Expression of lactase–phlorizin hydrolase in sheep is regulated at the RNA level

Stephen W. LACEY,*¶ Hassan Y. NAIM,† Ronald R. MAGNESS,‡ Mary-Jane GETHING§ and Joseph F. SAMBROOK||

*Department of Internal Medicine, 5323 Harry Hines Blvd., UT-Southwestern, Dallas, TX 75235-8887, U.S.A., †Heinrich Heine Universität, 40225 Dusseldorf, Germany,

‡Departments of Pediatrics and Obstetrics/Gynecology, UT-Southwestern, Dallas, TX, U.S.A., §Department of Biochemistry, Howard Hughes Medical Institute,

UT-Southwestern, Dallas, TX, U.S.A., and ||Department of Biochemistry, UT-Southwestern, Dallas, TX, U.S.A.

Lactase–phlorizin hydrolase (LPH) is expressed on the intestinal brush border and is responsible for the hydrolysis of lactose, the chief sugar in mammalian milk. The enzymic activity of LPH peaks soon after birth in most mammals and declines to much lower levels before adolescence. The molecular basis of this pattern of expression has not been clearly established. We have measured relative amounts of LPH mRNA in intestine from sheep with ages across a developmental spectrum, including third trimester fetal lambs, newborn lambs and adult sheep. LPH mRNA levels in the jejunum decline ~ 50-fold between infancy

and adulthood, in parallel with the reduction in both lactase specific activity and immunologically reactive lactase protein expression in sheep jejunum. LPH mRNA is present in high concentration in the duodenum of newborn lambs, but steadily declines by day 34 and is dramatically reduced in adults. Because the changes in LPH mRNA, protein, and enzymic activity are generally parallel, we conclude that the developmental regulation of LPH in sheep is probably mediated primarily at the mRNA level.

INTRODUCTION

Lactose is the major sugar in mammalian milk and must be hydrolysed to its component monosaccharides, glucose and galactose, before absorption. The enzyme responsible for this hydrolysis, lactase–phlorizin hydrolase (LPH EC 3.2.1.23–62), is located in the brush border of the small intestine [1–7]. In most mammalian species, the ability to hydrolyse lactose diminishes after weaning, when the disaccharide essentially disappears from the diet. Re-feeding this sugar to adult humans does not restore their ability to digest lactose [8,9]. Indeed, no specific dietary substrate has been convincingly shown to be the primary regulator of any brush-border hydrolase [3]. For example, in rats, sucrase–isomaltase (SI) activity increases 1000-fold around the time of weaning while LPH activity falls [10]. These changes occur even if sucrose is withheld from the diet and feeding of lactose is maintained [11,12].

Unlike most other mammalian species, in which the ability to hydrolyse lactose diminishes, most Caucasoid humans retain their ability to digest this sugar for their entire lives [7]. This trait, which allows them to consume milk products with equanimity, is inherited in an autosomal dominant fashion [1]. The developmental regulation of LPH is therefore both complex and unique among the enzymes of the brush border.

Recently, it has been suggested that both pre- and post-translational mechanisms are involved in this regulation. Sebastio et al. [13] have reported that the level of LPH mRNA declines in weanling Wistar rats, but returns to its original level in adult animals, even though LPH activity is not restored. By contrast, Buller et al. [14] have reported that the level of LPH mRNA in Sprague–Dawley rats declines as a function of age and does not increase in adult life. Freund et al. [15] suggested that the rat regulates LPH expression differently in the jejunum and ileum because ileal LPH mRNA levels fall during development while jejunal levels remain constant. Lloyd et al. [16], Escher et al. [17],

and Fajardo et al. [18] have shown that humans regulate LPH at the mRNA level. Keller et al. have recently suggested that lactase regulation in the rabbit occurs at both pre- and post-translational levels [19]. Troelsen et al. identified a trans-acting nuclear factor that binds the LPH promoter and is more abundant in infant than in adult pigs [20]. We decided to examine LPH expression in the domesticated sheep as an example of a mammal known to exhibit a dramatic fall in lactase associated with the development of the rumen [21,22].

By analysing samples of sheep intestine at eight stages of development from fetus to adult, we have been able to show co-ordinate regulation of LPH protein, enzymic activity and mRNA. The results are consistent with the hypothesis that ovine regulation of LPH occurs exclusively, or in large part, at the level of mRNA synthesis, processing or degradation.

