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A STUDY FOR THE DETECTION OF KNOWN AND NOVEL MUTATIONS IN THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR GENE

by

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B.S. in Biology, Boğaziçi University, 1994

Submitted to the Institute for Graduate Studies in Science and Engineering in partial fulfillment of the requirements for the degree of

Master of Science

in

Molecular Biology and Genetics

Bogazici University Library

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ACKNOWLEDGMENTS

This study was carried out between September 94 and July 96 in the Department of Molecular Biology and Genetics at Boğaziçi University. It was supervised by Prof. Aslı Tolun and supported by the Boğaziçi University Research Fund (Project No. 96B0151).

I wish to express my sincere gratitute to my thesis supervisor Prof. AslıTolun for encouragement, continuous guidance and support she has given during the years I have known her. She always put lots of effort to motivate me throughout my thesis studies.

I wish to thank the other members of my jury, Prof. Beki Kan and Assoc. Prof. Kuyaş Buğra for their valuable criticisms and comments on my thesis.

I am grateful to my teachers for their contributions to my formation as a scientist. In particular I would like to thank Prof. Nazlı Başak, Assoc. Prof. Hande Çağlayan and Assist. Prof. Esra Battaloğlu.

I would like to thank all of the clinicians for their contribution in providing the blood samples and the clinical data.

My very special thanks go to my friends in the department and for keeping me in high spirits, their willingness to help and the continuous support in the preparation of this thesis.

I am grateful to Dr.Elizabeth Girodon in Centre Hospitalier Henri Mondor Laboratoire De Biochimie (France) for her interest, support and help.

Finally, this work would not have been possible without the patience and everlasting support of my parents and my wife.

ABSTRACT

Cystic fibrosis (CF) is one of the most common and severe autosomal recessive genetic disorders worldwide. It results from mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR). The gene has 27 exons which encode a 1480 amino acid transmembrane ion channel protein. The major CF mutation ΔF508 accounts for about 67% of the 30000 CF Caucasian chromosomes screened worldwide. Moreover, more than 600 other mutations have been identified, each with a frequency of at the most a few percent. The mutations responsible for CF in the Turkish population are not yet known. In the Turkish patients, ΔF508 is found at a significantly lower frequency (13%), which indicates that CF is caused predominantly by other mutations. A systematic study was initiated to characterize the CF mutations. First, we screened for four mutations which are frequent in neighboring geographical areas, and found them to be infrequent. It became obvious that, an efficient, quick and reliable mutation screening method which would cover all of the coding region was needed. We applied to 124 CF chromosomes the DGGE technique to analyze all of the gene except for the first and last exons with the purpose of detecting any variants in the gene. These variants would later be subjected to DNA sequence analysis to determine mutations or polymorphisms which they represent. Our previous hypothesis that the profile of CF mutations in the Turkish population shows great heterogeneity is consistent with the data obtained. As expected, several mutations and variants were identified which were distributed randomly throughout 14 exons in the coding regions of the CFTR gene, each of which would account for a relatively small fraction of the CF mutations.

