CFTR Cl^- Channel Function in Native Human Colon Correlates With the Genotype and Phenotype in Cystic Fibrosis

STEPHANIE HIRTZ,* TANJA GONSKA,* HANS H. SEYDEWITZ,* JÖRG THOMAS,* PETER GREINER,* JOACHIM KUEHR,* MATTHIAS BRANDIS,* IRMGARD EICHLER,† HERCULANO ROCHA,§ ANA–ISABEL LOPES,‖ CELESTE BARRETO,‖ ANABELA RAMALHO,¶ MARGARIDA D. AMARAL,¶,# KARL KUNZELMANN,** and MARCUS MALL*,††

*Department of Pediatrics and Adolescent Medicine, Albert Ludwigs University, Freiburg, Germany; †Department of Pediatrics and Adolescent Medicine, University of Vienna, Vienna, Austria; §Hospital Maria Pia, Porto, Portugal; ‖Department of Pediatrics, Hospital de Santa Maria, Lisbon, Portugal; ¶Centre of Human Genetics, National Institute of Health Dr. Ricardo Jorge, Lisbon, Portugal; #Department of Chemistry and Biochemistry, Faculty of Sciences, University of Lisbon, Lisbon, Portugal; **Institute of Physiology, University of Regensburg, Regensburg, Germany; and the ††Cystic Fibrosis/Pulmonary Research and Treatment Center, School of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina

Background & Aims: **Cystic fibrosis (CF) is caused by over 1000 mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene and presents with a widely variable phenotype. Genotype-phenotype studies identified *CFTR* mutations that were associated with pancreatic sufficiency (PS). Residual Cl^- channel function was shown for selected PS mutations in heterologous cells. However, the functional consequences of most *CFTR* mutations in native epithelia are not well established.** ***Methods:*** **To elucidate the relationships between epithelial CFTR function, *CFTR* genotype, and patient phenotype, we measured cyclic adenosine monophosphate (cAMP)-mediated Cl^- secretion in rectal biopsy specimens from 45 CF patients who had at least 1 non-ΔF508 mutation carrying a wide spectrum of *CFTR* mutations. We compared *CFTR* genotypes and clinical manifestations of CF patients who expressed residual CFTR-mediated Cl^- secretion with patients in whom Cl^- secretion was absent.** ***Results:*** **Residual anion secretion was detected in 40% of CF patients, and was associated with later disease onset ($P < 0.0001$), higher frequency of PS ($P < 0.0001$), and less severe lung disease ($P < 0.05$). Clinical outcomes correlated with the magnitude of residual CFTR activity, which was in the range of ~12%–54% of controls.** ***Conclusions:*** **Specific *CFTR* mutations confer residual CFTR function to rectal epithelia, which is related closely to a mild disease phenotype. Quantification of rectal CFTR-mediated Cl^- secretion may be a sensitive test to predict the prognosis of CF disease and identify CF patients who would benefit from therapeutic strategies that would increase residual CFTR activity.**

Cystic fibrosis (CF) is the most frequent, lethal, autosomal-recessive disease in the Caucasian population and is caused by more than 1000 different mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene[1,2] (cystic fibrosis mutation database: https://www.genet.sickkids.on.ca/cftr/). In part as a consequence of this wide spectrum of *CFTR* mutations, the clinical presentation of CF varies widely from monosymptomatic disease to multiorgan involvement.[2–4]

CFTR encodes a cyclic adenosine monophosphate (cAMP)-regulated Cl^- channel expressed in the apical membrane of many epithelia.[2,5–7] Biochemical and functional studies in heterologous cells identified several molecular mechanisms by which *CFTR* mutations can disrupt CFTR Cl^- channel function, including defects in protein production (class I), processing (class II), Cl^- channel regulation (class III), altered single-channel properties (class IV), and decreased abundance of CFTR Cl^- channels (class V).[4,8–11] The mechanisms by which *CFTR* mutations can reduce the number of functional membrane Cl^- channels are complex and include incomplete processing defects[12,13] and alternative splicing with reduced levels of full-length *CFTR* messenger RNA.[14] Based on these studies it was predicted that class I–III mutations result in a lack of functional CFTR Cl^- channels at the cell surface, whereas class IV and V mutations may retain significant residual function.

Abbreviations used in this paper: cAMP, cyclic adenosine monophosphate; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; FEV_1, forced expiratory volume in 1 second; Isc, short-circuit current; IBMX, 3-isobutyl-1-methylxanthine; PCR, polymerase chain reaction; PI, pancreatic insufficiency; PS, pancreatic sufficiency; R_{te}, transepithelial resistance; V_{te}, transepithelial voltage.

0016-5085/04/$30.00
doi:10.1053/j.gastro.2004.07.006

Previous genotype-phenotype correlation studies established a close association between the *CFTR* genotype and exocrine pancreatic function and showed that specific mutations were related closely with pancreatic sufficiency (PS).[15] Furthermore, a recent study identified a panel of *CFTR* mutations that were associated with mild clinical manifestations and lower mortality.[16] Based on these results and on functional studies of a limited number of PS mutations in heterologous cells, it was hypothesized that PS reflects residual CFTR function.[10,12,13] However, information on the relationship between CFTR function in native epithelia, the CF genotype, and the clinical phenotype is limited. For many *CFTR* mutations, detailed clinical and/or functional data are not available and it is not known what level of CFTR activity is required to ameliorate disease severity. Furthermore, functional properties in heterologous cells transfected with mutant CFTR may not always reflect the in vivo situation in which endogenous CFTR is expressed at low levels in native epithelial cells.

Several previous studies used nasal potential difference measurements to examine the relationship between Cl^- secretory responses in a native epithelium, *CFTR* mutations, and clinical features of CF. These studies, however, produced conflicting results. Although one study reported differences in mean nasal Cl^- conductance in patients carrying at least 1 mild CF allele compared with patients with 2 severe CF alleles,[17] other studies were not able to reproduce these findings. Instead, these groups reported either a lack of residual Cl^- conductance in patients with mild *CFTR* mutations,[18] or detection of residual Cl^- secretory responses in both groups.[19] In part, the difficulty in detecting residual CFTR-mediated Cl^- secretion in nasal epithelia from individual patients with mild *CFTR* mutations may reflect the relatively low levels of CFTR expression in the nose (e.g., compared with the colon[20]). Further, alternative Ca^{2+}-dependent Cl^- channels expressed in nasal epithelia may contribute to Cl^- secretory responses in this tissue, which may explain why these studies did not find a correlation between nasal Cl^- secretory responses, *CFTR* mutation type, and clinical status.[17–19]

We previously developed a sensitive functional protocol for detection of CFTR-mediated Cl^- secretion in small human rectal biopsy specimens.[21,22] In the present study, we used this approach to elucidate further the relationship between CFTR function in native human colon, genotype, and phenotype in CF. CF patients who have at least 1 non-ΔF508 mutation were chosen for this study based on the consideration that clinical genotype-phenotype studies estimated that ~15%–20% of CF patients express mild CFTR mutants that confer PS.[2,15,23] Approximately 44% of CF patients are ΔF508 homozygous, present with severe pancreatic insufficiency (PI), and lack CFTR function and mature protein in native colon.[2,4,15,20] Accordingly, it is expected that ~30%–40% of CF patients who are not ΔF508 homozygous carry at least 1 mild *CFTR* mutation, allowing us to compare functional properties of mild vs. severe *CFTR* mutations. We determined CFTR function from cAMP-dependent and cholinergic Cl^- secretion across freshly excised rectal biopsy specimens from CF patients carrying a wide spectrum of *CFTR* mutations, and compared disease severity in CF patients who expressed residual CFTR Cl^- channel function and CF patients in whom Cl^- secretion was absent.

