

Molecular Cloning of a Novel cDNA From *Mus Muscular* BALB/c Mice Encoding Glycosyl Hydrolase Family 1: A Homolog of Human Lactase-Phlorizin Hydrolase¹

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Objective To study the mechanism of lactose intolerance (LI) by cloning the mouse lactase cDNA and recombining a vector. **Methods** Total murine RNA was isolated from the small intestine of a 4-week-old BALB/c mouse (♂). Gene-specific primers were designed and synthesized according to the cDNA sequences of lactase-phlorizin hydrolase (LPH) in human, rat, and rabbit. A coding sequence (CDS) fragment was obtained using RT-PCR, and inserted into a clone vector pNEB-193, then the cDNA was sequenced and analyzed using bioinformatics. **Results** The cDNA from the BALB/c mouse with 912 bp encoding 303 amino acid residues. Analysis of the deduced amino acid sequence using bioinformatics revealed that this cDNA shared extensive sequence homology with human LPH containing a conserved glycosyl hydrolase family 1 motif important for regulating lactase intolerance. **Conclusion** BALB/c mouse LPH cDNA (GenBank accession No: AY751548) provides a necessary foundation for study of the biological function and regulatory mechanism of the lactose intolerance in mice.

Key words: BALB/c mice; LPH; cDNA; pNEB 193; Cloning; Sequence

INTRODUCTION

Lactase-phlorizin hydrolase (LPH) is an enzyme responsible for hydrolysis of lactose in the small intestine. Studies in humans have shown that low expression of LPH causes lactose intolerance^[1]. In contrast to congenital hypolactasia, adult-type hypolactasia is a very common condition in humans, characterized by a decreased intestinal lactase level of 5%-10% in childhood and adolescence^[2-3]. The post-weaning decline in LPH expression is genetically determined^[4-5] but its molecular basis is unknown, although intensive efforts have been made to solve the problem^[6-8].

There are numerous reports on the post-weaning decline of intestinal lactase activity in humans and mammals, including rat, sheep and pig^[9]. Surprisingly, there is little information on this in mice. Mouse has been served as a model organism for mammals due to its numerous advantages including its economically controlled genetic background and sequenced genome. With the available mouse

genomics data and the help of bioinformatics, studies on the protein function of mouse to understand the biological processes in health and disease of related species can be carried out. This prompted us to choose the BALB/c inbred strain mouse as a suitable animal model for studying the molecular mechanism underlying the mammalian lactose intolerance. The present study was designed to obtain information on the mouse LPH cDNA sequence.

METHODS AND MATERIALS

Materials

Four-week-old BALB/c mice were supplied by the Laboratory Animal Center of Henan Province. Trizol reagent was purchased from Invitrogen Life Technologies (Invitrogen Beijing Office, China). RevertAid first strand cDNA synthesis kit was purchased from Fermentas Life Sciences Company (Burlington, Canada). *E. coli* TB1 and pNEB193 vector were purchased from NEB Company (Beverly,

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MA) and 1 kb DNA ladder was purchased from Sangon (Shanghai, China). Pyrobest DNA polymerase, dNTP, 100 bp DNA ladder, *Sal* I, *Eco*R I, *T*₄ DNA ligase, IPTG, and *Xgal* were purchased from Takara Company (Dalian, China). Qiaquick nucleotide removal kit was obtained from Qiagen (Hilden, Germany). All reagents were of molecular grade. Oligo (dT), Ribolock ribonuclease inhibitor and dNTP were in ready-to-use forms from suppliers.

Total Murine RNA Extraction

Total RNA was isolated by using the Trizol reagent according to the manufacturer's instructions.

First Strand cDNA Synthesis

For the first strand cDNA synthesis, the following items were added to a RNase free 1.5 mL centrifuge tube containing: 500 ng total RNA, 500 ng oligo (dT)18 and 12 μ L DEPC water. The contents were gently mixed, centrifuged for 3-5 sec and incubated at 70 °C for 5 min. The tube was immediately chilled on ice and centrifuged. The tube was placed on ice again and 4 μ L of 5 \times reaction buffer, 1 μ L of Ribolock ribonuclease inhibitor and 2 μ L of 10 mmol/L dNTP mix were added. After a gentle mix and brief centrifugation, the tube was incubated at 37°C for 5 min. One μ L of RevertAid M-MuLV reverse Transcriptase was added, the tube was incubated at 42°C for 60 min. The reaction was stopped by placing the tube at 70°C for 10 min. The resulting first strand cDNA was used for subsequent polymerase chain reaction (PCR).

