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**Research Article**

In congenital bilateral absence of the vas deferens patients, the T5 allele at the polymorphic Tn locus in the CFTR (cystic fibrosis transmembrane conductance regulator) gene is a frequent disease mutation with incomplete penetrance. This T5 allele will result in a high proportion of CFTR transcripts that lack exon 9, whose translation products will not contribute to apical chloride channel activity. Besides the polymorphic Tn locus, more than 120 polymorphisms have been described in the CFTR gene. We hypothesized that the combination of particular alleles at several polymorphic loci might result in less functional or even insufficient CFTR protein. Analysis of three polymorphic loci with frequent alleles in the general population showed that, in addition to the known effect of the Tn locus, the quantity and quality of CFTR transcripts and/or proteins was affected by two other polymorphic loci: (TG)m and M470V. On a T7 background, the (TG)11 allele gave a 2.8-fold increase in the proportion of CFTR transcripts that lacked exon 9, and (TG)12 gave a sixfold increase, compared with the (TG)10 allele. T5 CFTR genes derived from patients were found to carry a high number of TG repeats, while T5 CFTR genes derived from healthy CF fathers harbored a low number of TG repeats. Moreover, it was found that M470 CFTR proteins matured more slowly, and that they [...]

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# Polyvariant Mutant Cystic Fibrosis Transmembrane Conductance Regulator Genes

## The Polymorphic (TG)<sub>m</sub> Locus Explains the Partial Penetrance of the T5 Polymorphism as a Disease Mutation

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### Abstract

In congenital bilateral absence of the vas deferens patients, the T5 allele at the polymorphic Tn locus in the *CFTR* (cystic fibrosis transmembrane conductance regulator) gene is a frequent disease mutation with incomplete penetrance. This T5 allele will result in a high proportion of *CFTR* transcripts that lack exon 9, whose translation products will not contribute to apical chloride channel activity. Besides the polymorphic Tn locus, more than 120 polymorphisms have been described in the *CFTR* gene. We hypothesized that the combination of particular alleles at several polymorphic loci might result in less functional or even insufficient *CFTR* protein. Analysis of three polymorphic loci with frequent alleles in the general population showed that, in addition to the known effect of the Tn locus, the quantity and quality of *CFTR* transcripts and/or proteins was affected by two other polymorphic loci: (TG)<sub>m</sub> and M470V. On a T7 background, the (TG)<sub>11</sub> allele gave a 2.8-fold increase in the proportion of *CFTR* transcripts that lacked exon 9, and (TG)<sub>12</sub> gave a sixfold increase, compared with the (TG)<sub>10</sub> allele. T5 *CFTR* genes derived from patients were found to carry a high number of TG repeats, while T5 *CFTR* genes derived from healthy CF fathers harbored a low number of TG repeats. Moreover, it was found that M470 *CFTR* proteins matured more slowly, and that they had a 1.7-fold increased intrinsic chloride channel activity compared with V470 *CFTR* proteins, suggesting that the M470V locus might also play a role in the partial penetrance of T5 as a disease mutation. Such polyvariant mutant genes could explain why apparently normal *CFTR* genes cause disease. Moreover, they might be responsible for variation in the phenotypic expression of *CFTR* mutations, and be of relevance in other genetic diseases. (*J. Clin. Invest.* 1998. 101:487–496.) Key words: cystic fibrosis trans-

membrane conductance regulator • cystic fibrosis • congenital bilateral absence of the vas deferens • splicing • haplotype background

### Introduction

Mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*)<sup>1</sup> gene (1), which encodes a chloride channel (2), cause cystic fibrosis (CF; 3–6). The disease phenotype of CF patients is quite variable, however, going from severe pulmonary disease with pancreatic insufficiency to severe pulmonary disease with pancreatic sufficiency (PS), to atypical CF (i.e., CF-like pulmonary disease with normal sweat test). *CFTR* mutations, however, are also involved in diseases that only share part of the symptoms observed in CF patients, such as congenital bilateral absence of the vas deferens (CBAVD; 7–10). Finally, *CFTR* mutations have been found at significantly higher than expected frequencies in patients with disseminated bronchiectasis (11) and allergic bronchopulmonary aspergillosis (12). *CFTR* mutations, therefore, appear to be associated with a broad spectrum of clinical phenotypes.

Patients that present with a milder phenotype usually are heterozygotes for a mild mutation on one *CFTR* allele and a severe one on the second allele, or may even carry mild mutations on both *CFTR* genes. Milder mutations, such as the 3849+10kbC→T mutation (13), could be located in regions recognized by the splicing machinery when some normal splicing still occurs. Mutations that affect the conductivity properties of *CFTR*, such as R117H, R334W, and R347P, are other examples of mild mutations (6, 14). Some mutations can be responsible for different phenotypes. For example, the R117H mutation is found in PS-CF patients as well as in CBAVD patients (15), and the difference in phenotypic expression has been explained by the polymorphic Tn locus in front of exon 9. Three alleles (T5, T7, and T9) can be found at this polymorphic locus (16). These alleles determine the efficiency by which the intron 8 splice acceptor site is used (16, 17). The efficiency will decrease when a shorter stretch of thymidine residues is found. A higher proportion of *CFTR* transcripts that lack exon 9 sequences, which encode part of the functionally important first nucleotide-binding domain, will therefore be found when a shorter stretch of thymidine residues is present (16, 17). Such transcripts are known to be translated in *CFTR* proteins that do not mature, and will therefore not result in apical chloride channel activity (18, 19). If a R117H *CFTR* gene harbors a T5

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1. Abbreviations used in this paper: CBAVD, congenital bilateral absence of the vas deferens; CF, cystic fibrosis; *CFTR*, cystic fibrosis transmembrane conductance regulator; PKA, protein kinase A; PS, pancreatic sufficiency.

allele, the mutant gene will be responsible for CF (15). A R117H mutant CFTR gene that harbors a T7 allele can either result in CF or CBAVD (15).

However, the T5 allele alone can be responsible for disease, such as CBAVD. Indeed, compared with control individuals, the T5 allele is found at an increased frequency in CBAVD patients (8–10). A considerable number of CBAVD patients are compound heterozygotes for a true CFTR disease mutation and a T5 CFTR gene (8–10). Some CBAVD patients are even homozygous for two T5 CFTR genes (8). However, the number of male individuals that carry one of the latter two genotypes is much higher than the frequency of CBAVD in the general population (10). Among these are fathers of the patients, who are clearly not affected but harbor Tn CFTR genotypes that are also observed in CBAVD individuals (8, 10, 20). The T5 allele has therefore been classified as a CBAVD disease mutation with partial penetrance (10). The fact that T5 can either result in disease or no disease makes genetic counseling of these individuals complex.

Besides the polymorphic Tn locus, more than 120 polymorphic loci have been described in the CFTR gene (4, 5). Some of the alleles found at polymorphic loci located in exons coding for the first nucleotide-binding domain, as well as in their exon/intron splice junctions, reach high frequencies in the general population. These include polymorphisms found at the (TG)m and M470V loci (9, 20). Although a particular allele may not by itself have deleterious consequences, we hypothesized that the combination of particular alleles at several polymorphic loci might result in less functional, or even insufficient CFTR protein. Here we show that particular alleles at these polymorphic loci do indeed affect the final quantity and/or quality of CFTR. Moreover, the combination of particular alleles are found to explain the partial penetrance of T5 as a disease mutation. For these reasons we would name these genes polyvariant mutant CFTR genes.

