Isolation, Characterization, and Expression of cDNAs Encoding Murine α-Mannosidase II, a Golgi Enzyme That Controls Conversion of High Mannose to Complex N-Glycans

Kelley W. Moremen and Phillips W. Robbins

Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Abstract. Golgi α-mannosidase II (GlcNAc transferase I-dependent α1,3[α1,6] mannosidase, EC 3.2.1.114) catalyzes the final hydrolytic step in the N-glycan maturation pathway acting as the committed step in the conversion of high mannose to complex type structures. We have isolated overlapping clones from a murine cDNA library encoding the full length α-mannosidase II open reading frame and most of the 5′ and 3′ untranslated region. The coding sequence predicts a type II transmembrane protein with a short cytoplasmic tail (five amino acids), a single transmembrane domain (21 amino acids), and a large COOH-terminal catalytic domain (1,124 amino acids). This domain organization which is shared with the Golgi glycosyltransferases suggests that the common structural motifs may have a functional role in Golgi enzyme function or localization.

Three sets of polyadenylated clones were isolated extending 3′ beyond the open reading frame by as much as 2,543 bp. Northern blots suggest that these polyadenylated clones totaling 6.1 kb in length correspond to minor message species smaller than the full length message. The largest and predominant message on Northern blots (7.5 kb) presumably extends another ~1.4-kb downstream beyond the longest of the isolated clones. Transient expression of the α-mannosidase II cDNA in COS cells resulted in 8–12-fold overexpression of enzyme activity, and the appearance of cross-reactive material in a perinuclear membrane array consistent with a Golgi localization. A region within the catalytic domain of the α-mannosidase II open reading frame bears a strong similarity to a corresponding sequence in the rat liver endoplasmic reticulum α-mannosidase and the vacuolar α-mannosidase of *Saccharomyces cerevisiae*. Partial human α-mannosidase II cDNA clones were also isolated and the gene was localized to human chromosome 5.

N- AND O-GLYCAN structures are increasingly being found to contribute to biological recognition events during development, oncogenic transformation, and cell adhesion (3, 10, 11, 37, 38, 40). The enzymes involved in the maturation of cell surface and intracellular N-glycans are found in the endoplasmic reticulum and Golgi complex where they act upon newly synthesized glycoproteins to generate an array of different structures from a common oligosaccharide precursor (18). The N-glycan processing pathway consists of three stages: (*a*) the initial synthesis of a dolichol-linked precursor oligosaccharide and the en bloc transfer of the oligosaccharide to newly synthesized polypeptide Asn-X-Ser/Thr sequons on the lumenal face of the ER; (*b*) the trimming of the high mannose structures by α-glucosidases and α-mannosidases in the ER and Golgi complex; and (*c*) the elaboration of the branched oligosaccharide chains by Golgi glycosyltransferases. The trimming phase of the pathway is accomplished by α-glucosidases I and II as well as a collection of processing α1,2-mannosidases (29) in the ER and Golgi complex. The resulting $Man_5GlcNAc_2$ structure is then modified by the addition of a single GlcNAc by GlcNAc transferase I (GnT I),[1] before the final hydrolytic steps in the pathway are accomplished by α-mannosidase II (Man II), catalyzing the removal of α1,3- and α1,6-mannosyl residues (50). The trimming and elongation phases of the pathway overlap at the GnT I/Man II steps, with each reaction being obligatory for further processing steps. GnT I is essential for processing to hybrid or complex type structures (34), while the absence of Man II activity, either by inhibition with the alkaloid, swainsonine (52), or in the human autosomal genetic disease hereditary erythroblastic multinuclearity associated with positive acidified serum (HEMPAS), characterized by the reduced expression of Man II (12), results in the accumulation of Asn-linked hybrid oligosaccharides in lieu of the standard array of complex type structures. The cleavage of glycoprotein processing in-

Dr. Moremen's present address is Complex Carbohydrate Research Center, University of Georgia, 220 Riverbend Rd., Athens, GA 30602.

1. *Abbreviations used in this paper*: GnT I, GlcNAc transferase I; Man II, α-mannosidase II; PCR, polymerase chain reaction.

termediates by Man II also confers resistance to cleavage by endoglycosidase H, a commonly used marker for transit through the Golgi complex (42).

A number of the mammalian Golgi glycosyltransferases have been cloned recently (1, 7, 17, 21, 25, 32, 45, 46, 53, 56) allowing a comparison of the polypeptide structures and the ability to examine the regulation of transferase expression in relation to terminal oligosaccharide processing events. Among these enzymes a common domain motif has been described (37) of a type II transmembrane structure with a small NH_2-terminal cytoplasmic tail, a single transmembrane domain, a variable stem region, and a large COOH-terminal catalytic domain. Among the processing hydrolases, only an α-mannosidase presumably responsible for the removal of a single α1,2-mannosyl linkage in the ER has been fully cloned (2). This enzyme bears no resemblance to the glycosyltransferase domain structure, but it does share extensive homology to the yeast vacuolar α-mannosidase. Both enzymes contain no classical signal sequence or membrane-spanning domain. Characterization of the primary structures and molecular aspects of the early processing steps therefore await the cloning and characterization of the remainder of the processing α-glucosidases and α-mannosidases.

The most well characterized of the processing α-mannosidases is Man II. The enzyme has been purified and extensively characterized from rat liver (for review see reference 29). It is a transmembrane glycoprotein with an apparent molecular mass of 124 kD by SDS-PAGE and a catalytic domain facing the lumen of the Golgi complex. Release of a 110-kD catalytically active soluble form of the enzyme can be accomplished by a mild chymotrypsin digestion of permeabilized or solubilized Golgi membranes (28). The chymotrypsin-cleaved form of the enzyme has been purified and is catalytically indistinguishable from the intact enzyme (30), while it differs in NH_2-terminal sequence and hydrophobic character. NH_2-terminal sequence data from the 110-kD soluble form of the enzyme along with internal peptide sequences have allowed us to generate a Man II–specific cDNA probe by mixed oligonucleotide-primed amplification of cDNA (26). A partial cDNA clone was isolated from an oligo(dT)-primed rat liver cDNA library which spanned ~40% of the Man II open reading frame. We present here the isolation of cDNA clones which span the entire open reading frame and much of the 5′ and 3′ untranslated regions of Man II from a random- and oligo(dT)-primed murine cDNA library. The open reading frame confirms the map of peptide sequence data from the purified rat protein demonstrating that Man II conforms to the domain structure model common among the Golgi glycosyltransferases. Transient expression of a full length murine Man II cDNA clone in COS cells directs the overexpression of enzyme activity and the synthesis of immunoreactive material in a perinuclear membrane array consistent with the localization of the Man II polypeptide in the Golgi complex of the transfected cells.

Materials and Methods

Materials

Restriction enzymes were purchased from New England Biolabs (Beverly, MA) or Bethesda Research Laboratories (Gaithersburg, MD). 4-methylumbelliferyl α-D-mannoside, and trypsin were from Sigma Chemical Co. (St. Louis, MO). 150-200 g male Sprague-Dawley rats were from Charles River Breeding Laboratory (Wilmington, MA). Bincinchoninic acid (BCA) protein assay reagent was from Pierce Chemical Company (Rockford, IL). λZAP II cloning vector and the in vitro transcription/capping kit were from Stratagene (La Jolla, CA). Sequenase was from United States Biochemical (Cleveland, OH). The Cyclone Biosystem M13 deletion kit was from International Biotechnologies Inc. (New Haven, CT). Zetaprobe membranes were from Bio-Rad (Richmond, CA). Rabbit reticulocyte lysates, microsomal membranes and RNasin were from Promega (Madison, WI). Tran-[^{35}S]-label (1,024 Ci/mmol) was from ICN Radiochemicals (Irvine, CA). N-glycanase was from Genzyme (Cambridge, MA). FITC-conjugated goat anti-rabbit antibody was from Cappel Laboratories (Cochranville, PA). DNA from a human/hamster hybrid somatic cell panel was from BIOS Corp. (New Haven, CT). *Taq* polymerase was from Perkin Elmer (Norwalk, CT). The pXM vector was a gift from Harvey Lodish (Massachusetts Institute of Technology, Cambridge, MA). All other reagents were of at least reagent grade and obtained from standard suppliers.

Assays and Preparation of Cell Extracts

Man II was assayed using 4-methylumbelliferyl α-D-mannoside as substrate as described (27). NaCl-washed microsomal membranes from COS cell monolayers were prepared from four 100-mm culture dishes (90% confluent) exactly as previously described for 3T3 cells (27). Protein concentration was determined using the BCA protein assay reagent as described by Pierce Chemical Co. (Rockford, IL) using BSA as standard.

