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# Region-specific expression of multiple lactase-phlorizin hydrolase genes in intestine of rabbit

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We have identified a total of 4 sequences coding for lactase-phlorizin hydrolase (LPH) in the rabbit. Individual rabbits each yielded three different LPH cDNA sequences, or three chromosomal segments containing exon 1 of the LPH gene, representing either three genes or allelic variants of two genes. The three sequences were found in differing proportions in intestinal mRNA depending on the position along the small intestine from which the RNA was isolated. If all these mRNAs are translated, posttranslational mechanisms previously implicated in the regulation of LPH in the rabbit will be acting on different enzyme species in different parts of the intestine. However, we find no evidence for more than one LPH gene in the rat, and have previously shown that humans have only one LPH gene.

Lactase-phlorizin hydrolase; Developmental regulation; Small intestine; Gene family

## 1. INTRODUCTION

Small intestinal lactase-phlorizin hydrolase (LPH; EC 3.2.1.23–62) is anchored in the brush border membrane of enterocytes, where it digests lactose, the sugar of milk. The protein also exhibits phlorizin hydrolase activity [1–3], for which the natural substrates are more likely glycosylceramides [4]. The sequence of cloned LPH cDNA [5] and further biochemical data show that LPH has five domains (Fig. 1, top): (i) a signal sequence of 19 amino acids, (ii) a large pro region of 847 amino acids (in rabbit), (iii) an extracellular domain of 1016 amino acids, (iv) a stretch of 19 hydrophobic amino acids serving as a membrane anchor [6], and (v) a cytoplasmic domain of 25 amino acids at the carboxy terminus. The sequence contains four internal repeats sharing 35–50% identical residues [5]. Two of the repeats are in the pro region, while the active sites of lactase and phlorizin hydrolase are in the third and fourth repeats, respectively [6].

The specific lactase activity (U lactase per g mucosal protein) is high in sucklings, and except in some human populations declines thereafter to reach a level some 3- to 20-fold lower in the adult. The decline very likely has multiple causes, whereby the importance of the LPH mRNA level may vary with position along the small intestine. In adult compared to baby rats and rabbits, the disappearance of lactase from the distal ileum can be explained by the almost total disappearance of LPH mRNA, whereas the approximately 2-fold drop in the jejunal mRNA level is much less than the decline in enzyme activity ([7–9], P. Keller, unpublished results). We have previously shown that although the rate of biosynthesis of LPH follows the mRNA level along the length of the small intestine of rabbit, the steady-state level of enzyme activity per $\mu$g LPH mRNA is much lower in duodenum and proximal jejunum compared to the middle of the intestine [8], implicating a posttranslational element in the decline of lactase activity in proximal intestine. Similar conclusions have been reached by Rossi et al. [10]. An increase in proteolytic degradation is an attractive possibility [11,12] consistent with the known increase in pancreatic protease activity occurring at weaning [13]. We now show that in rabbit intestine the situation may be even more complex: mRNA from proximal and medial intestine contains varying proportions of three different LPH mRNAs, potentially coding for lactases differing in stability or other properties.

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*Abbreviations:* LPH, lactase-phlorizin hydrolase.

The nucleotide sequences reported here will be available in the GenBank, EMBL, and DDBJ data banks under the accession numbers Z27166 to Z27169, and Z27223.

## 2. MATERIALS AND METHODS

### 2.1. *Cloning of LPH DNA*

A 4–8 kb fraction of cDNA was prepared [14] from intestinal mucosa RNA of a 12-day-old rabbit, or from proximal and medial intestine of a 3.5-year-old rabbit, and cloned in the $\lambda$ZAP vector (Stratagene) as described previously [5]. The library from total mucosa was screened with a 5′-proximal *Eco*RI–*Pst*I fragment (138 bp) from the rabbit lactase plasmid pRL8 (ref. [5]), and inserts subcloned into pBluescriptSK(-) after excision with *Eco*RI. For the proximal and medial libraries a 3.6 kb *Eco*RI–*Hin*dIII fragment from pRL8 was used as a probe and subcloning was performed with a 'plasmid rescue' procedure [15] using 'Exassist' helper phage (Stratagene) according to the manufacturer's protocol. (In our hands many of the rescued clones

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gave very low yields of plasmid DNA until the plasmids were retransfected into fresh *E. coli*, suggesting the original rescued colonies may have been latently infected with helper phage.)

