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CLONING A cDNA FOR THE LYSOSOMAL α-GLUCOSIDASE

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Messenger RNA was isolated from monkey testes and size-fractionated on sucrose gradients. *In vitro* translation of these mRNA fractions resulted in nascent, labeled α-glucosidase that could be precipitated with anti human α-glucosidase antiserum. A cDNA library was constructed from the most enriched fraction. The library was screened with cDNA made from mRNA obtained from immunoselected polysomes. Five cross-hybridizing clones were isolated and identified by their selection of α-glucosidase mRNA, as shown by hybrid released translation and further by their ability to hybridize with DNA from human chromosome 17, on which the gene coding for acid α-glucosidase is located.

Lysosomal α-glucosidase (EC 3.2.1.20) hydrolyzes 1,4 and 1,6 glycosidic bonds as present in maltose, glycogen and some oligosaccharides to yield free glucose (1). Deficiency of this enzyme in Glycogenosis type II (Pompe's disease) results in lysosomal accumulation of glycogen (2,3). Glycogenosis type II is clinically heterogeneous and known in several forms including conditions with early onset, which are fatal in the first two years of life and forms with adult onset and slow progression in which the symptoms are restricted to muscle weakness. It has been suggested that a higher level of residual acid α-glucosidase activity, found in adult forms compared to infantile forms, could in part explain the clinical heterogeneity (4).

Recently, the biochemical studies were extended by investigating the synthesis and molecular processing of acid α-glucosidase in fibroblasts from patients with infantile and adult forms of the disease (5,6,7). In control human fibroblasts acid α-glucosidase is synthesized as a precursor of M_r 110,000 and is then gradually processed to mature forms of M_r 76,000 and 70,000. By studying the formation of the enzyme in fibroblasts from patients, evidence was obtained that defects became particularly apparent during the early stages of enzyme realization. Some patients with the infantile form of Glycogenosis type II did not produce any detectable acid α-glucosidase at all, whereas labile precursors were reported to be formed in fibroblasts from some adult patients (7).

abbreviations: bp, base pairs; kb, kilobase pairs; kD, kilo daltons; SDS, sodium dodecyl sulfate; SSC, 150 mM NaCl/15 mM Na_3 citrate, pH 6.4.

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These various molecular abnormalities of the enzyme indicate that different forms of Glycogenosis type II originate from distinctive gene mutations. However, complementation studies failed to provide evidence; apparently because these mutations reside in the same gene. To further investigate what type of mutations are responsible for the variety of clinical forms we have undertaken to clone the gene coding for α-glucosidase. We present here the method that proved to be successful for cloning the cDNA from a messenger that, estimated on its colony representation and gradient purification, represents less than 0.002% of the total mRNA.

MATERIALS AND METHODS

Construction of a cDNA library. Macaque testes RNA was isolated by the LiCl method (8), poly(A^+) selected by oligo(dT)cellulose (P.L. biochemicals) chromatography and centrifuged through a linear sucrose gradient (9). mRNA fractions were translated in a rabbit reticulocyte lysate (N.E.N. biochemicals) according suppliers recommendations. The [^{35}S]-labeled products were immunoprecipitated with anti human α-glucosidase (6) according to the procedure described by Maccecchini et al. (10) and applied on an SDS-polyacrylamide gel. Products were electrophoresed and visualized as described by d'Azzo et al. (11). mRNA from the fraction containing the highest amount of acid α-glucosidase messenger was transcribed into cDNA by the method described by Rowekamp & Firtel (9). The hairpin loops of 500ng double stranded cDNA were removed by 5 units of mung bean nuclease (P.L. biochemicals) in 100mM NaOAc pH 5.6/14mM β-mercaptoethanol/0.01mM $ZnSO_4$/0.001% Triton X-100 at 30°C for 30 min. After phenol extraction and ethanol precipitation the cDNA was electrophoresed through a 0.7% low melting point agarose gel and material larger than 300 bp was cut out form the gel and the DNA recovered by phenol extraction (12). The double stranded cDNA was C-tailed and inserted into the G-tailed Pst I site of pBR327 (9) and used to transform Escherichia coli C600 according to Valerio et al. (13). The library was constructed with 146ng C-tailed cDNA and consisted of 30,000 members of which 8% contained the wildtype pBR327. Transformed cells were plated, replicated and lysed as described by Grosveld et al. (14).

