

## Tay-Sachs disease in Jacob sheep

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### ABSTRACT

Autopsy studies of four Jacob sheep dying within their first 6–8 months of a progressive neurodegenerative disorder suggested the presence of a neuronal storage disease. Lysosomal enzyme studies of brain and liver from an affected animal revealed diminished activity of hexosaminidase A (Hex A) measured with an artificial substrate specific for this component of  $\beta$ -hexosaminidase. Absence of Hex A activity was confirmed by cellulose acetate electrophoresis. Brain lipid analyses demonstrated the presence of increased concentrations of G<sub>M2</sub>-ganglioside and asialo-G<sub>M2</sub>-ganglioside. The *hexa* cDNA of Jacob sheep was cloned and sequenced revealing an identical number of nucleotides and exons as in human *HexA* and 86% homology in nucleotide sequence. A missense mutation was found in the *hexa* cDNA of the affected sheep caused by a single nucleotide change at the end of exon 11 resulting in skipping of exon 11. Transfection of normal sheep *hexa* cDNA into COS1 cells and human Hex A-deficient cells led to expression of Hex S but no increase in Hex A indicating absence of cross-species dimerization of sheep Hex  $\alpha$ -subunit with human Hex  $\beta$ -subunits. Using restriction site analysis, the heterozygote frequency of this mutation in Jacob sheep was determined in three geographically separate flocks to average 14%. This large naturally occurring animal model of Tay-Sachs disease is the first to offer promise as a means for trials of gene therapy applicable to human infants.

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### 1. Introduction

Tay-Sachs disease (TSD, MIM 272800) is a rare autosomal recessive neurodegenerative disorder characterized by progressive accumulation of G<sub>M2</sub>-ganglioside within nerve cells of the central nervous system [1]. It is due to a deficiency of the lysosomal enzyme  $\beta$ -N-acetylhexosaminidase A (Hex A). Clinically, it begins in infancy with a startle response, developmental delay, visual inattention, and failure to thrive and progresses to seizures and a vegetative state before the age of 2 years. The brain enlarges disproportionately due to neuronal storage of the ganglioside and death results in 3 to 5 years.

TSD is generally regarded as the classical variant of the G<sub>M2</sub>-gangliosidoses, each of which is associated with a defect in the intralysosomal hydrolysis of G<sub>M2</sub>-ganglioside [2]. This glycosphingolipid is normally metabolized by Hex A, a heterodimer composed of both  $\alpha$ - and  $\beta$ -subunits. Each of these peptides is encoded by a separate gene, the  $\alpha$ -subunit by *HEXA* on chromosome 15 and the  $\beta$ -subunit by *HEXB* on chromosome 5. In addition to late-infantile TSD, juvenile and adult forms of G<sub>M2</sub>-gangliosidosis are encountered

resulting from Hex A deficiency due to mutations in the  $\alpha$ -subunit gene, *HEXA*.  $\beta$ -N-acetylhexosaminidase B (Hex B) is a homodimer of  $\beta$ -subunits and mutations in the *HEXB* gene cause deficiency of both Hex A and Hex B (total hexosaminidase deficiency). While Hex B cannot degrade G<sub>M2</sub>-ganglioside, the deficiency of Hex A in patients with the combined deficiency also causes G<sub>M2</sub>-ganglioside storage. These individuals are referred to as Sandhoff disease whether they are of late-infantile, juvenile, or adult onset. Another component, G<sub>M2</sub>-activator protein, also encoded on chromosome 5, is also required for the action of Hex A on G<sub>M2</sub>-ganglioside. In a few very rare instances, G<sub>M2</sub>-gangliosidosis has been encountered due to deficiency of the activator protein. These patients have normal Hex A activity when measured with artificial substrates.

Many lysosomal storage diseases (LSDs) that occur in humans have also been found spontaneously in animals. Multiple examples of Sandhoff disease (G<sub>M2</sub>-gangliosidosis type O) have been reported in cats [3–11], dogs [12–16], and pigs [17–19]. G<sub>M2</sub>-activator deficiency (G<sub>M2</sub>-gangliosidosis type AB) has also been found in the cat [20] and dog [21]. However, naturally occurring Tay-Sachs disease (G<sub>M2</sub>-gangliosidosis type B) in animals has been observed only twice, in two Muntjak deer from the same dam and sire [22] and in two American flamingos [23]. In both instances, the animals were available for study because they were captives within a zoological park. Attempts to create a mouse model of TSD in the laboratory by

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gene knockout has not been entirely successful due to an active alternative pathway for  $G_{M2}$ -ganglioside catabolism utilizing a sialidase in place of the deficient hexosaminidase [24,25]. This report describes the biochemical and molecular characteristics of Tay-Sachs disease in Jacob sheep, a rare primitive breed that is believed to have originated many centuries ago in the Middle East. The existence of a large animal model of late-infantile onset Tay-Sachs disease now provides a practical opportunity to explore the utility of gene therapy for the treatment of this and possibly other currently fatal neurodegenerative LSDs of infancy.

