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Crypt/villus site of substrate-dependent regulation of mouse intestinal glucose transporters

(induction/carbohydrates/phlorizin/active transport)

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ABSTRACT **The intestinal epithelium is in a constant state of turnover, with cells differentiating at the crypts and then migrating toward the tips of the villi. Does substrate-dependent regulation of intestinal Na^+/D-glucose cotransporters occur only in crypt cells, or can transport activity be subsequently reprogrammed in mature enterocytes? We used *in situ*, glucose-protectable specific phlorizin binding to determine site density of brush border glucose transporters in enterocytes fractionated along the crypt/villus axis of mice that were killed shortly after drastic changes in carbohydrate levels of their diets. Dietary carbohydrate-induced changes in site density of specific phlorizin binding initially appeared only in crypt cells before spreading, over the course of several days, to the villus tips. Thus, only crypt cells perceive the signal for glucose transporter regulation, and the observed time lag of diet-induced changes in intestinal glucose uptake is due mainly to cell migration times.**

Within a day, a high-carbohydrate (HC) diet stimulates intestinal D-glucose transport, and a high-protein diet stimulates intestinal amino acid transport. Increased intestinal absorption of D-glucose is due solely to specific, parallel increases in number of intestinal brush border Na^+/D-glucose cotransporters (1, 2) and in number and activity of basolateral, facilitated D-glucose transporters (3); no other mechanism such as a change in intestinal mass, solvent drag, passive permeability, or Na^+ gradient need be involved (4, 5). Does induction of transporters take place only in developing enterocytes at the base of the intestinal crypts, or can it also occur in mature enterocytes near the tips of the villi? A similar question has already been asked for substrate-dependent induction of sucrase (6, 7). This uncertainty about substrate-dependent regulation of transporters arises because of the rapid state of turnover of small intestinal cells. The absorptive cells of the small intestine are heterogeneous: stem cells at the base of the crypt simultaneously differentiate and migrate up the villus column before eventually being sloughed off at the tip (Fig. 1). In mouse intestinal epithelium, the migration from crypt base to villus tip takes 2–3 days (8).

The time lag between switching a mouse onto a HC diet and the first appearance of enhanced sugar transport is between 12 and 24 h (4) and is consistent with two alternative hypotheses: (*i*) induction of transporters only in developing crypt cells and not in mature villus cells, or (*ii*) induction of transporters in cells of all ages. By the first hypothesis, induction would be rapid and the lag would be due to cell migration times; by the second hypothesis, the lag would be due to induction itself. Determining site density of transporters in enterocytes at short times after a diet switch would enable us to distinguish between the two hypotheses. If only crypt cells respond to changes in luminal carbohydrates, we expect a higher number of transporters to appear first in crypt cells and to spread gradually along the axis toward the villus tips. If glucose transporter induction by dietary substrate can occur in all cells, then we expect changes in transporter number to appear simultaneously at different positions along the crypt/villus axis.

To determine the site of induction of the brush border glucose transporter, we performed a series of four experiments. First, we validated a method (9) that separated the enterocytes along the crypt/villus axis (Fig. 1). This allowed us to study diet-induced changes in transporter number of cells from different regions of the villus. Second, we modified the phlorizin binding method, previously applied to determine the site density of Na^+/D-glucose cotransporters in intact mucosa (10) and brush border membrane vesicles (11), to apply it to isolated mouse enterocytes (12). We fed mice a HC or no-carbohydrate (NC) diet for 2 weeks and then quantified diet-induced, steady-state variations in site density of specific phlorizin binding in each region of the villus column. Third, we demonstrated that intestinal mass, villus height, and enterocyte migration rates as well as lifetimes of enterocytes were all independent of dietary carbohydrate (8). A dietary effect on these parameters could have changed intestinal D-glucose transport nonspecifically. Finally, we determined diet-induced, transient variations in glucose transporter site density along the crypt/villus axis of mice whose diets were switched at a series of short times from a NC to a HC diet or vice versa. We found that dietary carbohydrate-induced changes in site density of transporters initially appeared in crypt cells and only later appeared in upper villus regions as those crypt cells migrated toward the villus tip.

MATERIALS AND METHODS

Animals and Diets. We used adult male white Swiss Webster mice (average body weight, 35.1 ± 1.2 g) with ad libitum access to food and water.

