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Regulatory Regions Driving Developmental and Tissue-Specific Expression of the Essential Pancreatic Gene *pdx1*

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***pdx1* (*pancreatic and duodenal homeobox gene-1*), which is expressed broadly in the embryonic pancreas and, later, in a more restricted manner in the mature β cells in the islets of Langerhans, is essential both for organ formation and β cell gene expression and function. We carried out a transgenic reporter gene analysis to identify region- and cell type-specific regulatory regions in *pdx1*. A 14.5-kb *pdx1* genomic fragment corrected the glucose intolerance of $pdx1^{+/-}$ animals but, moreover, fully rescued the severe gut and pancreas defects in $pdx1^{-/-}$ embryos. Sequences sufficient to direct reporter expression to the entire endogenous *pdx1* expression domain lie within 4.3 kb of 5′ flanking DNA. In this region, we identified two distinct fragments that drive reporter gene expression to different sets of islet neuroendocrine cells. One shows pan-endocrine cell specificity, the other is selectively activated in insulin-producing β cells. The endocrine-specific regulatory regions overlap a localized region of 5′ flanking DNA that is remarkably conserved in sequence between vertebrate *pdx1* genes, and which has been associated with β cell-selective expression in cultured cell lines. This region contains potential binding sites for several transcription factors implicated in endodermal development and the pathogenesis of some forms of type-2 diabetes. These results are consistent with our previous proposal that conserved upstream *pdx1* sequences exert control over *pdx1* during embryonic organogenesis and islet endocrine cell differentiation. We propose that mutations affecting the expression and/or activity of transcription factors operating via these sequences may predispose towards diabetes, at least in part by direct effects on endocrine *pdx1* expression.**

Key Words: pancreas; islet; diabetes; organogenesis; transgenic; *pdx1*; β cell.

INTRODUCTION

Organogenesis involves multiple interactions among interdependent cell types and, concomitantly, the precise orchestration of cellular differentiation programs by complex batteries of transcription factors. Many genes have been linked to the differentiation of specific embryonic cell types. There are fewer examples where the development of a whole organ is affected by single gene mutations, including the spleen, which requires *Hox11* (Roberts *et al.*, 1994), and the pancreas, which requires both *pdx1* and PTF^{p48} (Jonsson *et al.*, 1994; Offield *et al.*, 1996; Krapp *et al.*, 1998).

The pancreas develops from separate dorsal and ventral outgrowths of the endodermal epithelium, which later fuse. Dorsal bud outgrowth depends on inductive signals from the nearby notochord (Kim *et al.*, 1997; Hebrok *et al.*, 1998), and its subsequent expansion and differentiation require continuous signals from the surrounding mesenchyme (Wessels *et al.*, 1967; Ahlgren *et al.*, 1996). Gene-inactivation studies reveal that dorsal and ventral bud development occur independently and require different sets of transcription factors. For example, mutations in *Isl1* or *Hlxb9* block dorsal, but not ventral, bud outgrowth (Ahlgren *et al.*, 1997; Li *et al.*, 1999; Harrison *et al.*, 1999). Morphogenesis of the pancreatic epithelium yields a highly branched ductal network within which multipotent precursors give rise to both exocrine and endocrine cells in a process involving cell–cell interactions and Notch signaling (Apelqvist *et al.*, 1999; Jensen *et al.*, 2000; Schwitzgebel *et al.*, 2000). The exocrine (acinar) cells produce digestive enzymes. Endocrine cells located within the islets of Langerhans produce peptide hormones involved in glucose

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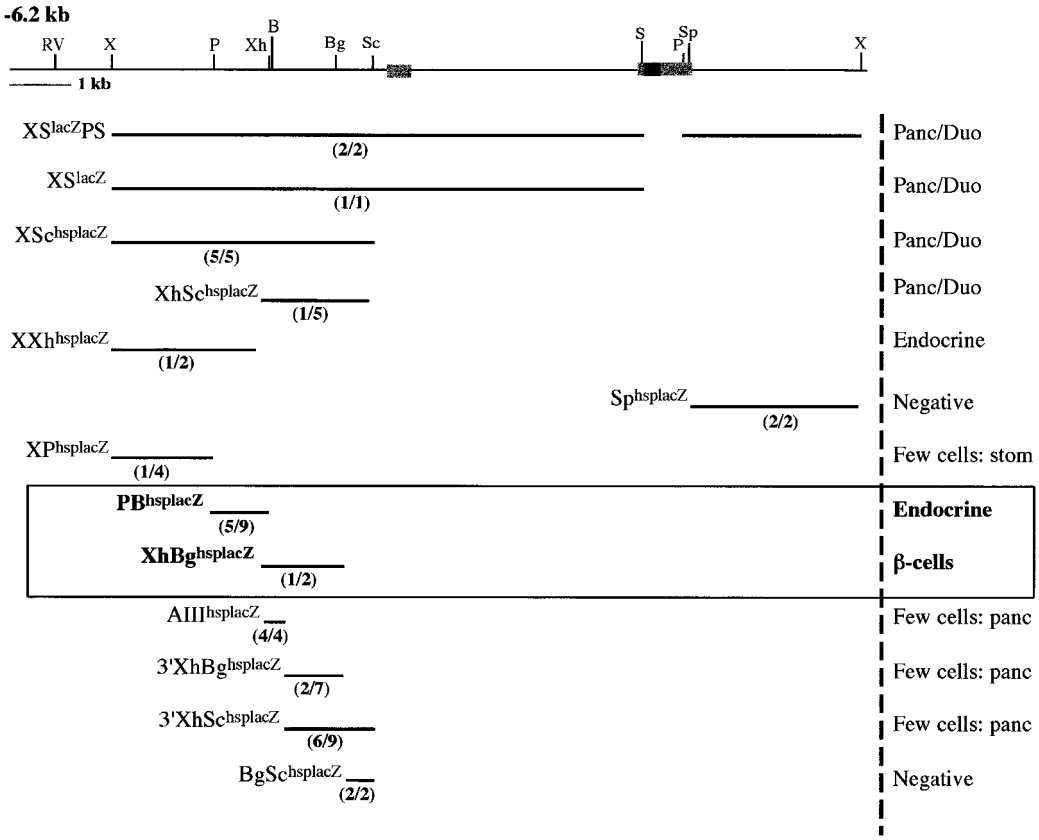

FIG. 1. Diagram of *pdx1* locus showing relevant restriction enzyme sites. Thin lines, *pdx1* genomic sequences; gray boxes, *pdx1* exons; black box, homeodomain. Transgenic constructs are named according to restriction enzyme sites and are drawn below the locus. Numbers in parentheses indicate number of expressing lines (numerator) out of total lines examined (denominator). Expression pattern for each transgene is given at the right. B, *Bst*EII; Bg, *Bgl*II; P, *Pst*I; RV, *Eco*RV; S, *Sma*I; Sc, *Sac*I; Sp, *Spe*I; X, *Xba*I; Xh, *Xho*I.

homeostasis—glucagon, insulin, somatostatin, and pancreatic polypeptide—in α, β, δ, and PP cells, respectively. As for other organs of the digestive system, a substantial amount of pancreas differentiation and morphogenesis occurs after birth, with the exocrine and endocrine compartments becoming fully functional by weaning (Lebenthal and Lebenthal, 1999; Menard *et al.*, 1994). Consequently, while mutations in genes that affect the differentiation of specific pancreatic cell types may have dramatic embryonic phenotypes, some of their effects may not be manifested physiologically until the neonatal period or even into adulthood.

Our laboratory focuses on *pdx1* (*pancreas and duodenum homeobox gene 1*), which may represent a nodal point in a network of transcriptional regulators that directs pancreas formation, maintenance, and function. In all vertebrates examined, *pdx1* is expressed in the region of the endoderm that ultimately gives rise to stomach, pancreas, and duodenum (Gannon and Wright, 1999) and its function is critical for posterior foregut development. Null mutations in *pdx1* result in defects in most, if not all, of the different cell types in which it is expressed, including malformations of the pylorus and rostral duodenum, absence of Brunner's glands, and reduced numbers of specific enteroendocrine cell types in the stomach and intestine (Jonsson *et al.*, 1994; Offield *et al.*, 1996; Larsson *et al.*, 1996). Most dramatically, loss of *pdx1* function results in pancreatic agenesis in mice and humans (Jonsson *et al.*, 1994; Offield *et al.*, 1996; Stoffers *et al.*, 1997b).

Late aspects of pancreatic differentiation and maintenance of mature organ function also require *pdx1*. Tissue-specific gene-inactivation experiments show that deletion of *pdx1* function from β cells causes dramatic decreases in insulin expression and overt diabetes (Ahlgren *et al.*, 1998; M.G. and C.V.E.W., unpublished observations). In addition to the insulin gene, PDX1 can activate transcription of several genes involved in glucose sensing and metabolism such as GLUT2 and glucokinase (Watada *et al.*, 1996a,b; Waeber *et al.*, 1996; Wu *et al.*, 1999; MacFarlane *et al.*, 2000b; Sayo *et al.*, 2000; Glick *et al.*, 2000). $pdx1^{+/-}$ mice are glucose intolerant (Dutta *et al.*, 1998; Ahlgren *et al.*, 1998), suggesting that gene dosage is critical for maintaining normal blood glucose levels. Con-

