Am. J. Hum. Genet. 60:87–94, 1997

The Molecular Basis of Partial Penetrance of Splicing Mutations in Cystic Fibrosis

Naama Rave-Harel,[1] Eitan Kerem,[3] Malka Nissim-Rafinia,[1] Igal Madjar,[5] Ran Goshen,[2] Arie Augarten,[6] Ayelet Rahat,[1] Arye Hurwitz,[4] Ariel Darvasi,[1] and Batsheva Kerem[1]

Departments of [1]Genetics and [2]Biochemistry, The Life Sciences Institute, The Hebrew University, [3]Department of Pediatrics and Cystic Fibrosis Clinics, Shaare Zedek Medical Center, and [4]Department of Obstetrics and Gynaecology, Hadassah University Hospital, Jerusalem; and Departments of [5]Urology and [6]Pediatrics, Sheeba Medical Center, Ramat-Gan, Israel

Summary

The splicing variant, 5T allele, in intron 8 of the cystic fibrosis transmembrane conductance regulator (CFTR) gene was shown to be associated with partial penetrance of the clinical expression. This splicing variant leads to two possible transcripts: one normal and the other aberrantly spliced that lacks exon 9. The aim of this study was to analyze the molecular basis of the partial penetrance in individuals carrying the 5T allele. We analyzed the level of the correctly spliced RNA transcribed from the 5T allele in nasal and epididymal epithelium and correlated it with disease expression. Semiquantitative nondifferential reverse-transcriptase–PCR showed a considerable variability (6%–37%) in the total level of correctly spliced RNA transcribed from the 5T allele in nasal epithelium from 11 patients. A significant nonlinear correlation ($r = .82$, $P = .002$) between the level of the normal CFTR transcripts and the severity of lung disease was shown. No individuals with normal lung function and minimal or no lung disease (FEV_1 >80% predicted) had <25% of normal transcripts, and individuals with <15% of normal transcripts did not have FEV_1 >80%. The level of normal transcripts in epididymal epithelial cells from four infertile males with congenital bilateral absence of the vas deferens was low (6%–24%). In infertile males with normal lung function the level of correctly spliced transcripts in the nasal epithelium was higher than the level in the epididymal epithelium. These results indicate that there is variability in the efficiency of the splicing mechanism, among different individuals and between different organs of the same individual. This variability provides the molecular basis of the partial penetrance of cystic fibrosis disease in patients carrying the 5T allele.

Received June 19, 1996; accepted for publication September 20, 1996.

Address for correspondence and reprints: Dr. Batsheva Kerem, Department of Genetics, The Life Sciences Institute, The Hebrew University of Jerusalem, Jerusalem 91904, Israel. E-mail: kerem@leonardo.ls.huji.ac.il

0002-9297/97/6001-0014$02.00

Introduction

In many human monogenic diseases high variability in disease expression is found among patients carrying the same genetic defect. The molecular basis for this variability has been suggested to be allelic heterogeneity, additional genetic loci, and/or environmental factors. Cystic fibrosis (CF) is a common severe autosomal recessive disease caused by mutations in the CF transmembrane conductance regulator (CFTR) gene (Kerem et al. 1989*b*; Riordan et al. 1989; Rommens et al. 1989). The disease is characterized by wide variability in the clinical presentation and organ involvement (Welsh et al. 1995). Genotype-phenotype and haplotype analyses have shown that there are mutations associated with pancreatic insufficiency and a severe form of the disease, as well as mutations associated with pancreatic sufficiency and a milder form of the disease (Kerem et al. 1989*a*; see review in Kerem and Kerem 1996). However, patients and even siblings carrying the same CFTR mutations have shown variability in severity of the disease (Welsh et al. 1995). Furthermore, although almost all men with CF are infertile as a result of congenital bilateral absence of the vas deferens (CBAVD), CBAVD can be found among otherwise healthy males (Schellen and van Stratten 1980). The frequency of CFTR mutations is increased among men with CBAVD (Anguiano et al. 1992; Osborne et al. 1993; Patrizio et al. 1993; Culard et al. 1994; Oates and Amos 1994; Casals et al. 1995; Chillon et al. 1995*a*; Costes et al. 1995; Jarvi et al. 1995; Mercier et al. 1995; Rave-Harel et al. 1995), indicating that in many cases CBAVD is a CFTR-associated disease with incomplete CF expression. Significantly higher frequencies of CFTR mutations also were found among men with congenital unilateral absence of the vas deferens (CUAVD) and among men with absence of the epididymis (Chillon et al. 1995*a*; Jarvi et al. 1995). Thus, many cases of obstructive azoospermia are caused by defective CFTR alleles and might be considered cases of CFTR-associated disease with atypical expression. The most common CFTR defect among men with CBAVD is the 5T variant at the branch/acceptor site of intron 8 (Chillon et al. 1995*a*; Jarvi et al. 1995). This site has

three alleles, with 5, 7, or 9 thymidines (the 5T, 7T, and 9T alleles, respectively). Normal individuals, CF patients, and patients with other respiratory abnormalities have different levels of aberrantly spliced mRNA lacking exon 9 in respiratory epithelial cells (Chu et al. 1993). The level is inversely correlated with the length of the poly(T) tract. The frequency of the 5T allele is increased among patients with atypical CF expression (Pignatti et al. 1996; Kerem et al., in press) and also has been found among healthy fathers of CF patients (Chillon et al. 1995; Jarvi et al. 1995). Thus, the 5T allele is a mutation with partial penetrance, associated with wide variability in disease expression.

