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Pax6 and Cdx2/3 form a functional complex on the rat glucagon gene promoter G1-element

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Abstract **α-cell specific transcription of the glucagon gene is mainly conferred by the glucagon promoter G1-element, while additional elements G2, G3, and G4 have broad islet cell specificity. Transcription of the glucagon gene has been shown to be stimulated by Pax6 through binding to the glucagon gene promoter G3-element. In this report, we show that Pax6 additionally binds the glucagon gene promoter G1-element and forms a transcriptionally active complex with another homeodomain protein, Cdx2/3. Two distinct mutations in the G1-element, that both reduce promoter activity by 85–90%, is shown to eliminate binding of either Pax6 or Cdx2/3. Additionally, Pax6 enhanced Cdx2/3 mediated activation of a glucagon reporter in heterologous cells. We discuss how Pax6 may contribute to cell-type specific transcription in the pancreatic islets by complex formation with different transcription factors.**

Key words: Pax6; Cdx2; Glucagon; Pancreatic islet; Transcription

1. Introduction

The cell specific production of glucagon, insulin, somatostatin, and pancreatic polypeptide by the four classical cell types of the islets of Langerhans is regulated at the transcriptional level [1–5]. The promoters of the glucagon, insulin, and somatostatin genes are composed of a combination of positively and negatively acting *cis*-elements [6–12]. When isolated, positively acting *cis*-elements confer tissue (islet) specific or cell-type (α, β, δ) specific expression [13–17]. Thus, the glucagon gene G1-element confers α-cell specific expression [15,18] and the G2-, G3-, and G4-elements confer islet cell specific activity [15,19,20]. Many of the transcription factors regulating the activity of the glucagon gene have been identified. A NeuroD/Beta2 containing complex binds the G4-element [21], Pax6 binds the G3-element [22], and HNF-3β binds to the G2-element [23]. Cdx2 (also known as Cdx3 [24] and henceforth called Cdx2/3) that is expressed at low levels in both exocrine and endocrine cells, and Brain-4 (Brn4) which is expressed at high levels in α-cells have been surmised to play a role in α-cell specific glucagon gene activation through binding to the G1-element [25–28]. In spite of their different cell-type specificity the G1- and G3-elements contain a similar sequence motif, the so-called PISCES (Pancreatic ISlet Cell Enhancer Sequence) motif [13] suggesting that Pax6 might also bind the G1-element. To begin to unravel the mechanism by which these two elements confer different cell-type specificity we analyzed the factors binding to these elements in a glucagon-producing cell line. We found by antibody supershift experiments that the major difference between the two elements was the formation of a Cdx2/3-Pax6 complex specific for the G1-element. This complex was also formed in the presence of recombinant Cdx2/3 and Pax6 protein. Small mutations in the G1-element that are known to impair promoter activity selectively abolished binding of either Cdx2/3 or Pax6. Furthermore, Pax6 enhanced Cdx2/3 mediated activity of a glucagon reporter lacking the G3-element in heterologous cells. Together, these data suggest that the Cdx2/3-Pax6 complex is functional and required for full promoter activity.

2. Materials and methods

2.1. Plasmids

pBAT7-Cdx3 [24] containing the hamster Cdx2/3 cDNA under control of the cytomegalovirus (CMV) promoter was a kind gift from M. German, pSG5-Pax6 containing the quail Pax6 cDNA under control of the SV40 promoter was a gift from S. Saule. The glucagon promoter (−254 to +58) luciferase reporter plasmid p-254GLU-Luc was constructed by replacing the SV40 promoter in the pGL2-Promoter (Promega) with a 312 bp *Hin*cII-*Bam*HI fragment cloned in p0CAT [15].

