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Regulation of *CFTR* expression and function during differentiation of intestinal epithelial cells

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CFTR, the protein defective in cystic fibrosis is regulated during differentiation of intestinal epithelial cells. The undifferentiated cells (Caco-2 and HT-29) show a lower level of CFTR mRNA, while a 10-fold increase is seen in differentiated cells. These differences correlate well with those of other intestinal-specific genes, including sucrase-isomaltase, villin and α 1-antitrypsin, indicating that the regulation is cell specific. In Caco-2 cells the increase in CFTR mRNA cannot be accounted for by increased transcription of the gene. These data indicate that CFTR mRNA stabilizing factor(s) might be present in differentiated cells. The higher levels of CFTR mRNA in differentiated cells are accompanied by decreased protein levels, indicating, as well, involvement of translational control in the regulation of CFTR in these cells. Finally, fully differentiated cells show lowered levels of cyclic AMP-activated Cl^- transport, the characteristic function of CFTR. Thus, CFTR function in differentiated cells is modulated by a complex interaction of regulatory elements. Caco-2 and HT-29 cells provide a suitable *in vitro* system in which to study the mechanism of regulation of CFTR.

Key words: caco-2 cells/cystic fibrosis/gene expression/ HT-29 cells/ion transport

Introduction

Cystic fibrosis (CF) is the most common lethal disease in the Caucasian population. The disorder presents a complex phenotype resulting from the malfunction of the epithelial layer in most, if not all, exocrine glands (Boat *et al.*, 1989). The gene defective in the disease has been cloned (Kerem *et al.*, 1989; Riordan *et al.*, 1989; Rommens *et al.*, 1989) and, on the basis of the predicted structure of the protein, the gene product (CFTR-cystic fibrosis transmembrane conductance regulator) has been assigned to the superfamily of ATP binding transporters (Riordan *et al.*, 1989). The function of the protein is currently being defined by *in vitro* expression studies with the full length cDNA. Introduction of the cDNA into epithelial cells derived from CF patients corrects their inability to transport Cl^- after treatment with agents that increase intracellular cAMP levels (Drumm *et al.*, 1990; Rich *et al.*, 1990). Furthermore, expression of the cDNA in heterologous, non-epithelial cells leads to the appearance of a small (6-10 pS), linear Cl^- channel (Anderson *et al.*, 1991a; Kartner *et al.*, 1991; Rommens *et al.*, 1991; Tabcharani *et al.*, 1991). The ion selectivity of the channel can be modulated by altering charged residues in putative transmembrane domains (Anderson *et al.*, 1991b), the requirement for activation by cAMP can be largely eliminated by the deletion of a region in the middle of the protein corresponding to part of the R domain that contains sequences characteristic of phosphorylation sites for protein kinase A (Rich *et al.*, 1991). Mutagenesis of some of these sites also removes activation by protein kinase A (Cheng *et al.*, 1991). Finally, reconstitution of purified CFTR into planar lipid bilayers results in Cl^- channels with the same properties as those observed after cDNA transfection (Bear *et al.*, 1992). These results validate some aspects of the model presented in the original description of the putative protein and are consistent with the view that CFTR is a Cl^- channel, albeit one with structural features different from those of other channels from excitable tissues.

Less is known about the function of CFTR *in vivo*. Studies using RNA *in situ* hybridization have shown that in the rat the gene is strongly expressed in the ducts of salivary glands and of the pancreas, where Cl^- and fluid reabsorption takes place, as well as in the crypts of the intestine, where Cl^- and fluid secretion occur (Trezise and Buchwald, 1991). The bidirectional movement of Cl^- in tissues expressing CFTR is consistent with the evidence from *in vitro* studies showing that CFTR is a Cl^- channel. Little expression is seen in the villi of the intestine, where fluid reabsorption takes place, suggesting that in this tissue CFTR does not play a role in reabsorption at that site. These data are consistent with clinical and physiological evidence that CF patients have defects in intestinal Cl^- secretion (Gowen *et al.*, 1991) and with evidence that the Cl^- channel in guinea pig villi has properties different than those produced by expression of CFTR (Sepulveda *et al.*, 1991). Little, if any, information is available about *in vivo* CFTR protein abundance or function or the cells in which it is present.

Defining the regulation of expression and function of CFTR in the intestine may help to understand the precise role of the protien *in vivo* and its effect in the disease process. In this organ, stem cells first appear in the crypts and migrate to the villi concomitantly with their differentiation into enterocytes, goblet cells and enteroendocrine cells (see review by Smith, 1985). Given the marked differences in CFTR mRNA abundance between crypts and villi, it is possible that the expression of CFTR is regulated by or in conjunction with the differentiation of intestinal epithelial cells. *In vitro* models of intestinal epithelial cell differentiation, the adenocarcinoma cell lines Caco-2 and HT-29, are available to test this hypothesis (Rousset, 1986). These cells show both morphological and biochemical differentiation characteristic of fetal colonic cells, Caco-2 cells when grown post-confluency (Pinto *et al.*, 1983) and HT-29 cells when deprived of glucose (Pinto *et al.*, 1982).

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Transmission and scanning electron microscopic analysis has shown that during the process of differentiation the morphology of the cells changes from a flattened appearance with few microvilli to a columnar shape with well developed apical brush borders. The marked increase in microvilli is accompanied by an increase in villin synthesis (Robine *et al.*, 1985) and microvillar hydrolases, such as sucrase–isomaltase (SI), alkaline phosphatase and aminopeptidase (Pinto *et al.*, 1982, 1983).

