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Increased Expression of the Secretory Na^+-K^+-$2Cl^-$ Cotransporter with Differentiation of a Human Intestinal Cell Line

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We studied the expression of the secretory Na^+-K^+-$2Cl^-$ cotransporter during epithelial differentiation using the clonal human adenocarcinoma cell line HT29-18. Differentiation of HT29-18 cells was accompanied by up to 7-fold increases in cotransporter protein levels, ~3-fold increases in cotransporter mRNA levels, and ~2.5-fold increases in cotransporter functional expression. No apparent change in cotransporter mRNA stability was observed with differentiation, suggesting that these effects may be due to differences in mRNA transcription rate. Confocal immunofluorescence microscopy showed that undifferentiated cells grew in multilayers and exhibited a diffuse, apparently unlocalized membrane labeling by anti-Na^+-K^+-$2Cl^-$ cotransporter antibody. In contrast, differentiated cells grew in monolayers with strong cotransporter labeling localized to the basal and lateral membranes. Taken together with previous studies demonstrating that expression of the cystic fibrosis transmembrane regulator is also increased following HT29-18 cell differentiation, our results suggest that these cells provide a promising model for studying epithelial differentiation to a Cl^- secretory phenotype.

Key Words: **HT-29 cells; salt and water secretion; epithelial differentiation.**

Little is known about the processes involved in the generation and maintenance of epithelial differentiation. The multi potent human colonic adenocarcinoma cell line HT29 and its subclones, such as the HT29-18 cells employed here, have proven to be useful model systems for studying these events since their degree of differentiation and polarization can be modulated *in vitro* by simple changes in culture conditions. Thus, for example, in 'normal' culture medium (Dulbecco's Modified Eagles Medium containing 25 mM glucose) these cells display a poorly differentiated epithelial phenotype, but when they are switched to glucose-free medium they differentiate and acquire the morphological characteristics of a highly polarized ion transporting epithelium (1-4). This change can also be induced by several other stimuli such as incubation with the short chain fatty acid butyrate (5), or with the tumor growth factor inhibitor suramin (6,7). This phenomenon is thought to mirror events occurring during epithelial differentiation such as the differentiation of intestinal stem cells into mature enterocytes (8).

In the present work we studied the expression of the secretory Na^+-K^+-$2Cl^-$ cotransporter during differentiation of the HT29-18 cells. This transporter is known to play a major role in salt and water movements in a number of secretory tissues including intestinal and exocrine epithelia (9). In these tissues the Na^+-K^+-$2Cl^-$ cotransporter, which is localized to the basolateral membrane, concentrates Cl^- in the cytoplasm above its electrochemical equilibrium using the extracellular to intracellular Na^+ gradient generated by Na^+/K^+ ATPase. Secretory stimuli typically cause the opening of an apical Cl^- channel resulting in a flux of Cl^- ions into the lumen. Na^+ then follows Cl^- by leaking from the interstitium through the tight junctions between the cells in order to preserve electroneutrality, and the resulting osmotic gradient for NaCl causes a transepithelial movement of water from interstitium to lumen. Our data show that both the expression and the basolateral polarization of the secretory Na^+-K^+-$2Cl^-$ cotransporter are markedly enhanced during differentiation of HT29-18 cells. Previous studies have demonstrated that expression of the cystic fibrosis transmembrane regulator (an apical Cl^- channel) is also elevated under these conditions (10). Thus the levels of both ion pathways involved in active Cl^- transport by Cl^- secretory epithelia are increased by differentiation of these cells, consistent with their progression toward a Cl^- secretory phenotype.

