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Mechanisms of Development 79 (1998) 153–159

Pax 4 and 6 regulate gastrointestinal endocrine cell development

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Received 21 September 1998; accepted 25 September 1998

Abstract

The mechanisms behind the cell-specific and compartmentalized expression of gut and pancreatic hormones is largely unknown. We hereby report that deletion of the Pax 4 gene virtually eliminates duodenal and jejunal hormone-secreting cells, as well as serotonin and somatostatin cells of the distal stomach, while deletion of the Pax 6 gene eliminates duodenal GIP cells as well as gastrin and somatostatin cells of the distal stomach. Thus, together, these two genes regulate the differentiation of all proximal gastrointestinal endocrine cells and reflect common pathways for pancreatic and gastrointestinal endocrine cell differentiation.

Keywords: Pax genes; Gastrointestinal hormones; Pdx-1; NeuroD; Beta2; Gastrointestinal development

1. Introduction

The gastrointestinal tract is subdivided into compartments involved in the sequential digestion, absorption and disposal of fuels. These functions are regulated by an extensive system of gastrointestinal endocrine cells which produce numerous different hormones and together constitute the largest endocrine gland in the body (Solcia et al., 1981). With few exceptions gut endocrine cells show a strictly compartmentalized distribution in the gut (Solcia et al., 1981). The mechanisms behind such compartmentalized and cell-specific hormone expression are largely unknown, but must involve both rostrocaudal and transverse gradients, hormones and locally acting factors (Gordon, 1989; Rawdon and Andrew, 1993). Recent findings indicate that transactivating factors that are important for pancreatic formation and cell specification also are important to endocrine cell fate in the stomach and proximal small intestines. Thus, the pancreatic-duodenal homeobox1 (Pdx1) which is needed for the formation of a pancreas (Jonsson et al., 1994; Offield et al., 1996) has also been found to be needed for the differentiation of gastric gastrin-producing G cells (Larsson et al., 1996) and for the differentiation of endocrine cells and epithelium of the most rostral part of the duodenum (Larsson et al., 1996; Offield et al., 1996). In addition, the basic helix loop helix (bHLH) factor Beta2/NeuroD which is required for pancreatic islet formation and insulin cell differentiation is also needed for differentiation of duodenal secretin and cholecystokinin cells (Mutoh et al., 1997; Naya et al., 1997). These overlaps in function may reflect the fact that the pancreas, stomach and duodenum develop from closely adjacent regions of the primitive foregut (Pictet and Rutter, 1972).

Very recently, two paired box (Pax) genes were reported to be essential for pancreatic endocrine cell differentiation. Thus, Pax 4 was found to be necessary for the development of pancreatic insulin and somatostatin cells (Sosa-Pineda et al., 1997) while Pax 6 was needed for pancreatic glucagon cell development (St-Onge et al., 1997). In addition, Pax 6 is known to regulate also eye and brain development (Walther and Gruss, 1991) while no other function has yet been described for Pax 4. Studies in mice with spontaneous mutations in the Pax 6 gene moreover indicated that this gene also affected the expression of other hormones produced in the islets of Langerhans (Sander et al., 1997). Mice with deletions of both the Pax 4 and 6 genes were, moreover,

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0925-4773/98/$ - see front matter
PII S0925-4773(98)00182-8

devoid of all islet hormone-producing cells (St-Onge et al., 1997).

We have now examined the effect of targeted deletion of Pax 4 and 6 genes on gastrointestinal hormone expression using immunocytochemistry and reverse transcriptase-polymerase chain reaction.

2. Results

2.1. Gastrointestinal expression of Pax 4 and 6

In both Pax 4 and 6 constructs, the β-galactosidase (βGAL) gene was inserted under the control of the respective promoter. Enzyme histochemistry (LacZ staining) for studying Pax 4 or 6 expression was, however, precluded by high endogenous βGAL activity in the intestines. In the stomach, such endogenous activity was not present, and here LacZ staining identified βGAL-expressing endocrine-like cells in distal stomach (antrum) of Pax 6, but not Pax 4, heterozygous animals. Immunocytochemistry with an antibody to bacterial βGAL permitted selective detection of expressed βGAL in both stomachs and intestines. With immunocytochemistry, βGAL expression was detected throughout the gastrointestinal tract of both Pax 4 and 6 heterozygotes and mutants (Fig. 1). There was no significant difference in numbers of βGAL positive cells between mutants or heterozygotes in either Pax 4 or Pax 6 animals. No staining occurred in wild-type animals. The immunopositive cells occurred scattered in-between non-immunoreactive epithelial cells and possessed the characteristic flask-like shape of endocrine cells.

