

# Multiple Levels of Control of the Stage- and Region-Specific Expression of Rat Intestinal Lactase

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**Abstract.** To elucidate the mechanisms leading to the functional regionalization of the digestive epithelium, lactase expression was analyzed at the protein, mRNA and gene levels, along the intestinal tract at various stages of the rat postnatal development. In the colon of neonates, the transient expression of mRNA and enzyme correlated well with gene transcription. In contrast to the colon, complex patterns were observed in the small intestine. In suckling animals, the mRNA was present at a high level despite the progressive decline of enzyme activity. Crypts were devoid of mRNA and the transcript mainly accumulated in the lower half of the villi. From weaning onwards, a functional regionalization of the epithelium was defined, characterized by the modification of the longitudinal distribution of lactase mRNA. Indeed the transcript remained abundant in the distal duodenum, jejunum and proximal ileum, but decreased in the proximal duodenum and became virtually absent in the distal ileum. Concomitantly, the mRNA and enzyme distribution along the villi changed in the different segments of the small intestine. Patterns similar to those described in sucklings were retained in the adult jejunum. In contrast, mRNA and enzyme could no longer be detected in the distal ileum, while mosaicism appeared in the proximal duodenum. In vitro transcrip-

tion assays carried out with isolated nuclei suggested that the decay of lactase mRNA in the proximal duodenum at weaning was associated with a decreasing rate of transcription of the gene. However active gene transcription was retained in the nuclei of the adult jejunum and ileum. The loss of mRNA in the adult distal ileum despite the maintenance of active transcription did not result from an intragenic block of pre-RNA elongation, as shown by transcription assays carried out at various positions of the lactase gene. In addition, we looked for the ontogenic decline of lactase protein despite the maintenance of a high amount of mRNA in the jejunum, and it became evident that the fraction of mRNA present in polysomes was constant with age. Taken together, these data indicate that lactase constitutes an unusual marker of development and of functional regionalization of the intestinal tract which exhibits a complex time- and space-specific pattern of gene, mRNA, and protein expression. The distinct patterns occurring in the duodenum, jejunum, ileum, and the colon of pre- and postweaned rats depend on a combination of transcriptional, posttranscriptional, and posttranslational levels of regulation, and are associated with a different mRNA distribution along villi in each intestinal segment.

**T**HE intestinal epithelium of mammals represents an attractive model for studying cell differentiation because it is continuously renewed from pluripotent stem cells (Gordon, 1989). Once intestinal organogenesis is achieved in embryos from the association of endodermal and mesodermal sheets, the epithelium exhibits a similar organization all along the antero-posterior axis of the intestinal tract, with two structurally and functionally distinct compartments: invaginated crypts or glands containing stem

cells and proliferative cells, and evaginated villi (in the small intestine) or polygonal cuffs (in the colon) lined with differentiated cells specialized for the digestion and absorption of nutrients. The continuous renewal of the intestinal epithelium leads to the emergence from crypts of ribbons of differentiated enterocytes that migrate toward the villus tip. Crypts are polyclonal when they form in fetuses or at birth as invaginations of the intervilli regions into the lamina propria. From this point, they progressively become monoclonal as shown from studies of X-inactivation mosaic mice or using mouse aggregation chimeras (Ponder et al., 1985; Griffiths et al., 1988; Schmidt et al., 1988). Yet despite the similar organization of the digestive mucosa seen throughout

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the length of the intestinal tract, functional differences arise during development leading to a regional specialization of the epithelium which is well exemplified in the case of the colon compared to the small intestine.

The ontogeny of the intestinal tract has been widely studied in altricial species such as the rat because of their immaturity at birth which implies considerable changes up to weaning (Henning, 1987; Dauça et al., 1990). These studies found that the enzyme activity involved in the digestion of milk lactose and glycosylceramides, i.e., Lactase-Phlorizin Hydrolase (LPH,<sup>1</sup> EC 3.2.1.23–62), declines at weaning, at the stage otherwise characterized by the appearance or the rise of most digestive enzymes required for the assimilation of the solid food of adult animals. In addition, it is worth noting that functional differences among regions of the intestinal tract become more pronounced at weaning, especially at the level of the small intestine.

The aim of the present work was to investigate the molecular mechanisms involved in the regulation of the spatial and temporal expression of a marker of enterocyte differentiation. For this purpose, LPH was chosen on the basis of three criteria: the enzyme is already expressed at a high level at birth; its mRNA remains abundant at the adult stage despite the ontogenic decline of enzyme activity (Freund et al., 1989; Sebastio et al., 1989); regional differences in its pattern of expression emerge during postnatal development (Foltzer-Jourdainne et al., 1989; Freund et al., 1990a, 1991a). For this study, measurements of gene transcription and rates of mRNA and enzyme accumulation were combined with analyses of the *in situ* distribution of the transcript and protein. Experiments were conducted along the small intestine and in the colon of newborns, sucklings, and adult rats. The results show that LPH expression is regulated by multiple molecular mechanisms which act at the transcriptional, posttranscriptional, and posttranslational levels depending on the developmental stage and the region of the intestinal tract. Furthermore, the quantitative changes in lactase expression occurring during postnatal development are accompanied by the emergence of proper cellular patterns of mRNA and enzyme distribution along the crypt-villus axis in each region of the intestinal tract.

## Materials and Methods

### Animals and Tissues

4 day-old neonates, 14 day-old sucklings, and 3 month-old adult Wistar rats from our own breeding colony were used. Intestinal segments were carefully recovered in each animal as follows: the duodenum extending from the pylorus to the ligament of Treitz was subdivided into proximal and distal halves; the jejunoleum from the ligament of Treitz to the ileocecal junction, was divided into four segments of identical length that corresponded to the proximal and distal jejunum and to the proximal and distal ileum; and finally, the proximal half of the colon was taken. The whole intestinal tissue was used in neonates and in one group of sucklings, whereas the mucosa was scraped with glass slides in the second group of sucklings and in adults. Similar data were obtained in both groups of sucklings so that the results illustrated here were only those coming from animals for which the mucosa was scraped. Experiments were conducted on pools of 10–12 neonates, 5–7 sucklings, and 2 adult animals.

1. *Abbreviations used in this paper:* C, colon; DI, distal ileum; LPH, lactase-phlorizin hydrolase; PD, proximal duodenum; PJ, proximal jejunum; TPI, triosephosphate isomerase.

