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Molecular Cloning of Mouse Paired-Box-Containing Gene (Pax)-4 from an Islet β Cell Line and Deduced Sequence of Human Pax-4 [1]

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A mouse cDNA encoding paired-box-containing gene (Pax-4) was cloned from a cDNA library of a mouse pancreatic islet β cell line of MIN6. The predicted open reading frame encodes a protein of 349 amino acids with a calculated molecular weight (MW) of 38-kD. A human Pax-4 cDNA sequence encoding 350 amino acids was deduced from a human cosmid clone. The mouse nucleotide and deduced amino acid sequences exhibit 83.4 and 80.0% identity with those of deduced human Pax-4, respectively. Southern blot analysis suggested that the mouse Pax-4 gene exists as a single copy in the genome. Reverse transcription (RT)-PCR analysis suggested that the mouse Pax-4 gene is expressed in pancreatic islets, cultured islet β cell lines of MIN6, βTC, and NIT-1 cells, but not detectable in any of 13 adult mouse organs examined.

Key Words: **paired box containing gene; Pax-4.**

The paired box was originally identified in the *Drosophila* segmentation genes *paired* (1), *gooseberry distal* (*gsb-d*), and *gooseberry proximal* (*gsb-p*). The paired box has been highly conserved during evolution, being present in such divergent organisms as nematode, zebrafish, *Xenopus,* chicken, rodent, and man. It encodes a protein domain of 128 amino acids containing a DNA-binding motif. Based on *gsb-d* paired box, a mouse multigene family of paired-box-containing 9 genes has been isolated (2, 3). All 9 Pax genes in the Pax multigne family encode paired-box containing transcriptional factors and exhibit temporally and spatially restricted expression patterns during embryogenesis (4, 5).

Nine Pax genes are classified into four subgroups; Pax-1/9, Pax-2/5/7, Pax-3/7, and Pax-4/6. Pax-4/6 was shown to be involved in pancreatic islet development in mice; Pax-4-deficient mice lack pancreatic islet β and δ cells (6), and Pax-6-deficient mice lack pancreatic islet α cells (7). Although most of the Pax gene family including mouse and human Pax-6 cDNAs (8, 9) have been cloned, only a 613 bp partial mouse Pax-4 cDNA sequence (GenBank accession number Y09584, corresponding to the nucleotide number of 337 to 949 in our cDNA sequence) and a partial 383 bp genomic sequence corresponding to the paired domain (3) which turned out to be 131 and 36 bp exons separated by one 216 bp intron were reported. No human Pax-4 sequence except the 240 base pair (bp) genomic STS sequence of G31758 (10) was reported. In this study, we cloned mouse Pax-4 cDNA encompassing the full coding sequence from MIN6 cDNA library and deduced human Pax-4 cDNA sequence from a human cosmid clone obtained by homology search. We report the nucleotide and amino acid sequences of mouse Pax-4 and the deduced nucleotide and amino acid sequences of human Pax-4.

MATERIALS AND METHODS

Cloning of mouse Pax-4 cDNA from MIN6 cDNA library. Total RNA was prepared from MIN6 (11) cells by ISOGEN (NIPPONGENE, Tokyo, Japan). A cDNA library was constructed in a phage vector, λTriplEx (CLONTECH, Palo Alto, CA). Oligonucleotides of oRB4A and oRB5S were synthesized based on the published partial mouse cDNA sequence (GenBank accession number Y09584) corresponding to an antisense sequence of bases 52-71 and a sense

[1] The nucleotide sequences reported in this manuscript were deposited in DDBJ, EMBL, and GenBank under accession number AB008912 (mouse sequence) and AB008913 (human sequence).

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Abbreviation used: Paired-Box-Containing Gene (Pax), Reverse transcription (RT), molecular weight (MW), base pair (bp), Dulbecco's modified Eagle's medium (DMEM), dithiothreitol (DTT).

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sequence of bases 580-599, for the 5′- and 3′-RACE, respectively. PCR was carried out using a pair of λTriplEx vector primers corresponding to the left arm (λTriplEx 3′ LD-insert amplimer; ATACGACTCACTATAGGGCGAATTGGCC) and the right arm (λTriplEx 5′ LD-insert amplimer; CTCGGGAAGCGCGCCATTGTGTTGGT).