We have previously shown that the levels of CFTR mRNA increase as Caco-2 cells grow to confluency and differentiate and that these changes are not seen in T_{84} cells that do not differentiate in culture (Buchwald *et al.*, 1991). Montrose-Rafizadez *et al.* (1991) studied the levels of CFTR mRNA in a series of HT-29 sublines and found that whereas differentiation in galactose increased the levels of the message, it did not change the levels of forskolin-stimulated Cl^- transport. Thus, the precise relationship between CFTR mRNA, protein and function in differentiating epithelial cells is poorly understood. We describe here a detailed analysis of these three aspects of CFTR in both intestinal cell lines. These studies define a model system to study the factors involved in the regulation of CFTR.

Results

CFTR mRNA expression

To examine whether the expression of CFTR varies with the differentiation state of the cell, we measured the amount of CFTR mRNA in two human intestinal epithelial cells, Caco-2 and HT-29, as they diferentiated in culture. CFTR mRNA signal intensity was normalized against that of pyruvate dehydrogenase (PDH), a ubiquitously expressed housekeeping gene (Koike *et al.*, 1988), to account for the variation in RNA loading. No change in levels of CFTR mRNA had been detected in T_{84} cells that are fully differentiated at all stages of growth, indicating that growth in culture *per se* does not affect gene expression (Buchwald

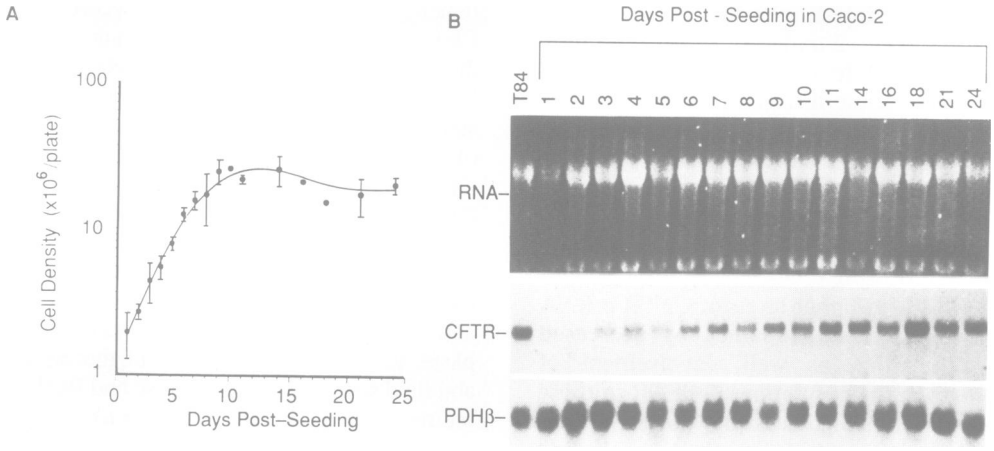

Fig. 1. Relationship of Caco-2 differentiation to expression of CFTR. (**A**) Growth of Caco-2 cells. The cells were seeded at 4.5×10^4 cells/cm^2 and cultured for a period of 24 days with feeding. (**B**) CFTR mRNA levels in Caco-2 cells. The upper panel (RNA) and the bottom panel (PDH) show the variation in RNA loaded in each lane. Middle panel (CFTR) shows the changes in levels of CFTR expression at the above-mentioned time points.

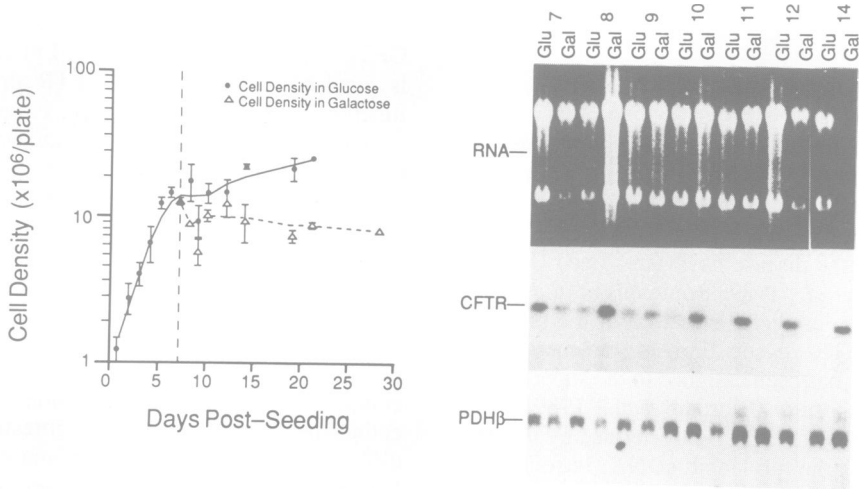

Fig. 2. Relationship of CFTR expression to differentiation of HT-29 cells. **Left**. Growth of HT-29 cells. The cells were grown to confluency in glucose medium and then maintained in either glucose or galactose medium. **Right**. CFTR mRNA levels in HT-29 cells. The upper panel (RNA) and the bottom panel (PDH) show the variation in RNA loaded in each lane. The middle panel (CFTR) shows the changes in levels of CFTR expression at various time points in the two different media.

et al., 1991). In contrast, CFTR mRNA increased by an order of magnitude in Caco-2 cells as they grew to confluency and differentiated. Low levels were present during the active phase of growth (Figure 1A) and reached a maximum at confluency (Figure 1B). The mRNA levels remained stable thereafter while the cells were differentiating. CFTR expression remained stable in HT-29 cells maintained in galactose, where they differentiated, but decreased by ~10-fold when cultured continuously in glucose, where they remained undifferentiated (Figure 2, days 10–14). A detailed analysis of CFTR mRNA level in post-confluency cells maintained in glucose indicated a gradual decrease over a period of 3–4 days. The above results were the same in four such experiments on both cell lines when mRNA levels were also examined by RNase protection (not shown).