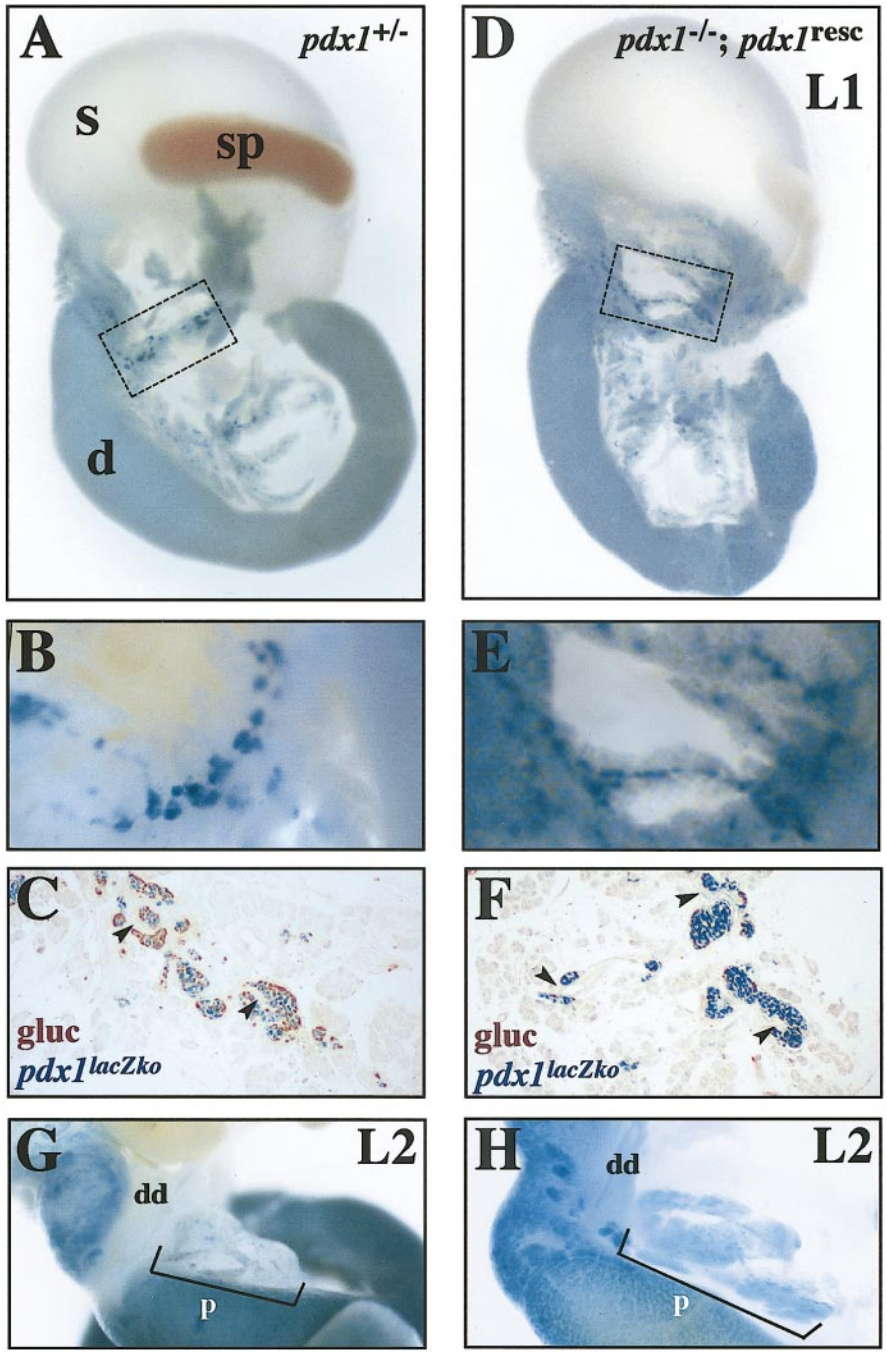

FIG. 2. A *pdx1* transgene rescues pancreatic development in $pdx1^{-/-}$ mice. Digestive organs from $pdx1^{+/-}$ (A), and $pdx1^{-/-}$;$pdx1^{resc}$ L1 (D) and L2 (G, H) neonates. *pdx1*-driven *lacZ* expression from the $pdx1^{lacZKO}$ null allele is detected by X-gal staining (blue). The presence of two copies of the $pdx1^{lacZKO}$ null allele results in darker X-gal staining in $pdx1^{-/-}$;$pdx1^{resc}$ animals. Boxed areas in (A) and (D) indicate pancreatic tissue magnified in (B) and (E), respectively. Islets are present along the pancreatic duct (arrowheads in C and F). Sections in (C) and (F) were immunolabelled for glucagon (brown) to outline islets. (G, H) Two examples of the extent of pancreatic rescue observed in L2 transgenics. s, stomach; sp, spleen; d, duodenum; dd, dorsal ductile; p, pancreas. A, D: 3.75×; B, E, G, H: 12×; C, F: 25×.

sistent with this, humans heterozygous for inactivating mutations of *pdx1* are predisposed to diabetes (Stoffers *et al.*, 1998) and other mutations in human *pdx1* (*Ipf1*) are associated with adult onset (Type II) diabetes (Macfarlane *et al.*, 2000a) and maturity onset diabetes of the young type 4 (MODY4; Stoffers *et al.*, 1997a).

In addition to *pdx1*, mutations in other homeobox genes (*Isl1*, *Pax4* and *-6*, *Nkx2.2*, *Hlxb9*, *HNF6*), or genes encoding basic helix–loop–helix proteins (*Beta2/NeuroD*, *PTF1*^{p48}, *neurogenin3*), abrogate pancreatic development, or cause loss of or dramatic reduction in specific pancreatic cell types (Ahlgren *et al.*, 1997; Sosa-Pineda *et al.*, 1997; St-Onge *et al.*, 1997; Naya *et al.*, 1997; Krapp *et al.*, 1998; Sussel *et al.*, 1998; Harrison *et al.*, 1999; Li *et al.*, 1999; Jacquemin *et al.*, 2000; Gradwohl *et al.*, 2000). Mutations in other endodermal transcription factors are also associated with subtypes of human diabetes, for example, HNF1α (MODY3), HNF1β, (MODY5), and HNF4α (MODY1) (Yamagata *et al.*, 1996a,b; Horikawa *et al.*, 1997).

The evolutionarily conserved expression pattern and function of *pdx1* make it an attractive entry point for addressing the general issue of endodermal regionalization and the mechanisms driving pancreas outgrowth. Within the posterior foregut, the anterior limit of *pdx1* expression in the stomach is sharp, but the posterior boundary within the duodenum is much less definite. *pdx1* expression within the pancreas itself is dynamic. Initially it is detected throughout the epithelium, but becomes downregulated in exocrine and ductal tissue, although cells within the ductal epithelium are capable of reactivating *pdx1* during pancreatic regeneration and islet neogenesis (Song *et al.*, 1999; Sharma *et al.*, 1999). In neonates and adults, *pdx1* expression is highest in insulin-producing β cells with lower levels in subpopulations of acinar cells (Guz *et al.*, 1995; Wu *et al.*, 1997). Little is known about the processes that regulate *pdx1* expression along the anteroposterior axis of the primitive gut, during organogenesis, or during the differentiation, maintenance, and function of specific cell types. We have used a transgenic reporter gene assay to identify regulatory regions that drive *pdx1* expression in distinct patterns during embryonic development and in the adult organ. Our data suggest that separate regulatory modules direct duodenal versus pancreatic expression, and that multiple elements contribute to the complex spatiotemporal expression pattern of *pdx1* within the islet endocrine cells.

MATERIALS AND METHODS

pdx1 Transgenic Constructs and Generation of Transgenic Mice

(See Fig. 1 for location of restriction enzyme sites.) *pdx1*^{resc} was generated by using an approximately 14.5-kb lambda genomic clone (Offield *et al.*, 1996). The *Sal*I site is located within the lambda vector. Construction of *XS*^{lacZ}*PS*, *XS*^{lacZ}, and *PB*^{hsplacZ} has been previously described (Wu *et al.*, 1997). All constructs designated "hsp.lacZ" contain *pdx1* promoter fragments blunt-end ligated into the *Sma*I site of the modified *hsp68lacZpA* vector (a gift from B. Hogan; Sasaki and Hogan, 1996), which contains the heat shock protein minimal promoter and a bacterial *lacZ* cDNA. XSc^{hsplacZ}, 4.3 kb 5′ *Xba*I to 5′ *Sac*I; XXh^{hsplacZ}, 2.5 kb 5′ *Xba*I to *Xho*I; XhSc^{hsplacZ}, 1.8 kb *Xho*I to *Sac*I; Sp^{hsplacZ}, 3.0 kb *Spe*I site within 3′ UTR to *Sal*I; XP^{hsplacZ}, 1.6 kb 5′ *Xba*I to *Pst*I; XhBg^{hsplacZ}, 1.1 kb *Xho*I to *Bgl*II; BgSc^{hsplacZ}, 690 bp *Bgl*II to *Sac*I.

Other genomic fragments were generated by PCR. Lower-case nucleotides indicate base changes introduced to produce 5′ *Hind*III or 3′ *Pst*I restriction enzyme sites for directional cloning into the *hsplacZ* vector. PCR products were sequenced to ensure fidelity of amplification. *5′XhBg*^{hsplacZ}, 360 bp, F, 5′-GGTGaaGCTtGAGGAACAGCAGGGGGTC-3′; R, 5′-GTCTCTGcagTCTTCAGGGAAAAGAGCCAC-3′. *3′XhBg*^{hsplacZ}, 720 bp, F, 5′-CAGGaaGCTtTTTTCCCTGAAGAAATCAGAG-3′; R, 5′-GGGTcTgCAGATCTGTGACCATCCTGGCGTC-3′. *3′XhSc*^{hsplacZ}, 1.4 kb, F, forward primer of *3′XhBg*^{hsplacZ}, R, 5′-GCTTGCTGCaGTGGAGCTCTCCAAAACG-3′.

Plasmid DNA was CsCl-purified; the insert isolated by low-melt agarose gel electrophoresis following *Sal*I digestion, and purified using Gelase (Epicentre Technologies). Pronuclei of one-cell embryos from B6D2 females were injected with 1–5 pl DNA (3 ng/μl) and embryos implanted into psuedopregnant ICR females (Hogan, 1994). Some F_0 founders were sacrificed at embryonic stages to analyze the *pdx1*-driven β-galactosidase (β-gal) expression pattern, although transgenic lines were generated for most constructs. Genotyping was by Southern blot analysis of DNA isolated from brain (embryos and neonates) or tail (adults) tissue. *pdx1*$^{+/-}$ mice were identified as in Offield *et al.* (1996). *pdx1*^{resc} animals were identified by probing *Pst*I and *Sal*I digested DNA with a 550-bp *Xba*I–*Eco*RI 3′ fragment from the *pdx-1* locus (Offield *et al.*, 1996). *hsplacZ* transgenics were identified by probing *Pvu*II-digested DNA with a 726-bp *Pvu*I fragment from *lacZ*. To determine transgene copy number, hybridization of 10 μg digested genomic DNA with the appropriate probe was compared with known copy number equivalents of the parent transgene plasmid run in parallel.

