The Paired Homeodomain Transcription Factor Pax-2 Is Expressed in the Endocrine Pancreas and Transactivates the Glucagon Gene Promoter*

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Glucagon gene expression is controlled by at least four DNA elements within the promoter; G2, G3, and G4 confer islet-specific expression, while G1 restricts glucagon transcription to α-cells. Two islet-specific complexes are formed on G3, the insulin-responsive element of the glucagon gene; one of these corresponds to the paired homeodomain protein Pax-6, a major glucagon gene transactivator that plays a crucial role in α-cell development. We describe here the identification of the second complex. Pax-2A and Pax-2B, another member of the paired box family. Pax-2 is known to be crucial for the development of the urogenital tract and of the central nervous system, but its presence in the endocrine pancreas has not been reported. We detected Pax-2 gene expression by RT-PCR; in islets, Pax-2 is present as two alternative splicing isoforms, Pax-2A and Pax-2B, whereas in the glucagon- and insulin-producing cell lines αTC1 and Min6, a distinct isoform, Pax-2D2, is found in addition to Pax-2B. Both islet-specific isoforms bind to the enhancer element G3 and to the α-specific promoter element G1 that also interacts with Pax-6. Pax-2A and Pax-2B dose-dependently activate transcription from the G3 and the G1 elements both in heterologous and in glucagon-producing cells. Our data indicate that Pax-2 is the third paired domain protein present in the endocrine pancreas and that one of its roles may be the regulation of glucagon gene expression.

The pax gene family of transcription factors is characterized by a 128-amino acid DNA binding motif, the paired domain. Encoded by the paired box and originally identified in the Drosophila segmentation gene paired (1), this motif is highly conserved throughout evolution in organisms from jellyfish to humans (2, 3). In vertebrates, the pax gene family consists of nine members, Pax-1 to Pax-9; these proteins are classified into four groups based on the presence of three conserved sequence motifs, paired domain homology, and similar expression pattern (reviewed in Refs. 4 and 5). Group I, represented by Pax-1, has been studied extensively in kidney development, where Pax-2 expression is required for the conversion of the mesenchyme to epithelium and its subsequent down-regulation allows for terminal differentiation of the renal tubule epithelium (15–17). Its specific induction function during organ formation has been studied extensively in kidney development, where Pax-2 expression is required for the conversion of the mesenchyme to epithelium and its subsequent down-regulation allows for terminal differentiation of the renal tubule epithelium (15, 18, 19). Patients with Pax-2 mutations display defects of optic nerves and kidneys (renal-coboloma syndrome); spontaneous or targeted Pax-2 mutations in mice severely affect the development of the optic nerve and of the inner ear and lead to a failure in cerebellum, posterior mesencephalon, and urogenital tract development (20–23).

We demonstrate that two Pax-2 isoforms, Pax-2A and Pax-2B, are present in rat pancreatic islets and bind to the insulin-responsive element G3 and to the proximal promoter element G1 of the glucagon gene. Both elements also interact with Pax-2; whereas both Pax-2A and Pax-2B interact with the proximal promoter element G1 of the glucagon gene. Both elements also interact with Pax-6; whereas both Pax-2A and Pax-2B interact with the proximal promoter element G1 of the glucagon gene.

Experimental Procedures

Plasmids and Oligonucleotides—Expression vectors containing the mouse Pax-2A or Pax-2B (6) or quail Pax-6 cDNA were kindly provided by

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Drs. G. Dressler (Howard Hughes Medical Institute, University of Michigan) and S. Saule (Institut Curie, Orsay, France). Reporter plasmids comprised the CAT gene driven by the rat insulin I gene promoter (241InsCAT) (24) or by different fragments of the rat glucagon gene promoter (2292GluCAT, 2292GluCAT, G3–31GluCAT, G3M6–31GluCAT) (25, 26); plasmid G1–31GluCAT was constructed by insertion of oligonucleotide G1–56 into the blunted BamHI site of pcCAT (27). Oligonucleotides used in this study are listed in Table I.