The PCR template from the MIN6 phage library was heated at 65°C for 15 min to extract DNA from phage capsids. The PCR mixture contained 3 μl of a template/200 μM each of dNTPs/0.25 μM each of primers/2.5 units of ExTaq polymerase (Takara, Shiga, Japan)/1× ExTaq buffer in a 100 μl reaction volume. After incubating the mixture at 94°C for 5 min, PCR proceeded 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. PCR primer sets of oRB4A and λTriplEx 5′ LD-insert amplimer, oRB5S and λTriplEx 3;pr LD-insert amplimer were used to obtain 5′ and 3′ sequences of the Pax-4 cDNA, respectively.

The amplified 5′ and 3′ sequences of Pax-4 cDNA were cloned into pCRII (Invitrogen, San Diego, CA) (Pax-4-5′ and Pax-4-3′, respectively), and overnight cultures of bacterial colonies were screened by the blue/white color selection. After confirming the insert sizes, DNA sequencing was carried out using the Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Perkin Elmer/Division of Applied Biosystems, Foster city, CA). M13 vector primers were used to sequence four independent clones of the Pax-4-5′ and Pax-4-3′, respectively.

Oligonucleotide PCR primers of oRB1S (CTGTCCTTCTGTGAGGAG-TA) and oRB2A (AAGAAAAGGTTTTTATTACT) were synthesized based on the determined mouse cDNA sequence to amplify the open reading frame of mouse Pax-4. PCR was carried out as described above except for the extension time of 2 min. The amplified 1,118 bp cDNA out of the determined 1,324 bp sequence was cloned into pCRII (Pax-4ORF). The 1,118 bp sequence was determined by sequencing Pax-4ORF. M13 vector primers and 6 internal primers were used to sequence twelve independent clones. The 1,324 bp sequence was generated from determined sequences of Pax-4-5′, Pax-4-3′, and Pax-4ORF.

Southern blot analysis. Ten micrograms of genomic DNA isolated from mouse tail were digested with one of the following 6 restriction enzymes recognizing 6 base sequences; *Eco*RI, *Kpn*I, *Pst*I, *Sac*I, *Spe*I, and *Xho*I. Digested samples were subjected to 1.0% agarose gel electrophoresis and transferred to nylon membrane (GeneScreen *plus*, MEN Life Science Products, Boston, MA) by the method of Southern (12). The 358 bp DNA fragment (Pax-4PD) from nucleotide numbers 340 to 697 of mouse Pax-4 obtained RT-PCR from MIN6 total RNA was cut out and gel-purified. This fragment was radiolabeled with [α-^{32}P]dCTP (3,000 Ci/mmol; Amersham, Buckinghamshire, UK) using a Megaprime DNA labeling system (Amersham) and used as a probe. Hybridization was carried out in 1 M NaCl containing 50% formamide, 1% SDS, 100 μg/ml of salmon sperm DNA, 10% dextran sulfate, and a radiolabeled probe at 42°C overnight.

RT-PCR analysis. Various mouse tissues were obtained by necropsy. Mouse islet were obtained by collagenase method (13). Mouse pancreatic β cell lines of MIN6 (11), βTC (14), and NIT-1 (15) were

