An Improved Cell Culture Model Based on 2/4/A1 Cell Monolayers for Studies of Intestinal Drug Transport: Characterization of Transport Routes

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Purpose. To improve the viability of the 2/4/A1 cell culture model and to investigate different routes of drug transport in this cell line.
Methods. Two approaches were taken to decrease apoptosis. First, rat intestinal 2/4/A1 cells were transfected to overexpress the antiapoptotic protein Bcl-2. Second, normal 2/4/A1 cells were cultivated under conditions that stimulate differentiation and limit apoptosis. The monolayer integrity was investigated by transepithelial electrical resistance, permeability, and microscopy. The expression of drug transporters was investigated by RT-PCR, and transport function was assessed using specific markers.
Results. Normal 2/4/A1 cells died by apoptosis at 39°C. Bcl-2–expressing 2/4/A1 cells were viable but adopted a morphology of less-differentiated epithelial cells. Optimization of the culture conditions for 2/4/A1 cells inhibited cell death. The integrity was comparable to that of the human jejunum (50 $\Omega \times cm^2$), making this approach preferable to Bcl-2 overexpression. Transcriptional analysis showed that some (e.g., MDR1), but not all (e.g., PepT1), transporters were found in 2/4/A1 cells. Studies using substrates for PepT1, P-gp, MRP2, and BCRP showed that none of the transporters were functional in 2/4/A1.
Conclusions. The improved culture procedure will facilitate the use of 2/4/A1 cells. 2/4/A1 lack several transporters, which makes them a promising alternative to Caco-2 cells and artificial membranes in studies of passive drug transport.

KEY WORDS: Drug transport; Caco-2; Bcl-2; 2/4/A1; G418.

INTRODUCTION

We have recently introduced 2/4/A1, a conditionally immortalized rat intestinal cell line, as an alternative model to Caco-2 for studies of drug absorption (1). We showed that 2/4/A1 cell monolayers are more permeable to incompletely absorbed drugs than Caco-2 cell monolayers. This makes 2/4/A1 cells an excellent model for determination of drug permeability coefficients that are of the same order of magnitude as those found in the human jejunum. 2/4/A1 cells, which have been conditionally immortalized with a temperature-sensitive mutant of SV40 large T antigen (tsSV40T), proliferate at 33°C but stop proliferation and enter a differentiation program when the temperature is increased to 39°C (2). However, in our hands, the 2/4/A1 cells entered cell death when cultured at 39°C. We solved this problem by cultivating the cells at an intermediate temperature of 37°C, at which the tsSV40T remains partly active (1). At this temperature the 2/4/A1 cell monolayers reached transepithelial electrical resistance (TER) values of 25 $\Omega \times cm^2$, a value that is comparable to those obtained in rat small intestinal segments (3).

Unfortunately, this approach has two drawbacks. First, at 37°C, the tsSV40T is partly active, and small changes in temperature may therefore introduce variability e.g., with regard to cell monolayer formation and integrity of the cell monolayers. Second, the 2/4/A1 cells grown at 37°C are more poorly differentiated than the fraction that survives at 39°C, as judged from the expression levels of brush border enzymes (1). Therefore, we set out to reduce the variability and improve the differentiation of the 2/4/A1 model by establishing culture conditions under which 2/4/A1 cells form confluent cell monolayers at 39°C.

We determined that the cell death of 2/4/A1 cells at 39°C is caused by apoptosis. We then used two strategies to overcome the apoptosis of 2/4/A1 cells. First, we transfected 2/4/A1 cells to overexpress the antiapoptotic protein Bcl-2. Bcl-2 protects various cell lines from apoptosis induced by oxidative stress and apoptosis-inducing agents (4,5). Second, we developed a modified culture protocol for 2/4/A1 cells in which we excluded the selection drug G418 (which eradicates cells that have lost the expression of the transgene tsSV40T) (6), and in which we added a small amount of serum to the cell culture medium. We then compared the mRNA expression of nine transporters and drug efflux systems in 2/4/A1 cells grown at 33°C and at 39°C with that in rat small intestine. Finally, we investigated the functions of one drug transport protein (PepT1) and of three drug efflux systems (P-gp, MRP2, and BCRP) using established substrates.

MATERIALS AND METHODS

Cell Culture

Culture media and supplements were purchased from Gibco BRL Life Technologies AB (Täby, Sweden) unless otherwise stated.

2/4/A1 cells originating from fetal rat intestine were expanded as described previously (1). These cells have been conditionally immortalized with a pZipSVtsa58 plasmid containing a temperature-sensitive mutant of SV40T antigen (2). The medium was changed every second day, and the cells were passaged at 80% confluence, approximately every fourth day. For functional studies, the 2/4/A1 cells at a seeding density of 100,000 cells/cm^2 were grown in RPMI 1640 supplemented with growth factors, 0–8% fetal calf serum (FCS) with or without G418 on permeable supports at 100,000 cells/cm^2 (Transwell Costar, Badhoevedorp, The Netherlands, 0.4 μm pore size, 12 mm in diameter) coated with EHS matrix (Promega Corporation, Madison, WI) at 33, 37, or 39°C. Cells at passage number 23 through 43 were used. The 2/4/A1 cells were cultivated for 6–8 days before they were used in drug transport experiments.

Caco-2 cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). The cells were

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maintained as described elsewhere (7). Cells at passage number 90–105 were seeded at 440,000 cells/cm^2 on permeable supports (Transwell Costar, 0.4 μm pore size, 12 mm in diameter) and cultivated at 37°C for 21–35 days before the Caco-2 cell monolayers were used in drug transport experiments.

MDCK II and LLC-PK1 cells were obtained from ATCC. LLC-PK1 cells expressing murine BCRP (LLC-BCRP) (8) were a generous gift from Professor Alfred Schinkel. MDCK II cells expressing human MDR1 (MDCK-MDR1) (9) and MDCK II cells expressing human MRP2 (MDCK-MRP2) (10) were generous gifts from Professor Piet Borst. MDCK II and MDCK II clones at passage 6–15 were seeded at 440,000 cells/cm^2 on permeable supports and cultivated for 4 days in DMEM supplemented with 10% FCS, 1 mM Na-pyruvate, 2 mM glutamax, and 1% penicillin–streptomycin at 10% CO_2 and 37°C. LLC-PK1 and LLC-BCRP at passage 6 were grown as described for MDCK II in Medium 199 supplemented with 10% FCS, 4 mM glutamax, and 1% penicillin–streptomycin at 5% CO_2 and 37°C.