**Cell Culture, DNA Transfection, and Reporter Assays**—InRIG9 (28), Min6 (29), αTC1 (30), and BHK-21 cell lines were grown as described (26, 29). BHK-21 cells were transfected by the calcium phosphate precipitation technique (31) and InR1G9 cells by the DEAE-dextran method (32). pSV2A pap encoding the placental alkaline phosphatase was added to monitor transfection efficiency in BHK-21 cells (33). Cell extracts were prepared 48 h after transfection and analyzed for CAT and alkaline phosphatase activities as described previously (25). A minimum of three independent transfections was performed; each of them was carried out in duplicate.

**Reverse Transcription and PCR**—cDNA was generated from 2 μg of total RNA isolated by the guanidine thiocyanate method followed by a cesium chloride gradient (34) and 200 ng of random hexamer primers using the SuperscriptII reverse transcriptase (Life Technologies, Inc.). Mouse paired domain cDNAs were amplified using Min6 cDNA and degenerate primers PaxPDup and PaxPDown (Table I); the resulting fragment was cloned into the BamHI and HindIII sites of pBluescriptII KS+ (Stratagene), sequenced, and analyzed using the BLAST Network Service at the National Center for Biotechnology Information. To iden-

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**FIG. 1.** Pax-2 is present in glucagon- and insulin-producing cell lines and binds to the G3 element of the glucagon gene promoter. EMSA using nuclear extracts from glucagon-producing InR1G9 and αTC1 cells or from insulin-producing Min6 cells and 32P-labeled oligonucleotide G3. Pax-2-containing complexes are recognized by anti-Pax-2 antibodies and also by antibodies raised against the paired domain (PD) of Pax-6 but not by antibodies against the junction between Pax-6 paired domain and homeodomain (JCT).

**FIG. 2.** Pax-2 isoforms in rat islets and insulin- and glucagon-producing cell lines. A, schematic representation of the mouse Pax-2 gene with exons numbered 1–12. Hatched boxes mark the paired domain (PD) and a conserved octapeptide (OP). Pax-2 isoforms A and B both contain exon 10 but differ by the inclusion of exon 6. Distinct patterning of exons 11 and 12 in isoforms Pax-2C and Pax-2D, respectively, designate identical nucleotide but different amino acid sequence. The arrows indicate oligonucleotide primers Pax2–1 and Pax2–2 rev used for RT-PCR, which hybridize to all Pax-2 cDNAs. B, Southern blot of Pax-2 RT-PCRs from rat islets, Min6, and αTC1 cells. Control amplifications were performed with plasmids containing the Pax-2A and Pax-2B cDNA, and the PCR products were hybridized with mouse Pax-2 cDNA.

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**TABLE I**

| Name       | Gene, application | Sequence (5’-3’) | Reference |
|------------|-------------------|-----------------|-----------|
| PaxPDup    | Pax proteins, degenerate paired domain PCR primer | ACTGAGATCC (A/C) (A/C)G (G/C)C (A/C/G) (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C | 15 |
| PaxPDown   | Pax proteins, degenerate paired domain PCR primer | ACTAAGGTCC (A/C) (A/C)G (G/C)C (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C (G/C) (A/G) (A/C/G)C | 15 |
| Pax2–1     | Pax-2, amplification of Pax2 isoforms | CTCGCTTTTCCACCCTACC | 15 |
| Pax2–2rev  | Pax-2, amplification of Pax2 isoforms | GTTGGCGGCTATAGCCGAC | 15 |
| Pax-2 ex6  | Pax-2A, Pax-2D1 (hybridization probe specific for exon 6) | TCGAGGCTATACACTGATCCTGC | 15 |
| Pax-2 Δex6 | Pax-2B, Pax-2C, Pax-2D2 (hybridization probe specific for the deletion of exon 6) | AAGCCGGAGGAGATGTGCTGAG | 15 |
| Pax-2 ex10 | Pax-2C (hybridization probe specific for exon 10) | AGGAAGCTTGCAAGATGCC | 38 |
| Pax-2 ex12 | Pax-2D1, Pax-2D2 (hybridization probe specific for a deletion in exon 12) | CTTTACTTATGCCTCC | 39 |
| G1–56      | Rat glucagon, gel shift | GTTGAATCATTGTGAACAAACCCCAATTATTTACAGTAGAATGAAATTTAT | 11 |
| G3         | Rat glucagon, gel shift | GCTGAAGATTTTTTCCAGGCTGACTGAGATGAAAGGCGGTTACTC | 11 |
tify Pax-2 isoforms expressed in the endocrine pancreas, primers Pax2–1 and Pax2–2rev (Table I) hybridizing to all known Pax-2 isoforms were used to amplify cDNAs from rat islets and brain, Min6, and αTC1 cells. PCR products from Min6 and αTC1 cells were analyzed by sequencing. Pax-2 amplification products generated from rat islets were subcloned in pCR2.1-TOPO (Invitrogen) and transformed into Escherichia coli HB101. The resulting colonies were hybridized with 32p-labeled oligonucleotides specific for exon 6 (in Pax-2a and Pax-2-d1), the deletion of exon 6 (in Pax-2b, Pax-2c, and Pax-2-d2), exon 10 (in Pax-2c), or a deletion in exon 12 (in Pax-2d1 and Pax-2-d2) (Table I).

