Identification of differentially expressed genes induced in pancreatic islet neogenesis

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Abstract Cellophane wrapping of the hamster pancreas induces islet neogenesis. We have used the mRNA differential display technique to select for genes expressed during islet neogenesis but not in control pancreata. Ten candidate clones have been identified. Upon sequencing, 6 clones showed a high degree of homology to known genes, 1 showed some, and 3 showed no homology to genes of known sequence. Thus, mRNA differential display is a useful technique to identify genes induced during islet neogenesis, and in combination with screening hamster pancreatic cDNA libraries for full length clones, will enhance the likelihood of capturing the participants in this process.

Key words: Islet neogenesis; Cellophane wrap; mRNA differential display; Hamster

1. Introduction

We have reported previously that cellophane wrapping (CW) of the hamster pancreas induces islet neogenesis from pancreatic ductal cells which are capable of proliferating and differentiating upon stimulation into adult islets capable of secreting insulin in a fully regulated manner [1–3]. The appearance of islet cell hormones in the duct of CW pancreata reiterates that seen during normal pancreatic embryogenesis (glucagon message preceding insulin message, and glucagon protein preceding insulin protein) [4]. This process involves activation of genes to initiate proliferation, differentiation and growth of stem cells. Much is known about other cell types such as bone marrow stem cells [5], but little is known about pancreatic islets. Elucidation of the genes that are activated in the pancreatic islet regenerative process has only begun. It is hoped that identifying these genes will provide new insights into the mechanisms involved in the islet regenerative process.

One approach to identifying and characterizing the genes responsible for regeneration is to compare those expressed in sham-operated versus CW pancreata. The identification of alterations in gene expression induced by CW has been undertaken primarily by the 'candidate gene' approach [1,6]. These methods only permit screening of known molecules on an individual basis but fail to identify new genes that function to regulate growth in these specific circumstances. A promising new technique called mRNA differential display (DD) has been recently described and used to identify differences in subsets of mRNA samples [7–9]. The key element of this technique is to use sets of anchored and arbitrary primers to generate cDNA fragments by reverse transcription, followed by polymerase chain reaction (RT-PCR). The cDNA fragments are resolved and compared on sequencing gels. The resulting cDNA patterns reflect differences in the mRNA levels and composition between control and experimental animals. Theoretical calculations and experimental results confirm that the method can indeed generate patterns of bands which might represent almost all expressed genes in a particular cell [10]. Differentially displayed cDNAs are isolated, cloned, sequenced and used as probes on Northern blots to confirm differences in the particular mRNA level. This method has the advantage of being very sensitive, reproducible and can detect quantitative as well as qualitative changes in gene expression [11,12].

We report here the use and validation of this method to study differential gene expression in the CW-induced pancreatic islet regeneration and the isolation of 10 clones uniquely or differentially expressed in the regenerating pancreas.

2. Materials and methods

2.1. Experimental approach

We used reverse transcription-PCR of genes differentially expressed in pancreata undergoing islet neogenesis. The templates used were total RNA pooled from both control and wrapped hamster pancreata. Pooled pancreata were used to exclude differences in the level of gene expression resulting from variable degrees of islet regeneration in the different animals. Those cDNA bands demonstrating differential expression were isolated and used directly from PCR reamplification as probes on Northern blots to confirm the differential expression between CW and control non-regenerating tissue. The samples in which differential expression was confirmed were subcloned and Northern results reconfirmed. Northern blots shown in the text are derived from these subclones. The differentially expressed clones were further subjected to sequence analysis.