MATERIALS AND METHODS

Tissue acquisition

Proximal jejunal tissue was obtained from sheep undergoing non-survival surgery using pentobarbital sodium (50 mg/kg) as anaesthetic. This procedure was approved by the Institutional Review Board for Animal Research. Strips of small intestine (~ 75 mm × 75 mm) were snap-frozen in liquid nitrogen for RNA isolation, and identical samples were stored on ice before freezing at –20 °C for activity and Western blot analyses.

RNA isolation

RNA was prepared by homogenizing tissue samples in 4 M guanidinium thiocyanate/Tris followed by sedimentation through a CsCl cushion [23,24]. RNAs were quantified by measuring the absorbance at 260 nm and compared for equivalence by electrophoresis through formaldehyde/agarose gels.

Oligonucleotides used in the assays

Synthetic oligonucleotides

All sequences presented 5'→3'. The numbering convention for the LPH oligonucleotides is that the number of the base in the human LPH cDNA [25] corresponding to the extreme 5' base of each oligonucleotide appears in the name. The names of antisense oligonucleotides begin with c for complementary.

LPH 3883– 5'(sense)amplification oligonucleotide for LPH:

AAAGGATCCTACCACAAAACCTATATCAACGAGGCT

*Bam*HI

cLPH 4117– 3'(antisense)amplification oligonucleotide for LPH:

AAACTGCAGCCCGGCCAGGGGCATGCCATTGTTGG-
CAATGACCTC

*Pst*I

LPH 4044– Probe oligonucleotide for LPH:

CCTCGCACAGCAAGAGCCTC

v1063– 5'(sense)amplification oligonucleotide for Villin [1]:

AAAGGATCCCAGAAGTGGACAGCGTCCAACCGGACC

*Bam*HI

cv1437– 3'(antisense)amplification oligonucleotide for Villin [1]:

AAACTGCAGATTGTACTTCTGGTCCAGGATGACGGC

*Pst*I

v1324– Probe oligonucleotide for Villin [1]:

CTCATCGGCGAGAAGCAGCA

Staging the animals

The ages of the animals are stated as adult or in days relative to known or predicted birth. The three adults were > 3 years old and were multiparous. The first adult biopsied underwent RNA measurement only and therefore appears in Figure 2, but not Table 1. The two fetal specimens were obtained at 110 and 128 days gestation. Since the females in our flock characteristically deliver at 144±2 days gestation, we expressed the 110 and 128 day gestations as –34 and –16 days, respectively.

RNA-PCR assays

The RNA-PCR assay we used was developed in our laboratory and is described and validated in detail elsewhere [18]. Briefly, 1 or 10 µl aliquots of total RNA were subjected to reverse transcription and PCR, using LPH 3883 and cLPH 4117 in parallel with synthetic standards for quantitation. LPH amplification products were detected by Southern analysis using LPH 4044 as a probe. The bands were excised from the Southern blots and counted as described previously [18]. The c.p.m. for the

standard Southern blots were then plotted against the log in attomoles of synthetic RNA and fitted to quadratic equations using Macintosh Quadra 700 and Delta Graph software. The c.p.m. from the experimental bands were then converted into human LPH RNA attomole equivalents using the standard curve for each experiment. We compared the measurements of sheep LPH mRNA with a standard curve using synthetic human LPH RNA. We previously used the assay to measure attomoles of human LPH mRNA [18]. Since we used a standard curve here with human RNA, the sheep results are related to products generated from human LPH RNA (human LPH RNA attomole equivalents Figures 2 and 5). We plotted c.p.m. against log (attomol LPH RNA) because the assay becomes limiting at higher amounts of RNA. The limiting step could either be the RNA-PCR step or the Southern hybridization. We performed Southern hybridization with serial dilution of a high-abundance sample and showed that the Southern hybridization step is not limiting for the amounts of PCR product in the samples presented here. Therefore, the limiting step must be the RNA-PCR. In spite of the limiting character of the assay, results were consistent from experiment to experiment.

Single samples from each sheep RNA and control were assayed because handling constraints limit the practical number of tubes per assay to 16–18. The experimental results presented in Figures 2 and 5 therefore, are each one of three showing the same relative patterns by Southern blot. The representatives presented were chosen for quantification because they had the cleanest standard curves.

Control experiments were performed with oligonucleotides (v1063 and cv1437) designed to detect villin mRNA by the same method. V1324 was used as the probe for the Southern blots.