Genomic DNA from rabbit intestine was cloned into λ-EMBL3-*cos-Not* (ref. [16]) as described previously [17]. For 'gene 3' (see section 3), a 1.6–2.2 kb fraction of rabbit DNA totally digested with *Eco*RI was cloned into λZAP. Clones containing exon 1 of the LPH gene were isolated from both libraries by screening with a 430-bp *Eco*RI–*Nco*I fragment from pRL8.

Poly(A)$^+$-RNA from intestinal mucosa was reverse transcribed with random hexanucleotide primers as described [17]. Nucleotides 372–569 of the LPH sequence were amplified with the primers gccgaattCGGTGCTACCGGCAGCTCCTT and gccggaTCCAAGTCACTAAAGGTGAGCC (plus and minus strands, respectively; small letters indicate linker sequences), using 30 cycles of 45 s at 95°C, primer hybridization for 2 min at 50°C for the first 5 cycles and 60°C thereafter, and 2 min at 72°C. The amplified products were isolated by gel electrophoresis, digested with *Eco*RI and *Bam*HI, and cloned into M13mp18.

### 2.2. *DNA sequencing and analysis*

Sequencing was performed with a T7 sequencing kit from Pharmacia, using single-stranded M13 DNA or alkali-denatured plasmid DNA as template. Full length LPH cDNAs pBL20 and pBL70 were sequenced according to a 'semi-random' strategy [5] on both strands, except that part of the pBL70 sequence was determined on one strand in parallel with the already known pRL8 sequence using a series of LPH-specific primers (primers as in ref. [17], and T. Oberholzer and E. Zwicker, unpublished results). Exon 1 sequences were determined by subcloning restriction fragments predicted from the pRL8 sequence. A segment of the LPH sequence in clones derived from mRNA of proximal and medial intestine was determined with the primer CCATTGTGCAGAGGTTC (nucleotides 4565–4581 in the pRL8 sequence); in a few cases these sequences were verified by use of a minus strand primer complementary to nucleotides 4932–4916.

Sequences were analyzed with the GCG program package [18]. The significance of the variation in occurrence of LPH sequences in different cDNA banks was assessed by contingency table analysis with the program StatView for Macintosh.

Southern blotting was performed as described previously [17], with final washing at 55°C in 1× saline/sodium phosphate/EDTA (ref. [19]), 0.2% sodium dodecyl sulfate.

## 3. RESULTS

We isolated several LPH cDNA clones from a baby rabbit intestine cDNA bank and compared them with the previously described rabbit LPH cDNA clone pRL8 (ref. [5]) Two of the new clones, pBL20 and pBL70, were sequenced, and proved to differ from pRL8 over much of their length. However, the differences are very small: the three DNA sequences share more than 94% identical bases, and the predicted amino acid sequences are >97% identical (Fig. 1). Unexpectedly, the differences are greater in the mature LPH domain than in the pro region, but even here stretches of more than 100 amino acids are identical in all three sequences. There are no differences in the signal sequence or the membrane anchor.

Do these sequences represent alleles of one gene or multiple genes? Southern blotting was performed with DNA from a single rabbit and with a probe comprising nucleotides 1 to 430 of the pRL8 sequence, a sequence that in the human case is completely within exon 1 [17] All of 5 enzymes which do not cut within this region of the cDNA sequence gave 3 bands, and *Pst*I, which cuts in the cDNA, gave 6 bands (Fig. 2A). These data and further Southern blots with more downstream probes (not shown) suggest there are 3 LPH genes. A similar analysis with rat DNA suggested that by contrast there is only one LPH gene in the rat (Fig. 2B).

