IPF1, a homeodomain protein with a dual function in pancreas development

JÖRGEN JONSSON, ULF AHLGREN, THOMAS EDLUND and HELENA EDLUND*

Department of Microbiology, University of Umeå, Umeå, Sweden

ABSTRACT Insulin promoter factor 1 (IPF1), is a homeodomain protein which, in the adult mouse pancreas, is selectively expressed in ß-cells, and which binds to, and transactivates, the insulin promoter via the P1 element. In mouse embryos, IPF1 expression is initiated when the foregut endoderm commits to a pancreatic fate, i.e. prior to both morphogenesis and hormone specific gene expression. At later stages of development the expression is restricted to the dorsal and ventral walls of the primitive foregut at the positions where the pancreases will form. Mice homozygous for a targeted mutation in the *Ipf1* gene selectively lack the pancreas. The mutant pups develop to term and are born alive, but die after a few days. The gastrointestinal tract with its associated organs shows no obvious malformations. No pancreatic tissue and no ectopic expression of insulin or pancreatic amylase could be detected in this region in mutant neonates or embryos. These findings demonstrate that IPF1 is needed for the formation of the pancreas, and suggest that IPF1 acts to determine the fate of common pancreatic precursor cells and/or to regulate their propagation. The lack of a pancreas in the *Ipf1*-deficient mutants, the pattern of IPF1 expression and its ability to stimulate insulin gene transcription, strongly suggest that IPF1 functions both in the early specification of the primitive gut to a pancreatic fate and in the maturation of the pancreatic ß-cell.

KEY WORDS: *homeodomain, IPF1, endoderm, ß-cell specific, apancreatic*

Introduction

The mammalian pancreas is a mixed exocrine and endocrine gland that, in most species, arises from ventral and dorsal buds which subsequently merge to form the definitive pancreas. In both mouse and rat, the first histological sign of morphogenesis of the dorsal pancreas is a dorsal evagination of the duodenum at the level of the liver at the 22-25 somite stage (Wessels and Cohen, 1967; Spooner *et al.,* 1970). Shortly thereafter a ventral evagination appears as a derivative of the liver diverticulum. The epithelial cells lining these pancreatic rudiments subsequently differentiate and segregate into duct, acinar and endocrine cells, secreting fluid/electrolytes, digestive enzymes and hormones, respectively. These two pancreatic rudiments later fuse to form a single pancreatic mass and their ducts usually fuse to form the main duct of the mature pancreas. (Wessells and Cohen, 1967; Pictet and Rutter, 1972). Fate mapping experiments suggest that the different pancreatic cell types have a common endodermal origin but the precise lineage relationship among these cell types remains unknown (Pictet *et al.,* 1976; Le Douarin, 1978). Using mouse *in vitro* explant cultures it has been shown that the gut acquires the ability to form a pancreas around the 10 somite stage (Wessels and Cohen, 1967; Spooner *et al.,* 1970). *In vivo*, low levels of insulin gene transcripts are already present and restricted to the dorsal foregut endoderm at 20 somites, providing evidence of a premorphogenetic phase of pancreas development (Gittes and Rutter, 1992). The early onset of insulin gene transcription suggests that pancreas- or insulin gene-specific transcriptional factors may be present in this region prior to the onset of morphogenesis, and it is plausible that commitment of the endoderm to a pancreatic fate involves the selective expression of such transcriptional regulatory molecules.

In our search for transactivators of the insulin gene we isolated a cDNA encoding a homeodomain protein, denoted insulin promoter factor 1 (IPF1). In the normal adult mouse pancreas IPF1 is only expressed in the ß-cells, where it binds to and transactivates the insulin promoter (Ohlsson *et al.,* 1993). In mouse embryos, the IPF1 protein is detected only in the developing pancreas and IPF1 expression is initiated at around the 10 somite stage which correlates temporally with the pancreatic commitment of the endoderm. To test the hypothesis that IPF1 plays a role in the pancreatic commitment of the foregut endoderm we have generated IPF1-deficient mice. The homozygous mutant mice develop to term, but die 2-4 days after birth. These mice show no morphological signs of a pancreas, and no pancreatic exocrine or endocrine tissue or markers can be detected in the region of the duodenum from which the dorsal and ventral

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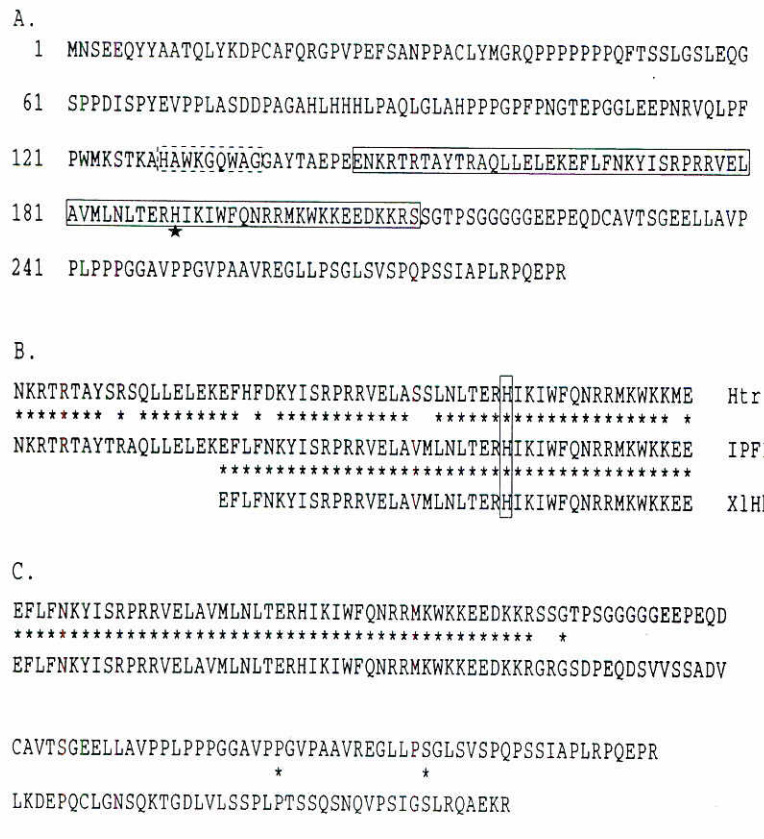

