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Cloning of a cDNA encoding the human cation-dependent mannose 6-phosphate-specific receptor

(lysosomal enzyme transport/transmembrane protein/chromosomal assignment)

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ABSTRACT **Complementary DNA clones for the human cation-dependent mannose 6-phosphate-specific receptor have been isolated from a human placenta library in λgt11. The nucleotide sequence of the 2463-base-pair cDNA insert includes a 145-base-pair 5′ untranslated region, an open reading frame of 831 base pairs corresponding to 277 amino acids (M_r = 30,993), and a 1487-base-pair 3′ untranslated region. The deduced amino acid sequence is colinear with that determined by amino acid sequencing of the N-terminus peptide (41 residues) and nine tryptic peptides (93 additional residues). The receptor is synthesized as a precursor with a signal peptide of 20 amino acids. The hydrophobicity profile of the receptor indicates a single membrane-spanning domain, which separates an N-terminal region containing five potential N-glycosylation sites from a C-terminal region lacking N-glycosylation sites. Thus the N-terminal (M_r = 18,299) and C-terminal ($M_r \leq 7648$) segments of the mature receptor are assumed to be exposed to the extracytosolic and cytosolic sides of the membrane, respectively. Analysis of a panel of somatic cell (mouse–human) hybrids shows that the gene for the receptor is located on human chromosome 12.**

Targeting of newly synthesized lysosomal enzymes in many cell types depends on formation of mannose 6-phosphate residues on lysosomal enzymes and recognition of these residues by specific receptors (Man6*P*R). Two species of Man6*P*R have been described. First, a receptor with a subunit M_r of 215,000 has been found in a variety of tissues (for reviews, see refs. 1 and 2). This receptor binds its ligands in a pH-dependent manner (3) and participates in targeting of endogenous lysosomal enzymes and in endocytosis of exogenous lysosomal enzymes (4). A second receptor with a subunit M_r of 46,000 has recently been isolated from bovine liver and murine macrophages (5), and its presence has been demonstrated in several human and rat cell types (31). Binding of ligands to this receptor depends on divalent cations (5) and pH (6). This receptor is used for intracellular targeting but not for endocytosis of lysosomal enzymes (32).

Human M_r 46,000 Man6*P*R is a transmembrane protein that consists of two identical subunits, as concluded from cross-linking experiments (33). The deglycosylated receptor has a M_r of 26,500–28,000 (5), and its susceptibility to proteinases indicates that the cytosolic portion has a M_r of ≈5000 and contains the C terminus (31). The mannose 6-phosphate binding site probably contains a histidine residue in addition to an essential arginine residue (33). In the present study, we report the cloning and sequencing of a cDNA encoding the entire human M_r 46,000 Man6*P*R‖ and the chromosomal assignment of the gene for the M_r 46,000 Man6*P*R to human chromosome 12.

MATERIALS AND METHODS

Materials. The human hepatoma cDNA library (7) in λgt11 (8×10^6 independent clones) was kindly supplied by J. O'Brien and J. de Wet. For construction of a human liver cDNA library in λgt11 (1×10^6 independent clones), cDNA synthesis was random-primed (8), and second strand synthesis was performed according to Gubler and Hoffmann (9). The human placenta cDNA library in λgt11 (1×10^6 independent clones) was from Clontech (Palo Alto, CA). Restriction endonucleases, T4 polynucleotide kinase, Klenow fragment, T4 DNA ligase, and M13-derived 15-mer primer were from Boehringer Mannheim; cloned Moloney murine leukemia virus reverse transcriptase was from Bethesda Research Laboratories; [γ-^{32}P]ATP (222 TBq/mmol) was from New England Nuclear; and [α-^{32}P]dATP (111 TBq/mmol) and dATP[α-^{35}S] (>22 TBq/mmol) were from Amersham. Dideoxy- and deoxynucleotide triphosphates were from Pharmacia-PL Biochemicals.

