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Differentiation phenotypes of pancreatic islet β- and α-cells are closely related with homeotic genes and a group of differentially expressed genes

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Abstract

To identify the genes that determine differentiation phenotypes, we compared gene expression of pancreatic islet β- and α-cells, which are derived from the common precursor and secrete insulin and glucagon, respectively. The expression levels of homeotic genes including Hox genes known to determine region specificity in the antero-posterior (AP) body axis, tissue-specific homeobox genes, and other 8,734 genes were compared in a β- and α-cell line of MIN6 and αTC1.6. The expression of homeotic genes were surveyed with reverse transcription-polymerase chain reaction (RT-PCR) using degenerate primers corresponding to invariant amino acid sequences within the homeodomain and subsequently with specific primers. Expression of Hoxc6, Hoxc9, Hoxc10, Pdx1, Cdx2, Gbx2, Pax4, and Hlxb9 genes in MIN6 was higher than those in αTC1.6, while expression of Hoxa2, Hoxa3, Hoxa5, Hoxa6, Hoxa7, Hoxa9, Hoxa10, Hoxa13, Hoxb3, Hoxb5, Hoxb6, Hoxb13, Hoxb8, and Brain4 genes in αTC1.6 was higher than those in MIN6. Out of 8,734 mouse genes screened with high-density mouse cDNA microarrays for MIN6- and αTC1.6-derived cDNA, 58 and 25 genes were differentially over- and under-expressed in MIN6, respectively. GLUTag, which is derived from a large bowel tumor and expresses the proglucagon gene, showed a comparatively similar expression profile to that of αTC1.6 in both homeotic and other genes analyzed in cDNA microarray.

Our results are consistent with the interpretation that not only the tissue-specific homeotic genes, but also Hox genes are related to differentiation phenotypes of pancreatic β- and α-cells rather than their regional specification of the body in vertebrates.

Keywords: Pancreatic islet; β-cell; α-cell; Homeotic gene; Hox gene; Microarray

Abbreviations: IAPP, islet amyloid polypeptide; Pdx1, pancreatic-duodenal homeobox 1; PCR, polymerase chain reaction; AP, antero-posterior; RT, reverse transcription; GLP-1, glucagon-like peptide-1; D-MEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; G6PDH, glucose-6-phosphate dehydrogenase; ITF2A, immunoglobulin transcription factor 2A; TBP, TATA-binding protein; EST, expression sequence tag; CAM, cell adhesion molecule; RDA, representational difference analysis; IGFII, insulin-like growth factor II; PRKAR1A, regulatory subunit RIα of protein kinase A; Msx1, homeo box, msh-like 1; Gbx2, gastulation brain homeo box 2; Hlxb9, homeobox gene HB9; Ptf1a, pancreatic specific transcription factor, 1a; Nkx2.1, thyroid transcription factor 1; FHL1, four and a half LIM domains 1; ERO1-Lβ, endoplasmic reticulum oxidoreductin 1-L beta; Meox2, mesenchyme homeobox 2; Rian, RNA imprinted and accumulated in nucleus.

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1. Introduction

Among four pancreatic cell types derived from a common endocrine precursor including α-, β-, δ-, and PP cells, β- and α-cells are the two main islet cell types. To produce and secrete insulin in response to metabolic needs, β-cells must use a specialized set of proteins exclusively or predominantly expressed in β-cells. In addition to the hormones such as insulin and islet amyloid polypeptide (IAPP), well-characterized β-cell enriched proteins include pancreatic-duodenal homeobox 1 (Pdx1), glucose transporter type 2, and glucokinase. To better understand the development and function of β-cells, many studies have focused on identifying pancreatic β-cell specific genes (Neophytou et al., 1996; Niwa et al., 1997; Arava et al., 1999), while the gene expression in α-cells has received less attention. As a model system to define differentiation phenotype through β- and α-cell specific expression, we analyzed expression of homeotic genes and 8,734 cDNAs with cDNA microarray in simian virus 40 T antigen-transformed mouse cell lines of MIN6 and αTC1.6. They secrete insulin and glucagon, respectively, and have relatively differentiated functions as islet β- and α-cells (Ishihara et al., 1993; Hamaguchi and Leiter, 1990).

Little direct evidence on differences in gene expression between MIN6 and αTC1.6 has been documented. Polymerase chain reaction (PCR)-based subtractive hybridization and representational difference analysis between βTC and αTC suggest differences in expression of several genes, but comprehensive gene expression profile in βTC and αTC has not been tested (Neophytou et al., 1996; Niwa et al., 1997; Arava et al., 1999). Genes which are induced in β-cells by glucose in rat islets (MacDonald, 1996), human islets (Shalev et al., 2002), and mouse insulinoma cell lines (Yamato et al., 1996; Josefsen et al., 1999; Webb et al., 2000) were analyzed. It is important to obtain further information on differentially expressed genes between MIN6 and αTC1.6 to understand the factors that regulate the initiation of differentiation of pancreatic islet α- and β-cells and maintain cell-specific hormone production.

Homeotic genes include Hox genes and tissue-specific homeobox genes. Hox genes are involved in the specification of each body part along the antero-posterior (AP) body axis during embryogenesis (reviewed in Krumlauf, 1994). The chromosomal order of mouse Hox genes is co-linear as to the relative positions of their expression domains along the AP body axis of the embryo. Although some Hox genes are reported to be expressed in pancreatic islets and islet cell lines, there is little information about the expression of Hox genes in β- and α-cells. We have now used reverse transcription (RT)-PCR, with a set of degenerate oligonucleotide primers, to identify a subset of homeotic genes that are expressed in MIN6 or αTC1.6. In addition, expression of Hox genes in MIN6, βTC1, αTC1.6, and GLUTag (Drucker et al., 1994) was analyzed by RT-PCR with specific primers to each Hox gene.

Microarray technology represents a potentially powerful approach to identify genes specifically expressed in different cell or tissue types (Brown and Botstein, 1999). To our knowledge, no comprehensive study has been performed on the difference in gene expression between pancreatic β- and α-cells using cDNA microarray. In this study, we used cDNA microarray analysis to compare expression profiles of 8,734 genes in mouse MIN6 versus αTC1.6. We identified 83 genes that were differentially expressed by 4.0-fold or above 4.0-fold between two cell lines. We verified the expression levels of some of these genes by Northern blot analysis. The ability to detect differentially expressed genes with cDNA microarrays should enable us to identify those genes which determine differentiation phenotypes of pancreatic islet cells. The goal of this study is to understand the molecular basis of the phenotype differentiation in β- and α-cells by identifying genes with cell type-specific expression.

