# *In vitro* translation of RNA to lactase during postnatal development of rat intestine

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mRNA levels encoding lactase were detected by Northern blot analysis using two different probes in developing rat intestine. Probe I and probe II corresponding to second half of prolactase gene showed a 6·8 kb mRNA transcript in 7, 14, 21 and 30 day old rat intestine. There was no change in quantity of lactase mRNA detected using probe II, but hybridization with probe I showed a progressive decrease in mRNA transcript encoding lactase with age. At day 7 and 14 of postnatal development, the lactase mRNA was quite high, but it reduced upon weaning. The *in vitro* translation products of RNA detected by Western blot analysis using brush border lactase antibodies showed several isoforms of lactase antigen with molecular weight ranging from 100–220 kDa. Analysed at days 7 and 30 of postnatal development, lactase isoforms of molecular weight 130 kDa and 220 kDa were similar to those found in purified brush border membranes. The translation of RNA to 220 kDa lactase protein was high in 7 and 14 day old pups, but it was markedly reduced in 30 day old animals. These findings support the contention that translation of mRNA to lactase is impaired in weaned animals, which may also be responsible for the maturational decline in lactase activity in adult rat intestine.

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## 1. Introduction

Lactase phlorizin hydrolase (LPH, EC 3·2·1·23·62) is a membrane-associated glycoprotein localized on the epithelial surface of enterocytes in intestine. The deficiency of the enzyme leads to milk intolerance, a phenomenon prevalent in humans worldwide (Vrese *et al* 2001; Kuokkanen *et al* 2003). In rodent intestine, postnatal development of LPH follows a specific pattern such that the enzyme activity is quite high in the perinatal period, but it declines considerably upon maturation (Lee *et al* 1997). The observed maturational decline in LPH activity is similar to adult-type hypolactasia observed in humans, but the underlying mechanism of this phenomenon remains largely unknown. A number of hypotheses have been put forward to explain maturational decline of lactase activity: such as (i) decreased amount of lactase protein (Lloyd *et al* 1992), (ii) defect in post-translational modification of precursor lactase to the mature enzyme (Rings *et al* 1992) and (iii) synthesis of an inactive, high molecular weight lactase with altered glycosylation, rather than the active form found in young animals (Nsi-Emvo *et al* 1987). Büller *et al* (1989) observed similar enzyme kinetics of immunoprecipitated lactase in suckling and adult rat intestine. However, they also described coordination between decline in lactase activity and mRNA levels encoding lactase and concluded that lactase is regulated predominantly by transcriptional mechanism consistent with a hypothesis of declining lactase activity and decreased synthesis during maturation (Büller *et al* 1989). In con-

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Abbreviations used: LPH, Lactase phlorizin hydrolase; MOPS, 3-(N-morpholino) propane-sulphonic acid.

trast, Freund *et al* (1990) found maintenance of appreciable amounts of lactase mRNA together with low enzyme levels in 3 and 24 month old adult rat and thus did not find any association between the cognate mRNA, catalytic activity and lactase protein throughout the developmental period. Based on observed parallelism between lactase activity and mRNA content and transcription rate experiments, Krasinski *et al* (1994) suggested that LPH activity is regulated mainly at the level of gene transcription. More recently Wering *et al* (2002) have shown that association between GATA-5 and HNF-*a* both *in vitro* and *in vivo* is necessary for the activation of LPH promoter indicating transcriptional regulation of the enzyme activity in cell culture system. To understand whether the observed decline in lactase activity in adult animals is a consequence of reduced mRNA levels or impaired translation to mature enzyme, we investigated the *in vitro* translation of total RNA isolated from different ages of rat intestine to lactase. These findings suggest that translation of mRNA to lactase protein is impaired with age, which may be an additional mechanism responsible for the observed decline in lactase levels in mature rat intestine.