Sequencing of PCR products

PCR products were chromatographed over G-50 spun columns equilibrated in 1× restriction buffer to remove free nucleotides and Taq DNA polymerase, and to prepare the DNA for digestion with the restriction enzymes *Bam*HI and *Pst*I [24]. The digested PCR products were separated from the enzymes and oligonucleotides by agarose-gel electrophoresis and purified using NA45 filters (Schliecher & Schuell). These fragments were then subcloned into pGEM4Z (Promega, Madison, WI, U.S.A.) or pSL7 and sequenced using the dideoxy-chain termination method of Sanger [24,26]. The sequencing primers were designed to hybridize to plasmid sequences flanking the polycloning sites. Thus, the sequencing reactions confirmed the designed oligonucleotide sequences and generated the intervening sheep LPH cDNA. Both LPH strands were completely sequenced using this method.

Activity

LPH and SI activities were measured on homogenized tissue samples using the method of Dahlqvist and Asp [27]. Assays were performed in duplicate, giving the means presented in Table 1. Activity is given in units where 1 unit = µmol glucose liberated/min. Preparation of membranes for activity measurements was performed at 4 °C. Tissues were homogenized for 30 s in 3 ml of 25 mM Tris/HCl, pH 8.1, supplemented with 50 mM NaCl, 1 mM phenylmethylsulphonyl fluoride, 1 mg of pepstatin/ml, 5 mg of leupeptin/ml, 5 mg of antipain/ml and 1 mg of aprotinin/ml (buffer A). The homogenates were centrifuged at 1000 g for 15 min to remove nuclei, debris and non-homogenizable tissue. The supernatant was retained and centrifuged at 10000 g for 15 min. The pellets containing crude

Table 1 Comparison of lactase specific activity with relative LPH mRNA levels

All samples from proximal jejunum.

Age (days)	Lactase activity (units/g protein)	Relative sheep LPH RNA (human LPH RNA attomol equivalents)
—34	629	112
—16	732	~ 100
2.5	349	2672
5	1026	289
13	547	1956
25	349	457
34	570	1195
Maternal	9.7	< 20
Non-pregnant	18.1	< 20

membranes were resuspended in 2 ml of buffer A. The amount of protein was estimated by the method of Bradford [28] to permit calculation of specific activity.

Western blot analysis

Protein (10 µg) from each tissue sample was electrophoresed through a 6% (w/v) polyacrylamide gel [29]. Proteins were transferred to nitrocellulose membranes (Schliecher & Schuell) in a standard Tris/glycine transfer buffer [24]. Confirmation of transfer was assessed with pre-stained protein markers and by staining the blot with Ponceau S (Sigma, St. Louis, MO, U.S.A.) according to the specifications of the manufacturers. Non-specific binding sites were blocked by incubating the blots with 0.02% (v/v) Tween-20 in Tris-buffered saline (TBS) (20 mM Tris/HCl, pH 7.5, 500 mM NaCl) for a minimum of 2 h at 25 °C. Blots were then incubated with anti-human LPH antiserum at 1:200 dilution for detection of LPH. Some blots were subsequently stained with a mixture of monoclonal antibodies to human SI [30] as control. Antibody staining was detected with goat anti-rabbit immunoglobulin conjugated to horseradish peroxidase (HRP).

RESULTS AND DISCUSSION

Development of the LPH mRNA assay

We developed a rapid, simple reproducible assay to measure relative amounts of LPH mRNA in tissues using PCR. The oligonucleotides used to amplify the LPH sequence were chosen for high levels of conservation among rat, rabbit and human LPH. These oligonucleotides have successfully amplified LPH mRNA from human, rat, mouse and sheep small intestine (data from rat and mouse not shown). The relationship between the amount of specific hybridization and the log of moles of LPH sequences present is easily and reproducibly fitted to a simple quadratic equation using standard plotting software. This permits construction of a standard curve for quantifying experimental samples. We have used this assay previously to quantify LPH mRNA from humans [18].

When this work was begun, the nucleotide sequence for sheep

LPH was unknown. However, the sequence of a human and rabbit, full-length cDNA had been published [25], and we had previously used this sequence to isolate a full-length cDNA encoding human lactase [31]. A partial sequence of the rat LPH was presented in abstract form and confirmed the conservation of the sequences we had chosen for the amplification oligonucleotides. The complete sequence for the rat LPH cDNA has now been published [32]. By comparing the sequences of these cDNAs, we were able to identify highly-conserved regions and generate synthetic oligonucleotides that could serve as primers for amplification of LPH sequences from multiple mammalian species. The assay was standardized using human LPH RNA synthesized *in vitro* [18]. Sequences of the oligonucleotides used for the work described in this paper are included in Materials and methods.

