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Research Article

Cystic fibrosis (CF) patients bearing the premature translation termination mutation (nonsense mutation) W1282X present severe pulmonary and pancreatic disease, whereas patients carrying other nonsense mutations such as G542X, R553X, S1255X, R1162X, and W1316X show a severe pancreatic but mild pulmonary illness. CF gene expression was found absent in respiratory tissues with mutations R553X and W1316X, which led to the hypothesis that the absence of the gene product in the lung is more favorable than the presence of an altered one. We asked whether or not all the nonsense mutations characterized by mild pulmonary disease phenotypes do present the absence of CF gene expression. We therefore investigated gene expression at the mRNA level in respiratory cells obtained from nasal polyps from a patient homozygous for the R1162X mutation. Gene expression was studied by amplification with polymerase chain reaction of segments of the CF transmembrane conductance regulator cDNA that was obtained by reverse transcription of RNA. Semiquantitative analysis was performed by Northern analysis. By comparing the data obtained from polyps deriving from non-CF subjects and a CF patient homozygous for dF508 mutation, it is shown that no reduction of CF gene expression is evident in R1162X respiratory tissue. We conclude that CF nonsense mutations have heterogeneous mechanisms of gene expression.

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Nonsense Mutation R1162X of the Cystic Fibrosis Transmembrane Conductance Regulator Gene Does Not Reduce Messenger RNA Expression in Nasal Epithelial Tissue

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Abstract

Cystic fibrosis (CF) patients bearing the premature translation termination mutation (nonsense mutation) W1282X present severe pulmonary and pancreatic disease, whereas patients carrying other nonsense mutations such as G542X, R553X, S1255X, R1162X, and W1316X show a severe pancreatic but mild pulmonary illness. CF gene expression was found absent in respiratory tissues with mutations R553X and W1316X, which led to the hypothesis that the absence of the gene product in the lung is more favorable than the presence of an altered one. We asked whether or not all the nonsense mutations characterized by mild pulmonary disease phenotypes do present the absence of CF gene expression. We therefore investigated gene expression at the mRNA level in respiratory cells obtained from nasal polyps from a patient homozygous for the R1162X mutation. Gene expression was studied by amplification with polymerase chain reaction of segments of the CF transmembrane conductance regulator cDNA that was obtained by reverse transcription of RNA. Semiquantitative analysis was performed by Northern analysis. By comparing the data obtained from polyps deriving from non-CF subjects and a CF patient homozygous for dF508 mutation, it is shown that no reduction of CF gene expression is evident in R1162X respiratory tissue. We conclude that CF nonsense mutations have heterogeneous mechanisms of gene expression. (*J. Clin. Invest.* 1993. 92:2683–2687.) Key words: cystic fibrosis • reverse transcription • polymerase chain reaction • genotype • phenotype

Introduction

Cystic fibrosis (CF)¹ is the most common severe autosomal recessive disorder in Caucasians. The pathophysiology of the disease is associated with the abnormal regulation of epithelial chloride channels (1), which determines accumulation of vis-

cous secretions in the lungs and pancreas and abnormal electrolyte composition in the sweat (2). CF is caused by mutations in the so-termed cystic fibrosis transmembrane conductance regulator (CFTR) gene, which encompasses 27 exons encoding a 1,480-amino acid protein (3–5). Structurally, CFTR is similar to the multidrug resistance P-glycoprotein, and is considered a member of the ATP-binding cassette superfamily of membrane transporters (4, 6). CFTR presents two ATP-binding sites, two transmembrane-spanning domains, and a regulatory domain, containing phosphorylation sites, which links the two halves of the protein (4). Several evidence point out that CFTR is a protein kinase-regulated Cl[−] channel (7–13).

So far 250 mutations have been identified in the CF gene (CF Genetic Analysis Consortium, unpublished data), and 27 of these (13%) produce stop-codons (nonsense mutations). Patients bearing nonsense mutations, such as S1255X, G542X, G553X, and W1316X, present severe pancreatic insufficiency but mild to moderate pulmonary illness (14–17), whereas other patients carrying the W1282X present differences in the severity of lung involvement (18, 19). Pancreatic insufficiency and mild to moderate pulmonary disease have also been shown in patients homozygous for a very common nonsense mutation in northeastern Italy (10.2% of the CF chromosomes), which is R1162X (20, 21). This mutation is located in exon 19 between the second transmembrane domain and the second nucleotide binding fold, and it is characterized by the substitution of the amino acid arginine in position 1162 of CFTR with a UGA termination codon. So far there is no basis for claiming a relationship between the type of nonsense mutation and CF phenotype, which is a crucial question that has important implications on both drug and gene therapy.

