Screening for Novel Pancreatic Genes Expressed During Embryogenesis

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We have combined suppressive subtractive hybridization with in situ hybridization to identify genes expressed at early stages of pancreas development. By using polymerase chain reaction amplification and subtractive hybridization, this protocol for screening can be applied when the amount of RNA is limited. Seven genes expressed in or adjacent to the pancreas anlage were isolated, three of which show similarity to known genes. The expression pattern and sequence information indicate that some of the genes could govern pancreas development. *Diabetes* 48:1550–1556, 1999

Before morphogenesis, the dorsal endoderm caudal to the stomach anlage is committed to pancreas development and receives essential permissive signals from adjacent mesodermal structures, namely the notochord, to initiate pancreas organogenesis (1,2). The first morphological sign of pancreas development in vertebrate embryos is an endodermal evagination that forms caudal to the stomach and dorsal to the liver anlage (3,4). Soon thereafter ventral pancreas buds form at the ventral side of the duodenum, adjacent to the liver diverticulum. These dorsal and ventral epithelial evaginations grow while branching into the surrounding mesenchyme and later fuse to form the mature pancreas. Subsequent development, dependent on mesenchymal-epithelial interactions, involves yet unknown genes governing morphogenesis and cytodifferentiation.

Some of the genes necessary for pancreas development are already known, including the transcription factors *Pdx-1*, *Isl-1*, *Pax6*, *Pax4*, *Nkx2.2*, and *NeuroD*, as well as extracellular and transmembrane proteins (5,6). For example, mice lacking the gene encoding *Pdx-1*, which is expressed throughout pancreas development in wild-type embryos, are born apancreatic (7,8). Isl-1 is necessary for proper function of pancreatic mesenchyme and β-cell differentiation. Similarly, inactivation studies of *Pax6*, *Pax4*, *Nkx2.2*, and *NeuroD* show that these genes also play roles during differentiation of specific endocrine cell lineages (9–14). E-cadherin is required for β-cell aggregation and islet formation (15). Perturbation of islet architecture in certain forms of diabetes suggests that cell organization in these microorgans is important for proper function (16). Furthermore, perlecan, a heparan sulfate proteoglycan, is likely involved in the pathogenesis of islet amyloidosis, a deposition and accumulation of amylin in β-cells observed in 90% of patients with type 2 diabetes (17). Other proteins, including Sonic hedgehog (SHH), a member of the Hedgehog family of secreted factors, interfere with pancreas organogenesis and are excluded from pancreatic tissue (2,9). Overexpression of SHH under the control of the Pdx-1 promoter disturbs pancreas morphogenesis (18).

Many of the genes noted above play important roles during late stages of pancreas formation or function. The purpose of this study was to identify novel genes expressed at a developmental stage when endoderm acquires the ability to express pancreas-specific markers and the first morphological signs of organogenesis become apparent. Several different methods have been described to isolate novel gene products, including differential display (19), genetic screens, or hybridization of cDNA libraries with subtracted probes. To directly obtain potentially informative expression patterns of the genes isolated, we have combined subtractive hybridization with an in situ hybridization screen.

Various subtractive hybridization approaches have been used previously to isolate differentially expressed gene products. The application of most of these techniques is limited because of their requirement to isolate relatively large amounts of RNA from tissues of interest (20,21). To circumvent these problems, we have included an additional polymerase chain reaction (PCR) amplification step before a combined suppressive subtractive hybridization/in situ hybridization screen. During this initial procedure, small amounts of total RNA are transcribed into cDNA and subsequently amplified by PCR to yield sufficient amounts of starting material for subtraction. Here, we report the use of this method to identify genes specifically expressed in pancreas mesenchyme or epithelium during chick organogenesis. Two different subtractions were performed to enrich for either gene products expressed predominately at early stages or genes whose expression is maintained during later stages of development. Gene fragments isolated from this screen were tested by in situ hybridization, and a small percentage of these showed expression patterns confined to pancreas epithelium or mesenchyme. Sequence information revealed that the obtained fragments include chick homologues of known extracellular matrix factors and a transcription factor. Other fragments are not similar to any known genes. The results demonstrate that this combined subtraction/in situ

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EDS, Ehlers-Danlos syndrome; HHMI, Howard Hughes Medical Institute; PCR, polymerase chain reaction; SHH, Sonic hedgehog.

hybridization screen can be used to isolate novel tissue-specific gene products.

