

1 kb of the lactase–phlorizin hydrolase promoter directs post-weaning decline and small intestinal-specific expression in transgenic mice

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Abstract

Adult-type hypolactasia is a genetic condition making approximately one half of the human population intolerant to milk because of abdominal symptoms. The cause is a post-weaning down-regulation of the intestinal-specific enzyme lactase-phlorizin hydrolase (LPH) reducing the intestinal capacity to hydrolyze lactose. We here demonstrate that the stretch –17 to –994 in the pig LPH-promoter carries cis-elements which direct a small intestinal-specific expression and a post-weaning decline of a linked rabbit β -globin gene. These data demonstrate that the post-weaning decline of LPH is mainly due to a transcriptional down-regulation.

Key words: Lactase-phlorizin hydrolase; Tissue specificity; Small intestine; Enterocyte; Hypolactasia; Lactose intolerance

1. Introduction

Reduced levels of LPH in adult life (adult-type hypolactasia) are responsible for the most common inherited condition in the human population [1]. Adult-type hypolactasia is characterized by reduced ability to digest lactose [2] caused by a post-weaning decline of the intestinal lactase activity. In contrast lactase-persistent humans (mainly Northern Europeans and their descendants) have a high lactase activity throughout their lifetime.

The post-weaning decline of LPH is a general event occurring in most mammals. The decline is coordinated with an increase of sucrase–isomaltase activity, and it is also correlated with the change from having milk as main carbohydrate source to a diet dominated by polysaccharides (starch and glycogen) and sucrose.

The mechanism of the post-weaning decline of LPH is under intense investigation [3]. Results from measurements of LPH mRNA levels in lactase-persistent and hypolactasic persons as well as in newborn and adult animals have been contradictory. The initial reports from such measurements pointed to a post-transcriptional regulation mechanism [4,5] while later reports suggest a pre-translational mechanism [6,7,8].

LPH mRNA is exclusively expressed in enterocytes of the small intestine of mammals [9] and is present in very

small amounts in the fetal colon [10]. The LPH mRNA levels are furthermore regulated along the longitudinal axis of the small intestine [6,11,12]. In the proximal jejunum and the distal ileum relatively low levels of LPH mRNA are present, whereas the distal jejunum and the proximal ileum contain high levels of LPH mRNA.

Transfection experiments of the pig LPH promoter region (position –17 to –994) into the colon carcinoma cell line Caco2 have demonstrated that this region is able to promote the transcription of a reporter gene [13]. Furthermore, a nuclear factor (NF-LPH1) binds to a sequence in the LPH promoter that is in close proximity to a putative TATA-box.

In order to investigate the role of transcription in the post-weaning decline of LPH expression and to search for the genomic region directing the specific small intestinal LPH-expression a transgenic mouse model has been established. Other transgenic mouse models have earlier proven their great value in studies on developmental events and tissue-specific promoter activity [14].

2. Materials and methods

2.1. Production and genotype-analysis of transgenic mice

Fertilized F1 mouse oocytes (C57Bl/6J × DBA/J2) were microinjected [15] with a DNA construct containing the pig lactase phlorizin hydrolase (LPH)-promoter (–17 to –994) [13] in front of a reporter gene (rabbit β -globin gene [16]). The resulting mice were analyzed for genomic integration of the transgene: tail DNA was prepared according to Laird et al. [17]. DNA (5 μ g) was analyzed by Southern blot analysis

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using a random primed 32 P-labelled rabbit β -globin DNA probe. Two male transgenic mice were identified (F1-generation) and mated with non-transgenic females to establish transgenic mouse-lines. The offspring (F2 generation) was analyzed for the presence of the transgene as described for the founder mice. One fourth of the offspring had inherited the transgene.