The aim of this study was to analyze the molecular basis for the partial penetrance of the CF disease among individuals carrying the 5T allele. We analyzed the level of the correctly spliced RNA transcribed from the 5T allele in respiratory and epididymal epithelium and correlated it with disease expression.

Subjects and Methods

Patients and Controls

Eleven individuals (9 males and 2 females) each carrying at least one allele with the 5T variant were studied. Sequence and haplotype analyses revealed no additional CFTR mutations on the 5T alleles in these individuals. Two of the individuals were homozygous for the 5T allele, and 9 were heterozygotes, in 8 of whom an additional CFTR mutation on the other chromosome was identified: 3 carried the G85E mutation (Zielenski et al. 1991*a*), 3 carried the ΔF508 mutation (Kerem et al. 1989*a*), 1 carried the N1303K mutation (Osborne et al. 1991), and 1 carried the W1282X mutation (Vidaud et al. 1990) (table 1). Three individuals (1474, 658, and 607) either homozygous for the 5T allele or compound heterozygous for the 5T allele and the G85E mutation were from the same family carrying the same 5T allele, as evidenced by their pedigree and by an extended haplotype analysis of the CFTR locus (Kerem et al., in press). Mutations were identified as described elsewhere (Kerem et al., in press).

Eight male individuals were infertile, with CBAVD diagnosed by semen analysis (low volume, low pH, and azospermia) and transrectal ultrasound. One male (G85E/5T) suffered from typical CF respiratory disease characterized by diffuse bronchiectasis, nasal polyposis, and chronic sinusitis. The two healthy females were identified during a screen for CF carriers in their village. Pulmonary function was assessed in all individuals, by forced expiratory volume in 1 s (FEV_1), expressed as a percentage of predicted values for height and sex, by use of standard pulmonary equations (Polgar and Promadhart 1971). Nasal polyps from three non-CF individuals were used as controls.

RNA Extraction and Single-Strand cDNA Synthesis

Nasal epithelial cells were scraped from 11 individuals. The scraped cells were suspended in 300 μl RNAzol B buffer (BIOTECX Laboratories). Total RNA was extracted from the scraped nasal cells by use of the acid-phenol-chloroform method, according to the manufacturer's instructions. RNA was solubilized in 10 μl diethylpyrocarbonate (DEPC)-treated RNAse-free double-distilled water. RNA was extracted from the polyp biopsies of the control individuals by the guanidinium thiocyanate method. The RNA was purified by centrifugation through a CsCl cushion (Chirgwin et al. 1979).

Biopsies from epithelial epididymis were obtained

Table 1

Levels of Normally Spliced RNA Transcribed from the 5T Allele in Nasal and Epididymal Epithelium, and Clinical and Genetic Features

					Nasal Epithelium (% Normal RNA)		Epididymal Epithelium (% Normal RNA)	
Patient	Sex/Age	Diagnosis	Genotype	FEV_1	From 5T[a]	From Total	From 5T[a]	From Total
607[b]	M/41	CBAVD	5T/5T	88	37	37	24	24
1549	F/19	Healthy	5T/5T	83	31	31	...	...
658[b]	M/36	CBAVD	5T/G85E	78	28	14	...	...
1703	F/33	Healthy	5T/G85E	92	62	31	...	...
1474[b]	M/17	CF	5T/G85E	40	16	8	...	...
660	M/29	CBAVD	5T/ΔF508	91	72	36	20	10
666	M/31	CBAVD	5T/ΔF508	83	52	26	32	16
662	M/33	CBAVD	5T/ΔF508	71	24	12	...	...
628	M/35	CBAVD	5T/N1303K	57	12	6	12	6
642	M/39	CBAVD	5T/W1282X	72	12	6	...	...
610	M/36	CBAVD	5T/(unknown mutation)	86	50	25	...	...

[a] Values are means of repeated experiments (n = 2–6); variability between experiments was <10% of the mean.
[b] Individuals from the same family.

from four infertile males with CBAVD while they underwent sperm aspiration for in vitro fertilization. The cells were suspended in 2 ml RNAzol for 100 mg tissue. RNA was extracted by the same method used for the nasal epithelial cells (see above).

cDNA was synthesized by use of 2.5 μM random-hexamer mix (Pharmacia Fine Chemicals), 5 mM $MgCl_2$, 1 mM dNTP mix (Pharmacia), 2.5 units Moloney murine leukemia virus reverse transcriptase (RT) (BRL), and 4 units RNase inhibitor (Boehringer). The tubes were incubated at room temperature for 10 min, at 42°C for 20 min, at 99°C for 5 min, and at 4°C for 5 min. Each cDNA-synthesis experiment included a control sample in which all reagents except RNA were present.

