

# In vitro biosynthesis of lactase in preweaning and adult rabbit

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Lactase is synthesized as a high-mannose large precursor (200 kDa) which is subsequently complex-glycosylated (215 kDa) and split into the 150 kDa mature form. The regulatory mechanisms responsible for the decline of activity at weaning are not yet known. We have set up in vitro cultures of intestinal mucosa from suckling and adult rabbit and found that suckling and adult animals synthesize the same four forms of lactase-phlorizin hydrolase (LPH) but with a different distribution. In the proximal adult small intestine there is very little 180 kDa form, which is most probably a product of the 215 kDa complex-glycosylated precursor. The 180 kDa form comprises a greater percentage of total LPH in the middle of the small intestine in adult and particularly in suckling rabbits. In the latter tissue this form is apparently more stable than in the adult tissue. Posttranscriptional control of lactase synthesis is therefore different in the various parts of the adult small intestine, and it is different in the suckling as compared to adult tissue.

Lactase-phlorizin hydrolase biosynthesis; Lactase-phlorizin hydrolase processing; Preweaning (rabbit); Adult (rabbit)

## 1. INTRODUCTION

In mammals lactase activity is very high at birth and declines to low levels after weaning (for a review see [1]). In vivo and in vitro studies have demonstrated that the enzyme is synthesized as a single-chain high molecular weight high-mannose precursor (approximately 200 kDa), which is transformed into a complex-glycosylated form and then split into the 150–160 kDa mature form [2–5]. The primary structure of the lactase precursor and of the mature lactase was deduced by rabbit and human cDNA sequence analysis [6]. The proteolytic processing of prolactase to lactase appears to take place, at least to a very large extent, intracellularly [7,8]. The complex-glycosylated precursor is also present in the brush border (M. Rossi et al., unpublished results), where the conversion into the mature form may also occur, due to intraluminal proteases [9]. An intermediate 180 kDa form has been demonstrated in in vitro studies in suckling rats [5].

Conflicting results on the molecular basis of adult-type hypolactasia in mammals have been reported in in vivo studies; lactase biosynthesis has been found to be decreased [10] or not significantly changed at weaning [11–13]. An enzymatically inactive, immunologically cross-reacting 100 kDa polypeptide occurring in the brush border membrane of the adult rat small intestine

has been indicated as a possible degradation product [11], whereas in a different study comparable protein patterns were precipitated by antilactase monoclonal antibodies from extracts of small intestine of preweaning and postweaning rats [14].

In the present study, we have set up in vitro cultures of the suckling and adult rabbit intestine. No attempt has been made to quantitatively compare the biosynthesis rate in the adult and newborn intestine. On the contrary, we have compared the processing of the lactase proteins in suckling and in various parts of the adult small intestine.

## 2. MATERIALS AND METHODS

### 2.1. Animals

New Zealand white rabbits 15 days or six months old were used.

### 2.2. Antibodies

Monoclonal antibodies raised against human lactase (mlac 5) have been previously found to be able to cross-react with rabbit lactase [15].

### 2.3. Organ culture

In the suckling animal mucosal specimens were obtained from the middle region of the small intestine; in the adult animal they were obtained from the middle region of the small intestine, from the proximal jejunum, just after the Treitz ligament, and from the distal ileum, 3–4 cm before the ileum-coecal valve.

The explants were placed under the stereomicroscope with the mucosal surface downwards, and the serosal and muscularis layers were stripped. Explants (20 mg) were cut and placed on grids. They were preincubated for 1 h in Eagle's Minimum Essential Medium (modified) with Earle's Salts (EMEM) in the absence of methionine and supplemented with 10% dialyzed fetal calf serum. 150  $\mu$ Ci/ml [<sup>35</sup>S]methionine was subsequently added for continuous labeling or pulse-chase experiments.

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*Abbreviation:* LPH, lactase-phlorizin hydrolase.