## Methods

**Studied samples.** Nasal biopsies were obtained from patients suffering from chronic nasal obstruction or sinusitis. These patients did not suffer from disseminated bronchiectasis or chronic obstructive pulmonary disease (COPD). T5 CFTR genes were derived from CBAVD, CF patients, parents of CF patients, and random fertile people. The majority of CBAVD patients have been described previously (9). The PS-CF patient was the only patient of the more than 100 CF patients that were completely sequenced over the complete coding region and the exon/intron junctions of CFTR that was found to be a compound heterozygote for the T5 allele and a severe CFTR mutation ( $\Delta F508$ ). Given the low frequency of the T5 allele (0.06) in the general population (20), only a limited number of T5 CFTR genes derived from random fertile people could be studied.

**Genomic analysis.** The  $\Delta I507$ ,  $\Delta F508$ , 1717-1G $\rightarrow$ A, G542X, G551D, R553X, W1282X, and N1303K mutations were screened by means of the INNO-LiPA CF2 assay (Innogenetics, Zwijndrecht, Belgium) according to protocols provided by the manufacturer. The 3849+10kbC $\rightarrow$ T mutation was screened as described by Highsmith et al. (13). Sequencing of exons 8–10 and their exon/intron junctions was performed as previously described (21), which allowed both screening for mutations and genotyping at the (TG)m and Tn loci. The (TG)m locus immediately precedes the Tn locus, both of which are located at the end of intron 8. The alleles at these loci were designated according to the number of repeats. Amplification across the M470V locus was also performed as previously described (21). The alleles present at these loci, which were designated according to

the amino acid found, were determined by means of HphI restriction enzyme analysis. The M470V locus is located in exon 10 and encodes part of the first nucleotide-binding domain. The alleles found at these polymorphic loci are rather frequent in the general population (9, 20).

**Determination of CFTR haplotypes.** The haplotypes, when possible, were determined by segregation analysis. If no relatives were available and the individuals were compound heterozygotes for a true CF mutant CFTR gene, the haplotypes could be deduced according to previous haplotype analysis of known CF mutations (20). Indeed, a particular CF mutation is almost always found to be associated with one particular allele at associated polymorphic loci (20).

**Qualitative and quantitative CFTR transcript analysis.** Nasal epithelial cells were cultured for one or two passages as previously described (22). Total RNA was extracted from the cultured nasal epithelial cells with the TRIzol™ reagent and converted into cDNA with M-MLV reverse transcriptase, using pd(T)<sub>12–18</sub> primers (Pharmacia Biotech Inc., Piscataway, NJ) according to protocols provided by the manufacturer (Life Technologies). Qualitative and quantitative CFTR transcript analysis was performed as previously described (23). For samples heterozygous for the alleles at the M470V locus, PCR products were also digested with HphI and analyzed by qualitative and quantitative means. The proportion of each type of transcript derived from each allele was calculated as follows: the proportion of M470 exon 9+ transcripts is equal to the proportion contributed by the 357-bp signal in the digested sample; the proportion of V470 exon 9+ transcripts is equal to the proportion contributed by the 559-bp signal in the undigested sample minus the proportion of M470 exon 9+ transcripts calculated above; the proportion of M470 exon 9– transcripts is equal to the proportion contributed by the 376-bp signal in the digested sample; and the proportion of V470 exon 9– transcripts is equal to the proportion contributed by the 376-bp signal in the undigested sample minus the proportion of M470 exon 9– transcripts calculated above.

**Plasmid constructions and transfections.** A Kpn I Xho I CFTR cDNA fragment was isolated from the prokaryotic vector pTG5960 (Transgene, Strasbourg, France), which contains a CFTR cDNA construct in which the cryptic bacterial promoter (24) has been inactivated (T $\rightarrow$ C at 930, A $\rightarrow$ G at 933, T $\rightarrow$ C at 936) and ligated into the pcDNA3 expression vector (Invitrogen Corp., San Diego, CA). Subsequently, the construct was digested with Kpn I and Eco RV in order to remove a potential ATG start codon that was derived from the polycloning site of pTG5960. The termini were subsequently blunt-ended, and the construct was religated. Sequencing the complete CFTR coding region of this construct did not reveal any differences apart from V470 and silent mutations introduced for inactivation of the cryptic bacterial promoter from the CFTR amino acid sequence (1). From this construct, a M470 CFTR cDNA/pcDNA3 construct was made by means of the Transformer™ Site-directed Mutagenesis Kit (Clontech, Palo Alto, CA) according to protocols provided by the manufacturer (mutagenesis primer: 5'-TTC ACT TCT AAT GAT GAT TAT GGG A-3'; selection primer: 5'-CTC TGG GGT CCG GAA TGA CCG AC-3', which will destroy a BstBI site). The complete CFTR coding region of this construct was also verified by sequencing. Only the mutagenesis event was observed; no other mutations had been introduced. Both constructs were then transiently transfected in COS-1 cells or stably transfected in CHO-K1 cells according to protocols previously described (25).

**Pulse-chase experiments.** Transfected cells were preincubated for 30 min in methionine- and cysteine-free RPMI 1640 medium, supplemented with L-leucine, L-arginine, glucose, L-glutamine, I-inositol (Life Technologies), and 10 mM Hepes for 30 min at 37°C. The transfected cells were labeled in the same medium containing 100  $\mu$ Ci/ml [<sup>35</sup>S]methionine and [<sup>35</sup>S]cysteine (ICN Pharmaceuticals Inc., Costa Mesa, CA) during a pulse of 15 min, and then chased for different time periods at 37°C. For chasing, the labeling medium was replaced with DME-F12 medium (Life Technologies) supplemented with 10% FBS (HyClone, Logan, UT). The cell monolayers were scraped in ice-cold Tris-NaCl buffer (20 mM Tris-HCl, 150 mM NaCl; pH 7.4) sup-

plemented with protease inhibitors (antipain, chymostatin, leupeptin, and pepstatin A at 0.5  $\mu\text{g/ml}$ , 0.5 mM phenylmethylsulfonylfluoride, 10  $\mu\text{g/ml}$  soybean trypsin inhibitor). The cells were solubilized at 4°C in 750  $\mu\text{l}$  IPPA buffer (Tris-NaCl buffer, 1% Na<sup>+</sup>-desoxycholate, 1% Triton X-100, 0.1% SDS; pH 7.4), supplemented with protease inhibitors, and sonicated briefly. After centrifugation at 4°C, the supernatant was precleared on protein A Sepharose CL-4B beads (Pharmacia Biotech Inc.) at 4°C. The precleared supernatant was then incubated at 4°C with 1.5  $\mu\text{g}$  COOH terminus-specific monoclonal mouse antihuman CFTR antibody (Genzyme Corp., Boston, MA) or HCF703 monoclonal antibody directed against the R domain (26). The immune complexes were precipitated with protein A Sepharose CL-4B beads at 4°C followed by four washes in IPPA buffer supplemented with protease inhibitors. Immunoprecipitated proteins were eluted and denatured in sample buffer (13 mM Tris-HCl, 3.25% SDS, 1%  $\beta$ -mercaptoethanol, 25% glycerol; pH 6.8) for 15 min at room temperature. The samples were analyzed by electrophoresis on a 4–12% Tris-glycine PAGE gel (Novex, San Diego, CA) and autoradiography.

