The promoter element G1, critical for α-cell-specific expression of the glucagon gene, contains two AT-rich sequences important for transcriptional activity. Pax-6, a paired homeodomain protein previously shown to be required for normal α-cell development and to interact with the enhancer element G3 of the glucagon gene, binds as a monomer to the distal AT-rich site of G1. However, although the paired domain of Pax-6 is sufficient for interaction with the G3 element, the paired domain and the homeodomain are required for high affinity binding to G1. In addition to monomer formation, Pax-6 interacts with Cdx-2/3, a caudal-related homeodomain protein binding to the proximal AT-rich site, to form a heterodimer on G1. Both proteins are capable of directly interacting in the absence of DNA. In BHK-21 cells, Pax-6 activates glucagon gene transcription both through G3 and G1, and heterodimerization with Cdx-2/3 on G1 leads to more than additive transcriptional activation. In glucagon-producing cells, both G1 and G3 are critical for basal transcription, and the Pax-6 and Cdx-2/3 binding sites are required for activation. We conclude that Pax-6 is not only critical for α-cell development but also for glucagon gene transcription by its independent interaction with the two DNA control elements, G1 and G3.

The glucagon gene is expressed in the α-cells of the pancreatic islets, the L cells of the intestine, and specific areas of the brain (1). The factors controlling glucagon gene expression are still poorly understood. Pancreas-specific expression of the glucagon gene is conferred by the islet-specific enhancer elements G2, G3, and G4 (2–5), and the α-cell-specific proximal promoter element G1 (2, 3, 5–7). G1 contains two nearly identical 7-bp AT-rich sequences forming a direct repeat that are candidate binding sites for homeodomain transcription factors. At least three protein complexes (B1, B2, and B3) interact with G1, and the integrity of the AT-rich direct repeat is critical for their binding and for transcriptional activity (2, 6, 7). We and others have recently characterized the transcription factor that binds to the proximal 7-bp site as Cdx-2/3, which is encoded by a caudal-related gene encoded in the endocrine pancreas and the intestine (7, 8). Cdx-2/3 is able to bind with high affinity to the 7-bp proximal AT-rich site of G1 when isolated but binds intact G1 in glucagon-producing cells preferentially as a multiprotein complex, B3 (6, 7), when both AT-rich sites are present.

We report here that the factor that interacts with Cdx-2/3 to form the B2 complex is Pax-6, a member of the pax family of vertebrate genes that contain conserved paired and homeobox encoding DNA-binding domains (9). Pax-6 has previously been reported to be expressed in the endocrine pancreas (10), to be critical for α-cell development (11, 12), and to bind to the enhancer element G3 of the glucagon gene (11). Our results indicate that the paired domain of Pax-6 is sufficient for interaction with the G3 element, whereas both paired domain and homeodomain are important for interaction with the G1 element. Furthermore, Pax-6 binds to the promoter element G1 preferentially as a monomer but also as a heterodimer with Cdx-2/3. When both Pax-6 and Cdx-2/3 are overexpressed in BHK-21 cells, we observe more than additive effects on transcriptional activation of glucagon gene expression, suggesting that Pax-6-Cdx-2/3 interactions have functional consequences on transcription. We conclude that Pax-6 not only is a key regulator of α-cell development but is also critical for glucagon gene expression through its independent interaction with the promoter and enhancer elements, G1 and G3, respectively.