## 2. Materials and methods

### 2.1. Animals

The clinical and pathologic findings in four affected lambs from the same flock of Jacob sheep are described in a separate manuscript [26]. Two were 6-month-old males and two were 8-month-old females. Ataxia, proprioceptive defects, and cortical blindness were present in each. On histological examination, the neurons were enlarged and contained cytoplasm distended by foamy to granular material staining positively with Luxol fast blue and Sudan black B. Widespread astrocytosis, microgliosis, and scattered spheroids were also present. Electron microscopy demonstrated membranous cytoplasmic bodies within neurons.

### 2.2. Blood and tissue samples

Brain and liver tissue from Jacob sheep were provided by the Department of Veterinary Pathobiology of the Texas A&M College of Veterinary Medicine and Biomedical Sciences. In addition, blood specimens from 104 Jacob sheep were kindly provided by the Jacob sheep registries. Leukocytes and plasma were obtained from heparinized blood. Leukocytes were isolated by a differential sedimentation procedure using 4% dextran (molecular weight [MW] 180,000–200,000), dextrose-ACD solution in physiological saline [27]. For genotyping, blood spots were collected on filter paper using a lancet to puncture the skin in the ear.

### 2.3. Enzyme assays

Brain and liver tissue stored at  $-80^{\circ}\text{C}$  from an affected and control sheep were thawed, homogenized in water (10% wt./vol.), and centrifuged at  $30,000 \times g$  for 20 min. Protein concentration was determined using the Lowry method [28]. Enzymatic activities of Hex components were performed using the synthetic substrates, 4-methylumbelliferyl-2-acetoamido-2-deoxy- $\beta$ -D-glucopyranoside (4-MUG) and its 6-sulfate derivative (4-MUGS) obtained from Sigma-Aldrich (St. Louis, MO). The reaction mixture consisted of 100  $\mu\text{l}$  of a 3 mM 4-MUG or 4-MUGS solution dissolved in citrate-phosphate buffer, pH 4.0 and 10  $\mu\text{l}$  of sonicated sample equivalent to 10  $\mu\text{g}$  of protein. The reaction mixture was incubated at  $37^{\circ}\text{C}$  for 30 min and subsequently stopped with 2 ml of 0.2 M glycine buffer, pH 10.3. Fluorescence was determined in a fluorescence spectrophotometer (Hitachi F-2500) at an excitation wavelength of 365 nm and an emission wavelength of 448 nm. Samples were compared against a 4-methylumbelliferone (4-MU) standard curve prepared in 0.2 M glycine buffer. In addition, enzymatic activity of the lysosomal enzymes  $\beta$ -galactosidase,  $\alpha$ -mannosidase,  $\alpha$ -fucosidase, and  $\beta$ -glucuronidase was determined using their corresponding 4-MU glycoside substrates (obtained from Sigma-Aldrich). The reaction mixture was incubated for 2 hours, and the product was determined as described above for analysis with 4-MUG and 4-MUGS.

### 2.4. Analysis of Hex by electrophoresis

Leukocyte lysates and plasma were prepared by sonication and centrifugation to yield 1  $\mu\text{g}$  protein/ $\mu\text{l}$ . Electrophoresis of both plasma and leukocyte lysates was carried out at pH 6.5 in 0.02 M citrate-phosphate buffer on cellulose acetate membranes (Scie-Plas, Warwickshire, England) [29]. In summary, 3–5  $\mu\text{l}$  aliquots of the lysates and plasma were applied to the membrane and run for 90 min in a Ca-Sys electrophoresis apparatus at 2 mA/cm width of the membrane at 100 V. Subsequently, the membrane was incubated at  $37^{\circ}\text{C}$  for 60 min between two filter papers impregnated with substrate solution (2 mM 4-MUG or 4-MUGS in 0.1 M citrate-phosphate buffer, pH 4.4). After incubation, the membrane was developed with concentrated ammonium hydroxide and illuminated with ultraviolet light (312 nm). The bands of fluorescent-free 4-MU representing Hex activity were then photographed.

### 2.5. Glycolipid analysis

Brain tissue (~0.2 g) was homogenized in 2 ml of water with a Brinkman homogenizer (high speed, 30 s, 2–3 times), and their lipids were extracted overnight with 40 ml chloroform-methanol (C/M, 2:1). The extract was filtered and the solvent removed under pressure. The residue was re-extracted with 20 ml of the same solvent [30]. The combined material was dried under a Brinkman Rotavac. The sample was resolubilized in 5 ml of C/M, 2:1 and washed. Upper phase gangliosides and long-chain glycosphingolipids (GSL) were extracted using a Bond Elut C18 cartridge column (Varian, Inc., Harbor City, CA) containing 500 mg of adsorbent and solubilized in 5 ml of methanol [31]. The extract was taken to dryness and redissolved in C/M 2:1. Aliquots were removed for lipid-bound sialic acid determination by the thiobarbituric acid procedure [32]. Total lower-phase lipids were fractionated by chromatography using 500 mg of silicic acid packed in chloroform. Cholesterol was eluted with 3.5 ml of chloroform, GSL were eluted with 5 ml of acetone and phospholipids were eluted with 5 ml of methanol. Cerebroside and asialo- $G_{M2}$  were separated from sulfatides by chromatography with DEAE-Sephadex [33].

