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Pancreas Development in Zebrafish: Early Dispersed Appearance of Endocrine Hormone Expressing Cells and Their Convergence to Form the Definitive Islet

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To begin to understand pancreas development and the control of endocrine lineage formation in zebrafish, we have examined the expression pattern of several genes shown to act in vertebrate pancreatic development: pdx-1, insulin (W. M. Milewski et al., 1998, Endocrinology 139, 1440–1449), glucagon, somatostatin (F. Argenton et al., 1999, Mech. Dev. 87, 217–221), islet-1 (Korzh et al., 1993, Development 118, 417–425), nkx2.2 (Barth and Wilson, 1995, Development 121, 1755–1768), and pax6.2 (Nornes et al., 1998, Mech. Dev. 77, 185–196). To determine the spatial relationship between the exocrine and the endocrine compartments, we have cloned the zebrafish trypsin gene, a digestive enzyme expressed in differentiated pancreatic exocrine cells. We found expression of all these genes in the developing pancreas throughout organogenesis. Endocrine cells first appear in a scattered fashion in two bilateral rows close to the midline during mid-somitogenesis and converge during late-somitogenesis to form a single islet dorsal to the nascent duodenum. We have examined development of the endocrine lineage in a number of previously described zebrafish mutations. Deletion of chordamesoderm in floating head (Xnot homolog) mutants reduces islet formation to small remnants, but does not delete the pancreas, indicating that notochord is involved in proper pancreas development, but not required for differentiation of pancreatic cell fates. In the absence of knypek gene function, which is involved in convergence movements, the bilateral endocrine primordia do not merge. Presence of trunk paraxial mesoderm also appears to be instrumental for convergence since the bilateral endocrine primordia do not merge in spadetail mutants. We discuss our findings on zebrafish pancreatogenesis in the light of evolution of the pancreas in chordates.

Key Words: **zebrafish; pancreas; endoderm; insulin.**

INTRODUCTION

The vertebrate pancreas exerts its exocrine and endocrine functions through two distinct tissue components. The exocrine component is composed of acinar glands that release digestive enzymes into the intestine, whereas the endocrine component is composed of four distinct cell types that secrete hormones into the bloodstream in order to control glucose homeostasis (reviewed by Slack, 1995). Dysfunction of endocrine β-cells may lead to diabetes mellitus, a widespread disease affecting more than 150 million people worldwide. Despite the availability of insulin as a treatment to temporarily restore glucostasis, diabetes is still incurable. Future regenerative or restorative therapies will depend on a better understanding of the development of the endocrine pancreas, and β-cells specifically. The recent emergence of the zebrafish as a highly suitable genetic model organism for vertebrate development (Driever *et al.*, 1996; Haffter *et al.*, 1996) prompted us to investigate its potential to study pancreas organogenesis.

In higher vertebrates, the Islets of Langerhans constitute the endocrine tissue compartment of the pancreas and are found embedded in the exocrine tissue. Every islet is composed of four major cell types: insulin-producing β-cells form the core while somatostatin-producing δ-cells,

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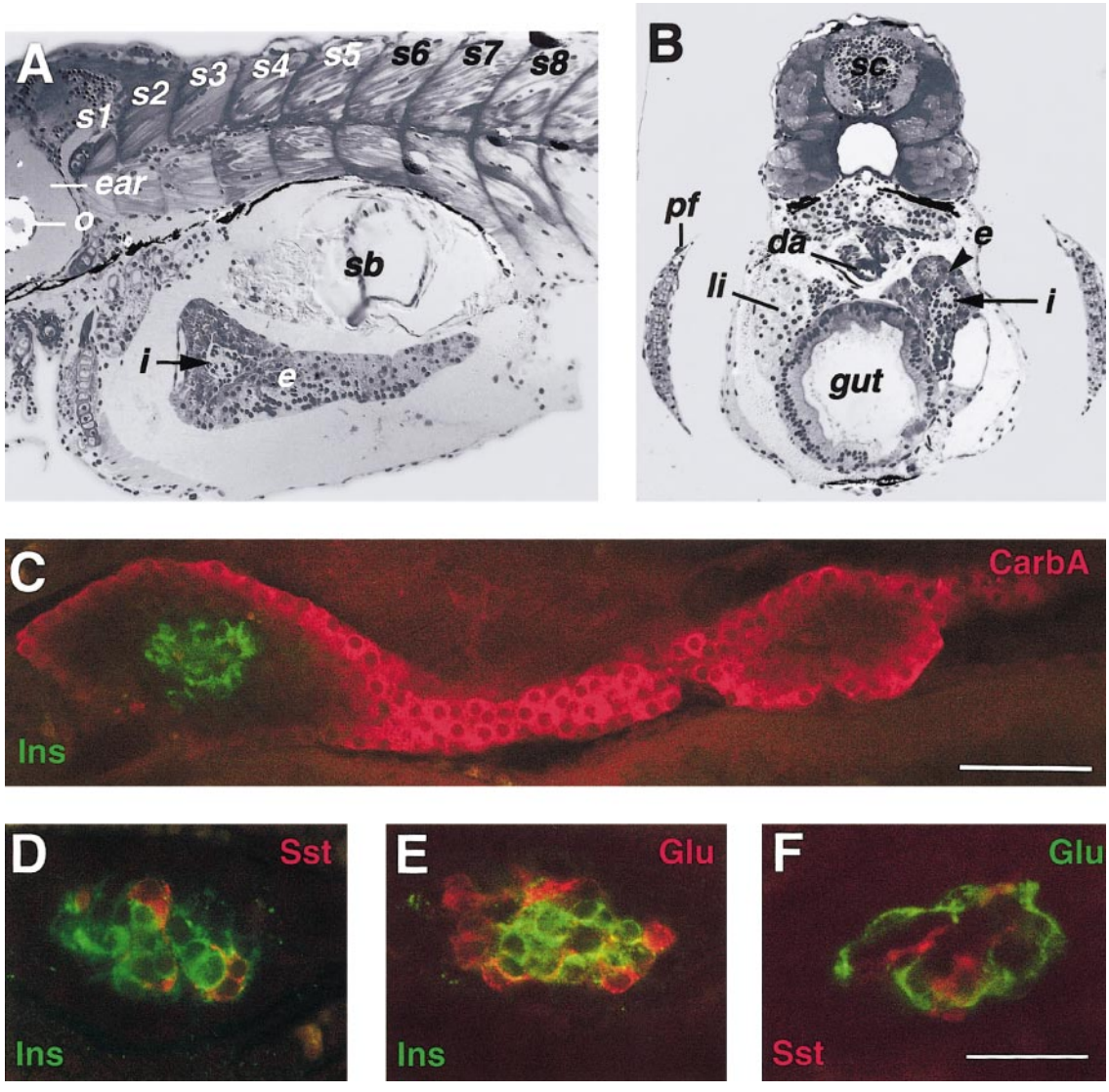

FIG. 1. Morphology of the exocrine and endocrine pancreas in the zebrafish larva. (A) Sagittal and (B) cross sections of 6-day-old larvae showing the overall organization of the endocrine (i, arrow) and exocrine pancreatic tissues (e in A, and e, arrowhead in B). (C–F) Confocal images of pancreatic islet tissue showing whole-mount immunofluorescence staining performed on (C) 5.5-day-old, (D) 4-days, and (E, F) 3-day-old embryos with a combination of (C) anti-carboxypeptidase A and anti-insulin; (D) anti-insulin and anti-somatostatin; (E) anti-insulin and anti-glucagon; (F) anti-somatostatin and anti-glucagon antisera. In A and C, anterior is oriented to the left. da, dorsal aorta; e; exocrine tissue; i, islet; li, liver; o, otolith; pf, pectoral fin; sb, swim bladder; sc, spinal cord; s1–8, somite 1 to 8. Scale bar, 50 μm in C and 25 μm in F (same magnification as D and E).

glucagon-producing α-cells, and pancreatic polypeptide secreting PP-cells are located at the periphery. Both exocrine and endocrine pancreatic tissues are of endodermal origin and develop from dorsal and ventral epithelial evaginations, arising from the gut tube caudal to the stomach (reviewed in Slack, 1995).