We confirmed the blot analysis of rabbit DNA by cloning segments of the chromosomal LPH genes from

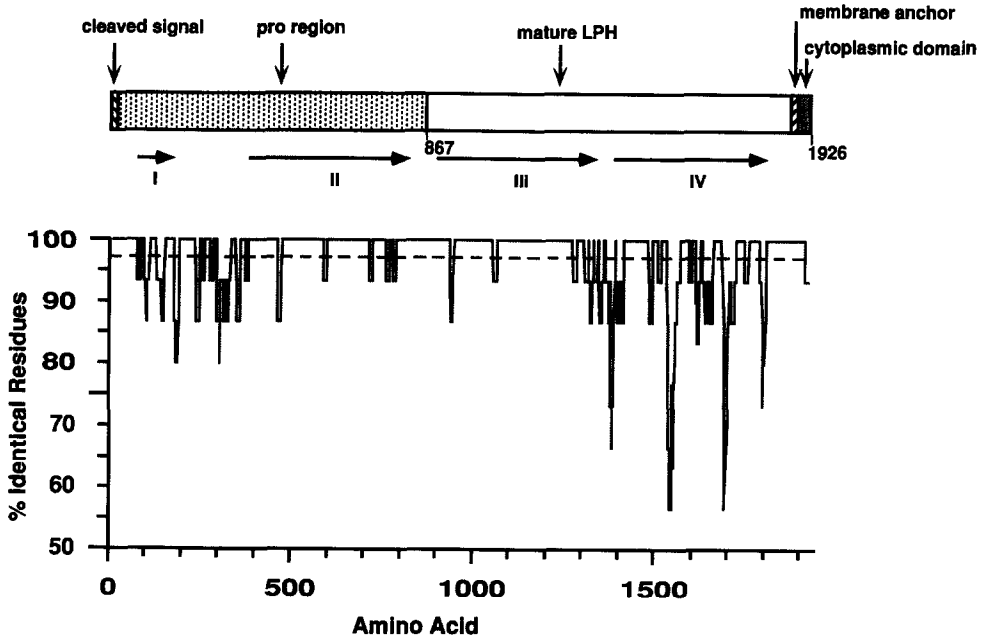


Fig. 1. The amino acid sequences coded by pBL20, pBL70, and pRL8 are compared with the program PlotSimilarity, part of the GCG program package. The % identical amino acids averaged over all pairwise comparisons within a window of 10 amino acids is plotted as a function of position of the window. The dotted line shows the overall average % of identical residues. The scheme at the top shows the domain structure of LPH at the same horizontal scale; horizontal arrows indicate internal homologies.

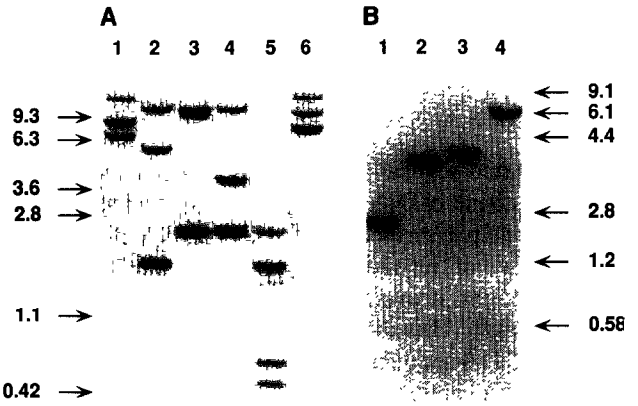


Fig. 2. Southern blot analysis of chromosomal LPH sequences in rabbit and rat. (A) Rabbit DNA (10 μg) digested with *Bam*HI (lane 1), *Eco*RI (lane 2), *Eco*RV (lane 3), *Hin*dIII (lane 4), *Pst*I (lane 5), or *Xba*I (lane 6) was hybridized with a $^{32}$P-labelled *Eco*RI–*Nco*I fragment from the 5′ end of the LPH cDNA in plasmid pRL8. (B) Rat DNA digested with *Eco*RI (lane 1), *Bam*HI (lane 2), *Hin*cII (lane 3) or *Pst*I (lane 4) was hybridized with a $^{32}$P-labelled *Hin*dIII–*Nco*I fragment isolated from plasmid pRLU6 (ref. [20]); the probe contains nucleotides 30 to 397 of the rat LPH cDNA sequence [21]. Sizes of marker fragments in kilobases are indicated by arrows.