The cell cultures tested negative for *Mycoplasma* contamination every second month throughout this study.

Establishment of 2/4/A1-Bcl-2 Clones

2/4/A1 cells at 80% confluence were trypsinized and pelleted, harvested by centrifugation, and washed twice with Hanks' balanced salt solution (HBSS). Immediately before transfection, the cells were resuspended in PBS. The suspension was divided into 1-ml fractions of 10^7cells each. Two plasmids were added to each fraction; 3 μg/1 × 10^6 cells of human Bcl-2 cDNA-containing plasmid (11) and 0.15 μg/1 × 10^6 cells of hygromycin B resistance plasmid (P3′SS). Electroporation (The ElectroPorator; Invitrogen, Life Technologies, Carlsbad, CA) was performed at room temperature (250–500 μF, 300 V, 0.4-cm cuvette). After electroporation the cells were plated onto culture dishes containing 50% conditioned RPMI 1640. The dishes were placed in an incubator (33°C, 5% CO_2) and left overnight. Twenty-four hours after transfection, 200 U/ml hygromycin B was added to the conditioned medium. Clones were immediately isolated with cloning cylinders when they appeared, in order to avoid the spreading and mixing of clones. The clones were harvested by local trypsinization and transferred to a 96-well plate in 50% conditioned medium followed by successive expansion to 175-cm^2 culture flasks and frozen in liquid N_2 pending characterization.

For the characterization of Bcl-2 clones, approximately 7 × 10^6 of flask-grown cells were harvested, washed twice in PBS, and used for the preparation of RNA. Total RNA was isolated using the RNeasy minikit (Qiagen GmbH, Hilden, Germany) following the instructions provided by the manufacturer, with an additional on-column DNase treatment step (Qiagen). Cells were homogenized using a QiaShredder (Qiagen). RNA was quantified using UV absorption at 260/280 nm, and RNA integrity was checked by assessing the sharpness of ribosomal RNA bands on a native 1% agarose gel using a 1 × TBE running buffer (0.09 M Tris-borate, 0.002 M EDTA, pH 7.8). The gel was run at 2.5 V/cm for 40 min.

Bcl-2 clones were investigated for Bcl-2 expression using Northern blot analysis, with a ^{32}P-labeled full-length (1.9 kbp) Bcl-2 cDNA probe, and using a human Bcl-2 ELISA kit (Calbiochem, CA, USA). ELISA and Northern blot analysis were carried out according to the manufacturer's instructions and standard molecular biology protocols (12), respectively.

RT-PCR of Transport and Efflux Systems

For the characterization of transport and efflux systems, approximately 7 × 10^6 filter-grown 2/4/A1 cells were harvested using ice-cold phosphate-buffered saline solution and a cell scraper. Cells were kept on ice during the scraping procedure and subsequently recovered by centrifugation. 2/4/A1 cells and the rat ileal tissue (positive control) were homogenized using a Heidolph Diax 900 tissue homogenizer equipped with a 6G tool (Heidolph Instruments, Cinnaminson, NJ). RNA was extracted and checked for absence of contaminating genomic DNA, as previously described (13). Also, PCR primer design (Table I) and reaction conditions were as previously described by Taipalensuu *et al.* (13), except that no additional 5′ sequence was added to the PCR primers, and all PCR cycles were conducted at an annealing temperature of 56°C. Samples were analyzed after 25, 29, 33, and 37 PCR cycles.

Cell Viability

The effect of G418 on cell viability was assessed by measuring the intracellular dehydrogenase activity of 2/4/A1 cells using the MTT method (14) as described previously (15). 2/4/A1 cells were seeded at 100,000 cells/cm^2 in EHS-coated 96-well plates in the presence of different concentrations of G418 in the culture medium and incubated at 39°C for 48 h before the MTT assay was done.

Fluorescence Microscopy

Cells grown on EHS-coated permeable supports were washed with PBS and fixed with 3% paraformaldehyde in PBS at pH 7.4 for 10 min at room temperature. The cells were then permeabilized using 0.1% Triton X-100 in PBS for 10 min. The ZO-1 protein distribution was studied by indirect immunofluorescence using a rabbit polyclonal antibody to the ZO-1 protein (1:1000) (Zymed Laboratories Inc., San Francisco, CA). The samples were washed with PBS, mounted in Dako fluorescent mounting medium, sealed, and examined in a confocal laser-scanning microscope (Leica TCS 4D; Leica LT, Heidelberg, Germany).

Transepithelial Electrical Resistance

The transepithelial electrical resistance (TER) of 2/4/A1 cell monolayers grown on EHS-coated permeable supports was measured at 37°C with an Endohm tissue resistance measurement chamber connected to an Evohm resistance meter (World Precision Instruments, Sarasota, FL). The cell culture medium was replaced by HBSS (pH 7.4; containing 25 mM HEPES) preheated to 37°C, and the 2/4/A1 cell monolayers were allowed to equilibrate for 20 to 25 min before TER measurements. The resistance of EHS-coated filters without cells (4 Ω × cm^2) was subtracted from each TER value.

Drugs

Alprenolol, digoxin, glycylsarcosine, mannitol, mitoxantrone, and vinblastine were purchased from Sigma (St Louis, MO). [^{14}C]Mannitol was obtained from New England Nuclear (Boston, MA). [^{3}H]Digoxin was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). [^{3}H]Glycylsarcosine, [^{3}H]mitoxantrone, and [^{3}H]vinblastine were obtained from Moravek Biochemicals (Brea, CA).

[^{14}C]PEG (MW 4000) was purchased from Amersham Biosciences (Uppsala, Sweden).

Permeability of Passively Transported Drugs in 2/4/A1 Cell Monolayers

The drug transport experiments were performed in 6- to 8-day-old 2/4/A1 cell monolayers as described previously (1). At the start of the experiment 4 ml HBSS containing the drug was added to the donor chamber, either 0.1 mM unlabeled drug spiked with 5000 Bq/ml of the corresponding radioactively labeled drug or 0.1–1 mM unlabeled drug only. Samples were withdrawn from the receiver side at regular time intervals. The samples were replaced with fresh preheated HBSS. In order to obtain drug permeability coefficients that were unbiased by the unstirred water layer (UWL), the filters were agitated on a calibrated plate shaker (IKA Shüttler MTS4) at two different stirring rates, 100 rpm and 500 rpm, as described previously (16).