PCR Amplification of Murine Lactase cDNA.

Lactase gene-specific primers were designed using software Primer 5.0 according to cDNA sequences of human, rat and rabbit lactase-phlorizin hydrolase (LPH) gene database published on Genbank. The primers synthesized by Takara Company were: 5'-CCGAATTCATGGGGGGCTGGTTTGCA C-3' and 5'-ACGTCGACTCAGAAGGAAGAGGT TTGA-3'. The second strand cDNA synthesis and PCR amplification were carried out using Pyrobest DNA polymerase with standard PCR procedures. A total of 20 μ L PCR reaction volume consisted of 2 μ L 10 \times Buffer, 2 μ L 4 \times dNTP, 2 μ L first strand cDNA template, 1 μ L (0.1 μ mol/L) P₁, 1 μ L (0.1 μ mol/L) P₂, 0.125 μ L (0.65 U) Pyrobest DNA polymerase, and 11.875 μ L Milli-Q H₂O. After the cDNA template was denatured at 95°C for 5 min, the reaction tube was processed for PCR at 94°C for 30 s, at 55°C for 30 s, and at 72°C for 1 min. After 30 cycles, the PCR product was elongated at 72 °C for 5 min. The

amplified cDNA fragment was tested by 1% agarose gel electrophoresis.

Construction of Recombinant Plasmid pNEB-lac

The pNEB193 vector DNA digested with *Sal* I and *Eco*R I, and the PCR fragment also digested with the same restriction enzymes, were ligated by using *T*₄ DNA ligase.

Transformation and Positive Clone Screening

Competent cells were prepared as previously described^[10]. The ligation product (2 μ L) was mixed with 25 μ L competent TB1 cells, and incubated on ice for 5 min, then at 42°C for 2 min. One hundred μ L of LB broth was added to the reaction tube and incubated at 37°C for 20 min. The cells were spread on a LB agar plate containing 100 mg/L ampicillin, 80 mg/L *Xgal* and 0.1 mmol/L IPTG. The plate was incubated at 37°C overnight. On the next day, white clones on the LB plate were selected. Plasmid DNA was obtained using Qiagen miniprep kits, and the PCR fragment was identified by restriction digestion using *Sal* I and *Eco*R I, then by agarose gel electrophoresis.

Sequencing by BLAST

DNA sequencing was performed by Takara Company. The DNA sequence and its deduced amino acid sequence were compared with those of human, rat and rabbit lactase-phlorizin hydrolase (LPH) gene/protein, and mouse inbred strain C57BL/6J genome by using the Basic Local Alignment Search Tool (BLAST) (<http://www.ncbi.nlm.nih.gov>).

Enzymatic Characterization Evaluated By Bioinformatics

Analysis on the features of the target gene and its encoded product characteristics was performed by using software Omega 2.0 and a Conserved Domain Architecture Retrieval Tool (CDART).

RESULTS

Total RNA Extraction From Mouse Small Intestine Tissue

Figure 1 shows the agarose gel electrophoresis assay of total RNA extracted by Trizol reagent from the BALB/c mouse intestine tissue. It revealed that there are 3 main components (28S, 18S, 5S) of total mouse RNA.

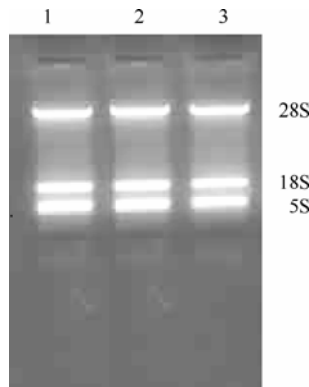


FIG. 1. Total RNA extracted from BALB/c mouse intestine tissue.