**Data Analysis**—Data are presented as mean ± S.E., and statistical significance was tested by analysis of variance and Student’s t test where applicable. The threshold for statistical significance was p < 0.05.

**RESULTS**

The Paired Domain Protein Pax-2 Is Expressed in Pancreatic Islets and Binds to the Glucagon Gene Promoter—Using nuclear extracts from InR1G9 cells, four protein complexes are formed on G3, the insulin-response element of the glucagon gene promoter. Two complexes, C2 and C3, contain widely expressed proteins that may represent CCAAT binding factors, and two correspond to islet-specific proteins (Fig. 1; Ref. 26); we and others have recently identified one of the islet-specific complexes as the paired homeodomain protein Pax-6 (10, 11). The second, slightly slower migrating complex, C1B, was supershifted with antibodies raised against the paired domain of Pax-6 but not with antibodies specific for the Pax-6 homeodomain or the junction between paired domain and homeodomain, indicating that C1B may represent a protein antigenically related to Pax-6 (Fig. 1; Ref. 11). To identify C1B, we performed RT-PCR reactions of total RNA prepared from InR1G9 and Min6 cell lines with degenerate primers to conserved domains of the paired box. We were able to amplify not only Pax-6 cDNA sequences but also cDNAs coding for the paired domain protein Pax-2 that comprises a paired domain, the conserved octapeptide, and a partial paired-type homeodomain (5). To confirm the presence of Pax-2 in InR1G9 cells, anti-Pax-2 antibodies were added to EMSA reactions on G3 and indeed recognized complex C1B (Fig. 1); the same results were obtained with nuclear extracts from Min6 and αTC1 cells. We thus conclude that a third paired domain protein, Pax-2, in addition to Pax-6 and Pax-4, is expressed in islet cell lines and interacts with the G3 element of the glucagon gene promoter.

Since five Pax-2 isoforms generated by alternative splicing have been described (Fig. 2A; Refs. 38–40), we analyzed Pax-2 mRNAs in the endocrine pancreas by RT-PCR. Using mRNA from rat islets, Min6, and αTC1 cells, we obtained multiple amplification products hybridizing with a Pax-2 probe (Fig. 2B). To identify specific isoforms, Pax-2 cDNAs from rat islets were therefore subcloned and hybridized with oligonucleotides discriminating alternatively spliced cDNAs (Table I). 69% (127 out of 184) and 31% (57 out of 184) of these clones corresponded to Pax-2a and Pax-2b, respectively; other isoforms were not detected in rat islets. In contrast, Min6 and αTC1 cells expressed Pax-2b as major and Pax-2d2 as minor alternative splicing products as revealed by direct sequencing of PCR products.

