Five Percent of Normal Cystic Fibrosis Transmembrane Conductance Regulator mRNA Ameliorates the Severity of Pulmonary Disease in Cystic Fibrosis

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Estimates of the level of transcripts from the cystic fibrosis (CF) transmembrane conductance regulator (*CFTR*) gene required to develop a CF phenotype range from 4–20% of normal. Due to the importance of obtaining reliable data on this issue for therapeutic strategies, we developed a novel polymerase chain reaction–based method to quantify CFTR transcripts and applied it to the analysis of nasal epithelium RNA of five patients with CF and the 3272-26A>G/F508del genotype. We calculated that 8.2 ± 0.84% of the total CFTR RNA present in these five patients is normal full-length CFTR mRNA. We then demonstrated (in nasal samples from F508del carriers, *n* = 30) that the abundance of full-length F508del CFTR transcripts is reduced compared with wild-type transcripts, and estimated that the average ratio of F508del/wild-type transcripts is 0.87 ± 0.06. To determine the amount of full-length transcripts relative to levels found in normal individuals, we corrected for the lower abundance of the F508del transcripts and calculated that the five patients with CF have, on average, 4.7 ± 0.45% of the normal level of wild-type CFTR mRNA. Because these patients have mild CF compared with F508del homozygotes, this CFTR mRNA level appears to be sufficient to avoid the severe complications of the disease.

Mutations affecting the splicing of the cystic fibrosis (CF) transmembrane conductance regulator (*CFTR*) gene that lead to the simultaneous presence of correctly and aberrantly spliced transcripts (included in class I [1], previously class V) cause CF disease due to insufficient levels of the normal mRNA, and hence, functional protein. Previous studies have reported a wide range of normal CFTR transcripts, from less than 4% (2) to less than 20%, associated with "leaky" splicing mutations (3). However, it is important to obtain reliable data on this issue for the design of therapies aiming at restoring lung function by increasing the levels of normal CFTR, namely gene therapy.

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Abbreviations: base pairs, bp; cystic fibrosis, CF; CF transmembrane conductance regulator, CFTR; polymerase chain reaction, PCR; reverse transcription, RT.

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The splicing mutation 3272-26A>G is widespread in Europe (4). Compound heterozygotes with this mutation and a severe mutation have milder CF disease in terms of pancreatic function, pulmonary involvement, and incidence of *Pseudomonas aeruginosa* colonization than F508del-homozygotes who have classic CF (4). In Portugal, the frequency of 3272-26A>G is ∽ 2% (5). We have previously shown that this mutation, located in intron 17a, activates a cryptic acceptor splice site by competing with the normal one, resulting in a transcript with 25 extra nucleotides and a premature stop codon (5).

The objectives of the present study were to quantify the relative amount of normal full-length CFTR transcripts in patients with the 3272-26A>G/F508del genotype, and to estimate the percentage of these full-length CFTR transcripts, relative to normal individuals. To attain this goal, the level of three species of RNA encoding nonfunctional protein had to be determined from the total CFTR RNA present in these patients. These included CFTR transcripts with the F508del mutation, transcripts alternatively spliced at intron 17a, because this product contains premature stop codon (5), and CFTR mRNA lacking exon 9, a common event which occurs in variable levels both in CF and non-CF invividuals (6). Furthermore, the level of normally spliced CFTR transcripts had to be corrected for the reduced abundance of CFTR transcripts bearing the F508del mutation when compared with wild-type (wt) transcripts. After incorporating a rigorous control for polymerase chain reaction (PCR) amplification efficiency (see Figure 1), we achieved consistent and reproducible results on five patients with identical *CFTR* genotypes.

Materials and Methods

Individuals, Genotypes, and Nasal Brushings

Cells from nasal brushings were obtained from five patients with CF and the 3272-26A>G/F508del genotype, and four carriers of the 3272-26A>G mutation. Clinical data as well as haplotypes of the above-mentioned five patients with CF were described elsewhere (4). After informed consent, nasal brushings were performed as previously described (5).

mRNA Analysis

Immediately after brushing, nasal epithelial cells were put into extraction buffer and stored at –80°C until use. RNA extraction by the RNeasy method (Qiagen, Hilden, Germany), hexanucleotide-

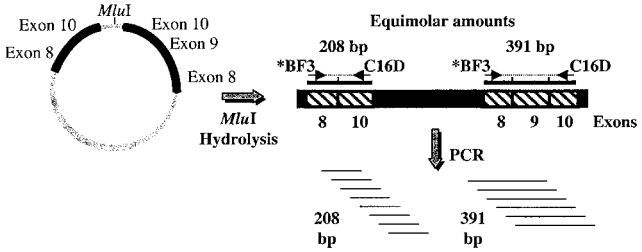

Figure 1. Schematic representation of the control plasmid used as external control. The plasmid contains two copies of CFTR cDNA in the region of exons 7–12 on the same molecule, one including and another excluding exon 9. Following *Mlu*I hydrolysis for linearization, the plasmid was used as a template in PCR reactions performed in parallel with those of the patient-derived materials and using the same primers. As the two templates were initially in equimolar amounts in the plasmid, the ratio of the amounts of the two PCR products (with and without exon 9) was used to correct size-specific amplifications in the PCR products from patient-derived materials.

primed reverse transcription (RT) reaction and PCR amplification of regions 16–17b, were performed as previously described (5), except that the 5′ primer was Fam-labeled. PCR amplification of region 8–10 was performed using primers: B3F: 5′-AATGTAAC AGCCTTCTGGGAG-3′ (Fam-labeled) in exon 8 (positions 1318–1338) and C16D: 5′-GTTGGCATGCTTTGATGACGCTTC-3′ in exon 10 (positions 1685–1708). cDNA samples were heated at 94°C for 5 min and then subjected to amplification cycles of denaturation at 94°C for 1 min, primer annealing at 60°C for 1 min, and extension at 72°C for 2 min, followed by a final extension at 72°C for 30 min.