Screening of the Murine and Human cDNA Libraries

An unamplified BALB/c 3T3 cDNA library primed with a mixture of oligo(dT) and random hexamers and packaged into a λZAP II cloning vector (Stratagene) was obtained from D. J. G. Rees (Massachusetts Institute of Technology) (41). An amplified version of the same library was used in the second round of screening. A similarly prepared amplified HepG2 cDNA library was obtained from E. Marcantonio (Columbia University, New York, N.Y.). The packaged libraries were plated on XL1-Blue host cells and screened by plaque hybridization using standard procedures. The probe that was used for library screening and blot hybridizations (see below) was a 1,170 bp Man II PCR amplification fragment (PCR-1) generated from a rat liver cDNA preparation by amplification with Man II–specific degenerate and inosine-substituted oligonucleotide primers designed using protein sequence data from the purified Man II polypeptide (26).

DNA Sequencing

Selected λZAP II clones were subcloned into M13 and sequenced by the "dideoxy" chain termination method (43) using deoxyinosine triphosphate in place of dGTP and Sequenase (United States Biochemical) as described by the manufacturer. Sequence data were obtained from successive deletions in M13 using T4 polymerase (Cyclone Biosystem, International Biotechnologies Inc., New Haven, CT) or using synthetic oligonucleotide primers. Oligonucleotides were synthesized on an Applied Biosystems (model 380B) DNA synthesizer.

RNA Hybridization

RNA was prepared from adult male rat tissues as described (2). Indicated quantities of poly(A^+) RNA were resolved on a 1% formaldehyde/agarose gel (26) and transferred by capillary blotting to a Zetaprobe membrane (Bio-Rad Laboratories). Filters were prehybridized, hybridized, and washed as described (6) using the rat PCR-1 cDNA amplification product (26) as the radiolabeled probe.

In Vitro Transcription and Translation

Clone MII-8 containing the entire Man II open reading frame inserted into the EcoRI site of pBluescript II (see Fig. 1) was linearized by digestion with BamHI and transcribed in vitro using the T7 polymerase promoter of pBluescript II and the in vitro transcription kit from Stratagene. RNA synthesis was carried out for 2 h at 37°C in a 40 μl reaction volume containing 40 mM Tris HCl (pH 7.5), 50 mM NaCl, 8 mM $MgCl_2$, 2 mM spermidine, 0.3 mM $M^7G(5')ppp(5')G$, 30 mM DTT, 80 U RNasin, 0.5 mM each ATP, UTP, and CTP, 0.17 mM GTP, 10 U of T7 polymerase, and 5 μg of the linearized clone MII-8 template. After the addition of GTP to 0.5 mM the reaction was continued for another 16 h at 37°C. The reaction mixture was extracted twice with phenol/chloroform/isoamyl alcohol followed by

ethanol precipitation. Control reactions contained plasmid without insert or plasmid with a noncoding insert.

Translation in vitro was carried out for 1.5 h at 30°C in a 25 μl reaction volume containing all amino acids except methionine (1 mM each), 40 U RNasin, 25 μCi of a mixture of [^{35}S]L-cysteine and [^{35}S]L-methionine (Tran ^{35}S-label, ICN Radiochemicals, 1,024 Ci/mmol), the RNA synthesized above, and a rabbit reticulocyte lysate preparation from Promega. Incubations containing dog pancreas microsomal membranes were carried out by addition of 1.8 μl of microsomal membranes (Promega)/25-μl reaction either before or following the translation reaction. Posttranslational addition of microsomal membranes was followed by a 45-min incubation at 30°C. Samples were either denatured directly in SDS sample buffer or processed for proteolysis by the addition of trypsin (Sigma Chemical Co.) to 100 μg/ml, ± 0.1% Triton X-100, followed by incubation at 0°C for 1 h. Proteolysis was terminated by addition of 30 KIU of aprotinin and boiling in SDS sample buffer. SDS-PAGE and autoradiography were carried out as described (26).

Transient Transfection of COS Cells

COS cells were grown in 100-mm culture dishes in a humidified incubator at 37°C in 5% CO_2 with DME media containing 0.1 μg/ml penicillin and streptomycin and 10% FCS (DME/10% FCS). Confluent cell monolayers were trypsinized and split 1:8 two days before transfection. The large EcoRI fragment from clone MII-8 containing the entire Man II open reading frame was excised and ligated into the EcoRI site of the pXM COS cell expression vector (58). Recombinant plasmids were checked by restriction mapping to confirm the correct orientation of the insert. COS cells (80% confluent) were transfected either with the pXM without an insert, pXM containing the Man II insert in the correct orientation (MII-pXM), or pXM containing the Man II insert in the antisense orientation (asMII-pXM) by liposome-mediated transfection (Lipofectin; Bethesda Research Labs, Gaithersburg, MD) as described by the manufacturer. Briefly, 20 μg of the relevant plasmid were mixed with 50 μl of Lipofectin reagent in 3 ml of serum-free medium (Opti-MEM; Bethesda Research Labs) and incubated at room temperature for 15 min. Monolayer cultures were rinsed twice with Opti-MEM and incubated with the 3 ml media containing the plasmid/lipofectin mixture for 6–8 h at 37°C. The cultures were rinsed twice with DME/10% FCS and incubated with DME/10% FCS at 37°C for 24–72 h. Cells prepared for immunofluorescence studies were plated on coverslips placed in 24-well microtiter plates, grown to 50% confluency, and transfected at the same plasmid/Lipofectin/Opti-MEM ratio as in the larger scale transfections (total volume of 200 μl/well).

Biosynthetic Labeling of NIH-3T3 Cells and COS Cells

NIH-3T3 cells were grown in 100-mm culture dishes in DME containing 10% calf serum. For biosynthetic labeling 3T3 or COS cells in 100-mm plates were washed twice with Met-free DME and incubated in Met-free DME for 30 min. Cells were then labeled for 60 min at 37°C with 4 ml Met-free DME containing 120 μCi/ml [^{35}S]-labeled mixture of methionine and cysteine (Tran ^{35}S-label, 1,024 Ci/mmol; ICN Radiochemicals). Cell monolayers were washed with DME/10% FCS and chased for 60 min with the unlabeled medium. Cells were collected by trypsinization, washed twice in PBS, and vigorously resuspended in lysis buffer containing 1% Triton X-100, 0.5 M NaCl, 20 mM Tris HCl, pH 7.5. The extract was clarified by centrifugation at 200,000 *g* for 30 min in a centrifuge (TL-100; Beckman Instruments, Inc., Palo Alto, CA) using a TLA 100.3 rotor at 2°C. The clarified extracts were preadsorbed with 100 μl of a 50% (vol/vol) slurry of protein A-Sepharose beads (Pharmacia Fine Chemicals, Piscataway, NJ) for 1 h at 4°C. After the removal of the beads by centrifugation, 5 μl of anti-Man II antiserum (27) was added and incubated at 4°C for 4 h with constant mixing. Protein A-Sepharose was added (50 μl of the 50% slurry) and the mixture was incubated for an additional 2 h at 4°C with mixing. The immunoprecipitates were washed, eluted, and resolved by SDS-PAGE as previously described (27). Samples digested with N-glycanase (Genzyme) were processed as described (27).

Indirect Immunofluorescence

Cell monolayers were fixed at the indicated time posttransfection by washing three times with PBS and incubating for 15 min at 37°C in a 3.7% solution of formaldehyde in PBS. Coverslips were removed from the wells, washed with PBS, and permeabilized by incubation for 1 min in a solution of 100% methanol at −20°C. After washing with PBS the cells were incubated in a solution containing 1% FCS in PBS for 30 min at room temperature followed by a 30-min incubation at 30°C with a 1:2,000 dilution of anti-Man II antibody (27) in PBS. The coverslips were washed again in PBS and incubated for 30 min at 30°C with a 1:1,000 dilution of a FITC-conjugated goat anti-rabbit antibody (Cappel Laboratories) in PBS containing 1% FCS. Cells were observed and photographed using an Axioplan microscope (Carl Zeiss, Oberkochen, Germany).

Chromosome Mapping

Human Man II-specific primers were synthesized to assay for the presence of the human Man II gene in a human/hamster somatic cell hybrid panel. DNA isolated from a panel of 25 human/hamster hybrids (50 μg/ml, BIOS Corp.) was screened by PCR using primers with the following sequence: sense primer-GCTCGGATGCTACTAGA (3/17 mismatch with murine Man II); antisense primer-TCTTAACTTTAAACTTGGA (7/19 mismatch with murine Man II) bracketing a 178 bp of the human Man II gene which was not interrupted by introns (see Fig. 1). Amplification reactions (25 μl) containing 50 mM KCl, 10 mM Tris HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM each dNTP, 50 ng genomic DNA, 0.5 μM each primer, and 0.6 U *Taq* polymerase. A temperature cycle of 92°C (1 min), 55°C (1 min), and 72°C (2 min) was repeated for 35 cycles followed by an extension of 4 min at 72°C. Amplification products were resolved on a 2% agarose gel containing ethidium bromide and scored for the presence of the 178-bp human Man II amplification fragment. A faint 178-pb band as well as several characteristic nonspecific bands were found in hamster controls and in human/hamster hybrids lacking chromosome 5, but the human amplification product could be unambiguously detected above this background by a greater yield, the presence of unique HindIII and EcoRI sites, and the DNA sequence of the human Man II cDNA (data not shown).

