BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **224,** 619–624 (1996)
ARTICLE NO. 1075

Expression of Human Lysosomal Alpha-Mannosidase Activity in Transfected Murine Cells and Human Alpha-Mannosidase Deficient Fibroblasts

W. Wang,* V. L. Nebes,† M. C. Schmidt,‡ and J. A. Barranger*,[1]

Department of Human Genetics, University of Pittsburgh, Pittsburgh, Pennsylvania 15261; †Department of Ophthalmology, Allegheny-Singer Research Institute, Pittsburgh, Pennsylvania 15212; and ‡Department of Molecular Genetics and Biochemistry, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania 15261

Received June 20, 1996

We studied the human lysosomal alpha-mannosidase (MANB) by expressing the putative cDNA in mammalian cells, using the eucaryotic expression vector pCDE. The construct pCDE-MANB and pSV2-Neo were cotransfected into human α-mannosidase deficient fibroblasts and into a murine cell line and selected by culture in the presence of G418. Six G418 resistant 3T3 clones had increased α-mannosidase activity 2 to 3 times above the controls. Two clones from transfected human fibroblasts showed a 2 fold increase in enzyme activity. The human MANB cDNA gene was demonstrated in the target cells by Southern blot analysis and the expression of the gene was shown by RT-PCR analysis. This study is the first to successfully express the MANB gene in a human and a murine cell line. The results confirm that the putative MANB cDNA encodes the full length of lysosomal α-mannosidase. Molecular characterization of mannosidosis and approaches to gene therapy are now possible using this cDNA. © 1996 Academic Press, Inc.

Deficiency of lysosomal α-mannosidase results in mannosidosis, an inherited autosomal recessive disease (1). Human lysosomal α-mannosidase (α-D-mannoside mannohydrolase, EC 3.2.1.24) participates in the catabolism of glycoproteins and complex polysaccharides in lysosomes. The enzymes hydrolyze terminal mannose residues from endogenous and exogenous glycoproteins and complex polysaccharides(2, 3). Three of human lysosomal isoenzymes of α-mannosidase are known and two of them (A and B) are apparently encoded by the same gene MANB (4, 5). These two isoenzymes are derived from a common 110 kDa polypeptide precursor, which is post-translationally, proteolytically processed to form isoenzymes of different molecular weight (6, 7). The gene encoding the lysosomal α-mannosidase, MANB, had been mapped to chromosome 19 by analyzing α-mannosidase activity in human and rodent somatic cell hybrid mapping panels (8–11).

Previously, degenerate primers were used to amplify the sequence from a HeLa cDNA library. This resulted in the isolation of a cDNA clone which mapped to chromosome 19q concordant with the location of the lysosomal α-mannosidase gene (MANB). The indirect evidence suggested that the cDNA encoded the lysosomal alpha-mannosidase, including deduced amino acid sequence of the cDNA showing a 38% sequence similarity to the amino acid sequence of *Dictyostelium discoideum* lysosomal α-mannosidase (12). Recently, Emiliani et al. reported that the partial amino acid sequence of the 30 kDa band of purified human α-mannosidase was identical with residues 551-569 of the sequence deduced from MANB cDNA (13). To confirm the cDNA encoding the full length lysosomal alpha-mannosidase, we performed the expression analysis. Here, we report the expression of MANB gene in human

[1] Corresponding author. Fax: (412) 624-1032.

0006-291X/96 $18.00
Copyright © 1996 by Academic Press, Inc.
All rights of reproduction in any form reserved.

fibroblasts deficient in lysosomal α-mannosidase and in NIH3T3 cells. The MANB gene will be useful in characterizing mannosidosis and in approaching gene therapy strategies.

MATERIALS AND METHODS

Construction of pCDE-MANB. The plasmid, pCDE, is an eucaryotic expression vector, having a polylinker sequence in the Bam HI site of the vector, pCD, (14). The vector contains the simian virus 40 (SV40) early region promoter, upstream of polylinker, and an SV40 late region polyadenylation sequence downstream of the cDNA cloning site. The vector, pCDE, was used to construct pCDE-MANB. The 3.1 Kb MANB cDNA was cut by the two restriction enzymes, EcoR I and BamH I, from the plasmid, pHAM31, which is a 2.96 Kb phagemid vector, bluescript SK-, containing the full length MANB cDNA in its MCS (multiple cloning site). The cohesive end of the MANB cDNA fragment was blunt ended by Klenow fragment; then the EcoR I linkers were added to both ends of the fragment by T4 DNA ligase, followed by EcoR I enzyme digestion. The cDNA fragment with cohesive EcoR I ends was ligated into EcoR I linearized pCDE vector to construct the plasmid, pCDE-MANB.

pCDE-MANB DNA preparation. E. coli, DH5α, competent cells were transformed with pCDE-MANB and were grown on agar plates in the presence of 50 μg/ml ampicillin. DNA mini-preparation was performed and clones containing MANB cDNA were picked. To select the plasmids containing correct orientation of MANB cDNA, the enzyme, Alf III, was used to digest the plasmid and agarose gel electrophoresis was performed. The plasmid, pCDE-MANB #4, containing MANB cDNA with correct orientation was then chosen, and large-scale plasmid preparation was performed.

