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DETERMINATION OF THE DEVELOPMENTAL PROFILES OF LYSOSOMAL ENZYME ACTIVITIES IN NORMAL GOATS AND CLONING AND SEQUENCING THE BOVINE β-MANNOSIDASE GENE PROMOTER

By

MEI ZHU

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ABSTRACT

DETERMINATION OF THE DEVELOPMENTAL PROFILES OF LYSOSOMAL ENZYME ACTIVITIES IN NORMAL GOATS AND CLONING AND SEQUENCING THE BOVINE β-MANNOSIDASE GENE PROMOTER

By

MEI ZHU

β-Mannosidosis, an autosomal recessive disorder of glycoprotein metabolism caused by a deficiency of β-mannosidase, is associated with early prenatal pathological changes, including cytoplasmic vacuolation in the nervous system and viscera. The first goal of this study was to investigate the developmental profiles of caprine β-mannosidase activities in various organs. Four lysosomal enzyme activities were assayed at different gestation stages. The results showed tissue-specific and enzyme-specific developmental patterns. In thyroid, β-mannosidase specific activities significantly increased during the second half of gestation and had the highest activity compared with other tissues, suggesting that there may be cell-specific transcription factors involved in the regulation of gene expression. The second goal of this study was to clone and sequence the promoter region of the bovine β-mannosidase gene. The 5'-end of bovine β-mannosidase cDNA (203bp) was used as a probe to screen the bovine genomic library by PCR. Further analysis of this promoter region by computer search showed the common characteristics of a housekeeping gene promoter: no TATA box, but highly GC rich with potential Sp1 binding sites. In order to understand the mechanism of the cell-specific gene expression of β-mannosidase, further characterization of this promoter region is needed.

To my parents, Yulan Chen and Xiucheng Zhu, and my sisters, Tong, Li and Ping for their love, support, and encouragement; also to my dear husband Hongwei and our lovely daughter Anqi for giving me strength and always be there for me.

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LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS

Abbreviations	Full Description
4-MU	4-methylumbelliferone
ASN	asparagine
AIRS	artificial introduction of restriction sites
bp	base pair
cDNA	complementary DNA
g	gram
GlcNAc	N-acetylglucosamine
hr	hour
min	minutes
ml	milliliter
s	second
µl	microliter
PCR	polymerasse chain reaction
BSA	bovine serum albumin
vol.	volume
Cfu	colony forming unit
dg	days of gestation

INTRODUCTION

INTRODUCTION

Lysosomal storage disease

Lysosomes are cytoplasmic organelles that contain many different acid hydrolases which are responsible for the degradation of a variety of macromolecules, such as proteins, lipids, complex carbohydrates, and nucleic acids (Berman, 1994). These hydrolases that function at acidic pH are referred to as lysosomal enzymes. The deficiency or dysfunction of any one of these enzymes leads to the accumulation of undigested substrate in the lysosomes, which progressively increases in size and number, and eventually leads to disruption of cellular function. The concept of "lysosomal storage diseases" was first introduced by Hers (Hers, 1965) when he described how genetically determined absence of α-glucosidase could lead to the fatal condition known as Pompe disease. Later on, this concept led to the discovery of several dozen lysosomal storage disorders. Most of them are caused by deficiency of a single lysosomal enzyme; some may be caused by the failure to synthesize, transport and process the lysosomal enzyme (Durand, 1987).

There are more than fifty lysosomal enzymes and all of them are glycoproteins. Lysosomal enzymes are synthesized as preproproteins on membrane-bound ribosomes attached to the rough endoplasmic reticulum. Then they undergo a series of posttranslational modifications involving protein and carbohydrate recognition signals that enable them to reach their final destination in lysosomes. Defects in any of these steps during the whole process of enzyme biosynthesis could lead to deficiency of the

specific enzyme activity, which would lead to the specific accumulation of undigested substrates in the organelles of cells, called lysosomal storage disease.

Lysosomal enzymes are relatively stable and can be assayed by different methods. Some studies have shown certain lysosomal enzyme activities that varied with ages and the developmental periods of the animals (Verity et al., 1968; Shailubhai et al., 1990; Verdugo, M., and Ray, J., 1997). Studies also have shown that lysosomal enzyme activities in normal animals exhibit species variation and tissue-specific expression (Freysz et al., 1979; Abe et al., 1979; Reiner and Horowitz, 1988; Aronson et al., 1989). A deficiency of one lysosomal enzyme activity usually causes an increase in several other lysosomal enzyme activities (Healy et al., 1981; Jones et al., 1981; Pearce et al., 1987; Jolly et al., 1990; Embury et al., 1985; and Vandevelde et al., 1982).

There are two categories of oligosaccharides: (1) the O-linked oligosaccharides, in which oligosaccharides are linked O-glycosidically to serine or threonine; (2) the N-linked oligosaccharides, in which oligosaccharides are linked N-glycosidically to asparagine(ASN). The oligosaccharides are degraded in the lysosome by (1) a group of exoglycosidases acting at the non-reducing termini, (2) endo-β-N-acetylglucosaminidase, and (3) aspartylglucosaminidase. In the lysosome the N-linked oligosaccharides of glycoproteins are sequentially catabolized from the non-reducing end by at least six different exoglycosidases. The lysosomal enzymes involved in cleaving steps include neuraminidase (sialidase), β-galactosidase, β-N-acetylhexosaminidase, α-mannosidase, β-mannosidase, and α-fucosidase. The stepwise removal of sugars is illustrated in Figure 1.

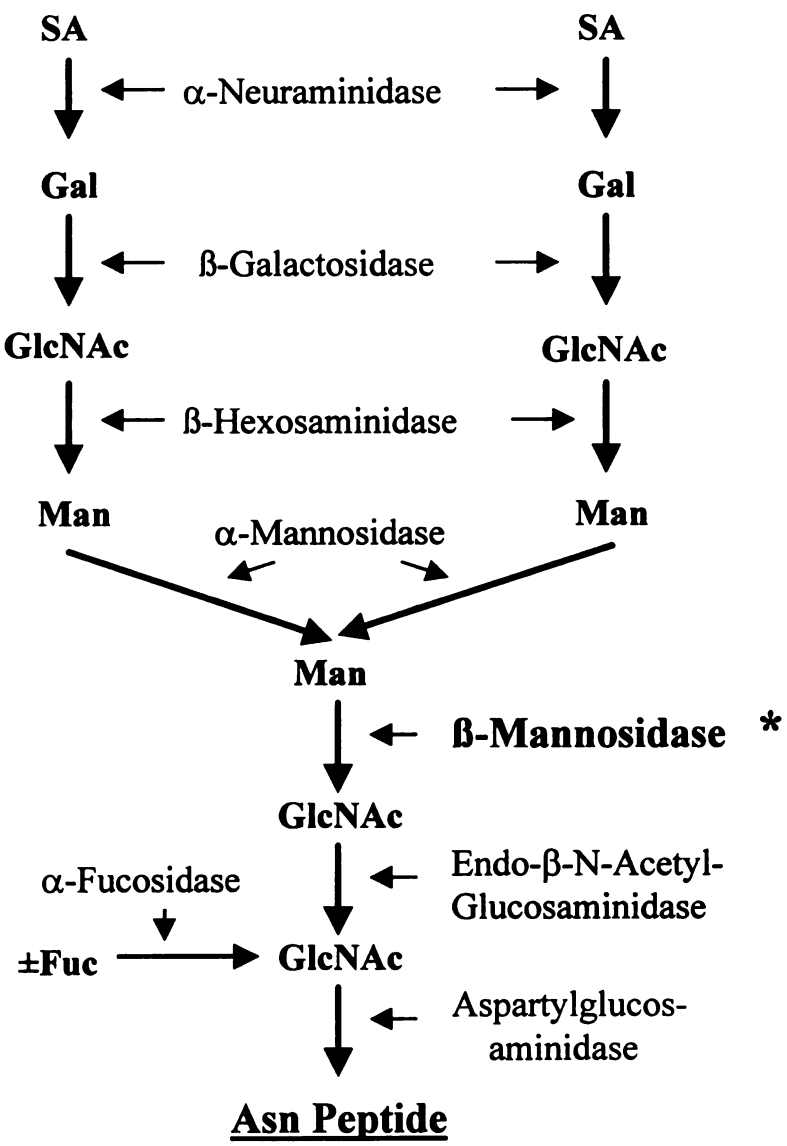

Figure 1. The degradative pathway for the oligosaccharide portion of Asn-linked glycoproteins.

Several animal models have been employed in studying human lysosomal storage diseases, including cat, dog, cow, goat, sheep, and mouse. Animal models play a central role in unraveling the molecular pathology of specific lysosomal enzyme deficiencies, and also are especially suited for testing new therapeutic approaches that are either impractical or difficult to conduct in humans. In the study of caprine β-mannosidosis, Jones and colleagues (Jones and Kennedy, 1993; Jones et al., 1993) found that early intervention is effective in altering the phenotype. For example, a caprine β-mannosidosis genotype, with a much milder disease expression, occurred in a chimera resulting from prenatal transfusion of hematopoietic stem cells from an unaffected to an affected sibling. This with other examples (Walkley et al., 1994; Snyder et al., 1995) suggested that early supply of normal cells to organs, including brain, may be a good way to supply missing enzyme. Other strategies include enzyme replacement, bone marrow transplantation, and gene therapy. The discovery of lysosomal storage disease was accompanied by the suggestion that this class of disorders could be treated by administration of exogenous enzymes, which might find their way to lysosomes by the process of endocytosis. One major problem of this treatment is the minute amounts of enzymes that can be administered. The ideal treatment for inherited metabolic disorders would be gene therapy. In principle such treatment would entail not only insertion of DNA containing the normal gene into the defective host genome but also appropriate management of its expression in host tissues; and this strategy may only be tested first in animal models.

The molecular approach for studying lysosomal enzymes involved in lysosomal storage disease has been successful in the past decade, especially with the rapid

development of recombinant DNA technologies. Over two dozen complementary DNAs encoding lysosomal enzymes have been cloned and characterized, and most of the promoter regions have also been analyzed. Lysosomal enzyme genes have been thought to be housekeeping genes. Typical housekeeping gene promoters are characterized by no TATA box and /or CAAT box, and high GC content with potential Sp1 binding sites. Most promoters of lysosomal enzymes have the typical characteristics expected for a housekeeping gene, and these genes include: human acid phosphatase (Geier et al., 1989), human α-glucosidase (Hoefsloot et al., 1990), human arylsulfatase A (Kreysing et al.,1990), human α-N-acetylgalactosaminidase (Wang et al., 1990), canine IDUA (Menon, 1992), human α-L-iduronidase (Moskowitz et al., 1992), human α and β-subunit of β-hexosaminisidase (Neote et al., 1988; Norflus et al., 1996), mouse α-galactosidase A (Ohshima et al., 1995), human α-mannosidase (Riise et al., 1997), and human galactocerebrosidase (Sakai et al., 1998). However, some lysosomal enzyme gene promoters do contain TATA box and potential Sp1 binding sites, including genes for human acid β-glucosidase (Doll et al., 1994), α-subunit of β-hexosaminidase (Proia et al.1987), cathepsin D (Cavailles et al., 1991,1993), β-subunit of murine β-hexosaminidase (Yamanaka et al., 1994), and murine α-D-mannosidase (Stinchi et al., 1998). The gene encoding glucocerebrosidase has a TATA box, no Sp1 binding sites, and causes differential expression of a reporter gene in different cell types, similar to the expression level of endogenous glucocerebrosidase in the same cells (Reiner et al., 1988). All these indicated that the genes encoded for lysosomal enzymes are differentially regulated by different mechanisms.

β-Mannosidosis

β-Mannosidosis is an autosomal recessive inherited disorder (Fisher et al., 1986) of glycoprotein catabolism, which is caused by deficiency of β-mannosidase (EC 3.2.1.25). This disease was first decribed in Nubian goats (Jones and Dawson, 1981; Healy et al., 1981; Jones and Laine, 1981) and later on was also found in humans (Cooper et al., 1988; Wenger et al., 1986; Dorland et al., 1988 Kleijer et al., 1990), and cattle (Jolly, et al., 1990; Abbitt et al., 1991; and Patterson et al., 1991). The disease in all species is characterized by decreased β-mannosidase activity, but is phenotypically variable.