**Experimental Procedures**

**Cell Culture and DNA Transfection**—The glucagon-producing hamster InRIG9 (13) and the non-islet Syrian baby hamster kidney (BHK-21) cell lines were grown in RPMI 1640 (Seromed, Basel, Switzerland) supplemented with 5% heat-inactivated fetal calf serum and 5% heat-inactivated newborn calf serum, 2 mM glutamine, 100 units/ml of penicillin, and 100 μg/ml of streptomycin. InRIG9 cells were generously provided by Dr. R. Takaki (Medical College of Oita, Oita, Japan). BHK-21 cells were transfected by the calcium phosphate precipitation technique (14) using 10–12 μg of total plasmid DNA/10-cm Petri dish. 1 μg of pSV2A pap, a plasmid containing the human placent al alkaline phosphatase gene, driven by the SV40 long terminal repeat was added to monitor transfection efficiency (15). Transfection of glucagon-producing InRIG9 cells was done using the DEAE-dextran method as described previously (16). Expression vectors consisted of the hamster Cdx-2/3 cDNA, kindly provided by Dr. M. German (University of California, San Francisco, CA), and the quail pax-6 cDNA (17), both cloned in pBSG (Stratagene). Reporter plasmids consisted of 1) wild-type and mutated (at nucleotides –90 to –91 or –71 to –72) 408-bp fragments (nucleotides 254 to 274 G3 sequence) (nucleotides –350 to –254) 408-bp fragments (nucleotides –350 to –254) linked to oligonucleotides containing the wild-type or mutated (nucleotides –254 to –258) G1 sequence (nucleotides –274 to –234 relative to the transcriptional start site) (G3–138 CAT and G3M6–138 CAT); 3) a 128-bp fragment (nucleotides –75 to +58) of the 5′-flanking sequence of the rat glucagon gene linked to the wild-type oligonucleotide G3 (G3–75 CAT) (Refs. 2, 5, and 6; see Fig. 1). These fragments were subcloned into pBLCAT3 (6). RSVCAT (18) served as positive control.

**CAT and Protein Assays**—Cell extracts were prepared 36–48 h after transfection and analyzed for CAT and alkaline phosphatase activities as described previously (6). Quantification of acetylated and nonacyl-
lated forms was done with a PhosphorImager (Molecular Dynamics). A minimum of three independent transfections were performed, each of them carried out in duplicate. Protein concentrations were determined with a Bio-Rad protein assay kit.

**RESULTS**

We previously reported that G1 is a large, 50-bp-long proximal upstream promoter element critical for a-cell-specific expression that binds at least three protein complexes, B1, B2, and B3 (Ref. 6 and Fig. 1). The more distal enhancer element G3 can be separated into an A and a B domain; G3 binds four complexes, C1A and C1B (A domain), which are islet-specific, and C2 and C3 (B domain), representing ubiquitous proteins (Refs. 5 and 20 and Fig. 1). From our previous studies, we concluded that the protein complexes B1 and C1A interacting with the G1 and G3 elements, respectively, were similar or identical inasmuch as they displayed closely related binding affinities for both G1 and G3 and migration characteristics in EMSAs (5, 6). Recently, Sander et al. (12) provided evidence that the paired homeodomain transcription factor Pax-6 interacts with the A domain of G3 and is critical for a-cell development. We thus investigated the nature of C1A and their relationship with B1. We first performed EMSAs with nuclear extracts from the glucagon-producing hamster cell line INRlG9 and 32P-labeled oligonucleotides containing either G1 (G1–56), G1–33, G1–33r5, and G1–33r3; see Fig. 1) or G3 (see Fig. 1) sequences have previously been described (5, 6). Anti-Cdx-2/3 and anti-Isl-1 antibodies were generously provided by Drs. M. German (University of California) and D. Drucker (Toronto University, Toronto, ON, Canada), respectively; anti-Pax-6 antibodies were raised against the paired domain or the homeodomain of the quail protein (serum 11 and 13, respectively, in Ref. 17).