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   1  TGAAGGCTCTGTGAAGCTCTGGACCCCCTGGCAGGACTGAAGCAGCTGGAGGCTGTTACA  60
  61  AGACCAGACCACCAGCAAACCCTGGAGCCTGCACAGGACCCTGAGACCTCTTCCTGGAAT 120
 121  TCCCACCTTTTTTCCTCCATCCAGAACCAGTCCCAAAGAGAAACTTCCAGAAGGAGCTCT 180
 181  CCGTTTTCAGTTTGCCAGTTGGCTTCCTGTCCTTCTGTGAGGAGTACCAGTGTGAAGCAT 240
                                                          M
 241  GCAGCAGGACGGACTCAGCAGTGTGAATCAGCTAGGGGGACTCTTTGTGAATGGCCGGCC 300
       Q  Q  D  G  L  S  S  V  N  Q  L  G  G  L  F  V  N  G  R  P
 301  CCTTCCTCTGGACACCAGGCAGCAGATTGTGCAGCTAGCAATAAGAGGGATGCGACCCTG 360
       L  P  L  D  T  R  Q  Q  I  V  Q  L  A  I  R  G  M  R  P  C
 361  TGACATTTCACGGAGCCTTAAGGTATCTAATGGCTGTGTGAGCAAGATCCTAGGACGCTA 420
       D  I  S  R  S  L  K  V  S  N  G  C  V  S  K  I  L  G  R  Y
 421  CTACCGCACAGGTGTCTTGGAACCCAAGTGTATTGGGGGAAGCAAACCACGTCTGGCCAC 480
       Y  R  T  G  V  L  E  P  K  C  I  G  G  S  K  P  R  L  A  T
 481  ACCTGCTGTGGTGGCTCGAATTGCCCAGCTAAAGGATGAGTACCCTGCTCTTTTTGCCTG 540
       P  A  V  V  A  R  I  A  Q  L  K  D  E  Y  P  A  L  F  A  W
 541  GGAGATCCAACACCAGCTTTGCACTGAAGGGCTTTGTACCCAGGACAAGGCTCCCAGTGT 600
       E  I  Q  H  Q  L  C  T  E  G  L  C  T  Q  D  K  A  P  S  V
 601  GTCCTCTATCAATCGAGTACTTCGGGCACTTCAGGAAGACCAGAGCTTGCACTGGACTCA 660
       S  S  I  N  R  V  L  R  A  L  Q  E  D  Q  S  L  H  W  T  Q
 661  ACTCAGATCACCAGCTGTGTTGGCTCCAGTTCTTCCCAGTCCCCACAGTAACTGTGGGGC 720
       L  R  S  P  A  V  L  A  P  V  L  P  S  P  H  S  N  C  G  A
 721  TCCCCGAGGTCCCCACCCAGGAACCAGCCACAGGAATCGGACTATCTTCTCCCCGGGACA 780
       P  R  G  P  H  P  G  T  S  H  R  N  R  T  I  F  S  P  G  Q
 781  AGCCGAGGCACTGGAGAAAGAGTTTCAGCGTGGGCAGTATCCAGATTCAGTGGCCCGTGG 840
       A  E  A  L  E  K  E  F  Q  R  G  Q  Y  P  D  S  V  A  R  G
 841  GAAGCTGGCTGCTGCCACCTCTCTGCCTGAAGACACGGTGAGGGTTTGGTTTTCTAACAG 900
       K  L  A  A  A  T  S  L  P  E  D  T  V  R  V  W  F  S  N  R
 901  AAGAGCCAAATGGCGCAGGCAAGAGAAGCTGAAATGGGAAGCACAGCTGCCAGGTGCTTC 960
       R  A  K  W  R  R  Q  E  K  L  K  W  E  A  Q  L  P  G  A  S
 961  CCAGGACCTGACAGTACCAAAAAATTCTCCAGGGATCATCTCTGCACAGCAGTCCCCCGG 1020
       Q  D  L  T  V  P  K  N  S  P  G  I  I  S  A  Q  Q  S  P  G
1021  CAGTGTACCCTCAGCTGCCTTGCCTGTGCTGGAACCATTGAGTCCTTCCTTCTGTCAGCT 1080
       S  V  P  S  A  A  L  P  V  L  E  P  L  S  P  S  F  C  Q  L
1081  ATGCTGTGGGACAGCACCAGGCAGATGTTCCAGTGACACCTCATCCCAGGCCTATCTCCA 1140
       C  C  G  T  A  P  G  R  C  S  S  D  T  S  S  Q  A  Y  L  Q
1141  ACCCTACTGGGACTGCCAATCCCTCCTTCCTGTGGCTTCCTCCTCATATGTGGAATTTGC 1200
       P  Y  W  D  C  Q  S  L  L  P  V  A  S  S  S  Y  V  E  F  A
1201  CTGGCCCTGCCTCACCACCCATCCTGTGCATCATCTGATTGGAGGCCCAGGACAAGTGCC 1260
       W  P  C  L  T  T  H  P  V  H  H  L  I  G  G  P  G  Q  V  P
1261  ATCAACCCATTGCTCAAACTGGCCATAAGAGGCCTCTATTTGACAGTAATAAAAACCTTT 1320
       S  T  H  C  S  N  W  P  *
1321  TCTT  1324
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FIG. 1. The nucleotide sequence of mouse Pax-4 cDNA and its deduced amino acid sequence. The 1,324 nucleotide sequence is numbered from the nucleotide at the 5′-end. The 349 amino acid sequence shown by one letter code corresponding to the 1,047 bp coding sequence is numbered from the amino terminus. The polyadenylation signal is underlined. An asterisk marks stop codon, UAA.