Affected goats and cattle have very similar clinical features which include inability to stand, facial dysmorphism (dome-shaped skulls, small palpebral fissures, depressed nasal bridge, and elongated, narrow muzzle), intention tremors, carpal flexion contractures, and pastern joint hyperextension (Jones et al., 1983; and Abbitt et al., 1991). Affected animals usually die in the neonatal period if intensive care is not provided. Gross pathological characteristics include ventricular dilatation with a marked paucity of myelin in cerebral hemispheres, cerebellum, and brain stem. Microscopic examination reveals ubiquitous cytoplasmic vacuolation and myelin deficiency in the central nervous system but not in peripheral nerves (Jones et al., 1983; Lovell and Jones, 1983; and Patterson et al, 1991). Affected goats and calves are hypothyroid, possibly contributing to the central nervous system hypomyelination (Boyer et al., 1990; Lovell et al., 1991). In contrast with the ruminant β-mannosidosis, the human cases have a milder and more heterogeneous clinical expression. The most severe cases are associated with mental retardation, developmental delay and dysmorphology, and hearing loss (Wenger et al.,

1986; Kleijer et al.1990; and Cooper et al., 1991). The reason for the difference between ruminants and humans is not known. However it was found that the accumulated substrates were different in these two species. In both affected goats and calves the predominant accumulated substrates are the trisaccharide Manβ1-4GlcNAcβ1-4GlcNAc with lesser amounts of the disaccharide Manβ1-4GlcNAc (Jones et al., 1981; Jones et al., 1992; Matsuura et al., 1981; and Cavanagh et al., 1982), whereas in humans the major accumulated substrates are disaccharide (Van Pelt et al., 1990; Cooper et al., 1988).

β-Mannosidase catalyzes the penultimate step in N-linked oligosaccharide catabolism (Figure 1), cleaving the single β-linked mannose residue. β-mannosidase activity has been measured in various tissues and body fluids, such as brain, thyroid, kidney, liver, spleen, urine, and plasma (Cavanagh et al., 1982; Bernard et al., 1986; Pearce et al., 1987; Jones et al., 1984). In newborn goats, the activity of β-mannosidase is highest in thyroid, with decreasing activity in the order: kidney, liver, muscle, and brain (Pearce et al., 1987; Lovell et al., 1994). A regional difference of β-mannosidase specific activity in the central nervous system has also been observed by Lovell et al. (1994): β-mannosidase as well as α-mannosidase, β-hexosaminidase, α-fucosidase, and β-glucuronidase all have higher specific activities in white matter than in gray matter in normal goats, which suggested that a high level of enzyme activities are needed in white matter, and may relate to turnover of glycoproteins in myelin or axonal membranes. The purification of β-mannosidase from caprine and bovine kidney (Sopher et al., 1992; 1993) by using monoclonal and polyclonal antibodies permitted successful cloning and sequencing of bovine and caprine β-mannosidase cDNAs (Chen et al., 1995; Leipprandt

et al., 1996). Human β-mannosidase cDNA has also been characterized in this laboratory by Alkhayat et al. (1998).

Current research

This research was designed to investigate the developmental pattern of β-mannosidase activity during gestation in various normal goat tissues, which may provide baseline information for testing new prenatal therapies in goats, and provide information related to pathogenesis of lesions. Two other lysosomal enzymes, α-mannosidase (EC 3.2.1.24) and total β-hexosaminidase (EC 3.2.1.52), which are also involved in the pathway of N-linked glycoprotein catabolism, were examined. Acid phosphatase (EC 3.1.3.2), which is not in this pathway but usually used as a marker, was also studied. In order to understand the regulatory mechanism of expression of normal β-mannosidase which may contribute to the cell-specific and developmental enzyme activities, we further characterized this gene by cloning and sequencing the 5'-flanking region of the bovine β-mannosidase gene, and then compared it with the promoter regions of other lysosomal enzymes.

CHAPTER 1

DETERMINATIOIN OF THE DEVELOPMENTAL PROFILES OF LYSOSOMAL ENZYME ACTIVITIES IN NORMAL GOATS

INTRODUCTION

β-Mannosidosis, associated with deficiency of β-mannosidase, is an autosomal recessive inherited lysosomal storage disease involving N-linked glycoprotein catabolism. It was first described in Nubian goats (Jones and Dawson, 1981; Healy et al., 1981; Jones and Laine, 1981), and has also been found in humans (Cooper et al., 1988; Wenger et al., 1986; Dorland et al., 1988; Kleijer et al.,1990), and cattle (Jolly et al., 1990; Patterson et al.,1991). The caprine model has been used to study this disease for approximately 18 years, including investigation of clinical features and pathological changes (Jones et al., 1983; Lovell et al., 1983; and Lovell et al., 1997), the molecular level studies (Leipprandt et al., 1996), and prenatal therapy. In order to understand the pathogenetic mechanisms and provide therapy for this disease, further studies of the nature of β-mannosidase activity and the mechanism involving regulation of its expression is needed. The current study was originally designed to determine the developmental profiles of β-mannosidase specific activities in selected tissues of goats, which may provide information for the optimal time for therapy. Three other lysosomal enzymes, α-mannosidase, total β-hexosaminidase and acid phosphatase, were also assayed for comparison.

MATERIALS AND METHODS

Materials

Most artificial substrates and protease inhibitors were purchased from Sigma Chemical Company (St. Louis, MO, USA), including 4-methylumbelliferone (4-MU), free acid (M 1381) as standard curve; 4-methylumbelliferyl-α-D-mannopyranoside (M 3657); 4-methylumbelliferyl-β-D-mannopyranoside (M 0905); 4-methylumbelliferyl phosphate (M 8883); and Leupeptin and Pepstatin A. 4-Methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside (# 474502) was purchased from Calbiochem (La Jolla, CA). Ampli Taq® DNA polymerase was purchased from Perkin Elmer (Branchburg, New Jersey, USA).

DNA test for selecting normal tissues

Animals used in this study included 18 normal goats and 1 β-mannosidosis carrier goat, which were identified by a PCR based assay for artificial introduction of restriction sites (AIRS) (Cotton, 1993). This method was developed by creating modified PCR primers that flank the mutation site. The antisense primer, which elongates from a position immediately adjacent to the mutation site, had two bases that were mismatched with the template such that the resulting amplicons from normal genes had Ban I restriction sites (recognition sequence:GGPyPuCC). The amplicons produced from mutant genes did not have the site. The sense primer also had two mismatched bases to introduce a different Ban I site in both the normal and the mutant amplicons, to act as an internal control for monitoring the completeness of the subsequent restriction cutting. In this study, most of the animal DNAs were extracted from liver (relatively high yield of DNA), except two from kidney, using a Puregene Gentra Kit. The DNA preparation was

diluted to 1:100, and then 1 μl was used as template for PCR. The total reaction volume was 20 μl, and the primers used for PCR were MJ179 (5'ACGTCCGGTGCCTGAAATCT 3') and MJ180 (5'AGCCGGGCTTTGTATGGTAC 3'). Following PCR, the amplicons were cut by restriction enzyme Ban I at 37°C for 1 hr, and then analyzed by 4% agarose gel electrophoresis (Figure 2).

Tissue Extraction

Animals selected from goat research herds at MSU ranged from 65 days of gestation to 3 days of age. After euthanasia tissue samples were stored at –80°C. Tissues were removed from the freezer individually and weighed. After weighing, the tissue was minced using a razor blade and suspended in extraction buffer giving a final concentration of 0.2 g/ml. Extraction buffer (pH 5.5) contained 0.01M citrate, 0.05M NaCl, 1mM $MnCl_2$, 1 mM $CaCl_2$, 0.02% NaN_3, 10% glycerol, with final concentration 0.5mg/l of both leupeptin and pepstatin A (protease inhibitors). Tissues were disrupted by sonication (Micro Ultrasonic Cell Disrupter, Model 50-Watt) for 15-60 s at setting output control 40; the sonication time varied between 15-60 s depends on different tissues: the brain needed a shorter time, while the thyroid needed a longer time. A 10 s period of sonication was followed by a 20 s cooling time. Sonication was continued until a homogeneous solution was attained, or a total of 60 s. Samples were kept on ice during the processing. The tissue solutions were centrifuged for 10 min at maximum speed (Micro Centrifuge, Model 235c) at 4°C; and the supernatants were removed and stored at –80°C.

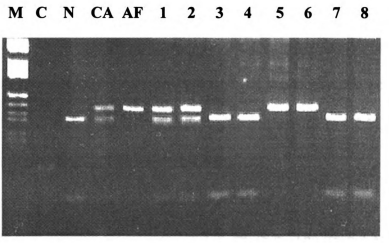

Figure 2. DNA test for normal tissue selection. (4 % agarose gel). M: Marker V, C: negative control (H_2O), N: normal animal control, CA: carrier animal control, AF: affected animal control. Samples were run in duplicate. Lane 1,2 are from a carrier; lane 3,4 and 7,8 are from two normal animals; lane 5,6 are from an affected animal.

Substrate Preparation

4-MU substrates were first dissolved in 200-400 µl of dimethyl sulfoxide (DMSO), heated for 1 min at 50-55°C and then brought to volume in the appropriate buffer in a volumetric flask. The substrate for β-mannosidase was 2 mM 4-methylumbelliferyl-β-D-mannopyranoside in 24 mM citrate-51 mM phosphate buffer, pH 5.0, with BSA of final concentration 0.2 mg/ml. The substrate for α-mannosidase was 2 mM 4-methylumbelliferyl-α-D-mannopyranoside in 31 mM citrate-37 mM phosphate buffer, pH 4.0. The substrate for acid phosphatase was 2 mM 4-methylumbelliferyl phosphate in 0.1 M sodium acetate buffer, pH 4.8. The substrate for β-hexosaminidase was 4 mM 4-methylumbelliferyl-2-acetamido-2-deoxyl-β-D-glucopyranoside in 0.1 M citrate buffer, pH 4.4. All the substrate solutions were stored at –20°C.

Standard Curve

4-MU free acid is stable for 30 days once in solution. The fresh 4-MU free acid was made monthly and each time the standard curve was checked for consistency. 4-MU free acid (2 mM) was prepared directly in methanol and stored in a dark bottle at 4°C. Table 1 shows the serial dilutions. In this study, we used B→H dilution solution as standard curve.

Enzyme Assay

In order to make the fluorimeter reading within range of the standard curve, some samples needed appropriate dilution (Table 2). The dilution buffer contained 0.2 mg/ml BSA in extraction buffer (pH 5.5). The fluorescence of the liberated 4-MU was measured by Luminescence Spectrometer (Model LS 50B) using 4-MU method program.

Table 1. 4-MU Dilutions for Standard Curve.

Tube (#)	Citrate buffer (µl)	4-MU volume (µl)	Final [4-MU] (mol)	4-MU nmol/20µl dilution solutions
AA	800	200 "stock"	4×10^{-4}	8
A	500	500 "AA"	2×10^{-4}	4
B	800	200 "A"	4×10^{-5}	0.8
C	500	500 "B"	2×10^{-5}	0.4
D	800	200 "C"	4×10^{-6}	0.08
E	500	500 "D"	2×10^{-6}	0.04
F	800	200 "E"	4×10^{-7}	0.008
G	500	500 "F"	2×10^{-7}	0.004
H	800	200 "G"	4×10^{-8}	0.0008

4-MU stock concentration is 2×10^{-3} M.

Table 2. Tissue Dilutions for Enzyme Assay.

Tissue	β-mannosidase	α-mannosidase	β-hexosaminidase	acid phosphatase
Thyroid	1:20	1:20	1:100	1:100
Kidney	1:5	1:20	1:100	1:50
Spleen	1:5	1:5	1:50	1:50
Epididymis	none	none	1:10	1:20
Testis	1:5	1:5	1:50	1:20
White matter*	none	none	1:5	1:20
Gray matter*	none	none	1:5	1:20
Spinal cord	none	none	1:10	1:50
Plasma	none	1:10	none	1:10

* - Cerebral hemisphere white matter and gray matter. Tissue extract was diluted in series in dilution buffer: extraction buffer (pH 5.5) with BSA (0.2 mg/ml).

The samples were loaded in a 96-well plate using a multichannel pipetter. Each tissue extract and standard curve was run in duplicate. For the standard curve, 20 μl of 4-MU standard dilutions (B→H) was added to 10 μl citrate-phosphate buffer (pH 5.0); for the unknown tissue extracts, 20 μl of the substrate was added to 10 μl of the tissue extracts, then incubated at 37°C for 5 min for α-mannosidase, β-hexosaminidase, acid phosphatase, and 30 min for β-mannosidase. Following incubation, 170 μl 0.1M glycine stop buffer (pH ≥ 10.8) was added to stop the reaction. Substrate blank and plasma controls were used during the assay. The substrate blank control was used for the free fluorescence from the artificial substrate, and the value was subtracted from the fluorimeter reading of samples when doing the calculation for specific activity. A plasma control was used for the consistency of the assay system. Plasma samples were prepared from a normal goat, and stored at –80°C in aliquots. If the plasma values were out of a specified range (10% of difference), then the enzyme assay was repeated until the plasma control values were consistent.