Library Screening. Immunological screening was performed with an affinity-purified polyclonal rabbit anti-human M_r 46,000 Man6*P*R antibody. Detection of fusion proteins was accomplished by using a modification of the procedure described by de Wet *et al.* (7). After induction of fusion proteins with isopropyl β-thiogalactoside at 37°C, the nitrocellulose filters (Schleicher & Schüll) were saturated with Blotto (10) for 4 hr at 4°C. Incubation with the affinity-purified polyclonal antibodies was for 14–16 hr at 4°C, and subsequent incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:5000 dilution; Tago, Burlingame, CA) was for 2 hr at 4°C. The staining was performed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (11). An *Eco*RI–*Hin*dIII fragment was prepared from the 5′ end of the insert P4a and was nick-translated by the method of Feinberg and Vogelstein (12). This probe was used to screen 10^6 plaque-forming units (pfu) of the placenta library (13).

Oligonucleotides. The partial amino acid sequences Asn-Trp-Ile-Met-Leu and Met-Glu-Gln-Phe-Pro of tryptic peptides of the M_r 46,000 Man6*P*R were used to construct the mixed oligonucleotides 280 (5′ARCATDATCCARTT3′) and 281 (5′GGRAAYTGYTCCAT3′) (where R = an unspecified

Abbreviations: pfu, plaque-forming units; Man6*P*R, mannose 6-phosphate-specific receptor(s).
¶To whom reprint requests should be addressed.
‖This sequence is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg, F.R.G.) (accession no. J02937).

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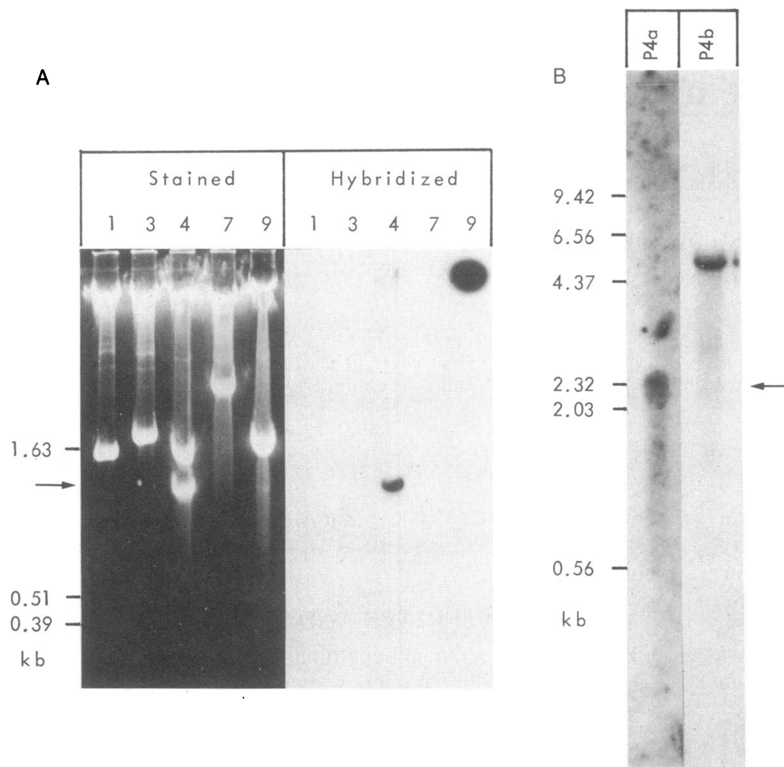

FIG. 1. Analysis of recombinant clones for the M_r 46,000 Man6*PR* and blot-hybridization analysis of poly(A)$^+$ RNA. (*A*) DNA from clones isolated by immunological screening of a human placenta cDNA library was digested with *Eco*RI, and the fragments were separated by electrophoresis. The DNA was stained with ethidium bromide and after being transferred to a nitrocellulose filter was hybridized to ^{32}P-labeled oligonucleotide 281. Positions and sizes (in kb) of DNA markers are indicated. The identification numbers assigned to the placenta clones are given above the lanes. The insert P4 contains an internal *Eco*RI site and is cleaved into fragments of 1.2 kb (P4a) and 1.5 kb (P4b). The arrow indicates the P4a fragment that hybridizes with oligonucleotide 280. (*B*) Human liver mRNA (10 μg) was hybridized with ^{32}P-labeled P4a and P4b as indicated above the lanes. The arrow indicates the mRNA species hybridizing with P4a.

purine nucleoside, Y = an unspecified pyrimidine nucleoside, and D = G, A, or T), which specify all possible combinations of codon choices. The oligonucleotides were purified by polyacrylamide gel electrophoresis and were labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (13).