Cell culture. Human fibroblasts (NIGMS GM00654) were maintained in Dulbecco's modified Eagle's medium (DMEM, high glucose, Gibco BRL) with 20% fetal bovine serum (FBS, Gibco BRL), 1$\times$ L-Glutamine and 1$\times$ Penicillin-Streptomycin; NIH3T3 cells were maintained in DMEM, (high glucose, Gibco BRL), with heat-inactivated 10% calf serum (CS, Gibco BRL), 1$\times$ L-Glutamine and 1$\times$ Penicillin-Streptomycin. All cells are incubated at 37°C under 5% CO_2, and 90% humidity, except as indicated.

Transfer of pCDE-MANB into mammalian cell lines. The construct, pCDE-MANB, was transfected into a human α-mannosidase deficient cell line (NIGMS GM00654) and into murine fibroblast cells (NIH3T3) by two different methods: 1) Lipofectamine reagent (Gibco BRL) was used as medium according to the manufacturer's instruction (Gibco BRL); 2) the calcium phosphate method was also used to perform transfection according to Chen and Okayama (15). For calcium phosphate transfection, 5×10^5 cells were plated in 10 cm dishes and incubated overnight at 37°C under 5% CO_2. Then 20 μg DNA (construct: pSV2-Neo, 20:1 ratio) was mixed with 0.5 ml of 0.25 M $CaCl_2$ and 0.5 ml of 2 $\times$ BBS (50 mM Bes, pH 6.95, 280 mM NaCl, 1.5mM Na_2HPO_4). The mixture was incubated at room temperature for 15 min. and the calcium phosphate-DNA solution 1 ml was added to cells in a dropwise manner. The cells were incubated overnight at 35°C under 3% CO_2. The next day, cells were washed with fresh growth medium 2 times and incubated overnight at 37°C under 5% CO_2. After 24 h, the cells were split at 1:10, 1:20 and 1:30 and incubated overnight at 37°C under 5% CO_2. To select stable transfected clones, G418 was added at a concentration of 400 μg/ml and the G418 resistant clones were selected in 2 to 3 weeks.

Enzyme assay. The α-mannosidase enzyme assay was conducted by using 10 mM 4-methylumbelliferyl α-D-mannopyranoside (Sigma) as the substrate according to the modified protocol described by Lee, et al. (2). Briefly, 50 μl of cell lysate supernatant (10^6 cells/ml) was added to 150 μl of a cold reaction mixture containing 10 mM substrate, 0.025% bovine serum albumin, and 30 mM Na_2HPO_4/20 mM citric acid, pH 4.5. After incubation at 37°C for 30 min., the reaction was terminated with 4 ml of glycine carbonate buffer (170 mM glycine/Na_2CO_3), pH 10.4. Fluorescence of the liberated 4-methylumbelliferone was measured in a fluorometer (Turner, Fluorometer, Model 112). Total protein was measured using BCA protein assay reagent (Pierce) according to the manufacturer's instruction. One unit is defined as the amount of enzyme that hydrolyzes one nanomole (nmol) substrate per hour at 37°C. Specific activity was expressed as enzyme units/mg protein.

Southern blot analysis. Genomic DNA was extracted from G418 resistant clones selected from pCDE-MANB and pSV2-Neo cotransfected cells and non-transfected control cells. Equal amounts of various genomic DNA samples were cut by EcoR I restriction enzyme and loaded into 1% agarose gels. Following electrophoreses at 35V overnight, gels were blotted to nylon membranes and hybridized to the 3.1 Kb MANB cDNA labeled with [^{32}P]dATP, using a randomly primed DNA labeling kit (Boehringer Mannheim).