Protein Assay

Protein concentrations were determined by using bicinchoninic acid (BCA) and bovine serum albumin as a standard (Smith et al., 1985). A BCA Protein Assay Kit was purchased from Pierce company. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu^{+1}) using a unique reagent containing bicinchoninic acid. Tissue extracts were run in duplicate without dilution. The standard curve contains a serial dilution (0,10,25,50,100, 150 μg) of BSA. 0.5 μl tissue extracts were added to 9.5 μl sterile water, then 2 ml BCA working buffer

was added to both standard and unknown samples, and the whole reaction was incubated at 37°C for 30 min. The sample absorbances were read by Beckman DU 64 spectrophotometer with absorbance at wavelength 562 nm. All samples had to be read in 10 min, otherwise the results would not be consistent due to the color developing.

Statistical analysis

Comparisons between groups were performed using the Mann-Whitney nonparametric test (Conover, 1971).

RESULTS

The prenatal profiles of α-mannosidase, β-mannosidase, total β-hexosaminidase, and acid phosphatase specific activities (nmol /hr per mg of protein) were determined in selected normal goat tissues. Our results showed tissue-specific and enzyme-specific developmental patterns. The data are summarized in Tables 3-6 and the developmental patterns are shown in Figures 3-6. One of the significant findings in this study is the dramatic increase of β-mannosidase activity in thyroid during development. In newborn goats the highest activity for β-mannosidase in thyroid is consistent with previous reports (Pearce et al., 1987 et al., Lovell et al., 1994). The developmental patterns for different enzymes are not the same (Figures 3-6). For instance, in thyroid, β-mannosidase activity significantly ($P< 0.005$) increased from 85 dg group to 113-120 dg group, and from 113-120 dg group to newborn, but a significant increase of α-mannosidase activity was observed only between 85 dg group and 113-120 dg group ($P< 0.005$), there were no significant changes between 113-120 dg and newborn group. No statistical differences

Table 3. β-Mannosidase Specific Activities In Selected Goat Tissues.

	Enzyme specific activities (nmol 4-MU/hr per mg of protein)						
Age	**Thyroid**	**Kidney**	**Spleen**	**Epididymis**	**Testis**	**Brain**	**Spinal cord**
65-69 dg	**ND**	**11.3** (3) [3.76] {8.27-10.1}	**ND**	**ND**	**ND**	**1.56** (2) {1.46, 1.45}	**ND**
85 dg	**17.3** (4) [4.39] {14.3-23.8}	**9.55** (5) [1.08] {8.11-11.0}	**3.70** (5) [0.35] {3.31-4.22}	**6.48** (3) [0.42] {6.13-6.95}	**10.4** (3) [0.77] {9.54-11.0}	**1.35** (2) {1.13, 1.57}	**2.45** (1)
113-120 dg	**36.2** (7) [11.3] {23.9-59.1}	**17.4** (5) [1.48] {15.9-19.2}	**5.41** (4) [0.47] {5.04-6.09}	**6.70** (2) {6.60, 7.39}	**16.7** (2) {14.0, 19.4}	**1.68** (5) [0.47] {1.22-2.78}	**4.53** (5) [1.21] {3.08-6.09}
Newborn	**54.4** (4) [9.85] {43.6-67.4}	**7.64** (5) [1.06] {6.38-8.94}	**4.29** (4) [1.10] {3.27-5.82}	**5.60** (2) {3.80, 7.40}	**14.0** (2) {11.0, 16.9}	**1.88** (4) [0.80] {1.01-3.19}	**2.79** (4) [1.01] {1.80-2.63}

dg – days of gestation. ND – not determined. Values represent mean (in bold) ± [SD]. The number of animals studied is shown in ( ). The range of values for each age group is listed in { }.

Table 4. α-Mannosidase Specific Activities In Selected Goat Tissues.

Age	Enzyme specific activities (nmol 4-MU/hr per mg of protein)						
	Thyroid	**Kidney**	**Spleen**	**Epididymis**	**Testis**	**Brain**	**Spinal cord**
65-69 dg	**ND**	**235.5** (3) [56.94] {193.9-300.4}	**ND**	**ND**	**ND**	**27.16** (2) {22.83, 31.49}	**ND**
85 dg	**170.3** (4) [44.28] {124.1-230.1}	**320.0** (5) [78.91] {223.6-431.2}	**20.81** (5) [6.21] {13.23-30.27}	**53.17** (3) [15.69] {36.02-66.81}	**109.2** (3) [21.49] {84.54-124.0}	**21.57** (2) {15.86, 27.28}	**16.43** (1)
113-120 dg	**301.7** (7) [94.98] {191.0-456.9}	**294.4** (5) [27.05] {267.2-331.7}	**19.25** (4) [2.22] {17.56-22.45}	**45.64** (2) {40.48, 50.80}	**111.0** (2) {108.3, 113.7}	**28.94** (5) [3.37] {23.39-34.87}	**59.59** (5) [10.16] {45.09-70.46}
Newborn	**367.8** (4) [56.34] {321.1-449.3}	**323.6** (6) [113.0] {213.4-529.4}	**22.42** (5) [8.78] {17.13-37.70}	**27.12** (3) [4.70] {24.11-32.53}	**70.41** (2) {67.27, 73.53}	**26.39** (5) [11.7] {12.54-37.79}	**36.68** (4) [16.25] {18.18-57.33}

dg – days of gestation. ND – not determined. Values represent mean (in bold) ± [SD]. The number of animals studied is shown in ( ). The range of values for each age group is listed in { }.

Table 5. Total β-Hexosaminidase Specific Activities In Selected Goat Tissues.

	Enzyme specific activities (nmol 4-MU/hr per mg of protein)						
Age	**Thyroid**	**Kidney**	**Spleen**	**Epididymis**	**Testis**	**Brain**	**Spinal cord**
65-69 dg	**ND**	**1692** (3) [607.1] {1161-2354}	**ND**	**ND**	**ND**	**215.5** (2) {191.0, 240.0}	**ND**
85 dg	**1157** (4) [331.4] {712.2-1449}	**2044** (5) [395.0] {1719-2712}	**422.6** (5) [113.6] {323.5-603.0}	**928.0** (3) [380.4] {517.3-1268}	**1401** (3) [244.4] {1155-1644}	**157.7** (2) {130.7, 184.7}	**333.2** (1)
113-120 dg	**1570** (7) [478.5] {806.8-2095}	**1994** (5) [116.4] {1869-2124}	**542.8** (4) [94.23] {487.6-683.8}	**490.4** (2) {340.4, 640.5}	**1275** (2) {1047, 1475}	**158.9** (5) [17.05] {127.9-189.8}	**380.9** (5) [144.7] {263.2-623.8}
Newborn	**1735** (4) [551.2] {1335-2545}	**1871** (6) [529.0] {1389-2888}	**774.6** (5) [276.6] {442.5-1144}	**343.5** (3) [106.4] {258.0-462.7}	**878.5** (2) {699.0, 1058}	**163.3** (5) [38.49] {126.6-231.2}	**365.8** (4) [191.1] {205.9-613.0}

dg – days of gestation. ND – not determined. Values represent mean (in bold) ± [SD]. The number of animals studied is shown in ( ). The range of values for each age group is listed in { }.

Table 6. Acid Phosphatase Specific Activities In Selected Goat Tissues.

	Enzyme specific activities (nmol 4-MU/hr per mg of protein)						
Age	**Thyroid**	**Kidney**	**Spleen**	**Epididymis**	**Testis**	**Brain**	**Spinal cord**
65-69 dg	**ND**	**1480 (3)** [336.0] {1030-1673}	**ND**	**ND**	**ND**	**665.8 (2)** {623.9, 707.6}	**ND**
85 dg	**873.1 (4)** [141.5] {664.1-973.0}	**983.8 (5)** [95.96] {831.1-1093}	**721.9 (5)** [76.15] {616.2-798.2}	**750.5 (3)** [209.7] {585.9-986.5}	**800.2 (3)** [124.2] {718.9-943.2}	**604.4 (2)** {511.8-696.9}	**693.4 (1)**
113-120 dg	**1140 (7)** [470.5] {873.4-2189}	**1287 (5)** [296.7] {930.7-1722}	**650.6 (4)** [61.51] {583.9-709.4}	**830.9 (2)** {652.1, 1010}	**651.4 (2)** {623.5, 679.3}	**493.6 (5)** [85.97] {359.4-637.3}	**820.0 (5)** [78.37] {726.9-935.0}
newborn	**993.3 (4)** [168.8] {785.7-1149}	**1469 (6)** [219.6] {1163-1807}	**809.4 (5)** [139.5] {608.7-897.6}	**679.4 (3)** [65.01] {609.5-738.0}	**496.7 (2)** {433.9, 559.4}	**467.5 (5)** [76.78] {364.9-547.2}	**673.9 (4)** [162.1] {493.0-885.2}

dg – days of gestation. ND – not determined. Values represent mean (in bold) ± [SD]. The number of animals studied is shown in ( ). The range of values for each age group is listed in { }.

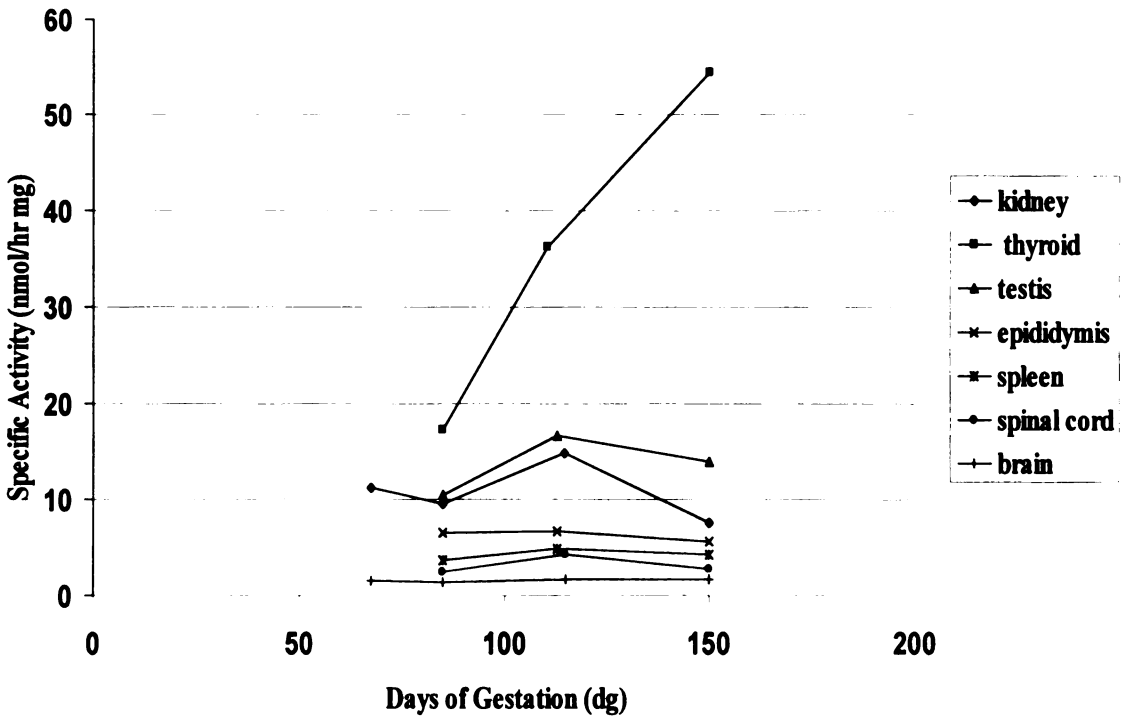

Figure 3. Developmental pattern of β-mannosidase specific activity in selected goat tissues. Each point represents the mean of values of a group (see corresponding table for n and SD for each group). There were significant differences ($P<0.005$) in thyroid and kidney respectively between 85 dg group and 113-120 dg group, and between 113-120 dg and newborn group. No significant differences for other tissues were observed during development.