All PCR reactions were performed in triplicate under exactly the same conditions but with different numbers of cycles (generally n, n + 2, and n + 4) to certify that it was still in the log-phase (Figures 2C and 2E). All experiments were repeated from different RT reactions (*see* Table 1, first column): three (Patient 3) or four (the other four patients). For three patients (Patients 1, 4, and 5), measurements were from two different nasal brushings, collected on different occasions. PCR products were separated by polyacrylamide gel electrophoresis in the ABI Prism 377 automatic sequencer (Applied Biosystems, Foster City, CA), and quantitative analysis of peaks was performed with the GeneScan software (Applied Biosystems). This allows a direct integration of the peak area corresponding to the number of Fam-labeled molecules synthesized by PCR to determine the relative percentages of CFTR mRNA specimens present.

To correct size-specific differences in amplification efficiency for RT-PCR products obtained in the region of exons 8–10 (i.e., with and without exon 9) for patients, parallel PCR reactions were performed using the same primers and a control plasmid as template. This plasmid contains two copies of CFTR cDNA (exons 7–12) on the same molecule (*see* Figure 1), one including and another excluding exon 9. Following *Mlu*I hydrolysis for linearization, 5 μl of a 3.3×10^{-5} μg/ml solution of the plasmid ($\backsim$ 100,000 copies) per reaction was used as a template in PCR reactions performed in parallel with samples from patients using the same primers and conditions. Because the plasmid has the two templates in equimolar amounts, the ratio of the amounts of the two PCR products (with and without exon 9) was used to correct size-specific amplifications in the PCR products from patients' samples. To determine whether amplification efficiency varies with the initial ratio of the two templates, the exon 9^-/exon 9^+ plasmid (1 copy each on the same molecule) was combined in different ratios with a plasmid containing full-length CFTR cDNA in the following proportions: 1:1 ($9^-/9^+$ plasmid alone, no full-length CFTR); 1:2; 1:3; 1:4; 1:5; 1:6; 1:7; 1:8; 1:9; 1:10; and 1:11. Using each of these mixtures as template, PCR reactions were performed and aliquots removed at different numbers of cycles, namely: 19, 20, 21, 22, and 24. Kinetic analyses were performed by plotting the logarithm of peak areas against the respective number of cycles (data not shown) for each of the two products (exon 9^- and exon 9^+), according to the following equation: $\text{Log A} = [\text{Log}(1 + \text{E})] \cdot n + \text{Log A}_0$, where A_0 = the starting amount of RNA; A = the amount of amplified product (peak areas); E = the amplification efficiency; and n = the number of amplification cycles.

From the slopes of lines plotting Log A (peak areas) versus n, the number of PCR cycles (determined by linear regression from this equation), the efficiency of amplification (E) can be estimated for each exon 9^-/exon 9^+ ratio as follows: linear regression of Log A (peak areas) versus the number of PCR cycles (n), for each exon 9^-/exon 9^+ ratio (see Figure 3), allowing estimation of the of amplification efficiencies (E) through the equation: slope = Log (1 + E).

Estimate of Levels of Full-Length CFTR Transcripts

A schematic representation of the calculations used is shown in Figure 4. Raw data obtained from integration of peak areas (*see* RESULTS) from independent analysis of the same patient were used separately to estimate levels of full-length CFTR transcripts and averaged in the end for each of the five 3272-26A>G patients. All values shown are approximate averages (*see* Table 1) for exact values and respective SD. Calculations made are schematically represented in Figure 4 and next briefly described.

Peaks P1–P6 (*see* Figure 4) are defined as: P1, F508del, exon 9^-; P2, non-F508del, exon 9^-; P3, F508del, exon 9^+; P4, non-F508del, exon 9^+; P5, normally spliced (17a) + F508del; P6, alternatively spliced (17a); and P_{deg}, assumed to be degraded (not observed).

Step 1: Use of plasmid to correct exon $9^-/9^+$ unequal amplification. To correct peak areas (P_{corr}) for size-specific amplification differences (in the region of exons 8–10), raw values for peak area integration obtained with the GeneScan software were multiplied by their respective correction factors. These were estimated from the parallel amplification of exon $9^-/9^+$ plasmid and found to be $\sim$ 0.4 for peaks corresponding to exon 9^- products (amplified at higher efficiency) and 0.6 for peaks corresponding to exon 9^+ products (amplified at lower efficiency). Corrected peak areas were calculated as follows: $P1_{corr} = P1 \times 0.4$; $P2_{corr} = P2 \times 0.4$; $P3_{corr} = P3 \times 0.6$; and $P4_{corr} = P4 \times 0.6$.

Step 2: Estimate of percentage of transcripts relative to total CFTR present. The second step was to estimate the percentage of each of the six different CFTR transcripts present (*see* Figures 4 and 5) in the patients relative to the total CFTR RNA present (Total_{pres}), using the corrected values determined in Step 1. Total_{pres} can be estimated from RT-PCR amplifications in the region of exons 8–10 as follows: $\text{Total}_{pres} = \Sigma\ \text{Pn}_{corr}$ (n = 1–4); or from RT-PCR amplifications in the region of exons 16–17b as follows: $\text{Total}_{pres} = P5 + P6$. The percentage for each of the six different CFTR transcripts present is thus: $\%\ \text{Pn} = (\text{Pn}_{corr}/\ \text{Total}_{pres}) \times 100$.

The total percentage of F508del transcripts without (P1) or with (P3) exon 9 in the five patients was estimated from the following formula: $\%\ \text{F508del} = (\text{P1}_{corr} + \text{P3}_{corr}\ /\ \text{Total}_{pres}) \times 100 \approx 77.1\%$.