Mice were fed chow (Wayne Lab Blox; Allied Mills, Chicago; 50% carbohydrate/24.5% protein) for 1 week until use in diet experiments. The HC diet consisted of 55% soluble carbohydrate (sucrose)/15% protein; the NC diet had 70% protein. Both diets contained 7% fat, 16% fiber, 4% mineral mix, 1% vitamin mix, and 2% brewer's yeast (ICN) [see Diamond and Karasov (4) for detailed composition].

Feeding Protocol. There were two series of experiments. In the first series, a total of 16 mice were given a NC diet for 2 weeks and segregated into four groups of four mice each; then their diet was switched to HC. One group was then sacrificed at each of the following time intervals: 0 (control), 12, 36, and 156 h after the diet switch. This procedure was repeated four

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Abbreviations: HC, high carbohydrate; NC, no carbohydrate.

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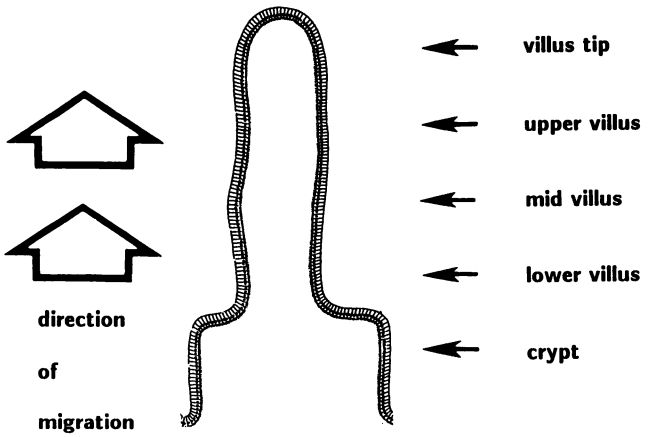

FIG. 1. Sketch of an intestinal villus, with the terms used to describe arbitrarily defined regions along the crypt/villus axis.

times, so that a total of four groups were sacrificed for each time interval. In the second series, 16 mice were initially fed a HC diet for 2 weeks, segregated into four groups of four mice each as described above, and their diet was switched to NC. One group was sacrificed at each of the following times after the diet switch: 0 (control), 24, 72, and 168 h. This procedure was also repeated four times.

Cell Fractionation. We separated cells along the crypt/villus axis by the Weiser (9) method as modified by Ferraris *et al.* (12). Briefly, the everted jejunums of three mice (a fourth was held in reserve) were incubated in 1 mM EDTA-containing phosphate-buffered solutions (PBS; 130 mM NaCl/2.7 mM KCl/8.1 mM Na_2HPO_4/1.5 mM KH_2PO_4, pH 7.3) placed in a shaker/water bath set at 37°C and 90 Hz. Dissociated cells were collected in ice-cold PBS after the jejunal tissues were transferred from one solution to the next; the time of incubation per solution depended on the amount of cells dissociated. Enterocytes were fractionated into 10 fractions. Adjacent fractions were combined, and cells in the resulting 5 fractions were considered sequentially removed from villus tip, upper villus, midvillus, crypt/villus junction, and crypt (Fig. 1).

Phlorizin Binding. The plant glycoside phlorizin is an impermeant, competitive inhibitor of active glucose transport in the apical membrane of intestinal cells. It has a much greater affinity for the glucose carrier than does glucose itself, and phlorizin binding is an excellent measure of glucose transport site density. We followed the method described in detail by Ferraris *et al.* (12). Briefly, we diluted cells to a density of ≈80 μg/10 μl and incubated them in [^{3}H]phlorizin plus 50 mM D-fructose to determine total phlorizin binding. We then incubated cells from the same fraction in [^{3}H]phlorizin plus 50 mM D-glucose to determine nonspecific phlorizin binding. By subtracting nonspecific from total binding, we obtained specific glucose-protectable phlorizin binding to the glucose transporter. We used seven phlorizin concentrations (0.0045, 0.014, 0.036, 0.09, 0.29, 0.91, and 2.7 μM) known to span the values of affinity constants for specific phlorizin binding as determined in previous work (12). Cells were rinsed in ice-cold PBS, dissolved in Solvable (NEN/DuPont), and then assayed for radioactive content. Excess cells were used for determination of protein content (Bio-Rad protein assay) and alkaline phosphatase activity (8).