2.2. Transfections

Transient transfection assays were done using Lipofectamine (Gibco-BRL) according to the manufacturer's instructions. Briefly, approximately 2.5×10^5 NIH-3T3 cells were seeded 4 to 8 h before transfection in a 24-well tissue culture dish (Nunc). 150 ng of reporter plasmid was cotransfected with 2 ng of the pRL-CMV renilla luciferase (Promega) as an internal standard, 5 ng of pSG5-Pax6, and 0.1 ng of pBAT7-Cdx3. The same amount of empty expression vectors were used for controls. All transfections were adjusted to a total of 500 ng DNA per well using pBluescript SKII+ (Stratagene). 5–10 min before adding transfection mix to the cells, cells were washed using 1 ml opti-MEM per well. Cells were incubated overnight in a total volume of 500 μl of transfection mix after which the same volume of normal culture media with 20% serum was added. Cells were cultured for an additional 24 h before harvesting. Luciferase and CAT activities were measured as previously described [29]. Quantification of CAT assays was done using a PhosphoImager (Molecular Dynamics). Dual luciferase was measured using the Dual-Luciferase Reporter Assay System (Promega) and a TD-20/20 luminometer (Turner Designs). All reporter activities were normalized to the internal standard.

2.3. Cell culture

NIH-3T3-B (obtained from R. Schumacher) and InR1-G9 cells [30] were cultured in DMEM 1000 mg/l glucose containing 10% fetal calf serum, 100 U penicillin/ml, and 100 μg/ml streptomycin.

2.4. Cell extracts

Nuclear extracts were prepared by the method of Schreiber et al. [31] except that buffer C contained 500 mM KCl instead of 400 mM NaCl. Furthermore, leupeptin (1 μg/ml), aprotenin (1 μg/ml), 1 mM

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4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), and 1 mM sodium-orthovanadate were included. The recombinant proteins were produced by transiently transfecting 2×10^6 NIH-3T3 cells in 6-well tissue culture dishes (Nunc) with 3–6 μg of the relevant expression plasmid. Whole cell extract was prepared as follows: Cells were washed in PBS and lysed in whole cell extract buffer (2.5 mM DDT, 20 mM HEPES pH 7.8, 10 mM KCl, 1 mM $MgCl_2$, 10 mM EGTA, 0.2% Triton X-100 supplemented with leupeptin (1 μg/ml), aprotenin (1 μg/ml), AEBSF (1 mM), and 1 mM sodium-orthovanadate) using 175 μl per well. After shaking for 10 min at room temperature, 33 μl 2.1 M KCl and 46% glycerol buffer was added and shaking was allowed to proceed for 10 min at room temperature. The contents of the wells were adjusted to 15% glycerol using a 50% stock, collected and centrifuged at $20\,000\times g$ for 10 min at 4°C. The supernatant was collected and stored at −80°C. Protein content was measured using the Bradford assay (Biorad).

2.5. Antisera and electrophoretic mobility shift assays

The bg11 anti-Pax6 antiserum was a gift from S. Saule and has been described previously [32]. Rabbit anti-Cdx2/3 antiserum 1277 was raised against the synthetic peptide NH_3-**SPPPQPSQPQPGSLRS**C-COOH (bold face indicates amino acids 262–277 of hamster Cdx2/3) coupled to keyhole limpet hemocyanin as previously described [33]. The anti-Cdx2/3 antiserum was determined to be specific based on its ability to recognize Cdx2/3 but not Pdx1 or Pax6 expressed in NIH-3T3 cells. Oligonucleotide sequences for glucagon probes are shown in Figs. 1 and 3. Five μg of nuclear extract was used in each standard EMSA. The reaction buffer contained 12.5% glycerol, 30 mM Tris-HCl, 75–100 mM KCl, 20 mM NaCl, 1 mM MgCl, 20–50 μg/ml poly-dIdC, 20–50 μg/ml poly-dGdC, 2–4 μg/ml herring sperm DNA, 0.1% Nonidet NP-40 and, 3 mM DTT. For recombinant proteins up to 10 μg of whole cell extract was used and all reactions were adjusted to the same protein concentration using extracts from untransfected cells. Antisera were used at a 1/10 dilution in the presence of 15 mM NaN_3 using 2–3 μl per binding reaction as described previously [29]. Binding reactions were separated on a 5% polyacrylamide gel as previously described [29]. The gels were dried and analyzed by autoradiography. Gel images were scanned using Adobe Photoshop 3.0 and figures prepared in Canvas 5.0.2.