Similarly, the proportion of transcripts resulting from the 3272-26A>G allele that are abnormally spliced at intron 17a (alt_{17a}) was estimated to be (Table 1, second column): $\%\ \text{alt}_{17a} = (\text{P6}_{corr}\ /\ \text{Total}_{pres}) \times 100 \approx 14.1\%$, and the percentage of transcripts resulting

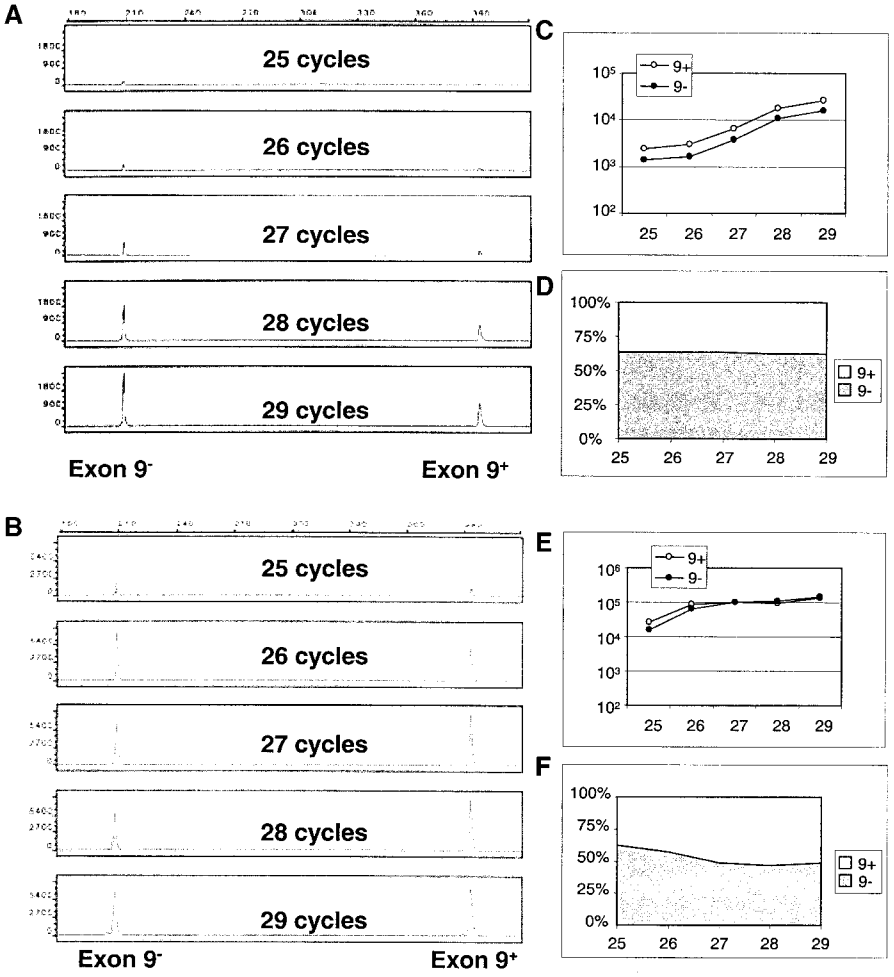

Figure 2. Relative efficiency of amplification for exon 9^+/exon 9^- products using the control plasmid. Results from electrophoresis of Fam-labeled products from PCR reactions with different number of cycles (indicated in *electrophorams*) and using as template 16.5×10^{-5} ng (*A*) or 16.5×10^{-4} ng (*B*) of the control plasmid per reaction. The peak areas shown in *A* and *B* were calculated by software integration (*see* MATERIALS AND METHODS) and plotted on a logarithmic scale against the respective number of cycles (*C* and *E*, respectively). Also, from integration of peak areas shown in *A* and *B*, the relative percentages of products obtained with (9^+) and without (9^-) exon 9 were estimated and plotted against the number of cycles used (*D* and *F*, respectively). By comparison of *C* with *D*, and *E* with *F*, it can be observed that the relative percentages of 9^+ and 9^- products are only maintained while the amplification is in the exponential phase. The *scale on the left* is in arbitrary units and the one above *electrophorams* is calibrated to base pairs (bp).

from the 3272-26A>G allele that are normally spliced at intron 17a ($norm_{17a}$) was estimated from the difference between the total (100%) and the two above-mentioned percentages (of total F508del transcripts and of abnormally spliced transcripts at intron 17a, Table 1, third column): % $norm_{17a}$ = 100% - % F508del - % alt_{17a} = 100% - 77.1% - 14.1% ≈ 8.8%.

Step 3: Estimate proportion of exon $9^-/9^+$ CFTR transcripts. The percentage of normally spliced transcripts at intron 17a estimated above did not take into consideration the fact that part of these lack exon 9. Accordingly, the proportion of exon 9^- transcripts within % $norm_{17a}$ (assuming that splicings of introns 8 and 17a occur as independent events) could be estimated from the ratio of non-F508del peaks in the 8–10 region, without (P2) and with (P4) exon 9, as follows: (% $norm_{17a}$ ex 9^-) / (% $norm_{17a}$ ex 9^+) = P2 / P4 = $^1/_{17}$.

