Cell-specific posttranscriptional regulation of CFTR gene expression via influence of MAPK cascades on 3′UTR part of transcripts

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Baudouin-Legros, Maryvonne, Alexandre Hinzpeter, Amandine Jaulmes, Franck Brouillard, Bruno Costes, Pascale Fanen, and Aleksander Edelman. Cell-specific posttranscriptional regulation of CFTR gene expression via influence of MAPK cascades on 3′UTR part of transcripts. Am J Physiol Cell Physiol 289: C1240–C1250, 2005. First published June 8, 2005; doi:10.1152/ajpcell.00595.2004.—Expression of the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) gene, which contains the mutations responsible for CF, is regulated by cytokines (TNF-α and IL-1β) in a cell-specific manner. TNF-α decreases CFTR mRNA in human colon cell lines (HT-29), but not in pulmonary cell lines (Calu-3), and IL-1β increases it only in Calu-3 cells. We looked for the cytokine-induced posttranscriptional regulation of CFTR gene expression and studied the modulation of CFTR mRNA stability linked to its 3′ untranslated sequence (3′UTR) in HT-29 and Calu-3 cells. The stability of CFTR mRNA was analyzed by Northern blot after in vitro incubation of total RNAs from CFTR-expressing cells with cytosolic proteins extracted from control or cytokine-treated HT-29 and Calu-3 cells. CFTR mRNA was degraded only by extracts of TNF-α-treated HT-29 cells and not by cytosolic proteins from untreated or IL-1β-treated HT-29 cells. In contrast, extracts of untreated Calu-3 cells enhanced CFTR mRNA degradation, and IL-1β treatment inhibited this; TNF-α had no significant effect. The 3′UTR part of CFTR mRNA was found to be required for this posttranscriptional regulation. The 5′ part of the 3′UTR (the 217 first bases), which contains two AUUUA sequences, was implicated in CFTR mRNA destabilization and the following 136 bases, containing several C-repeats in U-rich environment, in its protection. The proteins, which reacted with the U- and C-repeats of CFTR mRNA 3′UTR, were mainly controlled by stimulation of the p42/p44 and p38 MAP kinase cascades with interaction between these pathways. This posttranscriptional control of gene expression is a common feature of CFTR and many proteins of inflammation.

cytokines; HT-29; Calu-3

THE CYSTIC FIBROSIS (CF) transmembrane conductance regulator (CFTR) protein (36) is altered by several mutations of its gene, whose genetic dysfunctions lead to CF, the most common autosomal genetic disease in Caucasians. CFTR is a cAMP-activated chloride channel that is primarily located in the apical membrane of some epithelial cells; the main symptoms of CF are inflammation and enhanced secretion of inflammatory cytokines. As demonstrated by several studies performed on various human cell lines, CFTR gene expression and protein synthesis are modulated by various cytokines, and molecular mechanisms of this regulation have been described. CFTR gene expression is stimulated by IL-1β in Calu-3 pulmonary cells via the activation of the κB system, a transduction pathway triggered by many excitatory stimuli in most cells, and binding of NF-κB to the CFTR promoter (7, 9). On the other hand, in HT-29 and T-84 colon cells, TNF-α and IFN-γ downregulate CFTR gene expression by enhancing its mRNA degradation (3, 12). However, it is unknown whether IL-1β vs. TNF-α and IFN-γ exert the same effect in all the CFTR-expressing cells, and whether IL-1β controls the sole gene transcription or mRNA stability.

Posttranscriptional reactions often participate in the control of gene expression by extracellular stimuli. They proceed from the modulation of mRNA stability and/or translation efficiency via the binding of activated heterogeneous nuclear ribonucleoprotein (hnRNP) to specific ribonucleotide sequences, which are often present in the 3′ untranslated part (3′UTR) of the mRNA (for review, see Refs. 4, 15, 18). The “AU-rich elements” (ARE sequences) are the best-known ribonucleotide sequences of the 3′UTR. They can bind many proteins that either stabilize the mRNA, such as HuR (6, 29), or accelerate their degradation (AUF1 protein and tristetraprolin) (26, 8), thereby enhancing or decreasing the corresponding protein synthesis. Posttranscriptional regulations involving reactions with ARE sequences are particularly important in the response of the cell to inflammatory stimuli involving genes encoding mediators of inflammation, such as cyclooxygenase-2 and cytokines (1, 2, 14, 31–34). The stability of these mRNAs is also controlled by other cis-elements rich in U- and C-repeats in their 3′UTR. These repeats are also present in mRNAs encoding proteins, which are not involved in inflammation (25, 28, 35, 37). They react with specific poly(C)-binding proteins and with some ARE-binding ones, such as HuR (37), with possible competition between the ARE and U/C sequences. In this case, the posttranscriptional regulation results from the activation of diverse hnRNP, and this diversity participates in the cell specificity of the response (4, 5, 20). Several mRNAs encoding proteins with very different functions may present the same ribonucleotide sequence(s) in their 3′UTR, and thus may be simultaneously regulated by the activation of the corresponding hnRNP acting as “posttranscriptional operons” (21), which contributes to harmonious cell response (1). Activation and binding of the various hnRNP on the ribonucleotide sequences depend on their phosphorylation, which is often controlled by stimulus- and cell-specific stimulation of the MAP kinase cascades (10, 22). These cytoplasmic transduction

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pathways are thus deeply involved in the cell-specific regulation of gene expression (1, 10, 22, 23, 31, 34).

