BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **248,** 153–156 (1998)
ARTICLE NO. RC988925

Molecular Cloning of Rat Pax4: Identification of Four Isoforms in Rat Insulinoma Cells

Yoshiharu Tokuyama,[1] Kazuo Yagui, Ken-ichi Sakurai, Naotake Hashimoto, Yasushi Saito, and Azuma Kanatsuka

Second Department of Internal Medicine, Chiba University, School of Medicine, 1-8-1 Inohana, Chuo-ku, Chiba 260-0856, Japan

Received June 4, 1998

Molecular cloning of rat Pax4 cDNA from a rat insulinoma cell line, RINm5F, library by PCR-based cloning strategy revealed four isoforms of the protein. Analysis of tissue distribution using Northern blotting and RT-PCR showed specific expression of Pax4 mRNA in pancreatic islets and RIN cells. RT-PCR confirmed that the mRNAs of four isoforms are expressed in RIN cells. These Pax4 variants may regulate the transcriptional activity of Pax4 during the development of pancreatic islets. © 1998 Academic Press

The Pax protein constitute a family of developmental regulators which are characterized by the presence of the paired domain (PD), a conserved DNA binding motif of 128 amino acids and are involved in pattern formation during embryogenesis, possibly by determining the time and place of organ initiation or morphogenesis (1, 2, 3). The mammalian genome contains nine Pax genes which have been classified into four different groups (1, 2, 3). Members within a group are characterized by a specific assembly of three structural motifs; PD, homeodomain (HD), and octapeptide (OP). Pax4 and Pax6 constitute a same group which contains PD and HD but does not have OP. Recently, Pax4 and Pax6 knockout mice exhibited the lack of β and δ cells, and α cells in pancreatic islets, respectively (4, 5), demonstrating that these proteins are profoundly involved in the differentiation of pancreatic islets. Mouse Pax4 cDNA has been recently cloned and deduced human Pax4 sequence has been published (6). Alternative splicing generates multiple gene transcripts in Pax3, 5, 6, 7 and 8 by inclusion or exclusion of exon sequences and have the potential to mediate distinct functions in the developing embryo (7,8,9,10,11,12). In this study, we cloned the rat Pax4 cDNA from rat insulinoma cell line, RINm5F, library and report four isoforms of this protein.

[1] Corresponding author. Fax: 81-43-226-2095. E-mail: yoshit@intmed02.m.chiba-u.ac.jp.

MATERIALS AND METHODS

Cloning of rat Pax4 cDNA from RINm5F cDNA library. Based on the published mouse cDNA sequence (6), PCR primer, prm1: GACTCAGCAGTGTGAATCAGC, sense, nt 252-272 of AB008912 (nucleotide positions are represented by GenBank accession number in this manuscript), and primer, prm2: TTATTACTGTCAAATAGAGGC, antisense, nt 1312-1292 of AB008912 were synthesized. PCR was carried out using these sense and antisense primers, λgt10 RINm5F cDNA library (kindly provided by S. Seino; Chiba University, Japan) as a template, and Pfu DNA polymerase (Stratagene, La Jolla, CA). PCR products were subcloned into pBluescript SK(−)(Stratagene) and at least 4 independently isolated clones were sequenced on both strands using a Sequenase Version 2.0 sequencing kit (United States Biochemical, Cleveland, OH). To obtain 5′ and 3′ sequences, PCR were performed using a λgt10 reverse (pRev: GAGGTGGCTTATGAGTATTTCTTC) and forward primers (pFor: CTTTTGAGCAAGTTCAGCCTGGT), antisense primer, prr1: TACTCACACAGCCGTTAGATAC, nt 215-194 of AF053100), and sense primer, prr2: CAGTTACCAGATTCAGTTGTC, nt 626-646 of AF053100, and λgt10 RINm5F cDNA library as a template. PCR products were subcloned and sequenced as mentioned above.

RNA extraction and Northern blotting. Various tissues were obtained from the adult rat weighing 400g. Rat islets were isolated by collagenase method (13). Total RNA was extracted from these tissues and RINm5F cells using Trizol reagent (Gibco BRL, Tokyo, Japan). Aliquots of 10-20 μg total RNA were denatured in formamide and formaldehyde, separated by electrophoresis, transferred to nylon membrane and hybridized in 50% formamide at 42°C to [α-^{32}P]dCTP labeled DNA probe (mapping sequence from nucleotide position from 63 to 687 of AF053100). The membrane was washed in 0.1×SSC and 0.1% sodium dodecyl sulfate at 55°C for 30 min.