### Protein and RNA Analyses

Lactase activity was assayed on intestinal homogenates in the presence of 0.1 mM *p*-chloro-mercuri-benzoate, and expressed as specific activity (mU/mg protein) where 1 mU hydrolyzed 1 nmol of lactose per min at 37°C (Freund et al., 1991a). The proteins of the homogenates were suspended in denaturing buffer, separated by SDS-PAGE, and transferred to nitro-cellulose membranes (Schleicher and Schuell, Kassel, Germany) as described by Quan et al. (1990). They were incubated with rabbit anti-rat-LPH antiserum diluted to 1:1,500 (Nsi-Emvo et al., 1986) or with mouse anti-actin monoclonal antibodies used at a 1:2,000 dilution (Amersham, Les Ulis, France). The primary antibodies bound on the membranes were revealed by autoradiography using a polyclonal anti-rabbit-immunoglobulin antibody or a polyclonal anti-mouse-immunoglobulin antibody labeled with <sup>125</sup>I (28-111TBq/mmol, Amersham, Les Ulis, France). RNA preparation, Northern blotting, and hybridization with <sup>32</sup>P-labeled DNA probes for LPH and  $\beta$ -actin were performed according to standard procedures (Freund et al., 1990a, 1991a).

### Nuclear Transcription Assays

Intestinal nuclei were prepared according to Schibler et al. (1983) and stored at –80°C in 20 mM Tris-HCl pH 8, 75 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, 0.125 mM PMSF, 50% glycerol. Before use, they were observed by light microscopy in the presence of Trypan blue. Newly synthesized transcripts were radiolabeled and processed as described by Wicker and Puigserver (1990) with some modifications. One volume of transcription buffer containing 50 mM Tris-HCl pH 8, 5 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>, 4 mM DTT, 300 mM NH<sub>4</sub>Cl, 1 mg/ml BSA, 5 U/ml RNasin (Appligène, Strasbourg, France), 1 mM ATP, GTP, CTP, 3.7MBq [ $\alpha$ -<sup>32</sup>P]UTP (30TBq/mmol, Amersham, Les Ulis, France) was added to the nuclei. The reaction was incubated 30 min at 25°C and stopped by the addition of nine volumes of 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM KCl, 1 mM EDTA and 0.5% SDS. The solution was incubated 30 min at 37°C with 100  $\mu$ g/ml Proteinase K (Sigma, St Quentin, France), and then with 200 U/ml RNase-free DNase (Boehringer, Mannheim, Germany). Nucleic acids were extracted with phenol/chloroform, taken up in 0.5 M NaCl, 50 mM Pipes pH 7, 35% formamide, 0.1% SDS, 2 mM EDTA, and hybridized 48 h to 5  $\mu$ g of denatured DNA spotted on nitro-cellulose filters. The filters were washed twice 15 min in 2 $\times$  SSC (1 $\times$  SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.2), 0.1% SDS at 30°C, twice 30 min in 1 $\times$  SSC, 0.1% SDS at 50°C and incubated 1 h at 37°C in 2 $\times$  SSC, 1  $\mu$ g/ml RNase A (Sigma, St Quentin, France). The radioactivity retained on the filters was revealed by autoradiography. Spotted DNA used for this study corresponded to the vector pBluescript (Stratagene, La Jolla, CA) and to the plasmids pHF $\beta$ A3UT, pRLU6, and p3T, containing respectively, a fragment of the  $\beta$ -actin cDNA (Ponte et al., 1983), the nearly full-length LPH cDNA (Duluc et al., 1991) and the pseudogene for Triosephosphate Isomerase (TPI; Mehtali, 1988). For some experiments, restriction fragments of the LPH cDNA and the 0.7 kb EcoRI fragment of plasmid pRL5E were eluted from agarose gels and spotted separately on filters. pRL5E contains a segment of the first intron of the rat LPH gene (Boukamel and Freund, 1992).

### Preparation of Polysomes

Polysomal RNA was prepared as described by Clements (1984). Briefly, jejunal tissue samples were homogenized in 25 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mg/ml heparin, 2% Triton X-100, 25 mM Tris-HCl pH 7.5, and centrifuged at 27,000 g. The supernatant was suspended in the previous solution supplemented with 0.2 M MgCl<sub>2</sub>, placed on ice for 1 h, and loaded on a 1 M sucrose solution with 25 mM NaCl, 0.1 mM MgCl<sub>2</sub>, 25 mM Tris-HCl pH 7.5. After centrifugation at 27,000 g, the pellet containing the polysomes was suspended in 1% SDS, 0.2 M NaCl, 40 mM EDTA, and 20 mM Tris-HCl pH 7.5. The polysomal fraction and the cytoplasmic supernatant were heated 2 min at 100°C, incubated 10 min at 30°C with 0.5 mg/ml Proteinase K (Sigma, St Quentin, France), and the ribonucleic acids were extracted with phenol/chloroform. They were suspended in 5 $\times$  SSC, 50% formamide and spotted on nitro-cellulose filters. Hybridizations with <sup>32</sup>P-labeled DNA probes for LPH and  $\beta$ -actin were carried out as for the Northern blots.

### Quantification of the Results

Nitro-cellulose pieces with blotted proteins, mRNAs or newly synthesized pre-RNAs for LPH and  $\beta$ -actin were identified by autoradiography, cut out, and the radioactivity was counted by liquid scintillation spectrometry. The amounts of LPH pre-RNA, mRNA, and protein were related to those of ac-

tin and expressed as a percentage of the values obtained in the proximal jejunum of neonates. Mean values were calculated from three independent pools of samples at each developmental stage.