The Weiser method removed villus tip cells first and crypt cells last. Delaying the removal of villus cells until the normal release time of crypt cells by reducing the rate of mechanical agitation showed that incubation time did not affect phlorizin binding kinetics; hence, the difference in kinetics between villus and crypt cells was presumably real.

Statistics. We estimated dissociation constants (K_d) and site densities by nonlinear regression analysis (10) and then expressed results as regression estimate ± SE of the estimate. We compared site densities by a one-tailed t test because we hypothesized that site densities would increase with dietary carbohydrate level. We compared K_d values by a two-tailed t test because we had no hypothesis regarding effect of diet (if any) on K_d.

We analyzed body weights and intestinal lengths by two-way ANOVA. We considered the level $P < 0.05$ as significant for all statistical comparisons.

RESULTS AND DISCUSSION

Body Weight and Intestinal Length. As in previous studies, our experimental diets maintain body weights and yield similar intestinal lengths. Two-way ANOVA showed that body weight at the time of sacrifice was independent of diet ($P = 0.69$) and time after diet switch ($P = 0.40$). Body weights regardless of diet were 37.2 ± 0.8 ($n = 24$) g for mice switched at $t = 0$; 37.6 ± 0.8 ($n = 24$) g at $t = 12$ and 24 h; 36.7 ± 0.6 ($n = 24$) g at $t = 36$ and 72 h; and 38.4 ± 0.8 ($n = 24$) g for $t = 156$ and 168 h. Intestinal length was independent of diet ($P = 0.64$) and time after diet switch ($P = 0.84$). Average intestinal length for all mice used in this experiment was 46.2 ± 1.0 ($n = 80$) cm.

Sequential Removal of Crypt/Villus Cells. *Alkaline phosphatase activity along the crypt/villus axis.* We assayed the alkaline phosphatase activity of each cell fraction to demonstrate successful sequential removal of cells along the crypt/villus axis (Fig. 2). Two-way ANOVA showed that in mice switched from NC to HC, there was a highly significant effect of crypt/villus position ($P < 0.0001$) and time after diet switch ($P < 0.0001$) on specific alkaline phosphatase activity. Mean specific alkaline phosphatase activity (μmol per min per mg of protein) regardless of crypt/villus position was greater at $t = 0$ (0.84 ± 0.10) than at other times (for $t = 12$ h, 0.49 ± 0.09; for $t = 36$ h, 0.34 ± 0.04; and for $t = 156$ h, 0.49 ± 0.06) ($n = 20$ in each case).

In mice switched from HC to NC, there were also highly significant effects of crypt/villus position ($P < 0.0001$) and time after switch in diet ($P < 0.0001$) on specific activity of alkaline phosphatase. Mean specific activity (μmol per min per mg of protein) regardless of crypt/villus position was 0.48 ± 0.06 ($n = 19$) at $t = 0$, 0.47 ± 0.07 ($n = 20$) at $t = 24$ h, 0.38 ± 0.05 ($n = 20$) at $t = 72$ h, and 0.89 ± 0.10 ($n = 20$) at $t = 168$ h. Mean specific activity (μmol per min per mg of protein) of all fractions for NC-to-HC mice [0.54 ± 0.03 ($n = 80$)] did not differ significantly ($P > 0.50$) from mean specific activity of all fractions from HC-to-NC mice [0.56 ± 0.03 ($n = 79$)]. These specific activities and a tendency for NC diets (which contain high protein) to stimulate alkaline phosphatase activity are similar to those reported earlier for mouse small intestine (8).

