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The expression of the Na^+/glucose cotransporter (SGLT1) gene in lamb small intestine during postnatal development

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We have shown previously that the activity and abundance of the intestinal Na^+/glucose cotransporter (SGLT1) declines dramatically during the postnatal development of lambs, and that it can be restored in the intestine of ruminant sheep by intra-luminal infusion of D-glucose. The work presented in this paper has followed the expression of the SGLT1 gene along the vertical and horizontal axes of the ovine small intestine during early development, using quantitative in situ hybridisation histochemistry. Along the vertical axis, SGLT1 mRNA was first detectable just below the crypt-villus junction and rose rapidly to a peak level approx. 150 μm above this point. After reaching a maximum, the amount of message gradually declined towards the villus tip. This pattern of mRNA accumulation along the crypt-villus axis was similar in all intestinal positions and age groups. Along the length of the small intestine (horizontal axis), a decline in the level of SGLT1 mRNA was observed first in the distal intestine. This decrease in SGLT1 mRNA was significant in the intestine (75% of length) of 5-week-old lambs when compared to tissue taken from 25 and 50% of length ($P < 0.01$ and $P < 0.02$, respectively). However, the observed fall in the expression of this gene during weaning did not coincide with the fall in activity and amount of SGLT1. In adult animals, where the activity of SGLT1 is very low, the amount of message was greatly reduced. This work supports the finding that the expression of SGLT1 is primarily controlled at the post-transcriptional level during the postnatal development of ovine intestine.

Introduction

The primary source of dietary carbohydrate during the early postnatal development of all mammals is the milk sugar lactose. Hydrolysis of lactose into its constituent monosaccharides by the brush border enzyme lactase, liberates large amounts of D-glucose and D-galactose into the lumen of the small intestine. With the onset of weaning, this source of glucose/galactose begins to decline. However, in non-ruminants, including man, the level of sugars reaching the intestinal lumen is maintained during adult life from other sources of dietary carbohydrate. This situation is in contrast to the ruminant intestinal tract. Sheep are normally weaned between 3–8 weeks of age which coincides with the maturation of the rumen. In this organ, dietary carbohydrates are fermented into volatile fatty acids by the rumen microflora. As a consequence of this, the amount of hexoses reaching the small intestine gradually declines during weaning and are undetectable in the intestine of adult sheep [1].

The Na^+/glucose cotransporter (SGLT1) is present in the brush border membrane of the epithelial cells (enterocytes) lining the small intestine where it serves to absorb luminal D-glucose and D-galactose. We have previously investigated the expression of SGLT1 in the ovine small intestine during postnatal development and shown that the activity and the abundance of this cotransporter dramatically declines during weaning. The initial rate of uptake by brush-border membrane vesicles prepared from the proximal intestine of preruminant lambs (birth to 3 weeks old) was high (347 ± 130 pmol/mg per s, mean ± S.E.). The rates of glucose uptake in vesicles prepared from the proximal intestine of 5-week-old lambs was significantly less (79 ± 26 pmol/mg per s), and uptake into vesicles prepared from the intestine of 10–12-week-old ruminant lambs

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Abbreviations: SGLT1, Na^+/glucose cotransporter; SSC, standard sodium citrate; PBS, phosphate-buffered saline.

or adult sheep was barely measurable (4 ± 1.5 pmol/mg per s) [2,3]. This decline in the glucose transport capacity of the small intestine appears to be largely due to substrate deprivation as (a) lambs kept on a milk replacer diet beyond the weaning period did not show the normal decrease in activity and (b) ruminant animals whose small intestines were infused with a solution of 30 mM D-glucose for 4 days showed an increase in activity of SGLT1 between 40–80-fold above controls [3]. The ovine intestine therefore provides a unique model system to study the regulation of intestinal sugar transport by dietary sugars.

The activity of this cotransporter has also been demonstrated to be regulated by dietary substrate levels in many other species [4]. The mechanisms by which glucose regulates the abundance and activity of the protein that transports it, are unknown. Sequencing of the rabbit SGLT1 gene [5] and more recently the human gene [6] has now made it possible to examine the expression of this cotransporter at the mRNA level. This study has followed the expression of the SGLT1 gene during the first 5 weeks of ovine development using quantitative in situ hybridisation histochemistry. This approach has allowed the examination of the pattern of SGLT1 mRNA accumulation along the vertical and horizontal axes of the ovine small intestine, during this interesting developmental period.

Materials and Methods

Materials

Materials were obtained from BDH Ltd., Poole, Dorset, UK, unless otherwise stated.

Collection and storage of tissue

All animals were Clun Forest, they had been reared normally, and allowed to run with their ewes and have access to grass. Animals were aged 1 day ($n = 6$), 14 days ($n = 6$), 35 days ($n = 6$) and adult (1–3 years, $n = 4$). Prior to the removal of the tissue animals were injected intravenously with a lethal dose of 10–20 ml of pentobarbitone (Lethobarb, Duphar Veterinary Ltd., Southampton, UK). The abdomen was opened and the intestine promptly removed. Tissue was taken from 25, 50 and 75% along the length of the small intestine and washed with ice-cold phosphate-buffered saline (PBS) containing 0.5 mM dithiothreitol and 1 U/ml RNase inhibitor (Sigma, Gillingham, Dorset, UK). The intestine was then opened out, embedded in pigs liver and rapidly frozen in isopentane cooled with solid carbon dioxide. Tissue was stored at −80°C prior to use.

In situ hybridisation

(a) Using oligonucleotide probes.