**Pax-2A and Pax-2B Interact with the G3 and G1 Elements of the Glucagon Gene Promoter**—To investigate the DNA binding properties of Pax-2 on the glucagon gene promoter, we concentrated on the two isoforms present in rat islets. Pax-2A and Pax-2B were overexpressed in BHK-21 cells, and the resulting nuclear extracts were used for EMSA with the G3 element. Pax-2A and Pax-2B both bound to G3 and comigrated with the Pax-2 (C1B) complex present in nuclear extracts from InR1G9 cells (Fig. 3A). In addition to its interaction with G3, Pax-6 also binds to the proximal promoter G1 (11). Since Pax-2 and Pax-6 form complexes of equal intensity on G3 using InR1G9 nuclear extracts, we tested whether Pax-2 was also able to bind to G1. Pax-2A or Pax-2B-containing nuclear extracts from BHK-21 cells formed a complex with G1–56 that was recognized by anti-Pax-2 antibodies (Fig. 3B). However, about 4–10-fold more nuclear extracts were necessary to obtain a Pax-2 complex of similar intensity on G1 as compared with G3, indicating a lower binding affinity of Pax-2 for this site. To test for the presence of Pax-2 in the complexes formed with nuclear ex-
**FIG. 4. Binding affinity of Pax-2 and Pax-6 for G1 and G3.** A, protein-DNA complexes formed with nuclear extracts from InR1G9 cells and G3 were competed for by the indicated molar excess of cold oligonucleotides G1–56 or G3. B and C, competition experiments using nuclear extracts from BHK-21 cells overexpressing Pax-2A (C) or Pax-2B (B) mixed with Pax-6 containing extracts and labeled oligonucleotide G1–56. Due to the lower affinity of Pax-2 for G1 compared with Pax-6, the absolute quantity of nuclear extract containing Pax-2 versus Pax-6 was about 10-fold higher in order to obtain complexes of equal intensity. C, when Pax-6 was in excess over Pax-2 binding activity, Pax-2 complexes could only be detected when Pax-6 was competed for by oligonucleotide G1–56. D, sequence alignment of the rat glucagon gene elements G3 and G1 and consensus sequences for Pax-6 and Pax-2 binding sites. Asterisks and hatched symbols indicate nucleotides corresponding to the Pax-6 PD and the Pax-2 PD binding site consensus (41), and underlined nucleotides may interact with the Pax-6 homeodomain.
tracts from glucagon-producing cells on G1, we added anti-Pax-2 antibodies to EMSA reactions. Three protein complexes were detected: Pax-6 as a monomer, Pax-6 and Cdx-2/3 as a heterodimer, and B2, an as yet unidentified complex (11). The Pax-2 complex formed with Pax-2-containing extracts from BHK-21 cells did not comigrate with any of the complexes detected with InRIG9 nuclear extracts, and the addition of anti-Pax-2 antibodies did not affect any of these complexes. Thus, although Pax-2A and Pax-2B bind to the G3 and, with a lower affinity, to the G3 element of the glucagon gene promoter, Pax-2 binding activity from glucagon-producing cells is restricted to the G3 element in vitro.

Since both potential Pax-2 target sites on the glucagon gene promoter are also bound by Pax-6, we compared the relative affinity of both proteins for G1 and G3 by gel shift competition experiments. The Pax-2 complex formed with InRIG9 nuclear extracts on labeled G3 was competed for by a 10-fold excess of cold oligonucleotide G3, while a 500-fold excess of G1–56 was required for the same reduction. In contrast, competition of Pax-6 by G3 required a 5-fold higher excess of cold oligonucleotide than competition by G1–56 (Fig. 4A). These data indicate that Pax-2 has a slightly better affinity for G3 as compared with Pax-6, whereas G1 is a much better target site for Pax-6 than for Pax-2. To test the binding affinity of Pax-2A and Pax-2B individually on G1, we performed competition experiments with nuclear extracts from BHK-21 cells overexpressing Pax-6, Pax-2A, or Pax-2B. When Pax-6-containing extracts were mixed with an excess of Pax-2B-containing extracts, both proteins formed complexes of similar intensity on G1–56 as compared with the individual binding reactions (Fig. 4B). Competition of the Pax-2B and Pax-6 complexes on G1–56 revealed a better affinity of Pax-2B for G3 than for G1, whereas the opposite was observed for Pax-6. Similar qualitative results were obtained using Pax-2A (Fig. 4C). When a relative excess of Pax-6 versus Pax-2A binding activity was used for EMSA, the Pax-2A complex was no longer detected in the combined reaction, and it reappeared only when Pax-6 was competed for by cold G1–56 oligonucleotides (Fig. 4C, right panel). We conclude that the differential affinity of Pax-2 and Pax-6 for G1 and G3, as observed in these competition experiments, provides an explanation of why both proteins form complexes of similar intensity on the G3 element using nuclear extracts from InRIG9 cells, whereas with the same extracts, binding of Pax-2 cannot be detected on G1–56 (Fig. 3). The consensus binding sequences of the Pax-2 and Pax-6 paired domains (41–43) are highly similar and correspond well to the G3 element (Fig. 4D). Although G1 is a lower affinity binding site for both paired domains, this binding site is preceded by an ATTA sequence that may interact with the homeodomain of Pax-6. We indeed previously demonstrated that a Pax-6 protein containing the paired domain, linker domain, and homeodomain has a significantly better binding affinity for G1 compared with the paired domain alone (11). Pax-2 contains only a partial homeodomain that might be unable to interact with DNA (44), thus explaining its weaker interaction with G1 as compared with Pax-6.