Fig. 1. Deduced amino acid sequence of IPF1. (A). *The homeodomain is boxed with a solid line, the unique histidine in helix 3 is indicated by a star, a homology to the immediate early proteins of herpes class viruses (Cheung, 1989) is boxed with a dashed line.* **(B)** *Alignment of the homeodomains of Htr-A2, IPF1 and XlHbox8, the stars represent identities. The unique histidine is boxed.* **(C)** *Alignment of the known amino acid sequence of XlHbox8 with the C-terminal half of IPF1 the, stars represent identities. (From Ohlsson et al., 1993. Reprinted with kind permission from EMBO J.).*

pancreas normally develop. No other parts of the gastrointestinal tract, including the liver, show any obvious abnormalities. IPF1 is thus required for pancreas formation and these results suggest that IPF1 acts in the pancreatic precursor cells and that IPF1 is involved in the determination of these cells, their propagation, or both.

Results

Cloning of cDNAs encoding IPF1

The ß-cell specific expression of the rat insulin I gene is dependent on 350 bp of 5' flanking DNA which harbors both a strong ß-cell specific enhancer and a promoter element with low intrinsic activity (Edlund *et al.*, 1985; Karlsson *et al.*, 1987). DNA-protein interaction studies have shown that at least four different nuclear DNA-binding proteins interact with five distinct regions within the enhancer and that one protein, IPF1, interacts with the conserved P1 promoter site, TAATGGG, which is located at position -80 to -74 (Ohlsson and Edlund, 1986; Ohlsson *et al.*, 1988, 1991). The P1 element, which is a very well conserved element of the promoter region of various mammalian insulin genes (Steiner *et al.*, 1985), resembles a binding site for homeodomain proteins. The previously isolated LIM homeodomain protein Isl-1 has been shown to bind to the P1 element *in vitro* in EMSA assays. However, by using anti-Isl-1 antibodies it was demonstrated that nuclear IPF1 was not antigenically related to Isl-1 (Karlsson *et al.*, 1990; Ohlsson *et al.*, 1991). The cDNAs encoding IPF1 were isolated from a ßTC1 λgt11 library. The full-length cDNA encodes a protein of 284 amino acids with a calculated molecular weight of 31 kDa. The deduced amino acid sequence reveals a homeodomain which is divergent from the Antennapedia prototype and which contained a unique histidine in position 45 of helix 3 (Fig. 1A, Ohlsson *et al.*, 1993). This homeodomain is not identical to any previously isolated mammalian homeodomain protein, but part of the homeodomain is highly homologous to the known part of the homeodomains of the *XlHbox8* (100%) protein from *Xenopus laevis* and the *Htr-A2* (86%) protein from the leech *Helobdella triserialis* (Fig. 1B, Ohlsson *et al.*, 1993), defining a specific class of homeotic proteins (Wright *et al.*, 1988; Weeden *et al.*, 1990). All three of these proteins contain a unique histidine in position 45 of helix 3. The rat homologue of IPF1, STF-1/IDX-1, has also been isolated (Wright *et al.*, 1988; Leonard *et al.*, 1993; Miller *et al.*, 1994).

IPF1 binds to and transactivates the insulin promoter

In vitro translated IPF1 RNA was shown to bind to the insulin promoter P1 element when analyzed in an EMSA assay (Fig. 2A, Ohlsson *et al.*, 1993). Competition studies with wild-type and mutant P1 sites (Ohlsson *et al.*, 1991, 1993) showed that the *in vitro* translation product (Fig. 2B, Ohlsson *et al.*, 1993) had the same binding specificity as the endogenous IPF1 (Ohlsson *et al.*, 1991). Anti-IPF1 antibodies were shown to block binding of nuclear IPF1 to the P1 site, but did not recognize other homeodomain proteins including Isl-1 (Fig. 2C, Ohlsson *et al.*, 1993).

Sequences immediately upstream of the insulin gene TATA box, which include the P1 promoter site, have previously been shown to be important for the transcriptional activity of the insulin 5'flanking DNA and to be preferentially active in pancreatic