the same rabbit. A gene bank of partially *Sau*3a-digested genomic DNA was prepared and screened with the 5′-proximal probe described above, leading to the isolation of two types of clones, named LPH gene 1 (carries the 6 kb *Eco*RI fragment seen in Fig. 2A, lane 2) and LPH gene 2 (12 kb *Eco*RI fragment). The third sequence, corresponding to the 1.9 kb *Eco*RI fragment in Fig. 2, lane 2 was isolated by screening a bank of size-selected *Eco*RI fragments cloned in the λZAP vector. Sequence analysis of all three clones showed that the boundary between exon 1 and intron 1 is at the same relative position as found in the human sequence [17], i.e. between nucleotides 682 and 683 of the pRL8 sequence. In a comparison of the exon 1 sequences with the cDNA sequences, pBL70 can be identified as arising from gene 1 and pBL20 from gene 2 (Table I). Although the pRL8 sequence is more similar to that of gene 3 than to genes 1 or 2, gene 3 is almost equidistant from the three cDNA sequences. Gene 3 and pRL8 may therefore represent a third and fourth type of LPH sequence.

We then asked whether all three LPH sequences appear in mRNA. In an initial experiment, intestinal mucosa RNA from the same animal used for the chromosomal cloning was reverse-transcribed, and a segment of the LPH cDNA sequence coded by part of exon 1 was amplified by PCR. Control experiments with cloned DNA from LPH genes 1, 2, and 3 as template showed that under these conditions all three genes could be amplified to the same extent. The amplified cDNA was cloned into M13mp18 and inserts of 23 clones were sequenced, whereby 14 corresponded to gene 1, 1 to gene 2, and 8 to gene 3. All chromosomal sequences found in this rabbit therefore do appear in intestinal RNA.

In view of our previous finding that in adult rabbits the lactase activity per μg LPH mRNA is lower in proximal than in mid-intestine [8] we wished to determine whether the relative amounts of the three mRNA sequences vary between these two regions. Two cDNA banks were prepared, starting with RNA from a proximal and a medial part of the intestine of the same 3.5 year old rabbit studied previously (proximal segments 1a to 2a and medial segments 5b to 6b as shown in Fig. 1 of ref. [8]). We chose cDNA cloning rather than a PCR-based method to avoid the danger of overlooking a sequence variant because of mismatches in a primer. LPH clones were isolated by plaque hybridisation and converted to plasmid form by the plasmid rescue procedure. The sequence of a particularly variable segment in the cDNA sequence, from nucleotides 4631 to 4770 in the pRL8 coordinates, was determined for 20 clones from the proximal segment and 21 from the medial segment. Three different sequences were found, corresponding to those of pRL8, pBL20, and pBL70 (Fig. 3). A single variable nucleotide at position 4702 in the pRL8-type sequences is attributed to allelic variation; further single nucleotide differences found in three other clones may be cloning artefacts. As shown in Table II, the pBL70 sequence is almost equally represented in both segments, occurring in about 40% of the clones. In contrast, the frequency of the pBL20 and pRL8 sequences varies strongly, with pBL20 found only in the medial segment and pRL8 being far more frequent in the proximal part. The overall difference between the proximal and medial segments is highly significant ($\chi^2 = 18.3$; 2 D.F.; $P = 0.0001$).

## 4. DISCUSSION

We have found three different rabbit LPH cDNAs which share > 94% identical nucleotides and code for LPH proteins with > 97% identical amino acids. These clones stem from different rabbits, and it appeared possible that they represented merely different alleles of one rabbit LPH gene. However, the cloning of three different exon 1 sequences from one rabbit conclusively dem-

Table I

Comparison of exon 1 sequences

| Chromosomal sequences | cDNA sequences | | |
|---|---|---|---|
| | pRL8 | pBL20 | pBL70 |
| Gene 1 | 14/682 | 11/623 | 1/624 |
| Gene 2 | 12/682 | 0/623 | 11/624 |
| Gene 3 | 9/682 | 6/623 | 8/624 |

