Specific Gene Expression in Pancreatic β-Cells

Cloning and Characterization of Differentially Expressed Genes

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Identification and characterization of genes expressed preferentially in pancreatic β-cells will clarify the mechanisms involved in the specialized properties of these cells, as well as providing new markers of the development of type 1 diabetes. Despite major efforts, relatively few β-cell–specific genes have been characterized. We applied representational difference analysis to identify genes expressed selectively in the pancreatic β-cell line βTC1 compared with the pancreatic α-cell line αTC1 and isolated 26 clones expressed at higher levels in the β-cells than in the α-cells. DNA sequencing revealed that 14 corresponded to known genes (that is, present in GenBank). Only four of those genes had been shown previously to be expressed at higher levels in β-cells (insulin, islet amyloid polypeptide, neuronatin, and protein kinase A regulatory subunit [RIα]). The known genes include transcription factors (STAT6) and mediators of signal transduction (guanylate cyclase). The remaining 12 genes are absent from the GenBank database or are present as expressed sequence tag (EST) sequences (4 clones). Some of the genes are expressed in a highly specific pattern-expression in βTC1 and islet cells and in relatively few of the non–β-cell types examined; others are expressed in most cell types tested. The identification of these differentially expressed genes may aid in attaining a clearer understanding of the mechanisms involved in β-cell function and of the possible immunogens involved in development of type 1 diabetes. *Diabetes* 48:552–556, 1999

Pancreatic β-cells have the unique capacity for production and regulated secretion of insulin in response to metabolic needs. To fulfill these functions, they use specialized proteins found exclusively or predominantly in β-cells; relatively few such proteins have been characterized to date. In addition to the hormones insulin and islet amyloid polypeptide (IAPP), well-characterized β-cell–enriched proteins include the insulin gene transcription factors PDX1/IPF1 (1–3) and BETA2/NeuroD (4,5) and proteins involved in glucose transport and sensing, such as GLUT2 and glucokinase (6).

Type 1 diabetes is an autoimmune disease in which β-cells are specifically targeted for destruction; both cell- and humoral-mediated immunities are involved, with T-cells directly responsible for the process of β-cell killing (7). Circulating islet cell–specific autoantibodies can be detected in newly diagnosed type 1 patients and in the sera of those healthy relatives who will eventually progress to overt type 1 diabetes (8,9). This finding has opened the way to approaches aimed at prediction and prevention of β-cell autoimmune destruction and type 1 diabetes. Various type 1 preventive trials suggest that the autoimmune destructive process can be delayed or prevented if treatment is begun early enough in the course of the disease (10). There is therefore a need to identify additional autoantigens to improve early detection of the disease.

With a view to better understanding the development, function, and pathology of β-cells, a number of studies have focused on identifying pancreatic β-cell–specific genes (11–13), yet relatively few genes have thus far been identified and characterized. We have applied representational difference analysis (RDA) to preparations of mRNA derived from the established cell lines αTC1 (14) and βTC1 (15). As a result, we identified 26 cDNA clones expressed preferentially in β-cells. In this report, we describe the initial characterization of those clones.

RESEARCH DESIGN AND METHODS

Cell lines. αTC1 (mouse glucagon-producing cells) (14) and βTC1 (mouse insulin-producing cells) (15) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% horse serum, 2.5% fetal calf serum, and penicillin/streptomycin.

RNA preparation and cDNA-RDA protocol. RNA was prepared by using the TRI reagent kit (Molecular Research Center) followed by DNase I treatment for 20 min at 37°C. PolyA$^+$ RNA was selected on an oligo-dT cellulose column (Boehringer Mannheim). RNA from mouse islets was prepared by collagenase digestion followed by fractionation on a density gradient of Histopaque (16) and extraction of RNA using the guanidinium thiocyanate procedure (17). For cDNA synthesis, two rounds of polyA$^+$ selection were performed, and 10 μg RNA was used to synthesize double-strand (ds)-cDNA (17). Two micrograms ds-cDNA was used for the RDA procedure, which was performed as described by Hubank and Schatz (18). The DP3 fraction was digested with *Dpn*II, separated on a 1.5% low-melting-point agarose gel, excised from the gel with Jetsorb resin (Genomed), and cloned into the *Bam*HI site of the vector pBS-KS, which was transformed into *Escherichia coli* DH5α or XL-1 blue.