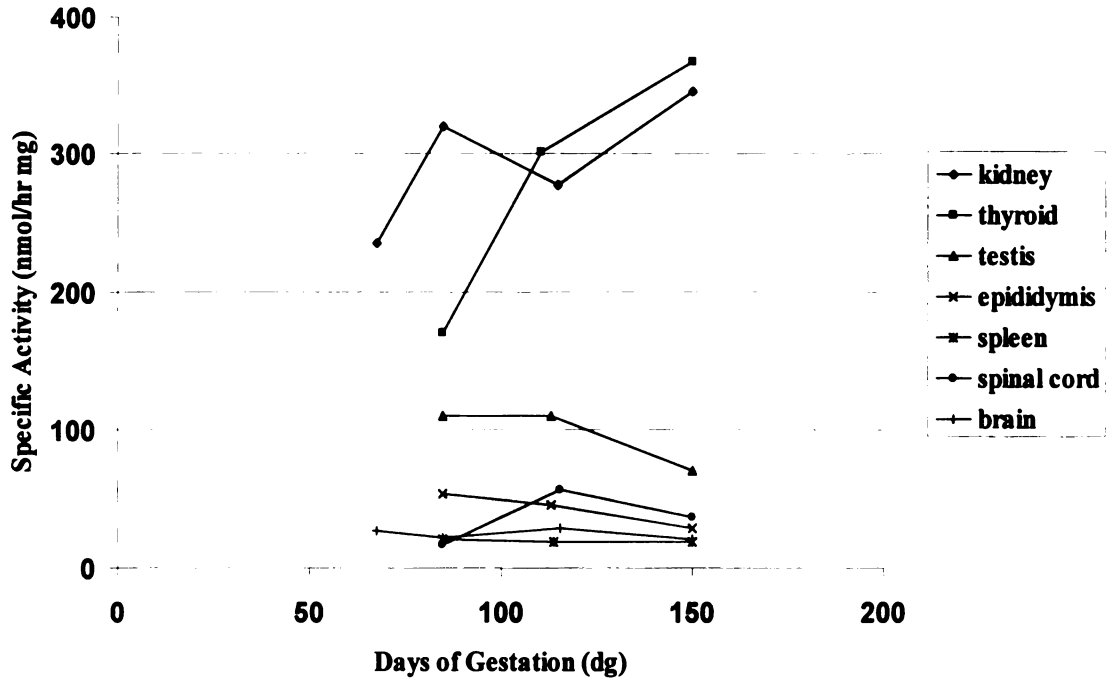

Figure 4. Developmental pattern of α-mannosidase specific activity in selected goat tissues. Each point represents the mean of values of a group (see corresponding table for n and SD for each group). There was significant difference between 85 dg and 113-120 dg group ($P<0.005$) in thyroid, and significant difference between 113-120 dg and newborn group ($P<0.05$) in testis. No significant differences for other tissues were observed during development.

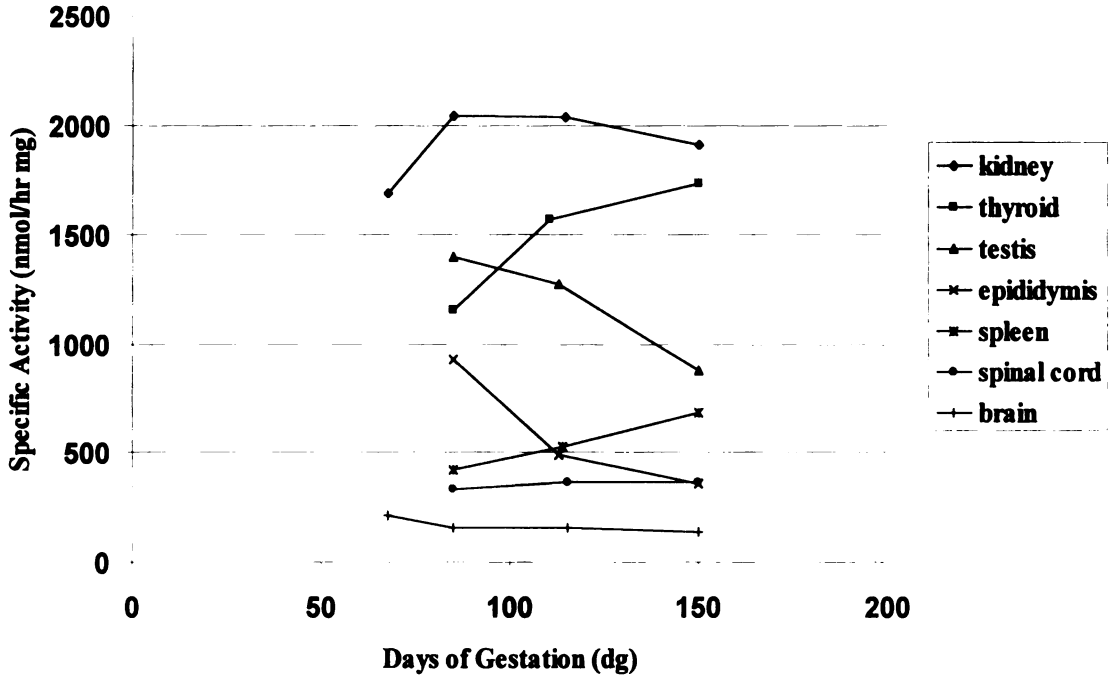

Figure 5. Developmental pattern of β-hexosaminidase specific activity in selected goat tissues. Each point represents the mean of values of a group (see corresponding table for n and SD for each group). No significant differences ($P<0.05$ or 0.01) were observed in all tissues during development.

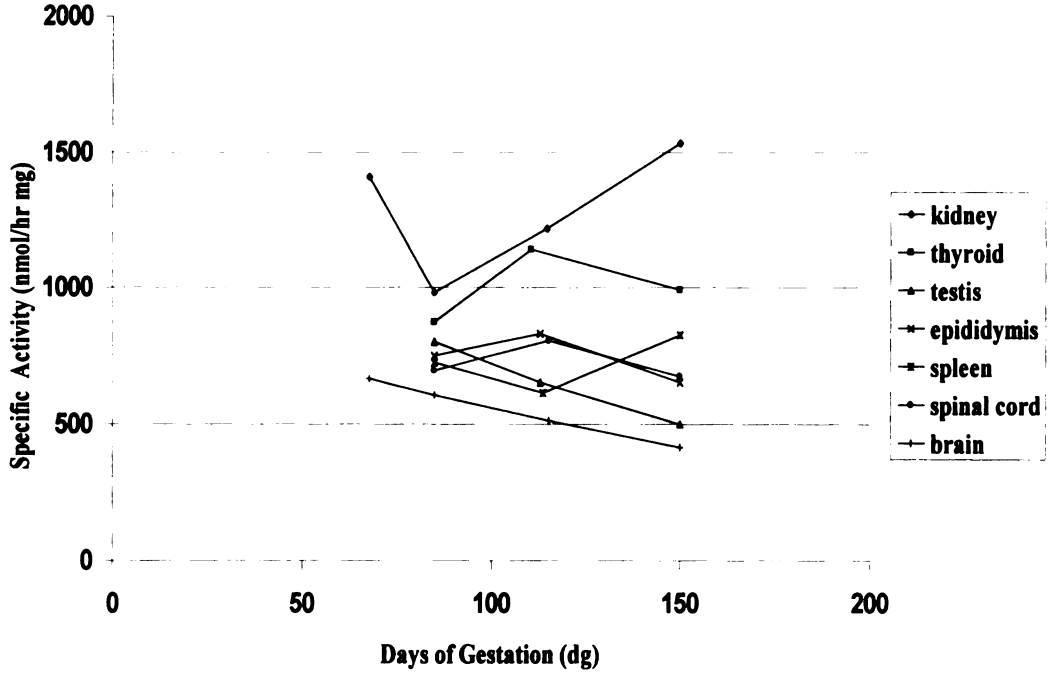

Figure 6. Developmental pattern of acid phosphatase specific activity in selected goat tissues. Each point represents the mean of values of a group (see corresponding table for n and SD for each group). There were significant differences between 85 dg group and 113-120 dg group ($P<0.005$) and between113-120 dg and newborn group ($P<0.05$) in kidney; and significant difference between 113-120 dg and newborn group ($P<0.05$) in spleen. No significant differences for other tissues were observed during development.

were noted for total β-hexosaminidase and acid phosphatase activities during development in thyroid. In kidney, β-mannosidase activity also showed a significant change. The enzyme activity first increased to reach a peak around 113-120 days of gestation, and then decreased by the time of birth. Thus the pattern of prenatal β-mannosidase activity change is different in kidney and thyroid. For acid phosphatase, the enzyme activity significantly ($P< 0.05$) increased from 85 dg to 113-120 dg group. There were no significant differences between 65 dg and 85 dg groups and between113-120 dg and newborn groups. There were no statistical differences for α-mannosidase and β-hexosaminidase activities during development in kidney. All other tissues being examined did not show significant changes for all four enzyme activities during development except that in testis, α-mannosidase activity significantly ($P< 0.05$) decreased from 113-120 dg to newborn, and in spleen, acid phosphatase activity significantly ($P< 0.05$) increased from 113-120 dg to newborn. In brain all four enzyme activities were steady and lowest compared to other tissues.

DISCUSSION

This study has determined the specific activities (nmol /h per mg protein) of β-mannosidase, α-mannosidase, β-hexosaminidase, and acid phosphatase in a wide range of tissue types and gestation stages (65 dg - newborn). Our results have demonstrated that the specific activities of each enzyme in various tissues had different developmental patterns (Figures 3-6), suggesting tissue-specific and enzyme-specific regulation of expression of enzyme activity. Variations among tissues have been previously

demonstrated (Cook et al., 1984; Pearce et al., 1987; Lovell et al., 1994; Cingle et al., 1995; Verdugo et al., 1997), but the patterns of developmental expression have not been compared.

One of the most important findings in this study is the significant ($P < 0.005$) increase of β-mannosidase activity in thyroid from 85 dg to newborn (Figure 3) compared to all other examined tissues. The highest activity in thyroid in newborn goats was consistent with previous reports (Pearce et al., 1987; Lovell et al., 1994). These results suggested that high level activity of β-mannosidase may be needed for thyroid function. Thyroid hormone is synthesized as a prohormone, thyroglobulin, which is a huge glycoprotein (660 kda) and contains many mannose residues, including Manβ1-4GlcNAc and α1-3(6) Man, bond in its N-linked oligosaccharides (Yamamoto et al., 1981; Burrow et al., 1989). Thyroid hormones are known to be important for the growth and differentiation of many tissues. For example, during brain development, thyroid hormones are essential for cell migration, dendrite and axon outgrouth, myelination and gliogenesis (Porterfield & Hendrich, 1993; Bernal & Nunez, 1995). Boyer et al. (1990) and Lovell et al. (1991) have reported that in caprine and bovine β-mannosidosis severe morphological thyroid lesions are accompanied by decreased thyroid hormone concentration, possibly contributing to the severe paucity of myelin in the central nervous system (CNS) characteristic of β-mannosidosis. The unique developmental pattern and the high expression of β-mannosidase in thyroid may be related to the requirement for the synthesis of thyroid hormone during developmental stages, which is necessary for growth and differentiation. Although the β-mannosidase gene, presumed to be a housekeeping gene, exists in all types of tissues, the highest activity and substantial increase during

prenatal development was observed only in thyroid. The reasonable explanation would be the tissue-specific regulation of gene expression.

In the present research, some tissues such as thyroid, kidney, brain and spleen have enough animals available to study and are well studied, however, for epididymis and testis we do not have enough tissues to study at this time, therefore they may need further examination. Another limitation of this study is that we could not get the tissues from very early gestational stages, especially for thyroid (too little to be extracted). To assay earlier stages new technique or methods need to be developed.

The current study was initially designed to investigate the prenatal profiles of β-mannosidase in various goat organs in different gestation stages. However, three other lysosomal enzymes (α-mannosidase, β-hexosaminidase, and acid phosphatase) were measured at the same time for comparison. All the results from this study supported one conclusion: enzyme activities were differentially regulated in different tissues. To understand the molecular basis of such variations, further studies were undertaken.

CHAPTER 2

CLONING AND SEQUENCING THE BOVINE β-MANNOSIDASE GENE PROMOTER

INTRODUCTION

Housekeeping genes encode products required for growth, metabolism, or replication of all cell types, and therefore are expressed ubiquitously. Lysosomal enzymes and lysosomal enzyme deficiency can be found in every type of cell except for the organelle-less mature erythrocyte, so they have been considered to be "housekeeping gene" enzymes. There are several lysosomal gene families summarized by Neufeld (1991). Genes in each family presumably originated from the same ancestral gene. Most housekeeping genes share the common characteristics of the promoter region: lack a recognizable TATA box but contain multiple GC boxes acting as putative binding sites for the transcription factor Sp1(Dynan, 1986; Blake et al., 1990). Housekeeping genes usually have multiple initiation sites of transcription (Cavailles et al., 1993).