RT-PCR analysis. Total RNA were extracted from G418 resistant clones selected from pCDE-MANB and pSV2-Neo cotransfected cells and non-transfected control cells using a trizol reagent, a mono-phasic solution of phenol and guanidine isothiocyanate (Gibco BRL). The first strand cDNA was synthesized at 42°C for 1 h in a reaction mixture containing 1 μg of total RNA, 2.5 U/μl MuLv reverse transcriptase (Perkin Elmer), 5 mM $MgCl_2$, 1 $\times$ PCR buffer II (Perkin Elmer), 1 mM each dNTP and 2.5 μM oligo-dT. For PCR, 10 μl of cDNA synthesis reaction mixture was added directly to 40 μl RT-PCR mix containing 2 mM $MgCl_2$, 1 $\times$ buffer II (Perkin Elmer), 0.3 μM each sense and anti-sense primer, and 2.5 U/100 μl AmpliTag DNA polymerase (Perkin Elmer). The pair of primers specific for human MANB gene, (sense, 5′ GCCAGCACCAGCCTGAAGCC, and anti-sense, 5′ GCAGGAGTTGCTGATTCATG) was used to detect the human MANB gene expression in the G418 resistant NIH3T3 cells. The PCR conditions were as

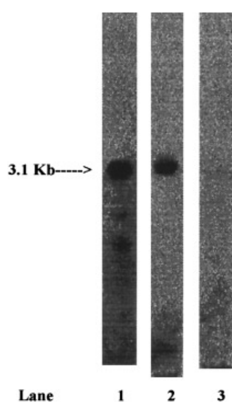

FIG. 1. Southern blot analysis of transferred MANB cDNA gene in the target cell lines. Genomic DNA was extracted from G418 resistant clones selected from pCDE-MANB and pSV2-Neo cotransfected NIH3T3 cells (lane 1 and lane 2), and non-transfected control NIH3T3 cells (lane 3). Equal amounts of genomic DNA samples (10 μg) were cut by restriction enzymes EcoR I and were resolved on a 1% agarose gels. Gels were blotted to nylon membranes and hybridized to the 3.1 Kb MANB cDNA labeled with [^{32}P]dATP by randomly priming.

follows for 35 cycles: denaturation at 95°C for 30 s., annealing at 60°C for 20 s, synthesis at 72°C for 1 min. After PCR, 10 μl of RT-PCR product was analyzed on a 2% agarose gel.

RESULTS

Southern blot analysis. The presence of the human MANB cDNA gene in the transferred NIH 3T3 cells was detected by Southern blot analysis, using 3.1 Kb human MANB cDNA as probe at high stringency hybridization conditions. The results indicated that the MANB gene was present in the transfected NIH3T3 cells (figure 1). The predicted 3.1 Kb band of the human MANB gene was shown in genomic DNA extracted from selected clones (lane 1 and lane 2) but not in the DNA of non-transfected NIH3T3 control cells (lane 3).

RT-PCR analysis. The expression of human MANB gene in NIH3T3 cells cotransfected with pCDE-MANB and pSV2-Neo was also studied by RT-PCR analysis. The results showed in the figure 2 and the specific transcripts of human MANB gene presented in the transfected clones (lane 1, lane 2 and lane 3), but not in the non-transfected NIH3T3 control cells (lane 4).

Human MANB gene expression in clones selected from transfected NIH3T3 cells. Murine fibroblast cells (NIH3T3) were cotransfected with pCDE-MANB and pSV2-Neo, and selected by G418. MANB gene expression was analyzed on the G418 resistant clones by the enzyme assay described in material and methods. Six G418 resistant clones (A to E) have increased human α-mannosidase activity 2 to 3 times above the background (non-transfected control NIH 3T3 cells) (figure 3). Statistical analysis (two tails student t-test) indicated that the human α-mannosidase activity in these clones were significantly higher than non-transfected control ($p \approx 0$).

Enzyme activity in clones selected from transfected human mannosidosis fibroblasts. The clones resistant to G418 were obtained from human α-mannosidase deficient fibroblasts (GM00654) cotransfected with pCDE-MANB and pSV2-Neo. Two clones have shown increased enzyme activity when compared with non-transfected GM00654 cells (figure 4). The two clones (#1 and #2) showed a 91% and 43% increase in the enzyme activity respectively. The results of statistical analysis (two tails student t-test) demonstrated that the human enzyme activity in these clones were significantly higher than the non-transfected fibroblasts ($p=0.0015$ and $p=0.021$ respectively).