Relationship of CFTR mRNA expression to that of other genes regulated during differentiation

To investigate the relationship of CFTR expression with differentiation, the cells were examined for both morphological and biochemical markers of differentiation. Scanning and electron micrographs of both Caco-2 and HT29 cells confirmed that the cells differentiated under appropriate culture conditions as previously demonstrated by Pinto *et al.* (1982, 1983) (data not shown). Villin, sucrase–isomaltase and α1-antitrypsin were used as biochemical markers of differentiation and were analyzed using corresponding cDNA or genomic probes on the same Northern blots prepared from Caco-2 and HT-29 cells. No changes were detected in the expression of villin mRNA in Caco-2 cells (Figure 3A), whereas in HT-29 cells the pattern of villin expression was similar to that of CFTR (Figure 3b). Sucrase isomaltase expression (Green *et al.*, 1987) in Caco-2 cells was as expected from the enzyme activity assays of Pinto *et al.* (1983) and was similar to the expression of CFTR (Figure 4). No hybridization could be detected with HT-29 RNA, although in several studies enzyme activity has been detected in these cells (Pinto *et al.*, 1982; Zweibaum *et al.*, 1983; Trugnan *et al.*, 1987).

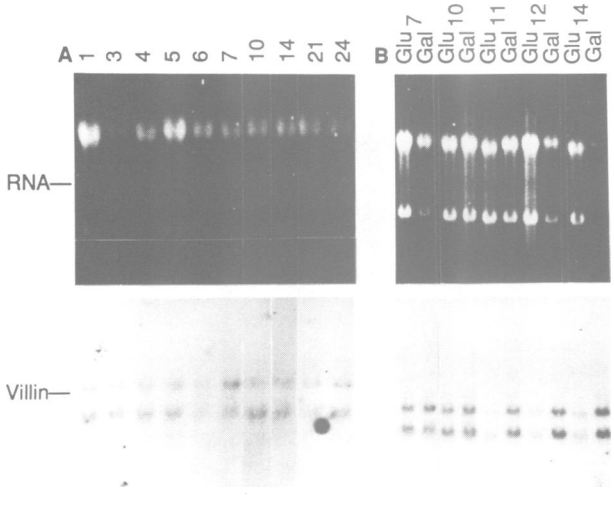

Fig. 3. Relationship of CFTR expression to villin expression in Caco-2 (**A**) and HT-29 (**B**) cells. Samples were taken at the time points (in days) indicated at the top of the figure after seeding for Caco-2 cells and after growth in glucose (Glu) or galactose (Gal) for HT-29 cells. The upper panel (RNA) shows the variation in RNA loading and the bottom panel (villin) the signal generated using a villin probe.

Caco-2 cells were also examined for the expression of α1-antitrypsin (Perlmutter *et al.*, 1989). While α1-antitrypsin mRNA appeared at the same time as CFTR mRNA (Figure 4), it continued to increase after cells reached confluence, while CFTR mRNA amounts levelled off. No changes were observed in α1-antitrypsin expression in HT-29 cells (not shown). These experiments confirmed that Caco-2 and HT-29 cells differentiate under our culture conditions and that the regulation of CFTR expression during this process may be related to that of other regulated genes.

Regulation of CFTR mRNA expression

The above results suggest that CFTR mRNA is regulated in differentiating Caco-2 and HT-29 cells, either by a change in the rate of transcription or at the post-transcriptional level, i.e. by changes in the stability or processing of the transcript or in the transport of mRNA to the cytoplasm. To test the first possibility, nuclear run-on transcriptional assays were performed on Caco-2 cells at various points during growth and on HT-29 cells grown in glucose or galactose post-confluency. Pyruvate dehydrogenase cDNA was used as a positive control by hybridization whereas Bluescript (Stratagene) plasmid DNA was used as a negative control. No significant variation occurred in transcriptional activity of CFTR irrespective of the growth state of the Caco-2 cells (Figure 5). Table I summarizes results of two such experiments. Transcriptional activity of CFTR appeared to be low in HT-29 cells and therefore no conclusions could be reached about changes under different culture conditions.

Changes in protein levels

To test whether the changes in CFTR mRNA are also reflected at the protein level, we prepared membranes from cells at indicated time points and analyzed these by Western blotting using antibodies described in Kartner *et al.* (1991).

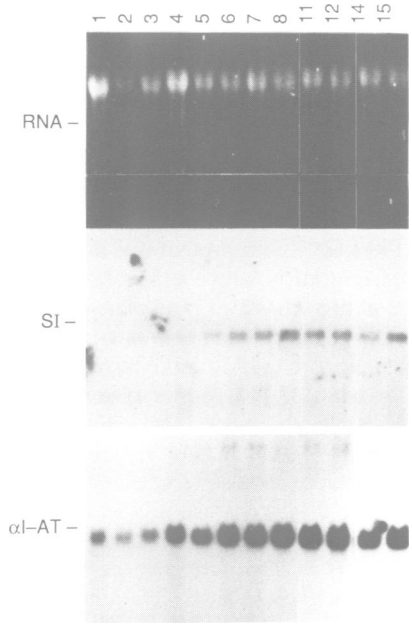

Fig. 4. Relationship of CFTR expression to sucrase–isomaltase and α1-antitrypsin expression in Caco-2 cells. Samples were taken at the indicated days after seeding. The upper panel (RNA) indicates the variation in RNA loading, the middle panel (SI) the signal generated using a sucrase–isomaltase probe and the bottom panel (α-AT) the signal produced by an α1-antitrypsin probe.