Preparation of hybridization probe. Polysomes from 50gr Macaque testes were prepared and immunoselected following the modified procedure of Shapiro & Young (15,16). For this purpose affinity purified antibodies against α-glucosidase were immobilized by coupling to Sepharose 4B (Pharmacia) to permit multiple use of the same batch of antibodies. The poly(A^+) enriched RNA, obtained after immuno- and oligo(dT)selection was transcribed into cDNA for 3hrs at 42°C in the presence of 100μCi α[^{32}P]dATP, 100μCi α[^{32}P]dCTP (Amersham) and 3 units of AMV reverse transcriptase (Life Sciences) in a total volume of 5μl. The library was hybridized under described conditions (14) with the addition of 5μg/ml poly(U) in the hybridization mixture. The positively hybridizing clones were picked and rescreened (14).

Hybrid released translation. Plasmid DNA was purified by isopycnic centrifugation in CsCl/EtBr gradients (17). Twenty μg of DNA was digested with Eco RI, heat denatured and coupled to 1 cm^2 discs of activated APT paper (BioRad)(18,19). The coupled DNA was hybridized with 100μg poly(A^+)RNA from monkey testes in 50% formamide/0.9M NaCl/20mM PIPES pH 6.4/0.2% SDS/1mM EDTA for 16hrs at 37°C in a total volume of 2 ml (16 discs). The discs were then washed 5 times in 50% formamide/20mM NaCl/8mM Nacitrate/0.2% SDS/1mM EDTA at 37°C for 30 min. periods. Hybridized RNA was eluted in 90% formamide/20mM PIPES pH 6.4/0.5% SDS/1mM EDTA. After dilution with 2 volumes water and addition of 10μg calf liver tRNA, the RNA was ethanolprecipitated. Before translation, the mRNA was treated with DNase (20μg/ml) in a solution containing 10mM Tris/Hcl pH 7.5/10mM $MgCl_2$ for 30 min. at 37°C. After phenol extraction the ethanol precipitated RNA was washed extensively with 70% ethanol before *in vitro* translation.

DNA analysis. Southern blot hybridization with DNA from somatic cell hybrids between man and mouse was performed as described (20,21). The human chromosome content of these hybrids was deduced from direct chromosome analysis.

RESULTS

Construction of library. Poly(A^+)RNA, 620µg, isolated from approximately 10gr of monkey testes was equally divided over 6 sucrose gradients of 5 ml each. Twenty fractions were collected from each gradient and 3 fractions, containing the larger messenger were translated *in vitro*. The nascent proteins were immunoprecipitated with anti human α-glucosidase (fig. 1). The specificity of the antiserum was proven by its lack to precipitate any material from fibroblasts from patients with the infantile form of Glycogenosis type II. Moreover, no labeled proteins were precipitated from an *in vitro* translation reaction carried out in the presence of saturating amounts of purified unlabeled human α-glucosidase (data not shown). mRNA from fraction 2 (fig. 1; lane D) was used to prepare the cDNA library. Two µg of this poly(A^+)RNA served for cDNA synthesis and resulted in 344ng single stranded cDNA. After second strand synthesis and removal of the single stranded loop, the resulting cDNA was electrophoresed

