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Molecular cloning and expression of cDNAs encoding human α-mannosidase II and a previously unrecognized α-mannosidase II^x isozyme

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ABSTRACT **Golgi α-mannosidase II (α-MII) is an enzyme involved in the processing of N-linked glycans. Using a previously isolated murine cDNA clone as a probe, we have isolated cDNA clones encompassing the human α-MII cDNA open reading frame and initiated isolation of human genomic clones. During the isolation of genomic clones, genes related to that encoding α-MII were isolated. One such gene was found to encode an isozyme, designated α-MII^x. A 5-kb cDNA clone encoding α-MII^x was then isolated from a human melanoma cDNA library. However, comparison between α-MII^x and α-MII cDNAs suggested that the cloned cDNA encodes a truncated polypeptide with 796 amino acid residues, while α-MII consists of 1144 amino acid residues. To reevaluate the sequence of α-MII^x cDNA, polymerase chain reaction (PCR) was performed with lymphocyte mRNAs. Comparison of the sequence of PCR products with the α-MII^x genomic sequence revealed that alternative splicing of the α-MII^x transcript can result in an additional transcript encoding a 1139-amino acid polypeptide. Northern analysis showed transcription of α-MII^x in various tissues, suggesting that the α-MII^x gene is a housekeeping gene. COS cells transfected with α-MII^x cDNA containing the full-length open reading frame showed an increase of α-mannosidase activity. The α-MII^x gene was mapped to human chromosome 15q25, whereas the α-MII gene was mapped to 5q21-22.**

α-Mannosidase (α-M) activities are involved in both biosynthesis and catabolism of N-linked glycans (1, 2). These enzyme activities are present in cells ranging from yeast to human. There are different forms of α-Ms: lysosomal α-Ms are soluble and involved in degradation of N-glycans, endoplasmic reticulum (ER) and Golgi α-Ms are involved in processing of newly synthesized N-glycans, and cytoplasmic α-Ms may be involved in degradation of dolichol intermediates that are not needed for protein glycosylation or oligosaccharides derived from glycoprotein turnover in the ER (1). Substrate specificities of these α-Ms differ from each other, and Golgi α-MII specifically hydrolyzes two peripheral mannosyl residues from Manα1→6(Manα1→3)Manα1→6(GlcNAcβ1→2Manα1→3)[Manβ1→4GlcNAcβ1→4GlcNAcβ1→]asparagine structure. Several α-Ms have been cloned to date. These include Golgi α-MII (3, 4), ER/cytosolic α-MI (5), two isozymes of Golgi α-MI (6–8), lysosomal α-M (9), *Dictyostelium* α-M (10), and yeast α-M (11) (for a recent review, see ref. 2).

α-MII is a type II membrane protein mainly residing in the medial Golgi cisternae, while its localization is cell-type dependent (12, 13). A genetic defect of α-MII in humans causes congenital dyserythropoietic anemia type II or HEMPAS (hereditary erythroblastic multinuclearity with positive acidified serum lysis test) (14). Thus, the reduction of α-MII activity results in a failure of polylactosaminoglycan formation in erythrocyte membrane proteins, leading to clustering of membrane proteins and formation of unstable erythrocytes (14–17). Since α-MII is normally expressed in a variety of cells in tissues, the HEMPAS defect is not restricted to erythroid cells (18, 19). However, there are cells and tissues that are not affected by HEMPAS genetic defect. This predicts the existence of one or more tissue-type-specific α-MII isozymes (16, 17) that compensate for the α-MII defect in certain cell types. The present report describes the cloning of human α-MII and the genomic and cDNA cloning of an α-MII isozyme designated α-MII^x and their sequences.¶

MATERIALS AND METHODS

Materials. Full-length mouse α-MII cDNA clones and cDNA clones containing the partial human α-MII open reading frame have been isolated (3). Additional human α-MII cDNA clones were isolated from a human liver cDNA library in the Uni-ZAP XR vector (Stratagene). A human cosmid genomic library constructed in pWE15 cosmid vector (Stratagene) was provided by T. Sato, La Jolla Cancer Research Foundation. A melanoma cDNA library constructed from human melanoma cell line SK-Mel-28 (ATCC HTB 72) in the pcDNAI vector was purchased from Invitrogen.

