif in the proximal promoter of Cdx-2/3—When the GST-Cdx-2/3 fusion protein was applied to the probe PE, two major complexes and one minor complex were observed, indicating that within the proximal element, there is/are additional binding site(s) for Cdx-2/3 (lane 2, Fig. 6A). The formation of all these three complexes could be supershifted by anti-Cdx-2/3 antibody (data not shown), competed by unlabeled PE (data not shown), or the GC element in the rat proglucagon gene promoter (lanes 9–11), indicating that all three complexes represent the Cdx-2/3-binding events. One may suggest that C3 represents the monomer binding, and C2 represents the dimer binding, as observed in the binding of Cdx-2/3 to the SIF1 element of the sucrase-isomaltase promoter (12). However, such an explanation is not plausible. The TATA probe (USE) only provides one complex, or one binding site. The overall structure of PE is different from that of SIF1, which contains an inverted repeat of ATAAA separated by only two nucleotides (12). In PE, the other binding site must be downstream of −15; and the TAAT motif from −6 to −3 is a good candidate (Fig. 1B). This TAAT motif is separated by 18 base pairs from the TATA box. Thus it is unlikely that a dimer molecule of Cdx-2/3 is able to contact these two well separated AT-rich regions. In addition, if C2 represents the binding of one PE molecule and two separated Cdx-2/3 molecules, reducing the amount of GST-Cdx-2/3 by adding unlabeled USE probe should first interfere with the formation of this slower migrating complex. However, by increasing unlabeled USE probe, we observed gradual disappearance of the faster migrating complex C3 (lanes 3–5). We therefore propose that the TATA box is one binding site for Cdx-2/3, and C2 represents this binding event. A downstate motif designated as PBSD, in DSE (downstream sub-element of PE). Four ng of GST-Cdx-2/3 fusion protein was used against DSE and its mutants (DSE1, DSE2, DSE3, M4, and M5, see Fig. 1B for detail). PP, free probe.

Fig. 5. Cdx-2/3 binds to its own TATA box. Four ng of GST-Cdx-2/3 was incubated with USE (or its mutants, M1, M2, and M3, see Fig. 1B) in the absence (lane 2) or presence (lane 3) of 2.5 μl of Cdx-2/3 antisem. Comp., 20–200-fold unlabeled USE or NR (a non-related competitor). FP, free probe.

Fig. 6. GST-Cdx-2/3 forms two major complexes with the proximal element (PE). A. 4 ng of GST-Cdx-2/3 fusion protein was incubated with the PE probe. Competition assay was conducted by using 20–200-fold unlabeled probe. USE, upstream sub-element of PE (see Fig. 2B); NR, a non-related competitor; and GC, a sub-element of G1 enhancer in rat proglucagon gene promoter that binds to native Cdx-2/3 (see Ref. 13). FP, free probe. B. GST/Cdx-2/3 binds to another TAAT motif, designated as DBS, in DSE (downstream sub-element of PE). Four ng of GST-Cdx-2/3 fusion protein was used against DSE and its mutants (DSE1, DSE2, DSE3, M4, and M5, see Fig. 1B for detail). FP, free probe.

![Diagram of complex formation](image-url)
may represent an event where one copy of PE is bound to two separated Cdx-2/3 molecules.

To examine the above hypothesis, a probe designated as DSE (Downstream sub-element of PE) containing the TAAT motif from -6 to -3 was examined. It forms a complex with GST-Cdx-2/3 (lane 2 in Fig. 6B). Deleting the 3'-TAAG (+3 to +6, DSE.1) or the 5'-GTAAA (-18 to -14, DSE.3) had no substantial effect on the complex formation. However, when this TATA motif was deleted (DSE.2, M4) or mutated (M5), the formation of the complex was abrogated (lanes 4, 6, and 7).