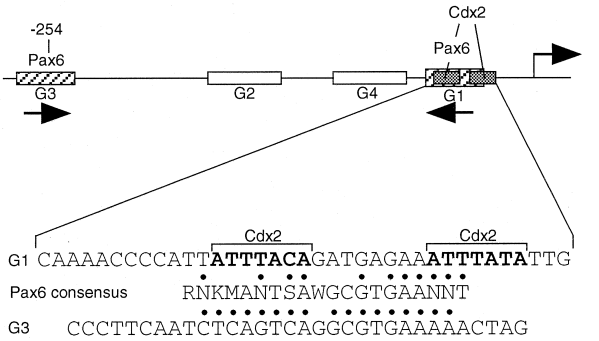

Fig. 1. Schematic representation of the rat glucagon promoter. Pax6 binding sites are indicated by hatched boxes and Cdx2/3 binding sites as shaded boxes. Arrows indicate the orientation of the Pax6 binding sites. The sequence of the G1-element with Cdx2/3 binding sites [26] and the G3-element is shown aligned with the Pax6 paired domain consensus binding site [43].

3. Results

3.1. Pax6 and Cdx2/3 form a ternary complex with the glucagon G1 promoter element

We initially confirmed that Pax6 bound to the G3-element of the rat glucagon promoter. Electrophoretic mobility shift assays (EMSA) using the G3 probe and InR1-G9 nuclear extracts (Fig. 2A) revealed two complexes G3-A and G3-B.