Nondifferential PCR of cDNA Products

Nondifferential RT-PCR reactions in which the normally spliced transcripts, containing exon 9, and the aberrantly spliced transcripts, lacking exon 9, produce products of the same size were designed:

RT-PCR 1.—Amplification of the region between exon 11 and the junction of either exons 10/9 (for amplification of correctly spliced transcripts) or exons 10/8 (for amplification of aberrantly spliced transcripts) was performed by use of the oligonucleotide primers 11Ri3 5′ TTCTTGCTCGTTGACCTCCA 3′, 8/10i5 5′ CTTCTGGGAGGAGACTTCAC 3′, and 9/10i5 5′ TGGAGCAGGCAAGACTTCAC 3′, respectively (fig. 1*A*).

RT-PCR 2.—Amplification of the region between exon 3 and the junction of either exons 8/9 or 8/10 was performed by use of the oligonucleotide primers 3Ri5 5′ GGATAGAGAGCTGGCTTCAAAGAAA 3′, 8/9Ri3 5′ AAATAATTCCCCAAATCCCTCCTCC 3′, and 8/10Ri3 5′ CATCATTAGAAGTGAAGTCTCCTCC 3′, respectively (fig. 1*B*).

RT-PCR 3.—The stability of transcripts lacking exon 9 relative to correctly spliced transcripts was studied in an individual heterozygous for the 5T allele and the ΔF508 mutation and homozygous for the M470 allele. RT-PCR was performed between exons 10 and 11 by use of the oligonucleotide primers 11Ri3 (see RT-PCR 1) and M470 5′ CTTCTAATGATGATTATGGG 3′.

RT-PCR 4.—A differential RT-PCR system in which the correctly spliced transcripts and transcripts lacking exon 9 produce products of different size was performed by use of primers 7Bi5s 5′ ACTTCAATAGCTCAGCCTTC and 11Ri3 (same as in RT-PCR 1) in exons 7 and 11, respectively.

The cDNA samples were heated at 94°C for 3 min and then were subjected to 35 cycles of denaturation at 94°C for 60 s and primer annealing for 30 s, at 55°C for RT-PCRs 1 and 4, at 60°c for RT-PCR 2, and at 51°C for RT-PCR 3. In RT-PCRs 1, 3, and 4 the extension was performed at 65°C for 60 s, in RT-PCR 2 at 60°C for 120 s, followed by a final extension of 7 min at 65°C. RNA-less samples were used as controls. Semiquantitative PCR conditions, which reflect the initial relative amounts of the normal and aberrantly spliced transcripts, were established by serial dilutions (1:3–1:5) of the cDNA products prior to the nondifferential PCR.

Hybridization to RT-PCR Products

Fifty microliters of each differential RT-PCR reaction were subjected to electrophoresis and subsequently were blotted. RT-PCR 1 products were hybridized to the oligonucleotide M470 5′ CTTCTAATGATGATTATGGG 3′, which identified the M allele, and were washed at 52.5°C. The membranes were deprobed and rehybridized to the oligonucleotide 470V 5′ CTTCTAATGGTGATTATGGG 3′, which identified the V allele, and were washed at 53.5°C. Hybridization to oligonucleotides specific for the ΔF508 and to the normal sequence was performed as described elsewhere (Kerem et al. 1989*a*). RT-PCR 2 products hybridized to the exon 3 oligonucleotide G85E-N 5′ GTTCTATGGAATCTTT 3′, which identified the normal sequence, washed at 42°C, and to G85E-M 5′ GTTCTATGAAATCTTT 3′, which identified the G85E mutation, washed at 40°C. The RT-PCR 3 products were hybridized in the same way as was RT-PCR 1. The intensity of the RT-PCR products was measured by PhosphorImager.

Detection of Polypyrimidine-Tract Length Variants at the Acceptor/Branch Site of Exon 9

The genomic region flanking the polythymidine tract was amplified by PCR using the primers 9i-5 and 9i-3s (Zielenski et al. 1991*b*). Nested PCR subsequently was performed with primers TT-i5 (5′ GTGTGTGTGTGTGTTTTT 3′) and TT-i3 (5′ CTGTCCTCTTTTCTATCTTG 3′). The PCR conditions were 94°C for 6 min, followed by 35 cycles of 94°C for 30 s, 54°C for 30 s, 74°C for 40 s, and 74°C for 6 min. The PCR products were visualized on 12% nondenaturing polyacrylamide gels (sequencing format), which were electrophoresed at room temperature at 600 V for 24 h and subsequently were silver stained. Assignment of the splice-variant alleles was performed by analysis of available family members. In cases in which family members were not available the assignment was performed by the complete correlation between the 9T allele and the ΔF508 and N1303K mutations and between the 7T allele and the W1282X and G85E mutations.

Analysis of the M470V Polymorphic Site

DNA analysis of the polymorphic site M470V was performed by *Hph*I digestion as described elsewhere (Chehab et al. 1991).