### Immunocytochemistry

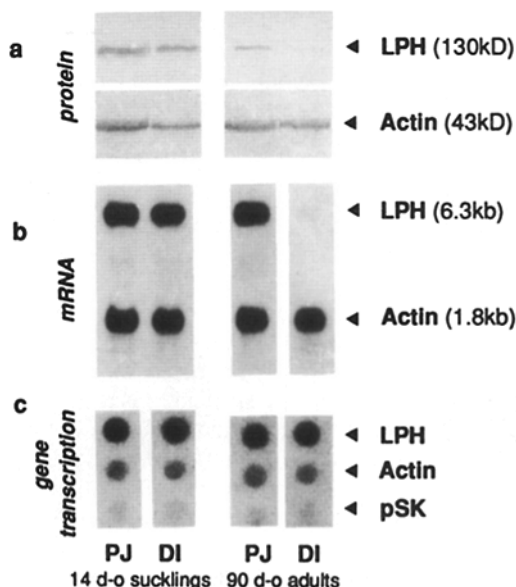
Samples originating from the midpoint of the proximal duodenum, of the proximal jejunum, and of the distal ileum were collected in 14 d-old and in 3 month-old rats. They were fixed with 2% paraformaldehyde in 0.1 M piperazine-1,4 (2-ethanesulfonic acid) buffer pH 7 at 4°C for 1 h, embedded in Tissue-Tek (Miles Inc., Elkhart, IN) and frozen in liquid nitrogen. Cryosections of 5  $\mu$ m were incubated 2 h at room temperature with a 1:50 dilution of the murine monoclonal antibody FBB3421 raised against rat LPH (Quaroni and Isselbacher, 1985), rinsed three times in PBS, and then immunolabeled for 2 h with a 1:100 dilution of goat anti-mouse-immunoglobulin antibody coupled to fluorescein isothiocyanate (Pasteur, Paris, France). Slides were rinsed in PBS, mounted in glycerol/PBS/phenylene-diamine under coverslips and observed with an Axiophot Microscope (Carl Zeiss, Thornwood, NY). Control sections were processed identically except that the monoclonal antibody FBB3421 was omitted. No specific immunofluorescent staining was detected under this condition (not illustrated).

### In Situ Hybridization

Samples were collected as described above for immunocytochemistry, fixed in 4% paraformaldehyde for 48 h at 4°C, and embedded in paraffin. 5- $\mu$ m sections were deparaffinized in toluene, rehydrated, and treated for 1/2 h at 37°C with 10–40  $\mu$ g/ml Proteinase K (Sigma, St Quentin, France). They were rinsed in PBS, fixed again with 4% paraformaldehyde in PBS for 15 min, dehydrated in graded series of ethanol, and air-dried. Slides were pre-hybridized 1 h at 37°C in 4 $\times$  SSC, 50% deionized formamide, 0.2 mg/ml salmon sperm DNA, 0.5 mg/ml *E. coli* tRNA, 0.02% polyvinylpyrrolidone, 0.02% Ficoll. They were then hybridized overnight at 37°C in the previous solution supplemented with 5% sulphate dextran and with 0.2  $\mu$ g/ml digoxigenin-labeled DNA probe. The sections were successively washed 20 min at 37°C in 4 $\times$  SSC/30% formamide, 2 $\times$  SSC/30% formamide, and 1 $\times$  SSC/30% formamide. Subsequently they were placed for 1/2 h in PBS, 0.3% BSA, 0.3% Tween 20, and then incubated overnight at room temperature with a 1:1,500 dilution of anti-digoxigenin antibody coupled to alkaline phosphatase (Boehringer, Mannheim, Germany). Slides were washed three times in PBS, 0.3% Tween 20, and alkaline phosphatase activity was revealed by incubation with 0.3 mg/ml 4-nitroblue-tetrazolium chloride, 0.15 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (Boehringer, Mannheim, Germany) in 0.1 M carbonate buffer pH 9.8. We used as probe the 6.3-kb LPH cDNA contained in plasmid pRLU6 (Duluc et al., 1991). This cDNA was eluted from agarose gels, cut into small fragments by incubation with the restriction enzyme *Sau*3A, and labeled with digoxigenin-11-2'-deoxyuridine-5'-triphosphate (Boehringer, Mannheim, Germany). Controls for in situ hybridization were conducted in parallel for all the experimental series. They included in situ hybridizations carried out (a) in the absence of LPH cDNA probe, (b) using as labeled probe the vector pBluescript (Stratagene, La Jolla, CA), and (c) using the LPH cDNA probe after the tissue sections were preincubated for 30 min at 37°C with 50  $\mu$ g/ml RNase A (Sigma, St Quentin, France). No significant staining could be detected under these conditions (not shown). In particular, the endogenous activity of alkaline phosphatase present in the intestinal brush border membrane was almost completely destroyed by the treatment with Proteinase K.

### Results

LPH expression was analyzed in the consecutive segments of the intestinal tract in 4 d-old neonates, 14 d-old sucklings, and 3 month-old adult rats. In this study we determined the specific activity of lactase, the rate of accumulation of the mature LPH protein, the rate of accumulation of the corresponding mRNA, and the rate of transcription of the *LPH* gene by transcription assays conducted in vitro with purified cell nuclei.  $\beta$ -actin was chosen as internal standard, thus allowing simultaneous measurements for LPH and actin at each of the protein, mRNA, and gene level. The distribution of the enzyme and of its transcript was also investigated



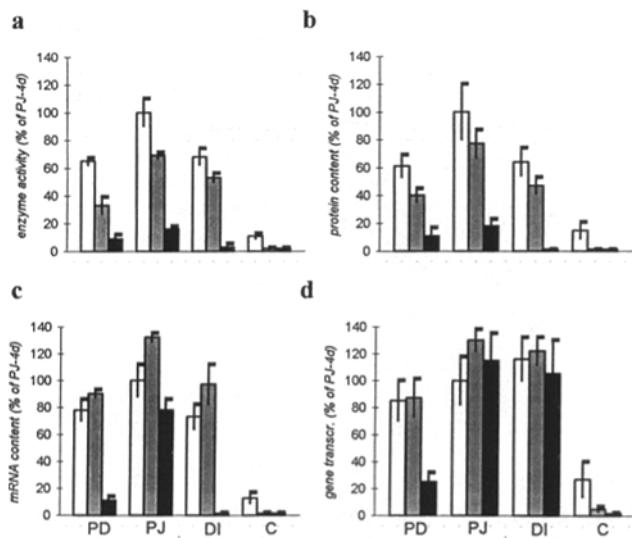
**Figure 1.** Protein accumulation (a), mRNA accumulation (b), and pre-RNA synthesis (c) of LPH and actin. (a) Intestinal proteins (70  $\mu$ g/lane) were separated by SDS-PAGE. LPH and actin were detected with the respective antibodies and revealed using second antibodies labeled with  $^{125}$ I. (b) Cytoplasmic RNA (20  $\mu$ g/lane) was run on denaturing agarose gels and hybridized to  $^{32}$ P-labeled cDNA probes for LPH and actin. (c) Nuclear transcripts were labeled in vitro in intestinal cell nuclei with  $^{32}$ P-UTP and hybridized to the cDNA for LPH, actin (*Act*), and to the plasmid pBluescript (*pSK*). The experiments were carried out in the proximal jejunum (*PJ*) and distal ileum (*DI*) of 14 day-old sucklings and 90 day-old adult rats.

along the crypt-villus axis by immunocytochemistry and in situ hybridization. Similar patterns of LPH expression were obtained in the distal duodenum, proximal and distal jejunum, and the proximal ileum, whereas differences appeared in the proximal duodenum and the distal ileum. The colon exhibited a distinct pattern compared to the small intestine. Thus, the results reported here are restricted to those coming from the proximal duodenum (PD), proximal jejunum (PJ), distal ileum (DI), and the colon (C). Fig. 1 shows an example of the experimental data obtained for lactase and actin in the PJ and in the DI of sucklings and adult rats. Quantitative measurements are given in Fig. 2 and discussed below.