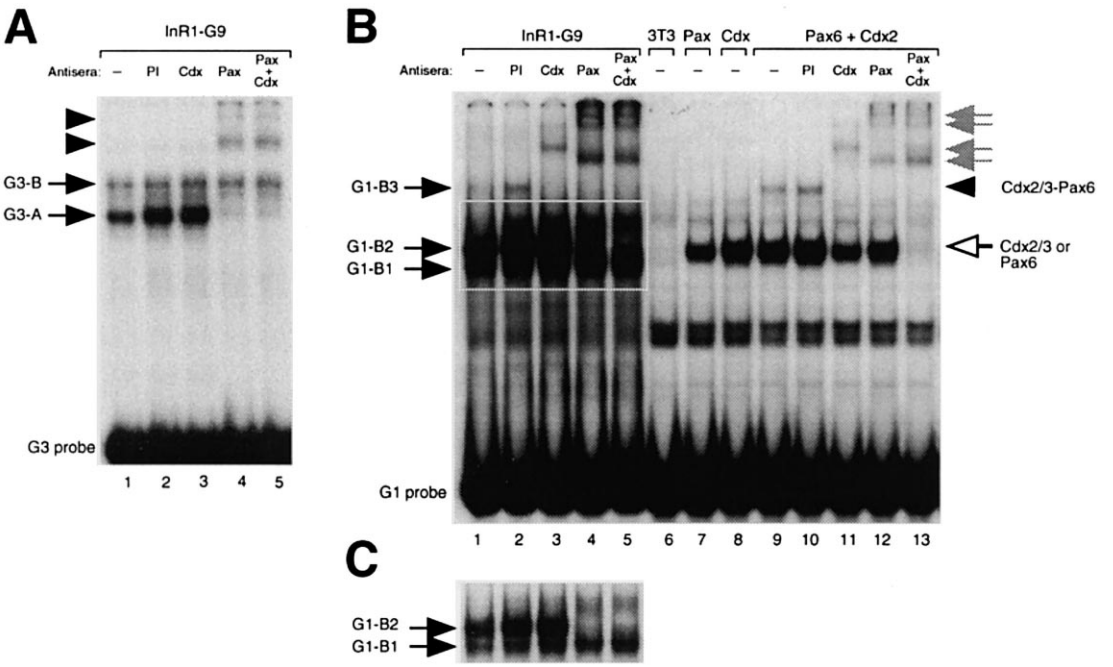

Fig. 2. Pax6 and Cdx2/3 form a complex specifically on the G1-element. A: Pax6 forms a single complex with the glucagon G3-element. ^{32}P-labelled G3 oligonucleotide was incubated with nuclear extract from InR1-G9 glucagonoma cells and anti-Pax6 and anti-Cdx2/3 antiserum as indicated. Arrows indicate the two specific complexes G3-A and G3-B. G3-A is Pax6 immunoreactive. Arrowheads indicate supershifted complexes. B: Pax6 and Cdx2/3 form a ternary complex with the G1-element. ^{32}P-labelled G1 oligonucleotide was incubated with nuclear extract from either InR1-G9 glucagonoma cells or recombinant Pax6 and Cdx2/3. Anti-Pax6 and anti-Cdx2/3 antisera were added as indicated. PI equals a Pdx1 preimmune serum. Arrows indicate the G1-B1, G1-B2, and G1-B3 complexes. Open arrow indicates Cdx2/3 and Pax6 monomers. The arrowhead indicates the Pax6-Cdx2/3 immunoreative complex (G1-B3). Grey arrows indicate supershifted complexes (note that supershifts are identical with InR1-G9 extract and recombinant Pax6 and Cdx2/3). C: Lower exposure of the region boxed in B.

The G3-A complex was identified as Pax6 since addition of a Pax6 antiserum supershifted this complex (Fig. 2A). The identity of the G3-B complex remains unknown. We next established that the G1-element also contained a Pax6 binding site. Using the G1 probe (Fig. 1) we observed binding of two prominent complexes G1-B1 and G1-B2 (Fig. 2B and C) as well as a faint low mobility complex G1-B3 (Fig. 2B). The intensity of these three bands was increased by the addition of a preimmune serum (Fig. 2B, lane 2). Complexes G1-B2 and G1-B3 were found to contain immunoreactive Pax6 (Fig. 2B and C, lane 4). The G1-B2 complex formed with InR1-G9 extract migrated the same as recombinant Pax6 protein (Fig. 2B, lane 7) indicating that this complex represents a Pax6 monomer. Previously reported binding of Cdx2/3 to the G1-element [26,27] prompted us to examine whether the G1-B3 complex also contained Cdx2/3. As seen in Fig. 2B, the G1-B3 complex was supershifted by anti-Cdx2/3 antiserum. A complex of identical mobility was created when mixing recombinant Pax6 and Cdx2/3, demonstrating that Pax6 and Cdx2/3 form a ternary complex with DNA on the G1-element (Fig. 2B, lane 9). In this experiment, we failed to detect the Cdx2/3 dimer reported by Laser et al. [26] due to the low amounts of recombinant Cdx2/3 used. Higher amounts did produce a Cdx2/3 dimer (see Fig. 3B). The supershift pattern generated with the recombinant proteins was identical to the pattern seen when using InR1-G9 extracts (Fig. 2B, compare lanes 3–5 with lanes 11–13). Using InR1-G9 nuclear extracts we could not detect Cdx2/3 monomer binding to the G1 probe. This was possibly due to a combination of low amounts of Cdx2/3 in the extract and that Cdx2/3 monomeric binding was obscured by other complexes.

