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Tissue specific expression of the splice variants of the mouse vacuolar proton–translocating ATPase a4 subunit

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Abstract

We have identified splicing variants of the mouse a4 subunit which have the same open reading frame but have a different 5′-noncoding sequence. Further determination of the 5′-upstream region of the a4 gene in mouse indicated the presence of two first exons (exon1a and exon1b) which are including the 5′-noncoding sequence of each variant. The mRNAs of both splicing variants (a4-I and a4-II) show a similar expression pattern in mouse kidney by *in situ* hybridization. However, tissue and developmental expression patterns of the variants are different. In addition to strong expression in kidney, a4-1 expression was detected in heart, lung, skeletal muscle and testis, whereas, a4-II is expressed in lung, liver and testis. During development, a4-I was expressed beginning with the early embryonic stage, but a4-II mRNA was detected from day17. These results suggest that each a4 variant has both a tissue and developmental stage specific function.

Keywords

Vacuolar Proton ATPase; Kidney; tissue specific; splicing variant; isoform

The kidney plays an essential role in ion homeostasis for mammals[1]. Many ion channels and transporters contribute to this regulatory role [1]. In renal intercalated cells, plasma membrane vacuolar (H^+)-ATPases (V-AT Pases) localized to the apical membrane are essential for controlling acid secretion in the distal tubule and collecting duct [1,2]. Mutations of the "kidney-specific" isoforms of the V-ATPase subunits (B1 and a4) lead to renal tubular acidosis [3,4]. The V-ATPases are a family of ATP-dependent proton pumps found in many intracellular compartments as well as the plasma membrane of specialized cells including renal intercalated cells and osteoclasts [2,5]. Acidification of intracellular compartments and the extracellular space is essential for many cellular processes (see reviews, ref.5).

The V-ATPases from fungi, plants and animals are structurally very similar and are composed of two domains, V1 and Vo [5–9]. In yeast, the cytosolic V_1 complex, composed of eight different subunits of molecular weight 70-14 kDa (subunits A-H), is responsible for ATP hydrolysis. The Vo domain is a membrane integral complex composed of six subunits of

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[1]The abbreviations used are: ATPase, adenosine triphosphatase; ORF, open reading frame

molecular weight 100-10 kDa (subunits a, d, e, c, c′ and c″) that is responsible for proton translocation. The a-subunit is the largest subunit of the V-ATPase complex and is composed of an amino-terminal hydrophilic domain and a carboxyl-terminal hydrophobic domain containing multiple putative membrane spanning segments [5–9]. The a-subunit serves several essential functions [10–12]. In yeast, the a-subunit is encoded by two genes, *VPH1* and *STV1* [13,14]. The proteins encoded by these genes show distinct intracellular localization, with Vph1p localized to the vacuole and Stv1p localized to a late-Golgi compartment [14, 15]. Kinetic analysis of the enzyme containing each of these isoforms indicates that Stv1p-containing complexes show less efficient coupling of ATP hydrolysis and proton transport than Vph1p-containing complexes [16]. These results demonstrate that a-subunit isoforms can directly contribute to the regulation of pH in intracellular compartments [16]. Furthermore, analysis of chimeric proteins of Vph1p and Stv1p demonstrate that the carboxyl-terminal domain helps to control coupling efficiency whereas the amino-terminal domain contains the targeting signals necessary to determine the final cellular destination [15]. In mammals, four a-subunit isoforms have been identified (a1-a4), [17–19]. Among these isoforms, the a3 and a4 isoforms are essential for born resorption by osteoclasts and acid secretion in the kidney, respectively [3,10]. In this study, we have isolated additional splice variants of the a4 isoform and showed that these variants are expressed in a tissue and developmental stage specific manner.

Materials and methods

Materials

E. coli culture media was purchased from Difco Laboratories. Restriction endonucleases, T4 DNA ligase and other molecular biology reagents were obtained from Invitrogen, Promega and New England Biolabs. Most other chemicals were purchased from Sigma Chemical Co.