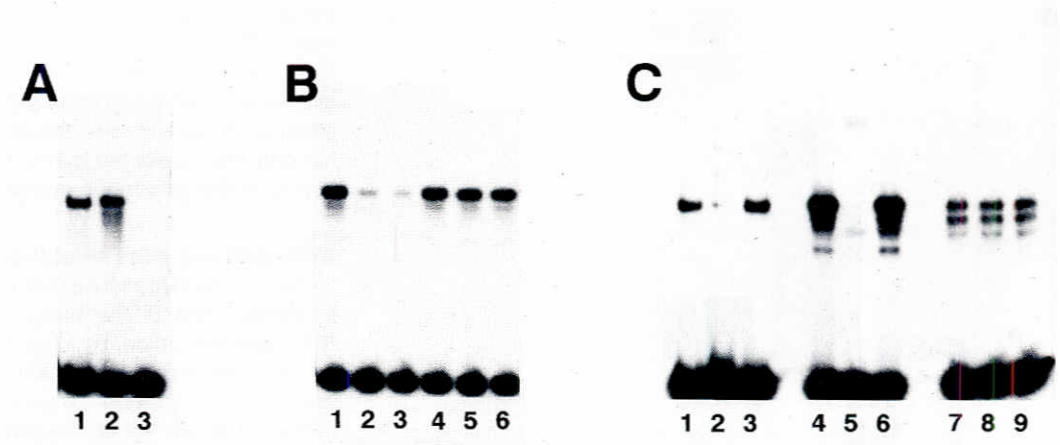

Fig. 2. DNA-binding of in vitro translated IPF1 and endogenous nuclear IPF1. (A) *Mobility of in vitro translated recombinant IPF1 and nuclear IPF1 in EMSA using the wtP1 insulin promoter site as probe (see Materials and Methods). Lane 1, nuclear extracts (3 ug) from ßTC1 cells; lane 2, in vitro translation mixture (2 ul) programmed with sense RNA of the IPF1 cDNA, and lane 3, in vitro translation mixture (2 ul) programmed with anti-sense RNA of the IPF1 cloned cDNA.* **(B)** *DNA-binding specificity of in vitro translated IPF1. Competition analysis using the wt and mutant variants of the P1 site (Ohlsson et al., 1991) as a probe and in vitro translation mixture programmed with sense IPF1 RNA. Lane 1, no competitor; lane 2, wildtype P1 site as competitor; lane 3, mutant 1 as competitor; lane 4, mutant 2 as competitor; lane 5, mutant 3 as competitor and lane 6, the insulin enhancer E2 site as competitor.* **(C)** *The protein encoded by the cloned cDNA is antigenically related to endogenous nuclear IPF1. Antisera raised against a bacterially produced trpE/IPF1 fusion protein product recognize endogenous IPF1 in ßTC1 nuclear extract. The P1 site was used in EMSA with βTC nuclear extract (lanes 1-3) and in vitro translation mixture programmed with either sense IPF1 RNA (lanes 4-6) or sense Isl-1 RNA (7-9). Lanes 1, 4 and 7 no addition of anti-sera; lanes 2, 5 and 8 antisera (2 ul) raised against the C-terminal part (including a part of the homeodomain) of the cloned protein; lanes 3, 6 and 9 preimmunesera (4 ul).*

endocrine cell lines (Edlund *et al.,* 1985). Mutations within this P1 element were shown to result in a 2.5 fold drop in activity compared to the wild type 5'flank in ßTC1 cells when analyzed in a transfection assay (Fig. 3A, Ohlsson *et al.,* 1993). The activity of the wildtype insulin 5'flank in ßTC1 cells could also be further increased by co-transfection with a vector which overexpresses IPF1 (Fig. 3A, Ohlsson *et al.,* 1993) but, as expected, the mutant 5´flank could not be transactivated by IPF1. In heterologous, non-pancreatic cells, such as CHO, IPF1 was able to transactive a construct carrying 5 copies of the P1 site linked to the ß-globin TATA box (Fig. 3B, Ohlsson *et al.,* 1993) but this construct on its own was shown to be 3-fold more active in the ßTC1 cells than in the CHO cells (Fig. 3B and C, Ohlsson *et al.,* 1993) and could be further increased by the overexpression of IPF1 in the ßTC1 cells.

IPF1 is selectively expressed in the adult pancreatic ß-cells

Northern blots reveal that IPF1 mRNA is restricted to ßTC1 cells and is undetectable in a variety of other cell lines and tissues (Ohlsson *et al.,* 1993). By performing immunohistochemical analysis on the adult mouse pancreas using anti-IPF1 antibodies, we showed that no positive IPF1 staining could be detected in the exocrine pancreas or within the ducts cells (Fig. 4A, Ohlsson *et al.,* 1993), but rather the expression was restricted to the islets. Within the islets the staining is nuclear and parallels the typical pattern for insulin producing cells, since the majority of the cells are positive and these cells are all located in the center of the islets. Double immunostaining using anti-IPF1 and anti-hormone antibodies showed that IPF1 is not present in glucagon and somatostatin producing cells (Fig. 4B and C, Ohlsson *et al.,* 1993). In summary, in the adult mouse IPF1 is restricted to the pancreatic ß-cells where it binds to and transactivates the insulin promoter, suggesting that IPF1 is directly involved in the control of the ß-cell specific activity of the insulin gene.

The early onset of IPF1 expression is correlated to pancreatic commitment of the foregut endoderm

To correlate the temporal pattern of IPF1 expression in the foregut endoderm to the stage at which the gut is assumed to commit to a pancreatic fate, we used anti-IPF1 antibodies in whole-mount immunohistochemistry on mouse embryos at different stages of development. *In vitro* explant studies, in both the rat and the mouse, suggest that the primitive gut acquires sufficient ability to develop into a pancreas around the 10-12 somite stage (Wessells and Cohen, 1967). Using whole-mount immunohistochemistry, a few IPF1 positive cells can first be detected in the gut region at around the 10 somite stage (Fig. 5A). The number of IPF1 expressing cells increases as embryogenesis proceeds. At the 15 somite stage IPF1 expression is evident in both dorsal and ventral foregut endoderm (Fig. 5B), and at 18-20 somites IPF1 is strongly expressed in both pancreas primordia which now appear as protrusions of the dorsal and ventral foregut (Fig. 5C). At this stage, the gut epithelium consists of only one cell layer and, since the majority of the cells in the pancreatic anlagen seemed to express IPF1 (Fig. 5C), we analyzed this in more detail by staining transversal sections of the gut from a 20 somite embryo with the anti-IPF1 antibodies. A representative section is shown in Fig. 5D, and the analysis of consecutive sections strongly suggests that, at this stage of development, all the cells in the dorsal and ventral pancreatic primordia express IPF1. High level IPF1 expression is maintained in the majority of the cells in the pancreatic buds at the 27 somite stage (Fig. 5E). At the 35 somite stage, when insertion of the dorsal pancreas on the gut narrows and the stalk is being formed, a large proportion