2. Materials and methods

2.1. Cell lines

The insulin-producing MIN6 and βTC1, and the glucagon-like peptide-1 (GLP-1)-producing GLUTag were grown in Dulbecco's Modified Eagle Medium (D-MEM) (Sigma, St. Louis, MO) with 25 mM glucose, and 10% fetal bovine serum (FBS). The glucagon-producing αTC1.6 and a fibroblast cell line of NIH3T3 were cultured in D-MEM with 5.5 mM glucose and supplemented with 10% FBS. MIN6, βTC1, αTC1.6, and GLUTag were generously provided by Drs. Miyazaki, Hanahan, Hamaguchi, and Drucker, respectively.

2.2. RNA isolation

RNA isolation was carried out by lysing cells or tissues in guanidinium thiocyanate/phenol buffer (ISOGEN, NIPPON GENE, Tokyo, Japan) according to manufacturer's instructions.

2.3. cDNA synthesis

Total RNA in 3 μg treated with RNase-free DNase (Promega, Madison, WI) was reverse-transcribed with ThermoScript™ RT-PCR system (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Total RNA and random hexamer primers were denatured at 65 °C for 3 min, and quickly chilled on ice. Ten μl of the total RNA were mixed with 10 μl of cDNA synthesis mix and left at room temperature for 10 min, and subsequently incubated at 50 °C for 50 min. The reaction mixture was heated at 85 °C for 5 min followed by

RNase H treatment at 37 °C for 20 min. cDNA was stored at −20 °C until use.

2.4. Amplification of homeotic genes by using degenerate primers

Two blocks of conserved amino acids, QT(L/F)ELEKE and WFQN(S/R)(S/R)MKW based on highly conserved sequences of the *Antennapedia* homeodomain class of transcription factors, were chosen for degenerate primers (Gehring et al., 1994). The degenerate primers used for PCR amplification were shown in Table 1. PCR conditions consisted of 3 cycles of amplification (94 °C, 30 s; 37 °C, 30 s; 72 °C, 1 min) followed by 30 cycles of amplification (94 °C, 30 s; 40 °C, 30 s; 72 °C, 1 min) with the final incubation at 72 °C for 10 min. A band of 120 base pairs (bp) was gel-isolated and cloned into a TA-vector (Invitrogen). Each clone was sequenced using a sequencing kit (Big Dye Terminator Cycle Sequencing Kit, Applied Biosystems, Foster City, CA). Sequence comparisons were performed with the BLAST program.

2.5. RT-PCR of homeotic genes

The oligonucleotides used for PCR are listed in Table 1. The primer pairs that flank at least one intron were designed to avoid the amplification from contaminated genomic DNA. Standard thermal cycle profile was as follows. A single denaturing step at 95 °C for 10 min was followed by 35 cycles as given: 95 °C for 30 s; 56 °C for 1 min; 72 °C for 1 min, with the final extension for 10 min. PCR products were electrophoresed on a 10% polyacrylamide gel, followed by ethidium bromide staining. Gels were photographed with an ultraviolet transilluminator. Negative controls included PCR with samples without RT or a water control instead of cDNA as templates in PCR. Genes of β-actin, glucose-6-phosphate dehydrogenase (G6PDH), immunoglobulin transcription factor 2A (ITF2A), and TATA-binding protein (TBP) were amplified at 30 cycles as internal standards.

2.6. Microarray preparation and hybridization

Poly $(A)^+$ RNA was purified using Oligotex-dT30 super mRNA purification kit (TaKaRa, Kyoto, Japan) and labeled with Cy3 and Cy5 fluorescent dyes for microarray hybridization on mouse GEM I (Incyte Genomics, St. Louis, MO). The arrays consisted of 8,734 cDNAs representing clones from several cDNA libraries, including expressed sequence tag (EST) clones. Fluorescent labeling of probes, hybridization, and scanning of the GEM I microarray, and data collection were performed by the company and transferred electronically for analysis in our lab. Briefly, mRNA was reverse-transcribed with random 9-mers labeled with 5′ Cy3 dye (mRNA from αTC1.6) or Cy5 dye (mRNA from MIN6). The probe was applied to the array. After hybridization at 60 °C for 6.5 h, slides were washed in three consecutive washes of decreasing ionic strength. After washing, the GEM I microarray was scanned to detect Cy3 (αTC1.6) and Cy5 (MIN6) fluorescence. Background-subtracted element signals were used to calculate Cy3:Cy5 ratios. The average of the resulting total Cy3 and Cy5 signals gave a ratio that was used to balance or normalize the signals. In addition, cDNA microarray analysis of two pairs of cell lines (MIN6 and GLUTag, αTC1.6 and GLUTag) were performed on Mouse UniGene 1 (Incyte Genomics), which consisted of 9,514 cDNAs clones.

2.7. Northern blot analysis

For Northern blot analysis, 10 μg of total RNA were separated by electrophoresis on 1% denaturing agarose gels and transferred to GeneScreen membranes (Biotechnology Systems NEN Research Products, Boston, MA). To confirm integrity and amounts of loaded RNA, the gels were stained with ethidium bromide and photographed under a UV transilluminator. Probes were labeled with [α-^{32}P]dCTP using the Megaprime Labeling kit (Amersham Biosciences, Tokyo), according to the manufacturer's protocol. Hybridization was carried out at 42 °C overnight in 6× SSC−10 mM EDTA−5× Denhart's solution−0.5% SDS−100 μg/ml denatured salmon sperm DNA−10% dextran sulphate−50% formamide. The membranes were washed twice in 2× SSC−0.1% SDS at room temperature for 15 min, and twice in 0.5× SSC−0.1% SDS at 65 °C for 15 min. The membranes were exposed to Kodak BIOMAX MS film at −70 °C with intensifying screen. I.M.A.G.E. clones used as probes were as follows: No. 2 in Table 4 (GenBank accession no., AI892149), No. 5 in Table 4 (W89392), No. 10 in Table 4 (AA242626), No. 19 in Table 4 (W91135), No. 41 in Table 4 (AA231293), No. 44 in Table 4 (AA000829), No. 5 in Table 5 (AA269699), No. 6 in Table 5 (AA172519), No. 7 in Table 5 (AI893491), No. 8 in Table 5 (AI892342), No. 11 in Table 5 (W97514), No. 22 in Table 5 (AA068436). Following cDNA clones were also used as probes: mouse protein phosphatase inhibitor 1 (provided from Dr. McLaren, University of Edinburgh), mouse alcohol dehydrogenase (Dr. Edenberg, Indiana University School of Medicine), rat monoamine oxidase B (Dr. Ito, Kyusyu University), mouse Williams–Beuren syndrome chromosome region 14 (Wbscr14) (Dr. Jurado, Universitat Pompeu Fabra), rat carboxypeptidase E (Dr. Fricker, Albert Einstein College of Medicine), mouse CD9 (Dr. Boucheix, INSERM), mouse solute carrier family 40 (iron-regulated transporter), member 1 (Dr. Zon, Children's Hospital, Boston), rat ATP-citrate lyase (Dr. Kim, Yonsei University College of Medicine), growth factor receptor bound protein 10 (Dr. Margolis, The University of Michigan Medical Center), mouse fructose

