Cystic fibrosis, the most commonly inherited lethal pulmonary disorder in Caucasians, is caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR). To identify genomic responses to the presence or absence of CFTR in pulmonary tissues in vivo, microarray analyses of lung mRNA were performed on whole lung tissue from mice lacking (CFTR(-/-)) or expressing mouse CFTR (CFTR(+)). Whereas the histology of lungs from CFTR(-/-) and CFTR(+) mice was indistinguishable, statistically significant increases in the relative abundance of 29 and decreases in 25 RNAs were identified by RNA microarray analysis. Of RNAs whose expression was consistently altered by the absence of CFTR, functional classes of genes influencing gene transcription, inflammation, intracellular trafficking, signal transduction, and ion transport were identified. RNAs encoding the transcription factor CCAAT enhancer-binding protein (CEBP) δ and interleukin (IL) 1β, both known to regulate CFTR expression, were induced, perhaps indicating adaptation to the lack of CFTR. RNAs mediating lung inflammation including calgrulin-S100 family members, IL-1β and IL-4, were increased. Likewise, expression of several membrane transport proteins that interact directly with CFTR were increased, suggesting that CFTR protein complexes initiate genomic responses. Absence of CFTR influenced the expression of genes modulating diverse pulmonary cell functions that may ameliorate or contribute to the pathogenesis of CF.

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Transcriptional Adaptation to Cystic Fibrosis Transmembrane Conductance Regulator Deficiency*

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Cystic fibrosis (CF)1 is the most common lethal, inherited pulmonary disorder and is caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR) (1). More than 800 distinct mutations in the CFTR gene have been associated with clinical diseases characteristic of cystic fibrosis (2). Whereas many organs are affected in CF, morbidity and mortality in the disease is primarily related to mucus accumulation, recurrent infections, and excessive inflammation in the lung. Whereas the pathogenesis of CF is not fully understood, abnormalities in cyclic AMP-dependent chloride secretion and excessive sodium reuptake by epithelial cells related to CFTR deficiency are thought to alter fluid homeostasis at the airway surface liquid leading to its dehydration, impaired mucociliary clearance, and infection (see Ref. 3 for review). Because the elucidation of the primary structure of CFTR, a myriad of functions and numerous interactions with other cellular proteins have been ascribed to CFTR. Thus, in addition to the role of CFTR in the regulation of cAMP-dependent chloride transport, this protein may play pleotropic roles in many cellular processes by interacting with the cytoskeleton, membrane transport proteins, as well as receptors, protein routing and degradation machinery (2). A number of studies support the concept that the excessive inflammatory responses occur in the CF lung, but the mechanisms underlying these abnormalities have not been clarified. Changes in levels of IL-8 and other proteins mediating inflammatory signaling including NFκB and iNOS have been associated with CF, in the presence or absence of infection, raising the possibility that abnormalities in CFTR may constitutively alter pathways mediating inflammation (4–6).

In the lung, CFTR is distributed primarily in apical regions of airway and submucosal gland epithelial cells (7). Abundance and cellular sites of expression of CFTR are strongly influenced by developmental, spatial, and humoral factors, supporting the concept that the expression and function of CFTR are regulated at both transcriptional and post-transcriptional levels. Despite extensive study, the precise role of CFTR in the pathogenesis of CF disease remains poorly understood. At the clinical level, severity of CF disease is highly variable even among individuals bearing identical mutations, supporting the concept that environmental and hereditary factors may influence the severity of the disorder (2). These clinical observations, and observations demonstrating strain differences in the severity of CF phenotype after CFTR gene targeting or mutation in mice (8), support the concept that the expression of CFTR and its function in cellular processes may be influenced by many genes or pathways intensifying or mollifying CF disease in various organs. Morbidity and mortality in patients with CF is strongly associated with pulmonary disease caused by mucous accumulation, inflammation, and infection; however, deletion of CFTR mice does not cause significant pulmonary disease suggesting that expression of alternative channels or other complementary genes maintains pulmonary homeostasis in the mouse. Whereas numerous in vitro and in vivo models have been developed for study of CFTR, analysis of genomic responses to the presence or absence of CFTR are complicated by heterogeneity of cell models, and culture conditions that may influence

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† The abbreviations used are: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; IL, interleukin; iFABP, intestinal fatty acid-binding protein gene promotor; RT, reverse transcriptase; CEBP, CCAAT enhancer-binding protein; TNF, tumor necrosis factor; CSF, colony stimulating factor; Npr-3, natriuretic peptide receptor 3; ADRB, β-adrenergic receptor.
cell function and gene expression independently of CFTR. Direct RNA analysis of pulmonary tissue from humans with CF is complicated by the nearly ubiquitous, severe pulmonary infections that may secondarily modify cellular responses and gene expression, complicating identification of responses to CFTR in vivo.