Statistical Analysis

The correlation between the levels of aberrantly spliced CFTR transcripts and the pulmonary function,

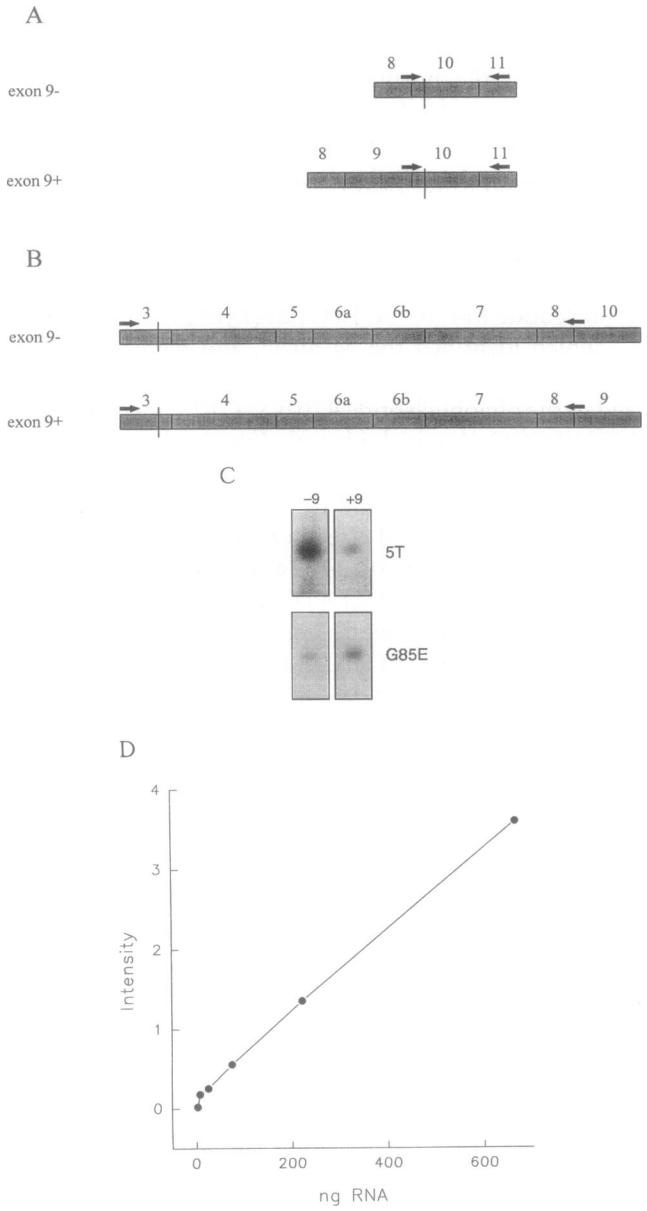

Figure 1 *A*, Schematic presentation of nondifferential RT-PCR 1. Correctly and aberrantly spliced transcripts were amplified by use of a common primer in exon 11 and primers either in the junction between exons 9/10 (for the amplification of correctly spliced transcripts) or in the junction between exons 8/10 (for the amplification of aberrantly spliced transcripts). The vertical line represents the M470V polymorphic site. The RT-PCR products were of 288 bp. *B*, Schematic presentation of RT-PCR 2. An alternative RT-PCR system was used to confirm that the partial difference between the primers used in RT-PCR 1 did not introduce a bias. Correctly and aberrantly spliced transcripts were amplified by use of a common primer in exon 3 and primers either in the junction between exons 8/9 (for the amplification of the correctly spliced transcripts) or in the junction between exons 8/10 (for the amplification of aberrantly spliced transcripts). The reaction was performed on a patient heterozygous for the 5T allele and the G85E mutation. The vertical line represents the G85E site. The RT-PCR products were 1,057 bp. *C*, Analysis of RNA transcribed from the 5T allele in heterozygotes. Heterozygotes for the 5T allele also were heterozygous for either a silent polymorphism in exon 10 (M470V) or the ΔF508 mutation. Hybridization of RT-PCR-1 products to oligonucleotides specific for the different polymorphic alleles revealed four transcripts: the correctly and aberrantly spliced transcripts from each of the two alleles. The example is from patient 1474, heterozygous for the 5T allele, the G85E mutation, and the M470V polymorphism. *D*, Semiquantitative nondifferential RT-PCR conditions. RT-PCR of a series of RNA 1:3 dilutions, from a control sample, was performed in each RT-PCR experiment. Only results from reactions in the linear phase were included. In the presented example the linear range was 70–670 ng RNA.

presented as FEV_1, was analyzed by standard linear regression (*SAS User's Guide to Statistics* 1985). The factor of difference between RT-PCR 1 and RT-PCR 4 was calculated as follows: $K = P_2(1 - P_1)/P_1(1 - P_2)$, where P_1 is the level of the aberrantly spliced transcript transcribed from a specific allele in the nondifferential RT-PCR and P_2 is the level of the aberrantly spliced transcript (the shorter transcript) in the differential RT-PCR.

Results

Levels of Correctly Spliced CFTR RNA Transcribed from the 5T Allele in Respiratory Epithelium

The level of correctly spliced RNA transcribed from the 5T allele was analyzed by use of semiquantitative nondifferential RT-PCR (RT-PCR 1; fig. 1*A*, *C*, and *D*). An alternative set of primers (RT-PCR 2; fig. 1*B*) was used to confirm that the partial differences between the primers used in RT-PCR 1 did not introduce a bias. The level of the correctly spliced RNA transcribed from the 5T allele in individual 1474 was 16% (range 14%–18% in three independent experiments) in RT-PCR-1 and 14% (in two independent experiments) in RT-PCR 2. These similar levels indicate that RT-PCR 1 is a suitable semiquantitative system for the analysis of the level of correctly spliced RNA transcribed from the 5T allele. RT-PCR 1 was performed on respiratory epithelium from 11 individuals who carried at least one 5T allele. There was considerable variability among different individuals, in the levels of correctly spliced RNA transcribed from the 5T allele (12%–72%) (table 1 and fig. 2*A*). High variability also was found among individuals from the same extended family carrying the same 5T allele (table 1).

