Thyroid hormone regulation of the Na^+/glucose cotransporter SGLT1 in Caco-2 cells

Miriam MATOSIN-MATEKALO*, José E. MESONERO*, Olivier DELEZAY†, Jean Claude POIREE‡, Anunciacion Ana ILUNDAIN§ and Edith BROT-LAROCHE*[1]

*Unité de Recherches sur la Différenciation Cellulaire Intestinale, INSERM U178, 16 avenue Paul-Vaillant-Couturier, 94807 Villejuif Cedex, France, †Laboratoire de Génie Génétique, Fac de Pharmacie, Bd Jean Moulin, 13385 Marseille Cedex 5, France, ‡Laboratoire de Biochimie, Faculté de Médecine, Av de Valombrose, 06107 Nice Cedex 2, France, and §Depto. Fisiologià y Biologia Animal, Facultad de Farmacia, c) Tramontana s/n, 41012 Sevilla, Spain

The expression of the Na^+/glucose cotransporter (SGLT1) in response to thyroid hormone [3,5,3′-tri-iodo-L-thyronine (T3)] was investigated in the enterocytic model cell line Caco-2/TC7. In differentiated cells, T3 treatment induces an average 10-fold increase in glucose consumption as well as a T3 dose-dependent increase in SGLT1 mRNA abundance. Only cells grown on glucose-containing media, but not on the non-metabolizable glucose analogue α-methylglucose (AMG), could respond to T3-treatment. The V_{max} parameter of AMG transport was enhanced 6-fold by T3 treatment, whereas the protein abundance of SGLT1 was unchanged. The role of Na^+ recycling in the T3-related activation of SGLT1 activity was suggested by both the large increase in Na^+/K^+ATPase protein abundance and the inhibition, down to control levels, of AMG uptake in ouabain-treated cells. Further investigations aimed at identifying the presence of a second cotransporter that could be expressed erroneously in the colon cancer cell line were unsuccessful: T3-treatment did not modify the sugar-specificity profile of AMG transport and did not induce the expression of SGLT2 as assessed by reverse transcription-PCR. Our results show that T3 can stimulate the SGLT1 cotransport activity in Caco-2 cells. Both transcriptional and translational levels of regulation are involved. Finally, glucose metabolism is required for SGLT1 expression, a result that contrasts with the *in vivo* situation and may be related to the fetal phenotype of the cells.

INTRODUCTION

Sodium–glucose cotransport is a secondary active process involving the Na^+/glucose cotransporter in the apical membrane of mammalian enterocytes and the presence of Na^+/K^+ ATPase in the basolateral membrane to maintain an inwardly directed Na^+-electrochemical gradient [1]. The Na^+/glucose cotransporter SGLT1 was first cloned in 1987 [2,3]; it elicits, in *Xenopus laevis* oocytes, the expression of a protein with the expected properties of a native Na^+/glucose cotransporter. SGLT1 is expressed in mammalian small intestine and kidney absorbing cells (for a review see [3,4]), sheep parotid acinar gland [5], mammalian neurons [6], Caco-2 [7,8], HT-29-Glc- [9], HT29 cl.19A [10] and HT-29 D4 [11] cells. A second isoform (SGLT2) of the Na^+/glucose cotransporter has been identified in kidney. Identical cDNA sequences were found for SGLT1 cDNA from normal human and Caco-2 enterocytes [12]. In normal small intestine the expression of SGLT1 mRNA is undetectable in the crypts where enterocytes are mainly undifferentiated, and it appears as soon as the cells migrate and mature along the villus axis [13–15]. The mechanisms underlying the control of SGLT1 expression are poorly understood. Two interrelated levels of regulation may coexist: (i) a differentiation-related control due to the induction, in crypt cells, of SGLT1 expression by glucose [16–18]; (ii) a modulation of SGLT1 mRNA and protein abundances occurring in already matured enterocytes, i.e. in mid-villus, a hypothesis still to be demonstrated.

Caco-2 cells spontaneously differentiate into enterocytic-like cells and acquire, after confluency, the morphological and functional features of human enterocytes [19,20]. This cell line is therefore widely used as an *in vitro* model to study the regulation of enterocyte functions. Caco-2 cells express GLUT1 and GLUT3 and contain large glycogen stores, thus exhibiting some characteristics of fetal enterocytes [8,20]. SGLT1 mRNAs are only expressed in differentiated Caco-2 cells. Using Caco-2 clones we could show that those differentiated cells that consume the least glucose expressed the most SGLT1 mRNA. Moreover, glucose deprivation [8] or treatment of the cells with xenobiotics (β-naphthoflavone or omeprazol), which increases the glucose consumption rate of the cells [21], downregulate the expression of the gene. The metabolism of glucose may therefore be involved in the control of the expression of the SGLT1 gene.

Thyroid hormone [3,5,3′-tri-iodo-L-thyronine (T3)] accelerates the turnover and rate of glucose utilization in many cell types [22] and affects the expression of key enzymes of hepatic glucose metabolism, i.e. glucokinase [23], phosphoenolpyruvate carboxykinase (PEPCK) [24] and glycogen phosphorylase [25]. Na^+/K^+ ATPase activity and mRNA are strongly increased by T3 in Caco-2 cells [26]. *In vivo*, hyperthyroidism stimulates the expression of GLUT1 in Sertoli cells [27] and of GLUT2 in the liver [28]. T3 therefore increases the expression of proteins involved in the entry and utilization of glucose by the cells.

In this study, we examined the mechanisms by which Caco-2/TC7 cells control the expression of human SGLT1 (hSGLT1). T3-treated cells were studied. We measured the mRNA abundance and stability and assayed the protein abundance and function of SGLT1 in relation to glucose supplies and glucose consumption rates of the cells. The respective roles of the modulation of the expression of Na^+/K^+ ATPase by T3 and of the presence of an unexpected expression in intestinal cells of the

Abbreviations used: AMG, α-methylglucose; hSGLT1, human SGLT1; PEPCK, phosphoenolpyruvate carboxykinase; DRB, 5,6-dichloro-1-β-D-ribofuranosyl-benzimidazol; T3, thyroid hormone (3,5,3′-tri-iodo-L-thyronine).

[1] To whom correspondence should be addressed (e-mail laroche@infobiogen.fr).

kidney-specific isoform of Na^+/glucose cotransporter (SGLT2) were evaluated.

EXPERIMENTAL

Cell culture

The parental Caco-2 population [19] was obtained from the late Dr. Jorgen Fogh (Memorial Sloan Kettering Cancer Center, Rye, NY, U.S.A.) and has been cloned in the laboratory [29]. In the present study, clones of the cell line (Caco-2/TC7 and -/PD7) were used because of their high expression of SGLT1 [8]. Because the results obtained were identical, only the results from Caco-2/TC7 cells are presented in this paper. The cells were seeded at 12×10^3 cells/cm² in 25 cm² plastic flasks (Corning Glassworks, Corning, NY, U.S.A.) and cultured in a 10% CO_2/90% air atmosphere in Dulbecco's modified Eagle's medium (Eurobio, Paris, France) containing 25 mM glucose and 20% heat-inactivated (30 min, 56 °C) fetal bovine serum (Boehringer, Mannheim, Germany), 1% (w/v) non-essential amino acids (Gibco, Glasgow, Scotland, U.K.), 100 units/ml penicillin and 100 μg/ml streptomycin. Under all culture conditions, the medium (0.2 ml/cm²) was changed 48 h after seeding and daily thereafter. The cell line was passaged every 6 days. All cells were harvested 24 h after a change of medium.