To standardize the assay for LPH mRNA, we used a synthetic template of human LPH RNA [18], 869 bases in length, that was made *in vitro* by bacteriophage T7 DNA-dependent RNA polymerase. The double-stranded template DNA was a linearized plasmid (pBIII) that carried a fragment of the human LPH cDNA [31] downstream of the bacteriophage T7 late promoter. The amount of synthetic LPH mRNA was measured in two ways: (i) by including in the transcription reaction, radiolabelled precursors, permitting accurate calculation of RNA synthesized; and (ii) by measuring the absorbance at 260 nm (A_{260}) of purified RNA product. Serial dilutions of synthetic LPH mRNA containing between 100 amol (10^{-16} mol) and 10000 amol (10^{-14} mol) were used to produce a standard curve for each experiment. To confirm that the assay measures the concentration of LPH mRNA, the sequence of the 242 bp DNA obtained by amplification of sheep intestinal RNA was determined. Figure 1 shows a comparison of the nucleotide sequences of authentic human, rabbit and rat LPH cDNAs with that of the amplified sheep product between the amplification oligonucleotides. Approximately 85% of the nucleotides of the amplified ovine product are identical with the corresponding regions of the human, rabbit and rat LPHs. The Figure includes only the

	1				50
SHEEP	TGAAAGCCTA	CAGaCTTGAT	GGTGTcaACC	TTCGAGGaTA	TgcTGcCTGG
HUMAN	TGAAAGCCTA	CAGGCTcGAT	GGTaTagACC	TTCGAGGGTA	TgtcGCCTGG
RABBIT	TGAAAGCCTA	CAGGCTTGAT	GGTGTcaACC	TTCGAGGTA	TttTGcCTGG
RAT	TGAAAGCCTA	CaaGCTgGAT	GGTGTggACC	TTCGAGGGTA	ctcTGcCTGG
	51				100
SHEEP	TCcCTGATGG	ACAaCTTTGA	GTGGCTgaAT	GGCTACaCGg	TCAaATTtGG
HUMAN	TCTCTGATGG	ACAaCTTTGA	GTGGCTaaAT	GGCTACaCGg	TCAaGTTtGG
RABBIT	TCTCTGATGG	ACAaCTTTGA	GTGGCTtcaA	GGCTACActa	TCAaGTTtGG
RAT	aCgCTGATGG	AcgACTTcGA	GTGGCTGctt	GGCTACACca	TgAgaTTtGG
	101				150
SHEEP	ACTGTaCCAT	GTTGAtTTca	ATgAcGTGAA	CAGGtCTCGC	ACAGCAAGAG
HUMAN	ACTGTaCCAT	GTTGAtTTca	AcAAcacGAA	CAGGCCTCGC	ACAGCAAGAG
RABBIT	ACTGTaCCAT	GTTGAcTTtg	AaaATGTGAA	CAGGCCTCGC	ACAGCAAGaa
RAT	AtTGATaCAC	GTTGAcTTTa	ATcAtGTGAg	CAGaCCTCGC	ACAGCAAGAG
	151				170
SHEEP	CCTCAGCCAG	GTACTACACA			
HUMAN	CCTCcGCCAG	GTACTACACA			
RABBIT	tCTCAGCCAG	tTACTACACA			
RAT	CCTCAGCCAG	aTACTAtgCA			

Figure 1 Sequence comparisons

PCR products were separated from nucleotides and enzymes by passing them over a 1 ml G-50 spun column, equilibrated in a 1 × restriction buffer. Products were digested with *Bam*HI and *Pst*I, gel purified, subcloned and sequenced using vector-specific oligonucleotides flanking the polycloning site. The sequence alignment begins at base 3878 of the human LPH cDNA sequence, base 3903 of the rabbit LPH cDNA sequence, base 3914 of the rat cDNA and the first matching base of sheep LPH PCR product sequence. The sequences of the amplification oligonucleotides are not shown here (see Materials and methods). The rat LPH sequence is reproduced in a best fit with the other three. Best consensus sequences are capitalized. The degree of identity with all possible pair-wise comparisons is approximately 85%.