Table 1
Primer sequences used in RT-PCR

	Name	Forward primer sequence (5′–3′)	Name	Reverse primer sequence (5′–3′)	Size (bp)
Degenerate primers	2136*	CARACNYTNGARCTVGARAARGARTT	2138*	CCAYTTCATNCKNCKRTTYTGRAACCA	120
Hoxa1	Y400	AAGTTAAAAGAAACCCTCCC	Y401	TTTCTCATCGCTGCCAGGAG	293
Hoxa2	Y396	CTGCCTGCCTCGGCCACAAA	Y397	ACTTTGTCCGAGTCCTCCAG	297
Hoxa3	Y477	ACTCTCCCACCGTGGGCAAA	Y478	AGACGAGGTCAGCATGCCTT	338
Hoxa4	Y330	CTGGATGAAGAAGATCCACG	Y450	GTGAGTTTGTGCTTTCCCAG	312
Hoxa5	Y332	ACCCACATCAGCAGCAGAGA	Y333	CGGCCATACTCATGCTTTTC	384
Hoxa6	Y433	TGCAGCGGATGAATTCCTGT	Y434	CTGCGTGGAGTTGATGAGTT	244
Hoxa7	Y378	TTCCGCATCTACCCCTGGAT	Y379	GGAGCCTGGCTCTCATCTTT	238
Hoxa9	Y392	AATGAGAGCGGCGGAGACAA	Y393	CCTAAAAGGCTCACTCGTCT	285
Hoxa10	Y289	GTGTCAAGTCCTGAATGGGC	Y290	AGAGAAACCAGGCCTGGACT	243
Hoxa11	Y479	AGTCGTCTTCCGGCCACACT	Y480	TTCACATGTATGAAGCCCCC	321
Hoxa13	Y435	CACCTCTGGAAGTCCACTCT	Y436	TCTCAGAGAGGTTTGTCGTG	193
Hoxb1	Y398	TCGACTGGATGAAGGTCAAG	Y399	ACTGGTCAGAGGCATCTCCA	320
Hoxb3	Y437	CAGTACCACTAGCAACAGCA	Y438	CGCCACCACCACAACCTTCT	178
Hoxb4	Y475	CCAGAACCCCCTGCATCCCA	Y476	CATGTTCGAACTCCTGCTTG	178
Hoxb5	Y338	GGATGAGGAAGCTTCACATC	Y339	GCCAGACTCATACTTTTCAG	246
Hoxb6	Y336	AGAGACCGAGGAGCAGAAGT	Y337	TCACTCGGCTGGCTTTTCCT	328
Hoxb7	Y406	CGAGAGTAACTTCCGGATCT	Y407	TCCCGGTCCTGAGGTTTTGT	247
Hoxb8	Y277	CAGTACGCAGACTGCAAGCT	Y279	CTTCTCTTTCTCCAGCTCCT	361
Hoxb9	Y287	GGAAGCGAGGACAAAGAGAG	Y288	TACTCTTTGCCTGCTCCGTT	246
Hoxb13	Y545	CAGCCTATGGCCAGTTACCT	Y546	AGGAGGGTGCTGGACACT	212
Hoxc4	Y481	GCAAGCGAGGACAAAGAGAG	Y482	TGACCTCACTTTGGTGTTGG	280
Hoxc5	Y410	TGAACCCTGGGATGTACAGT	Y411	TAACTGGTTCGGGACCGCTT	195
Hoxc6	Y445	ACCAGAAAGCCAGTATCCAG	Y446	CTTTTCCTCTTTTCCGCCCA	329
Hoxc8	Y402	TGTTTCCATGGATGAGACCC	Y403	TCGGGCCCCAGGCAGTTTAT	238
Hoxc9	Y342	AAAGAGGAGAAGGCCGACCT	Y343	CAGGGCTTAGGATTGTTCCT	278
Hoxc10	Y483	AGTCCAGACACCTCGGATAA	Y484	ATGACGCTGGCTCAGGTGAA	320
Hoxc12	Y547	TACTCAACGAGGGCAATAAGA	Y548	GGCTTGCGCTTCTTTCGCGA	138
Hoxc13	Y549	ACGCGCTCATCCCTGTTGAA	Y550	TTCGGGCTGTAGAGGAACCA	142
Hoxd1	Y447	AAGAGGAACGCCCCCAAGAA	Y448	AGAGGCAGCTGTGGCCAGAA	264
Hoxd3	Y394	CGACAGAACTCCAAGCAGAA	Y395	AGAATGCAGGATGCCCTTAG	280
Hoxd4	Y485	TGAAAAAGGTGCACGTGAAT	Y486	GAAGAAGACCTGCCCTTGGT	262
Hoxd8	Y441	CTTAAATCAGAGCTCGTCTC	Y442	TTGGGGTCTCCATCCTTTGC	293
Hoxd9	Y443	CTAAAGTCTCCCAAGTGGAG	Y444	GCTGGTTGGAGTATCAGACT	142
Hoxd10	Y346	GTGCAGGAGAAGGAAAGCAA	Y347	GGTCAGTTCTCGGATTCGAT	276
Hoxd11	Y487	TTGATCAGTTCTACGAGGCG	Y488	GGTACATCCTGGAGTTCTCA	502
Hoxd12	Y404	TAAACAGTGCCCATGCTCCC	Y405	ATAGAGGGCCAGTGCTTGCT	268
Hoxd13	Y489	AGCCACAGGGTTCCCATTTT	Y490	GTGTCTTTGAGCTTGGAGAC	280
Pdx1	Y469	CCGGACATCTCCCCATACGAAGT	Y470	CGCACAATCTTGCTCCGGCTCTT	500
Cdx2	Y318	TGAAAACCAGGACAAAAGAC	Y320	ATTTTCCTCTCCTTGGCTCT	198
Gbx2	Y439	AGGGCTCGCTGCTCGCTTTC	Y440	GAGCTGTAATCCACATCGCT	154
Nkx6.1	Y322	TCTTCTGGCCTGGGGTGATG	Y323	GTGCTTCTTTCTCCACTTGGTCC	277
Nkx6.2	Y355	ATCTTCTGGCCTGGGGTGGT	Y356	TTTTAGCCGACGCCATCTCT	300
Nkx2.2	Y359	CATCTTGGACCTTCCGGACAC	Y361	GGCGTCACCTCCATACCTTT	545
Pax6	Y364	ACCAACGATAACATACCCAG	Y365	CTGAAGTCGCATCTGAGCTT	278
Pax4	Y357	ACCCTGTGACATTTCACGGAG	Y358	GTACTCGATTGATAGAGGAC	263
Isl1	Y324	CACTATTTGCCACCTAGCCAC	Y325	AAATACTGATTACACTCCGCAC	255
Hlxb9	Y368	AGCACCTTCCAACTGGACCA	Y369	AAAACGCTTGGGTCGAGACA	210
Brain4	Y370	CTGATGAAGAGACTCCAACC	Y371	ATAAACCTCGTGTGGCTGCT	493
Msx1	Y314	CATTTCTCAGTCGGAGGACT	Y316	CATCTTCAGCTTCTCCAGCT	314
Neurod1	BIIF1	GGATCCACATGACCAAATCATACAG	BIIR2	GGATCCTCTAATCGTGAAAGATGGCA	1000
Ptf1a	Y416	TGCAGTCCATCAACGACGC	Y417	GGACAGAGTTCTTCCAGTTC	710
Insulin2	IN-S	AACCACAAAGGTGCTGCTTGAC	IN-AS	CCTAAGTGATCCGCTACAAT	150
Glucagon	Y451	AGAAGGGCAGAGCTTGGGCCCA	Y452	TGCCAGCTGCCTTGCACCAGCA	159
N-CAM	Y311	TCGGATCCACTGGCGGCCCTCAACG	Y312	GTTCACCTTGATGGAGTTCCCG	136
β-actin	1634H	GTGGGCCGCTCTAGGCACCA	1635H	CGGTTGGCCTTAGGGTTCAGG	234
G6PDH	Y423	GACCTGCAGAGCTCCAATCAAC	Y424	CACGACCCTCAGTACCAAAGGG	214
ITF2A	IT-A3	GAAGCAAGGTAGCAACTTGG	IT-A4	GCTCAGGGTACGGAACTAGT	730
TBP	Y421	ACCCTTCACCAATGACTCCTATG	Y422	ATGATGACTGCAGCAAATCGC	190