RESEARCH DESIGN AND METHODS

Chick embryos, dissection, and RNA preparation. All experiments were performed on white Leghorn chick embryos from SPAFAS (Preston, Connecticut). Tissues for tester and driver DNA were isolated from embryos grown at 38°C and staged according to Hamburger and Hamilton (22). Endoderm from stage 12 embryos was isolated using needles and forceps and used as driver tissue for subtraction A. For subtraction B, dorsal pancreas bud derived from stage 24 embryos served as driver. The dorsal part of the forming gut tube including the pancreas anlage from stage 15 embryos was used as tester for both subtractions A and B. All dissected tissues were dissolved in Trizol (Gibco-BRL, Gaithersburg, MD), and total RNA was prepared according to the manufacturer's instructions.

Suppressive subtractive hybridization. Subtractions were performed using the PCR-Select cDNA Subtraction kit (Clontech Laboratories, Palo Alto, CA). Before subtraction, total RNA from driver and tester tissue was reverse-transcribed, and cDNA was amplified using Clontech's Capfinder cDNA synthesis kit (now named Smart PCR cDNA synthesis kit). After subtraction, PCR fragments from subtractions A and B were cloned into pCRII dual and pCRII vectors, respectively (Invitrogen, Carlsbad, CA). A fifth of the ligation of subtraction A was transformed into INVαF′ competent bacteria. A total of ~8,000 clones were obtained. Bacteria were grown on Luria broth–plates containing ampicillin; single colonies were picked with toothpicks and transferred first into PCR reaction tubes and then grown overnight in 3 ml of LB-ampicillin medium to generate bacteria stocks. Bacteria in PCR tubes were microwaved for 2 min, and fragments were amplified by PCR using vector-specific primers including SP6 and T7 RNA-polymerase recognition sites. The primers used were M13 reverse primer, located 5′ of the SP6-promoter site of the fragment, and T7 primer, located 3′ of the T7-polymerase recognition site; M13 reverse primer: GGAAACAGCTATGACCATG and T7 primer: GTAATACGACTCACTAATAGGGC.

After 20 cycles of amplification (denaturation step: 94°C, 1 min [first cycle 3 min]; annealing step: 60°C, 1 min; elongation step: 72°C, 1 min), a tenth of the reaction was separated by electrophoresis in 1.5% agarose gels. Only fragments with a minimal length of 250 bp were used to generate digoxigenin riboprobes. Of 8,000 clones, ~30–40% contained subtracted fragments large enough for in situ hybridization.

PCR and primers. To estimate the efficiency of subtraction, DNAs from subtracted and unsubtracted samples were amplified by PCR to compare the relative amount of specific marker genes. Aliquots of each reaction were taken after 18, 23, 28, and 33 cycles for β-tubulin and 23, 28, 33, and 38 cycles for insulin, *Pdx-1*, *Isl-1*, and *Prox-1*. Primers used are as follows: β-tubulin: GATGAGCAGGGT GAATTTGAAGAG and TGTTACATCGGACAGTCGAGGATAC; insulin: TCTTCT GGCTCTCCTTGTCTTTC and CGGCTTCTTGGCTAGTTGCAGTAG (23); *Pdx-1*: CAAGTACATCTCCCGACCGAGG and CCCCGCTTTTTGTCTTCTTCTTTC; *Isl-1*: GGAGGACCTGGTTCCAATTCTACC and TTTCATGGAGCGGTTCCTTGCC; *Prox-1*: GCGAATGACTTTGAGGTTCCAG and CGGTATCACTCCTTGGAGGTAGAAC.

In situ hybridization, microscopy, and photography. Sections of 6–10 μm were probed by in situ hybridization (24) using sense and antisense digoxigenin riboprobes (25) or stained by immunoperoxidase techniques. Guinea pig anti-insulin (Incstar, Stillwater, MN) was used in a 1:200 dilution as primary antibody. Secondary anti-guinea pig biotin IgG antibody (Jackson Immuno Research, West Grove, PA) was used at a 1:500 dilution. After rehydration, slides were incubated in 0.3% peroxide in methanol for 30 min at room temperature and treated with primary and secondary antibodies as described (26); peroxidase activity was detected with the ABC immunoperoxidase system (Vector Labs, Burlingame, CA). Slides were photographed on a Zeiss Axiophot microscope (Jena, Germany), and images were scanned and formatted using Adobe Photoshop 3.0. Images of ethidium bromide–stained DNA fragments were black/white inverted.