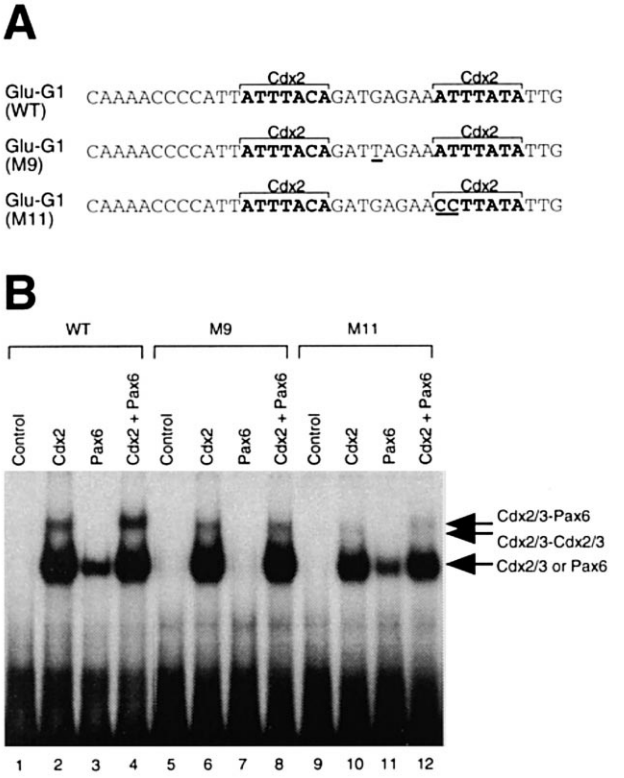

Fig. 3. Mutations that compromise glucagon promoter activity in InR1-G9 cells reduce ternary complex formation. A: Synthetic oligonucleotides used in EMSA. Mutations deviating from the wild-type rat glucagon gene sequence are underlined. B: Binding of recombinant Cdx2/3 and Pax6 to wild-type or mutant G1 oligonucleotides. Monomeric (Cdx2/3 or Pax6) and dimeric (Cdx2/3-Cdx2/3, Cdx2/3-Pax6) complexes are indicated. Cdx2/3 binding to the M11 mutant is mediated by the distal binding site [26].

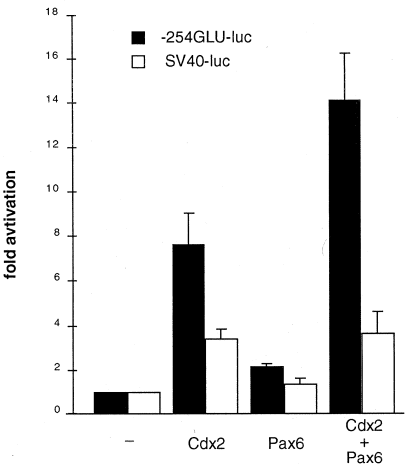

Fig. 4. Pax6 potentiates Cdx2/3 mediated transactivation of the rat glucagon promoter. NIH-3T3 cells were transfected with −254GLU-luc or SV40-luc (pGL2 promoter) and co-transfected with the indicated effector plasmids or empty expression vectors as control. The average of at least three independent experiments is presented. Error bars indicate S.E.M.

3.2. The M9 and M11 mutations target Pax6 and Cdx2/3 binding, respectively

The effects of mutations in the G1-element upon glucagon promoter activity in InR1-G9 hamster glucagonoma cells have been extensively studied by Philippe and co-workers [18,26]. The two most deleterious mutations found were: A single G to T substitution at position −79 (M9, Fig. 3A) which resulted in an approximately 90% decrease in promoter activity, and a double AT to CC substitution at positions −73/−74 (M11, Fig. 3A), that resulted in an 85% decrease in promoter activity [18]. To examine for a possible correlation between the effect of these mutations on promoter activity and the capability of Pax6 and Cdx2/3 to bind the G1-element we synthesized oligonucleotides carrying these two mutations and performed EMSA with recombinant Pax6 and Cdx2/3. As shown in Fig. 3B, the M9 mutation abolished Pax6 binding while leaving Cdx2/3 binding unaffected. Conversely, the M11 mutation reduced Cdx2/3 binding while only a slight reduction of Pax6 binding was observed. The residual Cdx2/3 binding is expected as the M11 probe still harbors the distal low affinity Cdx2/3 binding site and as the mutation does not completely abolish Cdx2/3 binding to the proximal site [26]. Formation of the Pax6-Cdx2/3-G1 ternary complex was diminished by both mutations (Fig. 3B).These data strongly suggest that simultaneous binding of Cdx2/3 and Pax6 to the proximal site is necessary for full promoter activity.