Isolation of α-MII cDNA Clones. The human liver cDNA library (≈1 × 10^6 plaques) was plated on XL-1-Blue host cells and screened by plaque hybridization using standard procedures (20). The probe used for the library screening was a ^{32}P-labeled 578-bp *Eco*RI restriction fragment derived from the 3′ end of the previously isolated human α-MII clone, HM-1 (3). Clones were excised from the Uni-ZAP vector as inserts in Bluescript II SK(−) and were sequenced. A single clone, designated HM-4, was completely sequenced and found to be 2825 bp long, to overlap the 3′ end of the HM-1 clone starting at base pair position 810 of HM-1, and to contain 163 bp of 3′ untranslated sequence followed by a poly(A) tail. The full open reading frame of human α-MII cDNA was assembled from those two clones by ligating the 5′ 1258-bp *Hin*dIII fragment of HM-1 with the 2383-bp *Hin*dIII fragment of HM-4.

Screening of the Human Genomic Library. About 1 × 10^6 colonies of the cosmid library were hybridized with a ^{32}P-labeled 1.4-kb *Eco*RI fragment of human α-MII cDNA as a

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Abbreviations: α-M, α-mannosidase; α-MI and α-MII, α-Ms I and II; HEMPAS, hereditary erythroblastic multinuclearity with positive acidified serum lysis test.

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‖The sequences reported in this paper have been deposited in the GenBank data base (accession nos. D55649 and L28821 for α-MII^x and U31520 for α-MII).

probe (20). Two positive clones with an insert size of about 40 kb were isolated and were designated clone 7 and clone 14.

Screening of a Human Melanome cDNA Library for α-MII^x Clones. A human melanoma cDNA library in the pcDNAI vector was screened with a 69-bp PCR amplimer probe derived from genomic αMII^x clone 7 (see Fig. 2). A clone with an insert size of 5 kb was isolated and sequenced (pcDNA-IMX).

Partial cDNA Amplification by Reverse Transcription (RT)–PCR. Poly(A)$^+$ RNAs were isolated from normal human lymphocytes, and cDNAs were synthesized by using a First-Strand cDNA synthesis kit (Pharmacia). PCR was performed with the following oligonucleotide primers: 5′-TTTCTTCTC-CTATGCGGACCG (nucleotides 1476–1496 in Fig. 3) and 5′-CCAGCTCCTTGTTGACGTAGTC (nucleotides 2652–2631 in Fig. 3). The PCR products were subcloned into Bluescript vector and designated pBS-LymRTPCR. Genomic clone 14 was also subjected to PCR with the primers 5′-GG-CCTGGGCGTGCAGCTA (nucleotides 2140–2160 in Fig. 3) and 5′-AGACAAGGACCTGCATGTCCA (nucleotides 2404–2384 in Fig. 3). A PCR product of about 500-bp was subcloned into pBluescript and named pBS-genomePCR.

Sequencing. Nucleotide sequencing of the cDNA and genomic clones encoding α-MII^x was performed by dideoxy-termination methods (21) using a Sequenase kit (United States Biochemical).

Construction of a Mammalian Expression Vector Harboring α-MII^x cDNA. Because the subclone pcDNAI-MX, isolated from the melanoma cDNA library encoded a truncated α-MII^x product, a cDNA encompassing the full coding region of α-MII^x cDNA was obtained by replacing the *Cpo* I fragment (1095 bp) of melanoma cDNA with a *Cpo* I fragment (1070 bp) of pBS-LymRTPCR. The resulting clone was named pcDNAI-MII^x. The large *Eco*RI fragment from pcDNAI-MII^x containing the entire α-MII^x cDNA open reading frame was excised and ligated into the *Eco*RI site of a mammalian expression vector, pXM (4).