Figure 2 Developmental profile of LPH mRNA expression in ovine proximal jejunum

A standard curve (**b**) was generated using synthetic human LPH RNA as template in the PCR assay. A developmental profile of LPH mRNA from ovine proximal jejunum near the ligament of Treitz is presented here expressed in human LPH RNA amol equivalents. The values under each sheep band in (**a**) are relative amounts of sheep LPH RNA compared with the PCR product generated concurrently using human LPH RNA template. The experiment presented here was chosen for quantification because it had the cleanest standard curve of three similar determinations. Relative amounts of sheep LPH RNA are shown for young animals ranging in age from 34 days prepartum (–34 days) to 34 days postpartum, along with three adults. The template input for the three adults was 10-fold greater than with the lambs to ensure that the amount of each PCR product was in the quantitative range of the assay. All the young animals but one expressed a 10- to 100-fold relative excess in LPH mRNA compared with adults.

Figure 3 Western blot

Intestinal samples were prepared for protein assays by homogenization and centrifugation at 1000 *g* for 10 min, and 10 μ g of protein from each supernatant was electrophoresed through 6% (w/v) polyacrylamide gels under denaturing conditions and transferred to nitrocellulose for Western blotting. Duplicate lanes were run for each sample side-by-side. Two gels were required to hold all the samples, and one lane for each animal was selected and incised for this composite Figure. Blots were treated with an anti-human LPH antiserum and binding was detected with goat anti-rabbit immunoglobulin conjugated to HRP. LPH is relatively abundant in the fetal and lamb proximal jejunum (PJ) samples and markedly decreased in lamb colon. No LPH band was detected in adult PJ. This is consistent with a marked developmental decline in LPH protein expression after day 34 post partum that parallels the LPH mRNA expression pattern in Figure 2.

170 bp that lie between the oligonucleotide sequences. This accounts for the difference in the number of bases shown here (170 bp) and the size of the PCR product (242 bp).

We wanted to determine whether a decline in content of LPH

mRNA could account for the post-weaning reduction of lactase activity in sheep. RNAs extracted from full-thickness biopsies of the proximal jejunum of lambs and adult sheep were used as templates for amplification of the LPH target sequence (Figure 2). This site was chosen because specimen selection relative to the longitudinal axis of the gut is reproducible and LPH activity tends to be high. The amount of RNA used as template in the various reactions was empirically adjusted to ensure that the final assay produced amounts in, or nearly in, the quantitative range. While the assay can become limiting in the Southern blot hybridization, the amounts of PCR products in the experiments presented were in the linear range of the Southern hybridization procedure. The reverse transcription PCR was the limiting step. This led us to use 10 μ g of RNA from adult small intestine and only 1 μ g from lamb small intestine as template in the assay. Figure 2 shows the results obtained when the products of 24 rounds of amplification were analysed by Southern hybridization. LPH mRNA sequences were detectable in intestinal tissue obtained from fetuses 34 days before parturition (144 days is term), and, despite sample-to-sample variation, these sequences persist in high concentration until at least 34 days after birth. There is considerable variation from animal to animal in the proximal jejunum. The following observations should, however, be noted: (i) all of the lamb and fetal samples but one have more than 100 relative amol LPH mRNA/ μ g RNA, and all the adult samples have < 20 amol/ μ g; (ii) several other studies have shown greater variability in jejunal samples than in mid small intestine; and (iii) these sheep were mixed Western breeds (not inbred), increasing expected variation. Lambs naturally wean between days 30 and 50 post partum [21]. In parallel, the concentration of LPH mRNA is dramatically reduced in adult intestinal tissue. Table 1 shows that on average, RNAs extracted from adult tissues contain 48-fold fewer LPH sequences than fetal or newborn tissue and display approximately 40-fold less LPH activity. Thus, the trend of high LPH activity and mRNA followed by low LPH activity and mRNA in adults is clear, in

Figure 4 Villin control for template suitability

To test the suitability of each RNA sample in our LPH PCR assay, we performed a similar assay using oligonucleotides directed to villin. Villin is a component of microvillar membranes. This is a typical assay using RNA from the proximal jejunum of four lambs (lanes 1–4), three adult sheep (lanes 5–7), adult sheep kidney (lane 8), and control lacking RNA (lane 9). All RNA templates reported in this manuscript were tested using the villin oligonucleotides and proved suitable in the PCR assay.

spite of considerable variation between young animals. Tissue homogenates from the same biopsies were examined by Western blotting for the presence of immunoreactive LPH protein expression. Figure 3 shows that mature (160 kDa) LPH protein is readily detectable in each of the homogenates prepared from fetal and lamb proximal jejunum, while no LPH protein can be detected in homogenates prepared from the proximal jejunum of adult sheep or from lamb colon.

