Is Congenital Bilateral Absence of Vas Deferens a Primary Form of Cystic Fibrosis? Analyses of the CFTR Gene in 67 Patients

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Summary
Congenital bilateral absence of the vas deferens (CBAVD) is an important cause of sterility in men. Although the genetic basis of this condition is still unclear, it has been shown recently that some of these patients carry mutations in their cystic fibrosis transmembrane conductance regulator (CFTR) genes. To extend this observation, we have analyzed the entire coding sequence of the CFTR gene in a cohort of 67 men with CBAVD, who are otherwise healthy. We have identified four novel missense mutations (A800G, G149R, R258G, and E193K). We have shown that 42% of subjects were carriers of one CFTR allele and that 24% were compound heterozygous for CFTR alleles. Thus, we have been unable to identify 76% of these patients as carrying two CFTR mutations. Furthermore, we have described the segregation of CFTR haplotypes in the family of one CBAVD male; in this family, there are two male siblings, with identical CFTR loci but displaying different phenotypes, one of them being fertile and the other sterile. The data presented in this family, indicating a discordance between the CBAVD phenotype and a marked carrier (ΔF508) chromosome, support the involvement of another gene(s), in the etiology of CBAVD.

Introduction
Congenital bilateral absence of the vas deferens (CBAVD) accounts for ~6% of male sterility (Holsclaw et al. 1971). The molecular basis of this condition is unknown, but recently it has been shown that a large proportion of these sterile men carry a mutation in at least one of their cystic fibrosis (CF) genes (Dumur et al. 1990). The CF transmembrane conductance regulator (CFTR) gene, which was cloned in 1989, has been analyzed extensively in the last four years (Kerem et al. 1989; Riordan et al. 1989; Rommens et al. 1989; Tsui 1992). The CF phenotype, which is characterized by lung disease and/or pancreatic exocrine insufficiency, can also include failure of reproductive function both in males and in females. More than 95% of males are sterile because of altered Wolfian ducts with atrophy of the vas deferens, tail and body of the epididymis, and seminal vesicles (Boat et al. 1989). The relationship between male sterility in CBAVD and CF patients was studied recently at the molecular level by Rigot and coworkers. They showed that in a cohort of asymptomatic azoospermic healthy males with CBAVD, there was a high frequency of ΔF508 carriers (Rigot et al. 1991). They also reported the frequent occurrence of a second mutation in their group of patients, the mutation R117H (Gervais et al. 1993). These observations have prompted the hypothesis that CBAVD men could represent an incomplete form of CF (Anguiano et al. 1992). To address the relationship between CBAVD and CF, we have conducted a large collaborative study including >60 CBAVD patients. We have analyzed the entire coding sequence of the CFTR gene for each individual, by a GC clamp denaturing gradient-gel electrophoresis (DGGE) assay and DNA sequencing. Where possible, we have performed segregation studies in families with at least one affected sibling. The results we obtained confirmed the implication of CFTR abnormalities in the pathogenesis of CBAVD, but our results also suggest that the genetic basis of CBAVD could be more heterogeneous than previously suggested and that at least a second gene could be involved in the etiology of the disease.

Patients, Material, and Methods
Patients
Sixty-seven unrelated azoospermic patients with congenital absence of the vas deferens were included in this study. Their diagnoses were initially suggested by the clinical observation of impalpable vasa and were subsequently confirmed by seminal analyses. These patients have no history of respiratory disease, nor have they any signs or
symptoms of CF. We have included only patients with bilateral absence of the vas deferens. Those patients with unilateral absence of the vas deferens were excluded, as were patients with renal malformations and other urogenital abnormalities. For each of these patients, a spermogram was performed, and all were azoospermic. Ninety percent of these patients had been included in a microsurgical epididymis sperm aspiration (MESA) program, and the diagnosis of CBAVD was confirmed at the time of the surgical sperm aspiration (Silber et al. 1990). While none of these patients had signs or symptoms of CF, a systematic measurement of steatorrhea was not performed for these patients. There was no difference in the phenotype presented by our three groups of patients, defined at the genotype level, particularly between the group of patients with two CF mutations and the group in which we were unable to find any mutation. However, we have not performed objective measurements of pancreatic status and pulmonary function. Sweat chloride tests were not routinely performed.