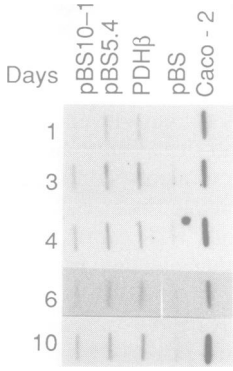

Fig. 5. Transcription rate of CFTR in Caco-2 cells. Filters containing 2 μg each of the following plasmids were hybridized with [^{32}P]RNA prepared as described in Materials and methods: pBS10-1, Bluescript plasmid containing 0.96 kb *Eco*RI fragment of CFTR cDNA. pBS5.4, Bluescript plasmid containing the entire length of CFTR cDNA and some poly(A)tail; phd, SP60 plasmid containing pyruvate dehydrogenase cDNA; pBS, Bluescript plasmid with no insert, used as a negative control; 200 ng of Caco-2 genomic DNA, used as a positive control.

Table I. Run-on transcriptional analysis of CFTR in Caco-2 cells

Day	pBS10-1 genomic		pBS5.4 genomic		pBS10-1 PDHβ	pBS5.4 PDHβ
	I	II	I	II		
1	0.48	0.16	0.78	0.19	0.86	0.98
2	0.83	–	0.39	–	–	–
3	0.73	0.46	0.80	0.32	1.25	0.88
4	–	0.28	–	0.20	2.09	1.46
6	0.78	0.30	0.79	0.27	0.88	0.80
10	–	0.23	–	0.15	1.37	0.90

The values represent the normalized rates of CFTR transcription in relation to that of total genomic or PDHβ transcription. pBS10-1 contains a 900 bp fragment of CFTR cDNA from the 5′ end while pBS5.4 contains 5.4 kbp of the cDNA including the complete coding region.

CFTR protein (~170 kDa in size) was detectable at all stages of growth in both Caco-2 and HT-29 cells. During differentiation of Caco-2 cells a slight decrease in the amounts of 170 kDa CFTR protein was detected despite the 10-fold increase in mRNA levels (Figure 6). Furthermore, at later stages of growth (day 12) an increase in the amounts of a degradation product was apparent. In HT-29 cells the amount of protein decreased markedly in cells maintained in glucose, similar to the results observed with CFTR mRNA. CFTR levels were also slightly reduced in differentiated HT-29 cells at later stages (day 21) (Figure 7). These results were consistent when either polyclonal or monoclonal antibodies were used.

Function of CFTR in differentiating cells

Recent evidence suggests that CFTR functions as a cyclic AMP-regulated Cl^- channel (Anderson *et al.*, 1991a; Kartner *et al.*, 1991; Tabcharani *et al.*, 1991; Bear *et al.*, 1992). Therefore, we measured cyclic AMP-stimulated Cl^- conductance as an assay for CFTR function in undifferentiated and differentiated Caco-2 cells. Cl^-

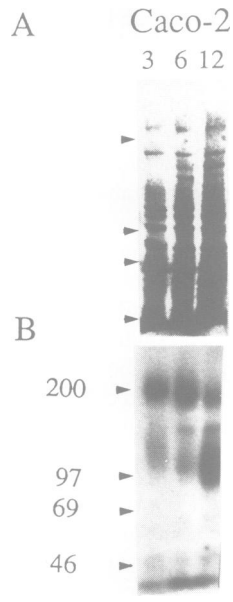

Fig. 6. Relationship of CFTR protein levels and differentiation in Caco-2 cells. The upper panel (**A**) shows the Pounceau-S stained filter for comparison of the total amount of protein loaded in each lane. The bottom panel (**B**) shows the X-ray film of the Western blot hybridized to alkaline phosphatase conjugated M3A7 anti-CFTR monoclonal antibody. The time points represent the number of days after plating.

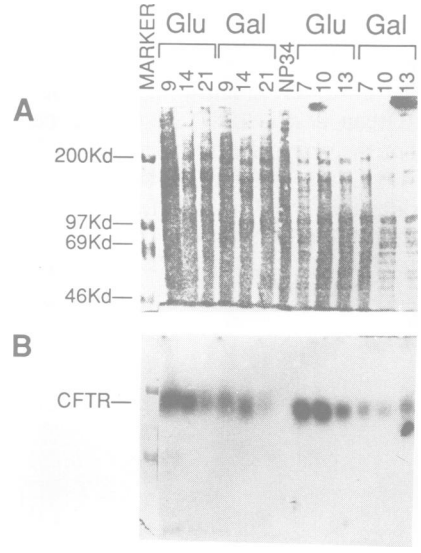

Fig. 7. Relationship of CFTR protein levels to differentiation of HT-29 cells. Cells were grown in either glucose (Glu) or galactose (Gal) for the indicated days after reaching confluence. Panels as in Figure 6. NP34 is a nasal polyp epithelial cell line that does not express CFTR (Buchanan *et al.*, 1990).

conductance was first measured as ^{125}I efflux from pre-loaded monolayers (Figure 8A). Since changes in ^{125}I efflux could result from alterations in the levels of anion cotransport or channel activity, we also measured conductance directly by whole-cell, patch clamp electrophysiology (Figure 8B). Cyclic AMP-activated ^{125}I efflux (Figure 8A) and whole-cell Cl^- current (Figure 8B) were significantly lower in day 12 (differentiated) cells than in day 2 (undifferentiated) cells ($P < 0.001$).

