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Porcine α-1,3-galactosyltransferase: full length cDNA cloning, genomic organization, and analysis of splicing variants

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Full length cDNA and genomic DNA of porcine α-1,3-galactosyltransferase were isolated, and their structures were analysed. The coding region was encoded by six exons as in the mouse, and the length of each exon was conserved between the two species. The porcine exons were designated Exon 4–9, since in the mouse coding exons started from Exon 4. Introns tended to be longer in the porcine gene; the distance from Exon 4 to the 3′-end of Exon 9 was 24 kb, while this region was 18 kb in the mouse gene. The cDNA structure was extended from the previous data to the 3′-end and to the 5′ side of the cDNA. In addition to a cDNA clone with all coding exons, clones lacking parts of these exons were isolated and their structures were determined. One variant lacked Exon 5; the second, Exons 5 and 6; and the third, Exons 5, 6 and 7. The last variant was not found in the mouse, and cDNA transfection of this variant yielded scarcely any enzymatic activity using asialo α1-acid glycoprotein as a substrate, and decreased activity using *N*-acetyllactosamine as a substrate.

***Keywords*: α1,3-galactosyltransferase gene, splicing variants, xenotransplantation**

Introduction

Due to the limited number of donor organs, xenotransplantation, e.g., from pig to human, is an alternative which should be seriously considered in transplantation surgery. One obstacle in xenotransplantation is the occurrence of hyperacute rejection which destroys vascular endothelial cells in the donor organ by a complement-dependent mechanism. A carbohydrate antigen, Galα1 → 3Gal, has been identified as the major antigen responsible for hyperacute rejection [1, 2]. Although most mammals have the Galα1 → 3Gal structure, it is lacking in man, and human sera have potent antibodies against the antigenic epitope [3, 4]. The antigenic structure is formed by α-1, 3-galactosyltransferase, which transfers galactose from UDP-galactose to *N*-acetyllactosamine (Galβ1 → 4GlcNAc), yielding Galα1 → 3Galβ1 → 4GlcNAc. α-1,3-Galactosyltransferase was initially cloned from mouse and cattle [5, 6]. Recently, mice lacking the α-1,3-galactosyltransferase gene have been generated [7, 8], and cells from these mice are less susceptible to cytotoxic attack by human sera [8].

Since the pig is the preferable source of xenotransplantable organs, removal of the α-1,3-galactosyltransferase gene from pigs has become an obvious method for minimizing host rejection. Only limited information is available concerning porcine α-1,3-galactosyltransferase. cDNA clones encompassing the coding region were obtained and their sequences were determined, and transfection analysis confirmed that these clones encoded functional α-1,3-galactosyltransferase [9, 10]. However, 3′- and 5′-untranslated regions of the cDNA have only been partially characterized, and the genomic organization of the gene encoding the enzyme has not been reported. The existence of splicing variants was demonstrated by PCR analysis [11], although their structure and enzymatic activities were not reported. In the present study, we investigated genomic organization, full length cDNA structure and splicing variants of porcine α-1,3-galactosyltransferase. This information is essential for the generation of pigs deficient in the gene encoding this enzyme. Furthermore, data concerning the enzymatic activities of some variants provided information relevant to the structural-functional relationship of the enzyme in general.

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Materials and methods

Materials

Restriction endonucleases, T4 DNA polymerase, Taq DNA polymerase, LA Taq DNA polymerase and T4 DNA ligase were from Takara, Japan. The pGEM-T vector and poly-ATtract mRNA isolation systems were purchased from Promega, USA. Reverse transcriptase was from Gibco BRL, USA, pBluescript II KS(+) vector was from Strategene, USA, Amplitaq DNA polymerase was from Perkin Elmer, USA, *Griffonia simplicifolia* (GS)-IB_4 lectin was from Sigma Chemical Co., USA, and radioactive nucleotides were from Amersham Corp., UK.