Family R

For individual III1, the diagnosis of CBAVD was made by clinical examination showing impalpable vasa and was confirmed at the time of surgical sperm aspiration, when this patient was enrolled in a MESA program for in vitro fertilization using epididymal sperm. He has undergone three trials in this program. One of the assays was successful until the pregnancy was terminated spontaneously at 6 wk. This patient has no history of upper and/or lower respiratory symptoms from childhood. Individual III1 was azoospermic and the FSH values were normal. The sweat chloride test performed recently showed a value of 48 mmol, which is within the normal range. As individual II4 is fertile and his paternity has been confirmed, no sperm count was carried out.

DNA Analysis

DGGE.—Oligonucleotides for amplification of exons and the amplification reactions for the DGGE analyses have been described elsewhere (Lerman et al. 1987; Myers et al. 1987). Amplification products were run on 6.5% polyacrylamide gels containing a linear denaturing gradient from 20% to 70%, where 100% denaturant equals 7 M urea with 40% formamide (v/v). Electrophoresis was performed at 75 V for 7–9 h, with respect to the melting map of the amplified sequence. PCR fragments displaying an altered behavior in the gel, indicating the presence of a mutation or a polymorphism in this particular part of the gene, were then sequenced. The Cystic Fibrosis Genetic Analysis Consortium rules for coding each nucleotide change, as well as the primers, have been described elsewhere (Tsui 1992; Audrézet et al. 1993).

Microsatellite Markers.—Oligonucleotides and amplification conditions for the analysis of the microsatellite markers have been described elsewhere (Morral et al. 1992). Amplification for the IVS 17b TA and IVS 17b CA repeat markers were carried out simultaneously. Primers were end labeled with T4 polynucleotide kinase in the presence of [γ-32P]ATP. Amplification was carried out for 25 cycles, using an annealing temperature of 50°C. A single PCR was performed for IVS8 CA, with an annealing temperature of 57°C for 35 cycles. Two microliters of PCR product were mixed with 2 μl of formamide, were loaded on a polyacrylamide gel, and electrophoresed for a period of 4.5 h at 2,000 V. Dried gels were then exposed to X-ray film overnight.

Results

Survey

We studied a group of CBAVD patients from Europe and the United States (23 patients from France, 7 from Italy, 14 from Belgium, and 23 from the United States). Altogether, we looked at 67 infertile men, none of whom had any of the classic symptoms of CF. We scanned the entire coding sequence of the CFTR gene of these patients, using DGGE, followed by DNA sequencing. This was made possible by the use of 37 primer pairs, which permitted the analysis of all 27 exons and their intron/exon boundaries (Audrézet et al. 1993).

We identified 28 ΔF508, 5 W1282X, 1 G542X, 1 R553X, and 2 N1303K mutations. These changes that were identified in CF patients are believed to be disease causing, this being determined either directly, by a study of their effect on the activity of the protein, or indirectly, by their exclusion from non-CF chromosomes. In addition, we identified the following missense mutations: four R668C, one A800G, one (G628R + S1235R, borne on the same chromosome), one (R74W + D1270N, borne on the same chromosome), six R117H, one F1052V, one R117C, one S1235R, one G149R, one R258G, two R347H, one R1066H, one R75L, and one E193K. Together with the four new alleles that we identified, these missense mutations correspond to a total of 23 different alleles (see table 1).

Moreover, we systematically looked for the transmission of CFTR mutations in pedigrees of CBAVD patients in which there were at least two affected males or an affected male with one or several nonsterile brothers. In order to define the phase of these chromosomes precisely, we employed polymorphic dinucleotide repeat markers, situated in introns 8 (IVS 8 CA) and 17b (IVS 17b TA and IVS 17b CA) (Morral and Estivill 1992; Morral et al. 1992). These dinucleotide markers allowed us to construct haplotypes for the individual chromosomes bearing each mutation. Among the families studied, we collected and analyzed data from three large families. In one family, there were two CBAVD brothers who shared the same genotype. In the second family, the microsatellites were noninformative. The third family is described below.
Table 1