The basic method used for in situ hybridisation has been described previously [7]. Briefly, non-serial cryostat sections (10 μm) were thaw-mounted onto poly(L-lysine) coated slides (3–5 sections/slide), fixed for 5 min in 4% phosphate-buffered paraformaldehyde on ice, and washed in PBS. Slides were dehydrated through alcohol and stored in 95% alcohol at 4°C until use. The oligodeoxyribonucleotide probes (41-mers, 'sense' and 'anti-sense') were synthesized on a Biosearch 8700 DNA synthesiser and purified on 8% polyacrylamide/8 M urea preparative sequencing gel. The sequence of the 'anti-sense' probe was complementary to nucleotides 1508–1548 (amino acids 500–513) of human SGLT1 cDNA [6]. Mixed site synthesis was used to allow for the two differences between the human and rabbit cDNAs [5] in this region. The sequence of the probe was unique to SGLT1 when examined on the GenBank and EMBL databases [8,9] (release 70 and 30, respectively). The probes were labelled using terminal deoxynucleotidyl transferase (Pharmacia, Milton Keynes, Bucks., UK) and [α-^{35}S]dATP (1300 Ci/mM, NEN, Stevenage, Herts., UK) at 32°C for 1 h. The

TABLE I

Median villus length and mean Na^+/glucose cotransporter (SGLT1) mRNA levels during ovine development

The mean villus mRNA level (absorbance, arbitrary units) has been used to compare the amount of SGLT1 mRNA between intestinal positions in the same age group. A paired *t*-test shows that the level of SGLT1 mRNA in the distal gut (75%) of 5-week-old animals is significantly lower than in the proximal and mid gut (25% and 50%, $P < 0.01$ and $P < 0.02$, respectively) of these lambs. Results were taken from 6 animals per group. The variation in villus length down the horizontal axis of the intestine and during development were not consistently significant when tested by the Mann-Whitney *U*-test.

Age	1 day			14 day			35 day		
Intestinal position	25%	50%	75%	25%	50%	75%	25%	50%	75%
Median villus length in μm (range)	1560 (1140–1860)	1380 (600–1860)	900 (840–1560)	1500 (1140–1860)	1170 (720–1680)	660 (540–780)	1080 (960–1440)	900 (600–1440)	540 (420–720)
Mean villus absorbance, A.U. ±S.E.	6.33 ±1.43	5.20 ±1.96	4.84 ±1.82	8.70 ±1.26	9.06 ±0.58	6.43 ±0.71	8.13 ±0.70	6.79 ±1.01	2.94 ±0.41

probe was applied to the sections in hybridisation buffer containing 4 × standard sodium citrate (SSC), 50% deionised formamide, 25 mM sodium phosphate (pH 7.0), 5 × Denhardt's solution, 200 μg/ml hydrolysed salmon sperm DNA, 120 μg/ml heparin, 100 μg/ml polyadenylic acid, 10% dextran sulphate and 40 mM dithiothreitol. The slides were left overnight at 42°C before being washed in 1 × SSC containing 20 mM β-mercaptoethanol for 1 h at 55°C and dehydrated through alcohol. Slides were exposed to X-ray film (Kodak XAR 5) for 5 days and then dipped in Ilford K5 emulsion and stored desiccated at 4°C for 2 weeks. After development, sections were stained with eosin and mounted with a coverslip.

(b) Using cDNA probe.

The full length cDNA for SGLT1 was a kind gift of Prof. E.M. Wright. The cDNA used was the protein coding region, approx. 2 kb, of rabbit intestinal SGLT1 [5]. This was isolated from its plasmid by digestion with *Eco*RI and *Mlu*I, gel purified and labelled with [α-^{35}S]dCTP (NEN) using a random prime method (Oligolabelling Kits, Pharmacia).

Sections (7 μm) of rabbit jejunum (10 weeks old) were prepared from tissue that had been fixed overnight in 4% phosphate-buffered paraformaldehyde and embedded in paraffin wax. The slides were dried at 60°C for 30 min, dewaxed and treated with 0.25% acetic anhydride in triethanolamine (0.1 M, pH 8.0) for 10 min. Slides were washed in 50 mM Tris buffer for 5 min and transferred to a solution of proteinase K (25 μg/ml, Sigma) for 1 h. After digestion, slides were washed in PBS and then water, and dehydrated through alcohol.

The labelled cDNA was applied to sections and the slides treated as described previously for the oligonucleotide probes. Slides were exposed to photographic emulsion for 3 days.

Northern blot analysis

Rabbit and lamb jejunal mucosal scrapings were used to extract total RNA [10]. Poly(A)$^+$ RNA was isolated by oligo(dT) cellulose chromatography [11]. The cDNA probe was prepared as described above but labelled with [α-^{32}P]dCTP (Amersham). The oligonucleotide probe was labelled with [γ-^{32}P]dATP using T4 DNA polynucleotide kinase [11]. Samples of poly(A)$^+$ RNA (2.5 μg per lane) were separated by electrophoresis on a 1% agarose gel (Seakem, FMC, Sittingbourne, Kent, UK) containing 2 M formaldehyde, transferred to Duralon-UV nylon membrane (Stratagene, Cambridge, Cambs., UK) and cross-linked by UV irradiation. Membranes were prehybridised for 4 h at 42°C in a hybridisation buffer containing 50% formamide, 5 × SSC, 3 × Denhardts, 0.2% SDS, 10% Dextran sulphate, 0.01% antifoam B (Sigma), 2.5 mM sodium pyrophosphate and 25 mM 2-(*N*-morpholino)-ethanesulfonic acid (Mes), pH 6.5. Following hybridisation with the cDNA probe, blots were washed for 10 min 5 × SSC/0.5% SDS/0.25% sarkosyl at 20°C and 55°C, followed by 1 × SSC/0.5% SDS at 55°C. Using the oligonucleotide probe the membranes were washed in 5 × SSC/0.5% SDS/0.25% sarkosyl at 42°C for 10 min and then in 1 × SSC/0.5% SDS for 10 min at 42°C. The membranes were apposed to Kodak XRP/5 film in cassettes containing intensifying screens (HI-speed, Hoefer, Newcastle, Staffs., UK) at −80°C for 1–8 days.

