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Expression of Dominant Negative Form of PAX4 in Human Insulinoma

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The paired-homeodomain transcription factor PAX4 is expressed in the early pancreas, but is later restricted to β cells and not expressed in mature islets, suggesting an important role of PAX4 in differentiation and development of pancreatic islet. Here we show that PAX4 mRNA was highly expressed in human insulinoma tissues, whereas little if any mRNA was expressed in normal islets. Furthermore, this insulinoma associated expression of PAX4 mRNA was accompanied with expression of its novel variant form (PAX4v). PAX4v was generated by alternative splicing lacking the exon 7, and containing intact paired and homeo domain followed by novel 35 amino acids. PAX4v reversed the wild-type PAX4 mediated repression of the insulin promoter in cotransfection assays. PAX4v may play a role to antagonize the wild-type PAX4 function in human insulinoma. These data imply a role of PAX4 and PAX4v expression in tumorigenesis and development of insulinoma.

Key Words: **PAX4; PAX4 variant; transcription; insulin promoter; pancreatic β-cell; insulinoma.**

The PAX gene family encodes highly conserved paired-box containing transcription factors that control the tissue specific expression of genes during embryogenesis (1, 2). To date, nine members of the PAX gene family have been identified in mammalian genomes and classified into four different groups characterized by a specific assembly of three structural domains: paired domain (PD), homeodomain (HD), and octapeptides (3). PAX4 and PAX6 that belong to a same group that contains PD and HD but does not have octapeptides, have been shown to be essential for normal differentiation and maturation of pancreatic endocrine precursor cells (4, 5). PAX4 and PAX6 are known to bind a common element in the glucagon, insulin, and somatostatin promoters; PAX6 transactivates, and PAX4 represses these promoters through the common DNA element. Inactivation of Pax4 by homologous recombination results in the absence of mature insulin- and somatostatin-producing cells (β and δ, respectively) in the pancreas (4), but glucagon-producing α cells are present in considerably higher numbers. It is proposed that the early expression of Pax4 in a subset of endocrine progenitors is essential for the differentiation of the β and δ cell lineages. A default pathway would explain the elevated number of α cells in the absence of Pax4. The Pax4 gene is expressed in the early pancreas, but is later restricted to β cells and not expressed in mature islets (6). Its absence from adult islets may suggest the role of PAX4 in maintaining the proliferative phenotype of the immature endocrine cells, although the precise mechanism of the development of pancreatic β cells has not been fully understood.

PAX genes are expressed in spatially and temporally restricted patterns during development (4). Several of these PAX genes have been implicated by genetic evidence in the control of early development, differentiation (2) and oncogenesis (7, 8), as they are frequently associated with mouse developmental mutant (9, 10), human diseases, or chromosomal translocations in human tumors. Overexpression of PAX proteins in rodent fibroblasts is oncogenic (7). In the pediatric alveolar rhabdomyosarcoma, chromosomal translocation results in fusions of the paired and homeo domains of PAX3 or PAX7 to the transactivation domain of a forkhead family of transcription factor (11, 12). Deregulated expression of PAX5 in a diffuse large-cell lymphoma has also been reported (13). Since PAX4 seems to have a pivotal role in developing β cells in pancreatic islets, it was therefore of interest to analyze its expression in pancreatic islet tumors. In this study, we demonstrated the elevated expression of PAX4 gene in

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pancreatic β-cell tumor and identification of a novel splice variant designated PAX4v which lacks exon7 resulting in COOH-terminal truncation with additional 35 amino acids. We also described the antagonizing function of PAX4v to release the PAX4 mediated transcriptional repression.