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MATERIALS AND METHODS

Cell culture. The HT29-18 cells (3) were generously provided by Dr. Marshall H. Montrose (Johns Hopkins University, Baltimore MD) and used at passages 18-25. Cells were routinely grown at 10% CO_2 in "glucose medium": Dulbecco's modified Eagles Medium containing 25mM glucose and supplemented with 10% fetal calf serum, ITS Premix (insulin, transferrin and selenium; Collaborative Research Incorporated, Bedford, MA), 2mM glutamine, and 100 mg/ml each of penicillin and streptomycin. Cells were split 1:10 with Trypsin/EDTA (0.005%/0.1mM in phosphate buffered saline) upon reaching confluence. Under all growth conditions described here media were changed daily.

Differentiation of HT29-18 cells in galactose (HT29-18gal cells). Cells were switched to "galactose medium" (identical to glucose medium except that 5 mM galactose replaced 25 mM glucose and 1 mM pyruvate) using a slight modification of previous methods (3). Briefly, on day zero cells were split (1:12) into glucose medium. The medium was then changed daily to the following mixtures of glucose medium:galactose medium: 4:1 (day 1), 3:2 (day 2), 2:3 (day 3), 1:4 (day 4). On day 5 the cells were switched to galactose medium and on day 6 they were split 1:8. These differentiated cells were maintained in galactose medium until use (typically at 5 days post confluence).

Differentiation of cells using butyrate (HT29-18but cells). Cells in glucose medium were split 1:10 then switched to medium containing 5 mM sodium butyrate after attaining 90-95% confluence. Cells were maintained in the presence of butyrate until use (5 days post confluence).

Differentiation of cells using suramin (HT29-18sur cells). Cells in glucose medium were switched to medium containing 100 μM suramin (Biomol Research Laboratories, Plymouth Meeting, PA) one day after splitting (1:10). After 5 days exposure to suramin cells were switched back to suramin-free medium and left for one day to recover before use (typically 2 days post confluence).

Membrane preparation. Cells growing in 10 cm culture dishes were washed twice in ice-cold phosphate buffered saline (PBS; pH 7.4), scraped into 1ml PBS/plate and centrifuged at 1000xg for 5 min. The pellet from this spin was suspended in the same volume of TEEA (20mM Tris-HCl pH 8.0 containing 1 mM EDTA, 3mM EGTA and 300μM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride [ICN, Aurora, OH]) and homogenized by passing 4 times through a 25g needle. This material was centrifuged at 1000×g for 5 min and the supernate saved. The pellet was resuspended in TEEA and rehomogenized as before. After a total of 3 homogenization steps the combined supernates were centrifuged at 100,000×g for 30 min. The resulting membrane pellet was resuspended in TEEA and assayed for protein using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA).

Western blotting. HT29-18 membranes were separated by SDS-PAGE on 4-20% gradient gels (Novex, San Diego, CA) and transferred to nitrocellulose. Immunoblotting was carried out in 50mM Tris-HCl pH 7.5 containing 136 mM NaCl, 0.05% Tween 20 and 4% BLOTTO (Tween 20 was omitted during incubation with the primary antibody). The primary antibody (used at 1-2 μg/ml) was an affinity purified rabbit anti-peptide antibody raised against a 22-mer unique to the secretory isoform of the Na^+-K^+-$2Cl^-$ cotransporter (11). The secondary antibody (used at 1:40,000 dilution) was a horse radish peroxidase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). Detection was carried out using the ECL kit from Amersham (Arlington Heights, IL).

Northern blotting. RNA was isolated by the phenol-acid method of Chomczynski (12) using Tri Reagent from Molecular Research Center (Cincinnati, OH). Cells on 10 cm culture dishes were washed twice with 6 ml of ice cold PBS then scraped into 3 ml of trizole reagent and stored at −80°C until use. Total RNA was isolated from this material according to the manufacturer's instructions. Equal amounts of total RNA were separated on 1.0% glyoxal gels and capillary blotted onto charged nylon membranes (Schleicher and Schuel, Keene, NH). Membranes were hybridized in 6×SSC, 5mM EDTA, 0.5mM pyrophosphate, 5× Denhardts, 0.5% SDS at 66°C with a ^{32}P-labeled 0.74-kb fragment of the rat parotid Na^+-K^+-$2Cl^-$ cotransporter cDNA corresponding to bp 2247-2991 of the coding region (in one experiment a 0.55-kb fragment corresponding to bp 263-816 was used). The blots were then washed under stringent conditions with 2×SSC, 0.1% SDS for 10 min at room temperature followed by 0.1×SSC, 0.005%SDS for 20 min at room temperature then 10 min with the same solution at 50°C. Blots were analyzed by autoradiography and densitometry (see below). Membranes were subsequently re-probed for β-actin and/or for glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA levels by the same method.