There is evidence that suggests that the most common CF mutation, dF508, results in a defective processing and localization of the CFTR protein (22–27). It was shown that the basis of CFTR dF508 failure was due both to a decreased amount of the protein on the apical membrane and to the reduced time of opening of the mutated CFTR channel (28). However, not all mutations of CFTR affect protein processing. For instance, the missense mutation G551D, which is also in the nucleotide binding domain like dF508, produces a normally processed CFTR (22) with a severely reduced function (29). Therefore CFTR mutations can either cause a defective processing of CFTR or directly decrease CFTR function.

A third different mechanism of CFTR alteration has been suggested by studies on some nonsense mutations, namely G553X (exon 11) and W1316X (exon 21), in which severe reduction of CFTR mRNA and undetectable protein have been reported in respiratory cells (30, 31). Also, for nonsense mutations of CFTR, the mechanism is probably not univocal, owing to the controversial data presented on the W1282X mutation (32, 33). It is already known that in vivo nonsense mu-

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1. Abbreviations used in this paper: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; RT, reverse transcription.

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tations in genes of other human diseases lead either to decreased mRNA accumulation (34–36) or to normal mRNA levels with production of truncated protein (37–39). In both cases the molecular mechanism is not clearly understood.

In order to better understand the pathway by which CFTR nonsense mutations lead to the CF disorder, we investigated the effect of the nonsense mutation R1162X on the expression of the CF gene. We analyzed the steady-state levels of CFTR mRNA in nasal polyps of a patient homozygous for R1162X, and we found a levels of transcript comparable to that detected in non-CF tissue.

Methods

Experimental subjects. Nasal polyps, excised to relieve airway obstruction, were obtained from a female CF patient homozygote for R1162X, a CF male homozygote for the phenylalanine deletion at codon 508 (dF508), and non-CF subjects. Tissue specimens were collected in the operating room immediately after the excision and placed in chilled Leibovitz-15 medium (ICN-Flow, Irvine, UK) for transport to the laboratory, where they were aliquoted, frozen in liquid nitrogen, and stored at -80°C until use.

Cell culture. T84 colon carcinoma cells (CCL 248, American Type Culture Collection, Rockville, MD) were grown to confluence in DME (ICN-Flow) and Ham's F12 (1:1) (Sigma Chemical Co., St. Louis, MO) supplemented with 5% FBS (ICN-Flow), 2 mM glutamine (ICN-Flow), and 40 $\mu\text{g}/\text{ml}$ gentamycin (Schering-Plough, Kenilworth, NJ). NIH 3T3 cells (American Type Culture Collection, CRL 1658) were grown to confluence in DME with 10% FBS, 2 mM glutamine, and 40 $\mu\text{g}/\text{ml}$ gentamycin. Cells were washed once with chilled PBS (ICN-Flow) before the total RNA extraction.

White blood cell isolation. White blood cells were isolated from buffy coats obtained from peripheral blood of healthy volunteer and red blood cells were disrupted by hypotonic lysis.

RNA extraction. Frozen polyps and washed cells were placed directly in the RNazol B solution (Biotecx Laboratories, Inc., Houston, TX), and homogenized with few strokes in a glass-Teflon homogenizer (B. Braun, Melsungen, FRG). Total RNA was isolated according to the supplier's instructions, redissolved in RNase-free 1 mM EDTA, 10 mM NaCl, 10 mM Tris-HCl (Carlo Erba, Milan, Italy) pH 8.0 solution, and measured by ultraviolet (UV) spectrophotometry. The quality of the extracted samples was also assessed in a nondenaturing 1.2% agarose (Sigma Chemical Co.)/89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA (Carlo Erba) gel electrophoresis (40) by visual examination of ethidium bromide-stained 18s and 28s ribosomal fractions as undegraded bands.