**GST Fusion Proteins and Protein-Protein Interaction in Vitro**—For construction of GST fusion proteins, pax-6 paired, homeo, and paired linker homeo boxes were generated from InRlG9 cells by reverse transcription-polymerase chain reaction using the primers Pax-6 PD5'9, actggatcc-cagcttggtggtctttg; Pax-6 PD3'9, actaagcttgctagccaggttgcaggaaac; Pax-6 HD5'9, actggatccggctgccagcaacaggaag; and Pax-6 HD3'9, actaagcttgtgttgctggcctgtcttc and inserted into the BamHI/HindIII sites of pGEX-4T3 (Amersham Pharmacia Biotech). GST fusion proteins were expressed in Escherichia coli and purified according to the manufacturers' recommendations. L-[^35S]Methionine-labeled Cdx-2/3 was generated in vitro using the TNT wheat germ extract system (Promega). For protein-protein interaction, 10 µg of GST or GST fusion proteins were bound to 25 µl of glutathione-Sepharose beads in a total volume of 50 µl of incubation buffer containing 12 mM Heps, pH 7.9, 4 mM Tris/HCl, pH 7.9, 50 mM NaCl, 10 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride for 20 min at room temperature. Beads were washed three times, resuspended in 20 µl of incubation buffer, and incubated with 10 µl of L-[^35S]methionine-labeled Cdx-2/3 for 40 min on ice. Beads were then washed five times at room temperature with 200 µl of washing buffer (20 mM Tris/HCl, pH 8, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40), recovered in SDS-polyacrylamide gel electrophoresis loading buffer, and bound proteins were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

**FIG. 1.** Protein complexes binding to the G1 promoter and G3 enhancer elements of the rat glucagon gene 5'-flanking sequence. Schematic representation of oligonucleotides used in EMSAs that correspond to the wild-type or mutated DNA control elements. Two 7-bp AT-rich sequences within G1 forming an imperfect direct repeat are underlined. In the G3 sequence, subdomains A and B are underlined.
G1 competed for complexes B1, B2, and B3 formed on G1–56 (Fig. 2A). Mutant G1–33r5 characterized by a mutation of the 5′ AT-rich site of G1 only competed for B3, whereas the 3′ AT-rich site mutant (G1–33r3) displayed the same competition characteristics as G1–33. Furthermore, oligonucleotide G1–54 containing the 5′ AT-rich site of G1 competed for complexes B2 and B3. These results indicate that B1 and B2 interact on overlapping sites within the distal part of G1–33 as previously reported (Ref. 6 and Fig. 1); however, B3 may interact on a more restricted site compared with B2 inasmuch as G1–54 efficiently competes for B3 but not for B1. Formation of B3, in contrast, involves both the distal and proximal parts of the core element oligonucleotide G1–33. Of note, oligonucleotide G3 competes for both B2 and B3 (Ref. 5 and Fig. 2A). We then applied the same competitor oligonucleotides as in Fig. 2A on the complexes formed with InR1G9 nuclear extracts and the 32P-labeled oligonucleotide G3 (Fig. 2B). Neither formation of the islet-specific complex C1B nor formation of the ubiquitous complexes C2 and C3 are significantly affected by addition of the competing oligonucleotides (Fig. 2B and data not shown). In contrast, all oligonucleotides competing for complex B1 on G1–56 (G1–56, G1–33, G1–33r3, and G3), do also compete for C1B formed on G3, and oligonucleotides that do not interfere with B1 (G1–33r5 and G1–54) do not affect C1A. These data show that C1A exhibits similar binding characteristics as B1 and suggest that C1A is capable of binding efficiently to the G1 element.

To assess the nature of complexes B1 and C1A, we added in the EMSA incubation reactions antisera raised against the paired or homeodomain of Pax-6 (Fig. 3A). Anti-paired domain antibodies displaced B2 and B3 (G1–56), as well as C1A and C1B (G3). Complexes B1 and C1A are also supershifted by antibodies raised against the homeodomain of Pax-6. Of note, addition of both anti-Pax-6 antibodies did not completely displace B1. The residual complex was, however, of variable intensity in different nuclear extracts of InR1G9 cells and also detected with nuclear extracts from insulin-producing cell lines. Whereas this complex was specifically competed for by G-56, it was less efficiently displaced by competition with G1–33 comprising the core element of G1, and not at all with unrelated oligonucleotides (data not shown); it thus may represent a protein present in insulin- and glucagon-producing cells that might play a role in glucagon gene expression via its interaction with G1. Incubation of oligonucleotide G3 with extracts from BHK-21 cells transfected with the avian pax-6 cDNA results in a complex that migrates similarly to C1A. This complex is supershifted with Pax-6 anti-paired and anti-homeodomain antibodies and absent from nuclear extracts from BHK-21 transfected with the vector alone; our data thus indicate that B1 and C1A represent Pax-6.