**Whole-cell and single-channel recordings.** Transfected cells selected for G418 resistance were seeded on glass coverslips and cultured overnight in DME-F12 medium supplemented with 10% FBS. For whole-cell and single-channel measurements, a conventional patch clamp device was used (EPC-7 amplifier; List, Darmstadt, Germany; pipette resistance 5–10 M $\Omega$ ; data acquisition by Clampex version 5.5.1 software package; Axon Instruments Inc., Foster City, CA). Whole-cell recordings were performed at room temperature (sampling rate 1 kHz, filter setting 200 Hz). Two second voltage ramps applied every 15 s from –100 to +100 mV were used for monitoring of I-V relationships (holding potential 0 mV). Currents were analyzed at +100 mV. In addition, voltage step protocols were used (duration 1 s, –100–+100 mV, increment 20 mV, holding potential 0 mV). Single-channel recordings were performed at 35°C and were analyzed by means of the ASCD-software program (27). Currents were digitized at 1 kHz and filtered at 500 Hz (holding potential of –60 mV).

For whole-cell recordings, the pipette (internal solution) contained (in mM): 100 Cs<sup>+</sup>- aspartate, 20 CsCl, 20 tetraethylammonium chloride, 5 Hepes, 4 EGTA, 4 Mg-ATP; pH 7.2. The bath solution contained (in mM): 150 NaCl, 10 glucose, 10 Hepes, 5.9 KCl, 1.2 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>; pH 7.4. To block K<sup>+</sup> currents, KCl was substituted by CsCl before activation of the Cl<sup>–</sup> currents. CFTR-mediated conductance was activated by a phosphorylation cocktail containing 1  $\mu\text{M}$  forskolin and 10  $\mu\text{M}$  IBMX (Sigma Chemical Co, St. Louis, MO). 50  $\mu\text{M}$  glibenclamide (Sigma Chemical Co.) was used for inhibition of channel activity.

For single-channel recordings, the pipette solution contained (in mM): 140 N-methyl-D-glucamine, 100 aspartate acid, 10 Hepes, 2 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 35 HCl; pH 7.3. The bath solution contained (in mM): 140 N-methyl-D-glucamine chloride, 10 NaF, 10 Hepes, 5 CsCl, 2 MgCl<sub>2</sub>, 1 Mg-ATP, 1 EGTA; pH 7.2. The channels were activated through addition of 75 nM protein kinase A (PKA; Promega Corp, Madison, WI).

**Oocytes and RNA injection.** Female toads (*Xenopus laevis*) were anaesthetized by immersion in ice water containing 2 g/liter 3-amino benzoic acid ethyl ester (Sigma Chemical Co.), and oocytes were removed via a small abdominal incision. The follicular membranes were removed and incubated for 1 h in a calcium-free solution containing 2 mg/ml collagenase A (Boehringer Mannheim Biochemicals, Indianapolis, IN). CFTR transcripts were in vitro-transcribed from the CFTR cDNA/pcDNA3 constructs using the T7 RiboMAX™ large-scale RNA production kit (Promega Corp.) according to the protocol provided by the manufacturer. Defolliculated oocytes were injected with 50 nl RNA (5  $\mu\text{g}/\mu\text{l}$ ).

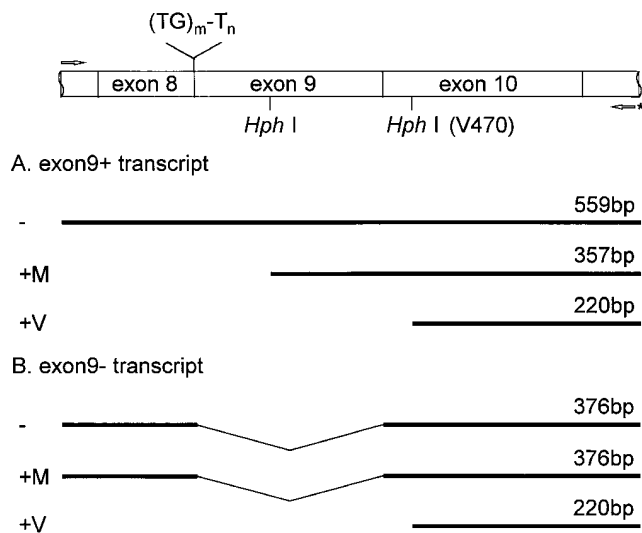
**Two-electrode voltage-clamp assays.** Oocytes injected 2–3 d previously were voltage-clamped with a two-electrode technique and continuously perfused with ND96 solution (96 mM NaCl, 5 mM Hepes, 2 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>; pH 7.4, 100  $\mu\text{M}$  niflumic

acid; Sigma Chemical Co.) to block endogenous Ca<sup>2+</sup>-activated Cl<sup>–</sup> currents. CFTR-mediated currents were activated by a cocktail containing 10  $\mu\text{M}$  forskolin and 1 mM IBMX. Membrane currents in un-injected, water-injected or mock-injected oocytes were unresponsive.

**Table I. Proportion of Exon9– CFTR Transcripts in Cultured Nasal Epithelial Cells**

Genotype	Haplotype	Sample no.	Proportion exon 9– transcripts	n	Proportion exon 9– transcripts/haplotype
I	D: TG10/T9/M470	1	0.03±0.01	19	
	B: TG10/T7/M470				
II	D: TG10/T9/M470	2	0.11±0.01	13	D: 0.02–A: 0.22
	B: TG10/T7/M470	4	0.05±0.01	9	
IV	B: TG10/T7/M470	5	0.08±0.01	15	B: 0.04–A: 0.14
	A: TG11/T7/V470	6	0.09±0.01	17	B: 0.06–A: 0.12
V		7	0.10±0.01	15	B: 0.07–A: 0.13
		8	0.10±0.01	12	B: 0.06–A: 0.15
	A: TG11/T7/V470	9	0.12±0.01	14	
	A: TG11/T7/V470	10	0.13±0.01	11	
		11	0.14±0.02	9	
		12	0.16±0.01	13	
		13	0.17±0.01	10	
		14	0.12±0.01	13	
		15	0.15±0.01	15	
		16	0.12±0.01	10	
VI		17	0.16±0.01	12	
		18	0.14±0.01	15	
		19	0.12±0.01	12	
		20	0.13±0.01	13	
		21	0.16±0.01	10	
		22	0.12±0.01	8	
	A: TG11/T7/V470	23	0.21±0.01	15	C: 0.29–A: 0.08
	C: TG12/T7/M470	24	0.20±0.01	14	C: 0.30–A: 0.05
		25	0.23±0.02	13	C: 0.32–A: 0.03
	VII	B: TG10/T7/M470	26	0.46±0.01	9
E: TG12/T5/V470					

Haplotypes (A–E) were built up by alleles found at the (TG)m, Tn, and M470V loci, respectively. For each type of transcript, the average proportion and the standard error of the mean was calculated. Only the quantitative data of exon 9– transcripts are given. For individuals heterozygous at the M470V locus, the proportion of exon 9– transcripts was calculated for the transcripts derived from each CFTR gene individually (haplotypes A–E are indicated). The total number of all independent measurements is given (n). This number comprises the number of RNA extractions (twice for samples 1, 6, 7 and 22; once for the remainder), the number of independent cDNA synthesis reactions from each of these RNA extractions (1–3), the number of independent PCR reactions from each of the cDNA synthesis reactions (3–6), and replicate loadings of the latter samples (1, 2). A further control was the use of duplicate PCR reactions, one being performed for two additional PCR cycles. The latter control was used to check if the different fragments were equally well amplified. Indeed, in the saturation phase of the PCR, smaller fragments could be more efficiently amplified than larger fragments. For each sample, the qualitative and quantitative measurements for the duplicate PCR reactions were not significantly different from each other, showing that the smaller fragments were not more efficiently amplified.