cDNA synthesis and PCR amplification. cDNA was synthesized by reverse transcription (RT) of total RNA and subsequently amplified in a single tube reaction by using the GeneAmp RNA PCR Kit and method (Perkin Elmer Cetus, Norwalk, CT). Briefly, 3 μg of total RNA suspended in 2 μl of 10 mM Tris-HCl, 10 mM NaCl, and 1 mM EDTA was added to 4 μl of 25 mM MgCl_2 , 2 μl of 500 mM KCl/100 mM Tris-HCl (10 \times PCR buffer II), 1 μl of sterile water, 2 μl of 10 mM (each) dNTP, 20 U of RNase inhibitor in 1 μl , 50 U of cloned Moloney murine leukemia virus reverse transcriptase in 1 μl , and 1 μl of 50 μM random hexamers. To reduce evaporation the reaction mixture was overlaid with 50 μl of mineral oil (Perkin Elmer Cetus, Norwalk, CT). The reaction tube was incubated at room temperature for 10 min, then in a Perkin-Elmer Cetus DNA thermal cycler at 42°C for 90 min, 99°C for 5 min, and 5°C for 5 min. The synthesized cDNA was amplified by adding 4 μl of 25 mM MgCl_2 , 8 μl of 10 \times PCR buffer II, 65.5 μl of sterile water, 2.5 U of AmpliTaq DNA polymerase in 0.5 μl , and 1 μl of each primer (15 mM). In order to exclude the presence of contaminants in the reagents, two negative controls were included, both in RT (water replacing RNA) and in the PCR amplification (water replacing cDNA). Fragments of CFTR cDNA from exons 5–7, and from exons 20–24 were generated by using sets of primers consisting respectively of

the sequences: 5'-ACTTTAAAGCTGTCAAGCCGTG-3' (sense) and 5'-CTGTATTTTGTATTGCTCCAA-3' (antisense), 5'-AAACTC-GAGGATCGATGGTGTGTCTTGGGATTC-3' (sense) and 5'-AAC-TGCAGCTAAAGCCTTGATCTTGCACCTCTTC-3' (antisense) (41). PCR amplification has been done by the following program: 30 repeated cycles, including 1 min of denaturation at 94°C , 1 min of annealing at 55°C , 1 min of extension at 72°C (41). To check for the quality of the RNA samples, we synthesized a set of primers to amplify a 523-bp fragment of human β -actin gene (41); their sequences were 5'-CATCGAGCACGGCATCGTCA-3' (sense) and 5'-GTCAGG-CAGCTCGTAGCTCT-3' (antisense) and they worked at the same PCR conditions of 5–7, 20–24 CFTR primers. To confirm the epithelial origin of the mRNA, a 588-bp fragment of the human cytokeratin 15 (HCK 15) was amplified (30) with the following steps of PCR reaction: after 6 min of denaturation at 94°C , 30 cycles of 45-s denaturation at 94°C , 45-s annealing at 58°C , 60-s extension at 72°C ; a final polymerization was carried out at 72°C for 10 min. HACK-15 primer sequences are: 5'-TGAAGGAGTTCAGCAGCCAGCTGG-3' (sense) and 5'-ACTGACTCTTCTACATTGATGTGG-3'. All primers were prepared with a model 392 DNA/RNA synthesizer (Applied Biosystems, Inc., Foster City, CA). 10 μl of each amplified cDNA sample was electrophoresed on 1% SeaKem GTG agarose (FMC Corp., Rockland, ME) ethidium bromide gel and photographed on a UV transilluminator (LKB-Pharmacia, Uppsala, Sweden). A 1-kb DNA ladder (Bethesda Research Laboratories, Life Technologies, Inc., Gaithersburg, MD) was used to determine the correct size of the produced fragments.

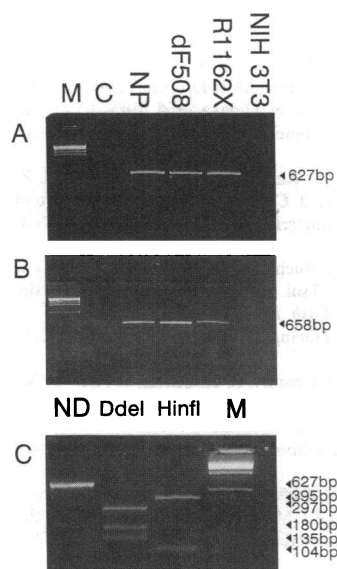
Restriction enzyme digestion. 10 μl of amplified CFTR cDNA fragments was digested with 20 U of appropriate restriction enzymes using protocols recommended by the manufacturer (Promega Corp., Madison, WI). Electrophoresis of digested cDNA fragments was carried out in a 2% Nusieve/1% SeaKem agarose (FMC Corp.) ethidium bromide gel. A 1-kb DNA ladder has been used for size determination.