The developmental patterns of lysosomal enzyme specific activities described previously (Chapter 1), especially that of β-mannosidase, suggested that tissue-specific gene expression may be differentially regulated in different cell types. If this is the case, the promoter for this gene would be expected to have elements that respond to tissue specific signals. We are most interested in the dramatic changes of β-mannosidase specific activity in thyroid, and this made our second goal to clone and sequence the β-mannosidase gene promoter and look for tissue-specific transcription binding sites, especially for thyroid-specific transcription factors.

To date, three thyroid-specific transcription factors (TTF) have been identified: TTF-1, TTF-2, and Pax-8 (Guazzi et al. 1990; Musti et al., 1987; Civitareale et al., 1989; Francis-Lang et al., 1992; Plachov et al., 1990; and Poleev et al., 1992). Thyroid

transcription factor 1 (TTF-1) was initially identified as a thyroid-specific factor for thyroglobulin (TG) gene expression (Musti et al., 1987). It is a homeodomain-containing protein (Guazzi et al., 1990), preferentially recognizing sequences having the 5'-CAAG-3' core motif. The homeodomain (HD) is the DNA-binding domain of several transcription regulators, and HD-containing proteins play important roles in regulating developmental programs (Scott et al., 1989). Damaute et al.(1994) have reported that TTF-1 is involved in the regulation of thyroid development and differentiation. It was later also found that TTF-1 was important in tissue-specific expression of thyroperoxidase (TPO) (Francis-Lang et al., 1992), thyrotropin receptor (TSHR) (Civitareale et al., 1993; Ohmori et al., 1995), and sodium iodide symporter (NIS) (Endo et al., 1997) genes, which all expressed specifically in thyroid. Additional studies, including gene targeting experiments, have shown that expression of TTF-1 is essential for organogenesis of lung, ventral forebrain, and pituitary, as well as thyroid, tissues in TTF-1 knockout experiments (Kimura et al., 1996). Therefore, TTF-1 seems to be able to regulate the expression of ubiquitous as well as tissue-specific genes during development. Several consensus TTF-1 binding sites core sequences from the rat TG, TPO, and TSHR genes have been identified (Francis-Lang et al., 1992; Guazzi et al., 1990; Ohmori et al., 1995). In this study, we were also interested in looking for these binding motifs in the bovine β-mannosidase promoter region, which may provide some information for further studies.

Although the activity studies were performed on goat tissues, the bovine promoter was chosen for this study for several reasons: (1) The phenotypic consequences of β-mannosidosis are nearly identical in the two ruminant species, suggesting similar

expression patterns (Jones et al., 1983; Abbitt et al., 1991). (2) Previous studies in this laboratory have shown that the caprine and bovine β-mannosidase cDNAs share 96.3 % homology at the nucleotide level and 95.2 % at the deduced amino acid level (Leipprandt et al., 1996), and have 86.4 % homology to human β-mannosidase cDNA (Alkhayat et al, 1998). (3) A bovine genomic library is available in the commercial market but no caprine library is available. In this study we used the 5'-end of bovine β-mannosidase cDNA (203 bp) as a probe to screen the bovine genomic library using a PCR based method, and the results show the characteristics of housekeeping gene promoter. Similar nucleotide sequences to the TTF-1 consensus motif were found in this promoter region, which raised the possibility that TTF-1 may be involved in the regulation of tissue-specific expression of β-mannosidase in the thyroid.

MATERIALS AND METHODS

Materials

A bovine genomic DNA library constructed in Lambda FIX II vector and XL1-Blue E. coli cells were obtained from Stratagene (La Jolla, CA). PCI-neo vector was a gift from Dr. McCabe's lab. Ampli Taq® DNA polymerase was from Perkin Elmer. Restriction enzyme EcoR I was purchased from GIBCO. All primers used in this study were synthesized in the MSU Macromolecular Structure Facility. All primers are listed in Table 7.

Genomic library screening

The library was screened using a PCR-based method (Israel, D.I.,1993). This method needed a piece of known sequence, or 3 oligonucleotides (two PCR primers, and the hybridization probe) as a probe. Briefly, a library was subdivided into 64 wells, each containing 1000 clones, and propagated in bacteria. Amplified phage from each of 8 wells across columns, and each of 8 wells down rows, were pooled. The pooled phage were screened for the sequence of interest by PCR using specific primers. A single well that contained the known sequence was identified by the synthesis of a PCR product of the correct size that hybridized to an internal oligonucleotide probe. This well was subdivided into 64 wells, each containing approximately 30 individual phage, reamplified, and rescreened utilizing the same protocol. A positive well was then screened a third time with about 2 phage per well. In the current research we used the 5'-end of bovine cDNA (203bp), which has been sequenced and characterized previously, as a probe, and two specific primers: MJ 120 and KF 204 (that flank the probe) were used.

To titer the library, 5 μl of the library was used and diluted in series: AA, $1:10^2$; A, $1:10^4$; B, $1:10^5$; C, $1:10^6$; and D, $1:10^7$, in SM buffer, 10 μl of each A, B, C, and D dilution was used to infect 200 μl of cultured XL1-Blue cells (A600 = 0.5). Phage and bacteria were incubated at 37°C for 15 min, then 2.5 ml of top agar (45°C) were added to the infected cells, mixed well and poured evenly on the NZY plate. Plates were incubated at 37°C overnight. The total number of plaques from plate C was 54 (the duplicated plate was 55), so the library concentration is about 5.4×10^9 pfu/ml. In order to get approximately 1000 pfu/100 μl per well for the first round screening and based on the titer of the library, we used 5 μl of the library dilution (1:100) to infect a 2.4 ml-cultured of XL1-Blue cells (A600=0.5) (followed the former experiment). To figure out the optimal ratio of phage/ cells for efficient infection, we performed an experiment, in which a fixed amount of phage (~3000 pfu) was used to infect a series amount of cells, 10 μl, 25 μl, 50 μl, 75 μl, 100 μl, 200 μl, 400 μl, and 600 μl (A600 = 0.5), and grown in 10 ml NZY broth at 37°C for overnight. The results showed all the cells were lysed well, especially in the infection culture with 50 μl of cells. Following the infection, 18 ml of NZY broth and 180 μl 1 M $MgSO_4$ (10mM $MgSO_4$ final concentration) were added to the infected cells and then the culture was subdivided into 64 x 2 wells in two 96-well plates (plate I and plate II) with 100μl per well. Concomitantly, 100 μl of the culture was used to do the titering for confirming the initial concentration of phage. The plates were sealed with GeNunc™ sealing tape, and grown at 37°C for 6 hr while shaking at 225 rpm. The culture was observed until there was a change from cloudy to clear, which indicated cells were completely lysed by phage. Following phage amplification, 25 μl of culture was removed from each well to pool across the rows (A→ H) and down the columns (1 → 8),

resulting in 16 pools for one plate with 200 µl/pool. To prepare phage DNA, 50 µl of phage lysates of 200 µl pool was added to 50 µl (equal vol.)100mM NaOH, heated at 90°C for 10 min, and then neutralized with 10 µl (1/10 vol.) 1 M Tris.HCL (pH 7.6). 2 µl was used as template for PCR. PCR program: denature templates at 94°C for 7 min, following 35 cycles: 94°C /30 s, 55°C /45 s, and 72°C /45 s; extension at 72°C for 10 min. The PCR products were analyzed for the correct size on 2.5 % agarose gel with 1x TBE buffer. For the secondary phage amplification, the positive well from the first screening was titered following the same protocol described previously (for the library titering). To get approximately 30 pfu/100 µl per well, the well was diluted to $1:10^4$ in SM buffer, then 15 µl of the dilution was used to infected 50 µl XL1-Blue cells (A600 = 0.5). Ten ml NZY of broth with 10 mM $MgSO_4$ were added to the infected cells, and then aliquoted to 8 x 8 wells in one plate with 100 µl per well, and another 100 µl was used for titering. Then following the same protocol used in the first screening. For the tertiary screen, again, the positive wells from the second round screening were titered, then we prepared plates containing ~ 100 pfu/plate to do the plaque lift hybridization experiment, plaques were transferred to Hybond-N nylon membrane and using biotin-labeled probe, PCR products of the 5'-end sequence of β-mannosidase cDNA (203 bp). Somehow, this system did not work out and we did not get any information from that. Since the actual low concentration (~18 pfu/well) was used for the secondary amplification, we decided to pick individual plaques for screening positive signals. A total of 64 plaques were picked from a plate containing ~100 plaques prepared from one positive well of the second screening. Individual phage was eluted in 400 µl of SM buffer at – 4 °C for overnight. After titering the elution (~2 $x10^7$ or 10^8 pfu/ml), 10 µl of

the eluted plaque phage was used to infect 10 µl cells in 96-well plate. Following the infection 100 µl of NZY broth with 10 mM $MgSO_4$ were added to each well containing the infected cells, and grown at 37°C for 6 hr. The amplified phages were then analyzed by PCR and gel electrophoresis following the same protocol used in the first two round screening. To purify the positive plaques (from the tertiary screening), we replated the phage from the positive plaques on NZY plates for overnight at 37°C. Then we picked all plaques from the plates to grow in 5 ml NZY broth for overnight, followed by PCR and gel electrophoresis analysis. After having pure positive phage clone we prepared phage stocks by performing both liquid and plate lysates. The titering results showed higher yield from plate lysates (1.1×10^{11} pfu/ml), so plate lysates were used to prepare phage DNA by polyethylene glycol precipitation (PEG) according to the method described in Current Protocols in Molecular Biology (Ausubel et al., 1994).

Subcloning

A positive phage clone was subcloned into two vectors, Ready-to-go™ pUC18 EcoR I /BAP + ligase (from Pharmacia) and PCI-neo expression vector (From Promega). Lambda FIX II vector contains Not I cloning sites that flank the insert region and also contains EcoR I cloning sites. To estimate the size of insert and look for the fragment for subcloning with EcoR I digestion, the positive phage DNA was digested by each of EcoR I, Not I, and EcoR I with Not I together. A 10 µl reaction, with 1 µl of each enzyme and 1 µl 10x Reac3 buffer, was incubated at 37°C overnight. The digestion reactions were resolved on 0.8 % agarose /1 x TAE gel. The insert contained three EcoR I sites and one Not I site. The total length of insert is approximate 15 kb. With EcoR I digestion the two fragments, ~7.7 kb and ~3.2 kb (estimated by computer analysis using Marker III as

standard), were cut out from a 0.8 % low melting agarose gel/1 x TAE buffer and purified using a Wizard Kit (Promega). Southern blot and also PCR, using the purified fragment from the gel as template, were used to identify the fragment containing the 5'-end of β-mannosidase sequence. The results revealed the smaller fragment (3.2 kb) contained the 5' sequence information. For subcloning, we first tried to put the 3.2 kb fragment into the Ready-to-go pUC18 vector, but the ligation reaction failed several times yielding unexplained strange bands, which did not match the size of either vector or insert. We thought that there might have some damage to the fragment DNA when we performed the gel purification. Then we directly used the mixture of digestion reaction of EcoR1, after phenol/chloroform extraction and ethanol precipitation (following the standard protocols), to do the ligation with the ratio of insert/vector at approximate 1:6 (referred to 3.2 kb fragment). Two μl of ligation reaction was used to transform 100 μl DH5α competent cells (2.48 x 10^7 cfu/μg) following the standard protocol. Such as: chill 30 min on ice, heat shock at 42 °C for 45 s, then add 900 μl SOC medium to grow 1 hr at 37°C with shaking. Then 2 x 200 μl of culture were spreaded on two LB plates containing ampicillin (100 μg/ml), and the plates were incubated at 37°C for overnight. Colonies were then randomly picked and grown in 3 ml LB broth containing ampicillin at 37°C for overnight. Plasmid DNA was prepared from overnight culture using a Promega's Wizard miniprep Kit. Three of six randomly picked colonies contained the insert of the 7.7 kb fragment (Figure 11). We still could not get the smaller size fragment into the pUC18 vector. Then we tried to subclone the 3.2 kb fragment using PCI-neo vector. Fragment DNA (3.2 kb) was purified from low melting agarose gel/1 x TAE

buffer and used to do the ligation, then total 10 μl ligation reaction was used to transform DH5α competent cells following the same proctocol described earlier.