**Figure 1.** Schematic representation of PCR products derived from *CFTR* transcripts, used for qualitative and quantitative analysis. The primer denoted with an asterisk carries a FITC moiety. Polymorphic sites are indicated. The ones shown (*top*) are located in intronic regions. M470V is located in the coding region. The *HphI* site in exon 10 is only present if the V allele is present. (A) PCR fragment derived from the exon 9+ transcript; (B) PCR fragment derived from the exon 9- transcript. -, FITC-labeled M470 or V470 fragment obtained after amplification; +M, FITC-labeled M470 fragment obtained after *HphI* digestion; +V, FITC-labeled V470 fragment obtained after *HphI* digestion.

## Results

**Characterization of *CFTR* genes.** Cultured epithelial cells of nasal biopsies obtained from 26 individuals were tested for the presence of one of the eight most common *CFTR* mutations, representing 85% of all CF mutations in our population. No mutation could be identified. The mild mutation, 3849+10kbC→T was also not found in any of these samples. Sequencing of exons 8–10 and their exon/intron junctions did not reveal any disease mutation apart from the polymorphic

sites described below. Next, the alleles present at the polymorphic (TG)<sub>m</sub>, T<sub>n</sub>, and M470V loci were determined, and the haplotypes composed of the different alleles of these polymorphic loci were constructed (Table I). The different haplotypes are given in Table I. The five haplotypes thus observed could be combined in seven different genotypes.

***CFTR* transcript analysis.** Since the nasal epithelium is in direct contact with the environment, expression of *CFTR* might be influenced by exogenous factors such as infections. Indeed, it has been shown that *CFTR* expression is different in injured and repairing tissue compared with the same tissue under normal physiological conditions (28). Culture of the nasal epithelial cells will remove infection and other environmental influences so that the cells can be studied under comparable conditions, and so that differences in the nature of the transcripts found during analysis could be completely attributed to the genetic constitution of the cells. Moreover, sufficient material could be obtained from these cultures to perform replicate measurements to test the findings for their significance. For these reasons, only cultured nasal epithelial cells derived from the individuals described in Table I were studied. Nevertheless, for samples 3, 4, and 21, we have previously shown that the qualitative and quantitative nature of the transcripts did not differ if they were derived from either fresh biopsies or from cultured cells.

Reverse transcription PCR was performed across sequences coding for the first nucleotide-binding domain such that alternative splicing of exon 9 could be studied (Fig. 1). This region also harbors the M470V locus. The M and V alleles can be discriminated by restriction enzyme analysis, which allows one to discriminate between the two types of transcripts derived from each *CFTR* gene in individuals heterozygous at the M470V locus (Fig. 1). The results are summarized in Table I.

In all samples studied, alternative splicing of exon 9 was observed. In agreement with previous studies (17), the amount of alternatively spliced transcripts increased when a shorter stretch of thymidine residues was present at the T<sub>n</sub> locus. Moreover, we found that the number of TG repeats also correlated with the proportion of exon 9- transcripts. Analysis of genotypes III-VI homozygous for the T7 allele revealed that increasing proportions of exon 9- transcripts were observed

**Table II.** Nucleotide Sequences of Intron 8 Splice Branch/Acceptor Sites of the Different *CFTR* Haplotypes' Buildup of Alleles at the (TG)<sub>m</sub> and T<sub>n</sub> loci

	sb	sa
(TG) 11-T9:	TTTTGATGTGTGTGTGTGTGTGTGTGTGTGTGTGT	TTTTTTTTTAACAG
(TG) 10-T9:	TTTTGATGTGTGTGTGTGTGTGTGTGTGTGTGTGT	TTTTTTTTTAACAG
(TG) 9-T9:	TTTTGATGTGTGTGTGTGTGTGTGTGTGTGTGTGT	TTTTTTTTTAACAG
(TG) 12-T7:	TTTTGATGTGTGTGTGTGTGTGTGTGTGTGTGTGT	TTTTTTTTTAACAG
(TG) 11-T7:	TTTTGATGTGTGTGTGTGTGTGTGTGTGTGTGTGT	TTTTTTTTTAACAG
(TG) 10-T7:	TTTTGATGTGTGTGTGTGTGTGTGTGTGTGTGTGT	TTTTTTTTTAACAG
(TG) 13-T5:	TTTTGATGTGTGTGTGTGTGTGTGTGTGTGTGTGT	TTTTTTTTTAACAG
(TG) 12-T5:	TTTTGATGTGTGTGTGTGTGTGTGTGTGTGTGTGT	TTTTTTTTTAACAG
(TG) 11-T5:	TTTTGATGTGTGTGTGTGTGTGTGTGTGTGTGTGT	TTTTTTTTTAACAG
Consensus:	YNYTRAY	(N) <sub>2-21</sub> YYYYYYYYYNYAG

The consensus splice branch/acceptor site (17) is also given. Nucleotides at the T<sub>n</sub> locus, which are part of the splice acceptor consensus sequence (17) that vary between the different haplotypes, are underlined. The branchpoint A nucleotide of the lariat formed during the splicing reaction is given in bold and is underlined. The TG repeats encode part of the splice branch site consensus sequence (17). sb, splice branch site consensus sequence; sa, splice acceptor site consensus sequence.

when a longer stretch of TG repeats was found independent of the allele present at the Tn locus. ANOVA showed that the mean proportion of exon 9– transcripts derived from haplotypes A–C were significantly different from each other ( $P < 10^{-6}$ ). Subsequent post-hoc pairwise comparison tests according to Bonferroni showed that the means for all tested haplotypes were significantly different from each other ( $P < 10^{-6}$ ). The Spearman rank correlation coefficient was 0.67, and the Pearson correlation coefficient was 0.84 ( $P < 10^{-9}$ ) by ranking for the number of TG repeats. On a T7 background, the (TG)11 allele increased the proportion of exon 9– transcripts 2.8-fold compared with a (TG)10 allele, and even sixfold in the presence of a (TG)12 allele. It should be noted that these haplotypes also differed for alleles at the M470V locus. The different alleles at the Tn locus affect the efficiency by which the splice acceptor consensus sequence is recognized, while the different alleles at the (TG)m locus place the branchpoint A nucleotide in a less favorable position for splicing (Table II).