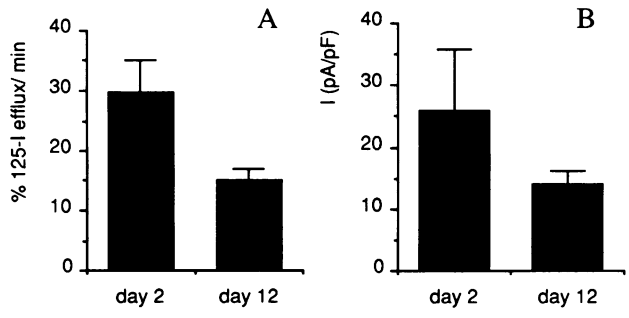

Fig. 8. Functional CFTR measurements in undifferentiated and differentiated Caco-2 cells. (**A**) cAMP-stimulated ^{125}I efflux in Caco-2 cells. The mean rate of ^{125}I efflux stimulated by cAMP was measured in eight paired experiments at 2 days (undifferentiated) and 12 days (differentiated). (**B**) Whole cell Cl^- currents stimulated by cyclic AMP in Caco-2 cells. Mean outward whole cell currents 3 min after addition of cAMP or DMSO (as control) at +40 mV expressed as a function of cell capacitance at days 2 (n = 19) and 12 (n = 8) after plating.

Discussion

Cellular differentiation is a complex phenomenon involving both spatial and temporal regulation of gene expression. Control of gene expression may be effected at several levels, including transcription of the gene, processing of the primary transcript, transport of the message, mRNA stability, translation of the RNA on polysomes and protein stability. For highly regulated genes, the factors involved, as well as the mechanisms of this regulation, need to be understood. In this paper, we have described an *in vitro* model system to study the regulation of CFTR.

CFTR expression and differentiation

Our data indicate that the maximum level of CFTR mRNA expression occurs in differentiated cells, i.e. at all stages of growth in T84 cells which are always differentiated (Buchwald *et al.*, 1991), in post-confluent Caco-2 cells and in HT-29 cells grown in the absence of glucose. Similar results were obtained by Montrose-Rafizadeh *et al.* (1991) using subclones of HT-29 that had undergone differentiation and were committed to either mucin-secreting or enterocyte-like phenotypes. Our more detailed analysis of CFTR mRNA levels in HT-29 cells during growth and differentiation has led to the unexpected observation that CFTR mRNA levels gradually disappear when post-confluence cells are maintained in glucose. Scanning and transmission electron microscopy has shown that under these conditions the cells remained undifferentiated for at least 1 week, after which they began to detach and, therefore, could not be examined by these methods. More recently, Chang *et al.* (1991) have reported that CFTR mRNA levels were the same 6 and 28 days post-confluence while the extent of cAMP- and Ca^{2+}-stimulated short circuit current decreased 4-fold. However, the conducting ion underlying the changes in short circuit current was not clearly identified. Furthermore, the probe used to detect CFTR also hybridized to a 4.3 kb band, bringing into question the reliability of these data.

An attempt has been made to determine the precise relationship of changes in steady state CFTR mRNA levels to the process of cellular differentiation using both structural (scanning and transmission electron microscopy) and biochemical (membrane proteins and enzymes) markers of enterocytic differentiation. The pattern of expression of other genes shown to be regulated during differentiation of these cells was compared with that of CFTR. In Caco-2 cells, CFTR mRNA expression correlates well with the expression of α1-antitrypsin and is similar to the expression of sucrase–isomaltase. Notwithstanding the fact that sucrase–isomaltase has been detected both antigenically and enzymatically in HT-29 cells (Zweibaum *et al.*, 1983), we could not detect hybridization in HT-29 using either the rabbit or human sucrase–isomaltase cDNA probes. A different isoform of sucrase–isomaltase may be present in these cells.

A cDNA probe encoding villin (Pringault *et al.*, 1986) was also used to hybridize our Northern blot since differentiation in both Caco-2 and HT-29 cells is accompanied by a marked increase in the number of microvilli (Rousett, 1986). Previous studies (Pringault *et al.*, 1986; Dudouet *et al.*, 1987) have shown that the rate of synthesis of villin increases by ~10-fold with the differentiation of HT-29 cells. Our data confirm these results and, in addition, show that villin mRNA levels decrease gradually in cells maintained post-confluency in glucose and, therefore, correlate well with CFTR expression. In contrast to the results observed with HT-29 cells, no change in villin mRNA was detected in Caco-2 cells. Thus, alterations in the patterns of mRNA expression appear to be both gene and cell specific.