For the purpose of this study, some cells were grown in heat-inactivated serum that was dialysed against saline (molecular-mass cut-off 6000–8000 kDa), yielding a final glucose concentration below 10 μM in the medium. Dialysed serum was not applied before day 10 of culture, so that the growth and differentiation processes, which depend on the presence of serum factors, are readily established [30]. T3 treatment was applied either in post-confluent cultures, i.e. after the onset of differentiation, or chronically, with identical results, so that the latter condition was used subsequently. Detailed culture protocols are given in the legends of the Figures.

Additions were made as follows: 200 μM 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; France-Biochem, Calbiochem) as 10 mM stock solutions in DMSO; to get rid of any trace amount of glucose, α-methylglucose (AMG; Sigma, St. Louis, MO, U.S.A.) was repurified on a Dowex 1-X2 200–400 mesh anion-exchange resin as described in [31]; and T3 was added as a 1 mM stock solution in 0.1 M NaOH (Sigma, St. Louis, MO, U.S.A.). Cell viability was unchanged.

cDNA probes and Northern blot analysis

The cDNA probe hNaGT2-2 (SGLT1, 2.0 kb insert) was obtained from G. I. Bell (Howard Hughes Medical Institute, University of Chicago, Chicago, IL, U.S.A.); and pBs-SGLT1, which was shown to promote Na^+/glucose cotransport in *Xenopus laevis* oocyte [12] was a kind gift of Dr M. Coady and P. Brunette (Université de Montréal, Montreal, Canada). Identical patterns of expression of SGLT1 transcripts were obtained (results not shown). DNA probes were obtained from the following sources: pGEM4-rata-1 Na^+/K^+ ATPase α1 subunit [32] from Sergio Gloor (ETH, Zurich, Switzerland); and V19 villin [33] from D. Louvard (Institut Pasteur, Paris). Probes were ^{32}P-labelled using a Megaprime DNA labelling kit (Amersham). Total RNA was isolated using guanidinium thiocyanate and centrifugation through a CsCl gradient [34]. Glyoxal-denatured RNA samples were electrophoresed on 1% agarose gels before transfer onto Hybond™-N membranes (Amersham). Hybridization with the ^{32}P-labelled probes was carried out as described [35]. High-stringency washing conditions were applied and, unless otherwise stated, a final 15 min wash in 0.1 × SSC (SSC = 0.15 M NaCl/0.015 M sodium citrate)/0.1% SDS at 60 °C before exposure. The films were scanned with a densitometer to quantify the hybridization signals.

Reverse transcription (RT)-PCR

Caco-2/TC7 cell total RNA (5 μg/50 μl) was reverse transcribed with the First Strand Synthesis for RT-PCR Kit (Amersham) and anchored dT^{25} primer. Oligonucleotides were designed to amplify genomic regions in exon 11, 12 and 13 of either hSGLT1 (1220–1573) or hSGLT2 (1230–1468) according to the cDNA sequences HUMSGLT1 (GenBank accession numbers M95549 and M95299) and HUMSGLCT (GenBank accession number M24847). A single sense (SGLT1/2) oligonucleotide (5′-CTCT-TCACCATGGACATCTAC-3′) and two antisense oligonucleotides specific for hSGLT1 (3′-TCGTTGACAGGGTGCTAA-TAG-5′) or hSGLT2 (3′-CACGGCGCGCAATTACTCGT-5′) respectively were used to amplify a 354 bp hSGLT1 or a 240 bp hSGLT2 DNA fragment in the coding regions. PCR was performed on 1 μl of the first-strand DNA product as follows: 94 °C, 7 min pre-denaturation; 30 cycles at 94 °C, 45 s denaturation; 50 °C, 1 min annealing; 72 °C, 3 min extension; followed by a 7 min final extension step. Reverse-transcribed DNA from total rat kidney was used in parallel as a positive control for SGLT2 using PCR conditions that differed in the annealing temperature (45 °C) due to the presence of three mismatches in the 20-mer sequence. Amplified PCR-DNA product (10 μl out of 25 μl) was Southern blotted and hybridized with a ^{32}P-labelled SGLT1 cDNA probe. Washings were designed to allow, first, the detection of both SGLT1 and SGLT2 (low-stringency: 50 °C, 2 × SSC/0.1% SDS) and, second, detection of PCR products fully homologous to the cDNA probe (high-stringency, 65 °C, 0.1 × SSC/0.1% SDS), thus eliminating any hybridization signal from heterologous DNA, i.e. SGLT2 with 30% identity in the common region.

Western blot analysis

Cell homogenates were prepared as described in [36]. Briefly, the cells were scrapped and homogenized in ice-cold glass grinding tubes in (mM): 2 Tris (pH 7.1), 50 mannitol, 1 PMSF, 0.02% (v/v) sodium azide and 25 μg/ml benzamidine as protease inhibitors. Further purification of the crude membrane fraction was made by diluting the homogenate with 1 M sucrose to generate a final 0.25 M sucrose cell suspension which was spun at 150 ***g*** for 10 min. The supernatant was further centrifuged at 15000 ***g*** for 30 min and the resulting membrane pellet was resuspended in the sucrose buffer and stored at −70 °C. Brush border membrane-enriched fractions were prepared using the $CaCl_2$ precipitation method [37]. Protein samples were solubilized in Laemmli buffer and electrophoresed under denaturating conditions in 7.5% or 10% acrylamide-slab gels. Molecular-mass markers (Rainbow Markers, Pharmacia) were run in parallel. Proteins were transferred to Hybond-P (PVDF) membranes (Amersham) by electroblotting, according to the manufacturer's protocol. The membranes were blocked with 5% (v/v) skimmed milk in PBS/0.05% Tween before incubation with a 1/500 dilution of polyclonal SGLT1-specific antibodies directed either against the Ser^{402}-Lys^{420} peptide sequence [11,38] or a purified 70 kDa glucose-protectable phlorizin-binding protein [39,40] or against Na^+/K^+ ATPase α1 subunit (CH64-6 IgG 1/k) [41]. The primary antibody was detected using goat anti-rabbit or anti-mouse secondary antibodies conjugated to horseradish peroxidase (working dilution: 1/2000). The labelled proteins were visualized with the enhanced chemiluminescence detection system (ECL; Amersham, France). In all instances the antibodies

were used in their linear range of response using 5–30 μg of protein loads with identical results for both the cell homogenates and brush border membrane-enriched samples.