Fetal pig genomic DNA was a kind gift from Meiji Health Science Institute. Landrace/Yorkshire crossbred female pigs (about 15 kg) were obtained from Oguri Chikusan, Japan. Genomic DNA [12] and total RNA [13] were isolated from the pigs according to the previously reported methods. Poly (A) + RNA was isolated using ISOGEN (Nippongene, Japan). Asialo α1-acid glycoprotein was prepared by treating α1-acid glycoprotein (Sigma Chemical Co., USA) with 0.04 N HCl at 80 °C for 1 h.

The following oligonucleotides were purchased from Biologica Co., USA.

PGT2, 5′-CATTGACAGAACCACTCTTCC-3′;
PGT3, 5′-GCTTGTCTCAACTGTAATGGTTGTGTTTTGGG-3′;
PGT4, 5′-GCCCCTCTGAGCACTGCTGCCAACTTCTGG-3′;
PGT5, 5′-GGTTTCCGAGCTGGTTTAAC-3′;
PGT8, 5′-TCAGGATTAAACCAGTCCAC-3′;
PGT11, 5′-ATGAATGTCAAAGGAAGAGTG-3′;
PGT12, 5′-TCAGATGTTATTTCTAACCA-3′;
PGT16, 5′-CCTCCAAGTAATGCTCAATG-3′;
PGT18, 5′-CTGGTATATCCAGAACAAAGAACCTTC-3′;
PGT19, 5′-GAAGGTTCTTTGTTCTGGATATACCAG-3′;
PGT20, 5′-GACTCACAGTTACCACGAAGAAGAAGACGC-3′;
PGT21, 5′-TCTAAGACGGCTCTGTTGTAAGTGCCTTCCC-3′;
PGT22, 5′-GCTCCAGTGGTATGGGAAGGCACTTACAAC-3′;
PGT25, 5′-GGTGGATGATATCTCCAGGATGCC-3′;
PGT26, 5′-GATAGAGCTGGGTCCTCTGCGTTCC-3′;

cDNA and genomic DNA analysis

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed to analyze mRNA expressed in the pig liver, spleen, kidney, heart and lung according to Saiki *et al.* [14]. The primers used were PGT11, PGT12 and PGT16, and PCR was performed with initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min. Long distance PCR was employed to obtain genomic DNA unless otherwise stated. Fetal pig genomic DNA (0.2 μg) was used as the template, and oligonucleotides PGT3, PGT4, PGT16, PGT18, PGT19, PGT20, PGT21 and PGT22 were used as primers. Long distance PCR was performed as described by Cheng *et al.* [15] using LA Taq DNA polymerase. PCR conditions were 94 °C for 1 min followed by 30 cycles of 94 °C for 30 sec and 66 °C for 6 min with final extension at 72 °C for 10 min. The genomic DNA fragment spanning from Exon 6 to Exon 7 was obtained by standard PCR using PGT5 and PGT8 as primers (94 °C, 1 min; 94 °C 1 min/55 °C 1 min/72 °C 1 min, 35 cycles).

These PCR products were subcloned into the pGEM-T vector or pBluescript II KS(+) vector and their sequences were analyzed by the di-deoxy chain termination method [16] using a DNA sequencer (Applied Biosystems 373A).

3′-RACE (Rapid Amplification of cDNA Ends) and 5′-RACE were performed according to Frohman *et al.* [17] using mRNA isolated from the pig spleen and PGT25, PGT26, PGT2 and PGT4 as primers.

Transfection of α-1,3-galactosyltransferase cDNA into COS-7 cells

cDNAs were subcloned into the pcDNA3 vector at *Bam*HI and *Eco*RI sites. COS-7 cells, cultured in a 10 cm Falcon 3003 dish with DMEM containing 10% fetal calf serum to reach 70% confluency, were incubated with a mixture of 5 ml DMEM with 10% Nu serum and 0.2 ml of 10 mg ml^{-1} DEAE-dextran containing 2.5 mM chloroquine and 15 μg of the plasmid DNAs for 3.5 h at 37 °C under a 5% CO_2 atmosphere. Then, the medium was replaced with 5 ml of PBS(−) (phosphate buffered saline without Ca^{2+} and Mg^{2+}) containing 10% dimethylsulfoxide. After 2 min at room temperature, the medium was changed to 10 ml DMEM with 10% fetal calf serum and the cells were cultured at 37 °C under 5% CO_2.

