Cystic fibrosis (CF) (MIM 219700) is one of the most common autosomal recessive diseases in Caucasians. It affects about 1 in 2500 births and approximately 1 in 25 individuals are heterozygotes, with marked regional variations (www.genet.sickkids.on.ca/cftr). It is caused by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR or ABCC7) gene (MIM 602421), which is also involved in a broad spectrum of phenotypes, including male infertility by congenital bilateral absence of the vas deferens (CBAVD), disseminated bronchiectasis (DB), and chronic pancreatitis. So far, over 1000 CFTR gene mutations have been described throughout the gene, along with geographic and ethnic variations in their distribution and frequency (www.genet.sickkids.on.ca/cftr). Of these variations, 99% consist of point mutations or micro-deletions/insertions (www.genet.sickkids.on.ca/cftr) but account for 33–98% of CF alleles, depending on the population. A number of cases remain unsolved after extensive and laborious screening of the 27 exons, thus making genetic counselling difficult for the patients and their families, particularly when the CF diagnosis is not certain. Unidentified CF mutations may lie in introns or in regulatory regions which are not routinely investigated, or correspond to gene rearrangements such as large deletions at the heterozygous state which escape detection using current PCR based techniques. Deletions have been suspected in a very few situations: upon failure of PCR amplification to target particular exons when the deletions were present in CF patients in the homozygous state, or in cases of abnormal segregation of a mutation or polymorphisms in a family. Two such deletions, CFTRdel2–31 and CFTRdel17a–18, which are now routinely tested for by conventional PCR using specific primers, were found in about 5% and 13% of CF alleles, respectively. In our population, unidentified CF alleles and for 1.3% of all CF mutations gene rearrangements thus accounted for 20% of unidentified CF alleles and for 1.3% of all CF mutations in our population.

The method is both simple and reliable, and is able to detect large rearrangements and single nucleotide deletions/insertions as well. Together these account for almost 24% of the CF mutations described, and makes this the method of choice for second line screening when frequent mutations are not found. This strategy has notable implications for genetic counselling and care of CF patients and their family.

Method

Patients

We investigated a total of 78 unrelated French patients or parents of deceased patients from mixed ethnic/geographic origins and subjected them to a complete CFTR gene screening. The subjects were divided into three groups according to the results of a previous screening: (i) 43 CF patients who fulfilled the diagnostic criteria of CF and who carried a CF mutation, and seven parents of deceased CF patients, a CF mutation having already been identified in the other parent (50 unidentified CF alleles); (ii) 12 CF patients with no identified CF mutation (24 unidentified CF alleles); and (iii) 16 patients apparently homozygous for a CFTR mutation and who had CF (F508del – n = 6, 2104insA–2109del10, S945L, 3210+1G→A, N1303K) or a CFTR related disease, that is, isolated CBAVD (D110H, R117H, L997F, R74W-D1270N) or DB (R334W, R668C-G576A-D443Y) (0–16 unidentified CF alleles). In these cases, status confirmation was not possible by family analysis.

Key points

• Cystic fibrosis (CF), one of the most frequent hereditary diseases in the Caucasian population, is mainly due to point mutations scattered over the whole cystic fibrosis transmembrane conductance regulator (CFTR) gene. CFTR gene deletions are rare, but their frequency may be underestimated as they remain undetected when using conventional PCR techniques.
• We studied 78 patients, including 62 CF patients or parents of CF patients bearing one or two unidentified CF alleles after an extensive CFTR gene study, and 16 patients apparently homozygous for a CFTR mutation.
• Making use of an assay based on semi-quantitative fluorescent PCR targeted on the 27 CFTR exons in a three multiplex format, we characterised gene rearrangements in 11 patients, including a recurrent deletion, a complete gene deletion, and the first CFTR gene duplication. Taking into account three known deletions previously characterised in other CF patients, gene rearrangements thus accounted for 20% of unidentified CF alleles and for 1.3% of all CF mutations in our population.