Northern analysis. 30 μg of denatured total RNA was electrophoresed for 5–6 h at 10 V/cm on 1.5% agarose gels containing 2.2 M formaldehyde, 20 mM Mops, pH 7.0, 8 mM sodium acetate, and 1 mM EDTA (42). 90 $\mu\text{g}/\text{ml}$ ethidium bromide was added to the samples before electrophoresis in order to assess the quality and the amounts of RNA in each lane by visual examination of ribosomal RNA fractions. As a molecular weight marker 3 μg of a 0.24–9.5-kb RNA ladder (Gibco BRL, Gaithersburg, MD) was loaded into the gels. The RNA was transferred to Hybond-C extra supported nitrocellulose membranes (Amersham International, Amersham, UK) by capillary blotting (42) for 15–16 h and immobilized on the membrane by incubation at 80°C for 2 h. The filters were prehybridized for 8 h at 42°C in a solution containing 50% formamide (vol/vol), 5 \times Denhardt's, 0.5% SDS, 5 \times SSPE (0.9 M NaCl, 50 mM sodium phosphate, pH 7.4, 5 mM EDTA), and 250 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA (Sigma Chemical Co.). The prehybridization mixture was replaced with fresh solution containing 2×10^6 cpm/ml of ^{32}P -labeled C1-1/5 CFTR cDNA probe (4) (American Type Culture Collection 61160, Rockville, MD) with a specific activity of 1.9×10^9 cpm/ μg . This probe, spanning exons 9–24 of CFTR cDNA, was radiolabeled with [α - ^{32}P]dCTP (3,000 Ci/mmol; Amersham International) by random priming using the Multiprime DNA labeling system kit according to the manufacturer's conditions (Amersham International). Hybridization was performed overnight at 42°C and then the blots were washed twice in 2 \times SSC ($1 \times = 0.15$ M NaCl, 15 mM sodium citrate, pH 7.4) solution containing 0.1% of SDS at room temperature for 10 min, followed by two washings in 0.5% SSC, 0.1% SDS at 65°C for 30 min, with continuous shaking. The colon carcinoma cell line T84 was used as a positive control for CFTR mRNA expression. Human β -actin probe cDNA (43) that hybridized to a 2.0-kb β -actin mRNA was used as a control probe and to estimate the RNA blotted on the filter. Membranes were exposed to Hyperfilm-MP films (Amersham International) at -80°C with intensifying screens. Laser densitometry, using a model 2202 Ultrascan (LKB-Pharmacia) was used to quantitate the relative signal intensity of the bands obtained and normalized on the basis of β -actin mRNA signal to correct for RNA loading.

Results

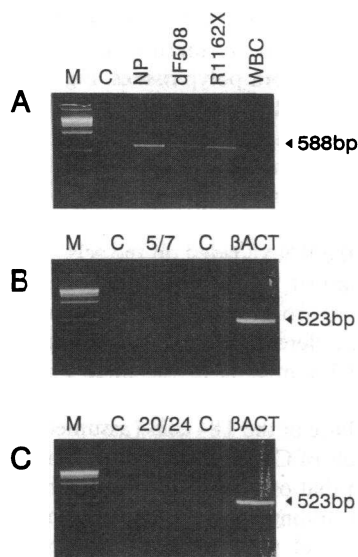
The expression of CFTR mRNA was initially studied by reverse transcription and PCR amplification. Two sets of primers amplifying fragments of CFTR cDNA spanning exons 5–7 and exons 20–24 were utilized in nasal polyps obtained from one patient homozygous for dF508, one patient homozygous for R1162X, and a non-CF subject. To confirm the specificity of the amplification of CFTR, RNA extracted from NIH 3T3 fibroblasts, in which CFTR is not expressed, was included. Fig. 1 *A* shows the presence of amplified fragments of the expected size from exons 5–7. The signal of the R1162X and dF508 samples is present and comparable to that of the non-CF polyp while no signal was detectable from the NIH 3T3 sample. The same results were observed utilizing the 20/24 primers, as shown in Fig. 1 *B*. To increase confidence that our primers were amplifying the expected fragments, restriction enzymes were used to cut the amplified product derived from the R1162X samples. Bands of the appropriate size were detected after digestion with Dde I (297, 180, 135 bp) and Hinf I (395, 104, 98 bp), giving further evidence to the specificity of the signal.