**Pax-2 and Pax-2B Transactivate the Glucagon Gene Promoter Elements G3 and G1**—To test the effect of Pax-2 on the transcriptional activation of the glucagon gene promoter, we cotransfected InRIG9 cells with a CAT reporter plasmid driven by the full-length promoter (−292Glu) and increasing amounts of Pax-2 expression plasmids. Pax-2A and Pax-2B dose-dependently increased basal CAT activity by up to 6- and 9-fold, respectively (Fig. 5A). Similar qualitative results were obtained with reporter plasmids containing the individual Pax-2 binding sites, but strikingly, maximal transactivation by Pax-2 was much higher, reaching 46-fold on G3 and 112-fold on G1 (−138Glu), the lower affinity binding site. These data indicate that Pax-2 strongly transactivates the glucagon promoter in glucagon-producing cells through its interaction with the G1 and G3 elements.

We then used the same reporter plasmids as above in the non-islet cell line BHK-21 to test whether Pax-2 directly activates the glucagon gene promoter. Cotransfection of increasing amounts of Pax-2A increased CAT activity of −292Glu, G3−31Glu, and −138Glu by up to 5-, 12-, and 10-fold respectively, whereas Pax-2B conferred an 18-, 18-, and 36-fold activation, respectively (Fig. 5B). These data indicate that, in InRIG9 and BHK-21 cells, 1) Pax-2B is a more potent transactivator than Pax-2A on the tested glucagon promoter constructs, particularly in BHK-21 cells and on −138GluCAT, and 2) transactivation of the full-length glucagon gene promoter by Pax-2A and Pax-2B is weaker than activation of either individual binding site. Since both G1 and G3 interact with Pax-2 and Pax-6, we compared the transactivation of different glucagon promoter constructs containing either one or both Pax binding sites (Fig. 6A). CAT activity driven by the G3 element was similar with Pax-6, Pax-2A, or Pax-2B factors, corresponding to their roughly equal binding affinity to this site. This effect was specific inasmuch as a mutation of G3 that interferes with Pax-2 and Pax-6 binding (G3M6Glu; Ref. 26) strongly reduced effector-induced CAT activity. Surprisingly, transactivation of G1−31Glu by Pax-2 was only 30–50% lower than that of Pax-6 despite a significantly lower binding affinity of Pax-2 for G1 compared with Pax-6; this might be accounted for by the large amounts of Pax-2 present in transfected cells. CAT activity conferred by Pax-6 on −138Glu, comprising G1, did not increase significantly compared with G1 alone, whereas Pax-2A- and Pax-2B-mediated activity increased by 2.2- and 2.9-fold, respectively. These data indicate that Pax-2 might interact with additional sites within −138Glu; we indeed observed some transcriptional activation (5–6-fold) of promoter fragments comprising either the first 75 bp or the fragment from base pairs −140 to −100 of the promoter (data not shown).

Using a reporter plasmid containing both Pax binding sites (G3−138Glu), additive transactivation by all three proteins as compared with the individual elements was observed. In contrast, CAT activity conferred by the full-length promoter (−292Glu) in the presence of Pax-6 or Pax-2 was less than 25% of that obtained with G3−138. We conclude that Pax-6 and the Pax-2 isoforms A and B transactivate the glucagon gene promoter through G1 and G3.