2.2. Isolation of differentially expressed mRNAs

Differential display was performed as described [9]. Total cellular RNA was isolated from control and CW hamster pancreas using the guanidine isothiocyanate method [13]. Four reverse transcription reactions were performed for each RNA sample using 0.2 μg of DNA-free total RNA in 1 × reverse transcription buffer, 25 μM each dGTP, dATP, dCTP, dTTP, 1 μM of either T12VG, T12VA, T12VC or T12VT (where V is 3-fold degenerate for G, A and C) and 100 units of moloney murine leukemia virus (M-MLV) reverse transcriptase (RT) and incubation at 37°C for 1 h. This reaction was performed in duplicate in order to minimize errors in the PCR procedure and appearance of false positive bands. The resulting cDNA was amplified using the PCR with 0.2 μM 10mer arbitrary primers, 1 × PCR buffer, 1 μM of the respective

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T12VN, 2 μM dNTPs, 2 units of AmpliTaq DNA polymerase (Boehringer-Mannheim, Indianapolis, IN) and 10 μCi [^{35}S]dATP. The cycling parameters were as follows: 94°C for 30 s, 40°C for 2 min, 72°C for 30 s for 40 cycles followed by 72°C for 7 min. To assure that the RNA samples did not have any remaining chromosomal DNA contamination we performed the PCR reaction in the absence of reverse transcriptase which resulted in complete disappearance of bands in the display. The amplified cDNAs were then separated on a 6% DNA sequencing gel. Bands showing differential expression were cut out and DNA was eluted and reamplified using appropriate primers and conditions described above except no radioisotope was added. The PCR product was visualized on a 1.5% agarose gel to verify size, eluted and used for subcloning or Northern blot analysis. Reamplified cDNAs were cloned into the PCR II vector using the TA cloning kit (Invitrogen, San Diego, CA) and digested with *Eco*RI to confirm the size of cDNA inserts. Clones that showed positive Northern blot analysis were sequenced using TaqTrack sequencing system (Promega, Madison, WI) and analyzed on a 8 M urea/6% acrylamide DNA sequencing gel. The cDNAs sequences were compared to those contained in the GENEBank and EMBL data base (PC/Gene by IntelliGenetics).

2.3. Quantification of gene expression by Northern blot analysis

For Northern blotting, 30 μg of heat denatured total RNA were separated on a 1.2% agarose, 0.6% formaldehyde/MOPS denaturing gel, transferred to nylon membrane (Nytran, Schleicher & Schuell, Keene, NH) and immobilized by baking at 80°C. cDNA probes used in Northern hybridization were labeled through the incorporation of [α^{32}P]dCTP (Dupont-New England Nuclear, Boston, MA) using random primed synthesis to a specific activity of $>1 \times 10^9$cpm/ug. A [γ^{32}P]ATP (Dupont-New England Nuclear) 5′-end-labeled 24mer synthetic oligonucleotide for 18S was used as a loading control. Prehybridization and hybridization buffers consisted of 50% formamide, 5 × SSPE, 1% SDS, 5 × Denhardt's, and 200 μg/ml denatured salmon sperm DNA. Prehybridizations were performed at 50°C for 4 h and hybridizations at 50°C for 16–18 h with $1–5 \times 10^6$ cpm/ml of the appropriate radiolabeled probe. Washes were performed twice for 15 min each in 6 × SSPE 0.1% SDS at 25°C; 1 × SSPE 0.1% SDS at 37°C; and under stringent conditions in 0.1 × SSPE 0.1% SDS at 65°C. Autoradiography was performed at −80°C for 1 h to 5 days by exposing scientific imaging film (X-Omat/AR, Kodak, Rochester, NY).