Quantification of in situ hybridisation

The system used to quantify the in situ hybridisation signal is similar to that used for the quantification of

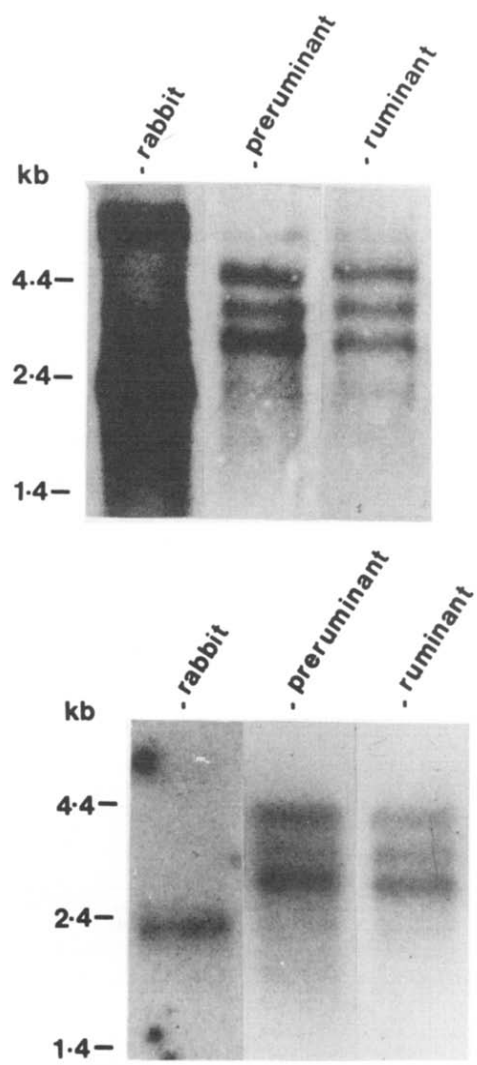

Fig. 1. Northern blot analysis of poly(A)$^+$ RNA (2.5 μg/lane) extracted from rabbit and ovine preruminant and ruminant small intestine. Fig. 1a (top) shows the pattern of hybridisation of a ^{32}P-labelled full length (2.2 kb) SGLT1 cDNA probe and Fig. 1b (bottom) the hybridisation of the 'anti-sense' oligonucleotide probe (41 mer) to the same poly(A)$^+$ RNA samples. Hybridisation was performed under conditions of similar stringency to that used for in situ hybridisation and blots were exposed for 1–8 days.

calbindin mRNA in chicken jejunum [12]. In the present study every attempt has been made to minimise variation due to localised differences in the intestine, photographic emulsion and other potential irregularities in assay conditions. The tissue from all animals was collected in as reproducible manner as possible and