**DBS Is Critical for Cdx-2/3 Autoregulation**—Three Cdx-2/3-Luc fusion genes carrying the mutations in the Cdx-2/3-binding site(s) were generated. The fusion genes and the wild type counterpart were transfected into the InR1-G9 cells (Fig. 7) and Caco-2 cells (not shown) with or without Cdx-2/3 cDNA co-transfection. Mutation of the TATA box abolished the activity of the promoter completely. However, activation by Cdx-2/3 cDNA co-transfection was still observed on this mutant (Fig. 7, M₅₇₅₇). Mutation of the DBS motif reduced the basal activity of the promoter about 50%, indicating that this motif is important for Cdx-2/3 expression. In addition, mutation of this motif substantially abolished the Cdx-2/3 autoactivation (Fig. 7, M₃₃₅₃). M₅₇₅₇DBS carries mutations on both the TATA box and the DBS motif. The promoter activity of this mutant is lower than both the wild type (-50 to -137) and M₅₇₅₇, but higher than M₅₇₅₇. Cdx-2/3 is able to activate this mutant promoter slightly higher than 2-fold. At the present time, we are unable to provide an appropriate explanation for this observation.

**Cdx-2/3 Activates the Expression of the Endogenous Cdx-2/3 Gene**—We demonstrated above that the Cdx-2/3 promoter could be activated by Cdx-2/3 cDNA in pancreatic and intestinal cells. Previously, we also obtained evidence indicating that in the pancreatic InR1-G9 cells, Cdx-2/3 transfection also activates the expression of endogenous Cdx-2/3 mRNA, although it was not appreciated at the time (15). We have generated several Cdx-2/3 overexpression clones after stable transfection of the InR1-G9 cells with Cdx-2/3 cDNA. In those clones, there were two Cdx-2/3 mRNA signals detected by Northern blot analysis (Fig. 8A). Based on their sizes, the signal below the 18 S rRNA (-1.8 kb, Cdx-2/3 T) should represent the mRNA expressed from the transfected Cdx-2/3 cDNA, whereas the signal above the 18 S rRNA (-2.3 kb, Cdx-2/3E) should represent the overexpressed endogenous Cdx-2/3. Therefore, the data indicate that the expression of the transfected Cdx-2/3 up-regulates the expression of the endogenous Cdx-2/3. To extend further this observation to Cdx-2/3 protein level, we inserted the GST-Cdx-2/3 into the pcDNA3 expression vector. InR1-G9 cells were then transiently transfected with different dosages of pcDNA3-GST-Cdx-2/3. InR1-G9 cells were then transiently transfected with either 5 μg of pcDNA3 (V) or 1, 2.5, or 10 μg of pcDNA3-GST-Cdx-2/3. Twenty five μg of nuclear protein were loaded for Western blot analyses.

**DISCUSSION**

The biological significance of Cdx-2/3 has been recognized within the last few years. It has been identified, or suggested, to regulate genes expressed in intestinal cells such as sucrase-isomaltase (12), phospholipase A/lysophospholipase (20), lactase-phlorizin hydrolase (21), carbonic anhydrase 1 (22), Calbindin-D₉k (19, 23), and vitamin D receptor (24). We and others (7, 13–16) have demonstrated previously that Cdx-2/3 is a transcriptional activator for rat insulin promoter and the proglucagon gene promoter. Cdx-2/3 also has been shown to bind to two well defined enhancer elements in another ho-
their pancreas. These results would suggest that the Cdx-2/3 transgene can be transferred to another, and no obvious abnormality was observed in Cdx-2/3 internal transcripts of Cdx-2/3 are essential for implantation (9). Embryos die between 3.5 and 5.5 d.p.c., suggesting that maternal proteins (27–33), may require co-factors to exert its multiple biological functions. In this study we isolated the mouse Cdx-2/3 promoter and examined the transcriptional property of this promoter. We examined the transcriptional property of this promoter. We found that Cdx-2/3 transfection activates its own promoter in cell lines that express endogenous Cdx-2/3. Therefore, the expression of Cdx-2/3, like many other HD proteins, could be autoregulated (34–37). However, in fibroblasts, which do not express Cdx-2/3, Cdx-2/3 transfection represses the expression of Cdx-2/3 promoter. This is in contrast to our previous observations in examining proglucagon gene activation where Cdx-2/3 is able to activate proglucagon gene promoter in both the pancreatic InR1-G9 cells (15) and the BHK fibroblasts (13). To investigate further whether Cdx-2/3 autoactivation, indeed, occurs in a cell type-specific manner, we examined the effect of Cdx-2/3 CDNA transfection over a very wide range of dosages on the expression of Cdx-2/3 and the proglucagon gene promoters in InR1-G9 cells and BHK fibroblasts. Our data clearly indicated that at all the dosages utilized, Cdx-2/3 activates both the Cdx-2/3 and the proglucagon gene promoters in InR1-G9 cells. In BHK fibroblasts, Cdx-2/3 transfection activates the proglucagon gene promoter but represses the Cdx-2/3 promoter. Therefore, we suggest that Cdx-2/3 requires some as yet to be determined cell type-specific component(s) to regulate selected downstream target genes, such as Cdx-2/3 itself.