Complex C1B, which displays binding characteristics that are different compared with B1 and C1A (Ref. 5 and Fig. 2), is supershifted with anti-paired domain but not with anti-homeodomain Pax-6 antibodies (Fig. 3A) and may thus represent a protein antigenically related to Pax-6 or a Pax-6 isoform with a deletion or modification in the homeodomain. Complex B3 formed on G1–56 has previously been shown by immunological criteria to consist of a protein complex containing Cdx-2/3 (7); its competition by oligonucleotide G3 and its interaction with both anti-Pax-6 antibodies now suggest that B3 may represent a Pax-6-Cdx-2/3 heterodimer.

To further characterize complexes B1 and B3, we transfected the avian pax-6 or hamster cdx-2/3 cDNA in BHK-21 cells, a cell line that does not express Pax-6 or Cdx-2/3. As shown in Fig. 3B, incubation of oligonucleotide G1–56 with extracts from BHK-21 cells overexpressing Pax-6 results in a complex that migrates similarly to B1 and that is absent from nuclear extracts from BHK-21 transfected with the vector alone. Of note, a nonspecific complex with similar migration mobility as Pax-6 and Cdx-2/3 can be observed in vector-transfected BHK-21 cells; this complex does not react with anti-Pax-6 or anti-Cdx-2/3 antibodies (data not shown). When extracts from BHK-21 cells overexpressing Cdx-2/3 are incubated with G1–56, a complex with a slightly lower electrophoretic mobility than B1 and corresponding to Cdx-2/3 appears (Fig. 3B) as previously reported (7). To test our hypothesis that B3 represents a Pax-6-Cdx-2/3 heterodimer, we incubated the labeled oligonucleotide G1–56 with a constant concentration of BHK-21 nuclear extracts overexpressing Pax-6 together with increasing amounts of Cdx-2/3 containing extracts or increasing amounts of Pax-6.
containing extracts at two different concentrations of Cdx-2/3 containing extracts; in these conditions, an additional complex is observed that migrates like B3 and supershifted by anti-Pax-6 and anti-Cdx-2/3 antibodies (Fig. 3B). Of note, the intensity of the reconstituted complex did not increase linearly; relatively high concentrations of either Pax-6 or Cdx-2/3 may be necessary for heterodimer versus homodimer formation in this in vitro system. In nuclear extracts from glucagon-producing cells, Cdx-2/3 appears to be present in much lower concentration than Pax-6 because it binds only as the heterodimer B3, whereas Pax-6 binds both as a monomer (B1) and a heterodimer (B3). Indeed, when anti-Pax-6 and anti-Cdx-2/3 anti-

bodies were added to InR1G9 nuclear extracts incubated with labeled G1–56, anti-Pax-6 antibodies displace B1 and B3, whereas anti-Cdx-2/3 antibodies displace only B3 (Fig. 4). To test whether the residual complex of low intensity after displacement of B1 by anti-Pax-6 antibodies corresponds to a Cdx-2/3 monomer, we added both anti-Pax-6 and anti-Cdx-2/3 antibodies to the reaction. However, no further displacement was seen, indicating that in InR1G9 cells, Cdx-2/3 binds G1 only as the heterodimer B3. By contrast to anti-Pax-6 and anti-Cdx-2/3 antibodies, anti-Isl-1 antisera (21) did not affect any of the complexes binding to G1–56.

To analyze the binding characteristics of Pax-6 on the G1 and G3 elements, we expressed the Pax-6 paired, homeo, and paired and homeodomains as GST fusion proteins (Fig. 5A). Whereas the Pax-6 homeodomain was unable to bind to G1 or G3, the paired domain interacted with both elements (Fig. 5B); however, the binding of the paired domain to G1–56 was very weak, and the resulting complex was readily competed for by unlabeled G3 oligonucleotides. In contrast, a fusion protein comprising both domains exhibited a high affinity for G1 and a relatively lower affinity for G3; the complex formed on G3 was highly competed for by unlabeled G1–56. We therefore conclude that although both the paired and homeodomains of Pax-6 are necessary for maximal interaction with the G1 and G3 elements of the glucagon gene, the paired domain alone is sufficient for about half-maximal binding to G3, whereas it displays very low affinity for G1.