Glucose consumption rates, glycogen content and cAMP concentrations

The glucose consumption rate, as estimated by the disappearance of glucose from the culture medium 24 h after a change in medium, was measured with an automated Beckman Glucose Analyzer 2. For glycogen assays, the cells were quickly rinsed with ice-cold Ca^{2+}/Mg^{2+}-free PBS and scrapped off the plastic support for subsequent extraction; measures were made with anthrone as previously reported [42]. cAMP concentrations were assayed as described [43] with the Biotrak Enzyme Immunoassay system (Amersham, Les Ulis, France). Protein assays were made with the bicinchoninic acid (BCA) assay kit using BSA as a standard (Pierce, Rockford, IL, U.S.A.).

Transport assays

The non-metabolizable analogue α-methylglucose (AMG), a specific substrate for Na^+/glucose cotransporters [1,31,44] was used to assess cotransport activity in control or T3-treated Caco-2/TC7 cell monolayers. Transport assays were performed in 24-well plates (Corning) essentially as described by Blais et al. [7]. The cells were seeded at a density of 4×10^4 cells per cm^2 and cultured for 20 days, at which time they are fully differentiated. Incubations were made at 25 °C in a transport medium which contained (mM): 137 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 10 Hepes (pH 7.2) and D-[U-^{14}C]AMG (197 mCi/mmol; Amersham). L-[1-^{3}H]Glucose (16.2 Ci/mmol; New England Nuclear/Dupont de Nemours) was used to evaluate the non-specific uptake of glucose. Inhibition of Na^+/K^+ ATPase was performed as described [45]. Cells were preincubated for 90 min with 10^{-4} M ouabain (Sigma) and further treated during the transport assay. Uptake of the labelled sugar was stopped by adding ice-cold transport medium containing 1 mM phlorizin. The monolayers were then rinsed and solubilized in (mM) 25 Tris/phosphate, pH 7.8/8 $MgCl_2$/1 dithiothreitol/1 EDTA/0.4 PMSF/1 % (v/v) Triton X-100 and counted. Triplicate cultures were assayed. Counts were transformed into pmol/μg of protein $\pm$ S.E.M.

RESULTS

Effects of T3 on morphology, growth, differentiation and biochemical parameters

Caco-2 cells are actively transporting epithelial cells and, as such, form domes that can be observed under a phase-contrast microscope as an accumulation of fluid between the monolayer and the plastic dish in localized areas [20]. In T3-treated monolayers, the number of these domes is higher and their surface larger, indicating increased electrolyte and water fluxes (results not shown). Protein contents and growth curves of T3-treated, as compared with control cells, were similar (Figures 1A and 1B respectively).

T3 affects glucose consumption rates (Figure 1C) in differentiated cells. The glucose consumption rate of T3-treated cells diverges from that of controls within 4–5 days after confluency and subsequently increases with time and differentiation, reaching a reproducible, $\sim$ 10-fold, average increase (11 ± 2; $n = 4$) at day 20. This effect is dose-dependent on T3 (Figure 1D) and glucose consumption rates were indicative of cell responses to T3. Glycogen contents are lower in T3-treated cells than in controls (1.40 ± 0.05 and 0.96 ± 0.01 mg of glucose equivs./mg of protein; $n = 3$), indicating lower rates of glycogenogenesis and/or higher rates of glycogenolysis. cAMP concentrations are similar in control and T3-treated cells (125 ± 43 and 153 ± 37 fmol/10^6 cells $\pm$ S.D. respectively; $n = 3$).

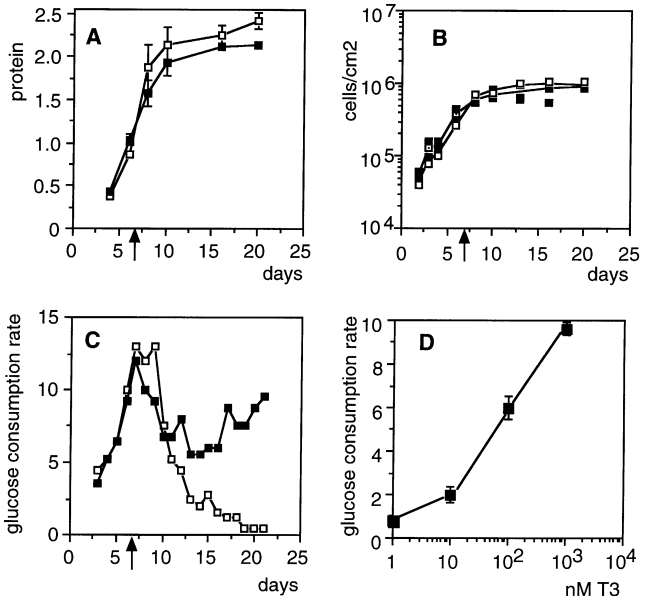

Figure 1 Growth curves, protein content and glucose consumption rates in controls and T3-treated Caco-2/TC7 cells

(**A**) Protein content (mg/cm^2), (**B**) growth curve (cells/cm^2) and (**C**) glucose consumption rates [mg glucose/h per flask $\pm$ S.E.M. ($n = 3$)] were assayed in control (□) and 1 μM T3-treated cells (■). (**D**) Glucose consumption rates as a function of the dose of T3 as measured at day 20. The results shown are representative of four experiments. In some instances the error bar is smaller than the symbol. The black arrows indicate the day of confluency.

mRNA expression as a function of cell growth and T3 treatment

PEPCK [24] and Na^+/K^+ ATPase [26,46] are known target genes for T3 regulation and their mRNA abundances are indeed increased (5- and 2-fold respectively) in T3-treated post-confluent Caco-2/TC7 cells (Figure 2, upper left). Because the expression of villin is insensitive to T3 treatment, villin mRNA abundance was used later to calibrate RNA loadings.

In Caco-2 cells the expression of the hexose transporters that are involved in the intestinal absorption of sugars *in vivo* is a differentiation-related process [8]. As in normal intestinal cells, three main SGLT1 mRNA transcripts (5.2, 3.0 and 2.4 kb respectively) can be detected. In Caco-2 cells, they are expressed after day 7 of culture and increase with differentiation (Figure 2, upper right). As for Na^+/K^+ ATPase and PEPCK, SGLT1 transcripts increase (2- to 5-fold) with time of T3 treatment, but cannot be stimulated before day 12 (results not shown). A 24 h treatment with the hormone does not affect SGLT1 mRNA expression (results not shown). However, a T3 post-confluent treatment for 4 days is long enough to give the same SGLT1 mRNA abundance as chronic or post-confluent treatment for 8 days (results not shown). T3 stimulates the expression of SGLT1 mRNA in a dose-dependent manner, and an unequivocal stimulation is observed in cells treated with 10^{-9} M T3, reaching a maximum at 10^{-7} M T3 (Figure 2, lower panel). Subsequent experiments were made using the maximum stimulatory concentration of T3.