Murine Lactase cDNA Amplification by RT-PCR

The agarose gel electrophoresis analysis on the lactase cDNA fragment obtained by RT-PCR is shown in Fig. 2. A 912 bp PCR fragment was obtained by using the lactase gene-specific primers. The most suitable temperature for the primers was 51 °C in this experiment.

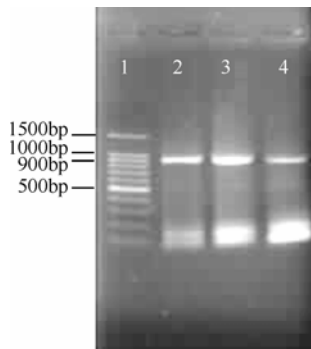


FIG. 2. 1% agarose gel electrophoresis of lactase PCR product.

Recombinant Plasmid Construction

Figure 3 illustrates the flowchart for the construction of recombinant plasmid pNEB193-lac, while Fig. 4 shows the restriction enzyme digestion analysis on pNEB193-lac. A band of approximately 900 bp, representing the 912 bp target fragment of lactase cDNA, was seen, indicating that this fragment was successfully inserted into the pNEB193 vector between the *EcoR* I and *Sal* I sites.

Nucleotide Sequencing and Analysis

The sequence of the murine lactase cDNA and its deduced amino acid sequence, shown in Fig. 6, were submitted to GenBank (Accession No: AY751548; Protein id: AAU95234.1). NCBI BLAST analyses indicated that the amplified DNA fragment was the

homologue of human lactase gene. The comparison of this mouse lactase gene sequence with several other lactase sequences revealed high degrees of identities (Table 1).

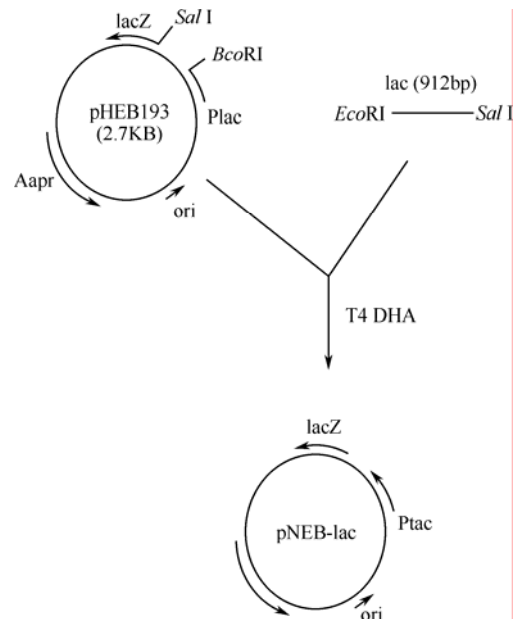
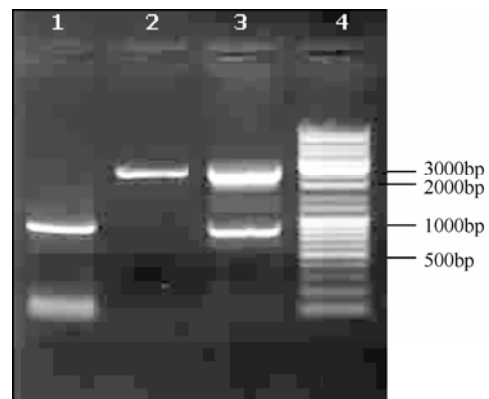


FIG. 3. Construction of recombinant plasmid pNEB193-lac.



Lane 1 lac PCR fragments
Lane 2 pNEB193 plasmid cut by *EcoR*I
Lane 3 pNEB-lac plasmid cut by *EcoR*I and *Sal*I
Lane 4 1kb DNA ladder

FIG. 4. Restriction assay of pNEB193-lac.