Another interesting observation was that the proportion of exon 9– transcripts derived from a haplotype A gene varied depending on the haplotype found in *trans*:  $0.18 \pm 0.06$  ( $n = 2$ ) for haplotype D;  $0.14 \pm 0.01$  ( $n = 4$ ) for haplotype B; and  $0.05 \pm 0.02$  ( $n = 3$ ) when haplotype C was found in *trans* (Table I). ANOVA showed that the mean proportion of exon 9– transcripts from haplotype A found in these three groups were significantly different ( $P < 0.02$ ). Subsequent post-hoc pairwise comparison tests according to Bonferroni showed that the mean proportion of haplotype A exon 9– transcripts for genotype A/C was significantly different from the ones found for the A/B ( $P < 0.05$ ) and A/D ( $P < 0.02$ ) genotypes. The Pearson correlation coefficient was  $-0.87$  ( $P < 0.01$ ).

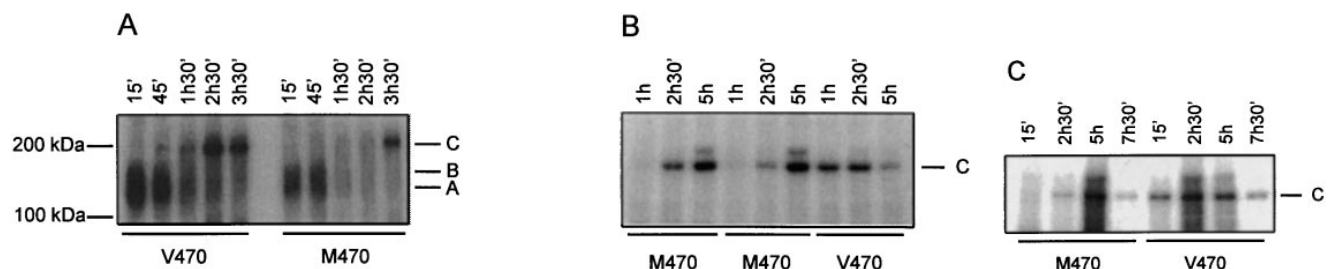
It can thus be concluded that another locus than the Tn locus alone determines the extent to which exon 9– transcripts are found.

**Maturation of M470 and V470 CFTR.** In contrast with the polymorphic loci (TG)m and Tn, the M470V locus is polymorphic at the amino acid level. Therefore, the kinetics of maturation and degradation of these two variant CFTR proteins were studied. M470 or V470 CFTR was transiently expressed in COS cells. After metabolic labeling of the transfected cells and subsequent chases during different time periods, the cells were lysed and CFTR was immunoprecipitated with a monoclonal antibody directed against the COOH-terminal part of CFTR (Fig. 2 A). Both M470 and V470 CFTR did mature up to the complex-glycosylated CFTR form (C-form). Since both alleles

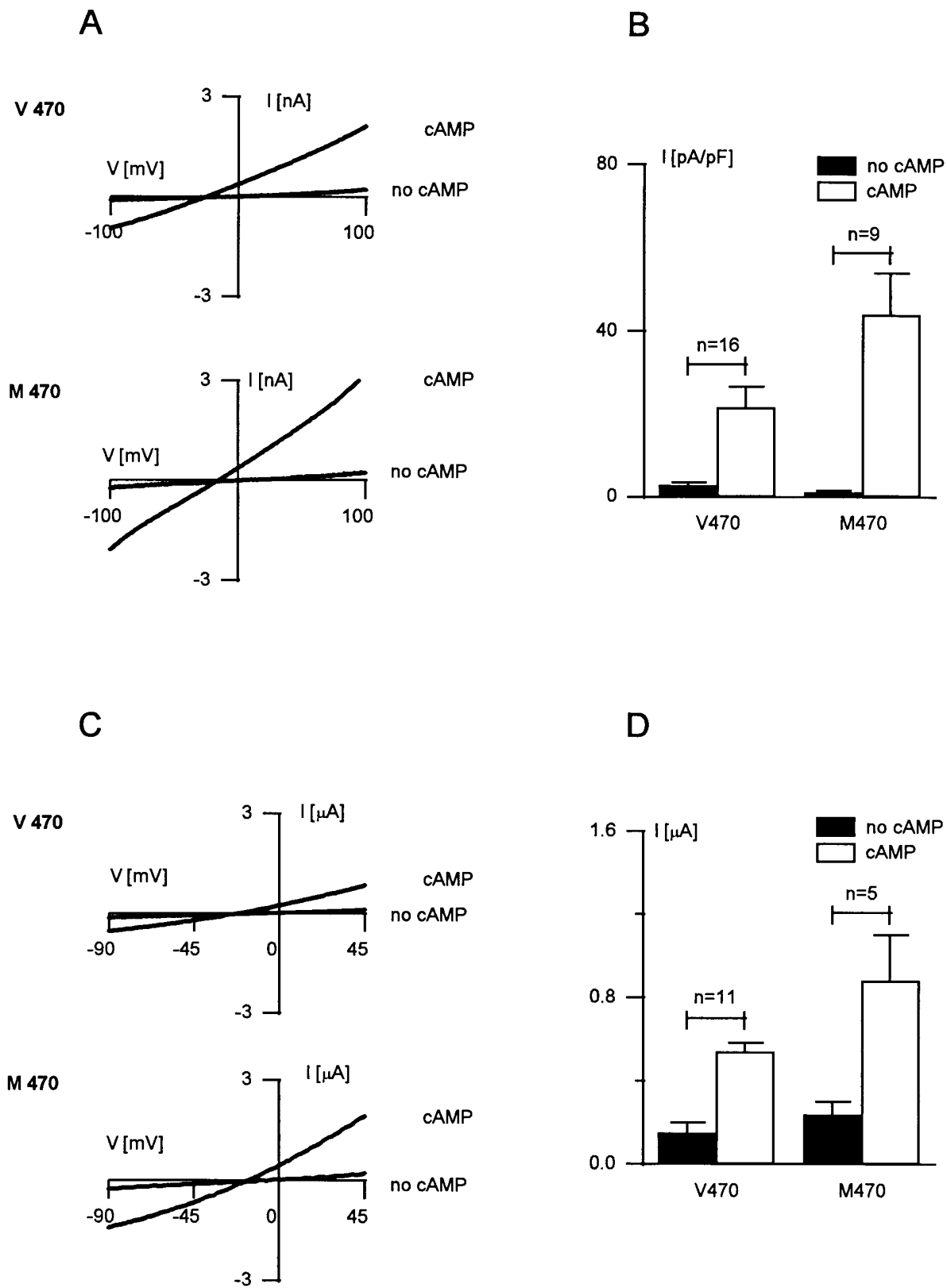
at the M470V locus are frequent in the general population and both are therefore found in individuals not affected by CF or its related diseases, this finding was not unexpected. As expected, both for M470 and V470 CFTR, a substantial proportion of the primary translation and core-glycosylated CFTR products was degraded (24, 29). However, M470 CFTR proteins matured more slowly than V470 CFTR. As shown in Fig. 2 A, small amounts of complex-glycosylated V470 CFTR protein were already found after a chase of 45 min, and most of the complex-glycosylated V470 CFTR protein appeared after a chase of 2 h and 30 min. In contrast, most of the complex-glycosylated M470 CFTR protein appeared only after a chase of 3 h and 30 min. This difference in kinetics did not depend on the cell type used for transfection since it was also observed in stable transfected CHO cells (Fig. 2 B). Moreover, similar observations were made when another monoclonal antibody directed against the R domain was used for immunoprecipitation of CFTR (Fig. 2 C). Within each experiment ( $n = 6$ ), relative differences in maturation were systematically observed between M470 and V470 CFTR.