In order to calculate the total levels of the normal transcripts in individuals heterozygous for the 5T allele, the stability of transcripts lacking exon 9 was compared with that of correctly spliced transcripts, by use of a third set of primers directed at exon 10 (RT-PCR 3). We studied a compound heterozygote ΔF508/5T (662) in whom there were very different levels of transcript lacking exon 9 from the two alleles (76% from the 5T

allele and 36% from the ΔF508 allele). The ratio between the total RNA, as calculated from RT-PCR 3 transcribed from the 5T allele and from that transcribed from the ΔF508 allele was similar to the ratio between exon 10 DNA-PCR products of the same individual (23.7 vs 24.1, respectively). This result indicates that the correctly and the aberrantly spliced transcripts are of similar stability. Since the 5T heterozygotes each carried a nonfunctional CFTR allele on the other chromosome, normal transcripts could be transcribed only from the 5T allele. In these patients, the amount of normal transcript, expressed as a percentage of total transcript, was assumed to be half the amount of normal transcript as measured from the 5T allele (table 1). As can be seen in table 1, the level of normal transcripts from respiratory epithelial cells was highly variable among individuals (range 6%–37% of the total CFTR transcripts).

Correlation between Aberrantly Spliced CFTR Transcripts and Pulmonary Function

A significant nonlinear correlation was found between the level of the normal CFTR transcripts and the severity of respiratory disease, expressed in terms of FEV_1 (r = .82, P = .002; fig. 3*A*). As can be seen in figure 3*A*, the patients fell into two subgroups: one with FEV_1 >80% predicted and the other with FEV_1 <80% predicted. Individuals with normal pulmonary function (FEV_1 >80% predicted) had significantly higher levels of normal RNA than did those with FEV_1 <80% (P < .000001). No patients with normal lung function (83%–92% predicted) and minimal or no lung disease had <25% normal CFTR transcripts, and patients with <15% of normal transcripts did not have FEV_1 >80% predicted. Within each of these subgroups, the sample size was too small to establish or reject a correlation between normal RNA and FEV_1.

Our nondifferential RT-PCR results differed from those obtained by Chu et al. (1993), who found only 10% normal transcripts in three healthy individuals homozygous for the 5T allele. That system employed a differential RT-PCR in which the PCR products lacking exon 9 were 183 bp smaller than the correctly spliced transcripts (509 bp). Since smaller PCR products might amplify more efficiently than larger products, such differential RT-PCR could lead to overestimation of the aberrantly spliced transcripts. To study this possibility we designed a differential RT-PCR system (RT-PCR 4) and analyzed CFTR transcripts from the same individual, using both the differential and the nondifferential RT-PCR systems. The individual studied was homozygous for an allele of 7T at the branch/acceptor site of intron 8 and heterozygous at the M470V locus. The nondifferential RT-PCR 1 results showed that 19% of the RNA transcribed from the M allele and 18% from the V allele were aberrantly spliced. The differential RT-PCR 4, designed to yield shorter products from the aberrantly spliced transcripts, indeed showed a higher level of aberrantly spliced RNA—37% from the M allele and 37% from the V allele. The factor of difference between

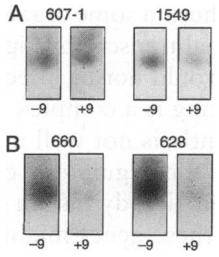

Figure 2 Correctly spliced (+9) and aberrantly spliced (−9) RNA transcribed from the 5T allele. RT-PCR 1 products were hybridized with primers specific for either the V or the M allele in amino acid 470. Examples from nasal (*A*) and epididymal (*B*) epithelium are shown.