Abbreviations: CBAVD, congenital bilateral absence of the vas deferens; CF, cystic fibrosis; CFTR gene, cystic fibrosis transmembrane conductance regulator gene; DB, disseminated bronchiectasis; DGGE, denaturing gradient gel electrophoresis; DHPLC, denaturing high pressure liquid phase chromatography; FISH, fluorescent in situ hybridisation; MP, multiplex PCR; QFM-PCR, semi-quantitative fluorescent multiplex PCR.
addition, we considered that the genotype could not explain the observed phenotypes in CBAVD and DB patients.

When available, parents of patients in whom an abnormal pattern had been detected were studied. All the patients were referred to our laboratory between 1989 and 2004. Informed consent to CTR studies had been previously obtained from the patients and/or their parents at the time of referral to the laboratory.

Previous extensive CTR gene analyses with a mutation detection rate of 95% included: (i) screening for 31 frequent mutations (CF OLA assay, Abbott, Rungis, France); (ii) scanning of the 27 exons and their boundaries using denaturing gradient gel electrophoresis (DGGE) and denaturing high pressure liquid phase chromatography (DHPLC), followed by sequencing to characterise the variants; (iii) screening for the intronic splicing mutations (CF OLA assay, Abbott, Rungis, France); (iv) screening for three previously characterised deletions using specific primers: CTRdele2,3, dele19. This last step led us to identify large deletions in four former patients: CTRdele2,3 (one allele), CTRdele17a–18 (two alleles), and CTRdele17a–18 (two alleles). These patients were thus not included in the cohort of patients studied here, but the data were taken into account to determine the proportion of CTR gene rearrangements among CF alleles.

DNA extraction
Most genomic DNAs were extracted from whole blood samples collected on EDTA using a phenol chloroform extraction protocol or a commercial kit (Nucleon, BACCC, Amersham Biosciences, Saclay, France). Some DNAs, received from other laboratories, were extracted with different protocols. DNA concentration and quality were determined for each sample.

Screening for CTR rearrangements by semi-quantitative fluorescent multiplex PCR (QFM-PCR)
The original protocol described by Yau et al. was adapted to screen for CTR rearrangements. Briefly, the principle is based on comparisons of the fluorescent profiles of multiplex PCR fragments obtained from different samples, the amplification being stopped at the exponential phase. This procedure allows the detection of heterozygous deletions (twofold reduction of fluorescence intensity) and heterozygous duplications (1.5-fold increase). The 27 CTR gene exons, a promoter region (18731-18868, GenBank AC000111.1), or 6035-6206 according to the current gene numbering) were amplified in three fluorescently labelled multiplex reactions, denoted multiplex PCR (MP): MP 1 (promoter, polyadenylation signal sequence, exons 1–6a and 11), MP 2 (exons 7–10 and 12–16), and MP 3 (exons 6b and 17a–24). Intrinsic specific primers (but exonic for the large exon 13) were chosen mostly among those designed for the cohort of patients studied here, but the data were taken into account to determine the proportion of CTR gene rearrangements among CF alleles.

Molecular characterisation of rearrangements
Several methods were used to characterise the rearrangements detected by QFM-PCR. When apparent deletions removed only one exon, mispriming at primer binding sites was first checked by amplification of the relevant exon using another set of primers bracketing the fragment amplified by QFM-PCR.

The size of rearrangements removing one or several exons was investigated by long range PCR experiments using the Expand Long Template PCR system (Roche Diagnostics, Meylan, France). The resulting fragments were sequenced to precisely define the deletion breakpoints and allow the design of primers suited to detect the deletions by conventional PCR.

In the case of complete CTR gene deletion, fluorescent in situ hybridisation (FISH) analysis with a CTR cDNA probe was performed by Genescan 3.7 software (Applied Biosystems) to obtain electropherograms for each sample. Each product was identified by its size, and fluorescence intensities were correlated to the copy number of the relevant exons. Four control DNAs were included in each experiment: two normal DNAs, that is, one from a woman and one from a man (twofold reduction of F9 exon 5 in men compared to women); a DNA for trisomy 21 (1.5-fold increase of DSCR 1 exon 4); and a DNA carrying a previously identified CTR deletion (CTRdele4–10 for MP 2, and CTRdele17a–18 or CTRdele19 for MP 3). The results were first analysed visually by superimposing fluorescent profiles of tested samples and normal controls, the normalisation being performed with exon 4 DSCR peaks. The peak height values were also imported into an Excel (Microsoft) spreadsheet and the copy number of each fragment was determined by calculating a dosage quotient (DQ) for each exon relative to all the other amplified exons in patients and controls (table 1).

