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RESEARCH ARTICLE

Cystic Fibrosis Patients With the 3272-26A→G Mutation Have Mild Disease, Leaky Alternative mRNA Splicing, and CFTR Protein at the Cell Membrane

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We characterized the 3272-26A→G mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, creating an alternative acceptor splice site in intron 17a, that competes with the normal one, although we predict from consensus values, with lower efficiency. We analyzed five Cystic Fibrosis (CF) Portuguese patients with the 3272-26A→G/F508del genotype. Besides clinical and haplotype characterization of those patients, we report here results from CFTR transcript analysis in nasal brushings from all five patients. RT-PCR analysis supports alternative splicing in all patients and carriers, but not in controls. By sequencing, we determined that the alternative transcript includes 25 nucleotides from intron 17a, which predictively cause frameshift and a premature stop codon. The use of this alternative splice site causes a reduction in the levels of normal transcripts from the allele with this mutation and, most probably, of normal protein as well. By immunocytochemistry of both epithelial primary cell cultures and slices from CF polyps, CFTR protein is detected at the cell membrane, with three different antibodies. Ussing chamber analysis of one nasal polyp shows a high sodium absorption, characteristic of CF. Altogether, the results suggest that the main defect caused by the 3272-26A→G mutation is a reduction in normal CFTR transcripts and protein and therefore this mutation should be included in class V, according to Zielenski and Tsui. Hum Mutat 14:133–144, 1999.

KEY WORDS: **cystic fibrosis; CFTR; splicing mutation; nasal epithelium**

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Abbreviations: ASO, allele-specific oligonucleotide; CF, cystic fibrosis; CFTR, CF conductance transmembrane regulator; CBAVD congenital bilateral absence of vas deferens; CM, cell membrane; C-terminus, carboxy-terminus; CV, consensus value; CVN, CV for the normal splice site sequence; CVA, CV for the activated (cryptic) splice site sequence; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; DPC, diphenylamine-2-carboxylate; I_{SC}, short-circuit current; PNU, potential for novel splice site utilization; RT, reverse transcription; TBE, Tris-borate EDTA electrophoresis buffer.

INTRODUCTION

Cystic fibrosis (CF; MIM# 219700) is a lethal autosomal recessive disease affecting 1 in 2,500 Caucasians [reviewed in Zielenski and Tsui, 1995]. The gene responsible for this disease [Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1989] encodes the cystic fibrosis transmembrane conductance regulator (CFTR; MIM# 602421) protein, which functions as a cyclic adenosine monophosphate (cAMP)-regulated chloride (Cl^-) channel in the apical membrane of secretory epithelial cells. Malfunction of this Cl^- channel in CF patients is associated with chronic pulmonary inflammation and obstruction, bacterial colonization of the airways, pancreatic enzyme insufficiency, elevated sweat electrolytes, and infertility in males [reviewed in Collins, 1992; Zielenski and Tsui, 1995].

More than 800 different mutations have been identified and linked to CF [Cystic Fibrosis Genetic Analysis Consortium, 1999]. The most common mutation associated with CF, the deletion of phenylalanine at position 508 (F508del) with a worldwide relative frequency of 68% is generally associated with severe disease [reviewed in Welsh et al., 1995]. Most reported mutations, including the most particular ones of the Portuguese population, have only been described at the DNA level and have not been studied at the biochemical or functional level. The functional consequences of many of the presumed pathogenic mutations are therefore unclear, but such knowledge is likely to be a rich source of information both for the structure and function of CFTR and for mutation-type-dependent therapy strategies [Delaney and Wainwright, 1996].

It was proposed that the 3272-26A→G mutation leads to the creation of an alternative acceptor splice site competing with the normal one during RNA processing [Bienvenu et al., 1995; Cuppens et al., 1995; Chomel et al., 1996]. This article describes the cellular and molecular effects of the 3272-26A→G mutation in intron 17a of CFTR gene, which accounts for about 2% of CF chromosomes in Portugal.

MATERIALS AND METHODS

Patients and Controls

Nasal polyps were collected from four CF patients, two with the 3272-26A→G/F508del genotype (patients 1 and 2 in Tables 1 and 2) and two homozygous for F508del, as well as from four non-CF individuals. Nasal brushings were from five unrelated CF patients with the 3272-26A→G/F508del genotype (including the above two), three carriers of the 3272-26A→G mutation (parents of CF patients) and two healthy controls. Clinical data on all five CF patients with the 3272-26A→G/F508del genotype are included in Table 1. None of the controls used in this study had clinical signs of CF, nor of F508del or 3272-26A→G mutations as determined by DNA analysis.