A potential interesting observation was that before reaching full glycosylation, i.e., after 1 h and 30 min of chase for V470 CFTR, and 1 h and 30 min and 2 h and 30 min for M470 CFTR, hardly any CFTR protein could be precipitated from COS cells (Fig. 2 A). The time period during which the majority of CFTR could not be detected was thus increased for M470 when compared with V470 CFTR proteins. This finding could not be studied in CHO cells since the primary translation and core-glycosylated CFTR products are not observed in this cell system. A possible explanation for the poor immunoprecipitation of CFTR could be that during these time periods CFTR adopted a configuration, or bound to another protein such that its COOH-terminal epitope was not accessible to this antibody. Taken together, these findings suggest that the allele present at the polymorphic locus M470V affects biogenesis of CFTR.

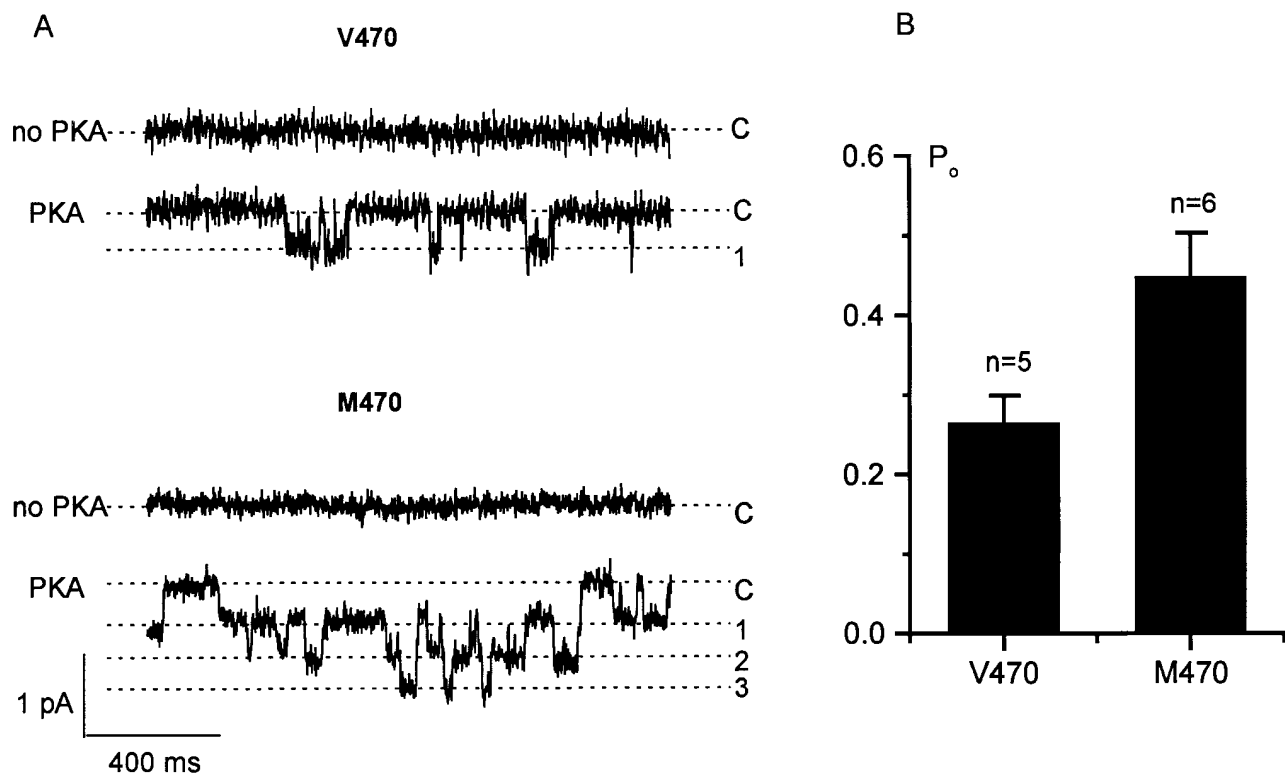
**Electrophysiological characterization of M470 and V470 CFTR.** Permeation and gating properties of M470 and V470 CFTR proteins, transiently expressed in COS cells, were analyzed by the patch clamp method in whole cell and single-channel configurations. In the whole cell configuration, expression of both V470 and M470 CFTR did result in the appearance of a current after stimulation of the cells with a phosphorylation cocktail. Nearly linear I-V relationships were obtained for the cAMP-activated currents (Fig. 3 A). The current carried by



**Figure 2.** Analysis of the biogenesis and degradation of M470 and V470 CFTR in transfected cells. (A) COS cells or (B, C) CHO cells transfected with either M470 or V470 CFTR were metabolically labeled and chased for the indicated times. The cells were then lysed, and CFTR was immunoprecipitated with monoclonal antibodies either directed to the COOH terminus (A, B) or the R domain (C) and analyzed by PAGE and autoradiography. Two M470 CHO clones are shown in B. The positions of the molecular weight markers and the primary translation (A-form), core-glycosylated (B-form), and complex-glycosylated (C-form) CFTR products are indicated.



**Figure 3.** Whole-cell currents of cells that transiently expressed M470 or V470 CFTR. (A) Current-voltage (I-V) relationships obtained from whole-cell currents of COS cells that transiently expressed V470 or M470 under basal conditions and after addition of cAMP activation cocktail to the bath solution. I-V relationships were obtained by 2 s voltage ramps. (B) Comparison of the current densities for COS cells transfected with V470 or M470 CFTR (with or without cAMP activation) at +100 mV. (C) I-V relationships of  $\text{Cl}^-$  currents obtained from oocytes injected with RNA for V470 or M470 CFTR (with or without cAMP stimulation). (D) Comparison of currents in RNA-injected oocytes with V470 or M470 CFTR constructs (with or without cAMP activation). Currents were measured from the ramp protocols at  $-90$  mV.



**Figure 4.** Activation of chloride channels in excised, inside-out patches from COS cells that transiently expressed M470 or V470 CFTR. (A) Representative current traces of single-channel recordings of V470 or M470 CFTR, with or without addition of PKA from the internal site of the membrane patch. C marks the closed state of the channel. 1–3 indicate open channel events. (B) Comparison of the probability of the channel being open ( $P_o$ ), pooled from all cells.  $P_o$  of V470 and M470 CFTR channels was measured at  $-60$  mV after application of PKA.  $P_o$  was calculated either from the probability density functions (amplitude histograms) or from the averaged mean currents divided by the single-channel current. The number of channels was estimated from the maximal number of overlapping opening events. This may even lead to an overestimation of  $P_o$ , given in the pooled data for V470 CFTR channels that open with a lower probability than M470 CFTR.