The reproducibility of the whole procedure was assessed by several operators who tested the same samples from patients, and normal and mutant controls, on the same day (duplicates) and on different days.

Table 1: Statistical profiles from the analysis of patient no. 7 carrying the CTRdele17a–17b deletion from multiplex PCR 2 (MP 2) and 3 (MP 3)

<table>
<thead>
<tr>
<th>Height value</th>
<th>DQSCR exon 4</th>
<th>DQexon 5</th>
<th>F9 exon 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient no. 7</td>
<td>565</td>
<td>461</td>
<td>0.98</td>
</tr>
<tr>
<td>Control</td>
<td>502</td>
<td>400</td>
<td>1.02</td>
</tr>
<tr>
<td>CTR exon</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Exon 16 MP 2</td>
<td>1156</td>
<td>1006</td>
<td>0.94</td>
</tr>
<tr>
<td>Exon 17a/MP 3</td>
<td>827</td>
<td>1292</td>
<td>0.52</td>
</tr>
<tr>
<td>Exon 17b/MP 3</td>
<td>360</td>
<td>690</td>
<td>0.73</td>
</tr>
<tr>
<td>Exon 18 MP 3</td>
<td>1052</td>
<td>827</td>
<td>1.04</td>
</tr>
</tbody>
</table>

The DQ values indicative of the 17a–17b deletion pattern are underlined. From our study, values of the DQ were within the range 0.75–1.31 (mean 0.95) for normal control samples, 0.38–0.64 (mean 0.48) for a heterozygous deletion control, and 1.40–1.79 (mean 1.55) for a heterozygous duplication control. They were concordant with those obtained by Yau et al. as an example (indicated in bold characters), the DQ for CTR exon 17a compared to DSCR 1 exon 4 is DQ (DSCR 1/CTR 17a) = (827/565)/(1292/461) = 0.52.
RESULTS

Detection of CFTR rearrangements

QFEM-PCR screening led to the detection of rearrangements in 11 families (table 2). Ten of them were found in the first group of 50 patients or parents of deceased patients where a mutation had been previously identified. None was detected in the second group of 12 patients who carried two unknown mutations. One further rearrangement was identified in the third group of 16 patients, in a CBAVD patient who was apparently R117H homozygous. No rearrangement was detected in the other patients of the third group, confirming that they were homozygous for a CFTR mutation. The results obtained in the third group thus added a CF allele to the total of unidentified alleles which were investigated for the presence of rearrangements. Overall, a rearrangement was found in 11/75 (14.7%) unidentified CF alleles. Taking into account the five CF alleles bearing known deletions that were previously identified, CFTR gene rearrangements accounted for 20% of 80 so called unidentified CF alleles, where a point mutation or a short deletion/insertion has not been found.

The 11 rearrangements detected consisted of eight different patterns. Most exhibited a simple deletion pattern involving one or several consecutive exons: 1; 2; 17b; 14a–17b; 17a–17b in four unrelated cases (fig 1A and table 1).

Two others consisted of a complex deletion removing exons 3–10 and 14b–16, and a whole cis 7q31 region and designed several additional sets of QFM-PCR primers to delineate the extent of this large deletion.

To determine the haplotypes associated with the rearrangements and potentially probe their limits, intragenic microsatellites were analysed in the patients and their families using conventional PCR or semi-quantitative PCR (when segregation study was not informative); IVS1(CA), IVS6a(TTGA), IVS8(CA), IVS8(TG)mTn, IVS17b(TA), and IVS17b(CA) along with single nucleotide polymorphisms (1540A/G and 2694T/G).