DNA Analysis

Haplotypes, detection of F508del mutation by dot-blot or by amplification refractory mutation system (ARMS) [Ferrie et al., 1992], and of 3272-26A→G mutation by denaturing gradient gel electrophoresis (DGGE) and direct sequencing were as previously described [Duarte et al., 1996]. The intron 8 $(TG)_nT_m$ polymorphic tract was analyzed as described [Costes et al., 1995] and sequenced with the following primer: 5′-GAAATTACTG-AAGAAGAGGC-3′.

mRNA Analysis

After brushing with interdental brushes (Paro-Isola, Thalwil, Switzerland), nasal epithelial cells were immediately put into extraction buffer, and

TABLE 1. Clinical Characterization of CF Patients at Date of Nasal Brushing for RNA Analysis

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Age (yr)	12	17	18	38	28
Sex	Female	Male	Female	Female	Female
Age at diagnosis (yrs)	9	2.5	3	32	27
Sweat test (mEq/L)[a]	110	103	100	96	114
FVC % predicted	93	86	103	38	77
FEV_1 % predicted	78	68	90	27	52
Bacteria	*Hi, Sa*	*Hi*	None	*Pa*	*Pa*
Pancreatic status	PS	Mild PI	PS	PS	PS
Nasal polyposis	Yes	Yes	Yes	Yes	No
Shwachman-Kulczycki score[b]	90	80	95	50	55

FVC, forced vital capacity; FEV_1, forced expiratory volume in 1 sec; *Hi*, *Haemophilus influenzae*; *Pa*; *Pseudomonas aeruginosa*; *Sa*, *Staphylococcus aureus*; PS, pancreatic sufficient; PI, pancreatic insufficient.
[a]Average vaue of several tests.
[b]Conway and Littlewood [1996].

TABLE 2. Genotype and Haplotype of CF Patient

Patient	CFTR Genotype[a]	Haplotypes[a]		
		XV-2c/KM19 (57)	Intron 9 $(CA)_n$	$(TG)_nT_m$
1	F508del/3272-26A→G	B/D	17/16	10.9/10.7
2	3272-26A→G/F508del	D/B	16/23	10.7/10.9
3	3272-26A→G/F508del	D/B	16/23	10.7/10.9
4	3272-26A→G/F508del	D/B or B/D	16/23	10.7/10.9
5	3272-26A→G/F508del	D/B	—	10.7/10.9

[a]Father-inherited allele/haplotype is mentioned first.

RNA was prepared by the RNeasy method (Qiagen, Hilden, Germany) according to the manufacturer's instructions. First-strand cDNA was synthesized using hexanucleotide primers and Superscript reverse transcriptase (Gibco-BRL, Gaithersburg, MD). Reverse transcription-polymerase chain reaction (RT-PCR) was performed as described [Chalkey and Harris, 1991] modified by using another PCR buffer (Pharmacia, Uppsala, Sweden). To amplify exons 16–17b, we used primer E2R [Chalkey and Harris, 1991] and 17bL with the following sequence: 5′-TAAATTCAGAGC-TTTGTGGAAC-3′.

Automatic Sequencing

The single band of the expected size was purified and the sequencing reaction was performed by the ABI PRISM™ Dye Terminator Cycle Sequencing system (Perkin Elmer, Norwalk, CA) using primer 17b-6L (allele-specific): 5′-AATT-GGACTCCTGCCTGTGA-3′, and according to the manufacturer's procedure.

Consensus Values

Consensus values (CVs) were calculated as defined [Shapiro and Senapathy, 1987] for the normal (CVN) and the activated cryptic (CVA) acceptor splice sites and for donor splice site, although the latter is not affected by this mutation. Estimates were based either on published nucleotide frequencies [Mount, 1982; Cooper and Krawczak; 1993], designated Mount's CVs, or on nucleotide frequencies at the 26 CFTR intron sequences for acceptor and donor (data not shown), referred as CFTR CVs. As branch site, we considered the adenine residue with highest CV (between –18 and –37 relative to AG), calculated according to a previous report [Penotti, 1991]. Adenines at –33 and –51 (–26 relative to the new exon border) were thus considered as the normal and the activated branch nucleotides, respectively. We used the potential for novel splice site utilization (PNU) as defined [Penotti, 1991], i.e., the CVA : CVN ratio, describing the probability value that best positively correlates with the relative proportion of cryptic acceptor splice site utilization.

By sequencing the 3′ end of intron 17a in all five patients with the 3272-26A→G mutation (data not shown), we detected three instead of the four adenines (residues –54 to –57 in intron 17a) originally reported [Zielenski et al., 1991]. This is not a polymorphism in the Portuguese population, but rather a mistake in the originally published sequence, as the same result was obtained for various non-CF individuals, as well as for the original CFTR subclone pTE33IIE4.5 [Zielenski et al., 1991]. For our estimates we used the three-adenine sequence.

Cell Cultures

Still in the operating room, nasal polyp tissue was washed in phosphate-buffered saline (PBS) and immediately placed in culture medium, 1:1 mixture of Ham's F12 and Dulbecco's modified essential media (DMEM) (Gibco-BRL) with 250 U/ml penicillin/125 μg/ml streptomycin and 2.5 μg/ml amphotericin B (Gibco-BRL) at room temperature. Epithelial cells were isolated by enzymatic digestion as described [Rodrigues et al., 1995]. Cells were seeded onto collagen-coated semipermeable membranes, 24-mm transwell from (Corning Costar Corp., Cambridge, MA) and maintained at 37°C in humidified atmosphere of 5% CO_2. Transepithelial electrical resistance was measured as an indicator of culture confluency [Rodrigues et al., 1995].