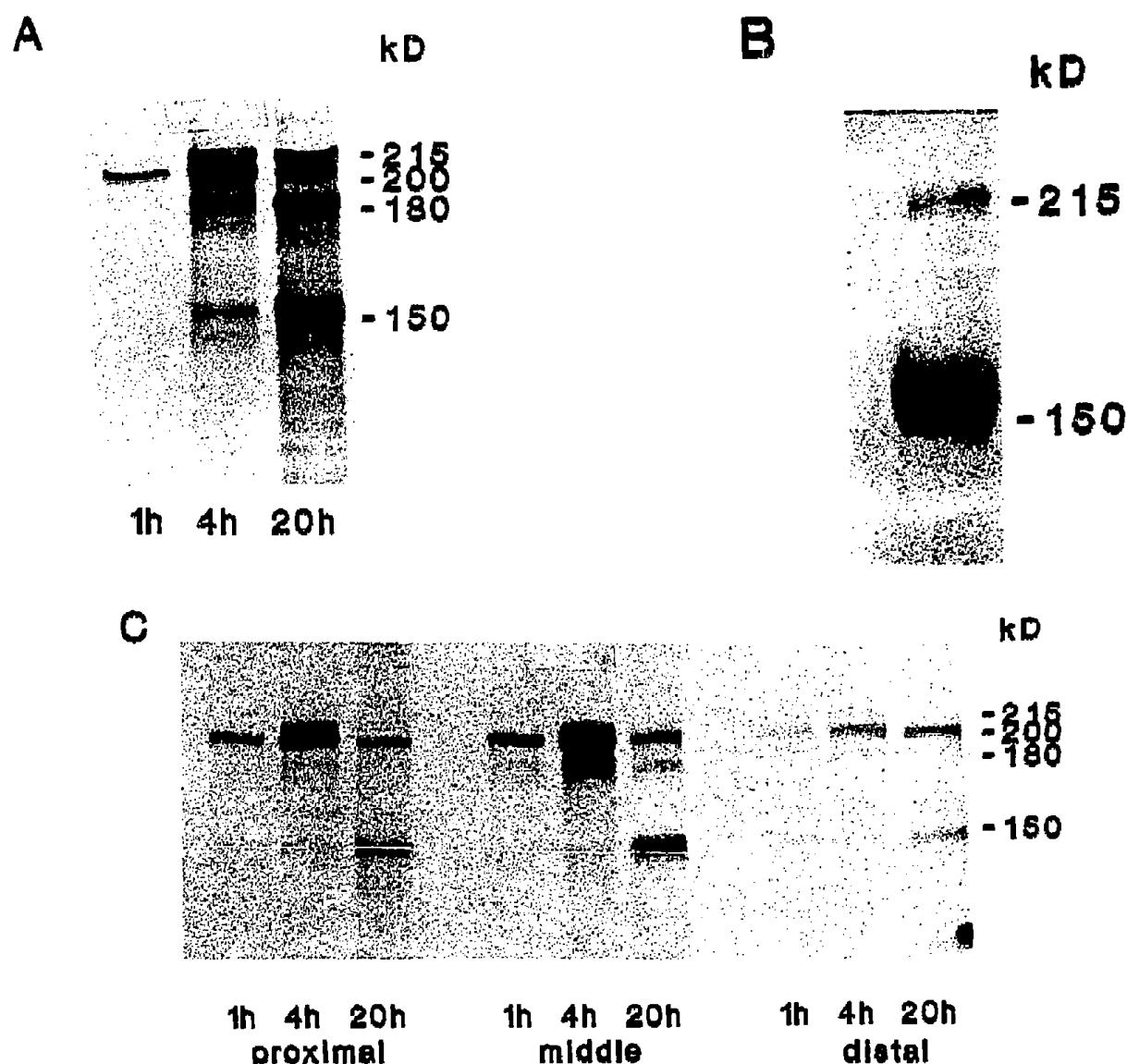


Fig. 1. Identification of LPH forms immunoadsorbed by the monoclonal antibody mlac 5. A. Fluorogram from a continuous labeling experiment of preweaning small intestine. B. Coomassie brilliant blue staining of LPH purified from preweaning mucosa. C. Fluorogram from continuous labeling experiments of different regions of adult small intestine.

#### 2.4. Immunoadsorption and fluorography

The radiolabeled explants were homogenized in 50 mM NaCl, 25 mM Tris, pH 8.1, containing 100  $\mu$ g/ml phenylmethylsulfonylfluoride (PMSF), 10  $\mu$ g/ml leupeptin, 17.4  $\mu$ g/ml aprotinin, 2  $\mu$ g/ml benzamidine, 50  $\mu$ g/ml soybean trypsin inhibitor and 380  $\mu$ g/ml EGTA as protease inhibitors and subsequently solubilized by 0.5% Nonidet-P40 and 0.5% deoxycholate. Lactase from fixed quantities of radiolabeled proteins (cpm), precleared with protein A-Sepharose (Pharmacia, Uppsala, Sweden), was quantitatively immunoadsorbed by addition of an excess of anti-lactase monoclonal antibodies coupled to (CNBr)-activated Sepharose for 2 h at 4°C. Two washing steps followed, one with 145 mM NaCl, 0.5% Nonidet P-40, 0.05% deoxycholate, 0.01% sodium dodecylsulphate (SDS), 20 mM phosphate pH 7.4, and a second one with 0.5 M NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 125 mM Tris pH 8.1. Finally the samples were subjected to SDS-PAGE [16] and fluorography by treatment of the gels with 2,5-diphenyloxazole (PPO) and exposure to preflashed Kodak X-Omat AR films