We then assessed whether Pax-2 and Pax-6 were capable of functionally interacting on the G1 and G3 elements. When both cDNAs were cotransfected with either G3−31Glu or −138Glu, no significant increase of CAT activity as compared with either cDNA was observed (Fig. 6B), except that Pax-2B was additive to Pax-6 on −138Glu, suggesting that Pax-2B might under these conditions interact with other sequences on −138Glu. We conclude from these experiments that Pax-2 and Pax-6 transactivate the glucagon promoter independently from the same binding site and without any functional interaction.

**Pax-2 Has No Effect on the Insulin Gene Promoter**—Since Pax-2 is expressed in glucagon- and insulin-producing cells, we tested whether it affected insulin gene transcription. Cotransfection of either Pax-2A or Pax-2B in BHK-21 cells had no effect on a reporter construct driven by 410 bp of the rat insulin I gene promoter (Fig. 7). Correspondingly, Pax-2 was unable to bind to the CII element, the Pax-6 binding site of the insulin I gene promoter (data not shown). We therefore conclude that Pax-2 transactivates the glucagon gene promoter but may have no role in insulin gene transcription.
DISCUSSION

The functional role of Pax-2 in organogenesis has been extensively studied during kidney and brain development (15, 18, 20, 21, 23); however, its molecular role is still poorly characterized. Only recently, a few Pax-2 in vivo target genes have been described. Pax-2 transcriptionally activates the Wilms tumor suppressor gene WT1, Engrailed 2, and, cooperatively with homeodomain proteins, the Pax-5 enhancer (45–47). In addition, two Pax-2 target genes with yet unknown function have been identified by chromatin precipitation using mouse embryonic spinal cord (43). Here we demonstrate that Pax-2 is expressed in the endocrine pancreas and that it transactivates the glucagon gene promoter through two cis-acting sequences, G1 and G3. Both elements interact also with Pax-6, another member of the paired homeobox family; however, the affinity of Pax-2 and Pax-6 for G1 and G3 differs considerably. Both proteins formed complexes of similar intensity on G3 with nuclear extracts from glucagon-producing InR1G9 cells, and, in gel shift competition assays, Pax-2 had a slightly better affinity for G3 as compared with Pax-6. In contrast, Pax-6 bound G1 with an about 50-fold higher affinity than Pax-2; correspondingly, Pax-2 could not be detected within the complexes formed on G1 with InR1G9 nuclear extracts, suggesting that Pax-2 may interact with G1 only in the absence of Pax-6. The differential properties of Pax-6 and Pax-2 can be explained by their DNA binding domains. Pax-6 comprises two functional DNA binding domains and has been shown to recognize target genes either with its paired domain (e.g. the neural cell adhesion molecule, N-CAM), its homeodomain (e.g. rhodopsin), or cooperatively by the paired domain and homeodomain (e.g. neural cell adhesion molecule L1, N-CAM L1) (48–50). We previously showed that Pax-6 interacts with the glucagon gene element G3 through the paired domain, whereas high affinity binding of the G1 element requires the paired domain and homeodomain (11). Pax-2 contains a paired domain for which the consensus binding sequence matches nearly perfectly the G3 element (Fig. 4D). The partial Pax-2 homeodomain, however, like its homologous domain in the Pax-5 protein, might not be capable of binding DNA but rather represent an interaction surface for the TATA-binding protein (44, 51). In glucagon-producing cells, Pax-2 might therefore predominantly interact with the glucagon gene element G3. Interestingly, G3 corresponds to the insulin response element of the glucagon gene (26, 35), and the identification of Pax-6 and Pax-2 as G3-interacting proteins will now allow analysis of the molecular mechanisms of insulin action on the glucagon gene promoter.