The sequence of the CFTR 3′UTR contains many U and C repeats, and particularly three AUUUA sequences and C repeats surrounded by several U (38), but its implication in the regulation of the gene expression has not been explored. The posttranscriptional regulation of CFTR gene expression by TNF-α, which was shown in HT-29 and T-84 colon cells, was not studied in pulmonary cells and under other stimulations, such as IL-1β treatment. The present research was designed to look for a possible cell-specific posttranscriptional regulation of CFTR gene expression triggered by TNF-α and IL-1β in HT-29 and Calu-3 cells. In this first physiological study, we chose not to use any gene manipulation of the colon and pulmonary cells to avoid artificial stimulation of the transduction cascades (11, 16), and chose to study the CFTR mRNA stability with an in vitro protocol. The results show that posttranscriptional modulations of CFTR gene expression occur in HT-29 and Calu-3 cells stimulated by IL-1β and TNF-α. The induced alterations of the CFTR mRNA stability depend on the activation of cytosolic proteins reacting with the CFTR mRNA 3′UTR, and the reactions are cell specific. These data provide a starting point for the identification of the precise proteins involved in the regulation and the other genes whose expression undergoes simultaneous analogous modulation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Treatment**

Calu-3, HT-29, and HeLa cells were obtained from the American Type Culture Collection and cultured on plastic in DMEM containing 10% fetal calf serum at 37°C in a 5% CO2-95% air atmosphere, except for HeLa cells, obtained from ATCC and cultured on plastic in DMEM containing 10% fetal calf serum at 37°C in a 5% CO2-95% air atmosphere, except when otherwise noted. The medium was enriched with 1 mM sodium pyruvate, nonessential amino acids for Calu-3 cells, and Zeocin (2.5 μg/ml) for the stably transfected HeLa cells. Subconfluent cells were placed in serum-free medium for 24 h before the cytokines were added in serum-free medium. The MAP kinase inhibitors (and the corresponding volume of 1 μM/ml DMSO added to controls) were added 30 min before the cytokines. In the experiments on the half-life of the CFTR mRNA, actinomycin D (5 μg/ml) was added to the medium 1 h after the cytokines.

**Plasmid Construction and HeLa Cell Transfection**

A plasmid vector for expressing the human CFTR gene in mammalian cells was constructed by placing the full-length CFTR cDNA coding sequence (4.5 kb from nucleotide position 96 to 4,578, obtained from pTG5960, a gift from Transgene) in the expression vector pTracer-cytomegalovirus (CMV) (Invitrogen), which is designed for the visual detection of transfected mammalian cells. The resulting plasmid was designated pTCF or “short CFTR construction.” Expression of the CFTR gene in pTCF is controlled by the CMV promoter, whereas synthesis of the green fluorescent protein-Zeocin fusion protein is controlled by the SV40 promoter. The plasmid containing the 3′UTR (“long CFTR” construction) was constructed by PCR and contained the full-length CFTR cDNA coding sequence and 3′ untranslated region (6.1 kb from nucleotide position 96 to 6129, with positions numbered from GenBank NM_000492 reference sequence).

The “short” (cDNA wild type) and “long” (cDNA + 3′ UTR) CFTR constructions were transfected into HeLa cells with the Lipofectamine Plus reagent from Invitrogen according to the producer’s instructions. The transfected cells were selected by culture in the presence of Zeocin.

**Preparation of Ribonucleotide Sequences**

Sequences corresponding to the proximal part of the CFTR 3′UTR were amplified by PCR amplification from the pCFTR3′UTR plasmid, using Taq polymerase (Finnzyme) and the following primers: 5′-GGTGCAAGATACAGAGTTTTAG-3′ and 5′-CACACATTT-GACTATTGCCAG-3′ for the oligo 1 sequence (from −19 before the TAG triplet to 217 bases after it), 5′-TGAGGACACTGTAT-GGGCTTT-3′ and 5′-TAGAATAAGCTGATCAACTA-3′ for the oligo 2 sequence (from base 161 after the TAG triplet to base 353), and finally 5′-GGTGCAAGATACAGAGTTTTAG-3′ and 5′-TAGAATAAGCTGATCAACTA-3′ for the oligo 3 sequence (from −19 to +353).

Both amplified fragments were cloned into the pCR2-TOPO vector (Invitrogen) and the resulting constructs, pCR2-TOPO-CFTR3′UTR1 (oligo 1), pCR2-TOPO-CFTR3′UTR2 (oligo 2), and pCR2-TOPO-CFTR3′UTR3 (oligo 3) were used as template for in vitro transcription.

RNA transcripts were synthesized from HaeIII-cut pCR2-TOPO-CFTR3′UTR1, pCR2-TOPO-CFTR3′UTR2, and pCR2-TOPO-CFTR3′UTR3 plasmids using the Ambion MEGashortscript T7 kit, and the reaction products were purified using the Ambion MegaClear kit, following the manufacturer’s recommendations. A nonspecific transcript was obtained from the control DNA supplied in the transcription kit.

**RNA Extraction and Northern Blot Analysis**

Total RNAs were isolated with phenol/chloroform with the use of TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. RNA was obtained from untreated Calu-3 and CFTR-transfected HeLa cells when they were used in in vitro incubation experiments to study the mRNA stability. Northern blot analysis was done with the use of total RNAs separated on 0.9% agarose gels (15 μg/well) and transferred to nylon membranes (Stratagene). The membranes were hybridized with 32P-labeled cDNA probes (specific activity >106 cpm/μg) with the Quik Hybridization solution provided by Stratagene. The CFTR probe was the 1.5-kb EcoRI-EcoRI fragment of human CFTR cDNA. The β-actin cDNA probe was purchased from Oncogene Science. The mRNAs were quantified by densitometry using an ImageMaster VSD (Pharmacia-Biotech-Amersham, Orsay, France), and the amounts of CFTR mRNA were normalized to those of β-actin. All experiments were repeated at least six times.

**Incubation Experiments.** All of the preparations and experiments were performed in an RNase-free environment with RNase-free water, buffers, and instruments.