at -80°C [17]. Quantification of bands was carried out by densitometric scanning using the laser densitometer Ultrosan XL (LKB, Bromma, Sweden).

#### 2.5. Endo H treatment

Immunoadsorbed lactase was boiled for 5 min in 0.2% dithiothreitol, 0.3% SDS, 0.1 M citrate buffer pH 5.2, and diluted twice with citrate buffer. 6.4 mU endoglycosidase H (Boehringer Mannheim, Mannheim, Germany) and 4 mM PMSF were added, and an overnight incubation at 37°C was performed.

#### 2.6. pNGase F treatment

Immunoadsorbed lactase was boiled for 5 min in 1.5% dithiothreitol, 0.3% SDS, 0.1 M phosphate pH 8.1 and diluted threefold with 0.1 M phosphate pH 8.6, 5 mg/ml albumin, 1.25% Nonidet P40, 4 mM PMSF, and 0.2 U glycopeptidase F (pNGase F) (Boehringer

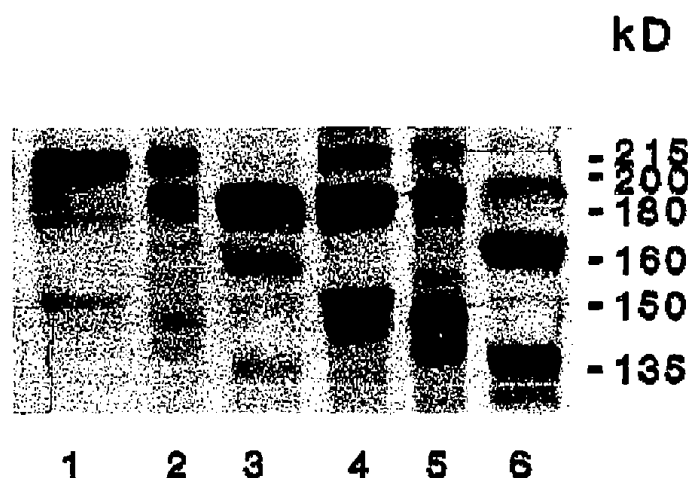


Fig. 2. Glycosidase treatment. Lactase detected in preweaning after 4 h (lanes 1–3) or 20 h (lanes 4–6) of continuous labeling was digested with endoglycosidase H (lanes 2 and 4) or pNGlycosidase F (lanes 3 and 6).

Mannheim, Mannheim, Germany) were added. The sample was incubated overnight at 37°C.

#### 2.7. Disaccharidases and protein assays

The enzymatic and protein assays were performed as previously described [15].

### 3. RESULTS AND DISCUSSION

Table I summarizes the disaccharidase activities and the label incorporation into total proteins in preweaning rabbits and in the proximal, middle and distal regions of the small intestine of adult rabbits. The linear increase of radioactivity into total proteins confirmed the applicability of the organ culture technique for studies of lactase synthesis. The tissue samples after 20 h of culture showed the presence of apparently normal villi with good preservation of the architecture of the surface epithelium (data not shown). Only the 200 kDa form of lactase was present in preweaning tissue after 1 h of labeling (Fig. 1A); at 4 h, 215, 180 and 150 kDa bands also appeared; at 20 h the 180 and 150 kDa were predominant. Only the 150 and 215 kDa bands were detected in steady-state conditions in preweaning intestinal mucosa (Fig. 1B), thus suggesting that the 200 and