Slot blot analysis. RNA was resuspended in a solution of 50% formamide, 7% formaldehyde, 1× SSC (150 mmol/l NaCl, 15 mmol/l Na citrate) and incubated at 68°C for 15 min. With a minifold II microsample filtration manifold (Schleicher & Schuell), the denatured sample was loaded on a Nylon membrane prewashed with 20× SSC, washed twice with 20× SSC, and ultraviolet (UV) cross-linked to the membrane. Radioactive probes were prepared by random priming using the hexanucleotide mix (Boehringer Mannheim), and filters were hybridized overnight at 42°C in a hybridization solution containing 50% formamide, 5× Denhardt's solution, 5× SSC, 0.1% SDS, and 250 μg/ml denatured salmon sperm DNA.

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DMEM, Dulbecco's modified Eagle's medium; ds, double-strand; EST, expressed sequence tag; IAPP, islet amyloid polypeptide; IL, interleukin; RDA, representational difference analysis; RT-PCR, reverse transcription–polymerase chain reaction; SSC, NaCl and Na citrate; UV, ultraviolet.

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Reverse transcription–polymerase chain reaction and Southern blotting. Reverse transcription–polymerase chain reaction (RT-PCR) was performed using the Access RT-PCR System (Promega) with 0.5 µg or 5 ng total RNA. Aliquots were removed every 5 cycles between cycles 20 and 30 to permit optimal comparison of expression levels. Samples were fractionated on 1.2% agarose gel, denatured by incubation in 0.5 mol/l NaOH, 1.5 mol/l NaCl (2 × 15 min), and renatured by incubation in 0.5 mol/l Tris-HCl, pH 7.4, 3 mol/l NaCl (2 × 15 min). Samples were applied to a Nylon membrane and UV cross-linked to the membrane. Hybridization with radioactive probes was performed as described above.

RESULTS

Products of RDA procedure. To clone genes that are expressed preferentially in β-cells, we used the cDNA-RDA protocol with ds-cDNA from βTC1 cells as a tester and ds-cDNA from αTC1 as a driver. Selection products were analyzed by agarose gel electrophoresis. Distinct bands became progressively stronger following successive rounds of subtraction and amplification. To maximize the number of differentially expressed genes isolated, we excised from the gel all detectable DP3 products and ligated this material to the vector pBS followed by transformation into *E. coli* to generate a β-cell–enriched library.

Analysis of β-cell–enriched library. Three hundred clones were picked at random from the library and subjected to Southern blot analysis (data not shown). Duplicate filters were hybridized with cDNA probes prepared from βTC1 and αTC1 mRNA. Clones that were positive for the βTC1 probe and negative for the αTC1 probe were in turn used to reprobe the Southern blots. These analyses indicated that the 300 clones included at least 26 distinct DNA fragments corresponding to differentially expressed mRNA species.

Sequence analysis and expression patterns of cloned genes. Clones identified by the above procedure were subjected to DNA sequencing analysis, and the resulting sequences were compared with the GenBank database by Blast search (19). Approximately half of the clones identified (14 of 26) corresponded to known genes (Table 1). These included genes known previously to be expressed preferentially in β-cells, such as insulin, IAPP, neuronatin (13), and protein kinase A regulatory subunit (RIα) (13,20), as well as known genes that had not previously been recognized to be differentially expressed in β-cells compared with α-cells, such as vinculin, guanylate cyclase, and STAT6. The remaining 12 clones are not represented in the database or are represented only as expressed sequence tag (EST) sequences (Table 1).

To analyze the expression pattern of the cloned genes, we performed RNA slot blot analysis with 10 µg total RNA from six different cell lines: Ltk^- (fibroblasts), L10 (lymphoid cells), βTC1 (pancreatic β-cells), αTC1 (pancreatic α-cells), C2 (muscle cells), and GH3 (pituitary cells) using the different inserts as probe (Fig. 1*A*). Inserts that failed to hybridize with 10 µg total RNA, presumably because of low abundance, were hybridized with blots containing 5 µg $polyA^+$ RNA from the indicated cells (Fig. 1*B*). All clones displayed a stronger signal in β-cells than in α-cells (confirming the low frequency of

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TABLE 1
Summary of clones isolated by RDA