2.2. Source of tissues

Hemizygotic transgenic and normal mice were killed by cervical dislocation. Small intestine, colon, liver, kidney lung and heart were dissected. For the examination of regional expression (Fig. 3) the small intestine was divided into segments. Small tissue samples taken from duodenum (I1), proximal jejunum (I3), proximal ileum (I5) and distal ileum (I7) were frozen in liquid nitrogen and later analyzed for lactase activity and protein concentration. The resulting 3 segments of equal length named proximal (I2), mid (I4) and distal small intestine (I6) were processed immediately for total RNA extraction. For examination of the developmental expression (Fig. 5) the small intestine was divided into a proximal (P) and a distal (D) part of equal length for RNA extraction. A small tissue sample was taken from the middle of the small intestine for lactase activity measurements.

2.3. RNA extractions and analysis

Total RNA was isolated by the guanidinium thiocyanate/acid phenol/chloroform method [18]. A 93-mer β -globin oligonucleotide was used to map the 5' end of the rabbit β -globin mRNA by S1 nuclease analysis. The 93-mer β -globin oligonucleotide was 5' end labelled by 32 P. 0.01 pmol β -globin oligonucleotide was hybridized with 10 μ g of total RNA at 37°C 16 h in 80% formamide, 0.4 M NaCl, 0.04 M PIPES, pH 6.4, 1 mM EDTA. The hybrids were treated with 100 U S1-nuclease (Promega). Protected fragments were separated by 10% polyacrylamide gel electrophoresis and the bands were visualized by autoradiography.

LPH and β -actin mRNA were quantified by slot blot hybridization of 20 μ g total RNA using a pig LPH cDNA and human β -actin cDNA random labelled probe. The mRNA levels of β -actin mRNA were used to normalize β -globin and LPH mRNA levels.

Quantitative estimations (Computing Densitometer, Molecular Dynamics) of the intensity of the hybridization signals were used to estimate the mRNA levels.

2.4. Lactase activity and protein measurements

For lactase activity and protein concentration measurements approximately 20 mg of small intestine were homogenized in 0.1% Triton X-100 solution. An aliquot was taken for protein concentration measurements before centrifugation (10,000 $\times$ g, 4°C, 15 min) and the lactase activity was measured in the supernatant using a glucose oxidase method [19]. Protein concentration was determined [20] using bovine serum albumin as standard.

2.5. In situ hybridization

The tissue was treated essentially as previously described [21]. A piece of the mid small intestine from an adult transgenic mouse was fixed in 4% paraformaldehyde in PBS, pH 7.4 overnight. The tissue was paraffin-embedded and 5 μ m sections were cut and mounted on aminoalkyl silane-coated slides [22]. The sections were deparaffinated in xylene and hydrated through graded series of ethanol in water. They were then incubated with 10 μ g/ml proteinase K in 0.1 M Tris-HCl, pH 8.0, 50 mM EDTA at 37°C for 30 min and then postfixed in 4% paraformaldehyde in PBS for 5 min. Prehybridization was carried out for 1 h at 45°C in 50% formamide, 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl, 0.015 M sodium-citrate, pH 7.2), 1 $\times$ Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin), 0.5% SDS and 100 μ g/ml denatured salmon sperm DNA. Hybridization was carried out in 20 μ l hybridization solution (50% formamide, 2 $\times$ SSC, 1 $\times$ Denhardt's solution, 0.5% SDS, 100 μ g/ml denatured salmon sperm DNA and 10% dextran sulphate) containing 5 ng/ μ l of digoxigenin-labelled antisense or sense β -globin RNA probe at 45°C for 16 h. A 180 nt. digoxigenin-labelled 5'-antisense β -globin RNA probe was produced by in vitro transcription with T7 RNA polymerase (Digoxigenin RNA Labelling Kit, Boehringer-Mannheim) using a *SalI*-digested plasmid containing the rabbit β -globin gene as template. Digoxigenin-labeled sense β -globin RNA served as a negative control. To avoid evaporation the hybridizations were covered with a parafilm cover slip. After hybridization the slides were washed in 2 $\times$ SSC for 5 min, 0.2 $\times$ SSC for 10 min