Characterization of Transgenics

The morning of vaginal plug was considered 0.5 d.p.c. Dissected internal organs were fixed, embedded, sectioned, and stained with X-gal to detect β-gal enzymatic activity as described (Wu *et al.*, 1997; Gannon *et al.*, 2000b). Immunoperoxidase staining for insulin, glucagon, somatostatin, and GLUT2 was performed as previously described (Gannon *et al.*, 2000b). Rabbit anti-amylase antibody (a gift from Ray MacDonald) was used at 1:500. Cholecystokinin (CCK), secretin (Peninsula Labs), and serotonin (Dako) were used at 1:2000, 1:1000, and 1:1000, respectively. Glucose tolerance tests (GTT) were performed as previously described (Gannon *et al.*, 2000b) on at least three animals of each genotype.

Western Blotting

Total pancreatic or kidney tissue from mice at 6 weeks of age was dissected free of other organs and homogenized on ice in 30 ml lysis buffer [10 mM Tris (pH 7.6), 10 mM NaCl, 2 mM $MgCl_2$, 1% Triton X-100] plus protease inhibitors (1 mM PMSF, 10 μM leupeptin, 10 μM pepstatin A). The nuclear fraction was pelleted by centrifugation (5 min; 500*g*), resuspended in 1.0 ml 1× sample buffer [62.5 mM Tris (pH 6.8), 2% SDS, 10% glycerol], and sonicated on ice for 7 s. Following centrifugation (8 min; 14,000 rpm), protein content of the supernatant was determined by comparison with a standard curve at 562 nm using BSA (BCA assay, Pierce). Total protein (75 μg) per lane was resolved on a 10% SDS polyacrylamide gel and transferred to nitrocellulose by using standard methods. Membranes were stained in 0.1% Ponceau S to

visualize protein and blocked overnight at 4°C in 3% BSA in 1× blot buffer (for details, see Backstrom *et al.*, 2000). The PDX1 antiserum was raised in rabbits against a fusion protein of GST with the N-terminal 75 amino acids of mouse PDX1 purified from bacteria. This antiserum detects PDX1 in tissues in an identical pattern to β-gal expression from $pdx1^{lacZKO}$ (data not shown), and specifically recognizes PDX1 on Western blots (Macfarlane *et al.*, 1999). Antibody incubations were in 3% BSA in blot buffer at room temperature. The blots were incubated with primary antibodies (rabbit anti-mouse PDX1, 1:2000; rabbit anti-β-gal, 1:250; Cappel) for 2 h, washed, and incubated with secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit, 1:1000; Dako) for 1 h. Blots were developed with BCIP and NBT (Pierce) and scanned with an Epson flat-bed scanner and Adobe Photoshop. Western blotting was performed by using tissue from two different groups of mice in four separate experiments with identical results. A representative blot is shown.

RESULTS

Identification of pdx1 Sequences Sufficient for Pancreatic Development

We have previously described a 14.5-kb region of DNA spanning the *pdx1* locus that was used to generate a targeting construct for *pdx1* gene inactivation (Offield *et al.*, 1996). This includes ~6.2 kb of sequence 5′ to the open reading frame, both exons and intron, and ~3 kb of 3′ sequence (Fig. 1). Since crucial regulatory elements can be located several kilobases 5′ (Goldhamer *et al.*, 1995) or 3′ to the coding region (Sasaki and Hogan, 1996), we used transgenesis to test if this 14.5-kb region contains regulatory sequences capable of recapitulating the normal temporal and spatial expression pattern of *pdx1*. We assessed the ability of this genomic fragment to direct expression to the endogenous *pdx1* domain in two ways: transgenic rescue of the *pdx1* homozygous null phenotype and expression analysis of a *lacZ* reporter transgene.

To attempt to rescue the null phenotype, the 14.5-kb genomic fragment was provided as a transgene ($pdx1^{resc}$) in the $pdx1^{lacZKO}$ null mutant background. $pdx1^{lacZKO}$ is a null allele containing *lacZ* within the endogenous *pdx1* locus (Offield *et al.*, 1996). $pdx1^{-/-}$ neonates usually die within 1 week of birth, lacking pancreatic tissue, but pancreatic development is essentially normal in $pdx1^{+/-}$ animals (Jonsson *et al.*, 1994; Offield *et al.*, 1996). Thus, *pdx1* null embryos that also carry the transgene should undergo normal foregut and pancreatic development. Two $pdx1^{resc}$ transgenic lines were generated (L1 and L2), each estimated by Southern blot analysis to harbor two copies of the transgene. Litters from crosses between *pdx1* heterozygotes ($pdx1^{+/-}$) and *pdx1* heterozygous mice carrying the $pdx1^{resc}$ transgene ($pdx1^{+/-}$;$pdx1^{resc}$) generated $pdx1^{-/-}$;$pdx1^{resc}$ pups at the expected frequency of 1 in 8 for both transgenic lines.

On analysis of neonatal $pdx1^{-/-}$;$pdx1^{resc}$ mice, L1 transgenics showed apparently complete rescue of posterior foregut development (Fig. 2D), and were outwardly indistinguishable from wild-type and heterozygous, nontransgenic littermates (data not shown). The L2 transgene partially rescued pancreas formation but failed to restore normal duodenal development (Figs. 2G and 2H). The partial rescue seen in L2 may be due to the effects of surrounding sequences at the site of integration or a failure of the transgene to integrate intact. With the exception of Fig. 3J, therefore, the data presented are from the L1 line.

As in newborn *pdx1* heterozygotes (Figs. 2B and 2C), β-gal expressing islet-like cell clusters were detected near the ducts in L1 $pdx1^{-/-}$;$pdx1^{resc}$ neonates (Figs. 2E and 2F), similar in size and shape to those present in wild-type and *pdx1* heterozygous mice. Exocrine and endocrine cells were both present in rescued pancreata (Fig. 3), and the normal islet architecture was observed: i.e., glucagon$^+$, somatostatin$^+$, and PP$^+$ cells surrounding a core of insulin$^+$ cells (Figs. 3F–3I). In addition, core islet cells showed normal expression of the β cell glucose transporter, GLUT2, at the membrane (data not shown).

Most L2 $pdx1^{-/-}$;$pdx1^{resc}$ pups died within 1 week of birth, similar to $pdx1^{-/-}$ animals, although some lived longer than 1 week, and two survived until weaning. Lifespan was correlated with the degree of pancreatic rescue. Pancreas formation in these animals varied between 10 and 30% of normal size (Figs. 2G and 2H), but differentiated pancreatic tissue could always be identified closely apposed to the rostral duodenum. Immunohistochemical analysis revealed the presence of acinar cells exhibiting normal apical/basal polarity around a central lumen (Fig. 3J), and endocrine cells clustered into small islet-like structures (data not shown). Currently, we believe that the pancreatic tissue restored in the L2 mice arose from the ventral bud, because a dorsal ductule, probably representing the developmentally arrested dorsal pancreatic bud that is seen in $pdx1^{-/-}$ animals (Offield *et al.*, 1996), was always observed (Figs. 2G and 2H). We conclude from these data that two independent $pdx1^{resc}$ transgenes can direct the formation of all pancreatic cell types in a *pdx1* null background.

pdx1 Transgene Rescues Pancreatic Function

L1 $pdx1^{-/-}$;$pdx1^{resc}$ mice were similar in body weight to their heterozygous and wild-type siblings at weaning and at 2 months of age, and survived to adulthood (Figs. 4A and 4B). Islet morphology and architecture were also normal in $pdx1^{-/-}$;$pdx1^{resc}$ adults (Fig. 4D). Thus, transgene-derived *pdx1* expression could support pancreatic organogenesis. To determine whether the *pdx1* transgene could restore glucose homeostasis, intraperitoneal glucose tolerance tests (GTT) were performed on $pdx1^{+/-}$, $pdx1^{+/-}$;$pdx1^{resc}$ and $pdx1^{-/-}$;$pdx1^{resc}$ mice to compare pancreatic function on a gross level. These studies were undertaken to assess whether this transgene, while sufficient for pancreatic development, nevertheless lacked regulatory elements required for aspects of *pdx1* regulation in mature β cells associated with their response to elevated blood glucose. A representative GTT is shown in Fig. 4E. The fasting blood glucose levels of all mice tested were in the normal

