INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

Genomic Organization and Chromosomal Mapping of the Murine α1,2-Mannosidase IB Gene Involved in N-Glycan Maturation

Nathalie V. Campbell Dyke, Department of Biochemistry
McGill University, Montreal

November 1996

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Master of Science

Table of Contents

List of Figures

List of Tables

List of Abbreviations

α1,2-Man	α1,2-mannosidase
BHK	baby hamster kidney
CAS	castanospermine
CDG	carbohydrate deficient glycoprotein
CnBr	cyanogen bromide
CsCl	cesium chloride
DAPI	4,6-diamidino-2-phenylindole
DNJ	1-deoxynojirimycin
dpc	days postcoitus
Dol	dolichol
Dol-P	dolichol phosphate
EDTA	ethylenediaminetetraacetate
Endo α-Man	endo α-mannosidase
ER	endoplasmic reticulum
FISH	fluorescence *in situ* hybridization
FITC	avidin-fluorescein isothiocyanate
Glc	glucose
Glc I	α-glucosidase I
Glc II	α-glucosidase II
GlcNAc	N-acetylglucosamine
GnTI	N-acetylglucosaminyltransferase I
GnTII	N-acetylglucosaminyltransferase II
HEMPAS	hereditary erythroblastic multinuclearity with a positive acidified serum lysis test
IPTG	isopropylthio-β-D-galactoside
KIF	kifunensine
Man	mannose
ManDNJ	1-deoxymannojirimycin
msc	multiple cloning site
N-CAM	neural cell adhesion molecule

N-linked	asparagine-linked
NK	natural killer
OST	oligosaccharyltransferase
PCR	polymerase chain reaction
SW	swainsonine
SDS	sodium dodecyl sulfate
SSC	sodium chloride / sodium citrate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
UTR	untranslated region
UV	ultraviolet

FOREWORD

The following manuscript is included as chapter 2 of this thesis. It was submitted for publication to Genomics on 4/10/96.

Genomic Organization and Chromosomal Mapping of the Murine α1,2-Mannosidase IB Gene Involved in N-Glycan Maturation.

Nathalie Campbell Dyke, Ariadni Athanassiadis, and Annette Herscovics
McGill Cancer Centre, McGill University, 3655 Drummond Street, Montréal, Québec, Canada, H3G 1Y6

The isolation and characterization of the cosmid clones, Cos.31.1 and Cos.25.1, were performed by Ariadni Athanassiadis. FISH was done by Dr. Barbara Beatty. P1 genomic library screening was done by Genome Systems, Inc. All other experiments were performed by the candidate.

ACKNOWLEDGMENTS

The work presented in this thesis would not have been possible without the contributions and encouragement of my supervisor, colleagues, friends and family.

First, I would like to thank Dr. Annette Herscovics for her advice and encouragement during the two years I worked in the laboratory.

Secondly, I would like to acknowledge the contribution of Ariadni Athanassiadis who isolated and characterized the cosmid clones used in this study, and the technical assistance of Homa Assar. I also would like to thank Dr. Philippe Gros for the gift of the mouse cosmid library, Dr. Barbara Beatty for the FISH localization, and Dr. Michel Tremblay for many helpful discussions.

Finally, I would like to thank the members of the laboratory (Barry, Pedro, Linda, Frank, Peng and Allison) who helped me out in many ways, and my family and Stu for their continuous support. I would also like to extend a special thanks to Patsy and Allan Perlman who provided me with a home away from home while I was writing this thesis.

ABSTRACT

α1,2-Mannosidases are a family of enzymes with similar amino acid sequences that are required for the formation of complex and hybrid asparagine-linked oligosaccharides in mammalian cells. We have determined the genomic structure of one member of this enzyme family, the murine α1,2-mannosidase IB gene. Two BALB/c cosmid genomic clones (Cos.31.1 and Cos.25.1) and three overlapping 129/*sv* P1 genomic clones were isolated. Analysis of Cos.31.1 and the P1 clones showed that the α1,2-mannosidase IB gene spans ≥80kb of the genome, and consists of 13 exons representing the complete open reading frame of the enzyme. Comparison of the intron/exon boundaries with those found in the partially characterized *D. melanogaster mas-1* gene indicates that there is some conservation of α1,2-mannosidase genomic structure between species. Fluorescence *in situ* hybridization with α1,2-mannosidase IB genomic DNA (Cos.31.1) localized the gene to mouse chromosome 3F2. Sequence analysis of the Cos.25.1 cosmid clone provided evidence for the existence of another related gene or pseudogene. Fluorescence *in situ* hybridization with Cos.25.1 localized this sequence to mouse chromosome 4A13.

RÉSUMÉ

Les α1,2-mannosidases, une famille d'enzymes contenant des similarités de séquence d'acides aminés, sont essentielles à la formation d'oligosaccharides complexes et hybrides liés à l'asparagine dans les cellules de mammifères. Nous avons determiné la structure génomique d'un membre de cette famille, l'α1,2-mannosidase IB de la souris. Deux clones cosmides génomiques de la souris BALB/c (Cos.31.1 et Cos.25.1) et trois clones P1 génomiques de la souris 129/*sv* ont été isolés. L'analyse du clone Cos.31.1 et des clones P1 indique que le gène de l'α1,2-mannosidase IB a plus que 80 kb de long et comprend 13 exons représentant la séquence codante complète de l'enzyme. Une comparaison des sites d'épissage du gène de l'α1,2-mannosidase IB avec ceux du gène de *D. melanogaster mas-1* indique que la structure de ces gènes a été conservée durant l'évolution des espèces. L'analyse des chromosomes par hybridation fluorescente *in situ* utilisant une sonde génomique de l'α1,2-mannosidase IB (Cos.31.1) indique que ce gène se trouve sur le chromosome 3F2 de la souris. L'analyse de la séquence du clone cosmide Cos.25.1 suggère qu'il existe un autre gène ou pseudogène, semblable mais non identique au gène de l'α1,2-mannosidase IB. Lorsque le clone Cos.25.1 est utilisé comme sonde pour l'hybridation fluorescente *in situ*, le signal se trouve sur le chromosome 4A13.

CHAPTER 1

1.0. Introduction.

N-linked oligosaccharides are found on many extracellular and cell-surface proteins of multicellular and unicellular organisms. The diverse array of N-linked oligosaccharides observed *in vivo* arises from the modification of an oligosaccharide precursor by enzymes that remove mannose and glucose (specific α-mannosidases and α-glucosidases) and enzymes that add sugars such as N-acetylglucosamine, galactose and fucose (glycosyltransferases). The oligosaccharide structures formed are species and cell-type dependent and can vary greatly between different glycoproteins and protein glycoforms. Studies with compounds that disrupt N-linked oligosaccharide maturation indicate that N-linked oligosaccharides are an intrinsic part of glycoprotein biological activity and play roles in numerous physiological events. In this introduction, the biological importance of N-linked oligosaccharides, their structure, and their biosynthesis, with particular emphasis on the α-mannosidases, will be discussed.

1.1. Biological importance of N-linked oligosaccharides.

Inhibition and genetic disruption of the enzymes involved in the biosynthesis of N-linked oligosaccharides suggests that many biological events of multicellular organisms require the presence of N-linked oligosaccharides. N-linked oligosaccharides have been shown to mediate events such as cell adhesion (both cell-cell interactions and cell-extracellular matrix interactions), ligand-receptor binding, protein folding, protein stability, and the modulation of protein function (Rademacher *et al.*, 1988; Varki, 1993). Since processes such as the immune response, embryonic and tissue development, and tumour metastasis involve many of these N-linked oligosaccharide mediated events, it is clear that N-linked oligosaccharides are required for normal biological function.

1.1.1. N-linked oligosaccharides in cell adhesion and receptor-ligand interactions.

Examples of protein-oligosaccharide mediated cell adhesion events and receptor-ligand interactions can be seen during many events of the immune response, such as natural killer cell lysis and leukocyte adhesion during inflammation. Natural killer (NK) cells are a population of lymphocytes that destroy certain tumour cells and virally infected cells

without prior sensitization or involvement of major histocompatibility antigens. Target cells for NK cell lysis are identified by the NK cell through a protein-oligosaccharide recognition event (Ahrens, 1993; Bezouska *et al.*, 1994). NK cell surface proteins, such as the C-type lectin NKR-P1, recognize and bind complex N-linked oligosaccharides expressed by target cells. This recognition is dependent on the structure of the target cell oligosaccharide. Altering the oligosaccharides by the use of inhibitors affects the sensitivity of the target cell, and can be used to target normally NK-resistant tumour cells for cell lysis (Bezouska *et al.*, 1994).

Similar recognition events are also observed during the early stages of inflammation (Smith, 1993). Inflammation begins with the recruitment of circulating leukocytes to the site of injury by their rolling adhesion on the surface of activated endothelial cells. The rolling adhesion phenomenon involves the direct interaction of endothelial cell-surface proteins called selectins (P, L, and E) with specific oligosaccharide structures found on circulating neutrophils. This adhesion is dependent on the type of oligosaccharide structures expressed by the circulating leukocytes, and can be significantly altered when the structure of the oligosaccharides is modified by inhibition of N-linked oligosaccharide maturation (Sriramarao *et al.*, 1993).

1.1.2. N-linked oligosaccharides in development.

Although the exact roles N-linked oligosaccharides play in embryonic development are not well defined, there is strong evidence that they are essential for this process. When oligosaccharide maturation is prevented by the genetic disruption of an enzyme essential for generating complex and hybrid oligosaccharides *in vivo*, normal embryonic development is impaired (Ioffe and Stanley, 1994; Metzler *et al.*, 1994). Disruption of the N-acetylglucosaminyltransferase I gene, which encodes a key enzyme in N-glycan maturation (see below), results in embryos that are not viable past 9.5 dpc. They are smaller than wild-type embryos, exhibit impaired vascularization, inverted left-right symmetry and have unclosed neural pores. The failure to generate the diverse array of complex and hybrid oligosaccharides observed *in vivo* is lethal, suggesting an essential role for these structures in post-implantation development.

Complex and hybrid oligosaccharide structures are also involved in angiogenesis, an essential process during embryonic development, wound healing and tumour growth.

In vitro studies using castanospermine (Pili *et al.*, 1995) and 1-deoxymannojirimycin (Nguyen *et al.*, 1992), both inhibitors of N-linked oligosaccharide processing, demonstrate that in the absence of complex and hybrid structures, capillary tubes do not form.

1.1.3. N-linked oligosaccharides in metastasis.

Dramatic changes in the composition of cell-surface N-linked oligosaccharides are believed to contribute to events such as tumour cell migration. An increase in tumour metastasis and tumour cell growth is correlated with the presence of highly sialylated and branched complex N-linked oligosaccharides on cell-surface glycoproteins. When the formation of these complex structures is prevented by the use of inhibitors of N-linked oligosaccharide processing, the rate of tumour growth and the metastatic potential of tumour cells can be significantly reduced (Dennis, 1986; Pili *et al.*, 1995). *In vitro* studies have also shown that the transformed phenotype of cells can be reversed by treatment of the cells with different N-oligosaccharide processing inhibitors (Nichols *et al.*, 1985; DeSantis *et al.*, 1987). These observations have led to the initiation of clinical trials with N-linked oligosaccharide processing inhibitors such as swainsonine for use as potential anti-cancer drugs (Goss *et al.*, 1994).

1.1.4. N-linked oligosaccharides in protein folding and stability.

Monoglucosylated N-linked oligosaccharides play an important role in the folding of glycoproteins and their quality control. Inhibitor studies demonstrate that incompletely folded and/or misfolded glycoproteins lacking these monoglucosylated structures are not transported to the Golgi, but instead accumulate in the ER (Lodish and Kong, 1984). This accumulation of glycoproteins in the ER results from the failure of calnexin, a chaperone which mediates glycoprotein folding, to recognize the incompletely folded and/or misfolded glycoproteins lacking the $Glc_1Man_9GlcNAc_2$ and to mediate their correct folding (Ou *et al.*, 1993; Ware *et al.*, 1995). The incompletely folded glycoproteins may then undergo rapid degradation (Ora and Helenius, 1995).

The stability and degradation of some glycoproteins is also dependent upon the composition of their oligosaccharide chains. Studies using inhibitors of glucose trimming indicate that the failure to remove the glucose residues results in rapid degradation of some glycoproteins in the ER (Moore and Spiro, 1993). It has been shown that modifications

made to the oligosaccharide structure at later stages of maturation can also affect glycoprotein stability. When the yeast prepro-α-factor is expressed in rodent cells, the newly synthesized glycoprotein is rapidly degraded in the ER or another pre-Golgi compartment. However, by inhibiting mannose trimming, this protein is stabilized (Su *et al.*, 1993).

1.1.5. N-linked oligosaccharides in intracellular targeting.

The presence of N-linked oligosaccharides on glycoproteins can also be essential for their correct intracellular targeting. Examples of this oligosaccharide mediated targeting are the targeting of the glucose transporter, GLUT1, to the plasma membrane and the targeting of newly synthesized lysosomal glycoproteins to the lysosomes. The GLUT1 transporter is normally targeted to the plasma membrane, however when it is deglycosylated, it is mistargeted to intracellular vesicles (Asano *et al.*, 1993). The delivery of newly synthesized soluble lysosomal glycoproteins to the lysosomes is mediated by mannose 6-phosphate receptor recognition of mannose 6-phosphate residues on their glycoproteins (Kornfeld, 1990). The mannose 6-phosphate receptor-glycoprotein complexes form in the Golgi and are sorted to the prelysosomal compartment where they are dissociated, allowing the glycoproteins to be delivered to the lysosome. If the glycoproteins do not bear mannose 6-phosphate residues on their oligosaccharides, they cannot be recognized by the mannose 6-phosphate receptors. In the absence of this recognition, the lysosomal glycoproteins fail to reach the lysosome and are secreted into the intracellular space.

1.1.6. Modulation of protein activity by N-linked oligosaccharides.

N-linked oligosaccharides also play important roles in modulating the activity of certain proteins. Recently, N-linked oligosaccharides have been implicated in the modulation of *in vivo* and *in vitro* thyroid hormone synthesis (Mallet *et al.*, 1995). Thyroid hormone synthesis occurs at specific sites of thyroglobulin upon iodotyrosine coupling. Studies with the N-terminal domain of thyroglobulin, which contains the preferred site for thyroxine synthesis, have shown that N-glycosylation is required for thyroxine formation. The N-terminal domain of thyroglobulin contains two glycosylation sites which must be fully or partially glycosylated for hormone synthesis to occur. In the absence of

glycosylation of these specific sites, thyroxine synthesis does not occur. Furthermore, it has been shown that certain oligosaccharide structures enhance hormone synthesis more than others. The N-linked oligosaccharides are thought to induce a conformational change in the structure of the N-terminal peptide affecting its ability to produce hormone.

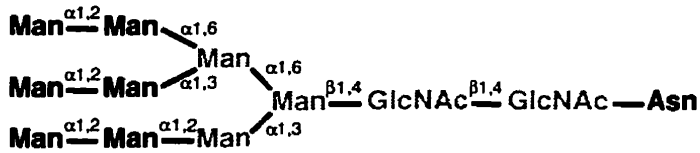
N-linked oligosaccharide chains are also involved in the modulation of neural cell adhesion molecule (N-CAM) homophilic binding (Krog and Bock, 1992). In the embryonic form, N-CAM carries large extended polysialic acid oligosaccharide chains that reduce its capacity for homophilic binding. During development, the length of these polysialic acid chains is modified, altering the homophilic adhesion of N-CAM. In the adult state, N-CAM carries shorter chains, and as a result exhibits an increased capacity for binding. The presence and regulation of polysialic acid on N-CAM has been associated with neuroplasticity, modulating such processes as neuron pathfinding, axon regeneration, and cell migration (Rutishauser and Landmesser, 1996).

1.2. The structure of mammalian N-linked oligosaccharides.

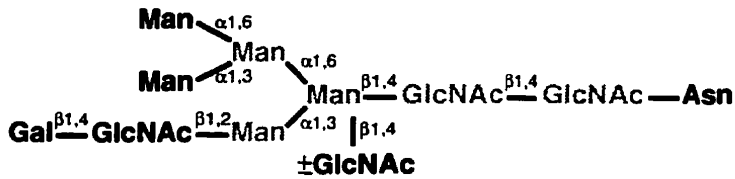
The N-linked oligosaccharide side chains of mammalian glycoproteins are characterized by their linkage via β-N-glycosidic bonds to the asparagine of Asn-X-Ser/Thr sequons (X≠Pro) found within membrane and secreted proteins (for review see Kornfeld and Kornfeld, 1985). The final structures of these oligosaccharides are very diverse, and can be classified as high mannose, complex or hybrid structures (Fig.1). Members of all three categories of N-linked oligosaccharides contain a common pentasaccharide core, $Man_3GlcNAc_2$, that is derived from an oligosaccharide precursor, usually $Glc_3Man_9GlcNAc_2$. In addition to this pentasaccharide core, the high mannose oligosaccharides typically contain two to six mannose residues linked α1,3, α1,6, and/or α1,2. The complex oligosaccharides are more heterogeneous, containing N-acetylglucosamine, fucose, galactose and sialic acid residues attached to the core structure. They can be very large, exist as bi-, tri-, or tetra-antennary forms, and can also be substituted with single sugars such as a fucose (linked α1,6 to the Asn-linked N-acetylglucosamine) and N-acetylglucosamine (linked β1,4 to the β-linked mannose of the pentasaccharide core). The hybrid oligosaccharides contain features of both high mannose and complex type oligosaccharides. Like the complex structures, hybrid oligosaccharides

Fig.1. Examples of the different types of N-linked oligosaccharides. A variety of structures ranging in size from the smaller high mannose oligosaccharides to the very large complex oligosaccharides such as the polylactosaminoglycans exist. The sugar residues of the common pentasaccharide core found in all N-linked oligosaccharides are shown in grey.

High mannose:

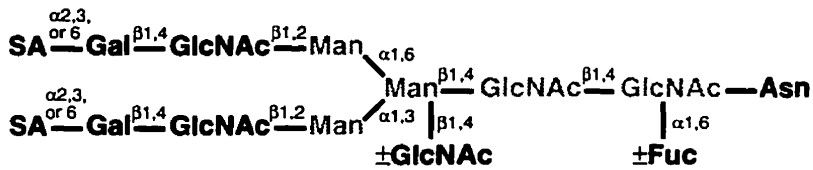


Hybrid:

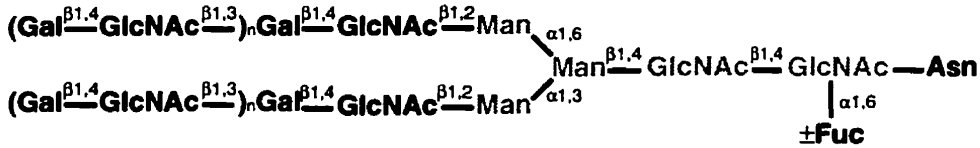


Complex:

a) Biantennary



b) Polylactosaminoglycan



can be bisected by a N-acetylglucosamine linked β1,4 to the β-mannose of the pentasaccharide core.

1.3. Biosynthesis of N-linked oligosaccharides.

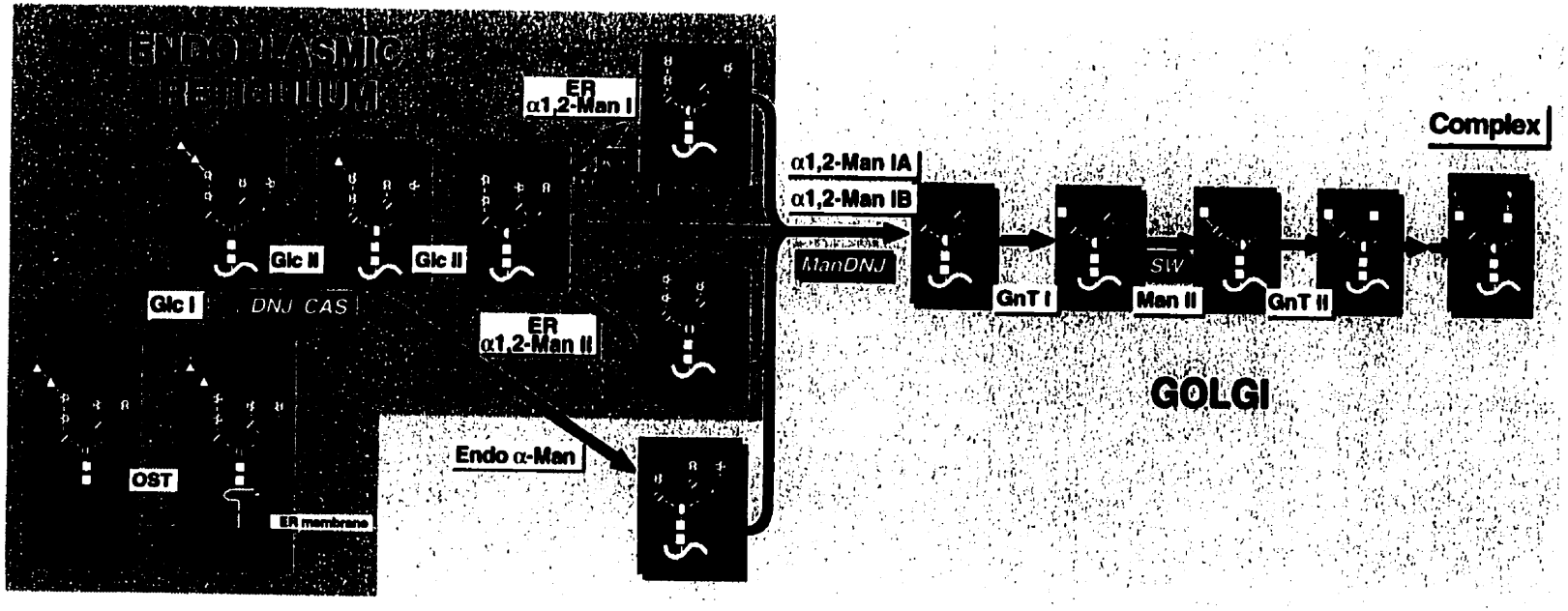
The modifications of the common precursor, $Glc_3Man_9GlcNAc_2$, to yield the diverse array of high mannose, complex and hybrid structures observed *in vivo* are catalyzed by a series of ER and Golgi resident enzymes. In higher eucaryotes, the biosynthesis of N-linked oligosaccharides can be divided into four major stages; 1) the synthesis of a dolichol-linked oligosaccharide precursor, 2) its subsequent transfer to protein, 3) the action of ER and Golgi processing enzymes to yield the oligosaccharide intermediate, $Man_5GlcNAc_2$, and 4) the action of α-mannosidase II and the glycosyltransferases in the Golgi to yield complex and hybrid structures.

The initial assembly of the oligosaccharide precursor begins by the formation of a lipid-linked oligosaccharide on the cytoplasmic face of the ER, where two N-acetylglucosamine and five mannose, derived from their respective nucleotide sugars (UDP-GlcNAc and GDP-Man) are sequentially added to dolichol phosphate by specific glycosyltransferases to yield $Man_5GlcNAc_2$-PP-dolichol (Hubbard and Ivatt, 1981; Snider and Robbins, 1982; Snider and Rogers, 1984). Once the $Man_5GlcNAc_2$-PP-dolichol precursor is formed, it is transferred across the ER membrane so that the oligosaccharide moiety faces the ER lumen. The mechanism by which this translocation occurs is unknown. Studies, however, indicate that dolichol derivatives do not spontaneously translocate across the lipid bilayer (McCloskey and Troy, 1980), and that their translocation may be protein mediated (Rush and Waechter, 1995). In the ER lumen, an additional seven sugars (four mannose, three glucose), derived from the lipid intermediates Dol-P-Man and Dol-P-Glc, are added to the precursor to yield $Glc_3Man_9GlcNAc_2$-PP-dolichol. The oligosaccharide moiety, $Glc_3Man_9GlcNAc_2$, is then transferred *en bloc* to the asparagine of the sequon Asn-X-Ser/Thr (where X≠Pro) of a nascent polypeptide chain. This transfer of the oligosaccharide moiety to protein is catalyzed by oligosaccharyltransferase, a large heteroligomeric membrane protein complex of at least three subunits in vertebrates (four in pig) and eight in yeast (for review see Silberstein and Gilmore, 1996), resulting in the formation of an N-C bond between the amide N of the asparagine and the C1 of the N-acetylglucosamine at the reducing end.

Once transfer of the oligosaccharide to the nascent peptide has occurred, the $Glc_3Man_9GlcNAc_2$ structure is subjected to processing by a series of ER and Golgi glycosidases and glycosyltransferases (Fig.2). The first modification made to the $Glc_3Man_9GlcNAc_2$ is the removal of the α1,2 glucose to yield $Glc_2Man_9GlcNAc_2$. This reaction is catalyzed by an ER resident enzyme, glucosidase I, which has been isolated and characterized in a number of species (Kilker *et al.*, 1981; Hettkamp *et al.*, 1984; Bause *et al.*, 1986; Schweden *et al.*, 1986; Shailubhai *et al.*, 1987; Bause *et al.*, 1989), and recently cloned from human hippocampus (Kalz-Füller *et al.*, 1995). Following glucosidase I trimming, glucosidase II catalyzes the removal of the two α1,3 linked glucose residues to yield $Man_9GlcNAc_2$ (Grinna and Robbins, 1979; Grinna and Robbins, 1980; Burns and Touster, 1982; Saunier *et al.*, 1982; Brada *et al.*, 1990). Although most of the glucose removal occurs by glucosidases, some deglucosylation also occurs by an alternate pathway involving a unique endo-α-mannosidase that can cleave the oligosaccharide chain internally, removing $Glc_{1-3}Man$ from $Glc_{1-3}Man_{9-4}GlcNAc_2$ to yield $Man_{8-3}GlcNAc_2$ (Lubas and Spiro, 1987; Lubas and Spiro, 1988; Moore and Spiro, 1990; Fujimoto and Kornfeld, 1991). Evidence for the existence of this pathway is supported by the observations that, when castanospermine or 1-deoxynojirimycin (inhibitors of glucosidase I) are added to normal cells, or when oligosaccharide biosynthesis is examined in glucosidase II deficient cell lines, some complex oligosaccharides are still formed. Addition of the endo-α-mannosidase specific inhibitor Glcα1,3(1-deoxy)mannojirimycin to either castanospermine inhibited cells or glucosidase deficient cells, abolishes the formation of complex oligosaccharides, and results in the accumulation of glucosylated polymannose structures (Hiraizumi *et al.*, 1993).