Mechanisms of regulation

Regardless of the precise relationship of CFTR expression to differentiation, variations in CFTR mRNA levels seem to be predominantly the result of post-transcriptional control. We observed little or no change in CFTR transcription rates when comparing exponentially growing and differentiated Caco-2 cells, despite the significant differences in steady-state mRNA levels under the same conditions (Figure 5). Given the slow decrease of CFTR mRNA levels of cells maintained in glucose, relative to those maintained in galactose, the higher levels of CFTR mRNA in the latter could either represent a higher level of mRNA synthesis or stabilization of pre-existing messages. We could not detect sufficient transcriptional activity in HT-29 cells to be able to answer this question. We now propose to test mRNA turnover as a mechanism of regulation by studying half life of CFTR mRNA in undifferentiated and differentiated Caco-2 and HT-29 cells. Alteration of mRNA stability as a mechanism of gene regulation has been reported previously for several genes (Thompson *et al.*, 1986; Paillard *et al.*, 1990). It is possible that certain factor(s) present only in differentiated cells interact with CFTR mRNA and increase its stability. These cells lines should, therefore, provide useful models with which to isolate and study such factor(s) and to define their interaction with structural elements of the gene or its mRNA.

The regulation of CFTR transcription has been measured by nuclear run-assays in HT-29 and T84 cells (Trapnell *et al.*, 1991; Yoshimura *et al.*, 1991a,b) but the effect of differentiation was not examined. These authors found low levels of CFTR transcription (~5% in comparison with β-actin) and that treatment of cells with phorbol myristate acetate (PMA) reduced transcription even further. In this regard it is interesting to note that Chou *et al.* (1991) have

shown that the 5′ upstream region of CFTR contains regulatory elements that either lead to inhibition or stimulation of transcription, when assayed using reporter gene constructs. It is possible that these negative and positive elements mediate the changes in CFTR mRNA levels we have observed in differentiating Caco-2 and HT-29 cells.

The levels of mature CFTR protein (170 kDa) and those of the mRNA did not correlate tightly, implying the existence of post-transcriptional or translational regulation. Protein levels decreased slightly in differentiated Caco-2 cells, though most of this change could be accounted for by increased protein degradation, since the antibodies used were specific for CFTR (Karter *et al.*, 1991). In HT-29 cells, mature protein was stable in cells cultured in the presence of galactose but amounts decreased in cells in glucose, paralleling the observations made at the level of mRNA. Thus, translational or post-translational regulation is not a likely mechanism under these circumstances.

The level of cAMP-activated Cl^- conductance Caco-2 cells correlates roughly with the level of CFTR protein detected. This finding supports the current hypothesis that CFTR functions as a regulated Cl^- channel. However, the decrease in cyclic AMP-activated Cl^- conductance with differentiation could also be due, in part, to altered levels of regulatory proteins for Cl^- channels in such cells. For example, Tabcharani *et al.* (1991) have shown that the Cl^- channel conferred by CFTR expression in CHO cells is activated by PKA- and PKC-mediated phosphorylation and deactivated by phosphatase. Variations in PKA, PKC or phosphatase levels during differentiation could account for our observations. While several reports have suggested that neither PKA nor PKC activities change with differentiation (de Jonge *et al.*, 1987; Rydell *et al.*, 1990), levels of alkaline phosphatase in Caco-2 increase during differentiation (Matsumoto *et al.*, 1990). Hence this increased alkaline phosphatase activity and consequent CFTR dephosphorylation could account, in part, for decreased Cl^- conductance in differentiated Caco-2 cells.

At least superficially, the increase in CFTR mRNA during differentiation of these cell lines does not seem to correspond to the observations made in rat intestinal tissues *in vivo* (Trezise and Buchwald, 1991). In the latter, CFTR mRNA levels, as detected by *in situ* hybridization, decrease gradually from crypt to villus, as the intestinal cells differentiate. Little CFTR mRNA is seen in the villus tips. In contrast, significant amounts of CFTR mRNA are present in both Caco-2 and HT-29 cells after differentiation. Thus *in vivo* and *in vitro* regulation of CFTR mRNA levels are probably different. On the other hand, CFTR function in both the *in vivo* and *in vitro* studies appears to be similar. Low levels of cyclic AMP-activated Cl^- transport have been detected in rodent villi (Sepulveda *et al.*, 1991), consistent with the absence of CFTR mRNA (Trezise and Buchwald, 1991). Similarly, cyclic AMP-activated Cl^- conductances are lower in differentiated than in undifferentiated Caco-2 cells. Thus, the phenotype of the differentiated cells is similar *in vivo* and *in vitro*, even though it apparently results from different mechanisms of regulating CFTR. Caco-2 and HT-29 cells thus provide us with useful models to study the elements that regulate CFTR expression, from transcription to transport activity.

Materials and methods

Cell culture

Cells from three colon carcinoma cells lines, T84 (ATCC # 248-CCL), Caco-2 (ATCC # HTB-37) and HT-29 (ATCC # HTB-38) (Fogh *et al.*, 1977) were obtained from the American Type Tissue Culture Collection (ATCC). All cultures were certified mycoplasma-free and were propagated by trypsinization when confluent, T84 as 1:2, Caco-2 as 1:6 and HT-29 as 1:8 subcultures, respectively. T84 cells were cultured in a 1:1 mixture of Ham's F12 medium and alpha MEM, (Gibco) with 5% Nu serum (Collaborative Research Inc.). Caco-2 cells were cultured in alpha MEM supplemented with 10% fetal bovine serum (Flow laboratories). HT-29 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose (Gibco) and 10% fetal bovine serum, unless mentioned otherwise. To determine the effect of growth and differentiation on the expression of CFTR, cells were seeded at 4.5×10^4 cells/cm^2 and grown to confluency, changing medium every other day. The medium was changed daily thereafter for all cells. However, HT-29 cells were grown in two different media at this point. Half of the dishes were maintained in the same medium, while the other half were switched to DMEM containing 25 mM galactose instead of glucose and supplemented with 10% fetal bovine serum dialysed against saline solution to remove glucose. Samples were taken at appropriate time points for counting (using a Coulter counter), RNA, nuclei and membrane preparations.