MATERIALS AND METHODS

Plasmid constructions. Full-length of Pax4 and Pax6 cDNA were isolated from human insulinoma tissues by RT-PCR. First-strand cDNA was synthesized from human insulinoma RNA by MMLV reverse transcriptase primed by a poly(dT) primer, and used as a template for PCR using primers that amplify full-length Pax4 (14) and Pax6 cDNA (5) (Pax4: forward primer containing *Xho*I site, 5′-atcctcgagctatgaaccagcttgggggct-3′; reverse primer containing *Xba*I site, 5′-atctctagattccaagccatacagtag-3′, Pax6: forward primer containing *Bam*HI site, 5′-atcggatcctaatgcagaacagtcacag-3′; reverse primer containing *Xba*I site, 5′-atctctagactgtaatcttggccagta-3′). After digestion by appropriate restriction enzymes, PCR products were cloned into pcDNA 3.1 His vector (Clonetech). One hundred picomoles of each primer were hybridized to 1.0 mg of first strand cDNA and amplified for 30 cycles using a Perkin-Elmer Gene Amp PCR System 2400. One cycle consisted of denaturation for 15 s at 94°C, annealing for 30 s at 55°C, and extension for 40 s at 72°C. Correct insertion was confirmed by dye-deoxy sequencing.

Cell culture. The hamster HIT-T15 insulinoma cells were obtained from American Type Culture Collection (Manassas) and grown at 37°C in 5% CO_2 in RPMI 1640 supplemented by 10% fetal calf serum (Gibco), 100 IU/ml penicillin and 100 mg/ml streptomycin.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was purified from the indicated tissue source by using a Qiagen RNeasy kit (Qiagen) and quantified spectrophotometry. Total RNA (0.5 mg) was then used as template to synthesized first-strand cDNA by MMLV reverse transcriptase primed by a poly(dT) primer. The cDNA produced was used as a template for PCR using primers that amplify fragments of the indicated cDNA. Each set of primers was designed to include at least one intron in order to allow the discrimination of contaminating genomic DNA from cDNA. The PCR condition was initial denaturation at 94°C for 105 s followed by 27 cycles of denaturation at 94°C for 15 s, annealing and extension at 60°C for 30 s, with a final extension at 72°C for 5 min. The PCR cycles were extended to 35 cycles to increase the sensitivity. The PCR products were separated by 1% agarose gel electrophoresis in TAE buffer and stained with ethidium bromide. Identity of the PCR products was confirmed by dye terminator cycle sequencing using ABI 310 sequencer.

Transcription assay. Hamster insulinoma cells, HIT-T15, were obtained from ATCC(CRL1777) as frozen stocks at passages 60, then thawed and grown in 10 cm plates in RPMI 1640 supplemented by 10% fetal calf serum (Gibco), 100 U/ml penicillin and 100 mg/ml streptomycin. Only HIT-T15 cells bellow passage 70 were used in experiments. Transfection was done in HIT-T15 cells using the standard calcium phosphate procedure. Typically, 0.25 mg luciferase reporter containing rat insulin II promoter (15) was cotransfected with 100 ng of the indicated expression vectors. Cells were incubated for 12 h, and the medium on the cells was replaced with fresh medium and indicated ligand was added. Cells were harvested after 24 h and b-galactosidase activity was measured by the method previously described using chlorophenolred-β-D-galactopyranoside (CPRG) as a substrate. Luciferase assays were performed using the PicaGene Luciferase Assay System (Toyo Inki, Tokyo). Luciferase activity was determined using Lumat LB9501 (Berthold Japan K.K., Tokyo, Japan) and expressed as RLU (Relative Light Units) normalized to the b-galactosidase activity.

In vitro transcription and translation. Coupled transcription and translation of PAX4 and PAX6 were carried out using a T7 TNT *in vitro* transcription/translation kit (Promega) according to the manufacturer's instructions.