The $Man_9GlcNAc_2$ can then be processed by number of different ER and Golgi α-mannosidases specific for the removal of up to four α1,2-linked mannose residues to yield $Man_{8-5}GlcNAc_2$. *In vivo* studies suggest that α1,2-mannosidase trimming is initiated in the ER of mammalian cells. Studies with the inhibitor 1-deoxymannojirimycin indicate that there are 1-deoxymannojirimycin sensitive ER α1,2-mannosidase activities that yield $Man_{6-5}GlcNAc_2$ in rat liver (Bischoff and Kornfeld, 1983) and $Man_6GlcNAc_2$ in pig liver (Schweden and Bause, 1989). In BHK cells, a specific isomer of $Man_8GlcNAc_2$ (isomer B), identical to the intermediate in yeast oligosaccharide processing, is formed by a 1-deoxymannojirimycin sensitive ER α-mannosidase (Rizzolo and Kornfeld, 1988).

Fig.2. Mammalian N-linked oligosaccharide processing pathway. The enzymes involved in processing the oligosaccharide precursor, $Glc_3Man_9GlcNAc_2$, to yield complex structures are shown in white boxes. Inhibitors of the pathway are shown in black boxes. The abbreviations used are as follows: OST, oligosaccharyltransferase; Glc I, α-glucosidase I; Glc II, α-glucosidase II; ER α1,2-Man I; ER α1,2-mannosidase I; ER α1,2-Man II, ER α1,2-mannosidase II; α1,2-Man IA, Golgi α1,2-mannosidase IA; α1,2-Man IB, Golgi α1,2-mannosidase IB; Endo α-Man, endo α-mannosidase; GnTI, N-acetylglucosaminyltransferse I; GnTII, N-acetylglucosaminyltransferase II; DNJ, 1-deoxynojirimycin; CAS, castanospermine; KIF, kifunensine; ManDNJ, 1-deoxymannojirimycin; SW, swainsonine.



Key:

□ β GlcNAc
◐ β 1, 4-Man

⊕ α 1, 2-Man
⊘ α 1, 3-Man

● α 1, 6-Man
▲ α 1, 2-Glc

△ α 1, 3-Glc

◍ α Fuc
β GlcNAc
α GalNAc

α or β Gal
α Sialic acid

The same $Man_8GlcNAc_2$ isomer is also formed by a 1-deoxymannojirimycin resistant α1,2-mannosidase activity in rat liver (Bischoff *et al.*, 1986). Recently, two other 1-deoxymannojirimycin sensitive ER mannosidase activities, ER mannosidase I and ER mannosidase II, have been described (Weng and Spiro, 1993; Weng and Spiro, 1996). ER mannosidase I is kifunensine sensitive, is inactive with *p*-nitrophenyl α-D-mannoside, and removes the α1,2-mannose on the middle branch yielding $Man_8GlcNAc_2$ (isomer B). This enzyme and the ER α-mannosidase activity described Rizzolo *et al.* (Rizzolo and Kornfeld, 1988) may be the same enzymes, but this has not yet been confirmed. In contrast, ER mannosidase II is kifunensine resistant, is active with *p*-nitrophenyl α-D-mannosidase, and removes the α1,2-mannose on the outer α1,6 branch yielding $Man_8GlcNAc_2$ (isomer C). A number of Golgi α1,2-mannosidase activities involved in the removal of α1,2-mannose to yield $Man_{6-5}GlcNAc_2$ have also been identified (Tabas and Kornfeld, 1979; Tulsiani *et al.*, 1982; Schweden *et al.*, 1986; Forsee *et al.*, 1989; Bause *et al.*, 1993; Herscovics *et al.*, 1994; Lal *et al.*, 1994; Inoue *et al.*, 1995; Kerscher *et al.*, 1995; Ren *et al.*, 1995; Yoshida and Ichishima, 1995; Hamagashira *et al.*, 1996). Biochemical evidence and cloning studies reveal that there are at least two Golgi α1,2-mannosidase activities in rat and mouse, that are involved in trimming to $Man_5GlcNAc_2$ (Tulsiani *et al.*, 1982; Herscovics *et al.*, 1994; Lal *et al.*, 1994).

The $Man_5GlcNAc_2$ produced by the α1,2-mannosidases is then modified by N-acetylglucosaminyltransferase I, an enzyme that transfers an N-acetylglucosamine residue to the α1,3 mannose of the pentasaccharide core (Kornfeld and Kornfeld, 1985; Kumar *et al.*, 1992). This transfer marks the beginning of the branching process that will eventually yield hybrid and complex oligosaccharide structures. The addition of N-acetylglucosamine by N-acetylglucosaminyltransferase I is followed by the action of α-mannosidase II, which removes the α1,3 and α1,6 mannose to yield the structure $GlcNAcMan_3GlcNAc_2$ (Moremen, 1989; Moremen and Robbins, 1991; Misago *et al.*, 1995). This structure is then modified by a series of specific glycosyltransferases that add monosaccharides such as galactose, fucose, and sialic acid derived from their respective nucleotide sugars (Kornfeld and Kornfeld, 1985; Paulson and Colley, 1989)

1.4. Genes of the glycosylation pathway.

Little is known about the regulation of the enzymes involved in N-linked oligosaccharide maturation, however the diverse array of N-linked oligosaccharides that are produced at different stages of development, differentiation and oncogenic transformation, suggest that these enzymes, the glycosidases and glycosyltransferases, are tightly regulated. As a first step in understanding this regulation, the genes for a number of these enzymes have been cloned and characterized.

The genomic organization of the glycosyltransferases has been the focus of much work in this field since many of their cDNAs have been cloned (Joziasse, 1992). The type of genomic organization observed for these enzymes can be divided into two categories, those which are encoded for by a single exon, and those that are multiexonic. A number of these enzymes, such N-acetylglucosaminyltransferase I (Kumar *et al.*, 1992), N-acetylglucosaminyltransferase II (Tan *et al.*, 1995), and human α1,3-fucosyltransferase (Lowe *et al.*, 1991; Weston *et al.*, 1992) have their coding regions contained within a single exon. In contrast, others, such as β1,4-galactosyltransferase (6 exons) (Hollis *et al.*, 1989; Mengle-Gaw *et al.*, 1991), N-acetylglucosaminyltransferase V(16 exons) (Saito *et al.*, 1995), α2,6 sialyltransferase (6 exons) (Svensson *et al.*, 1990; Wang *et al.*, 1990; Wen *et al.*, 1992), and α1,3-galactosyltransferase (9 exons) (Joziasse *et al.*, 1992) are large multiexonic genes spanning ≥35 kb (α1,3-galactosyltransferase) to ~140 kb (N-acetylglucosaminyltransferase V). Although the Golgi glycosyltransferases share a similar domain structure at the protein level, this structural similarity does not appear to be reflected in their genomic organization (Joziasse, 1992). Northern blot analysis of these enzymes reveals that they exhibit developmental and tissue-specifc patterns of expression (for review see Kleene and Berger, 1993).

Knowledge of the genomic structure for the glycosyltransferases has permitted investigators to gain insight into their regulation, and to determine that this regulation involves a number of different mechanisms. Studies on genes such as the β1,4-galactosyltransferase and the α2,6-sialyltransferase genes show that alternate promoter usage, alternative splicing, and differential usage of translational and transcriptional start sites may all play a role (for review see Dinter and Berger, 1995). For example, the multiple transcripts observed for β1,4-galactosyltransferase arise from the use of different transcriptional start sites and alternate promoters (Hollis *et al.*, 1989; Harduin-Lepers *et al.*,

1992; Harduin-Lepers *et al.*, 1993), while those transcripts observed for α2,6-sialyltransferase arise from the use of alternate promoters and alternative splicing (Wen *et al.*, 1992). Different enzymes have different mechanisms of regulation, however there is evidence that the homologous enzymes in different species may be similarly regulated.

The information gathered on genomic structure for different enzymes has also been extremely useful in the genetic studies aimed at evaluating the role of the enzymes *in vivo*. Transgenic experiments in which the N-acetylglucosaminyltransferase I gene is disrupted demonstrate that this enzyme and the formation of complex and hybrid oligosaccharides is essential for embryonic development (Ioffe and Stanley, 1994; Metzler *et al.*, 1994).

There is no information on the genomic organization of genes encoding the α-glucosidases and the α1,2-mannosidases, enzymes that act at the early stages of N-linked oligosaccharide processing.

1.5. Genetic diseases of glycosylation.

The existence of human genetic N-glycosylation diseases provides *in vivo* evidence that interfering with the processing and modification of N-linked oligosaccharides can have severe biological consequences. These genetic diseases have been shown to be caused by a number of different mechanisms, including defects in N-linked oligosaccharide processing enzymes, deficiencies in the metabolism of the nucleotide sugars, and the lack of oligosaccharide phosphorylation.

Congenital dyserythropoietic anaemia type II (HEMPAS) is an autosomal recessive disease that is characterized by erythroid hyperplasia in the bone marrow, with mature erythroblasts being bi- or multinucleated (Fukuda, 1990). The erythrocyte band 3 and 4.5 glycoproteins are underglycosylated, with their characteristic polylactosaminoglycan chains being replaced by shorter hybrid and high mannose structures. These shorter structures arise, in one case because of a lack of α-mannosidase II activity, and in others because of lowered levels of *N*-acetylglucosaminyltransferase II activity, both of which block oligosaccharide maturation. There are also HEMPAS patients for which the defect is unknown, suggesting that this disease may result from any of a heterogeneous collection of glycosylation deficiencies. As a result of these deficiencies, HEMPAS patients suffer from a variety of symptoms such as life long anaemia, splenomegaly, jaundice, hepatomegaly, cirrhosis of the liver, diabetes and gallstones.

The carbohydrate deficient glycoprotein (CDG) syndromes are another group of genetic diseases characterized by the aberrant N-glycosylation of secretory glycoproteins, lysosomal enzymes, and membrane bound glycoproteins (Charuk *et al.*, 1995). These syndromes are multisystemic congenital disorders that result in severe neurological symptoms and/or psychomotor retardation. CDG syndrome type I is characterized by a decreased number of oligosaccharide chains present on glycoproteins. The deficiency involved has been identified as a defect in phosphomannomutase, which catalyzes the conversion of mannose 6-phosphate to mannose 1-phosphate (Van Schaftingen and Jaeken, 1995). A defect in this enzyme results in the failure to generate mannose 1-phosphate, and hence the GDP-mannose needed in the assembly of the dolichol-linked oligosaccharide precursor. A deficiency of this enzyme in yeast is lethal (Kepes and Schekman, 1988). CDG syndrome type II, in contrast to CDG syndrome type I, is characterized by the absence of complex glycoforms of serum transferrin and other glycoproteins, rather than a decreased number of oligosaccharide chains (Charuk *et al.*, 1995). The absence of complex glycoforms has been attributed to a deficiency in N-acetylglucosaminyltransferase II activity. Although, CDG syndrome type II involves a deficiency in the same step of the pathway as in some HEMPAS patients, the pathology of these diseases is quite different.

A defect in fucose metabolism, specifically deficient biosynthesis of GDP-fucose, resulting in an absence of fucosylated glycoproteins has been shown to cause a congenital neutrophil disorder called leukocyte adhesion deficiency syndrome type II (Etzioni *et al.*, 1995). This disorder is characterized by the failure of neutrophils to express the fucosylated sialyl Lewis X oligosaccharide, which is essential for selectin binding (Phillips *et al.*, 1995). In the absence of sialyl Lewis X, the selectin mediated recruitment and adhesion of neutrophils is interrupted, interfering with events of the host defense mechanism. Patients lacking fucosylated glycoproteins are short in stature and suffer from mental retardation and recurrent bacterial infections.

The failure of lysosomal glycoproteins to be targeted to the lysosome is also the result of a genetic defect that alters the final structure of N-linked oligosaccharides. Targeting of newly synthesized lysosomal enzymes such as α-mannosidase, α-fucosidase, and β-galactosidase to the lysosomes is mediated by recognition of a phosphorylated mannose, mannose 6-phosphate, by mannose 6-phosphate receptors in the Golgi (Kornfeld, 1990). In I-cell disease, the mannose residues of the N-linked oligosaccharides

are not phosphorylated due to a deficiency in N-acetylglucosamine 1-phosphotransferase (Reitmann *et al.*, 1981). As a result, mannose 6-phosphate is not generated and the lysosomal glycoproteins cannot be recognized by the mannose 6-phosphate receptor. This results in a multiple lysosomal enzyme insufficiency due to a disruption of mannose 6-phosphate mediated lysosomal targeting.

1.6. The α-mannosidases.

The regulation of α-mannosidase trimming is poorly understood and appears to be more complicated than previously thought. Multiple ER and Golgi α-mannosidases with similar specificities have been purified and cloned from different species, showing that several α-mannosidases are involved in the trimming process. These α-mannosidases have been separated and characterized by a large number of techniques, including ion-exchange and affinity chromatography, subcellular fractionation, their affinity for synthetic substrates and inhibitors, and their ability to be stimulated or inhibited by cations. These biochemical studies reveal the existence of distinct types of α-mannosidases. There are α-mannosidases that remove four α1,2-linked mannose residues at early stages of the biosynthetic pathway, α-mannosidases that act at intermediate stages of the pathway to remove α1,3- and α1,6-linked mannose, and also α-mannosidases involved in the degradation of free oligosaccharides during glycoprotein turnover. Recent cloning studies of the α-mannosidases isolated from various tissues and species indicate that these enzymes can be grouped into two classes, Class I and Class II, based on amino acid sequence similarity [Moremen, 1994 #34]. Between the two classes, the enzymes can be distinguished not only by their unrelated sequences, but also by their differential sensitivity to inhibitors and by their differences in ability to hydrolyze aryl-α-mannosides. The Class I α-mannosidases hydrolyze only α1,2-mannosidic linkages. They range in size from 49-73 kDa, and can exist as both soluble or membrane bound enzymes. They are inhibited by 1-deoxymannojirimycin, but not by swainsonine, and cannot hydrolyze the synthetic substrate *p*-nitrophenyl α-D-mannoside. The Class II α-mannosidases are more heterogeneous in their specificity, hydrolyzing α1,2, α1,3, and α1,6 mannosidic linkages. They are inhibited by swainsonine, but not by 1-deoxymannojirimycin, and hydrolyze *p*-nitrophenyl α-D-mannoside. The enzymes of this class include the ER/cytosolic,

lysosomal, and vacuolar α-mannosidases, as well as the processing Golgi α-mannosidase II.

1.6.1. The Class I α-mannosidases.

The Class I α-mannosidases act at early stages of N-linked oligosaccharide processing, providing the substrate for the synthesis of complex and hybrid oligosaccharides. Although many of these enzymes have been purified, characterized, or cloned, little is known about their regulation and respective physiological roles. In the following sections, the properties of the Class I α-mannosidases, both those classified as Class I on the basis of sequence similarity, and those whose enzymatic properties suggest that they are Class I α-mannosidases, will be discussed.

1.6.1.a. Yeast ER α1,2-mannosidase.

The *Saccharomyces cerevisiae* specific α1,2-mannosidase activity was first described following pulse-labeling experiments with [2-^{3}H]mannose in yeast. This activity was capable of catalyzing the removal of a single α1,2-mannose to yield $Man_8GlcNAc_2$ (isomer B) as a transient intermediate in N-linked oligosaccharide processing (Byrd *et al.*, 1982). Partial purification and characterization of this enzyme showed that it produced the specific $Man_8GlcNAc$ (isomer B). This α1,2-mannosidase was found to be inactive with the synthetic substrate *p*-nitrophenyl α-D-mannopyranoside and 1-deoxymannojirimycin sensitive (Jelinek-Kelly *et al.*, 1985). Purification of the yeast α1,2-mannosidase to homogeneity confirmed these results and also showed that the enzyme was dependent on calcium for activity (Jelinek-Kelly and Herscovics, 1988; Ziegler and Trimble, 1991). Localization of the yeast α1,2-mannosidase to the ER was suggested by the observation that glycoproteins carrying $Man_8GlcNAc_2$ oligosaccharides accumulated in the ER in the sec 18 mutant, a yeast strain deficient in ER to Golgi transport (Esmon *et al.*, 1984). This localization has recently been established by immunofluorescence and immunoelectron microscopy of yeast cells (Burke *et al.*, 1996).

The yeast α1,2-mannosidase was the first member of the Class I α-mannosidase to be cloned (Camirand *et al.*, 1991). The intronless α1,2-mannosidase gene (*MNS1*) encodes a 63 kDa type II membrane protein. It contains a non-cleavable hydrophobic signal sequence, a putative EF hand-like calcium-binding consensus sequence, three

potential N-glycosylation sites, and no known ER retention motifs. Disruption of the *MNS1* gene in yeast has no apparent effect on cell growth (Camirand *et al.*, 1991), and *MNS1* activity is not essential for outer chain elongation (Puccia *et al.*, 1993). Using *Saccharomyces cerevisiae* and *Pichia pastoris* expression systems, Lipari and Herscovics (Lipari and Herscovics, 1994; Lipari and Herscovics, 1996) have been able to produce milligram quantities of an active secreted soluble form of the yeast α1,2-mannosidase without the transmembrane domain, allowing structure function studies to be performed. NMR experiments using this soluble form determined that this enzyme catalyzes removal of the α1,2-mannose by a mechanism of inversion (Lipari *et al.*, 1995). A recent study demonstrates that the formation of a disulfide bond between two conserved cysteine residues found in all Class I α-mannosidases is essential for enzyme activity (Lipari and Herscovics, 1996).

1.6.1.b. Fungal α1,2-mannosidases.

Two isozymes of a soluble α1,2-mannose specific mannosidase have been purified and characterized from the fungus, *Penicillium citrinum* (Yoshida *et al.*, 1993). Analysis of these isozymes, 1,2-α-D-mannosidase Ia and Ib, showed that they have identical molecular weights (53 kDa), and identical amino acid sequences at their C and N terminals. They are both inactive with *p*-nitrophenyl α-D-mannoside. Although they have sequence similarity to other Class I α-mannosidases isolated, they do not require calcium for activation and are not inhibited by EDTA. 1-deoxymannojirimycin inhibits these enzymes at high concentration, and has been shown to mask a catalytically important aspartic acid residue (Yoshida *et al.*, 1994). Despite their similarity, these isozymes differ in their pI by 0.1 and behave differently under alkaline or acidic conditions. The reason for these differences is still unknown, but may be due to posttranslational modifications. The *Penicillium citrinum* 1,2-α-D-mannosidase gene (*MsdC*) spans four exons of genomic DNA (Yoshida and Ichishima, 1995). Sequence analysis reveals that the soluble 53 kDa enzyme is originally produced as a 56 kDa protein and that its hydrophobic signal sequence is proteolytically removed during growth in culture. Although the sequence appears to contain the putative EF hand calcium-binding domain found in the yeast α1,2-mannosidase, it includes an extra amino acid. Yoshida and Ichishima (Yoshida and Ichishima, 1995) suggest that this insertion may disrupt the formation of one of the α-helices of the helix-loop-helix EF hand

by introducing a conformational change, and changes the ability of this motif to bind calcium. This however is speculation, as no direct evidence has yet been obtained confirming that the EF hand motif is actually the site of calcium binding in this class of enzymes.

A 51 kDa soluble α1,2-mannosidase has also been purified and characterized from *Aspergillus saitoi* (Yamashita *et al.*, 1980; Ichishima *et al.*, 1981). Like the *Penicillium citrinum* enzyme, *Aspergillus saitoi* 1,2-α-D-mannosidase does not require calcium for activity, does not hydrolyze *p*-nitrophenyl α-D-mannoside, and is inhibited by 1-deoxymannojirimycin. Cloning of this 1,2-α-D-mannosidase (*MsdS*) indicates that this protein is originally formed with a hydrophobic signal sequence, and that the signal sequence is cleaved, releasing a soluble protein (Inoue *et al.*, 1995). The *Aspergillus saitoi* and *Penicillium citrinum* 1,2-α-D-mannosidase share 70% identity in amino acid sequence, including the extra amino acid in the EF hand-like putative calcium-binding domain.

1.6.1.c. Rat Golgi α-mannosidase I.

The first mammalian Class I α-mannosidase activity to be described was Golgi α-mannosidase I isolated from rat liver Golgi membrane extracts (Tabas and Kornfeld, 1979). α-Mannosidase I catalyzes the removal of four α1,2-mannose residues from $Man_9GlcNAc_2$ to yield $Man_5GlcNAc_2$. This activity was distinguished from other Golgi mannosidases by its inability to hydrolyze *p*-nitrophenyl α-D-mannoside and its inhibition by EDTA. Further studies using ion exchange chromatography and gel filtration resolved the α-mannosidase I activity into two distinct components, α-mannosidase IA and α-mannosidase IB (Tulsiani *et al.*, 1982). Golgi α-mannosidase IA is unable to bind a cellulose phosphate column at pH 7.2, whereas Golgi α-mannosidase IB is retained on the column and is eluted at low salt concentrations. α-Mannosidase IA exists as a tetramer of 230 kDa with 57 kDa subunits. This enzyme is glycosylated as determined by its ability to bind to Con A and is inactive with *p*-nitrophenyl α-D-mannoside. α-Mannosidase IB differs from α-mannosidase IA in its ability to hydrolyze trace amounts of *p*-nitrophenyl α-D-mannoside, however this activity could be due to contamination with Golgi Class II α-mannosidases. Both α-mannosidase IA and α-mannosidase IB trim to $Man_5GlcNAc_2$, are 1-deoxymannojirimycin sensitive, and are inhibited by EDTA (Tulsiani *et al.*, 1982; Tulsiani and Touster, 1988). Whether or not these enzymes require calcium for activity has never been studied. Studies

with antibodies generated against α-mannosidase IA suggest that these two enzymes share antigenic determinants (Tulsiani and Touster, 1988).

1.6.1.d. Calf and pig Man_9-mannosidases.

Calf liver crude microsomes were also found to contain α1,2-mannosidase activity (Schweden *et al.*, 1986). The calf Man_9-mannosidase catalyzes the selective removal of three α1,2-linked mannose to yield a specific $Man_6GlcNAc_2$ isomer. Its inability to bind Con A indicates that it does not carry complex or high mannose oligosaccharides. A similar activity was also identified in crude pig liver microsomes (Schweden and Bause, 1989). Purification of the pig Man_9-mannosidase yields a 49 kDa soluble protein that is not N-glycosylated. A 65 kDa membrane bound form of the enzyme can also be detected by Western blot analysis, indicating that the hydrophobic N-terminal of the protein is proteolytically removed to release a soluble form. Like the calf Man_9-mannosidase, the pig enzyme removes only three α1,2-mannose from $Man_9GlcNAc_2$. It has been localized to the ER by immunogold labeling of pig hepatocytes (Roth *et al.*, 1990). Even though they only remove three mannose, the pig and calf liver Man_9-mannosidases behave similarly to other Class I α-mannosidases. They are 1-deoxymannojirimycin sensitive, calcium-dependent, and do not hydrolyze *p*-nitrophenyl α-D-mannoside.

Studies with the pig Man_9-mannosidase suggest that the apparently unique substrate specificity observed by the pig and calf enzymes may be determined by the substrate (Bause *et al.*, 1992). When $Man_9GlcNAc_2$ is used as substrate, the pig enzyme cleaves only to $Man_6GlcNAc_2$, however, if the terminal N-acetylglucosamine is reduced or removed, the enzyme to trims to $Man_5GlcNAc$. This suggests that the substrate used may impose structural constraints on the enzyme and determine its highly selective specificity. If this is the case, the differences observed in trimming among the α1,2-mannosidases (specifically trimming to $Man_6GlcNAc_2$ or $Man_5GlcNAc$) may be due to the use of different substrates, rather than a reflection of different specificities *in vivo*.

1.6.1.e. Rabbit α1,2-mannosidase.

Rabbit liver microsomes contain an α-mannosidase activity capable of removing four α1,2-linked mannose to yield $Man_5GlcNAc_2$ (Forsee and Schutzbach, 1981). This rabbit liver α1,2-mannosidase activity is calcium-dependent and does not hydrolyze *p*-

nitrophenyl α-D-mannoside. Optimal activity for this enzyme is obtained only when it is surrounded by a specific phospholipid environment (Forsee *et al.*, 1982). Purification of the rabbit liver α1,2-mannosidase to homogeneity confirmed these enzymatic properties (Forsee *et al.*, 1989). Inhibitor studies indicate that like other Class I α-mannosidases, the rabbit enzyme is sensitive to 1-deoxymannojirimycin. Binding of the enzyme to Con A Sepharose indicates that it is N-glycosylated. The purified protein has a molecular weight of 52 kDa by SDS-PAGE. On the basis of N-terminal and CnBr peptide sequences, oligonucleotide primers were designed to generate a probe for screening a rabbit liver cDNA library (Lal *et al.*, 1994). Analysis of the rabbit liver α1,2-mannosidase cDNAs isolated indicated that the full length protein undergoes proteolysis during purification to yield the 52 kDa purified form. The active 52 kDa form lacks ~26% of the protein including its transmembrane domain, suggesting that the N-terminal region is not essential for enzyme activity. Northern blot analysis reveals three full length α1,2-mannosidase transcripts of 2.5, 2.6 and 4.7 kb, which differ in their 3' ends as a result of alternate use of polyadenylation signals.