To investigate the functional role of Pax-6, we performed cotransfection experiments in BHK-21 cells using fragments of the 5′-flanking sequence of the rat glucagon gene linked to the CAT reporter gene and a SV40-driven expression vector containing the quail pax-6 cDNA. Using the first 350 bp of the glucagon gene promoter (−350 CAT) containing both Pax-6 binding sites (G1 and G3), we observed a dose-dependent in-
crease in CAT activity with increasing amounts of expression plasmids containing the pax-6 cDNA. The transcriptional activation observed was similar to that obtained with the transcription factor interacting with the proximal AT-rich site of G1, Cdx-2/3 (Figs. 6 and 7A). To characterize the relative functional importance of the proximal (G1) and the distal (G3) Pax-6 binding sites, we investigated the effects of Pax-6 overexpression on promoter constructs containing either one or both Pax-6 binding sites (Fig. 7A); we used the first 138 bp of the glucagon gene promoter either alone (∼138 CAT) or linked to oligonucleotides representing wild-type G3 (G3-138 CAT) or G3 specifically mutated at the Pax-6 binding site (G3M6–138 CAT). As shown in Fig. 7B, overexpression of Pax-6 resulted in a 98-fold activation of the basal activity obtained with G3-138 CAT, whereas only half of this activation was observed for either ∼138 CAT or G3M6–138 CAT (37- and 38-fold activation, respectively). These results indicate that both Pax-6 binding sites are necessary for maximal activation of the glucagon gene promoter in BHK-21 cells and that deletion of the G3 site results in a loss of 50% of the activation potential. When point mutations were directed to the Cdx-2/3 binding site (nucleotides −271/272, G1M1–350 CAT), Pax-6 induced a 78-fold activation. Our results thus indicate that in BHK-21 cells, each Pax-6 binding site of the rat glucagon gene 5′-flanking sequence accounts for about half of the full activation observed when both sites are present. Of note, we always obtained significantly lower stimulation by Pax-6 with the wild-type or mutated 350 first bp of the promoter compared with shorter promoters.

We then analyzed the respective activation potential of Pax-6 and Cdx-2/3 either alone or together on the various promoter mutants described above in BHK-21 cells. 0.25 μg of the Pax-6 or Cdx-2/3 expression plasmid was used for transfection because this concentration resulted in submaximal activation and dose-response curves with increasing amounts (from 0.125 to 1 μg) of the respective plasmids gave similar activation potentials (Fig. 6). Pax-6 and Cdx-2/3 alone activated ∼350 CAT by 21- and 13-fold, respectively; when both pax-6 and cdx-2/3 cDNAs were transfected, we observed an activation

\[ \text{activation}_{\text{pax-6 and cdx-2/3}} = \text{activation}_{\text{pax-6}} + \text{activation}_{\text{cdx-2/3}} \]

resulting in the loss of Pax-6 binding on G1 (G1M1–350 CAT) (Ref. 6 and Figs. 1 and 8A). A 40-fold stimulation of transcription by Pax-6 was observed for G3-75 CAT, which corresponds to half of the activation obtained with G3-138 CAT (Fig. 7B). With G1M1–350 CAT, transcription increased by 25-fold, which also corresponded to half of the activation seen with the respective control, ∼350 CAT. When point mutations were directed to the Cdx-2/3 binding site (nucleotides −71/72, G1M11–350 CAT), Pax-6 induced a 78-fold activation. Our results thus indicate that in BHK-21 cells, each Pax-6 binding site of the rat glucagon gene 5′-flanking sequence accounts for about half of the full activation observed when both sites are present. Of note, we always obtained significantly lower stimulation by Pax-6 with the wild-type or mutated 350 first bp of the promoter compared with shorter promoters.