V470 CFTR channels was characterized by a reversal potential of  $-24$  mV, which is close to the theoretical chloride equilibrium potential ( $E_{Cl} = -33$  mV, Fig. 3 A). Glybenclamide, in a concentration of  $50$   $\mu$ M, inhibited this current by  $78 \pm 5\%$  at a potential of  $+100$  mV ( $n = 3$ , data not shown). An anion permeability sequence of  $P_{Br^-} > P_{Cl^-} > P_{I^-} > P_{gluconate}$  ( $n = 5$ , data not shown) was observed. These characteristics are in agreement with previous findings (2, 30–33). The current density obtained for COS cells transfected with M470 CFTR ( $43.9 \pm 10.0$  pA/pF) was about twice as high as the current density obtained for COS cells transfected with V470 CFTR ( $21.6 \pm 5.1$  pA/pF;  $P < 0.05$ , Fig. 3 B). The functional properties of M470 and V470 CFTR were also studied in *Xenopus* oocytes by using a conventional two-microelectrode voltage clamp method. Again, larger membrane currents were obtained for oocytes that expressed M470 CFTR protein ( $0.9 \pm 0.2$   $\mu$ A,  $n = 5$ ) compared with cells that expressed V470 CFTR protein ( $0.5 \pm 0.04$   $\mu$ A,  $n = 11$ ;  $P < 0.05$ ; Fig. 3, C and D).

The increase in current density for the M470 variant compared with V470 can either be explained by the presence of an increased number of CFTR proteins in the membrane of the cells, an increased open probability of the expressed channels, or a combination of these. Therefore, single-channel patch-clamp analysis was also performed on COS cells that transiently expressed V470 or M470 CFTR protein (Fig. 4 A). A single-channel conductance of  $5.0 \pm 0.9$  pS was obtained for

V470 ( $n = 5$ ) and  $5.6 \pm 0.6$  pS for M470 CFTR protein ( $n = 6$ ). The probability of the channels being open was almost twice as high for M470 CFTR ( $0.45 \pm 0.05$ ,  $n = 6$ ) than for V470 CFTR ( $0.27 \pm 0.03$ ,  $n = 5$ ,  $P < 0.05$ ; Fig. 4 B). It can thus be concluded that the allele present at the polymorphic locus M470V affects gating of the CFTR channel.

**T5 haplotypes.** So far, the factors that determine the penetrance of T5 as a disease allele are unknown. Given our findings that particular alleles at polymorphic loci have functional consequences at the CFTR transcript or protein level, we wondered whether they would affect the penetrance of the T5 allele as a disease allele. T5 CFTR genes were derived from 1 PS-CF patient, 18 CBAVD patients, 13 parents of CF patients (their non-CF CFTR genes), and 5 random healthy fertile people. Due to lack of CFTR transcripts derived from these individuals, association studies were performed.

T5 was found on four different haplotypes (Table III). All T5 CFTR genes derived from parents of CF patients (5 paternal [ $P < 0.0002$ ] and 8 maternal [ $P < 10^{-5}$ ]) and 4 out of 5 T5 CFTR genes derived from random fertile people ( $P < 0.003$ ) were found on haplotype F, while only 1/18 T5 CFTR genes derived from a CBAVD patient was found on this haplotype background. Interestingly, based on the functional data mentioned above, haplotype F is associated with the most functional CFTR gene. Indeed, the highest proportion of exon 9+ CFTR transcripts is transcribed from this haplotype. Moreover, at the protein level this haplotype encodes channels with



Table III. T5 Haplotypes

Haplotypes	CBAVD <i>n</i> = 18	Parents <i>n</i> = 13	Random <i>n</i> = 5	CF <i>n</i> = 1
F: TG11/T5/M470	1	13	4	0
E: TG12/T5/V470	15	0	1	0
G: TG13/T5/M470	2	0	0	0
H: TG13/T5/V470	0	0	0	1

Haplotypes E–H were built from alleles found at the (TG)m, Tn, and M470V loci, respectively. *CFTR* genes were either derived from 1 PS-CF patient, random fertile people, parents (five paternal and eight maternal) of CF patients (non-CF *CFTR* genes), or CBAVD patients. One haplotype E *CFTR* gene derived from a CBAVD patient, and 1 haplotype G and the haplotype H *CFTR* gene were sequenced over the complete coding region and the exon/intron junctions. For the remainder CBAVD patients, the complete *CFTR* coding region and exon/intron junctions were analyzed by denaturing gradient gel electrophoresis (9). No other mutations were found. The numbers of each haplotype found in the four groups of individuals are given.

higher intrinsic chloride transport activities. Of control T5 *CFTR* genes, haplotype F turned out to be most common.

The majority of the T5 *CFTR* genes derived from CBAVD patients (15/18), and 1/5 derived from a random fertile person were found on haplotype E. This haplotype was not found at all among the normal *CFTR* genes of CF parents (paternal,  $P < 0.002$ ; maternal,  $P < 0.0002$ ). This haplotype is associated with a less functional *CFTR* gene: a lower proportion of exon 9+ transcripts that will be translated into CFTR proteins with lower intrinsic chloride transport activities are associated with this haplotype. Haplotype G was found twice on *CFTR* genes derived from CBAVD patients. These haplotypes are known to result in a dramatic high proportion of exon 9– transcripts. Finally, one T5 *CFTR* gene was found on haplotype H. This *CFTR* gene was derived from a 16-yr-old PS-CF patient who carried the  $\Delta F508$  on his other *CFTR* gene. This haplotype carries the less functional V470 allele, and will generate a very low proportion of exon 9+ *CFTR* transcripts.

## Discussion

We have shown that some of the more common polymorphisms in the *CFTR* gene have consequences at the functional level. In agreement with previous studies (17), the nature of the allele present at the Tn locus determined the amount of exon 9– transcripts. Moreover, in this study it was found that the (TG)m locus also affected the amount of exon 9– transcripts independently of the allele found at the Tn locus as illustrated for the T7 allele. A longer TG repeat would place the branchpoint A nucleotide of the lariat in a less favorable position for splicing. It should be noted that the qualitative and quantitative analysis was performed on samples derived from patients suffering from chronic nasal obstruction or sinusitis. In patients that present with milder pulmonary disease, such as in a small proportion of patients with disseminated bronchiectasis (11), *CFTR* mutations have been found. However, the patients studied here did not suffer from disseminated bronchiectasis or COPD. Moreover, no disease mutations

could be identified. First, no mutation was found when screening for the most common classical CF mutations that represent 85% of all CF mutations in our population. The majority of patients that present with milder disease phenotypes carry a mild mutation on one *CFTR* gene and a severe, classical CF mutation on their second allele (8–10, 12). The fact that the CF carrier frequency is not different from that found in the control population argues against an involvement of *CFTR* in the pathology of these patients. Second, the rather frequent 3849+10kbC→T mutation found in milder patients was not detected, also arguing against an involvement of *CFTR*. Third, no disease mutations were found after sequencing exons 8–10 and their exon/intron junctions. Indeed, splice site or nonsense mutations (34) could result in skipping exon 9. Therefore, it is highly unlikely that the qualitative and quantitative differences found were caused by disease mutations.

By means of association studies of T5 *CFTR* genes, only the shortest TG repeat number was found in healthy individuals, and was never found in patients. This result strongly favors the hypothesis that this polymorphic locus affects the nature of *CFTR*. A (TG)12 repeat in combination with a T7 stretch, a (TG)11-T9 haplotype, or a (TG)13 repeat in combination with a T5 stretch, would indeed place the branchpoint nucleotide in the same less favorable position (17; Table II). Thus, both the alleles at the (TG)m and Tn loci determine the proportion of transcripts from which functional CFTR proteins can be translated, and thereby affect net chloride transport activity of *CFTR*-expressing cells.