Step 4: Estimate of percentage of full-length relative to total CFTR present. By using the latter value in the following system

TABLE 1

Percentage of normal full-length CFTR mRNA present in patients with 3272-26A>G/F508Δ

Patient	Alt_{17} relative to total present (%)	Normal, relative to total present* (% ± *SD*)	Estimate of assumed degraded (± *SD*)	Normal relative to total in wt/wt individuals* (% ± *SD*)
1†	16.0 (15.1; 17.0)	7.0 ± 1.38 (*n* = 4)‡	43.1 ± 1.07	4.0 ± 0.85
2	12.2 (12.5; 11.9)	8.4 ± 5.41 (*n* = 4)	44.6 ± 4.11	4.8 ± 3.34
3	17.0 (16.6; 17.5)	7.7 ± 2.12 (*n* = 3)	41.6 ± 1.47	4.5 ± 1.37
4†	9.7 (9.5; 9.8)	9.0 ± 1.94 (*n* = 4)	46.1 ± 1.45	4.9 ± 1.18
5†	15.5 (15.4; 15.5)	8.8 ± 2.27 (*n* = 4)	41.8 ± 2.13	5.2 ± 1.47
Average ± SD	14.1 ± 3.1	8.2 ± 0.84	43.5% ± 1.9	4.7% ± 0.45

* Values corrected by use of exon 9^+/exon 9^- ratio obtained for control plasmid in parallel.
† Analysis repeated for different sample collections.
‡ *n* = number of times analysis was repeated.

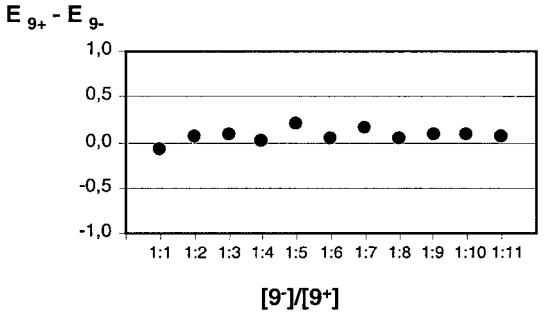

Figure 3. Variation of amplification efficiencies for exon 9^+ and exon 9^- products with increasing exon 9^-/exon 9^+ ratios of initial templates. The graph is a plot of the differences in efficiency values for exon 9^- and exon 9^+ products (y axis) for eleven different exon 9^-/exon 9^+ ratios (x axis). Amplification efficiencies (E) were estimated by linear regression of Log A (peak areas) versus the number of PCR cycles (n): slope = Log (1 + E). The differences in amplification efficiencies ranged from 0.01–0.19, indicating that the ratio of initial exon 9^-/exon 9^+ templates in the sample does not influence the amplification efficiency substantially.

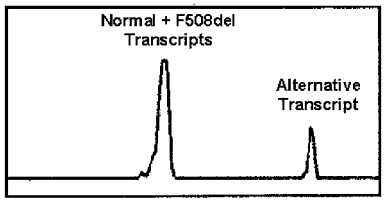

Figure 5. CFTR mRNA analysis from one patient with 3272-26A>G/F508del in the region of exons 16–17b. Following separation of Fam-labeled RT-PCR products (region of exons 16–17b) by polyacrylamide gel electrophoresis in the automatic sequencer, the peaks obtained by the GeneScan software (*see* MATERIALS AND METHODS) are shown here.

of equations: (% $norm_{17a}$ ex 9^-)/(% $norm_{17a}$ ex 9^+) = $^1/_{17}$ and (% $norm_{17a}$ ex 9^-) + (% $norm_{17a}$ ex 9^+) ≈ 8.8%, the two following values could be estimated: % $norm_{17a}$ ex 9^- = 0.6%; and % $norm_{17a}$ ex 9^+ = 8.2%.

The latter value is the percentage of full-length CFTR transcripts (i.e., normally spliced at intron 17a and containing exon 9) relative to the total CFTR RNA present in these patients.

Step 5: Estimate of total CFTR RNA present in normal individuals and corrections for percentage of assumed degraded trasncripts in 3272-26A>G/F508del patients. To quantify the level of full-length CFTR transcripts in the five 3272-26A>G/ F508del patients relative to normal individuals (*see* Figure 4), the amount of CFTR transcripts in the latter must be estimated. We term it "total in normal (wt/wt individuals)" ($Total_{norm}$). For that purpose, $Total_{norm}$ was assumed to be twice the average of wt-CFTR transcript levels found in F508del carriers (n = 30) estimated from the F508del/wt ratio: F508del/wt ≈ 0.87 ⇒ wt ≈ 1.15 × $F508del_{carr}$ (in F508del carriers, n = 30). The double of this average level is thus $Total_{norm}$: $Total_{norm}$ = 2 × wt ⇒ $Total_{norm}$ = 2 × (1.15 × $F508del_{carr}$).

The amount of RNA that was assumed to be degraded (P_{deg}) in the five patients (because of the stop codon in the alternative transcript) was estimated from the difference between levels of $Total_{norm}$ and $Total_{pres}$: $Total_{norm}$ = $Total_{pres}$ + P_{deg} ⇒ P_{deg} = $Total_{norm}$ − $Total_{pres}$ ⇒ P_{deg} = 2 × (1.15 × $F508del_{carr}$) − $Total_{pres}$.

Assuming that levels of $F508del_{carr}$ equal the levels of F508del transcripts in the 3272-26A>G/F508del patients, i.e., using the area of the F508del peak as reference, and from Step 2 above: $F508del_{carr}$ = F508del = $P3_{corr}$ + $P1_{corr}$.

Also from Step 2 above, the $Total_{pres}$ was: $Total_{pres}$ = Σ Pn_{corr} (n = 1–4). And, thus: P_{deg} = 2 × 1.15 × [$P3_{corr}$ + $P1_{corr}$] − [$P1_{corr}$ + $P2_{corr}$ + $P3_{corr}$ + $P4_{corr}$].

From here, we estimated the percentage of assumed degradation that occurred for 3272-26A>G-transcripts to be (Table 1, fourth column): % P_{deg} = P_{deg} / $Total_{norm}$ x 100 ≈ 43.5% of $Total_{norm}$.