ÖZET

Otozomal çekinik karakterde olan sistik fibrozis (CF) en ciddi ve yaygın kalıtsal hastalıklardan biridir. Hastalık, sistik fibroz transmembran regülatör proteinini (CFTR) kodlayan gendeki mutasyonlardan kaynaklanmaktadır. Yirmiyedi eksondan oluşan gen bölgesi 1480 amino asidin oluşturduğu bir iyon kanalı proteinini kodlamaktadır. Gendeki en yaygın mutasyon olan ΔF508 dünya genelinde taranmış olan 30.000 beyaz ırk kromozomunun yüzde 67'sinde gözlenmiştir. Bu güne kadar hastalığa neden olan 600'den fazla mutasyon tanımlanmıştır, ancak Türk toplumundaki mutasyonların tamamı henüz bilinmemektedir. En yaygın mutasyon olan ΔF508'in Türk hastalarındaki sıklığının az olduğu bilinmektedir. Bu çalışmada bu mutasyonun sıklığı yüzde 13 olarak belirlenmiştir. Mutasyonların tümünü belirlemek için sistematik bir çalışma başlattık. İlk olarak, komşu ülkelerin toplumlarında sık görülen bazı mutasyonlar (Q220X, L346P, N1303K ve W1282X) için hastaları taradık. Bu mutasyonların hastalarımızda yokluğu ya da sıklığının azlığı genin tüm eksonlarının mutasyon için taranmasının daha etkin bir strateji olacağını düşündürdü. Çalışma için denatüre edici gradientli gel elektroforezi (DGGE) tekniği seçildi. Altmışiki hastada genin ilk ve son eksonları dışındaki tüm kodlama bölgesinde baz değişiklikleri araştırıldı. Daha sonra DNA dizi analizi ile bu değişikliklerin moleküler temeli (mutasyon ya da polimofizm oldukları) kolayca belirlenebilecektir. Baz değişikliklerinin gen kodlama bölgesinde rastgele bir dağılım gösterdiği ve görülme sıklıklarının nispeten düşük oranlarda olduğu ortaya çıkmıştır. Dolayısı ile toplumda ΔF508'in dışında çok sık görülen bir mutasyonun var olmadığı anlaşılmıştır. Türk toplumunun çok heterojen bir yapı gösterdiği bu çalışma ile de kanıtlanmış olmaktadır.

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ABBREVIATIONS

ABC	ATP-binding cassette
APS	ammonium persulphate
ARMS	amplification refractory mutation system
ASO	allele specific oligonucleotide
ATP	adenosine triphosphate
bp	base pair
BPB	bromophenol blue
CBAVD	congenital bilateral absence of vas deferens
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulatory
DGGE	denaturing gradient gel electrophoresis
dNTP	2'-deoxynucleoside 5'-triphospate
dsDNA	double-stranded DNA
EDTA	ethylenediaminetetraaceticacid
EtBr	ethidium bromide
MSD	membrane spanning domain
NBF	nucleotide binding fold
ORF	open reading frame
PI	pancreatic insufficiency
PKA	protein kinase A
PS	pancreatic sufficiency
RFLP	restriction fragment length polymorphism
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SSCP	single strand conformational polymorphism
Temed	N',N',N',N',-tetramethylethylenediamine
XC	xylene cyanol

I. INTRODUCTION

A. Historical Background to Cystic Fibrosis

The gene responsible for cystic fibrosis (CF) was identified through molecular cloning studies after chromosomal localization of the disease locus (Rommens et al., 1989; Kerem et al., 1989). The most exciting aspect of the research was that the gene was identified before a biochemical description of the defect was available. Thus reverse genetics approach played the most important role. According to this approach, the disease gene is identified by virtue of its chromosomal map position. The encoded protein and the metabolic defect can then be determined.

One of the first major contributions to solving the nature of the disease came in 1938 from Dorothy H. Anderson of Colombia University. After performing autopsies on affected infants and children, she provided the first comprehensive description of the clinical symptoms and the changes that occurred in organs. Those changes, she noted, included destruction of the pancreas, and infection and damage to the lung airways. Anderson also gave the disease its name, "cystic fibrosis of the pancreas", on the basis of microscopic features she observed in pancreatic tissue. She also suggested for the first time an autosomal recessive inheritance pattern for CF. In 1945, Farber suggested that CF is a disease of exocrine glands, characterized largely by failure to clear the mucous secretory product. Chronic infection of the lungs was recognized early as a major contributing factor, and antibiotics were first used for the treatment of CF at that time. In 1953, DiSant Agnese and colleagues in Colombia University investigated salt depletion in children with CF during summertime and found that excessive salt loss occurred via sweating. This observation resulted in the development of a test that remained as the diagnostic criteria to this day: Picolocarpine iontophoresis method or, simply, sweat testing. Research into the biochemical understanding of cystic fibrosis progressed more slowly than the clinical work, despite extensive effort spent in the first half of the 1980's. During that time, scientists found out that in patients there was malfunction of the epithelial tissue, which is a sheet of cells that forms a barrier between different compartments of the body, lines the intestines and many ducts, and secretes mucus. Two observations at that time appeared to be particularly important. Knowles and coworkers described altered electrical properties of CF