Clone	Gene homology	Accession number	Frequency	βTC1/αTC1 ratio
63y	Dopamine receptor	X55674	3	2
59v	Glycogen synthase	J05446	1	3.7
10d	Guanylate cyclase	M22562	1	>50
23d	IAPP	M25389	>10	>50
28d	IgE	M10062	3	4
1v	Insulin	X04725	2	>50
46d	Neuronatin	X833569	2	>50
25d	ndr1	U60593	1	2
93v	Phosphorylase kinase	X74616	1	ND
1d	PKA Rlα	M17086	>10	>50
37x	P-type ATPase	AF038007	1	>50
20	STAT6	L47650	1	>50
48d	TALLA1	D26483	1	2.7
88v	Vinculin	L18889	1	28
41	Unknown	C88962 (EST)	4	ND
64k	Unknown	W10530 (EST)	1	16.9
31v	Unknown	AA217936 (EST)	1	2.2
61v	Unknown	Z47778 (EST)	1	11.5
32n	Unknown	—	2	>50
60	Unknown	—	1	2.6
92v	Unknown	—	1	>50
71k	Unknown	—	5	19
2n	Unknown	—	>5	13.4
15x	Unknown	—	1	>50
21y	Unknown	—	1	>50
24y	Unknown	—	1	2

Shown for each clone are the closest homolog found in the GenBank database, the accession number of the GenBank homolog, the number of times each clone was independently isolated, and the ratio of expression level observed in βTC1 cells compared with αTC1 cells as determined by phosphorimager quantitation of RNA slot blot. ND, not determined.