Materials and Methods

Patients

The study was approved by the Ethical Committee at the University Hospitals of Freiburg, Lisbon, and Vienna, and all patients gave their written informed consent. For children under 18, parents obtained detailed information and gave their signed informed consent. Forty-five CF patients who carry at least 1 non-ΔF508 mutation (29 PI, 16 PS; mean age, 16.5 $\pm$ 1.8 years; range, 5 months–47 years), 19 age-matched healthy controls (15.6 $\pm$ 3.7 years; range, 10 months–55 years), and 13 age-matched ΔF508 carriers (15.7 $\pm$ 5.5 years; range, 15 months–65 years) were enrolled in the study between 1997 and 2004 at the Children's Hospitals at the Universities of Freiburg, Lisbon, and Vienna. The diagnosis of CF was established by clinical signs of disease, increased sweat Cl^- concentrations ($\geq$60 mmol/L), and/or detection of 2 *CFTR* mutations.[24] In all control subjects, sweat Cl^- levels were normal ($<$30 mmol/L) and genetic screening for 20 of the most common *CFTR* mutations was negative (Elucigene CF20 kit; Cellmark, Abingdon, UK). At the time of the study, medical records of all CF patients were evaluated for weight, height, age at diagnosis, pancreatic function, and lung function measurements. The nutritional status was assessed by determining weight-for-height percentiles.[25] Shwachman–Kulczycki clinical scores were determined as described.[26] PI was defined by a history of malabsorption and fecal elastase E1 levels of $<$200 μg/g stool on 2 or more occasions. Elastase concentrations of $<$100 μg/g were considered as severe PI and concentrations between 100 and 200 μg/g were considered as moderate PI.[27] The most recent measurements of forced vital capacity and forced expiratory volume in 1 second (FEV_1) were evaluated from CF patients who were $>$6 years of age, and expressed as a percentage of predicted normal values for sex, age, and height.

Ussing Chamber Experiments

Superficial rectal mucosa specimens (~2–3 mm in diameter) were collected by forceps and immediately

stored in ice-cold buffer solution composed of the following (mmol/L): NaCl 127, KCl 5, D-glucose 5, $MgCl_2$ 1, Na-pyruvate 5, HEPES 10, $CaCl_2$ 1.25, and albumin (10 g/L). Rectal biopsy specimens were mounted in modified micro-Ussing chambers as previously described.[21,22] In brief, the luminal and basolateral surfaces of the epithelium were perfused continuously with a solution of the following composition (mmol/L): NaCl 145, KH_2PO_4 0.4, K_2HPO_4 1.6, D-glucose 5, $MgCl_2$ 1, Ca-gluconate 1.3, pH 7.4, at 37° C. HCO_3^--free buffer solutions were used to exclude a possible contribution of CFTR-independent electrogenic HCO_3^- secretion, which would be indistinguishable from electrogenic Cl^- secretion and thus may mimic residual Cl^- channel function in CF tissues.[20] Experiments were performed under open-circuit conditions. Values for the transepithelial voltage (V_{te}) were referenced to the serosal surface of the epithelium. Transepithelial resistance (R_{te}) was determined by applying intermittent (1 s) current pulses ($\Delta I = 0.5$ μA). The equivalent short-circuit current (Isc) was calculated according to Ohm's law from V_{te} and R_{te} ($Isc = V_{te}/R_{te}$) after appropriate correction for fluid resistance.

Experimental Protocol and Analysis of Ussing Chamber Experiments

Tissues were equilibrated for 60 minutes in the presence of indomethacin (10 μmol/L, basolateral) to inhibit endogenous cAMP formation. Amiloride (10 μmol/L, luminal) also was added to block electrogenic Na^+ absorption. To determine the magnitude of CFTR-mediated Cl^- secretion, we measured cAMP-induced lumen-negative (Cl^- secretory) responses after exposure to 3-isobutyl-1-methylxanthine ([IBMX] 100 μmol/L, basolateral) and forskolin (1 μmol/L, basolateral). In normal human colon, CFTR-mediated Cl^- secretion is augmented by cholinergic co-activation, which leads to stimulation of basolateral K^+ channels that increase the electrical driving force for luminal Cl^- exit via CFTR.[21] In CF tissues expressing *CFTR* loss of function mutations, cholinergic activation fails to induce Cl^- secretion and results in inverse lumen-positive responses, reflecting K^+ secretion that is unmasked in the absence of anion secretion.[7,22] To increase the driving force for Cl^- secretion by *CFTR* mutants that retain residual Cl^- channel function, we determined the responses to carbachol (100 μmol/L, basolateral) after stimulation with IBMX/forskolin. Three different response patterns were observed after stimulation with carbachol and analyzed as follows: (1) for monophasic lumen-positive (K^+ secretory) responses we determined the peak and the plateau, (2) for monophasic lumen-negative (Cl^- secretory) responses we determined the negative peak, and (3) for biphasic responses we determined the positive peak and the maximal negative deflection. To control for sample-to-sample variability, bioelectric measurements were performed on 2–5 biopsy specimens per individual, and data were averaged to obtain a single value for each individual. In most cases, Ussing chamber experiments were performed before genotyping (see later) (i.e., investigators were blinded for the genotype).

CFTR Genotyping

Screening for *CFTR* mutations was performed by allele-specific polymerase chain reaction (PCR)[28] (Elucigene CF20 kit; Cellmark) and denaturing gradient gel electrophoresis.[29] To detect *CFTR* mutations that were not identified by screening, we performed automatic DNA sequencing of all 27 exons of the *CFTR* gene. Exons and bordering intron sequences were amplified by PCR from genomic DNA as previously described,[30] with few modified primer pairs leading to shorter PCR products for exons 1, 2, 4, 9, 14a, and 21. PCR products were purified (QIAquick PCR purification kit; Qiagen, Hilden, Germany) and sequenced using the ABI Prism BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany). Homozygosity was confirmed by genotyping of parents.

Chemicals and Compounds

Amiloride, indomethacin, carbachol, IBMX, and forskolin all were obtained from Sigma (Deisenhofen, Germany). All chemicals used were of the highest grade of purity available.

Statistics

Unless otherwise stated, data are shown as mean ± SEM (n = number of individuals studied). Statistical analyses were performed using paired and unpaired Student t tests, the Mann–Whitney rank sum test, χ^2 test, or the Kruskal–Wallis 1-way analysis of variance on ranks, followed by the Dunn multiple comparison test as appropriate. The Pearson correlation coefficient was calculated between cAMP-mediated Isc responses and clinical outcomes. All P values are 2-tailed and $P < 0.05$ was accepted to indicate statistical significance.