Nucleotide sequence analysis

Sequencing was performed by dye terminator reaction in the DNA Sequencing Facility (MSU). Reactions consisted of 1 μg of plasmid DNA and 7.2 pMol (more than 6 pMol) of primer in 10 μl sterile water. New primers were designed as sequencing progressed. The strategy for sequencing is illustrated in Figure 13, and the primers used for sequencing were listed in Table 7. Contig assembly and sequence analysis were done by using Sequencher project and GCG computer program.

RESULTS

Genomic screening and subcloning

The bovine genomic library in lambda FIX II was first titered and found to contain approximately 5.4 x 10^9 pfu/ml. The first screening produced two positive pools, or one positive well (row E and column 3) (Figure 7). The second round yielded three positive pools, or two positive wells (row F, column 2 and 6) (Figure 8). Sixty-four plaques were picked for the tertiary screening and only two plaques #58 and #59 had the positive signal (Figure 9). The two positive plaques were then replated for purifying. The plate prepared from #58 plaque contained 6 plaques, all were picked and all came out positive. The total 10 plaques from the plate containing #59 plaque were all negative (PCR analysis data not shown). Thus, we assumed that we had one pure positive phage clone (#58). We amplified this positive phage clone using plate lysates and titered the lysates

Table 7. Oligonucleotides Used For Screening And Sequencing

Location*	Primer	Sequence 5'→ 3'
-44	MJ 120	CGCATCCCTCGGGTTCTT
159	KF 204	GAACAAGGCGCTGTGCACGC
-523	KF 354	AAACTGGGGAAAACTGAAGTGA
-360	KF 362	GCGAGAGCCATGCACGGTAA
-853	KF 389	CACAGCTACTGAGCGACAGA

* –oligonucleotides started from upstream (-) or downstream of the initiation codon (ATG). Antisense primers were indicated by underline.

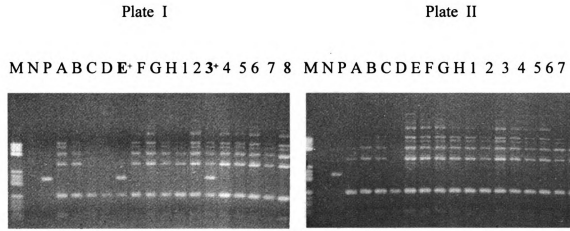

Figure 7. The first screening of bovine genomic library (PCR products were run on 2.5 % agarose gel/1 x TBE). M: Marker V, N: negative control (H_2O), P: positive control (203 bp probe). A→ B: 8 pools of rows, and 1→8: 8 pools of columns. The positive well is E3 in plate I.

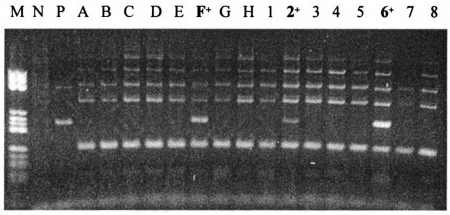

Figure 8. The second screening of bovine genomic library (PCR products were run on 2.5 % agarose gel/1 x TBE). M: Marker V. N: negative control (H_2O). P: positive control(203 bp probe). A→ H: 8 pools of rows, and 1→ 8: 8 pools of columns. The positive wells are F2 and F6.

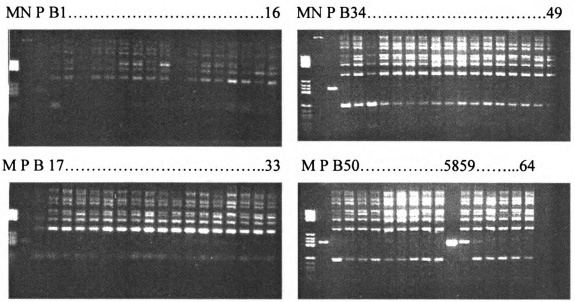

Figure 9. The third screening of bovine genomic library for positive clone. 64 plaques were picked, eluted in SM buffer, and then amplified in bacteria. PCR products were run on 2.5 % agarose gel/1 X TBE. M: Marker V, N: negative control (H_2O), P: positive control, B: bacteria control (no phage). The positive colonies are #58 and #59.

about 1.1 x 10^{11} pfu/ml, then we used this lysates to prepare the phage DNA using PEG precipitation method; 8 ml of plate lysates yielded approximate 6 μg of DNA. Direct sequencing of phage DNA did not yield good sequence information, therefore, the phage insert was subcloned into plasmid vectors. Figure 10 shows the restriction digest of the insert, three EcoR I sites and one Not I site were within the insert. The total length of insert is approximate15 kb. EcoR I digestion of insert created two fragments, ~7.7 kb and ~3.2 kb in addition to the vector arms plus insert. Not I yielded a 4.5 kb fragment and a fragment of 11.4 kb similar to the 10.7 kb arm, indicating a Not I site in the insert as well as at both sides of the cloning site. Double digest was compatible with the results of two single digests. Total seven fragments including the two arms of the vector and five fragments of the insert, 7.95 kb, 2.43 kb, 1.94 kb, 1.63 kb, and 0.857 kb, were produced from the double digest. Both Southern blot and PCR analysis revealed that 3.2 kb EcoR I fragment contained the 5'sequence information of β-mannosidase (data not shown). For subcloning, the 7.7 kb fragment was ligated into the Ready-to-go PUC18 vector, three out of six randomly picked colonies came out positive (Figure 11); and the 3.2 kb fragment was subcloned into the PCI-neo vector, four out of seven were positive (Figure 12). The results of restriction digest of the two subclones with EcoR I, Not I, and EcoR I with Not I, respectively, confirmed that the internal Not I site is within the 3.2 kb fragment (data not shown).

Nucleotide sequencing

In total 1182-bp of sequence, including 203-bp 5'-end of β-mannosidase exon1, 200-bp 3' single direction, and 779-bp two direction DNA sequence, of the promoter region was produced in this study (Figure 14). The nucleotide sequence and several

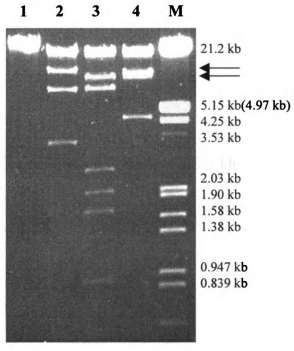

Figure 10. Insert analysis of lambda phage clone. The positive phage clone was digested by EcoR I (lane 2), EcoR I and Not I (lane 3), and Not I (lane 4). Lane 1 is the uncut control. M is 0.5 μg of Marker III. Two arrows indicates two bands are closely located in lane 4.

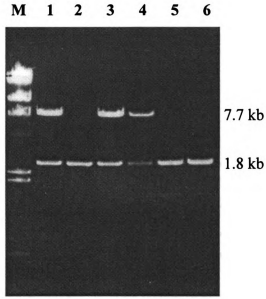

Figure 11. Subclone analysis (PUC18 vector). EcoR1 digest of 6 colonies from subclone using pUC18 vector, clones 1,3, and 4 have insert (7.7 kb). M is Marker II (0.5 μg).

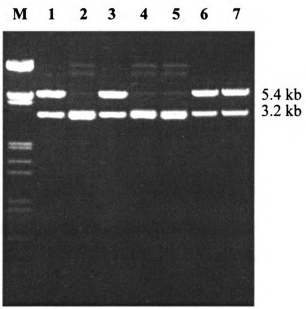

Figure 12. Subclone analysis (PCI-neo vector). EcoR1 digest of seven colonies, lane 1,3,6, and 7 show the positive clones. M is 0.5 μg Marker III.

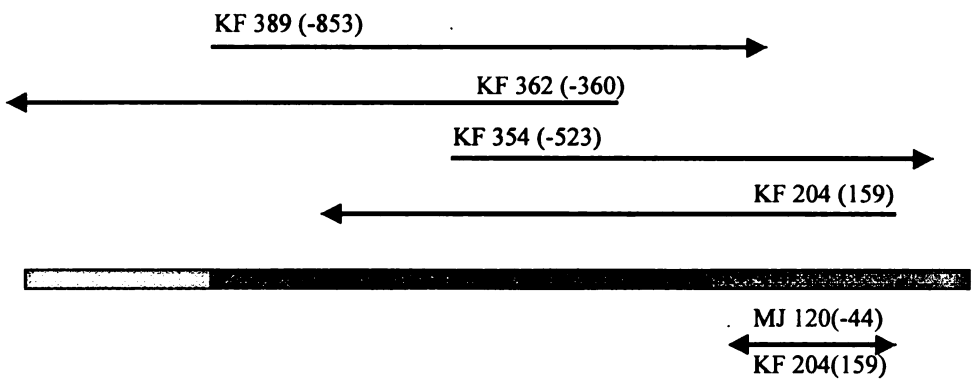

Figure 13. The strategy for sequencing the bovine β-mannosidase gene promoter region. The primers are indicated with ID# and with location shown in parenthesis. The two primers (MJ 120 and KF 204) flanking the probe (203-bp 5'-end of β-mannosidase cDNA), which was used for screening, were also displayed.

Figure 14. Sequence of the 5' flanking region of the bovine β-mannosidase gene. The bold portion represents the putative promoter region with strands sequenced in both direction; AP-2, putative binding sites for the AP-2 transcription factor; Sp-1, putative binding sites for the Sp-1 transcription factor; TTF-1 motif, putative binding sites for the thyroid transcription factor. An arrow indicates the 5'-end sequence of β-mannosidase cDNA.

potential promoter elements revealed by computer search are shown in Figure 14. The 200-bp region upstream from the initiation codon (ATG) was relatively high GC contents (75.5 %) with three potential Sp1 binding sites and two AP-2 binding sites. A CAAT box was noted at –382 upstream from the initiation codon. Over one hundred of other potential transcription factor binding sites were found in this promoter region by GCG computer program analysis. Two potential TTF-1 sites were identified using published consensus sites from other species as input into the sequencher homology search program.

DISCUSSION

We have cloned and sequenced the promoter region of β-mannosidase using a PCR based method, and this region was shown to share the common characteristics of a typical housekeeping promoter by computer database analysis. Lysosomal enzymes have been thought as housekeeping genes, which are expressed ubiquitously. Most of the promoters of lysosomal enzyme genes generally have housekeeping gene characteristics: they are strongly GC rich in the region directly upstream of the initiation codon (ATG) with potential Sp1 binding sites; they generally lack TATA and /or CAAT boxes; and their initiation of transcription occurs at multiple points (Geier et al., 1989; Hoefsloot et al., 1990; Kreysing et al., 1990; Wang and Desnick, 1990; Menon et al., 1992). Other lysosomal enzyme gene promoters do contain CAAT and TATA motifs in their 5' flanking regions as well as being GC rich (Park et al., 1991; Schuchman et al., 1992).

As shown in Figure 14, the 5' flanking sequence of β-mannosidsae is highly GC rich (75.5 %), containing three Sp1 binding sites at -116, -104, and –92 within a 200-bp region upstream from the initiation codon (ATG). This is consistent with the characteristics of housekeeping gene promoter. A CAAT box was found in the 5' proximal flanking region (-382) of β-mannosidase gene, which is probably too far from the transcription start site to be significant.

Tissue-specific expression of lysosomal enzyme has been demonstrated by a number of studies (Freysz et al., 1979; Abe et al., 1979; Reiner and Horowitz, 1988; Aronson et al., 1989) as well as our results presented earlier in this thesis. These results suggested that lysosomal enzyme activities may be under regulatory control. We are most interested in the results of the specific activity of β-mannosidase in thyroid where the highest expression levels are observed and with a sharp increase ($P < 0.005$, Mann-Whitney test) from gestation 85 days to newborn compared to other tissues. It was this interesting finding that prompted us to isolate the β-mannosidase promoter region and look for the thyroid-specific transcription factor binding sites. TTF-1 is a homeodomain (HD) protein that was initially identified as a thyroid-specific factor responsible for thyroglobulin (Tg) gene transcription. TTF-1 specifically recognizes oligonucleotide sequence containing the 5'-CAAG-3' core motif, contrary to other HDs that recognize a 5'-TAAT-3' core motif. One recent study has shown that two binding sites for TTF-1 in the bovine thyroglobulin gene upstream were essential for the activity of enhancer elements in this gene (Christophe-Hobertus et al., 1999). Several consensus TTF-1 binding site core motifs derived from TTF-1-specific binding sites in the rat TG, TPO, and TSHR genes were summarized by Suzuki et al. (1998). With the help of the

computer, we found two similar sequence, GTGCTGATCA (-944) and CACTCACGAC (-245) in the bovine β-mannosidase promoter region with two of the consensus TTF-1 binding site core sequence, GTGCTGAAGA and CACTCAAGTG, respectively. In addition, one consensus TTF-1 binding motif (CACTCAAGTG) is aligned to the human β-mannosidase promoter region (unpublished data) with 80% similarity (ATCTCAAGATG), and contains a 5'-CAAG-3' motif. This interesting finding raised the possibility that TTF-1 may be involved in the regulatory expression of β-mannosidase gene in thyroid in both ruminants and humans.