Analysis of Vectorial Drug Transport

[^{3}H]Digoxin, [^{3}H]glycylsarcosine (glysar), [^{3}H]mitoxantrone, and [^{3}H]vinblastine were chosen as markers to examine functional expression of P-glycoprotein (P-gp), oligopeptide transporter (PepT1), breast cancer resistance protein (BCRP), and canalicular multispecific organic anion transporter/multidrug resistance-associated protein 2 (cMOAT/MRP2), respectively. The transport studies were carried out as described above with the exception that the experiments with glysar (0.48 μM, 50 μM, and 50 mM spiked with 5000 Bq/ml [^{3}H]glysar) were carried out at a donor chamber pH of 6.0 and an acceptor chamber pH of 7.4. In order to measure vectorial transport, both apical-to-basolateral (a-b) and basolateral-to-apical (b-a) permeability coefficients were determined for digoxin, mitoxantrone, and vinblastine. As shown previously (10), an inhibitor of P-gp, GF12918 (at 2 μM apically and basolaterally), had to be used in the studies of vinblastine in order to study the influence of MRP2-mediated efflux unbiased by P-gp in MDCK II and in MDCK II cells overexpressing MRP2 (MDCK-MRP2).

Analytic Methods

Radioactive samples were analyzed using a liquid scintillation counter (Packard Instruments 1900CA TRI-CARB®; Canberra Packard Instruments, Downers Grove, IL). Alprenolol samples were analyzed using a binary HPLC system (Bischoff Analysentechnik und Geräte GmbH, Leonberg, Germany). The mobile phases were MilliQ:acetonitrile:TFA 99:1.0:0.1% (eluent A) and MilliQ:acetonitrile:TFA 1:99:0.1% (eluent B). The following gradient was programmed for each cycle: a linear gradient from A:B 95:5 to A:B 5:95 over 1 min followed by A:B 5:95 for 0.5 min and finally A:B 95:5 for 0.75 min. The analytic column was a Reprosil Pur C8-AQ 3 × 53 mm analysis column. The flow rate was 2.0 ml/min, and the absorbance was monitored at 210 nm.

Calculations

The apparent permeability coefficient (P_{app}, cm/s) was determined from the following equation:

$$P_{app} = \frac{K \cdot V_r}{A} \quad (1)$$

where K is the steady-state change in concentration in the receiver chamber (C_t/C_0) vs. time (s). C_t is the concentration in the receiver compartment at the end of each time interval, C_0 is the initial concentration in the apical chamber at each time interval (mole/ml), V_r is the volume in the receiver chamber (ml), and A is the surface area of the filter membrane (cm^2).

The cellular permeability (P_c) was calculated by determining the apparent permeability of the drugs in 2/4/A1 cell monolayers at two different stirring rates (V). P_c was calculated from the slope of the linear relationship between V/P_{app} and V as described previously (17).

$$\frac{V}{P_{app}} = \frac{1}{K} + \left(\frac{1}{P_c} + \frac{1}{P_f}\right) \cdot V \quad (2)$$

where P_f is the calculated permeability coefficient of the filter support and K is a constant.

Statistics

The experiments were carried out using four cell monolayers each time, unless otherwise stated. The results were expressed as mean values ± SD. Significance was tested using two-tailed Student's t-test or one-way ANOVA. A p value of < 0.05 was considered significant.

RESULTS

Determination of Apoptosis in 2/4/A1 Cell Monolayers Grown at 39°C

The amount of fragmented DNA in 2/4/A1 cells increased with increasing culture temperature (cultivated at 33, 37, and 39°C) (Fig. 1). We concluded that the cell death observed at 39°C was caused by apoptosis. We therefore overexpressed the antiapoptotic protein Bcl-2 in 2/4/A1 cells in an attempt to overcome the apoptosis.

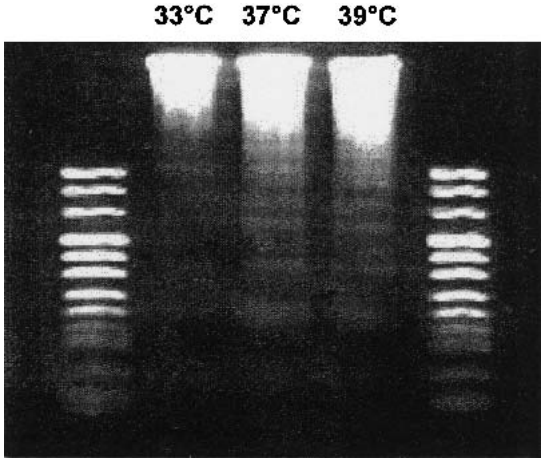

Fig. 1. DNA ladder showing increasing DNA fragmentation with increasing culture temperature in 2/4/A1 cell monolayers cultivated under the previously published culture conditions (1).

Establishment of 2/4/A1 Clones Overexpressing Bcl-2

Thirty-seven hygromycin B-resistant clones were isolated. Clones that had lost the ability to form cell–cell contacts were discarded. Northern blots (Fig. 2A) showed that three clones expressed Bcl-2 at different levels. Only the clone expressing the highest levels of Bcl-2 mRNA (2/4/A1/Bcl-2) showed detectable expression at the protein level as seen by ELISA (Fig. 2B).

Morphology and Integrity of the Bcl-2–Expressing 2/4/A1 Clone

Visual inspection in the confocal microscope showed that 2/4/A1/Bcl-2, in contrast to 2/4/A1, formed continuous cell monolayers at 39°C without signs of cell death (Fig. 3A). However, each 2/4/A1/Bcl-2 cell covered a large surface area and displayed a flat rather than cuboidal or columnar morphology at 33°C, 37°C, and 39°C (only cells cultivated at 39°C are shown in the figure).