## 2. Materials and methods

All the chemicals used were of molecular biology grade. The radiolabelled ¡ $^{32}$P-ATP was procured from Radioisotope Division, Bhabha Atomic Research Centre, Mumbai. The oligonucleotide primers were synthesized by Bangalore Genei (India). Goat anti-rabbit IgG was purchased from Bangalore Genei. Antibody against lactase was a gift from Professor D H Alpers of Washington University, School of Medicine, St. Louis, USA. Positively charged nitrocellulose membranes (polyvinylidene difluoride, PVDF) were from Bio-Rad, USA.

*In vitro* translation kit was obtained from Roche Molecular Biochemicals, Germany. ECL reagents 1 and 2 used for enhanced chemiluminescence (ECL) for Western blot analysis were obtained from Amersham (UK).

### 2.1 *Animals*

Inbred male albino rats (Wistar strain) of different ages: 7, 14, 21, 30 days and adults free of any infection were used. The animals were maintained as per the guidelines for care and use of experimental animals, Indian Council of Medical Research, New Delhi. There were 10–15 animals in each group. Rats were sacrificed under light ether anesthesia, intestinal tissues were taken out, cleaned by flushing with chilled normal saline. A segment of small intestine from 2–3 animals were pooled and immediately frozen in liquid nitrogen for RNA isolation. All the experiments were carried out at least 3 times.

### 2.2 *Western blotting*

Membrane proteins or *in vitro* RNA translation products were resolved on SDS-PAGE following the method of Lammeli (1970). Ten percent separating gel (lower gel) and 3% stacking gel (upper gel), freshly prepared were used. Western blot analysis was performed following the method described earlier (Mahmood *et al* 1993).

### 2.3 *Preparation of brush border membranes*

Brush border membranes (BBM) from proximal intestine were isolated by the method of Kessler *et al* (1978). The membranes were suspended in 20 mM Tris-maleate buffer, pH 6·8, and exhibited 10–12-fold enrichment of marker enzymes (alkaline phosphatase or lactase). Protein was determined by the method of Lowry *et al* (1951), using bovine serum albumin as the standard.

### 2.4 *Isolation of RNA*

The intestinal tissue (proximal jejunum portion: 100 mg) frozen in liquid nitrogen was used to isolate total RNA, following the method of Chomenzynski and Sacchi (1987). The isolated RNA was dissolved in 50 mM Tris-HCl (pH 7·4) containing 0·05% sodium dodecyl sulphate (SDS) and 1 mM EDTA.

### 2.5 In vitro *translation*

The RNA extracted from intestinal tissue was used as template and *in vitro* translation was performed using rabbit reticulocyte lysate in cell free reaction medium, following procedure recommended by the supplier of the *in vitro* translation kit (Roche Molecular Biochemicals, Germany). Assay tubes contained biotinylated reticulocyte, translation mixture from the kit (30 µl) and 2 µg of total RNA in a final volume of 50 µl made by the addition of sterile distilled water. Tubes were incubated at 30°C in water bath for 1 h. The reaction was stopped by snap chilling the mixture on ice. The protein products obtained by *in vitro* translation were resolved on 10% SDS-PAGE and immunostained using polyclonal rabbit anti-rat lactase antibody.

### 2.6 *Northern blot analysis*

Total RNA isolated from intestinal tissue was resolved on 1% agarose gel containing 2·2 M formaldehyde and ethidium bromide using 10X 3-(N-morpholino) propanesulphonic acid (MOPS) buffer (pH 7·0). RNA samples in a total volume of 20 µl [10 µl formamide, 5 µl formal-

dehyde, 3 µl 10X MOPS and 2 µl diethyl pyrocarbonate (DEPC) water] was applied to the gel and separated electrophoretically using MOPS as running buffer. Resolved RNA was transferred onto a positively charged nylon membrane (Zeta probe Biorad, USA) by alkaline capillary transfer. Transferred RNA on the membrane was fixed under UV cross-linker (Stratalinker, USA) and hybridized with $g^{32}$P-ATP labelled oligonucleotide probe I or probe II. An end labelled *b*-actin probe was used as the internal standard.