respiratory epithelium associated with abnormalities in both sodium and chloride transport. Soon chloride impermeability of CF sweat gland was documented. Paul M. Quinton at the University of California found that the epithelia lining the ducts of sweat glands failed to take up chloride efficiently from the cavity, or lumen, of glands. This finding finally explained why people with cystic fibrosis have unusually salty sweat. Sweat is produced at the base of the sweat glands. It then flows to the skin surface through a narrow duct. At the very beginning sweat is rich in sodium and chloride ions which are its constituents. However, as the fluid traverses the duct, the ions escape into the epithelium, leaving water behind. Thus, the sweat that emerges to cool the skin surface is only slightly salty. In patients with cystic fibrosis, in contrast, the inability of epithelial tissue to absorb chloride and the consequent impairment of sodium absorption from the duct lumen cause sweat to retain excess sodium and chloride, thus becoming abnormally salty.

As these studies on chloride transport were going on, many scientists were engaged in an intense race to find the gene responsible for CF.

B. Identification of the Gene For Cystic Fibrosis

The effort for isolation and identification of the CF gene came from a large group of collaborators led mainly by Lap-Chee Tsui. With the concept of an RFLP map for the human genome, the possibility of obtaining DNA markers linked to the CF locus became a major aim in research laboratories working in the field. The first evidence for the localization of the CF gene on chromosome 7 came from the usage of an anonymous unmapped DNA probe in linkage analysis in CF families. Following the result of this first linkage analysis, White et al. (1985) reported a very tight linkage between CF and the met oncogene locus, while Wainwright et al. (1985) reported another tight linkage to the DNA probe D7S8. As these analyses based on a large number of polymorphic DNA markers were being carried out, new DNA markers were identified and used in further mapping of the CF locus. The result was the precise localization of the gene locus to band 7q31. Then a collaborative study using these probes in over 200 families with CF gave a certain recombination fraction which was in turn used to detect the order of the locus with respect to the markers, the possible order being centromere/MET/D7S340/D7S122/CF/D7S8/ telomere (Rommens et al., 1989). The positioning of these markers relative to each other was facilitated by somatic cell hybrid mapping, linkage analysis and long range restriction mapping by pulsed field gel electrophoresis. Later the identification of closely linked flanking markers made it possible to use various gene cloning strategies to pinpoint the CF

gene. The strategy in short included chromosome walking and jumping from the loci of the flanking markers, cloning of DNA fragments from a defined physical region using pulsed field gel electrophoresis, a combination of somatic cell hybrid and molecular cloning techniques designed to isolate DNA fragments from undermethylated CpG islands near CF, and finally analysis of candidate genes.

At this stage, following the construction of a jumping library in which phage clones contained sequences that were widely separated between markers, overlapping clones were isolated by simply walking using this phage library. The following step was to test each clone for the presence of coding sequences within a 500 kb of DNA. The presence of CpG islands in one of the probes suggested the beginning of a gene. Results of further cDNA cloning experiments from epithelial cell libraries, sequencing and genetic analysis suggested that the homologous clone identified by this probe was most likely the gene responsible for CF (Riordan et al., 1989). A complete concordance of the restriction sites obtained from pulsed field gel analysis to those identified in the partially overlapping genomic DNA clones revealed that the size of the DNA locus was about 250 kb (Rommens et al., 1989). Riordan et al. (1989) provided strong supporting evidence that the isolated gene was indeed the CF gene by their studies which demonstrated an appropriate tissue distribution and predicted properties of the gene product. The tissue distribution of its mRNA was consistent with the predicted pathogenesis of CF. Also consistent was the deduced amino acid sequence which indicated that the gene product was related to membrane transport proteins. Hence, the protein was termed as the cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan et al., 1989). Final evidence that the isolated gene was indeed responsible for CF came from the identification of a specific mutation which was found in affected individuals, but never appeared in normal chromosomes. This abnormality in the DNA, which accounted for about seventy per cent of the cystic fibrosis cases in the patient population was denoted as the mutation ΔF508. The mutation is a deletion of three nucleotides from the gene and causes the protein product of the gene to lack a single amino acid: Phenylalanine at position 508.