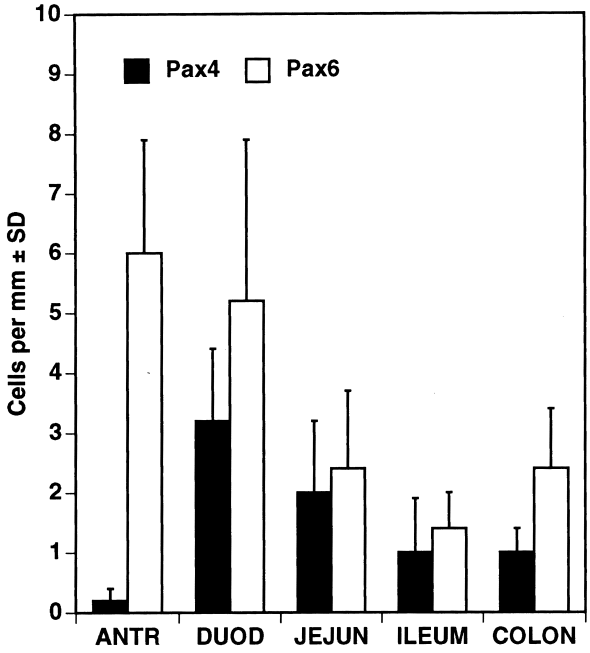

Fig. 1. Distribution of βGAL positive cells in the gastrointestinal tract of Pax 4 and Pax 6 mutants and heterozygotes. Notes that both genes are predominantly expressed in the proximal gastrointestinal tract.

2.2. Effects of Pax 4 and 6 deletion on gastrointestinal hormone expression

Immunocytochemistry for gastrointestinal hormones and RT-PCR for the corresponding mRNAs showed that deletion of either Pax 4 or 6 had pronounced effects on gastrointestinal endocrine cell differentiation and hormone expression (Figs. 2–4). In Pax 4 mutants the duodenum showed dramatic reductions in serotonin-, secretin-, CCK-, GIP- and PYY-immunoreactive cells (Figs 2 and 3). This corresponds to a near total elimination of all duodenal endocrine cells. Interestingly, Pax 4 heterozygous animals also tended to have reduced frequencies of most duodenal endocrine cell types, although the difference compared to wild-type animals did not reach statistical significance (data not shown). The decrease in endocrine cell numbers was also reflected by a decrease in the levels of hormone-encoding mRNA species (Fig. 4). In contrast, RT-PCR showed duodenal Beta2/NeuroD expression to be unchanged and immunocytochemistry revealed Pdx1 expressing cells in duodenum of wild-type, heterozygous and mutant animals (data not shown). The stomachs of Pax 4 mutants showed a significant and dramatic reduction in the numbers of somatostatin and serotonin cells, whereas gastrin cell numbers were not affected (Fig. 2). Similarly, with RT-PCR, somatostatin mRNA was undetectable, whereas gastrin mRNA levels were unaffected in the mutants (Fig. 4). In the jejunum, quantitations for serotonin and PYY cells documented dramatic reductions, comparable with those seen in the duodenum. However, more caudally, in the ileum and colon, no significant difference in endocrine (serotonin and PYY) cell numbers occurred between wild-type and Pax 4 mutant or heterozygous animals (Fig. 5). Concordant with this, RT-PCR showed no difference in glucagon and PYY mRNA levels between Pax 4 mutant, heterozygous or wild-type animals.

Pax 6 mutant mice had significant reductions in antral gastrin and somatostatin cell numbers and reduced gastrin and somatostatin mRNA levels (Figs. 2 and 4). Antral serotonin cell numbers were unaffected (Fig. 2). In the duodenum of Pax 6 mutants no significant changes occurred in secretin, PYY and serotonin cells while GIP cells were reduced in number (Fig. 2). With RT-PCR secretin mRNA levels were unchanged while GIP mRNA was undetectable in the Pax 6 mutants. In the colon of Pax 6 mutants, neither immunocytochemistry nor RT-PCR detected differences in PYY expression. Thus, the phenotypic alterations associated with deletions of Pax 4 and 6 are different and most marked in different regions of the gut. The alterations in the Pax 6 mutants predominated in the stomach, and were associated with severe reductions in gastrin and somatostatin cell numbers and mRNA levels. Immunocytochemical triple staining for βGAL, gastrin and somatostatin in Pax 6 heterozygotes revealed that both gastrin and somatostatin cells were βGAL positive and, hence, expressed the Pax 6 gene. In addition, cells that co-expressed gastrin and soma-