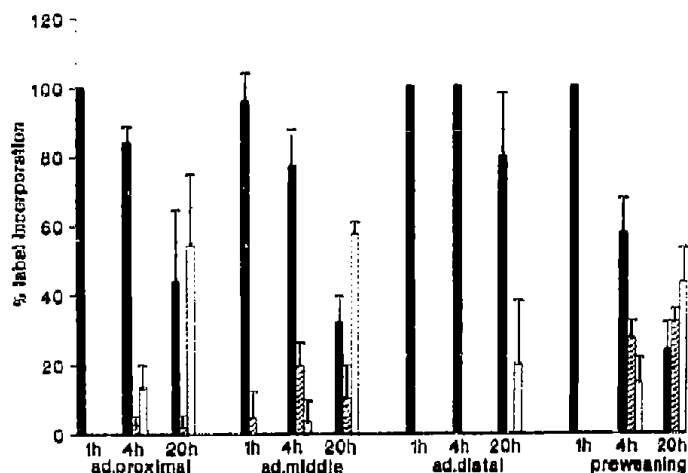


Fig. 3. Relative labeling distribution into LPH forms in different regions of adult small intestine and in preweaning tissue (continuous labeling experiments). Results are expressed as mean  $\pm$  S.D. (adults  $n = 3$ ; sucklings  $n = 4$ ). Full bars: 200–215 kDa; dashed bars: 180 kDa; dotted bars: 150 kDa.

180 kDa are transient forms. The 200 kDa was transformed by endo H treatment into a 180 kDa form while the mass of the other forms was only slightly modified (Fig. 2: lanes 2 and 5). The pNGase F treatment (lanes 3 and 6) transformed the 215 and 200 kDa into a 180 kDa form, the 180 kDa in a 160 kDa form, and the 150 kDa mature lactase into a 135 kDa product. These results suggest that the 180 kDa form is a proteolytic product of the 215 kDa form.

The continuous labeling experiments performed in different regions of adult small intestine (Fig. 1C), showed that the label incorporation in lactase proteins is higher in middle than in proximal jejunum and it is very low in distal ileum. The processing of lactase proteins was different in the various parts of the adult intestine and in the adult intestine as compared to the suckling tissue. In the adult intestine the 215 kDa form was present after 4 h and disappeared after 20 h of labeling whereas it was clearly detectable in the suckling tissue after 20 h (Fig. 1A). The mature 150 kDa species (Fig. 3) was present in about the same percentage both in the preweaning and in adult proximal and middle intestine at 20 h. Interestingly the 180 kDa form ac-

Table I  
Disaccharidases activities and label incorporation in preweaning and adult rabbit small intestine

Region	Specific activities (U/g protein: mean $\pm$ S.D.)			Label incorporation (cpm/mg protein $\times 10^{-6}$ : mean $\pm$ S.D.)		
	Lactase	Sucrase	Maltase	1 h	4 h	20 h
Preweaning ( $n = 6$ )	55.8 $\pm$ 23.7	7.3 $\pm$ 3.8	31.5 $\pm$ 9.6	1.9 $\pm$ 1.4	10.6 $\pm$ 10.5	30.0 $\pm$ 18.9
Adult ( $n = 4$ )	proximal	6.0 $\pm$ 2.4	87.7 $\pm$ 38.5	290.0 $\pm$ 79.6	2.8 $\pm$ 2.5	14.7 $\pm$ 8.3
	middle	6.4 $\pm$ 3.9	101.3 $\pm$ 35.4	302.0 $\pm$ 120.6	2.9 $\pm$ 1.6	11.9 $\pm$ 8.0
	distal	5.0 $\pm$ 3.5	75.5 $\pm$ 47.9	264.2 $\pm$ 136.9	5.6 $\pm$ 1.9	15.8 $\pm$ 6.2
						40.9 $\pm$ 9.5

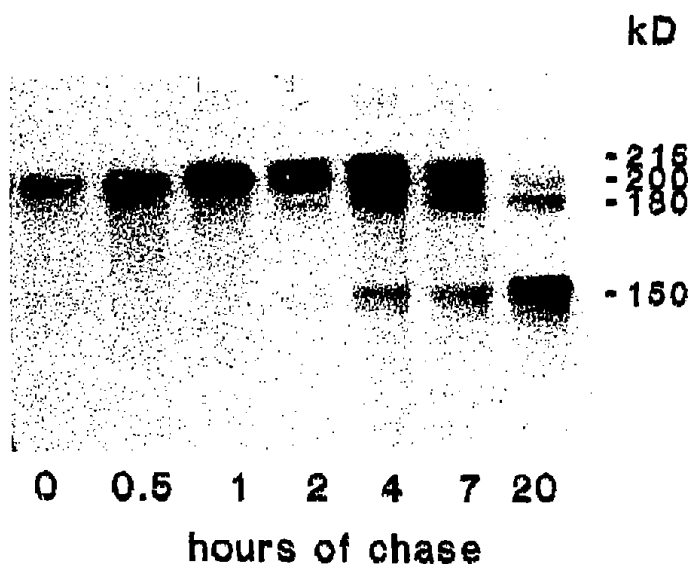


Fig. 4. Fluorogram from a pulse-chase experiment of preweaning tissue.

counted for a larger percent in preweaning both at 4 h (27%) and at 20 h (31.7%), while it was only 2.3% and 1.6% in proximal and 19.3% and 10% in middle jejunum at 4 h and 20 h respectively (Fig. 3). These results suggest that in preweaning tissue the processing is shifted toward the 180 kDa form more than in adult tissue or that this form is more stable in preweaning tissue. Finally in distal ileum a very slow processing was found, as the 200 kDa was predominant at all the examined labeling times.