RESULTS

Enrichment of tissue-specific gene fragments by suppressive subtractive hybridization. A combined PCR-based subtraction/in situ hybridization screen was used to identify novel genes expressed in or around the embryonic pancreas (Fig. 1). The tissues of interest are small, including a one cell-layer thick endodermal epithelium, and therefore it is difficult to recover RNA. Previous studies have used PCR amplification of cDNA before subtraction, although reports typically used a substantial amount of total RNA (~30 μg) or poly(A)$^+$ RNA (~4 μg) for cDNA amplification (21,27,28). We have overcome this limitation by using a

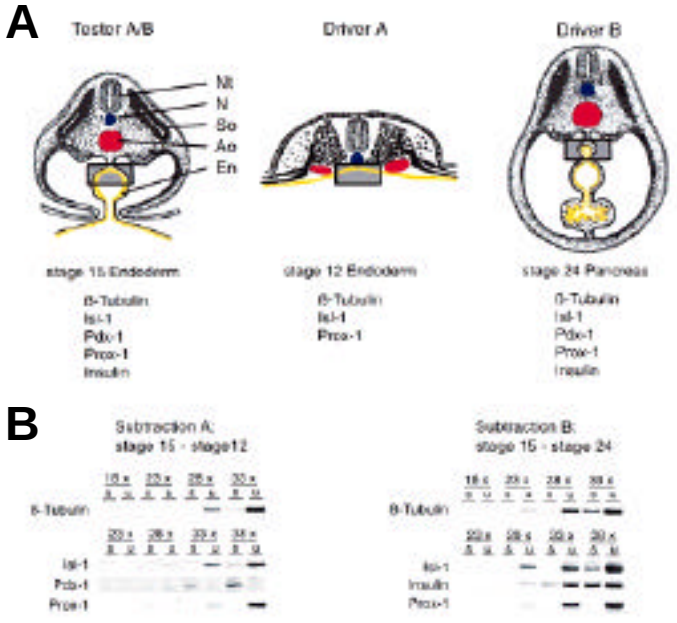

FIG. 1. *A*: Diagrams show transverse sections through chick embryos at different developmental stages. Tissues isolated for subtraction, including stage 15 dorsal endoderm (Tester A/B), stage 12 medial endoderm (Driver A), and stage 24 dorsal pancreas bud (Driver B), are shown in shaded boxes. Ao, aorta (red); En, endoderm (yellow); N, notochord (blue); Nt, neural tube; So, somite. *B*: Quantitative PCR was used to determine the efficiency of subtraction. During five cycles of amplification, PCR products are increased 10- to 20-fold. The intensities of PCR products amplified from subtracted (s) or unsubtracted (u) samples were compared to assess the efficiency of subtraction.

combination of established molecular approaches to generate sufficient cDNA for subtraction from minimal amounts of total RNA. These modifications allowed us to start subtractions with only 1 μg of total RNA, corresponding to ~3 dorsal pancreas buds isolated from stage 24 chick embryos. This can be compared with the 2 μg of poly(A)$^+$ RNA, isolated from ~200 μg of total RNA, that is normally required for these procedures (data not shown). This modified technique can be used to enrich for genes expressed in tissues, including epithelia or mesenchyme, where it is not feasible to isolate large amounts of tissue.

In general, cDNA subtraction methods rely on hybridization of cDNA derived from the tissue of interest (tester) to excess of cDNA from another tissue (driver) and the subsequent separation of hybridized common and unhybridized target sequences. We have used a modified procedure described by Diatchenko et al. (20) to enrich for genes expressed in pancreas anlage during embryogenesis, focusing on genes specifically expressed in either pancreas mesenchyme or epithelium or factors excluded from the immediate pancreas tissue. Subtraction A was designed to isolate genes that are expressed when pancreas-specific gene transcription is initiated. Subtraction B was used to enrich for genes transiently expressed during early stages of pancreas development.

In subtraction A, endoderm and adjacent mesenchyme isolated from stage 15 chick embryos served as tester tissue. As shown previously (1), stage 15 pancreatic endoderm expresses pancreas marker genes, including *Pdx-1*, insulin, and glucagon. Stage 12 endoderm, which is negative for *Pdx-1* and insulin but positive for other genes expressed in pancreas tissue, including homeobox transcription factors like *Isl-1* and

TABLE 1
Summary of sequenced clones

Degree of homology	Number of clones represented	
	Subtraction A	Subtraction B
High homology (>85%)	7 (33)	9 (19)
Low/no homology (<85%)	14 (67)	38 (81)

Data are *n* (%).

Prox-1 (9,29,30), was used as driver tissue (Fig. 1*A*). Stage 15 endoderm and stage 24 dorsal pancreas, including epithelium and mesenchyme, were used, respectively, as tester and driver for subtraction B (Fig. 1*A*).