Immunocytochemistry

Primary cells were grown on 8-well slides (Labtech, Uckfield, UK) equipped with Cellagen Discs (ICN Biomedicals, Costa Mesa, CA) as bicameral systems. After 11 days, cells were mildly fixed for 10 min in 4% (v/v) formaldehyde and 0.1 M sodium cacodylate. After two PBS washes, cells were incubated with two different CFTR specific antibodies: MATG 1031 (Transgène, Strasbourg, France), which specifically recognizes the first extracellular loop of CFTR [Demolombe et al., 1996],

or antibody M24-1 specific for the C-terminus of CFTR, residues 1377-1480 (Genzyme, Cambridge, MA) [Denning et al., 1992]. The first was incubated without permeabilization and the second after permeabilization for 30 min with 0.25% (v/v) Triton X-100 in PBS. Both antibodies were used diluted 1:20 in PBS with 0.1% (w/v) bovine serum albumin (BSA). Incubation time was overnight at 4°C. After two PBS washes, cells were incubated with FITC-conjugated goat anti-mouse IgG, as secondary antibody (Sigma Chemical Co., St. Louis, MO) diluted 1:100, for 1 hr at room temperature. After three 5-min washes at room temperature with PBS, slides were mounted with DABCO (diazabicyclo[2.2.2]octane) mounting medium from Sigma. Negative controls were performed using only the secondary antibody, following the same experimental procedure (data not shown). Specificity of CFTR antibodies under these conditions was confirmed in HeLa cells not expressing CFTR and expressing CFTR after transient transfection with a pCNA3-CFTR cDNA construction plasmid (data not shown).

Immunohistochemistry

Immediately after surgical removal, nasal polyps were rinsed in cold PBS and frozen in liquid nitrogen with OCT compound (Tissue Tek, Miles, Elkhart, IN). Immunohistochemical studies were carried out in cryosections (6–8 mm thick) using the anti-CFTR polyclonal antibody 169, recognizing the R-domain [Crawford et al., 1991], diluted 1:100. The secondary antibody, FITC-conjugated goat anti-rabbit IgG from Boehringer (Mannheim, Germany), was used in the above-mentioned conditions.

Transepithelial Parameters

Epithelial sheets dissected from a nasal polyp of one CF patient (# 2 in Tables 1 and 2) and of a non-CF individual were mounted in Ussing type chamber. They were bathed with a solution containing (in mM): 120 NaCl; 4 KCl; 3 $CaCl_2$; 1 $MgCl_2$; 20 $NaHCO_3$; and aerated with 5% CO_2. The preparations were voltage clamped at zero and the short circuit current (I_{SC}) measured. We tested the sensitivity of the measured currents to several chemicals (all from Sigma Chemical Co.): 0.1 mM amiloride (Na^+ channel blocker), 0.1 mM ouabain (Na^+/K^+ pump inhibitor), 1 mM DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid), a blocker of all Cl^- channels but not CFTR, and 1 mM DPC (diphenylamine-2-carboxylate), a blocker of all Cl^- channels, including CFTR [Anderson et al., 1992].

RESULTS

3272-26A→G/F508del Patients Have Mild CF Disease

The clinical phenotypes of CF patients analyzed in this study are summarized in Table 1. The data indicate that these patients are generally PS and have relatively mild to moderate pulmonary disease. Although sweat tests are consistent with the diagnosis of CF in these patients, most have been diagnosed at a late age. Extensive nasal polyposis seems to be a common complication in patients with this mutation.

Common Origin for the Portuguese 3272-26A→G Allele

The genotypes and haplotypes of CF patients analyzed in this study are summarized in Table 2. When informative, the same CFTR haplotype (D-16-10.7) is associated with the 3272-26A→G mutation, suggesting a common origin for this mutation in the Portuguese population.

Potential for Novel Splice Site Utilization

The CVs estimated using published frequencies [Mount, 1982] are CVN 0.891 and CVA 0.862 (see Table 3). CFTR CVs (see Methods) are 0.537 and 0.532 for the normal and cryptic acceptor splice sites, respectively (see Table 3). For the branch site (see Methods), the values of CVN 0.681 and CVA 0.640 were obtained (see Table 3). Interestingly, the CFTR CVs for the normal and cryptic acceptor sites of intron 17a (0.537 and 0.532, respectively) are much

TABLE 3. Estimated Parameters to Quantify Intensity of Normal and Cryptic Splice Sequences

Estimated parameters	Consensus used	CVN	CVA	PNU (CVA:CVN)
Acceptor site	Mount [1982]	0.891	0.862	0.967
	CFTR (this study)	0.537	0.532	0.990
Donor site	Mount [1982]	0.828	—	—
	CFTR (this study)	0.909	—	—
Branch site	Penotti [1991]	0.681 (–33)[a]	0.640 (–51)[a]	0.939

[a]Numbers in parentheses refer to the position of adenine residue considered as branch nucleotide relative to the normal splice junction.

lower than those calculated using Mount's frequencies (0.891 and 0.862). The opposite is observed for the donor site, for which the corresponding CVs calculated according to Mount's (0.828) or CFTR (0.909) frequencies are much closer and higher for the latter. The predicted strength, or PNU values (see Methods), of the alternative splice site when competing with the normal are 0.967 (Mount's) and 0.990 (CFTR) at the acceptor and 0.939 at the branch site.