Densitometry. Results of Western and Northern blotting experiments were quantitated using a Pharmacia Imagemaster Densitometer.

$^{86}Rb^+$ influx assay. Cells growing in 6-well culture dishes were washed twice with 2 ml PBS then incubated for 10 minutes in 0.9 ml of 10mM HEPES/Tris (10 mM HEPES buffered with Tris to pH 7.4) containing 5mM KCl, 136mM NaCl, 1mM $NaHPO_4$, 0.1mM ouabain and either 5mM galactose (HT29-18gal cells) or 25mM glucose (undifferentiated HT29-18 cells). 100 μl of the same buffer containing $^{86}Rb^+$ (2 μCi/ml) was then added. Uptake was terminated after 5 min by the addition of 2 ml of ice-cold 10mM HEPES/Tris containing 136 mM NaCl. Following aspiration of this medium the wells were washed four times with 1.5 ml of the same solution and the cells were solubilized in 1 ml of 0.5% Triton X100 and counted for radioactivity in a Beckman LS 3801 scintillation counter. The protein concentration of the solubilized cells was also determined and $^{86}Rb^+$ uptake was expressed as a percentage of the total $^{86}Rb^+$ added to the culture well divided by total cell protein recovered. In control experiments (not shown) we have established that $^{86}Rb^+$ uptake measured in this way is linear with time for up to 1 h.

Confocal microscopy. HT29-18 and HT29-18gal cells were grown as described above but on glass cover slips. Five days after reaching confluence the cells were washed twice in PBS and fixed in prechilled (−70°C) methanol for 15 minutes. Cells were then rehydrated with three 5 minute washes in PBS and blocked for 20 min in PBS containing 0.2% BSA and 5% donkey serum. Cells were incubated for 1 h with the anti-Na^+-K^+-$2Cl^-$ cotransporter antibody described above (~1-2 μg/ml in PBS plus 0.2% BSA), washed three times for 5 min in PBS containing 0.2% BSA and incubated for 20 min with lissamine-rhodamine-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). Following two washes in PBS plus 0.2% BSA and two washes in PBS alone the cells were mounted on Silanated glass slides (Digene, Beltsville, MD) in Citofluor glycerol/PBS mixture (Ted Pella, Redding, CA). Confocal microscopy was carried out as previously described (11).

RESULTS

To determine whether expression of the Na^+-K^+-$2Cl^-$ cotransporter is changed as a result of differentiation of HT29-18 cells we first examined membrane levels of cotransporter protein by Western blotting using an antibody specific for the secretory isoform of this transporter (see Methods). In these experiments membranes were harvested from cells differentiated in three different ways (see Methods): by growth in glucose-free medium (HT29-18gal cells; 3), by growth in the presence of butyrate (HT29-18but cells; 5) and by exposure to suramin (HT29-18sur cells; 7). Fig. 1 shows the results of a representative Western blot illustrating increases