The analysis of pancreas development in various fishes has raised questions concerning the physiological significance of the close spatial association between the endocrine and exocrine pancreatic tissue. In agnathans, both tissues are separate from each other, while in gnathostomes, although always associated, their distribution is variable. In teleosts, highly evolved fishes, the endocrine islets may be found scattered among the exocrine tissue as in mammals, or in a few large islets named the Brockmann bodies or "principal islets." In most cases, teleost islet tissue contains all four types of hormone producing cells, whereas only one (β-cells), two (β- and δ-cells), or three (β-, δ-, and

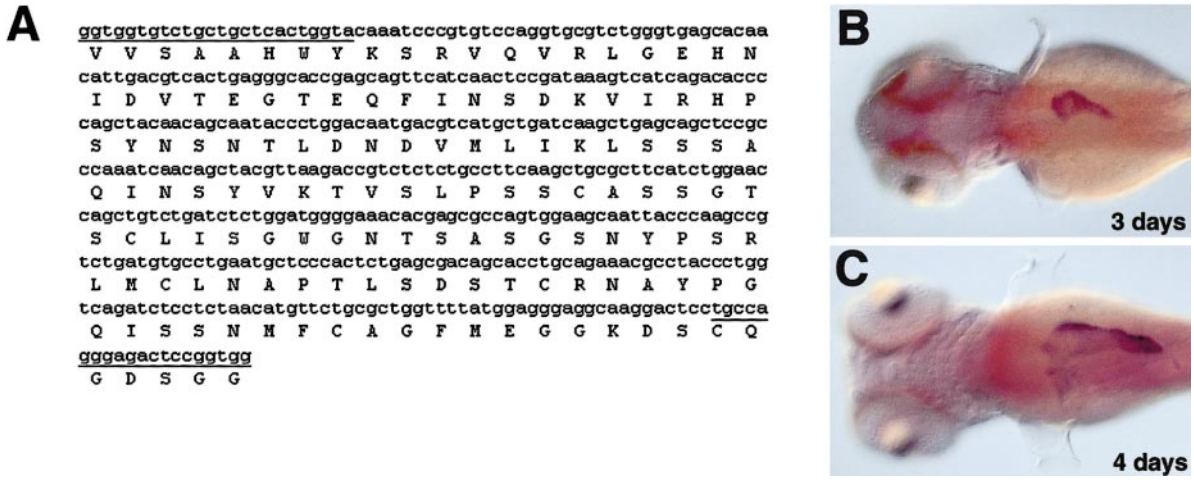

FIG. 2. Sequence and expression of the zebrafish trypsin homolog. (A) Nucleotide and deduced amino acid sequences of the partial cDNA fragment. PCR primers used are underlined. (B, C) Dorsal view of 3- and 4-day-old embryos after *in situ* hybridization for *trypsin*. Anterior is to the left.

PP-cells) cell types are usually found in the pancreas of more primitive fishes (Youson and Al-Mahrouki, 1999, and references therein).

Gene targeting studies in mice have revealed the functional importance of several transcription factors at various stages during endocrine pancreas development. These include the homeodomain protein Pdx-1 (Jonsson *et al*., 1994), the Lim homeodomain protein Isl-1 (Ahlgren *et al*., 1997), the paired box genes *pax4* and *pax6* (Sosa-Pineda *et al*., 1997; St-Onge *et al*., 1997), the bHLH gene *beta2/neuroD* (Malecki *et al*., 1999; Naya *et al*., 1997), the NK2 homeobox gene *nkx2.2* (Sussel *et al*., 1998), the homeobox gene *hb9* (Harrison *et al*., 1999; Li *et al*., 1999), and the cut homeodomain *hnf6* (Jacquemin *et al*., 2000). Numerous signaling molecules also act during pancreatic endocrine development, including Sonic hedgehog (Shh) (Apelqvist *et al*., 1997; Hebrok *et al*., 1998; Kim *et al*., 1997a; Kim and Melton, 1998), TGF-βs (Miralles *et al*., 1998a,b), FGFs (Le Bras *et al*., 1998; Miralles *et al*., 1999), and the Notch/Delta lateral inhibition system (Apelqvist *et al*., 1999; Gradwohl *et al*., 2000; Jensen *et al*., 2000a,b).

Beyond two pioneer studies (Pack *et al*., 1996; Milewski *et al*., 1998), zebrafish pancreatic development has received little attention. Study of both the cellular and molecular mechanisms that regulate early endoderm development and those that control organogenesis of the gut and its associated organs has only recently begun (Alexander *et al*., 1999; Alexander and Stainier, 1999; Reiter *et al*., 1999; Warga and Nüsslein-Volhard, 1999; Kikuchi *et al*., 2000). To begin to understand pancreas development and the control of endocrine lineage formation in zebrafish, we have assayed the expression of the following genes in the developing zebrafish pancreas: *pdx-1*, *insulin* (Milewski *et al*., 1998), *glucagon*, *somatostatin* (Argenton *et al*., 1999), *islet-1* (Korzh *et al*., 1993), *nkx2.2* (Barth and Wilson, 1995), and *pax6.2* (Nornes *et al*., 1998). We found expression of all these genes in the developing pancreatic primordium throughout organogenesis, similar to higher vertebrates. In addition we have cloned the zebrafish *trypsin* gene, coding for the homolog of the digestive enzyme Trypsin, which can serve as a marker for differentiated pancreatic exocrine cells. We further show evidences that the endocrine hormone expressing cells first appear scattered within the pancreatic primordium, possibly reflecting the involvement of a lateral inhibition system. They converge in a central position to form a single cluster, presumably representing the future islet of Langerhans. The analysis of zebrafish mutations affecting endoderm, axial, or paraxial mesoderm, or convergence movements during gastrulation suggests the contribution of defined genetic pathways to zebrafish pancreas development.

MATERIALS AND METHODS

Zebrafish Maintenance and Mutant Lines

Zebrafish (*Danio rerio*) were raised and cared for according to standard protocols (Westerfield, 1995). Wild-type embryos from the "AB" strain were used and staged according to Kimmel *et al.* (1995). Homozygous mutant embryos were obtained from matings between fish heterozygous for the *floating head*, *flh*^{tk241} (Odenthal *et al*., 1996); *knypek*, *kny*^{m818} (Solnica-Krezel *et al*., 1996; Marlow *et al*., 1998); *miles apart*, *mil*^{m93} (Stainier *et al*., 1996); *no tail*, *ntl*^{b160} (Halpern *et al*., 1993); *one-eyed pinhead*, *oep*^{m134} (Schier *et al*., 1996); *schmalspur*, *sur*^{m768} (Solnica-Krezel *et al*., 1996); *silberblick*, *slb*^{tz216} (Heisenberg *et al*., 1996); and *spadetail*, *spt*^{b104} (Kimmel *et al*., 1989) alleles, respectively. MZoep embryos were obtained as described in Gritsman *et al.* (1999).

Histology and Immunohistochemistry

Methacrylate sections were prepared using the JB-4 plus resin (Polyscience Inc.) according to the manufacturer's protocol. The 4-μm serial sections were stained with methylene blue–azure II. Whole-mount immunohistochemistry was performed as described previously (Argenton *et al*., 1999) using rabbit anti-bovine carboxypeptidase A (Chemicon), guinea pig anti-porcine insulin (Linco Inc.), rabbit anti-somatostatin-14 (Biotrend), mouse anti-porcine glucagon (Sigma), rabbit anti-porcine glucagon (Biotrend), and rabbit anti-human pancreatic polypeptide (Scytek) antisera. Appropriate combinations of Alexa Fluor™ 488 goat anti-rabbit IgG conjugate, Alexa Fluor™ 546 goat anti-rabbit IgG conjugate, Alexa Fluor™ 488 goat anti-guinea pig IgG conjugate, Alexa Fluor™ 546 goat anti-guinea pig IgG conjugate, and Alexa Fluor™ 488 goat anti-mouse IgG conjugate (Molecular Probes) were used as secondary antisera. Confocal images were taken on a Zeiss LSM 510 microscope.