Results

Assessment of *CFTR* Cl^- Channel Function

CFTR function was measured as cAMP-mediated Cl^- secretion in freshly excised rectal biopsy specimens from 45 CF patients who have at least 1 non-ΔF508 mutation, 19 age-matched controls, and 13 ΔF508 carriers. The magnitude of CFTR-mediated anion secretion was assessed after inhibition of endogenous cAMP formation by indomethacin (10 μmol/L, basolateral, 60 min) and electrogenic Na^+ absorption by amiloride (10 μmol/L, luminal). Under these conditions, V_{te} and Isc approached 0 and R_{te} was similar in control and CF tissues (control: $V_{te} = -0.2 \pm 0.0$ mV; $Isc = -7.6 \pm 1.1$ μA/cm^2; $R_{te} = 24.9 \pm 3.0$ Ωcm^2, $n = 19$; vs. CF: $V_{te} = 0.0 \pm 0.0$ mV, $Isc = -0.8 \pm 1.4$ μA/cm^2; $R_{te} = 23.9 \pm 1.8$ Ωcm^2, $n = 45$), showing that electrogenic ion transport was largely inhibited. Next, cAMP-dependent Cl^- secretion was activated with IBMX and forskolin (100 μmol/L and 1 μmol/L, basolateral). Based on bioelectric responses to IBMX/forskolin, 2 groups of CF

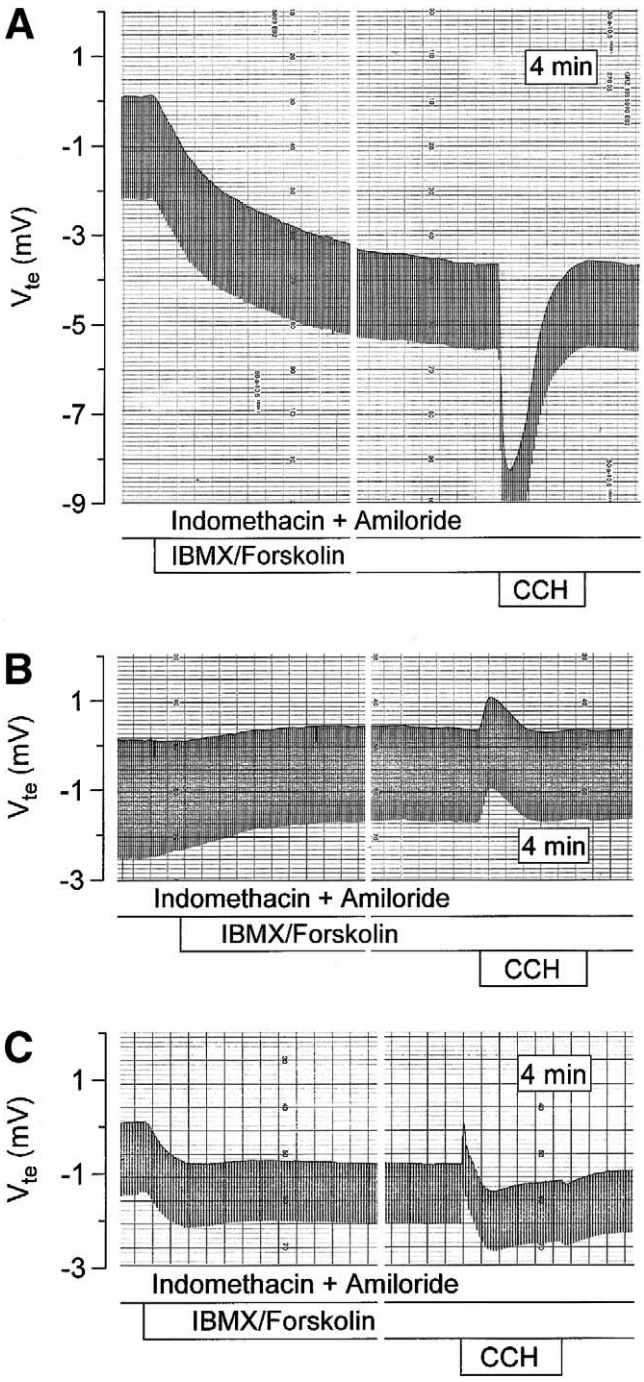

Figure 1. Original recordings of the effects of cAMP-dependent (100 μmol/L IBMX and 1 μmol/L forskolin, basolateral) and cholinergic (100 μmol/L carbachol and CCH, basolateral) activation on V_{te} and R_{te} in rectal tissues from (*A*) a control subject, (*B*) a CF patient with no detectable Cl^- secretion (R1162X/Q552X), and (*C*) a CF patient expressing residual Cl^- secretion (S1159F/S1159F), as evidenced by lumen-negative V_{te} responses. Experiments were performed in the presence of indomethacin and amiloride. R_{te} was determined from V_{te} deflections obtained by pulsed current injection.

patients could be distinguished. In the first group, cAMP-induced Cl^- secretion was absent (CF_{absent}); in the second group, we detected residual cAMP-induced Cl^- secretion ($CF_{residual}$). In control tissues, IBMX/forskolin induced a large lumen-negative Cl^- secretory response ($\Delta Isc = -94.3 \pm 8.6$ μA/cm^2, $n = 19$, $P < 0.0001$) (Figures 1*A* and 2). The mean difference in Cl^- secretory responses between rectal tissues from ΔF508 carriers and controls was not significant (Figure 2). (Data from 8 subjects in the group of ΔF508 carriers were included in a previous report.[20]) The CF_{absent} group ($n = 27$) was characterized by small lumen-positive responses to IBMX/forskolin ($\Delta Isc = 9.3 \pm 1.7$ μA/cm^2, $n = 27$, $P < 0.0001$) (Figures 1*B* and 2) that reflected K^+ secretion, which was revealed when CFTR-mediated Cl^- secretion was absent.[22] In contrast, in the $CF_{residual}$ group ($n = 18$), IBMX/forskolin induced sustained Cl^- secretory responses ($\Delta Isc = -28.1 \pm 2.9$ μA/cm^2, $n = 18$, $P < 0.0001$) of the mean magnitude of 29.8% ± 3.0% of control tissues (Figures 1*C* and 2). Interestingly, in a subgroup of CF patients ($n = 7$), we detected high residual Cl^- secretory responses in the range of 36%–54% of controls (Table 1, Figure 2).

Our previous studies showed that co-activation with cholinergic agonists increased the driving force for CFTR-mediated Cl^- secretion in non-CF colonic tissues.[7,21,22] To increase the sensitivity and assess for small residual Cl^- currents that may be

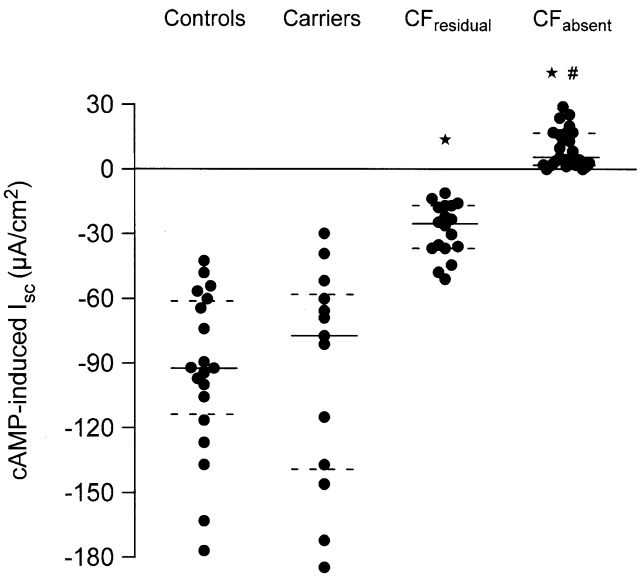

Figure 2. Summary of cAMP-induced (IBMX/forskolin) Isc in native rectal epithelia from controls, ΔF508 carriers, CF patients with residual Cl^- secretion ($CF_{residual}$), and CF patients with no detectable Cl^- secretion (CF_{absent}). All experiments were performed in the presence of indomethacin and amiloride. Individual data points represent the mean of measurements on 2–5 biopsy specimens per individual. *Significantly different from controls and ΔF508 carriers. #Significantly different from $CF_{residual}$ individuals (Kruskal–Wallis analysis of variance on ranks). Solid line, median; dashed line, 25th and 75th percentiles, respectively. Most data from ΔF508 carriers ($n = 8$) were reported previously.[20]

Table 1. Relationship Between the *CFTR* Genotype and Cl^- Channel Function in Native Rectal Epithelia