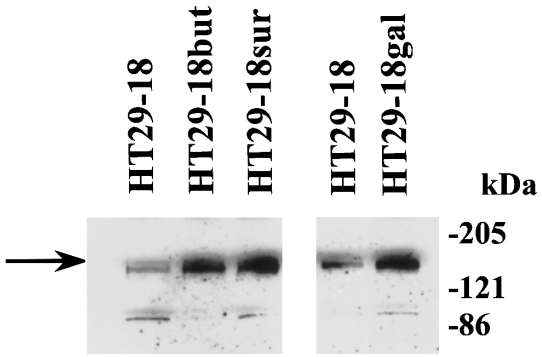

FIG. 1. Increase in Na^+-K^+-$2Cl^-$ cotransporter protein expression upon differentiation of HT29-18 cells. Ten μg of membrane protein prepared from undifferentiated or differentiated HT29-18 cells (see Methods) were run on SDS-PAGE, transferred to nitrocellulose and probed with a polyclonal antipeptide antibody specific for the secretory isoform of the Na^+-K^+-$2Cl^-$ cotransporter. Membranes from undifferentiated cells were from untreated cells carried in parallel in glucose medium and harvested 5 days post confluence. The arrow indicates the specific cotransporter band at ~170 kDa (18).

in cotransporter protein with all three of these treatments. Analysis of a number of experiments of this type indicated that membrane cotransporter levels were increased 7.0±2.1(n=7)-fold by growth in glucose-free medium, 2.0±0.8(n=2)-fold by treatment with 5 mM butyrate and 3.75±0.03(n=2)-fold by treatment with 100 μM suramin (increases were measured relative to levels in membranes from untreated cells carried in

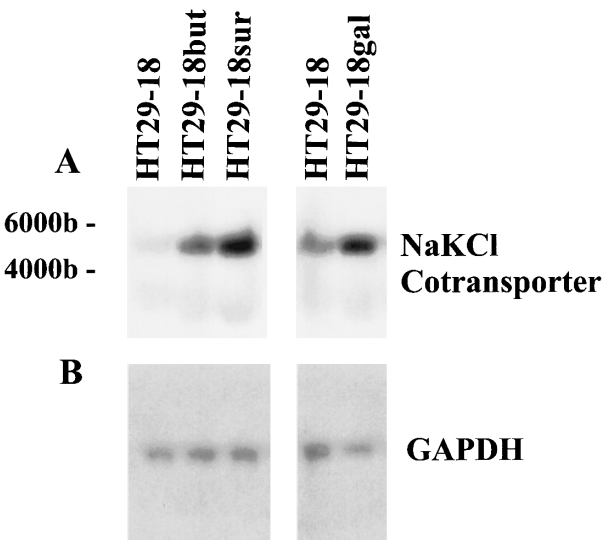

FIG. 2. Increase in Na^+-K^+-$2Cl^-$ cotransporter messenger RNA levels upon differentiation of HT29-18 cells. Total RNA was extracted from undifferentiated or differentiated HT29-18 cells (see Methods) and subjected to Northern blot analysis as described in Methods. (A) Blot probed for secretory Na^+-K^+-$2Cl^-$ cotransporter messenger RNA. (B) Same blot as in A but probed for GAPDH. RNA from undifferentiated cells was from untreated cells carried in parallel in glucose medium and harvested 5 days post confluence.