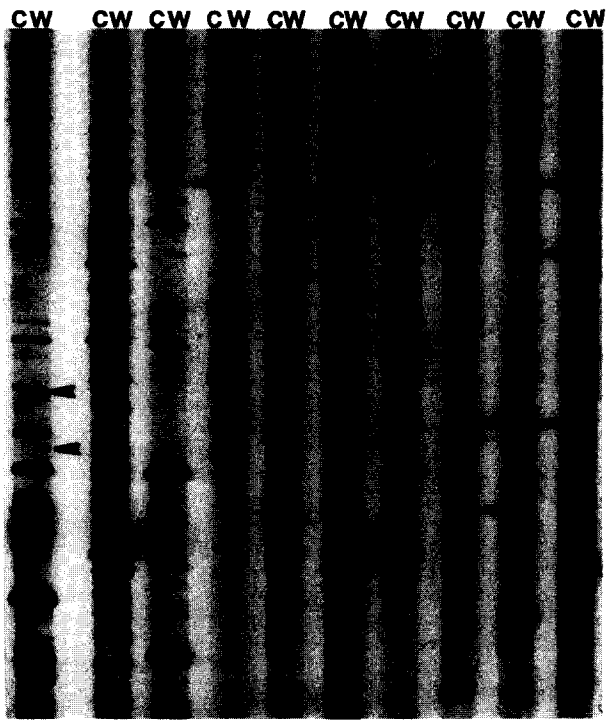

Fig. 1. mRNA differential display. Each primer combination displayed 100–200 bands. Candidate cDNAs which demonstrated altered expression between control (C) and cellophane wrapped (W) are marked by arrows.

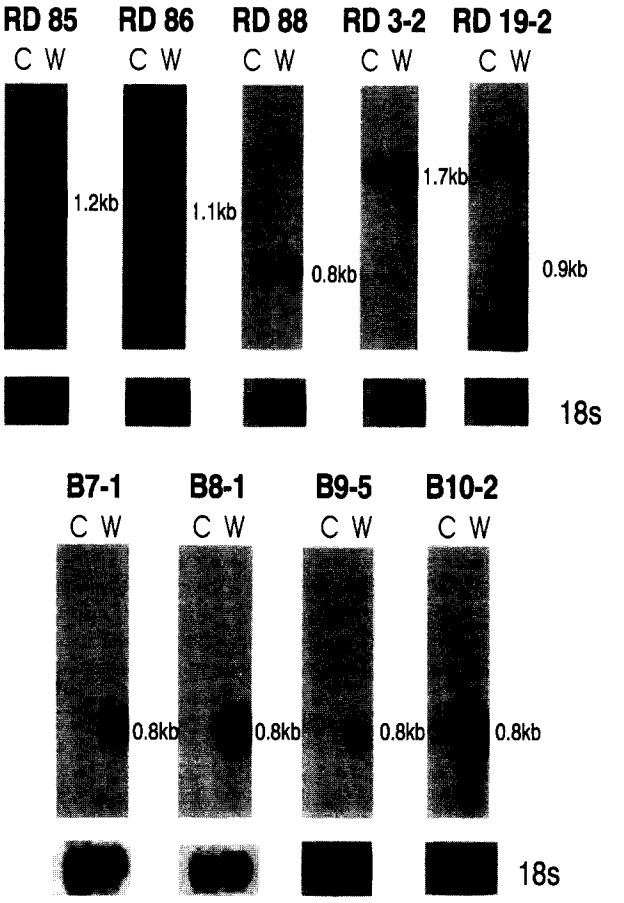

Fig. 2. Northern blot analysis of differential display clones. Heat-denatured total RNA derived from 10 pooled control (C) and cellophane wrapped (W) pancreata were separated on 1.2% agarose gel and transferred to Nytran nylon membrane. [^{32}P]dCTP-labeled cDNA probes were used to hybridize to the membrane. Bottom panel: the same membrane reprobed with a 5′-end-labeled 24mer synthetic oligonucleotide for the 18S ribosomal mRNA as a loading control. The location of the transcripts is indicated.

3. Results

3.1. Uniquely expressed mRNAs in the CW pancreas detected by mRNA differential display

RNA isolated from non-wrapped pancreas and 2-day wrapped pancreas were used in an attempt to isolate putative genes involved in pancreatic islet neogenesis. For each RNA, 80 combinations of primer sets made of 4 degenerate anchored oligo(dT) primers, T12MA, T12MC, T12MG, or T12MT, and 20 decamer arbitrary primers (AP1–20) were used for RT-PCR to obtain differential display bands. Reactions were performed in duplicate, and those showing uniquely expressed bands in the wrapped pancreas were repeated to ensure reproducibility. Twenty-five fragments were uniquely expressed in the 2-day wrapped pancreas (Fig. 1). These fragments were reamplified and used as probes in Northern hybridization.