TABLE 1

Four Blast Top Hits on BALB/c Lactase cDNA			
GenBank id	Organism	Identities (%)	Gaps
XM1294793	C57BL/6	99	3/915
XM341115.1	Rattus	89	3/915
X56747.1	Rat	88	5/916
NM002299.2	Homo Sapiens	83	9/873

Using software Omega 2.0 and the Conserved Domain Architecture Retrieval Tool (CDART), we obtained the following mouse LPH features: protein-translation of LPH[residues 1-909]; length: 303; molecular weight: 34.113 kDa; picoMoles per microgramme: 29.314 pMoles; Isoelectric point (pI):

8.676; charge at pH 7.0: 4.307; molar extinction coefficient at 280 nm: 52540; absorbance of 1 at 280 nm: 0.65 mg/mL; absorbance of 1 mg/mL at 280 nm: 1.540.

From Fig. 6, we could find that the LPH protein had a transmembrane peptide segment at C-terminal.

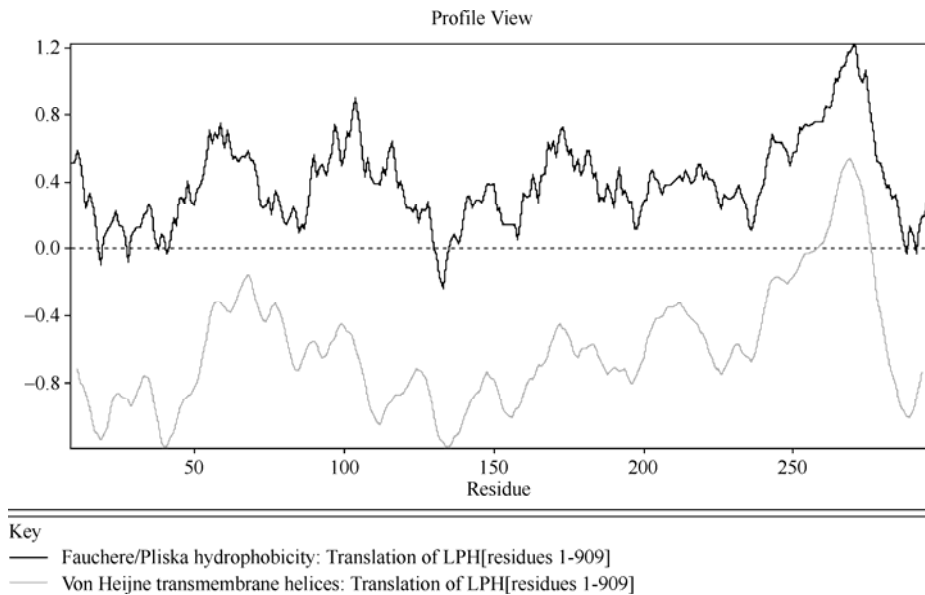


FIG. 5. Hydrophobicity and transmembrane helices of LPH by Omega 2.0.

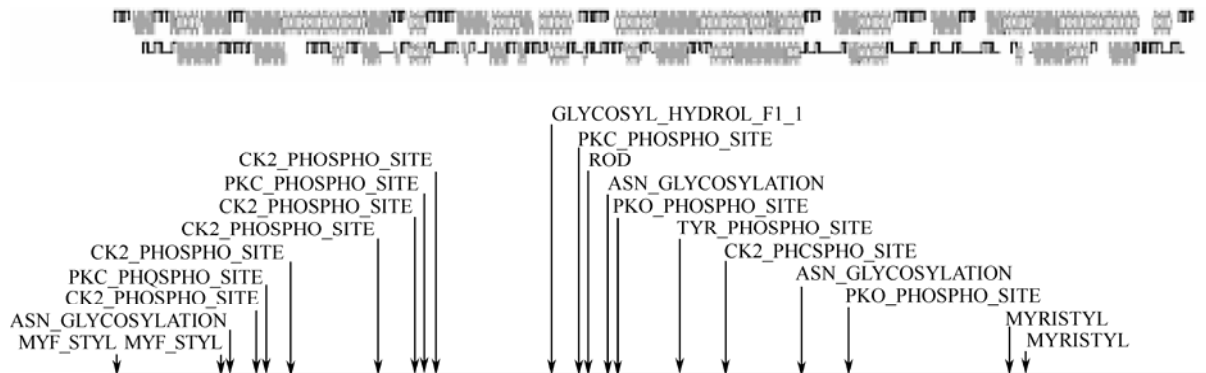


FIG. 6. Prediction of the second structure and conserved domain of LPH.