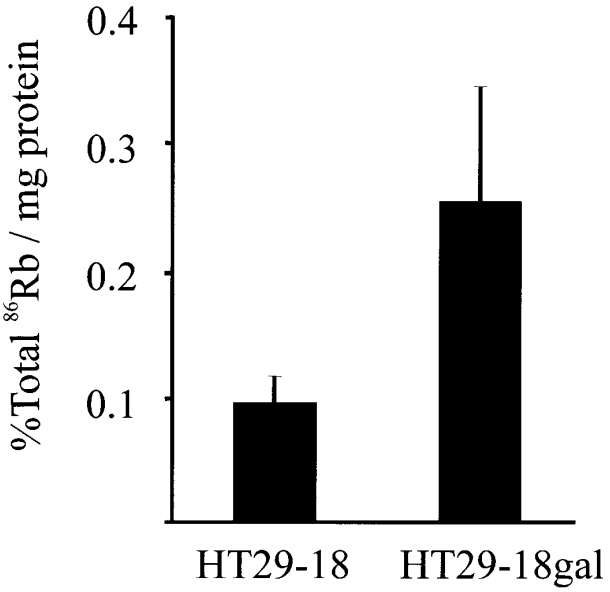

FIG. 3. Na^+-K^+-$2Cl^-$ cotransporter functional activity in HT29-18 and HT29-18gal cells. The uptake of $^{86}Rb^+$ into HT29-18 and HT29-18gal cells was measured as described in Methods in the presence and absence of 100 μM bumetanide. The bumetanide-sensitive components of $^{86}Rb^+$ uptake are illustrated (i.e., uptake measured in the absence of bumetanide minus that measured in its presence). The results of 3 independent experiments were averaged to produce the figure. Each experiment was carried out on the same day on HT29-18 and HT29-18gal cells carried in parallel in culture.

parallel in glucose medium and harvested five days post confluence).

We next investigated Na^+-K^+-$2Cl^-$ cotransporter mRNA levels in order to determine whether the above increase in protein expression was paralleled by an increase in message levels. Fig. 2A shows a representative Northern blot from undifferentiated and differentiated cells hybridized with a cDNA probe for the secretory Na^+-K^+-$2Cl^-$ cotransporter (see Methods). Analysis of a series of these experiments demonstrated that secretory Na^+-K^+-$2Cl^-$ cotransporter mRNA levels were increased approximately 3-fold by growth in glucose-free medium. More specifically we found 2.5±0.3 (n=6)-fold, 2.9±0.3 (n=6)-fold and 2.9±0.4 (n=3)-fold increases in Na^+-K^+-$2Cl^-$ cotransporter mRNA as measured versus total RNA, and mRNA for the "housekeeping" genes GAPDH and β-actin, respectively. Similar analyses showed that Na^+-K^+-$2Cl^-$ cotransporter mRNA was increased 2.7±0.5 (n=3)-fold by butyrate and 3.8±1.6 (n=3)-fold by suramin measured relative to total RNA, and 1.9±0.3 (n=3)-fold by butyrate and 2.9±1.0 (n=3)-fold by suramin measured relative to GAPDH mRNA.

To learn whether the increased mRNA levels observed in Fig. 2 for HT29-18gal cells was due to a difference in message stability in differentiated cells we incubated HT29-18 and HT29-18gal cells for various times