In the present study, we undertook experiments to identify RNA influenced by the presence and absence of CFTR in vivo, seeking to identify genes and pathways that interact with or compensate for the CFTR to maintain pulmonary function. In this study, stereotypic genomic responses to the lack of CFTR were observed in pulmonary tissues in the absence of infection or disease.

MATERIALS AND METHODS

Transgenic mice bearing a null mutation in CFTR (CFTR−/−), generally succumb to intestinal disease in the weaning period (9). To generate healthy mice deficient in CFTR, the human CFTR cDNA was expressed in the intestinal epithelium under control of the intestinal fatty acid-binding protein gene promoter (iFABP), fully correcting small intestinal pathology and supporting normal postnatal survival of CFTR−/− mice (10). The iFABP-hCFTR, mCFTR−/− mice have been maintained in a mixed FVB/N, C57BL/6 background without evidence of intestinal or pulmonary disease for nearly a decade in our laboratory. Histological and biochemical studies identified no overt pathology in lung tissue from these mice compared with CFTR expressing littermates (10, 11). Mice were maintained in filtered microisolation cages. Sentinel mice were free of mouse pathogens. Lungs of adult iFABP-hCFTR, mCFTR−/−, and control mice were free of bacterial pathogens or colonization as assessed by quantitative culture of lung tissue from these mice compared with CFTR expressing littermates (10). To measure intestinal pathology and supporting normal postnatal survival of CFTR−/− mice, the percentage of pups that normally succumb to intestinal disease in the weanling period (9).

To further characterize the genetic effect of CFTR on lung function, complications in this colony, lung RNA was isolated from sex-matched littermates at 3 weeks of age. The iFABP-hCFTR transgene. All CFTR−/+ mice were heterozygous for the targeted mCFTR gene. Lungs from sex-matched littermates were carefully dissected and the conducting airways and mediastinal structures removed. RNA, cDNA synthesis, and microarray analysis were performed in pairs to minimize technical variability related to RNA and isolation and hybridization conditions. Lungs were homogenized in TRIzol reagent (Invitrogen) using a 10 mm glass homogenizer. Total RNA was purified from the homogenate using the following primers: primers for mCFTR PCR: forward primer (exon 9), 5′-CTTTCGCTCAACGCAGCCAG-3′; reverse primer, 5′-ACAGGTCGAGTGCCGGCA-3′; and hCFTR PCR: forward primer (exon 9), 5′-AAATCTCTAATGGTATGACAGAC-3′; and reverse primer (exon 11), 5′-AGAAAGAATTCTGCTGCTGAGCG-3′.

Validation of RNAs by RT-PCR

For these studies, lung RNAs were isolated as described above. cDNAs were generated by reverse transcription using oligo(dT) with T7 promoter sequences attached, followed by second strand cDNA synthesis. Antisense cRNA was then amplified and biotinylated using T7 RNA polymerase, prior to hybridization to the Affymetrix genechip mouse U74Av2 using the Affymetrix recommended protocol (12, 13). Affymetrix Microarray Suite version 5.0 was used to scan and quantitate the genechips using default scan settings. Intensity data were collected from each chip, scaled to a target RNA sensitivity of 1500, and the results were analyzed using both Microarray Suite and GeneSpring 5.0 (Silicon Genetics, Inc., Redwood City, CA).

cDNAs were hybridized to U74Av2 chips (Affymetrix Inc.). Hybridization data were normalized in a CCAAT enhancer-binding protein two-step process to remove or minimize systemic sources of variation at both chip and gene level. Specifically, each chip was normalized to the distribution of all genes on the chip to control for variation between samples. Each RNA from mCFTR−/− mice was normalized to its specific control (i.e., sex and age-matched mCFTR+/+ littermates). Data were further transformed into log ratio for analysis and symmetry of distribution.

Changes in mRNAs were identified by the combination of a distribution analysis (JMP4, SAS Institute, Inc.), and the Welch analysis of variance. Outlier box and quartile box plots were used to identify outliers with the definition of up-outlier > upper quartile + 1.5 (interquartile range), and the down-outlier < lower quartile − 1.5 (interquartile range). Significant changes were calculated by the Welch t test at p < 0.05. Adjusted p values were calculated by Westfall and Young permutational correction for false positives (GeneSpring 4.2.1, Silicon Genetics). Comparisons between genotype and age groups were performed using one-way analysis of variance. To identify genes that were differentially expressed because of CFTR genotype regardless of age, hierarchical, and k-means clustering were used to identify consistent changes in gene expression in response to the lack of CFTR at all three time points. Candidate RNAs were further filtered on the basis of reproducibility and absolute intensity. Mean, standard deviation, and coefficient variation were calculated for each replicate. Replicates with coefficient variations > 50% were deleted from analysis. Genes whose expression was below the level of detection were eliminated as experimental noise.