and 0.1 $\times$ SSC for 15 min at 45°C and then incubated with blocking solution (PBS, pH 7.4, containing 0.5% (w/v) bovine serum albumin, 1% (w/v) fetal calf serum and 1% (w/v) blocking reagent (Boehringer Mannheim)) for 30 min. The sections were incubated with peroxidase-conjugated sheep anti-digoxigenin (Boehringer-Mannheim) (working dilution 1:50 in blocking solution). Peroxidase was visualized with diaminobenzidine-tetrahydrochloride (1.4 mM) and H₂O₂ (0.01%) and the sections were counterstained with Harris' hematoxylin, mounted and examined in a Zeiss light microscope.

3. Results and discussion

3.1. Production and identification of transgenic mice

We have used a rabbit β -globin gene linked to the pig LPH promoter region -17 to -994 [13] to examine the regulatory properties of this region. Two transgenic mouse lines were identified by Southern blotting (LPH 2.5 and LPH 2.8 (data not shown)). By examination of the restriction enzyme digestion pattern and by scanning the autoradiogram, LPH 2.8 mouse line was estimated to contain approximately five tandemly arranged transgenes. When mated with normal mice the transgene DNA was inherited in a Mendelian manner. It was shown by Northern blot analysis, that only the LPH 2.8 mouse line expressed the reporter gene, whereas no expression was detected in the LPH 2.5 mouse line (data not shown).

3.2. Small intestinal-specific transcription of β -globin reporter gene

S1 nuclease protection analysis of the 5' end of the rabbit β -globin mRNA showed that the transcription of the reporter gene was initiated correctly as the β -globin transcript protected a 75 nt. fragment of the 93 nt. antisense β -globin probe (Fig. 1).

To analyze whether the -17 to -994 pig promoter element confers tissue-specific expression the reporter gene mRNA levels were analyzed in different tissues by S1-nuclease mapping [16]. A high level of β -globin

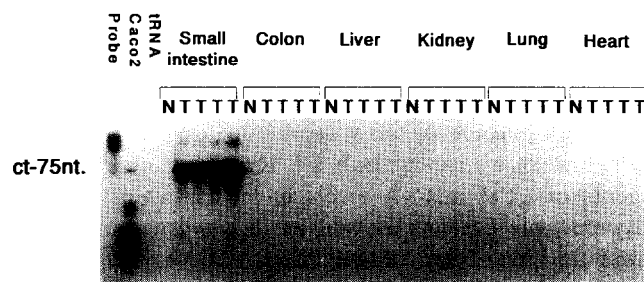


Fig. 1. Tissue-specific expression of the porcine LPH/rabbit β -globin gene in the small intestine of four hemizygotic transgenic mice analyzed by S1-nuclease mapping. Total RNA from small intestine, colon, liver, kidney, lung and heart were analyzed for the presence of reporter gene mRNA (rabbit β -globin mRNA). Correctly initiated mRNA generates a 75 bp protected band (ct). RNA from Caco2 cells stably expressing the reporter gene (75 bp band) and control transcripts [13] (48 bp and 54 bp bands) served as a positive control (Caco2). RNA from a normal mouse (N) and tRNA served as negative controls. T = transgenic mice.