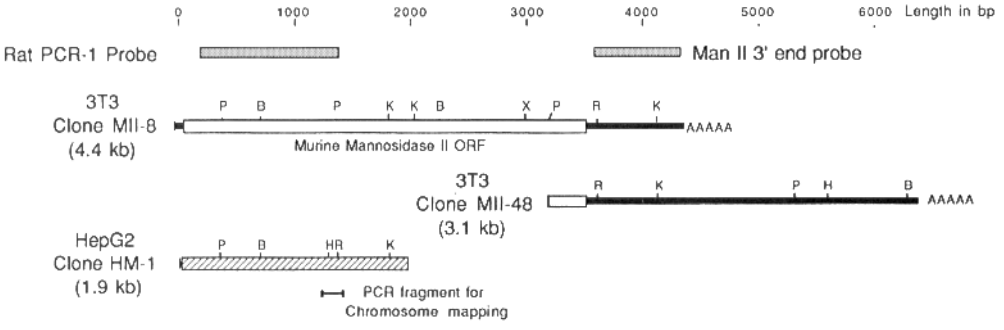

Figure 1. Schematic representation of murine and human Man II clones. The rat PCR-1 probe used to isolate murine Man II clone MII-8 and human Man II clone HM-1 was derived from mixed oligonucleotide primed amplification of cDNA (41). The stippled boxes represent the DNA fragments used as probes. The open boxes on the clone MII-8 and MII-48 diagrams represent the Man II open reading frame and the noncoding region is represented by the black line. The crosshatched box on the HM-1 clone diagram represents the portion of the human Man II open reading frame corresponding to the murine open reading frame. The segment of the human cDNA sequence used in chromosome mapping is shown at the bottom. Indicated restriction sites are: *P*, PstI; *B*, BglII; *K*, KpnI; *X*, XhoI; *R*, EcoRI; *H*, HindIII. A scale in bp is given at the top of the figure.

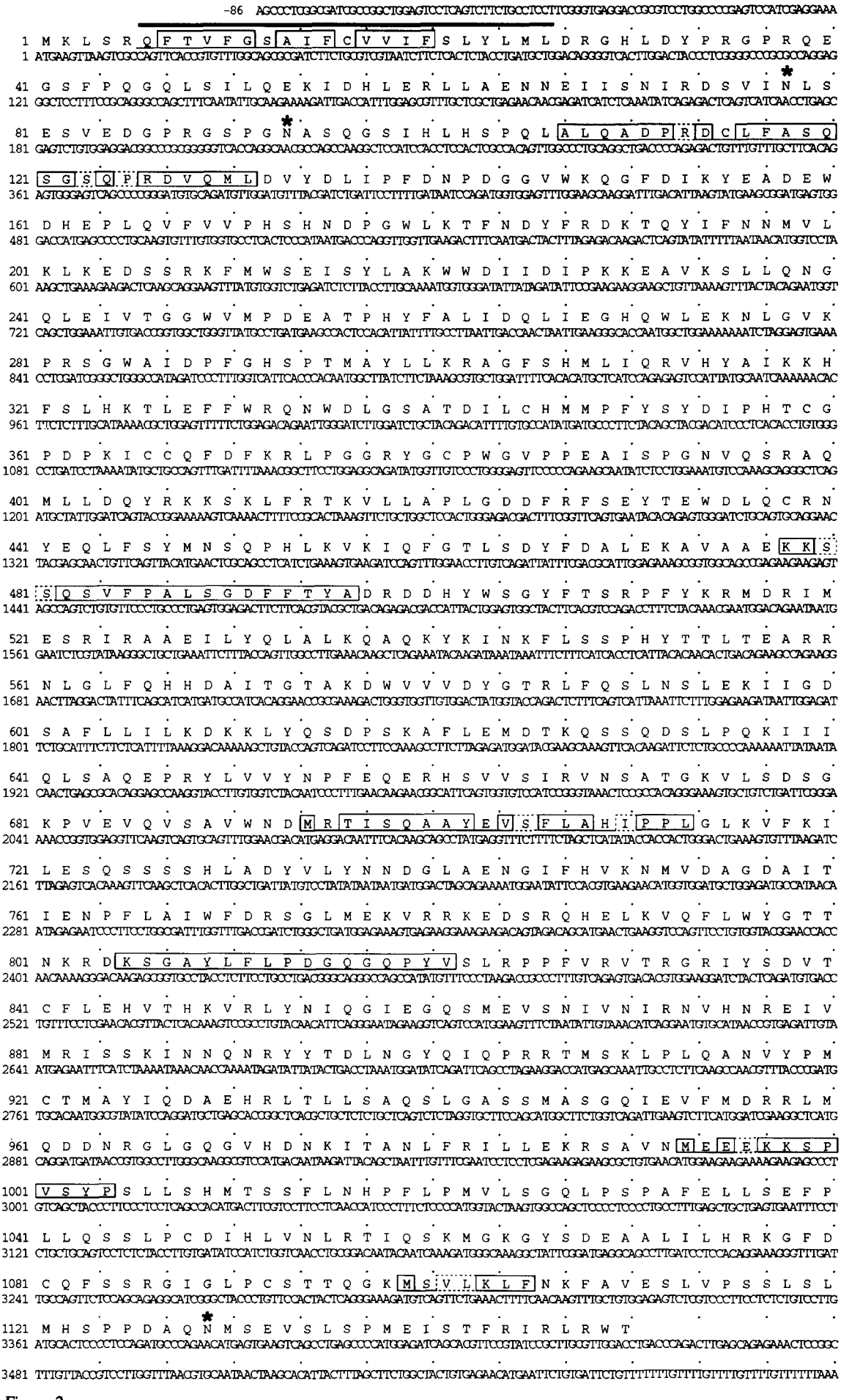

Figure 2.