Isolation of mouse a4 cDNA clones

A clone, number uj35a02 that contains the full-length a4 subunit cDNA, was identified in the expression sequence tag (EST) database and obtained from the American Tissue Culture Collection (ATCC) (Manassas, VA). The nucleotide sequence of the a4 subunit was determined and reported (gene bank accession number, **AF435090)**.

Identification of 5′-end of a4 subunit cDNA

To determine the transcription initiation sites for the a4 subunit mRNA, we performed 5′-RACE using the FirstChoice RACE-ready cDNA from mouse kidney (Ambion). Reactions were performed using the manufacturer's recommended protocol. Amplification of fragments was performed with the Elongase enzyme mixture (Gibco), 5′-RACE primers (Ambion) and a4 subunit gene-specific primers, a4R1; CAAGCCAAGCTCTCCCAGCTCAGCTAC and a4R2; AGCAATACGCAGCCTCCACCT. The resulting PCR products were cloned into the pCR-2.1-TOPO vector (Invitrogene) and sequenced. Amplification of the ORF region of the a4-I and a4-II was performed with each variant-specific primer, a4F6 or a4F5 (fig. 3) and the c-terminal primer; GCACCAGGATACAGACAGACCTACTC.

Identification of the mouse a4 isoform genomic sequence containing alternative exons 1a and 1b

Genomic sequence of the a4 gene, accession number, **AY137757**, was determined by the genomic walking method using mouse genomic DNA.

In situ hybridization

To determine the cellular expression of splice variants of the a4 subunit in kidney, we performed *in situ* hybridization using variant-specific probes. For the a4-I and a4-II variants, fragments containing −264 to +73 bp and −127 to + 73bp from the translation initiation codon were subcloned into pSK vector and used. Digoxigenine-labeled RNA probes were transcribed *in vitro* with the DIG RNA labeling kit (Rotch). RNA probes were hybridized with paraffin embedded mouse kidney slices (6μm thick) and developed with alkaline phosphatase labeled anti-digoxigenine antibody and NBT/BCIP, followed by Kernechtot staining to visualize the cells.

Tissue specific and developmental expression of alternatively spliced variants of the a4 subunit using RT-PCR

To analyze the expression of splice variants of the a4 subunit, we performed RT-PCR using multiple tissue cDNA panels (Clontech, CA). For the a4-I variant, a4-I-Fw (CCACTAGCAGAGTCCTCGCCATGTCA) and a4R2 (AGCAATACGCAGCCTCCACCT) were used. For the a4-II variant, Fw (CCAAGGTGGCCAAGAACTAGCTCAGA) and a4R2 were used. Detection of B1 or B2 subunit expression was performed with the following primers, B1-Fw; CCGGAAGCTTCGATGGCCACAACAGTAGACAGCAG, B1-Rv; CGGGGTACCTTAGAGCGCCGTGTCGGATGCGGGGTCC, B2-Fw; CCGGAAGCTTAAGATGGCGTTGCGAGCGATGCG and B2-Rv; CGGGGTACCCTAGTGTTTTGCAGAGTCTCGAGGGTAA. Amplified fragments were analyzed by separation on 1.2 % agarose gels.

Promoter Analysis

The 5′-upstream region of exon 1a or exon 1b of the a4 subunit gene was subcloned into the *Bgl* II and *Nco* I sites located upstream of the luciferase gene in the pGL3-Basic vector (Promega). Expression plasmids (0.2μg DNA) were transfected into NIH3T3 cells ($1x10^5$ cells per well on 12-well plates) using the Effecten transfection reagent (Qiagen). All cells also received 0.05 μg of the pRL-TK plasmid. After 24 hours, cells were lysed with Passive Lysis buffer (Promega) and luciferase activity was measured using a luminometer and the Dual Luciferase reporter system (Promega).