1.6.1.f. Human α1,2-mannosidases.

A human Man_9-mannosidase cDNA was isolated from a human kidney cDNA library using cDNA probes derived from peptide sequences of the pig Man_9-mannosidase (Bause *et al.*, 1993). The cDNA of the human Man_9-mannosidase encodes a 625 amino acid type II membrane glycoprotein. When transiently expressed in COS-1 cells, the 73 kDa human kidney Man_9-mannosidase can be detected with the pig Man_9-mannosidase antibodies, and is found to localize to the Golgi apparatus (Bieberich and Bause, 1995). Because of the cross-reactivity between the human and pig enzymes, the fact that their localization differed was unexpected. The reason for this difference is unclear, however it is most likely due to differences in N-terminal sequence which is not yet known for the pig enzyme. Characterization of human kidney Man_9-mannosidase reveals that this enzyme is 1-deoxymannojirimycin sensitive and requires calcium for activity. Although it is specific for α1,2-linked mannose residues, it is unclear whether it removes three or four mannose residues.

Recently, a second putative human α1,2-mannosidase cDNA has been isolated from human placenta (Tremblay *et al.*, 1996). This human placenta α1,2-mannosidase cDNA,

termed human α1,2-mannosidase IB, encodes a 73 kDa protein. Like other members of the Class I α-mannosidases, it contains the twelve amino acid EF hand like calcium-binding domain. The activity of human α1,2-mannosidase IB has not yet been studied. Sequence analysis reveals that this cDNA shares ~65% amino acid identity with the human kidney Man_9-mannosidase and ~94% amino acid identity with murine α1,2-mannosidase IB described below.

1.6.1.g. Mouse α1,2-mannosidases.

Two murine Golgi α1,2-mannosidases, α1,2-mannosidase IA and α1,2-mannosidase IB, have been shown to exist. The α1,2-mannosidase IA cDNA was isolated from a murine 3T3 cell cDNA library using the rabbit liver α1,2-mannosidase specific probe described above (Lal *et al.*, 1994), while the α1,2-mannosidase IB cDNA was isolated from a mouse liver cDNA library using a murine α1,2-mannosidase IB specific probe. The murine α1,2-mannosidase IB specific probe was generated by PCR amplification of cDNA using degenerate oligonucleotide primers derived from sequences of highly conserved regions in yeast and rabbit (Herscovics *et al.*, 1994). Both α1,2-mannosidase IA and α1,2-mannosidase IB are calcium-dependent 73 kDa type II membrane proteins sharing 65% amino acid identity. They are inactive with *p*-nitrophenyl α-D-mannoside and are inhibited by 1-deoxymannojirimycin (Herscovics *et al.*, 1994; Lal *et al.*, 1994). Both enzymes catalyze the removal of four α1,2-mannose from $Man_9GlcNAc$ to yield $Man_5GlcNAc$ (Schneikert and Herscovics, 1994; Lal *et al.*, 1996). Murine α1,2-mannosidase IA shares 90% amino acid identity with the human kidney Man_9-mannosidase, and is immunologically related to rat liver Golgi α-mannosidase IA (Lal *et al.*, 1994). Southern blot analysis of mouse genomic DNA using α1,2-mannosidase IA and α1,2-mannosidase IB specific probes clearly indicates that they arise from two distinct genes (Herscovics *et al.*, 1994). This is supported by Northern blot analysis which demonstrates that they exhibit different patterns of tissue-specific expression in adult mouse tissues and during embryonic development (Herscovics *et al.*, 1994; Herscovics, unpublished data). When α1,2-mannosidase IA is used as a probe in Northern blot analysis, two transcripts of 2.7 kb and 4.8 kb arising from the differential use of polyadenylation signals are detected (Lal *et al.*, 1994). In contrast, multiple transcripts

ranging in size from 4.2 to 8.7 kb are seen when an α1,2-mannosidase IB specific probe is used (Herscovics *et al.*, 1994).

1.6.1.h. Hen α1,2-mannosidase.

Recently, an α1,2-mannosidase with the same substrate specificity as the calf and pig Man_9-mannosidase has been purified from hen oviduct (Hamagashira *et al.*, 1996). Characterization of the hen oviduct α1,2-mannosidase indicates that the molecular weight of this protein is 42 kDa by SDS-PAGE, or 50 kDa by gel filtration. The hen α1,2-mannosidase activity can be inhibited by EDTA and then restored by calcium. Like all Class I α-mannosidases characterized to date, it is inhibited by 1-deoxymannojirimycin and does not hydrolyze *p*-nitrophenyl α-D-mannoside. Cloning of the gene for this enzyme is required before it can be truly classified as a member of the Class I α-mannosidases.

1.6.1.i. Insect α1,2-mannosidases.

A Class I α1,2-mannosidase gene, *mas-1*, was recently isolated from *Drosophila melanogaster* (Kerscher *et al.*, 1995). The *D. melanogaster mas-1* gene yields two proteins that differ at their 5' ends through the use of alternate promoters and the alternate usage of exons 1a and 1b. Transcripts containing the 1a exon represent a rare late embryonic and adult head specific class of transcripts. In contrast, transcripts containing exon 1b are ubiquitously expressed throughout development. Both *mas-1a* and *mas-1b* encode type II transmembrane proteins of 72.5 kDa and 75 kDa, respectively. The substrate specificity and cation dependency of these enzymes is not yet known. However like the calcium-dependent α1,2-mannosidases, *mas-1* contains the putative EF-hand calcium-binding domain. The *mas-1* gene is expressed in the preblastoderm stage as an abundant maternal message uniformly distributed in the egg cytoplasm. During formation of the cellular blastoderm, this signal becomes restricted to the basal part of the forming blastoderm cells and the egg cytoplasm and then eventually disappears. In the developing *D. melanogaster*, *mas-1* is expressed strongly in the central nervous system, the tracheal system, and to a lesser extent in the peripheral muscles and in a segmentally repeated pattern in the ventral nerve cord. The null mutation of *mas-1* produces a number of developmental defects. One sees intersegmental nerve pathfinding errors in the embryonic peripheral nervous system, and abnormal clusters of sensory organs. In the adult, the wing and longitudinal veins are

often incomplete and end in deltas. Mutant eyes are also observed, displaying patches with imperfectly aligned ommatidia and extra bristles. These observations indicate that the α1,2-mannosidases may play important roles in guidance mechanisms of the nervous system.

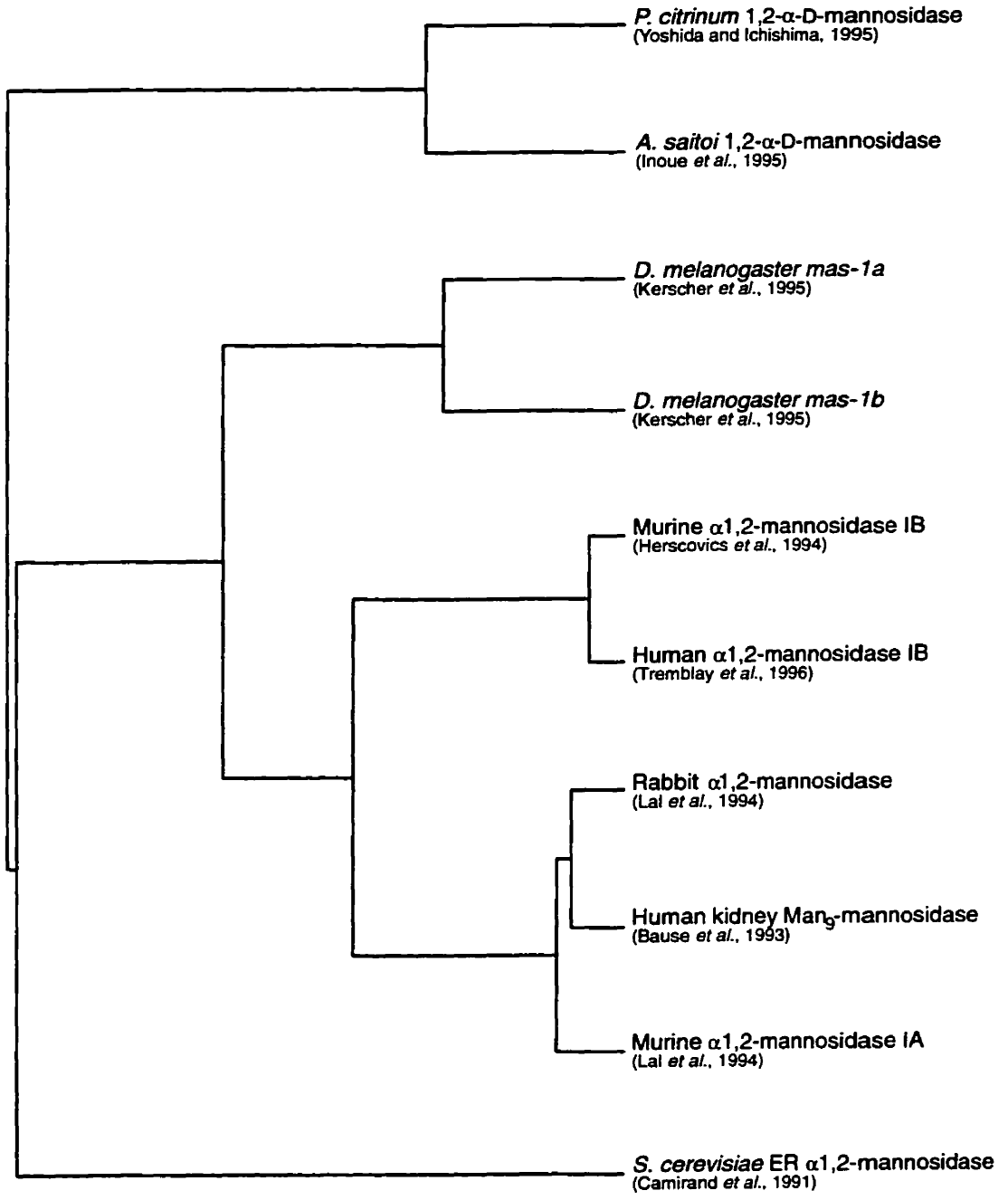
Evidence has also been gathered that lepidopterin insect cells (*Spodoptera frugiperda*) (IPLB-SF-21AE) contain a Class I α-mannosidase activity that is enhanced upon baculovirus infection (Ren *et al.*, 1995). The enzymatic properties of this 63 kDa Golgi membrane bound α1,2-mannosidase are similar to other Class I α-mannosidases. It is inhibited by 1-deoxymannojirimycin and by EDTA, and is inactive on *p*-nitrophenyl α-D-mannoside. It differs from the other Class I enzymes in its unique specificity. This enzyme cleaves the α1,2-mannose linkage from the specific Man_6 isomer Man (α1,2)Manα1,3[Manα1,3-[Manα1,6]Manα1,6Manβ1,4GlcNAc$_2$ with high affinity, and has weaker affinity for $Man_{9-7}GlcNAc_2$.

Recently, a second insect cell α1,2-mannosidase that shares ~46% identity with human kidney Man_9-mannosidase and ~57% identity with *D. melanogaster* mannosidase-1 has been isolated from lepidopterin Sf9 cells (Kwar *et al.*, 1996). This α1,2-mannosidase is a 670 amino acid type II membrane protein that is capable of trimming $Man_9GlcNAc$.

1.6.1.j. Comparison of the Class I α-mannosidases.

The α1,2-mannosidases described above, although differing in substrate specificity, cation dependency and subcellular localization, are strikingly similar between species. All of the Class I α-mannosidases described to date are sensitive to 1-deoxymannojirimycin and inactive against the substrate *p*-nitrophenyl α-D-mannoside. Sequence comparisons of the cloned α1,2-mannosidases (*S. cerevisiae*, rabbit, mouse, human, *D. melanogaster*, *P. citrinum*, and *A. saitoi*) indicate that these enzymes share a high degree of amino acid sequence similarity throughout the catalytic domain (Fig.3). The yeast ER α1,2-mannosidase exhibits ~36% identity at the amino acid level with murine α1,2-mannosidase IA, murine α1,2-mannosidase IB, rabbit α1,2-mannosidase, and human kidney Man_9-mannosidase, and exhibits ~25% identity with the fungal enzymes. The fungal and insect enzymes also share 30-37% identity with the murine α1,2-mannosidases and the human Man_9-mannosidase. Amino acid sequence alignment of the cloned

Fig.3. Dendrogram depicting the amino acid sequence relationships between the Class I α-mannosidases. All members of this class share significant amino acid identity. The yeast ER, *D. melanogaster*, and the fungal α–mannosidases share ~30-37% identity with the mammalian enzymes. Murine α1,2-mannosidase IB and α1,2-mannosidase IB share 65% identity. Human kidney Man_9-mannosidase is the human homologue of mouse α1,2-mannosidase IB, exhibiting ~90% identity, while the putative human α1,2-mannosidase IB shares >90% identity with mouse α1,2-mannosidase IB. Sequence comparison of the Class I α-mannosidases reveals two distinct groups of mammalian α1,2-mannosidases. The first group contains species variants of the α1,2-mannosidase IA enzymes, and includes α1,2-mannosidase IA, human kidney Man_9-mannosidase, and rabbit α1,2-mannosidase. The second group contains the α1,2-mannosidase IB enzymes, murine α1,2-mannosidase IB and human α1,2-mannosidase IB. This nomenclature stems from the observation that murine α1,2-mannosidase IA cross-reacts with an antibodies specific to rat liver Golgi α-mannosidase IA. Murine α1,2-mannosidase IB may be related to rat liver Golgi α-mannosidase IB, however this has not been determined. Between the α1,2-mannosidase IA and IB enzymes, there is ~65% amino acid identity. Sequence identity within these groups ~90%. The dendrogram was prepared using the Pileup program of the University of Wisconsin Genetics Computer Group software (version 8).


P. citrinum 1,2-α-D-mannosidase
(Yoshida and Ichishima, 1995)
A. saitoi 1,2-α-D-mannosidase
(Inoue *et al.*, 1995)
D. melanogaster mas-1a
(Kerscher *et al.*, 1995)
D. melanogaster mas-1b
(Kerscher *et al.*, 1995)
Murine α1,2-mannosidase IB
(Herscovics *et al.*, 1994)
Human α1,2-mannosidase IB
(Tremblay *et al.*, 1996)
Rabbit α1,2-mannosidase
(Lal *et al.*, 1994)
Human kidney Man_9-mannosidase
(Bause *et al.*, 1993)
Murine α1,2-mannosidase IA
(Lal *et al.*, 1994)
S. cerevisiae ER α1,2-mannosidase
(Camirand *et al.*, 1991)

α1,2-mannosidases reveals highly conserved sequence motifs such as FNTEAH, GDSFYEYLLK, and SFFLAETLK (Fig.4). The roles, if any, that these motifs play in the regulation or function of the α1,2-mannosidases are still unknown. All of the cloned calcium-dependent α1,2-mannosidases contain a twelve amino acid EF-hand like putative calcium-binding domain. Direct involvement of this domain in calcium binding has not been demonstrated, but it is disrupted in the calcium independent α1,2-mannosidases by the insertion of an extra amino acid. If this EF-hand is indeed the calcium-binding domain, this observation suggests that the requirement for calcium could have developed through evolution.

As previously mentioned, the mammalian Class I α-mannosidases have been localized to both the Golgi and the ER. While the pig Man_9-mannosidase is localized to the ER, the highly similar human Man_9-mannosidase is localized to the Golgi. The implications of these localization differences are important, as it suggests that members of the Class I α-mannosidases play a role in ER mannose trimming, and that there may be other ER Class I α-mannosidases that have not yet been described in other species.

1.6.2. Class II α-mannosidases.

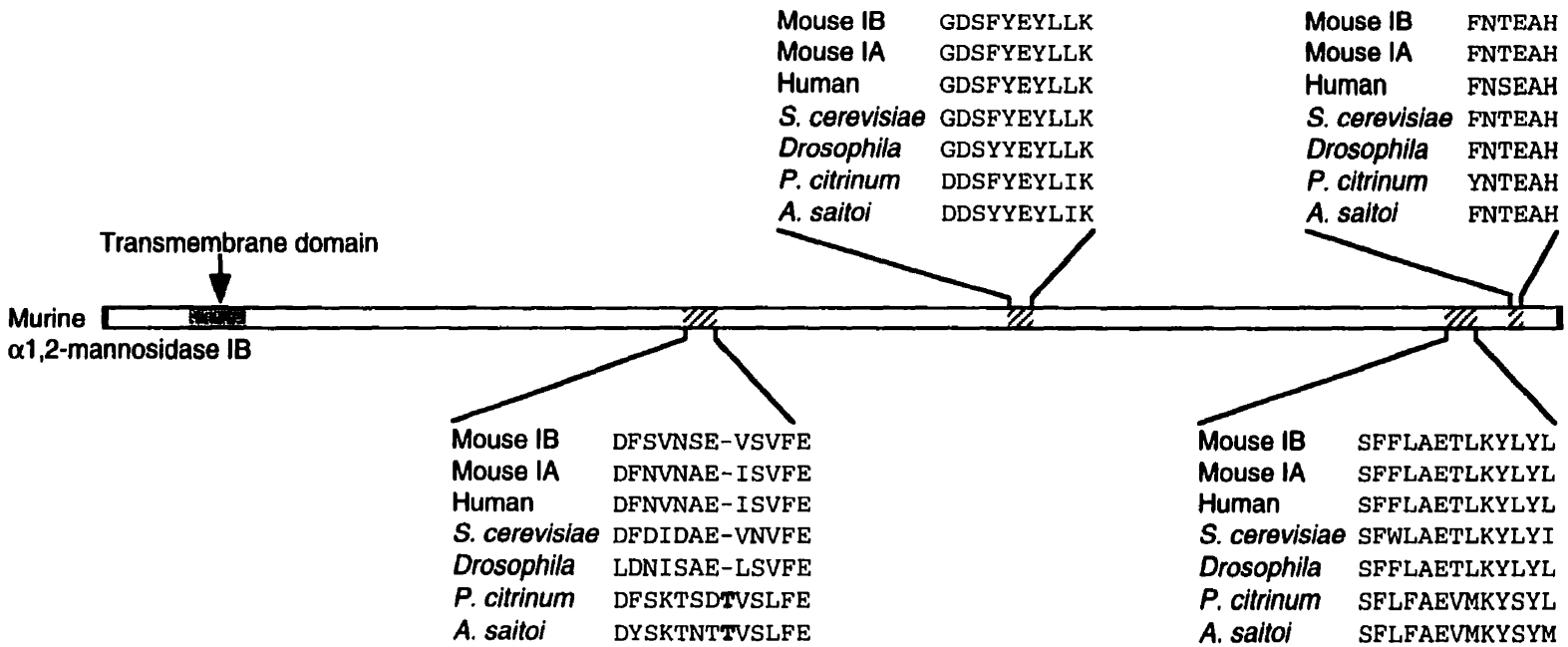
Unlike the Class I α-mannosidases, the activity of the Class II α-mannosidases is not limited to processing reactions during N-linked oligosaccharide maturation. Some members of this class also play roles in glycoprotein catabolism. The Class II α-mannosidases are heterogeneous in their oligosaccharide specificity, trimming α1,3-, α1,6- and α1,2-linked mannose, and in their subcellular localization, being found in the ER, cytosol, Golgi, lysosomes and yeast vacuoles. Despite these striking differences, they share regions of significant sequence similarity (Fig.5). In the following sections, the different class II α-mannosidases will be discussed.

1.6.2.a. Golgi α-mannosidase II.

α-Mannosidase II was first described in rat liver Golgi membranes as an enzyme capable of hydrolyzing the substrate *p*-nitrophenyl α-D-mannoside (Dewald and Touster, 1973). It has been purified to homogeneity from different sources (Tulsiani *et al.*, 1977;

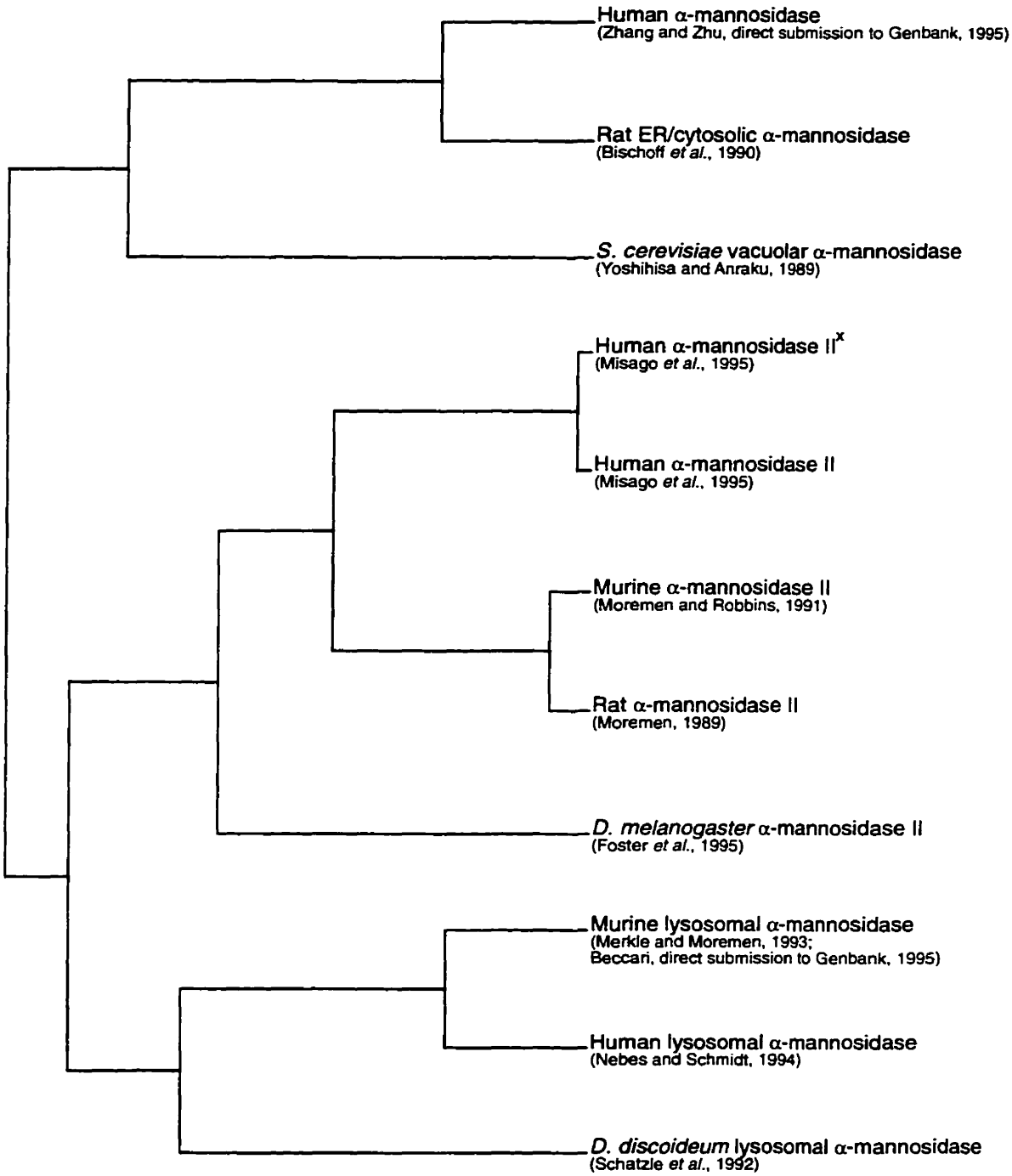
Fig.4. Highly conserved sequence motifs of the Class I α-mannosidases. Alignment of the amino acid sequences of the conserved motifs from different species is shown, and their position with respect to the murine α1,2-mannosidase IB cDNA is indicated. The extra amino acid found in EF hand putative calcium-binding domain of the fungal enzymes is indicated in bold.

(Mouse IA = murine α1,2-mannosidase IA; Mouse IB = murine α1,2-mannosidase IB; Human Man9 = human kidney Man9-mannosidase; *S. cerevisiae* = *S. cerevisiae* ER α1,2-mannosidase; *D. melanogaster* = *D. melanogaster mas-1*; *P. citrinum* = *P. citrinum* 1,2-α-D-mannosidase; *A. saitoi* = *A. saitoi* 1,2-α-D-mannosidase).



Putative EF hand Ca^{2+} binding domain

Fig.5. Dendrogram depicting the amino acid sequence relationships between members of the Class II α-mannosidases. The human lysosomal α-mannosidase and the *Dictyostelium discoideum* share share ~38% sequence identity, while the ER/cytosolic α-mannosidase and the *S. cerevisiae* vacuolar α-mannosidase share ~33% identity. The dendrogram was prepared using the Pileup program of the University of Wisconsin Genetics Computer Group software (version 8). Sequences representing the full length proteins were used.