The continuous 2/4/A1/Bcl-2 cell monolayers reached TER values of only 16 ± 3 Ω × cm^2 when grown for 4 days at 39°C (Fig. 3B). In contrast, 2/4/A1 and 2/4/A1/G7 cells did not form confluent cell monolayers as judged by their appearance in the confocal microscope (data not shown), and negligible TER values were obtained at this temperature (Fig. 3B), indicating that the Bcl-2 expression in 2/4/A1/G7 was insufficient to maintain complete integrity in this clone. Nor-

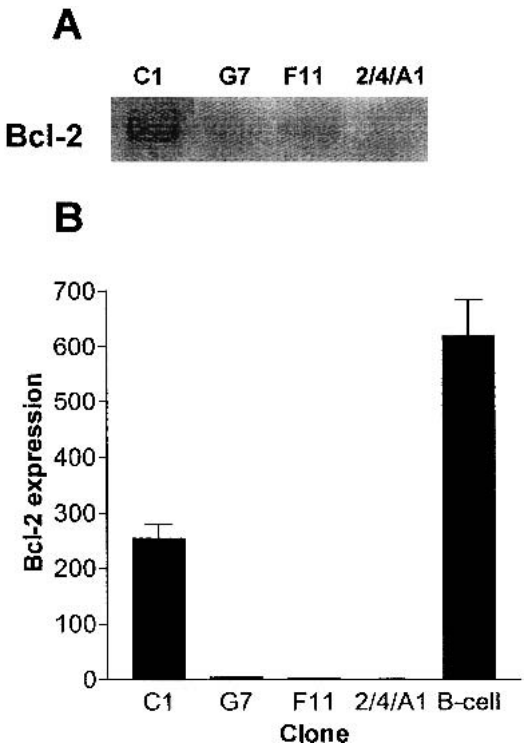

Fig. 2. (A) Northern Blot of 2/4/A1 clones expressing different levels of Bcl-2. The 2/4/A1/C1 (C1) clone expresses substantial levels of Bcl-2, whereas the 2/4/A1/G7 (G7) and 2/4/A1/F11 (F11) clones expresses low levels. Untransfected 2/4/A1 cells did not show any expression of Bcl-2. (B) ELISA showing the expression of Bcl-2. Expression of Bcl-2 was detected in 2/4/A1/C1. 2/4/A1/C1 will be denoted by 2/4/A1/Bcl-2 in the rest of this article. Each *bar* represents mean ± SD of four experiments.

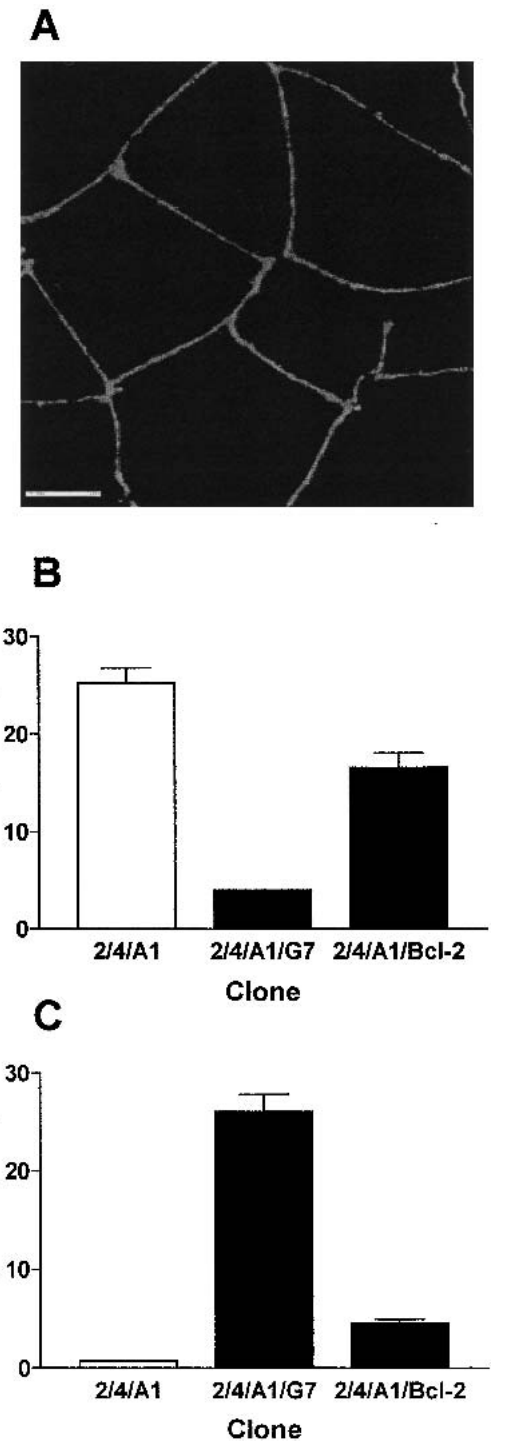

Fig. 3. (A) Immunofluorescence of ZO-1 of 2/4/A1/Bcl-2 grown at 39°C using previously published culture conditions (1). (B) Transepithelial electrical resistance (TER) of 2/4/A1 cell monolayers cultivated for 4 days at 37°C *(open bar)* and Bcl-2 clones cultivated for 4 days at 39°C *(closed bars)*. Untransfected 2/4/A1 cell monolayers reached a TER of approximately 25 Ω × cm^2 when cultivated at 37°C. The TER values of the Bcl-2 clone 2/4/A1/G7 at 39°C was only 4 Ω × cm^2, and the TER value of 2/4/A1/Bcl-2 at 39°C reached 16 Ω × cm^2 after 4 days. (C) Permeability coefficients for PEG 4000 in 2/4/A1 cell monolayers cultivated at 37°C *(open bar)* and Bcl-2 clones cultivated at 39°C *(closed bars)*. The permeability coefficient of PEG 4000 in 2/4/A1 cell monolayers was lower than in the Bcl-2 clones at 39°C. Each *bar* represents mean ± SD of four experiments.

mal 2/4/A1 cell monolayers grown for 4 days at 37°C reached significantly higher TER values (25 ± 3 Ω × cm^2) than 2/4/A1/Bcl-2 at 39°C ($p < 0.05$). Likewise, the permeability to the integrity marker PEG 4000 in 2/4/A1/Bcl-2 grown at 39°C was higher than that obtained in 2/4/A1 cell monolayers grown at 37°C (Fig. 3C). In summary, these results show that overexpression of Bcl-2 inhibited apoptosis and allowed the formation of intact cell monolayers. However, the undesirable change in morphology and the comparably low barrier function of the 2/4/A1/Bcl-2 prompted us to look for an alternative strategy to obtain 2/4/A1 cell monolayers suitable for drug transport studies.