Validation and Literature Analysis

Selected genes were subjected to intensive search to identify biological function and associated regulatory pathways. U74Av2 annotation data base with system identifiers was constructed for all the array elements and their associated GenBank accession numbers. Gene description, functional categories, biological processes, molecular functions, cellular components, protein domain, and literature information were identified. Information resources included NetAffy (www.affymetrix.com), Source Search genome-www5.stanford.edu/cgi-bin/SMD/source), BLAST NCBI, Locus Link, mouse-human homolog search (www.ncbi.nlm.nih.gov), and Gene Ontology data base (www.godatabase.org/chi-bin/go.cgi). Differentially expressed genes were classified into functional categories based on the annotation and literature information. To determine the gene ontology, Gene Ontology data were normalized in a CCAAT enhancer-binding protein consensus definition. To determine which functional category is overrepresented in the given population (U74Av2). Potential protein/protein interactions were identified using the published literature information.

Lung Histology and Immunohistochemistry

Lungs from postnatal animals were inflation fixed with 4% paraformaldehyde at 25 cm H2O pressure via a tracheal cannula. Lung tissue was processed according to the methods and embedding in paraffin. Sections were stained with hematoxylin and eosin. Antibodies for Mac-3 staining were previously described (14). Rabbit monoclonal antibody against the 110-kDa Mac-3 antigen was used at 1:40,000 to identify alveolar macrophages (BD Pharmingen, San Diego, CA).

RESULTS

Histology and Immunohistochemistry—Lung histology in adult iFABP-hCFTR, CFTR−/−; iFABP-hCFTR, CFTR+/+; and CFTR+/+ control mice was not different (Fig. 1). There was no evidence of pulmonary inflammation, infection, or remodeling. Mac-3 staining was used to identify alveolar macrophages.
Numbers and histology of alveolar macrophages were not altered by mCFTR.

Identification of Gene Responses to the Lung CFTR Deletion—To identify genes responsive to CFTR, lung RNAs from iFABP-hCFTR, mCFTR(H11002), iFABP-hCFTR, mCFTR(H11001), mCFTR(H11002), and mCFTR(H11001) littermates at the age of 3, 6, or 11 weeks of age were compared. Microarray analyses were performed in duplicate from RNA isolated at 3 and 6 weeks of age. Data from 10 Affymetrix Murine Genome U74Av2 chips were normalized and statistical differences between CFTR-deficient (CFTR(H11002)) and control (CFTR(H11001)) mice were identified. Differences related to age were identified by outlier analysis and/or unpaired t test. After normalization, normal distributions were observed in the intensity data from lung tissue obtained at all ages. Lung RNA data from 3-week-old mCFTR(H11001) and mCFTR(H11002) mice (lacking the iFABP-hCFTR transgene) were similarly distributed to those bearing the FABP-hCFTR gene and were, therefore, included in the analysis.

To identify RNAs that were differentially expressed in response to CFTR regardless of age, mCFTR(H11002) and mCFTR(H11001) data were separated into two groups. The log-ratio distribution and outlier plot of the combined data set are represented by Fig. 2. A total of 1977 outliers were identified from 12442 genes/expressed sequence tags analyzed. The abundance of 848 RNAs was increased; 1129 were decreased. Welch t test together with the Westfall and Young step-down permutation further narrowed the number of differentially expressed RNAs to 315. Hierarchical clustering was used to visualize and classify the data set (Fig. 3). Data are shown in a two-dimensional matrix to identify groups of genes with similar expression patterns and show remarkably ordered expression profiles of 315 selected genes. On the chip level (top dendrogram) RNAs influenced by CFTR formed two distinct groups. Within each group, the samples collected from age-matched pairs were more closely related than those from different ages, suggesting that age also influenced gene expression. At the RNA level (the dendrogram at the left side), genes were clearly separated into two major groups: those mRNAs increased or decreased in mCFTR(−) mice. Genes were further filtered for the consistency of differences in expression levels across all time points (coefficient variation < 50%) and for their absolute intensity above 243 (90% of genes called absent by Affymetrix software, 243 for this data set). Additional filters reduced the number of RNAs to 54, of which 29 were consistently increased and 25 were decreased in mCFTR(−) compared with their mCFTR(+) littermates (Tables I and II). The expression profiles of these 54 genes are shown in Fig. 4, demonstrating consistent patterns of expression of the CFTR-responsive RNAs regardless of age.

**Fig. 1.** Normal lung histology in CFTR(−) mice. Lung tissue was obtained after inflation fixation from iFABP-hCFTR, mCFTR(−) mice (B and D) and iFABP-hCFTR, mCFTR(+) littermates (A and C) at 3 months of age. Lung histology after staining with hematoxylin-eosin (A and B) or Mac-3 antibody (C and D), a marker of alveolar macrophages, were not altered in the CFTR(−) mice. Bar = 200 μm.