Another possible explanation for the variation in LPH mRNA was that some of the RNA samples were not suitable for amplification. We therefore tested all RNA samples for suitability by amplifying a larger (400 bp) region of villin mRNA. Villin was chosen because it is a structural component of microvilli and

is therefore present at all ages. A typical example of these control experiments is shown in Figure 4 including jejunum for some of the lambs, adult sheep, a positive control from adult kidney and an mRNA negative control. Clearly, all the template RNAs were suitable for our assay.

A second aim of this work was to investigate patterns of expression of LPH mRNA in different regions of small intestine from lambs of various ages. We focused on lambs because LPH mRNA and lactase activity were too low in the stomach, small intestine and colon in our adult animals to permit meaningful comparison (data not shown). Accordingly, the quantity of LPH mRNA was measured in samples of RNA prepared from duodenum, a second site in the jejunum 10 cm distal to the first, the mid-point of the small intestine, distal ileum and colon. The results obtained are shown in Figure 5 for duodenum, proximal jejunum and mid small intestine. Distal ileum and colon had levels that were too low to be quantified.

LPH mRNA was detectable in the duodenum of all the lambs studied. The levels were consistently lower than that seen in jejunum and mid small intestine. Furthermore, there was a clear age-related decline in LPH mRNA extracted from the lamb duodenum. The hallmark variability of LPH mRNA levels in the proximal jejunum is reproduced here. The levels were consistently high but have no other age-related pattern like that seen in the duodenum and mid small intestine. This variability in expression is reminiscent of the results obtained previously (Figure 2) with samples taken from a more proximal site in the jejunum. LPH sequences in the mid gut are abundant in young lambs and decline steadily, but slightly, with increasing age before weaning in lambs. Adult animals have neither lactase activity nor LPH mRNA detectable in the mid gut (data not shown). No LPH mRNA is detectable in RNA prepared from colon and extremely

Figure 5 Longitudinal profile of LPH mRNA expression in lamb small intestine

Relative levels of LPH mRNA from three additional sites in the ovine small intestine are presented for the lambs at the ages indicated and are expressed in human LPH RNA attomole equivalents. Duodenal samples were obtained halfway between the pylorus and ligament of Treitz. The jejunal samples were obtained 10 cm distal to the samples used in Figures 2 and 3, and the mid small intestine was selected halfway between the ligament of Treitz and the ileocecal valve. All assays began with 1 μ g total RNA. There is a marked age-related decline in LPH mRNA in the duodenum, variability in the proximal jejunum as was seen in Figure 2, and an age-related decline in the mid small intestine. As in Figure 2, the sheep LPH RNA determinations are relative amounts compared with PCR products generated in the standard assay using synthetic human LPH RNA as template. The experiment used for quantification had the cleanest standard curve of three similar assays.

low levels of LPH sequences are present in RNA purified from distal ileum (data not presented).

These data illustrate that the content of LPH mRNA in the small intestine of sheep undergoes both age-dependent and region-specific variation. It is unlikely that this apparent complexity results from variability in sample preparation or in the amplification process, since (i) the standard curves using synthetic LPH mRNA included in every experiment are reproducible; (ii) the age-independent variability of LPH mRNA content is confined to one region of the small intestine, i.e. the jejunum; and (iii) the templates were all shown to be suitable for the PCR assay with villin oligonucleotides. This variability is consistent with the observation of Maiuri et al. [33] that LPH expression is mosaic in the jejunum of humans. All other regions analysed, the mid-gut and duodenum for example, exhibit simple patterns of LPH expression that change in a consistent manner during development. The variability seen in the proximal jejunum could have been due to problems such as: (i) genomic DNA contamination of the RNA preparations, and (ii) unsuitability of RNA as template in some samples due to degradation. The first explanation was ruled out by performing the assays with and without reverse transcriptase. In no case was a 242 bp product generated without reverse transcriptase, confirming that the assay was absolutely dependent on RNA and not contaminating DNA. The second possibility was ruled out by using the same templates for synthesis of a 360 bp product based on villin sequences. Villin should be present in all intestinal biopsies, and indeed all templates were suitable for generating villin products.