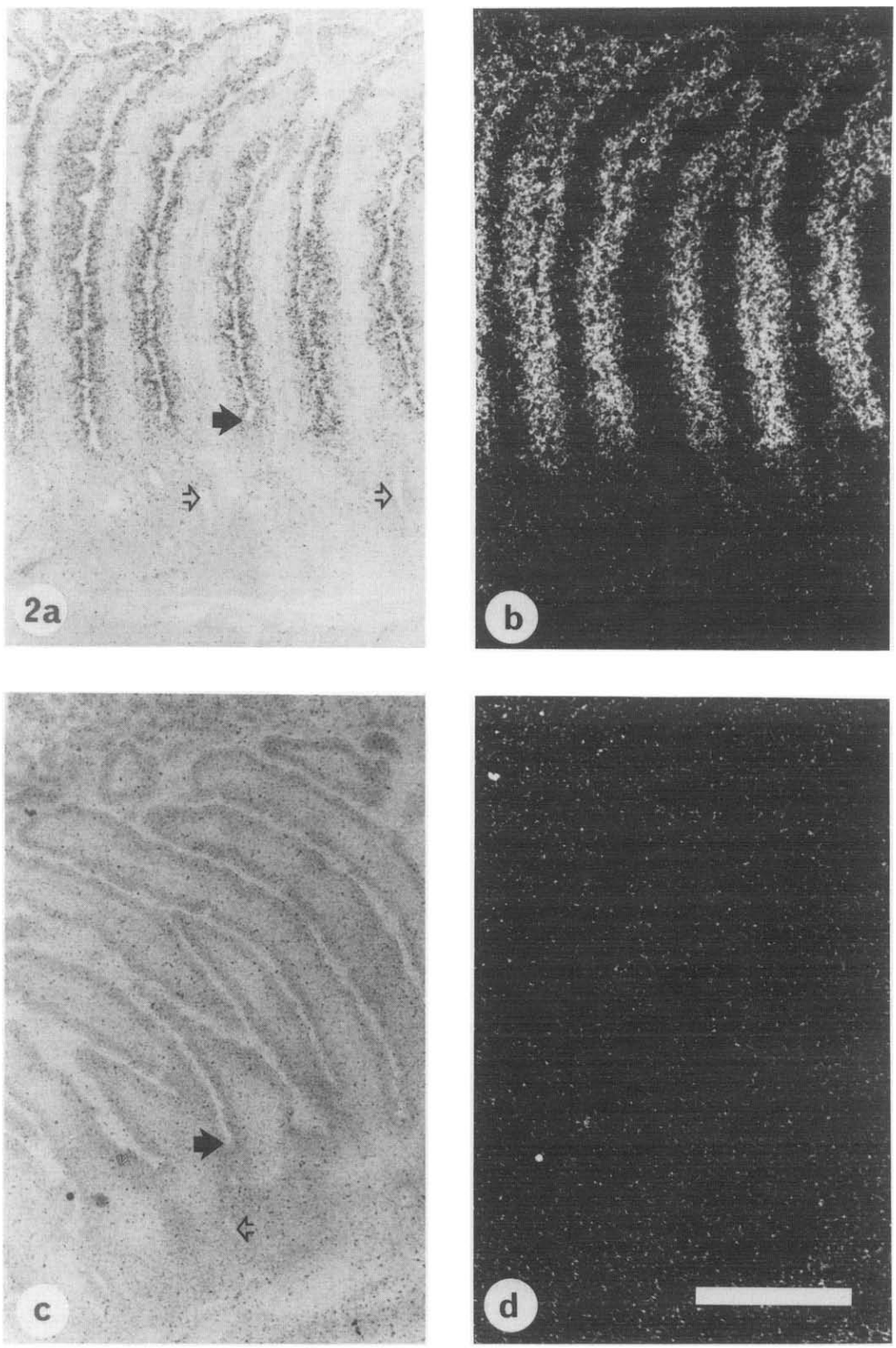

Fig. 2. Brightfield (a) and darkfield (b) photomicrographs of the hybridisation of the ^{35}S-labelled 'anti-sense' oligonucleotide probe to SGLT1 mRNA in frozen-fixed sections (10 μm) of 14-day-old lamb jejunum. Fig. 2c and d show similar pictures of the same intestinal tissue after incubation with the ^{35}S-labelled 'sense' probe performed under identical conditions as used for hybridisation of the 'anti-sense' probe. Sections were exposed to photographic emulsion for 14 days and counterstained with eosin. Solid arrows indicate the crypt-villus junction and open arrows crypts. Scale bar = 250 μm.

stored at −80°C prior to sectioning. When preparing slides, at least 50 μm was allowed between consecutive sections to minimise any local differences in the intestine and between 3–5 sections were placed on each slide. All in situ hybridisation was carried out in a single assay and slides were treated identically

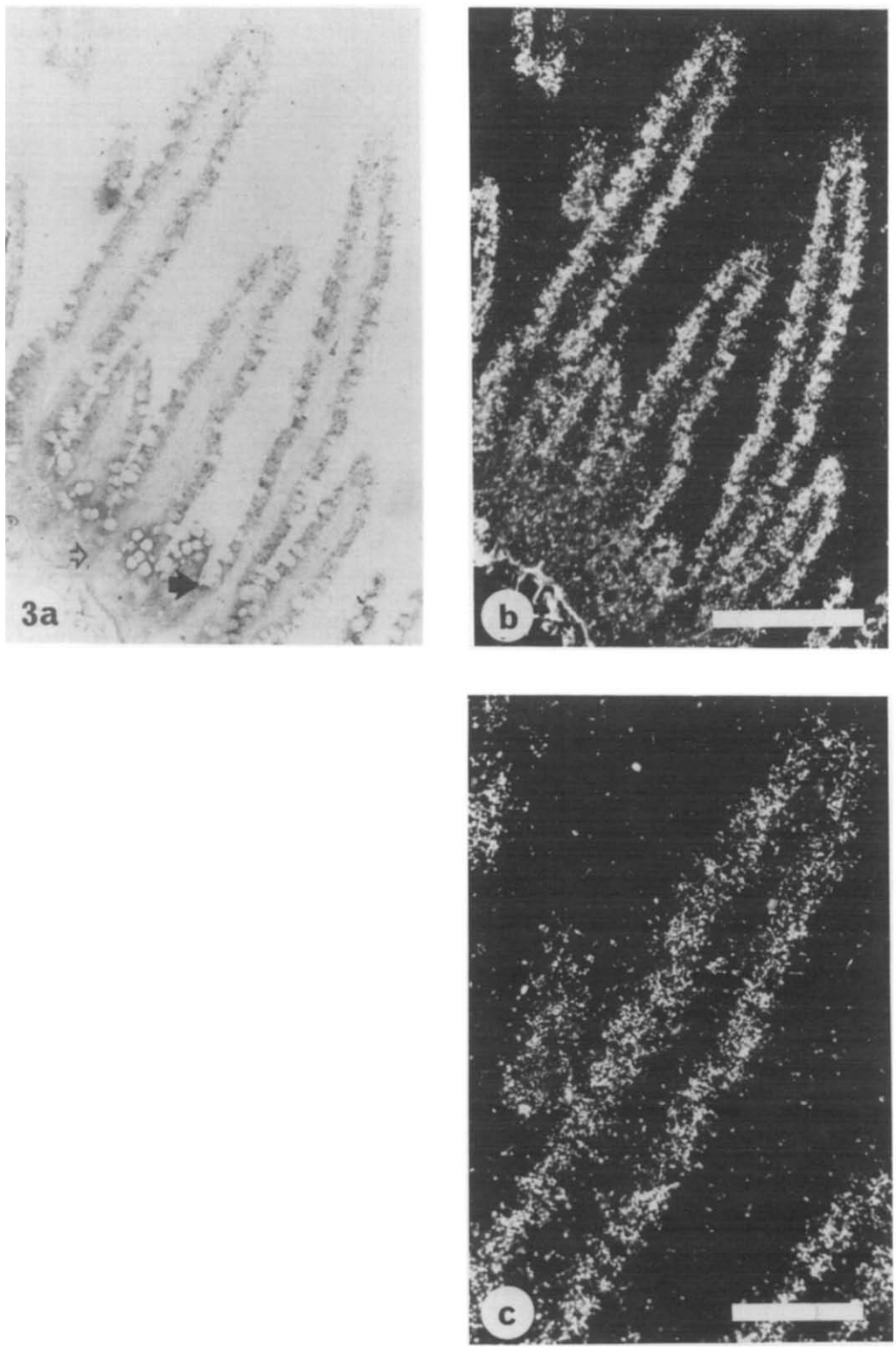

Fig. 3. Brightfield (a) and darkfield (b and c) photomicrographs of the hybridisation of ^{35}S-labelled SGLT1 cDNA to SGLT1 mRNA in rabbit jejunum (10-week-old) which had previously been fixed in 4% formaldehyde and embedded in paraffin wax. Sections were exposed to photographic emulsion for 3 days and counterstained with eosin. Solid arrows indicate the crypt-villus junction and open arrows crypts. Scale bar (a and b) = 250 μm and (c) = 50 μm.