Molecular characterisation of the rearrangements

The 11 rearrangements consisted of eight different anomalies, of which six are new (table 3). The two already reported involve exon 12 and exon 2 (Mekus and Tümmler, www.genet.sickkids.on.ca/cfr). We named the new rearrangements according to the nomenclature recommendations (www.hgvs.org), but the A of the ATG translation start codon was numbered +133 to be in accordance with the current CFTR gene numbering (GenBank NM_000492.2) and the CF mutation database, where the rearrangements were reported. The breakpoints of three new deletions involving intron 17b were determined. The 3’ breakpoints were concentrated in a small AT-rich region including the IVS17b(TA) microsatellite. Hence, abnormal segregation of this microsatellite was observed in four families where such a deletion was detected. The 3’ breakpoint of the partial deletion of exon 17b, 3413del355ins6, is located 9 bp after the IVS17b(TA) site, while those of CFTRdele414b–17b and CFTRdele17a–17b are located within it, leaving stretches of 13(TA) and 7(TA) repeats, respectively. The junction sequence of CFTRdele17a–17b was identical in the four patients carrying this anomaly, which was associated with the same extended haplotype (IVS1(CA), IVS6a(TTGA), IVS8(CA), IVS8(TG)mTn, 1540A/G, 2694T/G, IVS17b(CA); data partially shown in table 3). In all the cases characterised at the molecular level (nos. 1, 3–8), the rearrangements were inherited in a stable manner, as the breakpoints were identical in the patients and their parents.

The precise characterisation of the other rearrangements was hampered by various difficulties: (i) the size of the complete CFTR gene deletion, which was not detected by

Table 2: Phenotype and genotype data of patients carrying CFTR rearrangements

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Gender</th>
<th>Current age</th>
<th>Age at diagnosis</th>
<th>Pancr. status</th>
<th>Lung disease</th>
<th>Other</th>
<th>Sweet test</th>
<th>Allele 1</th>
<th>Allele 2</th>
<th>Allele 2 rearrangement involving exon(s)</th>
<th>Parental</th>
<th>Geographic</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>10 years</td>
<td>1 month</td>
<td>PI</td>
<td>Severe</td>
<td>114</td>
<td>F508del</td>
<td>1</td>
<td></td>
<td>-</td>
<td>Father</td>
<td>North eastern Italy</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>16 years</td>
<td>Birth</td>
<td>PI</td>
<td>Severe</td>
<td>130</td>
<td>A561E</td>
<td>2</td>
<td></td>
<td>-</td>
<td>Father</td>
<td>Southern Italy</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>10 years</td>
<td>1 year</td>
<td>Birth</td>
<td>Severe</td>
<td>+</td>
<td>E553X</td>
<td>17b</td>
<td></td>
<td>-</td>
<td>Father</td>
<td>France</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>13 years</td>
<td>4 years</td>
<td>PI</td>
<td>Severe</td>
<td>+</td>
<td>E553X</td>
<td>14b–17b</td>
<td>Mother</td>
<td>-</td>
<td>Father</td>
<td>Eastern France</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>24 years</td>
<td>1 month</td>
<td>PI</td>
<td>Severe</td>
<td>+</td>
<td>F508del</td>
<td>14b–17b</td>
<td>Mother</td>
<td>-</td>
<td>Mother</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>21 years</td>
<td>Childhood</td>
<td>PI</td>
<td>Moderate</td>
<td>+</td>
<td>F508del</td>
<td>17b–17b</td>
<td>Mother</td>
<td>-</td>
<td>Father</td>
<td>Eastern France</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>35 years</td>
<td>1 year</td>
<td>CI</td>
<td>CBVD, NP</td>
<td>+</td>
<td>F508del</td>
<td>17a–17b</td>
<td>Father</td>
<td>-</td>
<td>Father</td>
<td>Eastern France</td>
</tr>
<tr>
<td>8*</td>
<td>F</td>
<td>Deceased at 2 and 6 months</td>
<td>Birth</td>
<td>PI</td>
<td>Severe</td>
<td>ND</td>
<td>F508del</td>
<td>17b–17b</td>
<td>Father</td>
<td>-</td>
<td>Father</td>
<td>Eastern France</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>Deceased at 13 years</td>
<td>5 years</td>
<td>PI</td>
<td>Severe</td>
<td>300</td>
<td>1812–1G–A-1G–A</td>
<td>3–10,14b–16t</td>
<td>Mother</td>
<td>Kabylie (Algeria)/ Brittany (France)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>37 years</td>
<td>3 months</td>
<td>PS</td>
<td>None</td>
<td>90</td>
<td>G542X</td>
<td>2183AA</td>
<td>4–8</td>
<td>-</td>
<td>Mother</td>
<td>France</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>31 years</td>
<td></td>
<td>PI</td>
<td>Severe</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>Mother</td>
<td>Eastern France</td>
</tr>
</tbody>
</table>

CBAVD, congenital bilateral absence of the vas deferens; DB, disseminated bronchiectasis; dl, deletion; dup, duplication; F, female; M, male; NP, nasal polyposis; Pancr., pancreatic; PI, pancreatic insufficiency; PS, pancreatic sufficiency.