Whole-Mount in Situ Hybridization

Whole-mount *in situ* mRNA hybridizations were performed as described by Hauptmann and Gerster (1994). The following probes were used: *pdx-1*, *insulin* (Milewski *et al*., 1998), *glucagon*, *somatostatin* (Argenton *et al*., 1999), *islet-1* (Korzh *et al*., 1993), *nkx2.2* (Barth and Wilson, 1995), *pax6.2* (Nornes *et al*., 1998), *FoxA2/axial/fkd1* (Odenthal and Nüsslein-Volhard, 1998; Strähle *et al*., 1993), and *FoxA3/fkd2/Zffkh1* (Dirksen and Jamrich, 1995; Odenthal and Nüsslein-Volhard, 1998). For Fig. 3, panel O, two images were taken at the same focal plane, using a DIC filter transmitted light for the first one (black staining, *pdx-1*) and epifluorescence with a Rhodamine filter for the second one (fast-red fluorescent staining, *insulin*). The two pictures were then superimposed and processed using the OpenLab software (Improvision).

RESULTS

General Morphology of the Larval Zebrafish Pancreas

The pancreas of the zebrafish larva is composed of one single islet embedded in exocrine tissue. At 6 days post fertilization (dpf; Figs. 1A and 1B), the pancreas is located asymmetrically on the right side of the body. This asymmetry is established by 48 h post fertilization (hpf, data not shown). Differentiated exocrine cells, identified by immunoreactivity to Carboxypeptidase A, are first detected in the exocrine component of the pancreas at around 72 hpf (data not shown). By 5.5 dpf, exocrine tissue extends from the first to the sixth or seventh somite. Within this, the endocrine islet is located in the anterior-most portion, at the level of the third and fourth somite (Figs. 1A and 1C). As proposed for humans (reviewed in Slack, 1995) and chick (Kim *et al*., 1997b), the zebrafish pancreas can be divided into a "head" region followed by a "neck" region and, finally a "tail" region (Fig. 1C). While the endocrine islets are distributed throughout the pancreas of human and chick, in zebrafish, the head region contains the single islet of Langerhans. The zebrafish larval endocrine islet has a core composed of insulin- and somatostatin-immunoreactive cells (Fig. 1D) surrounded by glucagon- and pancreatic polypeptide-immunoreactive cells at the periphery (Figs. 1E, 1F, and data not shown). This organization is similar to mammalian islets, but differs in that somatostatin immunoreactive cells tend to be located at the periphery in mammals.

Trypsin Expression Marks the Exocrine Pancreas

Since so far no robust marker has been available to visualize the zebrafish exocrine pancreas at the RNA expression level, we cloned the zebrafish homolog of the *trypsin* gene. To design conserved oligonucleotides suitable for the amplification of zebrafish *trypsin* cDNA, the sequences of rat, chicken, *Xenopus*, sole, and salmon were aligned. Four strictly conserved regions were used to prepare two 5′ and two 3′ primers to be used in a set of nested PCRs. To amplify the core of zebrafish *trypsin* cDNA, we used mRNA isolated from pancreas and reverse-transcribed with AMV-reverse transcriptase. A 440-bp fragment obtained from the second (nested) PCR was cloned and sequenced. The translated cDNA sequence encodes an open reading frame with homology to Trypsin sequences in other vertebrate species. The overall homology at the amino acid level ranges from 76.6% for the rat sequence up to 81.4% for the *Xenopus* sequence. Expression analysis by whole-mount *in situ* hybridization of 24 and 48 (not shown) as well as 72 and 96 hpf old embryos (Fig. 2) revealed that *trypsin* expressing exocrine cells can first be detected between 48 and 72 hpf. At this time, the zebrafish pancreas is already located on the right side of the embryo, dorsal to the gut tube.

Zebrafish Pancreatic Primordia First Appear in Two Stripes Adjacent to the Midline

In mammals, birds, reptiles, and amphibians, the pancreas forms from dorsal and ventral evaginations of the posterior foregut that later join together (Pictet and Rutter, 1972). Soon after these two buds have formed, endodermal cells committed toward pancreatic or duodenal cell fates express the homeodomain transcription factor *pdx-1* (Guz *et al*., 1995; Ahlgren *et al*., 1996; Offield *et al*., 1996). The zebrafish homolog of *pdx-1* was cloned recently (Milewski *et al*., 1998) but little was revealed about its expression early in development. We therefore performed a detailed analysis of *pdx-1* expression to precisely determine the time of its initiation in the developing pancreas. *pdx-1* mRNA expression is first detected at the 10-somite stage in two bilateral rows of cells adjacent to the midline, located immediately above the large syncytial yolk cell at the level of the first two or three somites (Fig. 3A). Starting at the 14-somite stage, the rows of cells approach each other and eventually fuse to form a single field of *pdx-1* expressing cells (Fig. 3E). The convergence of the two bilateral stripes appears to be initiated at their posterior end. From the 18-somite stage, the field of *pdx-1* expressing cells is con-

tinuous and broadens as the number of *pdx-1* expressing cells increases (Fig. 3G). As development proceeds, the anterioposterior extent of the field of *pdx*-1 expressing cells appears to shorten, and a group of *pdx-1* expressing cells condenses at the posterior and dorsal margin of the original *pdx-1* expression domain (compare Figs. 3I, 3K, and 3M). Thus, in contrast to mammals and birds, zebrafish pancreatic primordia do not appear as dorsal and ventral protrusions of the gut tube but rather at left and right positions immediately adjacent to the midline within a field of endodermal cells.

Insulin Is the First Hormone Expressed within the Pancreatic Anlage

We investigated the initiation of pancreatic hormone expression in zebrafish, using the zebrafish *insulin* (Milewski *et al*., 1998), *glucagon*, and *somatostatin* (Argenton *et al*., 1999) cDNA probes. At the 10-somite stage, while *pdx-1* is already expressed in bilateral domains (Fig. 3A), *insulin* expression cannot be detected (Fig. 3B). At the 12-somite stage, while the *pdx-1* expression domains remain well separated (Fig. 3C), the first *insulin* expressing cells appear, scattered within the domains of *pdx-1* expression (Fig. 3D). As mentioned above, the fusion of the two *pdx-1* stripes begins at the 14-somite stage, starting from the posterior margin (Fig. 3E). A similar process is observed for the *insulin* expressing cells (Fig. 3F), which have already increased in number. At the 18-somite stage, the developing pancreas, represented by the *pdx-1* and *insulin* expression domains, is located in the midline, immediately dorsal to the yolk (Figs. 3G and 3H). By the 20-somite stage, the *insulin* positive cells have formed a one-cell-thick layer immediately above and adjacent to the yolk (Fig. 3J). From then on, the cells aggregate posteriorly into a cluster, presumably representing the prospective endocrine islet (Fig. 3L). By 24 hpf, the presumptive islet has formed and is located at the midline, dorsal to the yolk (Fig. 3N). The comparison with *pdx-1* expression at the 20-somite stage (Fig. 3I), 24-somite stage (Fig. 3K), and 24 hpf (Fig. 3M) suggests that a portion of the *pdx-1* expressing cells aggregates posteriorly into a compact cluster. To test whether this posterior cluster of *pdx-1* expressing cells corresponds to the forming endocrine islet, we performed double-labeling *in situ* hybridization using digoxigenin-labeled *pdx-1* and fluorescein-labeled *insulin* antisense riboprobes (Fig. 3O). As expected, *insulin* expression (red) is restricted to dorsal posterior *pdx-1* positive cells (black, see experimental procedures for details). The layer of *pdx-1* positive cells located immediately dorsal to the yolk is devoid of *insulin* staining. This indicates that the formation of the Islet of Langerhans in zebrafish begins by 24 hpf.