Since the nasal polyp is a dishomogeneous tissue, the expression of the human cytokeratin 15 (HCK15) RNA was checked to assess the epithelial origin of the RNA samples. We have chosen HCK15 as an epithelial marker, because the epithelial components of nasal polyps are very similar to those present in trachea (44) where HCK15 is expressed in substantial quantities (45). HCK15 is clearly expressed in all the polyp samples, as described in Fig. 2 *A*. In the same experiment no signal was detectable from white blood cell RNA that was used as an additional negative control for the primers. Therefore we can assume that our total RNA is at least in part deriving from respiratory epithelium. Since inflammatory cells deriving from circulating white blood cells could infiltrate the nasal polyp tissue and CFTR mRNA was detected also in lymphocytes by PCR amplification (46), we wanted to exclude that the primers utilized for the amplification of CFTR cDNA were detecting a



digested fragment. Dde I and Hinf I indicate the products of digestion with the two restriction enzymes.

Figure 1. Ethidium bromide staining of the PCR-amplified cDNA derived from nasal polyps. M is the 1-kb DNA ladder size marker; C is negative control (water instead of RNA in RT and amplification reactions). (A) Amplification of CFTR cDNA from exons 5–7. NP is polyp sample from a non-CF subject; dF508 is polyp sample from a dF508 homozygote. R1162X is polyp sample from a R1162X homozygote. NIH 3T3 is sample from murine fibroblasts. (B) Amplification of CFTR cDNA from exons 20–24. Same symbols as in A. (C) Digestion of the cDNA amplified from exons 5–7 of the polyp sample of the R1162X patient. ND is not



(5/7) and of human β -actin cDNA (*BACT*) in peripheral leukocytes. (C) Amplification of CFTR cDNA with primers from exons 20–24 (20/24) and of human β -actin cDNA (*BACT*) in peripheral leukocytes.

signal coming from white blood cells. The same primers and protocols were adopted to amplify the cDNA obtained by reverse transcription of 3 μ g of RNA extracted from circulating leukocytes. Fig. 2, *B* and *C*, demonstrates that no signal from CFTR is apparent from leukocytes. To check the quality of the RNA, primers to human β -actin were used and fragments of the expected size were obtained. Therefore we can assume with good confidence that the signal corresponding to CFTR mRNA in the polyp was not derived from leukocytes infiltrating the tissue.

The protocol adopted in the previous PCR experiments does not allow a reliable quantitative evaluation of the amount

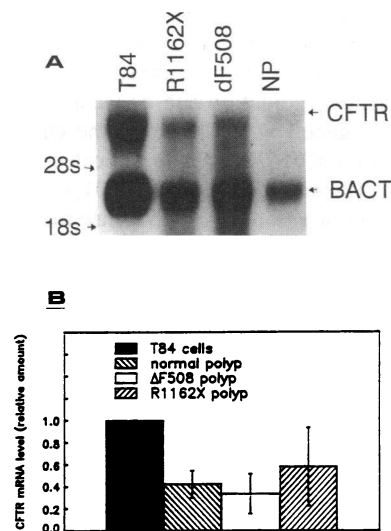


Figure 3. Northern analysis of total RNA. (A) Blots were hybridized with C1-1/5 CFTR probe and with human β -actin probe. NP is polyp sample from a non-CF subject, dF508 from a dF508 homozygote, R1162X from a R1162X homozygote. T84 is from a sample of T84 cells. CFTR and BACT indicate the position of the CFTR mRNA (6.5 kb) and of β -actin (2.0 kb) calculated from the migration of 9.5–0.25 RNA ladder size marker. 28s and 18s indicate the level of migration of the ribosomal fractions of RNA. (B) Steady-state level of CFTR mRNA expression in the same samples. Data are normalized to the level of T84 cells that was equal to 1. Mean and SD are reported ($n = 3$).

of CFTR mRNA expressed in the nasal polyps of the three different genotypes. For this reason a Northern analysis was performed on the RNA extracted from polyp tissues. Fig. 3 A confirms the presence of CFTR mRNA in all three samples. The data are validated by the detection of the expected 6.5-kb CFTR mRNA size that was clearly expressed in the T84 colon carcinoma cell line. The blots were simultaneously hybridized with a human β -actin probe to check for the RNA integrity and to correct for the amount of the RNA loaded on the gels.