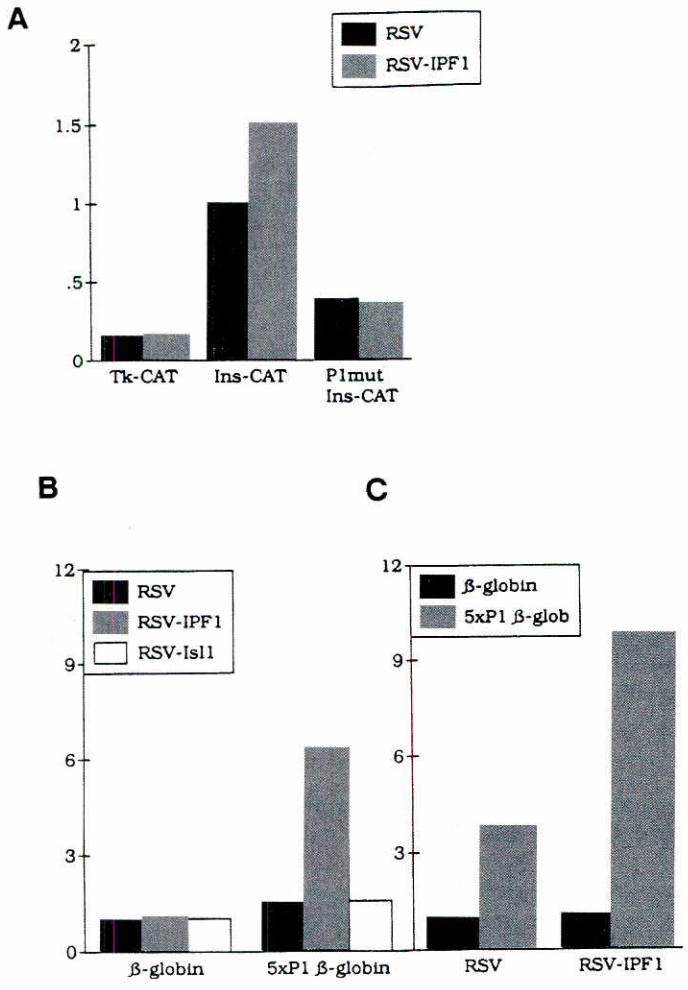

Fig. 3. Expression of the IPF1 gene in both ß-cells and non ß-cells transactivates a reporter construct via the P1-site. (A) *Mutation of the P1 promoter site results in a decreased activity of the rat insulin I 5' flank, and the stimulation of the activity of the 5'flank as a result of IPF1 expression is critically dependent on an intact P1-site. RSV and RSV/IPF1 recombinant expression vectors were cotransfected with the Tk-CAT, Ins-CAT and P1mutIns-CAT reporter genes into ßTC1 cells using an internal control ß-gal plasmid as described previously (Walker et al., 1983).* **(B)** *Multimers of the P1 site in front of a reporter gene is specifically trans-activated by the expression of the IPF1 gene in heterologous cells. RSV, RSV-IPF1 and RSV-Isl1 recombinant expression vectors were co-transfected with the ß-globin and 5xP1 ß-globin construct in CHO cells.* **(C)** *Overexpression of the IPF1 gene in ßTC1 cells results in a further up regulation of the activity of the P1 element. RSV and RSV-IPF1 recombinant expression vectors were co-transfected with the ß-globin and 5xP1 ß-globin construct in ßTC1 cells. The numbers given are normalized to the internal control and represent at least 5 independent transfection experiments. (From Ohlsson et al., 1993. Reprinted with kind permission from EMBO J.).*

of cells in the bud still express IPF1, whereas no or very few IPF1 containing cells are present in the duodenum or the stalk itself (Fig. 5F). We have also shown that at later stages of development, when the different differentiated cell types start to appear, the relative number of IPF1 expressing cells in the developing pancreas decreases dramatically (Ohlsson *et al.*, 1993). The early onset of IPF1 expression in the gut at around the time of pancreatic commitment, and the expression of IPF1 in probably all pancreatic precursor cells, suggest that IPF1 plays an important role in determination and/or differentiation of not only the ß-cells but rather in the establishment and propagation of all the pancreatic precursor cells.

IPF1-deficient mice selectively lack a pancreas

To test the hypothesis that IPF1 plays a role in the pancreatic commitment of the foregut endoderm we have generated IPF1-deficient mice by deleting exon 2, which encodes the homeodomain of IPF1 (J. Jonsson and H. Edlund, unpublished results), using homologous recombination in ESß-cells (Jonsson *et al.*, 1994). Newborn homozygous mutant mice do not show any morphological abnormalities, except that they appear slightly smaller than wildtype and heterozygous littermates (Fig. 6A, Jonsson *et al.*, 1994). The IPF1-deficient pups are initially able to feed as indicated by the presence of milk in their stomachs, but they soon become dehydrated and after a few days they become immobile and die. The homozygous *IPF1* mutants completely lack a pancreas, but there are no other apparent abnormalities. The duodenum from which the pancreas normally develops shows the normal C-shaped form (Fig. 6B and C, Jonsson *et al.*, 1994). The intestines as well as the liver and the common bile duct, which also develop from the same part of the primitive foregut as the pancreas, appear normal in size and occupy their normal positions (Fig. 7, Jonsson *et al.*, 1994). The spleen, which is, in part, thought to be derived from "pancreatic" mesoderm (Wessells and Cohen, 1967) also appears normal in size and position in the homozygous mutants (Fig. 7B, Jonsson *et al.*, 1994). Thus, we conclude that IPF1-deficiency leads to the selective loss of the pancreas. The newborn homozygotes showed elevated urine glucose levels (Jonsson *et al.*, 1994) suggesting that the symptoms observed and the cause of death are partly the result of insulin deficiency, which leads to the development of diabetes, dehydration, coma and death. The lack of the other islet hormones and exocrine digestive enzymes may also contribute to the pathology. A similar phenotype was previously observed using a diphtheria toxin gene to ablate the pancreas in transgenic mice (Palmiter *et al.*, 1987).

To exclude the possibility that in the homozygous *IPF1* mutants, morphogenesis but not cytodifferentiation, was arrested, we analyzed e15 embryos and neonates for ectopic expression of the pancreas-specific markers insulin and amylase. In normal mice, pancreatic amylase and insulin were highly and specifically expressed in the exocrine and endocrine pancreas, respectively, whereas no pancreatic tissue and no ectopic expression of insulin and amylase were detected in mutant e15 embryos or in neonates (Jonsson *et al.*, 1994) showing that both cytodifferentiation and morphogenesis of the pancreas are arrested in the homozygous mutants.

In summary, the early expression pattern of IPF1 where a few IPF1 positive cells can be detected in the gut region already at the 10 somite stage (Fig. 5A), i.e. when the foregut endoderm commits to a pancreatic fate, and the lack of a pancreas in the *Ipf1*-deficient mutants, strongly suggest that IPF1 functions in the determination and/or maintenance of the pancreatic identity

of common precursor cells, or in the regulation of their propagation.