Reverse transcription–polymerase chain reaction (RT-PCR). cDNAs were reverse transcribed from RNAs using random hexamer described previously (14). Since the sequence analysis suggested that there are four transcript variants of rat Pax4 cDNA, 5 PCR primers (prr3: CTCACGGAGCCTTAAGGTAGC, sense, nt 178-198 of AF053100; prr4: TGAATCTGGATACTGCCCACG, antisense, nt 640-620 of AF053100; prr5: GTCTCAGCAGTGTGAATC-

0006-291X/98 $25.00

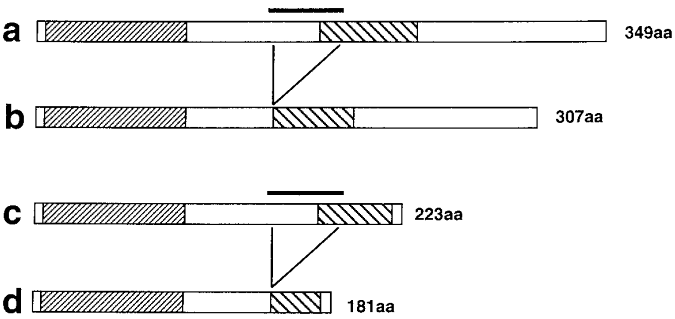

FIG. 1. Schematic expression of rat Pax4a, b, c, and d. Shaded box at N-terminus represents paired domain (PD). Shaded box near the C-terminus represents homeodomain (HD). Upper bar shows the deleted region in Pax4b and Pax4d.

AGC, sense, nt 63-83 of AF053100; prr6: CTCTTATGGCCAGTG-TAAGTA, antisense, nt 1102-1082 of AF053100; prr7: TTCCAC-CCTCTTGTTGCCATC, antisense, nt 798-778 of AF053102) were synthesized and subjected to PCR with taq polymerase (Takara, Shiga, Japan) to detect 4 variants (Fig. 4A). RT-PCR was carried out using islets, liver, kidney and RIN cell RNAs. PCR products of Pax4 were obtained from 100 ng of cDNA (i.e. the cDNA from 100 ng of RNA). The PCR condition was initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 45 sec and extension at 72°C for 1 min, with a final extension step at 72°C for 10 min. PCR products were separated by electrophoresis, transferred to nylon membrane, hybridized with ^{32}P-labeled probe (nt 63-687 of AF053100) and washed as described above. As an internal control, β actin was amplified using a primer set (CGTAAAGACCTCTATGCCAA) and (AGCCATGCCAAATGTGTCAT), and 10 ng of cDNA as a template, as described previously (14).

RESULTS

Cloning and sequence of rat Pax4 cDNA. Nucleotide sequences of PCR product using primer prm1 and prm2 revealed two different Pax4 cDNA fragments. The first one corresponds to the mouse Pax4 cDNA and second one lacks 126 bp in the middle of the first one. The 5′ sequence of rat Pax4 cDNA was obtained by the PCR using the primer set, pRev and prr1. The 3′ sequence of rat Pax4 cDNA obtained by PCR using the primer set, pFor and prr2, revealed a novel C-terminal sequence of Pax4 cDNA, which was substituted for the C-terminus of the mouse cDNA, resulting in 126 amino acids shorter than the corresponding mouse Pax4. In summary, PCR based cloning strategy revealed four variant forms of rat Pax4 protein (Pax4a, 4b, 4c and 4d), which are shown in Fig. 1. Pax4a protein is composed of two conserved motif, PD and HD, linker region of PD and HD, and C-terminal region. Pax4b protein is shorter than Pax4a protein, because of the deletion of 42 amino acids spanning the first α helix part in HD. Pax4c protein is 126 amino acids shorter than Pax4a protein, because of the replacement of the novel nucleotides sequence, resulting in the loss of the third α helix

```
                                                                        PD
rat     MQQDGLSSVN QLGGLFVNGR PLPLDTRQQI VQLAIRGMRP CDISRSLKVS NGCVSKILGR YYRTGVLEPK GIGGSKPRLA   80
mouse   ---------- ---------- ---------- ---------- ---------- ---------- ---------- C---------   80
human   -H---I--M- ---------- ---------- -R--VS---- -----I---- ---------- ---------- ----------   80

                                                                             ***** **********
rat     TPAVVARIAQ LKDEYPALFA WEIQRQLCAE GLCTQDKAPS VSSINRVLRA LQEDQRLHWT QLRSPAVLAP ALPSPHSNCE  160
mouse   ---------- ---------- ----H---T- ---------- ---------- -----S---- ---------- V--------G  160
human   --P------- --G-C----- ---------- -------T-- ---------- -----G-PC- R--------- -VLT---GS-  160
                                    HD
        ********** ********** *******                              ▼
rat     APRGPHPGTS HRNRTIFSPG QAEALEKEFQ RGQYPDSVVR GKLAAATSLP EDTVRVWFSN RRAKWRRQEK LKWETQMPGA  240
mouse   ---------- ---------- ---------- --------A- ---------- ---------- ---------- ----A-L---  240
human   T---T----G ---------S ---------- --------A- ----T----- ---------- ---------- ----M-L---  240

rat     SQDLMVPKDS PGIISAQQSP GSVPSAALPV LEQLNPSFCQ LCWGAVPDRC SSDTTSQACL QPYWECHS-L LPVASSSYME  319
mouse   ----T---N- ---------- ---------- --P-S----- --C-TA-G-- ----S---Y- ----D-Q--- --------V-  319
human   --G-T--RVA ---------- ----T----A --P-G--CY- ---ATA-E-- L---PPK--- K-C-D-G-F- ---IAP-CVD  320

rat     FAWPCLTTHP VHHLIGGPG QAPSTYYLHW P   349
mouse   ---------- --------- -V---HCSN- -   349
human   V-----DASL A------A- K-TP-HFS-- -   350
```