R = AG, N = ACGT, Y = CT, V = AGC, K = GT.

bisphosphatase 1, liver type (Dr. Eschrich, University of Leipzig), and mouse deafness dystonia protein 1 (translocase of inner mitochondrial membrane 8 homolog a) (Dr. Nakane, Shinsyu University).

3. Results

3.1. RT-PCR analysis of homeotic genes with degenerate primers

To rule out PCR biases, we first examined the amplification of various Hox genes using mouse genomic DNA as a template, because the amplified segment is uninterrupted by an intron. The result of genomic PCR amplification served as a control for RT-PCR (Table 2). PCR products of 120 bp were obtained from both MIN6 and αTC1.6. As shown in Table 2, 13 or 13 homeobox sequences were amplified from RNA derived from MIN6 or αTC1.6, respectively. The results, classed into paralogous groups, are shown in Table 2 as percentages. Only genes that gave products from either cDNA or genomic DNA are listed. The most abundantly expressed gene in MIN6 was Pdx1 (89.2%). Of note, Pdx1 expression was not exclusive in MIN6, but it was also expressed in the lesser amount in αTC1.6. Hox genes such as Hoxa4, Hoxa7, Hoxa10, Hoxb8, Hoxc6, Hoxc9, Hoxd1, Hoxd8, and Hoxd9 were amplified from MIN6. In addition, the degenerate RT-PCR detected such tissue-specific homeo-

Table 2
Expression of homeotic genes in MIN6 or αTC1.6

	MIN6 (%)	αTC1.6 (%)	Genome DNA(%)
Hoxa1			2.1
Hoxa4	0.2		2.1
Hoxa5		4.4	
Hoxa6			8.5
Hoxa7	1.7	2.2	10.6
Hoxa9		0.4	8.5
Hoxa10	0.5	38.2	
Hoxb1			2.1
Hoxb3		4.0	
Hoxb4			2.1
Hoxb5		6.2	4.2
Hoxb6		5.3	6.4
Hoxb7		0.8	4.2
Hoxb8	0.2	26.2	8.5
Hoxb9		4.4	10.6
Hoxc5			2.1
Hoxc6	0.2		2.1
Hoxc8			8.5
Hoxc9	0.5		6.4
Hoxd1	0.2		
Hoxd3			2.1
Hoxd8	0.2		
Hoxd9	2.4		2.1
Hoxd10			2.1
Hoxd12			2.1
Pdx1	89.2	6.2	2.1
Cdx2	4.0		
Nkx6.2		0.8	
Nkx6.1		0.4	
Gbx2	0.2		
Pax6	0.2		
Total clone numbers	418	225	47

Table 3
Homeotic gene expression by RT-PCR using specific primers

	Cell lines			
	MIN6	βTC1	αTC1.6	GLUTag
Hoxa1	–	–	–	+
Hoxa2	–	–	+	+
Hoxa3	–	+*	+	+
Hoxa4	–	–	–	+
Hoxa5	–	–	+	+
Hoxa6	–	+	+	+
Hoxa7	–	+	+	+
Hoxa9	+*	+	+	–
Hoxa10	–	–	+	+
Hoxa11	–	–	–	–
Hoxa13	–	+	+	+
Hoxb1	–	–	–	–
Hoxb3	–	–	+	+
Hoxb4	–	–	–	–
Hoxb5	–	+	+	+
Hoxb6	–	+*	+	+
Hoxb7	–	+	–	+
Hoxb8	–	+*	+	+
Hoxb9	–	+	–	–
Hoxb13	–	+	+	+
Hoxc4	–	–	–	–
Hoxc5	–	–	–	–
Hoxc6	+	+	+*	–
Hoxc8	+	+	+	+
Hoxc9	+	+	–	–
Hoxc10	+	+	+*	+*
Hoxc12	–	–	–	–
Hoxc13	–	+	–	+*
Hoxd1	–	–	–	–
Hoxd3	–	–	–	–
Hoxd4	–	–	–	–
Hoxd8	–	–	–	–
Hoxd9	–	–	–	+
Hoxd10	–	+	–	+
Hoxd11	–	–	–	–
Hoxd12	–	–	–	–
Hoxd13	–	–	–	–
Pdx1	+	+	–	+
Cdx2	+	nd	–	nd
Nkx6.2	+	+	+	+
Nkx6.1	+	+	+	+
Nkx2.2	+	+	+	+
Gbx2	+	–	–	–
Pax6	+	+	+	+
Pax4	+	+	+*	+
Isl1	+	+	+	+
Hlxb9	+	+	+*	+
Brain4 (25 cycles)	–	–	+	+*
Msx1	+	+	+	+

nd, not determined; +* denotes faint RT-PCR signal at 35 cycles of PCR.

box genes as Pdx1, Cdx2, Nkx6.2, Nkx6.1, Gbx2, and Pax6. The abundantly expressed Hox genes of Hoxa10 and Hoxb8, which accounted for 38.2% and 26.2%, respectively, of PCR clones in αTC1.6, suggested differences between MIN6 and αTC1.6. Cdx2 sequences were detected in MIN6, but not in αTC1.6. RT-PCR analyses and Northern blot analysis confirmed that mRNA level of Cdx2 in αTC1.6 was much lower than MIN6 and GLUTag (data not shown).