To determine the efficiency of subtraction, we performed PCR reactions with DNA of unsubtracted or subtracted samples. Gene-specific primers directed against ubiquitously expressed genes like β-tubulin or tissue-specific genes, including *Pdx-1*, insulin, *Isl-1*, and *Prox-1*, were used. Aliquots from PCR reactions were taken after 23, 28, 33, and 38 cycles (β-tubulin: 18, 23, 28, and 33 cycles), and amplified fragments were separated by gel electrophoresis (Fig. 1*B*). Under the conditions used, the number of PCR fragments increased 10- to 20-fold during five cycles of amplification. Therefore, subtraction efficiency was determined by comparing the intensities of specific PCR fragments amplified from DNA of subtracted and unsubtracted samples. Differences in intensities of PCR fragments, after the same number of PCR cycles, indicate relative reduction or enrichment of these genes. For example, in unsubtracted samples from subtraction A, the ubiquitous marker β-tubulin was detected after 28 cycles (Fig. 1*B*, 28x). Five additional PCR cycles were necessary to detect a β-tubulin signal from DNA of subtracted samples, and the resulting amount was still four- to fivefold less intense compared with the β-tubulin fragment amplified from DNA of unsubtracted samples (Fig. 1*B*, 33x). Therefore, it was estimated that relative abundance of β-tubulin was decreased 50- to 100-fold by subtraction. *Isl-1* and *Prox-1*, also expressed in both tester and driver tissue, were decreased ~100- to 400-fold in subtracted samples. In contrast, *Pdx-1*, which is only found in tester tissue, was increased 100- to 400-fold during subtraction (Fig. 1*B*). Similar subtraction efficiencies were observed for subtraction B (Fig. 1*B*).

Summary of fragments obtained through subtraction. Subtracted fragments were cloned into appropriate vectors, and bacterial libraries for both subtractions were generated. To assess the composition of gene transcripts remaining after subtraction, 50 randomly chosen fragments from subtraction B were sequenced. Blast searches showed that 81% of the clones had low or no similarity to sequences of known genes and that 19% matched with already described genes. None of the latter clones was homologous to ribosomal or mitochondrial genes. Eight of nine fragments used for in situ hybridization displayed either ubiquitous or no expression in pancreas tissue, whereas one showed specific expression in pancreatic epithelium. This clone, B1, has high similarity to reelin, an extracellular protein characterized in humans and mice (31) (Table 1). In the mouse, reelin can be detected in liver and kidney (32), but expression in pancreas tissue has not been reported.

To identify genes whose expression is initiated at early stages of pancreas formation and subsequently maintained during development, we examined the expression pattern of clones obtained through subtraction A. Sense and antisense probes of ~160 fragments were tested, and 21 clones with expression found within or adjacent to the pancreas anlage were sequenced. Of these, seven fragments (33%) showed high similarity either to known chick genes or to homologues in other species, including a transcription factor of the forkhead-related family, *Freac-1* (33,34), and a proteoglycan, lumican (35). The remaining 14 clones (67%) displayed little or no similarity to known genes (Tables 1 and 2). No fragments homologous to ribosomal or mitochondrial genes were found, indicating that during subtraction, the number of ubiquitously expressed genes is efficiently decreased, facilitating the identification of novel tissue-specific gene products.

Expression patterns of fragments isolated through subtraction. We were interested in identifying novel genes whose expression is restricted to either epithelium or mesenchyme in or around the pancreas anlage. At stage 19, the dorsal pancreas bud has formed caudal to the stomach anlage, and pancreatic epithelium and mesenchyme are easily distinguishable in sagittal sections (Fig. 2). Only 4% (seven clones) of the 170 fragments tested from subtractions A and B reproducibly showed expression in or around the pancreas of stage 19 chick embryos (Table 2; Fig. 2, A1–A5 and B1), whereas the expression of one fragment was restricted to the forming liver (Fig. 2, A6). The remaining clones either showed widespread expression or no signal at all. The isolated genes represented all possible combinations of mesenchymal/epithelial expression patterns. Clone A1 is expressed in pancreatic epithelium and surrounding mesenchyme, whereas fragments A4 (lumican) and A5 are restricted to mesen-chyme surrounding the pan-

TABLE 2
Summary of in situ hybrydization patterns

Clone size	Homology	Gene type	Expression in or around pancreas anlage
A1	None	Unknown/sulfatase?	Pancreatic mesenchyme/epithelium
A2	None	Unknown	Ventral pancreatic mesenchyme
A3	Freac-1	Transcription factor	Stomach and duodenal mesenchyme; excluded from pancreatic mesenchyme/epithelium
A4	Lumican	Proteoglycan	Pancreatic mesenchyme
A5	None	Unknown	Pancreatic mesenchyme
A6	None	Unknown	Liver
B1	Reelin	Extracellular matrix	Pancreatic epithelium