Other types of evidence. Fig. 2 shows that specific alkaline phosphatase decreases 3-fold from villus to crypt cells and suggests sequential removal of enterocytes along the crypt/villus gradient. In an earlier study, we used similar methods of cell fractionation and gave histological, enzymatic, histochemical, immunocytochemical, and autoradiographic evidence to prove sequential removal of enterocytes along the crypt/villus axis (8). First, histological sections were made from tissues incubated for 0, 10, 16, 35, 60, or 115 min. Only villus tip cells were removed after 10 min of incubation, but, after 35 min, cells from the upper villus and midvillus (but not crypt) regions were also removed. After 115 min, only a villus core of submucosal tissue remained, indicating that crypt cells were the last to be detached from the small intestine and that all enterocytes along the crypt/villus axis had been removed. About 69% ± 3% of the cells excluded trypan blue

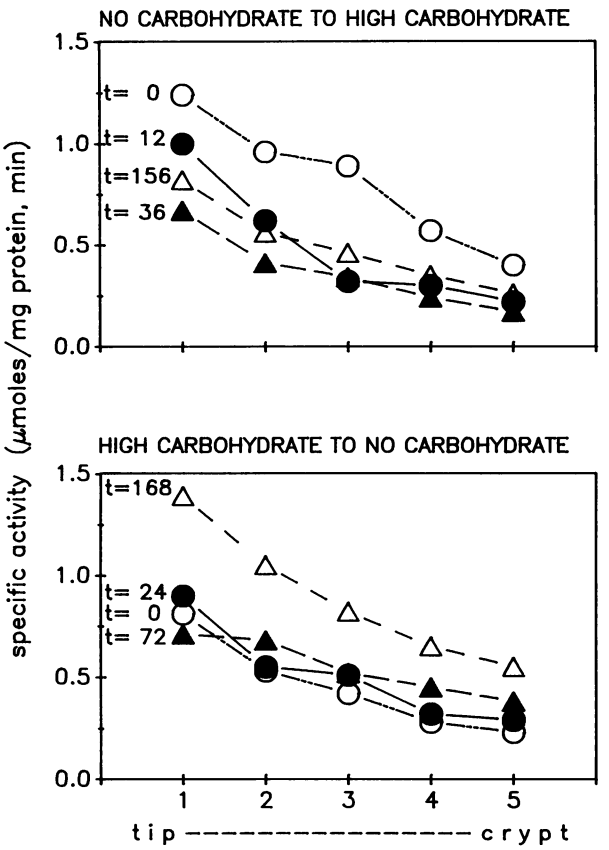

FIG. 2. Specific activity of alkaline phosphatase in enterocytes fractionated along the crypt/villus axis of mouse jejunum. Note that activity decreases 3-fold from villus tip to crypt (from left to right along the abscissa). Each symbol represents the mean of four experiments, with each experiment containing cells from three mice. Since this observed activity gradient is similar to observations on intact intestinal mucosa, it supports the validity of our procedure for fractionating enterocytes.

and hence were considered viable; cell viability was independent of incubation time. Second, *in situ* immunocytochemistry of sucrase and histochemistry of alkaline phosphatase revealed the distribution of these brush border enzymes along the crypt/villus axis. Subsequent crypt/villus profiles of the specific activities of three brush border enzymes (sucrase isomaltase, alkaline phosphatase, and γ-glutamyl transpeptidase) consistently showed higher specific activities in cells from early fractions (cells presumably collected from midvillus to villus tip regions) than in cells from later fractions (cells from crypt regions), a pattern similar to the *in situ* crypt/villus distribution of these enzymes. Third, morphometric evidence showed enterocyte length, enterocyte diameter, and protein per cell to be significantly lower in the last two fractions, indicating that the last fractions contained only crypt cells. Fourth, autoradiography of [^{3}H]thymidine incorporation in intestinal sections of mice sacrificed at various times after injection of [^{3}H]thymidine showed thymidine incorporation initially in crypt cells, followed by migration of thymidine-labeled cells to the villus regions. Subsequent cell fractionation using the modified Weiser method of mice injected with [^{3}H]thymidine showed that, 2.5 h after injection, only the last cell fraction (crypt cells) contained relatively significant amounts of radioactivity; 40 h after injection, the radioactivity had now advanced to the fraction containing upper villus cells. Thus, there was a tight correlation between [^{3}H]thymidine distribution in autoradiographic sections and in fractionated cells.

Steady-State Distribution of Glucose Transporters. We showed by [^{3}H]phlorizin autoradiography and specific phlorizin binding to isolated enterocytes that the steady-state distribution of brush border D-glucose transporters along the crypt/villus axis of mice fed a HC diet was ≈2-fold higher than in mice fed a NC diet for 2 weeks (12). By autoradiography, glucose-protectable phlorizin binding increased ($P < 0.05$ in each case) by 2.0 ± 0.5 times in the villus tips, by 2.9 ± 1.0 times in the upper villus, by 2.9 ± 0.6 times in the midvillus, by 4.0 ± 2.3 times in the lower villus, and by 18 ± 9 times in the crypt. Total specific binding along the whole HC villus was also higher by 2.9 ± 0.6 times that of the NC villus ($P < 0.001$).