### Lactase Activity, and LPH Protein, and mRNA Accumulation

We have determined the enzyme activity of lactase, the amount of lactase protein and the amount of LPH mRNA in intestinal samples pooled from 2 to 12 rats, depending on the age of the animals. Examination of data obtained from single rats indicated that intraindividual variations did not exceed 10% of the mean values.

The specific activity of lactase paralleled the amount of mature 130 kD enzyme at each developmental stage and in each region of the intestinal tract (Fig. 2, a and b). This observation corroborates the results of Tsuboi et al. (1985) suggesting that the ontogenic modification of lactase activity does not primarily result from changes in the kinetic proper-



**Figure 2.** Specific activity of lactase (a), LPH protein content (b), LPH mRNA content (c), and LPH gene transcription (d) during rat postnatal development. Values are expressed as a percentage of those obtained in the proximal jejunum of 4 day-old rat neonates (% of PJ-4d). (PD) Proximal duodenum; (PJ) proximal jejunum; (DI) distal ileum; (C) colon. The specific activity of lactase was determined in cell homogenates, and 100 percent of activity corresponded to 53 mU/mg of protein. Measurements were performed in 4 day-old neonates (□), 14 day-old sucklings (▨), and 90 day-old adults (■). SEM is given for three separated experiments performed on distinct samples.

ties of the enzyme. The 220-kD precursor form of LPH was not measured as it was barely detected by the procedure used in this study. The spatial and temporal patterns of lactase protein expression were in accordance with those commonly reported. Indeed, the enzyme was present in large quantities in all areas of the small intestine in neonates and progressively decreased during the suckling period. After weaning it reached a five- to tenfold lower level in the PD and PJ, and was virtually absent in the DI. In the colon, lactase was exclusively detected in rat neonates at a very low level.

Consistent with the protein data, the 6.3-kb LPH mRNA was more abundant in the small intestine than in the colon of neonates (Fig. 2 c). In the colon, the transcript disappeared thereafter as shown above for the enzyme. In the small intestine, the situation was different because a discrepancy appeared between the patterns of enzyme and mRNA accumulation. Thus, two temporally independent events might be distinguished: first, the amount of LPH mRNA slightly rose in all areas of the small intestine during the suckling period, despite the progressive decline of protein; second, the longitudinal distribution of the transcript was modified at weaning. Indeed, the LPH mRNA showed a slight reduction in the PJ of adult animals, whereas it exhibited a ninefold decrease in the PD and became almost completely absent in the DI. Thus, unlike the uniform distribution observed in preweaned animals, a high level of LPH mRNA was retained only in the middle part of the small intestine at the adult stage, while reduction and disappearance of this transcript occurred at the proximal end and in the most distal quarter. Our previous studies have shown that

the presence of abundant LPH mRNA was maintained in the jejunum throughout adulthood, except in old animals where a slight decrease was seen (Freund et al., 1991a).

### LPH Gene Transcription

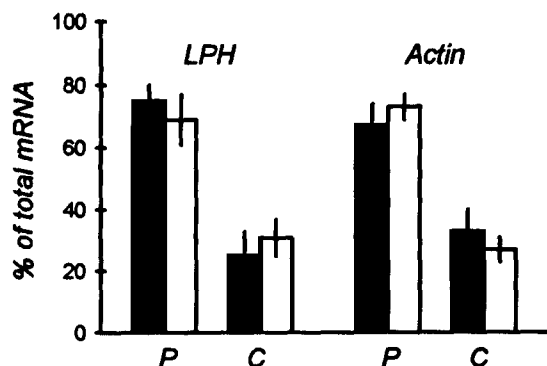
The rate of transcription of the LPH gene was determined by in vitro transcription assays conducted with cell nuclei prepared from the various segments derived from the intestinal tract (Fig. 2 d). The experiments were carried out on pools of 2–12 intestinal samples because the yield of isolated cell nuclei (especially in neonates) was insufficient to allow measurements on individual samples. As expected, the LPH gene was actively transcribed in all the regions of the small intestine and colon of rat neonates. Specific transcription could no longer be detected in the colon at later developmental stages. Unlike the colon, in vitro assays carried out with small intestinal samples showed that LPH gene transcription remained active in sucklings and in adult animals as well, except in the PD after weaning. Thus, the decline of LPH mRNA observed in the PD epithelium of postweaned rats correlated well with a drop of the rate of gene transcription in those cell nuclei. On the contrary, it should be emphasized that the loss of LPH mRNA in the DI at the adult stage occurred in spite of the maintenance of a high rate of gene transcription in the nuclei prepared from this part of the small intestine. Control experiments showed consistent hybridization of labeled nuclear transcripts to the DNA of the housekeeping gene encoding TPI (see Fig. 4 c), whereas hybridization was barely detected to the plasmid pBluescript (Figs. 1 and 4 c). Furthermore, we routinely recovered the labeled mixture containing the nuclear transcripts after hybridization to the nitro-cellulose filter and incubated it with a second filter on which LPH,  $\beta$ -actin, and TPI DNA has been spotted. In this case, a minor amount of radioactivity was retained on the second filter, indicating that the transcripts for LPH,  $\beta$ -actin, and TPI were almost completely extracted from the labeled mixture during the first round of hybridization (not shown).