CFTR genotype	Number of individuals	Sweat Cl^- concentration (*mmol/L*)[a]	cAMP-mediated response		Carbachol-induced plateau response or maximal lumen-negative response	
			Isc-cAMP ($\mu A/cm^2$)	Cl^- secretion (% of control)	Isc-carbachol ($\mu A/cm^2$)	Cl^- secretion (% of control)
Cl^- secretion absent						
R1162X/Q552X	1	71	17.1	0	0.7	0
W1282X/3121-2A>G	1	112	1.9	0	0.6	0
1898 + 1G > T/1609delCA	2[b]	114, 118	25.4, 13.4	0, 0	0, 0.7	0, 0
ΔF508/Q39X	2[b]	127, 129	2.6, 4.4	0, 0	1.7, 3.7	0, 0
ΔF508/G542X	1	102	29.0	0	6.6	0
ΔF508/R553X	3	112, 102, 109	13.1, 4.5, 23.8	0, 0, 0	1.5, 4.4, 1.0	0, 0, 0
ΔF508/E585X	1	115	1.4	0	1.1	0
ΔF508/Q637X	1	100	2.9	0	1.2	0
ΔF508/Y1092X	1	119	0.0	0	−0.3	0
ΔF508/120del23[c]	1	72	20.1	0	3.3	0
ΔF508/182delT	1	116	10.8	0	5.2	0
ΔF508/3905insT	2	88, 96	8.4, 5.6	0, 0	2.3, −1.1	0, 1
ΔF508/V520F	1	68	1.2	0	1.7	0
ΔF508/A561E	3	113, 146, 100	17.0, 17.0, 16.0	0, 0, 0	2.1, 1.5, 3.7	0, 0, 0
ΔF508/R1066C	1	138	0.0	0	0.0	0
ΔF508/N1303K	3	100, 117, 94	1.7, 4.1, 1.5	0, 0, 0	−0.6, 2.2, 0.8	0, 0, 0
A561E/A561E	2	101, 116	6.6, 2.0	0, 0	7.3, 3.3	0, 0
Residual Cl^- secretion[d]						
G542X/I148N	1	75	−50.1	54	−22.2	12
1898 + 3A > G/1898 + 3A > G	1	82	−36.8	39	−12.9	7
ΔF508/3272−26A > G	1	116	−17.8	19	−27.2	14
ΔF508/S108F	1	118	−15.8	17	−12.3	7
ΔF508/R117H	1	90	−35.9	38	−207.7	109
ΔF508/Y161C[c]	1	44	−35.1	37	−45.9	25
ΔF508/P205S	1	80	−23.3	25	−10.4	5
ΔF508/V232D	1	120	−16.9	18	−26.9	14
ΔF508/R334W	1	92	−22.1	23	−21.1	11
ΔF508/R334W	1	101	−24.5	26	−37.4	20
ΔF508/T338I	1	73	−44.4	47	−79.4	42
ΔF508/G576A	1	40	−16.9	18	−115.5	61
ΔF508/I1234V	1	113	−13.6	15	−8.6	5
G576A/G85E	1	95	−26.1	28	−61.6	32
F1052V/M1137R	1	47	−36.7	39	−146.6	77
M1101K/M1101K	1	94	−11.1	12	−4.8	3
S1159F/S1159F	1	67	−47.9	51	−38.7	21
N1303K/R334W	1	91	−30.3	32	−47.7	25

NOTE. CFTR Cl^- channel function was determined in rectal epithelia from Cl^- secretory responses induced by IBMX/forskolin (Isc-cAMP) and after co-activation with carbachol (Isc-carbachol). Data are expressed as absolute change in Isc and as percentage of mean Cl^- secretory responses observed in controls.

[a]Mean values of 2 or more sweat tests.

[b]Siblings.

[c]Novel mutation.

[d]The intron 8 5T allele occurred in only 2 tested CF patients with residual CFTR-mediated Cl^- secretion. One patient had TG10-9T/TG11-5T with G542X/1148N; the second patient had TG10-9T/TG12-5T with ΔF508/S108F.

mediated by mutant CFTR, we added carbachol in the presence of cAMP-dependent stimulation (Figure 1, Table 1). In the presence of cAMP-dependent activation (IBMX/forskolin), in control tissues, carbachol induced large, monophasic, lumen-negative (Cl^- secretory) responses ($\Delta Isc = -190.2 \pm 19.9$ $\mu A/cm^2$, $n = 19$) (Figure 2). In contrast, in the CF_{absent} group, cholinergic co-activation induced K^+ secretion, as revealed by a lumen-positive peak response ($\Delta Isc = 43.8 \pm 7.5$ $\mu A/cm^2$, $n = 27$) and a smaller lumen-positive plateau response ($\Delta Isc = 2.0 \pm 0.4$ $\mu A/cm^2$, $n = 27$), respectively. In the $CF_{residual}$ group, carbachol induced either biphasic responses with an initial lumen-positive peak ($\Delta Isc = 21.3 \pm 4.0$ $\mu A/cm^2$), followed by a lumen-negative deflection ($n = 13$), or monophasic lumen-negative responses ($n = 5$). Similar to cAMP-mediated responses, average cholinergic Cl^- secretory responses in $CF_{residual}$ tissues ($\Delta Isc = -51.5 \pm 12.9$

$\mu A/cm^2$, $n = 18$) were attenuated to 27.1% ± 6.8% of the mean response of control tissues (Table 1).

CFTR Function and Genotype

Table 1 summarizes the *CFTR* genotypes and corresponding CFTR Cl^- channel function in rectal epithelia. By extensive genotyping, 2 disease-causing *CFTR* mutations were identified in all 45 CF patients studied, including 2 novel mutations (120del23 and Y161C). Mutations were widespread throughout the *CFTR* gene, affecting the cytosolic N-terminus, membrane-spanning domains 1 and 2, nucleotide-binding domains 1 and 2, and the regulatory domain of the CFTR protein (Table 2). In agreement with previous in vitro studies,[8,9,11] compound heterozygosity for ΔF508, nonsense or frameshift mutations was associated with the absence of CFTR-mediated Cl^- secretion. CF patients carrying at least 1 missense or splice mutation were either in the CF_{absent} group or in the $CF_{residual}$ group. Importantly, CF patients with the same genotypes were always in the same functional group (i.e., either CF_{absent} or $CF_{residual}$).

CFTR Function and CF Phenotype

Table 3 compares clinical findings between CF_{absent} and $CF_{residual}$ individuals. $CF_{residual}$ patients were diagnosed at a later age, and exhibited better somatic development, as determined from weight-for-height percentiles and higher Shwachman–Kulczycki scores. Detection of residual CFTR function was associated closely with residual exocrine pancreatic function. All CF patients with loss of CFTR-mediated Cl^- secretion presented with severe PI (mean stool elastase concentration of 7.7 ± 2.2 μg/g), whereas all patients with residual CFTR function were either PS ($n = 16$) or had moderate PI with stool elastase concentrations between 100 and 200 μg/g ($n = 2$). CF patients with residual CFTR-mediated Cl^- channel function also had significantly lower sweat Cl^- concentrations (Table 3). Consistent with previous reports on CF patients with mild *CFTR* mutations,[14] 3 patients in this group had mean sweat Cl^- concentrations in the range of 40–60 mmol/L (Table 1). Furthermore, patients in the $CF_{residual}$ group presented with less severe lung disease, as determined from mean forced vital capacity percent and FEV_1 percent of predicted values (Table 3). Importantly, as shown in Figure 3, clinical outcomes including the age at diagnosis, sweat Cl^- concentrations, fecal elastase E1 concentrations, and FEV_1 % predicted were related directly to the magnitude of residual CFTR Cl^- channel activity (cAMP-induced Isc).