Oligonucleotide Hybridization. λgt11 clones were digested with *Eco*RI; fragments were separated by electrophoresis in a 1% agarose gel and were stained with ethidium bromide. Hybridization in the gel was performed according to Kidd (14) under high-stringency conditions.

Sequence Analysis. The inserts were subcloned into M13mp18 and M13mp19 vectors (15) and were sequenced by the Sanger *et al.* dideoxy chain termination method (16) with dATP[α-^{35}S] (17) and the Klenow fragment (16) or cloned Moloney murine leukemia virus reverse transcriptase (18). Primers were either a M13-derived 15-mer or sequence-derived oligonucleotides (17-mer).

RNA Blot Hybridization. Poly(A)$^+$ RNA from human liver was isolated by the guanidinium thiocyanate method (19) and selected by chromatography on an oligo(dT)-cellulose column (20). The RNA was denaturated with glyoxal (21) and was fractionated by electrophoresis in a 1% agarose gel. Transfer of the RNA to Hybond N membranes (Amersham) was performed according to the supplier's manual. Hybridization was carried out with nick-translated ^{32}P-labeled cDNA probes (12) by the method of Thomas (21).

Chromosomal Localization. Mouse–human cell hybrids were generated by fusion of mouse B82, RAG, or A9 cells with human fibroblasts. In the hybrid B82 MS2 1a-14-1, human chromosomes 2, 3, 4, 7, 8, 11, 13, 15, 16, 17, 18, 19, 21, 22, X, and Y were present. The other hybrids—RAG ANLY 1, RAG PI 7-2, RAG PI 5-15, RAG GM194 5-5, RAG GO 4, A9 SU 1-2, A9 1T 2-21-14, RAG GM610 5-23, and RAG GM610 4-5-1—were described previously (22–24). The presence of human chromosomes and chromosome fragments was determined by isozyme and cytogenetic analysis (24). DNA from hybrid cells was digested with *Eco*RI, separated by electrophoresis in 0.8% agarose, transferred to Biodyne A

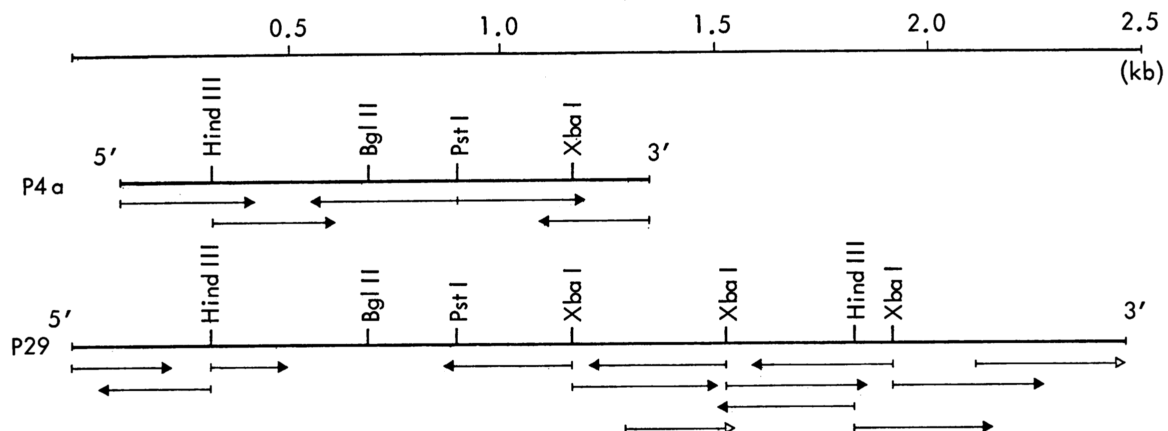

FIG. 2. Restriction map and sequencing strategy for the human M_r 46,000 Man6*PR* clones P4a and P29. Horizontal arrows indicate the direction and extent of sequence determination. Filled arrows refer to sequencing with the M13 primer, and open arrows refer to sequencing with the sequence-derived oligonucleotides.