The expression of *somatostatin* and *glucagon* mRNA in the developing zebrafish was reported previously (Argenton *et al*., 1999). However, since only three different stages were examined, we decided to investigate more time points in order to define the initiation of their transcription. We detected *somatostatin* expression at the 16-somite stage (Fig. 4A), while *glucagon* is only expressed later, at the 24-somite stage (Fig. 4D). By 24 hpf, both hormones are expressed in the presumptive islet (Figs. 4E and 4F).

Early Commitment to Endocrine Cell Fate in the Developing Zebrafish Pancreas

When expression of the zebrafish homolog of *nkx2.2* was characterized, Barth and Wilson (1995) noticed a "patch of *nkx2.2* positive cells ventral to the hypocord at the hindbrain/spinal cord boundary." Here we show that *nkx2.2* expressing cells can be detected as early as the 10-somite stage within the pancreatic primordium (Fig. 5A and A′) and is maintained during development (Fig. 5B at 24 hpf). Mice lacking Nkx2.2 do not develop properly differentiated α-, β-, and PP-cells and die of diabetes (Sussel *et al*., 1998). The conservation of pancreas specific expression of the zebrafish *nkx2.2* homolog indicates that the function of Nkx2.2 observed in mice may also have been conserved in zebrafish.

Early in pancreatic endocrine cell differentiation, postmitotic islet cells in mice initiate expression of the LIM homeodomain protein Isl1 (Ahlgren *et al*., 1997). We detected *islet-1* expression in the zebrafish pancreatic anlage as early as the 12-somite stage (Fig. 5C and C′) that continued past 24 hpf (Fig. 5D). These data suggest that commitment to endocrine cell fate, and the differentiation of at least one part of the endocrine cells is initiated relatively early in zebrafish, soon after the onset of *pdx-1* expression.

Targeted mutation of the *pax6* gene in mouse causes absence of glucagon producing cells, suggesting an important role of this protein in islets morphogenesis and in the differentiation of α-cells (Sander *et al*., 1997; St-Onge *et al*., 1997). It has recently been shown that the zebrafish genome contains two genes homologous to *pax6* (Nornes *et al*., 1998). We found initiation of *pax6.2* expression in the developing zebrafish pancreas at the 12-somite stage (Fig. 5E and E′). Similar to other pancreatic markers at this stage, the *pax6.2* expressing cells form a one-cell-thick layer immediately above the yolk, and expression is maintained throughout somitogenesis, still clearly visible by 24 hpf (Fig. 5F). We did not detect expression of the zebrafish *pax6.1* homolog in the pancreatic primordia at any stage.

Pancreatic Defects in Zebrafish Mutants Affecting Midline Specification

As experimental embryological studies in chick revealed an important inductive role for the notochord in pancreas development (Kim *et al*., 1997a), we investigated the influence on pancreas formation of various

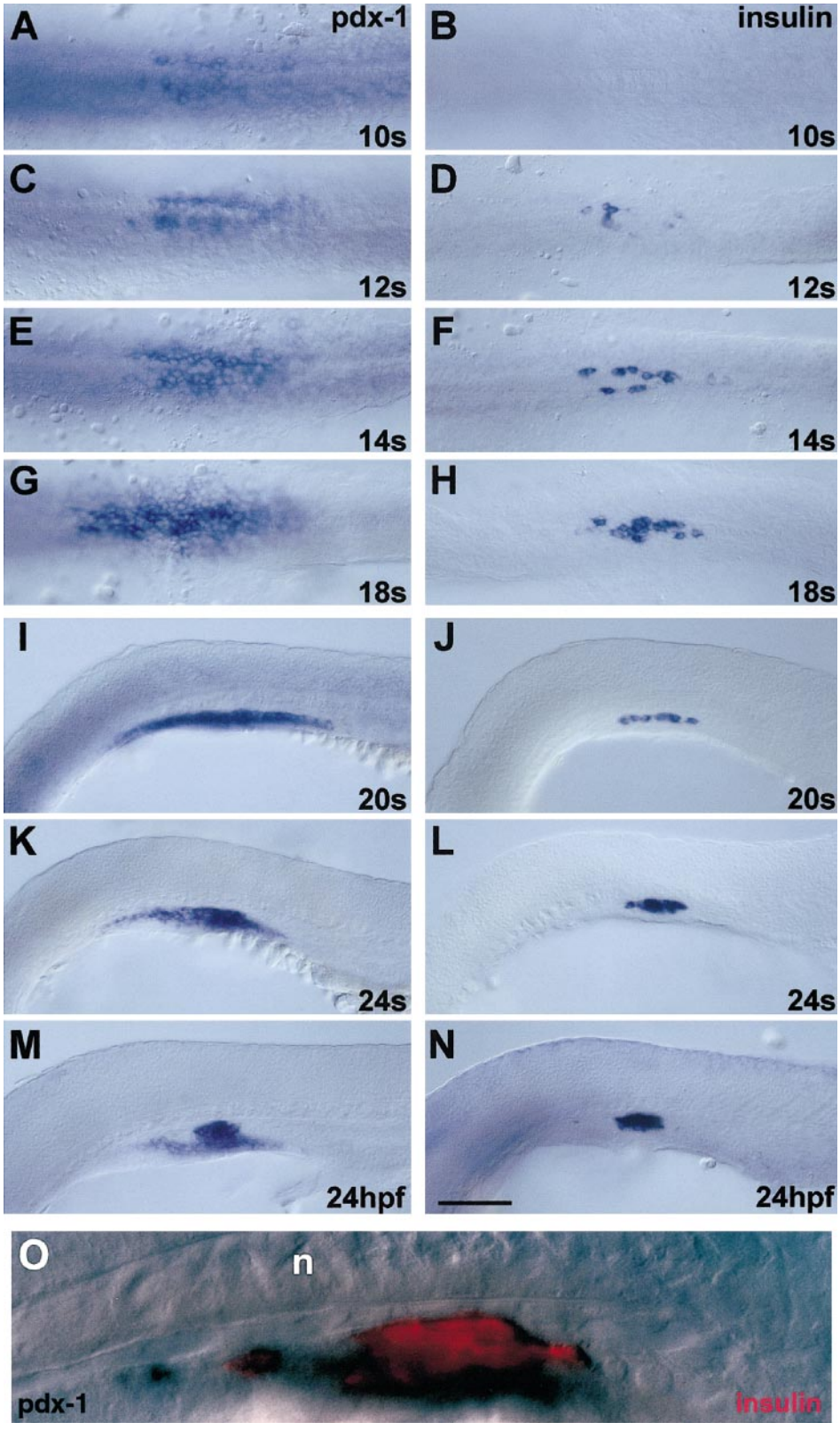

FIG. 3. Initiation of *pdx-1* and *insulin* expression in the pancreatic primordium. (A–H) Dorsal and (I–N) lateral views of wild-type zebrafish embryos after *in situ* hybridization with *pdx-1* (left panels) or *insulin* (right panels) antisense riboprobe at the (A, B) 10-somite stage; (C, D) 12-somite stage; (E, F) 14-somite stage; (G, H) 18-somite stage; (I, J) 20-somite stage; (K, L) 24-somite stage; and (M, N) 24 hpf. (O) 24 hpf embryo after *in situ* hybridization with both *pdx-1* and *insulin*; n, notochord. The yolk was manually removed; anterior is to the left. Scale bar, 100 μm (N).