Analysis of expression of α-galactosyl residue

COS-7 cells (1×10^6), which were cultured for 72 h after transfection and harvested with PBS(−) containing 0.5 mM EDTA were incubated with FITC-GS-IB_4 (20 μg ml^{-1}) in 100 μl of PBS(−) with 1% BSA at 4 °C for 30 min. After washing with PBS(−) twice, cells were analyzed with an Epics Profile flow cytometer (Coulter Electronics Corp., USA), and 1×10^4 cells were counted.

Enzyme assay

COS-7 cells cultured in a 10 cm dish for 48 h after transfection were harvested by scraping and washed with 25 mM MES [2-(N-Morpholino)ethanesulfonic acid] (pH6.0)/20 mM $MnCl_2$. After centrifugation at 15 000 rpm for 20 min, the supernatant was concentrated to 50 μl using Centricon 30 (Amicon). The reaction mixture (14 μl) contained 1 μl of the cell extract, 100 μg of bovine serum albumin, 20 μg of the acceptor (asialo α1-acid glycoprotein or

N-acetyllactosamine) and 1.6 nmole of UDP-[^{14}C]-galactose (0.1 μCi). After appropriate times at 37 °C, the reaction mixture was directly applied to 60 HPTLC silica gel plates (Merck) and developed in ethanol: pyridine: n-butanol: H_2O: acetate (100 : 10 : 10 : 30 : 3). The plates were dried and radioactivity remaining at the origin was determined by BAS 2000.

Results and Discussion

3′- and 5′-sequences of porcine α-1,3-galactosyltransferase cDNA

In order to obtain full length cDNA of porcine α-1,3-galactosyltransferase, RACE-PCR was performed. By 3′-RACE, we obtained a complete clone of 3′-region which contained the polyadenylation signal AATAAA and poly-A tail (Figure 1A). The length of the 3′-untranslated region before the start of the poly-A tail was about 1900 bp, of which only 222 bp was reported previously [9]. The 5′-untranslated region was also extended by 5′-RACE. We obtained five types of extended cDNA clones, 5′-A, 5′-B, 5′-C, 5′-D and 5′-E (Figure 1B). The major product, 5′-A, terminated at the [A] located 216 nucleotides upstream from the start codon ATG (six out of 13 clones). Thus, this site was considered as the major transcription initiation site (Figure 1A, 1B). All 5′-RACE products obtained shared 11 bp of sequence upstream from the start codon. Further upstream, there were partially conserved sequences, but no completely conserved sequences, indicating a complex mode of alternative splicing in the 5′-region. Previously Sandrin *et al.* reported 49 bp of 5′ upstream sequence [9], while Strahan *et al.* reported