**Fig. 2.** Distribution of lung RNAs from mCFTR(−) and mCFTR(+) mice. The left panel is a histogram of log ratio and gene frequency. The right panel is the outlier box plot. The ends of the dashed lines, denoted by an x and y markers, are the outliers identified from their respective quartiles.

**Fig. 3.** Two-dimensional hierarchical clustering of 315 genes/expressed sequence tag that were significantly altered in response to the presence or absence of mCFTR. Intensity in the red and green color range indicates up-regulated and down-regulated RNAs, respectively. Each row represents a single gene. Each column represents a particular experimental condition. Each box represents the normalized RNA intensity value. Clustering was performed by UP-GMA. Similarity measures were assessed utilizing Euclidean distance.
Differentially expressed genes were further classified according to their known or predicted functions. Each gene was annotated and assigned to a functional category. To simplify the calculation, we assumed that genes in each category could be fit to a binomial distribution. The binomial probability was calculated for each category using the entire U74Av2 as reference to a binomial distribution. The binomial probability was calculated for each category using the entire U74Av2 as reference to a binomial distribution. The binomial probability was calculated for each category using the entire U74Av2 as reference to a binomial distribution. The binomial probability was calculated for each category using the entire U74Av2 as reference to a binomial distribution. The binomial probability was calculated for each category using the entire U74Av2 as reference to a binomial distribution. The binomial probability was calculated for each category using the entire U74Av2 as reference to a binomial distribution. The binomial probability was calculated for each category using the entire U74Av2 as reference to a binomial distribution.
expression were related to CFTR and not to age or lung disease. Analysis of arrays prepared from pairs of mCFTR(−) mice and mCFTR(+) littermates (those lacking the iFABP-hCFTR transgene), confirmed the microarray findings, demonstrating both the lack of mCFTR mRNA in lungs of the mCFTR(−) and that RNA changes were independent of the iFABP-hCFTR transgene.

Expression of Genes Modulating CFTR—Expression of a number of genes known to influence CFTR expression was enhanced in the lungs of CFTR(−) mice, including CEBPβ and IL-1β suggesting that CFTR cells responded by enhancing levels of RNAs encoding transcription factors or pathways that may compensate for the lack of CFTR. IL-1β increased CFTR gene transcription in epithelial cells in vitro (15) and cis-acting elements binding CEBPβ (to CCAAT enhancer sites) and c-Fos (to AP-1 elements) are present in the promoter-enhancer region of the mCFTR gene (18). Like the S100 family of proteins, chitinase family members are a family of calcium-binding proteins termed the calgranulins (21). This family of peptides is expressed by alveolar macrophages in the lung (23). Thus, chitinase family members may compensate for the lack of CFTR. IL-1β mRNA was also consistently increased in the lungs of the mCFTR(−) mice (19). JAK-3, nuclear receptor subfamily 2, and interferon regulatory factor-1 RNAs were decreased in the mCFTR(−) mice. These RNAs encode transcription factors that regulate various pathways involved in inflammation and may, therefore, represent responses to the proinflammatory pathways induced in the CFTR(−) mice, in essence, being secondarily responsive to initial compensatory changes.

Enhanced Expression of Genes Modulating Inflammation—Genes involved in inflammation were overwhelmingly related to the increased expression of IL-1β and TNF. TNF-AIP-3 RNA was induced by either IL-1β or TNF. TNF-AIP-3 inhibited NFκB translocation in vitro, and may represent a compensatory response to the increased expression of IL-1β seen in the CFTR(−) mice (19). JAK-3, nuclear receptor subfamily 2, and interferon regulatory factor-1 RNAs were decreased in the mCFTR(−) mice. These RNAs encode transcription factors that regulate various pathways involved in inflammation and may, therefore, represent responses to the proinflammatory pathways induced in the CFTR(−) mice, in essence, being secondarily responsive to initial compensatory changes.