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To investigate the functional importance of the Pax-6 binding sites on G₁ and G₃, and the Cdx-2/3 binding site on G₃, in glucagon-producing cells, we transfected the reporter gene constructs analyzed in Figs. 7 and 8A, in InR1G9 cells. Construct G₃-138 CAT containing the G₃ element linked directly to the first 138 bp of the glucagon gene promoter conferred a CAT activity identical to the control plasmid −350 CAT. Mutation (G₃M6−138 CAT) or deletion (−138 CAT) of the enhancer element decreased this activity by about 80% (Fig. 9). Deletion (G₃−75 CAT) or mutation (G₃M1−350 CAT) of the Pax-6 binding site on G₁ resulted in 50–60% loss of activity. Surprisingly, mutation of the Cdx-2/3 binding site on G₁ (G₁M1−350 CAT), which did not affect the activated transcription conferred by overexpressed Pax-6 and Cdx-2/3 in BHK-21 cells (Fig. 8A), markedly decreased basal CAT activity in InR1G9 cells (80% reduction). This discrepancy may be due to the presence of additional, unidentified factors interacting with Pax-6 and Cdx-2/3 or with Pax6 alone on G₁ in glucagon-producing but not in heterologous cells; alternatively, interactions of the proteins binding to the distal enhancer G₀ or G₃ with those of G₁ that we suggested previously (22) may be impaired by the decreased Pax-6-Cdx-2/3 heterodimer formation.

The same wild-type and mutant reporter gene constructs were transfected along with Pax-6 and/or Cdx-2/3 expression plasmids in InR1G9 cells to observe the consequences of their overexpression on intact or mutated G₁ and G₃ elements; however, we found either no effect or, at the highest amounts of either cDNA transfected, an inhibition of basal activity (data not shown), compatible with a squelching phenomenon. We hypothesize from these results that proteins interacting with Pax-6, Cdx-2/3, or both may be rate-limiting for transcriptional activity and that overexpression of Pax-6 and Cdx-2/3 may decrease the availability of these proteins for transcription.

**DISCUSSION**

This study presents evidence that Pax-6 interacts with at least two DNA control elements of the rat glucagon gene promoter, G₀ and G₃, a critical element that confers a-cell specificity to the expression of the glucagon gene. Pax-6 is able to bind G₃ either as a monomer or as a heterodimer with Cdx-2/3. When both proteins are overexpressed in BHK-21 cells, they bind preferentially as monomers; formation of heterodimer B₃ is favored by increasing either Pax-6 or Cdx-2/3 concentration but in a nonlinear fashion (Fig. 3B). Binding assays with nuclear extracts from InR1G9 cells suggest that most of Pax-6 binds G₀ as a monomer, as evidenced by the ratio of complexes B₃/B₃. However, most if not all of Cdx-2/3 is contained within the heterodimer Pax-6-Cdx-2/3 because we have not been able to detect Cdx-2/3 monomer binding to intact G₀ using InR1G9 nuclear extracts (Ref. 7 and Fig. 4). This is most likely due to the relatively low amounts of Cdx-2/3, as compared with Pax-6, present in InR1G9 cells. Cdx-2/3 monomer binding to its recognition site is only observed when a mutation of the distal AT-rich site on G₀ prevents formation of B₀ (Pax-6), B₂, and B₃ (Pax-6-Cdx-2/3); the intensity of the monomer complex corresponds to that of the heterodimer in the presence of intact G₀ (7). Despite the relatively low abundance of the heterodimer Pax-6-Cdx-2/3 as compared with the Pax-6 monomer, interaction of Pax-6 with Cdx-2/3 may be functionally important because our results demonstrate that Pax-6 and Cdx-2/3 activate transcription in a more than additive manner in BHK-21 cells. This effect is entirely lost when Pax-6 binding to G₀ is impaired, such as on the mutant promoters G₃M1−350 and G₃−75. In this heterologous system, however, integrity of the Cdx-2/3 site is not required for interactive activation by Pax-6 and Cdx-2/3. Indeed, mutant promoter G₀M11−350 (6, 7), which decreases Cdx-2/3 binding, does not
affect activation by Pax-6 and Cdx-2/3 together in BHK-21 cells. In contrast, mutation of either the Pax-6 or the Cdx-2/3 binding site on G1 markedly decrease transactivation of the glucagon gene promoter in glucagon-producing cells. This phenomenon may be explained by additional contacts with the proximal AT-rich motif necessary for optimal conformation of the Pax-6-Cdx-2/3 heterodimer and its interaction with yet unidentified proteins in InR1G9 but not in BHK-21 cells. Different expression levels of Cdx-2/3 leading to a variable ratio of Pax-6 monomer/heterodimer formation may serve to modulate transcriptional activity of the glucagon gene during development or in response to physiological stimuli.