Results and Discussion

Identification of cDNAs encoding isoforms of a4 in mouse

We have previously shown important roles for isoforms of subunit a of the V-ATPase in yeast [5]. and have also identified isoforms of this subunit in mouse [17]. Like the B1 isoform, a4 has been reported to be “kidney specific” [2–4,19]. We now report identification the cDNA encoding a4 contains 264 bp of 5′-noncoding sequence, 2502 bp of open reading frame (ORF) and 542 bp of 3′-noncoding sequence (Accession Number, **AF435090,** data not shown). When we compared our a4 cDNA sequence with that previously identified from non-obese diabetic (NOD) mice [19], nine nucleotides were different in the ORF region. Seven of nine residues were silent mutations and were reported as a polymorphisms between NOD mice and normal B6 mice [19]. However, two additional nucleotide changes (T to C at 275 bp which gives the mutation V4A and G to A at 2137 bp which gives the mutation D625N) had not previously been reported. Both amino acid residues, Ala4 and Asn625, are not conserved between the four a subunit isoforms (Suppl. Fig. 1). These changes were also identified in cDNA and genomic sequences from C57BL6J mice. Because our a4 clone was isolated from an EST library from sugano mouse kidney library that was constructed from C57BL mouse kidney mRNA, these changes represent novel polymorphisms between the C57BL and other mouse strains.

The deduced amino acid sequences of this isoform displays approximately 62, 52 and 47% identity to that of the a1, a2 and a3 isoforms, respectively (Suppl. Fig. 1). Residues important for V–ATPase function from studies of the yeast -ATPase were conserved in all isoforms [12, 20, 21] (Suppl. Fig. 1), indicating a conservation of the proton transport mechanism from fungi to mammals.

Tissue expression of the a4 subunit

RT-PCR analysis of mRNAs isolated from several mouse tissues demonstrated that the a4 subunit is predominantly expressed in kidney (Fig. 1) (confirming previous reports [3,19]). We also compared the expression of a4 mRNA to that of the "kidney-specific" B1 subunit (the B2 isoform is ubiquitous [2,5]). The expression pattern of a4 was similar to that of B1 except that a4 is also detected in testis, skeletal muscle, brain and heart. In addition, a4 mRNA was detected from the day 7 embryonic stage of mouse whereas B1 mRNA is detected from day 17 (Fig. 1). Mouse kidney development is known to begin after day 14 and other kidney-specific V-ATPase subunits express from day 15 or later [22,23]. These results suggest that expression of the a4 subunit mRNA from early stages of mouse embryonic development may be related to its expression in tissues other than the kidney.

Identification of splicing variants of the mouse a4 subunit

5′-RACE was performed with a4 isoform-specific primers to determine the transcriptional initiation site. Using mouse kidney cDNA as a template, two different 5′-noncoding regions, a4–1 and a4-II, were amplified (Fig. 2-a). Nucleotide sequences of these fragments are different except for the −1 to −17bp region from the translational initiation codon (Fig. 2-a). These different 5′-noncoding regions correspond to the alternative splice variants of a4 isoform. To isolate the full length ORF of these splice variants, we performed RT-PCR using a4-I or a4-II specific primers (Fig. 2-a) as well as a4 subunit C-terminal primers as described in Materials and Methods. Both ORFs were successfully amplified from kidney cDNA and sequenced. The nucleotide sequences of these variants are identical (data not shown), suggesting that these variants encode the same protein.

To confirm the presence of two splice variants, we tried to identify the exons that correspond to the 5′-noncoding sequences of both cDNA variants in the mouse genome. Genomic walking analysis was performed in a region about 30kbp upstream of the a4 subunit gene (accession number, **AY137757**). Exon 1a for a4-I and exon 1b for a4-II were identified 30250bp and 15413bp upstream of exon 2 that contains the translational initiation site (Fig. 2-b). Splicing donor sites for exon 1a and 1b are conserved at their 3′ end (Fig. 2-c). These results indicate that the 5′-noncoding region of each splice variant is transcribed from the corresponding first exons in the mouse genome.