### 2.6. Thin-layer chromatography (TLC)

GSL were redissolved in C:M 2:1 and applied to an aluminum-backed silica gel high-performance thin-layer chromatograph (HPTLC) plate (Nano-SIL, Macherey-Nagel, Oensingen, Germany), developed with chloroform-methanol-water (C/M/W, 60:35:8) as solvent system and visualized by lightly spraying with orcinol-sulfuric acid (2% in 10% vol./vol. sulfuric acid in methanol) and then heating in an oven at  $100^{\circ}\text{C}$  for 20–30 min. Gangliosides were also dissolved in C/M (2:1), applied to HPTLC silica gel plates and developed with C/M 0.25% CaCl (C/M/CaCl, 55:45:10). Standards and samples were visualized by spraying with resorcinol (0.2% with 0.25 mM copper sulfate in 80% vol./vol. HCl). The plate is sprayed moderately, covered with a glass plate and heated in an oven at  $100^{\circ}\text{C}$  for 20–30 min [34].

Procedures for cell culture, extraction of total RNA and genomic DNA, amplification of cDNA, and genomic DNA were performed as previously described by us [23,35,36].

### 2.7. Sequence analysis of cDNA and genomic DNA

Genomic DNA and cDNA PCR products were either directly sequenced or cloned into pcDNA 3.1 DNA 5-His-TOPO vector by pcDNA 3.1 directional TOPO expression kit according to the manufacturer's suggested protocols and then sequenced (Invitrogen, Carlsbad, CA). Automated sequencing was used [23,35,36].

ReadyAmp™ genomic DNA purification system (Promega, Madison, WI) was used to extract genomic DNA from blood spots of filter paper (Whatman, Piscataway, NJ) under the conditions recommended by the manufacturer.

SMART™ RACE cDNA amplification kit (Clontech, Mountain View, CA) and Advantage-GC cDNA PCR kit (Clontech) were used for 5' extension of the Jacob sheep *hexa* cDNA under the conditions recommended by the manufacturer [23].

Advantage-HF™ PCR kit was used to amplify full-length cDNA of Jacob sheep under the conditions recommended by the manufacturer (Clontech). A pair of primers for amplification of full-length cDNA of Jacob sheep was designed by us on the basis of our sequence results of the *hexa* gene:

Forward primer (SHhexaATG-F: 5'CACCATGGCAGGCTCCACGCT-CAGG'3)

Reverse primer (SHhexaTGA-R: 5'TCAGGTTTGTCAAACCTCCATGTCACAGTAGCC'3)

In vitro mutagenesis and expression of the Jacob sheep and human *HEXA* cDNA including the entire coding region of normal *HEXA* cDNA was inserted into pcDNA 3.1 DNA 5-His-TOPO vector by pcDNA 3.1 directional TOPO expression kit according to the manufacturer's suggested protocols (Invitrogen). The site-specific mutation of the *hexa* cDNA was performed with the QuikChange Site-directed Mutagenesis kit and one set of complementary primers containing mutagenic nucleotides designed by us (5'-CCCCTGGCATTGAAACGTAGCCCT-GAGCA-3' and 5'-TGCTCAGGGCTACGTTCAAATGCCAGGG-3') under the conditions recommended by the manufacturer (Stratagene, La Jolla, CA). The *hexa* cDNA was sequenced completely to ensure that only the expected mutation was present. COS1 cells and affected human TSD fibroblasts were respectively transfected with 3 µg of vector containing either normal or mutated Jacob sheep and human cDNAs for the *HEXA* gene in conjunction with 10 µl of FuGENE 6 (Roche Diagnostics, Indianapolis, IN) in each T25 flask. The cells were harvested after 72 hours, and the Hex A activity in cell extracts of the different transfections were assayed using the synthetic substrate, 4-MUGS as previously described [23].

### 3. Results

#### 3.1. Enzymatic analysis

In Table 1, the activity of five lysosomal hydrolases assayed in brain tissue from two different control animals and a single affected animal are compared with the activity of the same enzymes examined using a single liver specimen from a control and affected animal respectively. β-Hexosaminidase activity measured using 4-MUGS as substrate was reduced in the affected sheep to 29% of control in brain and 6% of control in liver. As shown in Table 1, comparison of enzyme activity for four other lysosomal enzymes: α-fucosidase, β-galactosidase, β-glucuronidase and α-mannosidase showed no consistent differences

**Table 1**  
Lysosomal hydrolase activity in brain and in liver tissues from an affected and control sheep expressed as nanomoles per milligram of protein per hour.

Enzyme	Brain		Liver		
	Controls		Affected	Control	
	No. 1	No. 2		Affected	
α-Fucosidase	28	73	131	236	29
β-Galactosidase	41	38	15	277	243
β-Glucuronidase	12	15	43	27	18
β-Hexosaminidase (4-MUGS)	33	36	10	243	14
α-Mannosidase	24	69	112	280	114

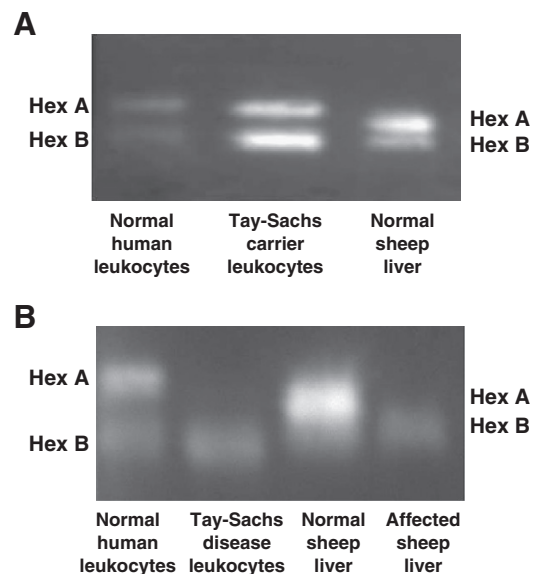
in the activity in both brain and liver between control and affected sheep.