By specific phlorizin binding in isolated enterocytes, we also showed that the HC diet increased ($P = 0.005$–0.06, depending on position along the crypt/villus axis; one-tailed *t* test) specific phlorizin binding by 2.0 ± 0.4 times in the villus tip, 2.7 ± 0.9 times in the upper villus, 1.8 ± 0.5 times in the midvillus, and 6.3 ± 4.8 times in the crypt. We could not get reasonably reproducible estimates of site density and K_d values in cells from the fourth (lower villus) fraction, probably because of a mixture of varying proportions of crypt and villus cells. K_d was independent ($P = 0.25$–0.8; two-tailed *t* test) of diet at each crypt/villus position (12).

There appeared to be two types of glucose-protectable phlorizin binding sites, distinguished by their binding kinetics. The site with the higher phlorizin affinity resided in cells from midvillus to villus tip and accounted for most of the specifically bound phlorizin. Based on our data, it cannot be determined whether there are really two unrelated brush border glucose transporters with differing affinity sites for phlorizin, or whether there is only one transporter whose function changes during enterocyte development from crypt to villus. On the one hand, only a single intestinal glucose transporter has been cloned (13), and a genetic defect in the cloned transporter was observed in a case of congenital glucose/galactose malabsorption in a human infant (14). On the other hand, most kinetic studies favor the coexistence of at least two types of intestinal glucose transporters, which differ in their (*i*) affinity for glucose and phlorizin; (*ii*) susceptibility to upregulation, Na^+ activation, and temperature sensitivity; and (*iii*) relative activities at different stages of development, at different positions along the crypt/villus axis, and at various regions of the small intestine (12).

Transient Distribution of Glucose Transporters. Having shown 2-fold, diet-induced changes in steady-state distribution of glucose transporters along the crypt/villus axis, we proceeded to determine transient changes in site density of glucose transporters after abrupt changes in dietary carbohydrate levels.

Dissociation constants. Our results again showed that the K_d values of specific phlorizin binding were independent of diet ($P = 0.81$) and time after switch in diet ($P = 0.11$) but varied greatly with position along the crypt/villus axis [$P < 0.001$ (15)]. The average K_d values were 0.41 ± 0.06 (average of 8 means from each time interval of each diet group), 0.47 ± 0.04 ($n = 8$), 0.41 ± 0.07 ($n = 7$), and 2.46 ± 0.58 ($n = 8$) μM for villus tip, upper villus, midvillus, and crypt regions, respectively. Because there were no statistically significant differences in the K_d values of the three terminal positions ($P > 0.50$), we pooled the average K_d values of the villus tip, upper villus, and midvillus cells. The overall mean K_d of specific phlorizin binding in enterocytes from midvillus to villus tip was 0.43 ± 0.03 μM (mean of 23 means) and was significantly different ($P < 0.001$) from that in the crypt. Both K_d values are well within the published range of 0.0005–2500 μM (12).

Site densities. For the group of mice switched from NC to HC diets, our experiments included two samples of mice—those switched at $t = 0$ and those switched at $t = 156$

h—whose glucose transport rates represented steady-state values for the NC and HC diet, respectively. At $t = 0$, site density was 26.7 ± 0.2 pmol per mg of protein (average of 3 means, since the three terminal villus regions had similar site densities; $P > 0.80$) along the terminal villus regions, and 45 ± 6 pmol per mg of protein in the crypt. These values were representative of steady-state values for a NC diet. At $t = 156$ h, site density was 70 ± 5 pmol per mg of protein (average of 3 means, as above) along the terminal villus, and 92 ± 50 in the crypt. These latter values were 2–3 times higher than those at $t = 0$ and were representative of steady-state values for a HC diet (Fig. 3).