Expression of a4 splice variants in kidney cells

As deduced from the nucleotide sequences, the a4 splice variants encode identical protein sequences. To elucidate the role of these variants in kidney function, we tested for their expression in mouse kidney cells by *in situ* hybridization using variant-specific RNA probes. Positive signals (blue staining) are observed in tubular cells in the kidney for both isoforms, with no significant differences between their expressions in different cell types in the kidney (Suppl. Fig. 2). This result suggests that these isoforms do not show cell- specific functions in mouse kidney.

Tissue and developmental stage expression of the a4 splicing variants

As discussed in Figure 1, the expression pattern of a4 mRNA is different from that of the "kidney specific "B1 isoform. To determine whether each a4 splice variant shows different

expression patterns in mouse tissues, RT-PCR was performed with variant-specific primers (Fig. 2-a). As shown in figure 3, the two splice variants showed different patterns of expression. In addition to its predominant expression in kidney, a4-I mRNA was detected in heart, lung skeletal muscle and testis. On the other hand, a4-II was expressed in lung, liver and testis, in addition to heavy expression in the kidney. In addition, embryonic expression of a4-II was embryonic day 17 or later, whereas a4-I was expressed from earliest stage of development tested (Fig. 3). Because heart and skeletal muscle, which express the a4-I variant, establish their function early in development, a4-I may be detectable earlier in development than a4-II. These results suggest that the expression of splice variants was controlled by tissue-specific promoters present in the 5′-upstream regions of their first exons. Comparison of the nucleotide sequences of the promoter regions did not reveal homologous regions or unique transcription factor binding sites (Suppl. Fig. 3).

Basal promoter activity of each splice variant

To test the properties of the promoter region of each splice variant, we analyzed the basal promoter activity of the 5′-upstream region of exon 1a (−1613 to +73bp) and exon 1b (−1765 to +73) that correspond to the a4-I and a4-II promoters, respectively (Fig. 2). We ligated different lengths of each promoter region upstream of the reporter luciferase gene (Suppl. Fig. 3), and transfected constructs into the mouse fibroblast cell line NIH3T3. As shown in Figure 4, the 5′-upstream region of exon 1b showed a significant amount of promoter activity in NIH3T3 cells, whereas the 5′-upstream region of exon 1a does not show detectable promoter activity. Compared with the promoter activity of the house keeping subunit genes (c and c″) of the V-ATPase, the promoter activity of a4-I is very low [24]. Because we could not detect endogenous expression of a4 in NIH3T3 (data not shown), it is possible that promoter activity of the 5′-upstream regions of each splice variant was repressed by silencer elements. When we deleted the −1507 bp to −623 bp region of the a4-II promoter, promoter activity was increased about two fold (Fig. 4), suggesting the presence of silencing elements in the −1507 bp to −623 bp region. In contrast, a series of deletions of the a4-I promoter region did not improve promoter activity, suggesting that regulation of promoter activity may be different for the two variants.

Previously, similar splice variants of the 5′-noncoding region were reported in the human a4 subunit [3]. However, such splice variants were not reported in mouse and no further analysis was performed of the human a4 splice variants [3,19]. Based on our current studies, human and mouse a4 subunit genes show a similar organization of their first exon which encodes the 5′-noncoding region. Our results further suggest that the a4 subunit splice variants play an important role in tissue or developmental specific functions common in mammals. Although functional defects of a4 cause distal renal tubular acidosis, morphological changes in the renal cells have not been reported [3]. Furthermore, obvious physiological defects in other tissues expressing a4 mRNA have not been observed. These results suggest that development may proceed normally even in the absence of functional a4 and that compensation for the absence of a4 from other a subunit isoforms may occur in tissues other than the kidney.