Modification of the Cell Culture Procedure for 2/4/A1

We investigated if extracellular factors that induce apoptosis could be identified in our serum-free cell culture medium for 2/4/A1 cells. We first studied the toxicity of the SV40T selection marker G418, using the MTT assay (Fig. 4). A concentration-dependent reduction of the intracellular dehydrogenase activity with an IC_{50} value of 18 μg/ml was observed for 2/4/A1 cells at 39°C, although G418 was nontoxic for 2/4/A1 cells at 33 or 37°C or to 2/4/A1/Bcl-2 cells at 39°C ($IC_{50} > 400$ μg/ml). G418 was therefore removed from the cell culture medium.

Serum deprivation may induce apoptosis (18), and so we next added increasing amounts of fetal calf serum to our serum-free medium. At serum concentrations equal to or greater than 6%, intact cell monolayers of fully viable cuboidal cells that survived at least 14 days at 39°C were obtained (Fig. 5A). In an attempt to improve the cell culture conditions even further, we investigated the role of the dexamethasone and EGF additives. Briefly, the concentration of dexamethasone could be reduced to half (65 ng/l) without detrimental effect on cell monolayer properties, although the previously selected concentration of EGF (20 ng/ml) was required to

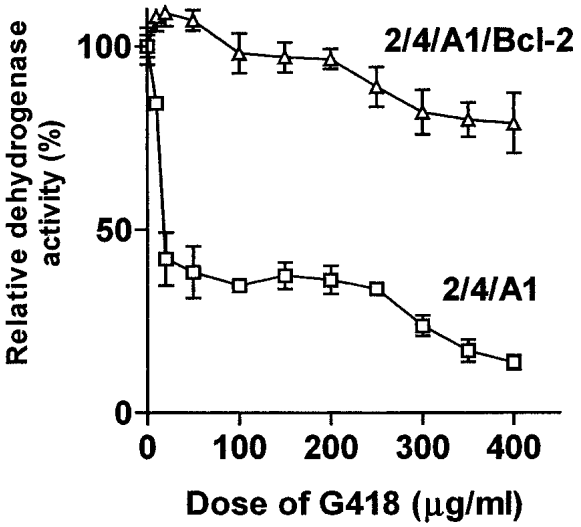

Fig. 4. Dose-dependent toxicity of G418 in 2/4/A1 cells cultivated at 39°C *(squares)* and 2/4/A1/Bcl-2 cultivated at 39°C *(triangles)*. G418 decreased the dehydrogenase activity of the 2/4/A1 cells at 10 μg/ml ($p < 0.05$), whereas the concentration of G418 had to be increased to 250 μg/ml before we could see significantly decreased dehydrogenase activity in 2/4/A1/Bcl-2 cells ($p < 0.05$). Each point represents the mean ± SD of four experiments.

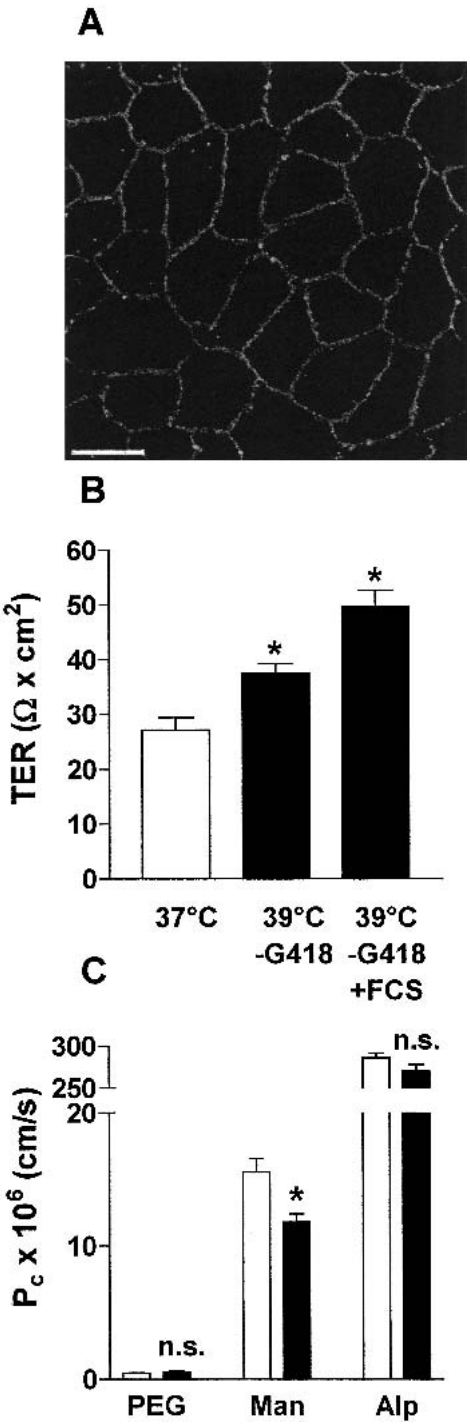

Fig. 5. (A) Immunofluorescence of ZO-1 of 2/4/A1 grown at 39°C under the new optimized conditions presented in this paper. (B) The TER in 2/4/A1 cell monolayers cultivated under serum-free conditions without G418 at 37°C and at 39°C reached approximately 27 and 37 Ω × cm^2 after 6 days, respectively. 2/4/A1 cell monolayers cultivated at 39°C in the presence of 6% FCS without G418 reached approximately 50 Ω × cm^2 after 6 days and maintained this value for at least another 8 days in culture (data not shown). (C) Permeability coefficients for the hydrophilic marker molecules mannitol and PEG 4000 and for the lipophilic drug alprenolol in 2/4/A1 cell monolayers cultivated under optimized conditions at 37°C *(open bars)* and at 39°C *(closed bars)*. Each *bar* represents the mean ± SD of four experiments. * and n.s. denote a significant ($p < 0.05$) and insignificant difference between 37°C and 39°C, respectively.

maintain cell monolayer integrity (data not shown). Further evidence for the improved integrity of the optimized cell cultures was obtained from the TER measurements (Fig. 5B). Thus, the highest TER value ($50 \pm 6.0\ \Omega \times cm^2$) was obtained for 2/4/A1 cells grown in the optimized cell culture medium at 39°C. Based on these results, 2/4/A1 cell monolayers grown without G418 in the presence of 6% FCS were selected for further studies.