RESULTS

Isolation of Human α-MII cDNA Clones. Partial cDNA clones containing the 5′ end of the human α-MII open reading frame have been previously isolated (4). Clones containing the remainder of the 3′ end of the open reading frame were isolated by plaque hybridization with a 578-bp *Eco*RI restriction fragment as a radiolabeled probe. The longest of the clones overlapped with the previous clones and extended downstream through the end of the open reading frame and 163 bp into the 3′ untranslated region before terminating in a poly(A) tract. A full-length human α-MII cDNA open reading frame was assembled from these clones. The open reading frame encompassed by this construct encoded a product that was 1144 amino acids in length and 80% identical to the product of the cDNA sequence of murine α-MII (Fig. 1), including a 97% identity over the NH_2-terminal 87 amino acids.

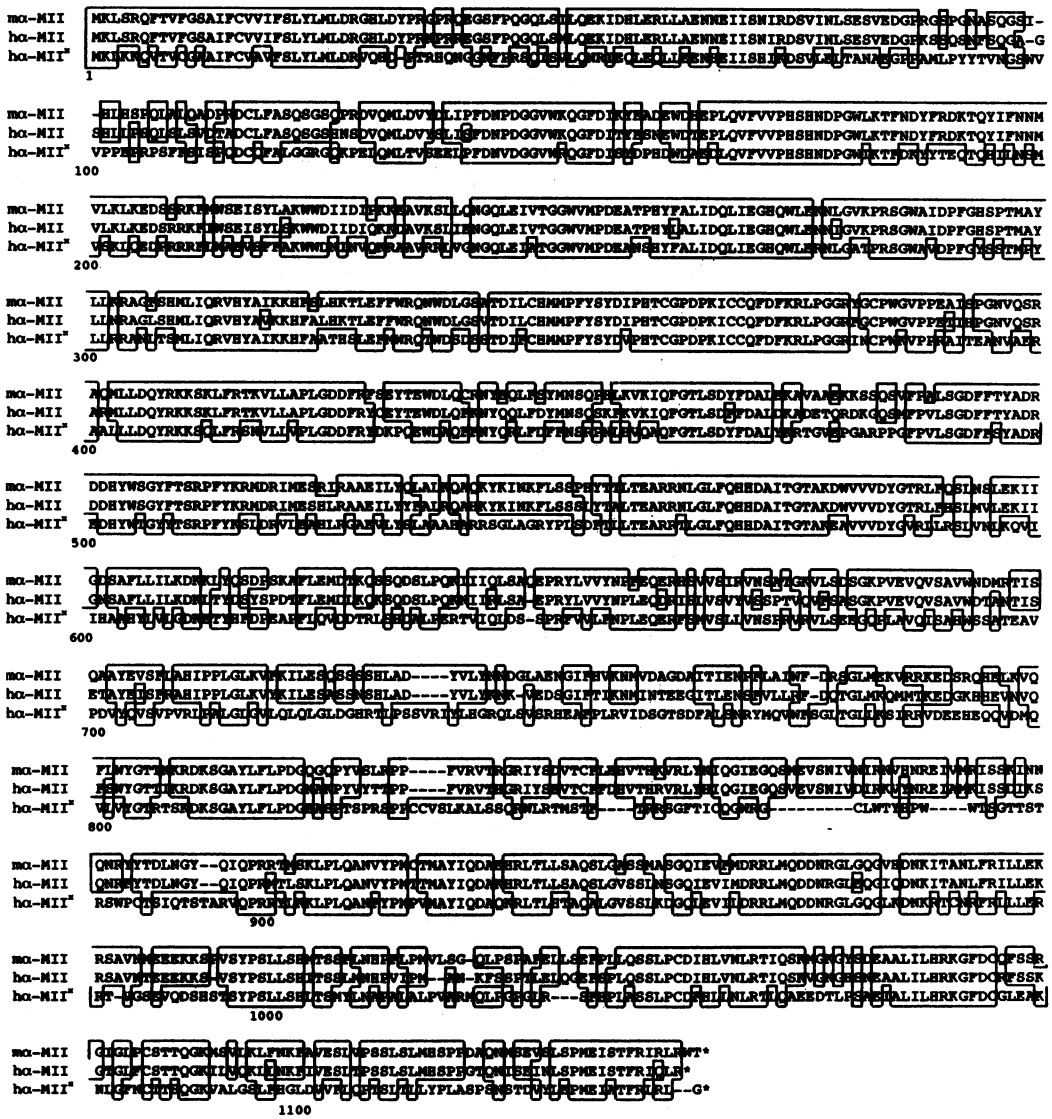

FIG. 1. Comparison of amino acid sequences between mouse and human α-MIIs and human α-MII^x. The boxes indicate identical residues between the lines of sequence.