Human α-mannosidase
(Zhang and Zhu, direct submission to Genbank, 1995)
Rat ER/cytosolic α-mannosidase
(Bischoff *et al.*, 1990)
S. cerevisiae vacuolar α-mannosidase
(Yoshihisa and Anraku, 1989)
Human α-mannosidase II[x]
(Misago *et al.*, 1995)
Human α-mannosidase II
(Misago *et al.*, 1995)
Murine α-mannosidase II
(Moremen and Robbins, 1991)
Rat α-mannosidase II
(Moremen, 1989)
D. melanogaster α-mannosidase II
(Foster *et al.*, 1995)
Murine lysosomal α-mannosidase
(Merkle and Moremen, 1993;
Beccari, direct submission to Genbank, 1995)
Human lysosomal α-mannosidase
(Nebes and Schmidt, 1994)
D. discoideum lysosomal α-mannosidase
(Schatzle *et al.*, 1992)

Kaushal *et al.*, 1990; Moremen *et al.*, 1991; Foster *et al.*, 1995). Rat liver α-mannosidase II differs from Golgi Class I α-mannosidases in its oligosaccharide specificity, hydrolyzing two terminal mannose residues, linked α1,3 and α1,6, from $GlcNAcMan_5GlcNAc_2$ to yield $GlcNAcMan_3GlcNAc_2$ (Tulsiani *et al.*, 1982). This trimming requires the prior addition of *N*-acetylglucosamine by *N*-acetylglucosaminyltransferase I.

Rat liver α-mannosidase II is a glycosylated homodimer with ~124kDa subunits as determined by SDS-PAGE (Moremen and Touster, 1985; Moremen *et al.*, 1991). It is resistant to inhibition by 1-deoxymannojirimycin and EDTA, but strongly inhibited by swainsonine (Tulsiani *et al.*, 1982; Tulsiani *et al.*, 1982). Proteolytic cleavage of this enzyme with chymotrypsin releases an active soluble peptide of 110 kDa (Moremen *et al.*, 1991). NH_2-terminal sequence data obtained from the 110 kDa peptide was used to generate probes to isolate an α-mannosidase II cDNA from a mouse 3T3 cDNA library (Moremen and Robbins, 1991). Sequence analysis reveals that the murine α-mannosidase II cDNA encodes a ~131 kDa type II membrane protein. The size discrepancy between the purified enzyme and the actual size of the cloned enzyme have been attributed to abnormal migration of the purified protein on SDS-PAGE. When murine α-mannosidase II is overexpressed in COS cells, detection with the anti-rat α-mannosidase II antibody confirms that it is Golgi localized.

An α-mannosidase II cDNA has also been isolated from human liver (Misago *et al.*, 1995). The human liver α-mannosidase II cDNA encodes a 1144 amino acid type II membrane protein. While screening for α-mannosidase II-containing genomic clones, a second related α-mannosidase II gene, α-mannosidase II^x, was identified (Misago *et al.*, 1995). Alternate splicing of the α-mannosidase II^x gene gives rise to two cDNAs, one encoding a truncated protein of 796 amino acids, and the other encoding a full length protein of 1139 amino acids. Both human α-mannosidase II and the full length α-mannosidase II^x are active towards *p*-nitrophenyl α-D-mannoside. Chromosomal localization of the human enzymes locates α-mannosidase II to chromosome 5q21-22 and α-mannosidase II^x to chromosome 15q25.

1.6.2.b. ER and cytosolic α-mannosidases.

Class II α-mannosidases have also been described in rat liver ER and cytosol. The first of these to be described was the cytosolic α-mannosidase (Shoup and Touster, 1976).

This soluble α-mannosidase exists as a tetramer composed of 110 kDa subunits. It hydrolyzes *p*-nitrophenyl α-D-mannoside and is resistant to inhibition by 1-deoxymannojirimycin (Bischoff and Kornfeld, 1984). An ER Class II α-mannosidase activity with enzymatic properties similar to the cytosolic enzyme was also detected in rat liver, and was purified by its ability to hydrolyze *p*-nitrophenyl α-D-mannoside (Bischoff and Kornfeld, 1983). Polyclonal antibodies raised against the cytosolic α-mannosidase cross-react with a 107 kDa soluble ER α-mannosidase and a 132 kDa protein believed to be the membrane bound ER α-mannosidase, suggesting that these two proteins are immunologically related to the cytosolic α-mannosidase (Bischoff and Kornfeld, 1986). Using sequence data obtained from the 107 kDa ER α-mannosidase, probes were generated to isolate its corresponding cDNA from a rat liver cDNA library. This ER α-mannosidase cDNA encodes a soluble protein lacking a transmembrane domain or signal peptide sequence (Bischoff *et al.*, 1990). Sequence analysis reveals that it shares 33% amino acid identity with the yeast vacuolar α-mannosidase. Because the soluble ER α-mannosidase and the cytosolic α-mannosidase are immunologically related, share similar enzymatic properties, and have similar molecular weights, they are often referred to as the ER/cytosolic α-mannosidase or cytosolic α-mannosidase leading to much confusion in the literature. Whether they are the same enzyme has never been confirmed.

An α-mannosidase of lower molecular weight, called ER α-mannosidase II can also be detected in rat liver ER. ER α-mannosidase II is an 82 kDa protein capable of hydrolyzing *p*-nitrophenyl α-D-mannoside (Weng and Spiro, 1996). Peptide antibodies raised against the ER/cytosolic α-mannosidase react with ER α-mannosidase II, suggesting that these two enzymes are immunologically related. Studies with antibodies specific to another peptide at the N-terminal of the ER/cytosolic enzyme suggest that ER mannosidase II does not contain this region. Both the ER α-mannosidase II and ER/cytosolic α-mannosidase are kifunensine resistant and are weakly inhibited by swainsonine. Studies by Weng and Spiro indicate that both enzymes are sensitive to 2mM 1-deoxymannojirimycin (Weng and Spiro, 1996), however earlier work done on the effect of 1-deoxymannojirimycin on ER α-mannosidase indicated that it was not (Bischoff and Kornfeld, 1984). This discrepancy is most like due to the fact that only 50μM 1-deoxymannojirimycin was used in the earlier studies. The relationship between ER α-mannosidase II and the ER/cytosolic enzyme has not been conclusively determined, but it

has been suggested that ER α-mannosidase II is a clipped form of ER/cytosolic α-mannnosidase that gets translocated into the ER lumen (Weng and Spiro, 1996).

In contrast to ER α-mannosidase II, which is involved in N-linked oligosaccharide processing on glycoproteins, the cytosolic α-mannosidase has been implicated in the catabolism of free oligosaccharides (Grard *et al.*, 1994). During glycoprotein turnover, free N-linked oligosaccharides are released from the ER into the cytosol, where they are trimmed to $Man_5GlcNAc$ before entering the lysosomes for further degradation (Moore and Spiro, 1994; Grard *et al.*, 1996). The cytosolic α-mannosidase trims $Man_9GlcNAc$ to yield a specific isomer of $Man_5GlcNAc$ that differs from the one generated by Golgi α1,2-mannosidase trimming.

1.6.2.c. Lysosomal and vacuolar α-mannosidases.

The Class II lysosomal α-mannosidases are involved in the lysosomal degradation of the free oligosaccharides released during glycoprotein turnover and of the products that result from cytosolic α-mannosidase trimming. Two types of lysosomal mannose trimming activities have been described (for review see Daniel *et al.*, 1994). The first is a major lysosomal activity that catalyzes the removal of all α1,2-, α1,3-, and α1,6-linked mannose from $Man_9GlcNAc_2$. The second activity is specific for the removal of the α1,6-mannose that is linked to the β-linked mannose of the N-glycan core. Sequence analysis of the cloned major lysosomal α-mannosidases from *Dictyostelium discoideum* (Schatzle *et al.*, 1992), mouse (Merkle and Moremen, 1993) and human (Nebes and Schmidt, 1994), indicates that these enzymes share significant sequence similarity with the rat ER/cytosolic α-mannosidase and the α-mannosidase II genes. No sequence has been reported for the α1,6-mannose specific lysosomal α-mannosidase. Sequence analysis of the *Saccharomyces cerevisiae* vacuolar α-mannosidase reveals that it also shares this sequence similarity (Yoshihisa and Anraku, 1989).

1.7. Objective of this thesis.

As described in the introduction, the correct biosynthesis and maturation of N-linked oligosaccharides is essential for many biological events. The various stages of the N-linked oligosaccharide biosynthetic pathway are reasonably well established, however little is known about their regulation. Each stage involves one or more enzymes, the

glycosidases and glycosyltransferases, whose activities and expression are individually regulated. The correct expression of these enzymes and the regulation of the order in which they act determines the final oligosaccharide structures observed *in vivo*. In order to understand this control of N-linked oligosaccharide maturation, it is necessary to characterize the regulation of the individual steps.

The research presented in this thesis is aimed at understanding the regulation of the early steps of the pathway, specifically the action of the α1,2-mannosidases. The α1,2-mannosidases catalyze the removal of four α1,2-mannose residues from $Man_9GlcNAc_2$ to yield $Man_5GlcNAc_2$. Numerous α1,2-mannosidases implicated in this trimming have been cloned and characterized from different species, however their exact physiological role and how they are regulated is not yet known. In mouse and human, there is evidence for the involvement of at least two Golgi α1,2-mannosidases, α1,2-mannosidase IA (Lal *et al.*, 1994) and α1,2-mannosidase IB (Herscovics *et al.*, 1994). The murine enzymes have been shown to have similar catalytic properties, but exhibit very distinct patterns of tissue-specific expression (Herscovics *et al.*, 1994; Lal *et al.*, 1994). They have also been shown to arise from two distinct genes (Herscovics *et al.*, 1994).

The focus of this thesis is on the gene encoding one of these murine α-mannosidases, namely α1,2-mannosidase IB (Herscovics *et al.*, 1994; Schneikert and Herscovics, 1994). The murine α1,2-mannosidase IB cDNA has been cloned and characterized, however little is known about the physiological role that this enzyme plays in protein glycosylation and how it is regulated. To understand these aspects of the murine α1,2-mannosidase IB, it will be necessary to undertake further genetic studies of the α1,2-mannosidase IB gene such as the development of transgenic mice bearing a null mutation of the α1,2-mannosidase IB gene and the characterization of the α1,2-mannosidase IB transcriptional start site and potential regulatory elements in the promoter regions. Such studies have been done on N-acetylglucosaminyltransferase I, the enzyme responsible for the next step of the pathway. Disruption of the N-acetylglucosaminyltransferase I gene in mouse results in the death of the embryo at 9.5 dpc due to the absence of complex and hybrid oligosaccharides. Since the action of α1,2-mannosidases is required prior to N-acetylglucosaminyltransferase I for the maturation of complex and hybrid oligosaccharides, disruption of this trimming activity may also have severe physiological consequences. However, there are at least two murine α1,2-mannosidase genes encoding

enzymes that exhibit similar catalytic properties, but different patterns of tissue-specific expression. It therefore seems likely that disruption of only one of the α1,2-mannosidase at a time may provide insight into the specific roles of these individual enzymes during embryonic development, protein glycosylation and some genetic diseases.

The aim of the present work is to characterize the genomic organization of the murine α1,2-mannosidase IB gene, in order to obtain the information required to undertake such genetic studies. In this thesis, the genomic organization of the α1,2-mannosidase IB gene, its chromosomal localization, and evidence for another α1,2-mannosidase IB related gene or pseudogene are presented and discussed.

CHAPTER 2

2.0. Background.

α-Mannosidases play an important role at different stages in the maturation of N-linked oligosaccharides from the common $Glc_3Man_9GlcNAc_2$ oligosaccharide precursor to the diverse array of complex and hybrid structures found in mammalian cells. In particular, α1,2-mannosidases are essential for the removal of the four α1,2-linked mannose residues from the oligosaccharide precursor following the action of glucosidases (Kornfeld and Kornfeld, 1985; Moremen *et al.*, 1994). The resulting $Man_5GlcNAc_2$ is the substrate for N-acetylglucosaminyltransferase I, the first glycosyltransferase essential for the elaboration of complex and hybrid N-glycans. Following the action of N-acetylglucosaminyltransferase I, α-mannosidase II catalyzes the removal of the α1,3 and α1,6 mannose to yield $GlcNAcMan_3GlcNAc_2$. This structure is further modified by various Golgi glycosyltransferases to yield complex and hybrid oligosaccharides.

Studies with specific inhibitors have demonstrated the importance of α-mannosidases in determining the types of oligosaccharides found on glycoproteins (Elbein, 1991). When α1,2-mannosidases are inhibited with compounds such as 1-deoxymannojirimycin, there is an accumulation of $Man_{8-9}GlcNAc_2$ oligosaccharides and a failure to form hybrid or complex oligosaccharides. On the other hand, when α1,2-mannosidase II is inhibited by swainsonine, there is an accumulation of hybrid N-glycans. Inhibition of the α-mannosidases with these compounds can have important biological consequences such as inhibition of angiogenesis (Nguyen *et al.*, 1992) and of tumor metastasis (Dennis, 1986). It seems likely therefore that the genetic regulation of expression of α-mannosidases plays a role in determining the structure and function of glycoproteins.

In recent years several α-mannosidases have been cloned, and based on amino acid sequence similarity, these are divided into two classes (Moremen *et al.*, 1994). Class I contains α1,2-mannosidases that have sequence similarity with the yeast ER α1,2-mannosidase, while Class II contains α-mannosidases similar to the α1,3/α1,6 specific α-mannosidase II. Class I α1,2-mannosidases have been biochemically characterized and cloned from a variety of species, beginning with the yeast ER α1,2-mannosidase (Camirand *et al.*, 1991). Since then, α1,2-mannosidases have also been cloned from human, mouse, rabbit, *Drosophila melanogaster*, *Penicillium citrinum*, and *Aspergillus*

saitoi (Bause *et al.*, 1993; Herscovics *et al.*, 1994; Lal *et al.*, 1994; Inoue *et al.*, 1995; Kerscher *et al.*, 1995; Yoshida and Ichishima, 1995). Although these enzymes share high amino acid similarity, they differ in molecular properties, substrate specificity, and subcellular localization (Moremen *et al.*, 1994).

Evidence for different murine α1,2-mannosidase cDNAs was obtained following PCR amplification of mouse liver cDNA using degenerate oligonucleotide primers derived from amino acid sequences highly conserved between yeast and rabbit α1,2-mannosidases (Herscovics *et al.*, 1994). The resulting PCR products were a mixture of two sequences having 65% identity at the amino acid level. The sequence of one of the PCR products was identical to the mouse α1,2-mannosidase IA cDNA (Lal *et al.*, 1994), while the second PCR product represented a novel cDNA. Using the second PCR product as a probe, a BALB/c 3T3 mouse liver cDNA library was screened and overlapping clones representing the α1,2-mannosidase IB cDNA were isolated (Herscovics *et al.*, 1994). Clone 4 contains the entire open reading frame of α1,2-mannosidase IB, encoding a protein of 641 amino acids, while clone 16 only codes for the last 471 amino acids. The overlapping sequences of clone 4 and clone 16 are identical except for three nucleotide positions, 1232, 1402, and 1775, which are thymidine in clone 16 and cytidine in clone 4. These point mutations result in three amino acid changes, from Met^{411}, Phe^{468}, Phe^{592} in clone 16 to Thr^{411}, Leu^{468} and Ser^{592} in clone 4. Clone 416 was generated by fusing the N-terminal of clone 4 and the C-terminal of clone 16 (including positions 1232,1402, and 1775) to obtain a full length cDNA. When α1,2-mannosidase IB is expressed as a secreted protein A fusion protein in COS7 cells, the mutations found in clone 4 prevent its secretion and inhibit enzyme activity. It was shown that the single mutation of Phe^{592} to Ser^{592} is sufficient to completely abolish α1,2-mannosidase IB activity (Schneikert and Herscovics, 1995). These studies demonstrated that the clone 4 cDNA does not encode an enzymatically active α1,2-mannosidase.

Murine α1,2-mannosidase IA (Lal *et al.*, 1994) and IB (Herscovics *et al.*, 1994) are 73 kDa calcium-dependent processing enzymes localized to the Golgi apparatus of transfected mammalian cells. They are type II membrane proteins, each with a cytoplasmic tail of about 35 amino acids and a large C terminal catalytic domain containing a number of amino acid sequences highly conserved among the different members of this enzyme family. One such domain is a twelve amino acid putative EF-hand calcium-binding

consensus sequence. Both enzymes are capable of trimming $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$ and are inhibited by 1-deoxymannojirimycin, but not by swainsonine. Although α1,2-mannosidase IA and IB have similar properties, Southern blot analysis clearly indicates that they arise from two distinct genes which have not yet been characterized (Herscovics *et al.*, 1994). Furthermore, Northern blot analysis shows that α1,2-mannosidase IA and IB exhibit very different patterns of tissue-specific expression and differential expression in adult mouse tissues and during mouse embryonic development (Herscovics *et al.*, 1994; Herscovics, unpublished data).

In order to understand the control of protein glycosylation, it is important to study the regulation and expression of the α1,2-mannosidase genes. As a first step in determining the role of α1,2-mannosidase IB, we have characterized the genomic structure of the murine α1,2-mannosidase IB gene. In the present work, we describe the isolation of P1 phage genomic clones representing the entire cDNA of murine α1,2-mannosidase IB. The gene spans at least 80kb of genomic DNA, contains 13 exons, and is localized to mouse chromosome 3. We also provide evidence for the existence of another α1,2-mannosidase IB related gene or pseudogene on mouse chromosome 4.

2.1. Materials and Methods.

2.1.1. Isolation of cosmid clones.

A pWEI5 cosmid genomic library made from BALB/c mouse liver obtained from Philippe Gros, McGill University was screened by hybridization with ^{32}P-labeled BstE II/Not I and Not I α1,2 mannosidase IB cDNA fragments derived from clones 4 and 16, respectively (Herscovics *et al.*, 1994). 1.2 x 10^6 colonies were transferred in duplicate to nylon membrane (Hybond-N, Amersham) or nitrocellulose membrane (Xymotech) according to standard methods (Sambrook *et al.*, 1989), followed by prehybridization at 42°C for 4 hrs in 50% formamide, 5x SSC, 5X Denhardt's solution, 0.1% SDS, and 100μg/ml denatured sonicated herring sperm DNA. Hybridization was carried out overnight at 42°C with 1.5 x 10^6 cpm/ml of denatured labeled probe. Filters were washed 3 x 20 min at RT in 2x SSC/0.1% SDS, followed by 1 x 20 min at 65°C in 0.1x SSC/0.1% SDS. Filters were exposed to Kodak XAR-5 film. After three additional rounds of screening as described above, three positive clones were identified.

Colonies containing individual cosmid clones were used to inoculate LB media containing 50μg/ml ampicillin and incubated overnight at 37°C. Cultures were diluted 1:100 with LB media containing 50μg/ml ampicillin and incubated overnight at 37°C. Bacteria were harvested and the cosmid DNA was isolated using alkaline lysis, followed by CsCl preparation according to standard methods (Ausubel *et al.*, 1987).

2.1.2. Isolation of P1 genomic clones.

P1 library screening was performed by Genome Systems on a 129/*sv* mouse genomic library. Screening of the library was performed using PCR and colony hybridization, using two sets of oligonucleotide primers. Sense primer position 324 (nucleotide position refers to sequence published in Herscovics *et al.*, 1994), 5' GCG TCT GAG AAA TAA GAT TAG 3', and antisense primer 529, 5' TCT CCA TGT CTT CTG GAT C 3', were used to screen for genomic clones containing the 5' end of the α1,2-mannosidase IB. Sense primer 1304, 5' TTA AGA AGT CCC GAG GAG 3', and antisense primer 1490, 5' GAC TCA TGA CAT GTT CGT GC 3', were used to screen for genomic clones containing the 3' end of the α1,2-mannosidase IB. Optimal PCR reaction conditions for screening were determined using 129/*sv* mouse genomic DNA prepared from 129/*sv* mouse blastocysts as template. For primer pair 324-529, the optimal reaction conditions were 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.64 mM of each dNTP, 2.5 mM $MgCl_2$, 12.5 pmol of each primer and 1.5U Taq DNA polymerase (Pharmacia). For primer pair 1304-1490, the final concentration of $MgCl_2$ was 3.0 mM. The reaction mixtures were overlaid with mineral oil, and 30 cycles of PCR were performed; 1 min at 94°C, 1 min at 55°C, 1 min at 72°C. Six positive clones were identified.

2.1.3. DNA isolation and Southern blot analysis of P1 clones.

To increase yields of DNA, the P1 clones obtained were transduced from the Cre+ host NS3529 to the Cre- host NS3516 as recommended by Genome Systems Inc. Bacterial colonies (NS3516) containing single P1 genomic clones were inoculated into LB medium containing 25μg/ml kanamycin, and were grown to stationary phase overnight at 37°C. The overnight cultures were diluted 1:30 with LB media containing 25μg/ml kanamycin and incubated 1.5 hrs at 37°C. The P1 lytic cycle was induced by the addition of IPTG to a final concentration of 0.5 mM and incubation was continued for 5 hrs. The

bacteria were harvested and the P1 plasmid DNA was extracted using a modified alkaline lysis protocol recommended by Genome Systems, Inc.

P1 genomic clones were analyzed by restriction endonuclease digestion and Southern blot analysis. P1 DNA was digested overnight with Eco RI and Bam HI and the products were fractionated by electrophoresis on a 0.6% agarose gel. The gels were treated successively with 0.25M HCl for 15 min, with 0.5 NaOH, 1.5M NaCl for 30 min, and with 0.5M Tris-Cl pH 8.0, 1.5M NaCl for 30 min, rinsing with water between solutions. The gels were equilibrated in 10x SSC , and transferred to nylon membrane (Hybond-N, Amersham) using a vacuutransfer apparatus (LKB-Vacugene XL, Pharmacia). Following the transfer, the membrane was rinsed in 2x SSC and the DNA was crosslinked to the membrane by exposure to UV light (Stratalinker, Stratagene). Prehybridization was performed for 1 hr at 37°C in 6x SSC, 0.05% $Na_4P_2O_7*10H_2O$, 0.5% SDS, 5x Denhardt's, and 100μg/ml denatured sonicated herring sperm DNA. Hybridization was performed overnight at 37°C in 6x SSC, 0.05% $Na_4P_2O_7*10H_2O$, 1x Denhardt's, and 100μg/ml denatured sonicated herring sperm DNA with 0.5-1.0 x 10^6 cpm/ml of ^{32}P-labeled exon specific oligonucleotide probes.

Genomic fragments hybridizing with exon specific probes were prepared by restriction endonuclease digestion of the P1 DNA, followed by electrophoretic separation on a 0.6% agarose gel. Fragments were purified using the Sephaglas kit (Pharmacia) and subcloned into pBluescript II KS(-). Subclones were analyzed by restriction endonuclease digestion and sequencing.

2.1.4. Southern analysis of 129/*sv* genomic DNA.

Southern blot analysis was performed on 129/*sv* genomic DNA. Genomic DNA was digested with Eco RI and BamHI and the digestion products were separated on a 0.8% agarose gel. The DNA was transferred to nylon membrane as described above. Prehybridization and hybridization were performed at 42°C in 50% formamide, 5x SSPE, 10x Denhardts', 2% SDS, and 100mg/ml denatured sonicated herring sperm DNA. 4 x 10 ^{6} cpm/ml of ^{32}P-labeled clone 416 was used as probe. Washes were performed 2 x 20 min at RT in 2x SSC, 0.1% SDS, followed by 1hr at 60°C in 0.1x SSC, 0.1% SDS. The Southern blot was exposed to a Fuji phosphoimager plate for 1 week.

2.1.5. PCR on genomic DNA from 129/*sv* and BALB/c mice.

PCR was performed on 100ng of genomic DNA from 129/*sv* and BALB/c mice to determine whether the genomic sequences contained either a thymine or cytosine at nucleotide position 1402. Primer pair 1304-1490 was used to amplify exon 10 of α1,2-mannosidase IB, using reaction conditions described for the P1 genomic library screening. The PCR products of 186 bp were subcloned into the TA vector (Invitrogen). Eighteen 129/*sv* genomic exon 10 clones and 9 BALB/c genomic exon 10 clones were sequenced.

2.1.6. Radiolabeling of cDNA and oligonucleotide probes.

The cDNA clones 4 and 16 were radiolabeled by [α-^{32}P]dCTP (3000 Ci/mmol, DuPont-NEN) incorporation with the Multiprime random labeling kit (Amersham). Oligonucleotide probes were end-labeled in a T4 polynucleotide kinase reaction containing 100mM Tris-Cl pH 7.5, 10 mM $MgCl_2$, and 20mM β-mercaptoethanol, 25μCi [γ^{32}P] dATP (3000Ci/mmol, DuPont-NEN) and 15U T4 polynucleotide kinase (New England Biolabs).

2.1.7. DNA sequencing.

DNA sequencing of the α1,2 mannosidase IB genomic subclones to determine the exons and intron/exon boundaries was performed by the dideoxynucleotide chain termination method using the T7 sequencing kit (Pharmacia) as described by the manufacturer. Regions of compression were resolved using the Deaza sequencing kit (Pharmacia). Sequence assembly was done using the SeqMan program of DNASTAR.

2.1.8. Fluorescence *in situ* hybridization (FISH).

Chromosomal mapping of the cosmid probes Cos.31.1 and Cos.25.1 was performed by FISH (Lichter *et al.*, 1990) to normal murine spleen chromosomes counterstained with propidium iodide and DAPI by the CGAT FISH Mapping Resource Centre, The Hospital for Sick Children, Toronto, Ontario. Biotinylated probe was detected with FITC and digoxigenin-labeled probe with anti-digoxigenin rhodamine. In the dual color FISH studies in which murine chromosomal paint and the cosmid probe were combined, the paint was labeled with biotin and detected with FITC, and the probe was labeled with digoxigenin and detected with rhodamine. For the dual color FISH the chromosome spreads were counterstained with DAPI only. Images of metaphase

preparations were captured by a thermoelectrically cooled charge coupled camera (Photometrics, Tuscon, AZ). Separate images of DAPI banded chromosomes (Heng and Tsui, 1993) and of FITC or rhodamine targeted chromosomes were obtained. Hybridization signals were acquired and merged using image analysis software and pseudo colored blue (DAPI), red (rhodamine) and yellow (FITC) as described by Boyle *et al.* (Boyle *et al.*, 1992) and overlaid electronically.