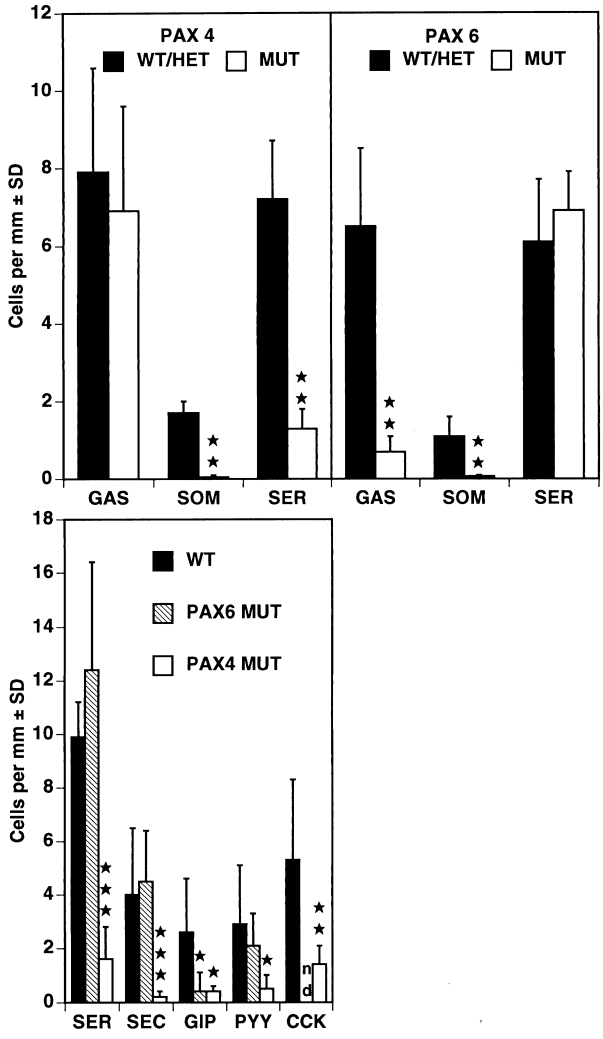

Fig. 2. Quantitations of endocrine cells in Pax 4 and 6 mutants (MUT), wild-types (WT) and heterozygotes (HET). The upper panel shows the effects of the Pax 4 and 6 deletions on gastric gastrin (Gas), somatostatin (Som) and serotonin (Ser) cells, while the lower panel shows the effects of Pax 4 and 6 on duodenal Ser, secretin (Sec), GIP, PYY and CCK cells. Between four and ten animals were studied for each point. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$; nd, not determined.

tostatin were also βGAL positive. It has previously been suggested that the co-expressing G/D cells represent precursors to mature gastrin and somatostatin cells (6) and the present data indicates that Pax 6 may be involved in the maturation of these precursor cells. Interestingly, the Pax 4 mutants showed a gastric phenotype different from that of the Pax 6 mutants, with reductions in antral somatostatin and serotonin cell numbers. Immunocytochemical identification of βGAL-expressing cells in Pax 4 heterozygotes has not been possible due to the weak expression of this gene in antrum. However, as it has been suggested that serotonin cells derive in part from somatostatin-serotonin positive intermediary precursor cells (Larsson et al., 1996), it is possible that Pax 4 may affect maturation of these cells. In the duodenum of Pax 4 heterozygotes βGAL expressing cells could be identified as serotonin cells (Fig. 6). Such double positive cells were present both on villi and in the intervillus/crypt epithelium. The duodenum of Pax 4 mutants showed no significant changes in βGAL expressing cells. However, double-stainings showed that serotonin expression was extinguished in these cells. Similar double-staining in Pax 6 heterozygotes and mutants showed that the cells expressing this gene also corresponded to serotonin cells.