Alternative Splicing Confirmed by RT-PCR and Sequencing

In order to observe the effect of the 3272-26A→G mutation upon splicing, we analyzed transcripts from the nasal epithelium of five patients, three healthy carriers, and two normal individuals (see Methods). The analysis of amplification products of nasal epithelium transcripts from CF patients (Fig. 1, lanes 2–6) and from carriers (Fig. 1, lanes 7–9) shows the presence of an extra band, with lower mobility, which is absent in samples from controls (Fig. 1, lanes 10 and 11). This extra band, consistent in size with the presence of 25 extra nucleotides, suggests the occurrence of the alternative splicing. To confirm it, both bands were analyzed by automatic sequencing. Figure 2B demonstrates that the upper band present in patients and carriers has 25 extra nucleotides by comparison with the normal band (Fig. 2A), matching exactly the last 25 nucleotides of intron 17a described in the original CFTR sequence [Zielenski et al., 1991]. This insertion causes a frameshift and a premature stop codon (TGA) in exon 17b.

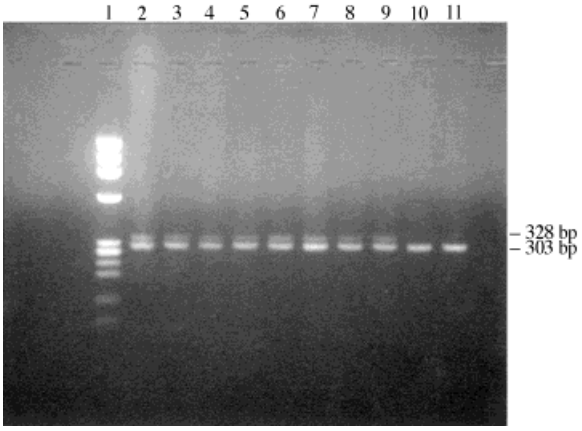

FIGURE 1. **mRNA analysis by reverse transcriptase-polymerase chain reaction (RT-PCR). mRNA was extracted from nasal cells of five CF patients, three CF carriers and two healthy controls (see Methods). After cDNA synthesis and PCR amplification as described in Methods, products were analyzed by 2% agarose gel electrophoresis. *Lane 1*, ϕ X174/*Hae*III DNA molecular-weight markers. *Lanes 2–6*, mRNA RT-PCR products from the five CF patients with the 3272-26A→G/F508del genotype (see Tables 1 and 2); *lanes 7–9*, mRNA RT-PCR products from three carriers of the 3272-26A→G mutation; *lanes 10, 11*, mRNA RT-PCR products from two healthy controls.**

CFTR is Detected at the Cell Membrane

Immunocytochemical studies, carried out in epithelial cell primary cultures from CF patient 2 (Fig. 3A) and from non-CF individuals (Fig. 3B) using the MATG 1031 anti-CFTR antibody without permeabilization (see Methods) show that in both cases, CFTR protein is present in the CM. It has been extensively described that very little, if any, F508del CFTR reaches the CM [Cheng et al., 1990; Gregory et al., 1991; Denning et al., 1992; Kartner et al., 1992].Therefore, most CFTR detected in the CM must result from the non-F508del allele, but it can result from the normal or of from the alternative transcript. Since the presence of CFTR at the CM was confirmed with the C-terminus-specific M24-1 antibody (Fig. 3C), which does not recognize the product of the alternative transcript, at least some of the protein at the CM in Figure 3A is normal CFTR.

In tissue slices of nasal polyps, the immunostain of CFTR with polyclonal antibody 169 [Crawford et al., 1991] shows marked differences between normal (Fig. 4A,B) and CF F508del/F508del (Fig. 4C,D) epithelia. CFTR stains as a very sharp line at the apical membrane of normal nasal polyp epithelial cells (white arrows, Fig. 4A,B) but appears with a rather diffuse distribution in F508del/F508del epithelia (white arrowheads, Fig. 4C,D). In the 3272-26A→G/F508del epithelia, CFTR distribution appears as a mixed pattern, resembling the sharp line of normal epithelia at some regions (white arrows, Fig. 4E,F), but also presenting diffuse distribution in some cells (white arrowheads, Fig. 4E,F). Negative controls were performed by following the same experimental procedure but omitting the primary antibodies (data not shown). Also, the specificity of these CFTR antibodies was tested under the same conditions by detecting labeling in HeLa cells expressing CFTR after transient transfection with the pCNA3-CFTR cDNA plasmid, but not in nontransfected HeLa cells (data not shown).

CF Typical Electrophysiology of Nasal Epithelium

Figure 5A,B shows the short-circuit current (I_{SC}) of two preliminary experiments performed on epithelial sheets obtained from nasal polyps (see