Table 2. Functional Classification and Protein Location of *CFTR* Mutations

Mutation type	Severe mutations (protein location)	Mild mutations (protein location)
Missense	V520F, A561E (NBD1) R1066C (MSD2, CL4) N1303K (NBD2)	G85E (MSD1, TM1) S108F, R117H (MSD1, EL1) I148N, Y161C[a] (MSD1, CL1) P205S (MSD1, TM3) V232D (MSD1, TM4) R334W, T338I (MSD1, TM6) G576A (NBD1) I1234V (NBD2) F1052V, M1101K (MSD2, CL4) M1137R (MSD2, TM12) S1159F (pre-NBD2)
Splice	1898 + 1G > T (R domain) 3121-2A > G (MSD2, TM9)	1898 + 3A > G (R domain) 3272-26A > G (MSD2, TM10)
Single amino acid deletion	ΔF508 (NBD1)	
Nonsense	Q39X (N-terminus) G542X, Q552X, R553X, E585X (NBD1) Q637X (R domain) Y1092X (MSD2, CL4) R1162X (pre-NBD2) W1282X (NBD2)	
Frameshift	120del23[a] 182delT (N-terminus) 1609delCA (NBD1) 3905insT (NBD2)	

NOTE. Severe mutation, Cl^- secretion absent; mild mutation, residual cAMP-mediated Cl^- secretion. Protein locations affected by mutations are given in parentheses and include the cytosolic N-terminus, transmembrane segments (TM), extracellular (EL) and cytoplasmatic (CL) loops of membrane-spanning domains (MSD 1, 2), nucleotide-binding domains (NBD 1, 2), and the regulatory (R) domain. Consequences include premature stop codons with protein truncation for nonsense and frameshift mutations, aberrant/alternative splicing for splice mutations, and single amino acid changes (point mutations) for missense mutations.

[a]Novel mutation.

Table 3. Comparison of Clinical Phenotypes Between CF Patients Without Detectable Cl^- Secretion (CF_{absent}) and CF Patients With Residual CFTR-Mediated Cl^- Secretion ($CF_{residual}$)

	CF_{absent}	$CF_{residual}$	*P* value
CF patients (*n*)	27	18	
Isc-cAMP ($\mu A/cm^2$) [% of control]	9.3 ± 1.7 [0]	−28.1 ± 9.8 [29.8 ± 3.0]	<0.0001[a]
Isc-carbachol ($\mu A/cm^2$) [% of control]	2.0 ± 0.4 [0]	−51.5 ± 12.8 [27.1 ± 6.8]	<0.0001[b]
Age at study (*yr*)	15.1 ± 2.5	18.6 ± 2.5	0.34[a]
Age at diagnosis (*yr*)	1.7 ± 0.3	12.2 ± 2.6	<0.0001[b]
Sweat Cl^- (*mmol/L*)	107.2 ± 3.9	85.4 ± 5.9	<0.01[a]
Weight for height (*wfh %*)	47.2 ± 6.6	68.5 ± 5.9	0.03[a]
Shwachman–Kulczycki score	70.7 ± 4.2	79.0 ± 6.3	<0.05[b]
Pancreatic sufficiency (*n* [%])	0 [0]	16[c] [89]	<0.0001[d]
Pulmonary function (*n*)[e]	19	15	
Age (*yr*)	20.4 ± 2.7	20.0 ± 2.3	0.93[a]
FVC % predicted	63.6 ± 6.0	81.7 ± 5.1	<0.05[a]
FEV_1 % predicted	54.6 ± 5.8	71.4 ± 5.6	<0.05[a]

NOTE. CFTR Cl^- channel function was determined in native rectal epithelia from Cl^- secretory responses induced by IBMX/forskolin (Isc-cAMP) and after co-activation with carbachol (Isc-carbachol). Data are shown as mean ± SEM and/or percentage.

[a]Unpaired *t* test.

[b]Mann–Whitney test.

[c]2 PI individuals in this group presented with moderate PI (mean stool elastase concentrations of 111 μg/g and 154 μg/g, respectively).

[d]χ^2 test.

[e]Pulmonary function tests were performed in all CF patients >6 years of age.

Discussion

The elucidation of the relationship between CFTR protein function and phenotypic traits for specific *CFTR* mutations is important for the understanding of CF pathophysiology and for the development of novel therapeutic strategies for patients with CF. Genotype-phenotype correlation studies estimated that ~15%–20% of CF patients express mild mutations that confer PS.[2,15,23] The most frequent *CFTR* mutation, ΔF508, accounts for ~66% of CF alleles and results in the loss of mature CFTR protein and apical membrane Cl^- channel function,[4,8,11,20] and ΔF508 homozygosity is associated with severe PI.[15] In contrast, most PS mutations occur at low allelic frequencies (≤0.1%) and data describing phenotypic traits and/or functional properties in native epithelia are not available for many rare *CFTR* mutations. In ΔF508 carriers, 50% of wild-type CFTR is sufficient to confer normal Cl^- channel function to native intestinal epithelia and prevent the symptoms of CF.[20,31] Furthermore, it has been shown that as little as 5%–10% of full-length *CFTR* messenger RNA is sufficient to confer a mild clinical phenotype to CF patients with *CFTR* splice mutations.[14,32] However, little is known about the threshold of CFTR function that is required to protect the host from CF, or ameliorate disease severity.

In an attempt to further elucidate the genotype function–phenotype relationship in CF, we performed sensitive functional measurements in rectal epithelia from a large group of CF patients who carry at least 1 non-ΔF508 mutation with a wide spectrum of *CFTR* genotypes and clinical presentation of disease. Previous studies on rectal biopsy specimens from partially genotyped CF patients relied exclusively on cholinergic (Ca^{2+}-dependent) activation to assess for anion secretion. These studies concluded that a subgroup of CF patients expressed alternative apical membrane Ca^{2+}-activated Cl^- channels and suggested that up-regulation of these alternative Cl^- channels in the intestinal epithelium may modify CF disease severity.[33] In our study, we used Cl^- secretory responses elicited by cAMP-mediated activation as a direct readout for CFTR-mediated Cl^- channel function. Residual CFTR activity was detected in 40% of CF patients in our study population ($CF_{residual}$), whereas no anion secretion was detectable in the remaining CF patients (CF_{absent}) (Figures 1 and 2, and Table 1). In agreement with our previous studies showing that Ca^{2+}- and cAMP-dependent signaling act cooperatively in determining the magnitude of CFTR-mediated Cl^- secretion in the colon,[21,22] co-activation with the Ca^{2+}-mediated agonist carbachol further enhanced cAMP-mediated Cl^- secretion in $CF_{residual}$ patients, but failed to induce Cl^- secretory responses in the CF_{absent} group (Figures 1 and 2, Table 1).