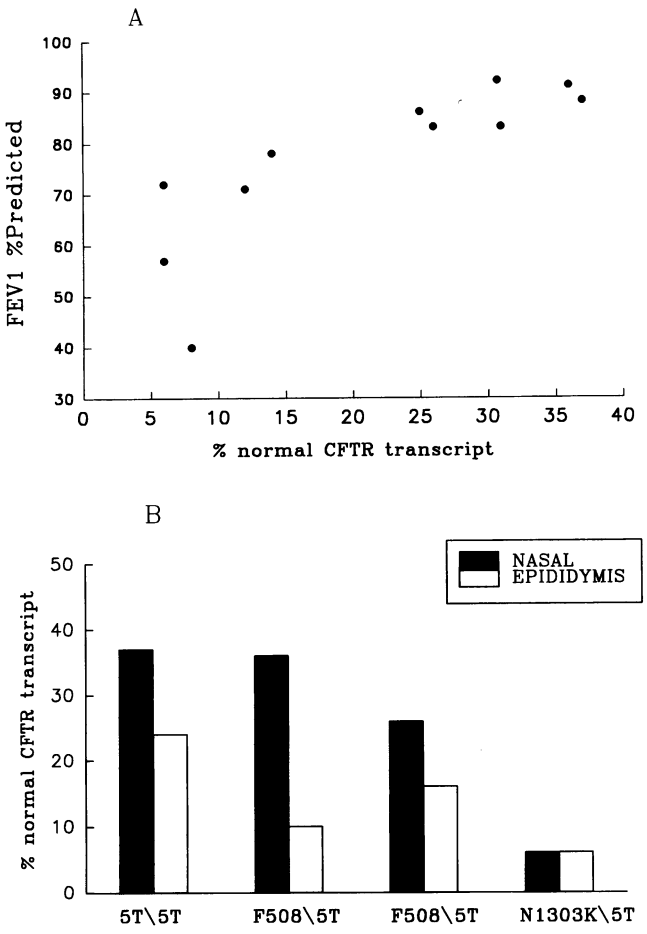

Figure 3 *A*, Correlation of normal CFTR transcripts and lung function, expressed as FEV_1 values. The correlation coefficient is r = .82, P = .002. *B*, Comparison of the level of normal CFTR transcripts from the 5T allele in respiratory and epididymal epithelium from the same individual.

the two RT-PCR systems was 2.5 (see Subjects and Methods).

Levels of Correctly Spliced CFTR RNA Transcribed from the 5T Allele in Epididymal Epithelium

RT-PCR 1 was performed on epididymal epithelium from four infertile males with CBAVD. Three of the patients were compound heterozygous for the 5T allele (table 1). The total level of normal transcripts in their epididymal epithelium was 6%, 10%, and 16% of total transcripts (table 1 and fig. 3*B*). In the fourth patient, homozygous for the 5T allele, the level of the normal RNA was 24% of total transcripts. The levels of normal transcripts in respiratory and epididymal epithelium from the same individual were compared. In patients with normal lung function (table 1 and fig. 3*B*) the level of normal transcripts in the respiratory epithelium was higher than the level in the epididymal epithelium. However, in a patient with moderate lung disease (patient 628; FEV_1 57% predicted) the level of the normal transcripts was low (6%) in both the respiratory and the epididymal epithelial cells.

Levels of Normally Spliced CFTR RNA Transcribed from the 9T Allele in Respiratory and Epididymal Epithelium

The level of correctly spliced RNA transcribed from a 9T allele at the branch/acceptor site of intron 8 of three heterozygous individuals (628, 660, and 666) was analyzed in the respiratory and the epididymal epithelium. In all three individuals the level in the epididymal epithelium was 57%. However, in two of them (666 and 660) the level in the respiratory epithelium, was higher, 75% and 82%, respectively. In individual 628 the level of correctly spliced transcripts from the 9T allele in the respiratory epithelium was 59%. Thus, although the number of studied individuals was small, these results might suggest that in the epididymal epithelium the general level of correctly spliced transcripts is lower than that in the respiratory epithelium, suggesting a less efficient splicing mechanism in this organ.

Discussion

This study further delineates the molecular mechanisms associated with the partial penetrance of the 5T allele. It shows variability, in the efficiency of the splicing mechanism, both between different organs of the same individual and among different individuals. Furthermore, the level of normal CFTR transcripts in respiratory epithelial cells correlated with the severity of lung disease. Individuals with normal lung function and minimal or no lung disease had >25% of normal transcripts, and individuals with moderate or severe lung disease had low levels (6%–14%) of normal transcripts. Our results imply that the critical level of normal CFTR transcripts that is required for maintenance of normal pulmonary function may be higher than previously reported. These data are important for the design of gene therapy aiming to restore normal lung function by increasing the level of normal CFTR. Chu et al. (1993) had reported that normal healthy individuals have 10% normal CFTR transcripts and had concluded that this low value is sufficient for maintenance of normal lung function. We believe that the differential RT-PCR system used in that study led to an underestimation of the level of normal transcripts and that the correct value was 22% (calculated from the factor of difference between the differential and nondifferential RT-PCR systems of 2.5). The level of normally spliced transcripts has been studied in two other CFTR splicing mutations: 3849+10kb C→T (Highsmith et al. 1994) and 1811+1.6kb A→G (Chillon et al. 1995*b*). In a patient homozygous for the 3849+10kb C→T mutation, with FEV_1 of 69% predicted, the level of normal transcript was 8% of the level measured in a normal control. In another patient with the 1811+1.6kb A→G mutation and with severe lung disease, the level of normal transcripts was 3%. These results are compatible with ours.

The level of normal transcripts in epididymal epithelial cells from infertile males with CBAVD was low (6%–24%). Elsewhere it has been shown that the CFTR is highly expressed in the epididymis, from early embryonic stages through adulthood (Trezise et al. 1993; Tizzano et al. 1994). The low level of normal transcripts found in the epididymis of adult males with CBAVD is likely to be associated with the infertility defect. However, since the vas deferens is already absent in utero, further studies are necessary to allow us to analyze both the efficiency of normal splicing in the fetus and its regulation during life.

Our study demonstrates that splicing efficiencies differ between organs of the same individual. In individuals with the 5T allele, partial disease expression might be a result of different efficiencies of exon 9 splicing in different tissues. Moreover, the threshold level required for maintenance of normal function might differ between different organs. Thus, in some organs a small reduction in the level of normal transcripts might lead to dysfunction while others would not be affected.