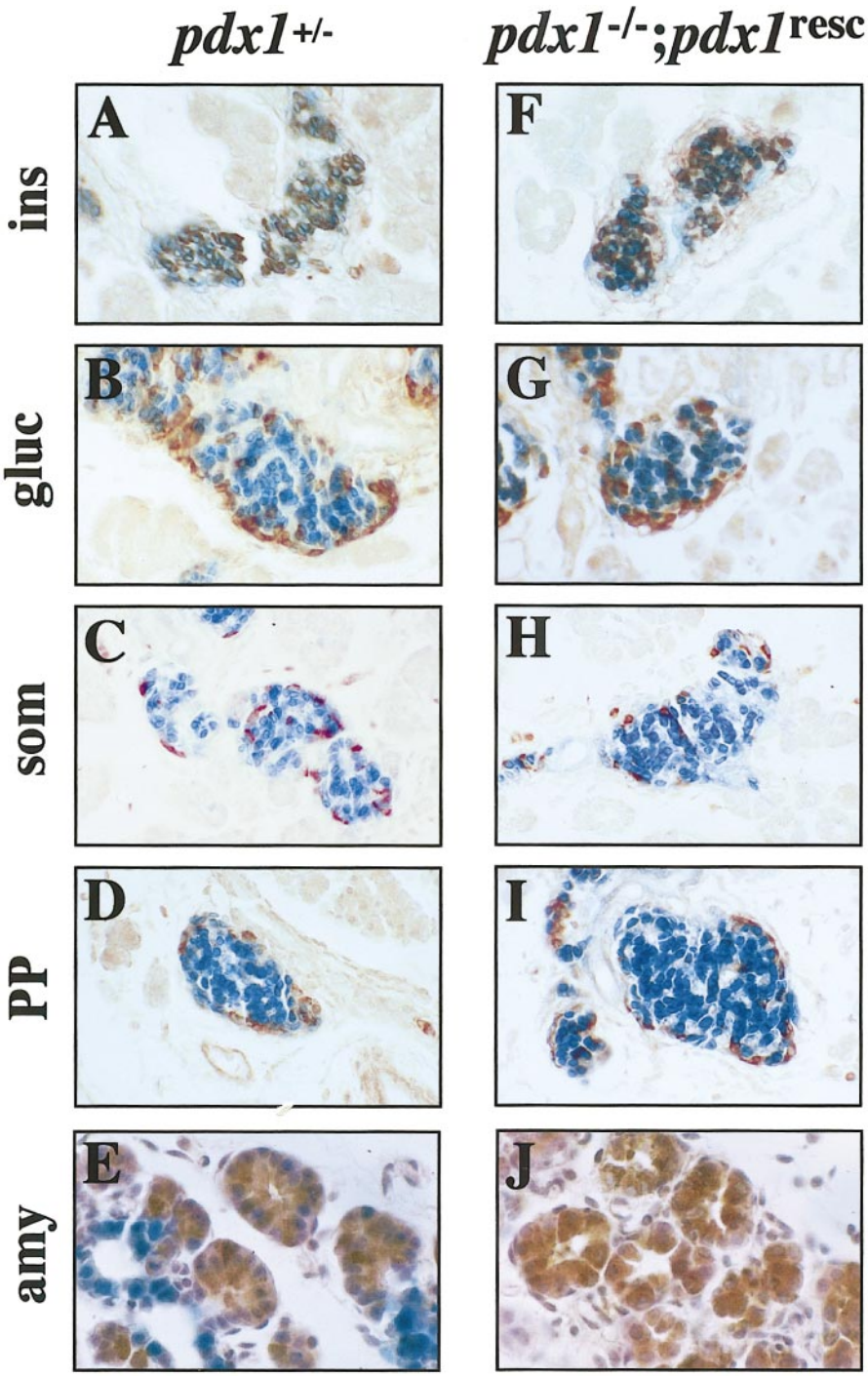

FIG. 3. All pancreatic cell types are rescued by the *pdx1*^{resc} transgene. Pancreatic sections from *pdx1*$^{+/-}$ (A–E) and *pdx1*$^{-/-}$;*pdx1*^{resc} (F–J) neonates. Blue color, *pdx1*-driven *lacZ* expression from the *pdx1*^{lacZKO} null allele. Immunolabeling of islet hormones (A–D, F–I) and exocrine cells (E, J) is shown in brown. ins, insulin; gluc, glucagon; som, somatostatin; PP, pancreatic polypeptide; amy, amylase. A-D, F-I: 100×; E, J: 200×.

range (below 150 mg/dl), and showed the expected increase within 15 min of glucose injection. At the next time point (30 min postinjection), blood glucose levels were beginning to return to normal for *pdx1*$^{+/-}$;*pdx1*^{resc} (n = 2) and *pdx1*$^{-/-}$;*pdx1*^{resc} (n = 3) mice, while *pdx1*$^{+/-}$ mice (n = 2; Fig. 4E) continued to display elevated blood

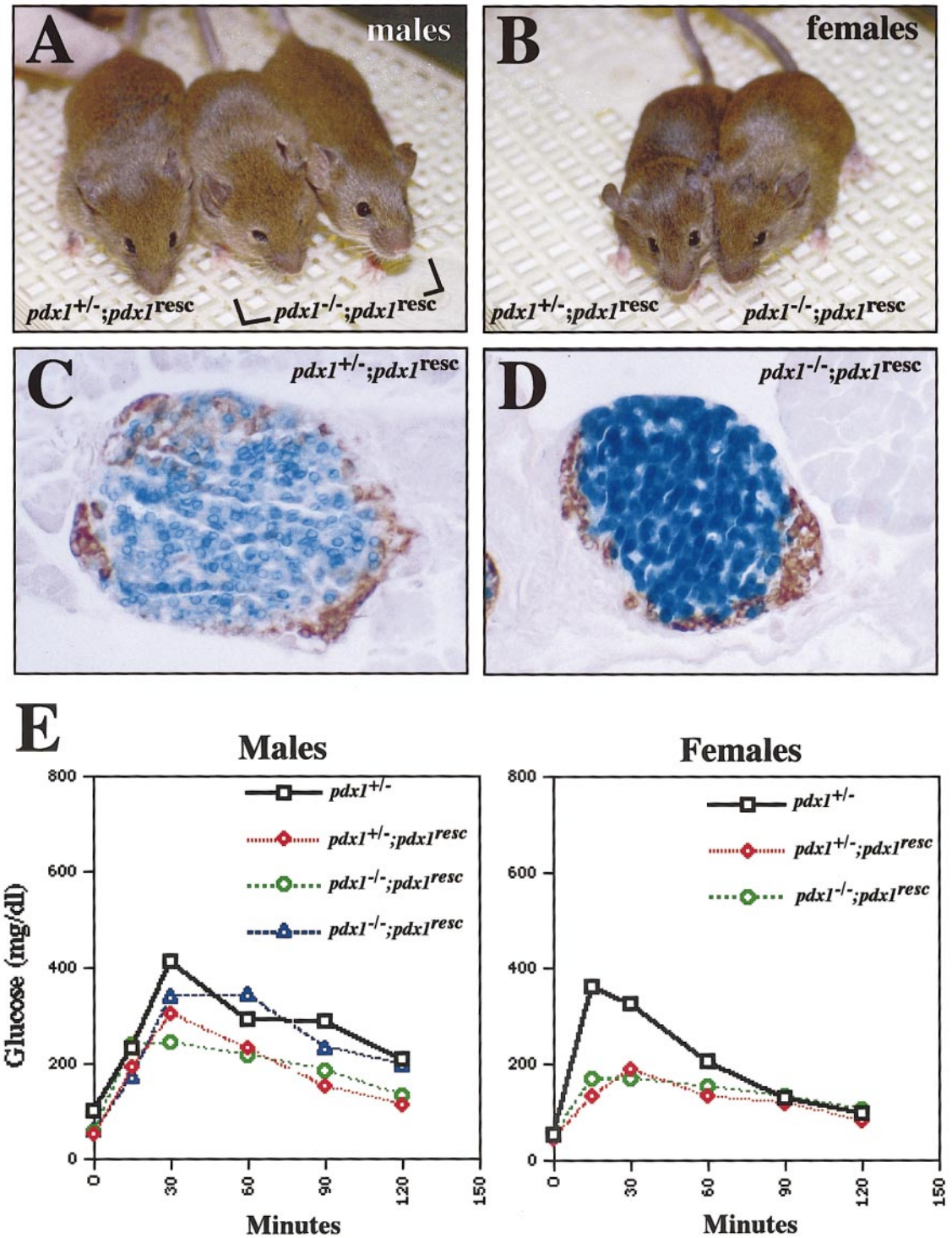

FIG. 4. The *pdx1*^{resc} transgene restores the normal physiological response to elevated blood glucose. Three-month-old *pdx1*$^{+/-}$;*pdx1*^{resc} and *pdx1*$^{-/-}$;*pdx1*^{resc} males (A) and females (B). Glucagon expression (brown) in islets from *pdx1*$^{+/-}$;*pdx1*^{resc} (C) and *pdx1*$^{-/-}$;*pdx1*^{resc} (D) adults is similar. The two copies of *pdx1*^{lacZKO} in *pdx1*$^{-/-}$;*pdx1*^{resc} islets result in darker X-gal staining (D). (E) Glucose tolerance test showing improved ability to clear glucose from the blood in animals carrying the *pdx1*^{resc} transgene as compared to *pdx1*$^{+/-}$ mice. C, D: 100×.

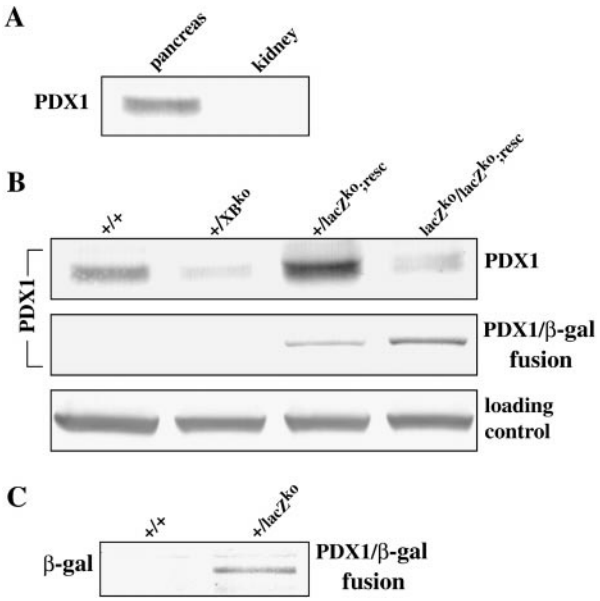

FIG. 5. Western blot analysis of PDX1 expression from the *pdx1*^{resc} transgene. The antibody used is indicated to the left of each panel. The PDX1 antiserum recognizes a specific band at 46 kDa in pancreas and not kidney, a nonexpressing tissue (A). PDX1 protein was detected at levels correlating with *pdx1* copy number (B, upper panel). In animals carrying the *pdx1*^{lacZKO} null allele, the PDX1 antiserum also recognizes a band at approximately 120 kDa, which represents the PDX1/β-gal fusion protein (B, middle panel). The identity of this protein was confirmed using an antibody against β-gal (C). Bottom panel in (B) shows nonspecific reactivity with a protein(s) at 100 kDa, demonstrating equal protein loading in all lanes. Genotypes are indicated above each lane. XB^{KO}, non-*lacZ*-containing *pdx1* null allele.

glucose. We conclude that the *pdx1*^{resc} transgene contains regulatory elements sufficient to direct appropriate expression of *pdx1* during pancreatic development and to support normal islet β cell function in adult mice, at least as measured by the GTT assay. Although our current sample size is small, glucose clearance during GTT was similar for both *pdx1*$^{+/-}$;*pdx1*^{resc} and *pdx1*$^{-/-}$;*pdx1*^{resc} mice, and improved compared to *pdx1* heterozygotes.