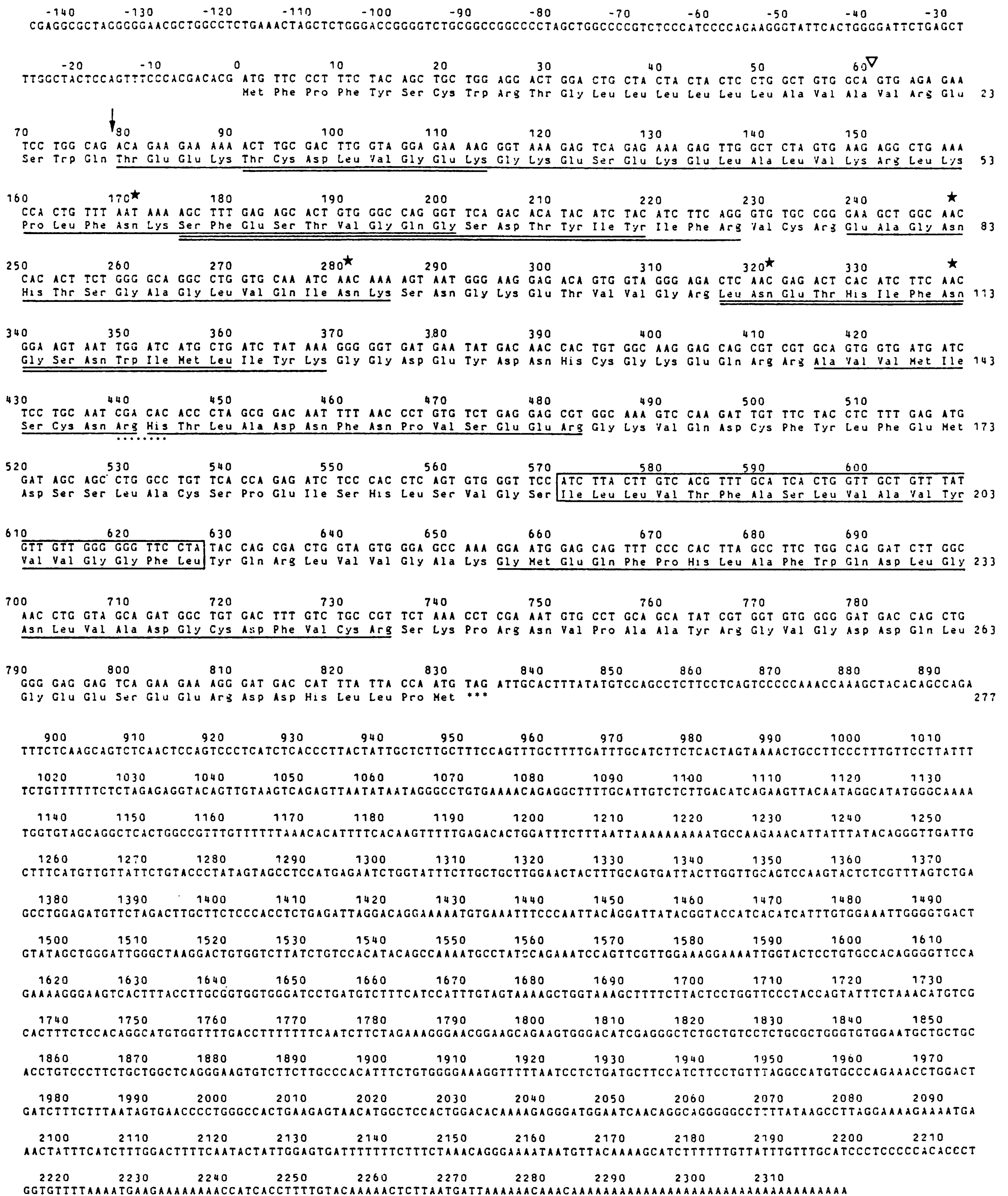

FIG. 3. Nucleotide sequence and deduced amino acid sequence of human M_r 46,000 Man6*P*R cDNA. The nucleotide sequence and the corresponding amino acid sequence of M_r 46,000 Man6*P*R were obtained by analysis of P4a and P29 as shown in Fig. 2. The numbering of the nucleotides starts at the adenosine of the initiator methionine. The arrow points to the N terminal of the mature M_r 46,000 Man6*P*R. The arrowhead marks the N terminus of the presumptive propeptide of the receptor. The potential N-glycosylation sites are indicated by stars. The lines indicate amino acid sequences that were determined by amino acid sequencing of the receptor and its tryptic fragments. The triple asterisk indicates the stop codon. The presumed membrane-spanning domain is boxed. The neighboring arginine and histidine residues presumably involved in the ligand binding are marked by a dotted line.

membranes (Pall Filtrationstechnik, Dreieich, F.R.G.), and hybridized to nick-translated P4a (13).

Computer Analysis. The hydrophobicity profiles of the amino acid sequences were generated by a program obtained

from Novotny and Auffray (25) using the hydrophobicity parameters given by Rose and Roy (26). Three smoothing cycles were used with a window size of 7. An homology search was performed with a program using the algorithm of Wilbur and Lipman (27), which evaluates direct amino acid matches and the coincidence of related amino acids as defined by the logarithm of odds score of Dayhoff *et al.* (28). The predicted amino acid sequence was compared with that of all the human receptor protein sequences stored in the Protein Sequence Database.**