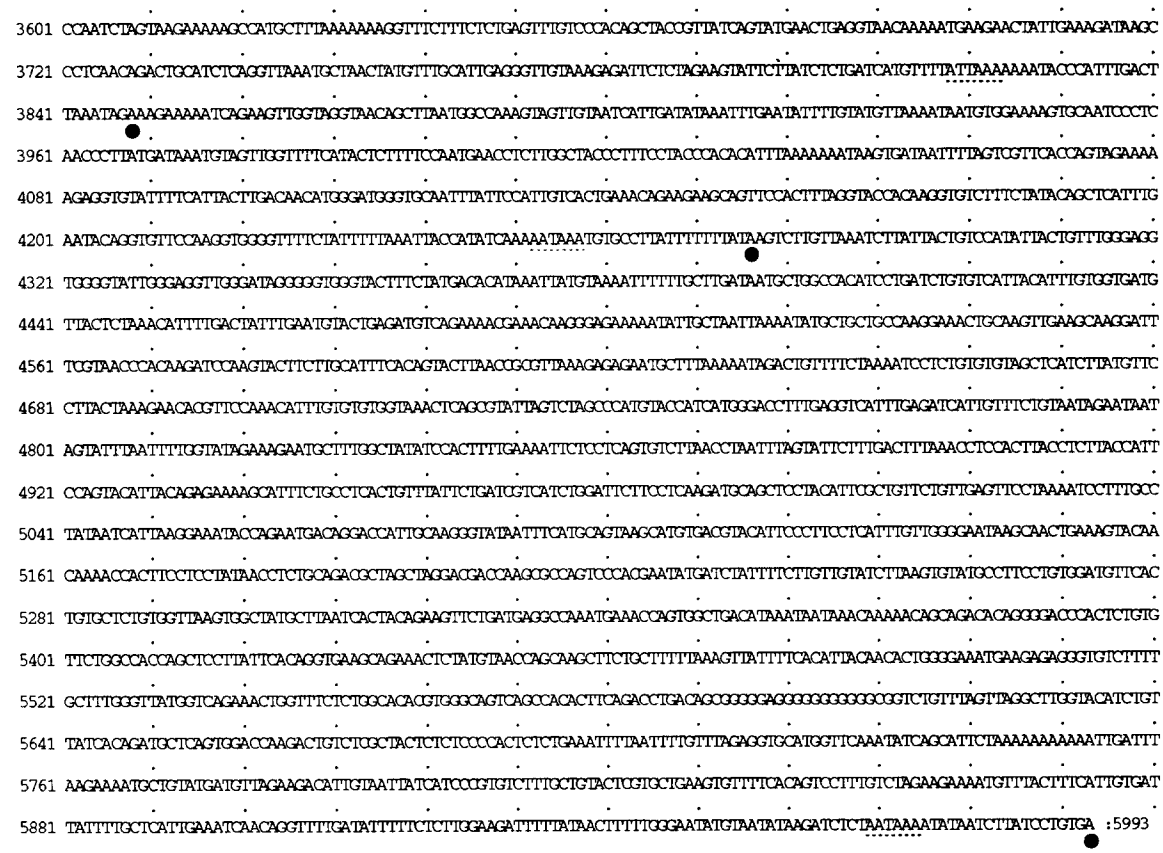

Figure 2. Nucleotide sequence of the cDNA encoding murine Man II and the predicted amino acid sequence. The single open reading frame is shown directly above the nucleotide sequence. Both nucleotides and amino acids are numbered from the beginning of the open reading frame. Nucleotides 5′ of the first in-frame ATG codon are given negative numbers. The putative membrane spanning domain is indicated by a solid line above the protein sequence. Amino acid residues indicated by a solid box are regions of the murine protein sequence that matches the rat Man II peptide sequence data. Amino acids residues indicated by a dashed box represent conservative substitutions from the rat peptide sequences. Underlined amino acid residues represent residues that could not be determined during peptide sequencing. Asterisks indicate potential glycosylation sites. In the 3′ untranslated region the putative polyadenylation signals are indicated by a dashed underline. A black dot is under the position where a poly(A) tract was found on a respective set of polyadenylated clones. These sequence data are available from EMBL/GenBank/DDBJ under accession number X61172.

Computer Methods

DNA sequence data was assembled into a contiguous sequence database by the method of Staden (49). Sequence comparisons against the GenBank or GenPept sequence databases were performed using the FASTA program (39). Statistical analysis of sequence similarity between two protein sequences was determined using the Bestfit program of the University of Wisconsin Genetics Computer Group (version 6.2) or the sequence similarity investigation program (DIAGON) of Staden (49).

Results

Isolation and Characterization of cDNA Clones Encoding Murine and Human α-Mannosidase II

We have previously isolated a partial cDNA clone encoding ~40% of the rat Man II open reading frame (26) using a probe isolated by mixed oligonucleotide-primed amplification of cDNA (PCR-1). Since Northern blots demonstrated a message size of ~7.5 kb (adjusted downward from the previously published ~8 kb; 26) we decided to perform subsequent rounds of screening using a library primed with both oligo(dT) and random hexamers (41). Approximately 1×10^6 independent recombinants from the unamplified 3T3 cDNA library were screened with the 1,170 bp rat Man II PCR probe (26). 36 independent positive clones were obtained, the longest being ~4.4 kb in length (clone MII-8, Fig. 1) and terminating at the 3′ end with a ~70-bp poly(A) tract 19 bp downstream from a consensus AATAAA polyadenylation signal (54) (base position 4253 in Fig. 2). This clone and several others in the open reading frame region were sequenced yielding the entire Man II open reading frame (3,550 bp) and 86 and 827 bp of 5′ and 3′ untranslated sequence, respectively (Fig. 2). Among the isolated clones several terminated at base position 3,848 followed by a poly(A) tract of >50 bp. 24 bases upstream from the poly(A) tract was another ATTAAA consensus polyadenylation signal (Fig. 2). Despite containing the entire Man II open reading frame and a poly(A) tail, clone MII-8 was ~3 kb smaller than the predominant message size on Northern blots. Based on the extended 3′ untranslated region of previously cloned Golgi enzymes (46, 53, 56) we hypothesized that the remaining message length extended further in the 3′ direction. The 730 bp EcoRI fragment on the 3′ end of clone MII-8 was used as a probe to re-screen the 3T3 cDNA library and an additional 16 independent clones were obtained. The longest of these clones (clone MII-48) extended an additional 1.7 kb in

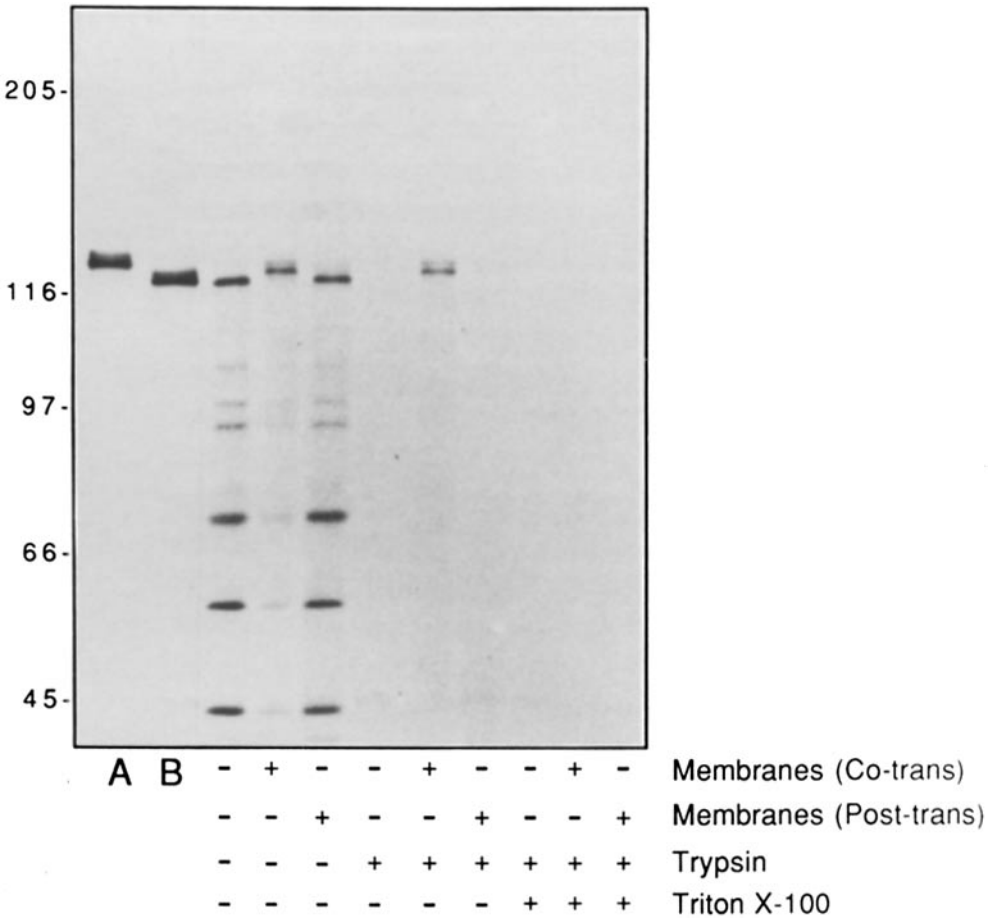

Figure 3. In vitro transcription/translation of Man II clone MII-8 and comparison with biosynthetically labeled Man II. Man II clone MII-8 was transcribed in vitro using T7 polymerase as described in Materials and Methods. In vitro translation was performed with or without the addition of microsomal membranes (co- or posttranslational addition of membranes) as indicated at the bottom of the figure. Aliquots of the translation were then digested or mock digested with trypsin in the presence or absence of Triton X-100 as indicated. Samples were resolved by SDS-PAGE and subjected to autoradiography. Lanes *A* and *B* represent cell extracts from biosynthetically labeled 3T3 cells which were immunoprecipitated with the anti-Man II antibody and either digested (*B*) or mock digested (*A*) with N-glycanase. Samples were resolved on the same gel as the in vitro translation samples. Lanes *A* and *B* were exposed to x-ray film for 3 d while the in vitro translation samples were exposed for 12 h. Identical results for the in vitro translation products were obtained if the samples were immunoprecipitated with anti-Man II antibody following the synthesis and processing (data not shown). Molecular weight markers (in kD) are indicated at the left of the figure.

the 3′ direction and terminated with a poly(A) tract 19 bp downstream from another AATAAA consensus polyadenylation signal. The size of the aggregate clone sequence is in close agreement with the ~6.1 kb minor message on Northern blots (see Fig. 5, lane *I*). The heterogeneity in termination and polyadenylation within the 3′ untranslated region suggests that the additional sequence necessary to generate the predominant ~7.5-kb message will likely result from an extension of an additional ~1.4 kb beyond the 3′ end of the MII-48 clone sequence.

We have also screened a human HepG2 cDNA library with the rat PCR amplification product (PCR-1) as a probe to isolate human cDNA clones for sequencing and for use as hybridization probes. These probes have been used to examine the expression of Man II in HEMPAS disease (12), a heterogeneous disease characterized in some individuals by a deficiency in Man II. The sequence has also been used to design human Man II specific primers for chromosome mapping by PCR (see below). The HepG2 library was screened (4×10^5 recombinants) and three independent clones were isolated. The longest of the clones (~1.9 kb) contained ~55% of the human Man II open reading frame and aligned to position −5 to +1,928 on the 3T3 sequence in Fig. 2 (Fig. 1).