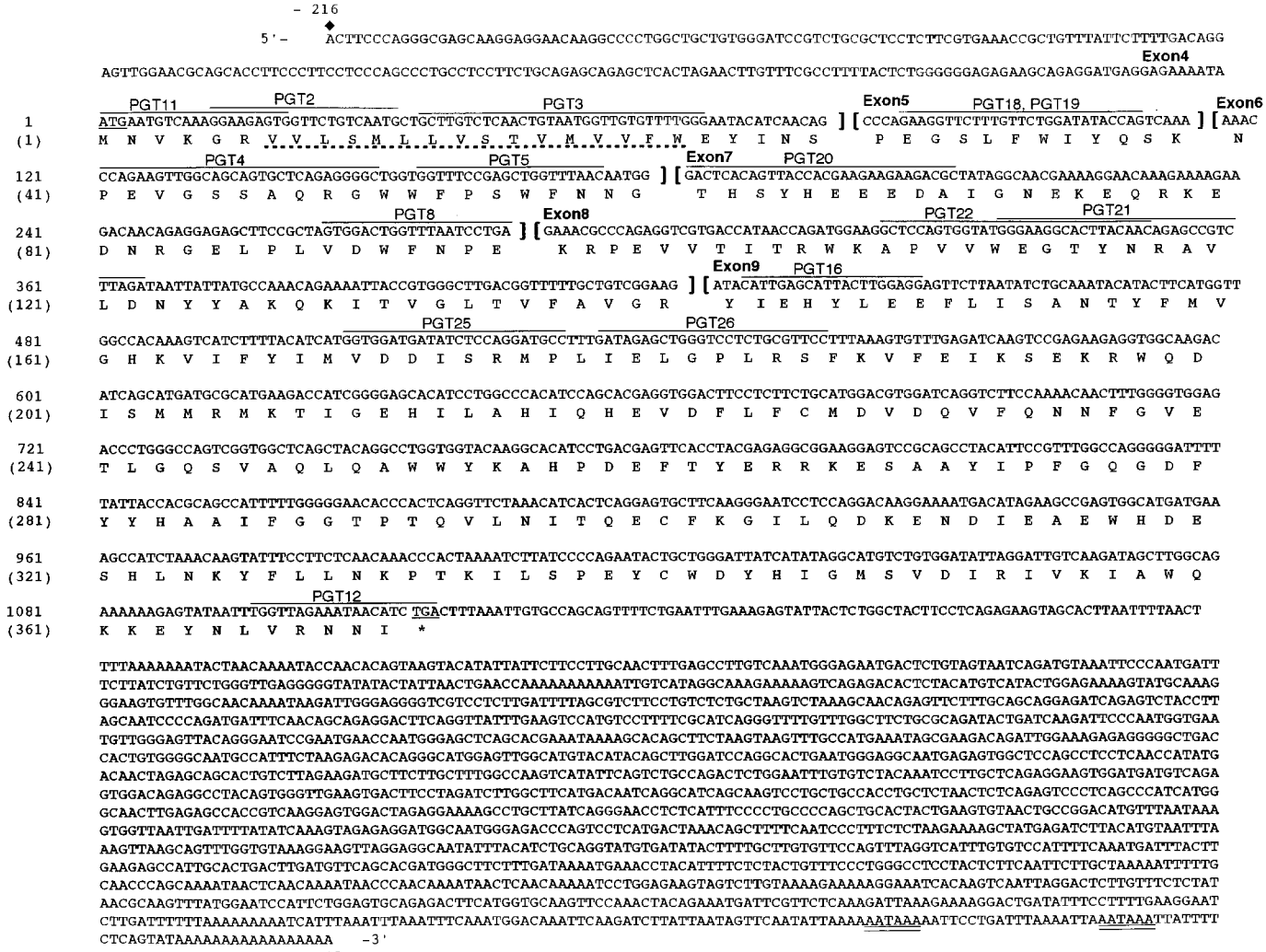

Figure 1A. Nucleotide sequence of porcine full length *α*-1,3-galactosyltransferase. The deduced amino acid sequence in the open reading frame is indicated below the nucleotide sequence. The start codon and termination codon are underlined. The primers used for PCR, long distance PCR, RT-PCR and RACE are indicated. The putative transmembrane domain is marked (– – – – – –). Brackets are used to show the start and end of each exon. ◆: the major transcription initiation site. **1B**. Nucleotide sequence of porcine *α*-1,3-galactosyltransferase in the 5′-untranslated region obtained by 5′-RACE. The start codon in Exon 4 is typed in bold letters. ◆: the major transcription initiation site, ∇: 5′-cDNA ends of 5′RACE clones. The clone number is indicated in parentheses in the case of plural clones. ——, ═══, – – – – – –, ======: homologous sequences.