The antisense oligonucleotide probes labelled with $g^{32}$P-ATP, complementary to cDNA sequence were used. The oligonucleotide probes synthesized from different regions of full-length cDNA sequence of precursor lactase gene (Mantei *et al* 1988; Duluc *et al* 1991) were used. The sequences of probe I (5′-----$^{3856}$TTGATATAG-GTTTTGTGGTAG$^{3876}$-----3′) and probe II (5′-----$^{4019}$TGA-GGCTCTTGCTGTGCGAGG$^{4039}$-----3′) were from the downstream region (amino acid 886 onward) of the second half of the full-length lactase cDNA (Duluc *et al* 1991). After hybridization, the membranes were washed 3 times with 2X saline-sodium citrate (SSC), 0·1% SDS for 10 min. The washed membranes were subjected to autoradiography at – 80°C.

## 3. Results

As shown in figure 1a, using probe I, mRNA signal corresponding to 6·8 kb fragment was observed in 7, 14, 21 and 30 day old rat intestine. Further, examination of this data revealed that lactase mRNA transcript exhibited a progressive decline in intensity with age. The signal was highest in 7–14 day old pups, but declined by 46% in 30 day old rat intestine (figure 1b). In contrast to these findings, when probe II was used to hybridize with the mRNA transcript at different ages of development, there was essentially no difference in the intensity of 6·8 kb mRNA transcript under these conditions.

*In vitro* translation of RNA isolated from 7 or 30 day old rat intestine to lactase was studied. The mRNA translated protein products were detected by Western blot analysis using lactase specific antibodies. These results were compared with lactase antigen in intact brush border from the corresponding age matched animals. As shown in figure 2a, lactase antibody picked protein bands of 130 kDa and 220 kDa in brush border membranes in 7 and 30 day old rat intestine. However, the intensity of protein signal was low in brush borders isolated from adult animals. Western blot analysis of *in vitro* translation products of RNA from 7 day and 30 day animals showed corresponding protein bands of 130 kDa and 220 kDa. The intensity of the protein bands was low in adult animals compared to that in pups. This is apparent