Discussion

The transcriptional activity of the rat insulin I 5'flanking DNA is to a large extent mediated by the enhancer element which contains binding sites for a number of transacting nuclear proteins, each contributing to the overall activity of the enhancer (Edlund *et al.,* 1985; Ohlsson and Edlund 1986, 1988; Karlsson *et al.,* 1987; German *et al.,* 1992). Although the enhancer element is dominant, it has previously been shown that the proximal "promoter" sequences have a low intrinsic cell specific activity (Edlund *et al.,* 1985). Since IPF1 is restricted to the ß-cells of adult pancreas and binds to and transactivates the insulin promoter via the P1 element (Ohlsson *et al.,* 1991, 1993) it is very likely that IPF1 *in vivo* contributes to the ß-cell specific activity of the insulin promoter. The P1 element is extremely well conserved in the promoter region of mammalian Ins genes and P1 like elements are also found within the regulatory regions of other ß-cell specific genes such as glucokinase and islet amyloid peptide (German *et al.,* 1992) suggesting that IPF1 may have a more general role in the regulation of ß-cell specific gene expression. The role of IPF1 in the development and maintenance of the mature ß-cell phenotype is presently being investigated.

The ability of gut endoderm and its adherent mesoderm (whole guts) to form a pancreas *in vitro* has been studied both in the rat and the mouse, and the results show that the morphological and biochemical changes that occur during *in vitro* organ culture correlate precisely with the events occurring *in vivo* (Wessells and Cohen, 1967; Spooner *et al.,* 1970). Whole guts from 12 somite rat embryos developed both dorsal and ventral pancreases (Spooner *et al.,* 1970). Using a similar protocol in the mouse it was shown that the capacity of the gut to form pancreatic tissue is acquired around the 10-somite stage (Wessells and Cohen, 1967). Taken together, these results suggest that the gut acquires sufficient ability to develop into a pancreas around the 10 to 12 -somite stage. It has also been shown that gut endoderm from 15-20 somite mouse embryos can develop into a pancreas if combined with "non-specific" salivary mesoderm, showing that at the 15 somite stage the endoderm is determined and needs no further specific extrinsic input (Wessells and Cohen, 1967). In the mouse, cells expressing the IPF1 protein can first be detected in the primitive foregut endoderm at around the 10 somite stage (Fig. 5A) which correlates with the time at which the gut explants acquire the ability to form pancreatic tissue *in vitro* (Wessells and Cohen, 1967). At the

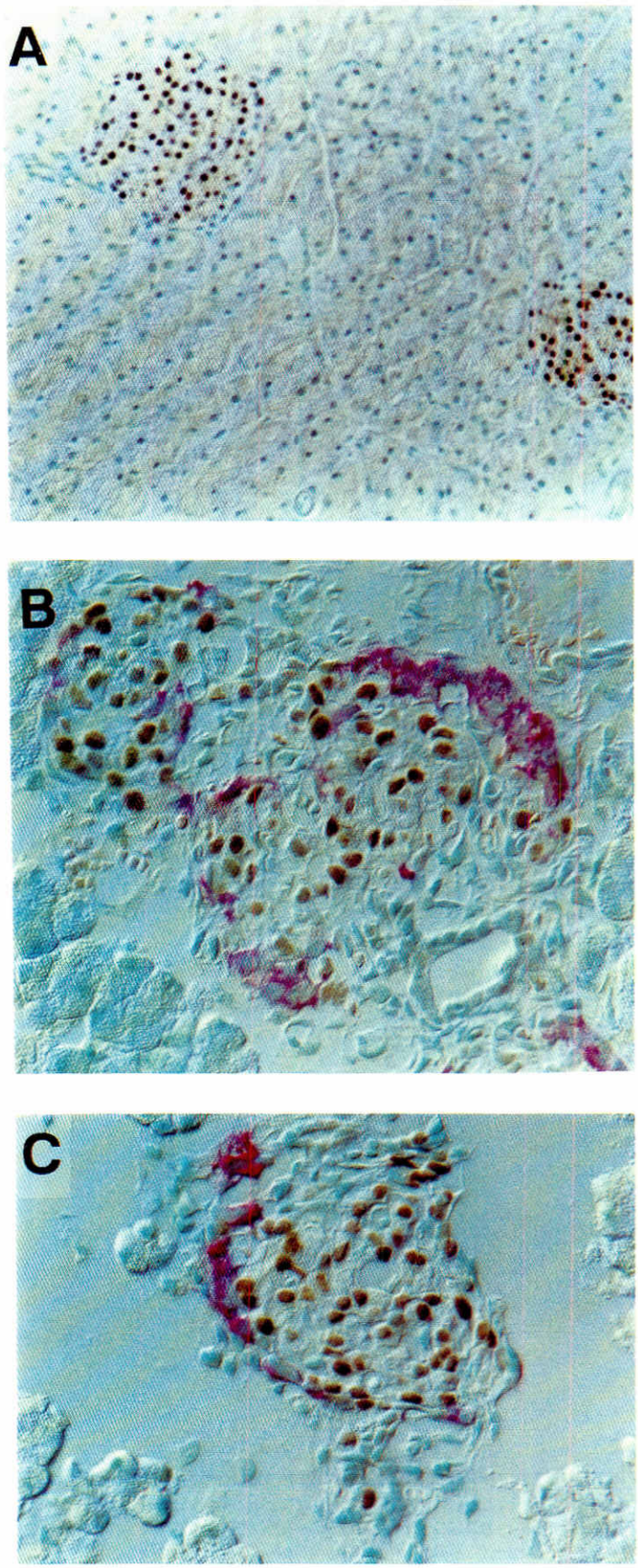

Fig. 4. IPF1 is restricted to the ß-cells of adult mouse pancreas. *Cryostat sections (8 um) from adult mouse pancreas were stained with affinity purified anti-IPF1 antibodies and anti-islet hormone antibodies.* **(A)** *Staining (dark brown) with anti-IPF1 antisera showing that IPF1 is restricted to the pancreatic islet cells and that IPF1 is localized to the nucleus.* **(B,C)** *Costaining with anti-IPF1 antisera and anti-islet hormone antibodies. The dark-brown nuclear staining represents the peroxidase staining of IPF1, and the red staining represents alkaline phosphatase staining of the different hormones.* **(B)** *Anti-glucagon antibodies.* **(C)** *Anti-somatostatin antibodies.*