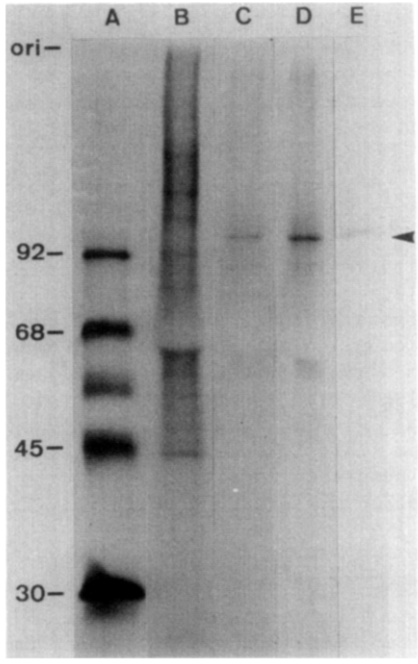

fig. 1. Autofluorography of immunoprecipitated [^{35}S]-labeled α-glucosidase after in vitro translation of the denser three mRNA fractions from the sucrose gradient. Lane A: markers. Lane B: 2% of the total translated products from fraction 2. Lanes C, D and E: immunoprecipitated α-glucosidase from fraction 1, 2 and 3 respectively. The origin of the separating gel is indicated by (ori). Arrow indicates position of the in vitro precursor of α-glucosidase.

through a low melting point agarose gel. Material larger than 300 bp was cut out from the gel, and 488ng of double stranded cDNA was recovered.

Screening the cDNA library for α-glucosidase clones. α-Glucosidase mRNA, obtained by immunoselection from 5gr of tissue, was used as template to synthesize radioactive labeled cDNA ($5x10^5$ cpm). After hybridization of the library with this probe, 14 positive clones were detected and isolated. Cross-hybridization experiments with the inserts of the clones revealed two distinct groups. One group consisting of 9 cDNA's, apparently contained repetitive sequences and produced a smear after hybridization on Southern blots of Eco RI digested human DNA. The other group of 5 inserts gave a distinctive hybridization pattern, indicating that they were derived from a unique sequence (fig. 3; lane A). Further experiments were performed with a clone from the latter group, pMAG126, which contains an insert of 680 bp.

Hybrid released translation. In order to identify this clone, pMAG126 DNA was coupled to APT paper and used in a hybridization selection assay. The bound RNA was eluted, translated *in vitro* and the product was precipitated with anti α-glucosidase antiserum. The result of this experiment is shown in fig. 2. Filters with bound pBR327 DNA (lane B) or DNA derived from cDNA clones from the group of 9 (lanes C and D) failed to bind any mRNA that directs the synthesis of α-glucosidase in an *in vitro* translation assay, pMAG

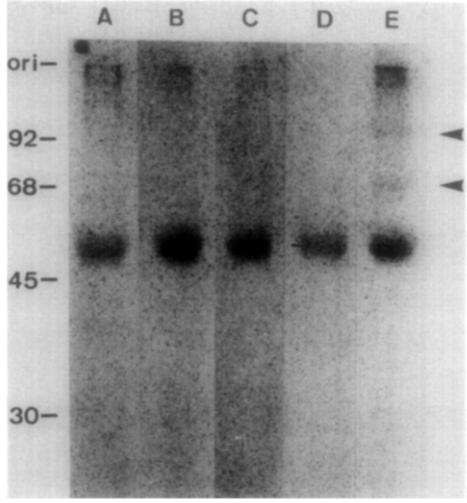

fig. 2. Autofluorography of in vitro translated proteins directed by hybrid released mRNA and immunoprecipitated by anti α-glucosidase antiserum. Lane A: blank from the in vitro translation without mRNA added. Lane B: products translated from mRNA that hybridized to pBR327. Lanes C and D: proteins translated from mRNA that was selected by cDNA clones 111 and 125 respectively. Lane E: translation products from mRNA that hybridized to pMAG126. The two arrows indicate the position of the 96 KD and 70 KD protein in lane E.

Table 1. Human chromosome content of human-mouse somatic cell hybrids. The letter between brackets denotes the lane of fig. 3

hybrid	human chromosomes																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y
14CB21(C)				+			+	+	+					+						+				
17CB13(D)																	+			+		+		
17CB21(E)				+			+		+			+		+			+			+	+		+	

126 DNA (lane E), however, selected for a messenger that directs the synthesis of a 96 kD protein, indicating that it contains an α-glucosidase mRNA-derived insert. In all lanes a prominent band of M_r 52,000 is shown, which must represent an endogeneous band from the *in vitro* translation, since it is also present in lane A, which contains the blank of the translation and therefore cannot contain any monkey α-glucosidase.