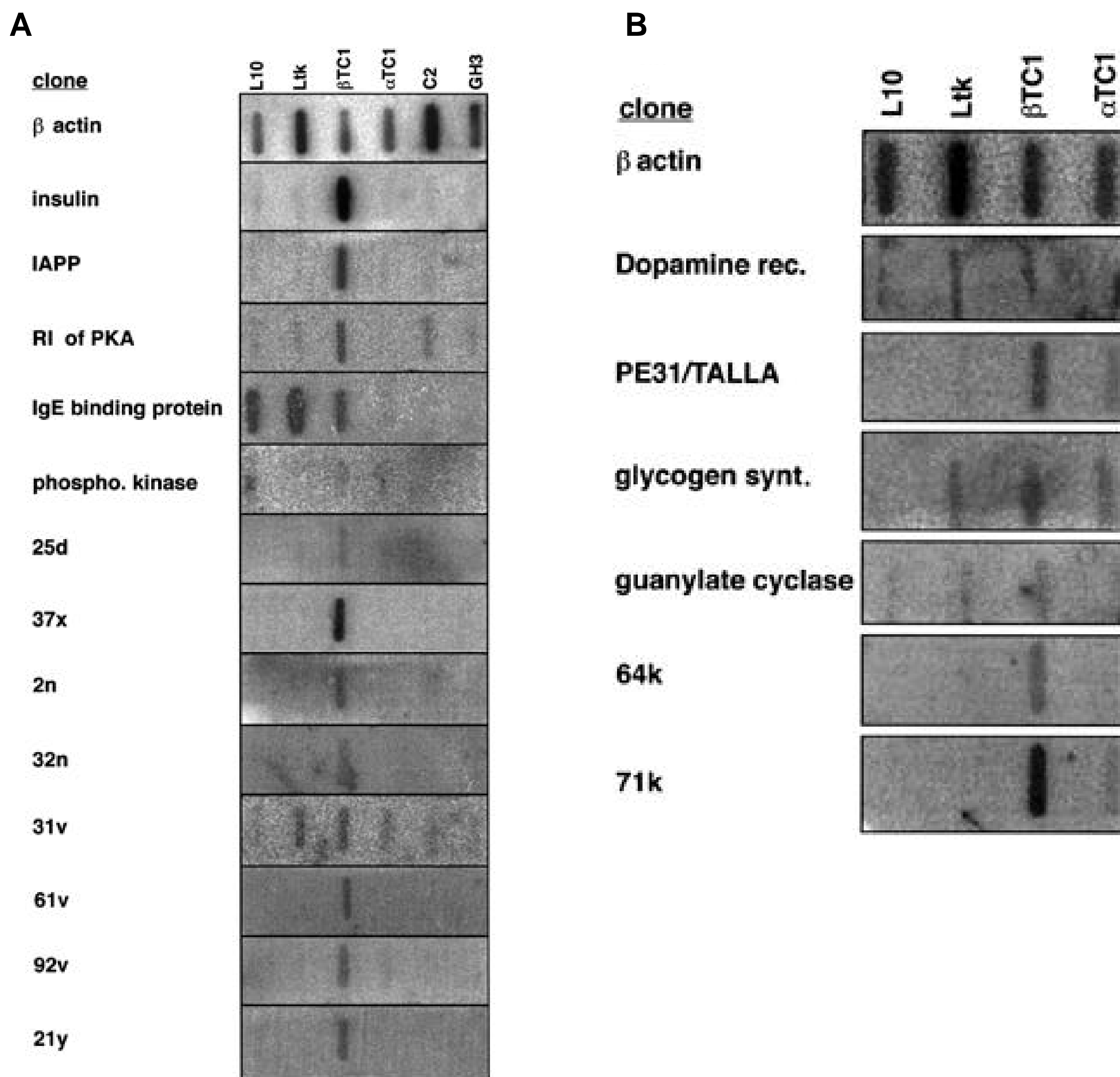

FIG. 1. RNA slot blot analysis: RNA was extracted from the indicated cell lines, and 10 µg of total RNA (*A*) or 5 µg of polyA$^+$ selected RNA (*B*) was applied to a nylon membrane. Each membrane strip was hybridized with probes prepared from the indicated RDA fragments or β-actin cDNA as positive control.

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false-positive clones isolated using this procedure). Most clones showed low expression in the non-pancreatic cell lines tested. Included in the collection were genes whose expression in β-cells was relatively high (such as insulin) and much lower (such as guanylate cyclase) and genes whose selectivity of expression in β-cells compared with α-cells was high (such as RIα) and low (such as TALLA1). Thus the RDA procedure is an effective and sensitive method for identifying differentially expressed β-cell genes. In our hands, experiments performed using differential display methodologies (21,22) failed to identify differentially expressed β-cell genes (data not shown).

To test the distribution of transcripts in different mouse tissues, we performed RT-PCR analysis using primers designed according to the sequence of the novel genes (Fig. 2*A*). As expected, signals were highest with βTC1 RNA. In addition, most clones showed high expression in the brain (clones 71k, 60, 32n, 37x, 41, 2n), consistent with previously observed similarities of gene expression between β-cells and brain. Only one of the clones (21y) appeared to be expressed exclusively in β-cells. Northern blot analysis showed that most or all of the clones represent different mRNAs (data not shown).

βTC1 and αTC1 cells are tumor cell lines whose patterns of gene expression may differ significantly from their natural counterparts. We wished to confirm that the clones isolated represent genes also expressed preferentially in normal β-cells. Since expression levels of the RDA clones were too low to permit detection by in situ hybridization analysis of islets (data not shown), we performed RT-PCR analysis of