In summary, this paper shows the presence of the a4 isoform splice variants (a4-I and a4-II) that are expressed in a tissue-specific manner. In particular, the a4-I variant shows a different expression pattern from that of kidney-specific V-ATPase genes, (B1, d2 and a4-II) [22,23]. It will be interesting to determine the roles of the a4-I variant in tissue-specific and developmental stage-specific functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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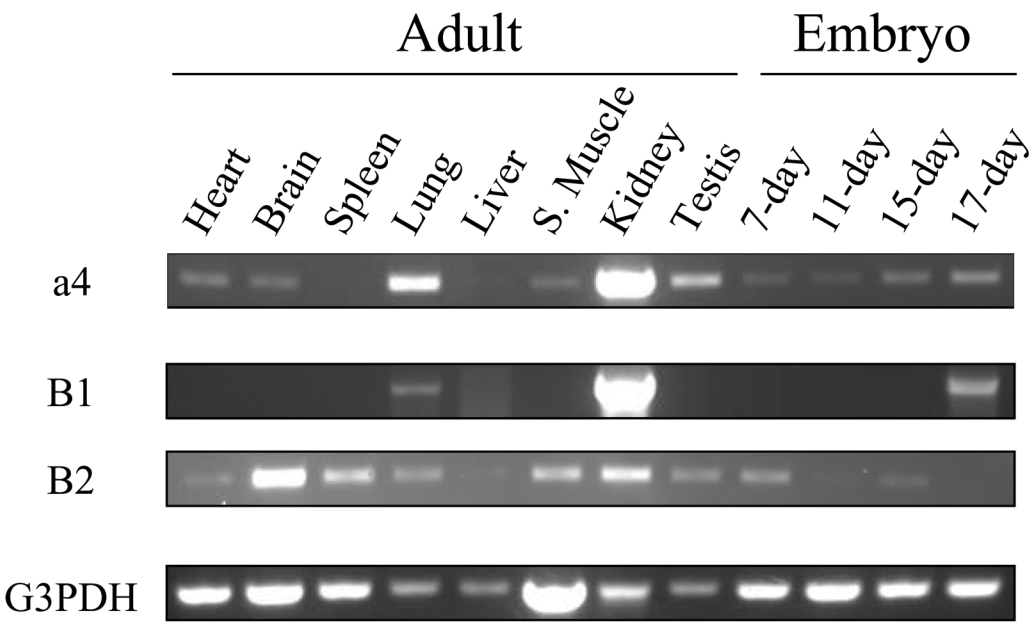
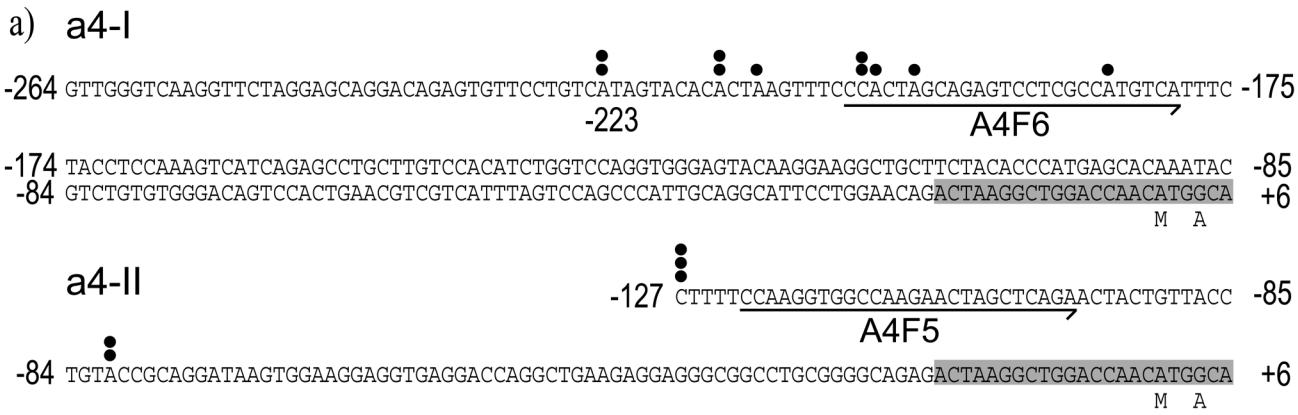
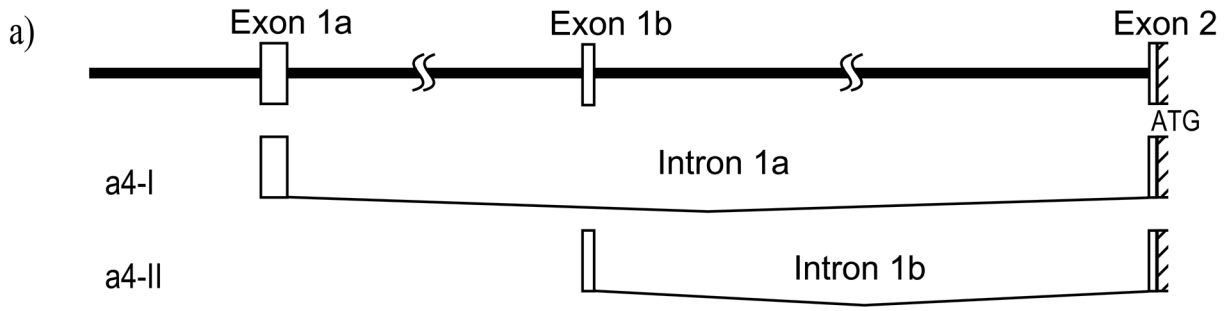