The relative deficiency of activity toward the 4-MUGS substrate, especially in the liver, suggested the possibility of a defect in HEX A. Attempts were made to measure both HEX A and HEX B activity using 4-MUG with heat inactivation of normal sheep liver homogenate. Rapid loss of total HEX activity resulted with less than 10% remaining after 2 hours at 50 °C. Enzyme activity was also determined at intervals up to 2 hours after exposure of the reaction mixture to 45 and 48 °C with only 20% of activity remaining after 1 hour at 48 °C and less than 30% of activity present after 2 hours at 45 °C. These findings indicated either that sheep HEX A and B differed significantly in thermostability characteristics from that of human HEX A and B or else that the HEX A component in sheep liver comprised nearly all of the lysosomal HEX A and B activity.

We therefore examined normal sheep liver by cellulose acetate electrophoresis (Fig. 1A). This technique revealed two bands of HEX activity with equal mobility of human leukocyte and sheep liver HEX B but with slightly greater intensity and less mobility of the HEX A band compared to HEX A from human leukocytes. Similar analysis of HEX activity from the liver of the affected sheep disclosed the presence of only a single band corresponding to HEX B with absence of the HEX A component as found in human Tay-Sachs disease leukocytes (Fig. 1B). Also, as expected, the intensity of fluorescence of the Hex B band derived from leukocytes of a Tay-Sachs heterozygote was greater than that of the Hex A band in keeping with inversion of the normal ratio of Hex A>Hex B in carriers of Tay-Sachs disease (Fig. 1A). In contrast, in both Figs. 1A and B, the intensity of the more rapidly migrating Hex A band from normal sheep liver homogenate predominated over the Hex B band.

#### 3.2. Lipid analysis of affected sheep brain

Lipid-bound sialic acid in the control sheep brain was 394 µg/g tissue which is close to that reported in sheep brain by Wang et al. [37] (280–366 µg/g of tissue). The concentration of lipid-bound sialic acid in the tissue from the affected sheep was 931 µg/g, a nearly three-fold increase over the normal control. HPTLC of gangliosides from brain of



**Fig. 1.** Cellulose acetate electrophoresis of β-hexosaminidase components from human leukocytes and sheep liver homogenates. Each lane represents 3 µg of total protein. (A) Normal and Tay-Sachs heterozygote leukocytes and normal sheep liver. (B) Normal and Tay-Sachs disease leukocytes and normal and affected sheep liver.



the affected sheep (Fig. 2) demonstrated a marked accumulation of  $G_{M2}$  in the disease sheep brain, the apparent source of the increased lipid-bound sialic acid. Analysis of the lower phase glycolipid fraction from the brain of the affected sheep by HPTLC developed with orcinol staining revealed a prominent band with the same mobility as the asialo- $G_{M2}$  standard. This band was not visible on HPTLC of the lower phase glycolipid fraction isolated from the control sheep brain (data not shown).

### 3.3. Isolation of full-length cDNA of Jacob sheep HEXA gene

To determine the sequence of the *hexa* gene in Jacob sheep initially, first-strand cDNA synthesis from total normal Jacob sheep RNA was obtained using the Super-Script™ II Reverse Transcriptase (Invitrogen). For PCR amplification of the sheep cDNA, we turned to the cattle *hexa* gene, the normal full cDNA sequence (1806 bp) of which was known (NM\_001075164). We reasoned that cattle and sheep are extremely close evolutionary relatives belonging to the family Bovidae and share a common ancestor that lived probably no more than 20 million years ago. Further, studies showed that less than 3% of the protein-coding nucleotide positions were different indicating that the prospect for successfully using cross-species strategies would be high. Therefore, we designed primers on the basis of homologous regions between human and cattle *hexa* gene sequences for PCR amplification of DNA fragments which were then sequenced. The three overlapping cDNA fragments of sheep *hexa* gene were amplified, respectively, with three sets of primers with PCR. The three sets of primers were as follows:

F1: 5'-AGGTTTTCGCTGCTGCTGGCGG-3'  
 R1: 5'-GTACGCCATGACATCCAGAGTGCCA-3'  
 F2: 5'-CTAGCATCCTGGACACTCTGGATGTCA-3'  
 R2: 5'-TACCTCAAATGCCAGGGGCTCC-3'  
 F3: 5'-TCTGCCCCCTGGTACCTGAACC-3'  
 R3: 5'-AGGGGCACGTGGCAAGGGCT-3'