To determine the relative level of PDX1 produced from the *pdx1*^{resc} transgene, Western blot analysis was performed on pancreatic nuclear extracts by using a PDX1-specific antiserum. This antiserum specifically recognizes a protein at 46 kDa (Figs. 5A and 5B), the size expected for nuclear localized PDX1 (Macfarlane *et al.*, 1999), that was not detected in nonexpressing tissues. In animals carrying the *pdx1*^{lacZko} allele, a specific band at ~120 kDa reacted with PDX1 (Fig. 5B) and β-gal antibodies (Fig. 5C), identifying this band as the PDX1/β-gal fusion protein encoded by the *pdx1*^{lacZko} allele (Offield *et al.*, 1996). The amount of PDX1/β-gal fusion protein was increased in *pdx1*$^{-/-}$;*pdx1*^{resc} animals compared to *pdx1*$^{+/-}$; *pdx1*^{resc} mice. The level of PDX1 generated from the *pdx1*^{resc} transgene is intermediate between that from one and two functional copies of the endogenous *pdx1* gene (Fig. 5B). As described above, this level of protein is sufficient to support normal pancreatic development and function. The fact that *pdx1* heterozygotes express less PDX1 than wild-type mice demonstrates that expression from the remaining functional allele is not upregulated in the heterozygous condition. The lower levels of PDX1 in *pdx1*$^{+/-}$ pancreata may be linked to the impaired physiological response to elevated blood glucose observed in these mice (Dutta *et al.*, 1998; Ahlgren *et al.*, 1998; see also Fig. 4E).

Localization of pdx1 Regulatory Sequences to the 5′ Region

Regulatory sequences within the 14.5-kb fragment of *pdx1* DNA were analyzed *in vivo* using *lacZ* reporter transgenes (Fig. 1), with β-gal expression being assayed in embryos, neonates, and adults. We previously showed that the *XS*^{lacZ}*PS* transgene (Fig. 1), which contains 4.3 kb of upstream sequence, intron, and 3′ sequences, recapitulates the endogenous *pdx1* expression pattern during early embryogenesis: the dorsal and ventral pancreatic buds, the duodenum, and common bile duct (Wu *et al.*, 1997 and see Fig. 6A). In adults, the highest levels of *XS*^{lacZ}*PS* expression are in islet β cells, and lower levels are observed in subpopulations of acinar cells (Wu *et al.*, 1997). Thus, the additional 1.9 kb of upstream sequence, which were included in the rescue transgene construct, are not absolutely essential to direct appropriate reporter gene expression during embryogenesis or in the adult. These data demonstrate that the *XS*^{lacZ}*PS* transgene contains regulatory elements sufficient to establish normal *pdx1* expression during development and in mature β cells.

The *XS*^{lacZ}*PS* transgene was then further dissected to determine which regions were critical for directing expression to the endogenous *pdx1* domain. Transgenes lacking the 3′ region (*XS*^{lacZ}; data not shown), or lacking both 3′ and intronic sequences (*XSc*^{hsplacZ}; Fig. 6B) directed β-gal expression throughout the *pdx1* domain at 11.5 d.p.c., similar to *XS*^{lacZ}*PS*. In neonates, the *XSc*^{hsplacZ} transgene was expressed at the highest levels in endocrine cells, which at this stage of development are either found as single cells or coalesced into primitive islets (Fig. 6B). This agreement with endogenous *pdx1* expression was maintained in adults: highest levels of transgene expression in β cells and lower levels in acinar cells (data not shown). All five *XSc*^{hsplacZ} lines showed the same expression pattern. All transgenes that were expressed in a "*pdx1*-like" pattern showed a sharp anterior boundary of reporter gene activity within the antral stomach epithelium and a more diffuse posterior boundary within the duodenal epithelium, consistent with that seen with the endogenous *pdx1* gene.

Intronic and 3′ sequences may not contribute to *pdx1* expression. For example, a transgene driven by only the 3 kb of sequence 3′ to the poly(A) tail (*Sp*^{hsplacZ}) failed to demon-

strate any β-gal activity within the digestive tract at 11.5 d.p.c in transient transgenics. We conclude that the 4.3-kb *Xba*I–*Sac*I region (Fig. 1) contains sequences sufficient to direct *pdx1* expression in the correct spatiotemporal pattern. These results are supported by studies of Stoffers *et al.* (1999) who used a similar fragment of the *pdx1* promoter to drive *lacZ* expression in transgenic mice.

Dissection of the pdx1 Upstream Region

Subregions of the 4.3-kb region were then tested for their ability to drive specific patterns of expression, as summarized in Fig. 1. The general hypothesis was that this region contains separable fragments capable of directing *lacZ* expression to subsets of *pdx1*-expressing cells, such as the stomach or duodenal epithelium, the islet endocrine cells, or β cells. Initially, the 4.3-kb region was divided into two pieces, a 5′ 2.5-kb fragment (*Xba*I–*Xho*I) and a 3′ 1.8-kb fragment (*Xho*I–*Sac*I). A transgene driven by the latter segment, $XhSc^{hsplacZ}$, was expressed throughout the pancreatic buds and duodenum at 11.5 d.p.c. (Fig. 6C). This 1.8-kb region is the smallest fragment so far identified that can direct expression to essentially the entire posterior foregut domain during embryogenesis. We have not yet tested whether this transgene behaves similarly to the larger $XSc^{hsplacZ}$ and endogenous *pdx1* at later stages.

Identification of Endocrine-Specific Regulatory Sequences

In dissecting the 1.8-kb *XhSc* fragment, we identified a 1.1-kb region (*Xho*I–*Bgl*II) within the 5′ end that drove β-gal expression to clusters of cells throughout the developing pancreas, both at 14.5 d.p.c. and at postnatal day 1 (Figs. 6D and 7). These were found to be insulin-producing cells (Figs. 7A and 7D; Table 1). At 14.5 d.p.c., the few somatostatin$^+$ cells that were present also expressed β-gal, but at birth only 4% of somatostatin$^+$ cells were also β-gal$^+$ (Table 1 and Fig. 7). The transient expression of $XhBg^{hsplacZ}$ in somatostatin$^+$ cells may reflect their putative common origin with β cells (Alpert *et al.*, 1988; Teitelman *et al.*, 1993; Sosa-Pineda *et al.*, 1997). $XhBg^{hsplacZ}$ was not expressed in glucagon$^+$ cells during embryogenesis and was expressed in only 6% of glucagon$^+$ cells in neonates (Table 1). No expression from this construct was detected in any other tissue, including forming acini and duodenal epithelium, which normally express *pdx1* at 14.5 d.p.c. Overall, expression from $XhBg^{hsplacZ}$ is highly restricted to β cells. Somewhat surprisingly, $XhBg^{hsplacZ}$ expression in β cells was absent from adult transgenics of the same line, suggesting that this region contributes exclusively to *pdx1* expression during embryonic and early postnatal stages. At present, we cannot exclude the possibility that the lack of $XhBg^{hsplacZ}$ expression in adults does not reflect the normal activity of this region within the endogenous locus, but rather that the transgene becomes silenced with age. We note, however, the absence of such silencing in any of our other transgenic lines. A transgene containing the 680 bp located at the 3′ end of the *XhSc* fragment ($BgSc^{hsplacZ}$) was not expressed in any tissue examined from neonates and adults from two separate lines.

In addition to the β cell-specific regulatory elements located within the *XhSc* region, the 2.5-kb *Xba*I–*Xho*I (*XbXh*) fragment (Fig. 1), also showed activity in endocrine cell clusters at 14.5 d.p.c (Fig. 6E), with no expression in the developing acinar pancreas, antral stomach, or rostral duodenum. We next tried to localize more precisely the putative endocrine cell enhancers within these two upstream *pdx1* fragments. The *XbXh* region was divided into a 1.5-kb *Xba*I–*Pst*I 5′ fragment ($XP^{hsplacZ}$) and a 1-kb *Pst*I–*Bst*EI 3′ fragment ($PB^{hsplacZ}$). Of four lines carrying the $XP^{hsplacZ}$ transgene, only one showed expression in visceral tissue of any type, which was localized to scattered cells within the gastric epithelium, mainly in the antral stomach (data not shown). The identity of these β-gal$^+$ cells has not yet been determined. In any case, we conclude that the 1.5-kb *XP* fragment does not contain an independently acting islet-specific regulatory element. We previously reported on the partial characterization of the 1-kb *PB* region (Wu *et al.*, 1997; Gannon *et al.*, 2000b). Combined with this previous work, the present study demonstrates that this region is capable of directing islet-specific transgene expression at neonatal and adult stages in 10 independent lines. Here, we have also addressed the timing and cell-type specificity of activity from this islet enhancer. As early as 11.5 d.p.c., $PB^{hsplacZ}$ was expressed weakly in insulin- or glucagon-producing cells dispersed through the embryonic pancreatic epithelium (data not shown). By 16.5 d.p.c., robust expression was detected within differentiating endocrine cells (Figs. 6F and 7). *PB*-driven islet expression appeared more intense and in a greater proportion of islet cells overall than that driven by the *XhBg* fragment located immediately downstream. This was confirmed by quantitation following immunohistochemical analysis, which showed that $PB^{hsplacZ}$ directs expression to significant numbers of insulin$^+$, glucagon$^+$, and somatostatin$^+$ cells during embryonic development and at birth (Figs. 7G–7I; Table 1). This pattern is quite different from that of the $XhBg^{hsplacZ}$ transgene, for which we detected only transient expression in somatostatin$^+$ cells and did not detect expression in the α cell lineage during embryonic development (Table 1).