Step 6: Estimate percentage of transcripts relative to total in normal individuals. Levels of all transcripts present were then re-estimated, now in relation to the total CFTR mRNA in normal individuals from both alleles (the total present plus the estimated assumed degraded), i.e., $Total_{norm}$ instead of $Total_{pres}$. So, Step 2 (*see above*) was repeated for $Total_{norm}$ and the new percentage of F508del transcripts is: % F508del = ($P1_{corr}$ + $P3_{corr}$)/$Total_{norm}$ × 100 ≈ 43.3%.

Similarly, for the two types of CFTR transcripts resulting from the 3272-26A>G allele: % alt_{17a} = ($P6_{corr}$/$Total_{norm}$) × 100 ≈ 8.2%;

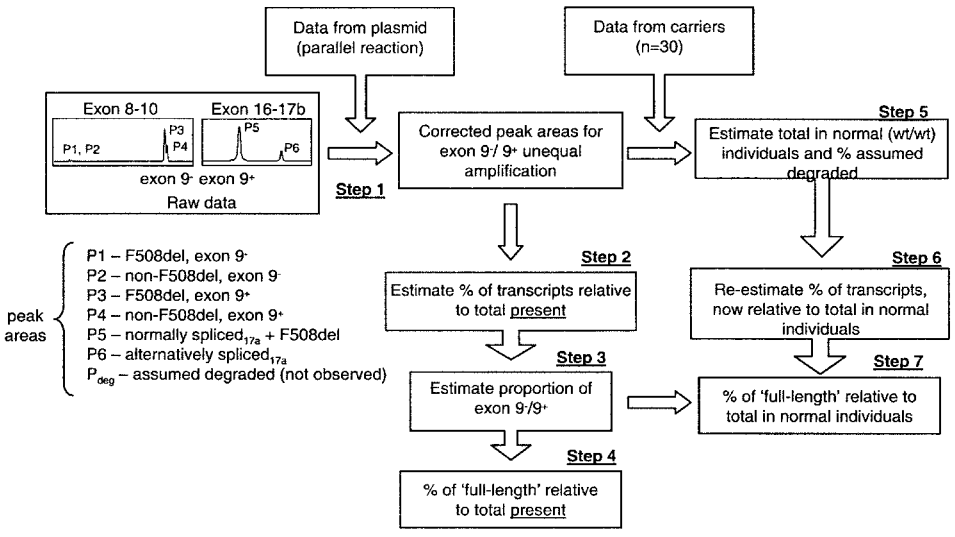

Figure 4. Schematic representation of calculations made from the raw data shown in Figure 6. For the calculations, data from independent analysis of the same patient was treated separately and averaged in the end. All values shown are approximate averages (*see* Table 1 for exact values ± SD, and MATERIALS AND METHODS for description).

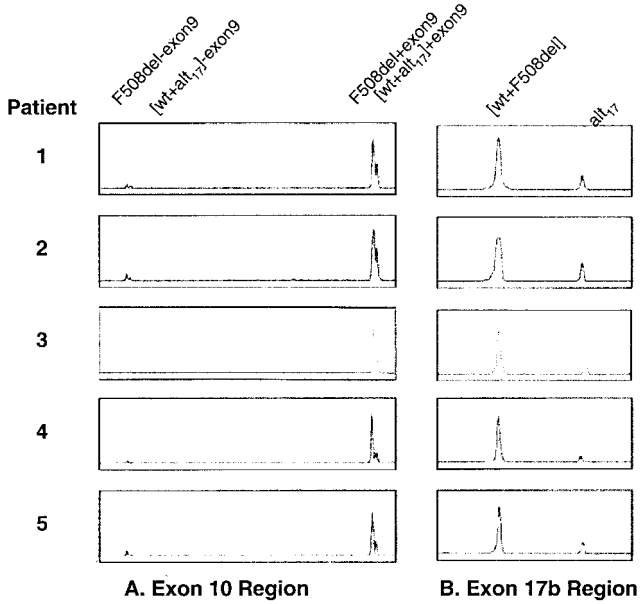

Figure 6. Combined CFTR mRNA analysis from the five patients with 3272-26A>G/F508del in the regions of exons 8–10 (*A*) and 16–17b (*B*). Raw data obtained from integration of these peaks as described for Figure 5 were used in calculations (as described in MATERIALS AND METHODS and schematically in Figure 4).

and % $norm_{17a}$ = 100%$_{norm}$ − % F508del − % alt_{17a} − % P_{deg} = 100% − 43.5% − 43.3% − 8.2% ≈ 5.0%.

Step 7: Estimate percentage of full-length relative to total in normal individuals. Similarly to Step 4 (*see above*), the $^1/_{17}$ ratio of CFTR transcripts without and with exon 9 obtained in Step 3 (*see above*) was used again, now in the following system of equations: (% $norm_{17a}$ ex 9^-)/(% $norm_{17a}$ ex 9^+) = $^1/_{17}$ (% $norm_{17a}$ ex 9^-) + (% $norm_{17a}$ ex 9^+) ≈ 5.0%.

From this, the following values could be obtained (Table 1, fifth column): % $norm_{17a}$ ex 9^- ≈ 0.3%; and % $norm_{17a}$ ex 9^+ ≈ 4.7%. The latter is the percentage of normal full-length CFTR transcripts (i.e., non-F508del transcripts, normally spliced at intron 17a and containing exon 9) relative to the estimated total amount of CFTR mRNA in normal individuals.