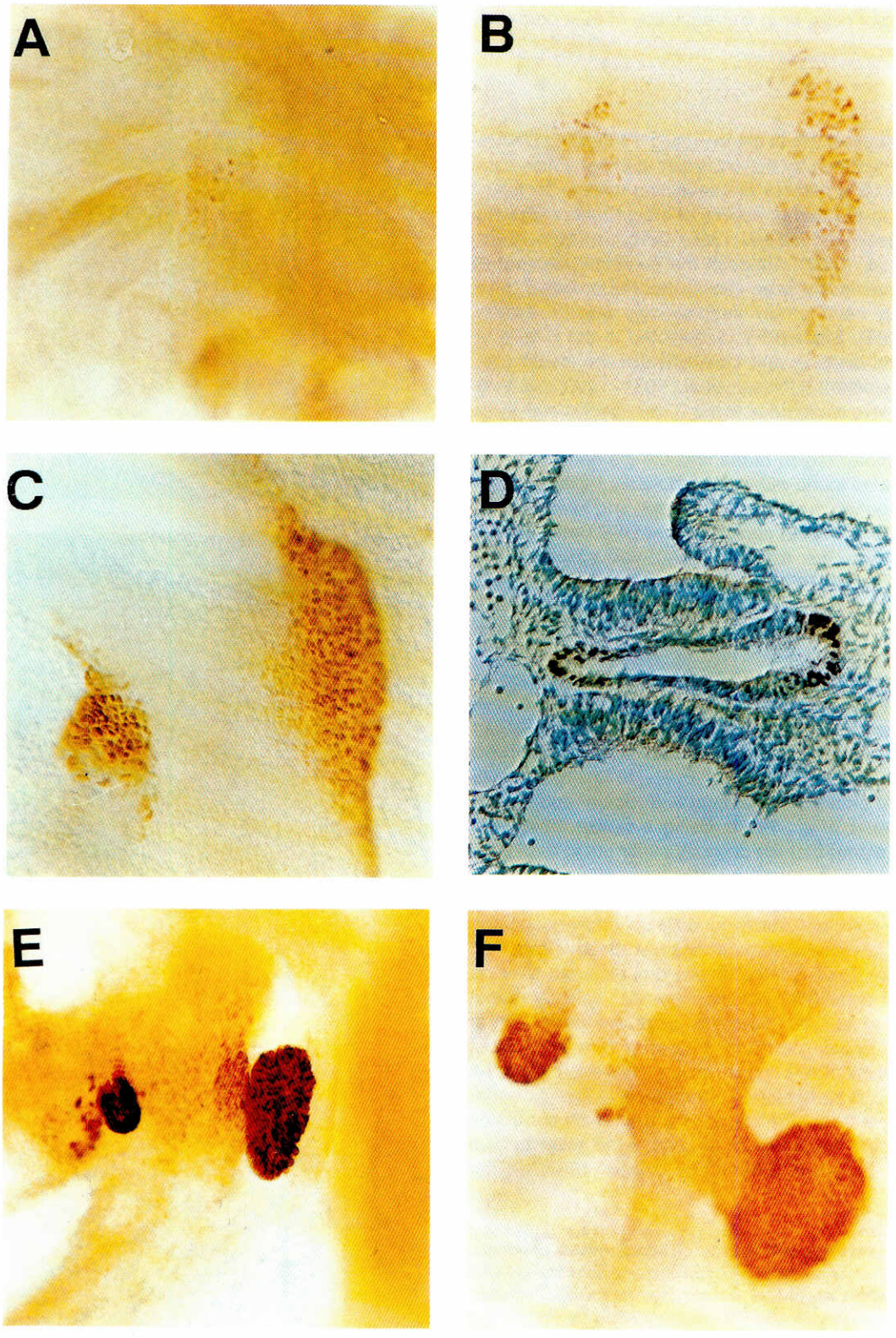

Fig. 5. IPF1 is specifically expressed in both the dorsal and ventral gut wall of early mouse embryos prior to morphogenesis and cytodifferentiation. *Whole-mount immunohistochemistry on wild-type mouse embryos* **(A,B,C,E,F)** *using anti-IPF1 antisera.* **(A)** *10-somite embryo,* **(B)** *15-somite embryo,* **(C)** *20-somite embryo,* **(E)** *27-somite embryo,* **(F)** *35-somite embryo.* **(D)** *Immunohistochemistry on a 10 μ cryostat section from a 20-somite embryo using anti-IPF1 antisera. Magnification x200.*