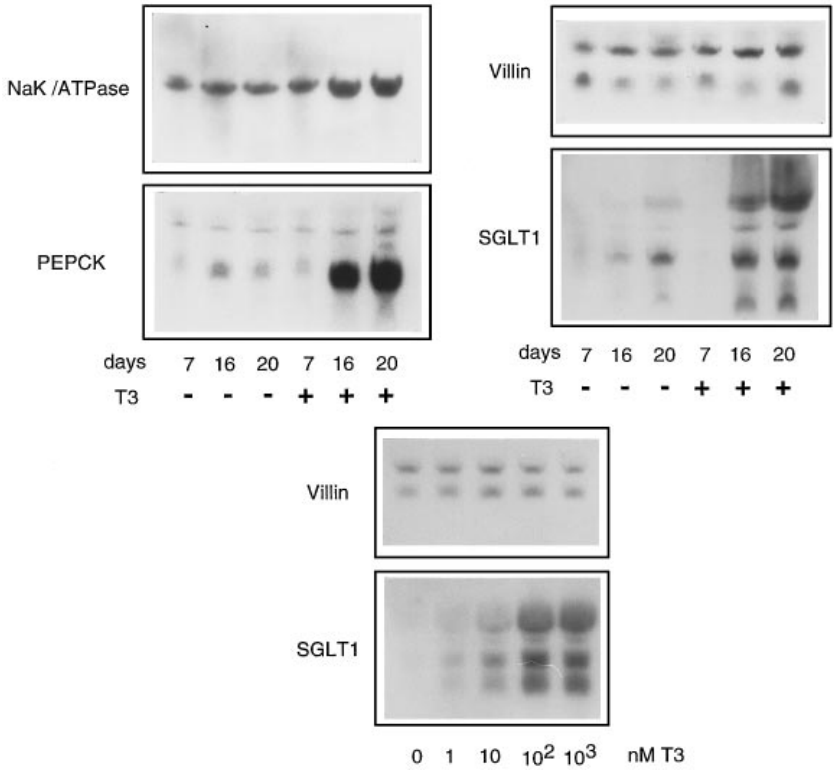

Figure 2 Northern blot analysis of the mRNA expression of Na^+/K^+ ATPase (α1 subunit, 4.8 kb), PEPCK (4.8 kb), villin (4.0 and 3.2 kb) and SGLT1 (5.2, 3.0 and 2.4 kb) as a function of growth (upper panels) or dose of T3 (lower panel)

Caco-2 cells were treated as described in Figure 1. Total RNA (20 μg) extracted from control and chronically T3-treated Caco-2/TC7 cells was loaded in each lane. Dose responses were evaluated in cells harvested at day 20 of culture. The X-ray films were exposed for 2 h (Na^+/K^+ ATPase, villin, PEPCK) and 23 h (SGLT1) respectively. Note that villin mRNA levels are independent of T3 treatment. Two independent experiments were performed with the same results.

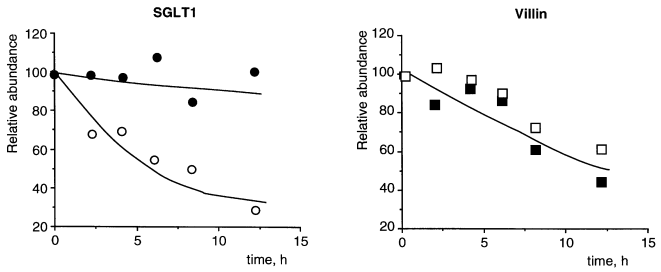

Figure 3 Effect of T3 treatment on the mRNA half-life of SGLT1 and villin

RNA was isolated from transcription-arrested (0.2 mM DRB) control (□,○) or 1 μM T3-treated (●,■) cells, blotted (15 μg RNA) and hybridized with SGLT1 (○,●) or villin (□,■) human cDNA probes. Transmission-scanning quantification of the X-ray film was plotted against time after the addition of DRB to the cultures and transformed into a percentage of the value obtained at $t = 0$. SGLT1 and villin densities have been followed for 15 h in control and T3-treated cells. Note that T3 does not affect villin mRNA stability.

T3 enhances the stability of SGLT1 mRNA

Increased mRNA abundance of a gene can be mediated by transcriptional and/or post-transcriptional events, including stabilization of the transcripts. Identical effects of T3 treatment on SGLT1 mRNA abundance were obtained with the transcription inhibitors DRB or actinomycin D, the former being preferred because cells tolerate longer exposure to the drug. T3 treatment evenly affects the stability of the different transcripts (results not shown). The mRNA half-life of the SGLT1 transcripts is increased significantly in T3-treated cells, thus differing from villin, whose mRNA half-life is unaffected (Figure 3). The mean half-life of SGLT1 transcripts in control cells is approx. 6 h, but such an estimate cannot be made accurately for T3-treated cells within the time of the experiment.

Glucose is required for T3 to increase SGLT1 mRNA abundance

Dividing cells have the highest glucose consumption rates and total glucose deprivation interferes with growth and differentiation. The glucose requirement diminishes rapidly after confluency [29] and differentiated cells can be maintained from then on in total glucose-free culture media and dialysed serum [43]. Cells cultured in the total absence of glucose do not express SGLT1 mRNA. Increasing the glucose concentration from 0.5 to 25 mM did not restore SGLT1 expression to any significant level (Figure 4, upper panel), suggesting that essential elements for the cellular utilization of glucose and/or for the expression of SGLT1 mRNA have been lost during dialysis. The addition of T3 to the culture media promotes the expression of SGLT1 mRNA, which appears to be dependent on the medium concentration of glucose, although the full expression of SGLT1 in 25 mM glucose and normal serum is not reached (Figure 4, upper panel). In contrast, the non-metabolizable analogue of glucose, AMG, failed to promote the expression of SGLT1 mRNA, even when T3 was present in the culture medium, indicating that metabolism of the

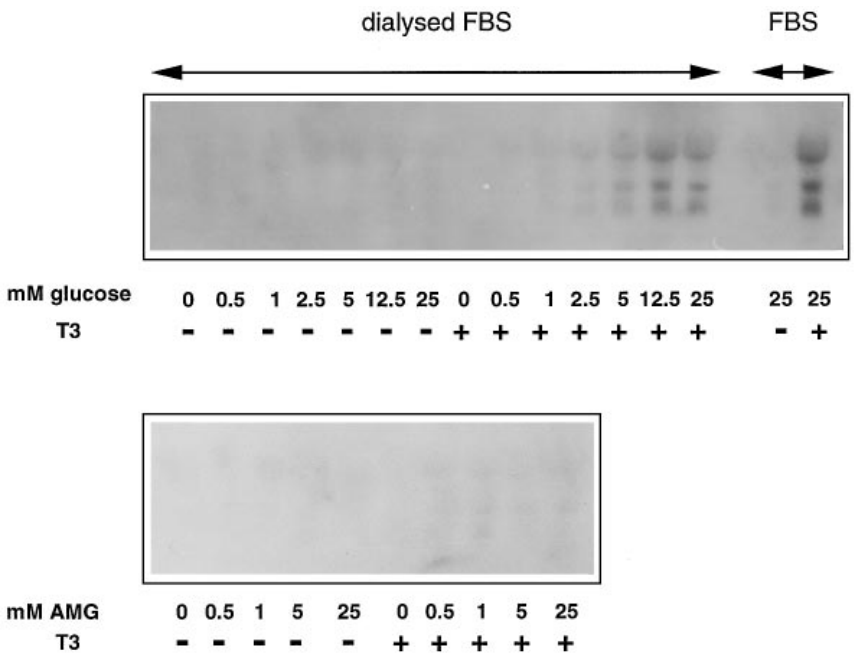

Figure 4 Role of glucose metabolism in the expression of SGLT1 mRNA in control and T3-treated cells

Cells were cultured in the standard medium [25 mM glucose and normal fetal bovine serum (FBS)] for 14 days and switched for another 5 days to dialysed serum and increasing concentrations (0.5–25 mM) of glucose or AMG. Northern blots were carried out with 20 μg of total RNA. Cells were chronically treated with 1 μM T3.

sugar is required for the gene to respond to T3 (Figure 4, lower panel).