3.2. Semi-quantitative RT-PCR analysis of homeotic genes with specific primers

RT-PCR with specific primers applied to MIN6 or αTC1.6 produced transcript signals of the predicted size of each gene, and the nucleotide sequences of PCR products were identical to the published sequences. The results of RT-PCR of homeotic genes were summarized in Table 3 and representative results were shown in Fig. 1. Transcripts of Hoxc8, Nkx6.2, Nkx6.1, Nkx2.2, Pax6, isl1, and homeo box, msh-like 1 (Msx1) were almost equally detected in both MIN6 and αTC1.6. Genes of Hoxc6, Hoxc9, Hoxc10, Pdx1, Cdx2, gastulation brain homeo box 2 (Gbx2), and homeobox gene HB9 (Hlxb9), however, showed preferential mRNA expression in MIN6 cells. The preferential expression of Hoxc6, Hoxc9, Hoxc10, and Pdx1 in MIN6 cells was confirmed in another β-cell line of βTC1. Genes of Hoxa2, Hoxa3, Hoxa5, Hoxa6, Hoxa7, Hoxa9, Hoxa10, Hoxa13, Hoxb3, Hoxb5, Hoxb6, Hoxb8, and Hoxb13 showed preferential mRNA expression in αTC1.6 cells. Among them, the expression of Hoxa3, Hoxa6, Hoxa7, Hoxa9, Hoxa13, Hoxb5, Hoxb6, Hoxb8, and Hoxb13 was observed in βTC1. The results of RT-PCR with specific primers did not always coincide with the results of RT-PCR with degenerate primers. Genes of Hoxa10, Hoxb8, Hoxb5, Hoxb6, Hoxa5, and Hoxb3, which showed high percentage in RT-PCR with degenerate primers in αTC1.6, were also detected by RT-PCR with specific primers in αTC1.6. The expression pattern of Hox genes in αTC1.6 was similar to that of GLUTag rather than MIN6. Transcripts of Hoxa2, Hoxa3, Hoxa5, Hoxa6, Hoxa7, Hoxa10, Hoxa13, Hoxb3, Hoxb5, Hoxb6, Hoxb8, and Hoxb13 were detected in both αTC1.6 and GLUTag, but not detected in MIN6. The Hoxd locus except for Hoxd9 and Hoxd10 was silent in the cell lines analyzed.

Non-homeotic gene of neurod1, which is important in development of pancreatic islets, was expressed in MIN6, βTC1, αTC1.6, and GLUTag (data not shown). The expression of pancreatic specific transcription factor, 1a (Ptf1a), which is important in development of pancreatic exocrine gland, was not found in four cell lines analyzed (data not shown).

The RT-PCR showed expression of the islet hormones such as insulin (Fig. 1) and IAPP in MIN6. Although expression of glucagon was scarcely observed in MIN6, strong signals of proglucagon gene expression were observed in αTC1.6 and GLUTag (Fig. 1).

3.3. cDNA microarray analysis between MIN6 and αTC1.6

Gene expression in MIN6 and αTC1.6 was compared using Incyte mouse GEM I cDNA microarrays. The Incyte's mouse GEM I microarray consists of a total of 8,374 clones. According to the Incyte Genomics' protocol, all balanced differential expression ratios between two samples equal to or higher than 2.0 were considered significant. We restricted our analysis to genes over-expressed or under-expressed at least 4.0-fold between two cell lines. With this stringency, 58 and 25 of the detected genes in MIN6 were differentially over- or under-expressed, respectively (Tables 4 and 5).

We examined the accuracy of the microarray analysis by selecting 23 genes for Northern blot analysis that encompassed a wide range of expression ratios. Fig. 2 shows the representative results of Northern blotting on these genes. Changes in the expression level of 23 genes from our array studies were confirmed by Northern blotting.

3.4. cDNA microarray analysis between αTC1.6 and GLUTag

Incyte's mouse Unigene1 microarray consists of a total of 9,514 clones. Out of the 9,514 mouse genes analyzed, only 17 showed significant changes in their expression by

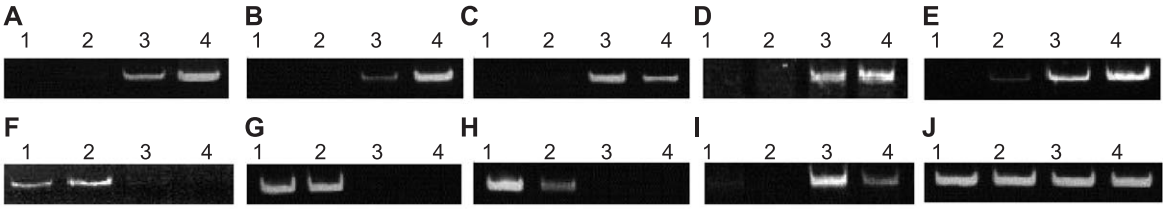

Fig. 1. Representative RT-PCR analysis of Hox genes. Total RNA isolated from MIN6 (lane 1), βTC1 (lane 2), αTC1.6 (lane 3), and GLUTag (lane 4) was reverse-transcribed and PCR was performed with specific primers. PCR cycles were 35 except for insulin2 (25 cycles) and glucagon (25 cycles). (A) Hoxa2; (B) Hoxa5; (C) Hoxa10; (D) Hoxb3; (E) Hoxb8; (F) Hoxc6; (G) Hoxc9; (H) insulin2; (I) glucagon; (J) TBP.

Table 4
Transcripts with their expression increased in MIN6 and fold increase relative to αTC1.6