In summary, we have shown the cloning and sequencing of the 5'flanking region of the bovine β-mannosidase gene. Analysis of the sequence yielded two findings: (1) This region shares the characteristics of a housekeeping promoter. (2) Nucleotide sequences similar to TTF-1 binding sites were found in this promoter region. The next step will be the characterization of this promoter and confirmation of these interesting findings.

CHAPTER 3

SUMMARY AND PROSPECTS

SUMMARY AND PROSPECTS

There are two objectives in this thesis: (1) determination of the developmental profile of lysosomal enzyme activities in normal goats: (2) cloning and sequencing the bovine β-mannosidase gene promoter. The two separate projects are complementary although we utilized two ruminant species, which have 96.3 % identical nucleotides of β-mannosidase cDNA and 95.4 % identity of the deduced amino acids (Leipprandt et al., 1996). The first project, at the biochemical function and developmental level, studied lysosomal enzyme activity. This was undertaken to provide information about disease development by determining the developmental requirements for lysosomal enzymes. Also this could give us information to design appropriate therapies, either enzyme replacement or gene therapy. The second project, at the molecular level of regulation of gene expression, investigated the basic mechanism that may explain the cell-specific and enzyme-specific developmental patterns found in the results of the first study.

The occurence of various patterns among different tissues and four lysosomal enzymes (β-mannosidase,α-mannosidase, β-hexosaminidase and acid phosphatase) is expected. The existence of variation in other enzyme activities is known as well (Verity et al., 1968; Zanetta et al., 1980; Cook et al., 1984; Cingle et al., 1995; Verdugo et al., 1997). But the developmental expression of β-mannosidase had not been studied previously. The significance of different patterns in developmental function of various organs is not yet determined.

Since the caprine genomic library is not currently available right now, and we also have been using the bovine as an animal model to study β-mannosidosis (Jones et al., 1992; Sopher et al., 1993, and Chen et al., 1995), we decided to clone and sequence the bovine β-mannosidase promoter region. PCR based screening method described earlier was used. In our case the probe size (203-bp) fell into a region with no non-specific amplification product (Figures 7- 9), so we did not even need to do the hybridization. The strategy for the sequencing is shown in Figure 13, and the total 5' flanking sequence was shown in Figure 14. From the sequence analysis by computer search, this region is a candidate promoter region: (1) It is located directly upstream of the transcription start site. (2) It shares the common characteristics of housekeeping gene promoter with no TATA box, but highly GC rich with three potential Sp1 binding sites. (3) Computer database analysis showed over 100 potential transcription factor binding sites in this region. However, further studies are strongly recommended to characterize this region and confirm these findings.

Tissue-specific transcription factors are important for the regulation of specific gene expression in specific tissues and during aging, or in the variation of enzyme activities. In order to look for cell-specific transcription factor binding sites, the functional characterization of genomic clones is a necessary step. And it is also useful for the gene and enzyme replacement therapies, in which vectors are made from naturally-regulated promoters rather than a promoter from a retrovirus; and also, the function analysis may lead us to further understand the biochemical and pathological relationship of this lysosomal storage disease. So the next step we are going to do is the function analysis of the bovine β-mannosidase gene, which may help us to understand tissue-specific

expression of enzyme activity and the development of pathogenesis. This may also contribute to regulation of enzyme activity with gene therapy approaches.

BIBLIOGRAPHY

BIBLIOGRAPHY

Abbitt, B., Jones, M.Z., Kasari, T.R., Storts, R.W., Templeton, J.W., Holland, P.S. and Castenson, P.E. (1991). β-mannosidosis in twelve Salers calves. J. Am. Vet. Med. Assoc. 198, 109-113.

Abe, T., Miyatake, T., Norton, W.T. and Suzuki, K. (1979). Activities of glycolipid hydrolases in neurons and astroglia from rat and calf brains and in oigodendroglia from calf brain. Brain Res. 161, 179-182.

Alkhayat, A.H., Kraemer, S.A., Leipprandt, J.R., Macek, M., Kleijer, W.J. and Friderici, K.H. (1998). Human beta-mannosidase cDNA characterization and first identification of a mutation associated with human beta-mannosidosis. Hum Mol Genet 7, 75-83.

Aronson, N.N. Jr. and Kuranda, M.J. (1989). Lysosomal degradation of Asn-linked glycoproteins. FASEB J. 3, 2615-2622.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K., eds. Current Protocols In Molecular Biology. Massachusetts General Hospital and Harvard Medical School: John Wiley and Sons, Inc., 1990: Vol 1, 1.13.7.

Berman, E.R. (1994). Retinal pigment epithelium: lysosomal enzymes and aging. Br. J. Ophthalmol. 78,82-83.

Bernal, J. and Nunez, J. (1995). Thyroid hormones and brain development. Eur. J. Endocrinol. 133, 390-398.

Bernard, M., Sioud, M., Percheron, F. and Foglietti, M.J. (1986). β-mannosidase in human serum and urine: a comparative study. Intl. J. Biochem. 18, 1065-1068.

Blake, M.C., Jambou, R.C., Swick, A.G., Kahn, J.W. and Azizkhan, J.C. (1990). Transcriptional initiation is controlled by upstream GC-box interaction in a TATAA-less promoter. Mol. Cell. Biol. 10, 6632-6641.

Boyer, P.J., Jones, M.Z., Nachreiner, R.F., Refsal, K.R., Common, R.S., Kelley, J. and Lovell, K.L., (1990). Caprine β-mannosidosis: Abnormal thyroid structure and function in a lysosomal storage disease. Lab. Invest. 63, 100-106.

Burrow, G.N. Thyroid gland and control. In: Burrow, G.N., Oppenheimer, J.H. and Volpe, R., ed. Thyroid Function and Disease. Philadelphia: W.B. Saunders, 1989: 11-14.

Cavanagh, K., Dunstan, R. W. and Jones, M.Z. (1982). Plasma α- and β-mannosidase activities in caprine β-mannosidosis. Am. J. Vet. Res. 43, 1058-1059.

Cavailles, V., Augereau, P. and Rochefort, H. (1991). Catheosin D gene of human MCF7 cells contains estrogen-responsive sequences in its 5' proximal flanking region. Biochem. Biophys. Res. Comm. 174, 816-824.

Cavailles, V., Augereau, P. and Rochefort, H. (1993). Cathepsin D gene is controlled by a mixed promoter, and estrogens stimulate only TATA-dependent transcription in breast cancer cells. Proc. Natl. Acad. Sci. USA , Vol. 90, 203-207.

Chen, H., Leipprandt, J.R., Traviss, C.E., Sopher, B.L., Jones, M.Z., Cavanagh, K.T. and Friderici, K.H. (1995). Molecular cloning and characterization of bovine β-mannosidase. J. Biol. Chem. 270, 3841-3848.

Christophe-Hobertus, C. and Christophe, D. (1999). Two binding sites for thyroid transcription factor 1 (TTF-1) determine the activity of the bovine thyroglobulin gene upstream enhancer element. Mol. Cell. Endocrinol. 149, 79-84.

Cingle, K.A., Kalski, R.S., Bruner, W.E., O'Brien, C.M., Erhard, P. and Wyszynski, R.E. (1995). Age-related changes of glycosidases in human retinal pigment epithelium. Curr. Eye Res. 433-438.

Civitareale, D., Lonigro, R., Sinchair, A.J. and Di Lauro, R. (1989). A thyroid-specific nuclear protein essential for tissue-specific expression of the thyroglobulin promoter. EMBO J. 8, 2537-42.

Civitareale, D., Castelli, M.P., Falasca, P. and Saiardi, A. (1993). Thyroid transcription factor 1 activates the promoter of the thyrotropin receptor gene. Mol. Endocrinol. 7, 1589-1595.

Conover, W.J. The use of ranks. In: Conover, W.J., ed. Practical Nonparametric Statistics. New York • London • Sydney • Toronto: John Wiley and Sons INC, 1971: 223-237.

Cook, N.J., Dontenwill, M., Meyer, A., Vincendon, G. and Zanetta, J.P. (1984). Postnatal variations of endo-β-D-N-acetyl-glucosaminidase in the developing rat cerebellum. Brain Res. 317, 298-301.

Cooper, A., Hatton, C., Thornley, M. and Sardharwalla, I. B. (1988). Human β-mannosidase deficiency: biochemical findings in plasma, fibroblasts, white cells and urine, J. Inher. Metab. Dis., 11, 17-29.

Cooper, A., Wraith, J.E., Savage, W.J., Thornley, M. and Noronha, M.J. (1991). β-Mannosidase deficiency in a female infant with epileptic encephalopathy. J. Inher. Metab. Dis. 14, 18-22.

Cotton, R.G.H. (1993). Current methods of mutation detection. Mutat. Res. 285, 125-144.

Damante, G. and Di Lauro, R. (1994). Thyroid-specific gene expression. Biochim. Biophys. Acta. 1218, 255-266.

Dynan, W.S. (1986). Promoters for housekeeping genes. Trends Genet. 2, 196-197.

Doll, R.F., Bruce, A. and Smith, F.I. (1994). Regulation of the human acid α-glucosidase promoter in multiple cell types. Biochim. Biophys. Acta. 1261, 57-67.

Dorland, L., Duran, M., Hoefnagels, F.E.T., Breg, J.N., Fabery de Jonge, H., van Eeghen-Cransberg, K., Van Sprang, F.J. and van Diggelen, O.P. (1988). β-mannosidosis in two brothers with hearing loss. J. Inher. Metab. Dis., 11, 255-258.

Durand, P. (1987). Recent progress of lysosomal disease. Enzyme 38, 256-261.

Embury, D.H. and Jerrett, I.V. (1985). Mannosidosis in Galloway calves. Vet. Pathol. 22, 548-551.

Endo, T., Kaneshige, M., Nakazato, M., Ohmori, M., Harii, N. and Onaya, T. (1997). Thyroid transcription factor-1 activates the promoter activity of rat thyroid Na^+/I^- symporter activity. Mol. Endocrinol. 11, 1747-1755.

Fisher, R.A., Rathke E. J. S., Kelley, J. A., Dunstan R. W., Cavanagh, K. and Jones, M.Z. (1986). Inherited β-mannosidosis in goats. Anim. Genet. 17, 183-190.

Francis-Lang, H., Price, M., Polycarpou-Schwarz, M. and Di Lauro, R. (1992). Cell-type-specific expression of the rat thyroperoxidase promoter indicates common mechanisms for thyroid-specific gene expression. Mol. Cell. Biol. 12, 576-588.

Freysz, L., Farooqui, A.A., Adamcczeewska-Goncerzewicz, Z. and Mandel, P. (1979) Lysosomal hydrolases in neuronal, astroglial, and oligodendroglial enriched fractions of rabbit and beef brain. J. Lipid Res. 20, 503-508.

Geier, C., von Figura, K. and Pohlmann, R. (1989). Structure of the human lysosomal acid phosphatase gene. Eur. J. Biochem. 183, 611-616.

Guazzi, S., Price, M., De Felice, M., Damante, G., Mattei, M.G. and Di Lauro, R. (1990). Thyroid nuclear factor 1 (TTF-1) contains a homeodomain and displays a novel DNA binding specificity. EMBO J. 9, 3631-3639.

Healy, P. J., Seaman, J. T., Gardner, I. A. and Sewell, C. A. (1981). β-mannosidase deficiency in Anglo-Nubian goats. Aust. Vet. J. 57, 504-507.

Hers, H.G. (1965). Progress in gastroenterology: inborn lysosomal disease. Gastroenterology 48, 625-633.

Hoefsloot, L.H., Hoogeveen-westerveld, M., Reuser, A.J.J. and Oostra, B.A. (1990). Characterization of the human lysosomal α-glucosidase gene. Biochem. J. 272, 493-497.

Istrael, D.I. (1993). A PCR-based method for high stringency screening of DNA libraries. Nucleic Acid Research, 1993, Vol. 21, No. 11, 2627-2631.