The pulse-chase experiments confirmed that the 200 kDa high-mannose precursor is first complex-glycosylated (215 kDa) at 1 h of chase and then split into the 180 kDa and 150 kDa bands at 2 h (Fig. 4). Both cleavage products appeared at 2 h of chase and similar results were obtained at 90 min (data not shown). On the basis of this experiment it is therefore impossible to know whether or not the 180 kDa product is an intermediate form on the way to the mature lactase. The same lactase protein forms have been found in similar pulse-chase experiments of proximal and middle parts of the adult small intestine; in distal ileum due to the very low label incorporation, pulse-chase studies were not performed.

The relative distribution of LPH forms as a function of time in pulse-chase experiments is reported in Fig. 5. In preweaning tissue the 180 kDa form represents an intermediate product that reached 32% of total label at 7 h and then slowly decreased to 20% of the total labeled forms after 20 h (Fig. 5A). The 180 kDa form is transient also in adult tissue, with a more rapid decline, as after 20 h of chase it is no longer evident. The 180 kDa form is therefore apparently more stable in preweaning tissue as compared to adult tissue. As the percentage of the mature form at 20 h in preweaning is comparable to that of adult tissue, we suppose that the 215 kDa

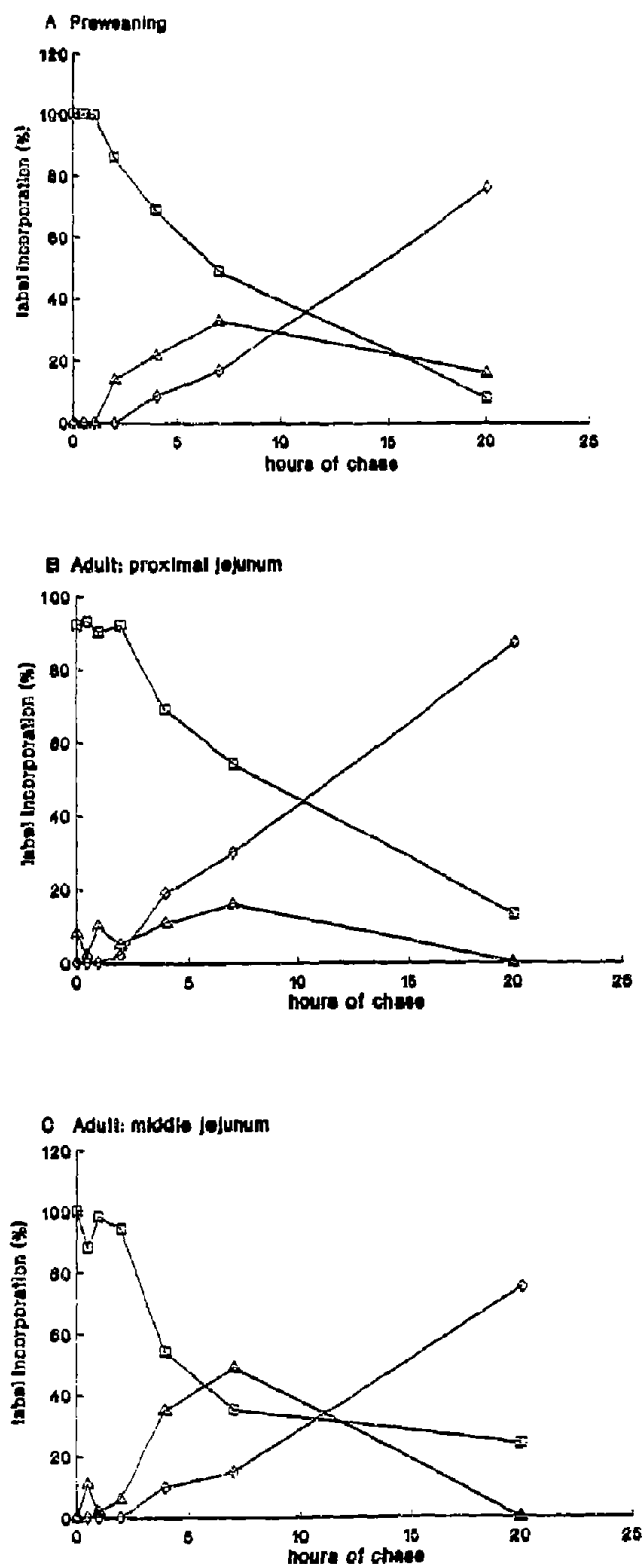


Fig. 5. Relative label distribution into LPH forms observed in pulse-chase experiments performed in preweaning (A) and in adult proximal (B) and middle jejunum (C). The same results were obtained in other four suckling and two adult animals. (□) 200-215 kDa; (△) 180 kDa; (○) 150 kDa.

form may be split either to the 180 kDa form and then into the mature form or directly into the 150 kDa form.

In conclusion, we have observed the same LPH forms in preweaning and adult rabbit. We have demonstrated the presence of a 180 kDa form in these tissues, which is probably a cleavage product of the 215 kDa form. In the adult distal ileum there is a reduced production of mature lactase with accumulation of the high-mannose precursor form. In the proximal adult small intestine there is very little 180 kDa form, which is more extensively produced in the middle small intestine and particularly in the