Figure 1. Tissue and developmental stage specific expression of a4 subunit and B subunit isoforms evaluated by RT-PCR

RT-PCR was performed using isoform specific primers against a4, B1, B2 or G3PDH and mRNA isolated from heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis as described in Material and Methods. RT-PCR was also performed using mRNA isolated from mouse embryos at day 7, day 11, day 15 and day 17.





b)

Exon	Size(bp)	Intron	Size(bp)	Doner	Acceptor
1a	247	1a	30,250	TGGAACAG/gtgagagggtttcta	
1b	108	1b	15,413	GGGCAGAG/gtgaggtgcccaggg	
2	>90				ttcttttccctccag/ACTAAGGCT

Figure 2. Determination of the 5′-end of the mRNA encoding the a4 isoform and the alternative spliced exons

a) 5′-RACE was performed using a4 isoform -specific primers and two different 5′-noncoding fragments were isolated. The start sites of each variant derived from several clones are indicated as the closed circles. Sequences identical between the two variants are indicated as shaded boxes.b) Schematic illustration of the 5′ portion of the mouse a4 subunit gene. Exons containing the 5′-noncoding region and ORF are indicated with open boxes and shaded boxes, respectively. c) Length of the exons and introns 1a, 1b. Splicing donor and acceptor site is underlined.