Permeability and Transport Characteristics of Optimized 2/4/A1 Cell Monolayers

Passive Permeability

The TER of the optimized cell cultures grown at 39°C was significantly higher than that of those grown at 37°C ($p < 0.05$) (Fig. 5B). However, the permeabilities of the optimized 2/4/A1 cell cultures grown at 39°C to hydrophilic markers and to the lipophilic drug alprenolol were comparable to those reported previously for 2/4/A1 cells grown at 37°C (Fig. 5C).

Active Transport

Some of the investigated intestinal membrane transporters were expressed in 2/4/A1 cells grown at 33 and 39°C (Fig. 6). The mRNA for the apically located sodium-independent hexose transporter (GLUT5) but not for the Na^+/glucose transporter (SGLT1) was expressed in 2/4/A1. A higher level of mRNA coding for the large neutral amino acid transporter (LAT) was detected in 2/4/A1 cells grown at 39°C than that detected at 33°C. On the other hand, only very faint smeared bands of mRNA corresponding to the oligopeptide transporter PepT1 were observed for the 2/4/A1 cells.

No significant H^+-coupled active transport of the PepT1 substrate glysar was observed in the 2/4/A1 cells ($p > 0.05$) (Fig. 7A). This result agrees with the results from the RT-PCR experiment showing that PepT1 is not expressed in these cells. In contrast, a clear concentration-dependent H^+-coupled active transport of glysar was observed in Caco-2 cells (Fig. 7B), which are known to express PepT1 (19,20). This observation was further supported by studies in the absence of a pH-gradient (pH_{apical} and $pH_{basolateral} = 7.4$). In 2/4/A1 the transport of glysar was comparable at an apical pH of 7.4 to that at an apical pH of 6.0, whereas in the case of Caco-2 cells, the transport of glysar was fourfold lower at an apical pH of 7.4 as compared to an apical pH of 6.0 ($p < 0.05$).

Efflux

Both genes that code for P-glycoprotein, *MDR1a* and *MDR1b,* were expressed in 2/4/A1. However, *MDR1b* ap-

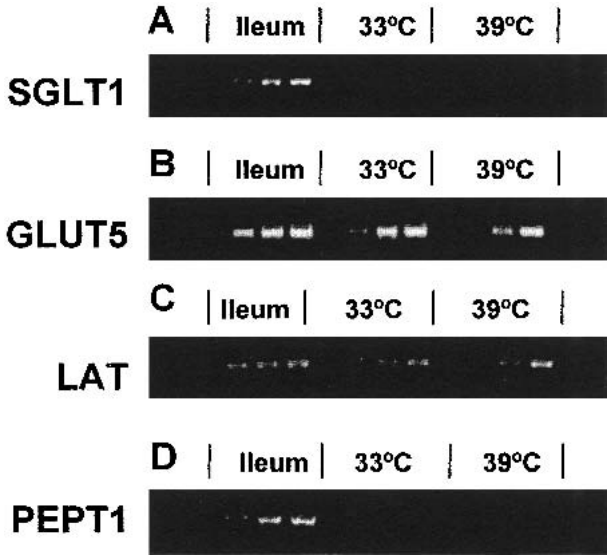

Fig. 6. RT-PCR detection of the Na^+/glucose transporter (SGLT1) (A), the Na^+-independent transporter (GLUT5) (B), the large neutral amino acid transporter (LAT) (C), and oligopeptide transporter PepT1 (D) in rat ileum and 2/4/A1 cell monolayers cultivated at 33°C and 39°C. The RT-PCR reaction was run 25, 29, 33, and 37 cycles for each of the three samples. The cDNA products were loaded on a 2.5% agarose gel, and gene-specific products were detected by ethidium bromide staining.

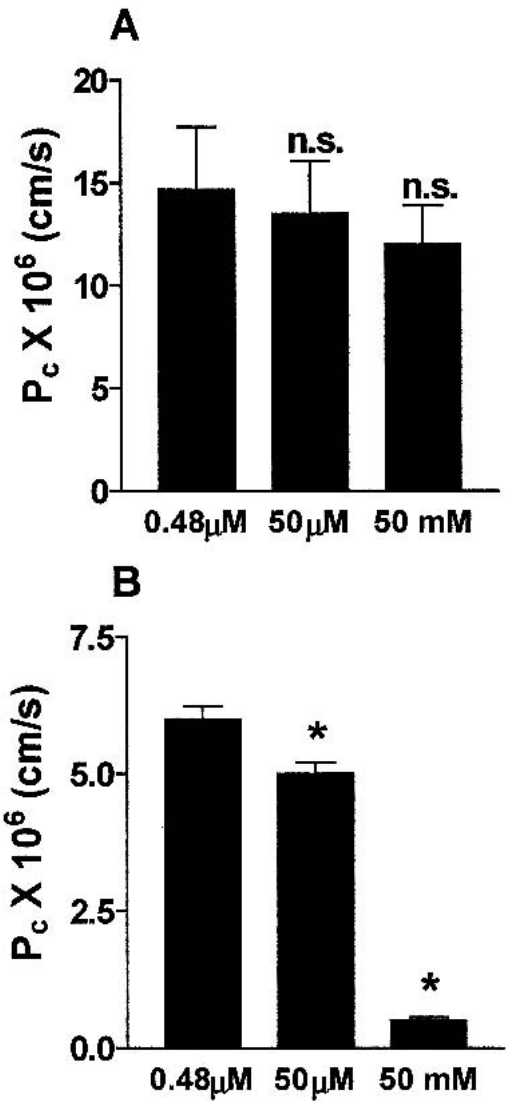

Fig. 7. Transport of the PepT1 substrate glysar in 2/4/A1 (A) and Caco-2 (B) cell monolayers. No significant dose-dependent decrease in permeability coefficient for glysar was observed in 2/4/A1 cell monolayers ($p > 0.05$) (A), although a dose-dependent decrease in permeability coefficient for glysar could be observed in Caco-2 cell monolayers ($p < 0.05$) (B). Each *bar* represents the mean ± SD of four experiments. * and n.s. denote significant ($p < 0.05$) and insignificant difference compared to transport at 0.48 μM glysar, respectively.

peared to be more abundantly expressed relative to *MDR1a* in 2/4/A1 cells in comparison to the rat ileum, also under conditions that are optimal for differentiation (39°C) (Fig. 8A,B).