C. The Cystic Fibrosis Transmembrane Conductance Regulator Gene

The gene was found to be approximately 250 kb in length and to contain 27 exons. The gene's open reading frame (ORF) is capable of encoding a polypeptide of 1480 amino acids via a messenger RNA of 6.5 kb. Sequence analysis of the 5' flanking region of CFTR

revealed some of the structural features specific to housekeeping genes. The 5' flanking region is highly GC rich. There is no TATA or CAAT box within 500 bp upstream of the major transcription start site which is designated as +1. However, several potential binding sites for the transcription factor Sp1 and Ap1 were detected. Furthermore, multiple transcription start sites were observed in cells expressing the CF gene between the positions -95 and -50. Despite the fact that the promoter shows characteristics of a housekeeping gene, the gene itself is tissue-specific. High levels of mRNA were detected in a number of organs, including pancreas, sweat glands, reproductive organs, nasal and airway epithelia, whereas little or no expression was found in some tissues, such as brain or fibroblasts. A number of repetitive elements, including five Alu and one Kpn families and simple repeats such as $(CA)_{17}$, $(GATT)_7$ and $(TA)_{14}$ were detected in close vicinity of some of the intron-exon boundaries (Zielenski et al., 1991). Four of the Alu repeats which are in introns 2, 8, 14a and 23, and the unique Kpn repeat in intron 16 are oriented in the same direction as the CFTR transcription. However, the fifth Alu repeat in intron 6a is in the opposite orientation. Some of other commonly occurring simple repeats which serve as polymorphic DNA markers convenient for linkage analysis are of $(CA)_n$ and $(TA)_n$ type. Two dinucleotide repeats of the $(CA)_n$ type were found in introns 8 and 17b and one of the (TA) type in again intron 17b (Morral et al., 1992). Another sequence repeat was a 4-bp repeat, namely $(GATT)_7$, which was found immediately preceding exon 6b. The sizes of the 27 exons vary greatly. Exon 14b was found to be the smallest (38 bp) and exon 13 (724 bp) the largest. The lengths of the introns, however, vary greatly from 1.1 kb (intron 6a) to 40 kb (intron 3) (Figure I.1).

D. The Cystic Fibrosis Transmembrane Conductance Regulator Protein

The linear sequence of amino acids in the protein, which was easily deduced once the gene was isolated, offered clues to the protein's function. The sequence of the CFTR gene was much like that found in a family of proteins called traffic ATPases or ABC transporters, because they carry what is known as an ATP binding cassette. The similarity implied that the CFTR protein might also resemble the family in its behavior and in its folded, three-dimensional structure (Hyde et al., 1990). When folded, the protein appears to be internally duplicated: There are two repeated motifs, each composed of six