The allele found at the M470V locus also affected *CFTR*. We found that M470 *CFTR* proteins have a 1.7-fold increased intrinsic chloride channel activity compared with V470 *CFTR* proteins. Also, differences were found in the biogenesis of these two *CFTR* proteins. The fully glycosylated form of M470 matured more slowly than V470. Whether the slower maturation of M470 *CFTR* is a result of its increased intrinsic chloride channel activity or vice versa, or is independent of it is not known.

Given our observations that alleles at polymorphic loci can affect *CFTR* at the transcript and/or protein level, it would be wise to study mutations on their respective haplotype background. This also holds for the mutations already studied (6). Indeed, while only a minority of the more than 720 *CFTR* disease mutations identified so far have been studied at the functional level, these studies have been performed irrespective of the haplotype background of these mutations.

Given the high frequency of the alleles at these polymorphic loci in individuals not affected by lung disease (9, 20), the allele present at each locus appears not to be deleterious by itself. However, the combination of particular alleles at these polymorphic loci does affect the amount of functional *CFTR* and can even lead to disease. Indeed, four different haplotype backgrounds for T5 *CFTR* genes were found. One of these (haplotype F) will generate the highest proportion of exon 9+ transcripts that will be translated into *CFTR* proteins with the highest intrinsic chloride transport activities. Only this fittest T5 haplotype background was found in fathers of CF patients, which could explain why these men do not have CBAVD. The other three T5 haplotype backgrounds were found in patients who carried a higher number of TG repeats. In view of the effect of the (TG)m locus on a T7 background on the proportion of exon 9+ transcripts, and of the analogous modifications caused by (TG)m alleles on T5 *CFTR* genes (Table II), the in-

involvement of the (TG)<sub>m</sub> locus in the penetrance of T5 as a disease mutation is obvious (haplotypes F and G only differ for the allele found at the (TG)<sub>m</sub> locus). Although the alleles found at the M470V locus affect the properties of CFTR, involvement of this locus in the penetrance of the T5 allele as a disease mutation was less clear than for the (TG)<sub>m</sub> locus. Indeed, for haplotype E, the presence of the (TG)<sub>12</sub> allele might by itself be sufficient to make T5 penetrant as a disease allele. In one individual, however, the proportion of exon 9+ transcripts derived from such a *CFTR* gene was found to be 0.28 (Table I). Given the low amount of CFTR that is required for normal chloride transport function (35, 36), this proportion is still considerable, suggesting that V470 is necessary for the penetrance of haplotype E as a disease haplotype. It is also interesting to note that the (TG)<sub>13</sub>/T5/M470 haplotype was found on *CFTR* genes derived from two CBAVD patients, while the (TG)<sub>13</sub>/T5/V470 haplotype was found on a *CFTR* gene derived from a PS-CF patient. Since these haplotypes only differed for the allele found at the M470V locus, these data again indicate a possible involvement of the M470V locus in the penetrance and severity of T5 as a disease allele. It should be noted, however, that the importance of the kinetics of CFTR maturation for overall CFTR-mediated chloride transport activity of cells that express CFTR has not been studied. It is therefore not known whether the slower maturation of M470 could compensate for its increased activity as a chloride channel, compared with V470 CFTR. However, in whole-cell recordings, both in COS cells and in oocytes, M470 was found to have about a twofold increased chloride transport activity compared with V470 CFTR, suggesting that the difference in maturation rate between M470 and V470 CFTR has no compensating effect. A larger number of T5 *CFTR* genes need to be studied before significant conclusions about the involvement of M470V on phenotypic expression can be drawn.

Apart from the fact that the nature of transcripts derived from a particular *CFTR* gene are affected by intragenic polymorphisms, a potential important observation was made during the course of the study. Indeed, our results suggest that the nature of these transcripts is also modulated by the *CFTR* gene in *trans*. The following significant inverse correlation was found: the proportion of exon 9– transcripts from haplotype A increased when the haplotype in *trans* produced a low proportion of exon 9– transcripts, and vice versa. To some extent, a lower or higher proportion of exon 9– transcripts derived from a particular *CFTR* gene was therefore compensated by the allele in *trans*. This compensation would further increase the complexity and variability by which a particular *CFTR* genotype confers a particular phenotype. This intriguing observation warrants further studies in order to confirm these findings, and to unravel the precise mechanisms involved and the physiological importance of this phenomenon.

At least one other polymorphic locus, (TG)<sub>m</sub> determines the partial penetrance and the severity of the T5 allele as a disease mutation. Such mutant *CFTR* genes that harbor a particular combination of alleles and/or variants at polymorphic loci could be classified as polyvariant mutant *CFTR* genes. In fact, one of the polyvariant mutant *CFTR* genes ([TG]<sub>12</sub>/T5) now turns out to be the most frequent CBAVD *CFTR* mutant. Polyvariant *CFTR* genes might also explain why no mutations can be detected in some *CFTR* genes derived from patients (8–10, 21, 37). While there is evidence that the CF phenotype can be modulated by other genes, as has been shown in mice

(38), and/or by environmental factors, the role of these intragenic polymorphisms should not be underestimated.

Polymorphisms have previously been shown to modulate the phenotypic expression of true disease mutations such as R117H (15). In the prion protein gene, the allele at the M129V polymorphic locus determines the fate of the N178D mutation, either as fatal familial insomnia or as a mutant gene that causes a subtype of familial Creutzfeldt-Jakob disease (39, 40). Rather than modulating the fate of a disease mutation such as R117H in *CFTR* and N178D in the prion protein gene, our results show that even two or more polymorphic loci alone can act in concert with each other to result in insufficient or less functional CFTR protein.

Knowledge of the molecular basis of the partial penetrance of the T5 allele as a CBAVD mutation should improve genetic counseling. Genetic testing for additional polymorphisms now allows one to identify the high-risk T5 *CFTR* genes. On the other hand, involvement of polymorphisms further complicates genetic testing for a disease caused by more than 720 mutations. New technologies such as high-density DNA array assays will become necessary to provide quick, accurate, and complete analysis of any mutation or polymorphism in any gene in a particular individual (41). However, they will not be able to construct haplotypes to detect such polyvariant genes, which in many cases will require segregation analysis. Therefore, further improvements in mutation detection assays and in their applications will be required before a foolproof mutation–polymorphism detection system will be available.

Apparently innocent allelic variants affect the phenotypic expression of particular genes. Combinations of polymorphisms in different genes clearly contribute to the development of multifactorial diseases. The apolipoprotein E and  $\alpha$ 1-antichymotrypsin polymorphisms have been shown to be associated with late-onset familial and sporadic forms of Alzheimer's disease (42–44). Apolipoprotein E polymorphisms also predispose to vascular disease (45). HLA polymorphisms in combination with polymorphisms at other loci are associated with diabetes and autoimmune diseases (46). Polyvariant mutant genes and possibly combinations of them might also be involved in multifactorial diseases. Current knowledge of polymorphisms and their role in genetic diseases, however, is still very limited. It is clear, therefore, that genetic and functional studies of polymorphisms in genetic diseases will become of major interest in the future.

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