2.2. Results.

2.2.1. Organization of the murine α1,2-mannosidase IB gene.

In previous work, two overlapping cDNA clones, clone 4 and clone 16, differing by three point mutations were isolated from a BALB/c 3T3 mouse liver cDNA library (Herscovics *et al.*, 1994). Clone 16 was shown to be a functional α1,2-mannosidase in protein A fusion protein expression studies, while clone 4 was inactive as an α1,2-mannosidase (Schneikert and Herscovics, 1994). In order to isolate genomic clones representing the α1,2-mannosidase IB gene, a mixture of clones 4 and 16 was used to screen a cosmid genomic library from BALB/c mouse liver. Three non-overlapping cosmid clones, Cos.31.1, Cos.25.1, and Cos.11.1 were isolated and characterized. Cos.31.1 is about 30 kb in size and contains sequences identical to the 5' region of the α1,2-mannosidase IB cDNA. Cos.25.1 is about 35 kb and contains sequences similar, but not identical, to the α1,2-mannosidase IB cDNA 3' region, with the similarity beginning at amino acid 560 of the α1,2-mannosidase IB cDNA. The corresponding regions of Cos.25.1 and the α1,2-mannosidase IB cDNA are 83% similar at the amino acid level, and 93% similar at the nucleotide level. When compared to the amino acid sequence of the α1,2-mannosidase IB cDNA and the P1 genomic clones described below, the sequence of Cos.25.1 contains a premature termination codon at position 566, an 9bp deletion (amino acids 601-603 of α1,2-mannosidase IB cDNA), multiple single base changes, and some unique sequences different from both intron and exon sequences upstream of position 560. Cos.11.1 had an identical restriction enzyme digestion pattern to Cos.25.1, and therefore only Cos.25.1 was characterized. Sequences identical to those of Cos.25.1 were also found in a λ phage clone isolated from a BALB/c mouse liver genomic library (Schneikert and Herscovics, unpublished). Since the cosmid clones did not represent the entire α1,2-

mannosidase IB cDNA, a 129/*sv* mouse P1 genomic library was screened by PCR and colony hybridization. Six clones were obtained, of which three unique overlapping clones were characterized: P1.E1-6, P1.E3-12, and P1.E9-13. The three other clones had similar restriction enzyme digestion patterns as the three clones that were characterized. P1.E1-6, P1.E3-12, and P1.E9-13 span ≥80kb of genomic DNA and contain sequences that are identical to clone 416 cDNA and include the complete α1,2-mannosidase IB open reading frame. By sequencing, restriction enzyme digestion and Southern blot analysis of the cosmid and P1 clones, the genomic structure of the α1,2-mannosidase IB gene was determined (Fig. 6). The intron/exon boundaries were mapped by sequencing the exons in their entirety and portions of the adjoining introns using oligonucleotide probes derived from the cDNA sequence. A schematic representation of the genomic structure of the α1,2-mannosidase IB gene is shown in Fig. 6. The 1926 bp open reading frame of the α1,2-mannosidase cDNA is distributed over 13 exons and spans at least 80kb. The exons range in size from 81bp (exon 5) to >891 (exon 1) (see Table 1). The size of the introns vary from about 2.0kb to >10.4kb in size. Exon 1 contains the initiating AUG, >589 bp of 5' UTR sequence, and the hydrophobic transmembrane domain. The conserved putative 12 amino acid EF-hand calcium-binding domain (^{255}DFSV/NSEVSVFE) spans exons 4 and 5 (a slash marks the intron/exon boundary). The highly conserved regions between species are found in exons 9 (^{398}GDSFYEYLLK), 12 (^{590}SFFLAETLK), and 13 (^{617}FNTEAH). Exon 13 also contains the termination of translation signal as well as 3' UTR sequences. Analysis of the DNA sequences spanning the intron/exon boundaries show that they all follow the 5' gt.....ag 3' splice junction consensus (Breathnach and Chambon, 1981; Shapiro and Senapathy, 1987)

2.2.2. Chromosomal localization of Cos.31.1 and Cos.25.1.

Sequencing of the genomic clones suggested that there may be more than one gene related to the α1,2-mannosidase IB cDNA. Cos.31.1 and the P1 clones contain sequences identical to the active form of α1,2-mannosidase IB, while Cos.25.1 contains sequences that differed from the α1,2-mannosidase IB cDNA, or any other α-mannosidase cDNA.

Fig.6. Schematic representation of the organization of the α1,2-mannosidase IB gene. Part A shows the arrangement of the 13 exons (depicted as vertical bars and numbers). The 5' and 3' UTR sequences are shown as white boxes. Part B and C show the positions of the BALB/c cosmid clones and the overlapping P1 129/*sv* clones, respectively, with respect to the α1,2-mannosidase IB gene. Cos.31.1 spans exons 2-5. Cos.25.1 contains sequences that are similar, but not identical to exons 12 and 13 of the α1,2-mannosidase IB gene (depicted as stippled grey bars and dashed lines). The sequence of Cos.25.1 contains a premature stop and is lacking intron sequence between the exon 12 and 13 sequences (see Fig. 11, Appendix). P1.E1-6 spans exons 1-6, P1.E3-12 spans exons 3-12, and P1.E9-13 spans exons 9-13. The clone length is indicated in parentheses. Part D shows a partial restriction endonuclease map of α1,2-mannosidase IB gene. Bam HI sites are indicated by triangles, Eco RI sites by ovals, and Hind III sites by crosses. The figure is drawn to scale. See Fig. 12, Appendix for further restriction endonuclease maps.

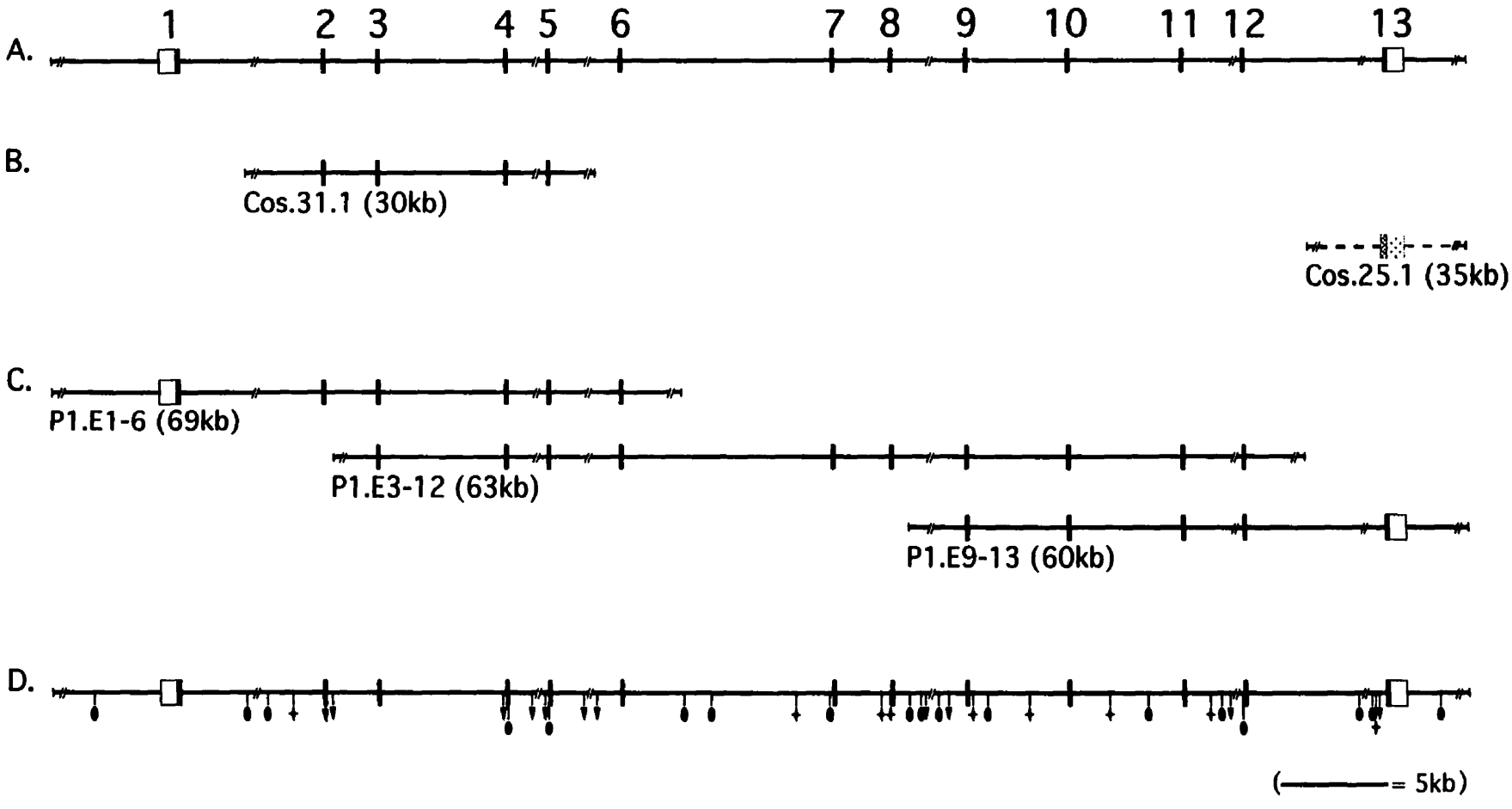

A.
1
2
3
4
5
6
7
8
9
10
11
12
13
B.
Cos.31.1 (30kb)
Cos.25.1 (35kb)
C.
P1.E1-6 (69kb)
P1.E3-12 (63kb)
P1.E9-13 (60kb)
D.
(——— = 5kb)

TABLE 1
Splice Junction Sequences, Exon Sizes, and Estimated Intron Sizes of the Murine α1,2-Mannosidase IB Gene

Exon	3'-Splice site					5'-Splice site					Exon size (bp)	Intron size (kb)
								101[a]				
1						.....	AGA	CAC	AG	gttcgtttgt	>891	>5.9
							R	H	R			
		102							186			
2	tatttcccag	G	GAA	GAA	GAA	.....	ATT	AAA	GAG	gtaataagct	256	~2.7
			E	E	E		I	K	E			
		187							218			
3	ctcttctcag		ATG	ATG	AAA	.....	AAC	ATA	TTC	G gtaaggtaac	97	~5.9
			M	M	K		N	I	F			
		219							258			
4	ctaaatatag		GA	AGC	TCA	.....	TTC	AGT	GTG	gtgtgtatac	119	>1.5
			G	S	S		F	S	V			
		259							285			
5	gtctccacag		AAT	TCA	GAA	.....	GGA	GAG	GAA	gtgagtagag	81	>2.6
			N	S	E		G	E	E			
		286							317			
6	ttcgtctcag		ATA	TTC	AAG	.....	CTG	AAA	AG	gtaaacagta	95	~10.4
			I	F	K		L	K	S			
		318							358			
7	tattactcag	T	GGA	GTA	GGT	.....	TAT	AAT	AAG	gttcgtctct	124	~2.5
			G	V	G		Y	N	K			
		359							389			
8	ttttgattag		GTC	ATG	CAC	.....	TGG	GGT	CAG	T gtaagtattt	94	>2.8
			V	M	H		W	G	Q			
		390							428			
9	tttgctacag		AT	CAC	ACA	.....	GCT	GTT	GAG	gtaactattg	116	~4.3
			Y	H	T		A	V	E			
		429							501			
10	tttcttgtag		GCT	ATA	GAA	.....	GAC	AGA	ACT	G gtaagaatat	220	~6.5
			A	I	E		D	R	T			
		502							559			
11	aatgttacag		CA	TTG	AAA	.....	GCA	GCA	CTG	gtaaatacgc	173	>2.5
			A	L	K		A	A	L			
		560							598			
12	tccctcacag		GCT	ATT	GAG	.....	ACA	TTA	AA	gtaagcacat	116	>7.3
			A	I	E		T	L	K			
		599							642			
13	ttctttttcag	A	TAC	TTG	TAC	.....	GTC	CGA	TGA	GCA CAG CCC		
			Y	L	Y		V	R	*			

[a] Amino acid designations are based on Herscovics *et al.*, 1994.

To determine whether these two cosmid clones were indeed derived from two different genes, chromosomal localization was performed with each clone. The 30 kb genomic probe derived from Cos.31.1, representing the α1,2 mannosidase IB gene exons 2-5, was used to determine the regional assignment of the α1,2 mannosidase IB gene within the genome. FISH analysis of 20 well spread metaphases from mouse cells assigned the α1,2 mannosidase IB to mouse chromosome 3 in the region of band F2. Positive hybridization signals were observed in 70-80% of the metaphase spreads, with no detectable signals on other chromosomes. Localization to chromosome 3 was confirmed by dual color FISH using chromosome specific biotinylated murine paints and the digoxigenin labeled Cos.31.1. probe (Fig. 7B).

The 35 kb genomic probe derived from Cos.25.1 was also mapped using FISH analysis to explore the possibility of a second gene or pseudogene. The regional assignment of this probe was determined by the analysis of 20 well-spread metaphases as for Cos.31.1. Positive hybridization signals were observed just below the centromere of mouse chromosome 4 at band A13 in 80% of the spreads. No detectable signals were observed on other chromosomes. The localization to chromosome 4 was confirmed using dual color FISH as for Cos.31.1 (Fig. 7A).

2.3. Discussion.

We have isolated a series of cosmid and P1 genomic clones representing the entire α1,2-mannosidase IB cDNA, and determined its genomic structure by restriction enzyme digest analysis, Southern blotting and sequencing. The α1,2-mannosidase IB gene, with an open reading frame of 1926 bp, consists of 13 exons distributed over ≥80kb of genomic DNA. The coding sequence is identical to that of clone 416, which is the active form of the enzyme (Schneikert and Herscovics, 1995). The 5' end of the gene, encoded by exon 1 and 2, is not required for enzyme activity as determined by protein A fusion protein expression studies (Schneikert and Herscovics, 1994). Comparison of the murine α1,2-mannosidase IB gene structure with the partial genomic structure of the *D. melanogaster mas -1* indicates that there is some conservation of intron/exon boundary positions between species (Fig. 8) (Kerscher *et al.*, 1995). The two splice sites identified in *D. melanogaster mas -1* have the same relative position in the sequence as the corresponding splice sites in

Fig.7. Chromosomal localization of Cos 25.1. and Cos 31.1. Fluorescence *in situ* hybridization (FISH) to mouse metaphase chromosomes using biotinylated inserts of (A) Cos.25.1 and (B) Cos.31.1 as probes was performed. Cos.25.1 was found to localize to just below the centromere of mouse chromosome 4 in the region A13, while the clone representing the active murine α1,2-mannosidase IB, Cos.31.1, was found to localize to mouse chromosome 3F2.

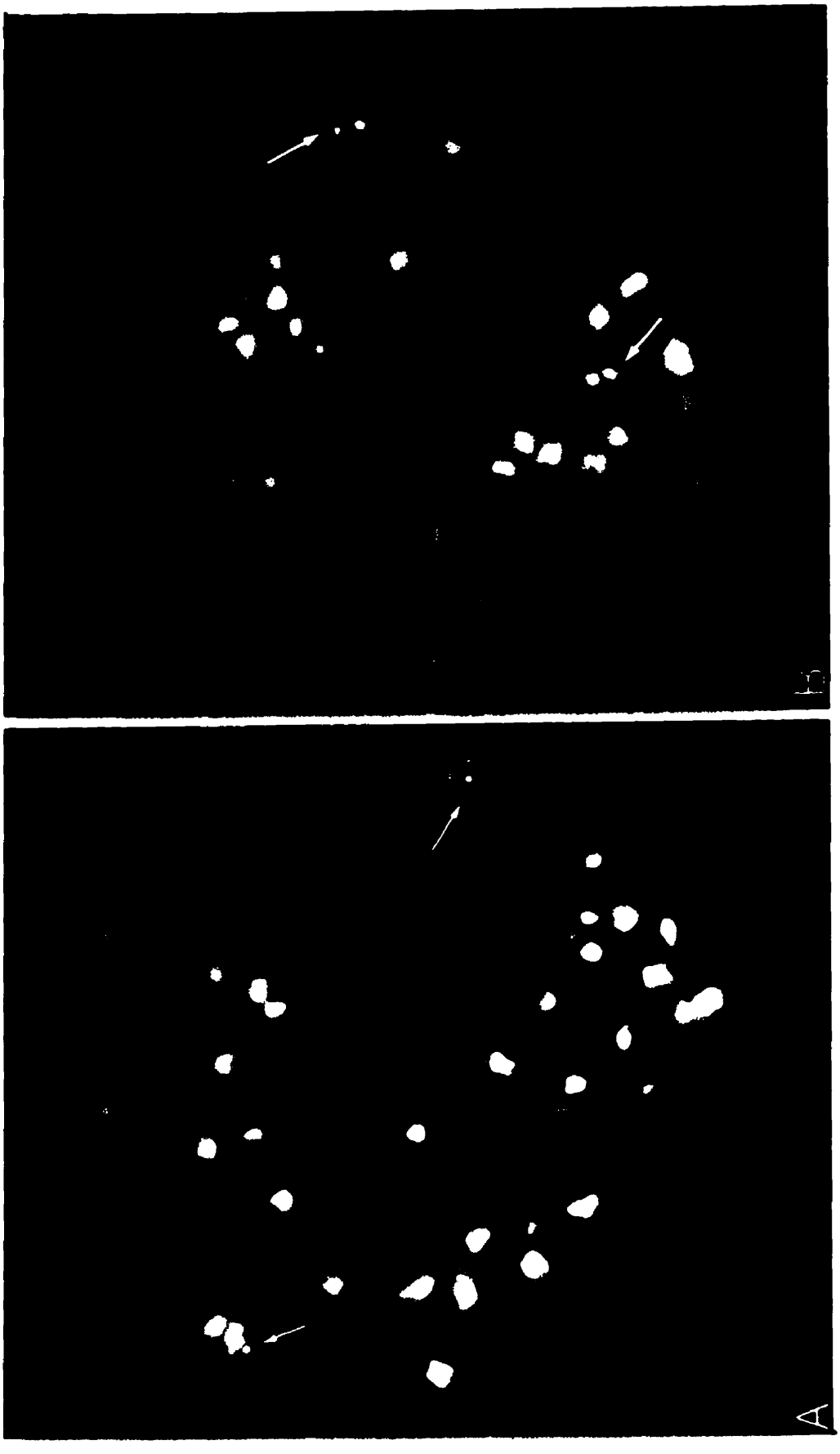
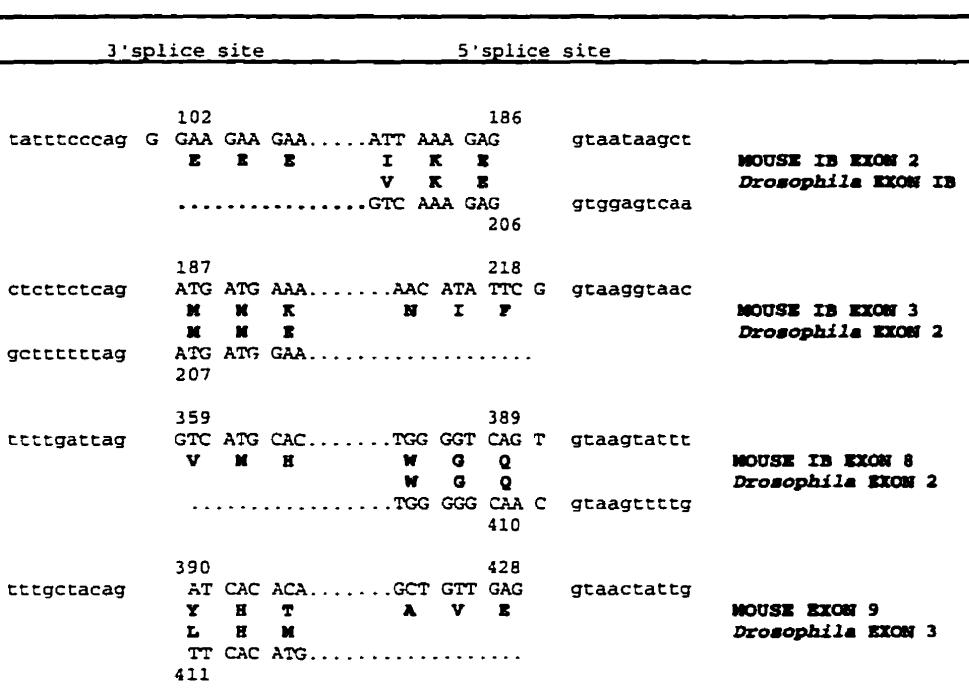

B
A

Fig.8. Comparison of the murine α1,2-mannosidase IB intron/exon boundaries with the boundaries identified in the *D. melanogaster mas-1* gene (Kerscher *et al.*, 1995). Amino acid positions correspond to those defined in Herscovics *et al.*, 1994 and Kerscher *et al.*, 1995.


3'splice site
5'splice site
102
186
tatttcccag G GAA GAA GAA.....ATT AAA GAG gtaataagct
E E E I K E
V K E
.....GTC AAA GAG gtggagtcaa
206
MOUSE IB EXON 2
Drosophila EXON IB
187
218
ctctttctcag ATG ATG AAA.....AAC ATA TTC G gtaaggtaac
M M K N I F
M M E
gctttttttag ATG ATG GAA.....
207
MOUSE IB EXON 3
Drosophila EXON 2
359
389
ttttgattag GTC ATG CAC.....TGG GGT CAG T gtaagtattt
V M H W G Q
W G Q
.....TGG GGG CAA C gtaagttttg
410
MOUSE IB EXON 8
Drosophila EXON 2
390
428
tttgctacag AT CAC ACA.....GCT GTT GAG gtaactattg
Y H T A V E
L H M
TT CAC ATG.....
411
MOUSE EXON 9
Drosophila EXON 3

the α1,2-mannosidase IB gene. The chromosomal localization of the α1,2-mannosidase IB gene was performed by FISH and was determined to map to mouse chromosome 3F2.

In characterizing the genomic clones for the α1,2-mannosidase IB gene, we did not detect any sequence corresponding to clone 4 cDNA. Previous work demonstrated that the cDNA clones 4 and 16, containing cytidine and thymidine respectively, are natural isoforms of the α1,2-mannosidase IB (Schneikert and Herscovics, 1995). The α1,2-mannosidase IB cDNA containing cytidine at positions 1232, 1402, and 1775 is inactive and poorly secreted when expressed as a protein A fusion protein, while the cDNA containing thymidine at these positions is active. Genomic DNA from BALB/c and 129/*sv* mice only contains thymidine at these positions, as determined by sequencing of genomic clones, and by PCR amplification of exon 10 (containing position 1402) using exon specific primers (data not shown). Southern blot analysis of genomic DNA does not reveal any bands that cannot be assigned to the α1,2-mannosidase IB gene. PCR amplification of exons 2-10 from genomic DNA yielded no product, indicating that there is no evidence for an improperly transcribed processed pseudogene. The origin of these T to C substitutions is unknown. Because at the present time there is no evidence for cytidine at these positions at the genomic level, in either 129/*sv* or BALB/c mice, one possible mechanism to be considered is RNA editing. This editing would involve three U to C conversion in the mRNA resulting in a transcript containing Thr, Leu and Ser at amino acid positions 411, 468 and 592 respectively, instead of Met, Phe and Phe. Similar U to C substitutions due to RNA editing have been observed in the Wilms' tumor suppressor gene (WT1), resulting in proteins containing proline (CCC) rather than Leu (CTC) at amino acid position 280 (Sharma *et al.*, 1994). Mammalian RNA editing has also been observed for α-galactosidase (U to A), human intestinal ApoB, and several subunits of the human and rat brain L-glutamate receptor (for review see Simpson and Emeson, 1996). If RNA editing is indeed occurring in α1,2-mannosidase IB mRNA, this would be the first example of multiple U to C substitutions in a mammalian RNA . Since the non conservative amino acid change from Phe^{592} to Ser^{592} is sufficient to abolish α1,2-mannosidase IB activity (Schneikert and Herscovics, 1995), RNA editing of the α1,2-mannosidase IB mRNA may be functionally relevant and play a role in the genetic regulation of α1,2-mannosidase IB gene expression *in vivo*.

Sequence analysis of the genomic clones that were isolated using the α1,2-mannosidase IB cDNAs as probes revealed the existence of another related gene or pseudogene. Cos.25.1 was found to contain sequences that are similar, but not identical, to α1,2-mannosidase IB and clearly different from any other α-mannosidase. The presence of a premature stop codon in the sequence of Cos.25.1 would result in the production of a transcript and/or protein truncated at a position corresponding to amino acid 566 of the α1,2-mannosidase IB cDNA. This truncation would eliminate two of the highly conserved amino acid regions, ^{617}FNTEAH and ^{590}SFFLAETLK, that may be essential for enzyme activity. Previous work demonstrated that a point mutation at amino acid position 592 is enough to abolish α1,2-mannosidase IB activity (Schneikert and Herscovics, 1995). Since Cos.25.1 is missing this region, it is unclear whether the protein, if translated, would have α1,2-mannosidase activity. Direct evidence that Cos.25.1 belongs to a second gene or pseudogene was obtained from FISH analysis using Cos.25.1 as a probe, which localized Cos.25.1 to just below the centromere of mouse chromosome 4 at band A13.