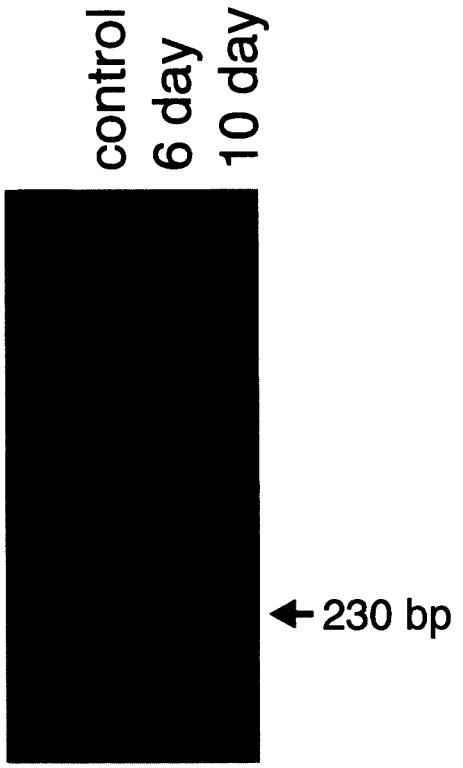

Fig. 3. Silver-stained polyacrylamide gel showing RD6-3 gene expression in whole pancreas from control animals and after 6 and 10 days of wrapping. PCR was performed using 2 mg of total RNA under conditions described in section 2. Aliquots of the amplified products were fractionated on a 5% polyacrylamide gel and silver stained.

3.2. *Quantification and sequence analysis of differentially expressed mRNAs in the CW pancreas*

Northern blot analysis confirmed unique or differential expression of 9 out of 25 candidate genes in wrapped pancreas: RD3-2, RD19-2, B7-1, B8-1, B9-5, B10-2, RD85, RD86 and RD88 (Fig. 2). Six failed to detect any signal, and the remaining 10 were false positive. The cDNAs which failed to detect any signal were sequenced and PCR amplified using specific primers; however, only 1 showed any signal after amplification, RD6-3 (Fig. 3). The remainder continued to be undetectable, presumable due to very low mRNA levels. The 10 candidate clones were individually subcloned into the TA cloning vector. For reconfirmation, Northern blots were screened using ^{32}P-labeled DNA from both the PCR fragments and subcloned inserts with equivalent results. Upon sequencing and comparison of the 10 clones with the GenBank database, 6 clones showed very high homology to known genes: RD3-2 corresponded to cytochrome *c* oxidase; RD6-3 corresponded to ubiquitin conjugating enzyme; RD85 corresponded to elastase I and B7-1, B8-1 and B10-2 corresponded to *Reg* family of genes. Due to the area of the sequence (being in the 3′-end) and size, we cannot conclude whether it is *Reg-I* or *Reg-II* gene. One clone, RD19-2, showed some homology to the *Reg* and PAP family of genes, and the remaining 3 showed no homology to any known genes.

3.3. *Temporal expression of the candidate genes in the hamster pancreas*

To delineate the phase of islet regeneration in which the candidate genes might be involved, we performed temporal gene expression experiments that demonstrated that there was a slightly increased expression of RD85 after wrapping which progressed with time (Fig. 4A). Expression of RD3-2 mRNA peaked at 2–4 days and decreased to control level thereafter (Fig. 4B). Expression of B7-1, B8-1 and B10-2 was increased at 12 h to 4 days, markedly decreased at 6 days and was absent from all other samples (Fig. 4C). B9-5 expression was absent from control non-wrapped samples but was increased at 1, 2 and 4 days after CW and disappeared thereafter (Fig. 4D). Increased expression of RD6-3 was observed at 6 and 10 days after cellophane wrapping (Fig. 3). Expression of RD19-2 was maximal at 1–2 days of CW without expression at any other time point (Fig. 5).