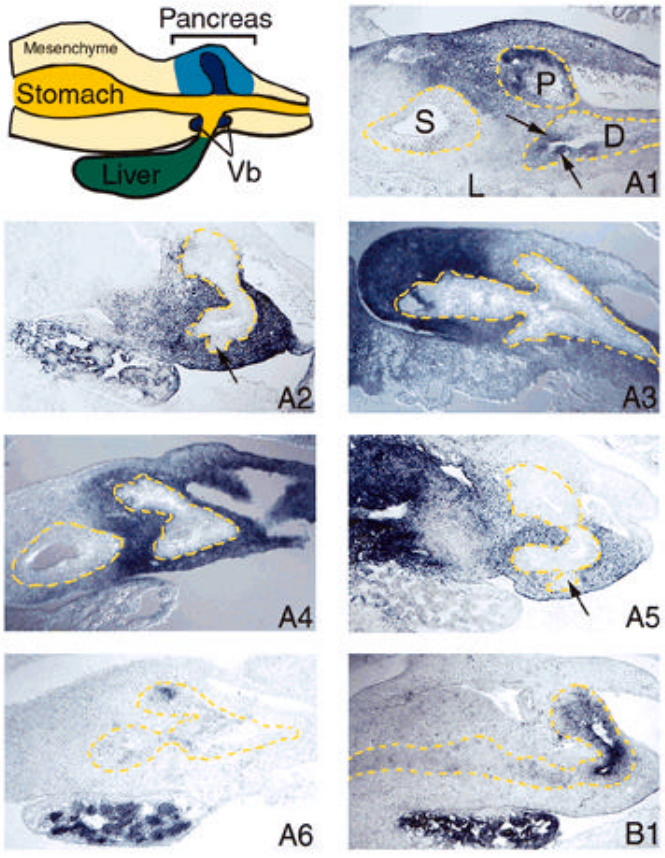

FIG. 2. In situ hybridization patterns of gene fragments obtained through subtraction. The *upper left panel* is a schematic of a sagittal section through the foregut/midgut region in stage 19 chick embryos. The dorsal pancreas bud develops immediately adjacent and posterior to the stomach, opposite the ventrally forming liver. The ventral buds are derived from the region adjacent to the liver diverticulum. At this developmental stage, epithelial and mesenchymal tissues of the foregut/midgut region are easily distinguishable. In the forming pancreas, including dorsal and ventral buds, the mesenchyme is shaded in light and the epithelial region in dark blue. Six gene fragments derived from subtraction A (A1–A6) and one gene from subtraction B (B1) are expressed in or around the pancreatic anlage. The dotted yellow line demarcates the epithelium of the stomach, pancreas, and intestine, and arrows indicate forming ventral pancreas buds. D, duodenum; L, liver; P, dorsal pancreas bud; S, stomach; Vb, ventral pancreas buds.

creatic anlage. The expression pattern of two other genes, A2 and A3, are also confined to mesenchyme. Clone A2 is restricted to the ventral parts of the pancreatic mesenchyme and cannot be detected in the tissue surrounding dorsal bud epithelium. Gene fragment A3 (*Freac-1*), which is expressed in mesenchyme anterior and posterior to the pancreas anlage, is excluded from mesenchymal tissue adjacent to the dorsal and ventral pancreas buds. In contrast, clone B1 (reelin), obtained through subtraction B, is expressed in pancreas epithelium of the forming dorsal and ventral buds, as well as in the forming liver. Interestingly, as predicted from the strategy of subtraction B, reelin is only detectable during early stages of pancreas development. Fragment A6 is not expressed in the pancreas tissue, but can be found immediately adjacent to the ventral pancreas buds in the developing liver.

In summary, the combination of subtraction with an in situ hybridization screen led to the isolation of genes expressed in pancreas anlage and provided information about possible function based on expression patterns.

Restricted expression patterns of A2 and A6. Clone A2 was further investigated because of its dynamic expression pattern at the foregut/midgut region of stage 19 chick embryos (Fig. 3*A*). In transverse sections anterior to the pancreas anlage, A2 expression is confined to ventral stomach mesenchyme (Fig. 3*A*, *left panel*). In more posterior sections, A2 is found throughout the mesenchyme adjacent to duodenal epithelium (Fig. 3*A*, *right panel*). At the level of the developing pancreas, expression is only seen in ventral mesenchyme surrounding duodenum and forming ventral pancreas bud(s), and no staining is detected in dorsal pancreas mesenchyme (Figs. 2 and 3*A*, *middle panel*). Therefore, A2 is expressed in a dorsoventral gradient at the foregut-to-midgut transition. Previous studies have reported differential expression of genes in dorsal and ventral bud formation. Isl-1 has been shown to be necessary for proper dorsal bud development, as well as for formation of endocrine lineages. In contrast to A2, Isl-1 is differentially expressed in dorsal, but not ventral, pancreas mesenchyme (9), further indicating that development of dorsal and ventral buds is regulated by differentially expressed gene products.