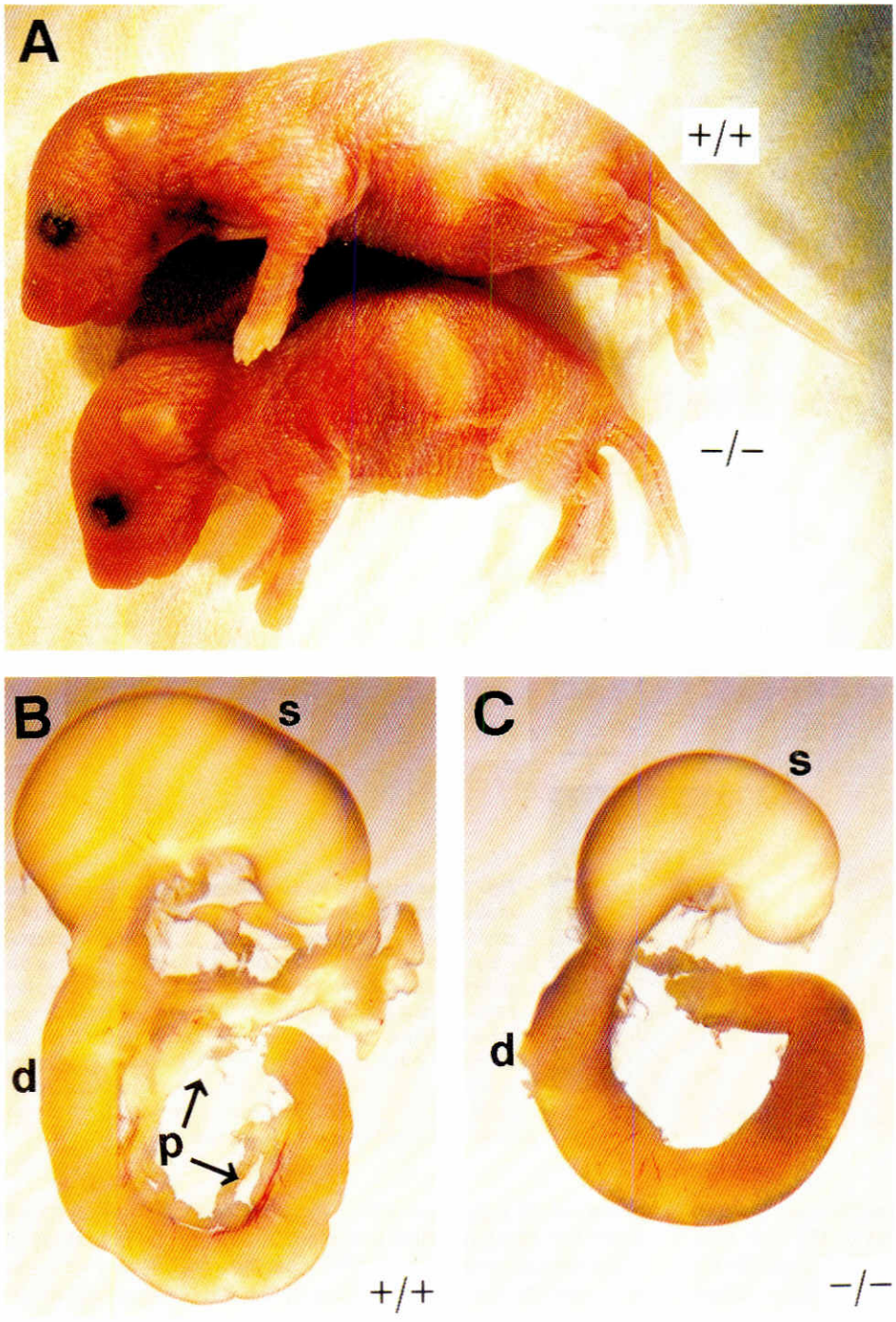

Fig. 6. Ipf1 deficient mice lack a pancreas. **(A)** *A pair of newborn littermates, wild-type (+/+) and Ipf1 (-/-) mutant.* **(B)** *Photograph of the stomach and duodenal region of a newborn wild-type mouse as it appears after removal from the abdomen. The arrows indicate the pancreas.* **(C)** *Same as in* **(B)** *but from an IPF1 (-/-) mouse. Note the complete absence of a pancreas. Abbreviations; s, stomach; d, duodenum; p, pancreas. (From Jonsson et al., 1994. Reprinted with permission from Nature. Copyright 1994 Macmillan Magazines Limited).*

early stages of pancreas development, prior to the onset of morphogenesis and cytodifferentiation, IPF1 is most likely expressed in all pancreatic precursor cells. This, and the selective loss of all pancreatic cell types in the mutant pups, suggest a function for IPF1 in the determination and/or maintenance of cell identity or propagation of a common pancreatic progenitor cell.

The structurally related *Xenopus XlHbox 8* (Wright *et al.*, 1988) and rat STF-1/IDX-1 (Leonard *et al.*, 1993; Miller *et al.*, 1994) proteins, are also selectively expressed in the endoderm of the duodenum and the pancreas but at present it is not known whether these proteins represent functional homologs of IPF1. *XlHbox8*, which, outside of the homeodomain is the most divergent of these structurally related proteins (Fig. 1C, Ohlsson *et al.*, 1993), is restricted to epithelial cells of the duodenum and the developing pancreas, but in the adults *XlHbox8* is only found in the nuclei of the pancreatic excretory ducts and no expression is observed in the pancreatic islet cells (Wright *et al.*,