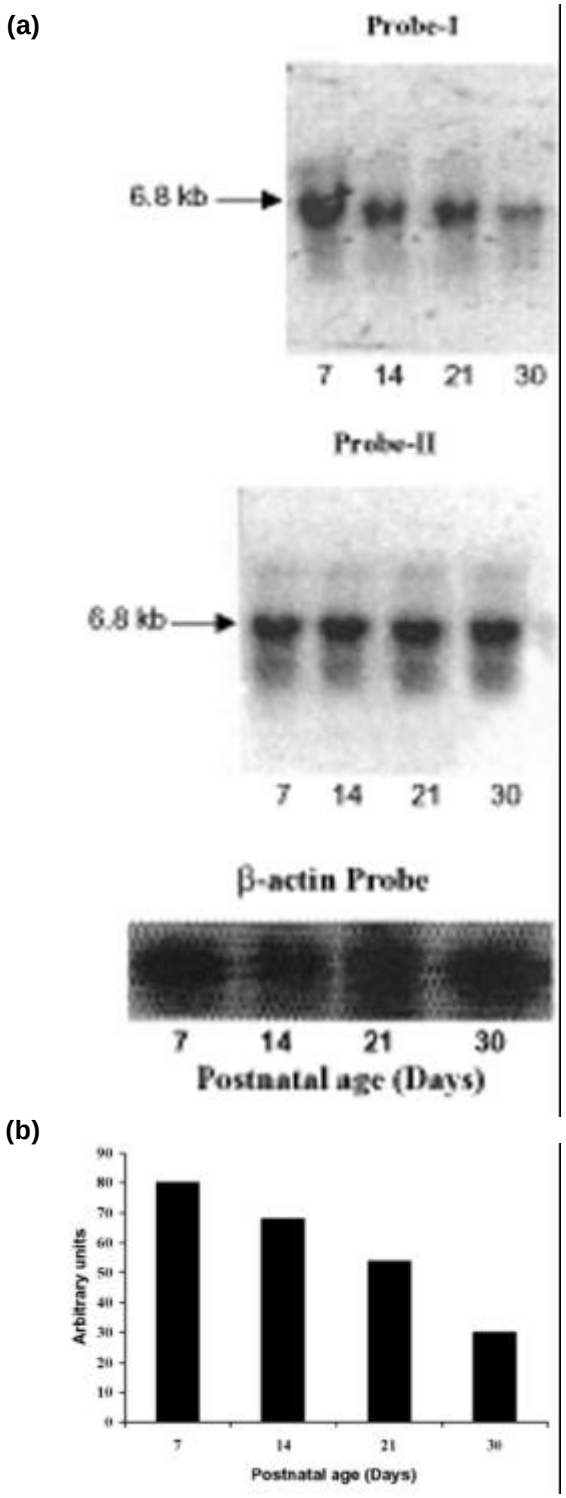


**Figure 1.** (**a**) Northern blot analysis of mRNA encoding intestinal brush border lactase from 7, 14, 21 and 30 day old rats using oligonucleotide probe I, probe II and *b*-actin probe from the regions of full length prolactase cDNA. Each lane was loaded with 10 µg of total RNA. Blots were hybridized with $g^{32}$P-ATP labelled oligonucleotide probes. (**b**) Densitometric scan of data given in (**a**) using probe I.

in densitometric scan of the data shown in figure 2b. These findings indicate that lactase species of 220 kDa and 130 kDa are synthesized by RNA *in vitro* both in suckling and adult animals which are similar to the enzyme present in the intact animal tissues. Lactase activity in *in vitro* translation samples could not be demonstrated, since the assay system contains considerable amounts of Tris, which is a powerful inhibitor of the disaccharidase activity (Vasseur *et al* 1990). Similar data was obtained with RNA from 7, 14, 21 and 30 day old rats (figure 3), which showed a progressive decline in the intensity of 220 kDa protein band, corresponding to lactase antigen in developing rat intestine.

## 4. Discussion

The results presented herein, indicate that *in vitro* translation of mRNA isolated from intestinal tissue produces several lactase species ranging in molecular weight 100–220 kDa as detected by Western blot analysis using lactase antibodies. These results are similar to earlier studies with the intact intestinal tissues, where presence of a number of immunoreactive lactase species has been reported (Lloyd *et al* 1992; Keller *et al* 1995; Lee *et al* 1997). These isoforms may represent lactase precursors generated during the course of mature enzyme formation. The detection of the translated protein products showed