*Case B: the deletion was identified in the father of two deceased children.

†The CFTRdele3–10,14b–16 deletion was identified in cis with the IVS54 variant.
conventional cytogenotyping but confirmed by FISH analysis (data not shown); (ii) the lack of DNA or cDNA to study the complex CFTRdel3–10,14b–16 deletion, which removes at least 54 kb, and the CFTRdup4–8 duplication, which is predicted to extend over a minimum of 11 kb.

**DISCUSSION**

**CFTR rearrangements account for 20% of unidentified CF alleles**

In the population studied, CFTR rearrangements were identified in 11 unrelated families. All these rearrangements were characterised in families where a CF mutation had been previously identified. Taking into account the number of alleles bearing one of three known deletions (CFTRdel2–3, CFTRdel17a–18, and CFTRdel19) in our population, large CFTR gene rearrangements represented 20% of CF alleles, where a point mutation or a short deletion/insertion has not been found, and 1.3% of overall CF anomalies. These figures indicate a slightly higher proportion of CFTR rearrangements in our larger and probably more heterogeneous population compared to that studied in related persons from Eastern France and is probably associated with the same haplotype, which suggests a founding effect. It would be interesting to screen for this particular deletion in patients from neighbouring countries. In this deletion and in those involving exon 17b and exons 14b–17b, an AT-rich environment, together with a short repeat at the breakpoints, may have favoured non-homologous recombination by slipped mispairing (table 3), as has been described for other CFTR deletions11 12 15 16 18 19 and deletions of other disease causing genes.20 The sequences bordering the breakpoints were screened for the presence of motifs known to be associated with site-specific recombination, mutation, cleavage, and gene rearrangements20; no such element was observed. Query to RepeatMasker2 (http://www.repeatmasker.org/) revealed no sequence homology that could have favoured homologous unequal recombination. In particular, the 5′ breakpoint of CFTRdel14b–17b is located within an Alu sequence, but no homologous sequence was found around the 3′ breakpoint, not even a partial core sequence.12 A mechanism of non-homologous recombination could also be invoked to explain the occurrence of the CFTRdup4–8, as has been demonstrated for duplications in other disease causing genes21 22 23 24 25 and the complex CFTRdel3–10,14b–16 anomaly, although homologous unequal recombination remains a possible hypothesis given the presence of a number of interspersed repeat elements in the introns involved in the rearrangements. Another complex deletion removing exons 4–7 and 11–18 (CFTR50kbdel) has been reported,26 but its breakpoints remain unknown so far. Interestingly, intron 10 is involved in both complex rearrangements. Unequal homologous recombination is more likely to have occurred for the complete CFTR gene deletion which removes at least 3 Mb.27

**Table 3** Molecular characterisation of the CFTR rearrangements

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Rearrangement</th>
<th>Simplified name</th>
<th>Exon(s) involved</th>
<th>Linked haplotype</th>
<th>Motif sequence at the breakpoints</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>c.136, c.185</td>
<td>136del119ins299</td>
<td>Part of 1 (codons 2-18)</td>
<td>CFTRdel1</td>
<td>23–16–29–13</td>
</tr>
<tr>
<td>2</td>
<td>c.186–192del1</td>
<td>2</td>
<td></td>
<td>CFTRdel2</td>
<td>Inverted CCATG</td>
</tr>
<tr>
<td>3</td>
<td>c.3413, c.3499</td>
<td>268del1355ins6</td>
<td>Part of 17b (codons 1094–1122)</td>
<td>CFTRdel14–17b</td>
<td>23–16–30–14</td>
</tr>
<tr>
<td>5–8</td>
<td>c.3121–3177, c.3499+</td>
<td>17a–17b</td>
<td></td>
<td>CFTRdel17a–17b</td>
<td>23–16–7–13</td>
</tr>
<tr>
<td>10</td>
<td>CFTRdel1–24</td>
<td>1–24</td>
<td></td>
<td>CFTRdel1–24</td>
<td>ND</td>
</tr>
<tr>
<td>11</td>
<td>c.406–7, c.1341–7dup</td>
<td>4–8</td>
<td></td>
<td>CFTRdup4–8</td>
<td>ND</td>
</tr>
</tbody>
</table>