### Polysomal LPH mRNA

The data reported above (Fig. 2) indicate that the ontogenic decline of lactase activity and protein in the middle part of the small intestine occurs despite the maintenance of active in vitro gene transcription and the maintenance of abundant mRNA in the digestive mucosa. To get further insight into the mechanism(s) leading to discrepancy between enzyme and mRNA patterns, we have compared the proportion of LPH transcript contained in the polysomal cell fraction in sucklings and adult rats. For this purpose, scraped mucosa of the PJ of 14 d-old sucklings and of 3 month-old adult animals were homogenized and the polysomes were separated from the cytosol by precipitation with  $Mg^{++}$  cations. As illustrated in Fig. 3, a similar amount of LPH and  $\beta$ -actin mRNA was recovered in the polysomal fraction in both groups of animals. These data suggest that the transcripts were equally committed into the translation machinery in pre- and postweaned animals.

### Transcription Along the LPH Gene

The present study shows that the LPH mRNA levels are very low in the DI epithelium from weaning onwards but in con-

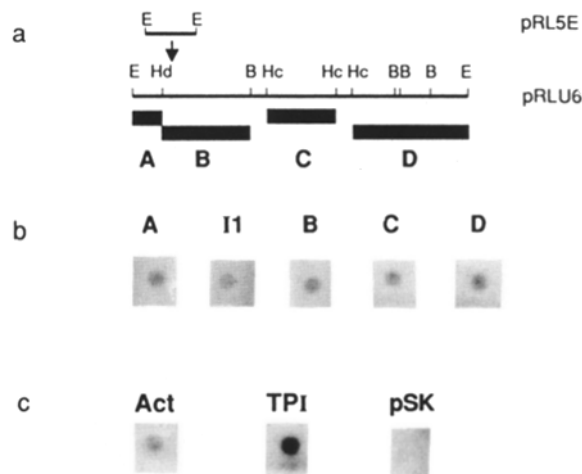


**Figure 3.** Polysome-associated LPH mRNA in jejunal enterocytes. The proportion of LPH and  $\beta$ -actin mRNA was determined in the polysome (P) and cytosol (C) cell fractions prepared from scraped mucosa of the proximal jejunum of 14 day-old suckling (■) and 3 month-old adult rats (□). Results are respectively given as a percentage of the total amount of LPH and  $\beta$ -actin mRNA. SEM is given for three separated experiments performed on distinct samples.

trast that the *LPH* gene remains actively transcribed in cell nuclei prepared from the DI of adult animals, as determined by hybridization of in vitro-synthesized nuclear transcripts to the nearly full-length LPH cDNA (see Fig. 2, c and d). Discrepancy between gene transcription and mRNA accumulation has already been shown to result either from an intragenic block of pre-RNA elongation (Spencer and Groudine, 1990) and/or from the rapid degradation of the transcript (Shapiro et al., 1987). To test these possibilities, experiments were conducted to analyze the elongation process of the pre-RNA through the *LPH* gene. For this purpose, transcription assays were performed with cell nuclei prepared from the DI of adult rats, and the newly synthesized transcripts were hybridized to various restriction fragments of the LPH cDNA and to a segment of the first intron of the *LPH* gene. An example of the results is illustrated in Fig. 4. It appears that a large amount of radioactivity was retained by all the DNA fragments, even those corresponding to the middle and to the 3' portions of the gene, suggesting that the full-length pre-RNA was actually synthesized during these experiments. Hence, these data support that the absence of cytoplasmic LPH mRNA in the DI epithelium of adult rats may not result from an intragenic block of pre-RNA elongation.

#### Distribution of the LPH Protein Along the Crypt-Villus Axis

The crypt-to-villus distribution of lactase protein was analyzed in the small intestine by immunocytochemistry using a monoclonal anti-LPH antibody prepared and characterized by Quaroni and Isselbacher (1985). Using similar methodology, Foltzer-Jourdainne et al. (1989) have reported that the transient expression of lactase in the neonatal colon correlated well with the presence of enzyme at the border of short villi. In suckling pups, the staining we obtained was similar in all the regions of the small intestine. As illustrated for the DI of 14 d-old animals (Fig. 5 A), the enzyme concentrated at the apical pole of the enterocytes and was present

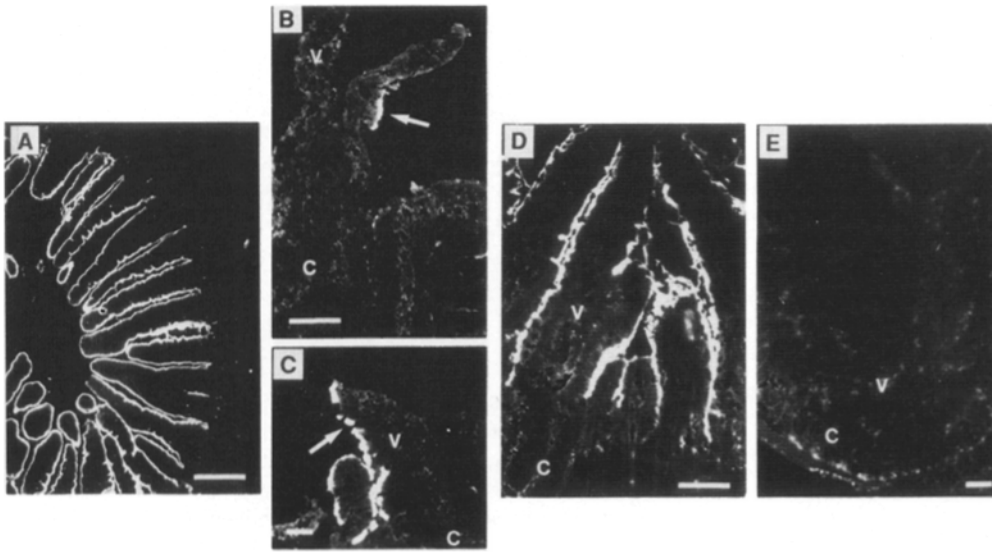


**Figure 4.** Elongation of the LPH pre-RNA in the nuclei prepared from the distal ileum of adult rats. (a) Restriction map of the LPH cDNA contained in the plasmid pRLU6 for the restriction enzymes BamHI (B), EcoRI (E), HincII (Hc), and HindIII (Hd). This cDNA lacks 29 bp at the 5' end to be full-length. The 0.7-kb EcoRI fragment of plasmid pRL5E contains a segment of the first intron of the rat *LPH* gene. The arrow denotes the position of this intron in the cDNA sequence. The cDNA fragments A-D, as well as the 0.7-kb intron fragment (I) were spotted on nitro-cellulose filters to hybridize to the labeled nuclear transcripts. (b) Hybridization of labeled transcripts synthesized in vitro in cell nuclei of the distal ileum of adult rats to the restriction fragments A-D of the LPH cDNA and to the fragment I1 of the first intron of the *LPH* gene. (c) Control hybridization with  $\beta$ -actin (*Act*) and TPI DNAs, and with the plasmid pBluescript (*pSK*).