RNA extraction

Total cellular RNA was isolated by the method of Chirgwin *et al.* (1979). All solutions were either prepared with diethyl pyrocarbonate (DEPC) treated water or treated with DEPC and autoclaved. Approximately 10^7 cells were chosen at each time point to yield ~100 μg of RNA. Cells were lysed with 1 ml/10 cm plate of denaturing solution (4 M guanidinium thiocyanate; 25 mM sodium citrate, pH 7.0; 0.5% *N*-lauroyl-sarcosine and 0.1 M 2-mercaptoethanol), scraped off plates and pooled on ice. The lysates were passed five times each through 18G and 21G needles to shear the DNA. The lysates were then layered over a cushion of 5.7 M cesium chloride/0.1 M ethylene diamine tetra-acetic acid (EDTA), pH 8, and centrifuged at 30 000 r.p.m., 20°C in an SW21 rotor for 18–24 h. The liquid was removed and the pellet was rinsed twice with ethanol, resuspended in TE (10 mM Tris–HCl, 1 mM EDTA, pH 7.5) and precipitated with two volumes of ethanol. The RNA was stored at −70°C, either at this stage or after resuspension in TE.

Quantitation of mRNA levels

Total RNA (20 μg/lane) was fractionated on 0.66 M formaldehyde, 1.0% agarose gels. Samples were heat-denatured in the presence of 0.6 M formaldehyde, 50% formamide, 0.05% bromophenol blue, 5% glycerol and 1 × MOPS–EDTA buffer [0.2 M MOPS [3-(*N*-morpholino)propanesulfonic acid], 50 mM sodium acetate, 10 mM EDTA, pH 7.0. Samples were electrophoresed in 1 × MOPS–EDTA buffer after the addition of 1 μl of 1 mg/ml ethidium bromide. Gels were photographed on a short wave transilliminator and then transferred onto Gene Screen plus membrane (DuPont) by capillary action in 10 × SSC (1 × SSC = 0.15 M sodium chloride, 0.015 M sodium citrate). Blots were rinsed in 2 × SSC, dried and baked at 80°C under vacuum for 2 h. Blots were prewashed in 0.1 × SSC, 0.5% SDS at 65°C for 1 h, prehybridized in 1% SDS, 1 M NaCl and 10% dextran sulphate at 65°C for 2–6 h and then hybridized overnight with [α-^{32}P]dCTP-labelled probes (Feinberg and Vogelstein, 1983). Blots were washed twice at room temperature in 2 × SSC, 0.1% SDS (10 min each), followed by a wash at 65°C in 0.1 × SSC, 0.1% SDS for 20 min and then autoradiographed on Dupont Cronex 4 X-ray film at −70°C. The probes used have been described before (CFTR: Riordan *et al.*, 1989; sucrase–isomaltase, Green *et al.*, 1987; villin, Prignault *et al.*, 1986; α-1-antitrypsin, Cox *et al.*, 1987; pyruvate dehydrogenase, Koike *et al.* (1988).

Levels of RNA were quantitated by laser densitometry (Molecular Dynamics, Model 300A) of the X-ray films. Care was taken to compare images that produced signals in the linear range of densitometer by using films of different exposures. The levels of RNA in each sample were compared with the amounts of pyruvate dehydrogenase RNA. In other experiments, RNA levels were measured by the RNase protection assay using the kit provided by Ambion. In these latter experiments cells were harvested simultaneously for nuclear run-on and Western blot analyses (see below).

Slot blots of DNA

Plasmids containing the cDNA of interest were linearized, denatured in 100 mM Tris−HCl, pH 7.4, 300 mM NaOH, 10 × SSC, by heating at 80°C for 20 min and neutralized with 250 mM Tris−HCl, pH 7.4, 2 μg DNA/slot was blotted onto ntirocellulose filters (Bio-Rad) using the Sleicher and Schuell Minifold II. The filters were then dried, baked and processed for nuclear run-on hybridization with [^{32}P]RNA as described below.

Nuclear run-on transcriptional analysis

Approximately 10^7 cells were washed with ice-cold phosphate-buffered saline (PBS) and collected by scraping into PBS. Cells were pelleted by centrifugation at 1500 r.p.m. The pellets were lysed in 500 μl NP-40 buffer (10 mM Tris−HCl, pH 7.4; 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40) and nuclei pelleted by centrifugation at 700 *g*, 5 min at 4°C. Nuclei were washed with NP-40 buffer, resuspended in glycerol buffer (40% glycerol, 50 mM Tris−HCl, pH 8.3, 5 mM $MgCl_2$, 0.1 mM EDTA) and stored at −70°C until used. For transcription, 100 μl of nuclei were incubated in a 200 μl reaction mixture containing 20% glycerol, 30 mM Tris−HCl pH 8.3, 5 M $MgCl_2$, 150 mM KCl, 0.25 mM ATP, GTP and CTP, and 250 μCi of [α-^{32}P]UTP (3000 Ci/mmol, Amersham or ICN) for 30 min at 30°C. The RNA was incubated with RNase-free DNase I (Boehringer Mannheim) at 37°C for 30 min and deproteinized by digestion with 100 μg of Proteinase K in 1 × SET (1% SDS, 10 mM Tris−HCl, pH 7.5, 5 mM EDTA) followed by phenol/chloroform extraction. The aqueous phase was precipitated twice in ethanol and the pellet was resuspended in 500 μl of hybridization buffer (10 mM Tris−HCl, pH 7.4, 0.2% SDS, 10 mM EDTA; 0.3 M NaCl, 1 × Denhardt's solution and 200 μg/ml yeast tRNA. Incorporation of ^{32}P into nascent RNA was determined by counting 5 μl aliquots. Each cDNA slot blot was hybridized with the same number of c.p.m. at 65°C for 36 h. The filters were washed in 2 × SSC, 0.1% SDS at room temperature twice for 30 min each and then at 60°C in 0.1 × SSC, 0.1% SDS for 30 min followed by autoradiography.