The production of lactose in milk by mothers, and suckling by neonates, distinguishes members of the order *mammalia* from other vertebrates. The emergence of lactose as a major source of calories in mammalian milk required evolution of an enzyme in breast tissue that joins glucose and galactose through a 1,4 β -galactosidic bond. In parallel, the neonatal small intestine evolved a disaccharidase (LPH, lactase) that hydrolyses this bond and converts lactose into its constituent sugars, which can be efficiently absorbed by enterocytes [7]. If lactose cannot be hydrolysed, the disaccharide is fermented by bacteria in the colon, producing painful cramps, flatus and diarrhoea.

Expression of lactase declines before adulthood in most mammalian species. The mechanism controlling the down-regulation of LPH has been suggested to be primarily post-translational by some authors [13,34], pre-translational by others [18,35], and mixed by Keller et al. [19]. Troelsen et al. [20] have identified a putative nuclear factor from pigs that may account for developmental gene regulation. We suspect that the following may account for these discrepancies. (i) Standard laboratory animals are at least partially inbred and may therefore exhibit strain specific phenotypes; our sheep have been outbred intentionally to avoid these effects. (ii) Each of the laboratories studying LPH mRNA regulation have differences in experimental design. (iii) If the 3–4-fold decline in lactase activity were exactly paralleled at the RNA level, demonstration of the decline in RNA could be difficult to reproduce. We chose to study sheep because they exhibit an unequivocal fall in lactase activity from the newborn period to adulthood and convert from a monogastric to a ruminant type of digestion. Our results suggest that pre-translational mechanisms are primary regulators of LPH expression in sheep.

All the investigators in this field face an experimental design problem when choosing a denominator for quantitation of mRNA levels. Several methods have been used and may account for much of the variability seen in published experiments. These include: (i) total small intestine RNA [35]; (ii) total RNA from a full thickness sample obtained from a fixed anatomical position;

(iii) total RNA from mucosal scrapings; (iv) α -actin comparison; (v) β -actin comparison [13]; and (vi) villin comparison [15]. All of these components of quantification strategies have strengths, but none is perfect. We chose full-thickness biopsies because the depth of biopsies is absolutely reproducible. We chose not to use the α -actin comparison because it has the theoretical objection that it is primarily made by smooth muscle and is therefore not an ideal control for enterocytes. On the surface, β -actin would appear to be better than α -actin because its expression is considered to be more restricted to non-muscle cells. Sebastio et al. [13] used β -actin to standardize their Northern blots and the β -actin appeared to increase dramatically during development. Both α - and β -actin appear to be developmentally regulated and therefore are not ideal denominators. We, therefore, chose to compare moles LPH mRNA to μ g total RNA. This approach avoids comparing LPH RNA to another regulated gene product. We used this method to demonstrate a linear relationship between human LPH mRNA levels and lactase activity [18].

We have learned the following about the developmental profile of expression of LPH mRNA in sheep. (i) There is a strong downward trend in expression in the proximal small intestine from infant to adult sheep. (ii) Expression in the lamb proximal jejunum is variable, but high, and has no clear age-specific pattern before the major decline after weaning. (iii) Expression in the duodenum begins lower than in proximal jejunum and declines steadily even before weaning. (iv) The level of expression in the mid-gut is as high as in proximal jejunum, but more consistent, and declines steadily before weaning. (v) LPH mRNA is barely detectable in the distal ileum of lambs and undetectable in the colon.

Finally, we conclude that LPH specific activity, protein and mRNA levels tend to be co-ordinately regulated in sheep, and the primary site of ovine LPH regulation is almost certainly at the RNA level.

The authors thank Kevin Kuehn for synthesis of high-quality oligonucleotides and David W. Russell for the plasmid pCMV2 from which pSL7 was derived. This work was supported by NIH Grant K11-DK01860 and the Ministry for Research and Technology (BMFT), Bonn, Germany.