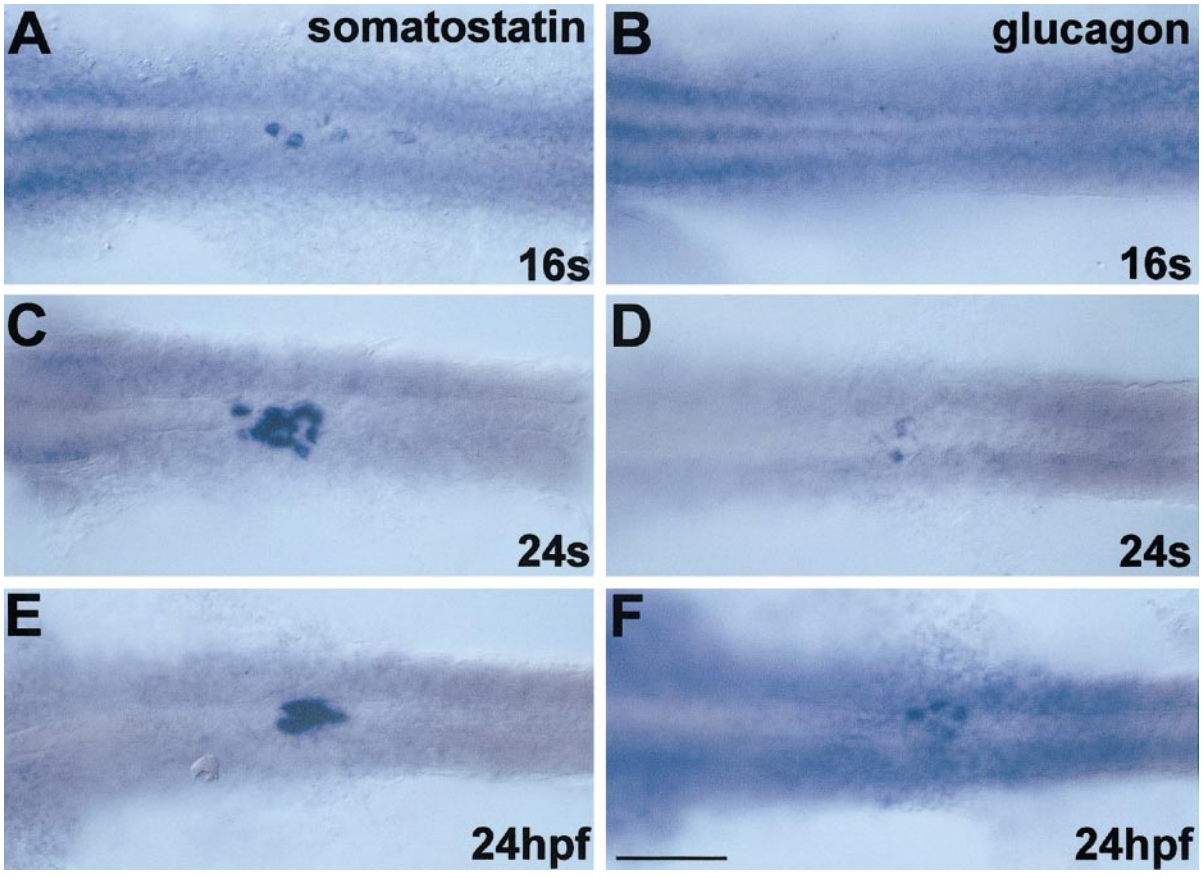

FIG. 4. Expression of *somatostatin* and *glucagon* in the developing zebrafish pancreas. Dorsal views of wild-type zebrafish embryos after *in situ* hybridization with *somatostatin* (left panels) or *glucagon* (right panels) antisense riboprobes at the (A, B) 16-somite stage; (C, D) 24-somite stage; and (E, F) 24 hpf. The yolk was manually removed; anterior is to the left. Scale bar, 100 μm.

mutations affecting zebrafish axial and nonaxial mesoderm. The *no tail* (*ntl*), *floating head* (*flh*), *schmalspur* (*sur*), and *one-eyed-pinhead* (*oep*) mutations all affect midline development, albeit to various degrees. Mutations at the *ntl/Brachyury* locus lead to defects in notochord differentiation, but during gastrulation chordamesodermal cells are present (Halpern *et al*., 1993; Schulte-Merker *et al*., 1994). In 24 hpf *ntl* mutant embryos, both *pdx-1* and *insulin* expression resemble that of wild-type embryos (data not shown). In contrast, *flh/Xnot* mutant embryos, in which the notochord (axial mesoderm) is replaced by muscle cells (paraxial mesoderm) (Halpern *et al*., 1995; Talbot *et al*., 1995), exhibit a much stronger pancreatic phenotype. While the duodenal, ventrally situated stripe of *pdx-1* expression is not affected, we found that the posterior cluster of *pdx-1* expressing cells, representing the presumptive islet, is absent (Fig. 6, compare A and E). *insulin* expressing cells are almost completely missing in the *flh* mutant pancreatic anlage (Fig. 6, compare B and F). Neither *sur* nor *oep* mutants specifically lack the notochord. However, both *oep/EGF-CFC*, the homolog of mouse *cripto* and *cryptic* genes (Zhang *et al*., 1998), and *sur* affect nodal signaling required for proper specification of endoderm and midline tissue, including notochord and floorplate (Schier *et al.*, 1997; Gritsman *et al.*, 1999; Pogoda *et al.*, 2000). The pancreatic phenotype displayed by *sur* mutant embryos at 24 hpf is intermediate between that of *ntl* and *flh* mutant embryos: a very small islet can be distinguished within the *pdx-1* positive cells and the number of *insulin* positive cells is only slightly reduced (data not shown). In contrast, in *oep* mutants which lack both maternal and zygotic expression of *oep* neither *pdx-1* or *insulin* expressing cells are found at 24 hpf. In some embryos lacking zygotic *oep* only, however, a few *pdx-1* positive cells are observed, indicating that some endoderm may still be formed in *oep* zygotic mutant embryos (Figs. 6C, 6D, 6G, and 6H). The differences in *sur* and *oep* phenotypes may be explained since *sur* predominantly affects maintenance of Nodal signaling (Pogoda *et al.*, 2000), while in the absence of maternal and zygotic *oep* function, Nodal signals cannot be perceived by responding

cells at all. Taken together, our data suggest that, while midline signaling is required for proper development of the pancreas, it may not be required to initiate a pancreatic domain within the forming gut epithelium in zebrafish (see discussion).

Pancreas Development in Mutants Affecting Convergence Movements

Pancreatic endocrine precursor cells in zebrafish initially appear as left and right bilateral rows of cells immediately adjacent to the midline that converge at the midline during late somitogenesis (see above). Thus, we investigated whether any of the known genetic components that contribute to convergence movements in zebrafish might be involved in pancreatic development. Mutations affecting four different aspect of convergence toward the dorsal midline were analyzed. The *spadetail* (*spt*) mutation affects formation and convergence of paraxial mesoderm in the trunk, causing severe defects in trunk somitic mesoderm (Ho and Kane, 1990; Amacher and Kimmel, 1998; Warga and Nüsslein-Volhard, 1998; Griffin *et al.*, 1998). *spt* encodes a T-box gene that may be involved in controlling both morphogenesis, by its effect on convergence, as well as cell differentiation of paraxial mesoderm. We find that the bilateral pancreas primordia fail to converge in *spt* mutant embryos: two clusters of *insulin* expressing cells remain on either side of the midline at 24 hpf (compare Figs. 6I and 6M, 6J and 6N).