Membrane preparations and Western blotting

Crude membranes containing mainly plasma membrane and endoplasmic reticulum were prepared by the method of Bell *et al.* (1985). Briefly, the method involved rapid swelling of cells in hyptonic lysis buffer (10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris−HCl, pH 7.4) containing 2 μg/ml each of the protease inhibitors aprotinin and leupeptin. Cells were homogenized manually in a Potter−Elvehjem homogenizer after the addition of 2 mM PMSF (phenylmethylsulfonyl fluoride) from a 200 mM stock solution in ethanol. The homogenate was centrifuged at 4000 *g* for 5 min at 4°C to remove cell debris and nuclei. Membranes were then collected by centrifugation of the supernatant for 15 min at 14 000 r.p.m. in a refrigerated microcentrifuge, resuspended in lysis buffer and stored at −70°C. Protein concentrations were determined by Pierce BCA (bicinchoninic acid) assay with bovine serum ablumin standards. Approximately 100 μg of proteins were electrophoresed on 5% SDS−polyacrylamide gels and electroblotted onto nitrocellulose filter paper. Protein profiles were visualized by ponceau S (Sigma) staining, the image saved by xeroxing and the gels were then destained with TTBS (Tris-buffered saline with Tween-20). The filter was blocked in TTBS containing 3% gelatin for 1 h, incubated with first antibody overnight, washed in TTBS and incubated with goat-anti rabbit IgG−alkaline phosphatase conjugate (Promega). The binding was visualized by colour development reaction using nitro blue tetreazolium and 5-bromo-4-chloro-3-indolyl phosphate (Promega). The blot was probed with an anti-CFTR mouse monoclonal antibody (M3A7) raised against an epitope between residues 1197 and 1408 (Kartner *et al.*, 1991).

^{125}Iodide efflux

The experimental protocol used for ^{125}I efflux measurements was similar to that described in studies of anion conductance in airway epithelial cells (Clancy *et al.*, 1990). Briefly, Caco-2 cells plated on 35 mm collagen-coated, semi-permeable culture-dish inserts (Costar) were loaded with ^{125}I (8 μCi/ml) for 2 h. The cells were subsequently washed by dipping the coverslip inserts three times into buffer for 10 s each. The rate of ^{125}I efflux was determined by transferring the cover slip through a series of 10 Petri dishes each containing 3 ml buffer. The coverslip was held in each Petri dish for 1 min. The ^{125}I efflux into the first two dishes was discarded as part of the washing procedure and the efflux into the third dish was considered the baseline measurement. The fourth and subsequent dishes contained varying experimental reagents. After each experiment, 1 ml of efflux buffer was taken from dishes 3−10 and the non-effluxed radioactivity remaining in the monolayer counted. As in the studies of Clancy *et al.* (1990), the results were expressed as % efflux/min = no. counts effluxed into each Petri dish/total available counts at the onset of each minute time interval. All the experiments were performed at room temperature and results presented as means ± SD.

Current recordings

Whole-cell currents were recorded according to Hamill *et al.* (1981) using a List EPC-7 patch-clamp amplifier (Medical Systems, Great Neck, NY). As in our previous experiments of cAMP Cl^- currents in Caco-2 cells (Bear and Reyes, 1992), whole cell currents were monitored continuously at 0 and +40 mV for 900 and 100 ms, respectively, prior to and following treatment with an activating cocktail containing: forskolin (10 μM), dibutryl cAMP (100 μM) and isobutyl methylxanthine (100 μM). Cell capacitance was compensated using the cancellation circuitry of the EPC-7 amplifier. Pipettes were fabricated from borosilicate glass type 7052 (Garner Glass Co.) using a two-stage Narishige pipette puller. When filled with Na^+−HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) solution, pipette resistances were ~2−5 MΩ. The bath electrode was a Ag−AgCl wire connected to the bathing solution via an agar bridge. Current output was monitored on a Tektronic oscilloscope and stored on video tape after A/D conversion by a video adaptor (PCM 2, Medical Systems). Records were sampled at 0.5−2.0 kHz. The standard bath solution contained: 140 mM NaCl or *N*-methyl D-glucamine (NMDG), 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES, pH adjusted to 7.2 using 1 N NaOH. The pipette solution contained 110 mM NaCl, 1 mM ethylene glycol-bis (β-aminoethyl ether)-*N,N,N'-N'*-tetraacetic acid (EGTA), 4 mM $MgCl_2$, 10 mM HEPES, 1 mM ATP (4), adjusted to pH 7.2. The pipette solution was made hypotonic to the bath to prevent the occurrence of cell swelling activated currents as previously described in Caco-2 and T_{84} cells (Bear and Reyes, 1992). Experiments were performed at 22−25°C.

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