Results

Summary of Quantification Process and Results

To quantify the levels of normal CFTR transcripts in the cells of the patients, two PCR reactions in the regions 8–10 and 16–17b were performed. Samples from patients are expected to produce four peaks in the exon 8–10 reaction (*see* peaks 1–4 in the "Raw data" *inset* of Figure 4) and two peaks in the exon 16–17b reaction (*see* peaks 5–6 in the *inset* of Figure 4). Based on these peak areas, calculations to obtain the levels of normal CFTR transcripts relative to a normal individual (wt/wt) were made using the following general strategy (*see also* Figure 4 and the detailed description in MATERIALS AND METHODS):

1. Amplification of exons 16–17b shows that 86% of CFTR transcripts are normally spliced, but part of these correspond to F508del (and are therefore non-functional) and part result from the 3272-26A>G allele.
2. To determine the relative contribution of each of the alleles to the normally spliced transcripts, the relative amount of F508del versus non-F508del transcripts was determined by performing exon 8–10 amplification.
3. Amplification in the exon 8–10 region evidences exon 9 skipping; to assess exon 9 skipping, correction for unequal amplification of 9^+ versus 9^- products had to be introduced.
4. The combination of Steps 2 and 3 showed that most of the transcripts (77%) were from the F508del allele.
5. The combination of Steps 1 and 4 showed that ∽ 8% of full-length transcripts were from the 3272-26A>G allele compared with F508del allele.
6. To assess the amount of wt transcripts in the patients compared with normal individuals, the relative amount of the F508del transcripts was assessed relative to transcripts from a normal allele (by using data from 30 F508del carriers).
7. Combination of Steps 5 and 6 revealed that these five patients with CF had ∽ 5% of normal levels.

Relative Efficiency of Amplification for exon 9^+/exon 9^- Products from the Control Plasmid

RT-PCR in the region of exons 8–10 of CFTR yields products with (9^+) and without (9^-) exon 9. Due to the difference in size between these products (183 nts), the amplification of exon 9^- product is expected to be more efficient than the amplification of the exon 9^+ product. To determine this difference of amplification efficiencies, we used a plasmid that provided *CFTR* sequences with and without exon 9 in equimolar amounts by virtue of being on the same molecule (Figure 1). As expected, the smaller product consistently produced a more intense signal while the amplification reaction was in exponential phase (Figure 2A and *first panel* of Figure 2B), which can be graphically controlled (Figures 2C and 2E). It is noteworthy that the relative percentages of the exon 9^+ and exon 9^- products from this external control change, even invert (*see graphs* in Figures 2D and 2F), when the reaction enters the plateau phase (*four lower panels* in Figure 2B)—i.e., for higher numbers of amplification cycles and/or higher amount of plasmid at the beginning (Figure 2B). Consequently, for quantification purposes, it is important to vary the cycle number to ensure that the amplifications are within the exponential range (Figures 2C and 2E).

Plotting the differences in efficiency values for exon 9^+ and exon 9^- products yields the graph shown in Figure 3. It is evident from the graph that amplification efficiencies do not vary substantially, with increasing amounts of $[Ex_{9-}]_0$/$[Ex_{9+}]_0$ ratio, up to 1:11. These data indicate that the unequal ratio of initial exon 9^-/exon 9^+ templates (of ∽ 1:10) in the sample does not influence the amplification efficiency. Thus, the amplification efficiencies obtained for the control plasmid (where initially the exon 9^-/exon 9^+ templates are in a 1:1 ratio) were used to correct values obtained experimentally for patients' samples. The relative intensities of the exon 9^+ and exon 9^- products from this external control (in the range 0.35–0.4/0.65–0.6) were therefore determined in parallel with each PCR reaction for patients' samples,

TABLE 2
Percentage of alternative transcripts present in 3272-26A>G carriers

Parent of Patient No.	Alternative transcripts, relative to total present (%)
1	11.4 (11.8; 11.)
2	8.8 (8.6; 8.9)
3	10.9 (10.0; 11.7)
5	11.1 (10.3; 11.9)
Average ± SD	10.5 ± 1.2

and were used to correct for their difference in amplification efficiencies (*see* Figure 4 and *note asterisk* in Table 1).

Transcripts from Patients with 3272-26A>G/F508del: Analysis of Exons 16–17b

RT-PCR analysis in the region of exons 16–17b for transcripts from patients with 3272-26A>G/F508del generated two peaks (Figure 5), corresponding to products from the alternatively spliced transcript, with the expected 25 additional nucleotides (*lower peak on the right*, 328 bp), and to products from the normal-sized transcripts (*higher peak on the left*, 303 bp), respectively. The latter peak, however, contains products both from correctly spliced transcripts from the −26 allele and F508del transcripts, as these are indistinguishable in this region. Results from quantification of the alternative transcripts (alt_{17}) for the five patients analyzed are shown in Table 1 (*second column*) and were obtained as described in MATERIALS AND METHODS (*see also* Figure 4). On average, the alternative transcripts accounted for 14.1 ± 3.1% of the total RNA present. Similar analyses were made for samples from the four 3272-26A>G-carriers of the mutation (Table 2), for which the alternative transcripts accounted for an average of 10.5 ± 1.2% of the total RNA present.

Analysis of Transcripts from patients with 3272-26A>G/F508del: Analysis of Exons 8–10

Following RT-PCR in the region of exons 8–10, four different peaks are detected in each RNA sample from the five patients with 3272-26A>G/F508del (Figure 6A). These correspond, from right to left, to amplification products of the following transcripts: wild-type (wt) plus alternative splicing at intron 17a− (alt_{17}), the latter appearing with normal size in this region (391 bp); F508del (388 bp); wt plus alt_{17}, without exon 9 (208 bp); and F508del without exon 9 (205 bp). The bands shown in Figure 5 for just one patient, are shown in Figure 6B for the five patients analyzed in this study, and correspond to the correctly (*left*) and alternatively (*right*) spliced transcript at intron 17a.