Sequence Analysis

The murine Man II coding sequence predicts a polypeptide of 1,150 amino acids (M_r 131,000) with a single type II transmembrane domain from amino acid residue 6–26. Several lines of evidence suggest that the coding region shown in Fig. 2 contains the correct initiation site for the Man II open reading frame. The Met codon in position 1 is the first ATG in the sequence and conforms to the consensus eucaryotic translation sequence with a purine at position −3, the most critical residue in the initiation sequence (19). The 5′ untranslated region is G/C rich (68%), a characteristic common to several of the Golgi glycosyltransferases (46, 53) and also a common feature among housekeeping genes (9). Finally, the NH_2-terminal peptide sequence of the purified intact rat liver enzyme starts at residue 6 of the predicted open reading frame (30). The purified enzyme is indistinguishable in size from the biosynthetically labeled 3T3 enzyme (27) and the enzyme detected by Western blots from freshly prepared Golgi membrane extracts (27), but the cleavage of five residues would be too small a change to detect on SDS gels during purification. Although the cleavage of the five residue cytoplasmic tail most likely reflects in vitro proteolysis during purification, it could also possibly represent the product of cleavage in vivo.

The in vitro transcription/translation of the clone MII-8 cDNA also resulted in a band of identical mobility to the deglycosylated 3T3 enzyme (Fig. 3). Translation in vitro in the presence of microsomal membranes resulted in the glycosylation of the polypeptide and a decrease in mobility to a doublet of identical size to that of the glycosylated 3T3

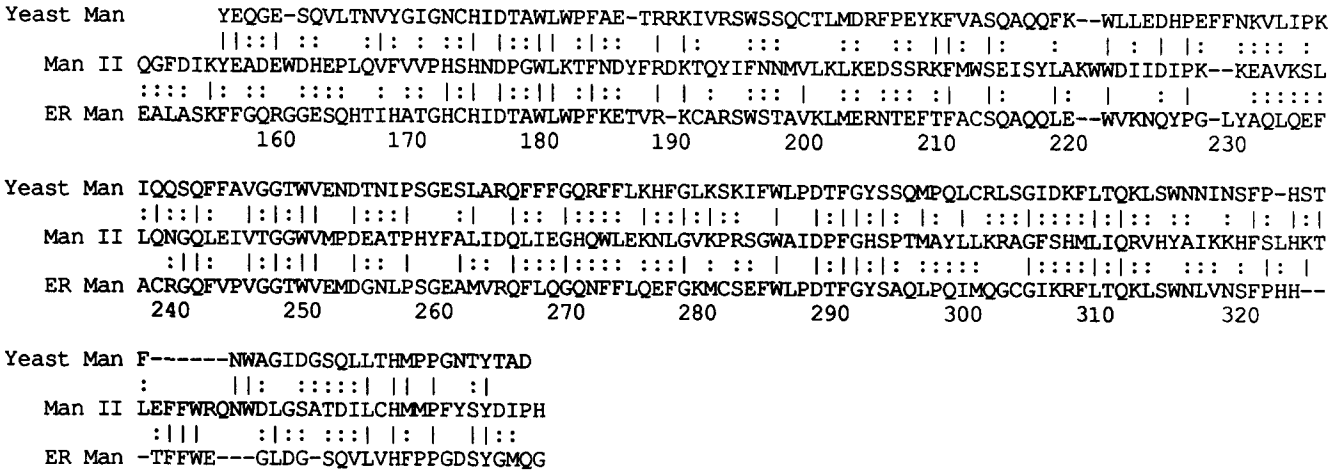

Figure 4. Comparison of murine Man II protein sequence with the yeast vacuolar α-mannosidase and the rat ER α-mannosidase. Murine Man II was aligned with the yeast vacuolar α-mannosidase and the rat ER α-mannosidase using the FASTA program (54). The sequences in the figure show the individual alignments of murine Man II (center sequence) with the yeast vacuolar α-mannosidase (59) (*Yeast Man*, upper sequence) and the rat ER α-mannosidase (24) (*ER Man*, lower sequence). The vertical bar indicates amino acid identities between the two lines of sequence. The double dots indicate conservative amino acid substitutions between the two lines of sequence. The numbering below the aligned sequences refers to the polypeptide numbering of 3T3 Man II in Fig. 2. When the indicated sequence pairs were subjected to statistical analysis using the Bestfit program from the University of Wisconsin Genetics Computer Group (comparison versus randomized sequences with the same amino acid content, gap weight = 3, gap length weight = 0.1) the sequence alignments were found to be 9.7 and 6.0 SDs above random for the Man II/yeast vacuolar α-mannosidase pair and the Man II/ER α-mannosidase, respectively.

biosynthetic product. The doublet likely results from the glycosylation of Asn residue 1,129 and a partial glycosylation at Asn residues 78 or 93, since the purified soluble form of the rat liver enzyme (residues 107–1,150) has been shown to be fully glycosylated at a single site (30). The apparent molecular weight on SDS gels is slightly less than is predicted for the open reading frame of the cDNA (~117 kD vs 131 kD for the deglycosylated forms, respectively). This presumably results from an anomalous gel migration since the in vitro translation product and the product of biosynthetic labeling run at an identical position by SDS-PAGE. Translation in the presence of membranes also resulted in the apparent co-translational translocation of the polypeptide across the lipid bilayer. Trypsin cleavage of the in vitro translation products in the presence of microsomal membranes did not alter the amount or the mobility of the glycosylated form but the smaller in vitro–synthesized products were fully susceptible to digestion. The lack of a mobility shift following trypsin digestion also confirms the orientation in the membrane and the location of the membrane–spanning domain. These results are also consistent with the inability to detect any cytoplasmically oriented polypeptide in protease protection experiments in intact Golgi membranes (27). Addition of membranes posttranslationally did not result in a significant production of glycosylated product suggesting a requirement for co-translational translocation of the enzyme.

In addition to the NH_2-terminal peptide sequence, the mouse equivalent of the six remaining rat Man II polypeptide sequences determined previously (30) were all found in the Man II open reading frame confirming the predicted peptide sequence map (Ref. 30, Fig. 8). Chymotrypsin has been used to cleave Man II in vitro generating a soluble catalytically active form of the enzyme (28, 30). The cleavage site is at residue 107 in the open reading frame and predicts that the cytoplasmic tail, transmembrane domain, and 80 residues of lumenally oriented polypeptide are not essential for enzyme activity.

Although Man II appears to have the domain structure common to the other Golgi glycosyltransferases no direct sequence homology was found with any of the glycosyltransferases. The previously described similarity between rat Man II and rabbit GnT I (45) was found to be marginally significant (4 SDs above random). When the murine and human Man II equivalents of this sequence were compared to either the rabbit or human (15) GnT I sequences the similarities were even less significant (2.1 and 1.7 SDs above random for the murine and human Man II sequences, respectively). Comparison of the Man II polypeptide to a translated form of the GenBank database (GenPept, version 64.3) revealed that a 215 amino acid region of Man II bears a statistically significant similarity to a corresponding region in the rat ER α-mannosidase (2) and the vacuolar α-mannosidase from *Saccharomyces cerevisiae* (59) (6.0 and 9.7 SDs above random, Fig. 4). The rat ER α-mannosidase and the yeast vacuolar α-mannosidase share extensive homology across their entire length (2), whereas Man II shares a more limited similarity within a region of the soluble catalytic domain of the enzyme. All three enzymes recognize α-mannoside linkages and cleave the synthetic substrates *p*-nitrophenyl α-D-mannoside and 4-methylumbelliferyl α-D-mannoside, but differ in their specificity toward natural high mannose oligosaccharides, inhibition by alkaloid inhibitors, antibody cross-reactivity, and subcellular localization (2, 29, 59, 60). The sequence similarity and the common activity toward synthetic substrates between the three enzymes would, however, suggest that this region may represent a portion of the active site involved in α-mannoside recognition.