3.3. Pax6 potentates Cdx2/3 activation of a glucagon reporter

To further test whether Pax6 and Cdx2/3 were capable of interacting functionally, we cotransfected a glucagon promoter luciferase reporter construct (−254/+55, Fig. 1) with Pax6 and Cdx2/3 expression vectors in NIH-3T3 cells and assayed the reporter activity. To exclude any Pax6 mediated

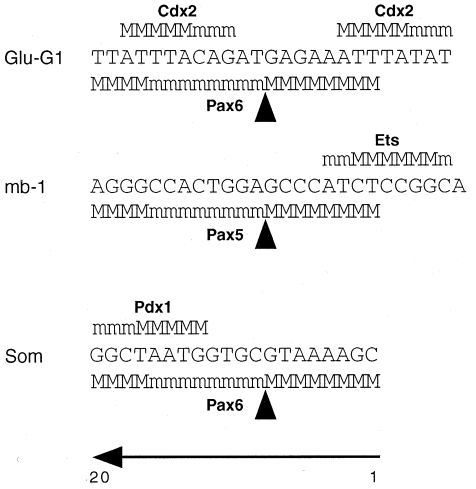

Fig. 5. Model of protein-DNA contacts in simultaneous binding of Pax6 to paired domain consensus binding sites overlapping homeodomain and Ets domain binding sites. Protein-DNA contacts are indicated as follows: m, minor groove contact; and M, major groove contact. Predictions of the protein-DNA contacts were based on the published three-dimensional structures of Drosophila *paired* [35] for Pax5 and Pax6, Drosophila *antennapedia* [36] for Cdx2 and Pdx1, and the Ets domain of PU.1 [37]. See main text for a discussion. The arrow indicates the orientation and numbering of nucleotide positions compared to the Pax6 paired domain consensus site [43]. Arrowheads indicate the equivalent position of the 20° bend in the *paired* DNA binding site induced by *paired* binding. Placement of Cdx2 contacts relative to its binding site supposes that the underlined nucleotides in the Cdx2 binding sites A$\underline{\text{TTTA}}^{\text{T}}/_{\text{C}}$A represent a degenerate ATTA core. Alternatively, a degenerate ATTA core can be visualized as $\underline{\text{ATTTA}}^{\text{T}}/_{\text{C}}$A, but this does not result in a sufficiently large change in spacing to avoid steric hindrance in the minor groove.

activity from the G3-element we used a reporter lacking the core sequence of the G3 Pax6 binding site. Pax6 and Cdx2/3 were both capable of activating the reporter construct individually (2- and 8-fold, respectively, Fig. 4). However, inclusion of both factors enhanced promoter activity 14-fold, more than additively compared to the individual factors (Fig. 4). A control SV40 promoter was weakly activated by Cdx2/3 but not by Pax6.