<table>
<thead>
<tr>
<th>No. of Patients</th>
<th>Genotype*</th>
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<tr>
<td>1</td>
<td>ΔF508 + (G628R + S1235R)</td>
</tr>
<tr>
<td>1</td>
<td>ΔF508 + (R74W + D1270N)</td>
</tr>
<tr>
<td>2</td>
<td>ΔF508 + R668C</td>
</tr>
<tr>
<td>4</td>
<td>ΔF508 + R117H</td>
</tr>
<tr>
<td>1</td>
<td>ΔF508 + R258G</td>
</tr>
<tr>
<td>1</td>
<td>ΔF508 + R751</td>
</tr>
<tr>
<td>1</td>
<td>E193K + N1303K</td>
</tr>
<tr>
<td>1</td>
<td>R347H + R1066H</td>
</tr>
<tr>
<td>1</td>
<td>R117C + W1282X</td>
</tr>
<tr>
<td>1</td>
<td>R553X + R668C</td>
</tr>
<tr>
<td>1</td>
<td>G149R + R668C</td>
</tr>
<tr>
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<tr>
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<td>N1303K/ unidentified</td>
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<td>1</td>
<td>S1235R/ unidentified</td>
</tr>
<tr>
<td>1</td>
<td>R347H/ unidentified</td>
</tr>
<tr>
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<td>A800G/ unidentified</td>
</tr>
<tr>
<td>1</td>
<td>F1052V/ unidentified</td>
</tr>
<tr>
<td>23</td>
<td>unidentified/ unidentified</td>
</tr>
</tbody>
</table>

*In parentheses are the two mutations located on the same haplotype.

Identification of Novel Mutations

Among the missense mutations, four have not been observed previously: (i) DGGE analysis of exon 13 showed one DNA sample displaying a modified pattern of migration, which was investigated by direct sequencing of amplified DNA. A nucleotide change, G→A at position 2531, was identified. The result of this change is the substitution of an alanine by a glycine at codon 800. This converts a nonpolar-lateral chain (A) to a polar-lateral chain (G). This amino acid modification occurs in the R domain, which regulates the opening of the chloride channel. This patient carries no mutation on the other allele, according to our analysis (fig. 1). (ii) The second change, situated in exon 4, is G→A at position 577 and corresponds to the substitution of a glycine for an arginine (G149R). It has been reported elsewhere that such an amino acid change could be considered as mild, as for G628R in exon 13 (Fanen et al. 1992) and G1061R in exon 17b (Mercier et al. 1993b) (fig. 1). (iii) The third novel mutation we found is R258G, and the nucleotide change is G→A at position 904 in exon 6b. It corresponds to the substitution of an arginine for a glycine (R258G) This mutation is situated in a region that encodes part of the transmembrane region of the protein, and the mutation results in a change of polarity at codon 258. (iv) The fourth novel mutation we have observed occurs at position 709 and corresponds to a G→A change that results in a glutamic acid codon instead of a lysine codon (E193K). This change modifies the polarity at this position (fig. 1).

The three latter mutations correspond to changes in amino acids that have been conserved throughout evolution, as observed in dogfish, Xenopus, bovine, mouse, and human species (Yorifuji et al. 1991; Tucker et al. 1992). This conservation suggests the functional importance of these amino acids.

For all four of these new mutations, a segregation analysis was performed in each family, allowing us to show that G149R, R258G, and E193K were carried by a particular allele and that these mutations were not de novo mutations. Moreover, we screened 250 CF patients and never observed such mutations. We also screened 300 normal chromosomes, so the possibility that these variations are polymorphisms is low.

Of the 67 patients analyzed, 16 were compound heterozygotes for two mutations in the coding portion of CFTR, including those changes we identified in this study (24%). Twenty-eight subjects (42%) were carriers of one CFTR allele. For 23 individuals (34%), we were unable to find any mutations in the coding region or splice junctions of CFTR, even with our comprehensive screening procedure (table 1).