Amino Acid Sequencing. Human M_r 46,000 Man6*P*R purified from liver (33) was reduced and carboxymethylated. Tryptic digestion of the purified protein was performed at a trypsin-to-protein ratio of 1:100. Peptides were separated by HPLC using a Baker RP18 wide-pore column and a solvent gradient of 0% to 70% acetonitrile in 0.1% trifluoroacetic acid. Single fractions were rechromatographed with the same acetonitrile gradient prepared in 0.1% ammonium acetate. The sequence analysis of the peptides was performed using an Applied Biosystems gas-phase sequencer with an on-line PTH analyzer (29).

RESULTS

Isolation of a Partial cDNA Clone for the Human M_r 46,000 Man6*P*R. Human cDNA libraries constructed in the expression vector λgt11 were screened with affinity-purified polyclonal antibodies against the human M_r 46,000 Man6*P*R. Screening of placenta (2×10^6 pfu), hepatoma (1.2×10^7 pfu), and liver (8×10^5 pfu) cDNA libraries yielded 10, 5, and 13 clones, respectively. The DNA of the clones was digested with *Eco*RI and was examined for hybridization with two 14-mer oligonucleotide probes (8- and 12-fold degenerate) that had been deduced from the partial sequences of two tryptic peptides of the receptor. Under high-stringency conditions, both probes hybridized to a 1.2-kilobase (kb) fragment of clone P4 from the human placenta cDNA library (shown for oligonucleotide 280 in Fig. 1*A*). The insert of clone P4 had an internal *Eco*RI site yielding *Eco*RI fragments of 1.2 kb (P4a) and 1.5 kb (P4b). In blot-hybridization analysis of poly(A)$^+$ RNA from human liver, the nick-translated inserts P4a and P4b hybridized to RNA species of 2.3 and 5.2 kb, respectively (Fig. 1*B*). Therefore, P4a and P4b are considered to be unrelated fragments that were ligated during construction of the cDNA library. P4a was subjected to sequencing (see below). In the longest open reading frame, 134 deduced amino acid residues were colinear with the N-terminus peptide and with nine tryptic peptides of the human liver M_r 46,000 receptor. This established the authenticity of P4a as the M_r 46,000 Man6*P*R.