MRP2 and MRP3 mRNA were not detected in 2/4/A1 samples (data not shown). However, similar levels of MRP5 mRNA were detected in rat ileum, in 2/4/A1 cells grown at 33°C, and in 2/4/A1 cells grown at 39°C (Fig. 8C), whereas MRP6 mRNA was detected in the rat ileum only (Fig. 8D). Despite a thorough database search, we were unable to find information on the sequence of rat BCRP. For this reason, no transcriptional analysis of BCRP was carried out.

There was no polarized transport in the b-a direction across 2/4/A1 cell monolayers for any of the substrates for the efflux systems that were investigated (P-gp, MRP2, and BCRP) (Fig. 9), and we concluded that these efflux systems are not functional in 2/4/A1 cell monolayers. In Caco-2 cell monolayers, on the other hand, all the substrates displayed severalfold higher permeability coefficients in the b-a than in the a-b direction.

Because Caco-2 cells express multiple drug efflux systems with overlapping substrate specificity, we also studied the efflux in more specific epithelial expression systems. Stable MDCK II and LLC-PK1 cell clones that overexpressed each of the efflux systems were used for this purpose. All of the typical substrates displayed higher efflux ratios (b-a/a-b) in the expression systems than in the corresponding untransfected cell lines (Fig. 9).

DISCUSSION

We present in this paper an improved culture protocol for the conditionally immortalized cell line 2/4/A1. Cell cultures grown using the new protocol at the nonpermissive temperature (39°C) form cell monolayers with an integrity that is comparable to that of the human small intestine *in vivo* (21). We also show that none of the more common drug efflux and transport systems are functionally expressed in this cell line. We suggest that the improved 2/4/A1 cell monolayers should be an excellent model in which to study passive drug transport.

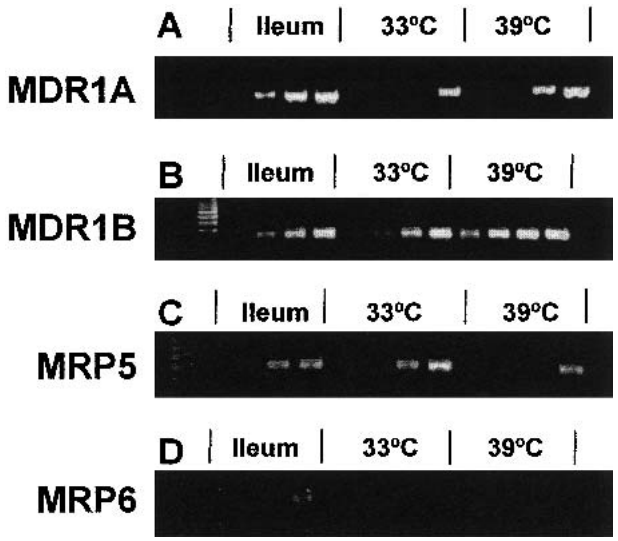

Fig. 8. RT-PCR detection of MDR1a (A), MDR1b (B), MRP5 (C) and MRP6 (D) in rat ileum and in 2/4/A1 cell monolayers cultivated at 33°C and 39°C. The RT-PCR reaction was run for 25, 29, 33, and 37 cycles for each of the three samples. The cDNA products were loaded onto a 2.5% agarose gel, and gene-specific products were detected by ethidium bromide staining.

We have previously shown that the 2/4/A1 cells died when grown at 39°C (1). The work presented here shows that this cell death is caused by apoptosis, which agrees with observations in other cell lines immortalized by tsSV40T (18,22–24). Cell death by apoptosis appears to be an inherent feature of these cell lines. The apoptosis in tsSV40T-expressing cell lines has been attributed to the binding of the wild-type p53 tumor suppression protein to tsSV40T at 33°C and the subsequent release of the tumor suppression protein when tsSV40T is inactivated at 39°C (22).

In the present study, we obtained intact 2/4/A1 cell monolayers at 39°C by the overexpression of Bcl-2 and by the modification of the culture conditions normally used for 2/4/A1 cells (1). Successful inhibition of apoptosis by overexpression of Bcl-2 in cell lines immortalized by tsSV40T has previously been shown in kidney tubule (25) and hippocampal neuronal (26) cell lines. 2/4/A1/Bcl-2 cells did form continuous monolayers when overexpressing Bcl-2, and this enables them to be used as a cell model for studying apoptotic processes in the intestinal epithelium (V. Milovic, unpublished results). However, the cells had a flat morphology and a TER value that was significantly lower than that of the small intestine *in vivo*, making them unsuitable for studies of drug transport.

We considered the cell culture medium and identified two possible reasons for the apoptotic behavior of 2/4/A1 at 39°C — the presence of G418 and the absence of serum. G418 is the compound that maintains the selection pressure for cells that express tsSV40T. We found that G418 was toxic to 2/4/A1 cells grown at 39°C even at a concentration of 10 μg/ml, which is 25-fold lower than the concentration that is nontoxic to 2/4/A1 cells grown at 33°C (250 μg/ml). We therefore removed G418 from the culture medium to prevent G418-induced toxicity for 2/4/A1 cells at 39°C. The second reason for the apoptotic behavior of 2/4/A1 cells grown at 39°C concerned the lack of serum in the culture medium. Serum deprivation has been shown to induce apoptosis in tsSV40-immortalized cell lines (18). The addition of FCS further stabilized the 2/4/A1 cell monolayers and resulted in TER values close to that of the human small intestine *in vivo* (21).

The TER of 2/4/A1 cell monolayers cultivated on EHS coated permeable supports at 39°C without G418 but with 6% FCS was approximately twice that of 2/4/A1 cell monolayers grown at 37°C. The twofold increase in TER did not result in a corresponding reduction in permeability to the paracellular marker mannitol. This discrepancy is in agreement with observations that an increase in TER does not always correlate with a reduction in permeability of paracellular markers (27). The reason for this difference may reside in the temporal difference between TER measurements (instant) and permeability determination of paracellular markers (minutes or hours) (28).