**Protein Extraction.** Serum-deprived cells were incubated with cytokines for 24 h, rapidly washed, and lysed at 4°C by incubation for 30 min in hypotonic medium (10 mM KCl, 10 mM HEPES, pH 7.4, and 1 mM MgCl2) containing cocktails of protease (Roche) and phosphatase (Sigma) inhibitors and homogenized in a glass/glass Potter homogenizer. Unbroken cells were removed by centrifugation at 2,500 g for 15 min; the resulting supernatant was centrifuged for 1 h at 100,000 g. The “cytosolic” proteins in the last supernatant were quantified and immediately used in the incubation experiments. Microscopic examinations of the control and treated cultures, always performed before extraction, did not show any cytokine-induced alteration in the cell viability. Furthermore, measurement of the protein content of the extracts confirmed the absence of any difference between control and treated cells. The total amounts of cytosolic proteins (in μg) obtained from 25 cm2 flask were, respectively, 394 ± 12 under control conditions, 408 ± 15 after IL-1β treatment, and 406 ± 11 with TNF-α for Calu-3 cells, and 774 ± 15, 822 ± 20, and 752 ± 18 for HT-29 cells (n = 12).
CONTROL OF CFTR mRNA DEGRADATION BY TNF-α AND IL-1β

Incubation Experiments

The proteins were incubated with total RNAs from CFTR gene-expressing cells (untreated Calu-3 or CFTR-transfected HeLa cells) with gentle stirring in buffered saline (300 mM NaCl, 10 mM Tris, pH 7.4, 5 mM EDTA; see, for example, Ref. 31a for a description of the RNase digestion mixture) for 20 min at 25°C. Preliminary experiments showed that these conditions prevented nonspecific RNA degradation. They also demonstrated that the protein extracts contained no CFTR mRNA, that their action on RNA stability depended on their amounts, and that the interpretation of the result was optimal when 20 μg RNA were incubated with 20 μg protein. In the competition experiments, commercial poly-U and -C preparations (Sigma; 100 μg/ml; 10 μg) or oligoribonucleotides (50 μg/ml; 5 μg) were incubated with the proteins for 10 min before adding the RNA.

All incubations were stopped by the addition of TRIZol (Invitrogen), the RNA was again extracted, and the CFTR mRNA content was estimated by Northern blot analysis.

Immunodetection of Phosphorylated MAP Kinases

Protein preparation. Serum-deprived cells were incubated with cytokine for 30 min, rapidly washed with PBS, and lysed by incubation for 30 min at 4°C in buffer (100 mM NaCl, 50 mM Tris, pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) containing cocktails of protease and phosphatase inhibitors, followed by sonication (2 X 1 min). The lysates were centrifuged (14,000 g for 30 min), and the proteins in the supernatant were measured by the Coomassie blue method.

Western blot analysis. Protein extracts were loaded onto 12% SDS-PAGE and electrophoresed for 1 h at 30 mA in Tris-glycine-SDS buffer. The separated proteins were electrotransferred to a nitrocellulose membrane (0.22 μm; Bio-Rad) at 2.5 mA/cm² for 90 min. Free sites on the membrane were blocked (incubation for 1 h at room temperature with Tris-buffered saline containing 0.1% Tween 20, 1% BSA, and 1% skim milk). The membranes were then incubated overnight at 4°C with polyclonal antibodies against phospho-p38, phospho-p42/p44, or phospho-JNK MAP kinases (Cellular Signaling) in the blocking solution, according to the supplier’s instructions. The secondary antibody was anti-goat anti-rabbit IgG, and the complexes were visualized using the ECL+ enhanced chemiluminescence kit (Amersham).

The membranes were then stripped and tested for unphosphorylated MAP kinases (Cellular Signaling), using the same experimental protocol. The stains were then numbered by densitometry (Image Master, Amersham) before the phosphorylated/nonphosphorylated signal ratios were calculated.

The linearity of the signals corresponding to both phosphorylated and nonphosphorylated MAP kinases and the constancy of the ratios were tested in preliminary experiments by loading 15, 30, and 45 μg of proteins extracted from IL-1β- and TNF-α-treated Calu-3 cells.

Statistical Analysis

When appropriate, the statistical significance of the results was checked with the use of ANOVA (completed with Tukey-Kramer post test) or Student’s t-test (paired or unpaired).

RESULTS

Modulation of CFTR mRNA Stability in Calu-3 and HT-29 Cells by TNF-α and IL-1β

CFTR gene expression was studied by Northern blot analysis of CFTR mRNA from Calu-3 and HT-29 cells incubated with IL-1β or TNF-α. Preliminary studies showed that maximal effects were obtained with 5 ng/ml IL-1β and 25 ng/ml TNF-α. IL-1β increased the CFTR mRNA only in the Calu-3 cells. TNF-α decreased it in the HT-29 cells, but had no effect on the Calu-3 cells (Fig. 1).

Participation of cell-specific regulation of CFTR mRNA stability in the different modulations of CFTR gene expression induced by TNF-α and IL-1β in HT-29 and Calu-3 cells was shown by measuring CFTR mRNAs in cells incubated with actinomycin D (5 μg/ml), which inhibits gene transcription (Fig. 2). In the absence of any cytokine, CFTR mRNA remaining after 24-h incubation with actinomycin D corresponded to ~50% of the initial amount in HT-29 cells, but <30% in Calu-3 cells. Furthermore, in HT-29 cells, the half-life of the CFTR messenger was significantly shortened by TNF-α but unaffected by IL-1β, whereas IL-1β (and to a lesser extent, TNF-α) increased the half-life of CFTR mRNA in Calu-3 cells, demonstrating its stabilizing effect. CFTR gene expression is thus differentially regulated posttranscriptionally in HT-29 and Calu-3 cells.

An analysis of the cytosolic compounds differentially involved in the process was performed in vitro. For this purpose, total RNAs from CFTR-expressing cells (untreated Calu-3 cells) were incubated in vitro with cytosolic proteins from Calu-3 and HT-29 cells that had been stimulated with either cytokine for 24 h. The mixtures were then analyzed by Northern blotting, and the degradation of the CFTR mRNA was assessed by changes in the CFTR mRNA-to-β-actin mRNA ratio. Cytosolic proteins from both control cells and from cells

![Image](http://ajpcell.physiology.org/DownloadedFrom/10.22033.5)
cytokine stimulated for 24 h modulated the stability of CFTR mRNA. Preliminary experiments showed that nuclear proteins were inactive.