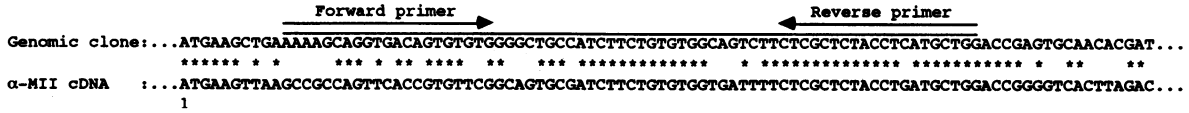

FIG. 2. Partial nucleotide sequence of genomic clone 7 and corresponding cDNA sequence of human α-MII. DNA sequence obtained from a subclone of the 2.4-kb fragment. Primers were designed of positions indicated by the arrows for amplification of the 69-bp probe used in cDNA library screening.

Hydropathy analysis on murine (4) and human α-MII and protease studies on the murine enzyme (22) indicate that α-MII from both species is a type II transmembrane protein with an NH_2-terminal 5-amino acid cytoplasmic tail and a single transmembrane domain within this highly conserved sequence region. Expression in COS cells (4) resulted in the overexpression of α-MII activity toward the synthetic substrate, *p*-nitrophenyl α-D-mannoside, and the appearance of anti-α-MII antibody cross-reactive material in a juxtanuclear membrane array consistent with the Golgi complex (data not shown).

Screening of a Human Genomic and cDNA Libraries and Identification of α-MII^x Gene. A human genomic library constructed in cosmid vector pWE15 was screened using ^{32}P-labeled human α-MII cDNA as a probe. Two positive clones, each with an insert size about 40-kb, were isolated. These two clones were designated clone 7 and 14, digested with *Eco*RI, and then analyzed by Southern blot hybridization using ^{32}P-labeled α-MII cDNA as a probe. *Eco*RI fragments of 2.4-kb and 4.0-kb from each clone were found to hybridize with the probe and were subcloned into pBluescript. The nucleotide sequence of these subclones showed that the sequences are similar but not identical to α-MII DNA (Fig. 2), suggesting that clone 7 encodes a new gene related to the α-MII gene. The product of this new gene was named α-MII^x. A human melanoma cDNA library was then screened by using a short (69-bp) DNA fragment as a probe (Fig. 2), and one positive clone with an insert size of 5-kb was obtained. The nucleotide sequence of the cDNA clone had an open reading frame encoding 796 amino acid residues (Fig. 3).

To determine if a different cDNA sequence encoding α-MII^x is present in different cells, RT–PCR was performed with poly(A)$^+$ RNAs isolated from normal human lymphocytes. PCR primers were designed so that the PCR products to be sequenced would cover the area between nucleotides 1476 and 2652, which is the region containing the stop codon at 2389. The sequences of lymphocyte PCR products revealed that there is an alternative α-MII^x cDNA sequence that has a 25-bp deletion when compared with the sequence of the α-MII^x cDNA clone isolated from the melanoma library (see Fig. 3). When the sequence obtained by PCR replaces the corresponding sequence in the melanoma cDNA clone, the resulting product of translation has 1139 amino acid residues (Fig. 4), which is comparable to the size of human α-MII (1144 amino acids). Hereafter, the cDNA product of 1139 amino acid residues will be referred to as α-MII^x.

A hydropathy plot (23) of the α-MII^x polypeptide showed an uncleavable membrane-spanning domain near the NH_2 terminus, suggesting that α-MII^x is a type II membrane protein. Alignment of peptide sequences shows a close similarity between human α-MII^x and human and mouse α-MII, respectively (Fig. 1).