Gene name	GenBank accession no.	Fold increase
(1) Neuropeptide Y precursor	W70782	33.4
(2) Keratin complex 2, basic, gene 7	AI892149	23.5
(3) Protein phosphatase 1, regulatory (inhibitor) subunit 1A	W75893	22.1
(4) Alcohol dehydrogenase1, complex	AA221141	20.6
(5) RNA imprinted and accumulated in nucleus	W89392	18.5
(6) Cholecystokinin	AI322505	17.9
(7) 3′-phosphoadenosine 5′-phosphosulfate synthase 2	AI390951	17.1
(8) Monoamine oxidase B	AA241899	13.1
(9) Maternally expressed gene 3	W97303	12.6
(10) ATPase, class I, type 8B, member 1	AA242626	11.6
(11) Annexin A4	AA397114	11.1
(12) Williams–Beuren syndrome chromosome region 14 homolog (human)	AA106263	10.6
(13) Selenoprotein P, plasma, 1	AA276440	10.5
(14) Endoplasmic reticulum oxidoreductin 1-Lbeta homolog (human)	AA217200	10.4
(15) RIKEN cDNA 3110032G18 gene	AA014375	10.3
(16) Insulin-like growth factor 2	AI322387	10.0
(17) Carboxypeptidase E	W83974	9.6
(18) Mus musculus transcribed sequence with moderate similarity to protein pir:A53436 (H.sapiens) A53436 3-alpha-hydroxysteroid/dihydrodiol dehydrogenase (EC 1.1.1.-)—human	W33809	9.1
(19) Erythrocyte protein band 4.1-like 4b	W91135	8.2
(20) Secretogranin III	AI021458	8.1
(21) Dipeptidylpeptidase 4	AA237541	7.3
(22) Cystathionine beta-synthase	AA239480	6.9
(23) CD24a antigen	W98974	6.9
(24) Solute carrier family 2 (facilitated glucose transporter), member 2	AA275871	6.8
(25) CD9 antigen	W98963	6.8
(26) Protein kinase, cAMP dependent regulatory, type 1, alpha	AA537355	6.7
(27) Deiodinase, iodothyronine, type I	AA212899	6.6
(28) Solute carrier family 40 (iron-regulated transporter), member 1	AA500296	6.1
(29) Thioesterase, adipose associated	AA036034	6.1
(30) Insulin-like growth factor 2, antisense	W97588	6.1
(31) RIKEN cDNA 2310039E09 gene	AA027653	6.0
(32) ATP citrate lyase	W33415	5.8
(33) Expressed sequence AW210596	AA268104	5.5
(34) DNA segment, Chr 11, ERATO Doi 498, expressed	AA138526	5.4
(35) ELL-related RNA polymerase II, elongation factor	AA545429	5.1
(36) Huntingtin-associated protein 1	AA254430	5.1
(37) Glycine amidinotransferase (L-arginine:glycine amidinotransferase)	AA049981	5.1
(38) Bone morphogenetic protein 1	W82677	5.0
(39) Pirin	W08720	4.8
(40) RIKEN cDNA 2600017H08 gene	AA184855	4.7
(41) Mesenchyme homeobox 2	AA231293	4.7
(42) EST	AI510251	4.7
(43) Protein kinase, cAMP dependent regulatory, type I beta	AA270948	4.6
(44) Pre B-cell leukemia transcription factor 3	AA000829	4.5
(45) Phosphoglucomutase 3	AI324878	4.5
(46) RIKEN cDNA 1300018J18 gene	AA217174	4.4
(47) SemaF cytoplasmic domain associated protein 2	AA023463	4.4
(48) Leucine rich repeat protein 1, neuronal	W40832	4.3
(49) Serine dehydratase related sequence 1	AI322392	4.3
(50) Transthyretin	W17647	4.3
(51) Sulfide quinone reductase-like (yeast)	AA266579	4.3
(52) Archain 1	AA404092	4.1
(53) RIKEN cDNA 5730592L21 gene	AA260520	4.1
(54) RIKEN cDNA F730017H24 gene	AA146110	4.1
(55) Expressed sequence AI662270	AA035956	4.1
(56) Phosphatidylinositol 4-kinase type 2 beta	AA276928	4.0
(57) REC8-like 1 (yeast)	AI426149	4.0
(58) RIKEN cDNA 2310020L09 gene	AA004070	4.0

4-fold or above 4-fold increase or decrease between αTC1.6 and GLUTag. Among them, 12 genes showed higher expression in αTC1.6 than in GLUTag, whereas 5

Table 5
Transcripts with their expression increased in αTC1.6 and fold increase relative to MIN6

Gene name	GenBank accession no.	Fold increase
(1) Growth factor receptor bound protein 10	AA260248	29.1
(2) Hypoxia induced gene 1	AA414831	27.2
(3) RIKEN cDNA 3930402G23 gene	W13316	9.4
(4) BM88 antigen	AA033029	8.4
(5) Neuropilin 2	AA269699	8.0
(6) Dynein, axon, heavy chain 11	AA172519	6.6
(7) RIKEN cDNA 1110036H21 gene	AI893491	6.5
(8) Cystathionase	AI892342	6.4
(9) Four and a half LIM domains 1	AA047966	6.2
(10) Potassium voltage-gated channel, subfamily Q, member 2	W97901	6.0
(11) N-myc downstream regulated 4	W97514	5.5
(12) MAP kinase-activated protein kinase 2	W45833	5.4
(13) Aquaporin 1	AA241281	5.1
(14) RIKEN cDNA 5730438N18 gene	W40994	4.9
(15) Brain protein 44-like	W08432	4.7
(16) Fructose bisphosphatase 1	AA276043	4.6
(17) WD repeat domain 12	AA467053	4.5
(18) AE-binding protein 2	AA416308	4.4
(19) RIKEN cDNA 1620401E04 gene	W16247	4.4
(20) Heat shock protein, 74 kDa, A	AA498713	4.3
(21) Translocase of inner mitochondrial membrane 8 homolog a (yeast)	W11535	4.2
(22) Basic leucine zipper and W2 domains 2	AA068436	4.2
(23) 3-oxoacid CoA transferase	AA230896	4.2
(24) StAR-related lipid transfer (START) domain containing 4	AA239481	4.1
(25) G elongation factor	AA498518	4.0