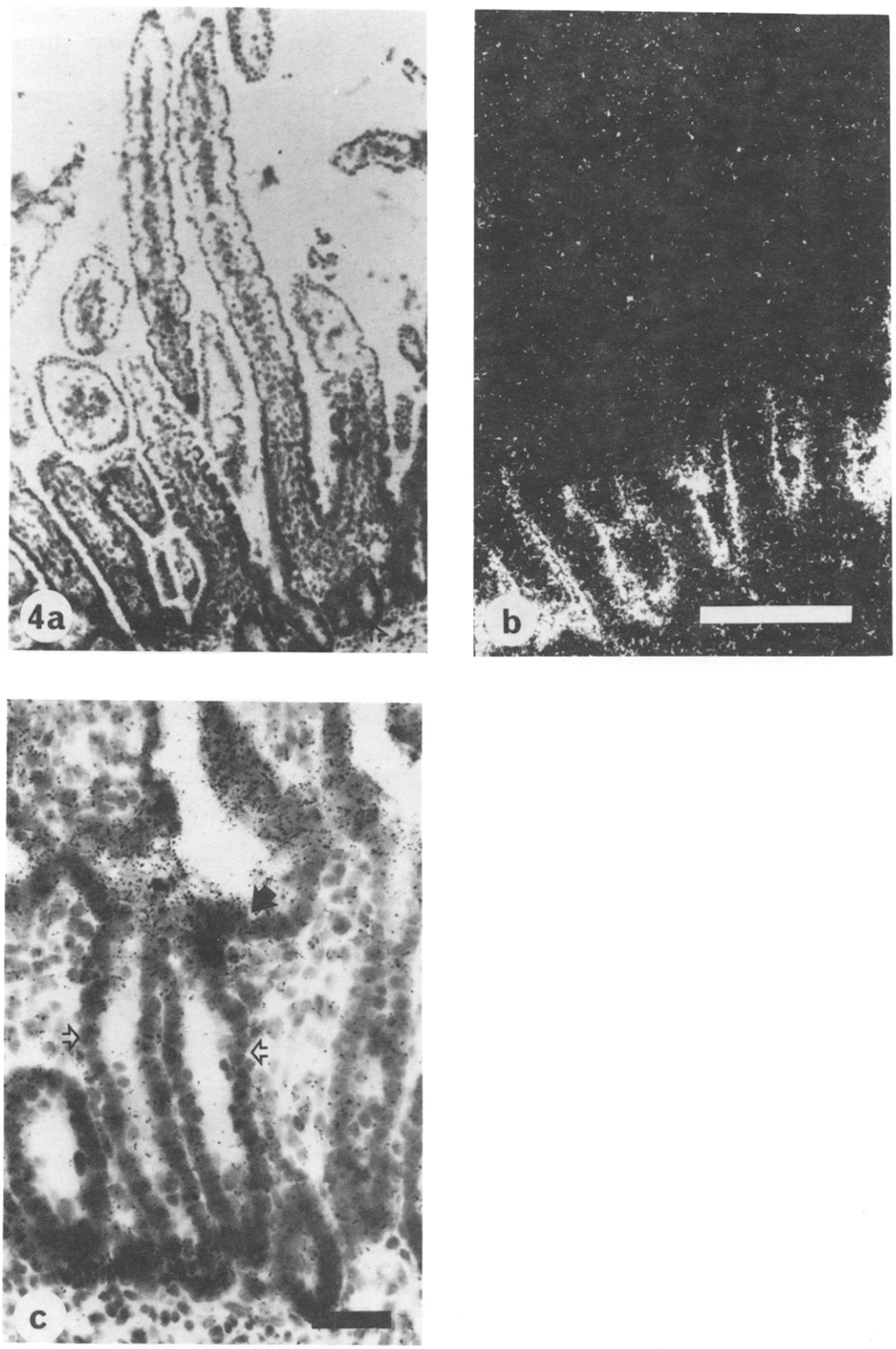

Fig. 4. Brightfield (a and c) and darkfield (b) photomicrographs of the hybridisation of the ^{35}S-labelled 'anti-sense' oligonucleotide probe to SGLT1 mRNA in frozen-fixed sections (10 μm) 1-day-old lamb jejunum. Fig. 4a and b clearly show the presence of SGLT1 mRNA only in the lower villus and Fig. 4c the start of SGLT1 mRNA accumulation at the crypt-villus junction. Sections were exposed to photographic emulsion for 14 days and counterstained with haematoxylin and eosin. Solid arrows indicate the crypt-villus junction and open arrows crypts. Scale bar = 250 μm.

throughout. Sections were examined using a MPV-3 microdensitometer (Leitz, Milton Keynes, Bucks., UK) at a magnification of ×400. The wavelength of the spectrophotometer was set at 640 nm. The measuring window (15 × 60 μm) was placed over the lamina propria near to the villus to be measured and the absorbance reading was set at zero. Consecutive density readings were then taken over the enterocyte population, from crypt base to villus tip, on five separate villi/slide.