Three other genetic loci have been shown to be involved in the control of convergence during gastrulation, or of specific cell populations during somitogenesis. Mutations at the *knypek* locus (*kny*; Solnica-Krezel *et al.*, 1996; Marlow *et al.*, 1998) result in embryos with a shortened body axis and broadened axial and paraxial mesoderm. We find that in *kny* mutants, the *pdx-1* domains fail to merge, and two separate, bilateral stripes of *insulin* expressing cells are maintained during development (compare at 24 hpf: Figs. 6K and 6O, 6L and 6P). However, similar to the variability in phenotype reported for the trunk defects in *kny* mutants, we observe variable expressivity of the phenotype affecting pancreas development. A second gene that has been demonstrated to contribute toward the orchestration of convergent extension movements during gastrulation is *silberblick* (*slb*; coding for *wnt11*; Heisenberg *et al.*, 2000). In *slb* mutants expression of *pdx-1* and *insulin* appears normal at 24 hpf (data not shown), so *slb* is not required for pancreas development. The mutation *miles apart* (*mil*) causes cardia bifida and has recently been shown to encode a sphingosin-2-phosphate receptor involved in the control of convergence of the bilateral heart primordia to the definitive heart (Kupperman *et al.*, 2000). Since *mil* affects convergence of cardiac precursors around the same time and only slightly anterior to where pancreatic precursors converge, we were curious about a potential contribution of *mil* to pancreatic development. However, our analysis revealed that convergence of pancreatic precursors in *mil* mutants, as judged from *pdx-1* and *insulin* expression at about 24 hpf, is not affected *(*data not shown).

DISCUSSION

The Zebrafish Pancreas: Lower versus Higher Vertebrate

Although previous studies reported the presence of several endocrine islets in the pancreatic parenchyma of adult zebrafish (Milewski *et al.*, 1998; Pack *et al.*, 1996), our results show no evidence for the existence of secondary, accessory islets during the first six days of larval development. These additional islets therefore most likely differentiate later during development. This would argue in favor of the persistence of a pool of endocrine precursor cells in the adult, as suggested in rat (Kaung, 1994; reviewed in Slack, 1995). The zebrafish larval islet exhibits the typical endocrine cell type distribution and hormone expression observed in other teleost species (Youson and Al-Mahrouki, 1999). The core of the islet is composed of both β- and δ-cells, whereas α- and PP-cells are found at the periphery. However, although the general organization of the endocrine cell types seems different in fish compared to other vertebrate models, where δ-cells may also be found at the periphery, the lineage relationships between the different endocrine cell types may be conserved. Gene targeting studies of *pax4* and *pax6* (Sosa-Pineda *et al.*, 1997; St-Onge *et al.*, 1997) in mouse suggest a close relationship between β- and δ-cells, while previous reports had proposed that α- and β-cell lineages are independent (Herrera *et al.*, 1998). Moreover, a recent study shows elegantly that α-and β-cells most likely belong to independent lineages (Herrera, 2000). The observation that endocrine cells of the 48 hpf (Argenton *et al.*, 1999) and 72 hpf (this study) developing islets never co-express insulin and glucagon or somatostatin and glucagon supports the current model of separate α and β lineages proposed for mammals (Herrera, 2000). Absence of co-expression of *insulin* with *glucagon* or *PP* was also observed for *Xenopus* (Kelly and Melton, 2000). A true cell lineage analysis would still be needed to confirm whether α and β lineages are also separated early in zebrafish.

A noticeable difference between zebrafish and other vertebrates concerns the initiation of pancreas development with the formation of the so called “pancreatic buds” in higher vertebrates. Developing zebrafish embryos do not appear to form equivalent bud structures during the developmental phase when expression of endocrine hormones begins. This may be explained by differences in gut tube morphogenesis between zebrafish and other vertebrates. In fact, as a result of gastrulation in zebrafish, a sheet of endodermal cells forms rather than a proper endodermal tube like in higher vertebrates. There is evidence that the gut lumen forms rather late, at around 36 hpf, most likely by a cavitation process (Pack *et al.*, 1996; data not shown), similar to the secondary formation of the lumen of the neural tube (Papan and Campos-Ortega, 1994). Hence, at

the time of the onset of *pdx-1* expression, the endodermal tissue constitutes a single sheet of cells dorsal to the large syncytial yolk cell. Thus, at this time no morphological structure like the gut tube exists as the basis for a mammal- or birdlike formation of pancreatic buds in zebrafish. Budding of epithelial sheets may, however, contribute to later pancreas morphogenesis in zebrafish.

In zebrafish, as in other vertebrates, the pancreatic primordia first appear in two distinct locations and fuse during embryogenesis to form a single pancreatic primordium by late-somitogenesis. The identity of the cells located between the two bilateral domains of *pdx-1* expression at the 10-somite stage is unclear. Whether these are endodermal cells not yet specified toward a pancreatic fate or cells that will acquire another developmental fate is unknown. Alternatively they may be nonpancreatic (endodermal or mesodermal) cells that will relocate to another position during late somitogenesis and the elongation of the body axis. The answer to this question awaits histological analysis, the identification of new molecular markers, and cell lineage studies.

An alternate interpretation for the observed pattern of appearance of pancreatic endocrine cells would be that it resembles to some degree early stages of evolution of the endocrine system. In protochordates, endocrine cells are dispersed in the epithelium of the simple gut tube (reviewed in Youson and Al-Mahrouki, 1999). This is similar to the distribution of endocrine cells in zebrafish between the 12- and 18-somite stages. During evolution, endocrine cells may have acquired the ability to detach from and exit the gut epithelium and enter a migratory phase. Then, "migrating" endocrine precursors, based on local guidance cues and development of novel cell adhesion properties, may have started to organize into islets. Migration and assembly may be represented in larval lampreys, in which "one-hormone islets" form, containing exclusively cells of the insulin-producing lineage. Over evolution, cells of the other lineages may have then assembled around this core, as reflected by the predominantly peripheral location of glucagon and PP producing cells in higher vertebrates. Whether the mediolateral convergence and anterioposterior condensation of endocrine cells that we observe between 18-somite stage and 24 hpf resemble events during pancreatic evolution remains to be determined. In light of the invasive character of pancreatic cancer, it may be of interest to learn more about such postulated migratory states of endocrine cells or their precursors.

Pancreatic Gene Expression in Zebrafish: Evidence for Conserved Functions

The cloning and developmental expression of pancreatic hormones has been described previously (Milewski *et al*., 1998; Argenton *et al*., 1999). In this study, we have reassessed the initiation of expression of the mRNAs encoding Insulin, Glucagon, and Somatostatin. We narrowed down the time frame of the earliest detectable expression for each hormone. Our results confirmed previous observations that *insulin* is the first pancreatic hormone to be expressed followed by *somatostatin* and *glucagon* (for Xenopus: Kelly and Melton, 2000). The late appearance of *trypsin*, after the three endocrine hormones analyzed in this work, is well correlated with a corresponding delayed onset of exocrine gene expression in the murine pancreas (Gittes and Rutter, 1992). While *insulin* and *glucagon* are first expressed in the mouse pancreatic primordium in a premorphogenetic phase (20-somite stage), exocrine specific products are expressed later, 24 h after the formation of the pancreatic diverticulum. In zebrafish, food uptake and requirement for digestive enzymes begin only at four dpf, presenting no need for earlier maturation of the pancreatic exocrine tissue.

Insulin expressing cells appear scattered within the prepatterned, *pdx-1* positive endodermal epithelium. All the pancreatic hormone-encoding mRNAs examined so far exhibit a similar scattered initiation of expression. This suggests that the zebrafish islet arises from the aggregation of differentiated cells rather than by monoclonal growth from individual progenitors, as described for mammals (Deltour *et al*., 1991; Percival and Slack, 1999). This also indicates that the process of lateral inhibition, involved in endocrine cell differentiation in mice (Apelqvist *et al*., 1999; Jensen *et al*., 2000b), may also be taking place in zebrafish.