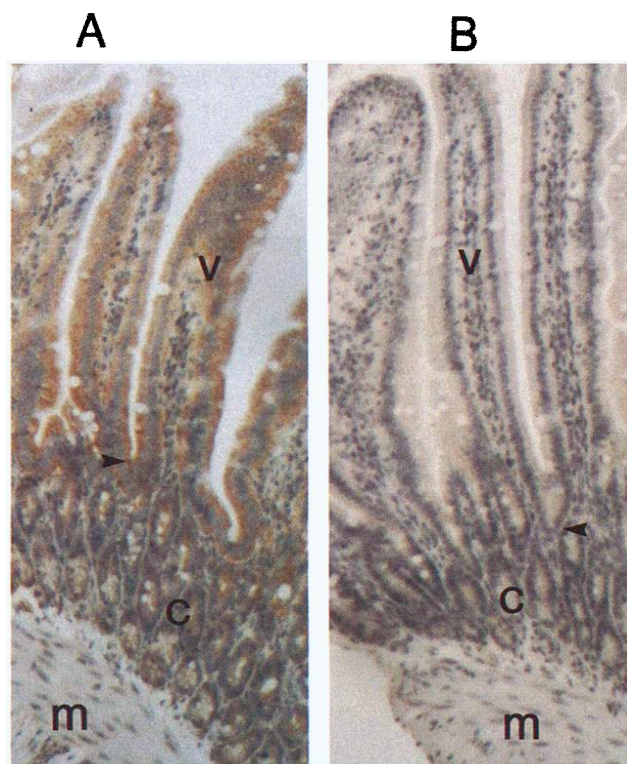


Fig. 2. In situ detection of rabbit β -globin mRNA in small intestine of a homozygotic transgenic adult mouse using a 180 nt. 5'-antisense digoxigenin-labeled RNA-probe (A). Hybridization was visualized by peroxidase immuno-detection using peroxidase conjugated anti-digoxigenin. A 5'-sense digoxigenin-labeled RNA was hybridized in parallel to a small intestinal section as negative control (B). v = villus, c = crypt area, m = muscle layer, arrowhead = crypt/villus junction.

mRNA was present in the small intestine (Fig. 1) while no expression was detected in colon, liver, kidney, lung and heart. This clearly shows that the region -17 to -994 of the pig promoter contains regulatory element(s) which direct the small intestinal-specific expression.

To further investigate the cell-specific expression of the transgene in situ hybridization was performed on small intestinal sections of an adult mouse (Fig. 2). The majority of the β -globin mRNA was detected in the enterocytes. Hybridization to a few blood vessels in the muscle layer was also seen. This can be explained as cross-hybridization to endogenous mouse β -globin mRNA. All other cell-types were free of hybridization. The hybridization signal was strongest at crypt/villus junction and decreased at the top of the villi. Some hybridization was also detected in enterocytes in the crypts.

Various genes have been expressed in a tissue-specific manner in transgenic mice [14]. Two examples of intestinal-specific expression have been reported so far [23, 24]. Thus, a 277 bp fragment of the intestinal fatty acid-binding protein promoter [23] and a 3,478 bp fragment of the sucrase-isomaltase promoter [24] have been shown to be able to drive the expression of a reporter gene both in the small intestine and in the colon. The pig LPH promoter activity is exclusively restricted to entero-

cytes of the small intestine (Figs. 1 and 2). Thus the -17 to -994 LPH pig promoter stretch constitutes the first reported element directing an enterocyte-specific expression. The enterocyte specificity is most probably executed by cell-specific transcription factors. We have previously shown, that an intestinal-specific DNA binding factor (NF-LPH1) binds to the pig LPH promoter in the region -40 to -54 (CE-LPH1) [13] and recently we have shown that the factor is of importance for LPH transcription (Spodsborg N., unpublished data). In parallel it has been shown for the promoter of another small intestinal-specific enzyme sucrase-isomaltase, that an intestinal transcription factor (SIF1 binding protein) binding close to a TATA-box can direct transient expression in the intestinal cell line Caco2 [25]. The SIF1 element and the CE-LPH1 element are competing for binding to factors from Caco2 nuclear extracts (see Troelsen et al. [29], accompanying paper). Thus the small intestinal-specific expression of LPH and sucrase-isomaltase seems to be mediated by closely related transcription-factors which bind in the proximity of a TATA-box.