| Gene | Symbol | Ratio | p value | Category |
|------|--------|-------|---------|----------|
| SA rat hypertension-associated homolog | Sah | -4.44 | 8.96E-04 | Blood pressure regulation |
| Adenylate cyclase 4 | Adcy4 | -1.34 | 3.72E-02 | cAMP biosynthesis |
| Formin-like | Fml1 | -2.21 | 1.04E-03 | Cell growth |
| Insulin-like growth factor binding protein 2 | Igfbp2 | -1.23 | 6.99E-04 | Cell growth |
| Insulin-like growth factor binding protein 2 | Igfbp7 | -1.38 | 4.23E-02 | Cell growth |
| Procollagen, type XV | Col5a1 | -2.07 | 4.30E-02 | Collagen |
| RAD51-like 1 (S. cerevisiae) | Rad511 | -1.56 | 1.32E-02 | DNA recombination/cell growth |
| Neurofilament, heavy polypeptide | Nfh | -1.92 | 4.86E-05 | Intermediate filament |
| Adaptor protein complex-2, alpha subunit | Ap2a1 | -1.52 | 3.72E-02 | Intracellular protein trafficking |
| Kinesin family member 3a | Kif3a | -1.48 | 3.72E-02 | Intracellular protein trafficking |
| ADP-ribosylation factor 5 | Arf5 | -1.34 | 1.06E-02 | Intracellular protein trafficking |
| Lipase, hormone sensitive | Lipe | -2.34 | 1.82E-02 | Lipid catabolism/cell growth |
| Ki antigen; Psme3 gene for PA28γ subunit | Psme3 | -1.57 | 5.44E-06 | Protein degradation |
| Metallocarboxypeptidase 1 | Cpx1 | -3.60 | 4.4E-03 | Protein degradation |
| Yolk sac gene 2 | Ysg2 | -3.21 | 1.38E-02 | Protein degradation |
| Parathyroid hormone precursor (Pth) gene | Pth | -2.13 | 2.68E-03 | Signal transduction |
| Tryptophan-2,3-dioxygenase | Tdo2 | -2.01 | 2.15E-02 | Signal transduction |
| β-3-Adrenergic receptor | Adrb-3 | -2.01 | 1.31E-02 | Signal transduction |
| Janus kinase 3 | Jak3 | -1.49 | 1.24E-02 | Signal transduction |
| Nuclear receptor subfamily 2, group F, member 1 | Nrzf1 | -1.46 | 3.85E-03 | Signal transduction |
| Interferon regulatory factor 1 | Irf1 | -1.19 | 8.51E-03 | Transcription regulation |
| Cystic fibrosis transmembrane conductance regulator homolog | Cfr | -2.80 | 3.63E-07 | Transport |
| Mouse gap junction gene connexin 37 | Gja4 | -1.81 | 2.12E-02 | Transport/cell communication |
| DNA segment, Chr 4, ERATO Doi 13 | D4Rtd13e | -2.07 | 4.45E-02 | Unknown |
| RIKEN cDNA 2700022J23 gene | 2700022J23Rik | -1.45 | 3.72E-02 | Unknown |
In the present study, IL-1β was increased and each may contribute to the proinflammatory response. In the present microarray analysis, CFTR deficiency was associated with increased claudin 8 and decreased gap junction connexin 37. Decreased expression of gap junction proteins (Cx43, 40, -37, and -32) and decreased gap junction communication were observed in various in vitro cell systems after exposure to proinflammatory cytokines (28). The observed changes in RNAs mediating cell adhesion and increased expression of proinflammatory molecules, such as IL-1β, IL-4, and CSF-1 receptor RNAs encoding a receptor that mediates monocytic cell migration, proliferation, and activity in response to CSF-3 (G-CSF) was increased 3–4-fold in the CFTR(−) mice (25). Thus, taken together, expression of a number of genes; many influenced by IL-1β and mediating inflammation, were induced in the lungs of CFTR(−) mice.

Fig. 4. Expression profile chart (A) and hierarchical clustering (B) of 54 selected RNAs that were consistently altered in response to the lack of CFTR regardless of age. Hierarchical clustering was performed by UPGMA. Data were normalized using Trimmed Mean and Z-score calculations. The y axis is normalized intensity (log scale) and the x axis represents experimental ages. Red lines represent the profiles of down-regulated RNAs. Green lines represent the profiles of up-regulated RNAs.

Increased IL-8 and neutrophilic infiltrates were observed in bronchoalveolar lavage fluid from CF patients in the absence of documented pulmonary infection (4). Although it remains possible that antecedent, but resolved, infections may have contributed to the increased inflammation observed in the CF, these observations support the concept that CF is associated with increased susceptibility to pulmonary inflammation. In the present study, IL-1β, IL-4, and CSF-1 receptor RNAs were increased and each may contribute to the proinflammatory milieu. IL-1β enhances CFTR gene transcription, induces inflammation, and is known to stimulate production of the S100-calgranulins, perhaps indicating a network of genes influenced by CFTR through IL-1β. IL-4 is a potent inflammatory mediator that enhances inflammation and mucous production in airway epithelia. Transgenic animals expressing IL-4 or animal models in which IL-4 is induced developed severe goblet cell hyperplasia, increased mucous production, and inflammatory cell infiltrates (24), findings typically found in patients with cystic fibrosis. CSF-3r RNA encoding a receptor that mediates monocytic cell migration, proliferation, and activity in response to CSF-3 increased 3–4-fold in the CFTR(−) mice (25). Thus, taken together, expression of a number of genes; many influenced by IL-1β and mediating inflammation, were induced in the lungs of CFTR(−) mice.