Percentage of Full-Length CFTR mRNA Relative to Total Present in the Five Patients

By combining quantitative results obtained in both amplifications, i.e., regions 8–10 and 16–17b (Figures 6A and 6B, respectively), after correction for unequal amplification (region 8–10) using the control plasmid (Figure 4, Step 1), we calculated the percentages of different CFTR mRNA species relative to total CFTR present for each of the five patients analyzed here (Figure 4, Step 2; *see also* MATERIALS AND METHODS). The level of transcripts lacking exon 9 was deduced from the previous estimates (Figure 4, Step 3) and percentage of full-length CFTR mRNA was obtained (Figure 4, Step 4). Results are shown in Table 1, *third column*. Calculations based on repeated experiments (*see* MATERIALS AND METHODS) indicate that on average 8.2 ± 0.84% of the total CFTR RNA present in these patients is full-length.

Percentage of Full-Length CFTR mRNA in the Five Patients Relative to Total in Normal Individuals

It is evident from peaks obtained in the analysis of exons 8–10 (Figure 6A) that the F508del-transcripts level is significantly higher than the level of [wt+alt_{17}] transcripts in these patients. Therefore, we determined the ratio of F508del-transcripts relative to a generic wt allele by analyzing nasal brushing RNA samples from F508del carriers (n = 30), using the same method described above (RT-PCR analysis of exons 8–10), and found it to be 0.87 ± 0.06. So, contrary to what is observed in patients with 3272-26A>G/F508del (Figure 6A), in F508del carriers, the ratio of 0.87 ± 0.06 indicates that the levels of F508del transcripts are consistently lower than non-F508del (i.e., wt) transcripts. We believe that the lower levels of the non-F508del transcripts in patients with 3272-26A>G/F508del must result from a dramatic decrease in the levels of alternative transcripts. Indeed, the latter most probably undergo degradation at increased rates due the presence of a premature stop codon (5), a generally described phenomenon (7).

To quantify the level of full-length CFTR transcripts present in the patients with 3272-26A>G/F508del in relation to the total in normal individuals (i.e., with a wt/wt *CFTR* genotype) we estimated the percentage of RNA that was assumed to be degraded. The latter is the difference between levels of expression of two generic wt-alleles (estimated from the F508del/wt ratio obtained in carriers; *see* Figure 4, Step 5), and the total level of CFTR transcripts present in the patients with 3272-26A>G/F508del, which we measure (Figure 4, Step 5). By using the average value of 0.87 ± 0.06 for the ratio F508del/generic-wt (*see* MATERIALS AND METHODS), we estimated the percentage of CFTR mRNA degradation assumed to have occurred in patients with 3272-26A>G/F508del (*see* Figure 4, Step 6) to be on average 43.5 ± 1.9% of the total in normal individuals (Table 1, *fourth column*). The levels of remaining normal full-length mRNA were then re-estimated, now in relation to the total present in normal individuals (Figure 4, Steps 5–7). Samples from patients with 3272-26A>G/F508del were thus found to have on average 4.7 ± 0.45% of normal CFTR mRNA relative to total in normal individuals (Table 1, *fifth column*).

Discussion

The 3272-26A>G mutation creates a novel acceptor in intron 17a that competes with the normal one, producing both correctly and aberrantly spliced mRNAs (5). As with

other class I *CFTR* mutations (previously class V) (8–10), the milder clinical phenotype of patients with 3272-26A>G (4) is due to the presence of reduced levels of functional CFTR resulting from transcripts correctly spliced at intron 17a. Here, we have quantified the levels of full-length mRNA present in these patients to address the question of the level of normal CFTR transcripts necessary to avoid classic CF.

Reproducibility of the Method

We developed and applied a sensitive and reproducible method that is based on the detection and quantification of fluorescently-labeled molecules resulting from RT-PCR amplification. The method accommodated the low number of CFTR mRNA copies present in nasal brushing samples (11), allowing simultaneous quantitation of low- and high-level transcripts. We demonstrated consistent differences in amplification efficiencies for exon 9^+/exon 9^- products (i.e., with 183 bp difference), by using a plasmid as control (Figures 2A and 2B), which to our knowledge has not been used before. These results demonstrated the need to introduce a correction for every PCR reaction performed to analyze exon $9^+/9^-$ products, and thus such a correction was used for values obtained in analysis of patient samples. Our results also show the need to obtain data in the exponential phase of the PCR (Figure 2), an issue that has been controversial (12, 13). Therefore, we performed, for each sample analyzed, three parallel reactions differing solely in the number of cycles. Only those values that resulted in the same peak area ratios were used (i.e., consistent results along a semilogarithmic line; *see graphs* in Figure 2), as this proved the reaction was still in exponential phase. We also demonstrated that the amplification efficiency of exon 9^+ and exon 9^- RT-PCR products does not vary substantially when the initial ratio of product varies by as much as one order of magnitude. This result is consistent with observations of other authors who have shown that PCR efficiency remains constant at ratios that differ by three, or even four, orders of magnitude (14–16).

After extensive experimentation to verify the accuracy and reproducibility of our methods, we obtained highly consistent results among samples from the same individual analyzed on different days, and among the five patients (and four carriers) analyzed. This optimized procedure distinguishes between F508del and non-F508del transcripts, thereby allowing comparative analysis of the CFTR mRNA transcript levels generated by any patients or individuals carrying F508del and any other allele.