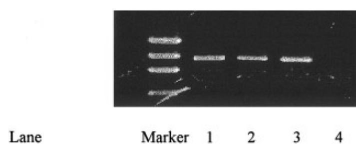

FIG. 2. RT-PCR analysis of the expression of MANB gene in cotransfected NIH3T3 cells. Total RNA were extracted from G418 resistant clones selected from pCDE-MANB and pSV2-Neo cotransfected cells and non-transfected control cells. The procedure for RT-PCR was described in the section on material and methods. The results showed the specific transcripts for the transfected cDNA in the cotransfected clones (lane 1, lane 2 and lane 3), but not in non-transfected NIH3T3 control cells (lane 4). Genetic marker is ϕX174 RF DNA/Hae III and the predicted size of transcripts is 1082 base pairs.

DISCUSSION

The MANB cDNA was isolated by amplifying a fragment of DNA from a HeLa cDNA library, using degenerate primers, followed by library screening methods. The similarity of the deduced amino acid sequence to other known α-mannosidase and the mapping data provided the indirect evidence which suggested that the cDNA encoded lysosomal α-mannosidase (12). Recently, Emiliani et al. have provided direct evidence of the cDNA encoding the α-mannosidase. They reported that the 19 N-terminal amino acids of the purified 30 kDa subunit of the human α-mannosidase has been shown to be a perfect match of the deduced amino acid sequence (13). In our report, we subcloned the putative human MANB cDNA gene into an

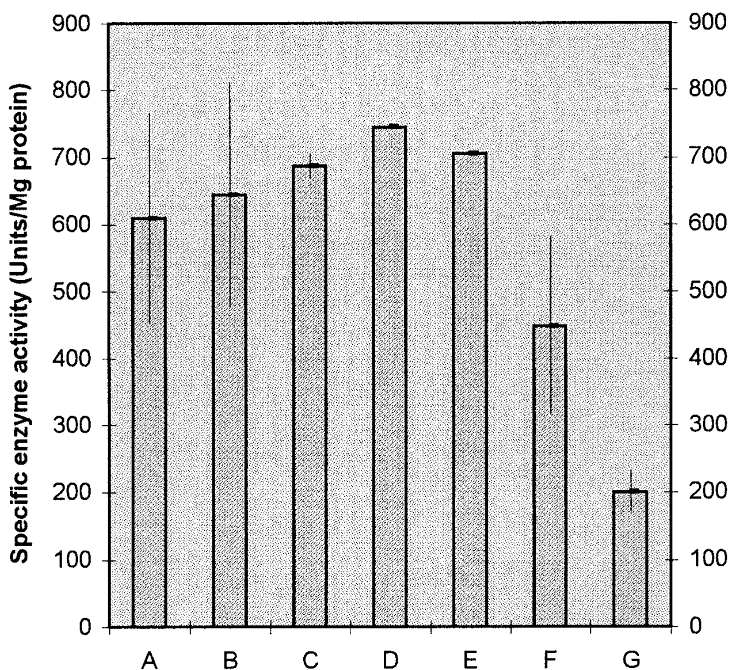

FIG. 3. Lysosomal α-mannosidase enzyme activity in murine fibroblast cells (NIH3T3). NIH3T3 cells were cotransfected by pCDE-MAN and pSV2-Neo and selected by G418. Six clones showed 2-3 times increased enzyme activity. Lanes A-F, G418 resistant clones selected from NIH3T3 cells cotransfected by pCDE-MANB and pSV2-Neo. Lane G, non-transfected NIH3T3 cells as control. Statistical analysis (two tail student t test) has shown enzyme activity of clones are significantly higher than non transfected NIH3T3 controls ($p \approx 0$, the average enzyme activities of clones were used to compare with controls). The lines extending through each bar represent standard error.