Analysis of data

The mean level of mRNA (absorbance) at each point along the crypt-villus axis was calculated from the mean of five readings of five separate villi from each animal in an age group. Since there were variations in the length of individual villi, mean absorbance values along the crypt-villus axis were included in the results only when over half the villi had been measured still gave readings. The crypt readings were divided into equal halves (lower and upper crypt) and the villus readings into equal thirds (lower, mid and upper villus), to allow for the comparison of mRNA levels along the crypt-villus axis in animals of different ages, with villi of variable length (Fig. 7). The crypt-villus junction was taken to be the point at which enterocytes reach the lumen. A paired *t*-test employing the mean villus mRNA level was used to compare enterocyte SGLT1 gene expression between different intestinal positions in an age group. The variation in villus length down the small intestine and during development were analysed using a Mann-Whitney *U*-test.

Results and Discussion

Changes in the intestinal structure during development

Many changes occur in the structure and function of the gastro-intestinal tract during postnatal development. This study has examined tissue taken from 25, 50 and 75% along the length of the ovine small intestine. These could be described as the proximal and mid jejunum and distal jejunum/proximal ileum, respectively; the exact jejunal-ileal junction is difficult to determine. Two trends were noticeable in the tissue used here (a) a decrease in villus length down the length of the small intestine, being most pronounced in the distal sampling position, (b) a decrease in villus length with age (see Table I). These changes were not however consistently significant, possibly due to the relatively small sample size. Previous studies have also observed a concurrent decrease in the enterocyte migration rate [13] and an increase in villus width and crypt depth [14], during this period of ovine development.

Specificity of hybridisation

Northern blot analysis of poly(A)$^+$ RNA extracted from ovine and rabbit intestine was used to establish the specificity of hybridisation of the 'anti-sense' oligonucleotide probe. Fig. 1 shows a Northern blot analysis of poly(A)$^+$ RNA isolated from the intestinal mucosa of rabbits, and preruminant and ruminant sheep. Fig. 1a shows the pattern of hybridisation of the cDNA probe, and Fig. 1b the hybridisation of the oligonucleotide probe to the same RNA samples. Both the SGLT1 cDNA and oligonucleotide probes recognise five RNA transcripts of approx. 6.0, 5.0, 4.0, 3.2 and 2.4 kb in ovine intestine. In addition, the level of SGLT1 mRNA was reduced in the ruminant animals compared to preruminant lambs. Both types of probe also hybridise to a 2.3 kb transcript from rabbit intestinal RNA. This pattern of hybridisation is consistent with previous studies [15,16].

The multiple mRNA species produced by this gene have been shown to possess the same coding region, but differ in the length of the 3′ non-translated region [6].

In situ hybridisation

We used quantitative in situ hybridisation technology to examine the cellular distribution of SGLT1 mRNA along (a) crypt-villus axis and (b) the length of the small intestine, during the transition from a suckling to a weaned lamb.

Radio-labelled probes were incubated with tissue sections cut from 25, 50 and 75% along the length of the small intestine of lambs aged 1, 14, and 35 days old and adult sheep (1–3 years). The hybridisation pattern of the 'anti-sense' probe to this tissue was largely consistent with the known distribution of SGLT1. The probe specifically hybridised to the villus enterocyte population in all lamb samples tested (Fig. 2a and b); no hybridisation was observed to the cells of the lower

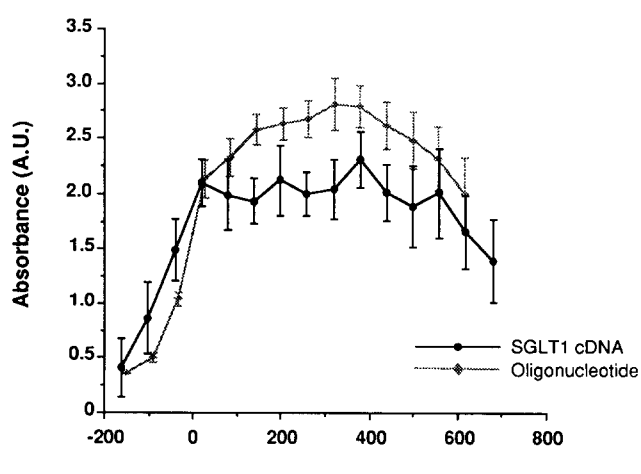

Fig. 5. A comparison of the profile of SGLT1 mRNA accumulation (arbitrary units) along the crypt-villus axis of rabbit jejunum as determined using SGLT1 cDNA and oligonucleotide probe [17]. Consecutive absorbance readings were taken along the crypt-villus axis at 60 μm intervals on 5 villi/slide.

crypts or any other cell type in the intestine. In addition, the level of signal was decreased in the adult sheep intestine which is known to possess very low levels of sodium-dependent transport activity [3] and significantly less (approx. 4-fold) SGLT1 mRNA, compared to lamb intestine [15]. No specific hybridisation was observed with the control 'sense' probe when labelled to the same specific radio-activity and used under identical hybridisation conditions (Fig. 2c and d). Finally, the profile of SGLT1 mRNA along the crypt-villus axis of rabbit small intestine was similar when in situ hybridisation was performed using either SGLT1 cDNA or the oligonucleotide probe [17] (Figs. 3a–c and 5).

(a) Distribution of SGLT1 mRNA along the crypt-villus axis

The distribution of SGLT1 mRNA along the crypt-villus axis was similar in all ages of lamb studied and at each position along the length of the small intestine (Figs. 6 and 7). The message was first detectable in crypt cells approx. 60 μm below the crypt-villus junction and rose rapidly to a peak level approx. 150 μm above this point. After reaching a maximum, the amount of message gradually declined towards the villus tip. Presentation of the data in Fig. 7 allows for the direct comparison of the relative distribution of mRNA along the crypt-villus axis of villi of different length.