FIG. 2. Alignment of rat, mouse, and human Pax4 amino acid sequences. The amino acid residues in mouse and human that differ from the rat sequence are shown. Dashes represent the amino acid residues identical to rat Pax4a. Paired domain (PD) and homeodomain (HD) are shown. Asterisks represent the deleted amino acid residues in Pax4b and Pax4d. The filled arrowhead points to the site which is followed by the novel amino acid sequence (SELWNTV.) in Pax4c and Pax4d.

of HD and C-terminal region of Pax4a. Pax4d is a shortened form of Pax4c by deletion of 42 amino acids in the linker region. These sequences have been deposited in the GenBank database with accession number AF053100, AF053101, AF053102 and AF053103. Fig. 2 shows the comparison among rat Pax4a, mouse and human Pax4 amino acid sequences. There were 93% nucleotide sequence identity between the sequences of rat Pax4a and mouse Pax4 cDNA and 91.7% identity between rat Pax4a and mouse Pax4 protein.

Northern blot analysis. To investigate the tissue distribution of Pax4 mRNA, Northern blot analysis was performed. As seen in Fig. 3, only RIN cell mRNA showed a single band corresponding to mRNA of ~2kb in size. Since RIN cells express 4 isoforms, Pax4a-d, it seems likely that one isoform is dominant among them.

RT-PCR analysis. As shown in Fig. 4B, two strong bands were detected in RIN cell and weak single band was detected in islets by RT-PCR using the primer set, prr3 and prr4, which can amplify all the four isoforms of rat Pax4 cDNA. RT-PCR using the primer sets, prr5 and prr6 (Fig. 4C), and prr5 and prr7 (Fig. 4D) revealed that 4 variants of Pax4 are expressed in RIN cells. Sequence analysis of these positive bands confirmed that their sequences are identical with those of rat Pax4 isoforms.

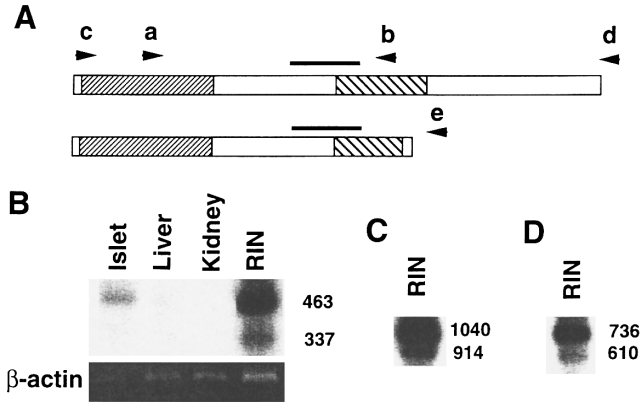

FIG. 4. (A) Schematic representation of rat Pax4a and Pax4c, and primers for RT-PCR. Left and right shaded boxes represent paired domain (PD) and homeodomain (HD). Upper bar represents deleted region in Pax4b and Pax4d. Short arrows a to e show the locations of the PCR primers used for RT-PCR. **a** (prr3) and **c** (prr5) are sense primers. **b** (prr4), **d** (prr6), and **e** (prr7) are antisense primers. **e** corresponds to the sequence in 3′-untranslated region of Pax4c. (B) RT-PCR analysis using the primer set, **a** and **b**. Lower picture shows the PCR product of β actin (349 bp in size) which was electrophoresed on the agarose gel as an internal control. (C) RT-PCR analysis using the primer set, **c** and **d**. (D) RT-PCR analysis using the primer set, **c** and **e**. PCR products were electrophoresed, transferred to nylon membrane, and hybridized with the ^{32}P-labeled probe (nt 63-687 of AF053100).