One advantage of the combined subtraction/in situ hybridization screen is the isolation of gene products expressed in specific regions outside the immediate pancreas anlage. It can be speculated that some of these factors, such as Freac-1 (Fig. 2, *A3*), interfere or restrict pancreas development, and therefore have to be excluded from the pancreas anlage. Others, such as A6, a ~500-bp fragment without similarity to described genes, were expressed in adjacent organs. In stage 17 chick embryos, A6 can be detected caudal to the heart primordium and ventral to the stomach, the region in which the liver forms (Fig. 3*B*). During later stages of development, expression of A6 is confined to liver (Fig. 3*B*) and may be a specific marker for developing liver.

A1 has highest similarity to sulfatase-like proteins and is expressed in pancreas at different stages of development. The expression pattern and possible function of A1 was further investigated because of its interesting expression at the border of pancreas epithelium and mesenchyme in stage 19 chick embryos (Fig. 2). In situ hybridization of transverse sections at this stage shows that A1 is also expressed in a proximomedial region of the forming limb buds and in the ventral floor plate of the neural tube (Fig. 4*A*).

Subtraction A was designed to isolate genes whose expression is maintained through pancreas development. In contrast to Pdx-1, which is expressed in epithelium of stomach and pancreas, A1 is detected in stomach mesenchyme but not epithelium (Figs. 2 and 4*B*). To investigate whether A1 expression is maintained when islets are formed, we performed in situ hybridizations on stage 35 pancreas tissue. When compared with insulin and *Pdx-1*, A1 appears to be expressed in some, but not all, islets (Fig. 4*C*).

The size of the full-length gene product of A1, as determined by Northern hybridization, is ~4.7 kb. National Center for Biotechnology Information–Blast analysis of a 2-kb partial clone isolated from a day-5 chick cDNA library revealed a stretch of 100 amino acids with highest similarity (64% identity) to a sulfatase-like protein from *Caenorhabditis elegans*. Furthermore, the same region shows 53 and 50% identity to the N-acetylglucosamine-6-sulfatase precursor proteins from goat and human, respectively (data not shown).

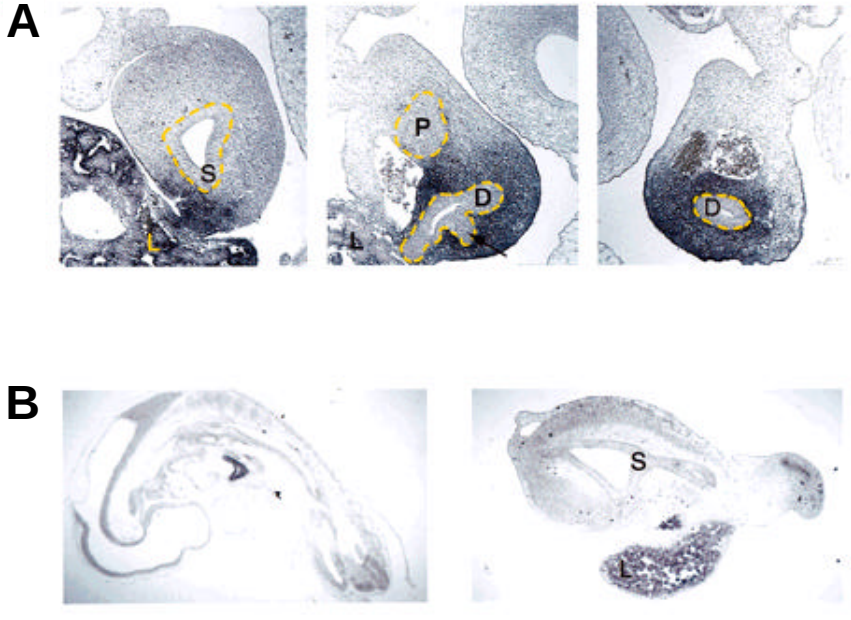

FIG. 3. ***A*****: Expression pattern of A2 along the anterior-posterior axis of the gut. Expression is confined to ventral mesenchyme at the stomach level (*left panel*) and extends dorsally at the level of the duodenum (*right panel*). A2 is expressed in ventral pancreas mesenchyme but excluded from dorsal pancreas mesenchyme (*middle panel*). Epithelial expression of A2 is not observed. The arrow points to the forming ventral bud. A blood vessel is apparent in the *middle* and *left panels* on the dorsal side (top) of the duodenum.** ***B*****: Expression of A6 at different stages of development is confined to liver tissue. A6 marks early liver cells at 3 days of development (*left panel*), whereas other areas, including stomach epithelium and mesenchyme, do not express A6. Also shown (*right panel*) is a sagittal section through stomach and liver at stage 24; A6 expression is limited to liver. D, duodenum; L, liver; P, pancreas; S, stomach.**