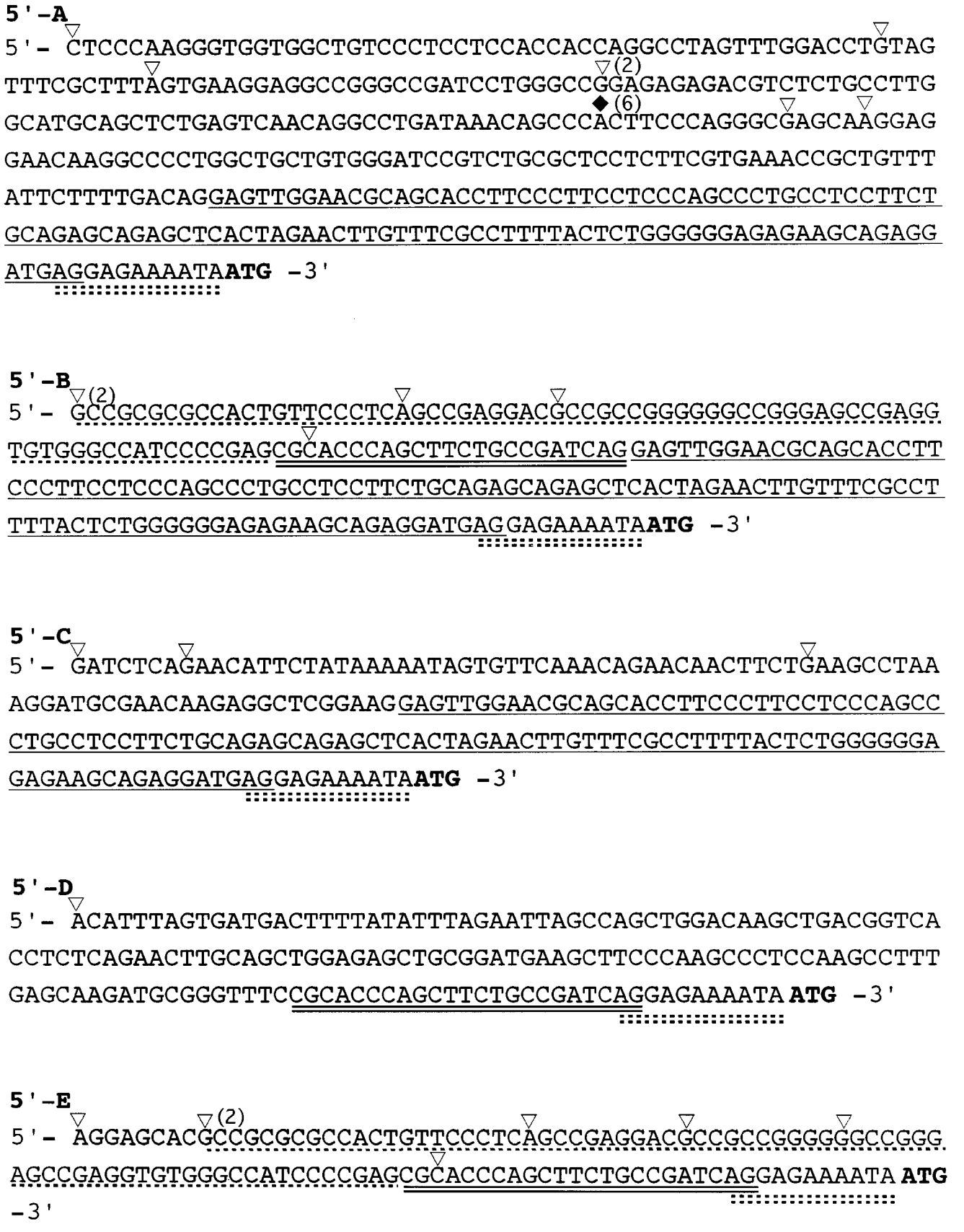

Figure 1. Continued.

15 bp of the upstream sequence [10]. Among the present 5′-sequences, three species (5′-A, B, C) shared the initial 14 bp sequence with that reported by Strahan *et al.* [10], while two species (5′-D, E) shared the initial 32 bp with that reported by Sandrin *et al.* [9].

Organization of genomic DNA of porcine α-1,3-galactosyltransferase

The present study dealt with the localization of coding sequence and the 3′-untranslated region (Figure 2). Genomic DNA containing the coding exons was obtained both by long distance PCR and conventional PCR (see Materials and Methods). From the size of the DNA fragments generated by restriction endonuclease digestion and partial sequence of the generated fragments, we determined the exon/intron organization of the gene (Figure 2). The porcine gene contained six exons in the region. The number of exons and the boundaries of exons in the coding sequence (cf. Figure 1) were completely conserved between the pig and mouse. However, the size of the introns tended to be larger in the pig. The structure of intron-exon boundaries in the genomic sequence shown in Figure 3 agreed with consensus splice sequences [18]. We named porcine exons Exon 4–9, since in the mouse coding exons started from Exon 4 [19]. This information enables us to prepare a knockout construct to delete a portion of the gene to render it unfunctional.