It should be pointed out that although cell type-specific autoregulation of homeobox gene Cdx-2/3 has not been previously studied, cell type-specific activation by Cdx-2/3 on heterologous promoters was recently reported by Taylor et al. (25). Cdx-2/3 was shown, by Suh et al. (12), to activate sucrase-isomaltase promoter via binding to its SIF1 enhancer cassette in the intestinal cells. Taylor et al. (25), however, found that Cdx-2/3 also activated a thymidine kinase promoter fused with one or more copies of the SIF1 enhancer cassette when transfected into intestinal Caco-2 cells. Such an activation, however, was not observed when examined in the NIH3T3 fibroblasts. Furthermore, Taylor et al. (25) found that if the Cdx-2/3 activation domain was linked to the Gal4 DNA binding domain, the chimeric protein was able to activate Gal4 enhancer constructs in the intestinal Caco-2 cell line but not in the NIH3T3 fibroblasts.

Haplo-insufficiency is not common for transcription factors, especially for homeobox genes (34). One explanation is that related homeobox genes may exert overlapping or redundant biological functions. In addition, the expression of many homeobox genes, like Cdx-2/3, could be autoregulated (34–37). Therefore, via a positive regulatory loop, the expression of a homeobox gene from only one functional allele is able to attain a physiologically required level. Other homeodomain proteins, such as Otx-2 and PAX6, do exhibit haplo-insufficiency for part of their biological functions (38, 39). An interesting question, therefore, is why one functional allele for a few homeobox genes is insufficient even though the expression of these genes can be autoregulated? Information from this study has provided one plausible explanation. In Cdx-2/3, some as yet to be defined cell type-specific components (a co-factor, and/or a signal) is critical in determining if the autoregulation will be effectively processed or not. If in an individual cell, autoregulation process failed due to, for example, the combination of loss of one functional allele and lack of a necessary signal, malfunctions including the formation of tumors may occur. It is quite possible for the existence of a critical threshold for triggering the autoregulation. In the Cdx-2/3−/− mice, since only one Cdx-2/3 wild type copy exists, the chance for the expression of Cdx-2/3 within some cells not reaching the threshold will be higher. If this hypothesis is correct, it will provide an explanation for why in the Cdx-2/3−/− mice, the various malfunctions observed vary, in degree, time, and location, from one mouse to another. To explore this hypothesis, a systematic examination of the mechanisms controlling Cdx-2/3 expression, using both in vivo and in vitro approaches, becomes an essential task.

We identified two Cdx-2/3-binding sites, TATA box and DBS, within the proximal element (PE, −53 to +18) of the mouse
Cdx-2/3 promoter. DNA sequence from −53 to +4, which covers both the TATA box and the DBS, is 100% conserved between mouse and human (10, 40). It is interesting that the autoregulation of the pituitary-specific POU domain transcription factor Pit-1 is also mediated by two Pit-1-binding sites within the proximal region of the promoter (35). These two binding sites are separated by 60 nucleotides. Mutation of the 5′ Pit-1-binding site (PitB1) abolished the positive autoregulation, whereas mutation of the other binding site that is located immediately 3′ of the cap site (PitB2) markedly increased the expression of the pit-1 promoter. Therefore, PitB2 is considered as an attenuating component for the autoregulatory loop (35).

In the Cdx-2/3 promoter, the overall organization of the two Cdx-2/3-binding sites is different from that of pit-1. The 3′ DBS motif is critical for autoactivation, whereas the other binding site is the TATA box per se. It is not surprising that mutation of the TATA box almost completely abolished the promoter activity. However, Cdx-2/3-LUC fusion gene with a mutated TATA box still responds to Cdx-2/3 transfection. We suggest that in the Cdx-2/3 promoter, the TATA box could act as an attenuating component in the autoregulatory loop. It should be pointed out that both general transcription factors and Cdx-2/3 bind to the proximal element of the Cdx-2/3 promoter. Based on our observation, one may propose that at a low concentration, Cdx-2/3 may compete with general transcription factors for the TATA box, resulting in repressing the expression of the promoter.

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