Identification of the chromosomal location of pMAG126. Since the gene for α-glucosidase is mapped on chromosome 17 (22), we performed experiments to show that pMAG126 DNA hybridizes human sequences on chromosome 17. To this end, pMAG126 was hybridized to DNA of an informative panel of mouse human hybrid cell lines. The human chromosome content of these hybrids is shown in table 1. In lane A of fig. 3 the hybridization pattern of pMAG126 with human DNA shows two bands, 18 kb and 7.5 kb respectively. The latter has approximately the same mobility as a band detected in mouse DNA (lane B). The 18 kb

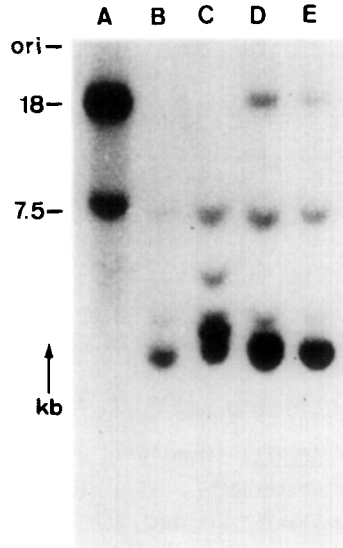

fig. 3. Autoradiography of a Southern blot hybridization of Eco R1-restricted DNA with pMAG126. Lane A contains human DNA and lane B from mouse. Lanes C, D and E contain DNA from hybrid 14CB21, 17CB13 and 17CB21 respectively.

band, however, is specific for human DNA and can be used for scoring the presence of the human hybridizing sequence. Lane D contains DNA from a hybrid with only the human chromosomes 17, 20 and 22 present and shows the 18 kb band. Lane E shows the same band but the hybrid used does not contain chromosome 22, which leads to the conclusion that pMAg126 hybridizes sequences present on chromosome 17 or 20. DNA in lane C, extracted from a hybrid containing human chromosome 20 but lacks 17, fails to show the specific 18 kb band. From this Southern blot hybridization we conclude that the cDNA clone pMAG126 shows homology to sequences on human chromosome 17.

DISCUSSION

The combination of two different techniques was used to clone a cDNA from a very rare mRNA. On the one hand a large cDNA library was constructed from size-fractionated mRNA, which was at least purified tenfold for the α-glucosidase mRNA. On the other hand immunoselection of polysomes provided mRNA that, transcribed into labeled cDNA, proved to be pure enough to screen this library directly. Five out of 14 clones, hybridizing with this cDNA probe, had a unique sequence and were eventually found to contain an α-glucosidase mRNA-derived insert.

The identification of clone pMAG126 to contain nucleotide sequences complementary to monkey α-glucosidase depends on hybrid released selection, assayed by *in vitro* translation and by hybridization to sequences present on human chromosome 17. The low signal obtained after immunoprecipitation of the *in vitro* translated α-glucosidase can be explained by the fact that this messenger is very rare, since the RNA preparations used in the hybrid selection was intact, as judged from the relative abundance of 28S and 18S ribosomal RNA in agarose gels.

Two of the protein bands that show after *in vitro* translation (fig. 2; lane E) are identified as α-glucosidase by the specificity of the antibodies used and by their molecular weight. The band with a M_r of about 96,000 represents the first (*in vitro*-) precursor form of the α-glucosidase. The second band with a M_r of 70,000 possibly resulted from proteolytic degradation. This degradation was observed with other proteins in reticulocyte lysate translation systems (14). Moreover a 70 kD protein is also precipitated from human placenta (5).

Southern blots of human DNA, cut with a number of restriction enzymes and hybridized with pMAG126, revealed in some instances a single hybridizing band (data not shown), indicating that this cDNA contains sequences unique to a single gene. The fact that the cDNA hybridizes to both human and mouse sequences, even after stringent washing of the Southern blots (0.1xSSC), suggests that these sequences are well conserved among these three species.

The availability of this cDNA clone will allow us to investigate possible defects in the realization of lysosomal α-glucosidase at the mRNA level, and ultimately we will gain insight in the genetic mutations that lead to the different clinical forms of Glycogenosis type II.

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