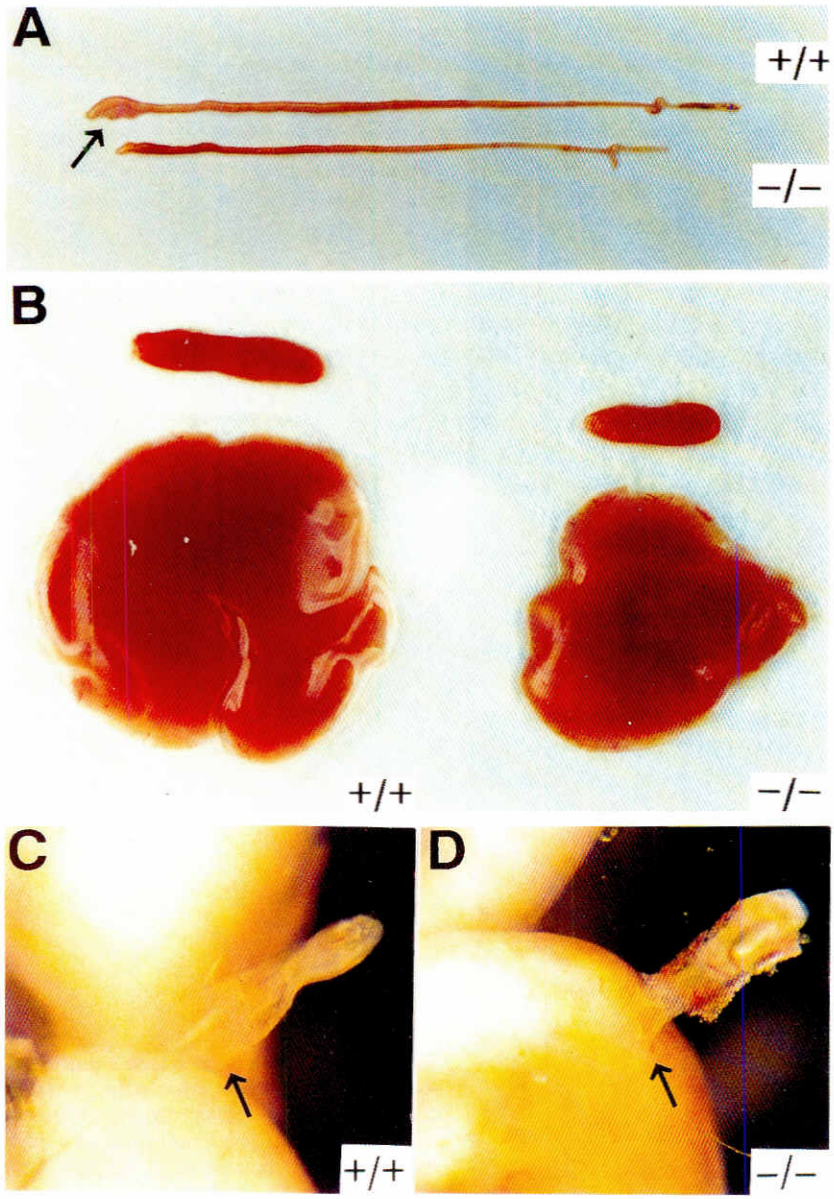

Fig. 7. The gut and its associated organs develop normally in Ipf1 -/- mice. (A) *The intestines from a pair of 2-day old wt (+/+) and mutant (-/-) pups. The arrow indicates the pancreas.* **(B)** *Spleen (top) and liver (below) from a pair of 2-day old wt (+/+) and mutant (-/-) pups.* **(C)** *Photograph showing the common bile duct of a newborn wild-type mouse. The arrow indicates the common bile duct.* **(D)** *Same as in* **(C)** *but from an IPF1 (-/-) mouse. The arrow indicates the common bile duct. (From Jonsson et al., 1994. Reprinted with permission from Nature. Copyright 1994 Macmillan Magazines Limited).*

1988). Using low stringency hybridization and PCR we have so far failed to detect any *Xenopus* counterpart of IPF1 or, conversely, a murine counterpart of *XlHbox8* (our unpublished data). The expression pattern reported for STF-1/IDX-1 differs slightly from that reported for IPF1 in that it is islet, rather than ß-cell, specific, but whether this is a true discrepancy or merely a consequence of the purity and/or specificity of the antibodies used remains to be elucidated. The LIM-homeo domain proteins Isl-1 (Karlsson *et al.*, 1990) and Imx-1 (German *et al.*, 1992), and the quail homolog of Pax-6, Pax-QNR (Turque *et al.*, 1994), are also expressed in the developing and adult pancreas. Recently a novel homeobox gene, HB9, which is also highly expressed in the pancreas has been isolated (Harrison *et al.*, 1994). The region-specific expression of IPF1, STF-1/IDX-1, XlHbox 8, Isl-1, Imx-1 and HB9 may permit the identification of putative inductive signalling pathways involved in the patterning of the primitive gut endoderm. The identification of target genes of IPF1 in these primitive endodermal cells will be important for our understanding of how IPF1 specifies the identity of these cells and for our understanding of the axial patterning of the endoderm.

It is interesting to note that when IPF1 expression is initiated around the 10 somite stage, no "pancreatic" mesoderm or even loose mesoderm is associated with the dorsal gut endoderm, which is instead in close proximity to the notochord (Wessells and Cohen, 1967). The notochord is known to be a source of inductive signals that contribute to the regionalization of the neural plate, possibly mediated by vertebrate members of the Hedgehog family of signalling molecules (Yamada *et al.*, 1991; Ericson *et al.*, 1992; Echelard *et al.*, 1993; Krauss *et al.*, 1993; Riddle *et al.*, 1993; Roelink *et al.*, 1994). A putative role of the notochord and/or Hedgehog proteins in the early inductive

events leading to IPF1 expression and the formation of the pancreas can now be studied. At later stages of development, IPF1 expression becomes gradually restricted and is only evident in the ß-cells of the adult pancreas, suggesting that identification of signalling pathways that regulate IPF1 expression may also contribute to our understanding of the generation and maturation of the pancreatic ß-cells.

Materials and Methods

Isolation of cDNA clones

PCR reactions were carried out on a ßTC1 λgt11 library using a set of degenerate oligonucleotides complementary to a consensus sequence of helix III of known homeobox genes, in combination with either of the two oligonucleotides included in the λgt11 insert screening amplimer set (Ohlsson *et al.,* 1993). The PCR product of interest was sequenced and subsequently labeled with ($a^{32}P$)dATP and used as a probe to screen the TC λgt11 in order to isolate a full-length cDNA clone.

Nuclear extract preparation, EMSA and DNA transfections

Nuclear extract was prepared from ßTC1 cells and the EMSA was carried out as previously described (Ohlsson and Edlund, 1986). The oligonucleotides used in the EMSA assays have been described (Ohlsson *et al.,* 1991, 1993). DNA transfections of ßTC1 and CHO cells were carried out as previously described (Walker *et al.,* 1983).

Immunohistochemistry

Immunohistochemistry on cryosections of mouse embryos (10 µm) and mouse tissue (8 µm) was performed as described previously (Ohlsson *et al.,* 1993; Jonsson *et al.,* 1994). IPF1 was detected with affinity-purified rabbit antibodies (Ohlsson *et al.,* 1993), glucagon with guinea pig anti-glucagon antibodies (Linco Inc), somatostatin with rabbit anti-somatostatin antibodies (Sera-Lab). The anti-IPF1 primary antibodies were detected with the ABC immunoperoxidase system (Vector Laboratories Inc.), the glucagon primary antibodies with alkaline phosphatase-conjugated goat anti guinea pig immunoglobulins (Southern Biotechnology Inc.) and the somatostatin primary antibodies with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulins (Dakopatt)

Whole-mount immunohistochemistry

Whole-mount immunohistochemistry was carried out on e8-e10 mouse embryos as described previously (Ohlsson *et al.,* 1993) but with the following modifications. Embryos were fixed in 4% paraformaldehyde in PB (0.1 M sodium-phosphate buffer pH 7.4) for 1-2 h, washed in TBST, transferred in steps into methanol, blocked for endogenous peroxidase activity in methanol containing 3% hydrogen peroxide for 30-60 min. The embryos were then transferred back to TBST. Non-specific binding was reduced by incubation in 10% normal goat serum in TBST. Antibodies were diluted in TBST with 10% normal goat serum. The primary antibodies were detected with the ABC immunoperoxidase system according to the manufacturers recommendation (Vector Laboratories Inc., USA) with the exception that the ABC complex was diluted 2-fold before incubation. After each antibody incubation, embryos were extensively washed in TBST for at least 4 h with 4-6 changes.

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