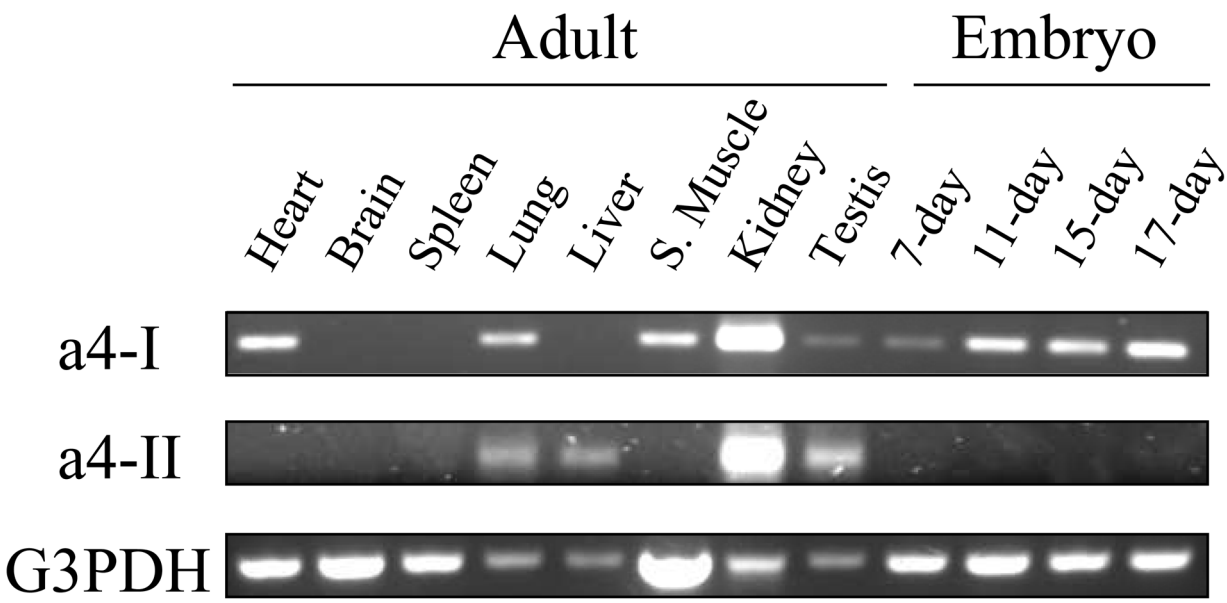


Figure 3. Tissue and stage-dependent expression of alternatively spliced variants of the a4 isoform

Confirmation of expression of each isoform by RT-PCR. RT-PCR was performed on poly A-mRNA isolated from either brain (lanes 1 and 3) or liver (lanes 2 and 4) using the primers indicated. Primers I-Fw and I-Rv were used for lanes 1 and 2 to detect the presence or absence of the amino-terminal insertion whereas primers II-Fw and II-Rv were used for lanes 3 and 4 to detect the presence or absence of the carboxyl-terminal insertion.

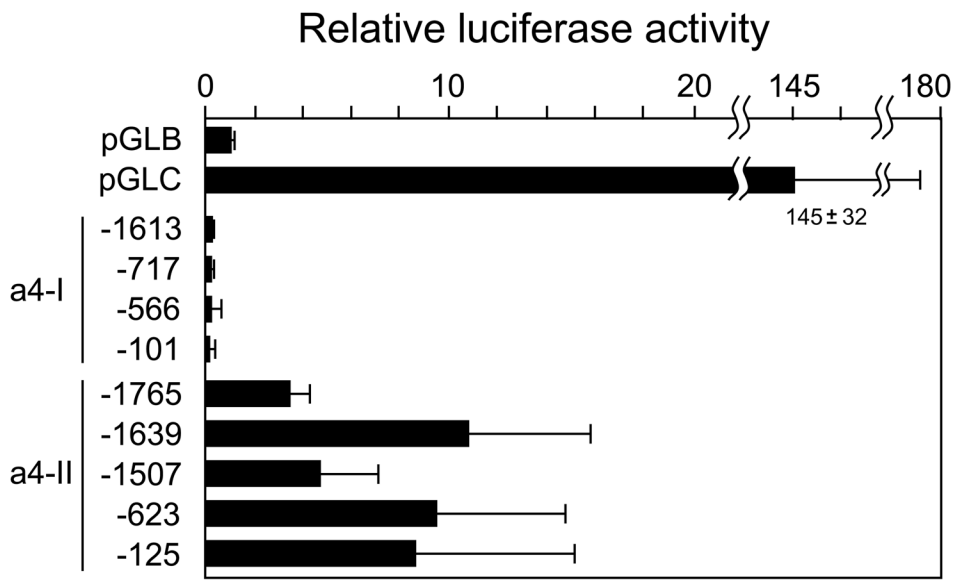


Figure 4. Promoter activity of the 5′-upstream region of each first exon

Various portions of the 5′-upstream region of each first exon were ligated in front of the luciferase gene in the pGL3-basic vector. Each of the resulting plasmids was introduced into NIH3T3 cells together with a constant amount of pRL-TK plasmid and cells were incubated for 24 hours. Promoter activity was measured and normalized using a dual luciferase assay system. Promoter activity is shown relative to that of the pGL3-basic vector. The number in each vector name corresponds to the number of bases upstream of each promoter (shown in Supplementary Fig. S3).