Comparison of the sequence of the 3′ untranslated region of murine Man II with the GenBank database (version 67.0) revealed that the extended 3′-untranslated region contains a

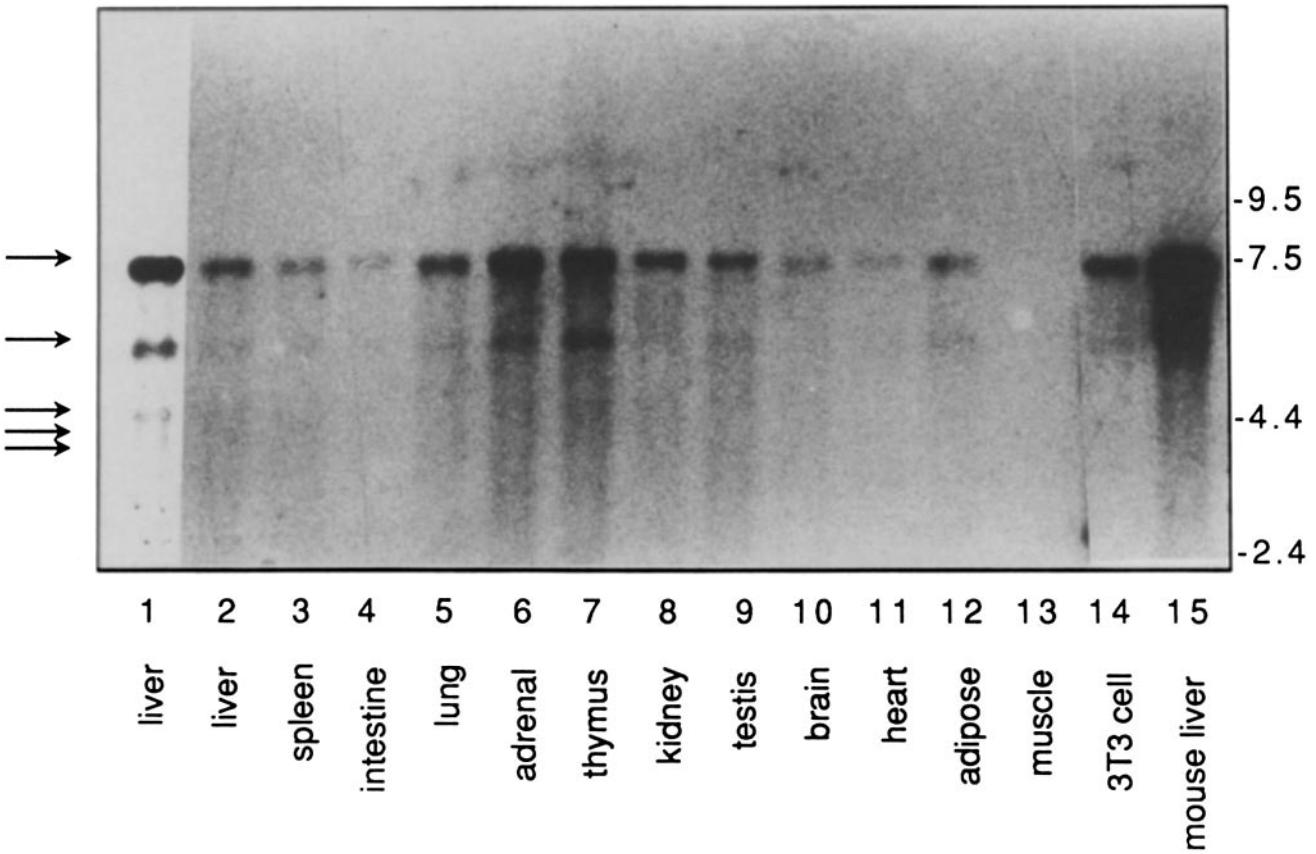

Figure 5. Tissue distribution of Man II mRNA. Poly(A) RNA (5 μg) was resolved on a 1% agarose/formaldehyde gel, except for lane *15* where 15 μg poly(A) RNA was loaded. The gel was blotted onto a nylon membrane and probed with a radiolabeled rat cDNA probe as described in Materials and Methods. Location of radiolabeled size standards (Bethesda Research Laboratories) are as indicated (in kb). Arrows indicate the positions of the major and minor message species in rat tissues. The blot in lane *1* was exposed to x-ray film for 8 d at −70°C. The gel in the remaining lanes was exposed for 2 d at −70°C.

single interrupted copy of a mouse B1 repetitive sequence (data not shown), the mouse equivalent of the human Alu consensus sequence (20, 48).

Tissue Distribution of Man II RNA

The isolation of several size classes of polyadenylated clones that differ in their degree of extension in the 3′ untranslated region demonstrates the heterogeneous nature of the termination and polyadenylation of the Man II message. Each of the separate polyadenylated clones contains an upstream consensus polyadenylation signal yet their abundance on Northern blots appears to be inversely proportional to their length; the 7.5-kb transcript being ~10-fold more abundant than the 6.1 kb transcript (Fig. 5, lane *1*) and ~50–100-fold greater than the 4.3- or 4.0-kb transcripts (barely visible in Fig. 5, lane *1* but readily visible with longer exposure). A Northern blot of poly(A$^+$) RNA from several rat and mouse tissues was prepared using samples identical to those used for the rat ER α-mannosidase (2). A predominant Man II

Table I. α-Mannosidase Activity in COS Cell Transfectants

	MII-pXM		pXM (no insert)		
	Activity	Specific activity	Activity	Specific activity	Increase
	μmol/min	*μmol/min/mg*	*μmol/min*	*μmol/min/mg*	*-fold*
Homogenate	53.4	21.4	13.8	5.5	3.9
Postnuclear supernatant	32.7	23.4	9.1	6.5	3.6
Salt-washed microsomes	26.5	52.9	2.2	4.3	12.3
Postmicrosomal supernatant	6.3	7.0	6.9	7.6	0.9

Assays were carried out as described in Materials and Methods for 40 min at pH 5.6 with 4-methylumbelliferyl α-D-mannoside as substrate. Activity at this pH also detects the lysosomal and ER α-mannosidases (27), both of which can be found predominantly in the postmicrosomal supernatant fraction. Four 100-mm plates of COS cell monolayers were used to prepare homogenates for each plasmid transfection. Parallel transfections and fractionations were carried out with COS cells mock transfected without the vector or with the Man II insert in pXM in the antisense orientation (asMII-pXM). Both samples gave results essentially identical to pXM without an insert.

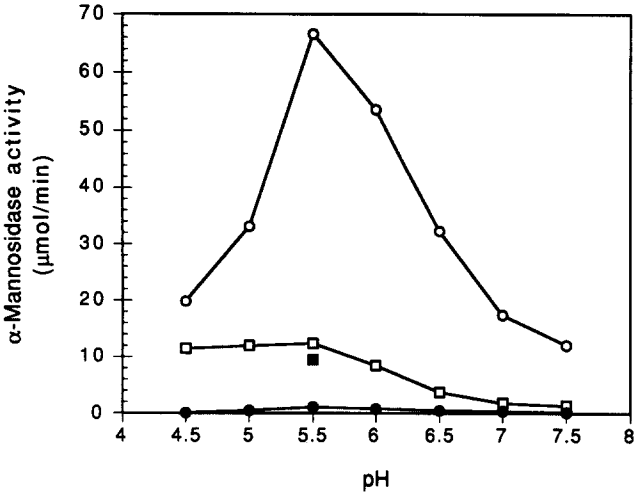

Figure 6. pH profiles of α-mannosidase activity in COS cells transfected with control (pXM, □) or Man II clone MII-8 (MII-pXM, ○) constructs. Salt-washed membranes were prepared and assayed as described in Materials and Methods with the 4-methylumbelliferyl α-D-mannoside substrate at the indicated pH. Membranes prepared from COS cells transfected with MII-pXM were also assayed at the indicated pH in the presence of 10 μM swainsonine (●). Residual α-mannosidase activity in the supernatant following immunoprecipitation of the salt-washed membrane extract with anti-rat Man II antibody is as indicated (■).

transcript of 7.5 kb was found in all tissues with the greatest enrichment being in adrenal and thymus. Most other tissues resulted in an autoradiographic band of ~3–10-fold lesser intensity except skeletal muscle which was undetectable except with prolonged exposure. The ratio of the multiple Man II message species appeared constant across the range of rat tissues despite the >100-fold difference in the level of expression between adrenal and muscle tissues. Comparison with the rat ER α-mannosidase (2), reveals that while the Man II transcript is most abundant in adrenal and thymus, the ER enzyme has a highest abundance level in adrenal and testis and minor differences in other tissues except spleen, intestinal epithelia, and muscle tissues which have a slightly reduced level of message. The differences with Man II message expression in the thymus, testis, and muscle suggests that there is a differential tissue expression of the two α-mannosidases at least in these tissues. A low but detectable level of Man II message was also found in rat brain tissues consistent with a report of reduced levels of Man II activity in rat brain and the presence of a novel brain α-mannosidase (51) which is not sensitive to inhibition by swainsonine and is able to directly cleave high mannose structures to $Man_3GlcNAc_2$ without the prior addition of a GlcNAc residue by GnT I. Whether the message levels adequately reflect the levels of the Man II enzyme activity in the brain or in the other tissues remains to be determined. Although there is a >100-fold difference in Man II autoradiographic intensity on Northern blots between thymus and muscle, it is also not clear if this merely reflects the biosynthetic level and the degree of elaboration of the Golgi complex and the secretory pathway/glycosylation machinery in the respective tissue types.