on the whole surface of the villi except at the very tip. Immunofluorescent staining was absent in the crypts. In adult rats, the situation differed in that each region exhibited a particular pattern. In the PJ, lactase protein was present on the whole surface of the villi (Fig. 5 D), whereas specific immunolabeling could no longer be detected in the DI (Fig. 5 E). In addition, the PD exhibited a complex pattern, as some villi were stained on their whole surface whereas others were not stained at all. In addition, a third group of villi exhibited mosaicism in that immunofluorescent staining appeared on one side only or in restricted areas at the villi surfaces (Fig. 5, B and C).

#### In Situ Hybridization of the LPH mRNA

The distribution of LPH mRNA along the crypt-villus axis was investigated in the various regions of the small intestine in suckling and adult rats by in situ hybridization to cDNA probes labeled with digoxigenin and revealed using anti-digoxigenin antibodies coupled to alkaline phosphatase. In these experiments, specific hybridization could never be detected in the crypts. As shown for the DI of 14 d-old animals, the LPH mRNA appeared at the crypt-villus junction, and was prominent at the villus base compared with the tip (Fig. 6 A). This vertical pattern recurred in all regions of the small intestine (not shown). However, in adult rats each small intestinal region exhibited a specific picture. Indeed, a similar expression pattern to the one described for sucklings was observed in the PJ (Fig. 6 D), whereas LPH mRNA could no

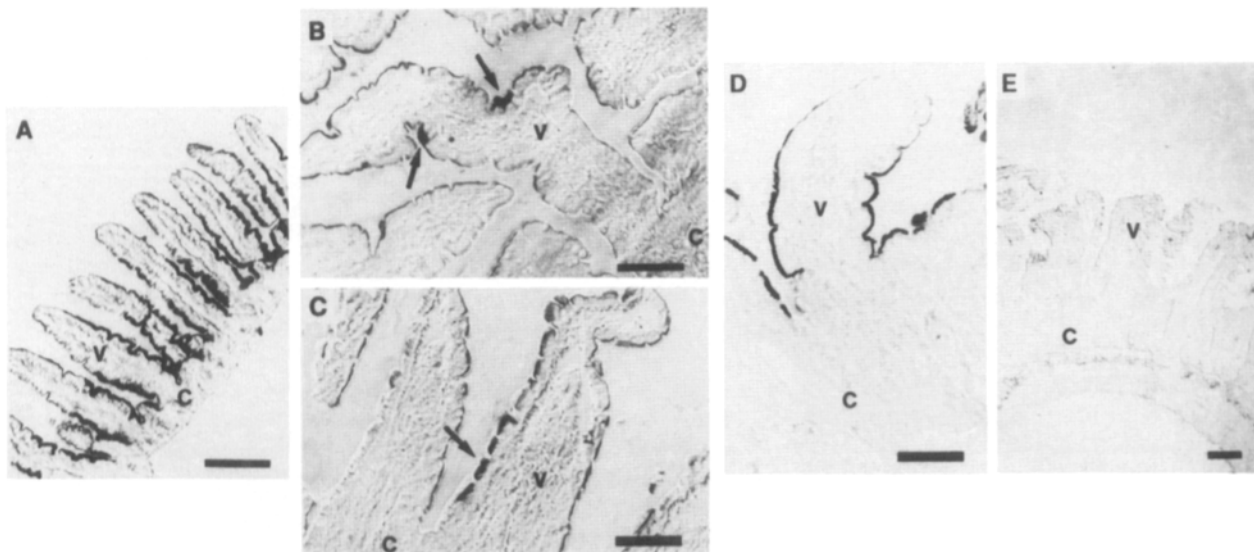


**Figure 5.** Distribution of the lactase protein along the crypt-villus axis. Lactase protein was revealed with the monoclonal antibody FBB3421. Whatever the small intestinal region and the developmental stage, immunofluorescent staining could never be detected in the crypts (c). In 14 day-old suckling rats, staining was present on the whole surface of the villi throughout the length of the small intestine, as illustrated for the distal ileum (A). In 3 month-old adult animals, all the enterocytes lining the villi (v)—except the tip—were immunostained in the proximal jejunum (D), whereas specific staining could no

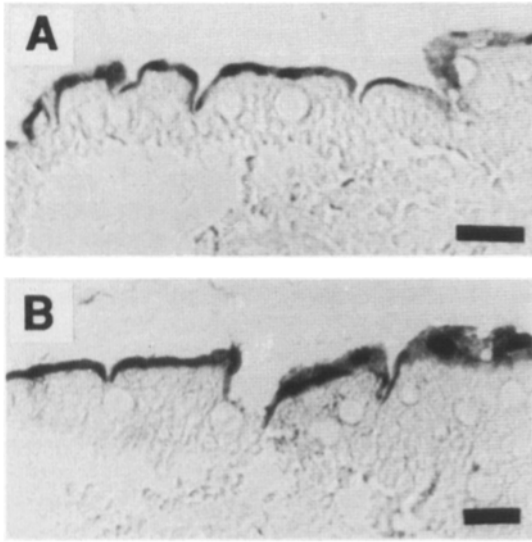
longer be detected in the distal ileum (E). In the proximal duodenum (B and C), some villi exhibited immunofluorescent staining on one side only, or in patches of immuno-reactive enterocytes (arrows). The signal observed in the lamina propria is due to the presence of immunocompetent cells that react with the second antibody labeled with FITC, as shown by the persistence of this aspecific labeling in controls in which the primary antibody was omitted. The exposure time of the photomicrographs was three times longer for samples of adult animals compared with sucklings, so that the results may not have quantitative implications. Bars, 100  $\mu\text{m}$ .

longer be detected in the epithelial cells of the DI (Fig. 6 E). In addition, mosaicism was found in the PD, as shown previously for the protein. Some villi were labeled on both sides as in the PJ, whereas others were completely devoid of LPH mRNA. In addition, a third population of villi showed labeling one side only, or at the level of restricted groups of enterocytes (Fig. 6, B and C).