Pax-6 has previously been shown to interact with other homeodomain proteins such as Engrailed (En-1); heterodimer formation with En-1, however, depends only on the Pax-6 paired domain and down-regulates the DNA binding and transactivation properties of Pax-6 (23). This is in contrast to the Pax-6-Cdx-2/3 heterodimer, whose formation implicates the Pax-6 paired and homeodomains resulting in a transactivation potential that is more than additive compared with both factors taken alone.

The role of Pax-6 in endocrine pancreatic development is well established (11, 12). Pax-6 is detected in glucagon-producing cells of the mouse embryonic pancreas at day 9.5 and in all pancreatic endocrine cells at later stages of development. In mice homozygous for a targeted null mutation in the Pax-6
gene, few if any α-cells are detected (12); similar findings are reported in the Sey Neu mice, which are characterized by a homozygous mutation of the Pax-6 gene (11). In the latter mice, insulin, somatostatin, and pancreatic polypeptide-producing cells are also markedly decreased, indicating a role for Pax-6 in the development of all islet cells. Mouse genetics has provided direct evidence for the developmental role of tissue-specific transcription factors. Pax-6 interacts with G1, a critical determinant of α-cell-specific expression of the glucagon gene, and may thus have a key role in its restricted cell-type expression. It has been proposed that Pax-4 and Pax-6 determine the islet cell fate of the pancreas during development; the onset of pax gene expression may then define the lineage of the different endocrine cells (11, 12). Cells expressing both Pax-4 and Pax-6 would differentiate into mature β, δ, and γ cells, whereas absence of Pax-4 would divert cells to the α-cell lineage. It is thus possible that the simultaneous presence of Pax-6 and Cdx-2/3 and the absence of Pax-4 or other β-cell-specific factors are the critical determinants for α-cell-specific expression of the glucagon gene. Several transcription factors present in β cells or other non-α-islet cells have been identified (PDX-1 (24), Pax-4 (25), and a recently characterized β-cell factor, IB1 (26)), whereas no α-cell-specific factor has been identified so far. Interestingly, PDX-1 has recently been shown to directly interact with Cdx-2/3 and to impair its transcriptional activation of the sucrase-isomaltase promoter (27). α-cell-specific expression of the glucagon gene might thus occur through a default mechanism whereby β-cell-specific factors like Pax-4 or PDX-1 suppress the glucagon promoter in non-α-islet cells through direct protein-protein interactions with Pax-6-Cdx-2/3, for example.

Pax-6 has previously been shown to interact with the G3 control element of the rat glucagon gene and to activate trans-
scription (12). We confirm and extend these findings to the G1 promoter element. Although the G2 and G3 Pax-6 binding sites are clearly homologous in their sequence, they only share 8 of 16 nucleotides (5), implicating functional differences in the DNA-protein interaction. It has indeed been shown that DNA binding induces conformational changes in the Pax-6 protein (28) and that different target sites might have allosteric effects on transcription factors (29). On the glucagon gene 5′-flanking sequence, the Pax-6 paired domain alone is sufficient for about half-maximal binding to G2, whereas it shows only weak affinity for G1. In contrast, a protein comprising the Pax-6 paired and homeodomains exhibits a higher affinity for G1 than for G3 (Fig. 5B). These different binding properties might then affect transcriptional potencies and interactions with additional proteins, e.g. Cdx-2/3.