Zebrafish homologs for many transcription factors expressed and required in the mouse pancreas all show expression in the pancreatic primordium defined by *pdx-1* expression: *islet-1*, *nkx2.2*, *pax6.2* (this report), *FoxA2* /*axial*, and *neuroD* (Korzh *et al*., 1998; see also Table 1). This suggests evolutionary conservation of the developmental mechanisms for pancreatic cell specification and differentiation and recommends the zebrafish as a valuable model for the study of pancreatic organogenesis. The expression of *pax6.2* but not *pax6.1* in the zebrafish pancreas provides us with a striking example of fractionation of expression domains (and of gene function) following gene duplication in the fish lineage leading to teleosts. Similar functional fractionation has already been reported in zebrafish for transcription factors of the *engrailed* (Amores *et al*., 1998) and *vsx* (Passini *et al*., 1998) families.

Mutant Analysis Identifies Steps in Pancreatic Development

Experimental work in chick has revealed a requirement for notochord derived signals in pancreas formation (Kim *et al*., 1997a). The availability of zebrafish mutations affecting axial mesoderm provides an opportunity to use genetic tests to assay for a role of notochord in pancreatic development. Our finding that the endocrine pancreas develops in *ntl* mutant embryos, in which chordamesoderm forms but does not differentiate properly, indicates that any signaling required for pancreas differentiation can already be accomplished by the chordamesoderm cells and does not require differentiated notochord. In *flh* mutant embryos, chordame-

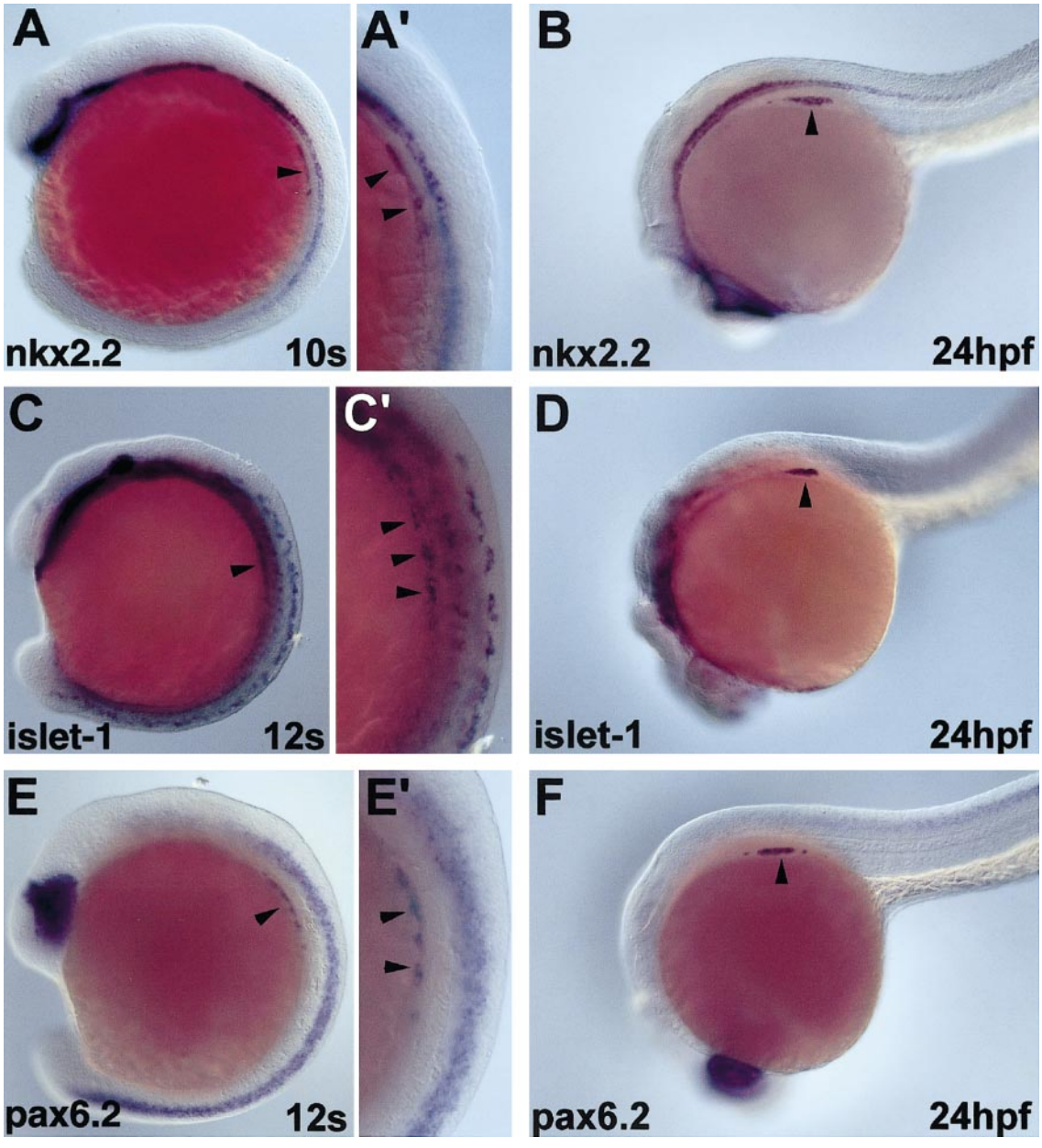

FIG. 5. Pancreatic specific expression of the zebrafish *nkx2.2*, *islet-1*, and *pax6.2* mRNAs. Whole-mount *in situ* hybridization of wild-type zebrafish embryos at the (A, A′) 10-somite stage; (C, C′, E, E′) 12-somite stage, and (B, D, F) 24 hpf performed with (A, A′, B) *nkx2.2*, (C, C′, D) *islet-1*, and (E, E′, F) *pax6.2* antisense mRNA probes. Lateral views, dorsal to the right (A, A′, C, C′, E, E′) or to the top (B, D, F). Pancreas-specific expression domain are indicated (arrowheads).

soderm does not form, and paraxial mesoderm is continuous in the midline of the embryo. Thus, notochord as a source for potential signals to pattern the endoderm does not exist in *flh* mutant embryos. Even without notochord, in *flh* mutants, we find both duodenal expression of *pdx-1* and small numbers of *insulin* expressing cells at proper morphological locations. This indicates that signaling sources other than the notochord might contribute to specification of the pancreatic primordium. We cannot exclude that floorplate cells, which appear dispersed in the spinal cord of *flh* mutants, share some of the inductive activities of notochord and are responsible for the induction of the small number of *insulin* expressing cells observed. In zebrafish, other than in chick for example, endoderm and