Gel retardation assay. Synthetic oligonucleotides representing each strand of sequences for PAX4 element glucagon G3 element (16, 17) (Sense strand: 5′-tcgagaaaattttcacgcttgagttcacagc-3′, Antisense strand: 5′-tcgagctgtgaactcaagcgtgaaaattttc-3′) were purified by polyacrylamide gel electrophoresis, eluted, and annealed. Double stranded oligonucleotides were radiolabeled with dCTP (>3300 Ci/mmol; ICN Biomedicals, Costa Mesa, CA) by fill in reactions using Klenow large fragment DNA polymerase. Radiolabeled probes (10 fmol, 20,000 to 30,000 cpm) were then incubated with binding proteins in 30 ml of reaction mixture containing 10 mM KPO4 pH 8.0 buffer, 1 mM EDTA, 80 mM KCl, 1 mg polydI-dC, 1 mM DTT, 0.5 mM $MgCl_2$, 5 mg BSA, 10% glycerol, 1 mM phenylmethylsulphonyl fluoride, 2 mg/ml aprotinin, 1 mM leupeptin, 1 mM pepstatin. These reactions were incubated for 30 min at room temperature and analyzed on a 5% nondenaturing polyacrylamide gel in TAE buffer. Electrophoresis was performed at a constant voltage of 200 V at 4°C in the same buffer.

RESULTS

Expression of Pax4 mRNA and Identification of Splicing Variant PAX4v mRNA in Human Insulinomas

We analyzed the expression of human PAX4 mRNA in normal islet cells and insulinoma tissues using reverse transcription-polymerase chain reaction (RT-PCR). In an attempt to isolate full-length PAX4 coding sequence from insulinoma tissues, we isolated a variant human Pax4 mRNA species that lacks the entire exon 7 due to alternative splicing (Fig. 1). This prompted us to screen for Pax4 variants with a series of human insulinoma tissues. In order to distinguish the PAX4 mRNA species, P1 and P2 primers were designed and used for RT-PCR (Fig. 2A). This primer set can amplify the two isoforms of human PAX4 cDNA which include and exclude exon 7 as 361 bp and 305 bp products, respectively. The RT-PCR assays were particularly important to show that isoform of PAX4 was real and not the result of artifact during cDNA synthesis, which can sometimes lead to hybrid clones. As shown in Fig. 2B, all of the six insulinomas analyzed had levels of PAX4 mRNA expression whereas normal islets had no detectable PAX4 mRNA. Similar levels of preproinsulin mRNAs were demonstrated in the same reactions to confirm that the equivalent amounts of RNA were loaded. Two strong bands were detected in each insulinoma by RT-PCR. Sequence analysis confirmed that both bands in each lane were identical to human PAX4 but all of smaller bands excluded exon 7.

PAX4v Protein Lacks the COOH-Terminus of PAX4

To examine the integrity of the encoded gene product, we allowed PAX4 and PAX4v mRNA to be transcribed and translated *in vitro* using a rabbit reticulocyte lysate system. As shown in Fig. 3, human PAX4