The crucial evidence in this study is found in the transient values at $t = 12$ h and at $t = 36$ h. At $t = 12$ h after a switch to a HC diet, crypt cells significantly ($P < 0.05$) doubled (99 ± 19 pmol per mg of protein) their number of D-glucose transporters, whereas that of villus tip cells remained close to the value at $t = 0$ (Fig. 3 *Upper*). At $t = 36$ h, cells from the crypt to upper villus doubled their number of transporters; cells at the tip still remained at NC values. The evidence suggests that upregulation of transporter number begins with cells from the crypt regions; it clearly does not show simultaneous upregulation of transporter number in cells from all five crypt/villus regions. Only at $t = 156$ h, when all villus cells at $t = 0$ have already been fully replaced by new cells, did site density of transporters become typically HC.

For the group switched from HC to NC, two data points ($t = 0$ and $t = 168$ h) represented steady-state values for the HC and NC diet, respectively. At $t = 0$, site density (pmol per mg of protein) was 61.0 ± 3.6 (average of 3 means in the terminal villus regions that did not differ from one another; $P > 0.50$) in the midvillus to villus tip region and 125 ± 50 in the crypt.

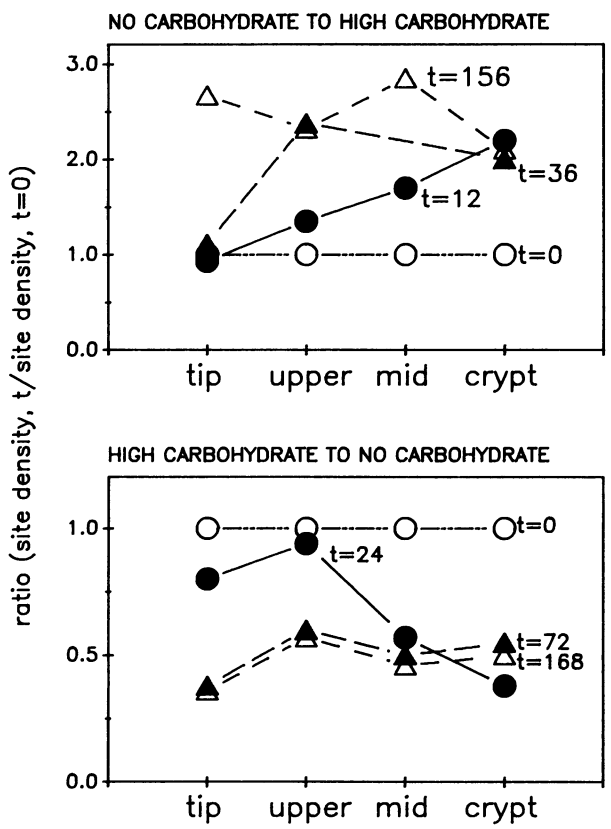

FIG. 3. Site density of specific, glucose-protectable phlorizin binding at time t after a switch in dietary carbohydrate level, divided by site density just before the switch ($t = 0$), at various positions along the crypt/villus axis. Changes in site density proceed with time from crypt to midvillus to upper villus to villus tips. See text for discussion.

These typically HC values were each similar ($P = 0.11$–0.70) to the corresponding values at $t = 156$ h in the NC-to-HC group. At 168 h, site density decreased to ≈29 ± 5 in the midvillus to villus tip regions and to 60 ± 28 in the crypt. These typically NC values were each similar ($P = 0.4$–0.8) to the corresponding values at $t = 0$ in the NC-to-HC group.

Transient values of site density (pmol per mg of protein) showed that at $t = 24$ h, only midvillus (34 ± 5; $P = 0.02$) and crypt (48 ± 9; $P = 0.08$) values decreased; cells from upper villus and villus tip contained transporters at typically HC values. These transient values again provide crucial evidence that regulation of transporter number does not occur simultaneously in all cells; rather, regulation is first expressed in crypt and/or lower villus cells. At $t = 72$ h, site density in villus tip was reduced to 22 ± 5 and in the upper villus it was reduced to 39 ± 8 pmol per mg of protein.

Site of Induction of Dietary Carbohydrate. Our results show that adaptive upregulation and downregulation of transporter site density progresses sequentially from crypt to villus. This main conclusion leads to other, related conclusions: that mature villus enterocytes cannot change their number of transporters, that crypt cells are programmed irreversibly by prevailing dietary carbohydrate levels, and that changes in villus cells occur only as a result of regulatory changes in crypt cells followed by migration of the crypt cells to the villus tips.