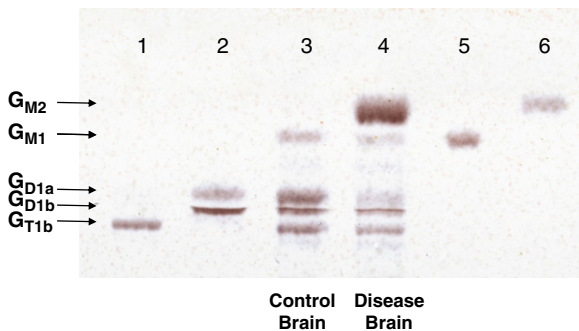
Then, a pair of internal primers (forward: 5'-CAGCGTTCGCTGGCGGCGGAC-3'; reverse: 5'-TCACAGTAGCCTACGCTGAGGGGCTGGG-3') were used to generate more specific cDNA. Then, 1770 bp of partial cDNA fragment, including the 3' encoding region but lacking the 5' encoding region, was generated by a two-stage nested PCR. The resulting sequence of the 1770 bp of partial cDNA fragment of the Jacob sheep *hexa* gene was found to be approximately 93% identical to the corresponding region of the cattle cDNA. Based on the sequence of the normal Jacob sheep *hexa* cDNA that we obtained, cDNA fragments for the 5' region of the Jacob sheep *hexa* cDNA were extended and

isolated from total Jacob sheep RNA. Two sets of nested primers for 5' extension (data not shown), as well as SMART™ RACE cDNA amplification kit and Advantage-GC cDNA PCR kit, were used for rapid amplification of G/C-rich cDNA ends. Approximately 500 bp of cDNA fragment of the 5' region was isolated and sequenced. Subsequently, the entire cDNA-coding region of the sheep *hexa* was isolated from total Jacob sheep RNA using a set of primers and Advantage-HF™ PCR kit. To further confirm the cDNA sequences of the 5' and 3' regions that we obtained, the 5' and 3' encoding regions of genomic DNA for the Jacob sheep *hexa* gene were also isolated and sequenced. The coding sequences obtained from the genomic DNA were identical with the cDNA sequence (data not shown).

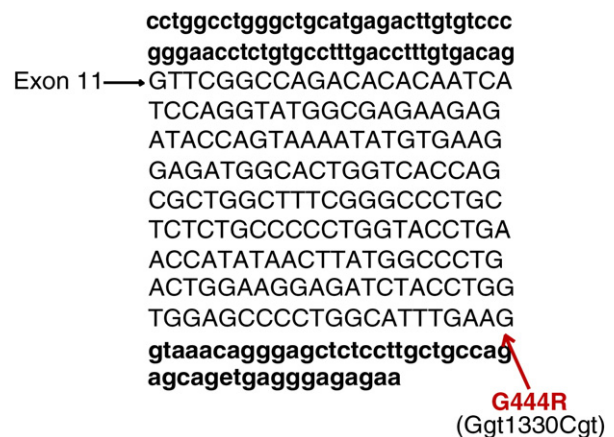
Our results showed that the full-length cDNA for the Jacob sheep *hexa* gene is a 1590-nucleotide sequence encoding a protein of 529 amino acids. It is 86% homologous with its human counterpart in nucleotide sequence and 89% in amino acid sequence with the same number of amino acids and also comprised of 14 exons as in the human *HEXA* gene. The deduced amino acid sequence includes three possible glycosylation sites (Asn-X-Thr or -Ser) located at positions 115, 157, and 295, as well as eight cysteine residues.

### 3.4. Mutation analysis of the *hexa* gene

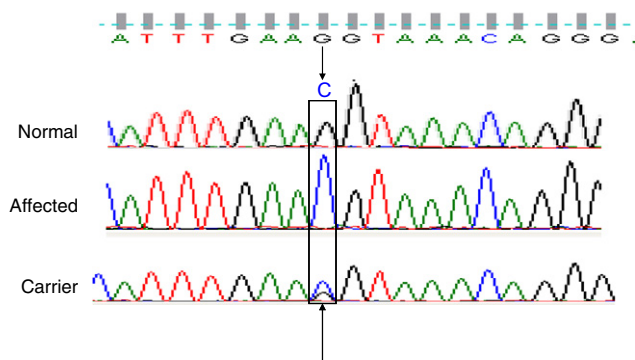
We next isolated and sequenced the full-length *hexa* cDNA from liver tissue of an affected Jacob sheep with Hex A deficiency. As compared with the full-length *hexa* cDNA of normal Jacob sheep, a shorter cDNA from the affected Jacob sheep (approximately 180 bp less than normal sheep cDNA) was amplified and sequenced. The sequencing result showed that exon 11 was skipped, which suggested either a possible mutation around a splice site or a deletion. A set of primers was then designed to amplify a genomic DNA fragment spanning the region from exon 10 to exon 12 by means of long PCR (Takara LA Taq™ polymerase; Clontech). An approximately 1.2-kb fragment of the genomic DNA was obtained and sequenced. The result demonstrated a homozygous recessive missense (G-to-C transition) mutation at nucleotide position 1330 ( $G^{1330} \rightarrow C$ ) which induces an amino acid change from Gly<sup>444</sup> to Arg (mutation G444R). This nucleotide transition is located on the last nucleotide of exon 11 within a conserved region of the *hexa* gene (Fig. 3) and subsequently resulted in skipping of exon 11. This mutation has not been reported in humans. Furthermore, this mutation was confirmed by analysis of genomic DNA of exon 11 from normal, heterozygous carriers, and affected Jacob sheep with TSD (Fig. 4). Also, the genomic sequence



**Fig. 2.** Thin-layer chromatography of sheep brain gangliosides. Following lipid extraction of brain tissue from a control animal (211 mg) and an affected sheep (180 mg), the Folch upper phase was brought to dryness and redissolved in 250  $\mu$ l C/M 2:1. Lane 3, 5  $\mu$ l of control brain upper phase extract; Lane 4, 5  $\mu$ l of disease brain upper phase extract; Lanes 1, 2, 5, and 6 contain 5  $\mu$ g each of individual ganglioside standards.