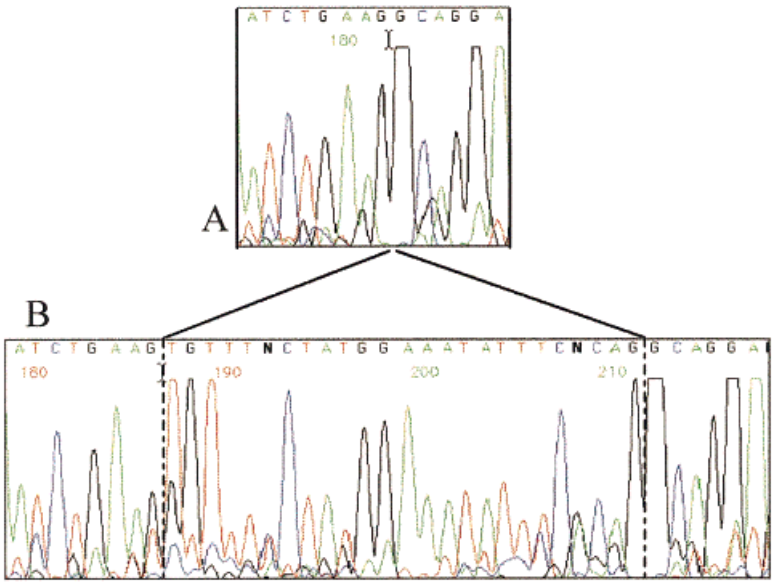

FIGURE 2. **Automatic sequencing of normal and alternative transcripts. Total RNA was extracted, cDNA synthesized, and transcript-specific polymerase chain reaction (PCR) performed in the exon 16–17b region with primer 17b-6L, specific for the intron 17a/exon 17b junction (see Methods), which failed to initiate PCR-amplification in normal controls (data not shown). The sequences of the normal (A) and alternative (B) transcripts are compared.**

Methods). It can be observed (Fig. 5A) that the I_{SC} recorded for the polyp from the CF patient (about 80 $\mu A/cm^2$) is almost twice the intensity of I_{SC} in control polyp (about 40 mA/cm^2) shown in Figure 5B. Furthermore, in CF polyp I_{SC} is almost totally inhibited by amiloride, whereas in control there is a substantial remaining current after amiloride inhibition, which is then inhibited by DIDS and finally by DPC. The current not inhibited by DIDS, but inhibited by DPC can be attributed to CFTR Cl^- channel [Anderson et al., 1992].

Altogether, these results are very clearcut and indicate that the high I_{SC} observed for the CF polyp was most probably exclusively due to a transepithelial (apical to basolateral) transport of sodium, which is consistently observed in CF epithelia [Boucher et al., 1986]. Indeed, it is known that CF, besides the default in cAMP-dependent Cl^- conductance, also causes an increase in the open probability of sodium channels, although the precise mechanism remains unknown [Chinet et al., 1994; Mall et al., 1998].

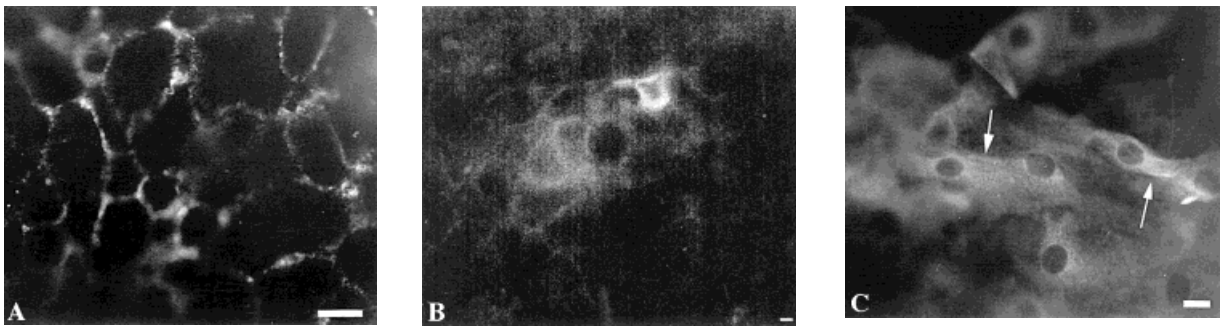

FIGURE 3. **Immunolocalization of cystic fibrosis transmembrane conductance regulator (CFTR). Primary cultures of epithelial cells from nasal polyps of one patient (# 2 in Tables 1 and 2) with the 3272-26A→G/F508del genotype CF (A) and of a non-CF individual (B) were stained by indirect immunofluorescence using the anti-CFTR antibody MATG1031 without cell permeabilization (see Methods). CFTR immunostain is clearly detected at CM of cells in both A and B. CFTR is also seen (C, *arrows*) at the CM of 3272-26A→G/F508del cells (as in A) when the C-terminus-specific M24-1 anti-CFTR antibody was used with cell permeabilization (see Methods). Non-CF epithelial cells (as in B) present similar staining with M24-1 antibody (data not shown). No signal was observed at the CM of nontransfected HeLa cells (see Methods) with either of these two antibodies (data not shown). Bar = 10 μm.**