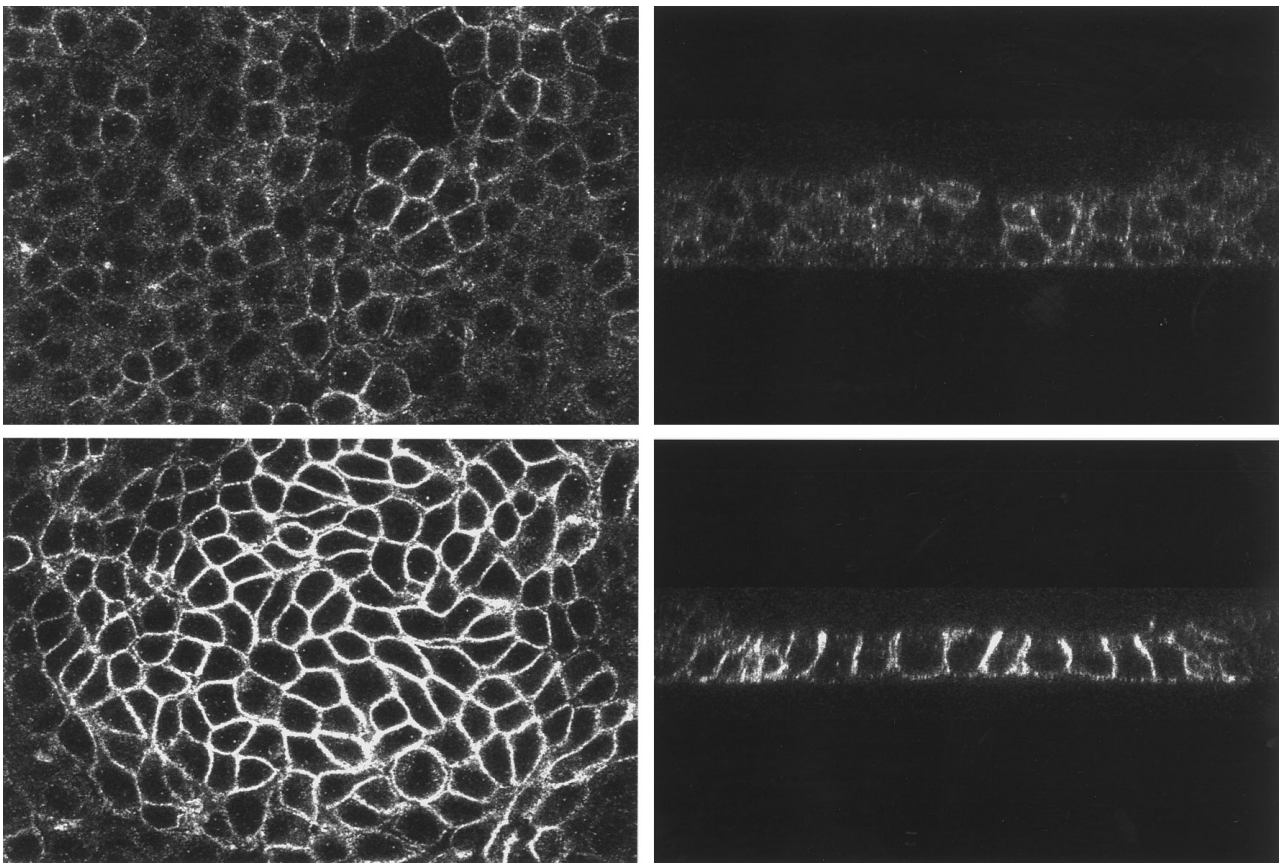

FIG. 4. Immunolocalization of the Na^+-K^+-$2Cl^-$ cotransporter in HT29-18 and HT29-18gal cells. Experimental procedures were as described in Methods. Cells were examined 5 days post confluence. The upper panels show top- and side-view confocal images (on the left and right, respectively) of HT29-18 cells growing on a glass coverslip while the lower panels show similar views of HT29-18gal cells.

(0-16 h) with the transcription inhibitor Actinomycin D (5 μg/ml). Total RNA was then isolated and probed for cotransporter mRNA by Northern blotting (not shown). In three independent experiments of this type we were unable to detect any significant difference in Na^+-K^+-$2Cl^-$ cotransporter mRNA stability between differentiated and undifferentiated cells (e.g., after 16 h incubation with Actinomycin D, Na^+-K^+-$2Cl^-$ cotransporter mRNA levels in HT29-18 cells were 79$\pm$24% of those in untreated cells while cotransporter mRNA levels in HT29-18gal cells were 72$\pm$19% of those in untreated cells).