Transient Expression of Man II in COS Cells

The large EcoRI fragment of clone MII-8 containing the entire Man II open reading frame was subcloned into the COS cell expression vector pXM. This is an SV-40–based expression vector driven by the adenovirus major late promoter (58). COS cells were transfected and assayed for Man II activity either in cell homogenates or in membrane fractions. Crude homogenates showed approximately threefold overexpression of 4-methylumbelliferyl α-mannoside activity (Table I), but Man II activity in crude homogenates is commonly masked by the lysosomal α-mannosidase or the ER α-mannosidase when using synthetic substrates. A salt washed membrane fraction was prepared which has been shown to result in the enrichment of Man II activity (27). Assays of this membrane fraction revealed a 8–12-fold overexpression of 4-methylumbelliferyl α-mannoside activity in COS cells transfected with MII-pXM when compared cells transfected with the vector alone or vector with an antisense insert (Table I). This degree of overexpression is similar to the activity levels seen for the β1,4-galactosyltransferase expressed in COS cells (24). The overexpressed Man II activity has a pH optimum of 5.5, identical to the endogenous enzyme in 3T3 cells (27), it is sensitive to inhibition by swainsonine, and is immunoprecipitable with antibody to the purified rat enzyme (Fig. 6). Biosynthetic labeling of transfected COS cells resulted in a >10-fold overexpression of immunoprecipitable polypeptide (data not shown) reflecting the high rate of biosynthesis of the vector encoded polypeptide 48 h after transfection.

Immunofluorescence of Transfected COS Cells

Immunocytochemistry at the EM level using antibodies to purified rat Man II has demonstrated the Golgi localization of the enzyme in rat tissues (44). The antibodies have also been used recently as a marker for Golgi membrane components in cell trafficking studies (8, 22; and C. Zuber, A. Nakano, K. W. Moremen and J. Roth, manuscript submitted for publication). Preliminary studies have shown that the antibody raised to the rat enzyme does not cross react with the COS cell enzyme (data not shown) allowing a minimal immunofluorescence background in the host cells. Transfection of COS cells with the pXM construct containing the Man II insert in the sense orientation (MII-pXM) resulted in an anti-Man II immunofluorescence pattern within 24 h of the initiation of transfection. The immunofluorescence was largely restricted to a crescent shaped reticular pattern adjacent to the nucleus, but occasionally this reticular structure would extend well into the cytoplasm (Fig. 7, *A* and *B*). This pattern of immunofluorescence has been previously shown to be characteristic of the Golgi complex (22, 31, 44). Mock transfections, transfections with vector alone, or with the Man II insert in the antisense orientation resulted in no detectable immunofluorescence signal (data not shown). Similarly, immunofluorescence of fixed but not permeabilized cells transfected with MII-pXM resulted in no detectable fluorescence signal. The transfection efficiency of the MII-pXM construct, as measured by the percentage of fluorescence-positive cells, was variable, but ranged from 5–25%. By 36 h posttransfection the intensity of the peri-nuclear immunofluorescence increased substantially with many cells generating an additional diffuse particulate pattern of fluorescence in the cytoplasm consistent with ER staining (8, 22, 31) (Fig. 7, *C–E*). By 48 h posttransfection immunofluorescence staining adjacent to the nucleus remained intense but the particulate cyto-

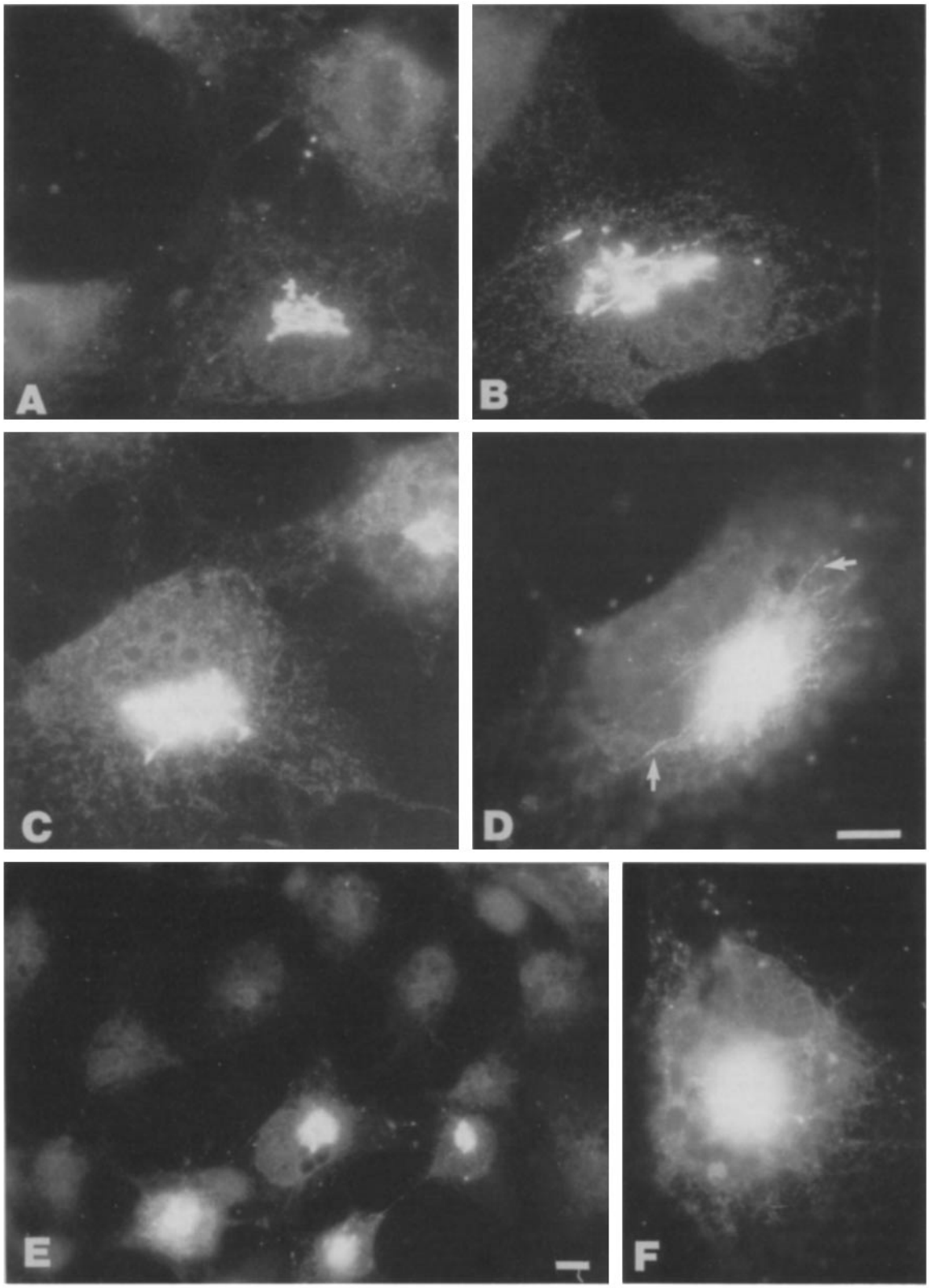

Figure 7. Immunofluorescence studies on COS cells transfected with Man II clone MII-8 in pXM (*MII-pXM*). Cells were transfected with MII-pXM and fixed with formaldehyde either 24 h (*A* and *B*), 36 h (*C–E*) or 48 h (*F*) posttransfection. Cells were permeabilized and stained with anti-rat Man II antibody. Within one day of transfection there was a strong fluorescence signal appearing as a cup or patch adjacent to one side of the nucleus in 5–25% of the transfected cells (*E*). After 36 h the fluorescence staining was more intense with occasional extensions (*arrows*) of fluorescent signal extending radially into the cytoplasm. By 48 h the fluorescence was quite intense, with the predominant signal adjacent to the nucleus but significant fluorescence in a particulate pattern throughout the cytoplasm. Bar, 10 μm.

Table II. Discordancy Analysis of a Panel of 25 Hamster/Human Hybrid Clones

	Human Man II amplification fragment/chromosome		
	Total		
Chromosome	Concordant	Discordant	Percent discordant
1	6	19	76
2	4	21	84
3	5	20	80
4	5	20	80
5	25	0	0
6	7	18	72
7	5	20	80
8	6	19	76
9	6	19	76
10	6	19	76
11	6	19	76
12	7	18	72
13	9	16	64
14	10	15	60
15	7	18	72
16	6	19	76
17	3	22	88
18	3	22	88
19	10	15	60
20	6	19	76
21	10	15	60
22	7	18	72
X	6	19	76
Y	7	18	72

The human chromosome compliment of each hybrid was compared with the ability to generate the 178-bp human Man II amplification fragment following PCR. Concordant segregation of the amplification fragment was observed only for chromosome 5. All other chromosomes were excluded as the site for the Man II gene by discordancies in at least 15 hybrids. Hybrid clones containing the indicated chromosome in at least 5% of the cells were considered positive for the chromosome in the discordancy analysis.

plasmic staining greatly increased. With the high level of Man II synthesis late in the transient expression, detection of early biosynthetic intermediates in the ER or an accumulation of aggregated enzyme in the ER might be expected.