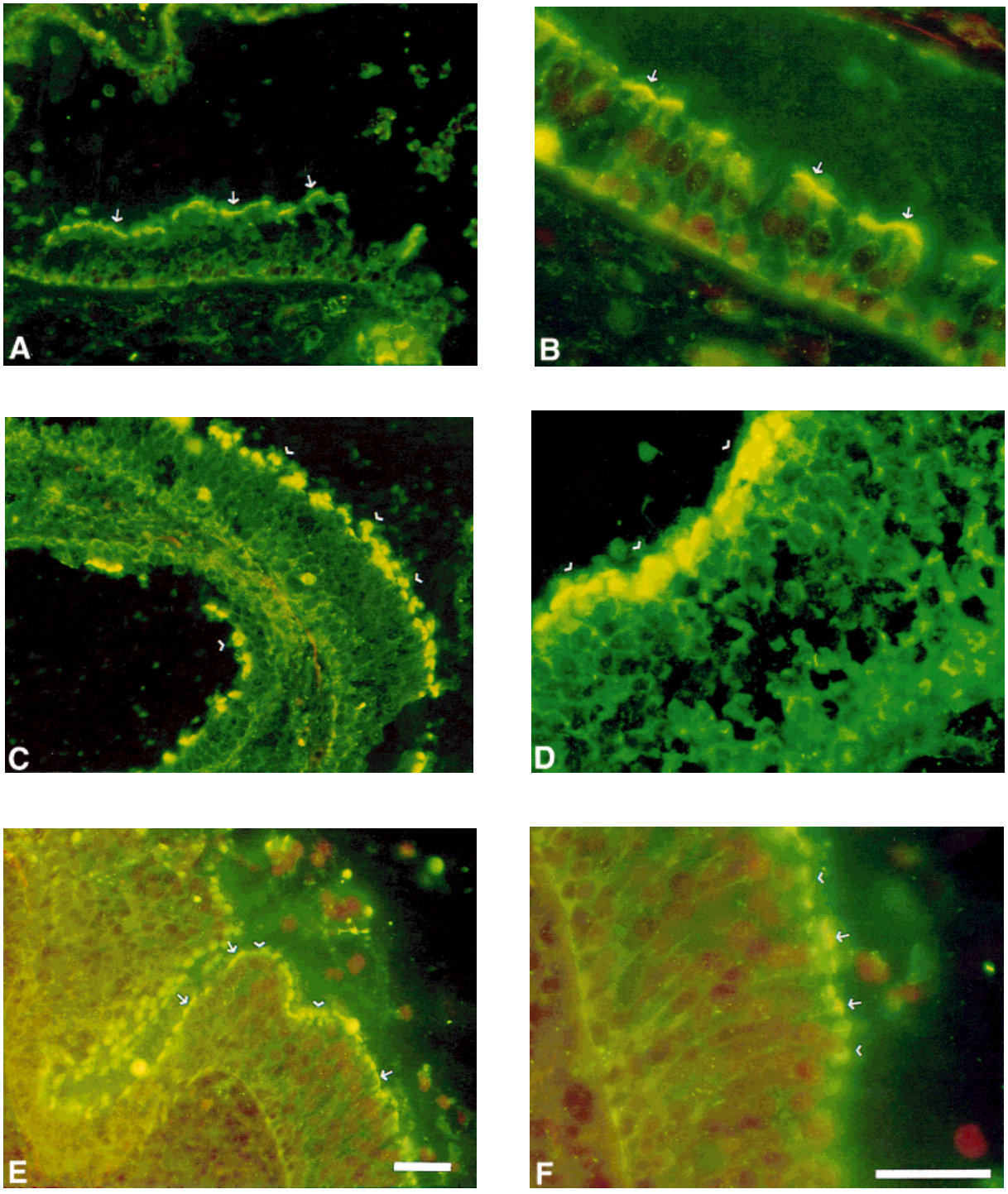

FIGURE 4. CFTR protein distribution in normal and cystic fibrosis (CF) nasal polyp tissues. Cryostat sections of unfixed nasal polyps tissues were obtained from: non-CF individuals (**A,B**); CF patients homozygous for the F508del mutation (**C,D**); a CF patient (# 1 in Tables 1 and 2) with the F508del/3272-26A→G genotype (**E,F**). Immunohistochemical studies were carried out in those sections using the anti-CFTR antibody 169 (see Methods). CFTR stained at the apical membrane very clearly in normal nasal polyp (*arrows*, Figs. 5A,B). A more diffuse distribution was observed in F508del/F508del (*arrowheads*, Figs. 5C,D). In the F508del/3272-26A→G polyp, a mixed pattern is observed (*arrows and arrowheads*, Fig. 5E,F). The same magnification was used for microphotographs in **A**, **C**, and **E**. **B**, **D**, and **F** were obtained at a higher magnification. Bar = 50 μm in all microphotographs.