3.3. Regional expression of the reporter gene

In order to determine whether the -17 to -994 LPH

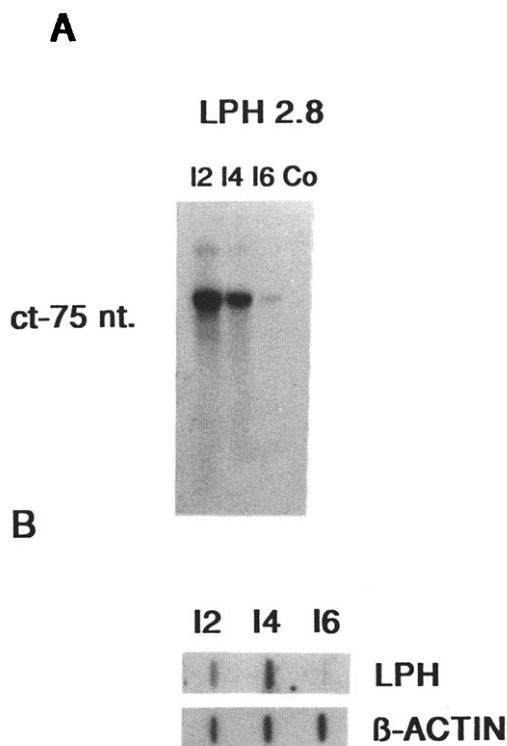


Fig. 3. (A) Regional expression of the porcine LPH/rabbit β -globin gene in the small intestine of a hemizygotic transgenic mouse (two months old) by S1-nuclease mapping. The small intestine was divided into segments as described in section 2. The resulting 3 segments of equal length named proximal (I2), mid (I4) and distal small intestine (I6) were used for rabbit β -globin mRNA analyses. Colon RNA (Co) was used as negative control. (B) Slot blot analysis of LPH mRNA and β -actin levels along the small intestine using a pig LPH cDNA probe and a human β -actin cDNA probe.

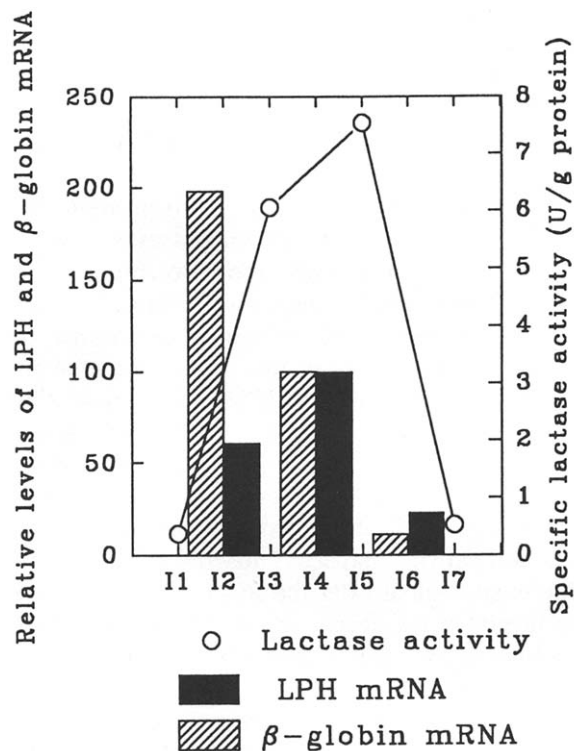


Fig. 4. LPH promoter activity, specific lactase activity and LPH mRNA along the proximal/distal axis of the small intestine of a two-month-old hemizygotic transgenic mouse (F2-generation). The small intestine was divided as described in section 2. Lactase activity was measured in segment duodenum (I1), proximal jejunum (I3), proximal ileum (I5) and distal ileum (I7). LPH mRNA, β -globin mRNA and actin mRNA levels were measured in proximal (I2), mid (I4) and distal small intestine (I6) (Fig. 3). Lactase activity is depicted as units/g protein, whereas LPH and β -globin mRNA levels are depicted relatively to the level in segment I4 (100%).

promoter also directs a regionally varied promoter activity thus explaining the high levels of lactase activity in the middle parts of the small intestine and the low levels in proximal and the distal parts of the small intestine [6,11,12] different regions of the small intestine of LPH 2.8 mice were analyzed for lactase activity, LPH-mRNA content and β -globin-mRNA expression (Figs. 3 and 4).