DISCUSSION

We have used a suppressive subtractive hybridization method (20) to isolate tissue-specific genes expressed at the onset of pancreas formation. The small size of our target tissues prohibited the isolation of sufficient amounts of poly(A)$^+$ RNA and required cDNA amplification by PCR before subtraction. This modification allows one to generate adequate quantities of cDNA from as little as 1 µg of total RNA and makes possible the isolation of genes with tissue-specific expression patterns when limited material is available.

The two subtractions performed were tested for the relative abundance or representation of specific genes present in the tester and the driver. During both subtractions A and B, products from these genes, including β-tubulin, Isl-1, and Prox-1, were decreased up to 400-fold. Other genes, including insulin, were only modestly affected and were depleted up to 20-fold in subtracted samples. In subtraction A, Pdx-1, which is exclusively expressed in the tester, was increased up to 400-fold, indicating that low abundant genes were enriched during subtraction (Fig. 1*B*). These differences in subtraction efficiency are consistent with the idea that certain sequences may not rehybridize or be amplified as well as others (27,36). It also has been noted that in subtractions in which the difference between tester and driver pool is small, a higher background of cDNAs common to both tissues occurs (37).

A total of 68 fragments from subtractions A and B were sequenced, and only 26% show similarity to known genes. This low percentage may be explained by the fact that the subtracted fragments were short in length and may correspond to regions, e.g., 5′ parts of known genes, which have not been fully sequenced and are absent in nucleotide databases. Furthermore, other studies have described similar results (38,39). Gubbay et al. (39) have analyzed a library generated from PCR-based subtractive hybridization of cDNA isolated from normal and regressing rat prostates and have reported that only 30% percent of the clones were similar to genes of known function. Notably, none of the 68 clones sequenced from subtractions A and B corresponded to ubiquitously expressed mitochondrial genes or ribosomal RNAs,

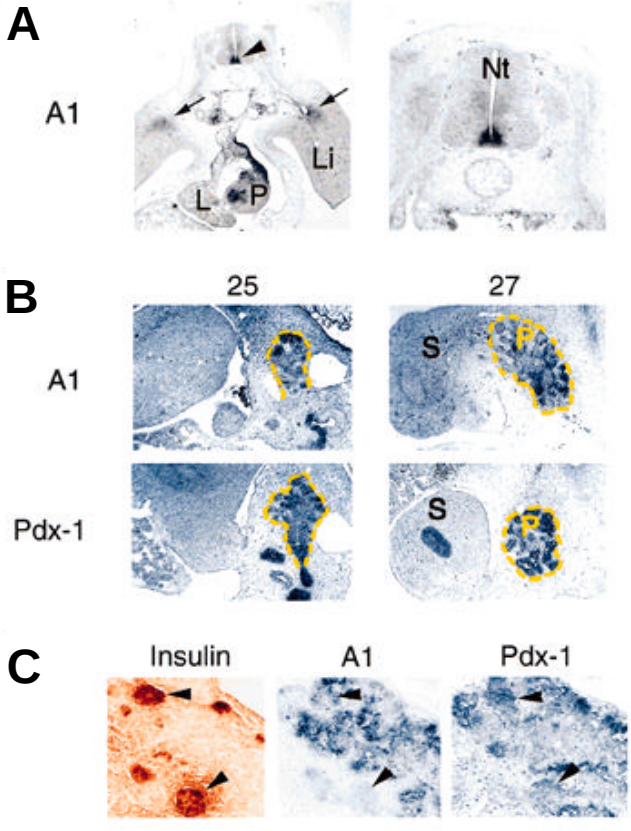

FIG. 4. A1 expression at different stages of development. ***A*****: Transverse section through the pancreas region of a stage 19 chick embryo (*left panel*). In addition to expression in the pancreas area (P), A1 can be detected in proximomedial parts of the forelimb buds (▶), as well as in neural tissue (▶). A close-up of the neural tube in the *right panel* shows expression of A1 in the ventral floor plate.** ***B*****: Expression of A1 at stage 25 and 27 is similar to, but distinct from, expression of Pdx-1 in the pancreas region. Both genes are expressed in pancreas epithelium, but Pdx is also found in stomach epithelium, whereas A1 is expressed in stomach mesenchyme.** ***C*****: At stage 35, when the first islets form during chick embryogenesis, A1 is colocalized with insulin and Pdx-1 in some, but not all, islets (▶). L, liver; Li, limb bud; Nt, neural tube; P, pancreas; S, stomach.**

suggesting that these sequences were efficiently depleted during subtraction.