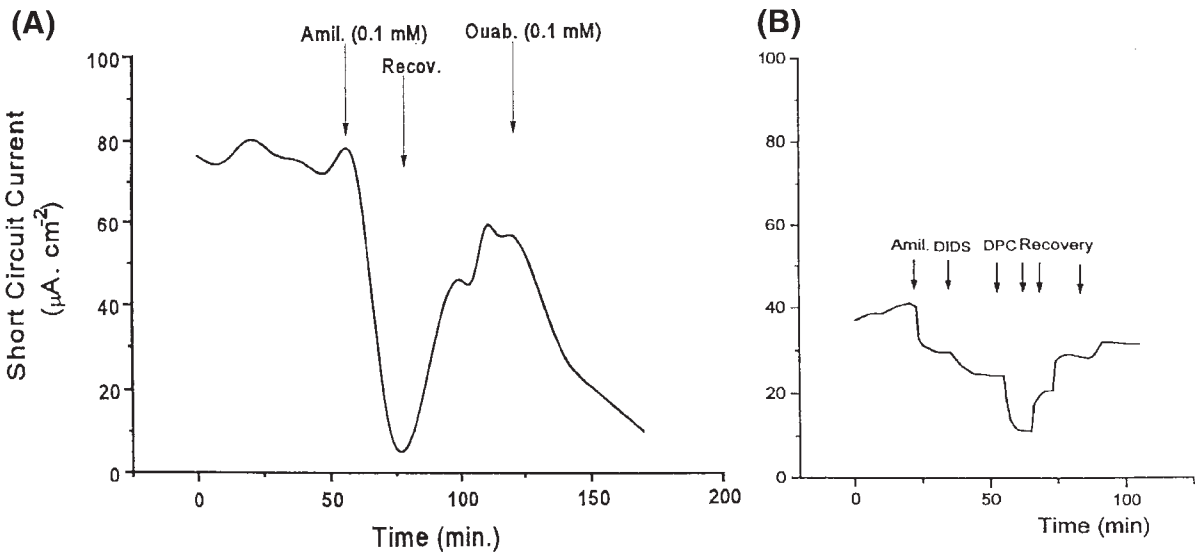

FIGURE 5. **Bioelectric properties of nasal polyps epithelia. Short-circuit currents (I_{SC}) were recorded during a period of 3 hr. A fragment of CF nasal polyp (A) from a patient with the 3272-26A→G/F508del genotype (#2 in Tables 1 and 2) was mounted on a Ussing chamber and a spontaneous potential 1.4 mV (basolateral positive) was measured. At voltage-clamp set up an I_{SC} of about 80 mA/cm^2 was observed. At the time indicated by the first arrow, amiloride (a potent Na^+ channel inhibitor) was added to the apical bath reducing drastically the I_{SC}. After a partial recovery, ouabain (a Na^+/K^+ pump inhibitor) was then added (at the time of the second arrow) to the serosal side and again the I_{SC} decreased. These results indicate that the I_{SC} was most probably due to a transepithelial (apical to basolateral) transport of sodium. B: Similar preparation of a non-CF polyp. A smaller I_{SC} was recorded (40 mA/cm^2). This was only partially inhibitable by amiloride, with the remaining current inhibitable by DIDS (a blocker of all Cl^- channels except CFTR), and DPC, a CFTR blocker of all Cl^- channels including CFTR (see Methods), showing evidence of the presence of active CFTR in these cells.**

DISCUSSION

From the 848 genetic alterations in the CFTR gene that have been described as CF mutations [Cystic Fibrosis Genetic Analysis Consortium, 1999], 136 (16%) are reported as splicing mutations. This percentage matches what was described, i.e., that point mutations causing a defect in mRNA splicing appeared to represent about 15% of all point mutations [Cooper and Krawczak, 1993].

The 3272-26A→G mutation was first described in compound heterozygosity with the severe mutation W846X in a patient with a mild phenotype [Fanen et al., 1992]. To date, a total of 44 patients with this mutation have been reported, or communicated to us: eleven patients in France [Bienvenu et al., 1995; Chomel et al., 1996], including the first reported [Fanen et al., 1992], nine in Greece [Kanavakis et al., 1995; Antoniadi et al., 1998], nine in Germany [Dörk et al., 1994], seven in Spain [Morral et al., 1996] (T. Casals, personal communication), two in Belgium [Cuppens et al., 1994], one in Canada [Morral et al., 1996], and the five Portuguese patients included in this study.

The haplotype analysis of the five Portuguese patients presented here suggests a common origin for the 3272A→G mutation in the Portuguese population. In another Portuguese patient, with congenital bilateral absence of vas deferens (CBAVD) that has the 5T allele on the other chromosome, the 3272-26A→G mutation is also associated with $TG_{10}T_7$ in intron 8 (P. Pacheco, unpublished data). However, patients studied in Germany [Dörk et al., 1994] and Belgium [Cuppens et al., 1994] have this mutation on different genetic backgrounds, which, together with its ubiquitous geographic distribution, suggests that 3272-26A→G has evolved from more than one mutational event.

The five unrelated Portuguese CF patients with the genotype 3272-26A→G/F508del present mild to moderate pulmonary disease, PS and extensive nasal polyposis. For another splicing mutation in the CFTR gene, the 3849+10kbC→T that introduces a novel exon, also by creating a novel acceptor splice site, a PS phenotype was described as well [Abeliovich et al., 1992; Highsmith et al., 1994]. From the clinical phenotypes of the other above-mentioned patients with the 3272-26A→G mutation, our impression is that, generally, patients have a mild clinical phenotype. The two patients described to have severe phenotypes, both with F508del in the other allele, one was PS [Bienvenu

et al., 1995] and the other is currently 28 years old and diagnosed at the age of 27 (T. Casals, personal communication).

Our analysis of CFTR cDNA reverse transcribed from nasal epithelial RNA, revealed the presence of an alternatively spliced transcript in all samples from CF patients and carriers of the 3272-26A→G mutation, but not in samples from controls. Other investigators have described alternative splicing as a result from this mutation but have not sequenced the alternative transcript [Cuppens et al., 1994; Chomel et al., 1996]. We confirmed the predicted insertion of 25 nucleotides from intron 17a into the alternative transcript by direct sequencing. We thus prove that the alternative acceptor splice site created by the 3272-26A→G mutation is used by the spliceosome.