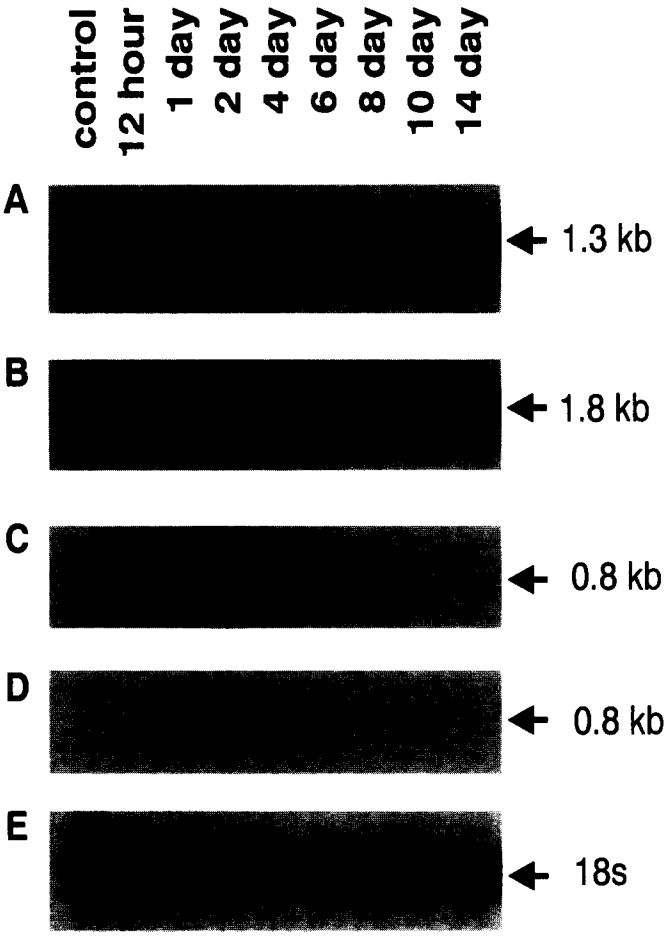

Fig. 4. Northern blot analysis of candidate genes in pancreatic tissue from control and CW hamster. Heat-denatured total RNA derived from control and cellophane wrapped pancreata were separated on 1.2% agarose gel and transferred to Nytran nylon membrane. [^{32}P]dCTP-labeled cDNA probes were used to hybridize to the membrane. Membranes were hybridized with a 490 bp hamster RD85 cDNA probe (A), a 300 bp hamster RD3-2 cDNA probe (B), a 326 bp hamster B8-1 cDNA probe (C), a 326 bp hamster B9-5 cDNA probe (D), and an 18S ribosomal 24mer synthetic oligonucleotide probe to control for RNA integrity and loading (E). The location of the transcripts is indicated.