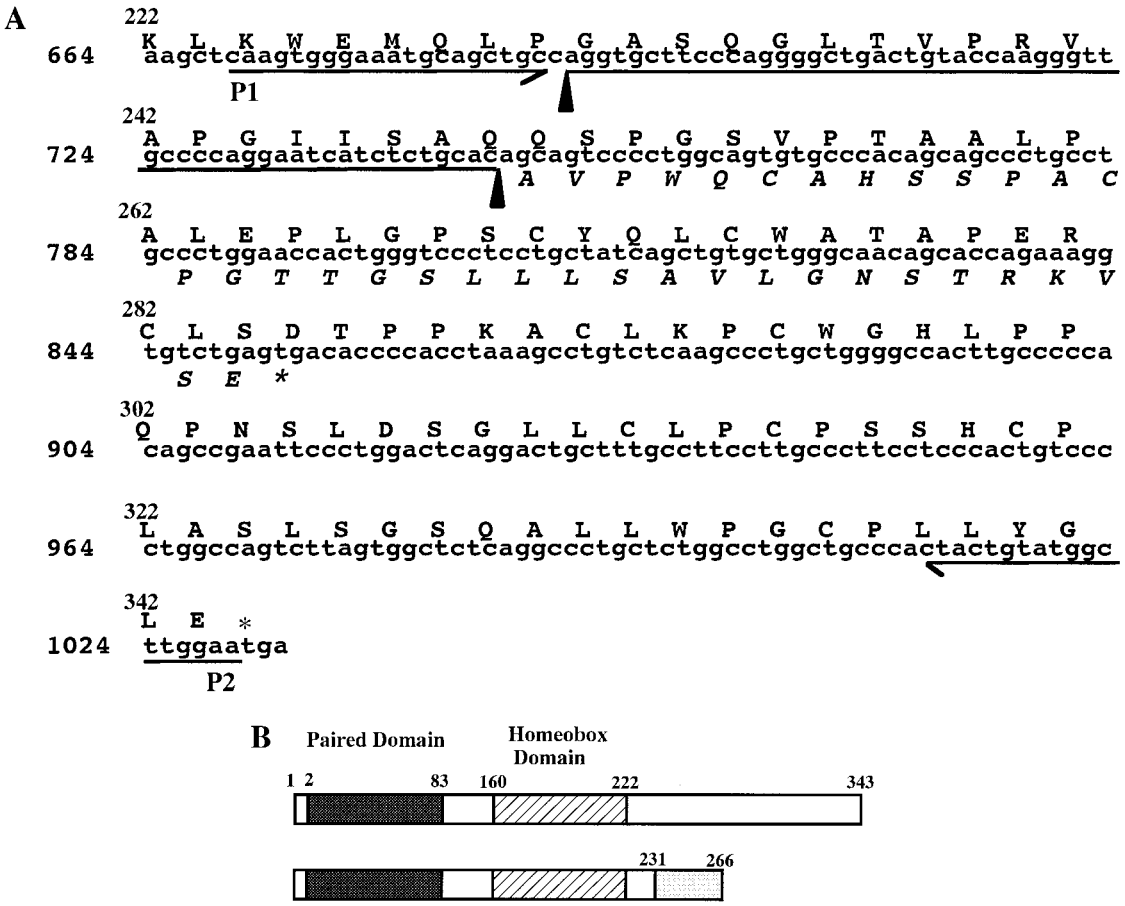

FIG. 1. (A) The nucleotide and amino acid sequence of PAX4 and PAX4v. Numbers of nucleotides from the first coding nucleotide were indicated at the beginning of each lane. Closed triangles indicate exon boundaries of exon 7 and the sequence of exon 7 was underlined. Arrows indicate primers used for RT-PCR, P1, and P2. Amino acids of wild-type PAX4 and PAX4v are shown in upper and lower lines, respectively. The in-frame stop codon at the end of the predicted coding region is denoted by an asterisk. (B) Schematic diagram illustrating the human PAX4 and PAX4v gene products. Shaded box at N-terminus represents paired domain (PD) and the one near the center represents homeodomain (HD).

mRNA could be translated into a single protein of 38 kDa in accordance with the size predicted by the nucleotide sequence. *In vitro* translation of the PAX4v mRNA resulted in a variant PAX4 protein of about 30 kDa (Fig. 3).

As shown in Fig. 1B, PAX4v was supposed to have intact paired and homeo domain and expected to possess similar DNA binding function. In order to investigate the DNA binding properties of the PAX4v, the G3 element of glucagon promoter was employed in gel shift assays (Fig. 4). *In vitro* translated PAX4 and PAX4v were incubated in parallel with the labeled DNA probes and the retarded protein–DNA complexes were analyzed on the gel. As shown in Fig. 4, PAX4v generated a retarded band migrating faster than that which was seen in wild-type PAX4. The different mobility was attributable to the difference in sizes of both proteins. PAX4v can bind DNA as efficiently as wild-type PAX4.