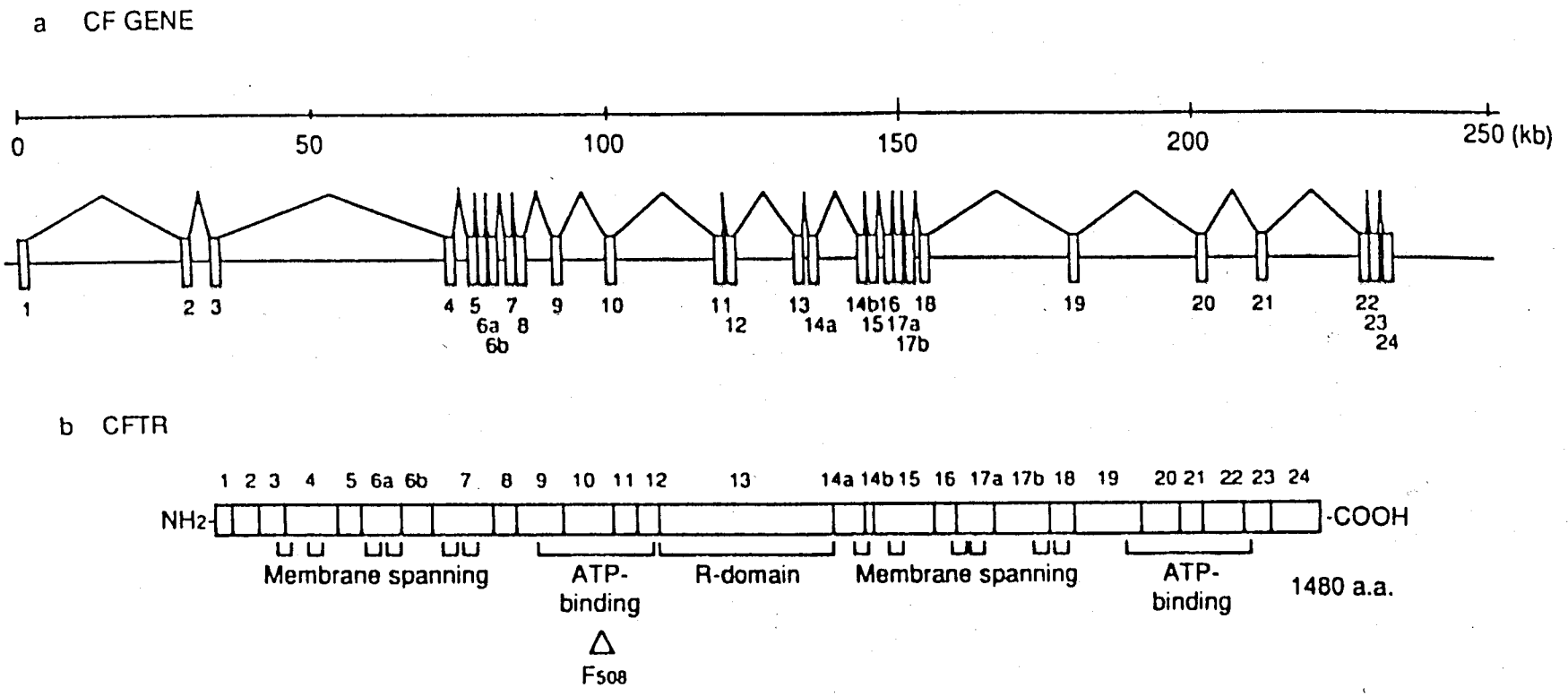

Figure I.1. A schematic diagram showing the organization of the CFTR gene and its protein product (Zielenski et al., 1991).

transmembrane segments and one region known to interact with ATP (take up and cleave ATP), termed as the nucleotide binding fold (NBF). These domains are also common to the traffic ATPase family proteins. In addition, CFTR has a unique hydrophilic regulatory domain called the R-domain which link the protein halves. Of the 241 amino acids encoded in this region, 69 are polar residues which are arranged in alternating clusters of positive and negative charges. In addition, nine of the ten potential sites for phosphorylation by protein kinase A and seven of the putative protein kinase C phosphorylation targets found in the gene are located in this domain (Rich et al., 1991; Rich et al., 1993). The first experimental evidence for the involvement of ATP in CFTR function came from a study which demonstrated the binding of ATP to a 67-residue synthetic peptide homologous to the central region of the first NBF (Thomas et al., 1991). The first NBF is encoded by the exons 9, 10, 11 and 12, the second NBF is encoded by the exons 20, 21 and 22, and the regulatory domain is encoded by the largest exon (exon 13) (Zielenski et al., 1991). Intact CFTR protein forms a chloride permeable channel in the outer membrane of many cells. The precise structure of the channel is yet to be determined, but movement of chloride through the pore is known to be regulated by three main domains of the protein. Passage is allowed only when the two nucleotide binding domains take up and cleave ATP, and when the regulatory domain becomes studded with phosphate groups. The two membrane-spanning domains form the pore through which chloride ions pass across the membrane. A recent model for the regulation of this protein is illustrated in Figure I.2. The evidence that supports this model came from various experiments, including the important study by Cheng et al., 1991. It showed that when R domain lacks phosphate groups, chloride ions cannot flow into the channel pore. But, when CFTR is phosphorylated by the cAMP-dependent protein kinase A (PKA), the opening of the channel is promoted. However, this is not sufficient to open the channel, also ATP must be bound and hydrolyzed either by one or both NBFs (Travis et al., 1993).