Two patients were carriers of two missense mutations on the same allele (G268R + S1235 R and R74W + D1270N) (table 1). The association of these two missense mutations on the same haplotype is interesting, as both S1235R and R74W have been independently reported elsewhere to be CFTR alleles (Claustres et al. 1993; Cuppens et al. 1993). We also had previously reported that haplotype R74W + D1270N was present in a healthy

![Figure 1](https://example.com/fig1.png)

**Figure 1** CFTR locus for brothers II-1 and II-4. Microsatellite markers (IVS 8 CA, IVS 17b TA, and IVS 17b CA) permitted the construction of four different phases (16/28/13, 16/31/13, 16/43/13, and 23/30/13), and their segregations show that the two brothers, II-1 and II-4, are identical at the CFTR locus. The number “16” corresponds to the number of CA repeats at the IVS 8 CA locus, “28” to the number of repeats at the 17b TA locus, and “30” to the IVS 17b CA, which in this case is polymorphic. The F508 is situated in exon 10, between IVS 8 CA and IVS 17b TA, as indicated. Both brothers II-1 and II-4 are identical at the CFTR locus. Individual II-1 is a CBAVD patient, and his brother (II-4) is fertile (his paternity was confirmed). The half-shading designates an individual heterozygote for F508.
mother of an affected child (Verlingue et al. 1993), her genotype being (R74W + D1270N) + 2183 AA→G. If we consider that CBAVD may be a mild form of CF, these observations suggest that a second mutation in a CFTR allele may result in a reversion or partial reversion of phenotype. Consistent with this possibility are two independent observations obtained first by mutation screening and second from studies in a yeast model system. Dörk and coworkers (1991) identified an individual bearing both the ΔF508 and R553Q mutations on the same allele. This CF patient presented with a relatively mild phenotype. In the experimental model system involving a yeast STE6 and human CFTR chimeric protein, a reversion of mating deficiency was obtained by introduction of a second alteration on the mutated allele (Teem et al. 1993). In both the mildly affected patient and the model system, the changes are predicted to be relatively close within a single working domain, the first nucleotide binding fold, of CFTR. It is reasonable to suspect that the first change disrupts structure, while the second change, at least partially, relieves this disruption. It is not clear how the double changes we identified may lead to a reversion, as the changes correspond to alterations that are predicted to be in the amino-terminal region of the second nucleotide binding fold. However, these alterations would both be predicted to be on the cytoplasmic surface of CFTR.

**Discordant Segregation of a Common CF Mutation and CBAVD**

In family R (fig. 2), subject II1 was a 45-year-old CBAVD male. Our analysis (table 1) showed him to be a carrier of the mutation ΔF508 that was inherited from his mother, I-2. His full brother, II-4, also had inherited the ΔF508 allele. The three polymorphic markers used to build the phase show complete informativity, the four haplotypes being clearly identified. ΔF508 was carried on the haplotype, 23/30/13, the most common haplotype associated with ΔF508 (Morral et al. 1994). We have established, without doubt, that the two brothers, II-1 and II-4, are identical at the CFTR genotype level (23/30/13 and 16/28/13). Subject II1 is fertile, having fathered two children. We performed a paternity test using polymorphic markers situated on different chromosomes (HLA DR DQ, polymorphism of intron 40 of factor von Willebrand [Gaucher et al. 1992], and microsatellite IVS 17b TA of the CFTR gene). Paternity could not be excluded for these two children, with a probability of paternity of >99.9%. A paternity test was also confirmed for I3 and his two children, II1 and II4. These results show clearly that these two brothers are carrying two identical CFTR haplotypes and suggest that another locus could account for the phenotype observed for subject II1.