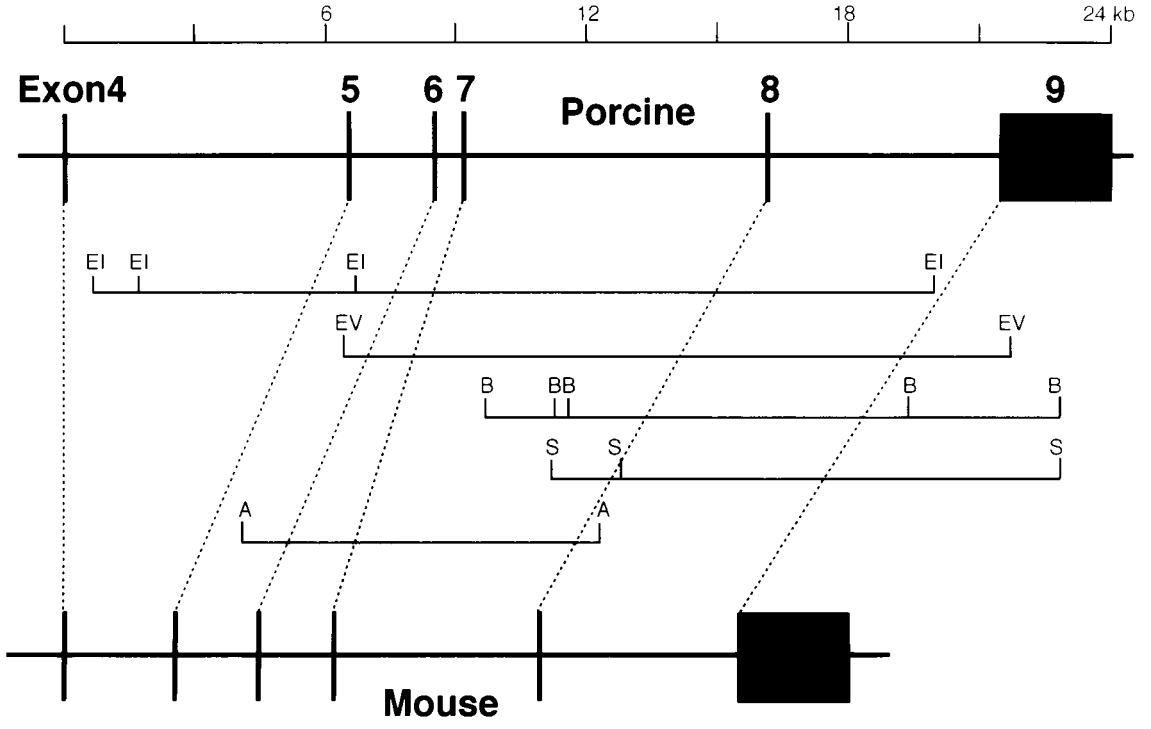

Figure 2. Genomic DNA organization of porcine *α*-1,3-galactosyltransferase and comparison to that of the mouse. The scale at the top shows the nucleotide position in kilobases from the beginning of Exon 4. The exon numbering refers to that of mouse *α*-1,3-galactosyltransferase. A partial restriction endonuclease map is shown: EI; EcoRI, EV; EcoRV, B; BamHI, S; SacI, A; ApaI.

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                                                exon4 ····· AACAG gtaat ····· intron4
intron4 ····· atttccatttgcttttag CCCAG ····· exon5 ····· TCAAA gtaag ····· intron5
intron5 ····· atctttttcatttcttag AAACC ····· exon6 ····· AATGG gtaag ····· intron6
intron6 ····· ttttccgtttgtacgtag GACTC ····· exon7 ····· CCTGA gtaag ····· intron7
intron7 ····· tctatgttttgtcaccag GAAAC ····· exon8 ····· GGAAG gtagg ····· intron8
intron8 ····· cattcttctttatttcag ATACA ····· exon9 ····· 3'-end
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Figure 3. The intron-exon boundaries of porcine *α*-1,3-galactosyltransferase.