4. Discussion

Cdx2/3 has previously been implicated in the control of glucagon promoter activity by binding to the glucagon G1 promoter element [26–28]. We now show that Cdx2/3, binding to its proximal binding site, forms a functional complex with Pax6 on the G1-element. We base our conclusion on several observations. First, the two most deleterious mutations described for the G1-element, M9 and M11 [18] selectively abolish binding of Pax6 and binding of Cdx2/3 to its proximal site, respectively. Second, both mutations abated the formation of the Cdx2/3-Pax6-DNA ternary complex. Lastly, a glucagon reporter construct lacking the upstream Pax6 binding G3-element was activated by Cdx2/3 in heterologous cells and this activity was enhanced by Pax6. Taken together these observations lead us to propose that simultaneous binding of Pax6 and Cdx2/3 to the G1-element is required for full activity of the glucagon promoter. The partial overlap between the proximal Cdx2/3 site and the Pax6 site is reminiscent of the composite Pax5/Ets domain site (Fig. 5) found in the early B-lymphocyte specific gene mb-1 [34]. In both cases the Pax protein and the second DNA binding protein seem to contact the major groove of the DNA at the same nucleotides but based on the published structures of the *paired* paired domain [35], the *antennapedia* homeodomain [36], and the Ets domain of PU.1 [37], the geometry of the paired domain and the homeodomain/Ets domain binding to these partially overlapping sites would place the DNA binding domains adjacent to each other. In contrast to the recruitment of Ets proteins by Pax5 on the mb-1 promoter we do not observe recruitment or even co-operative binding between Pax6 and Cdx2/3. Instead, the transcriptional co-operativity observed might result from independent contacts with the basal transcriptional machinery. The distal Cdx2/3 site is contained completely within the Pax6 site (Fig. 5), but simultaneous binding to these two sites is not observed. This is consistent with the relative insensitivity of glucagon promoter activity to mutagenesis of this Cdx2/3 site [18,26]. Moreover, structural considerations (see Fig. 5) would seem to prohibit simultaneous binding of Pax6 and of Cdx2/3 to the distal site due to steric hindrance between the N-terminal arm of the Cdx2/3 homeodomain and the Pax6 paired domain linker region, both contacting the minor groove of the DNA. Interestingly, an additional example of Pax6 binding simultaneously with another homeodomain protein is found on the somatostatin upstream enhancer (SMS-UE). The SMS-UE is a composite enhancer element that confers cell-type specific activity to the somatostatin gene [13,38]. In the case of the SMS-UE, Pax6 and Pdx1 bind to completely overlapping binding sites and together contribute to the β/δ-cell specificity of this enhancer element (see accompanying manuscript).

A model proposing simultaneous binding of Cdx2/3 and Pax6 is consistent with the observed reduced glucagon mRNA level in vivo in the homozygous $\text{Sey}^{1\text{Neu}}$ mouse, which lacks functional Pax6 protein [22]. Homozygous, *Cdx2/3* mutant mice do not survive the peri-implantation period and are thus not informative concerning effects on glucagon gene expression. However, heterozygotes show dosage dependent effects, evident as skeletal transformations. Additionally, the heterozygotes develop multiple intestinal tumors [39]. These mice would be interesting to examine for possible defects in the pancreas. The presence of an additional Pax6 binding site in the G3-element suggests that the glucagon gene might be particularly sensitive to the lack of Pax6. Accordingly, the severely reduced numbers of glucagon positive cells in the pancreas of Pax6 mutant mice [22,40] might be explained if disruption of the nucleosome structure of the glucagon gene is a stochastic process where the chance of an open chromatin structure is determined by the number of occupied binding sites. Such a mechanism appears to regulate the activity of the chicken erythroid specific $\beta^{\text{A}}/\varepsilon$ globin enhancer [41]. Cdx2/3 is expressed at very low levels in islet cells but does not appear to show preferential expression in the α-cells [25,26]. It would thus appear that additional factors are needed to restrict glucagon expression to the α-cell. One such factor could be the β-cell specific protein Pdx1, which has been reported to inhibit Cdx2/3 mediated activation of the glucagon gene through a direct protein-protein interaction [42]. Additionally, Brain-4 that is highly expressed in α-cells within the islet has recently been shown to activate the glucagon promoter through binding to the G1-element [25].

The presence of PISCES elements in β/δ-cell specific pro-

moter elements of the insulin and somatostatin genes [13] suggests that Pax6 might form different complexes on these element. Indeed, we have found that Pax6 forms a functional cell-type specific complex with Pdx1 on the somatostatin upstream enhancer (see accompanying manuscript).

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