This indicated that the cloned cDNA encoding a 303 aa polypeptide chain contained a glycosyl hydrolase family 1 regain IYVTENGVS between amino acid residues 121 to 129.

DISCUSSION

Birth defects and development disabilities remain an important public health issue worldwide. The adult-type hypolactasia was firstly described in 1963 by Aurichio *et al.*^[3] and Dahlquist *et al.*^[4].

Studies have shown that both Crohn's disease and irritable bowel syndrome are likely caused by lactose intolerance induced by lactase deficiency^[1,11]. Lactase deficiency is present in 15% of individuals of northern European descents, 80% of blacks and Latinos and 100% of American Indians and Asians^[1]. The post-weaning decline of intestinal lactase activity also occurs in mammals including rat, sheep and pig, *etc.* We found that the lactase activities of newly weaned mice was rather high, but few reports are available in this field. Up to now, there are only two records on house mouse lactase mRNA published on

Genbank. One is *Mus musculus* similar to lactase-phlorizin hydrolase precursor mRNA (GenBank accession No: XM-129479), the other is the lactase mRNA.

To investigate the molecular mechanisms of post-weaning decline in intestinal lactase activity which might lead to a better understanding of human adult-type hypolactasia, SPF house mouse BALB/c strain was selected as the animal model for their short living circle. Total RNA was purified from the small intestine tissue of 4-week-old BALB/c mice. According to the cDNA sequences of human, rat and rabbit and their LPH gene available on GenBank, the gene-specific primers were designed and synthesized. In this study, a 912 bp cDNA fragment was obtained by RT-PCR and a recombinant plasmid carrying target gene has been constructed.

A search by BLAST for all sequences in the databases of National Biomedical Research Foundation and SwissProt for homologous sequences indicated that the amplified DNA fragment was the homolog of human lactase cDNA. The comparison of sequences in UniGene with proteins supported by a complete genome showed that the alignment between the cloned gene product and human lactase-phlorizin hydrolase precursor was 79.57%. The alignment between the cloned gene product and rat lactase-phlorizin hydrolase precursor was 87.78%, suggesting that the function of the cloned gene is similar to that of human LPH gene.

Lloyd *et al.*^[12] reported that LPH is an integral microvillar membrane protein anchored by a transmembrane C-terminal peptide segment. It is synthesized on the rough endoplasmic reticulum which is then proteolytically converted to the final brush-border form. In construction of the deduced product from cloned gene in the present study, there was also a transmembrane peptide segment at C-terminal.

The role of the cloned novel cDNA in lactose intolerance remains to be further evaluated. Our findings suggest that mouse chromosomes carry one functional gene encoding glycosyl hydrolase family 1. The functional gene is assigned to murine chromosome 1E3, which is different from the region of human chromosome 2 where the human lactase gene is located at 2q21^[13-14].

It is the first paper reporting the cloning and sequencing of a homolog of human lactase-phlorizin hydrolase genes in BALB/c mice by comparative genomic approach and bioinformatics technology. The cDNA probe covering the conserved region has been prepared for the determination of lactase mRNA by *in situ* hybridization based on this study. The recombinant expression plasmid and the antibody against murine lactase could be used in future

experiments. This would greatly improve the understanding of the regulatory mechanism of lactose intolerance in mice.

In the past decade, great changes have taken place in the field of biologic research and computation biology with the advances in large-scale DNA sequencing technique. As the complete genome sequences of viruses, bacteria, and yeast have been obtained^[15-17], another milestone has been built up with the recent completion of the human genome project (HGP) and *Mus musculus* genome project. Anyway, a more powerful model of organisms is now available for biologists to study the functions, regulations, or interactions of genes by bioinformatical analysis. The comparative genomic approach extends the possibilities of using the sequencing data to identify new genes or conserved regulatory regions by means of nucleotide sequence alignment of the particular regions of mouse and human genomes, or to trace the evolutionary events resulting in the genome structure of modern mammals.

In conclusion, a comparative genomic approach for assisting functional gene discovery has been established. Critical information on important motifs and functions of proteins or on more complex genetic circuitry will soon be clear.

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