Differential effect of T3 on Na^+/glucose cotransport and protein abundances of SGLT1 and Na^+/K^+ ATPase

AMG and L-glucose uptakes were measured in fully differentiated cell-monolayers. The uptake of the diffusion marker L-glucose is unchanged in T3-treated cells (results not shown), indicating that the hormone does not alter the membrane permeability for monosaccharides. On the other hand, T3 increases AMG transport (Figure 5, top), resulting in a 6-fold increase in the V_{max} parameter. This suggests that either the SGLT1 protein content of the cells is increased and/or that T3 modifies the turnover number of AMG transport.

Western blots were made with cell homogenates, a 15000 ***g*** crude membrane fraction or with purified brush border membrane fractions from control and T3-treated cells. Use of either a polyclonal antibody directed against a purified 70 kDa phlorizin-binding protein [39,40] (Figure 6) or an affinity purified polyclonal antibody directed against the Ser^{402}-Lys^{420} peptide of hSGLT1 [11,38] (results not shown) allowed detection of a 68–70 kDa SGLT1 protein in both Caco-2/TC7 cell-homogenate and brush border membrane-enriched fractions. SGLT1 protein abundances were identical in T3-treated and control cells, a result that contrasts with the strong increase in Na^+/K^+ ATPase protein abundance in T3-treated differentiated cells (Figure 6).

To assess the role of Na^+ recycling and of electrical parameters on AMG transport we measured the impact of ouabain, a strong inhibitor of Na^+/K^+ ATPase, on the uptake of AMG in control or T3-treated cells. AMG uptake in control and T3-treated cells was inhibited down to the same residual levels, indicating that the increase in AMG transport was fully dependent on the increase in Na^+/K^+ ATPase activity. Passive diffusion and non-specific binding of sugar, estimated by the use of L-glucose as a labelled substrate, was not modified by ouabain treatment.

Absence of SGLT2 expression in Caco-2/TC7 cells

AMG is a highly specific non-metabolizable substrate of Na^+/glucose transporters in mammals. The sugar, however, does not discriminate between the isoforms of SGLT1 and SGLT2 cotransporters. Therefore enhanced AMG transport in Caco-2/TC7 cells may reveal the presence of an abnormal expression of the kidney-specific SGLT2 in the colon cancer cell line Caco-2/TC7. Because SGLT2 lacks the ability to transport galactose [44], [^{14}C]AMG transport was measured in the presence of saturating concentrations (1:150 and 1:300 substrate to inhibitor ratio) of either unlabelled AMG or galactose, L-glucose being used as an iso-osmotic replacer. A parallel uptake assay of labelled L-glucose was performed to correct AMG uptakes for non-specific uptake and binding. Our results (Figure 5) show that the 15 min uptake of [^{14}C]AMG is fully inhibited by galactose, AMG or by the potent and specific cotransport inhibitor phlorizin, a sugar-specificity pattern that does not account for the presence of any significant amount of SGLT2 in the cell membranes.

SGLT2 expression was also assessed by RT-PCR. Specific oligonucleotides were designed to amplify hSGLT1 and hSGLT2 cDNAs. SGLT1-PCR products (351 and 182 pb) were obtained in both control and T3-treated cells (Figure 7). Their abundance was higher in the T3 samples than in controls, a result that could be steadily observed between cycles 15–30 (results not shown) using Southern blotting and hybridization with the hSGLT1 cDNA probe. A single SGLT2 PCR product was detected in rat kidney samples after 30 cycles of PCR with SGLT2-specific primers (Figure 7). Regardless of the T3 status of Caco-2/TC7 cells (Figure 7, lanes 1–8), we never obtained an SGLT2 PCR

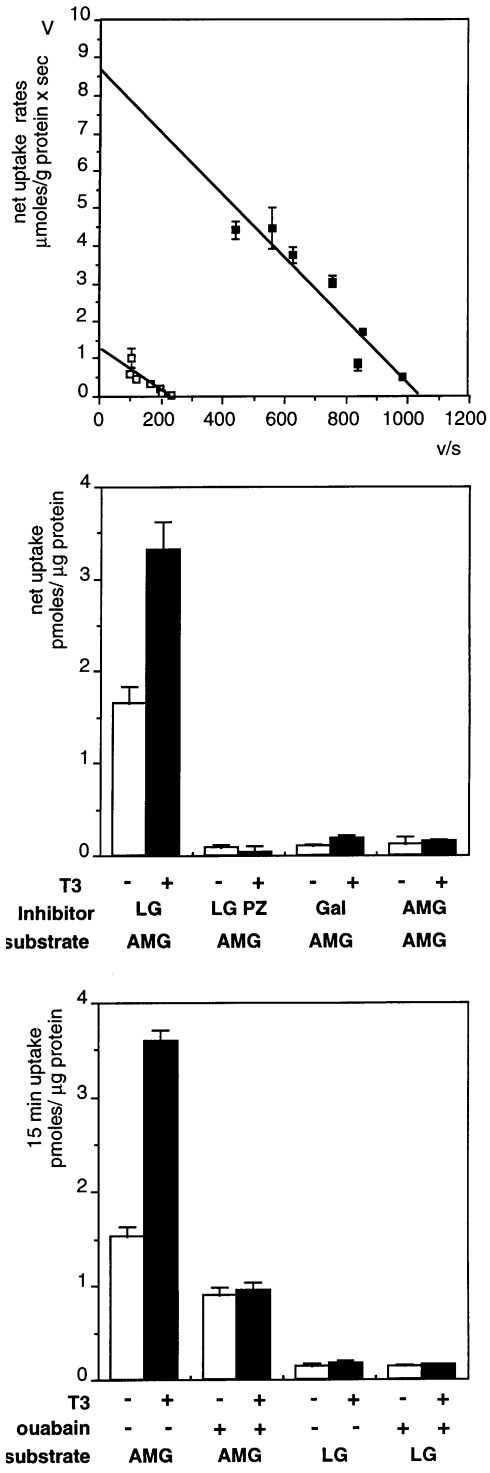

Figure 5 AMG transport in control and T3-treated Caco-2/TC7 cells

Uptake rates were measured in control (□) and 1 μM T3-treated (■) cell monolayers at day 20 as described in the Experimental section. Top: Eadie–Hoffstee transformation of the kinetics of [U-^{14}C]AMG uptake. Transport rates (μmol/s per g of protein $\pm$ S.D.; $n = 3$) were measured for 10 min at 25 °C. Middle: sugar specificity of [U-^{14}C]AMG uptake. Labelled 0.1 mM AMG uptake was challenged with 30 mM L-glucose (LG), D-galactose (Gal), AMG or 1 mM phlorizin (PZ). Bottom: [U-^{14}C]AMG uptake in ouabain-treated cells. Transport of 0.1 mM AMG and [1-^{3}H]L-glucose (LG) were measured in control and ouabain-treated cells. Cells were preincubated for 90 min with 10^{-4} M ouabain before transport measuments with ouabain. Net uptake rates (AMG-LG), expressed as pmol/μg of protein $\pm$ S.D. ($n = 6$) for 15 min, are from a representative experiment out of two, carried out using six independent culture wells.