Figure 3 shows the effect of 20-min incubation of RNA extracts containing CFTR transcripts with cytosolic proteins from untreated or treated HT-29 and Calu-3 cells, with a typical Northern blot in Fig. 3A and the corresponding quantification in Fig. 3B. Cytosol extracts of untreated HT-29 cells had relatively little RNase activity because the CFTR mRNA was reduced by only 25% after 20-min incubation with unstimulated HT-29 proteins (Fig. 3B, left). Cytosol proteins extracted from HT-29 cells treated with TNF-α more actively degraded CFTR mRNA (Fig. 3B, left), whereas those from HT-29 cells treated with IL-1β had about the same action as untreated controls (Fig. 3B, left). Extracts from untreated Calu-3 cells (Fig. 3B, right) degraded >50% of the original CFTR mRNA in 20 min. This was not modified by treatment with TNF-α (Fig. 3B, right), but extracts of IL-1β-treated cells (Fig. 3B, right) induced little or no loss of CFTR mRNA.

To test the importance of the CFTR mRNA 3’UTR in transcript stability we incubated cytosolic extracts from HT-29 or Calu-3 cells maintained under control conditions or treated with cytokine with total RNAs from HeLa cells transfected with either CFTR cDNA (“short CFTR”) or CFTR cDNA plus the 3’UTR part of the gene (“long CFTR”). CFTR mRNA transcribed from the CFTR cDNA (“short CFTR mRNAs”) was not affected by any of the protein extracts (Fig. 4, left). It was not significantly degraded by being incubated with extracts from either HT-29 cells treated with TNF-α (Fig 4A, solid bar), which had a large RNase activity on endogenous Calu-3 RNAs (see Fig. 3), or from untreated (Fig. 4B, open bar) or IL-1β-treated (Fig. 4B, gray bar) Calu-3 cells, which modulated the degradation of endogenous Calu-3 CFTR mRNA (see Fig. 3). On the other hand, when the coinoculation experiments were performed with the “long CFTR mRNAs,” the effects of the various protein extracts (Fig. 4, right) were analogous to those found with the endogenous CFTR mRNA in the total RNAs extracted from untreated Calu-3 cells. The long CFTR mRNAs from transfected HeLa cells were degraded by the extracts from both TNF-α-treated HT-29 cells and untreated or TNF-α-treated Calu-3 cells, unaffected by the extracts from either control or IL-1β-treated HT-29 cells, and protected by those from Calu-3 cells treated with IL-1β.

**Figure 2. Effects of TNF-α and IL-1β on CFTR mRNA half-life. TNF-α (25 ng/ml) or IL-1β (5 ng/ml) was added to the medium, followed by actinomycin D (5 µg/ml) 1 h later, and the transcripts from HT-29 (A) or Calu-3 (B) cells were analyzed by Northern blotting. The CFTR/β actin ratios are percentages of the ratio for control cells. Each value is the mean ± SE of 4 determinations. Difference between the result obtained with cytokines and with actinomycin D alone: *P < 0.01 for basal vs. cytokine values, ANOVA with Tukey-Kramer post test.**

**Figure 3. Properties of the cytosolic extracts from HT-29 and Calu-3 cells. Total RNA (20 µg) from untreated Calu-3 cells were incubated 20 min at 25°C with the experimental medium (control), then with 20 µg cytosolic proteins from untreated cells (basal), TNF-α (25 ng/ml)-treated cells (+TNF-α), and IL-1β (5 ng/ml)-treated cells (+IL-1β). A: typical Northern blot obtained with HT-29 cell extracts is shown at left, and that for Calu-3 cells is at right. B: results obtained after quantification of the autoradiograms and normalization of the intensities of the CFTR mRNA to that of β-actin. The resulting ratios are expressed as percentages of those obtained after incubation of the reference total RNA with the incubation medium alone (control C, hatched bars). Open bars, extracts from untreated cells (basal); solid bars, from TNF-α-treated cells; and gray bars, from IL-1β-treated cells. Each value is the mean ± SE of 6 determinations. •P < 0.05 for control vs. basal; oP < 0.02 for control vs. basal; *P < 0.05 for basal vs. cytokine, unpaired Student’s t-test.**
Ribonucleotide sequences rich in U- and C- repeats present in 3'UTR are often involved in the posttranscriptional regulation of gene expression, and CFTR mRNA possesses such sequences. Competition experiments using synthetic poly-U and -C preparations were performed to determine whether these ribonucleotide repeats were involved in the posttranscriptional regulation of CFTR gene expression. The cytosolic proteins extracted from HT-29 and Calu-3 cells were incubated for 10 min with large amounts (10 μg) of synthetic poly-U and poly-C before being incubated with total RNAs containing CFTR mRNA. Results obtained with total RNAs from HeLa cells transfected with long CFTR was then added for 20 min. The amount of CFTR mRNA still present at the end of the incubation was determined by Northern blot analysis, normalized to that of β-actin, and the results were expressed as percentages of the control value. The experimental protocol and the symbols are the same as those in Fig. 3. Each value is the mean ± SE of 6 determinations. *P < 0.05 for control vs. basal; •P < 0.02 for control vs. basal; *P < 0.05 for basal vs. cytokine, unpaired Student’s t-test.

Fig. 4. Results of incubating “short” and “long” CFTR mRNAs with proteins extracted from untreated and cytokine-treated HT-29 and Calu-3 cells. A: results obtained with proteins from HT-29 cells; B: results for Calu-3 extracts. The RNAs used were extracted from HeLa cells transfected with either CFTR cDNA (short mRNA) or CFTR cDNA, including its 3'-untranslated sequence (3'UTR; long mRNA). The amount of CFTR mRNA still present at the end of the incubation was determined by Northern blot analysis, normalized to that of β-actin, and the results are expressed as percentages of the control value. The experimental protocol and the symbols are the same as those in Fig. 3. Each value is the mean ± SE of 6 determinations. *P < 0.05 for control vs. basal; •P < 0.02 for control vs. basal; *P < 0.05 for basal vs. cytokine, unpaired Student’s t-test.