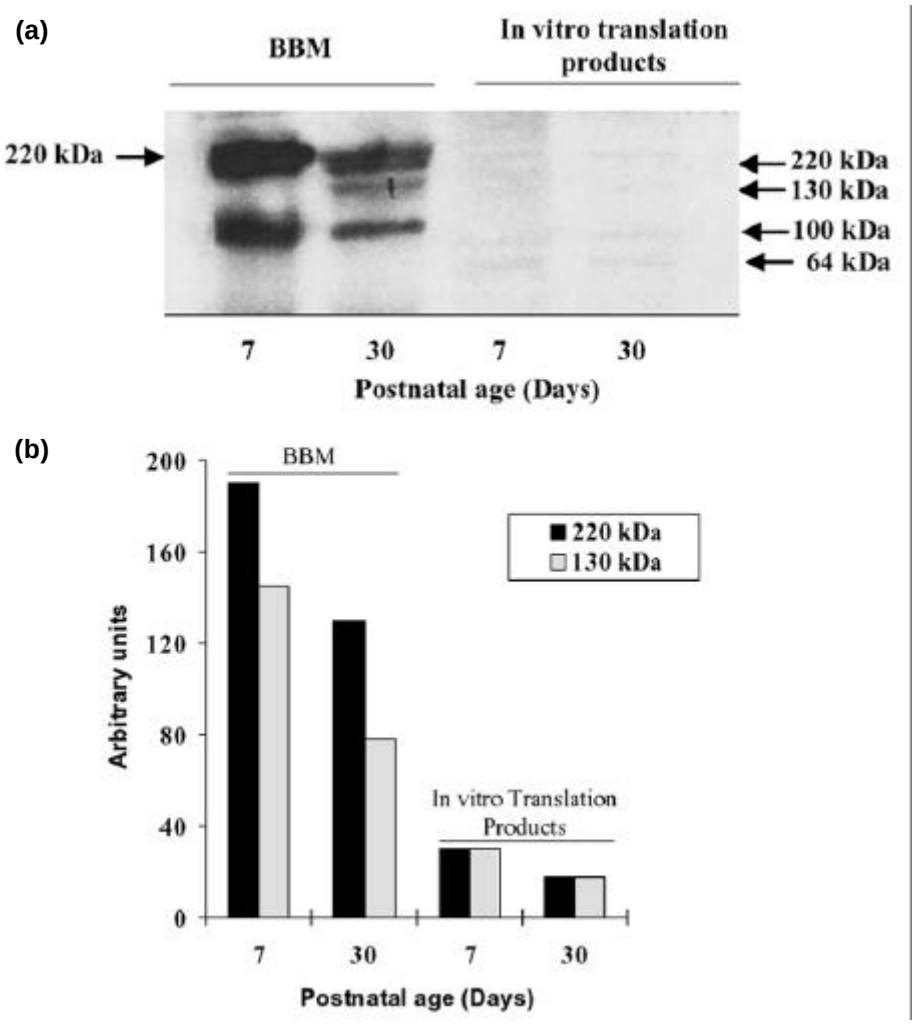


**Figure 2.** (**a**) Western blot of *in vitro* translation products (protein) of RNA isolated from rat intestine using lactase antibody (1 : 1000). Total RNA was isolated from intestine of 7 and 30 day old rats and compared with brush border lactase protein from 7 and 30 day old rats. 30 µg membrane protein was applied to each well. (**b**) Densitometric scan of data given in (**a**).