**Discussion**

It has been postulated since 1971 (Holsclaw et al. 1971) that CBAVD could represent an incomplete form of CF, as the majority of men suffering from CF are sterile because they lack the vas deferens. As soon as the CFTR gene was cloned, and in the following years, when the majority of CF mutations were identified, this hypothesis was reviewed by Rigot et al. (1991). They showed that 8/19 azoospermic men were carriers of ΔF508. This observed frequency was significantly higher than the expected frequency of 1/25. This high frequency has since been observed in additional studies (Patrizio et al. 1993). Further, a second mutation, R117H, known to lead to a mild phenotype in CF patients, was also found to occur at high frequency in these men (Gervais et al. 1993). More recently, the characterization of a large number of mutations in the CFTR gene has permitted Anguiano et al. to confirm these initial results, by describing 3/25 CBAVD patients to be compound heterozygotes for CFTR mutations and so to propose that CBAVD could be a primary form of CF (Anguiano et al. 1992). The results of CFTR mutation screenings in earlier studies have shown very similar results: Osborne et al. (1993) found that 2/26 men with CBAVD were compound heterozygotes (8 were carriers of one mutation, and no mutation was identified in the 16 other patients). Patrizio et al. (1993) reported 39% (26/44) of CBAVD subjects as carrying one CFTR allele, and Culard et al. (1994) found 50% among 12 CBAVD patients.

Different strategies have been used to scan the CFTR gene, but Osborne et al., Culard et al., and we have explored the coding sequence of the CFTR gene extensively, using either DGGE, SSCP, or direct DNA sequencing, and have obtained quite similar results. To date, evaluation of techniques allowing the identification of mutations in a cloned gene is still a matter of discussion. The DGGE tech-
nique has probably the highest sensitivity of detection (~99%) compared with the other available techniques (SSCP, chemical cleavage, and RNase protection assay) (Fodde and Losekoot 1994).

In this large study, in which we analyzed 67 CBAVD patients from different countries, only 24% could be identified as compound heterozygotes. One can assume that our analysis of the coding sequence of the CFTR gene was very thorough, as we have been able to identify ~98% of CFTR mutations in our cohort of CF patients (Férec et al. 1992) and ~95% of CFTR mutations in CF patients of various origins (Mercier et al. 1993a). Although we did not analyze the promoter and intronic regions of the CFTR, these results support the idea that CFTR plays a role in the genesis of CBAVD, but they are inconclusive, as 34% of these patients displayed no mutation at all in the coding sequence of the gene, and 42% were carriers of only one allele. This prompted us to look at segregation of CFTR alleles in families with at least one CBAVD male and a large number of siblings. One of our families, family R, (fig. 2) included two brothers who carry the common ΔF508 mutation (one of whom was obviously fertile, because he fathered two children). His full brother had CBAVD but had inherited identical chromosomes, as determined by microsatellite markers and haplotype analysis. The paternity of individual II1 to these two children has been confirmed using polymorphic markers. This transmission shows clearly, for the first time, to our knowledge, that at least one other gene is implicated in the pathophysiology of CBAVD.

The precise involvement of CFTR in the development of organs is poorly understood. In male sex organs, the CF pathway is observed as atrophy or absence of the body of the epididymis or of the vas deferens. The origin of this pathology may result either from defective development or from luminal obstruction of the ducts, as has been observed in the CF epididymis. It has been observed that 1%–2% of CF males have been able to father children (Feigelson et al. 1969).

In summary, after having studied this cohort of CBAVD patients, we have been able to show that ~42% of these subjects are carriers of one CFTR mutation and that 24% are compound heterozygotes for one severe and one mild mutation. These genotypes could be considered extremely mild forms of CF, the more frequent genotype for this condition being ΔF508/R117H. It has been shown by Kieseutter et al. (1993) that R117H occurs on two chromosomal backgrounds, one carrying a 5T variant with respect to a poly pyrimidine tract in intron 8, which alters exon 9 splicing, and the other carrying a 7T variant associated with normal exon 9 splicing. The R117H mutations in this study are associated only with the 7T variant, indicating that the chromosomal background associated with CBAVD may be specific. These data show that the CBAVD phenotype is determined by the association of the R117H with a particular chromosomal background.

Our results lead us to propose that CBAVD is a heterogeneous and complex genetic disorder. Between 20% and 25% of cases may actually represent very mild forms of CF, while 34% appear not to be associated with the CF gene. The incidence of CF mutations on just one of the alleles of the remaining proportion of CBAVD patients (43%) may implicate a role for CFTR, as suggested by the family we present. Carrier status is insufficient to lead to CBAVD, and additional factors or genes are necessary for CBAVD pathophysiology in these cases.

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References