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mouse Pax-4      1 MQQDGLSSVNQLGGLFVNGRPLPLDTRQQIVQLAIRGMRP    40
human Pax-4      1 MHQDGISSMNQLGGLFVNGRPLPLDTRQQIVRLAVSGMRP    40
                   * *** ** ********************** **  ****

mouse Pax-4     41 CDISRSLKVSNGCVSKILGRYYRTGVLEPKCIGGSKPRLA    80
human Pax-4     41 CDISRILKVSNGCVSKILGRYYRTGVLEPKGIGGSKPRLA    80
                   ***** ************************ *********

mouse Pax-4     81 TPAVVARIAQLKDEYPALFAWEIQHQLCTEGLCTQDKAPS   120
human Pax-4     81 TPPVVARIAQLKGECPALFAWEIQRQLCAEGLCTQDKTPS   120
                   ** ********* * ********* *** ******** **

mouse Pax-4    121 VSSINRVLRALQEDQSLHWTQLRSPAVLAPVLPSPHSNCG   160
human Pax-4    121 VSSINRVLRALQEDQGLPCTRLRSPAVLAPAVLTPHSGSE   160
                   *************** *  * *********    ***

mouse Pax-4    161 APRGPHPGTSHRNRTIFSPGQAEALEKEFQRGQYPDSVAR   200
human Pax-4    161 TPRGTHPGTGHRNRTIFSPSQAEALEKEFQRGQYPDSVAR   200
                    *** **** ********* ********************

mouse Pax-4    201 GKLAAATSLPEDTVRVWFSNRRAKWRRQEKLKWEAQLPGA   240
human Pax-4    201 GKLATATSLPEDTVRVWFSNRRAKWRRQEKLKWEMQLPGA   240
                   **** ***************************** *****

mouse Pax-4    241 SQDLTVPKNSPGIISAQQSPGSVPSAALPVLEPLSPSFCQ   280
human Pax-4    241 SQGLTVPRVAPGIISAQQSPGSVPTAALPALEPLGPSCYQ   280
                   ** ****   ************** **** **** **  *

mouse Pax-4    281 LCCGTAPGRCSSDTSSQAYLQPYWDCQS-LLPVASSSYVE   319
human Pax-4    281 LCWATAPERCLSDTPPKACLKPCWDCGSFLLPVIAPSCVD   320
                   **  *** ** ***   * * * *** * ****   * *

mouse Pax-4    320 FAWPCLTTHPVHHLIGGPGQVPSTHCSNWP             349
human Pax-4    321 VAWPCLDASLAHHLIGGAGKATPTHFSHWP            350
                    *****     ****** *    ** * **
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FIG. 2. Alignment of mouse and human Pax-4 amino acid sequence. Asterisks mark the conserved residues in mouse and human sequences.

grown on 90-mm dishes in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose with 10% fetal bovine serum in the atmosphere of 5% CO_2-95% air at 37°C. A mouse pancreatic α cell line of αTC (16), and a mouse fibroblast cell line of NIH/3T3 (17) were grown in DMEM with 5.5 mM glucose. Total RNA was prepared by ISOGEN (NIPPONGENE) from ICR mouse organs or five cultured mouse cell lines. AMV-RT reactions (LIFE SCIENCE, St. Petersburg, FL) were carried out with 10 μg of total RNA/50 ng of a random hexamer/10 mM dithiothreitol (DTT)/25 units of AMV-RTase/1× reaction buffer in a 25 μl reaction volume. The PCR mixture contained 0.5 μl of template cDNA/200 μM each of dNTPs/0.25 μM each of primers/0.5 unit of AmpliTaq Gold polymerase (Perkin-Elmer)/1× AmpliTaq Gold buffer in a 20 μl reaction volume. After incubating the mixture at 94°C for 5 min, PCR was carried out at the cycle number of 25, 30, 35, or 40 consisting of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec using a pair of primers oRB2S (TGTGAAGCATGCAGCAGGAC) and oRB4A. A sense (GTGGGCCGCTC-TAGGCACCA) and an antisense (CGGTTGGCCTTAGGGTTCAGG) PCR primers were used to amplify β-actin as an internal control.

RESULTS

Cloning of Mouse Pax-4 cDNA from MIN6 cDNA Library

PCR was used to clone mouse Pax-4 cDNA from MIN6 cDNA library. The 1,324 bp mouse Pax-4 cDNA contained a 1,047 bp open reading frame encoding 349 amino acids with the predicted MW of 38-kD (Fig. 1). The open reading frame starts at the first AUG codon at the nucleotide numbers 239-241, and ends with a UAA codon at 1,286-1,288. One polyadenylation signal is located at 1,308-1,313. The sequence surrounding the first AUG conform to Kozak's rules with A in position -3 of ATG (18).