Alternative Splicing of α-MII^x Gene. The sequences obtained from α-MII^x genomic clone 14 revealed that both cDNA sequences obtained from melanoma and lymphocyte cells are present in genomic clones. Melanoma cDNA contains an extra 25 nucleotides (see highlighted letters in Fig. 3) after residue 2347, leading to a frame shift with a stop codon at 2389. On the other hand, lymphocyte α-MII^x mRNA spliced out an additional 25-bp, yielding an open reading frame extending to nucleotide 3417, thus encoding a polypeptide with 1139 amino acid residues. RT–PCR analysis showed that these two forms of α-MII^x transcripts are present in various cell types (data not shown), suggesting that the α-MII^x gene generally produces two alternatively spliced mRNAs in normal human cells.

Expression of α-MII^x in Various Human Tissues. RNA hybridization (Northern) analysis of α-MII^x (Fig. 5 *Left*) showed that α-MII^x mRNA is seen as a single band at 5 kb in various types of adult tissues. Northern analysis of α-MII and α-MII^x showed that both enzymes are strongly expressed in the placenta and kidney; in heart, brain, and skeletal muscles, expression of α-MII^x is stronger than that of α-MII. Expression of these enzymes is very weak in lung.

Enzyme Activity of α-MII^x. COS-1 cells were transfected with plasmid constructs containing human α-MII or α-MII^x coding regions and assayed for overexpression of α-mannosidase activity as described (4). Cell lysates prepared from the COS-1 cells transfected with α-MII^x cDNA exhibited a 1.6-fold increase of α-mannosidase activity compared with those of controls (Table 1). These results indicate that α-MII^x polypeptide with 1139 amino acids is catalytically active and can hydrolyze *p*-nitrophenyl α-D-mannoside.

Chromosome Localization. α-MII^x gene and α-MII gene were mapped to human chromosomes 15q25 and 5q21-22, respectively (Fig. 6).

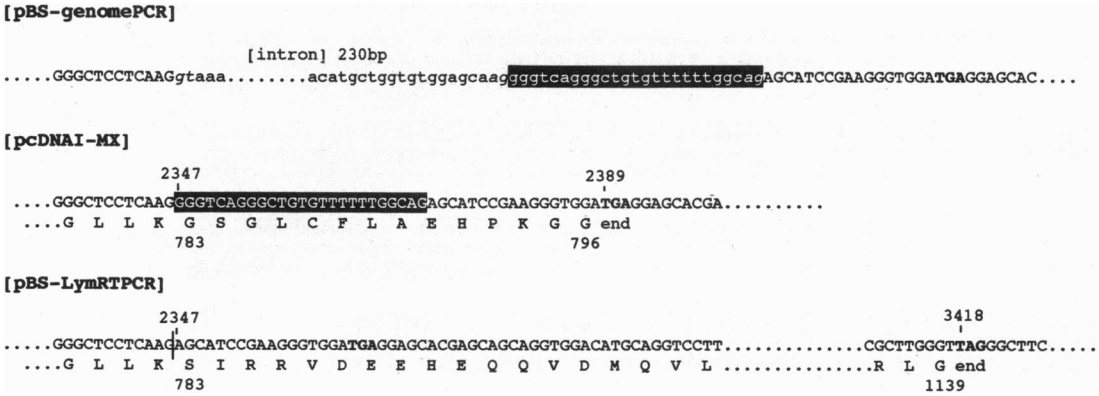

FIG. 3. Two alternative splicings of the genomic DNA encoding α-MII^x, resulting in either short (796 amino acid residues) or long (1139 amino acid residues) α-MII^x polypeptide. The sequence of 25-bp nucleotides (highlighted) is present in genomic clone 14 (top line). The sequence that becomes a part of the exon by an alternative splicing is highlighted (middle line).