Limitations of the Study

One of our objectives was to compare the levels of full-length transcripts present in these patients relative to normal individuals. Because quantification methods like Northern blotting or RNAse-protection assay (RPA) are not possible for CFTR transcripts because of their low abundance in native tissues (11), we used an indirect approach. The importance of taking into account transcript degradation, assumed to occur for alternative transcripts, is illustrated by the fact that the level of full-length CFTR mRNA in patients with 3272-26A>G/F508del decreases from 8.2 ± 0.84% (determined in relation to the total present) to 4.7 ± 0.45% (determined in relation to the total in normal individuals). This reduces our estimate by almost half. For determination of full-length CFTR mRNA, we also show that it is not critical in this case to deduce exon 9^- transcripts (corrected for amplification efficiency with plasmid), as these only account for $\backsim$0.3% reduction in the final fraction of remaining transcripts in the patients with 3272-26A>G/F508del. However, with other variants at the polymorphic region of intron 8 that favor exon 9 skipping, like the 5T allele (6), it may become critical to introduce this correction.

Although RT-PCR is an indirect approach to determine the level of full-length CFTR mRNA in patients relative to normal individuals, it is probably the only technique that is sufficiently sensitive for low-copy number transcripts such as CFTR. Indeed, RT-PCR has been used extensively for estimation of CFTR transcripts by other investigators (3, 6, 8, 17). However, in this study we have incorporated several key controls that were not used previously and have obtained consistent results from five patients. On the other hand, real-time PCR, which may have a number of applications, is only useful to quantify differences that lead to visible changes > 1/3 cycle. This means that for the present case of wt/F508del transcripts in F508del carriers, it would only detect differences above 42%/58%, i.e., 16%. Here we describe a 7% difference between F508del and wt transcripts (i.e., 46.5 and 53.5%, respectively; *see below*), which would thus will fail to be detected by real-time PCR.

CFTR mRNA Levels in Carriers

From results shown here, the aberrantly spliced transcripts present in the five patients with 3272-26A>G/F508del correspond on average to 14.1 ± 3.1% of the total RNA present. These transcripts constitute on average 10.5 ± 1.2% for the four 3272-26A>G carriers we analyzed. This difference may result from variation of splicing efficiencies at the cryptic and normal splice acceptors in intron 17a. Because the level of aberrant transcripts was significantly lower in parents than in their children ($P < 0.05$, paired Student's t test), we speculate that carriers may have higher total levels of CFTR mRNA than patients, due to the fact that they have wt, not F508del, in *trans* with 3272-26A>G, thus altering the relative percentages. Indeed, we demonstrated in 30 carriers that F508del transcripts are less abundant than wt transcripts. This result was intriguing because the level of F508del transcripts has been shown to be the same as wt in human carriers (5, 18–20), and in the CF-F508del mouse (21). Our finding that the F508del transcript level was consistently lower (7% on average) relative to wt is probably due to the higher sensitivity of the method we employed. The biologic reason for this difference is not understood at this point, as it may result from either diminished transcription (the F508del allele may be in *cis* with a less efficient variant of the promoter than wt) or enhanced degradation of the mutant transcripts. The CF-F508del mouse model, described to have equal levels of both transcripts, was produced by introducing the F508del mutation into a normal *CFTR* gene (21), an argument that favors the first hypothesis.

Comparisons with Other Studies and Implications for CF Therapy

Previous reports describing CFTR RNA quantifications did not correct for size-specific differences in amplification efficiency, and so comparisons were made between PCR products with and without exon 9 (6), or between products differing 84 bp in size (8). This could have led to underestimation of the (longer) products from normally and aberrantly spliced transcripts, respectively. Indeed, another study analyzed CFTR mRNA in individuals with the 5T allele, using both differential and nondifferential RT-PCR, and found that levels of PCR products obtained by the former method were 2.5-fold higher than in the latter (3). However, as the nondifferential method uses different 5′ primers to amplify the two different products, the question of variable primer efficiency should also be taken into consideration. Furthermore, the number of cycles used in PCR reactions ($n = 35$) is suggestive that products were no longer obtained in the exponential phase of amplification and, for most of the quantitative methods described in the literature, there is no description on how this issue was controlled.

Values obtained here for the percentage of full-length CFTR mRNA in patients with 3272-26A>G/F508del, relative to total present (8.2 ± 0.84%), are consistent with those described by Highsmith and coworkers (8) for the 3849+10kb C>T mutation, a CF allele associated with mild lung disease, pancreatic sufficiency, and male fertility (∽8%). The mutation 2789+5G>A, resulting in a similar pulmonary and pancreatic phenotype but with male infertility, is associated with lower levels of correctly spliced CFTR mRNA (4%) (10). However, neither of these studies took into consideration the possible degradation of F508del or misspliced transcripts. The latter issue is particularly important, because the alternatively spliced transcripts resulting from both 3849+10kb C>T and 2789+5G>A alleles bear premature termination signals. Enhanced degradation of messengers bearing premature stop codons is a recognized phenomenon, termed nonsense-mediated decay, or NMD (7), which was also described for CFTR transcripts (22). The possible degradation of the alternative transcripts should thus be evaluated, and if present, should be taken into account for the final estimates, as it causes a reduction in the overall CFTR levels present and therefore affects the resulting percentages of various mRNA species.

For other class I mutations, levels of full-length CFTR mRNA were described to be of 10% in three 5T/5T homozygous non-CF individuals (2), whereas another group reported that 5T/5T homozygous and compound heterozygous individuals for 5T with no lung disease (%FEV_1 > 80%) always have more than 25% of full-length CFTR mRNA (3). Our results are compatible with these data, although the latter value of 25% should perhaps be viewed with care, not only because percentages are relative to different totals, but also because the methods used were different, as discussed above.

In conclusion, by determining here that patients with 3272-26A>G/F508del have ∽ 5% of full-length mRNA relative to normal individuals, we postulate that this is enough to ameliorate the severity of CF disease, because in these patients most clinical symptoms, including lung function and pancreatic involvement, are attenuated by comparison to patients with two severe mutations (4). It should, however, be kept in mind that 5% is not enough to avoid the complications of CF, indicating that this threshold will probably have to be surpassed in therapeutic approaches aimed at increasing CFTR levels.

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