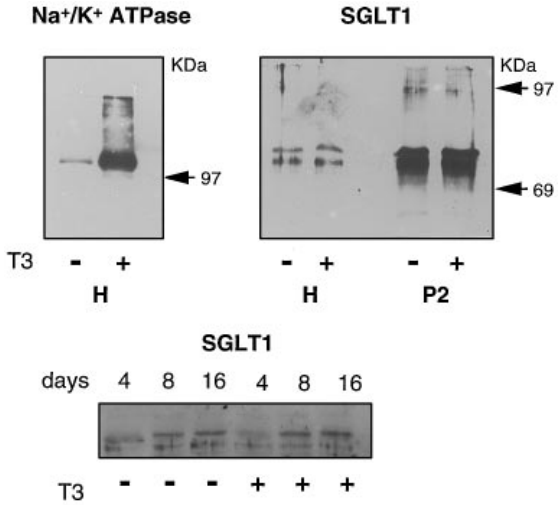

Figure 6 Protein abundances of Na^+/K^+ ATPase α1 and SGLT1 as a function of T3-treatment and cell differentiation

Representative Western blots from at least three independent experiments of: top right, SGLT1 (10% acrylamide slab gels) were carried out with 20 μg and 5 μg of protein for cell homogenates (H) and brush border membrane-enriched fraction (P2) (day 20) respectively; and top left, Na^+/K^+ ATPase (7.5% gels), using 10 μg of cell homogenate. Lower panel, blots of cell homogenates (20 μg) were carried out with cells harvested at days 4, 8 and 16 respectively.

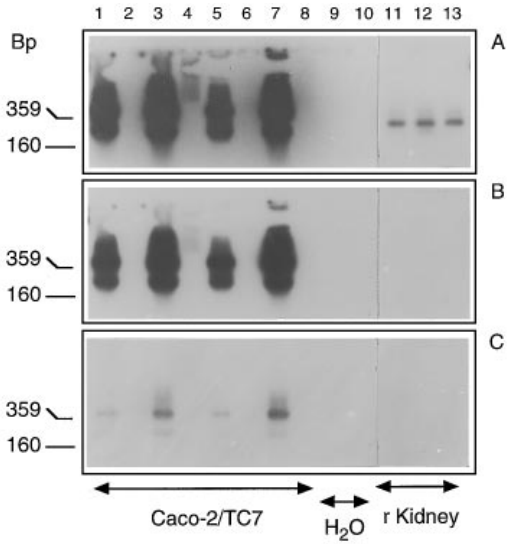

Figure 7 RT-PCR detection of SGLT1 and SGLT2 in Caco-2/TC7 cells

Control and T3-treated Caco-2/TC7 cells were cultured for 16 days. Total RNA extracts (5 μg) were used as templates for reverse transcription. A fraction of the RT products (one twenty-fifth) was used for PCR with hSGLT1-specific (SGLT1/2sens and SGLT1Rev) (lanes 1, 3, 5, 7 and 9) or hSGLT2-specific (SGLT1/2sens and SGLT2 AS) (lanes 2, 4, 6, 8, 10, 11, 12 and 13) primers. The starting material was from control (lanes 1, 2, 5 and 6) or T3-treated (lanes 3, 4, 7 and 8) Caco-2/TC7 cells or from whole rat kidney (lanes 11, 12 and 13). A 10^{-3} and 10^{-4} (lanes 12 and 13) dilution of the first PCR product was used for a second 30 cycle PCR. Lanes 9 and 10 are negative controls prepared with water. PCR products were Southern blotted and hybridized with a hSGLT1 cDNA probe. (**A**) Low-stringency washing (2 × SSC/0.1% SDS, 60 °C). (**B**, **C**) High stringency washing (0.1 × SSC/0.1% SDS, 65 °C). Note that the time of exposure of the X-ray film was 18 times longer in panel (**A**) and (**B**) (3 h) than in (**C**) (10 min).

product with the same oligonucleotides and PCR conditions, even though a second 30 cycle PCR was performed with a 10^{-3} and 10^{-4} dilution of the first PCR product.

DISCUSSION

Both Na^+/D-glucose cotransport activity and SGLT1 mRNA are found in differentiated Caco-2 cells [7,8]. T3 does not modify the growth curves of Caco-2/TC7 cells, nor does it allow a premature expression of SGLT1 mRNA. The T3-dependent overexpression of SGLT1 mRNA is obtained only upon treatment of differentiated cells. It is fully reversible within 3–4 days after the removal of the hormone. T3 concomitantly increases, although to different extents, the expression of SGLT1, Na^+/K^+ ATPase and PEPCK mRNAs, as well as the glucose consumption rates of the cells. PEPCK [24] and Na^+/K^+ ATPase [46] are known target genes for thyroid hormone. Their expression, as well as that of SGLT1, is enhanced by T3 post-confluently, further confirming that only differentiated Caco-2/TC7 cells can transduce T3 signals.

The expression of SGLT1 mRNA is highly sensitive to variations in the glucose supply and/or metabolic needs of Caco-2/TC7 cells [8,21]. Indeed, T3-treated cells exhibit a dose-dependent increase in glucose consumption and have smaller glycogen stores than control cells, a phenomenon that has been observed in Caco-2 cells that have been challenged with compounds that increase the glucose consumption of the cells [21,47]. To further understand the mechanism of T3 modulation involved in SGLT1 expression, we partially or totally deprived fully differentiated cells of glucose. Cells switched to a glucose-free medium (dialysed serum) downregulate the expression of SGLT1 to undetectable mRNA levels, indicating that glucose is essential to its expression. Increasing concentrations of glucose (0.5–25 mM) do not allow significant SGLT1 mRNA expression, unless T3 is present. Under these conditions, SGLT1 mRNA abundances are correlated with glucose concentrations, suggesting a co-regulation by glucose and T3. AMG-feeding does not sustain SGLT1 expression, indicating that T3 effects are mediated by glucose metabolism. The regulation of SGLT1 in Caco-2/TC7 cells thus differs from that observed in animal models *in vivo*, showing that non-metabolizable glucose analogues induce the expression of SGLT1 in crypt cells and that a functional cotransporter is found, inasmuch as cells migrate and mature along the villus [13,48,49].