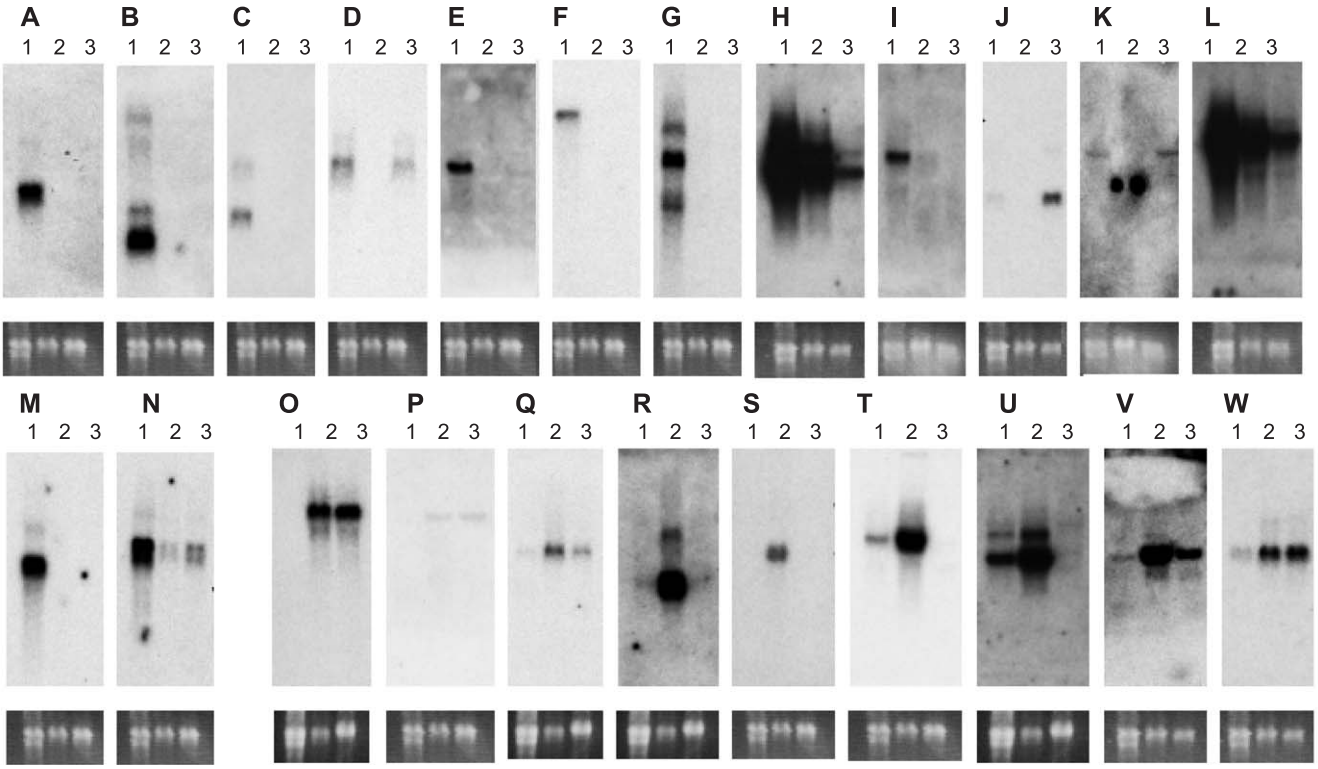

Fig. 2. Representative Northern blot analysis confirming changes in mRNA levels. Total RNA (10 μg) isolated from MIN6 (lane 1), αTC1.6 (lane 2), and NIH3T3 (lane 3) blotted to nylon membrane and probed with [α-^{32}P]-labeled cDNA. (A) Keratin complex 2, basic, gene 7; (B) protein phosphatase 1; (C) alcohol dehydrogenase1; (D) RNA imprinted and accumulated in nucleus; (E) monoamine oxidase B; (F) ATPase, class I, type 8B, member 1; (G) Wbscr14; (H) carboxypeptidase E; (I) erythrocyte protein band 4.1-like 4b; (J) CD9 antigen; (K) solute carrier family 40 (iron-regulated transporter), member 1; (L) ATP citrate lyase; (M) mesenchyme homeobox 2; (N) pre B-cell leukemia transcription factor 3; (O) growth factor receptor bound protein 10; (P) neuropilin 2; (Q) dynein, axon, heavy chain 11; (R) RIKEN cDNA 1110036H21 gene; (S) cystathionase; (T) N-myc downstream regulated 4; (U) fructose bisphosphatase 1; (V) translocase of inner mitochondrial membrane 8 homolog a; (W) basic leucine zipper and W2 domains 2. Ethidium bromide-stained 28S ribosomal RNAs were included to verify loading of similar amounts of RNA in the lower part.

were more expressed in GLUTag than in αTC1.6 (data are available on request).

4. Discussion

Identification of transcription factors that specify pancreatic β- and α-cell differentiation phenotypes are of major importance to understand the molecular basis of diabetes. Homeotic genes such as Pdx1 exemplify one class of transcription factors that govern pancreatic islet phenotypic diversity. Hox genes encode transcription factors which are involved in the establishment in regional identities along the AP body axis. In an attempt to identify difference in expression of Hox genes, we designed primers based on the sequences of *Drosophila* homeodomain protein *Antennapedia* and used these primers to amplify Hox genes by PCR from MIN6 or αTC1.6 cDNA. This method, initially used for mouse intestine (James and Kazenwadel, 1991), resulted in successful identification of homeotic genes expressed in pancreatic islets (Rudnick et al., 1994; Miller et al., 1994). The amplified products in our study included sequences encoding 16 distinct Hox genes. In addition, homeodomain transcription factors which share homeobox sequence with Hox genes and regulate islet cell differentiation were also obtained.

αTC1.6 and GLUTag secrete glucagon and GLP-1, respectively. Glucagon and GLP-1 are synthesized from a common precursor of proglucagon. Pancreatic glucagon is generated via the action of prohormone convertase 2. In the L cell of the intestine, proglucagon is processed by prohormone convertase 1/3, resulting in the formation of GLP-1. Expression of the proglucagon gene is highly restricted to α-cells, L cells of the intestine, and neuronal cell bodies in the brain stem (Drucker and Asa, 1988).

The Pdx1 gene is expressed in the duodenum and pancreatic islets. Our RT-PCR analysis suggested that Pdx1 was a predominant homeotic gene in MIN6, although it is also expressed in the intestinal neuroendocrine cell line of GLUTag. RT-PCR analysis of Hox genes with degenerate primers in rat pancreatic islets (Miller et al., 1994), insulin-producing cell lines (Rudnick et al., 1994), or glucagon-producing cell lines was performed, but numbers of clones analyzed were limited, and RT-PCR analysis with specific primers were not performed. Expression patterns of some Hox genes in βTC1 were different from those of MIN6, and

rather similar to those of αTC1.6. The minor difference in expression pattern of Hox genes between MIN6 and βTC1 might reflect different differentiation levels between two cell lines (Poitout et al., 1996). MIN6, βTC1, αTC1.6, and GLUTag were derived from C57BL/6, BDF1, BDF1, and CD-1 mouse, respectively. The difference in the expression profile may be in part dependent on the difference in the strain from which these cell lines were established. The expression pattern of Hox genes in αTC1.6 was similar to that of GLUTag rather than MIN6. Expression of 12 Hox genes were commonly detected in αTC1.6 and GLUTag, but not detected in MIN6. This may lead to the same phenotype of the proglucagon gene expression in both αTC1.6 and GLUTag.

A pair of neighboring murine Hox genes (Hoxb8 and Hoxb9) may define a molecular switch. The products of these two related Hox genes, which are located adjacent to each other in the Hox complex, can differentially modulate transcription from the promoter of the cell adhesion molecule (CAM) gene (Jones et al., 1992). Hoxb8 protein is an inhibitor, while Hoxb9 protein is an activator of the N-CAM gene. Hoxb8 was found to be over-expressed in αTC1.6. Although the N-CAM gene was expected to be over-expressed in MIN6 compared to αTC1.6, RT-PCR analysis did not show any difference in N-CAM expression between MIN6 and αTC1.6 (data not shown).