Fig. 5. Competition between CFTR mRNA and commercial poly-nucleotide preparations. Protein extracts from HT-29 (A, top) or Calu-3 (B, bottom) cells were incubated for 10 min in buffer (groups 1 and 1'), or with 10 μg of commercial poly-U (groups 2 and 2'), -C (groups 3 and 3'), -U and -C (groups 4 and 4'), or -G (groups 5 and 5'). Total RNA (20 μg) from HeLa cells transfected with long CFTR was then added for 20 min. The amount of CFTR mRNA still present at the end of the incubation was determined by Northern blot analysis, normalized to that of β-actin, and the results were expressed as percentages of the control value. Hatched bars, control; open bars, basal extracts; solid bars, extracts from TNF-α-treated cells; and gray bars, from IL-1β-treated cells. Each value is the mean ± SE of 5 determinations. NS, not significant. •P < 0.05 for control vs. basal; •P < 0.02 for control vs. basal; *P < 0.05 for basal vs. cytokine, unpaired Student’s t-test.
any competition (see Fig. 4). Poly-G had no effect (Fig. 5, A and B, groups 5 and 5').

We thus checked whether the two first 5′ parts of the CFTR RNA 3′ UTR, which respectively contain several U-repeats and two ARE sequences (oligo 1) and many C-repeats (oligo 2), were involved in the control of CFTR mRNA stability. For this purpose, the competition experiments were repeated with 5 μg of the corresponding oligoRNAs (oligo 1 and 2) obtained by in vitro transcription, which, as the poly-U and -C preparations, were preincubated with the protein extracts for 10 min before addition of RNA. As shown in Fig. 6, the presence of oligo 1 (groups 2 and 2′) qualitatively reproduced the effects of poly-U addition (Fig. 5, groups 2 and 2′) because it decreased the destabilizing effect of the protein extracts from HT-29 cells stimulated with TNF-α (Fig. 6A, group 2, solid bar) and reduced the protection afforded by the extracts from IL-1β-treated Calu-3 cells (Fig. 6B, group 2′, gray bar). As that with poly-C, the competition with oligo 2 (Fig. 6, A and B, groups 3 and 3′) conferred RNase activity on the extracts from control and IL-1β-treated HT-29 (Fig. 6A, group 3, white and gray columns) and inhibited the protective effect of the cytosolic proteins from Calu-3 cells treated with IL-1β (Fig. 6B, group 3′, gray bar). Furthermore, as the joint addition of poly-U and -C, the presence of oligo 3 (which covers both oligo 1 and oligo 2) protected the CFTR mRNA against the degradation induced by all the extracts (Fig. 6, A and B, groups 4 and 4′). Competition with the nonspecific ribonucleotide was ineffective (result not shown).

Altogether, these data show that the U- and C-repeats present in the 5′ part of the CFTR 3′ UTR play a major role in the control of posttranscriptional modulation of CFTR gene expression. They are involved in both the basal control of CFTR mRNA stability and its modulation by TNF-α in HT-29 cells and IL-1β in Calu-3 cells. CFTR mRNA stability seems to differ in HT-29 and Calu-3 cells because the differences in the responses of the two cell types disappear when the functions of either the U-repeats or the C-repeats are neutralized, and because there are differences in the synthesis or activation of cytosolic compounds that bind to and react with the ARE sequences and the C-repeats of the CFTR 3′ UTR.

**Activation of MAP Kinase Pathways and Control of CFTR mRNA Stability**

Activation of MAP kinase cascades is an important transduction mechanism of cytokine effects and of posttranscriptional regulation of gene expression (1). We thus looked for changes in the phosphorylation of p38, p42/p44 (ERK 1/2), and p46/p54 (JNK) MAP kinases, the three major MAP kinases in HT-29 and Calu-3 cells incubated with TNF-α and IL-1β. Preliminary experiments showed that the protein phosphorylation that could be detected in cells incubated with these cytokines for 15 min was maximal after incubation for 30–60 min, and decreased thereafter (data not shown). The cytokine effects were thus measured after 30 min of incubation. Phosphorylated JNK was not found in extracts from control or IL-1β-treated HT-29 and Calu-3 cells, but was present in both cells after incubation with TNF-α (result not shown), suggesting that it is not involved in cell-specific responses. The other immunoblots illustrate differences in the phosphorylation states of p38 and p42/p44 MAP kinases in HT-29 and Calu-3 cells (Fig. 7). Under control conditions (Fig. 7, A and B, open bars), p38 MAPK phosphorylation, though rather slight, was always more important in HT-29 cells than in Calu-3 cells, whereas the contrary was found for the p42/p44 MAPK, which was more intensively phosphorylated in Calu-3 than in HT-29 cells. Control Calu-3 cells (which possess important RNAsic properties) are thus characterized by, respectively, low and high phosphorylation of p38 and p42/p44 MAPK. The latter characteristic is conferred onto the HT-29 cells by TNF-α (Fig. 7, A and B, solid bars), which destabilizes CFTR mRNA in this model, via the stimulation of p42/p44 MAPK phosphorylation, whereas TNF-α stimulates the phosphorylation of both kinases, and particularly that of p38 MAPK, in Calu-3 cells (in
Activation. HT-29 and Calu-3 cells were incubated with TNF-α and IL-1β, which stabilizes CFTR mRNA in Calu-3 cells, but that of p42/p44 MAPK only in Calu-3 cells; and gray bars, IL-1β-treated cells (Fig. 8B, group 3′, gray bar). Finally, inhibiting either MAP kinase pathway in either cell type inhibited the posttranscriptional regulation produced by both TNF-α and IL-1β.