Isolation of a Clone Containing the Entire M_r 46,000 Man6*P*R Coding Sequence. The placenta cDNA library was rescreened using the nick-translated *Eco*RI–*Hin*dIII fragment from the 5′ end of P4a (Fig. 2). Plaque hybridization of 1×10^6 pfu yielded 28 positive clones. The restriction map and sequencing strategy for P29, the longest cDNA isolated from the placenta library, and for P4a are shown in Fig. 2. The nucleotide sequence and the deduced amino acid sequence are shown in Fig. 3. The P29 cDNA is 2463 base pairs (bp) long. It contains a 145-bp 5′ untranslated region and an 831-bp open reading frame that is initiated by a methionine-encoding triplet; it is terminated by a TAG stop codon and contains a 1487-bp 3′ untranslated region. P4a corresponds to nucleotides 36–1211 of P29. The 5′ untranslated region contains an in-frame termination codon at nucleotides −31 to −33. The 3′ untranslated region contains a poly(A) tail. A presumptive polyadenylylation signal, ATTAAA, is present 14 bp upstream of the poly(A) tail.

Predicted Amino Acid Sequence of Human M_r 46,000 Man6*P*R. The amino acid sequence predicted by the nucleotide sequence is shown in Fig. 3. The 20 residues at the N terminus contain a hydrophobic core and are assumed to function as a signal sequence for insertion of the nascent receptor polypeptide into the endoplasmic reticulum membrane. The hexapeptide located between the putative signal peptide and Thr-27, the amino terminal of the purified receptor, is removed during maturation *in vivo* or during purification. With the exception of asparagine/aspartic acid and glutamine/glutamic acid, the deduced amino acid composition of the receptor closely corresponds to that obtained by analysis of purified human liver receptor (Table 1).

The hydrophobicity profile of the sequence revealed a single hydrophobic stretch of 19 amino acids (residues 191–209) that is not interrupted by charged amino acids and is considered to be the membrane-spanning domain (Fig. 4). It separates the receptor into an N-terminal portion of 164 residues and a C-terminal portion of 68 residues.

Comparison of the amino acid sequence of the M_r 46,000 Man6*P*R with that of other human receptors revealed no homology greater than that seen for nonreceptor proteins.

Chromosomal Assignment. Nick-translated P4a hybridized with a 6.8-kb fragment of *Eco*RI-digested human DNA. Under the same conditions, P4a did not hybridize to mouse DNA (data not shown). The 6.8-kb fragment was present in all hybrids that contained human chromosome 12 (RAG ANLY 1, RAG PI 7-2, RAG PI 5-15, RAG GM194 5-5, A9 1T 2-21-14, and RAG GM610 4-5-1). It was absent from hybrids that lacked human chromosome 12.

DISCUSSION

A potentially full-length cDNA clone for the human M_r 46,000 Man6*P*R was isolated. The nucleotide sequence predicts a precursor receptor polypeptide of 277 amino acids. The first

Table 1. Amino acid composition of the human M_r 46,000 Man6*P*R

Amino acid	Residues per M_r 46,000 Man6*P*R	
	Protein*	Deduced from cDNA†
Asp	18.0 (Asx)	14
Asn		13
Glu	21.6 (Glx)	21
Gln		8
Ser	16.3	18
Gly	22.0	24
Thr	7.9	9
Ala	12.2	13
His	5.9	7
Pro	6.8	7
Arg	11.1	13
Tyr	6.8	8
Val	21.1	23
Met	3.5	5
Ile	9.7	9
Leu	21.4	23
Phe	13.0	12
Lys	11.9	14
Cys	ND	8
Trp	2	2

ND, not determined.
*Composition of the purified human liver receptor protein taken from ref. 33.
†Amino acid composition calculated from human placenta cDNA sequences for mature M_r 46,000 Man6*P*R (residues 27–277, Fig. 3).

**Protein Identification Resource (1986) Protein Sequence Database (Natl. Biomed. Res. Found., Washington, DC), Release 9.0.