Chromosome Mapping of the Human Man II Gene

Comparison of the murine Man II cDNA with the sequence of the partial human Man II clone revealed that the DNA sequences are 84% identical at the DNA level and 85% identical at the amino acid level (data not shown). Primers were designed to a region of the human Man II cDNA which was distinct from the murine cDNA sequence (3/17 and 7/19 mismatches, respectively) and produced a clear 178-bp PCR product with the human Man II cDNA sequence upon amplification with human genomic DNA. Amplification with hamster genomic DNA resulted in a faint band at 178 pb that was distinguishable from the human amplification product both in quantity and by the absence of the HindIII and EcoRI sites characteristic of the human Man II genomic sequence. DNA from a human/hamster somatic cell hybrid panel (4) was tested for the amplification of the 178-bp human Man II fragment. Cell lines which scored positive for the human Man II PCR fragment were also all found to contain human chromosome 5 (Table I). One of the lines (hybrid line 212) contained multiple deletions in 5q and several lines contained deletions in the 5p15.1–5p15.2 region. All of these lines scored positive for amplification of the human Man II fragment. All other chromosomes were excluded as the site for the Man II gene by discordancies in at least 15 of the hybrid lines.

Discussion

Man II occupies a central position in the Asn-linked oligosaccharide processing pathway acting as the committed step in the synthesis of complex type structures (29). The enzyme has long been considered a marker for the Golgi complex by cell fractionation (14) and immunocytochemistry (35), as well as being the biochemical marker responsible for the conversion of N-linked glycans to structures which are resistant to cleavage by endoglycosidase H (50). Intact Man II has been purified and characterized from rat liver (29) and shown to be a disulfide-linked homodimer (30) with a catalytic domain oriented toward the Golgi lumen. Protease protection experiments in intact Golgi membranes were unable to detect any cytoplasmically oriented polypeptide (28). The release of a soluble form of the enzyme following chymotrypsin digestion of solubilized Golgi membranes allowed us to purify and compare the intact and soluble forms of the enzyme demonstrating that the soluble catalytic domain retains all of the catalytic characteristics of the intact enzyme. The cloning of the full-length murine Man II open reading frame has allowed us to demonstrate that Man II shares several of the biochemical features of the Golgi glycosyltransferases (37), namely a type II transmembrane orientation with an NH_2-terminal membrane anchor domain and susceptibility to proteolytic release of a soluble, catalytically active form of the enzyme.

Several lines of evidence indicate that the isolated cDNA clone MII-8 encodes the authentic Man II polypeptide. The NH_2-terminal sequence of the intact purified enzyme aligns to amino acid position 6 of the translated cDNA at the junction of the cytoplasmic tail and the transmembrane domain. Whether the cleavage of the five amino acids segment represents an in vivo or in vitro event is difficult to determine since it is not possible to distinguish the cleaved from uncleaved forms based on size. The remaining six rat polypeptide sequences can be identified in the translation of the murine cDNA with several conservative amino acid substitutions between the two species. The Man II cDNA clone transcribed and translated in vitro was found to yield a polypeptide identical in size, glycosylation pattern, and topology in microsomal membranes to the rat liver (28) or biosynthetically labeled 3T3 enzyme (27). Expression of the Man II cDNA in COS cells resulted in a 8–12-fold overexpression of enzyme activity with the same pH optimum, sensitivity to inhibition by swainsonine, and crossreactivity with the anti-rat Man II antibody as the endogenous 3T3 enzyme (27). Finally, the enzyme expressed in COS cells was localized by immunofluorescence to a perinuclear membrane array characteristic of the Golgi complex suggesting an appropriate localization of the transfected cDNA translation product. The open reading frame predicts a type II transmembrane topology with a 5 amino acid cytoplasmic tail, a single transmembrane domain, a "stem" region of at least 80 amino acids based on proteolysis studies, and a 1,044 amino acid catalytic domain. Although the combined "stem" and catalytic domains are two to three times larger than most

of the glycosyltransferases, the general features of membrane topology are identical between the two classes of enzymes (37).

Transmembrane topologies of plasma membrane and ER proteins are quite varied (55) suggesting that there is no obvious selective advantage of a single topology for membrane protein function. Many of the Golgi glycosyltransferases (37), as well as Man II (28), retain full catalytic activity when released from their membrane anchoring domains by selective proteolysis either in vivo or in vitro suggesting that the cleaved regions exert little, if any, influence on the catalytic activities of these enzymes. The conservation of topological features between the collection of Golgi glycosyltransferases and a processing hydrolase, Man II, might therefore suggest a functional role for the common domain structure beyond a simple attachment of the catalytic domain to the membrane surface. One function that has been proposed (37) for the "stem" region is to confer flexibility to the catalytic domains of the Golgi enzymes in order to allow accessibility to lumenal and membrane associated substrates. Although this hypothesis provides a logical resolution to the problem of substrate accessibility, evidence for this "hinge" or "stem" has yet to be demonstrated either in vitro or in vivo. A Golgi GDPase from *S. cerevisiae* (57) which cleaves the soluble substrate GDP in the yeast Golgi lumen has recently been cloned and sequenced (C. Abeijon, K. Yanagisawa, K. W. Moremen, C. B. Hirschberg, and P. W. Robbins, unpublished results) and was found to contain a similar type II transmembrane structure. Since this enzyme presumably cleaves only soluble substrates it would suggest that the conserved topological features of Golgi enzymes might have a function distinct from providing flexibility to the catalytic domain.

Another potential role for the tail/transmembrane domain/stem regions of these Golgi enzymes would be in the recognition and retention of the polypeptides in the Golgi stacks. Recognition signals for soluble and membrane bound polypeptides in the ER have been described. The tetrapeptide recognition signal, KDEL, on the COOH termini of soluble, lumenal ER proteins results in their recognition and retention in the ER (31). Recently, a recognition signal for ER type I transmembrane proteins has also been described (16, 33, 36, 47) with the recognition sequence KKXX at the COOH termini of the polypeptide sequences being recognized on the cytoplasmic face of the ER (47). Deletions in the first of three transmembrane domains of the avian coronavirus E1 glycoprotein disrupted the *cis*-Golgi localization of this polypeptide suggesting that a membrane-associated region contains at least a portion of the information of Golgi targeting of this viral glycoprotein (23). The Golgi cisternae have an additional level of complexity, however, since many of the endogenous Golgi enzymes exhibit distinctive sub-Golgi compartmentation. The mechanistic requirements necessary to yield these subtle differences in Golgi localization may therefore be more complex than the simple linear sequences involved in ER protein retention.

The similarity in sequence between murine Man II, the rat ER α-mannosidase, and the yeast vacuolar α-mannosidase was surprising considering the distinctions between the enzymes on substrate specificity and sensitivity to alkaloid inhibitors. All three enzymes recognize the small synthetic substrates, *p*-nitrophenyl α-D-mannoside and 4-methylumbelliferyl α-D-mannoside, although with different K_ms (29). These results suggest that the region of similarity might reflect a portion of the catalytic domain involved in α-mannoside recognition. The mammalian lysosomal α-mannosidase (5) also cleaves the small synthetic α-mannoside substrates and, like Man II, is inhibited by swainsonine. When the lysosomal enzyme is cloned and the sequence is determined a comparison with the consensus sequence of the other α-mannosidases would be a critical test of the function of the consensus sequence. A match in the proposed substrate recognition region between the three disparate mammalian α-mannosidases would provide strong evidence that this region is involved in the active site of the enzymes.

The 3′ end of Man II was found to be heterogeneous in length with the isolation of three distinct size classes of polyadenylated clones. The longest class of clones extends 2,543 bp in the 3′ direction from the end of the open reading frame before terminating in a poly(A) tract. Comparison between the longest aggregate clone sequence and the message size on Northern blots suggests that the predominant transcript likely reads through the region containing the three polyadenylation signals and uses a termination and polyadenylation signal an additional ~1.4 kb further downstream. Repetitive element sequences were found in the long 3′ untranslated region of both murine Man II and murine β1,4-galactosyltransferase (46). Although this type of extended 3′ untranslated region is common among the glycosyltransferases the functional significance of this extended transcript is not clear.

The identification of a deficiency in Man II expression as the causative agent in one form of HEMPAS disease (12), a heterogeneous autosomal disease characterized by a defect in the synthesis of cellular and secreted glycoproteins (13), has focused our interest on the genomic structure of Man II and the regulation of gene expression. As a first step we have presented the cloning and expression of murine Man II cDNA. In addition we have localized the human Man II gene to chromosome 5. Further characterization of full length human Man II cDNA and genomic clones should allow us to determine the molecular basis of the Man II deficiency in HEMPAS disease and the regulatory features of the processing enzymes responsible for these maturation of cellular and secreted N-glycans.

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