Detection of splicing variants and their enzymatic activity

We performed RT-PCR analysis to detect porcine α-1,3-galactosyltransferase mRNA using PGT11 and PGT16 as primers. cDNA from the liver, spleen, kidney, heart and lung all yielded four products (Figure 4). They are a 445 bp product encoding Exons 4–9 (abbreviated Ex 4–9), a 409 bp one lacking Exon 5 (Δ Ex 5), a 346 bp one lacking Exons 5 and 6 (Δ Ex 5, 6) and a 241 bp one lacking Exons 5, 6 and 7 (Δ Ex 5, 6, 7) (Figure 5). Vanhove *et al.* reported splicing variants probably identical to Δ Ex 5 and Δ Ex 5, 6 [11], but not Δ Ex 5, 6, 7. This variant was also not reported in the mouse (Figure 5). From the intensity of the PCR bands, Δ Ex 5 is the most abundant one, followed by the full-size cDNA (Ex 4–9). Δ Ex 5, 6 and Δ Ex 5, 6, 7 were minor products (Figure 4). These relative amounts of RT-PCR products were observed in all the organs examined. Picking up the RT-PCR product cDNA from the spleen, the ratio of Δ Ex 5, Ex 4–9, Δ Ex 5, 6 and Δ Ex 5, 6, 7 was approximately 9 : 5 : 1 : 1.

Since Δ Ex 5, 6, 7 is a novel splicing variant, we examined whether it exhibited enzyme activity. Δ Ex 5, 6, 7 as well as Ex 4–9 and Δ Ex 5 were inserted into an expression vector and transfected into COS-7 cells. New expression of α-galactosyl epitope on the cell surface was analysed using FITC-labeled GS-IB_4 lectin, which specifically reacts with α-galactosyl residues. Flow cytometric analysis of the stained COS-7 cells revealed that about half of the cells transfected with the Ex 4–9 or Δ Ex 5 became to strongly express α-galactosyl epitope (Figure 6B, C), while cells transfected with vector alone did not (Figure 6A). Only a small portion of cells transfected with Δ Ex 5, 6, 7 expressed the α-galactosyl epitope, weakly (Figure 6D).

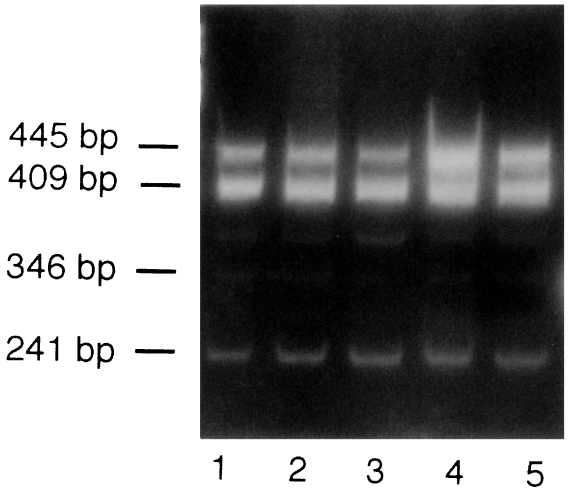

Figure 4. RT-PCR to detect splicing variants of porcine *α*-1,3-galactosyltransferase. PGT11 and PGT16 were used as primers. The sizes of the PCR products are shown at the left side of the figure. 1, liver; 2, spleen; 3, kidney; 4, heart; 5, lung.