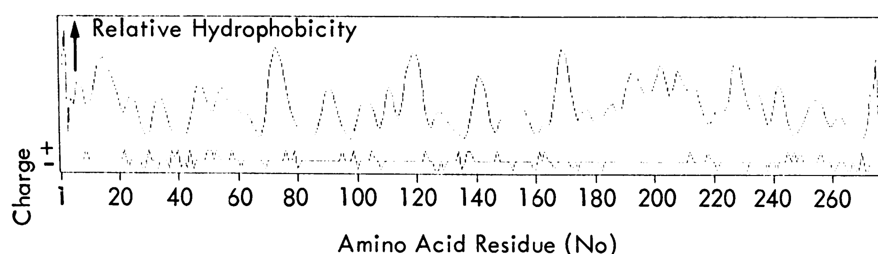

FIG. 4. Profile of relative hydrophobicity of the M_r 46,000 Man6*P*R and the distribution of positively and negatively charged amino acid residues. The numbers on the abscissa refer to the centers of the heptapeptides for which the relative hydrophobicity is shown on the ordinate.

20 residues of the N terminus have the characteristics of a signal peptide and terminate with Ala-Val-Ala. The latter conforms to the sequence Ala-Xaa-Ala, after which prepeptides are often cleaved by the signal peptidase (30). A hexapeptide is located between the signal peptide and the N terminus of the purified receptor. The gene for the M_r 46,000 Man6*P*R was localized to human chromosome 12.

The deduced amino acid sequence predicts a M_r of 27,910 for the mature receptor. The M_r of the deglycosylated receptor subunit determined in $NaDodSO_4$/polyacrylamide gel electrophoresis was 26,500–28,000 (5, 31), which is in good agreement with the predicted value. Therefore, it is unlikely that maturation of the receptor involves gross changes at the C terminus. Based on a decrease in M_r of 18,000–19,500 due to deglycosylation, it has been proposed that the receptor contains 5–8 N-linked oligosaccharides (5, 31). This may indicate that most, if not all, of the five potential N-glycosylation sites in the predicted amino acid sequence (Fig. 3) are utilized.

The exclusive occurrence of N-glycosylation sites in the N-terminal portion locates this part of the receptor on the extracytosolic side of the membrane. The calculated M_r values for the extracytosolic N-terminal, the membrane-spanning, and the C-terminal segments are 18,299, 1963, and 7648, respectively. In a study on the susceptibility of the M_r 46,000 Man6*P*R to proteinases, it was observed that the receptor is a transmembrane protein that has a large N-terminal portion exposed at the extracytosolic face and a smaller C-terminal portion at the cytosolic face of the membrane (31). The M_r of the C-terminal extension and that of the receptor portion containing the C-terminal extension plus the membrane-spanning domain were estimated to be $\geq$5000 and $\leq$8500, respectively.

Modification of arginine destroys the binding activity of the M_r 46,000 Man6*P*R (33). The binding activity is preserved if the treatment is done in the presence of mannose 6-phosphate, suggesting that arginine is a part of the binding site in the M_r 46,000 Man6*P*R. In the N-terminal portion of the receptor, which is assumed to contain the ligand binding site, eight arginine residues are found. One of these residues (Arg-147) is adjacent to a histidine (His-148). Interestingly, modification of histidine has also been shown to destroy the binding property of the receptor (33). However, presence of mannose 6-phosphate during modification of histidine does not protect the binding property of the receptor. Peptide-specific antibodies based on the sequence information provided by the receptor cDNA and site-directed mutagenesis may enable the identification of the ligand binding site in the M_r 46,000 Man6*P*R.

Most mammalian cells express a second Man6*P*R, which is cation-independent and has a subunit M_r of 215,000 (3). The two receptors are immunologically unrelated (33). The partial amino acid sequence of the M_r 215,000 receptor available to us (eight peptides comprising 111 amino acid residues) shows no homology to the sequence predicted for the M_r 46,000 Man6*P*R.

We would like to thank Drs. J. de Wet and J. O'Brien (University of California, San Diego) for providing us with their cDNA library and also Drs. H. Funke (Münster), K. Beyreuther (Köln), and D. Gallwitz (Göttingen) for the synthesis of oligonucleotides. This work was supported by the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie.

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