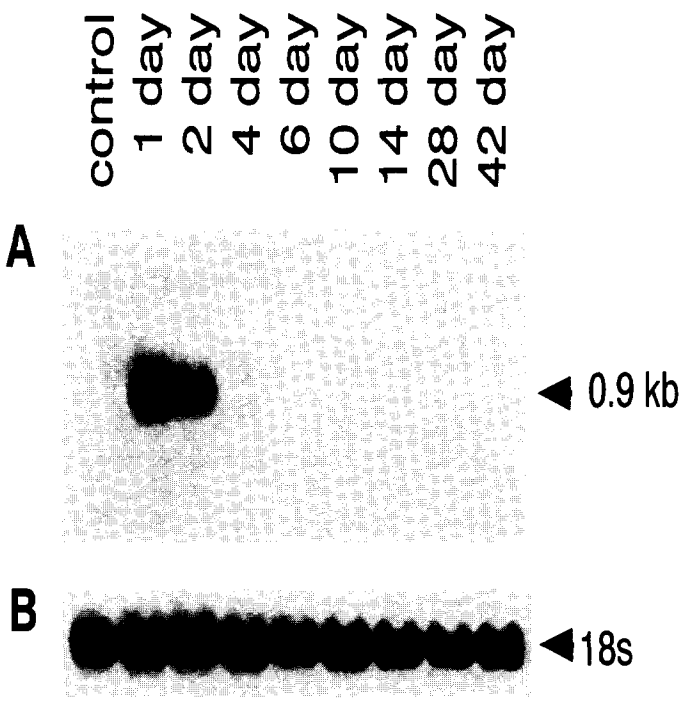

Fig. 5. Northern blot analysis of RD19-2 in pancreatic tissue from control and CW hamster. Heat-denatured total RNA derived from control and cellophane wrapped pancreata were separated on 1.2% agarose gel and transferred to Nytran nylon membrane. A 420 bp [^{32}P]dCTP-labeled hamster RD19-2 cDNA probe was used to hybridize to the membrane (A). Same membrane reprobed with a 5′-end-labeled 24mer synthetic oligonucleotide for the 18S ribosomal mRNA as a loading control (B). The location of the 0.9 kb RD19-2 transcript is indicated.

4. Discussion

Taken together, the results show that 80% (20/25) of candidate genes isolated by differential mRNA display resulted in transcripts detectable on Northern analysis. However, only 40% (10/25) were mRNA species differentially expressed in regenerating wrapped pancreas, while 40% (10/25) were false positive, the same level of expression being detected in control and regenerating pancreas. Thus, there were 10 mRNA species uniquely or differentially expressed in CW pancreata.

All 10 uniquely expressed clones were sequenced and compared to the GenBank and EMBL databases. Three of the 10 mRNA species identified showed no homology to any DNA sequence deposited in the DNA databases. The uniquely or differentially expressed cDNA clones were assigned the following identification numbers: RD19-2, B7-1, B8-1, B9-5, B10-2, RD85, RD86, RD88, RD3-2 and RD6-3. The unique expression of RD19-2, B7-1, B8-1, B9-5 and B10-2 clones in CW but not in control pancreata could indicate a particular role that these genes might play in the initiation or perpetuation of the regeneration process itself or simply as markers for cell proliferation.

Clones B7-1, B8-1 and B10-2 showed a high degree of homology to the 3′-end of *Reg* gene which has been found in regenerating islets [14,15] and regenerating wrapped pancreas [6]. Reg protein has been shown, under certain circumstances, to be able to initiate cell growth in the rat [16]. Reg is thought to be identical to the pancreatic stone protein (PSP) [17] and pancreatic thread protein (PTP) [18] whose putative function is to inhibit calcium lithiasis in the exocrine pancreas [19]. Thus, it is not clear that the increased expression is a function of growth or an anti-inflammatory response. Clone RD19-2 has shown some homology to the pancreatitis associated protein (PAP) and Reg families of genes [14–21], but further studies in our laboratory have shown it to be a novel gene associated with islet neogenesis whose protein product can initiate proliferation of proto-undifferentiated cells [22]. Clone RD85 was found to have very high homology to pancreatic elastase I gene and its expression was progressively increased with time after the surgical procedure. A possible explanation for the slight increase in elastase gene expression with time would be the fact that in response to CW there is also an increase in the acinar tissue mass along with the increase in islet tissue. Clone RD6-3 which showed higher expression in wrapped regenerating pancreas than in control, showed 90% homology to ubiquitin (Ub) conjugating enzyme which has been reported to play a crucial role in the progression of the cell cycle from the G1 to S phase [23].

It therefore appears that we have identified genes that may be implicated in the orchestrated transformation of proto-undifferentiated cells into mature adult islets. Since our model is one of induced cell proliferation and differentiation, such findings add validation to our approach for revealing genes involved in this entire process. The mRNA differential display is a useful technique for identifying differentially expressed genes in regenerating vs. non-regenerating pancreata which, in combination with screening hamster pancreatic libraries for full-length clones, enhances the likelihood of capturing the elusive participants in this process.

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