Dissection of β Cell-Specific Sequences

The $XhBg^{hsplacZ}$ transgene was highly β cell-enriched and active only during embryonic and perinatal stages. We therefore attempted to narrow down the *XhBg* β cell-specific activity by dividing it into two segments of 300 and 800 bp to generate the $5'XhBg^{hsplacZ}$ and $3'XhBg^{hsplacZ}$ transgenes, respectively (Figs. 1 and 8). We hypothesized that sequences that are highly conserved among vertebrate *pdx1* genes (see Discussion; Gerrish *et al.*, 2000a) were responsible for the β cell-specific activity of $XhBg^{hsplacZ}$. In both newborn and adult animals, in several independent lines,

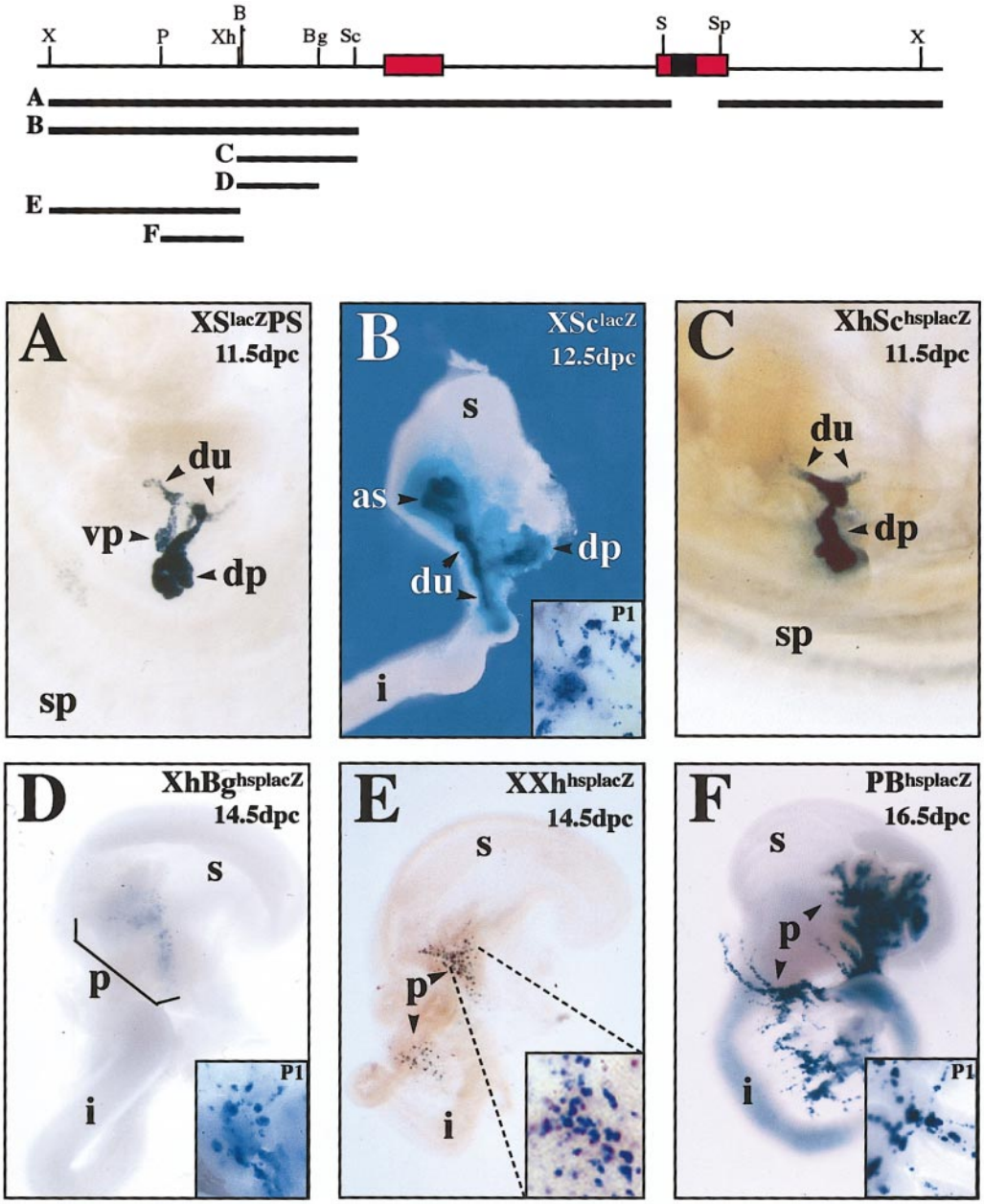

FIG. 6. Transgenic analysis of *pdx1* regulatory regions. The diagram shows the *pdx1* locus (thin line and red boxes) and location of genomic sequences used in transgene constructs (thick lines). Embryos or digestive organs were stained with X-gal in whole mount; transgene expression is indicated by blue staining. (A, C) Lateral views of posterior foregut staining in whole 11.5 d.p.c. embryos. (B) Dissected digestive organs from 12.5 d.p.c. embryo. (D, E) Dissected digestive organs from 14.5 d.p.c. embryos. (F) Dissected digestive organs from 16.5 d.p.c. embryo. dpc, days post coitum; du, duodenum; vp, ventral pancreas; dp, dorsal pancreas; sp, spinal cord; P1, postnatal day one; as, antral stomach; i, intestine; s, stomach; p, pancreas.

the $5'XhBg^{hsplacZ}$ and $3'XhBg^{hsplacZ}$ transgenes showed similar patterns of expression: occasional, scattered cells (one to several cells per total pancreas) in ductal epithelium, acini, and pancreatic blood vessels (Fig. 8). Expression was restricted to the pancreas, but not observed in islet endocrine cells in neonates or adults (Figs. 8C and 8F; data not shown). In 16.5-d.p.c. embryos, the $5'XhBg^{hsplacZ}$ transgene directed low levels of β-gal expression transiently to most insulin$^+$

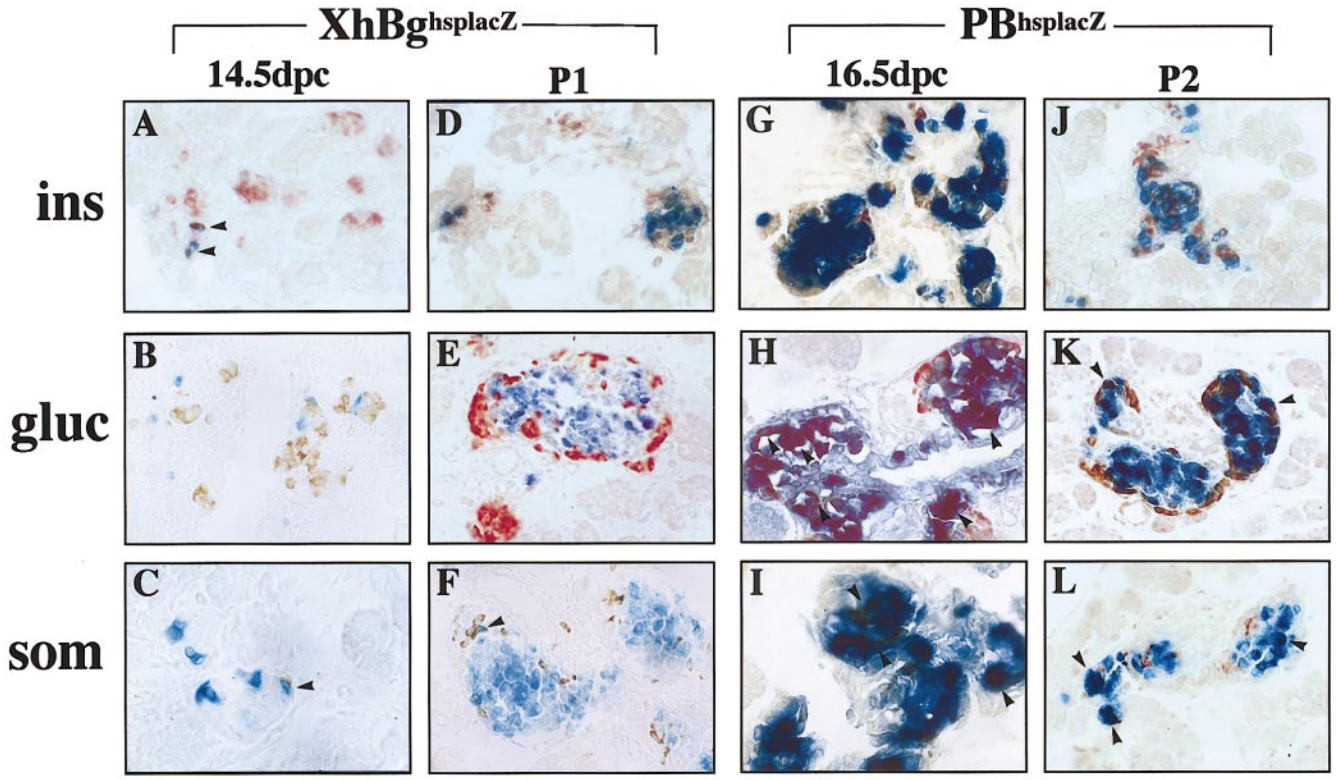

FIG. 7. Comparison of *XhBg*^{hsplacZ} (A–F) and *PB*^{hsplacZ} (G–L) expression in embryos and neonates. Brown color indicates immunolabelling for islet hormones. (A) In *XhBg*^{hsplacZ} transgenics, all β-gal$^+$ cells at 14.5 d.p.c. expressed insulin (arrowheads; see Table 1); not all insulin$^+$ cells are β-gal$^+$. (C) All somatostatin$^+$ cells at 14.5 d.p.c. were also β-gal$^+$ (arrowhead; see Table 1). (D) At P1, *XhBg*^{hsplacZ} is mainly expressed in insulin$^+$ cells, but also in a small percentage of somatostatin$^+$ cells (F, arrowhead). Expression is not detected in glucagon$^+$ cells at 14.5 d.p.c. (B). The great majority of glucagon$^+$ cells are β-gal$^-$ at P1 (E; see Table 1). *PB*^{hsplacZ} is expressed in the three islet cell types examined at 16.5 d.p.c. (G–I), but decreases in glucagon$^+$ cells after birth (K; see Table 1). Arrowheads mark some hormone$^+$ expressing cells. dpc, days post coitum; P, postnatal day; ins, insulin; gluc, glucagon; som, somatostatin. All panels are 100×.

and glucagon$^+$ cells in one line examined (Fig. 8 and data not shown). The *3′XhBg*^{hsplacZ} expression pattern in embryos was equivalent to that in neonates and adults (isolated β-gal$^+$ cells in acinar or ductal tissue, no endocrine cell expression). Thus, subdivision of the *XhBg* region results in a loss of β cell-specific activity (see Figs. 1 and 8).