PAX4 Variant Release the Wild-Type PAX4-Mediated Transcriptional Repression

To understand the functional significance of the expression of PAX4v, it is important to determine the effects of its expression on the transcriptional regulation of the target genes. It is recently reported that PAX4 could bind to the potential binding sites in insulin promoter and act as a transcriptional repressor (6, 17, 18). Since a primary role of PAX4 as a transcriptional repressor may involve the competitive inhibition of PAX6 function on a common DNA element, we next examined the effects of PAX4 and/or PAX4v expression on the transcriptional regulation of insulin gene by PAX6. Mammalian expression vectors encoding Pax4 and/or Pax4v complementary DNAs were transiently transfected along with an insulin promoter-luciferase (-695 InsLuc) reporter (15) into HIT cells. Expression of PAX4 repressed the transcriptional activity of the insulin promoter both in the presence or absence of the

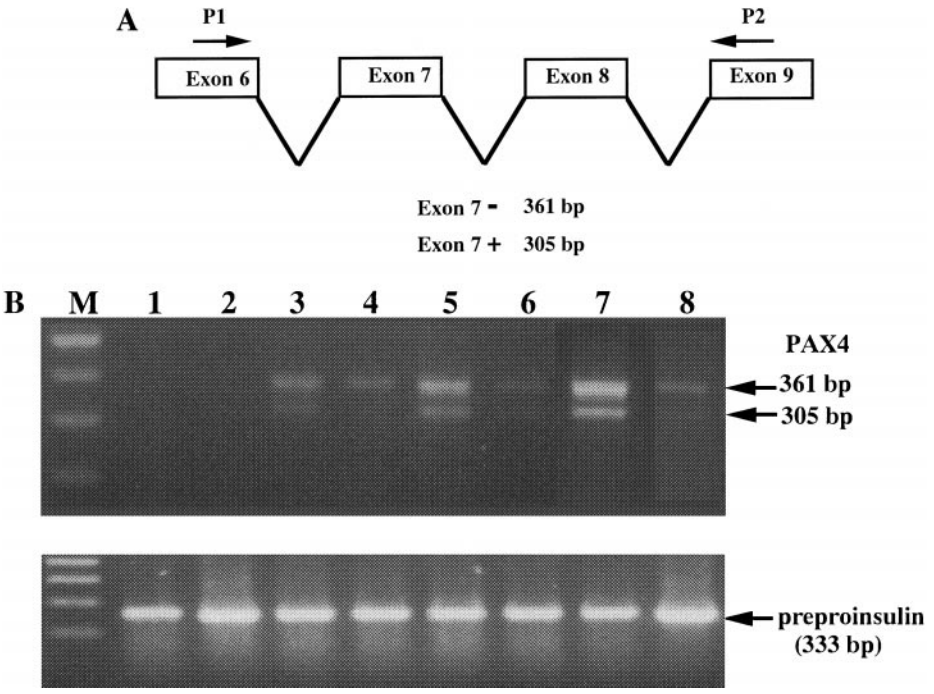

FIG. 2. (A) Schematic representation of RT-PCR strategy. Genomic structure of PAX4 including exon 6, 7, 8, 9 was illustrated. P1 and P2 primers were indicated by arrows. This primer set is expected to generate 361 bp and 305 bp PCR products for PAX4 mRNA including and excluding exon 7, respectively. P1: 5′-caagtgggaaatgcagctgc-3′, P2: 5′-ttccaagccatacagtag-3′. (B) Expression of PAX4 and PAX4v in human insulinoma tissues. Total RNA from normal islets (lanes 1 and 2) and insulinoma tissues (lanes 3–8) were purified and used as a template for reverse transcriptase. First strand cDNA were generated by oligo (dT) primer. PCR were performed using PAX4 or preproinsulin specific primers described under Materials and Methods. PCR products were separated by 1% agarose and visualized with ethidium bromide. Estimated length of PCR products for PAX4, PAX4v, or preproinsulin was 370, 314, or 333 bp, respectively. M, Marker was indicated.

transfected PAX6. The expression of PAX6 somehow appeared to enhance the repressive activity of PAX4, although PAX6 functioned as an activator for insulin promoter (Fig. 4A). The expression of Pax4v alone, however, slightly increased the reporter activity. Moreover, coexpression of PAX4v reversed the transcriptional repression caused by wild-type PAX4 either in the presence or absence of PAX6 expression. In contrast, control reporter such as CMV luciferase was not affected by the expression of PAX4 or PAX4v (Fig. 4B). Thus, PAX4v protein may function as a dominant negative variant that could antagonize the transcriptional activity of full-length PAX4 proteins on the target genes.