The lactase activity in the transgenic mice follows the pattern described for other mammals [6,11,12]: low lactase in the proximal jejunum and distal ileum, and high levels in the mid region of the small intestine. The LPH-mRNA essentially follows the same pattern, except in the proximal jejunum where the LPH-mRNA level is higher than expected from the lactase activity measurements. The explanation for this discrepancy is probably a faster turn-over of LPH-protein caused by pancreatic proteases in the proximal jejunum.

β -globin-mRNA expression follows a gradient from highest levels in the proximal jejunum to lowest levels in the distal ileum. In the mid and the distal parts of the small intestine a coordinate variation of LPH mRNA, lactase activity and β -globin mRNA expression is found.

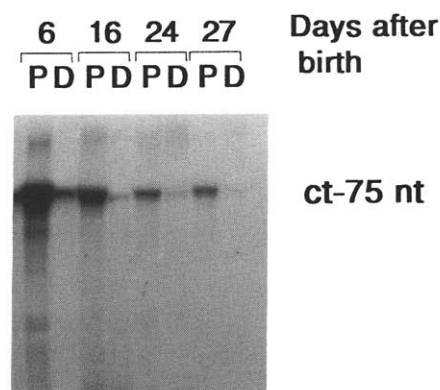
However, there is no correlation between the high β -globin mRNA level and the relatively low LPH mRNA level in the proximal part of the intestine (Figs. 3 and 4).

This difference may be explained by either a faster duodenal turn-over of the LPH mRNA which is not mimicked by the β -globin mRNA, or the existence of cis-elements important for a lower duodenal expression that are missing in the LPH promoter/ β -globin construct.

3.4. Post-weaning down-regulation of the reporter gene

To assess whether the -17 to -994 LPH promoter region confers post-weaning down-regulation of the reporter gene, sections of small intestines of transgenic mice obtained at different times (6, 16, 24, 27 days) after birth were analyzed for lactase activity, LPH mRNA content and β -globin mRNA content. These three parameters were measured both in the proximal and the distal

A



B

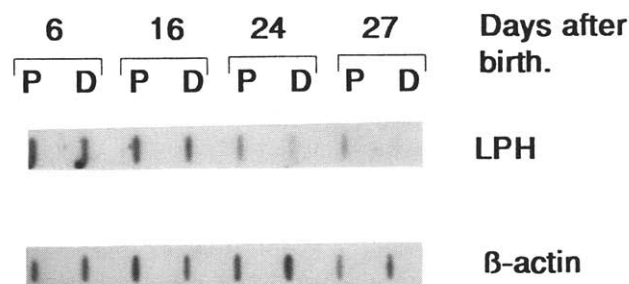


Fig. 5. (A) Post-weaning decline of the expression of the porcine LPH/rabbit β -globin gene by S1-nuclease mapping. Hemizygotic transgenic mice (F3 generation) (C57/6J/DBA/J2 $\times$ NMRI) were sacrificed 6, 16, 24 and 27 days after birth. Small intestines were divided into two parts of equal length (P = proximal small intestine, D = distal small intestine) and were analyzed for rabbit β -globin mRNA expression. (B) Slot blot analysis of LPH mRNA and β -actin levels during mouse development using a pig LPH cDNA probe and a human β -actin cDNA probe.