3. Discussion

In the proximal small intestines, the Pax 4 – but not the Pax 6 – mutants revealed a dramatic phenotype characterized by the virtual absence of cells immunopositive for gut hormones in the duodenum and jejunum. This suggests that the Pax 4 gene is essential for the maturation of duodenal and jejunal endocrine cell types. Two other genes Pdx1 (Larsson et al., 1996; Offield et al., 1996) and Beta2/NeuroD (Mutoh et al., 1997; Naya et al., 1997) have previously been found to be involved in gastrointestinal endocrine cell differentiation. Mutants deficient in these genes are, however, not associated with as marked gastrointestinal alterations as the Pax 4 mutants (Larsson et al., 1996; Naya et al., 1997; Offield et al., 1996). Thus, changes in the Pdx1 mutants include lack of differentiation of gastrin cells in the stomach as well as smaller decreases in several rostral duodenal endocrine cells (Larsson et al., 1996; Offield et al., 1996). In Beta2/NeuroD mutant animals the differentiation of small intestinal secretin cells and CCK cells was impeded, while cells expressing serotonin, glucagon, PYY, GIP and somatostatin were not noticeably affected (Naya et al., 1997). The Pax 4 gene therefore seems to act at an earlier stage during differentiation of primitive endocrine stem cells. As both Pdx1 and Beta2/NeuroD were expressed in the Pax 4 mutants it is evident that the changes recorded by us are not secondary to effects on these genes. Moreover, Pdx1 mutants lack a pancreas, lack gastrin cells and show intestinal defects restricted to the rostral duodenum (Jonsson et al., 1994; Offield et al., 1996; Larsson et al., 1996) – effects not seen in Pax 4 mutants. In contrast to the Beta2/NeuroD mutants, Pax 4 mutants are virtually devoid of all hormone-positive proximal intestinal cells. Together with other intestinal epithelial cells these cells are derived from stem cells present in the crypt epithelium (Cheng and Leblond, 1974; Ponder et al., 1985). Division of such stem cells assures continuous renewal of the epithelium throughout life with a turnover of 3–4 days in the mouse (Cheng and Leblond, 1974). This renewal is associated with migration and differentiation followed by cell death and extrusion. The basal two-thirds of the crypts contain dividing cells while the upper crypts and villi contain postmitotic, differentiating cells. Certain genes such as secretin, are expressed only by postmitotic cells situated on the villi (Larsson et al., 1977; Inokuchi et al., 1985) while other genes, including PYY, serotonin, and GIP also are expressed in the crypts (Roth and Gordon, 1990; Aiken et