In the in situ hybridization experiments carried out for this study, we have observed that the LPH mRNA was not evenly distributed in the cytoplasm of the epithelial cells. As illustrated for the DI of suckling rats and for the PJ of adults (Fig. 7), this transcript concentrated at the apical pole of the enterocytes whereas the cytoplasm surrounding the nucleus was not obviously labeled by the LPH cDNA probe.



**Figure 6.** Distribution of the LPH mRNA along the crypt-villus axis. The LPH mRNA was detected by in situ hybridization to the LPH cDNA labeled with digoxigenin and revealed with anti-digoxigenin antibody coupled to alkaline phosphatase. The mRNA was virtually absent from the crypts (c). As shown for the distal ileum of suckling rats (A), it appeared at the crypt-villus junction and accumulated predominantly at the villus base (v). In the adults, the pattern obtained in the proximal jejunum (D) was similar to that of the sucklings, whereas no mRNA could be detected in the distal ileum (E). In the proximal duodenum (B and C), some villi were labeled on one side only, or at the level of restricted groups of enterocytes (arrows). Bars, 100  $\mu\text{m}$ .



**Figure 7.** Intracellular distribution of the LPH mRNA. LPH mRNA revealed by in situ hybridization accumulates at the apical pole of the enterocytes as illustrated for samples originating from the distal ileum of suckling rats (*A*) and from the proximal jejunum of adult animals (*B*). Bars, 25  $\mu$ m.

## Discussion

This study is the first attempt to correlate comprehensively the pattern of lactase expression as a function of the developmental stage, the region of the intestinal tract, and the process of cell differentiation along the crypt-villus axis. LPH expression is associated with three events during postnatal development: first, the low and transient expression of enzyme and mRNA in the colon of neonates, at the stage where the mucosa temporarily shows villi structures (Foltzer-Jourdainne et al., 1989); second, the progressive decline of enzyme activity after birth in the small intestine; third, the rapid modification of mRNA distribution along the antero-posterior axis of the small intestine at weaning in that the transcript decreases in the proximal duodenum, becomes virtually absent in the distal ileum but remains abundant in the middle region comprising the distal duodenum, jejunum, and proximal ileum. Crypts are always devoid of lactase mRNA, and the expression patterns along the villi change with the modification of the longitudinal distribution of the mRNA at weaning. In addition, the primary mechanisms controlling LPH expression differ from one intestinal region to the other. In the proximal duodenum, the reduced enzyme activity in adult animals is accompanied by a decrease in mRNA content and by a decrease in gene transcription, which indicates a control mechanism at the transcriptional level. In the middle part of the small intestine, active gene transcription and a high level of mRNA are retained throughout development, while the fraction of LPH mRNA present in polysomes remains constant with age. This suggests that the ontogenic decline of enzyme is primarily dependent on a posttranslational regulation. In the distal ileum, the LPH mRNA is no longer detected after weaning despite the maintenance of active transcription all along the *LPH* gene, suggesting the intervention of a posttranscriptional mechanism. Finally, in the colon a transcriptional regulation is predicted

from the coordinated patterns of gene transcription, and mRNA and protein accumulation. Taken together, these results indicate that the regulation of LPH expression requires multiple mechanisms acting with stage- and region-specificity at the transcriptional, posttranscriptional, and posttranslational levels.

The regional specialization of the small intestine illustrated by the appearance of distinct patterns of LPH mRNA expression in the duodenum, jejunum and ileum takes place at weaning in rats, while the onset of expression occurring in parallel with the cytodifferentiation of the endoderm in fetuses (Rings et al., 1992a) leads at first to the emergence of a single phenotype all along the small intestine. Thus, changes at weaning have to be considered as a redifferentiation rather than a maturation of the intestinal epithelium when the animals adapt to the adult condition. Several features of the modification of the longitudinal expression of lactase at weaning should be emphasized: (*a*) except for the proximal duodenum, the occurrence of distinct expression patterns along the small intestine may not be related to a different frequency of specialized epithelial cells that would synthesize lactase, as in the case of molecules produced by entero-endocrine cells (Evans and Potten, 1988); (*b*) clear-cut differences between the jejunum and the ileum are specific for the LPH mRNA as other digestive hydrolases, i.e., sucrase-isomaltase and N-aminopeptidase, do not share this property (Freund et al., 1990b); (*c*) the emergence of distinct mRNA patterns at weaning occurs despite the common structural organization of the mucosa all along the small intestine; (*d*) whereas the onset of lactase expression is most likely associated with a transcriptional activation of the gene in fetuses, the expression pattern retained throughout adult life depends mainly on posttranscriptional mechanisms of regulation which drive the general decline of enzyme and the disappearance of the mRNA in the distal ileum. The finding of multiple mechanisms of regulation may be correlated with the fact that lactase expression is controlled by multiple factors. Indeed, the basic patterns of mRNA and enzyme are determined by the intrinsic program that governs intestinal ontogeny (manuscript submitted for preparation), but modulation also occurs by hormones and nutrients (Freund et al., 1991b; Duluc et al., 1992). The primary role of the ontogenetic program in the determination of the regional identity along the antero-posterior axis of the intestinal tract is supported by studies carried out on the *Fabp* gene family (Rubin et al., 1992). Homeogenes such as *Cdx1/2* have been proposed to play a crucial role in this program, yet their direct involvement in the control of the spatial and/or temporal expression of markers of terminal differentiation has not been clearly demonstrated (James and Kazenwadel, 1991; Freund et al., 1992).

It is well-known that lactase protein is virtually absent from the crypts and appears when cells reach the crypt-villus junction. Consistent with data obtained in baby rats (Rings et al., 1992a), in situ hybridizations shown here from suckling and adult animals indicate that this is also true for the mRNA. Moreover, the enzyme is present on the whole surface of the villi whereas the transcript accumulates in the lower half, which has also been demonstrated for other intestinal enzymes (Noren et al., 1989; Traber, 1990; Chandrasena et al., 1992). Thus enterocytes may be committed to synthesize these mRNA only at the villus base, while pro-

teins are stably anchored into the brush border membrane during cell progression toward the tip. Alternatively, a decay of the half-life of the transcripts when cells migrate may also occur. The in situ hybridizations carried out here with non-radioactive probes also suggest that the LPH mRNA concentrates at the apical pole of the enterocytes. A polar distribution has been described using radioactive probes (Rings et al., 1992b), which contrasts with the even distribution of the whole population of polyadenylated RNA in the cytoplasm (Talbot et al., 1989). The molecular basis of the intracellular pattern of LPH mRNA is unknown. Yet the colocalization of the transcript with cytoskeleton components of the apical network may be related to the fact that colchicine selectively inhibits lactase expression (Hudson and Smith, 1986). The loss of LPH mRNA in the adult distal ileum without turning off gene transcription may also be considered with respect to RNA sorting, and it would be of great interest to test whether the rate of accumulation or the stability of the LPH mRNA depend on the intracellular sorting process.