In our heterologous assay system using BHK-21 cells, both Pax-6 binding sites, G1 and G3, appear to be of equivalent functional importance inasmuch as deletions or mutations of either site result in a 50% loss of transcriptional activity. The respective importance of G1 and G3 in this heterologous assay does not, however, reflect the basal transcriptional activity of the glucagon gene in glucagon-producing cells. In these cells, deletion of G3 results in only mild decreases in transcriptional activity when the more proximal enhancer element G2 is present (2, 6), whereas a point mutation (G3M1) of the Pax-6 binding site within G1 leads to a 60% loss of activity (Fig. 9), indicating that the latter site is crucial for basal transcription. A second difference in the consequences of G1 mutations is observed between BHK-21 cells overexpressing Pax-6 and Cdx-2/3 and the glucagon-producing InR1G9 cells with a reporter gene construct containing a point mutation in the Cdx-2/3 binding site within G1 (G1M11). Whereas this mutation does not interfere with the more than additive transcriptional activation conferred by Pax-6 and Cdx-2/3, it decreases basal activation in InR1G9 cells by 80% (Figs. 8A and 9). The only change induced by mutation G1M11 in the complexes formed with InR1G9 nuclear extracts in EMSA is a reduction of B13, and a similar decrease is noted in BHK-21 nuclear extracts containing Pax-6 and Cdx-2/3 (Fig. 8B). Thus, despite the capacity of Pax-6 and Cdx-2/3 to directly interact in the absence of DNA, both binding sites may be important for optimal heterodimer formation and transcriptional activation in glucagon-producing cells; mutation G1M11 may attenuate the formation and change the conformation of the Pax-6-Cdx-2/3 heterodimer and thus impair its interaction with additional factors present in InR1G9 but not in BHK-21 cells. We cannot exclude, however, that the discrepancies in the consequences of G1 mutations observed between BHK-21 and InR1G9 cells are due to unidentified proteins interacting with Pax-6 and binding to the Cdx-2/3 site.

Pax-6 binds G3 as a monomer; our results suggest, however, that besides Pax-6, a related protein interacts with G3. Indeed, two complexes were previously shown to bind domain A of G3, C1A (Pax-6) and C1B (5); both complexes share very similar binding characteristics but display differences inasmuch as interference of methylation assays suggest that Pax-6 and C1B interact with different nucleotides of domain A (5); furthermore, competition for Pax-6 and C1B by the G1 binding site in EMSAs affects only Pax-6 interaction (Ref. 5 and Fig. 2B). C1B, which is recognized by Pax-6 anti-paired but not anti-homeodomain antibodies, displays slightly slower migration than Pax-6. Several isoforms of Pax-6 have been identified in the nervous system and the endocrine pancreas (10, 17, 30); however, functional tests on DNA binding and transactivation with these isoforms suggest that they do not interact with G2. Thus, C1B may represent either an as yet unidentified Pax-6 isoform or a related paired protein. Interestingly, the Pax-6 binding site on G2 corresponds to the insulin-response element of the glucagon gene and mutations impairing Pax-6 binding and C1B formation, result in a loss of regulation by insulin (3, 5). Formal characterization of C1B should lead to a better definition of the respective functional role of this Pax-6 related protein and Pax-6 on the insulin regulation of the glucagon gene.

We conclude from our studies that Pax-6 binds independently to at least two DNA control elements of the glucagon gene, G1 and G3. Pax-6 appears functionally important in both α-cell development and glucagon gene transcription; it may mediate α-cell-specific expression of the glucagon gene as the major transcription factor binding to G1 and by its interaction with Cdx-2/3 as well as by mediating the inhibitory effects of insulin on glucagon gene transcription by binding to G2. Further work will be necessary to appreciate the functional roles of Pax-6 in glucagon gene regulation.

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