When CFTR function and genotypes were compared (Table 1), it became apparent that our functional data from native colonic epithelia agreed well with previous functional studies in heterologous cells. CFTR-mediated Cl^- secretion was absent (or below the level of detection) in all CF patients compound heterozygous for class I and II mutations, including ΔF508, nonsense, frameshift, and missense mutations that result in defective processing (A561E, R1066C,

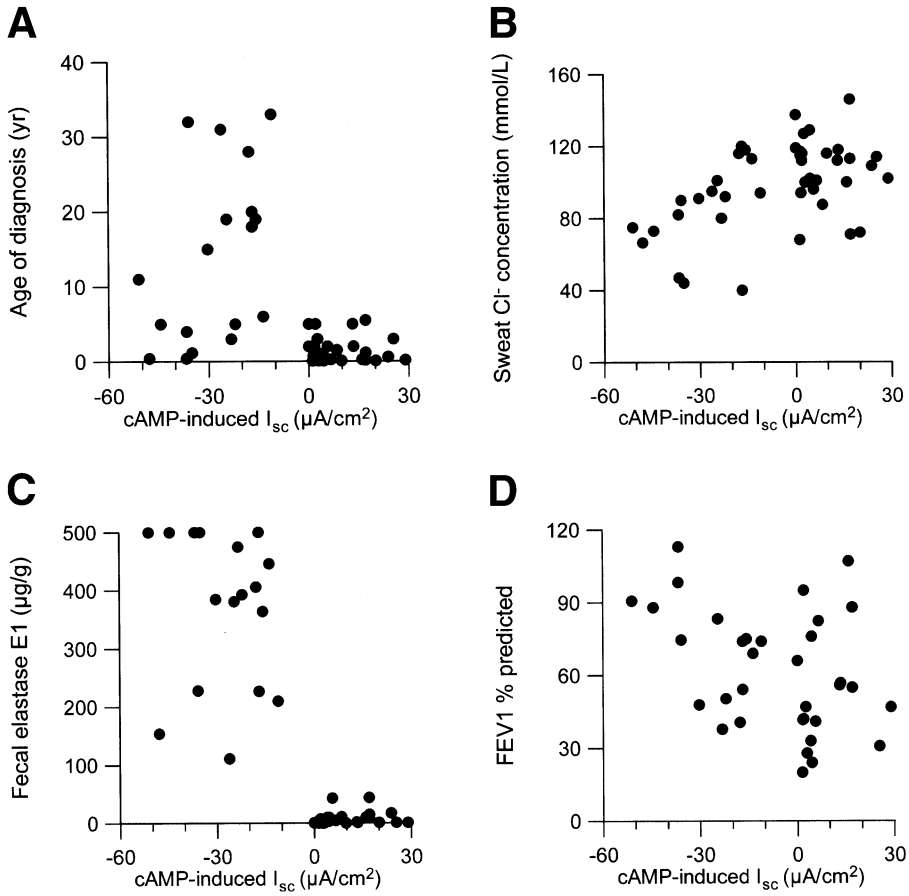

Figure 3. Relationship between clinical outcomes and cAMP-mediated Isc (IBMX/forskolin) in rectal tissues of CF patients. Lumen-negative Isc responses reflect Cl^- secretion, lumen-positive responses reflect K^+ secretion.[22] Correlation between cAMP-induced Isc and (*A*) age at diagnosis ($r = -0.44$, $P < 0.002$), (*B*) sweat Cl^- concentration ($r = 0.50$, $P < 0.0005$), (*C*) exocrine pancreatic function (fecal elastase E1 concentration) ($r = -0.82$, $P < 0.0001$), and (*D*) pulmonary function (FEV_1 %) ($r = -0.38$, $P < 0.03$) (Pearson's correlation).

N1303K).[8,9,11,34–36] Mutants that have been shown previously to form plasma membrane Cl^- channels with altered single-channel properties in heterologous cells (S108F, R117H, R334W, F1052V)[10,34,35,37] were associated with residual cAMP-mediated Cl^- secretion of ~12%–54% of control rectal epithelia. Residual function also was observed for mutants, which are expected to form membrane Cl^- channels that are reduced in number, either owing to improper protein maturation (P205S, M1137R) or owing to reduced levels of full-length *CFTR* messenger RNA (3272-26A>G, G576A).[32,38–40] The only exception was M1101K, which was reported as a loss of function mutation in heterologous cells[34] and was associated with residual CFTR function in the 1 homozygous patient studied (Table 1). No biochemical or functional data were available for some of the splice and missense mutations detected in our patients. According to our functional data, 3121-2A>G, 1898+1G>T, and V520F constitute severe mutations, whereas 1898+3A>G, I148N, Y161C, V232D, T338I, I1234V, and S1159F confer residual CFTR Cl^- channel function (Table 1). Furthermore, 2 patients in the $CF_{residual}$ group also carried the 5T allele, which constitutes a common polymorphism in intron 8 of the *CFTR* gene (Table 1). Previous studies have shown that the 5T allele, especially in conjunction with a high number of adjacent TG repeats, results in enhanced skipping of exon 9 and thus decreased levels of CFTR protein.[41,42] It is therefore possible that reduced posttranscriptional processing of messenger RNA transcripts from mild *CFTR* mutations (S108F and I148N) contributed to reduced CFTR-mediated Cl^- secretion, and the disease phenotype observed in these patients.

It is noteworthy that most *CFTR* mutations in the first nucleotide binding domain identified in our study population lacked functional activity. This finding is in agreement with previous reports that suggested that the structure of the first nucleotide binding domain is particularly sensitive to mutational changes.[43] In contrast to the study by Gregory et al.,[43] most nucleotide binding domain 2 (NDB2) mutants detected in our patients also were associated with an absence of CFTR-mediated secretion, whereas most mutations associated with residual CFTR function were located throughout the 2 membrane-spanning domains (membrane-spanning domain 1 and 2) of the

CFTR protein (Table 2). However, particularly in the case of missense and splice mutations, functional consequences could not be predicted from alterations in the DNA sequence.

Comparison of the clinical presentation for $CF_{residual}$ and CF_{absent} patients showed that residual CFTR function correlated with a milder disease phenotype (Table 3, Figure 3). CF patients with residual CFTR-mediated Cl^- secretion ($CF_{residual}$) were diagnosed at a later age, had better somatic development, and had higher Shwachman–Kulczycki clinical scores than CF patients with absence of Cl^- channel function (CF_{absent}). Furthermore, residual colonic Cl^- secretion was correlated with lower sweat Cl^- concentrations and exocrine pancreatic function. In agreement with previous studies of CF patients carrying specific PS mutations,[16,44,45] the severity of lung disease was variable in individuals within both groups ($CF_{residual}$ and CF_{absent}). Overall, however, lung disease was less severe in the $CF_{residual}$ compared with the CF_{absent} group. Importantly, we detected a direct correlation between the magnitude of residual CFTR-mediated Cl^- secretion and clinical outcomes (Figure 3). Collectively, these data indicate that residual function of specific CFTR mutants detected in the colon extended to other organ systems, including pancreas, sweat glands, and lung.

Interestingly, a number of patients in the $CF_{residual}$ group expressed high residual Cl^- secretory responses in the range of 36%–54% of controls (Table 1, Figure 2). Previous studies in heterologous cells showed that CFTR, besides its role as a cAMP-dependent Cl^- channel, also acts as a regulator of epithelial Na^+ channels[46,47] and epithelial HCO_3^- transport mediated by the SLC26 transporters.[48,49] The study by Choi et al.[48] identified specific *CFTR* mutations that retained normal Cl^- channel function, but resulted in deficient CFTR-dependent HCO_3^- transport in vitro, and were associated with a CF disease phenotype. In a transgenic mouse model with airway-specific overexpression of epithelial Na^+ channels, we recently showed that dysregulation of epithelial Na^+ channel–mediated Na^+ absorption alone (i.e., in the presence of normal Cl^- transport) is sufficient to cause CF-like lung disease in vivo.[50] These findings from heterologous cells and a murine model point to the possibility that aberrant regulation of other ion transport processes (e.g., HCO_3^- and Na^+ transport) may contribute to the heterogeneity of disease severity in CF patients with residual CFTR Cl^- channel function. However, further studies are required to determine the impact of specific *CFTR* mutations on CFTR-related functions other than Cl^- transport in native human tissues, and the role of defects in such functions in CF pathogenesis in humans.