Deduced Sequence of Human Pax-4

A human cosmid clone of AC000359 (GenBank accession number) and a human STS clone of G31758 (10) were found through searching GenBank database using mouse Pax-4 cDNA as a query sequence. First because the human STS clone of G31758 was reported to be in the human Pax-4 gene (10), second because the genomic 240 bp sequence of G31758 exhibited a 89% identity to the previously reported 613 bp partial mouse Pax-4 cDNA (GenBank accession number Y09584), and lastly because a part of AC000359 exhibited a very high identity to our mouse Pax-4 cDNA (the nucleotide identity of AC000359 with our full length 1,324 bp mouse Pax-4 cDNA turned out to be 83.4%), AC000359 was confirmed to include the human genomic Pax-4 sequence. Owing to the high level of nucleo-

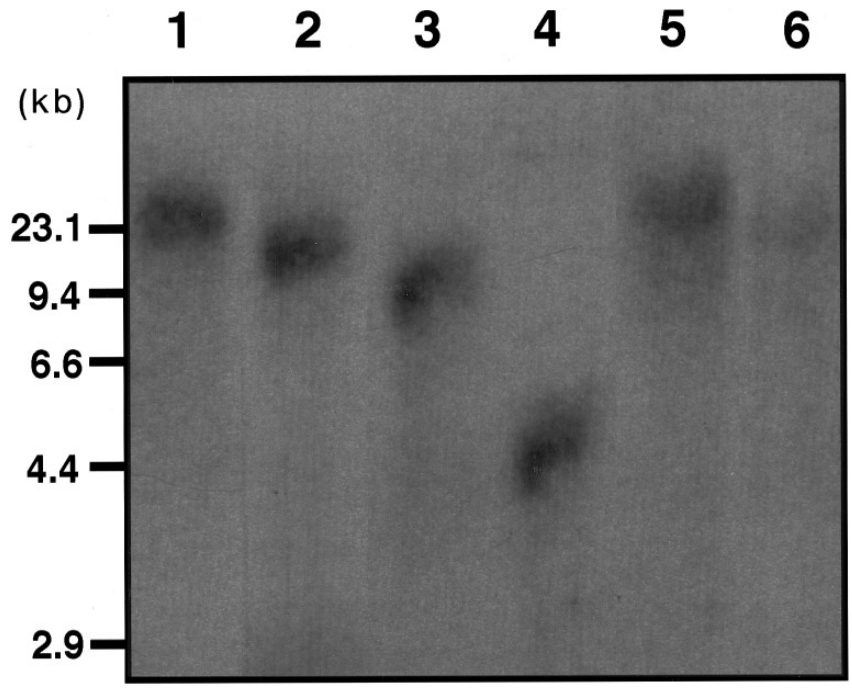

FIG. 3. Southern blot analysis. Mouse genome DNA in 10 μg was digested with lane 1; *Eco*RI, lane 2; *Kpn*I, lane 3; *Pst*I, lane 4; *Sac*I, lane 5; *Spe*I, and lane 6; *Xho*I. Digests were subjected to 1% agarose gel electrophoresis, transferred to nylon membrane, and hybridized with the probe containing a 358 bp DNA fragment from nucleotide number 340 to 697 of the mouse Pax-4 cDNA.

tide and amino acid identity, of which our final estimation turned out to be 83.4 and 80.0%, respectively, the human Pax-4 cDNA sequence and its genomic structure were effectively deduced by the computer homology search using the mouse Pax-4 cDNA sequence as the basis (Fig. 2).

Southern Blot Analysis

The genomic Southern blot which was probed with Pax-4PD exhibited a single band in each lane for 6 restriction enzymes, suggesting that the mouse Pax-4 gene exists as a single copy per haploid in the genome (Fig. 3).

RT-PCR Analysis

Ten micrograms of total RNA were prepared from various mouse organs and cell lines. A weakly amplified band was observed only in pancreatic islets at the PCR cycle number of 40 (Fig. 4, lane 6). Except for this, no band was observed in 13 organs examined including brain, lung, heart, thymus, muscle, stomach, intestine, colon, spleen, kidney, liver, ovary, and testis by RT-PCR. Although 5 cell lines did not exhibit a band at the cycle number of 25 or 30 (data not shown), MIN6, βTC, and NIT-1 exhibited a band at the cycle number of 35 (data not shown) or 40 (Fig. 4, lanes 1-3). Because RT-PCR of αTC or NIH/3T3 did not exhibit an amplified band, the mouse Pax-4 gene is suggested to be expressed in pancreatic islets, islet β cell lines of MIN6, βTC, and NIT-1. The expression level was far less than that of β-actin. Sequence analysis of the RT-PCR amplified 177 bp bands from MIN6, βTC, and NIT-1 total RNA confirmed that their sequences were identical with that of mouse Pax-4 cDNA.