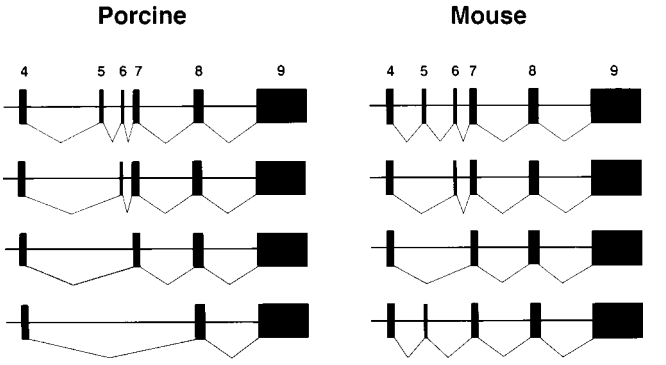

Figure 5. Comparison of porcine and mouse splicing variant forms of *α*-1,3-galactosyltransferase.

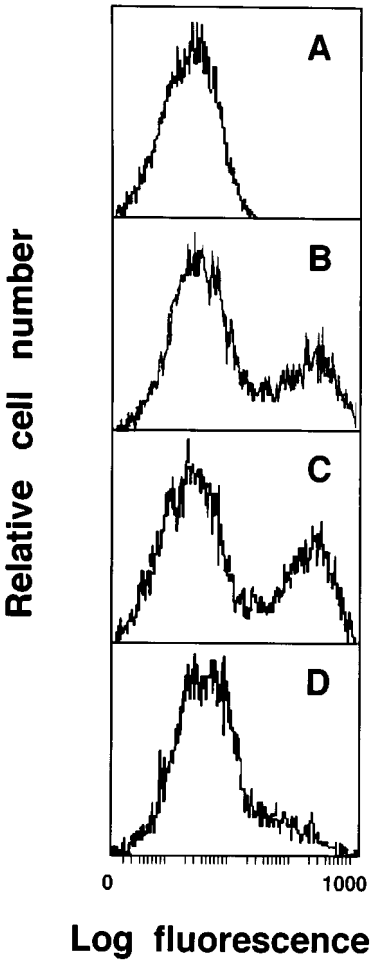

Figure 6. Expression of *α*-galactosyl residue in COS-7 cells transfected with porcine *α*-1,3-galactosyltransferase cDNAs. COS-7 cells were treated with FITC-GS-IB_4 and analysed by fluorescence-activated cell sorter. The cells were transfected with vector alone (A), the full-size cDNA (Ex 4–9) (B), Δ Ex 5 (C) or Δ Ex 5, 6, 7 (D).

Table 1. Comparison of the α-1,3-galactosyltransferase activities of splicing variants

	Acceptor (nmol mg protein^{-1} h^{-1})	
	Asialo α1-acid glycoprotein	N-*acetyllactosamine*
Control	< 0.1	< 0.1
Ex 4–9	3.5	11.1
Δ Ex 5	3.8	11.2
Δ Ex 5, 6, 7	0.1	1.7

α-1,3-Galactosyltransferase activity was also measured in cell extracts using asialo α1-acid glycoprotein or *N*-acetyllactosamine as a sugar acceptor. COS-7 cell extract transfected with Ex 4–9 or Δ Ex 5 cDNA efficiently catalyzed the transfer. The rates of those reactions were linear with time to 1 h. However, the level of activity in cells transfected with Δ Ex 5, 6, 7 cDNA was not different from that of cells transfected with vector alone when asialo α1-acid glycoprotein was used as a substrate (Table 1). Using *N*-acetyllactosamine as a substrate, a low level of enzymatic activity was found in the variant (Table 1). Thus, the deletion of Exons 5, 6 and 7 reduced the activity of α-1,3-galactosyltransferase. Since deletion of Exon 5 does not affect the mouse [6] or porcine (this study) enzyme, it appears that a peptide segment encoded by Exon 6 and/or 7 is important for the enzyme.

It is possible that deletion of the exons may influence expression or stability of the protein. However, our result is consistent with that of Henion *et al.* [20], who deleted the stem region stepwise from soluble primate α-1,3-galactosyltransferase. The enzyme composed of amino acids 97–376 scarcely showed the activity. They used only *N*-acetyllactosamine and lactose as substrates, and did not notice the change of specificity. The actual occurrence of mRNA encoding a protein with decreased and altered enzymatic activity raises an interesting question of whether the variant protein has a regulatory function by interfering with the fully active enzyme.

Acknowledgments

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