Inhibition of the p38 MAP kinase pathway with SB-203580 (Fig. 8, A and B, groups 3 and 3′) markedly enhanced degradation of the CFTR mRNA incubated with any extracts of both HT-29 and Calu-3 cells. The effect was particularly notable with extracts from untreated HT-29 cells (Fig. 8A, group 3, open bar), which blunted the destabilizing effect of the extracts of TNF-α-treated HT-29 cells (Fig. 8A, group 3, solid bar). The major action of SB-203580 cells in Calu-3 (Fig. 8B, group 3′) was to suppress the stabilizing properties of the extracts from the IL-1β-treated cells (Fig. 8B, group 3′, gray bar). These data suggest that the control of the stability of CFTR mRNA is linked to the stimulation of both cascades. We checked this by examining the stability of CFTR mRNA in Calu-3 cells treated for 15 min with 25 ng/ml of PD-98059 (Fig. 8A, open bar), which blunted the destabilizing effect of the extracts of TNF-α-treated HT-29 cells (Fig. 8A, solid bar). Finally, inhibiting either MAP kinase pathway in either cell type inhibited the posttranscriptional regulation produced by both TNF-α and IL-1β.

The opposing effects of PD-98059 and SB-203580, one favoring protection and the other degradation of the CFTR mRNA, reproduce the data obtained in the competition experiments with poly-U and -C or with oligoRNA 1 and 2 or (see Figs. 5 and 6). These two sets of results suggest relationships between p42/p44 MAP kinase activation and the U-rich sequences of the CFTR 3′ UTR, and between p38 MAP kinase activation and the C-rich sequences. This was checked in in vitro when mixed with protein extracts from cells treated with PD-98059 and SB-203580, the respective inhibitors of the activation of the p42/p44 and p38 MAP kinase pathways (Fig. 8), and then incubated with antibodies directed against p38 and p42/p44 MAP kinases. The membranes were then stripped and incubated with antibodies directed against p38 and p42/p44 MAP kinases. The data are representative of 4 different experiments with similar results. The histograms were obtained by quantifying the stains by densitometry before calculating the phosphorylated/nonphosphorylated signal ratios. They represent the mean of 4 independent determinations and are expressed as percentage of the result found in nonstimulated HT-29 cells.

In which it does not modify CFTR mRNA stability significantly). In contrast, IL-1β, which stabilizes CFTR mRNA in Calu-3 cells, stimulates p38 MAPK phosphorylation in both HT-29 and Calu-3 cells, but that of p42/p44 MAPK only in Calu-3 cells (Fig. 7, A and B, gray bars).

These data suggest that the control of the stability of CFTR mRNA is linked to the stimulation of both cascades. We checked this by examining the stability of CFTR mRNA in vitro when mixed with protein extracts from cells treated with PD-98059 and SB-203580, the respective inhibitors of the p42/p44 and p38 MAP kinase pathways (Fig. 8), and then stimulated with TNF-α and IL-1β. Inhibition of the p42/p44 MAP kinase pathway with PD-98059 (groups 2 and 2′) markedly enhanced degradation of CFTR mRNAs by the cytosolic protein extracts from HT-29 cells (Fig. 8A) stimulated with TNF-α (Fig. 8A, group 2, open bar) and the degradation induced by the extracts from untreated (Fig. 8B, group 2′, open bar) and TNF-α-stimulated (Fig. 8B, group 2′, solid bar) Calu-3 cells. The results obtained with extracts of IL-1β-treated cells (compare the gray bars of groups 1A and 2A, 1′B and 2′B) were not significantly affected by inhibiting the p42/p44 MAP kinase, but the increased stability of the CFTR mRNA induced by IL-1β in Calu-3 cells disappeared in the presence of PD-98059 (compare open and solid bars in groups 1′B and 2′B).
vitro competition studies using either poly-U and -C preparations or CFTR oligoRNA 1 and 2 and cytosolic extracts from cells treated with PD-98059 or SB-208580. Because treatment with the MAP kinase inhibitors and the in vitro competition experiments inhibited the effects of both TNF-α and IL-1β, Fig. 9 shows only the properties of unstimulated HT-29 and Calu-3 cell extracts, with the sole significant differences represented with bold lines. Inhibition of the p42/p44 MAPK pathway by PD-98059 (2 × 10⁻⁵ M) (groups 2 and 2') only suppressed the destabilizing effect of the extract from unstimulated Calu-3 cells (Fig. 9B, group 3', open bars) without changing the results of the competition experiments, either with poly-U/oligo 1 (Fig. 9, A and B, groups 2 and 2', striped bars) or poly-C/oligo 2 (Fig. 9, A and B, groups 2 and 2', dotted bars). Pretreating the cells with SB-203580 (2 × 10⁻⁵ M), which inhibits the effects of p38 MAPK activation (Fig. 9, A and B, groups 3 and 3'), decreased the CFTR mRNA stability found in the extracts from unstimulated HT-29 cells (Fig. 9A, group 3, open bar) and the stabilization induced by the competition with poly-U or oligo 1 in the extracts of Calu-3 cells (Fig. 9B, group 3', striped bars). Inhibiting p38 MAPK activation did not change the results of the competition experiments with poly-C/oligo 2 (Fig. 9, A and B, groups 3 and 3', dotted bars). However, binding to the CFTR U-repeats may also be controlled by activation of the p38 MAP kinase pathway, because the effect of the competition with poly-U and oligoRNA 1 was also inhibited in extracts from Calu-3 cells pretreated with SB-203580 (compare striped bars and open bar in group 3').