In the present work, we have reported the first complete genomic structure for a mammalian member of the Class I α1,2-mannosidases, α1,2-mannosidase IB, and provided evidence for the existence of another related gene or pseudogene. The characterization of the α1,2-mannosidase IB genomic structure will permit further genetic studies of α1,2-mannosidase IB, and allow for a better understanding of the regulation of mannose trimming in the cell.

CHAPTER 3

3.0. General discussion.

In this thesis, the genomic characterization of the murine α1,2-mannosidase IB gene, its chromosomal localization, and evidence for a second related gene or pseudogene have been presented. The α1,2-mannosidase IB gene, one of two known Golgi α1,2-mannosidase genes in the mouse, is a multiexonic gene containing 13 exons and spanning ≥ 80kb of genomic DNA. Cos.25.1 represents a previously undescribed gene or pseudogene that appears to share significant sequence identity with murine α1,2-mannosidase IB. FISH localization of the α1,2-mannosidase IB gene indicates that it maps to mouse chromosome 3F2, in a region syntenic to human chromosome 1, while the sequence represented by Cos.25.1 maps to mouse chromosome 4A13. In the following sections, these results and their use in future genetic studies will be discussed.

3.1. The murine α1,2-mannosidase IB gene as a possible model for other Class I α-mannosidases.

Since murine α1,2-mannosidase IB shares significant sequence and structural similarities with the mammalian Class I α-mannosidases (murine α1,2-mannosidase IA (Lal *et al.*, 1994), human Man_9-mannosidase (Bause *et al.*, 1993), rabbit α1,2-mannosidase IA (Lal *et al.*, 1994), and human α1,2-mannosidase IB (Tremblay *et al.*, 1996)), the information gathered from studying the murine α1,2-mannosidase IB genomic organization is relevant to future studies on the other mammalian α1,2-mannosidases. The mammalian Class I α-mannosidases are all type II membrane proteins, each with a short cytoplasmic tail, a hydrophobic transmembrane domain, a "stem" region, and a large catalytic domain. Sequence analysis indicates that they all contain an EF hand-like putative calcium-binding site and a number of amino acid motifs in their catalytic domain that are highly conserved. The significant sequence similarity of these enzymes, suggests that they may also exhibit similarities at the level of their genomic organization. Support for this hypothesis was obtained by the observation that some of the intron/exon splice junctions found in the murine α1,2-mannosidase IB gene are conserved in the partially characterized *D. melanogaster mas-1* gene (Kerscher *et al.*, 1995). Since the *D. melanogaster mas-1* and the murine α1,2-mannosidase IB genes, two enzymes which share only ~36% amino acid identity, exhibit similarities in their genomic organization, it is not unreasonable to expect the other mammalian Class I α-mannosidase genes, whose proteins share ≥65% amino acid

identity with murine α1,2-mannosidase IB, to have similar organization. The information obtained on murine α1,2-mannosidase IB genomic organization will therefore provide guidelines for studies on other mammalian Class I α-mannosidases.

3.2. Regulation of α1,2-mannosidase IB gene.

Northern blot analysis of murine α1,2-mannosidase IB expression in mouse tissues reveals multiple transcripts ranging in size from 4.2 kb to 8.7 kb (Herscovics *et al.*, 1994). In most tissues, the major transcripts observed were 4.2, 5.1, 6.4 and 8.7 kb, but minor transcripts of different sizes were also detected. The relative expression of these multiple transcripts varied between tissues, with the highest expression being observed in L-cells, followed by colon, ovary, thymus and brain. In brain, the major transcript was 8.7 kb, accompanied by a reduced expression of the shorter transcripts. In ovary, a transcript of 5.6 kb, which is a minor transcipt in other tissues, was found to be highly expressed. The origin of these transcripts and their regulation is still unknown.

The multiple transcripts observed for the murine α1,2-mannosidase IB gene could arise from a number of mechanisms including alternative splicing, the differential use of polyadenylation signals, different transcriptional start sites, and/or the alternate usage of promoters. The possibility that some of these transcripts correspond to an uncharacterized related gene that cross-hybridizes with the α1,2-mannosidase IB specific probe, and that some transcripts are incompletely spliced must also be considered.

In order to determine which of these mechanisms is responsible for the generation of this complex pattern of transcripts, it will be necessary to undertake further genetic studies. Knowledge of the intron/exon structure of the murine α1,2-mannosidase IB gene facilitates the prediction of possible alternatively spliced transcripts. Primer extension and reverse transcription PCR analyses aimed at characterizing the 5' and 3' untranslated sequences in order to identify transcriptional start sites, regulatory element binding sites, and potential polyadenylation signals will provide insight into the transcriptional regulation of this gene. *In vitro* transcription studies will also be useful in studying the transcription efficiencies of α1,2-mannosidase IB.

In order to fully understand the regulation of α1,2-mannosidase IB, it will also be necessary to study the control α1,2-mannosidase IB protein translation. The translation products of this gene have not yet been studied, however the presence of multiple

transcripts (Herscovics *et al.*, 1994) and the existence of two cDNAs differing in three point mutations (Schneikert and Herscovics, 1995) suggests that there may be different protein isoforms. *In vitro* translation studies will provide insight into the α1,2-mannosidase IB translation products and their efficiency of translation. Regulation at the level of translation could occur by the use of alternative translation start sites, resulting in the formation of different α1,2-mannosidase IB isoforms. It could also result from the regulation of translation efficiency. The secondary structure of the 5' flanking region of transcripts often plays a role in determining how efficiently a protein is translated. Transcripts with a 5' untranslated region that has a complex secondary structure are often translated inefficiently.

3.3. Why do multiple α1,2-mannosidases exist?

The reason for the existence of at least two functional murine Golgi α1,2-mannosidases, α1,2-mannosidase IA and α1,2-mannosidase IB, is unknown. The existence of multiple enzymes with similar specificities has also been described for other enzymes of the N-oligosaccharide biosynthetic pathway including α-mannosidase II (Misago *et al.*, 1995) and several of the glycosyltransferases (Joziasse, 1992; Dinter and Berger, 1995). One theory for their existence is that the production of multiple enzymes capable of catalyzing the same reaction ensures the production of oligosaccharide structures required for survival of the organism. Currently, there are no genetic diseases known that involve defects in α1,2-mannose trimming. It may be that a deficiency in α1,2-mannosidase activity, which would prevent the formation of complex and hybrid structures, could be lethal at early embryonic stages. It is also possible that the activity of one α1,2-mannosidase can replace the activity of another, allowing the organism to develop normally. Finally, it may be possible that diseases due to defects in the α1,2-mannosidase do exist, but have not yet been identified. Assuming that multiple α1,2-mannosidases with similar specificities exist as a mechanism of molecular redundancy to ensure organism survival, it is important to understand the regulation and expression of these enzymes, and their role in the control of protein glycosylation.

3.4. Generation of a mouse null α1,2-mannosidase IB mutant.

The generation of transgenic mice carrying a null mutation for the murine α1,2-mannosidase IB gene would be useful in evaluating the role of α1,2-mannosidase IB in embryonic development and in physiological events. It has already been established that the next step in N-linked oligosaccharide maturation, the action of N-acetylglucosaminyltransferase I, is essential for post-implantation development of the embryo (Ioffe and Stanley, 1994; Metzler *et al.*, 1994). Disruption of this enzyme, and hence a failure to form hybrid and complex oligosaccharides results in the death of the embryos at 9.5 dpc. Inhibition of α1,2-mannosidase activity with 1-deoxymannojirimycin or kifunensine also prevents the formation of complex and hybrid oligosaccharides in cells in culture (Elbein, 1991). It seems possible therefore that mice lacking this activity may also die during embryonic development. However, since at least two murine α1,2-mannosidases exist, each exhibiting tissue-specific expression, disruption of only one of these enzymes, murine α1,2-mannosidase IB, could yield one of three possible phenotypes.

The first possibility is that disruption of the α1,2-mannosidase IB gene would interfere with normal embryonic development and result in the death of the embryo due to a lack of complex and hybrid N-glycans, as observed for the N-acetylglucosaminyltransferase I disruption (Ioffe and Stanley, 1994; Metzler *et al.*, 1994). Since α1,2-mannosidase IB acts prior to N-acetylglucosaminyltransferase I in N-linked oligosaccharide maturation, one may speculate that disruption of this enzyme would interfere with earlier stages of embryonic development than disruption of N-acetylglucosaminyltransferase I.

It is also possible that disruption of α1,2-mannosidase IB would not be lethal, but would result in a more restricted pattern of abnormalities confined to tissues and cell-types in which this enzyme is expressed. Since the murine α1,2-mannosidase IB exhibits different levels of tissue-specific expression, the severity of the disruption may vary from tissue to tissue. This pattern of tissue-specific abnormalities is observed in *D. melanogaster* when *mas-1*, a *D. melanogaster* homologue of α1,2-mannosidase IB, is disrupted (Kerscher *et al.*, 1995). In the embryo of *D. melanogaster*, a disruption of *mas-1* results in intersegmental nerve pathfinding errors and abnormal clusters of sensory organs. In the adult *D. melanogaster*, the wing longitudinal veins are often incomplete and end in deltas.

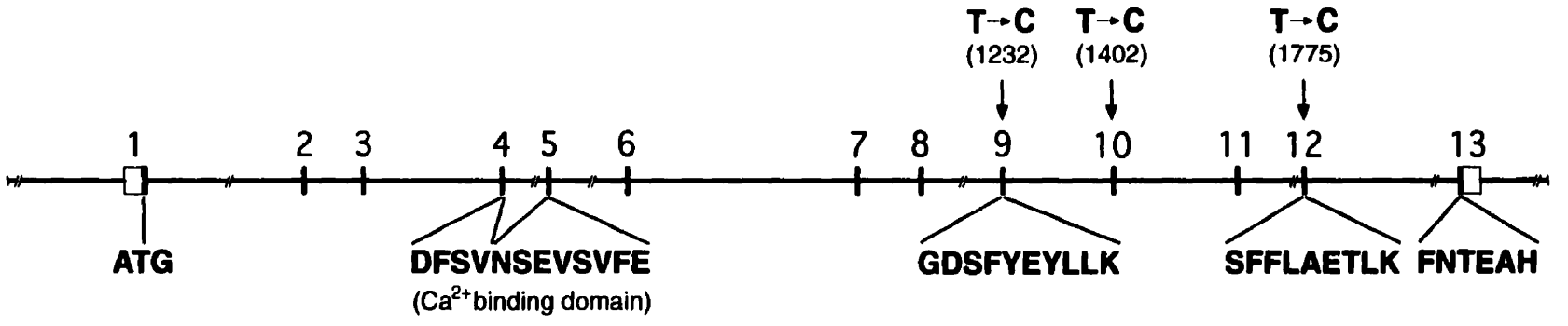
Their eyes have imperfectly aligned ommatidia and extra bristles. These observations, in combination with the high sequence similarity *mas-1* shares with murine α1,2-mannosidase IB, lead to the speculation that α1,2-mannosidase IB may be involved in the development of the nervous system and in angiogenesis in the mouse. The latter concept is also supported by the observation that α1,2-mannosidase activity is required for capillary tube formation *in vitro* (Nguyen *et al.*, 1992). If the phenotype of the *mas-1* disruption in *D. melanogaster* is any indication of the phenotype that would be observed for an α1,2-mannosidase IB disruption in mouse, the α1,2-mannosidase IB disruption may be extremely valuable in evaluating the role of α1,2-mannosidase IB *in vivo*. Should the resulting abnormalities resemble those observed in some human genetic diseases, studies of the phenotype resulting from an α1,2-mannosidase IB disruption may also be useful in understanding their cause.

The final possibility is that mice lacking α1,2-mannosidase IB would display no visible phenotype. This would occur if the expression of the undisrupted α1,2-mannosidase IA is adjusted in order to compensate for the disrupted α1,2-mannosidase IB. If this compensation were to occur, embryonic development could proceed normally and result in a viable normal adult mouse.

Knowledge of the genomic organization of the α1,2-mannosidase IB gene, and the results of α1,2-mannosidase IB expression studies can be used to identify regions of the gene that may be essential for enzyme expression and activity. Since the exact physiological role of the α1,2-mannosidase IB gene has not been studied, it is important to disrupt regions of the gene that not only abolish enzyme activity, but also prevent formation of the protein, which may have other unidentified roles *in vivo*. In the following section, regions of the α1,2-mannosidase IB gene that could be disrupted are discussed in light of the information that is presently known.

The first region of the α1,2-mannosidase IB gene that could be targeted for disruption is exon 1 (see Fig.9). Since exon 1 contains the site of initiation of translation, its disruption should prevent the transcription and translation of murine α1,2-mannosidase IB, assuming translation cannot be initiated at a downstream ATG. Although there is no evidence for the downstream initiation of α1,2-mannosidase IB transcription, the possibility cannot be excluded. Protein A fusion expression studies have shown that exon 1 and part of exon 2 are not required for enzyme activity (Schneikert and Herscovics,

Fig.9. Schematic representation of the α1,2-mannosidase IB indicating possible target sequences for disruption. Exon 1 and 2 are not required for enzyme activity. The putative EF hand-like putative calcium-binding domain spans exons 4 and 5. Exon 9, 12 and 13 contain regions that are highly conserved among all species, and that are thought to be essential for enzyme activity. The sites of the T to C point mutations observed in clone 4 are indicated by an arrow. Their nucleotide position relative to the α1,2-mannosidase IB cDNA is also indicated. Nucleotide positions are according to Herscovics *et al.*, 1994.


T→C
(1232)
T→C
(1402)
T→C
(1775)
1
2
3
4
5
6
7
8
9
10
11
12
13
ATG
DFSVNSEVSVFE
(Ca2+ binding domain)
GDSFYEYLLK
SFFLAETLK
FNTEAH

1994), suggesting that if translation were initiated at a downstream ATG, an active truncated α1,2-mannosidase IB protein could hypothetically be produced.

The second region of α1,2-mannosidase IB that could be disrupted is the conserved EF hand-like putative calcium-binding domain found in all calcium-dependent Class I α-mannosidases. EDTA inhibition studies have shown that murine α1,2-mannosidase IB requires calcium for activity. Disruption of this calcium-binding domain should therefore result in a protein, if produced, that cannot bind calcium. This possibility however is speculative, since no experimental evidence has yet been obtained to establish that the EF hand is the actual site of calcium binding in this particular protein.

The catalytic domain of α1,2-mannosidase IB gene also contains a number of regions that could be targeted for disruption. Previous work has shown that two naturally occurring isoforms of α1,2-mannosidase IB cDNA differing in three point mutations exist (Schneikert and Herscovics, 1995). Only one of these isoforms, clone 416, encodes an enzymatically active α1,2-mannosidase. Expression studies have demonstrated that the presence of cytidine instead of thymidine at position 1775 of clone 4 is sufficient to abolish enzyme activity. This point mutation changes Phe^{592} to Ser^{592}, and is found within the highly conserved seqeunce motif in exon 12 (^{590}SFFLAETLK). Conserved amino acid motifs are also found in exons 9 (^{398}GDSFYEYLLK) and exon 13 (^{617}FNTEAH). Although the role of these motifs is unknown, their high level of conservation, and the ability of a single point mutation in exon 12 to abolish enzyme activity, suggests that they may be essential for enzyme activity. These regions would likely be good candidates for disruption.

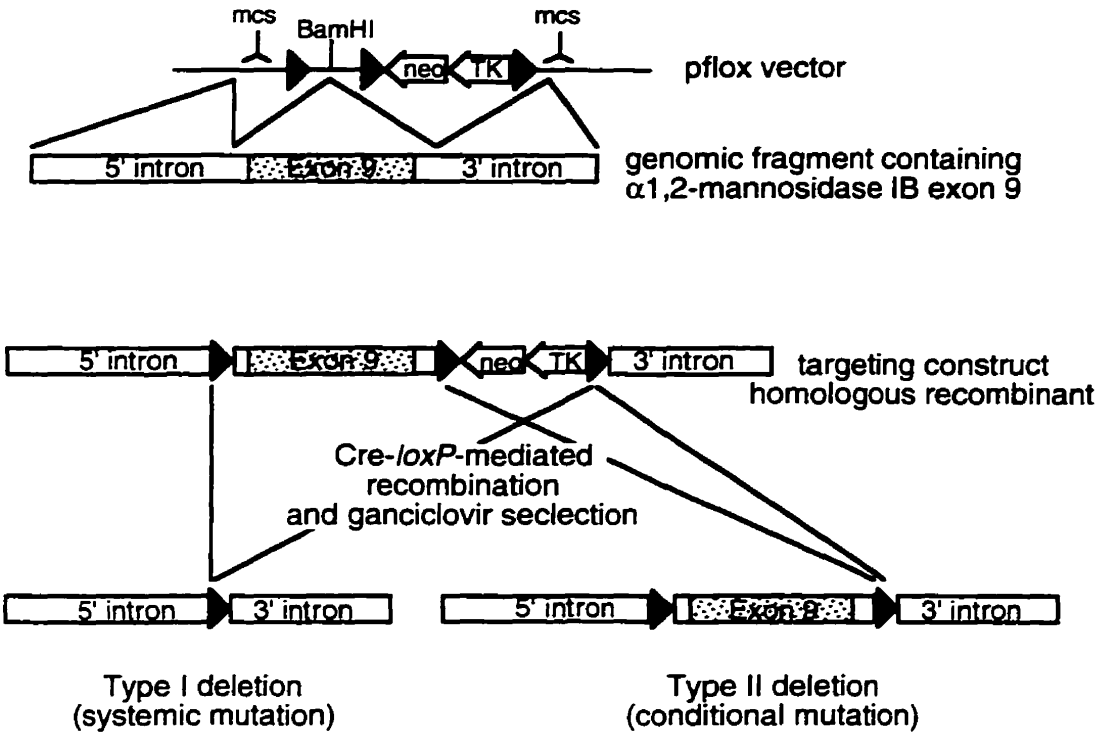
Of these three exons, exon 9 is probably the best candidate for disruption. It is 116 bp in size, is flanked by large introns, and contains the conserved region ^{398}GDSFYEYLLK, is the site of a T to C mutation in clone 4 which changes Met^{411} to Thr^{411}. The complete removal of this exon from the coding sequence causes a frameshift in α1,2-mannosidase IB reading frame, producing a truncated protein lacking any of the downstream conserved regions. If by chance the disrupted gene were transcribed and translated, a disruption of exon 9 would result in a protein lacking most of its catalytic domain and would most likely be inactive .

Regardless of which α1,2-mannosidase IB region is chosen for disruption, it will be important to introduce frameshifts and perhaps even point mutations into the targeting

construct to ensure that absolutely no protein is produced. The prevention of α1,2-mannosidase IB formation is extremely important, since α1,2-mannosidase IB may be involved in other physiological events besides mannose trimming.

If the disruption of α1,2-mannosidase IB is lethal at the embryonic stage, it will be difficult to evaluate the role of this enzyme *in vivo*. In this situation, the ability to generate a mouse model in which α1,2-mannosidase IB activity could be disrupted in specific tissues or cell-types would be useful. Recently, the development of Cre-*loxP* vectors has made these types of studies possible (Marth, 1996). These vectors are designed to take advantage of the ability of Cre recombinase of bacteriophage P1 to catalyze conservative reciprocal recombination events in mammalian cells. One such vector, pflox, contains three *loxP* sites arranged to allow the generation of two types of germline mutations by Cre recombination (Fig.10) (Gu *et al.*, 1994). If the α1,2-mannosidase IB targeting construct is designed using pflox as a vector, it will be possible to create two types of germline mutations, a Type I systemic mutation and a Type II conditional mutation, by homologous recombination. The Type I systemic mutation, in which the target α1,2-mannosidase IB sequence is completely excised from the genomic DNA, would result in the disruption of α1,2-mannosidase IB in all tissues and cell types at all ages of development. In contrast, the Type II deletion conditional mutation introduces *loxP* sites on either side of the targeted α1,2-mannosidase IB sequence, allowing the investigator to control the tissues in which the gene disruption occurs. The removal of the target sequence can then be induced in certain tissues, or at certain developmental stages by crossing a mouse expressing the Cre transgene with a mouse homozygous for the conditional mutation. The progeny produced will have a disrupted gene only in the cell types expressing the Cre transgene. Cre recombination can be temporally regulated and limited to certain cell types depending on the choice of regulatory sequences chosen to drive Cre transgene expression. The ability to target the α1,2-mannosidase IB disruption to specific cell types at different stages of development would be extremely useful in determining the physiological roles of α1,2-mannosidase IB, especially if disruption of this gene was lethal at early embryonic stages.

Fig.10. Targeting strategy for an α1,2-mannosidase IB disruption. The strategy used to generate two types of mutations, a Type I systemic mutation and a Type II conditional mutation by homologous recombination is shown. The Type I systemic mutation results in complete excision of the targeted genomic sequence found between the *loxP* sites. The Type II conditional mutation effectively flanks the genomic sequence to be targeted with *loxP* sites, enabling the investigator to induce recombination and excision of this fragment in specific tissues and cell-types. *LoxP* sites are indicated by triangles.


mcs
BamHI
mcs
neo
TK
pflox vector
5' intron
Exon 9
3' intron
genomic fragment containing
α1,2-mannosidase IB exon 9
5' intron
Exon 9
neo
TK
3' intron
targeting construct
homologous recombinant
Cre-loxP-mediated
recombination
and ganciclovir seclection
5' intron
3' intron
5' intron
Exon 9
3' intron
Type I deletion
(systemic mutation)
Type II deletion
(conditional mutation)

3.5. Evidence for another murine Class I α-mannosidase gene or pseudogene.

The results of this study also raise a number of intriguing questions about the possibility that other Class I α-mannosidases exist. Isolation of a novel genomic clone, Cos.25.1, that contains sequence with significant similarity to the murine α1,2-mannosidase IB cDNA (see Fig. 11, Appendix), provides evidence that other Class I α-mannosidases genes and/or pseudogenes exist within the genome. This existence of other α1,2-mannosidases is supported by biochemical evidence that indicates there are still uncloned α1,2-mannosidases, such as the rat liver ER α-mannosidase I (Weng and Spiro, 1993; Weng and Spiro, 1996). The properties of ER α-mannosidase I resemble those of the Class I α-mannosidases. It does not hydrolyze *p*-nitrophenyl α-D-mannoside, is kifunensine and 1-deoxymannojirimycin sensitive, and is resistant to inhibition by swainsonine. This enzyme has the same specificity as the yeast ER α1,2-mannosidase and yields $Man_8GlcNAc$ (isomer B). These observations suggest that this enzyme may be an uncloned member of the Class I α-mannosidases. Cos.25.1 could possibly represent the murine homologue of rat liver ER α1,2-mannosidase I, another previously unidentified α-mannosidase, or a pseudogene.

In order to understand the significance of Cos.25.1., it will be necessary to isolate the complete gene represented by this clone, and then undertake studies to determine if the gene is transcribed. If it is, further studies will be required to characterize the protein produced, the regulation of its expression and its physiological activity. If Cos.25.1 does not represent a functional gene, but rather a pseudogene, characterization of its structure will provide information as to its evolutionary origin.

3.6. A possible role for RNA editing in regulation of α1,2-mannosidase IB expression.

As previously described, two naturally occuring isoforms of α1,2-mannosidase IB cDNA, clone 416 and clone 4, differing in only three point mutations, have been isolated and characterized (Schneikert and Herscovics, 1995). Clone 416, the active form, is the product of the α1,2-mannosidase IB gene. The origin of clone 4 is still unknown. Because there is no evidence for a genomic sequence corresponding to clone 4, it is

suggested that the α1,2-mannosidase IB transcript may undergoe RNA editing to yield a cDNA with three point mutations. Since the point mutations introduced into the α1,2-mannosidase IB yield an inactive enzyme, it may be that RNA editing is a control mechanism governing α1,2-mannosidase IB activity. RNA editing has also been implicated in the regulation of another step in N-linked oligosaccharide biosynthesis. GlcNAc 1-phosphate transferase, an enzyme involved in the initial synthesis of the oligosaccharide dolichol-linked precursor, undergoes a G to A RNA editing event (Rajput *et al.*, 1994). Finally, the possibility that RNA editing may be a mechanism to alter the physiological function of murine α1,2-mannosidase IB must be considered. Although the edited form of α1,2-mannosidase IB lacks mannose trimming activity with $Man_9GlcNAc$ as substrate, it may have some other unknown function.

3.7. Conclusion.

Characterization of the genomic structure of the murine α1,2-mannosidase IB gene is the first step in achieving a complete understanding of the role and regulation of expression of the α1,2-mannosidases in N-linked oligosaccharide maturation. Knowledge of the genomic organization of this enzyme provides the basis for further genetic studies as described above. The completion of these genetic studies will provide insight into the mechanisms that regulate the invidual steps of N-linked oligosaccharide maturation and the overall control of protein glycsoylation.

APPENDIX

Fig.11. Comparison of the nucleotide and amino acid sequences from Cos.25.1 and exons 12 and 13 of murine α1,2-mannosidase IB (clone 416). The sequence similarity between Cos.25.1 and α1,2-mannosidase IB begins at amino acid position 560 of the α1,2-mannosidase IB cDNA. Sequence comparisons reveal that they are 83% similar at the amino acid level, and 93% similar at the nucleotide level. The sequence of Cos.25.1 contains a premature termination codon at position 566, an 9bp deletion (amino acids 601-603 of α1,2-mannosidase IB cDNA), and multiple base changes. The genomic organization also appears to be different. The amino acid and nucleotide differences found in Cos.25.1 are indicated in bold. The amino acid numbering of clone 416 is according to Herscovics *et al.*, 1994.