DISCUSSION

Mouse Pax-4 encodes a protein containing an N-terminally located paired box, a linker region, a paired type homeo domain, and a C-terminal region. The conserved octapeptide which is present in several Pax proteins is not found in the mouse Pax-4 protein. The C-terminal region in Pax-4 contains many leucines and prolines, but does not contain the proline-serine-threonine-rich region seen in Pax-6 (19).

Human Pax-4 cDNA encodes 350 amino acids with the predicted MW of 38-kD. The nucleotide and amino acid sequences of human Pax-4 exhibited 83.4 and 80.0% identity with those of mouse Pax-4, respectively. The paired domain and homeo domain of the human and mouse Pax-4 proteins are highly conserved, but the C-terminal regions share the amino acid identity of 67%.

The human Pax-4 translation unit contains 10 exons and 9 introns, which is the same as human Pax-6 (9).

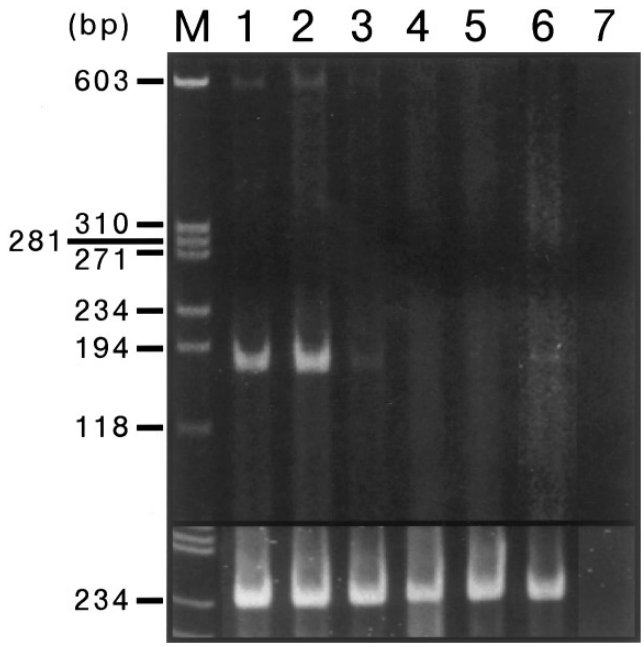

FIG. 4. RT-PCR analysis at the cycle number of 40. RT-PCR was performed with ten micrograms of total RNA isolated from indicated organs. A set of primers of oRB2S and oRB4A, or that of β-actin sense and antisense primers created 177 bp Pax-4 (upper panel) and 245 bp β-actin fragments (lower panel), respectively. The amplified DNA fragments were applied to electrophoresis on an 8% polyacrylamide gel and visualized with ethidium bromide staining. Lane M; molecular size marker, lane 1; MIN6, lane 2; βTC, lane 3; NIT-1, lane 4; αTC and lane 5; NIH/3T3, lane 6; pancreatic islets, and lane 7; negative control without template.

Human Pax-4 has one intron and two introns within the paired box and homeo domain sequence, respectively, where one and two introns are also located in the human Pax-6 gene within the paired box and homeo domain sequence, respectively.

The mouse Pax-4 gene presumably exists as a single copy in the genome, because all 6 genomic DNA samples in Southern blot analysis digested with 6 base recognition endonucleases showed single bands. The amplification of mouse Pax-4 is much less than β-actin and very limited in adult mouse organs including pancreatic islets in RT-PCR analysis. We tried to clone human Pax-4 using RT-PCR of total RNA obtained from a human nesidioblastosis cell line, but human Pax-4 was not amplified.

Our observations suggested that the expression of Pax-4 is limited to intact islets in adult organs and that the mouse Pax-4 is expressed in mouse pancreatic β cell lines of MIN6, βTC, and NIT-1, but not in a mouse pancreatic α cell line of αTC. Based on these, it is concluded that Pax-4 is expressed specifically in pancreatic islet β cells. The acquisition of Pax-4 cDNA is expected to facilitate the study on the role of Pax-4 on the organogenesis of pancreatic islet β cells.

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