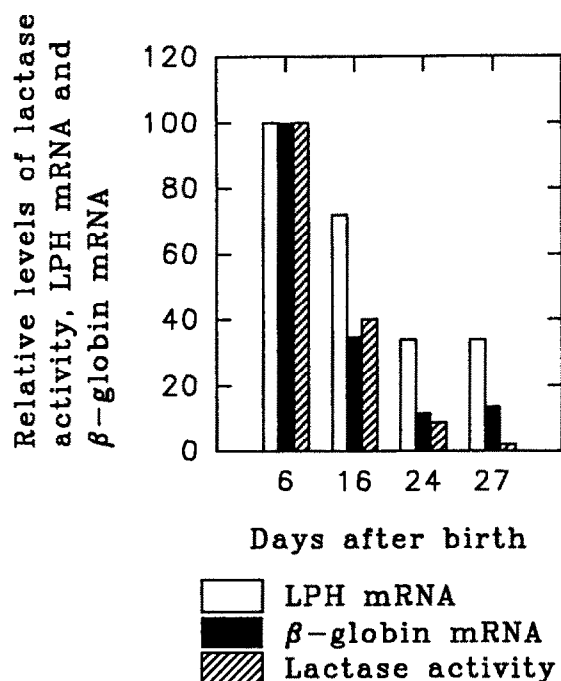


Fig. 6. Decline of the relative β -globin mRNA, lactase activity and LPH mRNA during mouse development of hemizygotic transgenic mice (F3-generation). β -globin and β -actin mRNA were measured in the proximal half of the small intestine. Lactase activity was measured in a small segment from the middle of the small intestine. All values were normalized to the corresponding values in the 6-day-old mouse.

part of the small intestine (Figs. 5 and 6). The lactase activity was highest 6 days after birth, then it diminished to 40% at day 16 to the lowest levels at day 24 and 27. Similar measurements of normal mice showed the same pattern (results not shown). This conforms with earlier data [26] reporting a 15-fold decrease between 14 and 20 days after birth of the mice. The presence of the transgenes in the transgenic mice thus does not affect the normal development of the lactase activity. The LPH-mRNA level also decreased during the weaning period, however the decrease in LPH-mRNA is not as pronounced as for the lactase activity. At 24 and 27 days after birth the LPH-mRNA level only declined to 34% of the level 6 days after birth as compared to 9% for the lactase activity (day 24). It has earlier been reported that the down-regulation of lactase activity and LPH-mRNA level are not correlated [11,12]. It was therefore suggested that the decline of LPH is regulated at both transcriptional and post-transcriptional levels [11].

The pig LPH promoter activity (β -globin mRNA level) in transgenic mice is coordinated with the decline in the lactase activity and mRNA (Fig. 6). However the β -globin mRNA level decreases more than the LPH mRNA level. The difference between the LPH mRNA level and the β -globin mRNA level might be explained by the repeated structure of the transgenes. The pig LPH promoter elements of the transgenes might act as en-

hancers influencing the β -globin expression of the adjacent transgene. The effect of the down-regulation might then be exaggerated as the expression of the tandemly ordered transgenes is not regulated linearly. However we can not exclude, that cis-acting elements are missing in the transgene.

We thus conclude, that transcriptional down-regulation of the LPH-gene is the major mechanism behind the post-weaning decline of LPH, and that down-regulation is regulated by transcription factors, which bind to cis-elements in the region -17 to -994. Both the proximal and the distal part of the small intestine are transcriptionally down-regulated during the weaning period (Fig. 5).

We have reported earlier that the level of the intestinal-specific factor NF-LPH1 co-varies with the lactase activity [13]. Thus NF-LPH1 is a candidate factor participating in the network of transcription factors responsible for the regulation of the LPH expression. It is very likely that the mechanism causing post-weaning decline of LPH and the β -globin reporter gene in the transgenic mouse is also the major mechanism responsible for the post-weaning decline causing adult-type hypolactasia in humans, although additional mechanisms modulate the final expression [27]. A high lactase activity in adult life is unique for lactase-persistent humans among the mammals. The mechanism behind this high lactase level is therefore probably an inability to down-regulate the lactase transcription. This implies that there are either sequence-differences in the LPH promoter of hypolactasic and lactase-persistent humans, i.e. mutations in cis-elements or differences in the expression or activity of trans-acting factors regulating the LPH expression. Even though the sequenced part of the human LPH promoter contains Alu-sequences [28] and therefore does not correspond to the entire pig LPH promoter region used in the present experiments, the absence of a consistent variation in the human promoter between hypolactasic and persistent humans [7] indicates differences in expression or activity of trans-factors.

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