PNU values estimated for the novel acceptor and branch splice sites (Table 3), based on either Mount's or CFTRs frequencies, indeed predicted that the novel splice site could effectively compete with the normal acceptor for the spliceosome, although CVAs are lower than CVNs (see Table 3). However, the CVN-CVA differences are smaller than 0.03 for the acceptor and less than 0.041 for the branch site. There are examples of other disease-causing mutations, in which the novel acceptor splice sites have lower PNUs and higher CVN-CVA differences than found here and for which alternative splicing has been shown to occur [Cooper and Krawczak, 1993]. Generally, the PNU value of mutations causing human disease through the creation of novel acceptor splice sites, seems to correlate positively with the amount of alternative transcript resulting from the novel site usage, being 100% when PNU ≥1 [Cooper and Krawczak, 1993].

The fact that CFTR CVs are lower than Mount's CVs for acceptor sites (Table 3) may be due either to a sampling bias (lower number of sequences used) or, more interestingly, to a lower conservation of acceptor site sequences within the CFTR gene than among acceptors in general. The opposite occurs for the donor site. The difference between donor and acceptor CFTR CVs (Table 3) may suggest a higher spliceosome stringency for donors than for acceptors, again if not due to small sampling bias. This idea is supported by the fact that for the CFTR gene a higher number of putative CF-associated mutations described at the 5′ of introns (71) than at the 3′ (59) [Cystic Fibrosis Genetic Analysis Consortium, 1999]. Acceptors in CFTR introns thus seem more permissive to genetic change. This fits into a more general rule, i.e., mutations in the 3′ splice site occur much less frequently than in 5′ splice sites [Cooper and Krawczak, 1993]. One explanation for this fact could be that mutations at acceptors could be compensated by the recently described splicing enhancers internal to exons that turn poor or leaky 3′ splice sites into strong acceptors [Cooper and Mattox, 1997].

Although PCR amplicons observed in ethidium bromide-stained gels should not be interpreted quantitatively, it is evident from this study that the alternative transcripts are formed in all five CF patients analyzed (upper band in Fig. 1). A preliminary semiquantitative analysis of allele-specific transcripts from a nasal polyp by ASO (data not shown), suggests that the levels of transcripts from the non-F508del allele (sum of normal and alternative transcripts) are reduced relative to transcripts from the F508del allele. This could result from partial degradation of the alternative transcript as a consequence of the *opal* codon, as nonsense mutations frequently cause reduction in mRNA levels, as described both for the CFTR gene [Hamosh et al., 1991; Will et al., 1995] as well as in other genes [Atwater et al., 1990]. To determine with some accuracy the levels of normal CFTR transcripts still present in these patients would be very important, even for (gene) therapy for CF [Delaney and Wainwright, 1996] and is our current research.

Even if partially degraded, the fact that some alternative transcript is present means that it must be translated into the respective protein product with still unknown functional properties. It might be predicted that this modified CFTR protein has some function, as it retains wild-type CFTR sequence until amino acid residue 1046, and it was reported that synthetic CFTR truncations distal to residue 836 [Sheppard et al., 1994], or to residue 370, were found to have single Cl^- channel conductances near to that of wild-type CFTR [Schwiebert et al., 1998].

However, beyond the end of exon 17a, the predicted product of the alternative transcript would have a 36-amino acid extension, with a hydrophobicity pattern (not shown) totally different from the wild-type CFTR C-terminus. This altered C-terminus could cause various effects upon CFTR folding, trafficking and function. We can, for instance, envisage that: (1) a different tail in CFTR would cause a change in stability, since truncation of the C-terminal cytoplasmic domain results in increased CFTR turnover [Loo et al., 1997]; (2) CFTR protein lacking the C-terminus would

miss the recently described [Wang et al., 1998; Short et al., 1998] interaction with PDZ-domain proteins such as EBP50 (ezrin-radixin-moesin binding phosphoprotein 50), with a probable influence in CFTR stability, Cl^- conductance regulation, or in its localization as well; and (3) a CFTR protein with an abnormal C-terminus can also speculated not to fold correctly within the cell causing it to be discarded by the cellular quality control, as it happens with the most common mutant F508del [Cheng et al., 1990; Yang et al., 1993; Pind et al., 1994].

We determined that at least part of the protein observed in the CM has the C-terminus of wild-type CFTR. However, it cannot be ruled out that some truncated CFTR, product of the alternative transcript, also reaches the cell membrane but has little or no function. The high sodium absorption presented here for a 3272-26A→G/F508del nasal polyp, which is typical of CF respiratory epithelia [Boucher et al., 1986; Chinet et al., 1994; Mall et al., 1998], together with the CF phenotype of the 44 patients described, lead us to postulate that the product of the alternative transcript either does not reach the apical CM or has an almost totally impaired function.

In conclusion, we can say that this mutation leads to the production of an alternatively spliced CFTR transcript, that causes a reduction in the amount of normal CFTR messages (and most probably of functional protein) in these patients. The 3272-26A→G mutation should thus be classified as a class V mutation, according to the Zielenski and Tsui classification [Zielenski and Tsui, 1995].

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