B1, a 400-bp fragment derived from subtraction B, shows high similarity to reelin, an extracellular matrix protein known in mice and humans. A spontaneous mutation occurred in Reeler mice in which a substantial part of the reelin gene has been removed. These mice have impaired motor coordination due to severed migration of neurons in the cerebellar and cerebral cortex (31). We have cloned a part of the mouse reelin gene and investigated whether the homologue is expressed in mouse pancreas at a comparable stage of development. So far, we have not been able to observe expression at early stages (data not shown). Considering the small size fragment of B1, an alternative explanation is that the isolated chick gene is not the homologue of mouse reelin.

Genes A2, A3, A4, and A5 are all mesenchyme-specific. A2 may specify an unknown factor expressed in a dynamic pattern at the foregut-midgut boundary. In transverse sections through the pancreas anlage, the expression of A2 is confined to the ventral part, surrounding the forming ventral pancreas buds, but excluded from mesenchyme immediately adjacent to the dorsal bud (Figs. 2 and 3*A*). The diversity of dorsal and ventral pancreatic mesenchyme has been described previously. Ahlgren et al. (9) have shown that Isl-1 expression is confined to dorsal and excluded from ventral mesenchyme. Dorsal mesenchyme does not form in Isl-1 deficient mutants, leading to failure of exocrine cell differentiation in dorsal pancreas tissue (9). In contrast to Isl-1, A2 is expressed in ventral, but not dorsal, mesenchyme and may be a useful marker for investigating ventral-specific pathways.

Gene A3 is excluded from pancreas mesenchyme but expressed in adjacent regions, including mesenchyme of stomach and duodenum (Fig. 2). A3 codes for Freac, a forkhead-related activator of transcription, with highest similarity to Freac-1 in humans and HFH-8 (HNF-3/forkhead homolog) in mice (33,34). Similar to A3 expression in chick embryos, HFH-8 has been detected in lung and intestinal tissue in mice. In humans and mice, Freac-1 is known to activate transcription of lung-specific genes, including pulmonary surfactant proteins (33,34). We are investigating whether exclusion of Freac-1 from prepancreatic tissues, as shown for SHH (2), is important for pancreas development.

In contrast to A3, lumican (A4), a keratan sulfate proteoglycan, is expressed in pancreas mesenchyme surrounding the dorsal bud and ventral anlage at stage 19 (Fig. 2). Chick lumican has been cloned previously and was shown to be expressed in cornea, intestine, and muscle (35). Mice homozygous for a null mutation in lumican display abnormalities resembling certain types of Ehlers-Danlos syndrome (EDS), a hereditary disease affecting connective tissue in humans (40). Fibrosis of islets of Langerhans in pancreas has been reported from a sporadic case of a variant of EDS (41), raising the possibility of a connection between lumican and pancreatic architecture.

The expression pattern of A1 is unique in that it marks both pancreatic mesenchyme and epithelium of dorsal and ventral buds (Figs. 2 and 4*A*). This expression pattern overlaps with that of Isl-1, which is also expressed in pancreas and neural tissue (9,42). Sequence analysis has shown that a part of the A1 gene has similarity to sulfatases that degrade extracellular matrix proteins, including keratan- and dermatansulfates (43). During normal development, dorsal and ventral pancreatic buds invade the surrounding mesenchyme, an event that may require regulated breakdown of extracellular matrix proteins, and the expression pattern of A1 suggests a role in this process (Figs. 2 and 4*B*). Interestingly, a specific heparan sulfate known as perlecan has been associated with fibrillar amylin deposition in the islets of Langerhans of type 2 diabetic patients. Islet amyloidosis is observed in 90% of type 2 diabetic patients, suggesting that perturbed degradation of extracellular matrix proteins might be involved in disease formation (17). Future experiments will show whether A1 is a new member of the sulfatase family and whether it functions in pancreas morphogenesis.

Recently, it has been shown that several proteins expressed during early stages of pancreas formation are necessary for proper function of the mature organ. Mutations in these genes, including Pdx-1 and Beta2/NeuroD, can lead to some forms of diabetes (10,44,45). Our approach using suppressive subtractive hybridizations combined with in situ hybridization has led to the isolation and characterization of novel and known genes expressed in or around pancreas tissue during organogenesis. Future experiments will cover further screening, as well as functional tests, including gain and loss of function studies in chick and mice. One goal of this work will be to determine whether the genes identified by this approach regulate pancreatic function in adults.

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