**Fig. 3.** Exon 11 of the sheep  $\beta$ -Hex showing flanking intronic sequence. Note that the mutation present in the affected sheep is in the last nucleotide of the exon resulting in skipping of the exon and a shorter PCR product for the mutant cDNA.



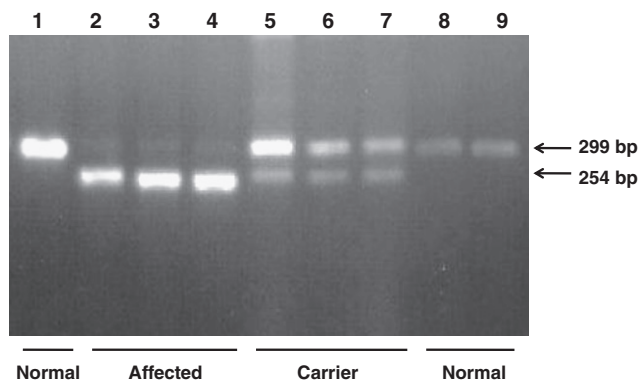
**Fig. 4.** Partial sequence of sheep  $\beta$ -Hex A genomic DNA sequence from liver of normal, affected, and carrier Jacob sheep indicating a G-to-C transition at nucleotide 1330 in heterozygotes and affected sheep.

information of exon 11 was found to be identical to the corresponding area of the cDNA sequence (data not shown).

The G444R mutation created a new restriction enzyme site for *Tail*. This permitted genotype analysis based on different band fragments observed following PCR amplification and restriction enzyme digestion of genomic DNA. The data shown in Fig. 5 confirmed that this mutation is recessively homozygous in affected Jacob sheep with TSD. With primers 5'-CTTGCATGAGACTGTGTCCC-3' and 5'-CTTCTCTCCCTCAGCTGCTCT-3', a 299-bp band is amplified (Fig. 5). After digestion with *Tail*, the affected HEX A-deficient Jacob sheep produces two fragments of 254 and 44 bp, respectively. Carriers produce three fragments of 299, 254, and 44 bp, whereas the normal DNA fragment of exon 11 is not digested by *Tail*, so that only one 299-bp band appears. Using DNA restriction site analysis for the G444R mutation, we then examined 104 genomic DNA samples from Jacob sheep in three geographically separate flocks. Each flock contained carriers with an overall incidence of 14.4% (range 12.5–18.5%).

### 3.5. Mutant gene expression

To confirm Hex A deficiency induced by the G444R mutation, we studied the *in vitro* expression of normal and mutant full-length cDNAs of the Jacob sheep and human *HEXA* genes in COS1 cells and human Hex A-deficient skin fibroblasts from TSD patients, respectively. Several considerations governed our use of two different cell

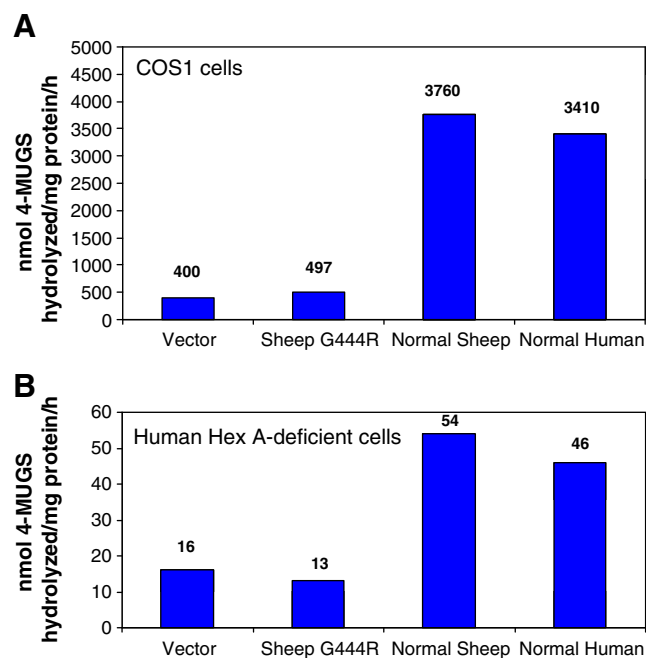


**Fig. 5.** Carrier identification of the the Jacob sheep *hexa* mutation by restriction enzyme analysis with *Tail*. Genomic DNA from affected sheep cut with *Tail* produces a single band of 254 bp, whereas two bands, 299 and 254 bp, respectively, are found in heterozygotes. *Tail* does not cut DNA from a homozygous normal animal so that only a single band of 299 bp is produced. The first lane contains a DNA molecular weight ladder.

lines to study mutant gene expression. 1) COS1 cell lines have been regularly utilized to study mutant gene expression of Hex A, providing a parameter with which to compare our study with other studies [38–43]. 2) The human Hex A-deficient fibroblast is a good model in which to investigate whether the Jacob sheep *hexa* gene can provide an  $\alpha$ -subunit product capable of generating activity of  $\beta$ -hexosaminidases including Hex A ( $\alpha\beta$ ) and Hex S ( $\alpha\alpha$ ).