Autoradiographs were scanned with a laser densitometer and the optical density deriving from CFTR was normalized with that of β -actin among the different samples. Fig. 3 B shows the relative expression of CFTR mRNA in the three polyp samples in comparison to that of the T84 cells. Normalizing the optical density values to those of the T84 cells (assumed as value 1), the steady-state levels of CFTR mRNA in the three polyp tissues were lower than that of T84 cells and no significant difference was observed among the polyps of the three investigated genotypes (normal polyp 0.42 ± 0.12 , dF508 polyp 0.33 ± 0.18 , R1162X 0.58 ± 0.35 , mean \pm SD, $n = 3$). Therefore we ruled out either the absence or the reduction of the expression of CFTR mRNA in presence of this type of nonsense mutation.

Discussion

Our data on the nonsense mutation R1162X clearly show that the CFTR mRNA is expressed to the same extent both in the CF and non-CF respiratory nasal epithelia, as demonstrated by RT-PCR and Northern analysis. In contrast, it has been already shown that the nonsense mutations R553X and W1316X determine severe reduction of CFTR mRNA (30).

Nonsense mutations have sometimes been associated with lack of mRNA and protein, as in human β -globin (34), human α 1-antitrypsin (35), and insulin receptor (36) genes, while in other cases such as that of the LDL receptor or the apolipoprotein C-II genes a stable truncated protein is synthesized (37, 39). It has been suggested that mRNAs presenting translation termination mutations might be unstable since they may not be protected by ribosomes from the endogenous RNAase digestion (34). On the contrary, mRNAs with premature termination codons located toward the 3' end of the transcript sometimes seem to escape the higher degradation rate, as occurs with the dihydrofolate reductase gene (47). Therefore, the effect produced by CFTR mutation R1162X, which is located toward the 3' end of the gene (amino acid 1162 of 1480), could be consistent with this latter condition. Since nonsense mutation W1316X, which is even more toward the 3' end, is characterized by a lack of mRNA, one might speculate that conformational modifications of the mRNA specific to each mutation rather than to the position of the mutation itself in the gene are responsible for the protection from RNAse digestion of the transcript.

These data may contribute to the further understanding of the molecular mechanisms by which mutations of CFTR gene eventually lead to defective chloride transport in CF epithelial cells. It is now known that CFTR protein is abnormally processed in the presence of deletion or missense mutations as it occurs with dF508, dI507, and S549I (22). On the contrary, a mature protein is present in the mutation G551D (22), in which a defective function is the molecular basis of the defect (29). Nonsense mutations of CFTR might therefore cause the

cellular defect either because of absence of the protein, as in R553X and W1316X (31), or possibly because of the presence of a truncated protein as could be hypothesized for the R1162X mutation.

Whether this putative truncated protein lacking the second nucleotide binding domain (NBD2) results in the cellular defect because of protein instability, altered processing, or reduced function is not yet understood. Even if the influence of genotype on phenotype is very controversial in this disease (48), it is noteworthy to mention that patients homozygous for the R1162X mutation present defective pancreatic function but mild to moderate lung disease (20). In parallel with data demonstrated in mutations characterized by a mild pulmonary phenotype such as G551S (29), it is possible that R1162X might express a partially functional protein, at least in the respiratory epithelia. We checked protein expression in the same nasal tissues by immunoprecipitation and Western analysis, but the level of protein in non-CF samples is below detectability (data not shown).

In conclusion, the mutation R1162X results in normally expressed CFTR mRNA, indicating that nonsense mutations of the CFTR gene have heterogeneous mechanisms of mRNA processing (e.g., degradation). The presence of the transcript in respiratory cells of patients with mild to moderate lung disease could suggest that a partially functional protein could be expressed at least in the airway tract.

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