DISCUSSION

In the case of transcription factor genes, alternative splicing frequently gives rise to protein isoforms with distinct or even opposing transcriptional activities (15). The 14 amino acid insertion in PD of Pax6 and extra amino acid residue insertion in PD of Pax3, 7 and 8 by alternative splicing were shown to alter the DNA binding activity and specificity (7, 10, 11). A potent transactivation domain has been identified within the C-terminal serine-threonine-proline rich region of Pax6 (16). Four splicing variants of Pax8 with defferent C-terminal regions exhibited distinct transactivation properties (12). Although we cannot tell whether four isoforms of rat Pax4 are generated by alternative splicing, the deletion in the part of HD may alter the DNA binding affinity and the loss of most of the C-terminal region of the protein may block the transactivation function of the other isoforms by binding the same sites of target DNA (15). The expression pattern of Pax genes observed during mouse embryogenesis and the phenotypic alterations in various Pax mutants reveal that major function of Pax gene is the control of organogenesis (1, 2, 3). Pax4 and Pax6 deficient mice lack mainly pancreatic islet β cells and α cells, respectively, suggesting that these transcription factors are essential for the differentiation of pancreatic islets in mice (4,5). Northern blotting and RT-PCR in this study revealed specific expression of Pax4 mRNA in pancreatic islets and RIN cells. As in the case of the six Pax8 mRNA isoforms which are spatially and temporally regulated during embryogenesis (12), expression of each isoform of Pax4 may be regulated during the development of pancreatic islets. A mutation in PDX1 (IPF1), another transcription factor involved in the development of pancreas and β cell (17), was detected in the patients with early onset of type 2 diabetes (18). Cloning of rat Pax4 cDNA and detection of its isoforms are expected to facilitate the study of the development and differentiation of pancreatic islets and related diseases including type 2 diabetes.

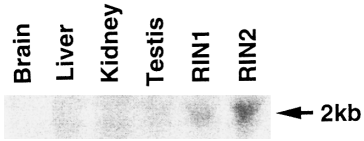

FIG. 3. Northern blot analysis of rat Pax4 mRNA. Total RNA (10 μg) from various rat tissues and total RNA (10 and 20 μg) from RIN cells (RIN1 and RIN2, respectively) were subjected to electrophoresis, blotted on nylon membrane, and hybridized with ^{32}P-labeled probe (nt 63-687 of AF053100).

REFERENCES

1. Stuart, E. T., Kioussi, C., and Gruss, P. (1993) *Annu. Rev. Genet.* **27,** 219–236.

2. Gruss, P., and Walther, C. (1992) *Cell* **69,** 719–722.
3. Dahl, E., Koseki, H., and Balling, R. (1997) *BioEssays* **19,** 755–765.
4. St-Onge, L., Sosa-Pineda, B., Chowdhury, K., Mansouri, A., and Gruss, P. (1997) *Nature* **387,** 406–409.
5. Sosa-Pineda, B., Chowdhury, K., Torres, M., Oliver, G., and Gruss, P. (1997) *Nature* **386,** 399–402.
6. Matsushita, T., Yamaoka, T., Otsuka, S., Moritani, M., Matsumoto, T., and Itakura, M. (1998) *Biochem. Biophys. Res. Commun.* **242,** 176–180.
7. Vogan, K. J., Underhill, D. A., and Gros, P. (1996) *Mol. Cell. Biol.* **16,** 6677–6686.
8. Busslinger, M., Klix, N., Pfeffer, P., Graninger, P. G., and Kozmik, Z. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 6129–6134.
9. Walter, C., and Gruss, P. (1991) *Development* **113,** 1435–1449.
10. Epstein, J. A., Glaser, T., Cai, J., Jepeal, L., Walton, D. S., and Maas, R. L. (1994) *Genes Dev.* **8,** 2022–2034.
11. Kozmik, Z., Czerny, T., and Busslinger, M. (1997) *EMBO J.* **16,** 6793–6803.
12. Kozmik, Z., Kurzbauer, R., Dorfler, P., and Busslinger, M. (1993) *Mol. Cell. Biol.* **13,** 6024–6035.
13. Tokuyama, Y., Kanatsuka, A., Ohsawa, H., Yamaguchi, T., Makino, H., Yoahida, S., Nagase, H., and Inoue, S. (1991) *Endocrinology* **128,** 2739–2744.
14. Tokuyama, Y., Sturis, J., DePaoli, A. M., Takeda, J., Stoffel, M., Tang, J., Sun, X., Polonsky, K. S., and Bell, G. I. (1995) *Diabetes* **44,** 1447–1457.
15. Foulkes, N. S., and Sassone-Corsi, P. (1992) *Cell* **68,** 411–414.
16. Czerny, T., and Busslinger, M. (1995) *Mol. Cell. Biol.* **15,** 2858–2871.
17. Jonsson, J., Carlsson, L., Edlund, T., and Edlund, H. (1994) *Nature* **371,** 606–609.
18. Stoffers, D. A., Ferrer, J., Clarke, W. L., and Habener, J. F. (1997) *Nature Genet.* **17,** 138–139.