We detected two Pax-2 isoforms, Pax-2A and Pax-2B, in roughly equivalent amounts in rat islets, whereas Min6 and αTC1 cells contained Pax-2B and Pax-2D2 as major and minor isoforms, respectively. This different distribution might reflect changes in alternative splicing occurring during development of the endocrine pancreas or being induced by the generation of
tumor cell lines. During kidney development and in Wilms tumor, Pax-2B is the major isoform with Pax-2A being 5-fold less abundant. Pax-2D represents a minor but nonnegligible fraction, and only traces of Pax-2C have been described (39, 40). Concentrating on the two splicing forms found in adult islets, we analyzed the binding and transactivation properties of Pax-2 on the glucagon and insulin gene promoters. Pax-2 acted as a potent transactivator of the glucagon gene. Consistent with the similar binding affinity of Pax-2A and Pax-2B for the isolated G3 and G1 elements, both isoforms transactivated G3–31Glu and G1–31Glu to a similar extent. Our data are consistent with previous reports observing similar binding and transactivation of Pax-2 and Pax-6 in glucagon gene expression. The proximal promoter element G1 by itself confers only weak transactivation activity and is dependent on either upstream enhancer element G2 or G3 for a high level of expression; on the other hand, G1 is required for G2 or G3 to enhance transcription, since deletion of G1 completely abolishes transcriptional activity (52). The major enhancer element is represented by G2, deletion of which leads to an about 40% decrease in transcriptional activity, whereas deletion of G3 results in a 25% loss of activity (52). In the full-length promoter, G2 may therefore confer basal enhancer activity, whereas G3 may predominantly act to regulate glucagon gene expression in response to insulin (26, 35). Pax-2 and Pax-6 display similar binding and transactivation of the G3 element; furthermore, although Pax-2 binds G1 with much lower affinity compared with Pax-6, it is able to transactivate this element to a similar extent. Inactivation of Pax-2 and Pax-6 in glucagon-producing cells will be necessary to evaluate their respective role in transactivation and regulation of the glucagon gene promoter.

Interestingly, we observed much lower activation of transcription by Pax-2 using the full-length promoter compared with G3–138Glu, suggesting the potential presence of negative acting cis elements either between base pairs −292 and −275 or between base pairs −230 and −138 of the glucagon gene promoter. Pax-2 has previously been shown to act as a transcriptional repressor on certain target elements including its own promoter; positive or negative effects on transcription seem to be determined by the sequence of the binding site with transcriptional repression requiring a consensus triple A motif (6, 53). We indeed detected two triple A motifs within this sequence that will be interesting to analyze in more detail.

Despite its presence in insulin-producing cells, we observed no direct effect of Pax-2 on insulin gene expression; we cannot, however, exclude the possibility that Pax-2 interacts with bind-

**FIG. 6.** Effect of Pax-2 and Pax-6 on different glucagon reporter gene constructs. A and B, BHK-21 cells were cotransfected with 10 μg of the respective reporter plasmid and 0.25 μg of Pax-6, Pax-2A, or Pax-2B expression vectors. In B, the total quantity of DNA was kept constant by adding appropriate amounts of empty expression vector pSG5. Data are presented relative to basal CAT activity observed in the presence of pSG5, and asterisks indicate statistical significance with p < 0.05.

**FIG. 7.** Pax-2 has no effect on the insulin gene promoter. BHK-21 cells were cotransfected with 10 μg of a reporter plasmid driven by 410 bp of the rat insulin I gene promoter and 0.5 μg of Pax-2A or Pax-2B expression vectors. Data are presented relative to basal CAT activity observed in the presence of pSG5.
ing sites distal from −410 bp. The functional role of Pax-2 in β cells remains to be elucidated.

In conclusion, we report the detection of a third Pax protein in the endocrine pancreas, Pax-2. Pax-6 and Pax-4 have been shown to be crucial for the development of specific cell lineages in the endocrine pancreas. Whereas mice lacking Pax-6 do not form glucagon-producing cells, inactivation of the Pax-4 gene has been shown to prevent β and δ cell differentiation. Furthermore, double mutant mice fail entirely to develop pancreatic islet cells (7, 9). Pax-2 mutant mice do exist and lead to severe malformations of the brain and a lack of the urogenital tract, but no pancreatic phenotype has so far been described. Preliminary data from our laboratory indicate that Pax-2 is expressed during development of the mouse pancreas, and we are currently investigating the potential role of Pax-2 in pancreatic development.

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