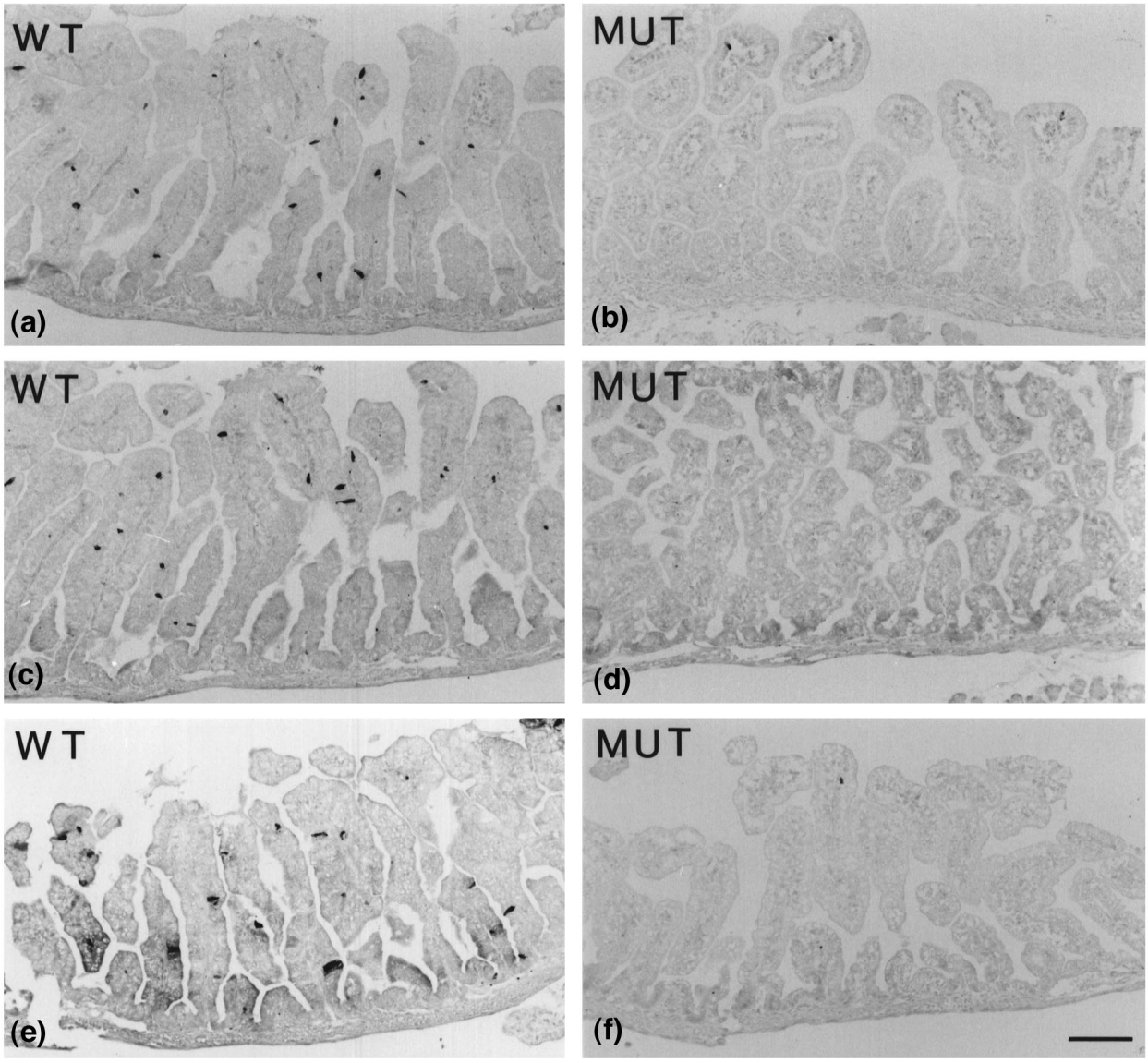

Fig. 3. Immunocytochemical staining for secretin (a,b), CCK (c,d) and serotonin (e,f) in duodenum of Pax 4 wild-type (WT) and mutant (MUT) animals. Note the near total elimination of immunoreactive endocrine cells in the mutants. Scale bar: 100 μm.

al., 1994; Lopez et al., 1995). Studies of transgenic animals and of the axial expression of endocrine markers have indeed suggested that secretin cells differentiate from serotonin-positive precursor cells (Roth and Gordon, 1990; Aiken et al., 1994; Lopez et al., 1995). In agreement with this, secretin and serotonin have been found to be co-expressed in early differentiating duodenal cells (Larsson and Mørch-Jørgensen, 1978; Roth and Gordon, 1990; Aiken et al., 1994; Lopez et al., 1995). Hence, much data indicate that secretin and, possibly, CCK cells may derive from a common serotonergic lineage (Roth and Gordon, 1990; Aiken et al., 1994; Lopez et al., 1995; Naya et al., 1997) while GIP cells and another part of the serotonin cells represent independent lineages (Aiken et al., 1994). Thus, from a common stem cell, several different lineages may evolve. The effects of the Pax 4 deletion affects all proximal intestinal endocrine cell populations. Thus, our data indicate that Pax 4 is indispensable for the differentiation of early endocrine stem cells before these diverge into different lineages. Moreover, together, Pax 4 and 6 are needed for the differentiation of all endocrine cells of the distal stomach, proximal intestines and pancreas (Fig. 7). The fact that βGAL expressing cells are not reduced in numbers in the Pax 4 or 6 mutants show that the Pax genes are not needed for the survival of these cells, but rather are needed for their differentiation. It is uncertain whether the Pax genes affect the maturation of the endocrine cell precursors or whether these genes are needed for the expression of all different hormones in the proximal gut. Our studies definitely show that Pax 4 is not needed for distal gut expression of serotonin and PYY. It is, however, not possible to exclude that these genes are differentially regulated in the proximal and distal gut.