**DISCUSSION**

Modulation of CFTR gene expression takes place inside general cell responses to extracellular stimulations or to differentiation processes. Some transcriptional mechanisms of action of cytokines have been clearly established as the stimulation of the gene expression induced by IL-1β via NF-κB activation in Calu-3 cells (7, 9). In contrast, little is known of cytokine-induced posttranscriptional regulations like that of TNF-α acting in colon cells (3). Yet variations in CFTR mRNA stability might play an important role in the modulation of CFTR gene expression. This has been shown in the context of cell differentiation, during which the half-life of CFTR transcripts is controlled by complex reactions that involve intronic sequences of the gene DNase I-hypersensitive sites and HNF1 binding sites in intestinal cells (27), 5' ORFs in

![Fig. 9](http://ajpcell.physiology.org/)

**Fig. 9.** Relationships between involvement of the U- and C-repeats of the CFTR 3'UTR and the p42/p44 and p38 MAP kinase cascade activation in the control of CFTR mRNA stability by extracts of cells without any cytokine stimulation (“basal” extracts). HT-29 (A) and Calu-3 (B) cells were treated with the p42/p44 (groups 2 and 2') and p38 (groups 3 and 3') MAP kinase inhibitors, as indicated in Fig. 7. Their incubation with RNA and the competition with commercial poly-U and -C preparations (10 μg) and with oligoribonucleotides 1 and 2 (5 μg) were performed as indicated in Fig. 5. The results are expressed as percentages of the control value. Open bars, no competition; horizontal bars, presence of poly-U or oligo 1; and dotted bars, presence of poly-C or oligo 2. Each value is the mean ± SE of 5 determinations. *P < 0.02 for control vs. basal; **P < 0.05 for basal vs. cytokine, unpaired Student’s t-test.
rabbit heart (13). Other cis-elements in the 3' UTR of the CFTR mRNA may contribute to cell-specific variations of CFTR gene expression induced by extracellular stimuli. They are the U-repeats, and especially the AUUUA sequences and the C-repeats (38), which bind proteins or protein complexes able to control stability of a large number of mRNAs that encode diverse proteins involved in the same global cell responses to either inflammatory stimuli or disturbed hydroelectrolytic composition (for reviews, see Refs. 1, 4, 18, 25). The simultaneous regulation of the expression of these genes and proteins is a major element of cell-specific adaptation to a particular extracellular stress (21). The present research was performed to explore the physiological occurrence of this type of modulation of CFTR gene expression. To demonstrate the different posttranscriptional reactions occurring in the two cell lines, we have chosen to use “noninvasive” protocols, and particularly in vitro incubation of RNA and protein extracts rather than gene manipulations that can stimulate the MAP kinase cascades or alter their normal cross-talk, or both (11, 16). We are aware that the cell lines used may differ from the native cells, but they are classic models of CFTR-producing cells, and the changes in CFTR gene expression described herein may apply to other studies performed with them.

Measuring the content in CFTR mRNA of HT-29 and Calu-3 cells submitted to TNF-α and IL-1β first showed that both cytokines did not have the same effect in the two types of cells. The experiments performed in the presence of actinomycin D showed that differential modulation of CFTR mRNA stability participates in the cell specificity of the regulation of CFTR gene expression by the cytokines. Furthermore, the different responses to the cytokines proceeds from a difference between nonstimulated HT-29 and Calu-3 cells, with a significant relative unstability of CFTR mRNA in untreated Calu-3 cells. The result was confirmed by the different stability of the CFTR transcript found by the in vitro incubation of RNA with protein extracted from the two types of cells treated or not with either TNF-α or IL-1β in the absence of actinomycin D.

The short half-life of CFTR mRNA found in Calu-3 cells is intriguing, because these cells contain a large amount of CFTR mRNA. A link may exist between the activity of some transcription factor and mRNA decay, as suggested by the control of posttranscriptional reactions by the activation of MAP kinase cascades, which also stimulate diverse transcription factors, including NF-κB (24, 30), which enhances CFTR gene transcription (7, 9).

The posttranscriptional effects of the cytokines on the cells in the presence of actinomycin D were also confirmed by the in vitro experiments, because the cytosol proteins extracted from IL-1β-treated Calu-3 cells stabilized the CFTR mRNA, and those from TNF-α-treated HT-29 cells destabilized it. However, in both series of results, the intrinsic effect of 24-h incubation with either cytokine is equivalent in HT-29 and Calu-3 cells; after TNF-α treatment, ~30% of the original CFTR mRNA remains, whereas >80% remains after IL-1β treatment. The major difference is that the TNF-α effect is observed in HT-29 cells, in which CFTR mRNA is not degraded under basal conditions, whereas the IL-1β protective effect is only apparent in Calu-3 cells, against a strong basal RNAse activity. This suggests that TNF-α and IL-1β stimulate molecular processes controlling CFTR mRNA stability, which are differently activated under basal conditions in HT-29 and Calu-3 cells.

These molecular processes control CFTR mRNA stability by acting on its 3' UTR because no cytosol extract affected the stability of the short CFTR mRNA, whereas the stability of the long CFTR mRNA was modulated in the same manner as that of the natural CFTR mRNA of Calu-3 cells. The stability of the short CFTR mRNAs was confirmed by determining that their half-life in transfected HeLa cells incubated with actinomycin D was ~24 h (result not shown). The data obtained on mRNA transcribed from CFTR cDNA linked to a strong promoter does not rule out a possible control of the CFTR mRNA stability linked to the presence of 5'ORF in the CFTR gene (13), and the posttranscriptional control we describe may even be modulated by 5'UTR-linked processes. However, the short mRNA stability agrees with the already-observed (27) absence of any important regulatory cis-element in the CFTR exons, and this might be important to consider in any future studies on gene therapy with CFTR cDNA.