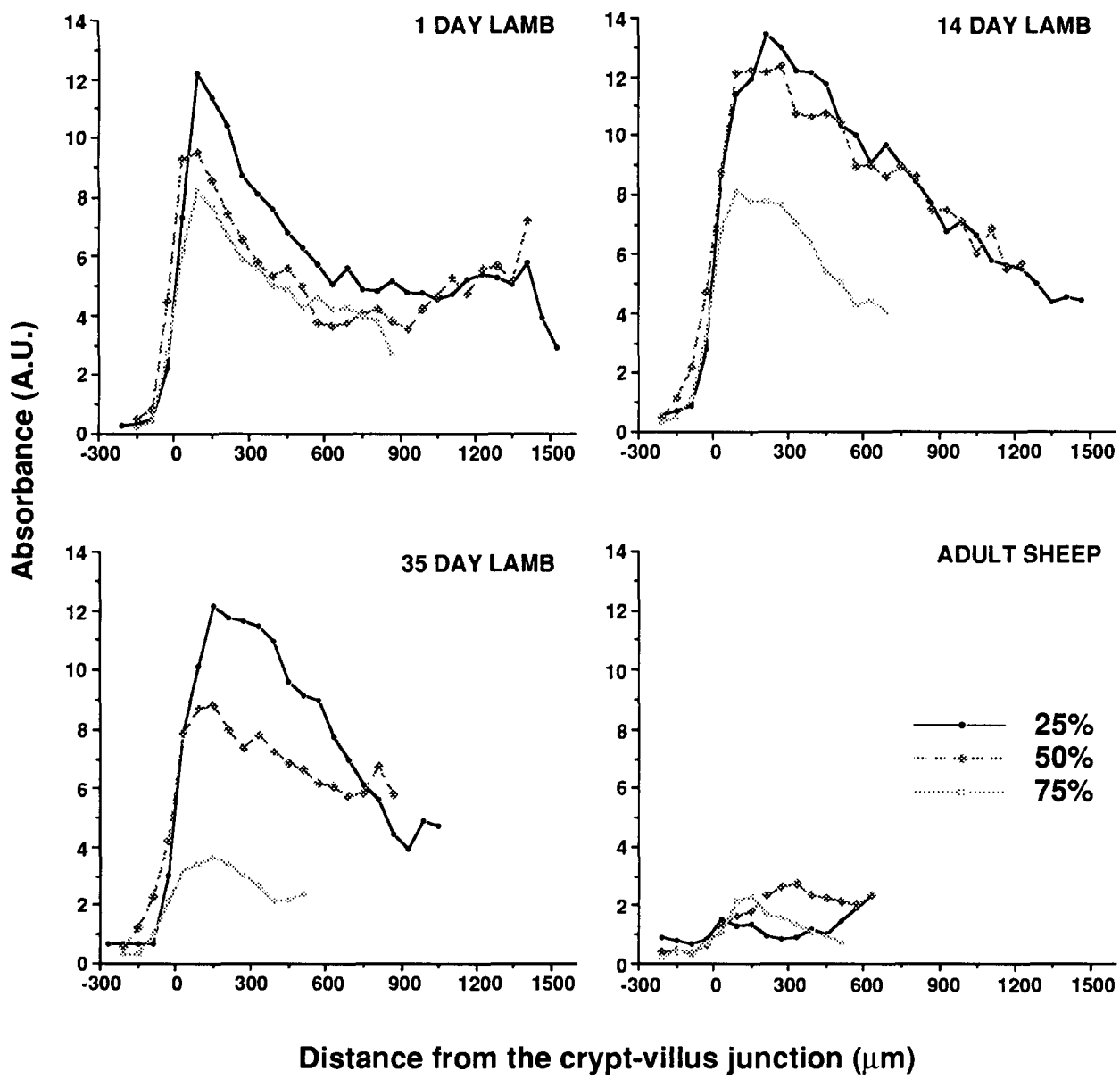

Fig. 6. The profile of SGLT1 mRNA along the crypt-villus axis of ovine small intestine during development. Tissue was taken from 25, 50 and 75% along the length of the small intestine of animals aged 1 ($n = 6$), 14 ($n = 6$) and 35 ($n = 6$) days old and 1–3-year-old adult sheep ($n = 4$). Consecutive absorbance readings were taken along the crypt-villus axis at 60 μm intervals on 5 villi/slide.

Similar work examining the distribution of mRNA for other proteins produced in villus enterocytes – villin [18], aminopeptidase N [19], sucrase-isomaltase [20,21], intestinal fatty acid binding protein (I-FABP) [22], intestine-specific anexin (ISA) [23] and calbindin [12] – all suggest a similar pattern to that seen here. Direct comparison is however difficult as results were not quantified in the majority of previous studies. It is interesting to note that the distribution of SGLT1 mRNA along the crypt-villus axis, as localised using both oligonucleotide and cDNA probes (Figs. 5 and 6), is not concurrent with the localisation of the functional activity of SGLT1, which is generally considered to be maximal in the upper villus [24–26]. Immunocytochemical demonstration of the presence of SGLT1 along the length of the villus in rat and rabbit intestine [27,28] raises the possibility that there may be a delay in the post-translational processing of the transporter, leading to the appearance of functional activity in the later stages of enterocyte migration along the villus. Localisation of SGLT1 in lamb intestine is necessary to test the above hypothesis in this model.