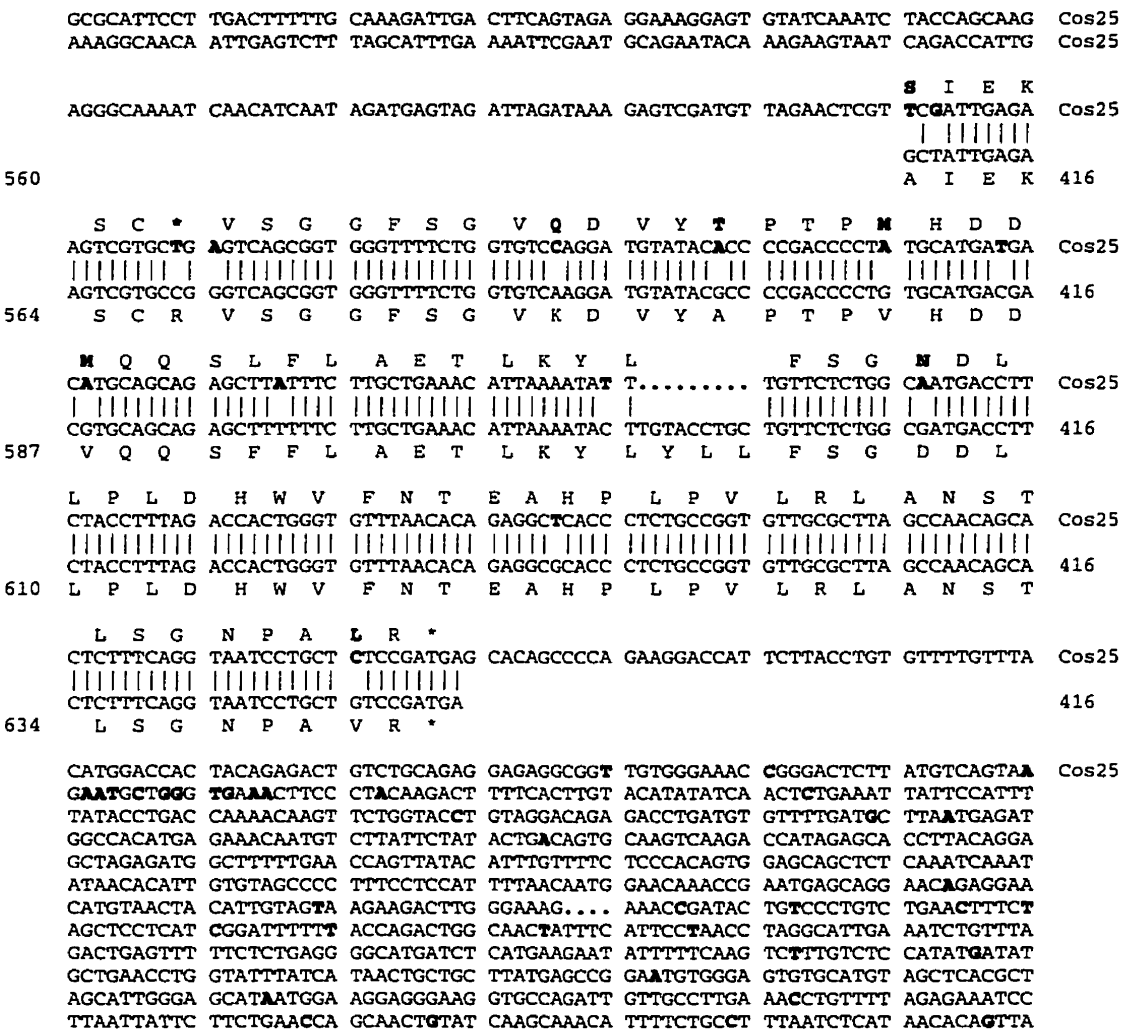
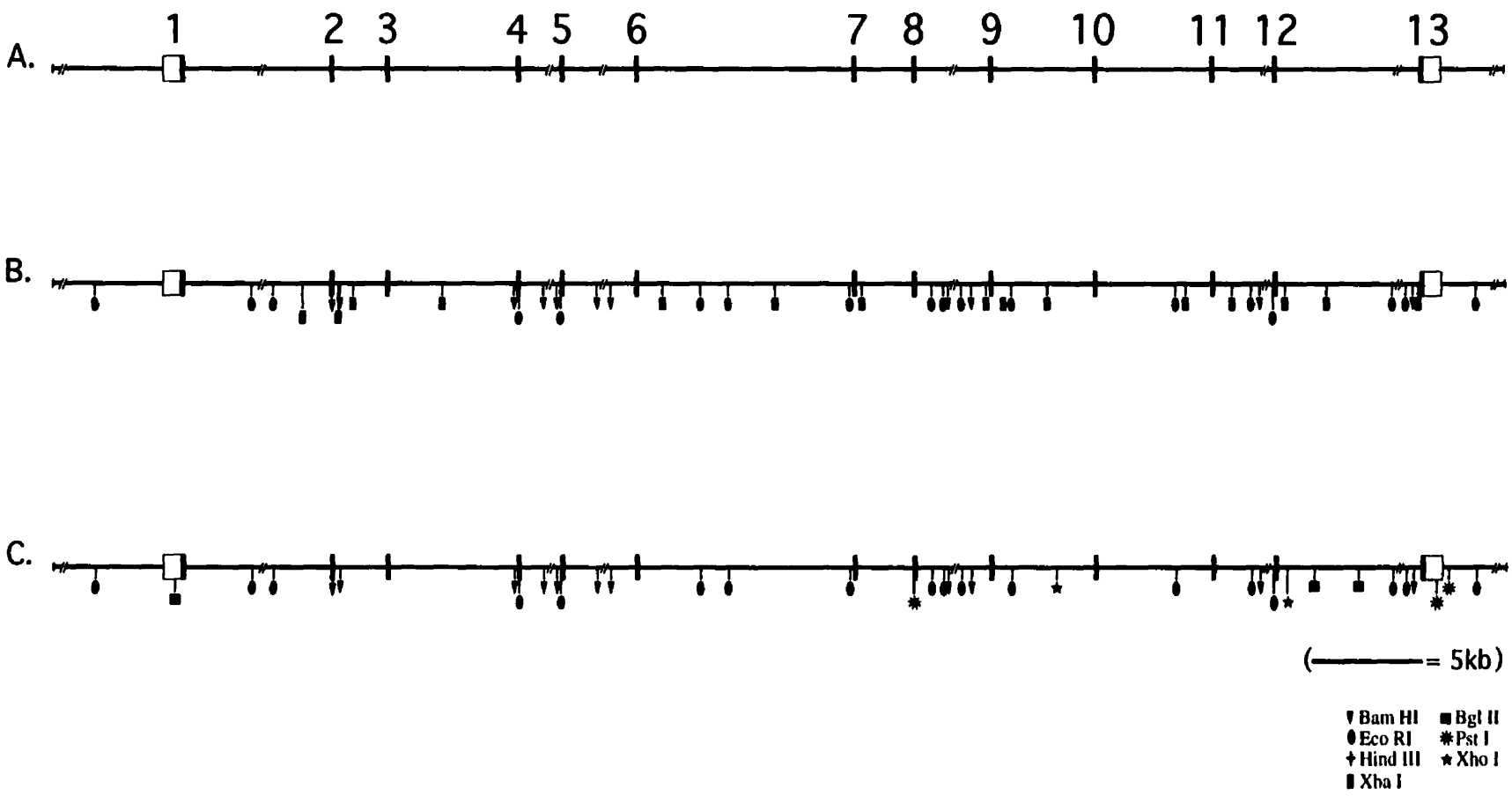

GCGCATTCCT TGACTTTTTG CAAAGATTGA CTTCAGTAGA GGAAAGGAGT GTATCAAATC TACCAGCAAG Cos25
AAAGGCAACA ATTGAGTCTT TAGCATTTGA AAATTCGAAT GCAGAATACA AAGAAGTAAT CAGACCATTG Cos25
S I E K
AGGGCAAAAT CAACATCAAT AGATGAGTAG ATTAGATAAA GAGTCGATGT TAGAACTCGT TCGATTGAGA Cos25
GCTATTGAGA
560 A I E K 416
S C * V S G G F S G V Q D V Y T P T P M H D D
AGTCGTGCTG AGTCAGCGGT GGGTTTTCTG GTGTCCAGGA TGTATACACC CCGACCCCTA TGCATGATGA Cos25
AGTCGTGCCG GGTCAGCGGT GGGTTTTCTG GTGTCAAGGA TGTATACGCC CCGACCCCTG TGCATGACGA 416
564 S C R V S G G F S G V K D V Y A P T P V H D D
M Q Q S L F L A E T L K Y L F S G N D L
CATGCAGCAG AGCTTATTTC TTGCTGAAAC ATTAAAATAT T..... TGTTCTCTGG CAATGACCTT Cos25
CGTGCAGCAG AGCTTTTTTC TTGCTGAAAC ATTAAAATAC TTGTACCTGC TGTTCTCTGG CGATGACCTT 416
587 V Q Q S F F L A E T L K Y L Y L L F S G D D L
L P L D H W V F N T E A H P L P V L R L A N S T
CTACCTTTAG ACCACTGGGT GTTTAACACA GAGGCTCACC CTCTGCCGGT GTTGCGCTTA GCCAACAGCA Cos25
CTACCTTTAG ACCACTGGGT GTTTAACACA GAGGCGCACC CTCTGCCGGT GTTGCGCTTA GCCAACAGCA 416
610 L P L D H W V F N T E A H P L P V L R L A N S T
L S G N P A L R *
CTCTTTCAGG TAATCCTGCT CTCCGATGAG CACAGCCCCA GAAGGACCAT TCTTACCTGT GTTTTGTTTA Cos25
CTCTTTCAGG TAATCCTGCT GTCCGATGA 416
634 L S G N P A V R *
CATGGACCAC TACAGAGACT GTCTGCAGAG GAGAGGCGGT TGTGGGAAAC CGGGACTCTT ATGTCAGTAA Cos25
GAATGCTGGG TGAAACTTCC CTACAAGACT TTTCACTTGT ACATATATCA ACTCTGAAAT TATTCCATTT
TATACCTGAC CAAAACAAGT TCTGGTACCT GTAGGACAGA GACCTGATGT GTTTTGATGC TTAATGAGAT
GGCCACATGA GAAACAATGT CTTATTCTAT ACTGACAGTG CAAGTCAAGA CCATAGAGCA CCTTACAGGA
GCTAGAGATG GCTTTTTGAA CCAGTTATAC ATTTGTTTTC TCCCACAGTG GAGCAGCTCT CAAATCAAAT
ATAACACATT GTGTAGCCCC TTTCCTCCAT TTTAACAATG GAACAAACCG AATGAGCAGG AACAGAGGAA
CATGTAACTA CATTGTAGTA AGAAGACTTG GGAAAG.... AAACCGATAC TGTCCCTGTC TGAACTTTCT
AGCTCCTCAT CGGATTTTTT ACCAGACTGG CAACTATTTC ATTCCTAACC TAGGCATTGA AATCTGTTTA
GACTGAGTTT TTCTCTGAGG GGCATGATCT CATGAAGAAT ATTTTTCAAG TCTTTGTCTC CATATGATAT
GCTGAACCTG GTATTTATCA TAACTGCTGC TTATGAGCCG GAATGTGGGA GTGTGCATGT AGCTCACGCT
AGCATTGGGA GCATAATGGA AGGAGGGAAG GTGCCAGATT GTTGCCTTGA AACCTGTTTT AGAGAAATCC
TTAATTATTC TTCTGAACCA GCAACTGTAT CAAGCAAACA TTTTCTGCCT TTAATCTCAT AACACAGTTA

Fig.12. Schematic representation of the α1,2-mannosidase IB gene with additional restriction endonuclease maps. Part A shows the arrangement of the 13 exons (depicted as vertical bars and numbers). The 5' and 3' UTR sequences are shown as white boxes. Part B shows a partial endonuclease restriction map for the enzymes BamHI (triangles), EcoRI (ovals), and Xba I (rectangles). Part C shows a partial endonuclease restriction map for the enzymes Bam HI (triangles), Eco RI (ovals), Bgl II (squares), Pst I (asterices), and Xho I (stars)


A.
1
2
3
4
5
6
7
8
9
10
11
12
13
B.
C.
(——— = 5kb)
Bam HI
Bgl II
Eco RI
Pst I
Hind III
Xho I
Xba I

REFERENCES

Ahrens, P. B. (1993). Role of target cell glycoproteins in sensitivity to natural killer cell lysis. *J. Biol. Chem.* **268**: 385-391.

Asano, T., K. Takata, H. Katagiri, H. Ishihara, K. Inukai, M. Anai, H. Hirano, Y. Yazaki, and Y. Oka (1993). The role of N-glycosylation in the targeting and stability of GLUT1 glucose transporter. *FEBS Lett.* **324**: 258-261.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl, Eds. (1987). Current Protocols in Molecular Biology. John Wiley and Sons, Inc.

Bause, E., E. Bieberich, A. Rolfs, C. Völker, and B. Schmidt (1993). Molecular cloning and primary structure of Man_9-mannosidase from human kidney. *Eur. J. Biochem.* **217**: 535-540.

Bause, E., W. Breuer, J. Schweden, R. Roeser, and R. Geyer (1992). Effect of substrate structure on the activity of Man_9-mannosidase from pig liver involved in N-linked oligosaccharide processing. *Eur. J. Biochem.* **208**: 451-457.

Bause, E., R. Erkens, J. Schweden, and L. Jaenicke (1986). Purification and characterization of trimming glucosidase I from Saccharomyces cerevisiae. *FEBS Lett.* **206**: 208-212.

Bause, E., J. Schweden, A. Gross, and B. Orthen (1989). Purification and characterization of trimming glucosidase I from pig liver. *Eur. J. Biochem.* **183**: 661-669.

Bezouska, K., C. Yuen, J. O'Brien, R. A. Childs, W. Chai, A. M. Lawson, K. Drbal, A. Fiserová, M. Pospísil, and T. Feizi (1994). Oligosaccharide ligands for NKR-P1 protein activate NK cells and cytotoxicity. *Nature* **372**: 150-157.

Bieberich, E. and E. Bause (1995). Man_9-mannosidase from human kidney is expressed in COS cells as Golgi-resident type II transmembrane N-glycoprotein. *Eur. J. Biochem.* **233**: 644-649.

Bischoff, J. and R. Kornfeld (1983). Evidence for an α-mannosidase in endoplasmic reticulum of rat liver. *J. Biol. Chem.* **258**: 7907-7910.

Bischoff, J. and R. Kornfeld (1984). The effect of 1-deoxymannojirimycin on rat liver α-mannosidases. *Biochem. Biophys. Res. Commun.* **125**: 324-331.

Bischoff, J. and R. Kornfeld (1986). The soluble form of rat liver α-mannosidase is immunologically related to the endoplasmic reticulum membrane bound α-mannosidase. *J. Biol. Chem.* **261**: 4758-4765.

Bischoff, J., L. Liscum, and R. Kornfeld (1986). The use of 1-deoxymannojirimycin to evaluate the role of various α-mannosidases in oligosaccharide processing in intact cells. *J. Biol. Chem.* **261**: 4766-4774.

Bischoff, J., K. Moremen, and H. F. Lodish (1990). Isolation, characterization and expression of cDNA encoding rat liver endoplasmic reticulum α-mannosidase. *J. Biol. Chem.* **265**: 17110-17117.

Boyle, A. L., D. M. Feltquite, N. C. Dracopoli, D. E. Housman, and D. C. Ward (1992). Rapid physical mapping of cloned DNA on banded mouse chromosomes by fluorescence in situ hybridization. *Genomics* **12**: 106-115.

Brada, D., D. Kerjaschki, and J. Roth (1990). Cell type-specific post-Golgi apparatus localization of a "resident" endoplasmic reticulum glycoprotein, glucosidase II. *J. Cell. Biol.* **110**: 309-318.

Breathnach, R. and P. Chambon (1981). Organization and expression of eucaryotic split genes coding for proteins. *Annu. Rev. Biochem.* **50**: 349-383.

Burke, J., F. Lipari, S. Igdoura, and A. Herscovics (1996). The Saccharomyces cerevisiae processing α1,2-mannosidase is localized in the endoplasmic reticulum, independently of known retrieval motifs. *Eur. J. Cell Biol.* **70**: 298-305.

Burns, D. M. and O. Touster (1982). Purification and characterization of glucosidase II, an endoplasmic reticulum hydrolase involved in glycoprotein biosynthesis. *J. Biol. Chem.* **257**: 9991-10000.

Byrd, J. C., A. L. Tarentino, F. Maley, P. H. Atkinson, and R. B. Trimble (1982). Glycoprotein synthesis in yeast: Identification of $Man_8GlcNAc_2$ as an essential intermediate in oligosaccharide processing. *J. Biol. Chem.* **257**: 14657-14666.

Camirand, A., A. Heysen, B. Grondin, and A. Herscovics (1991). Glycoprotein biosynthesis in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **266**: 15120-15127.

Charuk, J. H. M., J. Tan, M. Bernardini, S. Haddad, R. A. F. Reithmeier, J. Jaeken, and H. Schachter (1995). Carbohydrate-deficient glycoprotein syndrome type II. An autosomal recessive N-acetylglucosaminyltransferase II deficiency different from typical hereditary erthyroblastic multinuclearity, with a positive acidified-serum lysis test (HEMPAS). *Eur. J. Biochem.* **230**: 797-805.

Daniel, P. F., B. Winchester, and C. D. Warren (1994). Mammalian α-mannosidases-multiple forms but a common purpose? *Glycobiology* **4**:551-566.

Dennis, J. W. (1986). Effects of swainsonine and polyinosinic:polycytidylic acid on murine tumor cell growth and metastasis. *Cancer Res.* **46**: 5131-5136.

DeSantis, R., U. V. Santer, and M. C. Glick (1987). NIH 3T3 cells transfected with human tumor DNA lose the transformed phenotype when treated with swainsonine. *Biochem. Biophys. Res. Commun.* **142**: 348-353.

Dewald, B. and O. Touster (1973). A new α-D-mannosidase occurring in Golgi membranes. *J. Biol. Chem.* **248**:

Dinter, A. and E. G. Berger (1995). The regulation of cell- and tissue-specific expression of glycans by glycosyltransferases. *Adv. Exp. Med. Biol.* **376**: 53-82.

Elbein, A. D. (1991). Glycosidase inhibitors: inhibitors of *N*-linked oligosaccharide processing. *FASEB J.* **5**: 3055-3063.

Esmon, B., P. C. Esmon, and R. Schekman (1984). Early steps in processing of yeast glycoproteins. *J. Biol. Chem.* **259**: 10322-10327.

Etzioni, A., L. M. Phillips, J. C. Paulson, and J. M. Harlan (1995). Leukocyte adhesion deficiency (LAD) II. *Ciba Found. Symp.* **189**: 51-62.

Forsee, W. T., C. F. Palmer, and J. S. Schutzbach (1989). Purification and characterization of an α1,2-mannosidase involved in processing asparagine-linked oligosaccharides. *J. Biol. Chem.* **264**: 3869-3876.

Forsee, W. T. and J. S. Schutzbach (1981). Purification and characterization of a phospholipid-dependent α-mannosidase from rabbit liver. *J. Biol. Chem.* **256**: 6577-6582.

Forsee, W. T., J. D. Springfield, and J. S. Schutzbach (1982). Effect of phospholipids on α-1,2-mannosidase activity. *J. Biol. Chem.* **257**: 9963-9967.

Foster, J. M., B. Yudkin, A. E. Lockyer, and D. B. Roberts (1995). Cloning and sequence analysis of *Gm II*, a *D. melanogaster melanogaster* homologue of the cDNA encoding murine Golgi α-mannosidase II. *Gene* **154**: 183-186.

Fujimoto, K. and R. Kornfeld (1991). α-Glucosidase II deficient cell use endo-α-mannosidase as a bypass route for N-linked oligosaccharide processing. *J. Biol. Chem.* **266**: 3571-3578.

Fukuda, M. N. (1990). HEMPAS disease: genetic defect of glycosylation. *Glycobiology* **1**: 9-15.

Goss, P. E., J. Baptiste, B. Fernandes, M. Baker, and J. W. Dennis (1994). A phase I study of swainsonine in patients with advanced malignancies. *Cancer Res.* **54**: 1450-1457.

Grard, T., V. Herman, A. Saint-Pol, D. Kmiecik, O. Labiau, A. Mir, C. Alonso, A. Verbert, R. Cacan, and J. Michalaski (1996). Oligomannosides or oligosaccharide-lipids as potential substrates for rat liver cytosolic α-D-mannosidase. *Biochem. J.* **316**: 787-792.

Grard, T., A. Saint-Pol, J. Haeuw, C. Alonso, J. Wieruszeski, G. Strecker, and J. Michalski (1994). Soluble forms of α-D-mannosidases from rat liver. *Eur. J. Biochem.* **223**: 99-106.

Grinna, L. S. and P. W. Robbins (1979). Glycoprotein biosynthesis: Rat liver microsomal glucosidases which process oligosaccharides. *J. Biol. Chem.* **254**: 8814-8818.

Grinna, L. S. and P. W. Robbins (1980). Substrate specificities of rat liver microsomal glucosidases which process glycoproteins. *J. Biol. Chem.* **255**: 2255-2258.

Gu, H., J. D. Marth, P. C. Orban, H. Mossman, and K. Rajewksy (1994). Deletion of a DNA polymerase β gene segment in T cells using cell type-specific gene targeting. *Science* **265**: 103-106.

Hamagashira, N., H. Oku, T. Mega, and S. Hase (1996). Purification and characterization of hen oviduct α1,2-mannosidase. *J. Biochem.* **119**: 998-1003.

Harduin-Lepers, A., J. H. Shaper, and N: L. Shaper (1993). Characterization of two cis-regulatory regions in the murine β1,4-galactosyltransferase gene. Evidence for a negative regulatory element that controls intiation at the proximal site. *J. Biol. Chem.* **268**:14348-14359.

Harduin-Lepers, A., N. L. Shaper, J. A. Mahoney, and J. H. Shaper (1992). Murine β1,4-glactosyltransferase: round spermatid transcripts are characterized by an extended 5'-untranslated region. *Glycobiology* **2**:361-368.

Heng, H. H. Q. and L. Tsui (1993). Modes of DAPI banding and simultaneous in situ hybridization. *Chromosoma* **102**: 325-332.

Herscovics, A., J. Schneikert, A. Athanassiadis, and K. W. Moremen (1994). Isolation of a mouse Golgi mannosidase cDNA, a member of a gene family conserved from yeast to mammals. *J. Biol. Chem.* **269**: 9864-9871.

Hettkamp, H., G. Legler, and E. Bause (1984). Purification by affinity chromatography of glucosidase I, an endoplasmic reticulum hydrolase involved in the processing of asparagine-linked oligosaccharides. *Eur. J. Biochem.* **142**: 85-90.

Hiraizumi, S., U. Spohr, and R. G. Spiro (1993). Characterization of endomannosidase inhibitors and evaluation of their effect on N-linked oligosaccharide processing during glycoprotein biosynthesis. *J. Biol. Chem.* **268**: 9927-9935.

Hollis, G. F., J. G. Douglas, N. L. Shaper, J. H. Shaper, J. M. Stafford-Hollis, R. J. Evans, and I. R. Kirsch (1989). Genomic structure of murine β1,4-galactosyltransferase. *Biochem. Biophys. Res. Commun.* **162**: 1069-1075.

Hubbard, S. C. and R. J. Ivatt (1981). Synthesis and processing of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **50**: 555-583.

Ichishima, E., M. Arai, Y. Shigematsu, H. Kumagai, and R. Sumida-Tanaka (1981). Purification of an acidic α-D-mannosidase from *Aspergillus saitoi* and specific cleavage of 1,2-α-D-mannosidic linkage in yeast mannan. *Biochim. et Biophys. Acta* **658**: 45-53.

Inoue, T., T. Yoshida, and E. Ichishima (1995). Molecular cloning and nucleotide sequence of the 1,2-α-D-mannosidase gene, *msdS*, from *Aspergillus saitoi* and expression of the gene in yeast cells. *Biochim. et Biophys. Acta* **1253**: 141-145.

Ioffe, E. and P. Stanley (1994). Mice lacking *N*-acetylglucosaminyltransferase I activity die at mid-gestation, revealing an essential role for complex or hybrid N-linked carbohydrates. *Proc. Natl. Acad. Sci. USA* **91**: 728-732.

Jelinek-Kelly, S., T. Akiyama, B. Saunier, J. S. Tkacz, and A. Herscovics (1985). Characterization of a specific α-mannosidase involved in oligosaccharide processing in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **260**: 2253-2257.

Jelinek-Kelly, S. and A. Herscovics (1988). Glycoprotein biosynthesis in Saccharomyces cerevisiae. Purification of the α-mannosidase which removes one specific mannose residue from $Man_9GlcNAc$. *J. Biol. Chem.* **263**: 14757-14763.

Joziasse, D. H. (1992). Mammalian glycosyltransferases: genomic organization and protein structure. *Glycobiology* **2**: 271-277.

Joziasse, D. H., N. L. Shaper, D. Kim, D. H. Van den Eijnden, and J. H. Shaper (1992). Murine α1,3-galactosyltransferase. *J. Biol. Chem.* **267**: 5534-5541.