Following introduction of normal Jacob sheep and human *HEXA* cDNAs into COS1 cells and deficient human fibroblasts (Fig. 6), the enzyme activities increased markedly as compared with that of the parental cells and those transfected with vector alone. Expression of the normal Jacob sheep  $\alpha$ -subunit in the human deficient fibroblasts suggested that it may serve the same biological role in the ganglioside degradation pathway as in the human. In contrast, the enzymatic activity remained unchanged and undetectable in the cells transfected with the mutant Jacob sheep *hexa* cDNA containing the G444R mutation. Meanwhile, introduction of the human mutant *HEXA* cDNA containing this counterpart mutation into COS1 cells and human HEX A-deficient fibroblasts (Fig. 6) also did not increase enzymatic activity. In total, the data indicated that the G444R mutation was the cause of the Hex A deficiency in affected Jacob sheep.

It is also notable that Hex A consists of two subunits,  $\alpha$  and  $\beta$  encoded by the *HEXA* and *HEXB* genes, respectively. TSD and its variants are caused by defects of the  $\alpha$ -subunit induced by mutations of the *HEXA* gene or very rarely by mutations of the  $G_{M2}$  activator gene, *GM2A*. Transfection of the normal Jacob sheep *hexa* or the normal human *HEXA* cDNAs into COS cells can provide an  $\alpha$ -subunit product. The increase in enzymatic activities might have resulted from Hex A ( $\alpha\beta$ ) or Hex A-like activity. The Hex A-like activity could represent either the alpha homodimer Hex S ( $\alpha\alpha$ ) or a cross-species chimeric heterodimer Hex A ( $\alpha\beta$ ). To further characterize the increased enzymatic activities as either Hex A activity ( $\alpha\beta$ ) or other Hex A-like activity, cellulose acetate electrophoresis was done and developed with the synthetic substrates 4-MUG and 4-MUGS, respectively. 4-MUG is used for assaying enzymatic activities of Hex isoenzymes, while 4-MUGS is used specifically for assay of  $\alpha$ -subunit



**Fig. 6.** Activity of Hex A in cells transfected with various *hexa* cDNAs expressed as nanomoles of 4-MUGS hydrolyzed per milligram of protein per hour. (A) COS1 cells. (B) Human Hex A-deficient fibroblasts.

activity of  $\beta$ -hexosaminidases including Hex A ( $\alpha\beta$ ) and Hex S ( $\alpha\alpha$ ). As shown in Fig. 7, the higher level of expression of  $\alpha$ -subunit activity of Hex in COS1 cells transfected with the normal Jacob sheep *hexa* cDNA resulted from the formation of Jacob sheep Hex S ( $\alpha\alpha$ ), which was also shown in a previous study of TSD in the American flamingo [23]. In addition, a certain baseline level of endogenous Hex A activity existed in COS1 cells transfected with vector alone as shown by 4-MUGS hydrolysis [43]. The level of endogenous Hex A activity in COS1 cells between this study and another report was similar [44].

#### 4. Discussion

Analysis of lysosomal enzyme activity revealed a marked deficiency of Hex A activity in the liver tissue from an affected Jacob sheep (<6% of control) as determined with 4-MUGS, an artificial substrate specific for Hex A. Hydrolysis of 4-MUGS by brain of an affected sheep was also reduced (~29% of controls) but to a lesser extent compared to brain tissue from two control sheep. It is possible that the amount and expression of mutant full-length *hexa* mRNA may vary in different tissues from the same animal depending upon its stability and rapidity of degradation in each tissue.

Histologic studies of brain from four affected sheep originating from a single flock were highly suggested of a ganglioside storage disease [26]. We therefore investigated the ganglioside composition and neutral glycolipid fraction of sheep brain confirming the presence of a significant increase in  $G_{M2}$ -ganglioside and asialo- $G_{M2}$ -ganglioside typical of both Tay-Sachs disease and Sandhoff disease. However, the absence of Hex A activity and retention of Hex B activity, best shown by cellulose acetate electrophoresis (Fig. 1), supports the diagnosis of Tay-Sachs disease and excludes Sandhoff disease.

The bulk of  $\beta$ -hexosaminidase activity in Jacob sheep is heat-labile. This suggests that, analogous to human Hex A and B, the major Hex component in Jacob sheep is in the hex A form. Cellulose acetate electrophoresis of normal sheep liver (Fig. 1) demonstrates two bands with the more intense band as in human hex A, migrating toward the cathode. Taken together, these data are consistent with Hex A as the dominant component of  $\beta$ -hexosaminidase in Jacob sheep.