An interesting variation in the distribution of SGLT1 mRNA along the crypt-villus axis to that described above, was seen in the intestine of a number of 1-day-old lambs. In two lambs of this age used in this study, and in a number of animals studied subsequently, mRNA was only detectable in the lower villus; from the crypt-villus junction to between 100 and 300 μm above this point (Fig. 4). This pattern was the same in all regions of the intestine tested. It is still uncertain what causes such a striking change in the abundance of mRNA in the upper villus, or whether loss of mRNA in the upper villus is particular to the expression of SGLT1. Preliminary results however, suggest that the effect may be due to the disruption of enterocyte mRNA synthesis, due to the formation of large, protein rich intra-cellular vacuoles during the acquisition of maternal antibodies from colostrum [29]. Animals of this age in which these vacuoles were not present, possessed mRNA along the length of the villus.

(b) Distribution of SGLT1 mRNA along the length of the ovine small intestine

The mean villus enterocyte level of SGLT1 mRNA was not significantly different in the three regions, 25, 50 and 75% along the length of the small intestine, in 1-day-old animals. In 14-day-old animals the decrease in SGLT1 mRNA in the distal gut (75% of length) was more pronounced but not significantly so (compared with 25%). By 5 weeks old, SGLT1 mRNA in the distal gut (75% of length) was significantly reduced compared with 25% and 50% ($P < 0.01$ and $P < 0.02$, respectively, Table I). Therefore, it appears that the decrease in the abundance of SGLT1 mRNA during ovine development occurs first in the distal small intestine. In non-ruminant animals Na^+-dependent glucose transport is also lower in the distal gut [30]. This could be as a consequence of substrate gradients which are known to occur within the small intestine [31]. The amount of SGLT1 mRNA in the small intestine of adult sheep has been shown, using Northern blot analysis, to be approx. 4-fold lower than in the intestine of preruminant lambs [15]. This finding is comparable to the levels observed here using in situ hybridisation.

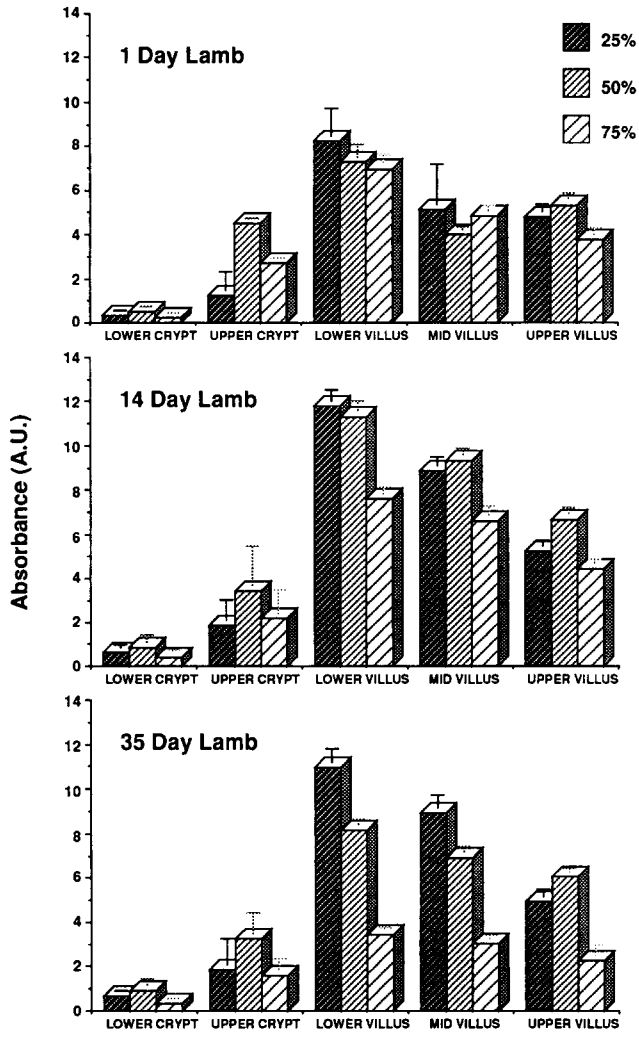

Fig. 7. Mean SGLT1 mRNA levels (±S.E.) at five positions along the crypt-villus axis; lower and upper crypt and lower, mid and upper villus at each sampling position along the small intestine. The presentation of the data in this form allows for the direct comparison of mRNA levels during the enterocyte life cycle from villi of different length.

Previous work examining the activity and amount of SGLT1 in brush-border membrane vesicle preparations during ovine postnatal development, observed a rapid fall in both parameters with the onset of weaning. The activity of the transporter was 4–5-fold lower in vesicles prepared from the proximal intestine of 35-day-old lambs than from 14-day-old animals [3]. However, this

study demonstrates that the level of SGLT1 mRNA in the proximal gut (25%) of animals of this age is almost identical. It would therefore appear that the control of SGLT1 activity during ovine development is primarily controlled by post-transcriptional events. Post-transcriptional regulation has also been inferred for the expression of lactase during the postnatal development of the rat intestine, as lactase mRNA remained elevated long after the amount of active protein had declined [32]. It is possible therefore that the activity of a number of the absorptive and digestive functions of the small intestine are controlled at both the transcriptional and translational levels. Transcription perhaps providing a crude control over protein synthesis, and post-transcriptional events providing the flexibility to 'fine tune' protein activity to meet the nutritional status of the animal.

In summary, this study has demonstrated that in the ovine small intestine, SGLT1 mRNA is absent, or at very low levels, in the lower crypts and accumulation of mRNA encoding for SGLT1 begins as the enterocytes approach the crypt-villus junction. Maximum levels are observed at approx. 150 μm above this point. The decline in SGLT1 mRNA to the level observed in adult ruminant small intestine, occurs first in the distal intestine. However, it appears that this decline in SGLT1 mRNA is not primarily responsible for the decrease in the abundance and activity of SGLT1 during this period. It may therefore be concluded that the activity of SGLT1 is controlled predominantly at the post-transcriptional level in the ovine small intestine during postnatal development. Further work will examine the mechanism of the post-transcriptional processing of SGLT1.

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