Jolly, R.D., Thompson, K.G., Bayliss, S.L., Vidler, B.M., Orr, M.B. and Healy, P.J. (1990). β-mannosidosis in a salers calf: a new storage disease of cattle. N. Z. Vet. J. 38, 102-105.

Jones, M.Z., Cavanagh, K.T., Kranich, R., Traviss, C., Fujita, Y., Ohta, M. and Matsuura, F. (1993). Possible β-mannosidosis chimera. Altered expression of metabolic perturbations. J. Inher. Metab. Dis. 16, 1012-1023.

Jones, M.Z., Cunningham, J.G., Dade, A.W., Alessi, D.W., Mostosky, U.V., Vorro, J.R., Benitez, J. T. and Lovell, K.L. (1983). Caprine β-mannosidosis: Clinical and pathological features. J. Neuropath. Exp. Neurol. 42, 268-285.

Jones, M. Z. and Dawson, G. (1981). Caprine β-mannosidosis. Inherited deficiency of β-mannosidase. J.Biol. Chem. 256, 5185-5188.

Jones, M.Z. and Kennedy, F.A. (1993). Caprine β-mannosidosis: aberrant phenotype in a 5-month-old euthyroid animal. J. Inher. Metab. Dis. 16, 910-911.

Jones, M. Z., and Laine, R. A. (1981). Caprine oligosaccharide storage disease. Accumulation of β-mannosyl (1-4) β-N-acetylglucosaminyl (1-4) β-N-acetylglucosamine in brain. J. Biol. Chem. 256, 5181-5184.

Jones, M.Z., Rathke, E.J.S. and Cavanagh, K. (1984). β-mannosidosis: prenatal biochemical and morphological characteristics. J. Inher. Metab. Dis. 7, 80-85.

Jones, M.Z., Rathke, E.J.S., Gage, D.A., Costello, C.E., Murakami, K., Ohta, M. and Matsuura, F. (1992). Oligosaccharides accumulated in the bovine β-mannosidosis kidney. J. Inher. Metab. Dis. 15, 57-67.

Kimura, S., Hara, Y., Pineau, T., Fernandez-Salguero, P., Fox, C.H., Ward, J.M. and Gonzalez, F. (1996). The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. Genes Dev., 10, 60-69.

Kleijer, W.J., Hu, P., Thoomes, R., Boer, M., Huijmans, G.M., Blom, W., van Diggelen, O.P., Seemanova, E. and Macek, M. (1990). β-Mannosidase deficiency:

heterogeneous manifestation in the first female patient and her brother, J. Inher. Metab. Dis., 13, 867-872.

Kreysing, J., von Figura, K. and Gieselmann, V. (1990). Structure of the arylsulfatase A gene. Eur. J. Biochem. 191, 627-31.

Leipprandt, J.R., Kraemer, S.A., Haithcock, B.E., chen, H., Dyme, J.L., Cavanagh, K.T., Friderici, K.H. and Jones, M.Z. (1996). Caprine β-mannosidase: sequencing and characterization of the cDNA and identification of the molecular defect of caprine β-mannosidase. Genomics 37, 51-56.

Lovell, K.L. and Jones, M.Z. (1983). Distribution of central nervous system lesions in β-mannosidosis. Acta Neuropathol 62, 121-126.

Lovell, K.L., Kranich, R.J. and Cavanagh, K.T. (1994). Biochemical and histochemical analysis of lysosomal enzyme activities in caprine β-mannosidosis. Mol. Chem. Neuropathol. Vol. 21,61-73.

Lovell, K.L., Jones, M.Z., Patterson, J., Abbit, B. and Castenson, P. (1991). Thyroid structure and function in bovine β-mannosidosis. J. Inher. Metab. Dis. 14, 228-230.

Lovell , K.L., Matsuura, F., Patterson, J., Baeverfjord, G., Ames, N.K. and Jones, M.Z. (1997). Biochemical and morphological expression of early prenatal caprine β-mannosidosis. Prenat. Diagn. Vol. 17, 551-557.

Matsuura, F., Laine, R.A. and Jones, M.Z. (1981). Oligosaccharides accumulated in the kidney of a goat with β-mannosidosis: Mass spectrometry of intact permethylated derivatives. Arch. Biochem. Biopohys. 211, 485-493.

Menon, K.P., Tieu, P.T. and Neufeld, E.F. (1992). Architecture of the canine IDUG gene and mutation underlying canine mucopolysaccharidosis I. Genomics 14, 763-768.

Moskkowitz, S.M., Menson, K.P., Tieu, P.T. and Neufeld, E.F. (1992). Architecture of the gene encoding alpha-L-iduronidase and some mutations underlying human and canine mucopolysaccharidosis I (MSP I). Am. J. Hum. Genet. 51, supple. A680.

Musti, A.M., Ursini, E.V., Avvedimento, E.V., Zimarino, V. and Di Lauro, R. (1987). A cell type specific factor recognizes the rat thyroglobulin promoter. Nucleic Acids Res. 15, 8149-8166.

Neote, K., Bapat, B., Dumbrille-Ross, A., Troxel, C., Schuster, S.M., Mahuran, D.J. and Gravel, R.A. (1988). Characterization of the human HEXB gene encoding lysosomal β-hexosaminidase. Genomics 3, 279-286.

Neufeld, E.F. (1991). Lysosomal storage diseases. Ann. Rev. Biochem. 60, 257-280.

Norflus, F., yamanaka, S. and Proia, R.L. (1996). Promoters for the human β-hexosaminidase genes, HEXA and HEXB. DNA cell Biol. 15, 89-97.

Ohmori, M., Shimura, H., Shimura, Y., Ikuyama, S. and Kohn, L.D. (1995). Characterization of an up-stream thyroid transcription factor-1-binding site in the thyrotropin receptor promoter. Endocrinology 136, 269-282.

Ohshima, T., Murray, G.J., Nagle, J.W., Quirk, J.M., Kraus, M.H., Barton, N.W., Brady, R.O. and Kulkarni, A.B. (1995). Structural organization and expression of the mouse gene encoding α-galactosidase A. Gene 166, 277-280.

Park, H., Fisher, K.J. and Aronson, N.N., Jr. (1991). Genomic structure of human lysosomal glycosylasparaginase. FEBS Lett. 288, 168-172.

Patterson, J. S., Jones, M. Z., Lovell, K.L. and Abbitt, B. (1991). Neuropathology of bovine β-mannosidosis. J. Neuropathol. Exp. Neurol., 50, 538-546.

Pearce, R.D., Callahan, J.W., Little, P.B., Armstrong, D.T., Kiehm, D. and Clarke, J.T.R. (1987). Properties and prenatal ontogeny of β-D-mannosidase in selected goat tissues. Biochem. J. 243, 603-609.

Plachov, D., Chowdhury, K., Walther, C., Simon, D., Guenet, J.L. and Gruss, P. (1990). Pax-8, a murine paired box gene expressed in the developing excretory system and thyroid gland. Development 110, 643-651.

Poleev, A., Fickenscher, H., Mundlos, S., Winterpacht, A., Zabel, B., Fidler, A., Gruss, P. and Plachov, D. (1992). PAX8, a human paired box gene: isolation and expression in developing thyroid, kidney and Wilms' tumors. Development 116, 611-623.

Porterfield, S.P. and Hendrich, C.E. (1993). The role of thyroid hormones in prenatal and neonatal neurological development: current perspectives. Endocrine Reviews 14, 94-106.

Proia, R.L. and Soravia, E. (1987). Organization of the gene encoding the human β-hexosaminidase α-chain. J. Biol. Chem. 262, 5677-5681.

Reiner, O. and Horowitz, M. (1988). Differential expression of the human glucocerebrosidase-coding gene. Gene 73, 469-478.

Riise, H.M., Berg, T., Nilssen, O., Romeo, G., Tollersrud, O.K. and Ceccherini, I. (1997). Genomic structure of the human lysosomal alpha-mannosidase gene (MANB). Genomics 42, 200-207.

Sakai, N., Fukushima, H., Inui, K., Fu, L., Nishigaki, T., Yanagihara, I., Tatsumi, N., Ozono, K. and Okada, S. (1998). Human galactocerebrosidase gene: promoter analysis of the 5'-flanking region and structural organization. Biochim Biophys Acta 1395, 62-67.

Schuchman, E.H., Levran, O., Pereira, L.V. and Desnick, R.J. (1992). Structural organization and complete nucleotide sequence of the gene encoding human acid sphingomylinase (SMPD1). Genomics 12, 197-205.

Scott, M.P., Tamkun, J.W. and Hartzell, G.W. 3d (1989). The structure and function of the homeodomain. Biochim. Biophys. Acta. 989, 25-48.

Shailubhai, K., Saxena, E.S., Balapure, A.K. and Vijay, I.K. (1990). Developmental regulation of glucosidase I, an enzyme involved in the processing of asparagine-linked glycoprotein in rat mammary gland. J. Biol. Chem. 265, 9701-9706.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. and Klenk, D.C. (1985). Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76-85.

Snyder, E.Y., Taylor, R.M. and Wolfe, J.H. (1995). Neural progenitor cell engraftment corrects lysosomal storage throughout the MPS VII mouse brain. Nature, 374, 367-370.

Sopher, B.L., Traviss, C.E., Cavanagh, K.T., Jones, M.Z. and Friderici, K.H. (1992). Purification and characterization of goat lysosomal β-mannosidase using monoclonal and polyclonal antibodies. J. Biol. Chem. 267, 6178-6182.

Sopher, B.L., Traviss, C.E., Cavanagh, K.T., Jones, M.Z. and Friderici, K.H. (1993). Bovine kidney β-mannosidase: purification and characterization. Biochem. J. 289, 343-347.

Stinchi, S., Orlacchio, A., Costanzi, E., Stirling, J.L., Menghini, A.R. and Beccari, T. (1998). Promoter characterization and structure of the gene encoding mouse lysosomal alpha-d-mannosidase. Mamm Genome 9, 869-873.

Suzuki, K., Lavaroni, S., Mori, A., Okajima, F., Kimura, S., Katoh, R., Kawaoi, A. and Kohn, L.D. (1998). Thyroid transcription factor 1 is calcium modulated and coordinately regulates genes involved in calcium homeostasis in C cells. Mol. Cell. Biol. 18, 7410-7422.

Vandevelde, M., Fankhauser, R. Bichsel, P., Wiesmann, U. and Herschkowitz, N. (1982). Hereditary neurovisceral mannosidosis associated with α-mannosidase deficiency in a family of Persian cats. Acta. Neuropathol. 58, 64-68.

Van Pelt, J., Hokke, C.H., Dorland, L., Duran, M., Kamerling, J.P. and Vliegenthart, J.F.G. (1990). Accumulation of mannosyl-β(1→4)-N-acetylglucosamine in fibroblast and leukocytes of patients with a deficiency of β-mannosidase. Clin. Chim. Acta. 187, 55-60.

Verdugo, M.E. and Ray, J., (1997). Age-related increase in activity of specific lysosomal enzymes in the human retinal pigment epithelium. Exp.Eye Res.65, 231-240.

Verity, M.A., Brown, W.J. and Reith, A. (1968). Structure-linked activity of lysosomal enzymes in the developing mouse brain. J. Neuorchem. 15, 69-80.

Walkley, S.U., Thrall, M.A., Dobrenis, K., Huang, M., March, P.A., Siegel, D.A. and Wurzelmann, S. (1994). Bone marrow transplantation corrects the enzyme defect in neurons of the central nervous system in a lysosomal storage disease. Proc. Natl. Acad. Sci. U.S.A. 91, 2970-2974.

Wang, A.M., Bishop, D.F. and Desnick, R.J. (1990). Human α-N-acetylgalactosaminidase: molecular cloning, nucleotide sequence, and expression of a full-length cDNA. J. Biol. Chem. 265, 21859-21866.

Wenger, D.A., Sujansky, E., Fennessey, P.V. and Thompson, J.N. (1986). Human β-mannosidase deficiency, N. Engl. J. Med. 315, 1201-1205.

Yamanaka, S., Johnson, O.N., Norflus, F., Boles, D.J. and Proia, R.L. (1994). Structure and expression of the mouse β-hexosaminidase genes, Hexa and Hexb. Genomics 21, 588-596.

Yamamoto, K., Tsuji, T. and Osawa, T. (1981). The structure of carbohydrate unit B of porcine thyroglobulin. Biochem J. 195, 701-713.

Zanetta, J.P., Federico, A. and Vincendon, G. (1980). Glycosidase and cerebellar ontogenesis in the rat. J. Neurochem. 34, 831-834.