Despite the increased expression of proinflammatory molecules, there is no evidence of inflammation in the lung of the CFTR(−) mice (2). Increased IL-8 and neutrophilic infiltrates were observed in bronchoalveolar lavage fluid from CF patients in the absence of documented pulmonary infection (4). Although it remains possible that antecedent, but resolved, infections may have contributed to the increased inflammation observed in the CF, these observations support the concept that CF is associated with increased susceptibility to pulmonary inflammation. In the present study, IL-1β, IL-4, and CSF-1 receptor RNAs were increased and each may contribute to the proinflammatory milieu. IL-1β enhances CFTR gene transcription, induces inflammation, and is known to stimulate production of the S100-calgranulins, perhaps indicating a network of genes influenced by CFTR through IL-1β. IL-4 is a potent inflammatory mediator that enhances inflammation and mucous production in airway epithelia. Transgenic animals expressing IL-4 or animal models in which IL-4 is induced developed severe goblet cell hyperplasia, increased mucous production, and inflammatory cell infiltrates (24), findings typically found in patients with cystic fibrosis. CSF-3r RNA encoding a receptor that mediates monocytic cell migration, proliferation, and activity in response to CSF-3 increased 3–4-fold in the CFTR(−) mice (25). Thus, taken together, expression of a number of genes; many influenced by IL-1β and mediating inflammation, were induced in the lungs of CFTR(−) mice.

Despite the increased expression of proinflammatory molecules, there is no evidence of inflammation in the lung of the CFTR(−) mice. Alternatively, the absence of CFTR in the alveolar macrophages may alter expression of genes mediating inflammation in those cells. It is of considerable interest that changes in RNAs modifying inflammation were altered in the lungs of mCFTR(−) mice in the absence of detectable bacterial infection or inflammation, supporting the concept that the transcriptional adjustment to CFTR deficiency suffices to maintain normal pulmonary homeostasis in the mouse in vivo. Alternatively, the levels of expression of the proinflammatory molecules may not be adequate to cause histologically detectable inflammation. It remains unclear whether these adjustments in gene expression may, in turn, render the CFTR(−) mice susceptible to inflammation following infection or injury.

Changes in NFκB- and TNF-α-dependent Pathways—RNAs encoding a number of proteins involved in TNF signaling and NFκB activation were also induced in the CFTR(−) mice. The abundance of TNF-AIP-3 mRNA, a zinc finger transcription protein, a protein whose expression is induced by both TNF-α and IL-1β, was increased in the CFTR(−) mice. TNF-AIP-3 inhibits NFκB activity at target genes (19) and may represent a response to the proinflammatory milieu established in the CFTR(−) lung. PEG-3, a protein that regulates the induction of NFκB following TNF stimulation, was also increased, providing for the support for transcriptional relationships between CFTR deficiency and activity of NFκB (26). Recent studies of Schroeder et al. (27) support the concept that the CFTR is required for regulation of NFκB, serving as a pattern-associated molecular recognition molecule, following pulmonary exposure to Pseudomonas aeruginosa. In that study, NFκB activation and its nuclear trafficking were deficient in CF cells. Taken together with the observation that IL-1β-mediated transcription of CFTR gene transcription is dependent upon NFκB, this important pathway mediating inflammation appears to be influenced by CFTR.
pression of IL-1β seen in the CFTR(−) mice are consistent with findings that in the absence of CFTR, IL-1β and TNF-α failed to inhibit cell communication via gap junctions. Previous studies demonstrated that CFTR is required for the uncoupling of gap junctions between epithelial cells during inflammation, a process that may restrict the spread of pathogens or signaling among adjacent cells. It will be of interest to test whether cell adhesion mediated by the claudins is also linked to the pathogenesis of CF.

Changes in Protein Degradation Pathways—Several genes involved in protein degradation were altered in the absence of CFTR compared with normal. Proteosome 26 S subunit (PSMC5) is the major proteolytic component of the ubiquitin-dependent proteosome. Proteosome 26 S regulates degradation of proteins influencing cell cycle, oncogenesis, transcription, and immunity, including CFTR itself (29, 30). The proteosome is composed of two subcomplexes, the 20 S proteosome and PA700. PSMC3 expression was modestly, but significantly increased (1.5-fold) in the absence of CFTR. In contrast, PA28γ (PSME3), an activator of the 20 S proteosome, was decreased 1.6-fold. These observations are consistent with previous findings that proinflammatory cytokines, including TNF-α or interferon-γ increased expression of the 26 S proteosome and its activators PA28α and β (31), whereas expression of PA28γ was decreased by interferon-γ (32). RNA encoding adaptor protein complex AP-2, α1 subunit (Ap2a1), a protein involved in the formation of intracellular transport vesicles was also decreased in the absence of CFTR. CFTR co-precipitates with α-adaptin (33). Recent studies demonstrated that a C-terminal domain of CFTR binds to the AP-2 adaptor complex to form clathrin-coated vesicles that mediates CFTR internalization (34). ADP-ribosylation factor 5 belongs to a family of GTP-binding proteins that play important roles in the control of membrane trafficking, including formation of secretory vesicles at the trans-Golgi network, endosomal and vesicle-plasma membrane fusion (35). Recent studies support the concept that CFTR regulates endosomal fusion and vesicular trafficking (36, 37) indicating potential relationships between CFTR and the actions of ADP-ribosylation factor 5. Another gene in this functional category is represented by kinesin3α, an mRNA that was decreased in the lungs of CFTR(−) mice.