Increased SGLT1 mRNA abundance is associated with mRNA stabilization of the three transcripts. Differential regulation of the half-life of SGLT1 transcripts has been observed in LLCPK1 cells, where the stabilization of the 3.9 kb transcript was shown to be cAMP dependent [50]. In Caco-2/TC7 cells, cAMP contents do not change with T3 treatment (results not shown). More work is clearly required to delineate the precise mechanism that is involved in the T3-specific, cAMP-independent, regulation of the half-life of SGLT1 transcripts.

Measurements of the uptake of AMG, a specific and non-metabolizable substrate for Na^+/glucose cotransporters, permit estimates of transport activities in living Caco-2 cell monolayers. Chronic treatment of the Caco-2/TC7 cells with T3 enhances the Na^+/AMG cotransporter activity affecting the V_{max} parameter (6-fold) of transport. Since Na^+/glucose cotransport activity depends on the functional expression of both SGLT1 and Na^+/K^+ ATPase (for reviews see [3,4]), we measured their protein abundances. The SGLT1 protein abundance is not modified upon T3 treatment of Caco-2/TC7 cells. Although very different from the situation described *in vivo*, these results add to the evidence that SGLT1 can be modulated post-transcriptionally in enterocytes, as has been proposed earlier [48]. On the other hand, T3-treated TC7 and PD7/Caco-2 cells do exhibit a strong increase in mRNA and protein abundances of Na^+/K^+ ATPase. This is in agreement with the results by Orlowski and co-workers [26,46] for parental Caco-2 cells, favouring the interpretation of an indirect effect of T3 on Na^+ recycling. The role of Na^+/K^+ATPase is further revealed by the fact that the T3-related enhancement of SGLT1 transport activity is lost upon ouabain treatment of Caco-2 cells, where the activity of the cation exchanger is inhibited [45].

Because Caco-2 cells are colon cancer cells, and even though they express an enterocytic phenotype of absorbing cells [20], AMG cotransport could reflect the activity of an aberrant expression of the kidney isoform of the glucose cotransporter SGLT2. The transport of [^{14}C]AMG was fully inhibited in the presence of saturating concentrations of unlabelled AMG or by adding phlorizin, a very potent inhibitor of Na^+/glucose cotransport; both results were expected in view of the ability of both AMG and phlorizin to inhibit both SGLT1- and SGLT2-mediated transport. The presence of equivalent amounts of galactose inhibits [^{14}C]AMG transport down to passive diffusion, indicating that no AMG is transported through the galactose-insensitive SGLT2 transporter, and thus confirming that SGLT1 is the only active cotransporter expressed in Caco-2/TC7 cells. The screening for SGLT1 and SGLT2 of total RNA preparations from control or T3-treated Caco-2/TC7 cells, as well as whole kidney preparations, was carried out using RT-PCR. Two PCR products (351 and 182 bp respectively) were detected with the SGLT1-specific oligonucleotides. The analysis of genomic sequence of SGLT1 between the two sites of priming strongly suggest that the most abundant band corresponds to the expected 351 bp size of the coding region, but that the smaller one (182 bp) is generated by skipping exon 12. The PCR products both increased with T3 treatment, as expected from the mRNA abundance pattern. On the other hand, control and T3-treated cells do not express SGLT2, although rat kidney samples used as positive controls for SGLT2 were detected under the same PCR conditions.

This paper provides evidence that the Na^+/glucose cotransport activity is enhanced by T3 in Caco-2 cells, without any change in protein abundance. The enhanced activity and protein abundance of the basolateral membrane-associated Na^+/K^+ ATPase suggest that T3 interferes with the recycling of Na^+ so that the capacity of cotransport is reinforced. This report further shows that sugar metabolism is required for the expression of SGLT1 in Caco-2 cells, suggesting that they behave differently than so called 'normal animal cells'. We feel that the modulation of SGLT1 expression in differentiated cells by glucose and T3 may result from the fetal phenotype of Caco-2 cells and therefore may represent a step in the development of intestinal cells. The regulation of SGLT1 mRNA is therefore probably subject to multiple, distinct control mechanisms that are still poorly understood, and work is needed to: (a) verify whether stability is the only mechanism involved in increased mRNA abundance or if transcription of the gene is altered; and (b) identify the mechanisms by which the cells counteract the increase in mRNA abundance to keep the protein abundance constant.

We wish to thank: Dr. M. Lacasa (INSERM 178, Villejuif Cedex, France); Dr. A. Muñoz (Instituto de Investigaciones Biomedicas, Madrid, Spain) for advice and help in the course of this work; Dr. E. M. Wright (UCLA School of Medicine, LA, U.S.A.) and Dr G. W. Gould (Institute of Biomedical and Life Sciences, University of Glasgow, Scotland, U.K.) for critically reading the manuscript; C. J. Gottardi and M. J. Kaplan (Department of Cellular and Molecular Physiology, Yale University, School of Medicine, New Haven, CT, U.S.A.) who provided the antibody to Na^+/K^+ ATPase, and S. M. GLOOR (Biochemistry II, ETH, Zurich, Switzerland) for the corresponding cDNA probes; and Dr. D. Lacasa (CJF INSERM 9402, Poissy, France) who provided the

reverse transcripts of rat kidney RNA. We are grateful to A. Barbat for the photographic work and to E. Dussaulx for skillfull assistance in cell culture. This work was supported by INSERM funds and by the Spanish DGICYT grant no. PB 96-1372.