DISCUSSION

We have pursued an *in vivo* characterization of *pdx1* regulatory sequences in the hope of focussing our future studies on core sequences through which critical upstream factors act on this gene. Such studies should provide valuable information on the position of *pdx1* in a regulatory network that is responsible for delineation of the posterior foregut domain within the endodermal germ layer, and the differentiation of specific organs such as the pancreas. In addition, identifying the transcription factors regulating pancreatic β cell specification, differentiation, and normal physiology is potentially medically relevant when one considers that dysfunction in these genes might contribute to the development of diabetes. For example, a full understanding of this network might facilitate *in vitro* production of islet endocrine cells, from stem or precursor cells, for transplantation therapies (Shapiro *et al.*, 2000).

Our transgenic analysis has identified adjacent, but separable enhancer-like elements that are likely to be major contributors to the normal expression of *pdx1* in pancreatic endocrine cells *in vivo*. Most likely, the activities of multiple factors acting in concert through these elements lead to robust *pdx1* expression within islets, and the maintenance of high levels of expression specifically in β cells. Consequently, we hypothesize that mutation of one of several of these DNA elements, or dysfunction of the corresponding *trans*-acting factor(s), might substantially abrogate *pdx1* expression and cause defects in organogenesis or mature organ function. A byproduct of our studies has been the generation of new tissue-specific promoter/

TABLE 1
Quantitation of Expression from *pdx-1* Islet-Specific Enhancers

e14.5		P1	
XhBg 1.1-kb β cell enhancer			
Insulin			
βgal$^+$ with Ins$^+$	100%	βgal$^+$ with Ins$^+$	88%
Ins$^+$ with βgal$^+$	5%	Ins$^+$ with βgal$^+$	48%
Glucagon			
βgal$^+$ with Gluc$^+$	0%	βgal$^+$ with Gluc$^+$	6%
Gluc$^+$ with βgal$^+$	0%	Gluc$^+$ with βgal$^+$	6%
Somatostatin			
βgal$^+$ with Som$^+$	6%	βgal$^+$ with Som$^+$	1%
Som$^+$ with βgal$^+$	100%	Som$^+$ with βgal$^+$	4%
e16.5		P1/P2	
PstBstE 1-kb islet enhancer			
Insulin			
βgal$^+$ with Ins$^+$	88%	βgal$^+$ with Ins$^+$	62%
Ins$^+$ with βgal$^+$	86%	Ins$^+$ with βgal$^+$	88%
Glucagon			
βgal$^+$ with Gluc$^+$	32%	βgal$^+$ with Gluc$^+$	13%
Gluc$^+$ with βgal$^+$	75%	Gluc$^+$ with βgal$^+$	22%
Somatostatin			
βgal$^+$ with Som$^+$	15%	βgal$^+$ with Som$^+$	10%
Som$^+$ with βgal$^+$	85%	Som$^+$ with βgal$^+$	57%

Note. At e14.5 and e16.5, there are likely to be some Ins$^+$/Som$^+$ cells accounting for the total number of βgal$^+$ cells adding up to more than 100%. By birth, islet lineages are segregated. *Pst*I–*Bst*EII directs expression to significant numbers of the three islet cell types examined during development and after birth. *Xho*I–*Bgl*II is activity is more restricted to β cells, but is transiently active in somatostatin cells during development, perhaps reflecting the common lineage of β and δ cells.

enhancer cassettes that can be used to assess the effect of misexpressing chosen factors within the pancreas and/or duodenum by transgenesis (Gannon *et al.*, 2000a,b).

Compact Organization of the pdx1 Gene

Many of the essential *pdx1* regulatory sequences are located close to the coding region. A genomic fragment spanning the coding region and 6.2 kb of sequence 5′ to the transcription start site can completely rescue the apancreatic *pdx1* null phenotype, and maintain glucose homeostasis during glucose challenge. A similar genomic region from rat *pdx1* containing 6.5 kb of upstream sequence is also able to rescue the *pdx1* homozygous null phenotype and the glucose intolerance seen in heterozygotes (Dutta *et al.*, 2001). Our Western blot analysis (Fig. 5) indicates that the steady-state level of PDX1 in *pdx1*$^{-/-}$;*pdx1*^{resc} (L1) pancreata is somewhere between that produced from one and two endogenous copies of *pdx1*, i.e., the levels in *pdx1*$^{+/-}$ and *pdx1*$^{+/+}$ mice, respectively. Because *pdx1*$^{+/-}$ mice show essentially normal pancreatic development but reduced glucose tolerance (this study; Dutta *et al.*, 1998; Ahlgren *et al.*, 1998), our observations are consistent with the hypothesis that the proper physiological function of β cells requires a specific threshold level of PDX1.

We propose, therefore, that these compactly organized regulatory sequences are sufficient for physiologically relevant levels of PDX1 expression, including any up- and downregulation of gene expression occurring during normal organogenesis, or in adult pancreatic tissue. A further delimitation of the key developmental regulatory sequences to a 4.3-kb (*XSc*) segment located immediately 5′ to exon 1 is supported by our finding, and that of Stoffers *et al.* (1999), that a reporter transgene driven by these sequences recapitulates the endogenous *pdx1* expression pattern. Future experiments will test whether this region is also sufficient to completely rescue pancreatic development and function in the *pdx1* null background, or if additional 5′ sequences contained within the rescue transgene are critical. Within the 4.3-kb region, we identified independent DNA modules that can direct reporter gene expression to *pdx1*-expressing pancreatic endocrine cells.

Separable Islet-Specific cis-Regulatory Regions in pdx1

Currently, much attention has been focussed on the role of *pdx1* in the mature pancreas as a critical transcriptional activator of insulin and other β cell-specific genes (see Introduction). Identifying the transcription factor binding motifs required for β cell expression of *pdx1* is a major goal of our combined *in vitro* and *in vivo* analyses. Although experiments in endocrine cell lines have quickly yielded information as to the location of potential β cell-specific regulatory elements (Wu *et al.*, 1997; Gerrish *et al.*, 2000a), a comprehensive *in vivo* analysis is likely to reveal how particular elements function during embryonic and postnatal pancreatic development, and in adult pancreata. Our *in vivo* dissection of *pdx1* has identified two different islet endocrine cell-specific *pdx1* regulatory activities located in adjacent ~1 kb fragments lying between −2.7 and −0.7 kb upstream of exon 1 (see Fig. 9). Although these sequences have been tested in the context of a heterologous minimal promoter (hsp68), and we have not shown that they are orientation/position-independent *cis*-regulatory sequences, we refer to them hereafter as enhancers for simplicity.

We have already partially described the *PB* endocrine cell enhancer (Wu *et al.*, 1997; Gannon *et al.*, 2000b). The extended analysis presented here demonstrates that this region directs expression to all islet endocrine cell types, and that the proportions of α and δ cells displaying reporter gene activity vary between embryonic, neonatal, and adult stages. The *PB* region can be considered a potent islet endocrine cell enhancer, since all expressing transgenic lines (10 of 14 total lines) showed strong endocrine-specific activity.

In contrast to the *PB* endocrine-specific enhancer, an almost exclusively β cell-specific activity was found in the