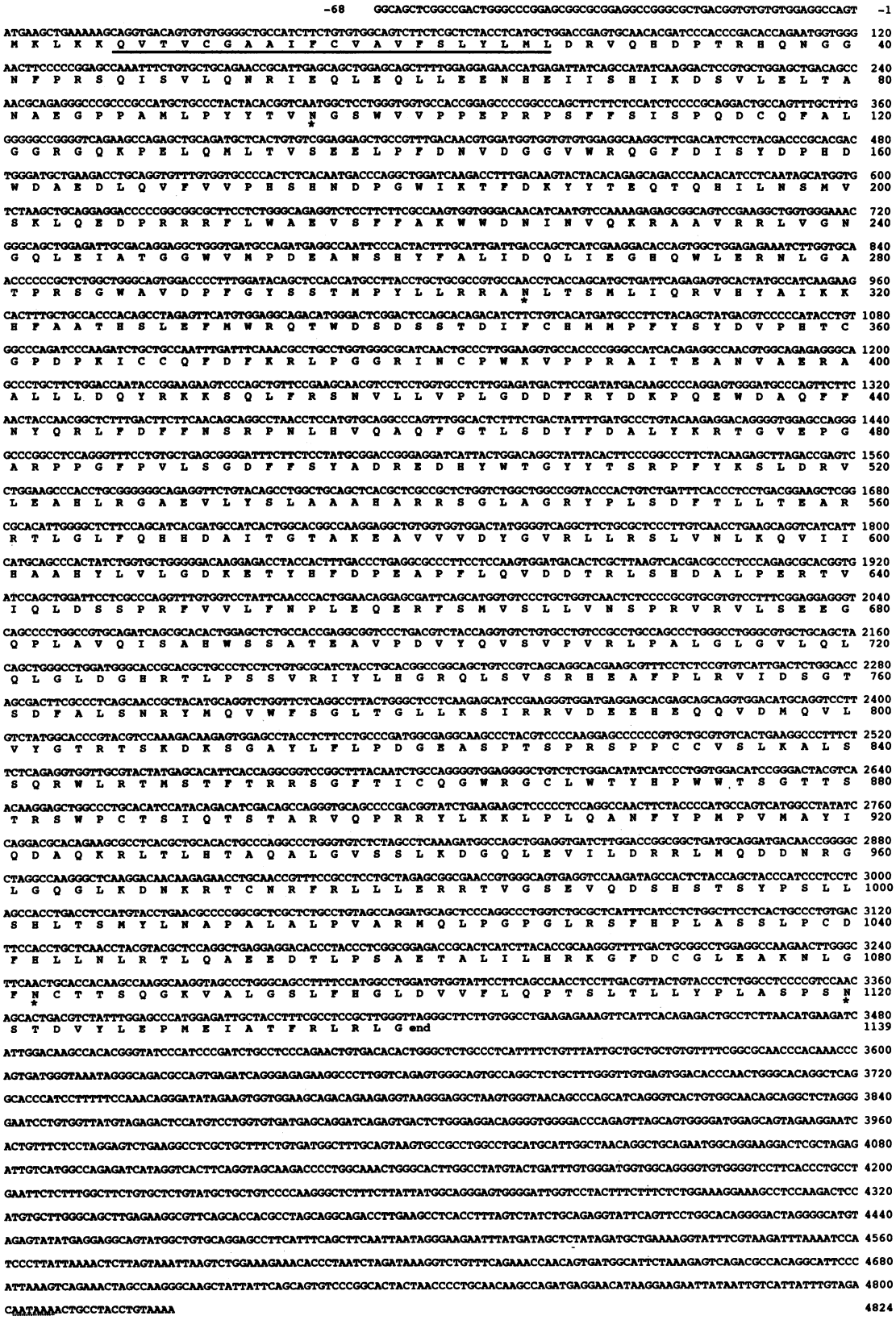

FIG. 4. Nucleotide sequence of α-MII^x cDNA and deduced amino acid sequence.

DISCUSSION

Recently it has become evident that trimming steps in the maturation of *N*-glycans (24) that were previously attributed to a single processing hydrolase can potentially be catalyzed by more than one isozyme in mammalian tissues (2). In particular, two highly related cDNAs encoding enzymes involved in the cleavage of α(1,2)-mannosyl units have recently been cloned (6, 8). We have also identified clones encoding a previously undisclosed α-MII-related gene. A 69-bp probe that was distinctive to this α-MII-related sequence was successfully used to isolate a full-length cDNA clone (Fig. 4).