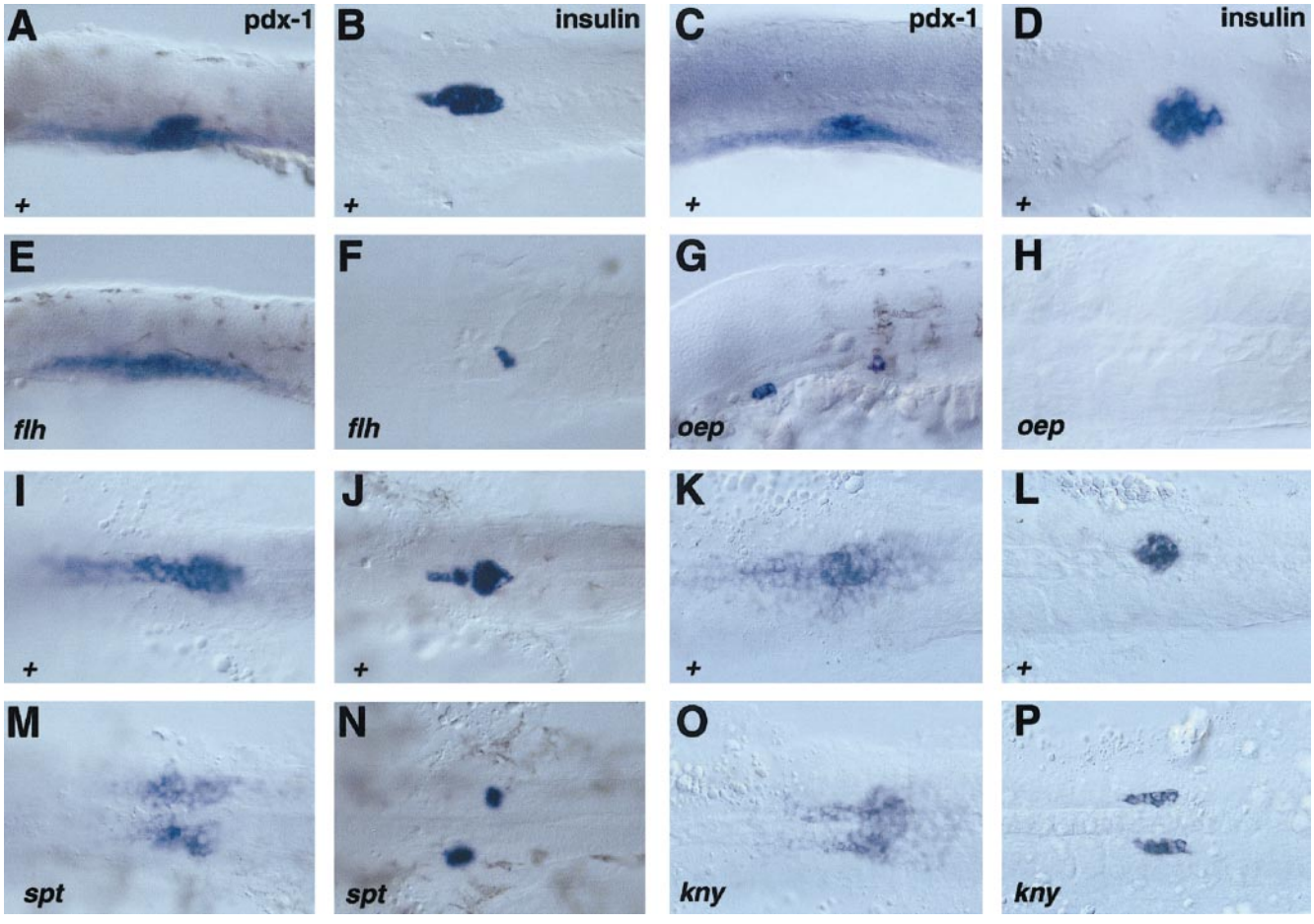

FIG. 6. Development of the endocrine pancreas in zebrafish mutations affecting midline development and convergence movements. Expression of *pdx-1* (A, C, E, G, I, K, M, O) and *insulin* (B, D, F, H, J, L, N, P) in wild-type and mutant embryos at 24 hpf. The following mutations are analyzed: (E, F) *floating head*; (G, H) *one-eyed pinhead* (*oep*); (M, N) *spade tail* (*spt*); and (O, P) *knypek* (*kny*). Lateral views in A, E, C, G, and dorsal views in all the other panels; anterior is to the left in all panels. All panels show the trunk of the embryo, between the posterior end of the hindbrain and about segment 10. The yolk has been removed from the embryos.

chordamesoderm are in close contact along a large portion of the anterioposterior axis during the developmental stage correlating with pancreas induction in chick. This observation makes it difficult to imagine a model in which local contact between notochord and endoderm induces pancreatic development. It has been suggested that a major function of notochord in pancreatic development is repression of hedgehog signaling (Kim *et al.*, 1997a; Kim and Melton, 1998). A detailed analysis of the role of the hedgehog signaling pathway during the ontogeny of the pancreas in zebrafish will be presented elsewhere.

Together these data indicate that patterning of the zebrafish endoderm and pancreas induction can proceed in the absence of axial mesoderm, although it is required for normal pancreatic development. In zebrafish, a prepattern might already exist in the endoderm, possibly established during gastrulation, or other tissues might be involved in pancreas induction. To test for involvement of paraxial mesoderm, we analyzed pancreas development in *spt* mutant embryos, which lack somitic mesoderm in the trunk. We found that duodenal as well as pancreatic primordia are located at the proper anterioposterior position in *spt* mutant embryos, indicating that paraxial mesoderm is not required for pancreas induction. However, the bilateral domains of *pdx-1* and *insulin* expression established during early somitogenesis fail to converge in *spt* mutants. Thus, paraxial mesoderm may be required for convergence of pancreatic precursors: It could provide a substrate on which the precursor cells converge to the midline, or it may be directly involved in signaling events leading to convergence. The *spt* mutation is also involved in the control of trunk paraxial mesoderm convergence (Warga and Nüsslein-Volhard, 1998). Since *spt* mRNA is expressed throughout the nonaxial marginal region during gastrula-

TABLE 1
Expression of Transcription Factors in the Zebrafish Pancreas

Transcription factors expressed in the mouse pancreas	Zebrafish homolog	Expression in the zebrafish pancreas	References
Pdx-1	pdx-1	Yes, this study	Milewski *et al.*, 1998
Isl-1	islet-1	Yes, this study	Korzh *et al.*, 1993
Pax6	pax6.1 & pax6.2	Yes, this study; pax6.2 but not pax6.1	Krauss *et al.*, 1991; Nornes *et al.*, 1998
Nkx2.2	NK2.2	Yes, this study	Barth and Wilson, 1995
NeuroD/Beta2	nrd	Yes	Korzh *et al.*, 1998
Foxa2 (HNF-3β)	FoxA2 (fkd1/axial)	Yes, this study, data not shown	Strähle *et al.*, 1993; Odenthal and Nüsslein-Volhard, 1998
Foxa3 (HNF-3γ)	FoxA3 (fkd2/Zffkh1)	Yes, this study, data not shown	Dirksen *et al.*, 1995; Odenthal and Nüsslein-Volhard, 1998
Prox-1	prox-1	Not determined	Glasgow and Tomarev, 1998

Note. Zebrafish homologs for the following transcription factors expressed in the mouse pancreas are not, to our knowledge, available: pax4, ngn3, hes1, nkx6.1, cdx-2/3, cdx-4, HNF6, HNF-4α, HNF-4γ, HNF-1α, HNF-1β, and PTF1-p48.

tion, we cannot exclude that tissues other than paraxial mesoderm contribute to the pancreatic phenotype observed in *spt*.

We investigated other mutations affecting convergence: *slb* (coding for Wnt11, Heisenberg *et al.*, 2000), *mil* (coding for a sphingosine-1-phosphate receptor, Kupperman *et al.*, 2000), and *kny* (not cloned; Marlow *et al.*, 1998), which are involved in specific aspects of convergence to the embryonic midline during gastrulation and organogenesis. Both mutants in *slb* and *mil* do not affect pancreatic development. However, *kny* mutant embryos, which have broadened axial and paraxial mesoderm and neural plate, suggesting a deficiency in convergence, fail to merge bilateral pancreatic primordia at the midline. Defined genetic pathways control different aspects of convergence. The pathway in which *kny* is involved controls convergence of a broad range of tissues including mesoderm and neurectoderm (Marlow *et al.*, 1998) and endodermal tissue in the pancreas, as this study shows. Observation of the convergence mutants suggests that merging of the bilateral pancreatic primordia results from active convergence of the pancreatic precursor cells, but not from a process that removes cells located between the primordia. Thus, at embryonic stages, endocrine pancreatic cells go through a migratory phase, which may relate to their proneness to invasive behavior in pancreatic cancer. Whether the convergence of pancreatic primordia we observe in zebrafish relates to the fusion of dorsal and ventral pancreatic buds in other vertebrates remains to be determined. However, we think that bud fusion, which involves repositioning of a whole organ component rather than that of individual cells or cell groups, might be controlled by mechanisms distinct from pancreatic primordia convergence in zebrafish and may have evolved separately in higher vertebrates.

Results from the analysis of zebrafish mutations indicate that genetics provides powerful tools to characterize crucial steps in the formation of the endocrine pancreatic lineages. Further genetic studies in zebrafish will hopefully contribute to our understanding of pancreas organogenesis.

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