suckling intestine; in this latter tissue this form is apparently more stable than in the adult tissue. Post-transcriptional control of lactase synthesis is therefore different in various parts of the adult small intestine and it is different in the suckling as compared to adult tissues. At the moment it is unknown if these differences are related to the decline of lactase activity occurring in mammals at weaning.

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## REFERENCES

- [1] Semenza, G. and Auricchio, S., in: *The Metabolic Basis of Inherited Diseases* (C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle, J.B. Stanbury, J.B. Wyngaarden and D.S. Fredrickson, Eds.) 6th edn., McGraw-Hill Information Science Company, New York, 1989, pp. 2975-2997.
- [2] Danielsen, E.M., Skovbjerg, H., Noren, O. and Sjoström, H. (1984) *Biochem. Biophys. Res. Commun.* 122, 82-90.
- [3] Nain, H.Y., Sterchi, E.E. and Lentze, M.J. (1987) *Biochem. J.* 241, 427-433.
- [4] Skovbjerg, H., Danielsen, E.M., Noren, O. and Sjoström, H. (1984) *Biochim. Biophys. Acta* 798, 247-251.
- [5] Buller, H.A., Montgomery, R.K., Sasak, W.V. and Grand, R.J. (1987) *J. Biol. Chem.* 262, 17206-17211.
- [6] Mantei, N., Villa, M., Enzler, T., Wacker, H., Boll, W., James, P., Hunziker, W. and Semenza, G. (1988) *EMBO J.* 7, 2705-2713.
- [7] Danielsen, E.M. and Cowell, G.M. (1984) *FEBS Lett.* 166, 28-32.
- [8] Danielsen, E.M. and Cowell, G.M. (1985) *Eur. J. Biochem.* 152, 493-499.
- [9] Yeh, K.Y., Yeh, M., Pan, P.C. and Holt, P.R. (1991) *Gastroenterology* 101, 312-318.
- [10] Jonas, M.M., Montgomery, R.K. and Grand, R.J. (1985) *Pediatr. Res.* 19, 956-962.
- [11] Quan, R., Santiago, N.A., Tsuboi, K.K. and Gray, G.M. (1990) *J. Biol. Chem.* 265, 6978-6983.
- [12] Castillo, R.O., Reisenauer, A.M., Kwong, L.K., Tsuboi, K.K., Quan, R. and Gray, G.M. (1990) *J. Biol. Chem.* 265, 15889-15893.
- [13] Tsuboi, K.K., Kwong, L.K., Sunshine, P. and Castillo, R.O. (1992) *Biochem. J.* 282, 107-113.
- [14] Quaroni, A. and Isselbacher, K.J. (1985) *Dev. Biol.* 111, 267-279.
- [15] Maiuri, L., Rossi, M., Raia, V., D'Auria, S., Swallow, D., Quaroni, A. and Auricchio, S., *Gastroenterology*, in press.
- [16] Laemmli, U.K. (1970) *Nature* 227, 680-685.
- [17] Bonner, W.M. and Laskey, R.A. (1974) *Eur. J. Biochem.* 46, 83-88.