REFERENCES

1 Semenza, G., Kessler, M., Weber, J. and Schmidt, U. (1984) Biochim. Biophys. Acta **779**, 343–379
2 Hediger, M., Coady, M. J., Ikeda, T. S. and Wright, E. M. (1987) Nature (London) **330**, 379–381
3 Hediger, M. A. and Rhoads, D. B. (1994) Physiol. Rev. **74**, 993–1026
4 Wright, E. M. (1993) Annu. Rev. Physiol. **55**, 575–589
5 Tarpey, P. S., Wood, I. S., Shirazi-Beechey, S. P. and Beechey, R. B. (1996) Biochem. J. **312**, 293–300
6 Poppe, R., Karbach, U., Gambaryan, S., Wiesinger, H., Lutzenburg, M., Kraemer, M., Witte, O. W. and Koepsell, H. (1997) J. Neurochem. **69**, 84–94
7 Blais, A., Bissonnette, T. and Berteloot, A. (1987) J. Membr. Biol. **99**, 113–125
8 Mahraoui, L., Rodolosse, A., Barbat, A., Dussaulx, E., Zweibaum, A., Rousset, M. and Brot-Laroche, E. (1994) Biochem. J. **298**, 629–633
9 Blais, A. (1991) Am. J. Physiol. (Cell. Physiol.) **29**, C1245–C1252
10 Hemlin, M. and Huang, X. (1997) Acta. Physiol. Scand. **160**, 185–194
11 Delézay, O., Baghdiguian, S. and Fantini, J. (1995) J. Biol. Chem. **270**, 12536–12541
12 Bissonette, P., Gagné, H., Coady, M. J., Benabdallah, K., Lapointe, J. Y. and Berteloot, A. (1996) Am. J. Physiol. (Gastrointest. Liver Physiol. **33**) **270**, G833–G843
13 Smith, M. W. (1993) Proc. Nutr. Soc. **52**, 293–300
14 Freeman, T. C., Wood, I. S., Sirinathsinghji, D. J. S., Beechey, R. B., Dyer, J. and Shirazi-Beechey, S. P. (1993) Biochim. Biophys. Acta **1146**, 203–212
15 Burant, C. F., Flink, S., Depaoli, A. M., Chen, J., Lee, W. S., Hediger, M. A., Buse, J. B. and Chang, E. B. (1994) J. Clin. Invest. **93**, 578–585
16 Ferraris, R. P. and Diamond, J. (1992) Am. J. Physiol. (Gastrointest. Liver Physiol. **25**) **262**, G1069–G1073
17 Ferraris, R. P., Villenas, S. A., Hirayama, B. A. and Diamond, J. (1992) Am. J. Physiol. (Gastrointest. Liver Physiol. 25) **262**, G1060–G1068
18 Shirazi-Beechey, S. P., Hirayama, B. A., Wang, Y., Scott, D., Smith, M. W. and Wright, E. M. (1991) J. Physiol. London **437**, 699–708
19 Pinto, M., Robine-Léon, S., Appay, M. D., Kedinger, M., Triadou, N., Dussaulx, E., Lacroix, B., Simon-Assmann, P., Haffen, K., Fogh, J. and Zweibaum, A. (1983) Biol. Cell. **47**, 323–330
20 Zweibaum, A., Laburthe, M., Grasset, E. and Louvard, D. (1991) in Intestinal Absorption and Secretion. Handbook of Physiology, Section 6. The Gastrointestinal System, vol. IV (Field, M. and Frizzell, R. A., eds.), pp. 223–255, American Physiological Society, Bethesda
21 Carrière, V., Barbat, A., Rousset, M., Brot-Laroche, E., Dussaulx, E., Cambier, D., de Waziers, I., Beaune, P. and Zweibaum, A. (1996) Am. J. Physiol. (Gastrointest. Liver Physiol. **33**) **270**, G976–G986
22 Muller, M. J., Acheson, K. J., Jequier, E. and Burger, A. G. (1988) Am. J. Physiol **255**, E146–E152
23 Hoppner, W. and Seitz, H. J. (1989) J. Biol. Chem. **264**, 20643–20647
24 Giralt, M., Park, E. A., Gurney, A. L., Liu, J., Hakimi, P. and Hanson, R. W. (1991) J. Biol. Chem. **266**, 21991–21996
25 Nebioglu, S., Wathanaronchai, P., Nebioglu, D., Pruden, E. L. and Gibson, D. M. (1990) Am. J. Physiol. (Endocrinol. Metab. **21**) **258**, E109–E116
26 Giannella, R. A., Orlowski, J., Jump, M. L. and Lingrel, J. B. (1993) Am. J. Physiol. **265**, G775–G782
27 Ulisse, S., Jannini, E. A., Pepe, M., De Matteis, S. and D'Armiento, M. (1992) Mol. Cell. Endocrinol. **87**, 131–137
28 Weinstein, S. P., O'Boyle, E., Fisher, M. and Haber, R. S. (1994) Endocrinology **135**, 649–654
29 Chantret, I., Rodolosse, A., Barbat, A., Dussaulx, E., Brot-Laroche, E., Zweibaum, A. and Rousset, M. (1994) J. Cell Sci. **107**, 213–225
30 Jumarie, C. and Malo, C. (1991) J. Cell. Physiol. **149**, 24–33
31 Brot-Laroche, E., Supplisson, S., Delhomme, B., Alcalde, A. I. and Alvarado, F. (1987) Biochim. Biophys. Acta **904**, 71–80
32 Devarajan, P., Gilmore-Herbert, M. and Benz, E. J. J. (1992) J. Biol. Chem. **267**, 22435–22439
33 Pringault, E., Arpin, M., Garcia, A., Finidori, J. and Louvard, D. (1986) EMBO J. **5**, 3119–3124
34 Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979) Biochemistry **18**, 5294–5299
35 Thomas, P. S. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 5201–5205
36 Mahraoui, L., Rousset, M., Dussaulx, E., Darmoul, D., Zweibaum, A. and Brot-Laroche, E. (1992) Am. J. Physiol. (Gastrointest. Liver Physiol. **26**) **263**, G312–G318
37 Schmitz, J., Preiser, H., Maestracci, D., Ghosh, B. K., Cerda, J. J. and Crane, R. K. (1973) Biochim. Biophys. Acta. **323**, 98–112
38 Delézay, O., Verrier, B., Mabrouk, K., Van Rietschoten, J., Fantini, J., Mauchamp, J. and Gérard, C. (1995) J. Cell. Physiol. **163**, 120–128
39 Gérardi-Laffin, C., Vittori, C., Sudaka, P. and Poirée, J. C. (1991) Biochim. Biophys. Acta **1063**, 21–26
40 Giudicelli, J., Bertrand, M. F., Bilski, S., Tran, T. T. and Poirée, J. C. (1998) Biochem. J. **330**, 733–736
41 Gottardi, C. J. and Caplan, M. J. (1993) J. Cell Biol. **121**, 283–293
42 Rousset, M., Robine-Léon, S., Dussaulx, E., Chevalier, G. and Zweibaum, A. (1979) Front. Gastrointest. Res. **4**, 80–85
43 Mesonero, J., Matosin, M., Cambier, D., Rodriguez-Yoldi, M.-J. and Brot-Laroche, E. (1995) Biochem. J. **312**, 757–762
44 Mackenzie, B., Loo, D. D. F., Panayotova-Heiermann, M. and Wright, E. M. (1996) J. Biol. Chem. **271**, 32678–32683
45 Ramond, M.-J., Martinot-Peignoux, M. and Erlinger, S. (1985) Biol. Cell. **54**, 89–92
46 Feng, J., Orlowski, J. and Lingrel, J. B. (1993) Nucleic Acids Res. **21**, 2619–2626
47 Rousset, M., Laburthe, M., Pinto, M., Triadou, N., Boige, N., Rouyer-Fessard, C., Dussaulx, E., Chevalier, G., Brun, J. L. and Zweibaum, A. (1984) 7th International Congress of Endocrinology, Quebec, Canada. Exerpta Med. p. 996 (Abstract)
48 Lescale-Matys, L., Dyer, J., Scott, D., Wright, E. M. and Shirazi-Beechey, S. P. (1993) Biochem. J. **291**, 435–440
49 Ferraris, R. P. and Diamond, J. M. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 5868–5872
50 Peng, H. and Lever, J. E. (1995) J. Biol. Chem. **270**, 20536–20542

Received 27 January 1998/1 June 1998; accepted 1 July 1998