Alternative splicing is a complex regulatory mechanism, which currently is not well understood. Several genes involved in the regulation of this mechanism have been identified already (Smith et al. 1989). Thus, allelic variants of these genes might contribute to the different efficiencies of alternative splicing found among different individuals. Such alleles might segregate independently from the CFTR alleles, leading to high variability of disease expression among individuals from the same family. Other, nongenetic factors—

such as viral infections or environmental factors—also might be involved.

In many human monogenic diseases high variability in disease expression is found among patients carrying the same genetic defect. Milder phenotypes of several human diseases have been reported to be associated with reduced production of normal transcripts that is due to mutations in conserved splicing sequences (Kishimoto et al. 1989; Weatherall et al. 1989; McInnes et al. 1992; Arredondo-Vega et al. 1994). Variations in the alternative splicing machinery may lead to differential expression of these splicing mutations and, hence, to different levels of the aberrantly spliced mRNA. Allelic differences in splicing factors, tissue-specific differences, and other nongenetic factors might contribute to the variability in both organ involvement and expression of human inherited diseases. Further understanding of the mechanisms regulating alternative splicing will contribute to potential therapy for patients carrying such splicing mutations.

Acknowledgments

This work was supported by grants from the Israel Ministry of Health and the Israel Academy of the Sciences and Humanities.

References

Anguiano A, Oates RD, Amos JA, Dean M, Gerrard B, Stewart C, Maher TA, et al (1992) Congenital bilateral absence of the vas deferens, a primarily genital form of cystic fibrosis. JAMA 267:1794–1797

Arredondo-Vega FX, Santisteban I, Kelly S, Schlossman CM, Umetsu DT, Hershfield MS (1994) Correct splicing despite mutation of the invariant first nucleotide of the 5′ splice site: a possible basis for disparate clinical phenotypes in siblings with adenosine deaminase deficiency. Am J Hum Genet 54:820–830

Casals T, Bassas L, Ruiz-Romero J, Chillon M, Gimenez J, Ramos MD, Tapia G, et al (1995) Extensive analysis of 40 infertile patients with congenital absence of the vas deferens: in 50% of cases only one CFTR allele could be detected. Hum Genet 95:205–211

Chehab FF, Johnson J, Louie E, Goossens M, Kawasaki E, Erlich H (1991) A dimorphic 4-bp repeat in the cystic fibrosis gene is in absolute linkage disequilibrium with the ΔF508 mutation: implications for prenatal diagnosis and mutation origin. Am J Hum Genet 48:223–226

Chillon M, Casals T, Mercier B, Bassas L, Lissens W, Silber S, Romey M-C (1995*a*) Mutations in the cystic fibrosis gene in patients with congenital absence of the vas deferens. N Engl J Med 332:1475–1480

Chillón M, Dörk T, Casals T, Giménez J, Fonknechten N, Will K, Ramos D, et al (1995*b*) A novel donor splice site in intron 11 of the *CFTR* gene, created by mutation 1811+1.6kbA→G, produces a new exon: high frequency in Spanish cystic fibrosis chromosomes and association with severe phenotype. Am J Hum Genet 56:623–629

Chirgwin JM, Przbyla AE, MacDonald RJ, Rutter W (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294–5299

Chu C-H, Trapnell BC, Curristin S, Cutting GR, Crystal RG (1993) Genetic basis of variable exon 9 skipping in cystic fibrosis transmembrane conductance regulator mRNA. Nat Genet 3:151–156

Costes B, Girodon E, Ghanem N, Flori E, Jardin A, Soufir JC, Goossens M (1995) Frequent occurrence of the CFTR intron 8$(TG)_n$ 5T allele in men with congenital bilateral absence of the vas deferens. Eur J Hum Genet 3:285–293

Culard J-F, Desgeorges M, Costa P, Laussel M, Razkatzara G, Navratil H, Demaille J, et al (1994) Analysis of the whole CFTR coding regions and splice junctions in azoospermic men with congenital bilateral aplasia of epididymis or vas deferens. Hum Genet 93:467–470

Highsmith WE, Burch LH, Zhou Z, Olsen JC, Boat TE, Spock A, Gorvoy JD, et al (1994) A novel mutation in the cystic fibrosis gene in patients with pulmonary disease but normal sweat chloride concentrations. N Engl J Med 331:974–980

Jarvi K, Zielenski J, Wilschanski M, Durie P, Buckspan M, Tullis E, Markievicz D, et al (1995) Cystic fibrosis transmembrane conductance regulator and obstructive azoospermia. Lancet 345:1578

Kerem B-S, Buchanan JA, Durie P, Corey ML, Levison H, Rommens JM, Buchwald M, et al (1989*a*) DNA marker haplotype association with pancreatic sufficiency in cystic fibrosis. Am J Hum Genet 44:827–834

Kerem B, Kerem E (1996) The molecular basis for genotype-phenotype correlation in cystic fibrosis. Eur J Hum Genet 4:65–73