Exon 1 sequences in three cloned rabbit LPH cDNAs are compared with the three different exon 1 sequences cloned from chromosomal DNA of one rabbit. The table shows the number of differences divided by the number of nucleotides compared (the latter values vary because not all the cDNAs extend equally far in the 5′ direction).

```
          4631
pBL20     --------- ----g----- g--t-a---c a---c-a--- -g--c--t---
pBL70     --------- ----a----- a--c-t---c a---t-a--- -t--c--c---
pRL8      --------- ----a----- a--c-t---t g---c-t--- -t--t--t---
Consensus TTTGGATCA CACT-AATGA -CC-T-TGT- -TTG-C-ACC A-GG-TA-GGC

          4681
pBL20     tac--agta -ca------- -g----c--- t--------- ----------
pBL70     aat--ggta -at------- -g----c--- g--------- ----------
pRL8      act--gctg -at------- -v----a--- t--------- ----------
Consensus ---GG---- T--GCCCCAG G-ATCT-CTT -AGGCCTGGC ACTGCTCCCTA

          4731
pBL20     c----t--- ---------- ---------- -c--------
pBL70     t----c--- ---------- ---------- -t--------
pRL8      c----t--- ---------- ---------- -c--------
Consensus -ATTG-TGG CCACAACTTA ATAAAGGCTC A-GCTGAGGC
```

Fig. 3. Sequences of LPH cDNA of the types found in plasmids pBL20, pBL70, and pRL8, as well as in 41 further LPH cDNA clones, from nucleotides 4631 to 4770 in the coordinates of the pRL8 sequence [5]. A consensus sequence is shown at the bottom, and nucleotides which are identical in all three sequences are indicated by dashes. The 'v' at position 4702 in the pRL8 sequence indicates that both G and C were found at this position (10 and 5 times, respectively; this work) as well as an A in the original pRL8 sequence.

onstrates that the rabbit has more than one LPH gene. Two of the exon 1 sequences match almost completely to the corresponding region of two of the cDNAs, whereas the third differs by about 1% (Table I). We suspect that there are three genes plus allelic variants of at least one of these, but can not exclude that there are only two genes with unusually high allelic variation.

The rabbit from which the three exon 1 clones were isolated also expressed these sequences in mucosal mRNA, as shown by analysis of PCR-amplified cDNA. Therefore none of the genes is a silent pseudogene. A similar conclusion was reached by analysis of LPH cDNA clones from another rabbit, where three different LPH sequences were again found (Fig. 3). Furthermore, the representation of the three sequences in the total LPH mRNA population was found to vary markedly along the length of the intestine. Finally, we have also compared a baby rabbit with its mother by direct sequencing of PCR-amplfied LPH cDNA, and seen that the major expressed LPH sequences were different (D. Brunschwiler, unpublished results). This may reflect a general difference between adult and baby rabbits, or simply the difference between the two individual animals studied.

Assuming that all mRNAs are translated, three or four different lactase-phlorizin hydrolase proteins will be produced in differing proportions, depending on the location along the intestine and on either the age of the animal or the particular animal studied. We do not yet know whether these forms of the enzyme differ in their biochemical or physiological properties, but differences in targeting, resistance to proteolysis, or intrinsic stability are certainly possible. Such differences could provide part of the explanation for the complex patterns of expression of lactase observed along the length of the intestine and during the development from suckling to adult rabbit ([8–10], and P. Keller, unpublished results). Unfortunately, there is no tissue culture model which even remotely approximates the conditions in the lumen of the intestine, so that definitive proof of this hypothesis may be difficult.

Study of human LPH is not complicated by this factor, as there is only a single human LPH gene [17]. We also did not find any evidence for more than one LPH gene in the rat, an animal in which the distribution of LPH mRNA along the intestine during development is similar to that in the rabbit.

Table II

LPH sequences in cDNA clones derived from proximal and medial intestine of rabbit

| Sequence type | Proximal | Medial |
|---|---|---|
| pBL20 | 0 | 10 |
| pBL70 | 7 | 9 |
| pRL8 | 13 | 2 |

The number of clones having each of the sequences shown in Fig. 3 is shown for collections of LPH cDNA clones isolated from proximal and medial rabbit intestine.

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