The localization of the protein is largely restricted to the apical membrane of specific epithelial cells. The highest mature protein levels are found in the pancreatic ducts, small intestine, liver, small bile ducts, epithelia lining the respiratory system, sweat gland ducts, and genital ducts. No CFTR activity was found in other tissues studied. CFTR expression in the former tissues throughout human embryonic development is characterized well by studies that used human fetal tissues (Trezise et al., 1993; Tizzano et al., 1993).

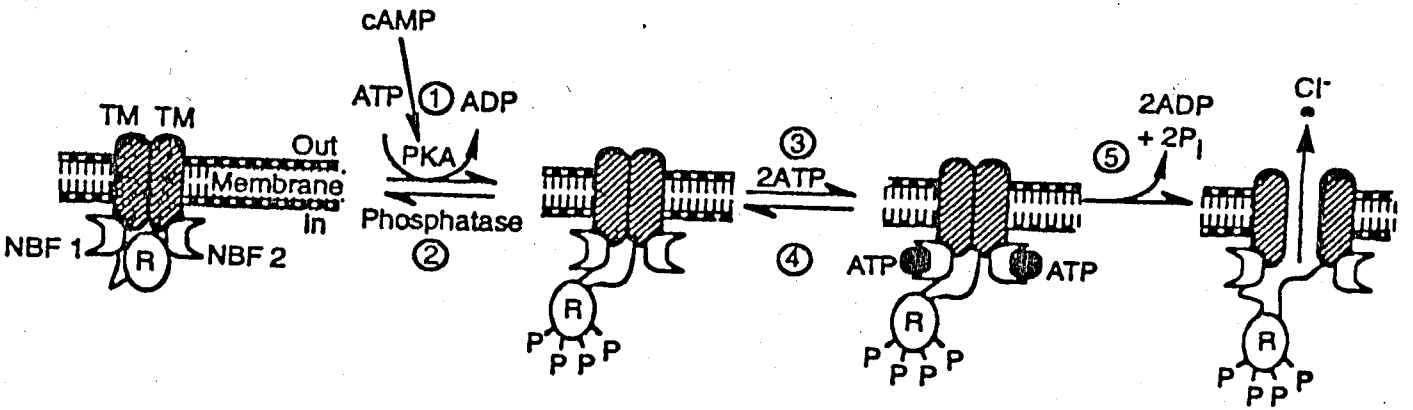

Figure I.2. A schematic model for the regulation of the CFTR protein. The precise structure of the channel is yet to be determined, but movement of chloride through the pore is known to be regulated by three main domains of the protein. Passage is allowed only when the R-domain is phosphorylated (step 1 and 2) and the two nucleotide binding domains take up and cleave ATP (step 3). Finally, cleavage induce a conformational change, opening the chloride channel (step 5). Pi; inorganic phosphate, cAMP; cyclic AMP, and TM; transmembrane domain (Collins et al., 1992).

Hence, the detection of CFTR in the tissue-specific organs is consistent with the clinical manifestations of CF and the function of CFTR as a chloride channel early in development.

E. Clinical Features

The typical diagnostic criteria for CF are: (1) a positive family history, which occurs in about 20% of patients; (2) neonatal intestinal obstruction, i.e., meconium ileus (20%); and (3) other disease manifestations at a variable age (Farrell, 1992), as described below. Once the characteristic signs and symptoms become evident, the diagnosis of CF can be readily established by performing a sweat test. During infancy or in early childhood, CF is usually suspected because of failure to thrive, steatorrhea, and/or chronic lung disease associated with cough and recurrent pneumonia. Although the lung is normal at birth, it eventually (but at a variable age) shows both infection and obstruction in a process whose onset and rate of progression vary widely among individuals. The manifestations in the respiratory system become increasingly important and ultimately dominate the clinical picture. They also generally determine the prognosis in a patient when irreversible lung disease is present. Chronic respiratory infection with *P. aeruginosa* and other pseudomonas species following the obstruction of the airways by thick and sticky mucus seems to be largely responsible for progressive pulmonary deterioration and mortality. There is also involvement of the gastrointestinal tract in most patients, with 85% of them showing pancreatic insufficiency as a result of obstruction of the pancreatic ducts and subsequent scarring and destruction of exocrine function (Boat et al., 1995).