DISCUSSION

To date, little is known about the molecular mechanism regulating the lineage of the pancreatic β cells. Recent results using gene targeting techniques revealed that PAX4 knockout mouse have a drastic reduction in insulin producing β cells and somatostatin producing δ cells. Therefore, Pax4 has been hypothesized to play an important role in the development of the pancreatic islet and to be profoundly involved in the pancreatic β cell differentiation and maturation. In the present study, using a RT-PCR approach, we demonstrated the elevated expression of PAX4 mRNA in human β cell tumors, insulinomas. All of the insulinomas analyzed (6 of 6) had levels of PAX4 mRNA expression while normal islets showed little if any expression of PAX4 in RT-PCR analysis. Our incapability of RT-PCR to detect the PAX4 mRNA in normal islets is consistent with previous reports showing that the Pax4 gene was expressed in the early pancreas, but later restricted to β cells and not expressed in mature islets (6). Transcriptional regulation by PAX4 apparently plays a role only early in islet development, since Pax4 mRNA as determined by RT-PCR peaks at embryonic day 13.5 in the fetal mouse pancreas and is undetectable in adult islets (6). Elevated expression of PAX4 in insulinoma may suggest the proliferating phenotype of the insulinoma, although further investigations will be required to elucidate the physiological role of PAX4 in insulinoma.

An oncogenic role has been proposed for PAX genes primarily based on the consistent involvement of PAX3 and PAX7 in the genesis of alveolar rhabdomyosarcoma (11, 12). In this pediatric muscle tumor, a specific translocation between one of two PAX loci and the fork head domain gene FKHR creates a novel fusion gene that codes for a potent chimeric transcription factor