In conclusion, our data establish that residual CFTR Cl^- channel function is a mechanistic link between specific *CFTR* mutations and a mild CF phenotype.[1,3,15,16,44,45] In contrast to previous nasal potential difference studies,[17–19] our Ussing chamber studies on rectal biopsy specimens discriminate between CF patients who express residual CFTR-mediated Cl^- channel function and patients in whom this function is absent. We show that residual colonic Cl^- secretion has a protective effect on the host and is associated with less severe organ disease. Because many *CFTR* mutations are not identified by standard screening tests, and functional data are not available for most rare mutations, measurements of CFTR function in rectal biopsy specimens serve as a sensitive test to predict the prognosis of CF. Furthermore, functional assessment of native colonic epithelia could assist in defining the role of factors other than *CFTR* (i.e., modifier genes or environmental factors[4,51,52]) that result in discordant phenotypes of CF patients carrying the same *CFTR* genotypes. Finally, detection of residual CFTR function identifies CF patients who may benefit from therapeutic strategies that increase residual CFTR activity.

References

1. Kerem B, Rommens JM, Buchanan JA, Markiewicz D, Cox TK, Chakravarti A, Buchwald M, Tsui LC. Identification of the cystic fibrosis gene: genetic analysis. Science 1989;245:1073–1080.
2. Welsh MJ, Ramsey BW, Accurso F, Cutting GR. Cystic fibrosis. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. The metabolic & molecular bases of inherited disease. 8th ed. New York: McGraw-Hill, 2001:5121–5188.
3. The Cystic Fibrosis Genotype-Phenotype Consortium. Correlation between genotype and phenotype in patients with cystic fibrosis. N Engl J Med 1993;329:1308–1313.
4. Tsui LC, Durie P. Genotype and phenotype in cystic fibrosis. Hosp Pract 1997;32:115–142.
5. Anderson MP, Gregory RJ, Thompson S, Souza DW, Paul S, Mulligan RC, Smith AE, Welsh MJ. Demonstration that CFTR is a chloride channel by alteration of its anion selectivity. Science 1991;253:202–205.
6. Bear CE, Li C, Kartner N, Bridges RJ, Jensen TJ, Ramjeesingh M, Riordan JR. Purification and functional reconstitution of the cystic fibrosis transmembrane conductance regulator (CFTR). Cell 1992;68:809–818.
7. Kunzelmann K, Mall M. Electrolyte transport in the mammalian colon: mechanisms and implications for disease. Physiol Rev 2002;82:245–289.
8. Cheng SH, Gregory RJ, Marshall J, Paul S, Souza DW, White GA, O'Riordan C, Smith AE. Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. Cell 1990;63:827–834.
9. Hamosh A, Trapnell BC, Zeitlin PL, Montrose-Rafizadeh C, Rosenstein BJ, Crystal RG, Cutting GR. Severe deficiency of cystic fibrosis transmembrane conductance regulator messenger RNA carrying nonsense mutations R553X and W1316X in respiratory epithelial cells of patients with cystic fibrosis. J Clin Invest 1991; 88:1880–1885.

10. Sheppard DN, Rich DP, Ostedgaard LS, Gregory RJ, Smith AE, Welsh MJ. Mutations in CFTR associated with mild-disease-form Cl^- channels with altered pore properties. Nature 1993; 362:160–164.
11. Welsh MJ, Smith AE. Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. Cell 1993;73:1251–1254.
12. Sheppard DN, Ostedgaard LS, Winter MC, Welsh MJ. Mechanism of dysfunction of two nucleotide binding domain mutations in cystic fibrosis transmembrane conductance regulator that are associated with pancreatic sufficiency. EMBO J 1995;14:876–883.
13. Champigny G, Imler JL, Puchelle E, Dalemans W, Gribkoff V, Hinnrasky J, Dott K, Barbry P, Pavirani A, Lazdunski M. A change in gating mode leading to increased intrinsic Cl- channel activity compensates for defective processing in a cystic fibrosis mutant corresponding to a mild form of the disease. EMBO J 1995;14: 2417–2423.
14. Highsmith WE, Burch LH, Zhou Z, Olsen JC, Boat TE, Spock A, Gorvoy JD, Quittel L, Friedman KJ, Silverman LM. A novel mutation in the cystic fibrosis gene in patients with pulmonary disease but normal sweat chloride concentrations. N Engl J Med 1994; 331:974–980.
15. Kristidis P, Bozon D, Corey M, Markiewicz D, Rommens J, Tsui L-C, Durie P. Genetic determination of exocrine pancreatic function in cystic fibrosis. Am J Hum Genet 1992;50:1178–1184.
16. McKone EF, Emerson SS, Edwards KL, Aitken ML. Effect of genotype on phenotype and mortality in cystic fibrosis: a retrospective cohort study. Lancet 2003;361:1671–1676.
17. Ho LP, Samways JM, Porteous DJ, Dorin JR, Carothers A, Greening AP, Innes JA. Correlation between nasal potential difference measurements, genotype and clinical condition in patients with cystic fibrosis. Eur Respir J 1997;10:2018–2022.
18. Walker LC, Venglarik CJ, Aubin G, Weatherly MR, McCarty NA, Lesnick B, Ruiz F, Clancy JP, Sorscher EJ. Relationship between airway ion transport and a mild pulmonary disease mutation in CFTR. Am J Respir Crit Care Med 1997;155:1684–1689.
19. Wallace HL, Barker PM, Southern KW. Nasal airway ion transport and lung function in young people with cystic fibrosis. Am J Respir Crit Care Med 2003;168:594–600.
20. Mall M, Kreda SM, Mengos A, Jensen TJ, Hirtz S, Seydewitz HH, Yankaskas J, Kunzelmann K, Riordan JR, Boucher RC. The DeltaF508 mutation results in loss of CFTR function and mature protein in native human colon. Gastroenterology 2004;126:32–41.
21. Mall M, Bleich M, Greger R, Schürlein M, Kühr J, Seydewitz HH, Brandis M, Kunzelmann K. Cholinergic ion secretion in human colon requires co-activation by cAMP. Am J Physiol 1998;275: G1274–G1281.
22. Mall M, Wissner A, Seydewitz HH, Kuehr J, Brandis M, Greger R, Kunzelmann K. Defective cholinergic Cl^- secretion and detection of K^+ secretion in rectal biopsies from cystic fibrosis patients. Am J Physiol 2000;278:G617–G624.
23. Kerem E, Corey M, Kerem BS, Rommens J, Markiewicz D, Levison H, Tsui LC, Durie P. The relation between genotype and phenotype in cystic fibrosis—analysis of the most common mutation (delta F508). N Engl J Med 1990;323:1517–1522.
24. Rosenstein BJ, Cutting GR. The diagnosis of cystic fibrosis: a consensus statement. Pediatrics 1998;132:589–595.
25. Prader A, Largo RH, Molinari L, Issler C. Physical growth of Swiss children from birth to 20 years of age. First Zurich longitudinal study of growth and development. Helv Paediatr Acta Suppl 1989;52:1–125.
26. Shwachman H, Kulczycki LL. Long-term study of one hundred five patients with cystic fibrosis: studies made over a five- to fourteen-year period. Am J Dis Child 1958;96:6–15.
27. Wallis C, Leung T, Cubitt D, Reynolds A. Stool elastase as a diagnostic test for pancreatic function in children with cystic fibrosis. Lancet 1997;350:1001.
28. Ferrie RM, Schwarz MJ, Robertson NH, Vaudin S, Super M, Malone G, Little S. Development, multiplexing, and application of ARMS tests for common mutations in the CFTR gene. Am J Hum Genet 1992;51:251–262.
29. Fanen P, Ghanem N, Vidaud M, Besmond C, Martin J, Costes B, Plassa F, Goossens M. Molecular characterization of cystic fibrosis: 16 novel mutations identified by analysis of the whole cystic fibrosis conductance transmembrane regulator (CFTR) coding regions and splice site junctions. Genomics 1992;13:770–776.
30. Zielenski J, Rozmahel R, Bozon D, Kerem B, Grzelczak Z, Riordan JR, Rommens J, Tsui LC. Genomic DNA sequence of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Genomics 1991;10:214–228.
31. Hogenauer C, Porter JL, Millard M, Gelfand A, Rosenblatt RL, Prestidge CB, Fordtran JS. Active intestinal chloride secretion in human carriers of cystic fibrosis mutations: an evaluation of the hypothesis that heterozygotes have subnormal active intestinal chloride secretion. Am J Hum Genet 2000;67:1422–1427.
32. Ramalho AS, Beck S, Meyer M, Penque D, Cutting GR, Amaral MD. Five percent of normal cystic fibrosis transmembrane conductance regulator mRNA ameliorates the severity of pulmonary disease in cystic fibrosis. Am J Respir Cell Mol Biol 2002;27: 619–627.
33. Veeze HJ, Halley DJ, Bijman J, De Jongste JC, De Jonge HR, Sinaasappel M. Determinants of mild symptoms in cystic fibrosis patients. J Clin Invest 1994;93:461–466.
34. Seibert FS, Linsdell P, Loo TW, Hanrahan JW, Clarke DM, Riordan JR. Disease-associated mutations in the fourth cytoplasmic loop of cystic fibrosis transmembrane conductance regulator compromise biosynthetic processing and chloride channel activity. J Biol Chem 1996;271:15139–15145.
35. Cotten JF, Ostedgaard LS, Carson MR, Welsh MJ. Effect of cystic fibrosis-associated mutations in the fourth intracellular loop of cystic fibrosis transmembrane conductance regulator. J Biol Chem 1996;271:21279–21284.
36. Mendes F, Roxo RM, Dragomir A, Farinha CM, Roomans GM, Amaral MD, Penque D. Unusually common cystic fibrosis mutation in Portugal encodes a misprocessed protein. Biochem Biophys Res Commun 2003;311:665–671.
37. Hammerle MM, Aleksandrov AA, Riordan JR. Disease-associated mutations in the extracytoplasmic loops of cystic fibrosis transmembrane conductance regulator do not impede biosynthetic processing but impair chloride channel stability. J Biol Chem 2001;276:14848–14854.
38. Sheppard DN, Travis SM, Ishihara H, Welsh MJ. Contribution of proline residues in the membrane-spanning domains of cystic fibrosis transmembrane conductance regulator to chloride channel function. J Biol Chem 1996;271:14995–15001.
39. Vankeerberghen A, Wei L, Teng H, Jaspers M, Cassiman JJ, Nilius B, Cuppens H. Characterization of mutations located in exon 18 of the CFTR gene. FEBS Lett 1998;437:1–4.
40. Pagani F, Stuani C, Tzetis M, Kanavakis E, Efthymiadou A, Doudounakis S, Casals T, Baralle FE. New type of disease causing mutations: the example of the composite exonic regulatory elements of splicing in CFTR exon 12. Hum Mol Genet 2003;12: 1111–1120.
41. Chu CS, Trapnell BC, Curristin S, Cutting GR, Crystal RG. Genetic basis of variable exon 9 skipping in cystic fibrosis transmembrane conductance regulator mRNA. Nat Genet 1993;3:151–156.
42. Cuppens H, Lin W, Jaspers M, Costes B, Teng H, Vankeerberghen A, Jorissen M, Droogmans G, Reynaert I, Goossens M, Nilius B, Cassiman JJ. Polyvariant mutant cystic fibrosis transmembrane conductance regulator genes. The polymorphic (Tg)m locus explains the partial penetrance of the T5 polymorphism as a disease mutation. J Clin Invest 1998;101:487–496.
43. Gregory RJ, Rich DP, Cheng SH, Souza DW, Paul S, Manavalan P, Anderson MP, Welsh MJ, Smith AE. Maturation and function of