Immunocytochemistry and in situ hybridizations carried out in the jejunoleum of suckling and adult rats corroborate the theory of crypt monoclonality (Ponder et al., 1985; Griffiths et al., 1988; Schmidt et al., 1988) in that all the enterocytes emerging from the crypts in a given intestinal segment share an identical phenotype. The situation is more complex in the adult duodenum where lactase mRNA and enzyme are restricted to groups of epithelial cells. Mosaicism has also been reported for the lactase protein in the duodenum of adult rabbits and humans (Maiuri et al., 1991, 1992; Lorenzsonn et al., 1993). The theory of crypt monoclonality remains valid when the mRNA and enzyme are restricted to one side of the duodenal villi, if one assumes that the mucosa contains a mixed population of crypts generating either LPH-producing or non-producing enterocytes. However crypt monoclonality is not obvious when LPH expression occurs in discontinuous patches of epithelial cells. Patches have been observed transiently at birth for the *Dlb-1* gene expression in mouse chimeras, suggesting that crypts are first polyclonal before the process of "purification" leads to monoclonality (Schmidt et al., 1988). They have also been described in adult rats for *Fabpl* expression in the ileum (Rubin et al., 1992). Thus, the results obtained for *LPH* and *Fabpl* in the adult duodenum and ileum may have some implications regarding the concept of crypt monoclonality. If crypts are actually monoclonal, regulatory factors modulating the local gene expression along the villus height may be expected. Although the origin of these factors is speculative, nutrients, secretions of other digestive organs and/or paracrine secretions can be proposed. Lateral extrusion of epithelial cells from the villi may also account for patches, as proposed for the duodenum of adult mice by Schmidt et al. (1985).

The maintenance of active *LPH* gene transcription in the jejunoleum of adult rats, the discrepancy arising at weaning between the enzyme and mRNA patterns, and the similar proportion of mRNA present in polysomes in pre- and post-weaned animals demonstrate that the ontogenic decline of lactase activity is primarily under posttranslational control. This contrasts with the conclusion made by Büller et al. (1990) who proposed a fundamental transcriptional control from the coordinated evolution of total lactase activity and total amount of LPH mRNA during development. However,

this work was not based on transcription assays, and the emergence of regional differences at weaning was not taken into account. On the other hand, the results reported here are consistent with data showing an equivalent rate of incorporation of labeled amino acids into the lactase pool in sucklings and adult rats (Tsuboi et al., 1992). The actual mechanism of the enzyme decay is under investigation. Alteration of the processing of the LPH precursor has been proposed in rats (Nsi-Emvo et al., 1987; Castillo et al., 1990; Quan et al., 1990) although this may not occur in rabbits (Keller et al., 1992). The increase in pancreatic proteases as a possible cause of the lactase decline remains a possibility.

The limited data obtained in rabbits (Sebastio et al., 1989; Keller et al., 1992) and pigs (Freund et al., 1989) are in accordance with the general developmental pattern of lactase enzyme vs mRNA established in rats. In pigs, the covariation of enzyme activity with the amount of a nuclear factor binding to the *LPH* gene promoter led to the conclusion that the ontogenic decline of lactase may be under transcriptional control (Troelsen et al., 1992). Yet, transcription assays have to confirm the conclusion and evidence should be provided that the DNA-binding factor identified in this study is the one and only required for the regulation of *LPH* gene transcription during development. In human, the situation is complicated by the fact that two phenotypes are commonly described at the adult stage, i.e., lactase persistence and nonpersistence (adult-type hypolactasia). Lactase nonpersistence occurs despite the maintenance of a high level of LPH mRNA in jejunal samples (Sebastio et al., 1989), whereas in most cases the mRNA content decreases in duodenal biopsies (Escher et al., 1992; Lloyd et al., 1992). Concomitantly, a mosaic expression of the enzyme appears in the proximal part of the small intestine (Maiuri et al., 1990; Lorenzsonn, 1993). Thus, the general pattern of lactase expression in human may resemble the one described in rats. In addition, lactase activity appears more distally in the duodenum of nonpersistent compared with persistent humans (Newcomer and McGill, 1966). Accordingly, one can hypothesize for an individual variation in the longitudinal extent of the proximal region of the small intestine exhibiting a low and coordinated expression of enzyme and mRNA at the adult stage. When this region is relatively long, a low mRNA content is expected in duodenal samples, as observed for many subjects with adult-type hypolactasia (Sterchi et al., 1990; Witte et al., 1990; Lloyd et al., 1992; Escher et al., 1992). Alternatively, when this region is short, maintenance of a high level of mRNA would occur in duodenal biopsies, as observed for lactase persistent subjects as well as for patients with hypolactasia who retain abundant mRNA, a high rate of synthesis of the LPH precursor but an altered conversion to the mature form (Sterchi et al., 1990; Witte et al., 1990; Lloyd et al., 1992). Thus, in the light of the complex pattern of LPH expression established along the antero-posterior axis of the rat small intestine, it would be fruitful to combine enzyme, mRNA and gene transcription analyses along the human gut to get a comprehensive view of adult-type hypolactasia. The ethnic origin of the patients should also be considered.

In conclusion, the present work shows that the cellular and molecular expression of lactase is regulated at multiple levels during the postnatal development, along the antero-posterior axis of the intestinal tract and along the crypt-villus



axis. Regional diversity in the gene, mRNA and enzyme patterns occurs despite the common structural organization of the digestive epithelium. Thus, lactase is a marker of development and of functional regionalization of the intestinal tract. It may help to approach questions related to the determination of the intestinal stem cells along the antero-posterior axis, to the lineage of the enterocytes that migrate and differentiate along the crypt-villus axis, and to the evolution of the population of stem cells during postnatal development, in particular when the colon loses its small-intestinal-like phenotype in neonates, and when the small intestine redifferentiates at weaning.

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