Transport Proteins Influenced by CFTR—RNAs encoding several transmembrane transport proteins and receptors were also altered in the lungs of CFTR(−) mice, including solute carrier 38 (member 4), the potassium inwardly rectifying channel (Kir 4.2 or Kcnj15), the glutamate receptor (Grin 2d), the natriuretic peptide receptor 3 (Npr3), and the β3-adrenergic receptor (ADRB3). Thus, expression of a number of membrane transport proteins was influenced by CFTR, perhaps representing compensatory responses to defects in CFTR-mediated transport activity. Kir 4.2 and Grin 2d RNAs were increased 2–3-fold in CFTR(−) mice. Kir 4.2 is expressed in respiratory epithelial cells at sites similar to that of CFTR (38). Kir 4.2 regulates cation transport upon which chloride transport via CFTR or other chloride channels may be influenced. Surprisingly, there is evidence for interactions between CFTR and Kir family members because they are both known to bind via PDZ binding domains to channel interacting PDZ domain protein (39). Likewise, there is precedence for PDZ-dependent interactions among glutamate receptors, CFTR, and Kir family members (40). Thus, the lack of CFTR enhanced the expression of a number of membrane proteins that may interact with CFTR via PDZ domains, perhaps indicating that CFTR-protein complexes may initiate changes in gene expression. These findings support the concept that CFTR interacts with numerous membrane transport proteins and do not support a model in which activity of an alternative Cl− transporter alone suffices to compensate for the lack of CFTR in pulmonary cells in the CFTR(−) mice.

Regulation of Cell Receptors by CFTR—Natriuretic peptide receptor C (Npr-3 or NprC) was increased more than 2-fold in CFTR(−) mice. Natriuretic peptides comprise a family of 3 structurally related molecules: atrial (ANP), brain (BNP), and C-type (CNP) whose functions are cGMP-dependent (41, 42). Among them, CNP increased ciliary beat, mucociliary clearance in airway epithelial cells, and activated CFTR-dependent chloride transport (42). Natriuretic peptides regulate cytokine-stimulated NO production via the binding of Npr-3 (43). Because deficient NO production was observed in respiratory epithelial cells of the iFABP-hCFTR, CFTR(−) mice, the increased expression of Npr-3 in CFTR(−) mice may represent a compensatory response influencing airway clearance and nitric oxide production via cGMP (44). In contrast to the transport/receptors that were induced, ADRB3, a G protein-coupled transmembrane protein mediating cAMP production was decreased in CFTR(−) mice. ADRB3 is co-expressed with CFTR in airway epithelium (45) and may be functionally coupled to CFTR via cAMP-independent pathways (46). β2-Adrenergic receptors directly interact with CFTR via the Na+(+)/H+(+) exchanger regulatory factor to form a signal transduction complex (47). Co-regulation of ADRB3 and CFTR may indicate that these proteins interact closely at both structural and functional levels, a finding that may be linked to the important role of β-adrenergic stimulation and cAMP in the activation of chloride transport mediated by CFTR.

Altered Expression of RNAs Encoding CFTR Interacting Proteins—Surprisingly, analysis of the RNA influenced by CFTR identified a number of proteins that directly or indirectly interact with CFTR via protein-protein interactions. This list included proteins involved in protein trafficking and degradation (proteosome 26 S and PA28 subunits and α-adaptin), ion transport (Kcnj15 and Grin 2d), and receptors (Npr-3 and ADRB3). Interaction of CFTR with many proteins occurs via PDZ binding domains that mediate protein-protein complex formation. The finding that expression of CFTR interacting proteins is altered in the lungs of CFTR(−) mice, suggests that CFTR influences networks of signaling and transport activities in the cell and that cells respond to CFTR deficiency via transcriptional responses to CFTR-protein complexes, rather than to CFTR per se.