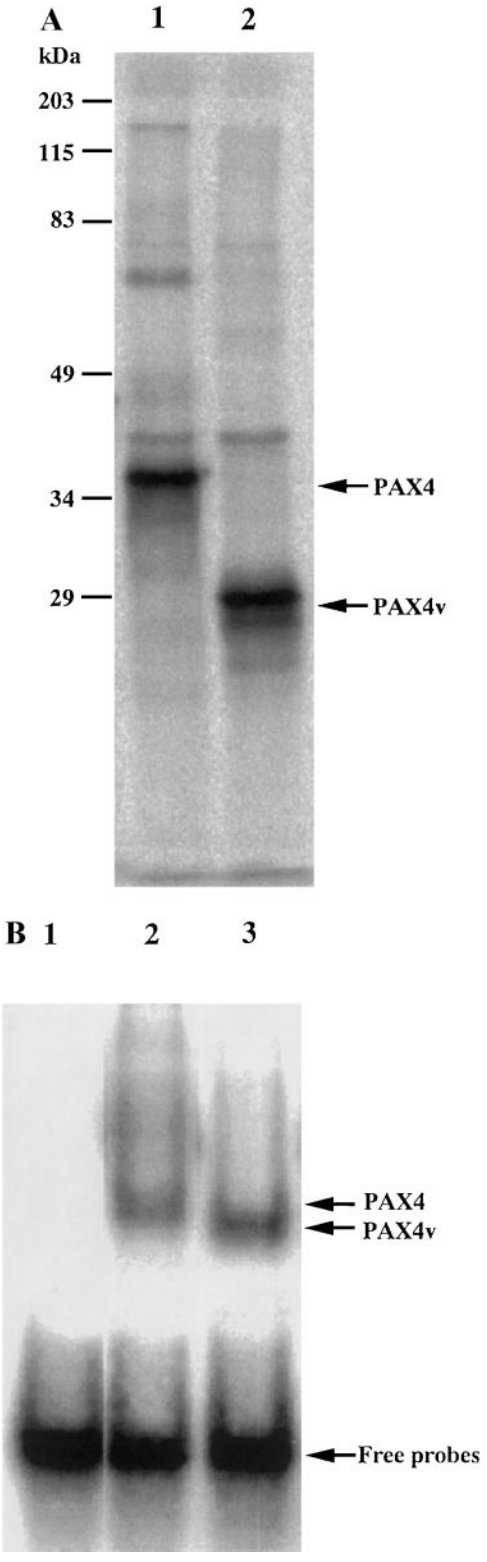

FIG. 3. (A) Analysis of *in vitro* translation of PAX4 and PAX4v protein. PAX4 and PAX4v proteins were synthesized by *in vitro* transcription and translation using TNT reticulocyte lysate. Autoradiograph of a SDS–polyacrylamide gel (10%) shows ^{35}S-methionine-labeled PAX4 and PAX4v proteins produced by coupled *in vitro* transcription and translation of two different cDNAs. The positions of the molecular weight markers are indicated at the left. The apparent molecular masses were 38 and 30 kDa for PAX4 and PAX4v, respectively. Two microliters of the samples were separated by 10% SDS–PAGE and visualized by phosphor imager (Fuji BAS 1500). (B) DNA binding activity of PAX4 and PAX4 variant. EMSAs were performed with *in vitro* synthesized PAX4 (lane 2) and PAX4v (lane 3) proteins. The ^{32}P-fill-in-labeled oligonucleotides representing the glucagon G3 element were employed as the binding probe. Unprogrammed lysate (lane 1) was included as a control. Arrows indicate the G3 probe associated with PAX4, and that associated with PAX4v, respectively. The position of the unbound probes is shown at the bottom of the gel.

(19). In addition to this, deregulated expression of PAX5 has been implicated in the formation of medulloblastoma (20) and in progression of malignant astrocytoma (21), although no molecular mechanism for oncogenic activation of PAX gene has yet emerged.

Another notable observation in this study is the identification of a variant Pax4 mRNA species that lacks the entire exon 7 due to alternative splicing. Amplification by RT-PCR of the Pax4 coding sequence from a series of human insulinomas and pancreas tissues revealed beside the expected Pax4 cDNA, a variant product which lacks exon 7. All of the insulinomas analyzed had similar proportion of PAX4v mRNA expression to wild-type PAX4. The exon 7 deletion leads to the frame shift resulting in the replacement of entire COOH-terminal region that is known to be a putative repression domain with 35 novel amino acids sequence but does not affect the N-terminal paired-homeodomain for DNA recognition. Alternative splicing of transcripts from a single gene is often used mechanism for generating protein variants with diverse function. Many tumor-associated genes are alternatively spliced; their expression leads to the production of multiple splice variants. Although the functions of most of these variants are not well defined, some have antagonistic activities to the wild-type genes. In the case of transcription factors, alternative splicing frequently gives rise to protein isoforms with distinct or even opposing transcriptional activities. In this study, we demonstrated a possible functional role of PAX4v to antagonize the wild-type PAX4 function. Similar to the novel isoform of PAX4 gene identified in this study, other PAX genes have also used alternative splicing to increase the functional diversity within the family. It is reported that alternative splicing generate multiple gene transcripts in PAX3, 5, 6, 7, and 8 by inclusion or exclusion of exons and have the potential to mediate distinct functions in the developing embryo (22, 23). In PAX8, multiple isoforms differing in their transactivation potential are generated by alternative splicing of exons downstream of the DNA-binding domains (24). Four isoforms of rat PAX4 protein were reported to be present in rat insulinoma cell line, RINm5F, although the functional significance of these multiple isoforms is not known.