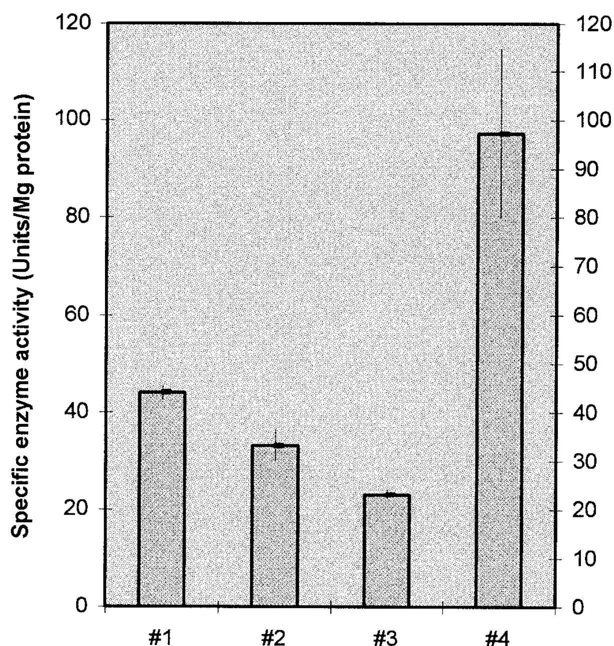

FIG. 4. Specific enzyme activity of human lysosomal α-mannosidase in human fibroblasts. Fibroblasts deficient in lysosomal α-mannosidase activity (GM00654) were cotransfected by pCDE-MANB and pSV2-Neo and selected by G418. #1 and #2, clones selected from transfected mannosidosis fibroblasts. #3, non-transfected mannosidosis fibroblasts. #4, normal human fibroblasts. #1 and #2 have shown increased enzyme activities of 91 and 43% respectively above deficient cell line (#3). The lines extending through each bar represent standard error.

eucaryotic expression vector, pCDE, and cotransfected murine fibroblast cells and human mannosidosis fibroblast cells with the construct, pCDE-MANB, and pSV2-Neo. The results demonstrated significantly increased α-mannosidase enzyme activity in the clones (A-F), indicating the expression of the human MANB gene in cotransfected NIH3T3 cells. This result provides direct evidence that cDNA encodes the full length of α-mannosidase gene and confirms that the sequence codes MANB.

We selected the G418 resistant clones from human mannosidosis fibroblast cells cotransfected with pCDE-MANB and pSV2-Neo and found increased lysosomal α-mannosidase activity in two clones. This suggests that the cells express both Neo and MANB genes. Further studies are needed to study normal human MANB gene expression in human fibroblasts which are deficient in α-mannosidase enzyme activity. It needs to be studied if the low expression of MANB gene in mannosidosis cell line is the function of cell line itself. Tzall and Martiniuk (16) found that the acid alpha glucosidase (GAA) deficient cell line expressed Neo gene one fifth to one tenth that of mouse 3T3 cells.

Mannosidosis is caused by the deficiency of α-mannosidase, but the molecular genetic basis for α-mannosidase deficiency is unknown. The relationship between structural gene mutations (MANB) and the enzyme (α-mannosidase) deficiency has not been explored. The identified MANB gene will provide the tools for further molecular genetic research on mannosidosis and the identification of mutation in the MANB gene. It is also possible to use the cDNA in gene therapy for mannosidosis.

REFERENCES

1. Beaudet, A. L., and Thomas, G. H. (1989) *in* The Metabolic Basis of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, A. L., and Valle, D., Eds.), pp. 1603–1621, McGraw-Hill, New York.

2. Phillips, N. C., Robinson, D., and Winchester, B. G. (1976) *Biochem. J.* **153,** 579–587.
3. Lee, J. E. S., Little, T. E., and Yoshida, A. (1982) *Enzyme* **28,** 33–40.
4. Gasperi, R. D., Daniel, P. F., and Warren, C. D. (1992) *J. Biol. Chem.* **267,** 9706–9712.
5. Winchester, B. (1984) *Biochem. Soc. Trans.* **12,** 522–524.
6. Pohlmann, R., Hasilik, A., Cheng, S., Pemble, S., Winchester, B., and von. Figura, K. (1983) *Biochem. Biophys. Res. Comm.* **115,** 1083–89.
7. Cheng, S. H., Malcolm, S., Pemble, S., and Winchester, B. (1986) *Biochem. J.* **233,** 65–72.
8. Champio, M. J., and Shoms, T. B. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 2968–2972.
9. Ingram, P. H., Bruns, G. A. P., Regina, V. M., Eisenman, R. E., and Gerald, P. S. (1977) *Biochem. Genet.* **15,** 455–476.
10. Martiniuk, F., Ellenbogen, A., Hirschhorn, K., and Hirschhorn, R. (1985) *Genet.* **69,** 109–111.
11. Bachinski, L. L., Krahe, R., White, B. F., Wieringa, B., Shaw, D. (1993) *Am. J. Hum. Genet.* **52,** 375–387.
12. Nebes, V. L., and Schmidt, M. C. (1994) *Biochem. Biophys. Res. Comm.* **200,** 239–245.
13. Emiliani, C., Martino, S., Stirling, J. L., Maras, B., and Orlacchio, A. (1995) *Biochem. J.* **305,** 363–366.
14. Okayama, H., and Berg, P. (1983) *Mole. and Cell Biol.* **3,** 280–289.
15. Chen, C., and Okayama, H. (1987) *Mole. and Cell Biol.* **7,** 2745–2752.
16. Tzall, S., and Martiniuk, M. (1991) *Biochem. Biophys. Res. Comm.* **176,** 1509–1515.