The results from the RT-PCR showed that PepT1 was not expressed in 2/4/A1 cells. One consequence of this is the absence of active uptake of the established marker for PepT1, glysar, in 2/4/A1 cell monolayers. We cannot rule out that the relatively high paracellular flux overshadowed a small active transport of glysar because glysar is a small (MW 146.1) hydrophilic molecule that might permeate the paracellular path-

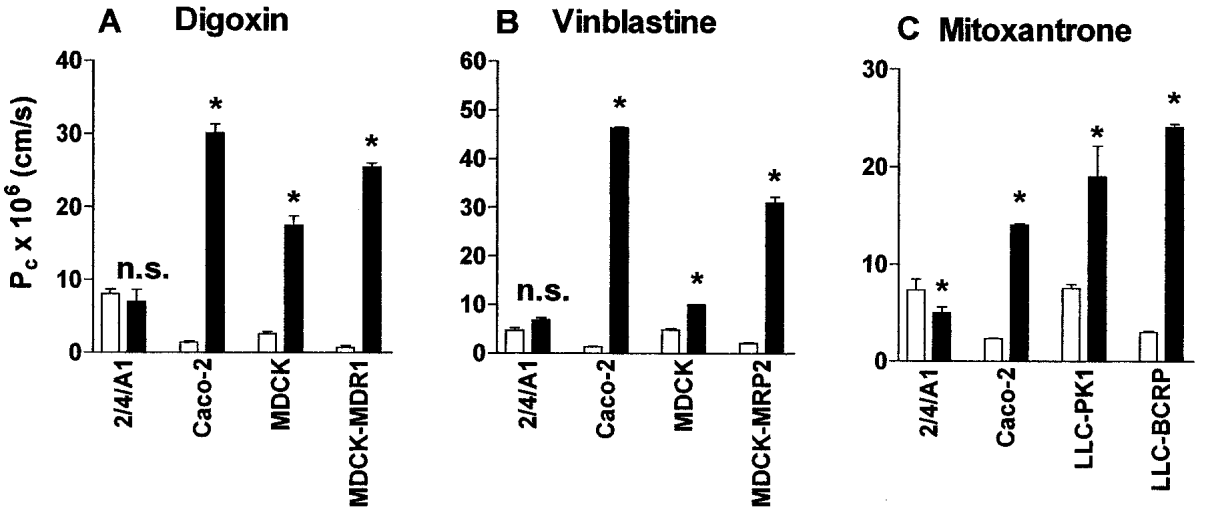

Fig. 9. Permeability coefficients in the apical to basolateral *(open bars)* and basolateral to apical *(closed bars)* directions for the P-gp substrate digoxin (A), the MRP2 substrate vinblastine (B), and the BCRP substrate mitoxantrone (C) in 2/4/A1, Caco-2 (A-C), MDCK II (A and B), MDCK-MDR1 (A), MDCK-MRP2 (B), LLC-PK1 (C), and LLC-BCRP (C) cell monolayers. The P-gp inhibitor GF120918 (2 μM) was added in the studies of vinblastine transport in MDCK II and MDCK-MRP2. Each *bar* represents mean ± SD of 3–6 experiments. * and n.s. denote a significant ($p < 0.05$) and insignificant difference between a-b and b-a transport, respectively.

way in 2/4/A1 cell monolayers. Interestingly, it has recently been shown that epidermal growth factor (EGF) down-regulates the expression of PepT1 in Caco-2 cells (29). This observation leads us to suggest that 2/4/A1 cells might express PepT1 in the absence of EGF (which is present in the culture medium). However, the integrity of 2/4/A1 cell monolayers diminished in the absence of EGF, and so we could not follow up this hypothesis.

There was a striking absence of functional activity of all the investigated efflux systems in 2/4/A1 cell monolayers. This may be because of a lack of expression in 2/4/A1 of the proteins involved in these systems at the transcriptional level (as may be the case for MRP2) or at the translational or post-translational level (as may be the case for P-gp). We tentatively conclude that 2/4/A1 cells most likely are not an appropriate system for studies of membrane transporters. When specific membrane transporters are to be studied, simple epithelial expression systems such as those based on MDCK and LLC-PK1 are more suitable, although not perfect (10).

Alternative short-term cultures for the studies of passive drug transport include MDCK (30) and short-term Caco-2 cultures (31). In contrast to 2/4/A1, several studies have shown that, depending on the cell culture procedure, both MDCK and short-term Caco-2 cells express varying amounts of functional transporters such as P-gp (usually at a lower level than traditional Caco-2) (32,33). Thus, studies of passive drug transport in these alternative cultures will likely be influenced by low and varying expression of transporters. In addition, short-term Caco-2 cells may generally be used in drug transport studies only during a very short time interval (approximately 1 to 2 days) because the integrity of these cultures decreases rapidly shortly after maximum TER is reached (34). In contrast, our studies show that 2/4/A1 cells can be used for drug transport studies 6–14 days postseeding.

In conclusion, the small-intestine-like paracellular permeability, together with the lack of functional expression of transport and efflux systems, make 2/4/A1 cell monolayers suitable for studies of passive drug permeability. In an accompanying paper we show that 2/4/A1 cell monolayers grown under the new cell culture conditions presented here display functional tight junctions comparable to those of the small intestine and that 2/4/A1 cell monolayers predict the *in vivo* absorption of low-permeability drugs better than Caco-2 cell monolayers (35). An additional advantage of the 2/4/A1 cell

Table I. Gene-Specific PCR Primers

Gene	Forward primer	Reverse primer	Expected fragment length
rMdr1a	5′-AGTGAAAGGGGCTACAGGGT	5′-AACTTCTGCTCCCGAGTCAA	276
rMdr1b	5′-TCTGCTGTTGTCTTTGGTGC	5′-TCTCGGGGATTTTCTCAATG	118
rMrp2	5′-CTGGTTGGAAACTTGGTCGT	5′-CAACTGCCACAATGTTGGTC	172
rMrp3	5′-TGTTCTATGGCTTCGTGCAG	5′-CTTGAAGTCTTGGACTCGGC	155
rMrp5	5′-CCTCTTTTATCCGGCAATGA	5′-ACTACGACAATGGGAGCCAC	252
rMrp6	5′-GTGGTATTTGGATCGATGGG	5′-TGGCCTGAGCACTCATACTG	232
rPepT1	5′-ACAGAGTCCTTGCCTGGAGA	5′-ACTTAGGAGGCAGAGGCAGA	225
rSGLT1	5′-CATCTTCAACAGCGCCAGTA	5′-TCAGGAAGCCGAGAATCAGT	296
rGLUT5	5′-TGTCAGCATCGTCTGTGTCA	5′-CAAGGCCCACTTGAATGAAT	191
rLAT	5′-GCTCATCTTGGAACACAGCA	5′-TACAGAGCTGCCTAGCACGA	135

model is that it will facilitate the establishment of structure–permeability relationships for the passive paracellular route.

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