cystic fibrosis transmembrane conductance regulator variants bearing mutations in putative nucleotide-binding domains 1 and 2. Mol Cell Biol 1991;11:3886–3893.
44. Gan KH, Veeze HJ, van den Ouweland AM, Halley DJ, Scheffer H, van der HA, Overbeek SE, De Jongste JC, Bakker W, Heijerman HG. A cystic fibrosis mutation associated with mild lung disease. N Engl J Med 1995;333:95–99.
45. Loubieres Y, Grenet D, Simon-Bouy B, Medioni J, Landais P, Ferec C, Stern M. Association between genetically determined pancreatic status and lung disease in adult cystic fibrosis patients. Chest 2002;121:73–80.
46. Stutts MJ, Canessa CM, Olsen JC, Hamrick M, Cohn JA, Rossier BC, Boucher RC. CFTR as a cAMP-dependent regulator of sodium channels. Science 1995;269:847–850.
47. Mall M, Hipper A, Greger R, Kunzelmann K. Wild type but not F508 CFTR inhibits Na^+ conductance when coexpressed in *Xenopus* oocytes. FEBS Lett 1996;381:47–52.
48. Choi JY, Muallem D, Kiselyov K, Lee MG, Thomas PJ, Muallem S. Aberrant CFTR-dependent HCO_3^- transport in mutations associated with cystic fibrosis. Nature 2001;410:94–97.
49. Ko SB, Shcheynikov N, Choi JY, Luo X, Ishibashi K, Thomas PJ, Kim JY, Kim KH, Lee MG, Naruse S, Muallem S. A molecular mechanism for aberrant CFTR-dependent HCO(3)(-) transport in cystic fibrosis. EMBO J 2002;21:5662–5672.
50. Mall M, Grubb BR, Harkema JR, O'Neal WK, Boucher RC. Increased airway epithelial Na^+ absorption produces cystic fibrosis-like lung disease in mice. Nat Med 2004;10:487–493.
51. Rozmahel R, Wilschanski M, Matin A, Plyte S, Oliver M, Auerbach W, Moore A, Forstner J, Durie P, Nadeau J, Bear C, Tsui LC. Modulation of disease severity in cystic fibrosis transmembrane conductance regulator deficient mice by a secondary genetic factor. Nat Genet 1996;12:280–287.
52. Zielenski J, Corey M, Rozmahel R, Markiewicz D, Aznarez I, Casals T, Larriba S, Mercier B, Cutting GR, Krebsova A, Macek MJ, Langfelder-Schwind E, Marshall BC, DeCelie-Germana J, Claustres M, Palacio A, Bal J, Nowakowska A, Ferec C, Estivill X, Durie P, Tsui LC. Detection of a cystic fibrosis modifier locus for meconium ileus on human chromosome 19q13. Nat Genet 1999;22:128–129.

Received November 16, 2003. Accepted June 24, 2004.

Address requests for reprints to: Marcus Mall, M.D., Department of Pediatrics III, Pediatrics Pulmonology and Infectious Diseases, University of Heidelberg, Im Neuenheimer Feld 153, D-69120 Heidelberg, Germany. e-mail: mmall@med.unc.edu; fax: (06221) 56-4559.

S.H. and T.G. contributed equally to this work.

Supported by the Mukoviszidose e.V., the Deutsche Forschungsgemeinschaft (DFG MA 2081/2-1 and KU 1228/1-1), the Zentrum Klinische Forschung 1 University of Freiburg (ZKF1, A2), and by POCTI/35737/MGI/2000 and POCTI/MGI/47382/2002 research grants (Fundacao Ciencia Tecnologia, Portugal).

The authors gratefully thank the CF patients and volunteers for their participation in this study, Andre Schmidt for technical assistance, and Deborah Penque for general support and assistance.