Are Changes in Gene Expression Bystander Effects?—Whereas genomic responses may compensate for the lack of CFTR mRNA and represent compensation for CFTR function, some responses may be secondary and mediated by pathways not directly related to the action of CFTR per se. Alterations in
Lung RNA samples from iFABP-hCFTR, CFTR(-) (i.e. gut corrected, GC) were compared with those from CFTR(+) (lacking the iFABP-hCFTR transgene, i.e. gut uncorrected, NGC). Ratio is defined as: R = GC/NGC, if (GC > NGC); R = −NGC/GC if (GC < NGC).

| Gene | p value | Ratio | Common | GeneBank™ | BioProcess |
|------|---------|-------|--------|-----------|-----------|
| Transmembrane 9 superfamily member 2 | 0.0381 | −1.13 | Tmn9f2 | NM_080556 | Transport |
| Mitochondrial ribosomal protein S24 | 0.0353 | −1.19 | Mrps24 | AA543858 | Unknown |
| Oncostatin receptor | 0.0257 | −1.26 | Osmr | NM_011019 | Signal transduction |
| RIKEN cDNA 4432409D24 gene | 0.0257 | −1.17 | 432409D24AK | AK014481 | Unknown |
| RIKEN cDNA 5730533P17 gene | 0.0257 | −1.32 | 5730533P17Rik | NM_027942 | Unknown |
| Expressed sequence A1323512 | 0.0257 | −1.22 | A1323512 | Unknown |
| Nuclear factor I/B | 0.0257 | −1.17 | Nfil | NM_008687 | Transcription regulation |
| Cold shock domain protein A | 0.0258 | −1.125 | CsdA | NM_139117 | Transcription regulation |
| Aldehyde dehydrogenase 9, subfamily A1 | 0.0258 | −1.25 | Aldh9a1 | NM_019993 | Oxidoreductase |
| Heat shock protein, 105 kDa | 0.0476 | −1.40 | Hsp105 | NM_013559 | Heat shock response |
| RIKEN cDNA 2610318I15 gene | 0.0258 | −1.14 | 2610318I15Rik | AK012045 | Serine/threonine kinase |
| Caspase 3, apoptosis related cysteine protease | 0.0476 | 1.13 | Casp3 | NM_009610 | Apoptosis |
| Ubiquitin-conjugating enzyme 8 | 0.0257 | 1.68 | Ubc8 | NM_019949 | Protein modification |
| T-cell receptor α chain | 0.0257 | 1.49 | TcrA | U07662 | Cellular defense response |
| Protein tyrosine kinase 2β | 0.0257 | 1.31 | Ptk2b | BF579309 | Kinase |
| Thymidine DNA glycosylase | 0.0257 | 1.17 | Tdg | NM_011561 | DNA repair |
| Caspase 7 | 0.0238 | 1.23 | Casp7 | NM_007611 | Apoptosis |
| Chemokine (C-C) receptor 7 | 0.0238 | 1.47 | Cmkbr7 | NM_007179 | Chemotaxis |

**Fig. 5.** Real time PCR analysis of Grin 2d (A), Kir4.2 (B), CEBPδ (C), and TNF-AIP-3 (D). Lung mRNA abundance was determined by RT-PCR using a Light Cycler™ in tissue from mCFTR(-) (open) and mCFTR(+) (black) mice (black). Values were normalized to β-actin or glyceraldehyde-3-phosphate dehydrogenase RNAs and represent mean ± S.E., n = 56 determinations and differences were assessed by paired t test.

Cytokine production or changes in transcription factor activity caused by the absence of CFTR may secondarily change the behavior of numerous cell types, that may, in turn, contribute to the pathogenesis of CF lung disease by processes that are not direct functions of CFTR. Because CFTR is known to form a membrane-associated cyclic nucleotide-activated Cl− channel and interacts with numerous other cellular proteins, changes in gene expression may indicate that cells sense the CFTR protein, CFTR-dependent activity, or CFTR-protein complexes that initiate transcriptional responses related to or unrelated to CFTR function. At present, it is unclear whether the “sensor” is chloride ion, the presence of CFTR, or CFTR-protein complexes in intracellular and membrane compartments or to other functions of CFTR. Likewise, the hierarchy and interrelatedness of the networks of RNA responses to CFTR deficiency remain to be unraveled.

Maintenance of pulmonary homeostasis in the mCFTR(-) mouse was associated with complex adaptive responses in gene expression. CFTR influenced RNAs encoding transcription factors, ion channels, membrane receptors, cytokines, and intracellular trafficking proteins. Finally, CFTR altered the expression of a number of proteins that interact with CFTR via protein-protein interactions perhaps representing transcriptional responses to functions mediated by CFTR(-) protein.
complexes (Fig. 6). The diversity of genes whose expression was altered by CFTR support the concept that, in addition to regulation of Cl⁻ transport, CFTR plays diverse roles in multiple cellular functions. The present findings support the hypothesis that pulmonary homeostasis in the CFTR⁻/⁻ mouse is maintained by complex genomic responses to the lack of CFTR rather than by the action of a single alternative Cl⁻ channel. Finally, the genes and pathways identified in this study provide new links between CFTR and cellular processes that may influence the pathogenesis of CP lung disease.

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