REFERENCES

- 1 Flatz, G. (1987) in *Advances in Human Genetics* (Harris, H. and Hirschhorn, K., eds.), pp. 1–77. Plenum Press, New York
- 2 Flatz, G. and Rothauwe, H. (1977) *Progr. Med. Genet.* **2**, 205–249
- 3 Henning, S. J. (1987) in *Physiology of the Gastrointestinal Tract* (Johnson, L., ed.), pp. 285–300. Raven Press, New York
- 4 Henning, S. J. (1985) *Annu. Rev. Physiol.* **47**, 231–245
- 5 Kretchmer, N. (1971) *Gastroenterology* **61**, 805–813
- 6 Montgomery, R. K., Buller, H. A., Rings, E. H. H. M. and Grand, R. J. (1991) *FASEB J.* **5**, 2824–2832
- 7 Paige, D. and Bayless, T. (1981) *Lactose Digestion: Clinical and Functional Implications*, Johns Hopkins University Press, Baltimore
- 8 Plimmer, R. H. A. (1906) *Am. J. Clin. Nutr.* **26**, 81–96
- 9 Rosensweig, N. S. (1973) *Am. J. Clin. Nutr.* **26**, 1166–1167
- 10 Leeper, L. L. and Henning, S. J. (1990) *Am. J. Physiol. Gastrointest. Liver Physiol.* **258**, G52–G58
- 11 Henning, S. J. and Sims, J. M. (1979) *Endocrinology* **104**, 1158–1163
- 12 Leberthal, E., Sunshine, P. and Kretchmer, N. (1973) *Gastroenterology* **64**, 1136–1141
- 13 Sebastio, G., Villa, M., Sartorio, R., Gussetta, V., Poggi, V., Auricchio, S., Boll, W., Mantei, N. and Semenza, G. (1989) *Am. J. Hum. Genet.* **45**, 489–497
- 14 Buller, H. A., Montgomery, R. K., Sasak, W. V. and Grand, R. J. (1987) *J. Biol. Chem.* **262**, 17206–17211
- 15 Freund, J.-N., Duluc, I. and Raul, F. (1991) *Gastroenterology* **100**, 388–394
- 16 Lloyd, M., Mevissen, G., Fisher, M., Olsen, W., Goodspeed, D., Genini, M., Boll, W., Semenza, G. and Mantei, N. (1992) *J. Clin. Invest.* **89**, 524–529
- 17 Escher, J. C., de Koning, N. D., van Engen, C. G. J., Arora, S., Bueller, H. A., Montgomery, R. K. and Grand, R. J. (1992) *J. Clin. Invest.* **89**, 480–483
- 18 Fajardo, O., Naim, H. Y. and Lacey, S. W. (1994) *Gastroenterology* **106**, 1233–1241

- 19 Keller, P., Zwicker, E., Mantei, N. and Semenza, G. (1992) *FEBS Lett.* **313**, 265–269
- 20 Troelsen, J. T., Olsen, J., Noren, O. and Sjostrom, H. (1992) *J. Biol. Chem.* **267**, 20407–20411
- 21 Shirazi-Beechey, S. P., Kemp, R. B., Dyer, J. and Beechey, R. B. (1989) *Comp. Biochem. Physiol.* **94**, 801–806
- 22 Shirazi-Beechey, S. P., Smith, M. W., Wang, Y. and James, P. S. (1991) *J. Physiol. (London)* **437**, 691–698
- 23 Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299
- 24 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn, vols. 1–3 (Ford, N., Nolan, C. and Ferguson, M., eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- 25 Mantei, N., Villa, M., Enzler, T., Wacker, H., Boll, W., James, P., Hunziker, W. and Semenza, G. (1988) *EMBO J.* **7**, 2705–2713
- 26 Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 5463–5467
- 27 Dahlqvist, A. and Asp, N.-G. (1971) *Anal. Biochem.* **44**, 654–657
- 28 Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
- 29 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 30 Hauri, H.-P., Sterchi, E. E., Bienz, D., Fransen, J. A. M. and Marxer, A. (1985) *J. Cell Biol.* **101**, 838–851
- 31 Naim, H. Y., Lacey, S. W., Sambrook, J. F. and Gething, M. J. H. (1991) *J. Biol. Chem.* **266**, 12313–12320
- 32 Dulac, I., Boukamel, R., Mantei, N., Semenza, G., Raul, F. and Freund, J.-N. (1991) *Gene* **103**, 275–276
- 33 Maiuri, L., Rossi, M., Raia, V., O'Auria, S., Swallow, D. and Quaroni, A. (1992) *Gastroenterology* **103**, 1739–1746
- 34 Freund, J.-N., Dulac, I., Foltzer-Jourdainne, C., Gosse, F. and Raul, F. (1990) *Biochem. J.* **268**, 99–103
- 35 Buller, H. A., Kothe, M. J. C., Goldman, D. A., Grubman, S. A., Sasak, W. V., Matsudaira, P. T., Montgomery, R. K. and Grand, R. J. (1990) *FEBS Lett.* **248**, 39–42