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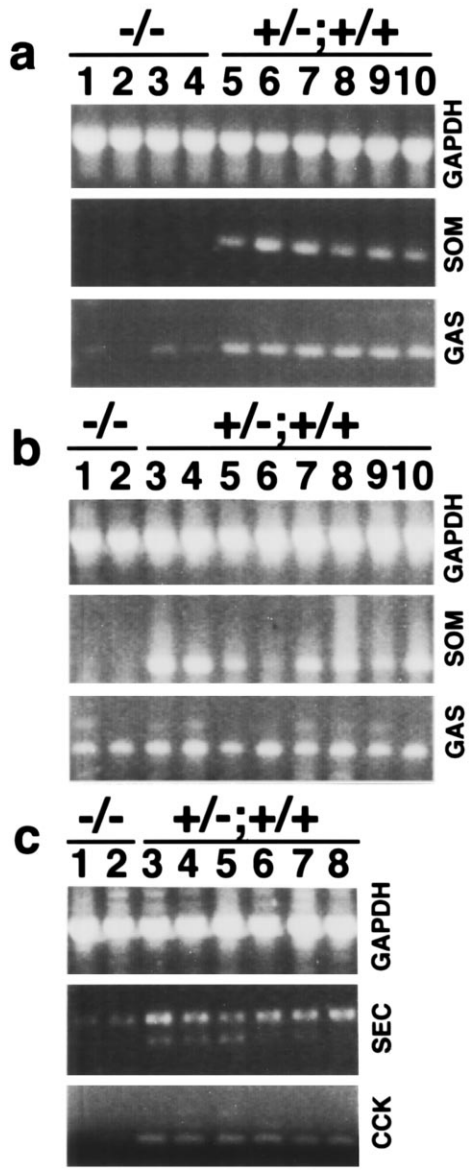

Fig. 4. RT-PCR analyses of GAPDH and peptide hormone mRNA expression in stomach (a,b) and duodenum (c). Note severely diminished expression of somatostatin (SOM) and gastrin (GAS) in distal stomach of Pax 6 mutant (–/–) animals (a), and severely diminished somatostatin, but not gastrin, expression in distal stomach of Pax 4 mutant animals. In the duo=-denum of Pax 4 mutant animals expression of secretin (SEC) and CCK is also severely diminished.

duodenum, and it is therefore possible that the pancreatic and gastro-duodenal endocrine cells may share common pathways for differentiation. This is underlined by the fact that Pax 4 and 6 deletions eliminate hormone expression in both the proximal gut and pancreas. In addition, gut hormones are transiently expressed during pancreatic differentiation, while pancreatic hormones are transiently expressed during gut differentiation (Larsson et al., 1976; Larsson, 1977; Wheeler et al., 1992; McGregor et al., 1995). Moreover, the presumptive pancreatic tumor stem cell line MSL-G2 that can differentiate into insulinoma (hypoglycemic) or glucagonoma (anorectic) variants produces several intestinal hormones (Madsen et al., 1986). It is, hence, possible that a common stem cell pathway is involved in the differentiation of both gastrointestinal and pancreatic endocrine cells and is under the control of the Pax 4 and 6 genes.

4. Experimental procedures

4.1. Animals

Pax 4 and Pax 6 LacZ mice were generated by homologous recombination as described (Sosa-Pineda et al., 1997; St-Onge et al., 1997). As Pax 4 and 6 mutations are lethal during the neonatal period, mice were collected by cesarean section just before birth (embryonal day 19). The entire fetal gastrointestinal tract was fixed by immersion in 4% paraformaldehyde or Bouin's fluid. Additional tissues were frozen in liquid nitrogen and stored at −80°C.

4.2. Histochemistry and quantitation

LacZ-staining was performed as described (Sosa-Pineda et al., 1997; St-Onge et al., 1997). Immunocytochemical stainings for gastrin/CCK, secretin, serotonin, GIP, somatostatin, PYY and Pdx1 employed antisera and procedures as described (Larsson, 1988; Larsson et al., 1996). In addition, commercial rabbit antisera to glucagon and somatostatin were used (Dako, Glostrup, Denmark) and antiserum to β-galactosidase was obtained from 5-prime-3-prime (Boulder, CO). The number of immunoreactive cells per millimeter length of gastrointestinal tract was counted in perfect transverse gut sections and expressed as means ± SD. Statistical treatment was by the Mann–Whitney *U*-test.

Fig. 5. Quantitations of serotonin and PYY cells in duodenum, jejunum, ileum and colon in Pax 4 mutants (MUT) and wild-types (WT). Note markedly diminished expression in duodenum and jejunum while smaller, non-significant changes occur in ileum and colon. Between four and 24 animals were studied for each point.