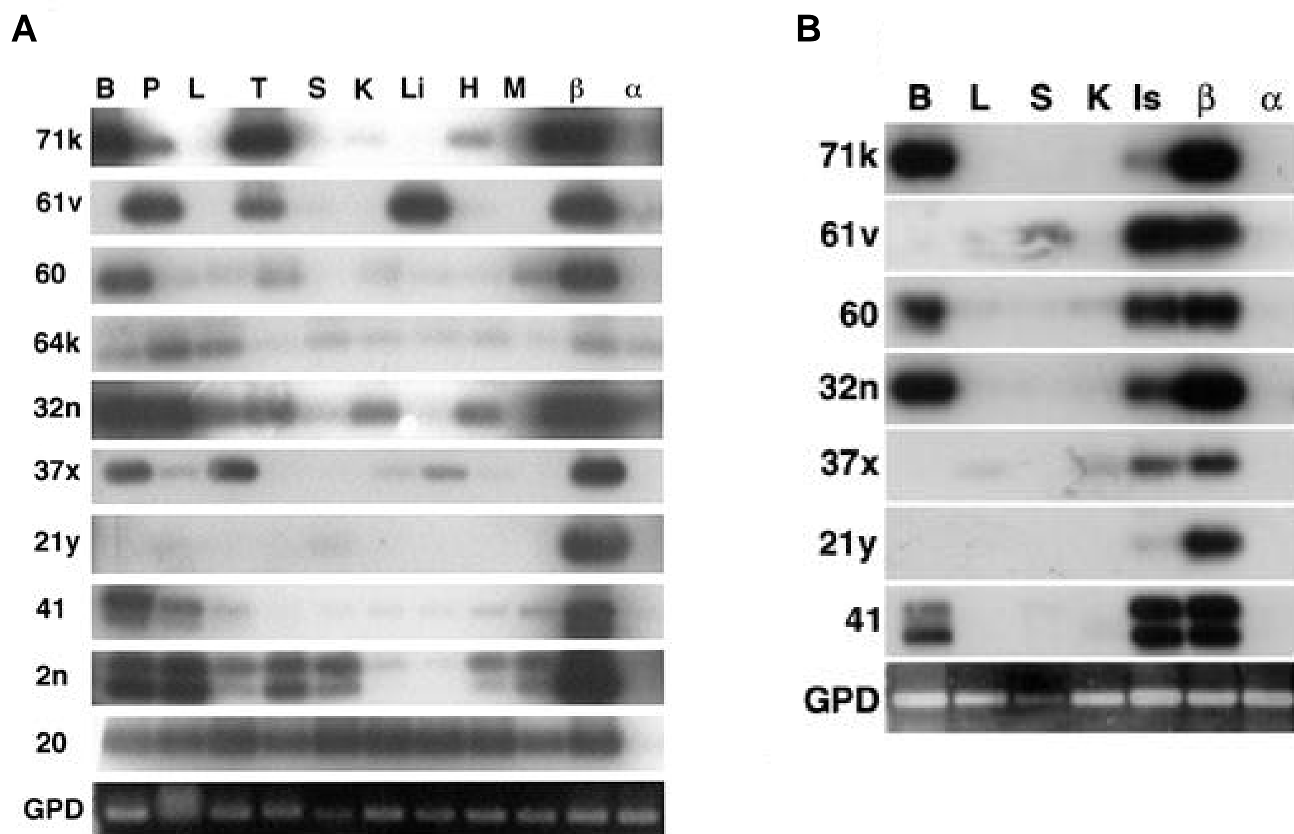

FIG. 2. RT-PCR analysis of RNA extracted from the following mouse tissues: brain (B), pancreas (P), lung (L), testis (T), spleen (S), kidney (K), liver (Li), heart (H), muscle (M), islets (Is), βTC1 (β), and αTC1 (α). Total RNA (*A*, 0.5 µg; *B*, 5 ng) was subjected to RT-PCR followed by hybridization with the indicated probes. The lower panel shows the ethidium bromide staining of PCR reactions performed with glyceraldehyde-3-phosphate dehydrogenase (GPD) primers, as a control for the efficiency of the RT-PCR reaction.

RNA isolated from mouse islets. All seven clones examined showed preferential expression in islet RNA (Fig. 2*B*). For five of the seven clones, expression in islets was similar to that for βTC1 cells (Fig. 2*B*); in the case of two clones, expression was significantly lower but still detectable in islet RNA, and higher than in other tissues (Fig. 2*B*). Thus these data indicate that the RDA clones show preferential expression in normal β-cells as well.

DISCUSSION

The procedure described in this report resulted in the isolation of clones corresponding to 26 genes. Of those, 14 correspond to known genes, including genes previously known to be expressed differentially in β-cells such as insulin and IAPP, thus validating the effectiveness of the procedure. Also among the known genes were several whose differential expression had not been previously described; included among this class are the genes for guanylate cyclase, dopamine receptor, glycogen synthase, phosphorylase kinase, IgE binding protein, TALLA1, STAT6, and vinculin. Although the reason for higher levels of expression of these proteins in β-cells is unclear at this stage, several of the genes have functions that might have important implications for β-cell activities.

The identification of guanylate cyclase in this screen may be relevant to the mechanism of β-cell destruction in type 1 diabetes. Cytosolic guanylate cyclases (23) can be activated by nitric oxide, which has been implicated in cytokine-mediated β-cell damage (24). Although the effects of elevated cGMP levels in β-cells are not known, studies in other cell systems have identified effects on Ca^{2+} channels (25), which play a central role in mediating nutrient-dependent insulin secretion in β-cells.

A second gene identified in this screen that may be implicated in cytokine-mediated effects on β-cells is STAT6. Members of the STAT (signal transducers and activators of transcription) family are cytosolic cryptic transcription factors that become activated on cytokine binding to the cognate membrane receptor and consequent activation of Janus family (Jak) tyrosine kinases (26). STAT6 has been shown to mediate the effects of interleukin (IL)-4 and IL-13 on gene expression (27). IL-4 is believed to have a protective effect on development of type 1 diabetes, though it is generally considered to function through direct action on lymphocytes (28). The presence of STAT6 in β-cells raises the possibility that IL-4 may also have direct protective effects on β-cells.

Database comparisons show that 12 of the genes are not represented in GenBank or (in 4 cases) are represented only as EST sequences. A number of clones show sufficient similarity to known proteins at either the protein or DNA level to indicate a possible function. For example, clone 24y may correspond to a novel transcription factor since it possesses a characteristic PHD finger domain (29). Clone 32n shows significant homology to the BMP1 family of

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growth factors (30). Further characterization of these clones will permit evaluation of their role in the function and pathology of β-cells.

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