Using primers derived from sequence of human *HEXA* cDNA, we were initially unable to clone the sheep *hexa* cDNA. However, we eventually obtained the full-length cDNA of Jacob sheep *hexa* using primers designed from the previously reported *hexa* cDNA sequence of cattle, a close evolutionary ancestor of sheep. The number of nucleotides and predicted number of amino acids are identical

to their human counterparts while the sequence of Jacob sheep cDNA is 86% homologous and of amino acids 89% homologous to the human. After we identified the normal full-length *hexa* cDNA sequence of Jacob sheep, we became aware of a report describing the *hexa* cDNA of Yunnan sheep from China [45]. A comparison of the Jacob sheep sequence with the Yunnan sheep indicates a difference of one amino acid in position no. 476 due to a single difference at nucleotide no. 1427.

The missense mutation we found in the *hexa* cDNA obtained from the tissue of the affected sheep, a G→C transition at the last nucleotide (no. 1330) in exon 11 causes a theoretical glycine to arginine substitution and induces skipping of exon 11 leading to loss of sheep Hex A activity. This was confirmed in both COS1 cells and human Hex A-deficient fibroblasts by comparing Hex expression after transfection with normal and mutated sheep cDNA. The major product after transfection with normal sheep cDNA was a  $\beta$ -hexosaminidase component that migrated further than Hex A and was visible with both 4-MUG and 4-MUGS. These are characteristics of Hex S, representing a dimer of  $\alpha$ -subunits ( $\alpha\alpha$ ). The appearance of Hex S as the sole enzyme product from transfection of normal sheep *hexa* cDNA is explainable by the production of sheep  $\alpha$ -subunits and the absence of sheep  $\beta$ -subunits with which they could dimerize to form Hex A. While chimeric heterodimerism with COS1 cell or human Hex  $\beta$ -subunits is theoretically possible, the evidence presented here does not support this alternative possibility.

The appearance of carriers of the G444R exon 11 mutation in three separate flocks of Jacob sheep suggest that this gene alteration may be widespread in Jacob sheep within North America. This possibility is being investigated through the cooperation of the Jacob sheep registries using blood spots for genotyping additional flocks. As the breed is rare and believed to have reached North America from England approximately 60 years ago, a founder effect would most likely explain the high incidence of this mutant gene and its current geographic dispersion.

The occurrence of gangliosidosis in Jacob sheep was first reported to two associations of Jacob sheep breeders in 2002 by two of the authors of this article (FH, JH), but the specific type of gangliosidosis remained unidentified. Annual reports of congenital defects in Jacob sheep provided by the Jacob Sheep Conservatory did include additional affected sheep and the USDA National Germplasm Program was made aware of a possible lysosomal problem in 2004. From conversations with several breeders of Jacob sheep, we have also learned of instances in which Jacob sheep have had to be euthanized

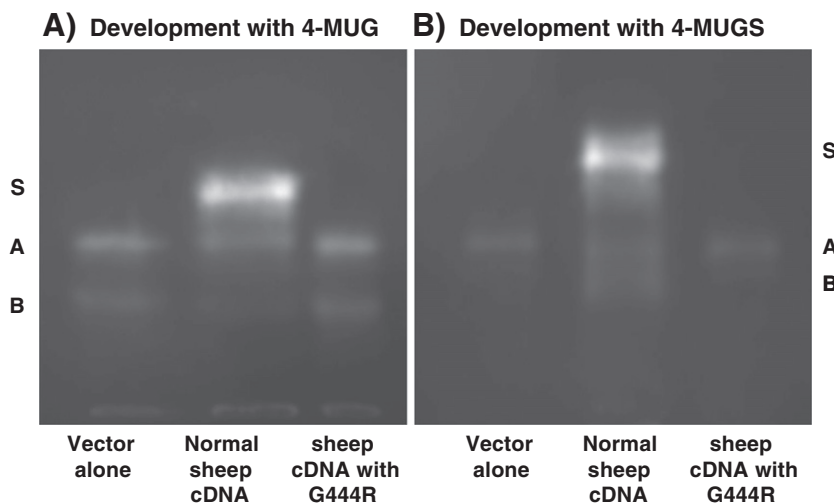


Fig. 7. Cellulose acetate electrophoresis of  $\beta$ -hexosaminidases produced by sheep cDNA transfected into COS1 cells (A) developed with 4-MUG and (B) developed with 4-MUGS. The major product of normal sheep *hexa* is the dimeric Hex S ( $\alpha\alpha$ ). Sheep *hexa* cDNA containing the G→C<sup>1330</sup> G444R mutation did not express Hex S.



because of progressive neurodegeneration. However, no previous attempts were apparently made to uncover the exact nature of the disorder.

The high incidence of carriers of this naturally occurring large animal model of human Tay-Sachs disease provides the first real opportunity to examine gene therapy for the Tay-Sachs variant of  $G_{M2}$ -gangliosidosis. The brain weight of a 4- to 6-month-old lamb is approximately 140 g or approximately one-quarter the size of an infant's brain (600 g) of the same age. Therefore, targeting, immunogenicity and toxicology studies in the sheep are much more likely to mirror the human experience than would be the case in a much smaller authentic Tay-Sachs disease rodent model were such a model actually available.

In summary, the clinical and biochemical features of the Jacob sheep model of TSD compares well with the human classical late-infantile form of this disorder and thus represents a possible new research tool for further study of the pathogenesis and treatment of TSD.

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