Within 12–24 h, luminal carbohydrate alters the number of low-affinity phlorizin sites in the crypt. Intestinal glucose transport does not change immediately upon alteration of the number of low-affinity sites because these sites do not contribute significantly to intestinal glucose transport (12). Either the future transport capabilities of enterocytes are somehow programmed irreversibly while they are in the crypts, or else the transporters are already induced in the crypts and synthesized there in the form of inactive precursors that become activated when the cells mature and reach the villi. Evidence regarding the correlation of crypt/villus distribution of phlorizin-sensitive transport sites with the crypt/villus distribution of its mRNA is conflicting. Hwang *et al.* (16) showed that mRNA for the sodium-dependent glucose transporter (SGLT1) is 6-fold more abundant in rabbit villus cells than in crypt cells, paralleling the crypt/villus gradient of glucose transport. Levels of specific sucrase activity along the crypt/villus axis are tightly correlated with levels of sucrase mRNA (17). In contrast, Smith *et al.* (18) found that phlorizin-sensitive glucose uptake along the rabbit crypt/villus axis is not correlated with SGLT1 mRNA levels.

The high-affinity phlorizin site responsible for most glucose transport begins to be expressed when those crypt cells reach the lower villus and becomes fully expressed in the midvillus. Only when the cell population in the terminal villus regions begins to change do we see a change in the transport capacity of the intact small intestine. Thus, the observed time lag for dietary regulation of glucose transport is due mainly to cell migration rates. At the end of a cell lifetime (≈68 h), the cell population of the whole length of villus has been renewed, and the change to a new set of cells with a different number of transporters is then completed.

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1. Ferraris, R. P. & Diamond, J. M. (1986) *J. Membr. Biol.* **94**, 77–82.
2. Shirazi-Beechey, S. P., Hirayama, B. A., Wang, Y., Scott, D., Smith, M. W. & Wright, E. M. (1991) *J. Physiol. (London)* **437**, 699–708.
3. Cheeseman, C. I. & Harley, B. (1991) *J. Physiol. (London)* **437**, 563–575.

4. Diamond, J. M. & Karasov, W. H. (1984) *J. Physiol. (London)* **349**, 419–440.
5. Ferraris, R. P., Yasharpour, S., Lloyd, K. C. K., Mirzayan, R. & Diamond, J. M. (1990) *Am. J. Physiol.* **259**, G822–G837.
6. Raul, F., Simon, P. M., Kedinger, M., Grenier, J. F. & Haffen, K. (1980) *Biochim. Biophys. Acta* **630**, 1–9.
7. Cezard, J. P., Broyart, J. P., Cuisinier-Gleizes, P. & Mathieu, H. (1983) *Gastroenterology* **84**, 18–25.
8. Ferraris, R. P., Villenas, S. A. & Diamond, J. (1992) *Am. J. Physiol.* **262**, G1047–G1059.
9. Weiser, M. M. (1973) *J. Biol. Chem.* **248**, 2536–2541.
10. Ferraris, R. P. & Diamond, J. M. (1986) *J. Membr. Biol.* **94**, 65–76.
11. Toggenburger, G. M., Kessler, M. & Semenza, G. (1982) *Biochim. Biophys. Acta* **668**, 557–571.
12. Ferraris, R. P., Villenas, S. A., Hirayama, B. A. & Diamond, J. (1992) *Am. J. Physiol.* **262**, G1060–G1068.
13. Hediger, M. A., Coady, M. J., Ikeda, T. S. & Wright, E. M. (1987) *Nature (London)* **330**, 379–383.
14. Turk, E., Zabel, B., Mundlos, S., Dyer, J. & Wright, E. M. (1991) *Nature (London)* **350**, 354–356.
15. Ferraris, R. P. & Diamond, J. (1992) *Am. J. Physiol.* **262**, G1069–G1073.
16. Hwang, E. S., Hirayama, B. A. & Wright, E. M. (1991) *Biochem. Biophys. Res. Commun.* **181**, 1208–1217.
17. Hoffman, L. R. & Chang, E. B. (1992) *Am. J. Physiol.* **262**, G983–G989.
18. Smith, M. W., Turvey, A. & Freeman, T. C. (1992) *Exp. Physiol.* **77**, 525–528.