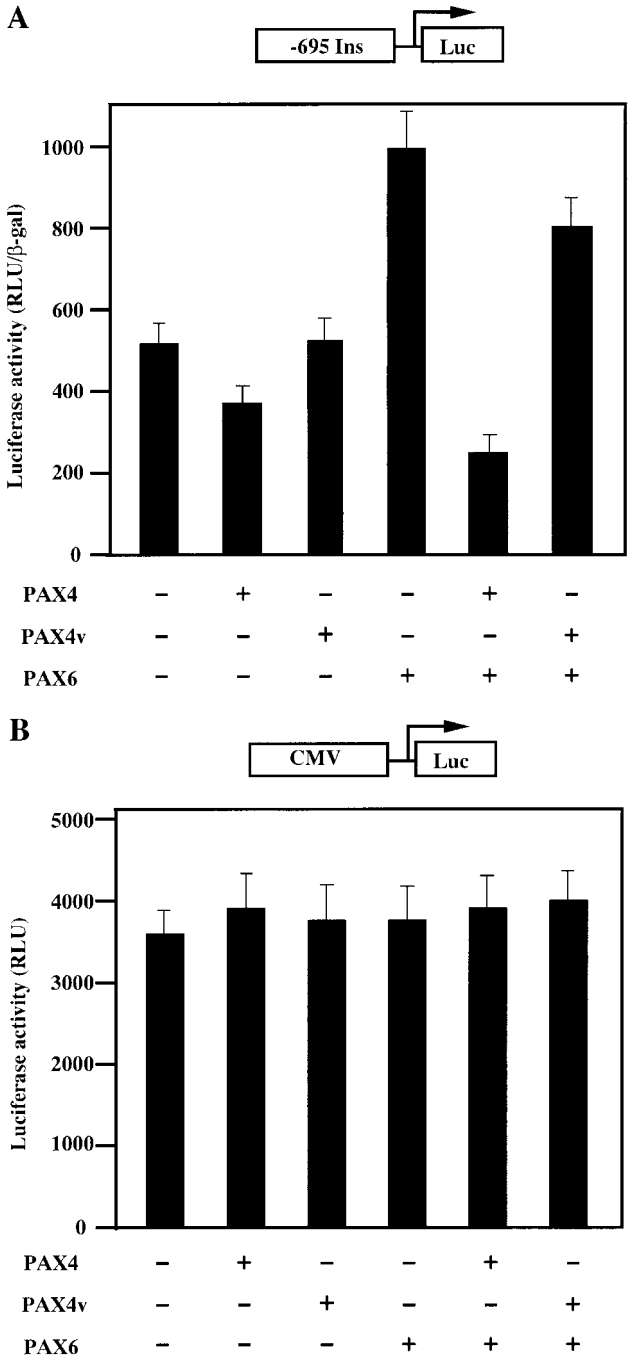

FIG. 4. PAX4 variant modified the transcriptional activity of PAX4. (A) Cells of the insulin-producing pancreatic tumor cell line (HIT-T15 cells) were transiently transfected with 0.25 μg of luciferase reporter plasmid under transcriptional control of rat insulin gene promoter (-695INS-luc) along with 100 ng of PAX6, PAX4, and/or PAX4 variant expression plasmids as indicated. The cells were collected for analysis of reporter gene activity 24 h later. Results were normalized to β-galactosidase activity derived from a cotransfected RSV-β-galactosidase expression plasmid. Total amount of transfected DNA was kept constant by adding empty expression vectors to the reaction. Luciferase activity was expressed as RLU (Relative Light Units) normalized to the β-galactosidase activity. Each transfection was conducted in triplicate, and data represent the mean $\pm$ S.D. of more than three individual experiments. (B) Similarly designed transfection experiments were performed as A, but CMV promoter luciferase was used instead of -695INS-luc.

Recently, the carboxy-terminal region of PAX4 were shown to function as repression domains when fused to GAL4 DNA binding domain (6, 17). PAX4v retains the paired-homeodomain for DNA binding but looses most part of the potential carboxy-terminal repression domain as a result of alternative splicing. Indeed, in transient transfection assays, PAX4v reversed the repressive function of PAX4. Thus the alternative splicing event appears to act as a molecular switch that regulates transcription of target genes by balance of wild-type and variant form of PAX4. It remains an interesting avenue for future study to investigate whether the alternative splicing event of PAX4 is regulated during tumorigenesis and β cell development. Further functional studies of PAX4 and its variant should provide new insights into development and progression of insulinoma.

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