Most often, posttranscriptional regulation of gene expression based on the mRNA 3' UTR involve ARE sequences or U-repeats and C-repeats, and our data show that this is the case for CFTR, which possesses 3 ARE sequences in an environment rich in U and C in its 3' UTR. The loss of the properties of the cytosolic extracts observed when poly-U and -C are allowed to compete with the long CFTR mRNAs shows that the U-repeats and C-repeats in the CFTR 3'UTR are most important in the regulation of long CFTR mRNA stability. The in vitro competition experiments suggest that U-repeats favor the formation of destabilizing ribonucleoprotein complex(es), particularly active in Calu-3 cells under basal conditions and TNF-α-stimulated HT-29 cells, whereas C-repeats are involved in stabilizing CFTR mRNA in untreated HT-29 and IL-1β-treated Calu-3 cells. Destabilizing protein(s), such as AUF1 or tristetraprolin, which bind 3' ARE sequences (1, 4, 5, 8), and stabilizing poly-C-binding protein(s) (25, 28, 35) are, respectively, good candidates for the negative and positive posttranscriptional regulation of CFTR gene expression. However, competition with poly-U and -C added together suppressed all regulation of CFTR mRNA stability by any extract from both cell types. This suggests the existence of complex posttranscriptional reactions, and this would not be unusual. Many posttranscriptional regulations of gene expression are due to the simultaneous formation and synergic or competitive action of several ribonucleoprotein complexes bound to both ARE and poly-C sequences (1, 15, 37). The formation of such huge complexes may involve very large ribonucleotide sequences, and this may be the case for CFTR. Analysis of the CFTR 3'UTR showed that many U-repeats and two ARE sequences were present in its 215 more proximal bases, and many C-repeats in the following 140 bases. Data from the competition experiments with the corresponding oligoribonucleotides 1 and 2 pointed to the involvement of the CFTR 3'UTR proximal part, which contains the two ARE sequences, in the degradation process, and that of the following sequence rich in C-repeats in the protective process. However, the competition with the oligoribonucleotide 3 (the 353 first bases of the CFTR 3'UTR) appeared to be less effective on basal CFTR mRNA stability than the combination of poly-U and -C preparations, suggesting the participation of some more distal part of the CFTR 3'UTR.
Activation of MAP kinase cascades is a major regulator of mRNA stability and is linked to complexes built on the ARE or U/C-repeats in the 3′UTR. Most often, activation of the p38 MAP kinase cascade stabilizes mRNA, and this is the case, for example, of TNF-α (31) and COX-2 (31, 34) mRNAs. In contrast, the role of the p42/p44 ERK pathway is more complex. Its activation may inhibit (8, 19) or enhance (5) mRNA degradation because the ERK cascade may control either protective or degradative ARE-related hnRNP. In HT-29 and Calu-3 cells, CFTR mRNA stability is controlled by both p38 and p42/p44 MAPK cascades, being enhanced by p38 MAPK activation, and downregulated by the stimulation of the p42/p44 ERK pathway. In both cells, the main effects of TNF-α and IL-1β were, respectively, on p42/p44 and p38 MAPK. However, CFTR mRNA is stable in nonstimulated HT-29 cells, in which p38 MAPK phosphorylation is weak, and no effect of TNF-α or IL-1β can be observed, in either cell type, when either MAPK pathway is inhibited. These data strongly suggest that the control of CFTR mRNA stability results from a balance between p42/p44 and p38 MAPK activation. The effect of p42/p44 MAPK activation predominates in Calu-3 cells under basal conditions and in both cells stimulated with TNF-α. The effect of p38 MAPK activation predominates in basal HT-29 cells and in both cells under IL-1β treatment. Comparison of the results noted after treating the cells with MAPK cascade inhibitors, and by adding competitive ribonucleic sequences during the in vitro coinjections, suggests dominant relationships between the poly-U sequences of the proximal 3′UTR, p42/p44 ERK activation, and subsequent destabilization of CFTR mRNA on one hand, and poly-C repeats between the bases 161 and 350 of the 3′UTR, p38 MAPK kinase stimulation, and stabilization of CFTR mRNA on the other. In the absence of stimulation, CFTR mRNA stabilization dominates in HT-29 cells, and its destabilization dominates in Calu-3 cells. This difference may be responsible for the down- and upregulation of CFTR gene expression, respectively, observed under TNF-α and IL-1β treatment in HT-29 and Calu-3 cells.

The present study thus shows that cis-elements in the CFTR 3′UTR participate in the modulation of the CFTR mRNA stability mediated by cell-specific activation of MAP kinase cascades. Other molecular reactions are involved in the regulation of CFTR gene expression induced by TNF-α and IL-1β in HT-29 and Calu-3 cells, and the MAP kinase cascades react to all the experimental protocols that could individualize each pathway. Thus the relative functional importance of the posttranscriptional regulation described in the present study could not be precisely defined. The important result is that cis-elements of the CFTR 3′UTR modulate gene expression via “eucaryotic posttranscriptional operons” (21) because they may adapt to the cell-specific cytoplasmic transduction pathways and regulate the expression of various genes simultaneously. This posttranscriptional regulation mechanism may be an important element of the coordinated cell responses to various stimuli. Analytical exploitation of our results should lead to the identification of hnRNP involved in the control of CFTR gene expression. In epithelial colon cells, the posttranscriptional mechanisms are dominant in the downregulation of CFTR gene expression induced by TNF-α. In this case, the decreased CFTR gene expression contrasts with the increased synthesis of inflammatory cytokines also induced by TNF-α (via the activation of the κB transcription factor, which stimulates CFTR gene expression in other cells) but occurs together with the downregulation of the expression of other transporter genes (12). This suggests that destabilization of mRNAs is involved in the TNF-α-induced phenotypic differentiation of colon cells (12). The involved hnRNPs might also be activated during the physiological differentiation of colon epithelium (27). As for the stabilization of the CFTR mRNA induced by IL-1β in Calu-3 cells, it reinforces the transcriptional action of the cytokine. In cell responses to inflammatory stimuli, such posttranscriptional regulations have been shown to prolong various stimuli (15, 21). This might be the case for the IL-1β effect on secretory Calu-3 cells: the enhanced MUC-1 production provoked by IL-1β (17) may benefit from an increased CFTR expression for optimal secretion. Therefore, the present data show that complex cell- and stimulus-specific posttranscriptional regulations of CFTR gene expression play an important role in integrating CFTR in various cell functions.

GRANTS

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REFERENCES

CONTROL OF CFTR mRNA DEGRADATION BY TNF-α AND IL-1β


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