It is well known that pancreatic endocrine development utilizes many transcription factors originally described in neural development (Wilson et al., 2003). The Nkx6.1 and Nkx2.2 genes are expressed in the central nervous system and involved in the terminal differentiation of pancreatic β-cells (Wilson et al., 2003). These genes were expressed equally in MIN6 and αTC1.6 in our study. The Nkx6.2, which was found to be expressed in MIN6 and αTC1.6, has a similar neuronal expression pattern as Nkx6.1. Although Nkx6.2 null mice were shown to have normal growth, detailed information on pancreatic development was not available (Cai et al., 2001). The Gbx2 gene is expressed mainly in embryonal brain (Wassarman et al., 1997). The Gbx2 null mice have failure of anterior hindbrain development. Although Gbx2 gene was found to be expressed in MIN6, but not αTC1.6 or GLUTag, detailed information on pancreatic development was not available. Our random cDNA sequencing study showed the expression of Msx1 gene in MIN6 (Tanaka et al., 1995). The Msx1 gene is expressed in a range of neural-crest-derived tissues and areas of putative epithelial–mesenchymal interactions during embryogenesis (Mackenzie et al., 1991). The expression of Msx1 was observed in all four cell lines analyzed. Although expression of Msx2 is elevated in the regenerating and developing pancreas of interferon-γ transgenic mice (Kritzik et al., 1999), the role of Msx1 in the development of pancreas remains unknown. The POU homeodomain factor Brain4 was originally described in the central nervous system. The Brain4 was found in the α-cell line of αTC1.6 and the enteroendocrine cell line of GLUTag where it plays a role of proglucagon gene expression. Thirty-five cycles of PCR detected faint signals of Brain4 even in MIN6 and βTC1. The report that the ectopic expression of Brain4 targeted to β-cells leads to the coexpression of insulin and glucagons suggests an important role of Brain4 in the proglucagon gene expression (Hussain et al., 2002).

To detect genes specifically expressed in pancreatic β-cells, Neophytou et al. (1996) used a subtractive cloning approach to identify specifically expressed mRNAs in pancreatic β-cells. Genes known to be highly expressed in βTC3 compared to αTC2 included insulin, IAPP, proinsulin convertase 1, and neuropeptide Y. In addition, they found a pancreatic islet-specific glucose-6-phosphatase-related protein (Arden et al., 1999). Niwa et al. applied the PCR-based subtractive hybridization technique of representational difference analysis (RDA) to βTC3 and αTC (Niwa et al., 1997). They found that insulin, IAPP, insulin-like growth factor II (IGFII), selenoprotein P, neuronatin, prohormone convertase, regulatory subunit RIα of protein kinase A (PRKAR1A) were over-expressed in βTC3. Arava et al. (1999) applied RDA to identify genes selectively expressed in βTC1 compared with αTC1. They isolated 26 clones expressed at the higher levels in βTC1 than in αTC1. Some genes such as insulin, IAPP, neuronatin, PRKAR1A, signal transducer and activator of transcription 6, guanylate cyclase, and vinculin were over-expressed in βTC1. Among these genes, expression of neuropeptide Y, IGFII, selenoprotein P, and PRKAR1A was confirmed to be higher in MIN6 than that in αTC1.6 in our experiment.

To identify the genes that determine differentiation phenotypes, we compared gene expression between MIN6 and αTC1.6 by DNA microarray. Because MIN6 is known to have more differentiated phenotypes of mature β-cells than βTC1, we selected MIN6 rather than βTC1 as a representative of β cell lines. Although cDNA clones on the Incyte's mouse GEM I lacked important β- or α-cell genes such as insulin, IAPP, glucokinase, Pdx1, neurogenin 3, neurod1, Isl1, Nkx2.2, Nkx6.1, Pax4, Pax6, Hlxb9, and Brain4 genes, DNA microarray hybridization effectively detected 83 differentially expressed genes between closely related cell types of MIN6 and αTC1.6. Among 83 differentially expressed genes, six genes were already known to be differentially expressed between MIN6 and αTC1.6. They were neuropeptide Y (Neophytou et al., 1996), selenoprotein P (Niwa et al., 1997), IGFII (Niwa et al., 1997), PRKAR1A (Arava et al., 1999), alcohol dehydrogenase (Neophytou et al., 1996; Niwa et al., 1997; Arava et al., 1999), and ATP citrate lyase (Niwa et al., 1997). Genes of Wbscr14, PRKAR1A, secretogranin III, ATP citrate-lyase, transthyretin were over-expressed in MIN6 than αTC1.6 or GLUTag.

Genes categorized as over-expressed in MIN6 than αTC1.6 included the endoplasmic reticulum oxidoreductin 1-L beta (ERO1-Lβ) homolog (human) gene. The ERO1-Lβ gene is detected with a frequency of 0.15% in RIKEN

full-length enriched, adult pancreatic islet library (http://www.ncbi.nlm.nih.gov/UniGene/). Because ERO-1 Lβ favors disulfide bond formation in the endoplasmic reticulum (Pagani et al., 2000), ERO-1 Lβ and selenoprotein P over-expressed in MIN6 might contribute to formation and maintenance of insulin disulfide bonds. The RNA imprinted and accumulated in nucleus (Rian) gene was detected in Melton Mouse E16.5 Pancreas Library 2 M16B2 with a frequency of 0.25% and in Kaestner ngn3 wt adult pancreas library with a frequency of 0.02% (http://www.ncbi.nlm.nih.gov/UniGene/). The Rian gene expresses maternally expressed brain-specific non-coding RNA (Hatada et al., 2001). The significance of over-expression of Rian gene in MIN6 than αTC1.6 remains unknown. Four and a half LIM domains 1 (FHL1) gene was over-expressed in αTC1.6 than MIN6. FHL1 contains four and a half LIM domains and is highly expressed in skeletal and cardiac muscle. Because a splicing isoform of FHL1 can interact and negatively regulate the activity of RBP-J, a transcription factor involved in Notch signaling pathway (Taniguchi et al., 1998), FHL1 might regulate activity of other transcriptional factors related to islet development or function in α-cells. Recently, Wang et al. (2003) reported that FHL1 gene was over-expressed in αTC1.6 than MIN6 by using oligonucleotide microarrays. Mesenchyme homeobox 2 (Meox2) is important regulator of vertebrate limb myogenesis. Meox2 was overexpressed in MIN6 than αTC1.6. The result was consistent with the data obtained by Wang et al. (2003). Homeotic genes of Msx and Meox families are coexpressed in the vertebrate embryo in regions of epithelial–mesenchymal interactions (Quinn et al., 2000). Because Msx1 and Meox2 were expressed in islet cell lines in our study, these genes might play a role on development of pancreatic islets.

In this study, we confirmed the difference in gene expression of homeotic and other genes between MIN6 and αTC1.6 with RT-PCR, Northern blot, and DNA microarray analysis, in spite of the differentiation from the common (neurogenin 3-expressing) precursor (Jensen et al., 2000; Schwitzgebel et al., 2000). In addition, GLUTag, which expresses the preproglucagon gene, showed a comparatively similar expression profile in regards to Hox, and other genes to that of αTC1.6. Our results are consistent with the interpretation that not only the tissue-specific homeotic genes, but also Hox genes are related to differentiation phenotypes of pancreatic β- and α-cells rather than their regional specification of the body in vertebrates.

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