We next compared the functional expression of the Na^+-K^+-$2Cl^-$ cotransporter in HT29-18 and HT29-18gal cells by measuring their uptake of $^{86}Rb^+$, a surrogate for K^+, in the presence and absence of bumetanide, a specific inhibitor of the cotransporter. All uptake assays were carried out in the presence of ouabain (see Methods) to block $^{86}Rb^+$ fluxes via Na^+/K^+-ATPase. In preliminary experiments (not shown) we established that preincubation of cells with ouabain did not significantly affect the magnitude of the bumetanide-sensitive component of $^{86}Rb^+$ uptake in HT29-18 or HT29-18gal cells. The averaged results of 3 independent flux experiments are shown in Fig. 3. In these experiments the bumetanide-sensitive component of $^{86}Rb^+$ uptake was increased $\sim$2.5-fold in differentiated cells. This somewhat smaller increase in functional expression of the cotransporter with differentiation as compared to its increase in protein expression (7- fold; see above) may reflect differences in the driving forces for Na^+-K^+-$2Cl^-$ cotransport (e.g., increased intracellular Cl^- levels due to the action of the cotransporter itself) or cotransporter regulation in differentiated cells. Indeed, the function of the Na^+-K^+-$2Cl^-$ cotransporter is known to be highly regulated (9). Recent studies suggest that cotransporter activity in secretory epithelia is down-regulated by increased intracellular Cl^- levels either directly (13) or via phosphorylation (14). In this way the cotransporter, which is mainly responsible for Cl^- entry in many of these tissues (see Introduction), is thought to act as the effector of a negative feedback control system that results in the maintenance of intracellular Cl^- levels within a narrow range (14). This phenomenon may play a role in coordinating Cl^- entry and exit during secretion.

Finally we examined the localization of the Na^+-K^+-$2Cl^-$ cotransporter in HT29-18 and HT29-18gal cells using confocal microscopy. The image in the upper right-hand panel of Fig. 4 is a side view of the HT29-18 cells fluorescently labeled with anti-Na^+-K^+-$2Cl^-$ cotransporter antibody (see Methods). This image confirms previous reports that undifferentiated HT29-18 cells grow in multilayers (3). In these cells (upper left and right panels of Fig. 4) there is a diffuse membrane labeling by the anti-Na^+-K^+-$2Cl^-$ cotransporter antibody with no apparent localization to any region of the cell surface. A similar side view of immunolabelled HT29-18gal cells is shown in the lower right-hand panel of Fig. 4. This image likewise confirms previous studies (3) illustrating that the HT29-18-gal cells grow in polarized monolayers. These cells (lower left and right panels of Fig. 4) show a much stronger labeling with the anti-Na^+-K^+-$2Cl^-$ cotransporter antibody than simultaneously processed HT29-18 cells (Fig. 4) and this labeling is localized to the basal and lateral surfaces of the cells.

DISCUSSION

The HT29 cells and its subclones have been used in a number of studies to explore the process of epithelial differentiation and its relationship to the development of epithelial polarity, enterocyte differentiation, embryonic development and neoplastic transformation (e.g., 2-7,10,15-17). At the present time these cells represent one of the few *in vitro* epithelial cell systems where substantial morphological and functional changes can be induced by relatively simple changes in culture conditions. In the present paper we examined changes in the expression of the secretory Na^+-K^+-$2Cl^-$ cotransporter with differentiation of the HT29-18 cells, a subclone of the HT29 cells (3) which retain the ability of the parental line to differentiate under a variety of growth conditions. Increased Na^+-K^+-$2Cl^-$ cotransporter expression following differentiation was observed at the protein (Fig. 1), mRNA (Fig. 2) and functional (Fig. 3) levels. No apparent difference in mRNA stability was found between differentiated and undifferentiated cells suggesting that increased transcription rate might underlie these phenomena. In addition we observed marked changes in cellular appearance and cotransporter localization with differentiation (Fig. 4). In undifferentiated cells, which grew in multilayers, we found a diffuse, apparently unlocalized, membrane labeling by anti-Na^+-K^+-$2Cl^-$ cotransporter antibody. In contrast, differentiated cells grew in monolayers with strong cotransporter labeling localized to the basal and lateral membranes. This, to our knowledge, is the first direct demonstration of the polarized redistribution of a membrane transport protein following differentiation of HT29 cells.

Taken together with previous studies demonstrating that expression of the cystic fibrosis transmembrane regulator is also increased following HT29-18 cell differentiation (10), the results presented here indicate that this cell line provides a promising and presently unique model for studying the morphologic and functional differentiation of epithelial stem cells to a Cl^- secretory phenotype.

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