The nomenclature recommendations were followed (www.hgvs.org) but the A of the ATG translation start codon was numbered as 1, according to the current CFTR gene numbering (GenBank NM_000492.2) and the CF mutation database. The new rearrangements are indicated in bold.

*The same indel was described in a CF patient with paternal isodisomy and further found in a French CF patient.*

†The same CFTRdel17a as that described by Mekus and Tümmler (www.genet.sickkids.on.ca/cftr) was identified using specific primers provided by T Dörk.

‡The precise IVS1(CA) allele could not be determined, as the father's DNA was not available.

**The linked haplotype was demonstrated in case nos 5 and 6 and hypothesised in case nos 7 and 8.**

*The precise IVS1(CA) allele could not be determined, as the father's DNA was not available.**

The linked haplotype was hypothesised, considering the most frequent haplotype IVS8(CA) linked to G542X.26

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10.1136/jmg.2006.040841
Considering the CFTR deletions already described and those we report here, it clearly appears that some CFTR sequences may be prone to rearrangements. Strikingly, the small intron 17b (2.8 kb) is involved in the 3’ breakpoint of five deletions (CFTRdele14b–17b, CFTRdele16–17b, CFTRdele17a–17b, 3413del355ins6, and the deletion removing the entire exon 17b described by Magnani et al.14), the IVS17b(TA) site appearing as a critical hotspot involved in the three described here. The CFTR introns 1, 3, and 18 are involved in the breakpoints of four, six, and five other deletions, respectively. However, these introns are large and it is not documented whether identical sequences are involved in the rearrangements.

Implications for genetic counselling

Identification of a CF rearrangement definitely confirmed the CF diagnosis in all cases but that of the CBAVD patient, although, at present, a moderate form of CF cannot be ruled out. Some of the patients were referred to our laboratory several years ago and, even if the diagnosis had been clearly established on the basis of clinical and biological findings,13 the presence of only one CF mutation after exhaustive screening of the CFTR gene coding regions could have thrown doubt upon the diagnosis. Moreover, in the context of a hereditary disease, identification of each parental CF anomaly allows the psychological burden to be shared in the family. Indeed, family studies showed that all the rearrangements were inherited from a parent (father in six cases, mother in the five others including that of the duplication). In other respects, it greatly facilitates genetic counselling and cascade screening in relatives and makes feasible prenatal diagnosis by direct analysis of the causative mutations. Precise identification of the breakpoints enables the design of primers for conventional PCR which may be easier and cheaper to use in these situations (in particular, the PCR primers and conditions for the detection of the common CFTRdele17a–17b are available upon request). The search for CFTR gene rearrangements should also be considered in patients presenting with a CFTR related disease and who carry a mild mutation, since they may have a severe CF mutation in trans.

Determination of CFTR copy number allows discrimination between true homozygotes for identified CF mutations and compound heterozygotes for a CF mutation and a deletion removing at least the relevant exon. In cases of apparent homozygosity for a CFTR mutation, ruling out a sequence variation at the primer binding sites can be performed first by sequencing the corresponding exon using a set of external primers.22 Then, determination of copy number, particularly useful when segregation analysis is difficult or not possible in the family, can be rapidly performed. Such a discrimination is crucial when cascade screening and prenatal diagnosis are requested. Geneticists must also be aware of correlations between genotype and phenotype. In the particular case of patient no. 10, we considered that a R117H–T7 homozygous genotype could not explain the CBAVD phenotype and suspected rather the presence of a severe CF anomaly in trans of R117H. Identification of a gene rearrangement in this patient had notable implications for genetic counselling for himself and his partner, as the couple had been referred for advice about assisted reproduction, and for his family. Indeed, once studied, the patient and his parents forwarded the genetic information to other family members. In other respects, the proven homoygous genotype for mild CFTR mutations found in CBAVD or DB patients of the third group, such as R74W-D127N71 or 1997F,14–16 is not considered as deleterious enough to account for their disease. Other CFTR mutations may have escaped detection or, alternatively, mutations in other disease causing genes may account for the phenotype, possibly acting in concert with CFTR mutations.