Kerem B, Rommens J, Buchanan JA, Markiewicz D, Cox TK, Chakravarti A, Buchwald M, et al (1989*b*) Identification of the cystic fibrosis gene: genetic analysis. Science 245 1073–1080

Kerem E, Harel-Rave N, Augarten A, Madjar I, Nissim-Rafinia M, Yahav Y, Goshen R, et al. A CFTR splice variant with partial penetrance associated with variable cystic fibrosis presentation. Am J Respir Crit Care Med (in press)

Kerem E, Kalman YM, Yahav Y, Shoshani T, Abeliovich D, Szeinberg A, Rivlin J, et al (1995) Highly variable incidence of cystic fibrosis and different mutation distribution among different Jewish groups in Israel. Hum Genet 96:193–197

Kishimoto TK, O'Connor K, Springer TA (1989) Leucocyte adhesion deficiency-aberrant splicing of a conserved integrin sequence causes a moderate phenotype. J Biol Chem 4: 3588–3595

McInnes B, Potier M, Wakamatsu N, Melançon SB, Klavins MH, Tsuji S, Mahuran DJ (1992) An unusual splicing mutation in the HEXB gene is associated with dramatically different phenotypes in patients from different racial backgrounds. J Clin Invest 90:306–314

Mercier B, Verlingue C, Lissens W, Silber SJ, Novelli G, Bonduelle M, Audrézet MP, et al (1995) Is congenital bilateral absence of vas deferens a primary form of cystic fibrosis? analyses of the CFTR gene in 67 patients. Am J Hum Genet 56:272–277

Oates RD, Amos JA (1994) The genetic basis of congenital

absence of the vas deferens and cystic fibrosis. J Androl 15: 1–8

Osborne L, Knight R, Santis G, Hodson M (1991) A mutation in the second nucleotide binding fold of the cystic fibrosis gene. Am J Hum Genet 48:608–612

Osborne LR, Lynch M, Middleton PG, Alton EWFW, Geddes DM, Pryor JR, Hodson ME, et al (1993) Nasal epithelial ion transport and genetic analysis of infertile men with congenital bilateral absence of the vas deferens. Hum Mol Genet 2:1605–1609

Patrizio P, Asch RH, Handelin B, Silber SJ (1993) Etiology of congenital absence of the vas deferens: genetic study of three generations. Hum Reprod 8:215–220

Pignatti PF, Bombieri C, Benetazzo M, Casartelli A, Trabetti E, Gile LS, Martinati LC, et al (1996) CFTR gene variant IVS8-5T in disseminated bronchiectasis. Am J Hum Genet 58:889–892

Polgar G, Promadhart V (1971) Pulmonary function testing in children: techniques and standards. WB Saunders, Philadelphia

Rave-Harel N, Madgar I, Goshen R, Nissim-Rafinia M, Ziadni A, Rahat A, Chiba O, et al (1995) CFTR haplotype analysis reveals genetic heterogeneity in the etiology of congenital bilateral aplasia of the vas deferens. Am J Hum Genet 56: 1359–1366

Riordan JR, Rommens J, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, et al (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 245:1066–1073

Rommens J, Iannuzzi MC, Kerem B, Drumm ML, Melmer G, Dean M, Rozmahel R, et al (1989) Identification of the cystic fibrosis gene: chromosome walking and jumping. Science 45:1059–1065

SAS User's Guide to Statistics, version 5 (1985) SAS Institute, Cary, NC

Schellen TMCM, van Stratten A (1980) Autosomal recessive heredity congenital aplasia of the vasa deferentia in four siblings. Fertil Steril 35:401–404

Smith CWJ, Patton JG, Nadal-Ginard B (1989) Alternative splicing in the control of gene expression. Annu Rev Genet 23:527–577

Tizzano EF, Siver MM, Chitayat D, Benichou J-C, Buchwald M (1994) Differential cellular expression of cystic fibrosis transmembrane regulator in the reproductive tissues. Am Pathol 144:906–914

Trezise AEO, Chambers JA, Wardle CJ, Gould S, Harris A (1993) Expression of the cystic fibrosis gene in human fetal tissues. Hum Mol Genet 2:213–218

Vidaud M, Fanen P, Martin J, Ghanem N, Nicolas S, Goossens M (1990) Three mutations in the CFTR gene in French cystic fibrosis patients: identification by denaturing gradient gel electrophoresis. Hum Genet 85:446–449

Weatherall DJ, Clegg JB, Higgs DR, Wood WG (1989) The hemoglobinopathies. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic basis of inherited disease, 6th ed. McGraw-Hill, New York, pp 2281–2339

Welsh MJ, Tsui L-C, Beaudet AL (1995) Cystic fibrosis. In: Scriver CL, Beaudet AL, Sly WS, Valle D (eds) The metabolic basis of inherited disease, xth ed. McGraw-Hill, New York, pp 3799–3876

Zielenski J, Bozon D, Kerem B, Markiewicz D, Rommens JM, Tsui L-C (1991*a*) Identification of mutations in exons 1 through 8 of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Genomics 10:229–235

Zielenski J, Rozmahel R, Bozon D, Kerem B, Grzelczak Z, Riordan JR, Rommens JM, et al (1991*b*) Genomic DNA sequences of the cystic fibrosis transmembrane conductance regulator. Genomics 10:214–228