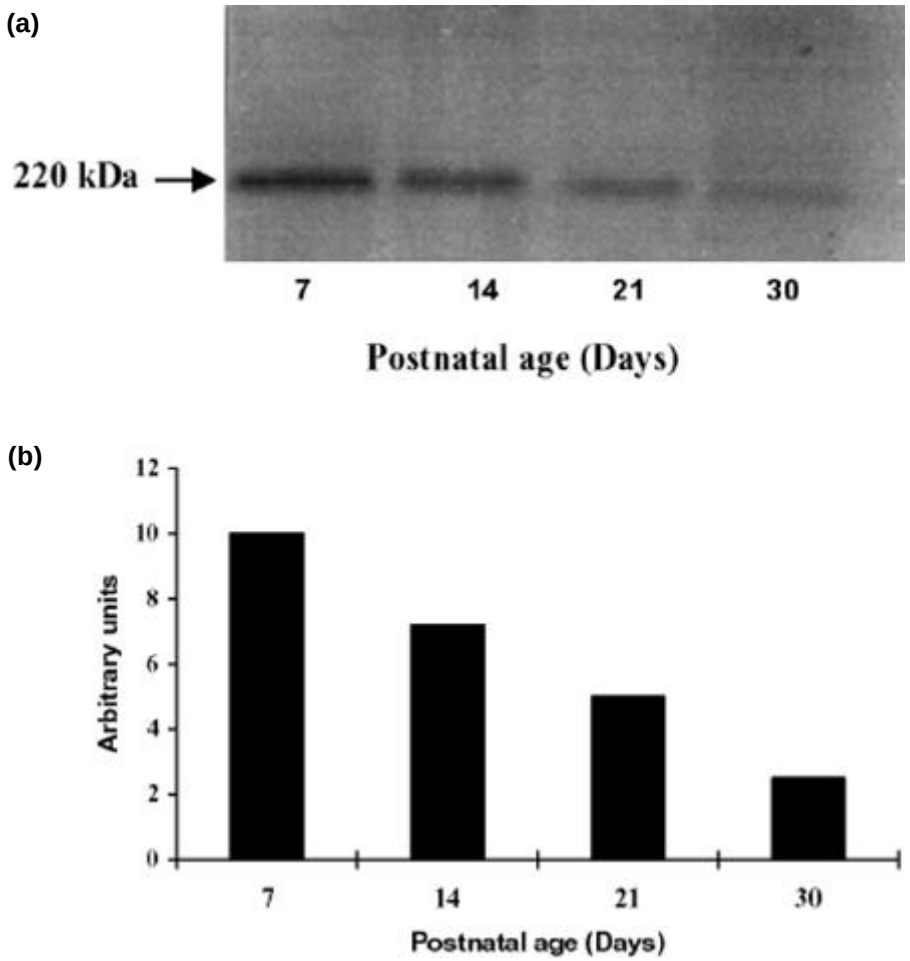


**Figure 3.** (**a**) Western blot of *in vitro* RNA translation product (220 kDa protein) in 7, 14, 21 and 30 day old rat intestine, using lactase antibody (1 : 1000). Horse-radish peroxidase labelled goat anti rabbit-IgG was used as the secondary Ab (1 : 4000 dilution). (**b**) Densitometric scan of data given in (**a**).

high lactase signal in 7 day old brush border membranes. Adult animals exhibited low levels of lactase protein in brush borders as well as *in vitro* translation products. These observations suggest that although RNA levels are apparently high in mature animals but its translation into 220 kDa lactase species is markedly diminished or the enzyme protein becomes unstable. The mRNA signal determined using probe I at day 7 was reduced by 46% compared to that at day 30 of postnatal development. However, the efficiency of RNA translation *in vitro* to 220 kDa lactase protein was impaired by 68% under these conditions. These findings corroborate the earlier contention, that high mRNA levels encoding lactase in mature rat intestine does not correlate with the enzyme protein (Nudell *et al* 1993). Goda *et al* (1999) also observed that mRNA level encoding LPH are persistently high in the upper villus region of crypt-villus axis. But immuno-reactive LPH protein decline at the apical region. This suggests that upper villus cells in intestine have characteristic reduced translation efficiency of LPH transcript or elevated degradation of LPH protein or both. Thus a negative correlation exists between lactase activity and mRNA levels encoding lactase protein. Administration of thyroxine has been shown to decrease lactase activity despite maintaining high quantities of its mRNA (Yeh *et al* 1991). Similarly, insulin (Shulman *et al* 1992) and hydroxycortisone (Villa *et al* 1992) treatments enhances lactase activity without a concomitant change in its mRNA. This may represent one of the mechanisms of post-translational regulation of lactase, in addition to the transcriptional regulatory mechanism of maturational decline in rat intestine as suggested by others (Kuokkanen *et al* 2003; Freund *et al* 1990; Krasinski *et al* 1994).

Nudell *et al* (1993) showed that quantity of lactase mRNA in the enterocyte during postnatal development is far out of proportion to the amounts of lactase protein.

The ratio of lactase specific mRNA to lactase protein (immuno protein), an estimate of the mRNA quantity required to support maintenance of a unit of lactase protein, increased 4–5-fold by 30 and 60 days of age, when lactase had declined considerably in the enterocyte. It was suggested that translation efficiency of mRNA becomes greatly curtailed during postnatal development.

Probe II designed corresponding to 5′ 4019–4039 downstream region of lactase gene, yielded strong signal corresponding to 6·8 kb fragment of lactase mRNA throughout the postnatal development of intestine in rats. These findings are in contrast to those obtained using probe I corresponding to 5′ 3856–3876 region of LPH gene (Duluc *et al* 1991). The underlying mechanism of these unusual findings is unknown, but it could be a consequence of altered secondary structure of mRNA encoding lactase, which hybridizes differently to these probes. The data from *in vitro* RNA translation study supports the contention that mRNA levels do not correlate with lactase protein in adult rat intestine. These results also indicate that quantity of mRNA detected using different cDNA probes for Northern blot analysis may yield different results. It suggests that to assess the true levels of mRNA encoding a protein, it may perhaps be necessary to use more than one probe corresponding to the different regions of a gene. This nevertheless, explains conflicting data on mRNA levels encoding lactase in developing rat intestine (Büller *et al* 1989, 1990; Freund *et al* 1990).

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