Further screening for rare mutations or rearrangements should also be considered when patients carry missense mutations whose deleterious effect is questionable with regard to the biochemical properties of the amino acids and the conservation of the CFTR protein sequence among species, and in comparison to other ABC proteins. We considered a possible complex allele in patient no. 9 on a V754M background, as the corresponding part of the R domain is not well conserved among species, residue V754 being a valine in primates but a methionine in rabbit and mouse species. In addition, residue V754 is not located in the refined functional R domain.17 V754M was described as a CF mutation, as it was found in a patient having classical CF (www.genet.sickkids.on.ca/cftr). The identification of the complex CFTRdele3–10,14b–16 in cis with V754M thus leads to the reconsideration of V754M as probably not disease causing, which will reassure individuals studied for carrier screening who are V754M heterozygotes but do not carry any other mutation/deletion. This observation highlights the need to achieve a complete CFTR gene analysis, including screening for rearrangements, when novel or rare missense mutations are found, since their deleterious effect cannot be easily proven.

A revised strategy for CFTR molecular studies

Among the gene quantification methods used to identify unknown gene deletions and duplications in hereditary

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Figure 1 Electropherograms from semi-quantitative fluorescent multiplex PCR experiments. The x axis displays the computed length of the PCR products in base pairs as determined by using an internal lane standard, which is indicated in black. The y axis shows fluorescent intensities in arbitrary units. Gene fragments are indicated at the top of the corresponding peaks. The electropherograms of the controls are in red and those of the patients are in blue. The profiles were superimposed and normalised using the exon 4 DSCR1 amplicon. The abnormal profiles have been highlighted by arrows and extended (windows). (A) CFTRdele17a–17b visualised from MP 3 in patient no. 7 (two-fold decrease in peak intensities for exons 17a and 17b). (B) CFTRdele1–24, visualised from MP 2 in patient no. 10 (two-fold decrease in peak intensity for all CFTR exons). For CFTR exon 9, the presence of a double peak in the control is attributable to the (TG)41Tn polymorphism. (C) CFTRdup4–8, visualised from MP 1 in patient no. 11’s mother (1.5-fold increase in peak intensity for exons 4–6a). She carries in trans the –912dupT polymorphism in the promoter region (double peak).

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Moreover, the use of genes located outside the CFTR locus serves as a single- or double-copy control depending on the control DNA; Thilo Do¨rk for providing primers for rereading the manuscript. Acknowledged for helpful discussion. David Kerridge is acknowledged for technical advice in implementing the QFM-PCR experiments.

Besides rearrangements detected by gene dosage, the technique described here is sensitive enough to detect length variations as small as 1 bp insertions/deletions, such as F508del, 394delTT, 1078delT, and 2184insA, as well as STS variants such as those at the IVS8(TG)nTn polymorphic site. Micro-deletions/insertions and large rearrangements thus account for 24% of the reported CF mutations (www.genet.sickkids.on.ca/cftr), while their cumulative frequency is above 70% of CF alleles in most Caucasian populations. After screening using a commercial kit for approximately 30 frequent mutations which account for 82% of French CF alleles, the use of our QFM-PCR system could contribute to identifying a further 4.5% of CF alleles, and possibly more in other populations. The simplicity and rapidity of such a procedure can be readily applied to the molecular diagnosis of many other hereditary diseases.

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ELECTRONIC-DATABASE INFORMATION

The URLs mentioned in this study are: www.genet.sickkids.on.ca/cftr, www.hgs.org, and http://www.repeatmasker.org/.

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Rapid detection of **CFTR** gene rearrangements impacts on genetic counselling in cystic fibrosis

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