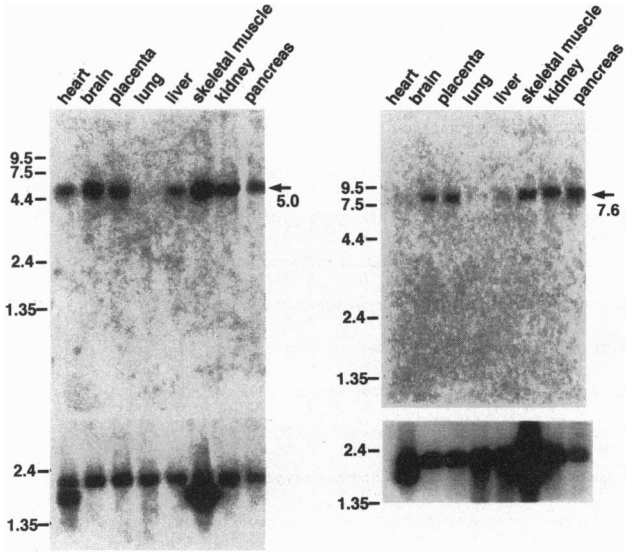

FIG. 5. Expression of α-MII and α-MII^x mRNA in normal human tissues. (*Upper*) The filter (multiple tissue Northern blots, Clontech) was hybridized with either human α-MII^x cDNA probe (*Upper Left*) or human α-MII cDNA probe (*Upper Right*). (*Lower*) Each filter was stripped of its probe and rehybridized with β-actin cDNA probe. The numbers show the position of standard size markers in kb.

The present study shows that expression of α-MII^x is influenced by alternative splicing. As described above, α-MII^x mRNA is spliced differently in the coding region, and such alternative splicing results in either active or inactive enzyme. PCR analysis of several tissues showed coexistence of two alternatively spliced mRNAs, suggesting that cells regularly produce both active and inactive α-MII^x. It would therefore be interesting to know whether any factor that affects the mode of splicing also controls the level of N-glycan processing *in vivo*.

We have predicted (16, 17) the existence of a fetal type α-MII isozyme. Such a hypothesis stemmed from an analysis of HEMPAS disease; HEMPAS patients do not show an obvious abnormality at the time of birth (18). This fact is intriguing, because *N*-glycans present in plasma membranes are involved in cell-to-cell interactions, and therefore may play important roles during embryonic development. In fact, "knock-out" of the *N*-acetylglucosaminyltransferase-I gene in mice resulted in embryonic lethality, presumably because the conversion of high mannose to complex type *N*-glycans is impaired (25). It is therefore possible that a fetal-type isozyme of α-MII is expressed in the human embryo, thus avoiding the genetic defect of α-MII in HEMPAS. Further analysis of α-MII-related genes will define an apparently developmental and tissue-specific manifestation of HEMPAS disease.

Table 1. α-Mannosidase activity in COS-1 cells transfected with α-MII- and α-MII^x-related cDNAs. Numbers presented are the average of quadruplicate analysis

	α-Mannosidase activity, % of control (mean ± SEM)
pXM	1.0
pXM-murine MII	2.5 ± 0.3
pXM-MII^x-sense	1.6 ± 0.2
pXM-MII^x-antisense	1.0 ± 0.1

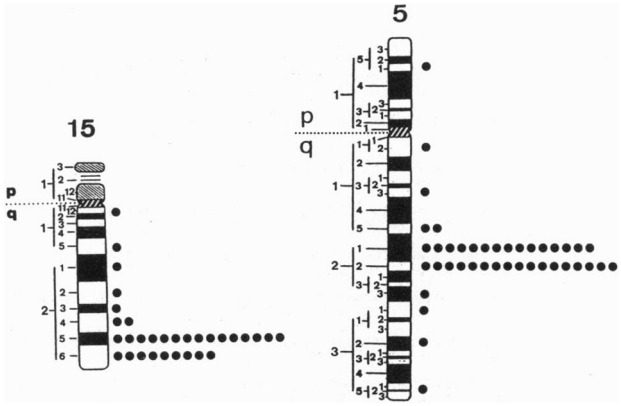

FIG. 6. Distribution of labeled sites on chromosome 15 for α-MII^x (*Left*) and chromosome 5 for α-MII (*Right*). Idiograms for the respective chromosomes were prepared as described (26).

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