4.3. Reverse transcriptase–polymerase chain reaction (RT-PCR)

Total RNA was extracted by the TRizol reagent (Life Technologies, Grand Island, NY), reversely transcribed to cDNA and submitted to PCR using the Gene Amp RNA PCR kit (Perkin Elmer, Branchburg, NJ) and primer sets specific for gastrin (upper primer position [upp] 99–122 and lower primer position [lpp] 428–449, gene bank accession number U58136), CCK (upp 25–45 and lpp 156–179, acc. no. M11739), secretin (upp 204–223 and lpp 630–653, acc. no. U07568), somatostatin (upp 941–962 and lpp 1822–1845, acc. no. X51468), glucagon (upp 173–193 and lpp 345–368, acc. no. Z46845), PYY (upp 164–184 and lpp 297–320, acc. no. AA238723), NeuroD(upp552–576 and lpp 729–751. acc. no. U28068) and glyceraldehyde-phosphate dehydrogenase (GAPDH) (upp 613–636 and lpp 1058–1078, acc. no. M32599). Some of the primer sets span known introns. 40 cycles of PCR were routinely performed, and the reaction products were size fractionated by agarose gel electrophoresis.

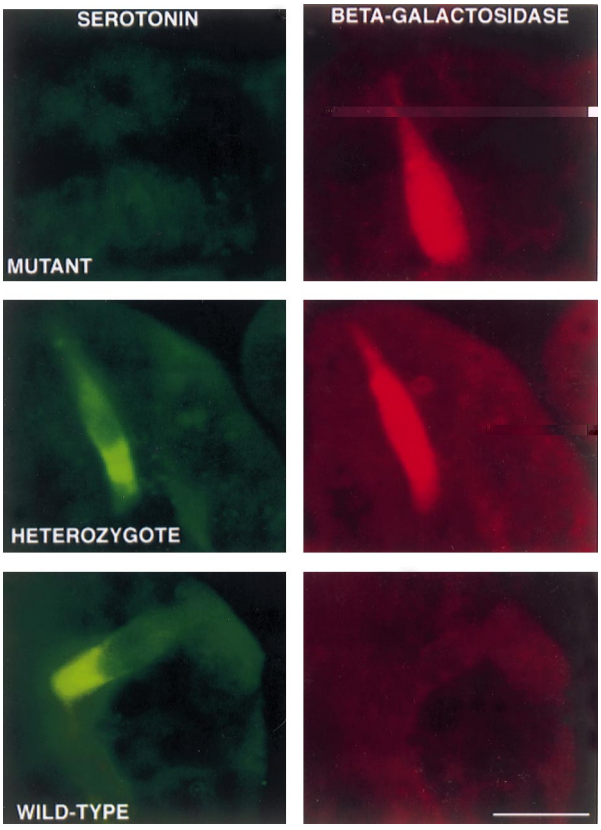

Fig. 6. Immunocytochemical double-stainings for serotonin and β-galactosidase in Pax 4 mutant, heterozygous and wild-type animals. Note that βGAL positive cells occur in both mutant and heterozygous animals, but are absent in wild-type animals. In heterozygous animals most positive cells react for both serotonin and βGAL. In mutant animals serotonin expression is virtually extinct, whereas βGAL positive cells can still be found. In wild-type animals no cells expressing βGAL are found, while numerous serotonin cells are present. Scale bar: 10 μm.

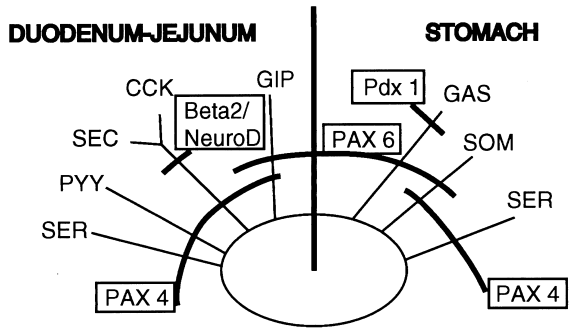

Fig. 7. Scheme summarizing the findings on Pax 4, Pax 6, Pdx1 and Beta2/NeuroD mutations on upper gastrointestinal endocrine cell development. Duodenal effects of Pdx1 mutations are restricted to the rostral duodenum and are therefore not included in this scheme. It is evident that Pax 4 and Pax 6 are needed for expression of all upper gastrointestinal hormones.

Acknowledgements

Grant support was from the Danish National Research Fund, Danish MRC and the Danish Biotechnology Program.

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