Kalz-Füller, B., E. Bieberich, and E. Bause (1995). Cloning and expression of glucosidase I from human hippocampus. *Eur. J. Biochem.* **231**: 344-351.

Kaushal, G. P., T. Szumilo, I. Pastuszak, and A. D. Elbein (1990). Purification to homogeneity and properties of mannosidase II from mung bean seedlings. *Biochem.* **29**: 2168-2176.

Kepes, F. and R. Schekman (1988). The yeast SEC53 gene encodes phosphomannomutase. *J. Biol. Chem.* **263**: 9155-9161.

Kerscher, S., S. Albert, D. Wucherpfennig, M. Heisenberg, and S. Schneuwly (1995). Molecular and genetic analysis of the *Drosophila mas-1 (mannosidase-1)* gene which encodes a glycoprotein processing α1,2-mannosidase. *Dev. Biol.* **168**: 613-626.

Kilker, J. D., B. Saunier, J.S. Tkacz, A. Herscovics (1981). Partial purification from *Saccharomyces cerevisiae* of a soluble glucosidase which removes the terminal glucose from the oligosaccharide $Glc_3Man_9GlcNAc_2$. *J. Biol. Chem.* **256**:5299-5303

Kleene, R. and E. G. Berger (1993). The molecular and cell biology of glycosyltransferases. *Biochim. Biophys. Acta* **1154**: 283-325.

Kornfeld, R. and S. Kornfeld (1985). Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **54**: 631-664.

Kornfeld, S. (1990). Lysosomal enzyme targeting. *Biochem. Soc. Trans.* **18**:367-374.

Krog, L. and E. Bock (1992). Glycosylation of neural cell adhesion molecules of the immunoglobulin superfamily. *APMIS Suppl.* **27**: 53-70.

Kumar, R., J. Yang, R. L. Eddy, M. G. Byers, T. B. Shows, and P. Stanley (1992). Cloning and expression of murine gene and chromosomal localization of the human gene encoding N-acetylglucosaminyltransferase I. *Glycobiology* **2**: 383-393.

Kwar, Z., A. Herscovics, and D. L. Jarvis (1996). Isolation and characterization of an α1,2-mannosidase from the lepidopteran insect cell line Sf9. *Glycobiology* **6**: 10.01.

Lal, A., A. Herscovics, and K. W. Moremen (1996). Biochemical characterization and substrate specificity of murine Class I mannosidases. *Glycobiology* **6**: 15.

Lal, A., J. S. Schutzbach, W. T. Forsee, P. J. Neame, and K. W. Moremen (1994). Isolation and expression of murine and rabbit cDNAs encoding an α1,2-mannosidase involved in the processing of asparagine-linked oligosaccharides. *J. Biol. Chem.* **269**: 9872-9881.

Lichter, P., C. J. Tang, K. Call, G. Hermanson, G. A. Evans, D. Housman, and D. C. Ward (1990). High-resolution mapping of human chromosome 11 by in situ hybridization with cosmid clones. *Science* **247**: 64-69.

Lipari, F., B. J. Gour-Salin, and A. Herscovics (1995). The *Saccharomyces cerevisiae* processing α1,2-mannosidase is an inverting glycosidase. *Biochem. Biophys. Res. Commun.* **209**: 322-326.

Lipari, F. and A. Herscovics (1994). Production, purification and characterization of recombinant yeast processing α1,2-mannosidase. *Glycobiology* **4**: 697-702.

Lipari, F. and A. Herscovics (1996). Role of the cysteine residues in the α1,2-mannosidase involved in N-glycan biosynthesis in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **in press**:

Lodish, H. F. and N. Kong (1984). Glucose removal from N-linked oligosaccharides is required for efficient maturation of certain secretory glycoproteins from the rough endoplasmic reticulum to the Golgi complex. *J. Cell. Biol.* **98**: 1720-1729.

Lowe, J. B., J. F. Kukowska-Latallo, R. P. Nair, R. D. Larson, R. M. Marks, B. A. Macher, R. J. Kelly, and L. K. Ernst (1991). Molecular cloning of a human fucosyltransferase gene that determines expression of the Lewis X and VIM-2 epitopes but not ELAM-1-dependent cell adhesion. *J. Biol. Chem.* **266**: 17467-17477.

Lubas, W. A. and R. G. Spiro (1987). Golgi endo-α-D-mannosidase from rat liver, a novel N-linked carbohydrate unit processing enzyme. *J. Biol. Chem.* **262**: 3775-3781.

Lubas, W. A. and R. G. Spiro (1988). Evaluation of the role of rat liver Golgi endo-α-D-mannosidase in processing N-linked oligosaccharides. *J. Biol. Chem.* **263**: 3990-3998.

Mallet, B., P. Lejeune, N. Baudry, P. Niccoli, P. Carayon, and J. Franc (1995). *N*-glycans modulate *in vivo* and *in vitro* thyroid hormone synthesis. *J. Biol. Chem.* **270**: 29881-29888.

Marth, J. D. (1996). Recent advances in gene mutagenesis by site-directed recombination. *J. Clin. Invest.* **97**: 1999-2002.

McCloskey, M. A. and F. A. Troy (1980). Paramagnetic isoprenoid carrier lipids. 2. Dispersion and dynamics in lipid membranes. *Biochem.* **18**: 2061-2066.

Mengle-Gaw, L., M. F. McCoy-Haman, and D. C. Tiemeier (1991). Genomic structure and expression of human β-1,4-galactosyltransferase. *Biochem. Biophys. Res. Commun.* **176**: 1269-1276.

Merkle, R. K. and K. W. Moremen (1993). Cloning of a murine homolog of the *Dictyostelium discoideum* lysosomal α-mannosidase. *Glycoconjugate J.* **10**: 240.

Metzler, M., A. Gertz, M. Sarkar, H. Schachter, J. W. Schrader, and J. D. Marth (1994). Complex asparagine-linked oligosaccharides are required for morphogenic events during post-implantation development. *EMBO J.* **13**: 2056-2065.

Misago, M., Y.-F. Liao, S. Kudo, S. Eto, M. Mattei, K. W. Moremen, and M. N. Fukuda (1995). Molecular cloning and expression of cDNAs encoding human α-mannosidase II and a previously unrecognized α-mannosidase II^x isozyme. *Proc. Natl. Acad. Sci. USA* **92**: 11766-11770.

Moore, S. E. and R. G. Spiro (1990). Demonstration that Golgi endo-α-D-mannosidase provides a glucosidase independent pathway for the formation of complex N-linked oligosaccharides of glycoproteins. *J. Biol. Chem.* **265**: 13104-13112.

Moore, S. E. H. and R. G. Spiro (1993). Inhibition of glucose trimming by castanospermine results in rapid degradation of unassembled major histocompatibility complex class I molecules. *J. Biol. Chem.* **268**: 3809-3812.

Moore, S. E. H. and R. G. Spiro (1994). Intracellular compartmentalization and degradation of free polymannose oligosaccharides release during glycoprotein biosynthesis. *J. Biol. Chem.* **269**: 12715-12721.

Moremen, K. W. (1989). Isolation of a rat liver Golgi mannosidase II clone by mixed oligonucleotide-primed amplification of cDNA. *Proc. Natl. Acad. Sci. USA* **86**: 5276-5280.

Moremen, K. W. and P. W. Robbins (1991). Isolation, characterization, and expression of cDNAs encoding murine α-mannosidase II, a Golgi enzyme that controls conversion of high mannose to complex N-glycans. *J. Cell. Biol.* **115**: 1521-1534.

Moremen, K. W. and O. Touster (1985). Biosynthesis and modification of Golgi mannosidase II in HeLa and 3T3 cells. *J. Biol. Chem.* **260**: 6654-6662.

Moremen, K. W., O. Touster, and P. W. Robbins (1991). Novel purification of the catalytic domain of Golgi α-mannosidase II. Characterization and comparison with the intact enzyme. *J. Biol. Chem.* **266**: 16876-16885.

Moremen, K. W., R. B. Trimble, and A. Herscovics (1994). Glycosidases of the asparagine-linked oligosaccharide processing pathway. *Glycobiology* **4**: 113-125.

Nebes, V. L. and M. C. Schmidt (1994). Human lysosomal alpha-mannosidase: Isolation and nucleotide sequence of the full-length cDNA. *Biochem. Biophys. Res. Commun.* **200**: 239-245.

Nguyen, M., J. Folkman, and J. Bischoff (1992). 1-Deoxymannojirimycin inhibits capillary tube formation *in vitro*. *J. Biol. Chem.* **267**: 26157-26165.

Nichols, E. J., R. Manger, S. Hakomori, A. Herscovics, and L. R. Rohrscheider (1985). Transformation by the v-fms oncogene product: role of glycosylational processing and cell surface expression. *Mol. Cell. Biol.* **5**:3467-3475.

Ora, A. and A. Helenius (1995). Calnexin fails to associate with substrate proteins in glucosidase-deficient cell lines. *J. Biol. Chem.* **270**: 26060-26062.

Ou, W., P. H. Cameron, D. Y. Thomas, and J. J. M. Bergeron (1993). Association of folding intermediates of glycoproteins with calnexin during protein maturation. *Nature* **364**: 771-776.

Paulson, J. C. and K. J. Colley (1989). Glycosyltransferases: Structure, localization, and control of cell type-specific glycosylation. *J. Biol. Chem.* **264**: 17615-17618.

Pili, R., J. Chang, R. A. Partis, R. A. Mueller, F. J. Chrest, and A. Passaniti (1995). The α-glucosidase I inhibitor castanospermine alters endothelial cell glycosylation, prevents angiogenesis, and inhibits tumor growth. *Cancer Res.* **55**: 2920-2926.

Puccia, R., B. Grondin, and A. Herscovics (1993). Disruption of the processing α-mannosidase gene does not prevent outer chain synthesis in *Saccharomyces cerevisiae*. *Biochem. J.* **290**: 21-26.

Rademacher, T. D., R. B. Parekh, and R. A. Dwek (1988). Glycobiology. *Annu. Rev. Biochem.* **57**: 785-838.

Rajput, B., J. Ma, and I. K. Vijay (1994). Structure and organization of mouse GlcNAc-1-phosphate transferase gene. *J. Biol. Chem.* **269**: 9590-9597.

Reitmann, M. L., A. Varki, S. Kornfeld (1981). Fibroblasts from patients with I-cell disease and pseudo-Hurler polydystrophy are deficient in uridine 5'-diphosphate-N-acetylglucosamine:glycoprotein N-acetylglucoaminylphosphotransferase activity. *J. Clin. Invest.* **67**:1574-1579.

Ren, J., R. K. Bretthauer, and F. J. Castellino (1995). Purification and properties of a Golgi-derived (α1,2)-mannosidase-I from baculovirus-infected lepidopteran insect cells (IPLB-SF21AE) with preferential activity toward mannose$_6$-*N*-acetylglucosamine$_2$. *Biochem.* **34**: 2489-2495.

Rizzolo, L. J. and R. Kornfeld (1988). Post-translational protein modification in the endoplasmic reticulum: Demonstration of fatty acylase and deoxymannojirimycin-sensitive α-mannosidase activities. *J. Biol. Chem.* **263**: 9520-9525.

Roth, J., D. Brada, P. M. Lackie, J. Schweden, and E. Bause (1990). Oligosaccharide trimming Man$_9$-mannosidase is a resident ER protein and exhibits a more restricted and local distribution than glucosidase II. *Eur. J. Cell Biol.* **53**: 131-141.

Rush, J. S. and C. J. Waechter (1995). Transmembrane movement of a water-soluble analogue of mannosylphosporyldolichol is mediated by an endoplasmic reticulum protein. *J. Cell. Biol.* **130**: 529-536.

Rutishauser, U. and L. Landmesser (1996). Polysialic acid in the vertebrate nervous system: a promoter of plasticity in cell-cell interactions. *Trends in Neuroscience* **19**: 422-427.

Saito, H., J. Gu, A. Nishikawa, Y. Ihara, J. Fujii, Y. Kohgo, and N. Taniguchi (1995). Organization of the human N-acetylglucosaminyltransferase V gene. *Eur. J. Biochem.* **233**: 18-26.

Sambrook, J., E. F. Fritsch, and T. Maniatis, Eds. (1989). Molecular cloning : a laboratory manual. Cold Spring Harbour, Cold Spring Harbour Laboratory Press.

Saunier, B., J. R. D. Kilker, J. S. Tkacz, A. Quaroni, and A. Herscovics (1982). Inhibition of N-linked complex oligosaccharide formation by 1-deoxynojirimycin, an inhibitor of processing glucosidases. *J. Biol. Chem.* **257**: 14155-14161.

Schatzle, J., J. Bush, and J. Cardelli (1992). Molecular cloning and characterization of the structural gene coding for the developmentally regulated lysosomal enzyme α-mannosidase in *Dictyostelium discoideum*. *J. Biol. Chem.* **267**: 4000-4007.

Schneikert, J. and A. Herscovics (1994). Characterization of a novel mouse recombinant processing α-mannosidase. *Glycobiology* **4**: 445-450.

Schneikert, J. and A. Herscovics (1995). Two naturally occurring mouse α1,2-mannosidase IB cDNA clones differ in three point mutations. *J. Biol. Chem.* **270**: 17736-17740.

Schweden, J. and E. Bause (1989). Characterization of trimming Man_9-mannosidase from pig liver. Purification of a catalytically active fragment and evidence for the transmembrane nature of the intact 65 kDa enzyme. *Biochem. J.* **264**: 347-355.

Schweden, J., C. Borgmann, G. Legler, and E. Bause (1986). Characterization of calf liver glucosidase I and its inhibition by basic sugar analogs. *Arch. Biochem. Biophys.* **248**: 335-340.

Schweden, J., G. Legler, and E. Bause (1986). Purification and characterization of a neutral processing mannosidase from calf liver acting on $(Man)_9(GlcNAc)_2$ oligosaccharides. *Eur. J. Biochem.* **157**: 563-570.

Shailubhai, K., M. A. Pratta, and I. K. Vijay (1987). Purification and characterization of glucosidase I involved in N-linked glycoprotein processing in bovine mammary gland. *Biochem. J.* **247**: 555-562.

Shapiro, M. B. and P. Senapathy (1987). RNA splice junctions of different classes of eukaryotes: Sequence statistics and functional implications in gene expression. *Nucleic Acids Res.* **15**: 7155-7174.

Sharma, P. M., M. Bowman, S. L. Madden, F. J. Rauscher III, and S. Sukumar (1994). RNA editing in the Wilms' tumor susceptibility gene, WT1. *Genes Dev.* **8**: 720-731.

Shoup, V. A. and O. Touster (1976). Purification and characterization of the α-D-mannosidase of rat liver cytosol. *J. Biol. Chem.* **251**: 3845-3852.

Silberstein, S. and R. Gilmore (1996). Biochemistry, molecular biology, and genetics of the oligosaccharyltransferase. *FASEB J.* **10**: 849-858.

Simpson, L. and R. B. Emeson (1996). RNA editing. *Annu. Rev. Neurosci.* **19**: 27-52.

Smith, C. W. (1993). Endothelial adhesion molecules and their role in inflammation. *Can. J. Physiol. Pharmacol.* **71**: 76-87.

Snider, M. D. and P. W. Robbins (1982). Transmembrane organization of protein glycosylation: Mature oligosaccharide-lipid is located on the lumenal side of microsomes from Chinese hamster ovary cells. *J. Biol. Chem.* **257**: 6796-6801.

Snider, M. D. and O. C. Rogers (1984). Transmembrane movement of oligosaccharide lipids during glycoprotein synthesis. *Cell* **36**: 753-761.

Sriramarao, P., E. Berger, J. D. Chambers, K. E. Arfors, and K. R. Gehlsen (1993). High mannose type N-linked oligosaccharides on endothelial cells may influence β2 integrin mediated neutrophil adhesion adherence in vitro. *J. Cell. Biochem.* **51**: 360-368.

Su, K., T. Stoller, J. Rocco, J. Zemsky, and R. Green (1993). Pre-Golgi degradation of yeast prepro-α-factor expressed in a mammalian cell. *J. Biol. Chem.* **268**: 14301-14309.

Svensson, E. C., B. Soreghan, and J. C. Paulson (1990). Organization of the β-galactoside α2,6-sialyltransferase gene. Evidence for the transcriptional regulation of terminal glycosylation. *J. Biol. Chem.* **265**: 20863-20868.

Tabas, I. and S. Kornfeld (1979). Purification and characterization of a rat liver Golgi α-mannosidase capable of processing asparagine-linked oligosaccharides. *J. Biol. Chem.* **254**: 11655-11663.

Tan, J., G. A. F. D'Agostaro, B. Bendiak, F. Reck, M. Sarkar, J. A. Squire, P. Leong, and H. Schachter (1995). The human UDP-N-acetylglucosaminyl: α-6-D-mannoside-β-1,2-N-acetylglucosaminyltransferase II gene. *Eur. J. Biochem.* **231**: 317-328.

Tremblay, L., N. Campbell Dyke, and A. Herscovics (1996). Characterization of human α1,2-mannosidase IB. *Glycobiology* **6**: 10.06.

Tulsiani, D. R. P., T. M. Harris, and O. Touster (1982). Swainsonine inhibits the biosynthesis of complex glycoproteins by inhibition of Golgi mannosidase II. *J. Biol. Chem.* **257**: 7936-7939.

Tulsiani, D. R. P., S. C. Hubbard, P. W. Robbins, and O. Touster (1982). α-D-mannosidases of rat liver Golgi membranes. *J. Biol. Chem.* **257**: 3660-3668.

Tulsiani, D. R. P., D. J. Opheim, and O. Touster (1977). Purification and characterization of α-D-mannosidase from rat liver golgi membranes. *J. Biol. Chem.* **252**: 3227-3233.

Tulsiani, D. R. P. and O. Touster (1988). The purification and characterization of mannosidase IA from rat liver Golgi membranes. *J. Biol. Chem.* **263**: 5408-5417.

Van Schaftingen, E. and J. Jaeken (1995). Phosphomannomutase deficiency is a cause of carbohydrate-deficient glycoprotein syndrome type I. *FEBS Lett.* **377**: 318-320.

Varki, A. (1993). Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology* **3**: 97-130.

Wang, X., T. P. O'Hanlon, R. F. Young, and J. T. Lau (1990). Rat β-galactoside α2,6-sialyltransferase genomic organization: alternate promoters direct the synthesis of liver and kidney transcripts. *Glycobiology* **1**: 25-31.

Ware, F. E., A. Vassilakos, P. A. Peterson, M. R. Jackson, M. A. Lehrman, and D. B. Williams (1995). The molecular calnexin binds $Glc_1Man_9GlcNAc_2$ oligosaccharide as an initial step in recognizing unfolded glycoproteins. *J. Biol. Chem.* **270**: 4697-4704.

Wen, D. X., E. C. Svensson, and J. C. Paulson (1992). Tissue-specific alternative splicing of the β-galactoside α2,6 sialyltransferase gene. *J. Biol. Chem.* **267**: 2512-2518.

Weng, S. and R. G. Spiro (1993). Demonstration that a kifunensine-resistant α-mannosidase with a unique processing action on *N*-linked oligosaccharides occurs in rat liver endoplasmic reticulum and various cultured cells. *J. Biol. Chem.* **268**: 25656-25663.

Weng, S. and R. G. Spiro (1996). Endoplasmic reticulum kifunensine-resistant α-mannosidase is enzymatically and immunologically related to the cytosolic α-mannosidase. *Arch. Biochem. Biophys.* **325**: 113-123.

Weston, B. W., R. P. Nair, R. D. Larson, and J. B. Lowe (1992). Isolation of a novel human α1,3-fucosyltransferase gene and molecular weight comparison to the human Lewis blood group α1,3/4-fucosyltransferase gene. *J. Biol. Chem.* **267**: 4152-4160.

Yamashita, K., E. Ichishima, M. Arai, and A. Kobata (1980). An α-mannosidase purified from *Aspergillus saitoi* is specific for α1,2-linkages. *Biochem. Biophys. Res. Commun.* **96**: 1335-1342.

Yoshida, T. and E. Ichishima (1995). Molecular cloning and nucleotide sequence of the genomic DNA for 1,2-α-D-mannosidase gene, *msdC* from *Penicillium citrinum*. *Biochim. et Biophys. Acta* **1263**: 159-162.

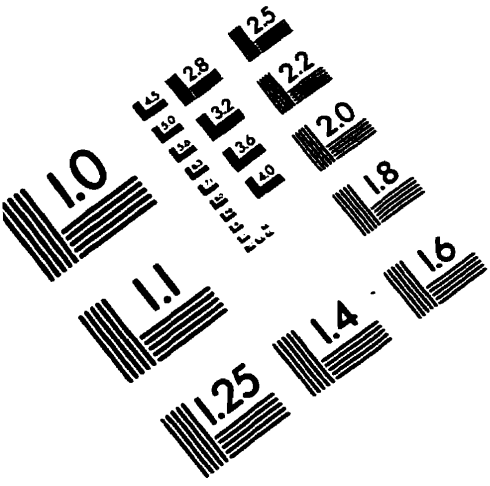
Yoshida, T., T. Inoue, and E. Ichishima (1993). 1,2-α-D-Mannosidase from *Penicillium citrinum*: molecular and enzymatic properties of two isoenzymes. *Biochem. J.* **290**: 349-354.

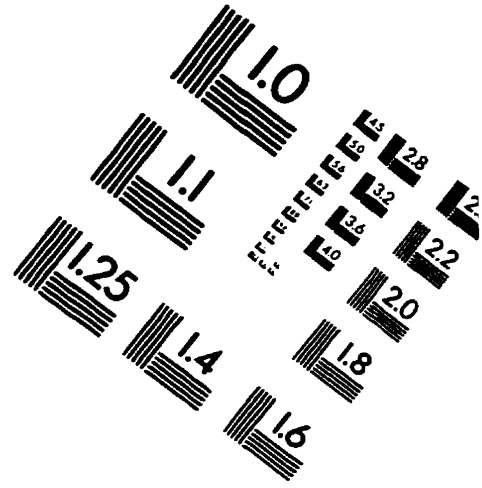
Yoshida, T., K. Maeda, M. Kobayashi, and E. Ichishima (1994). Chemical modification of *Penicillium* 1,2-α-D-mannosidase by water-soluble carbodi-imide: identification of a catalytically important aspartic acid residue. *Biochem. J.* **303**: 97-103.

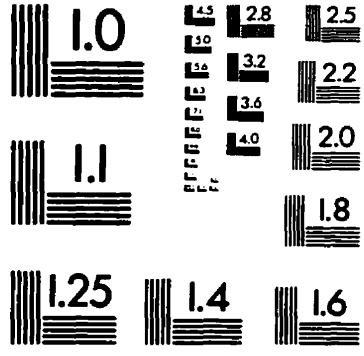
Yoshihisa, T. and Y. Anraku (1989). Nucleotide sequence of AMS1, the structure gene of vacuolar α-mannosidase of Saccharomyces cerevisiae. *Biochem. Biophys. Res. Commun.* **163**: 908-915.

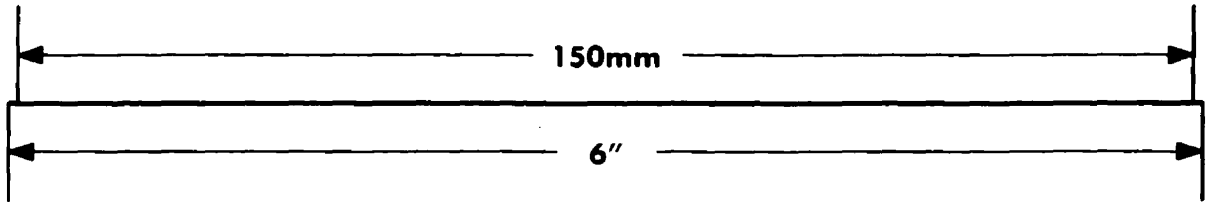
Ziegler, F. D. and R. B. Trimble (1991). Glycoprotein biosynthesis in yeast: purification and characterization of the endoplasmic reticulum Man_9 processing α-mannosidase. *Glycobiology* **1**: 605-614.

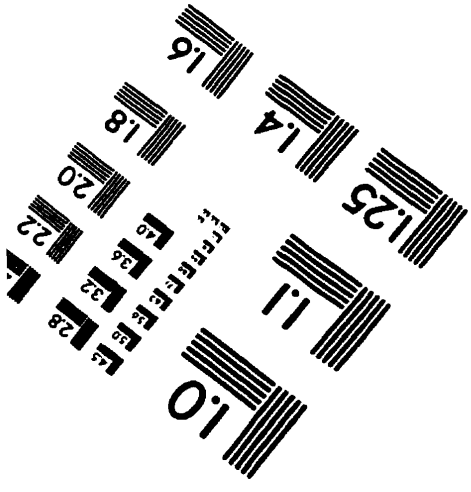
IMAGE EVALUATION TEST TARGET (QA-3)

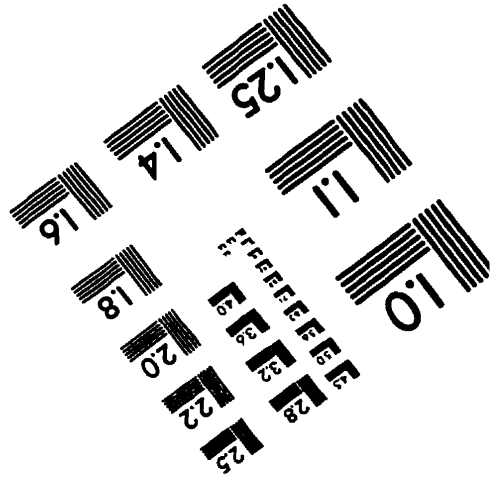












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989