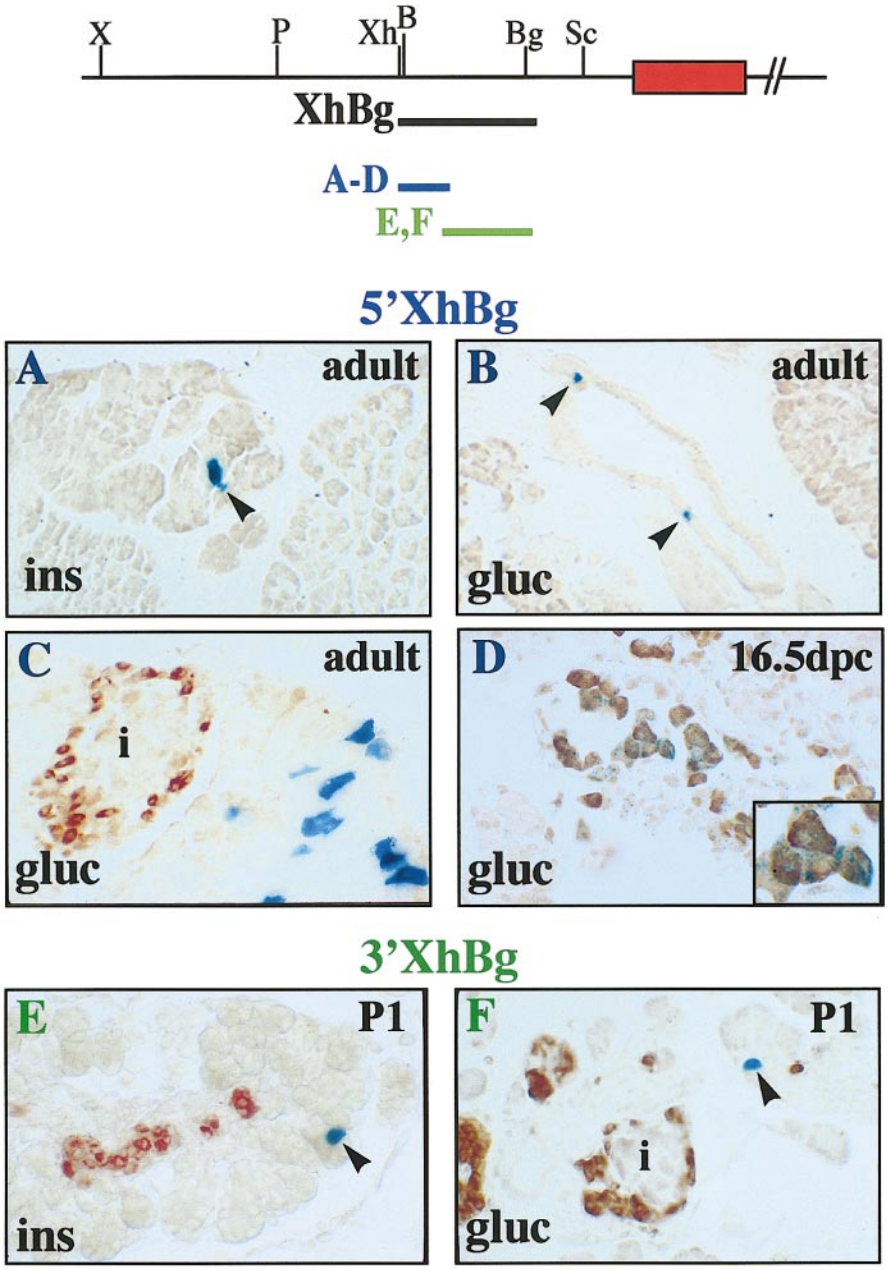

FIG. 8. Transgenic dissection of the *XB* β cell enhancer region. Diagram shows the *pdx1* locus (thin line, red box) and location of genomic sequences used in transgene constructs (thick lines). Constructs are color-coded with panels below. Blue color indicates transgene expression (arrowheads) in isloated acinar (A, C, E, F) and ductal (B) cells. At 16.5 d.p.c., low level β-gal expression is detected in hormone$^+$ cells in *5′XhBg* (Area III) transgenics (D). ins, insulin; gluc, glucagon; i, islets; dpc, days post coitum; P, postnatal day. A, C–H: 100×; B: 50×.

adjacent *XhBg* fragment. This activity was transient, and not present in adult pancreata from the same line. While the reason for silencing of this transgene is not clear, it suggests that the *XhBg* region plays a specific role in immature β cells, or perhaps provides critical developmental cues for the initiation of the mature β cell lineage and/or its precursors.

Two lines of evidence indicate that our transgenic findings are meaningful in relation to *pdx1* expression from the endogenous chromosomal context. First, the islet (*PB*) and β cell (*XhBg*) enhancers lie within regions of DNAse I hypersensitivity that were identified in β cell lines (Wu *et al.*, 1997; see Fig. 9). Second, the two enhancers overlap a localized segment of sequence that has a high degree of

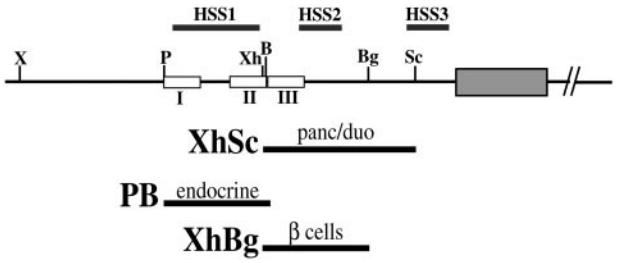

FIG. 9. Diagram showing the *pdx1* locus (thin line, dark gray box) with selected restriction enzyme sites, and genomic sequences that direct specific patterns of transgene expression (thick lines). The locations of the three previously identified DNase hypersensitive sites (HSS) are indicated above the locus (Wu *et al.,* 1997). The locations of the highly conserved Areas I, II, and III (Gerrish *et al.,* 2000a) are designated by the white boxes. Area I and Area II are separated by 300 bp of nonconserved sequences, while Area II and Area III sequences are continuous and were conceptually divided based on their location within the *PB* and *XB* regions, respectively.

evolutionary conservation among several vertebrates (Gerrish *et al.,* 2000a; see Fig. 9), potentially indicating the presence of core regions through which transcriptional control of *pdx1* is effected.

We have divided this region of conservation into three subdomains—Areas I, II, and III—that show between 78 and 89% sequence conservation in blocks of 200–300 nt between human, mouse, and chick (Gerrish *et al.,* 2000a); Fig. 9). Areas I and II are located within the *PB* islet enhancer, while Area III lies at the 5′ end of the *XhBg* β cell enhancer. Area I or Area II can independently direct β cell-specific expression in cell line transfection assays (Gerrish *et al.,* 2000a), suggesting that either the activity of Area I or Area II may explain the islet-selective enhancer activity of the *PB* region *in vivo*.

Database analysis of Areas I, II, and III has identified potential binding sites for endodermally expressed transcription factors, including recognition sequences for the products of other MODY genes (HNF1α, 1β, and HNF4α) (Gerrish, 2000b). This observation suggests significant inter-regulation between MODY genes. Accordingly, it is possible that a mutation in one MODY gene may result in diabetes, at least in part, by acting indirectly through other MODY genes such as *pdx1*. Support for this idea comes from the discovery that null mutations in HNF3α or HNF3β cause alterations in transcription of two MODY genes, HNF1α and HNF4α, in embryonic stem cells (Duncan *et al.,* 1998).

Comparison of in Vivo and in Vitro pdx1 Regulation

Valuable information on the potential regulatory activities of specific *pdx1* sequences has been obtained from transient transfection of immortalized cell lines *in vitro*. We tested the relevance of these sequences to *pdx1* regulation during organogenesis and in mature, differentiated cell types by placing them in the context of the whole animal. Frequently, the β cell-selective activities identified *in vitro* translated into active elements *in vivo*, but this was not always the case. For example, the *XhBg* fragment, containing the conserved Area III region, overlaps a DNAse I hypersensitive region (Fig. 9), suggesting a bona fide regulatory function that was confirmed in our transgenic analysis. Transfection assays in cultured β cell lines, however, did not detect cell type-selective regulatory activity in either *XhBg* (Wu *et al.,* 1997), or Area III (Gerrish *et al.,* 2000a). This discrepancy is, perhaps, not that surprising if one considers the possibility that immortalized cells have characteristics peculiar to a dedifferentiated or neoplastic state, and cannot be equated to any normal cell type *in vivo*. Nevertheless, because the endocrine cell-specific activity of *XhBg* and Area III *in vivo* is lost by the adult stage, it is tempting to speculate that the β cell lines are more closely related to mature β cells than embryonic cells.

Potential Redundancy in β Cell Enhancers

We have defined a genomic region that is apparently sufficient to completely rescue the *pdx1* null phenotype, although it remains possible that a subtle physiological function not tested here is not restored by this *pdx1* transgene. This analysis is also unable to rule out the possibility that enhancers located outside the −6.2 kb upstream region function, in the endogenous chromosomal context, to regulate specific aspects of *pdx1* expression.

Within the 6.2-kb upstream region, we found that the 3′-most 4.3 kb is capable of directing appropriate *pdx1* expression throughout development and in adults, and contains islet (*PB*) and β cell-specific (*XhBg*) enhancers. Results from studies of rat *pdx1* regulation in transfected β cell lines suggested that another endocrine-specific enhancer lies at approximately −6.0 to −6.5 kb (Sharma *et al.,* 1996, 1997). Most of this region was included in our rescue transgene, but was not present in the *lacZ* reporter transgenes, including those that could recapitulate the endogenous *pdx1* expression pattern, such as *XSc* and *XhSc* (Fig. 1).

Thus, there may be three independent regions of *pdx1* that display selective activity in endocrine cells *in vitro* and/or *in vivo*. It is possible that, *in vivo*, all three regions participate in directing *pdx1* expression to endocrine cells using different batteries of transcription factors. It remains to be seen, however, whether *in vivo* analysis of the −6.0 to −6.5 kb region in the mouse reveals a distinct function for this region in regulating *pdx1* expression. Establishing the requirement for each or any of these conserved endocrine-specific sequences in regulating this essential gene may require deletion or mutation of specific sites within the context of the endogenous *pdx1* locus.

Regional Specificity of pdx1 Expression in the Endoderm

The finding that *pdx1* is expressed within equivalent domains of the posterior foregut endoderm in all vertebrates so far examined implies a conserved role in the specification and differentiation of cells, tissues, and organs within this region. Our search for enhancer-like modules that direct *pdx1* expression to specific cell types or organs has so far, however, not found sequences that are able to direct expression exclusively to nonislet tissue. Because transgenes driven by the *XhSc* fragment showed activity throughout the *pdx1*-expression domain (Fig. 6C), and this fragment includes the β cell-specific *XhBg* enhancer, we hypothesized that separable gastric-, duodenal-, and/or acinar-specific activities would be contained within the *XhSc* region. The *3′XhBg* region, however, directed expression only to isolated acinar or ductal cells (Fig. 8). Furthermore, the $3'XhSc^{hsplacZ}$ transgene, which comprises the *3′XhBg* sequences and the 680 bp immediately downstream (*BgSc*), showed the same expression pattern as *3′XhBg* in embryos, neonates, and adults (data not shown). It is unlikely that this expression pattern is nonspecific and affected by the position of transgene insertion, given that all expressing lines, representing different insertion events, showed similar, albeit sparse, pancreas-restricted expression. Instead, we postulate that the *3′XhSc* region contains elements that are indeed important contributors to the overall pancreatic expression of *pdx1*, but that these elements were physically separated in the construction of the transgenes analyzed here, and act suboptimally in isolation. Similarly, expression of *pdx1* specifically in nonislet tissues might require combinations of DNA segments that are distributed throughout the *XhSc* region and therefore difficult to combine selectively in one transgene construct. There is precedent for cooperative interactions between nonadjacent enhancers: in the case of *HNF3β*, 1.5- and 0.4-kb fragments separated by 1.5 kb, drive neural tube floor plate expression only in combination (Sasaki and Hogan, 1996).

When beginning our experiments, we wondered whether negative regulatory interactions played a significant role in establishing the anterior and posterior expression boundaries of *pdx1* within the developing posterior foregut, and if removing such sequences would cause expansion of reporter gene expression outside the endogenous *pdx1* domain. However, none of the *pdx1* reporter transgenes that were expressed in the stomach, duodenum and pancreas (Fig. 1) showed altered anterior and posterior expression boundaries. It is possible that repressor sequences limiting the *pdx1* expression domain were present in all constructs tested, or that the spatiotemporal specificity of *pdx1* is established solely through positive-acting influences.

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