Infertility secondary to bilateral absence of the vas deferens is found in almost all male CF patients irrespective of the severity of their pancreatic or pulmonary disease (Anguiano et al., 1992). This suggests that CFTR has a role in the development of the vas deferens (CBAVD).

F. Phenotype-Genotype Correlation

By analyzing different clinical features resulting from various mutations, a strong correlation between genotype and phenotype has emerged. At present, the clinical variable that most readily discriminates different phenotypes is the state of pancreatic function. Most patients have pancreatic failure, requiring ingestion of supplemental pancreatic enzymes; such patients are referred to as pancreatic insufficient (PI). However, some patients retain significant pancreatic function and require little or no pancreatic enzyme supplementation. Those patients who are pancreatic sufficient (PS) make up about 15 per cent of all CF patients. This led to the classification of CF mutations as severe (S) or mild (M), respectively. Prior to the cloning of the CFTR gene, pancreatic status was found to be associated with certain haplotypes of closely linked markers. This suggested that the PI/PS phenotype is determined by specific CF mutations (Kristidis et al., 1992). The most common genotype in CF patients is homozygosity for ΔF508. Analysis of the patients with this mutation revealed that over 98 per cent are diagnosed as PI. Clinical studies show that when the protein is not produced, the phenotype is indistinguishable from that of ΔF508 (The Cystic Fibrosis Genotype-Phenotype Consortium, 1993). Suprisingly, some patients homozygous for severe mutations show a milder phenotype, which suggests the probable action of other genetic factors such as modifier genes (Rozmahel et al., 1996).

Amino acid substitutions provide useful information on the functions of the various CFTR domains and the genetic variability of the disease. Functional studies have suggested that missense mutations in the nucleotide binding or regulatory domains might be expected to produce a severe effect when combined in trans with a severe mutation. In contrast, defects in the membrane-spanning domains were predicted to cause a mild CF phenotype (Welsh, J.M., 1993). However, clinical data show that the severity of a mutation depends on its nature as well as its location. The level of functional CFTR protein depends on the nature of the mutations in the two CFTR alleles. Two severe mutations lead to a non-functional or absent CFTR protein and give rise to the classical CF phenotype with pancreatic insufficiency. For example, mutations which lead to the absence of protein production, or which prevent the protein from reaching the epithelial membrane are associated with severe phenotype. However, genotypes that preserve partial CFTR function

or low levels of normal CFTR protein produce a wide range of phenotypes (Figure I.3). There is now a large list of CFTR missense mutations associated with less severe phenotypes including obstructive azoospermia (Table I.1). The genital abnormality of CF includes related types of obstructive azoospermia which includes congenital bilateral absence of the vas deferens (CBAVD) and unilateral absence of vas deferens (CUAVD). The links which have slowly emerged between CBAVD and CF are now very well documented (Bienvenu et al., 1993; Meschede et al., 1993; Culard et al., 1994). Most of the cases of CBAVD indicate a mild form of the disease. Recently, three length variants of a polyprimidine tract within the splice acceptor site in intron 8 of the CFTR gene have been found to be associated with varying degrees of exon 9 splicing (Chu el al., 1993). Individuals with CBAVD are either homozygous for the 5T variant, or heterozygous for this variant but with a CF mutation on the other chromosome. It is interesting to note that compound heterozygous patients have a sweat chloride value higher than 60 mmol/l, whereas most of the patients having a 5T repeat or a genotype characterized by a 5T repeat and a ΔF508 mutation have a sweat chloride level in the intermediate range (between 40 and 60 mmol/l) (Dumur et al., 1996). There seems to be a relation between the genotype and the sweat chloride level. But what is more confusing is that some infertile patients have the same genotype as their fertile brothers, suggesting not surprisingly that additional factors influence patient fertility (Mercier et al., 1995).

The wide spectrum of phenotypes associated with pulmonary diseases again supports the idea that other factors interact with CFTR in causing the disease. The morbidity and mortality of CF are determined predominantly by the severity of lung disease. Despite the strong association between the cystic fibrosis genotype and pancreatic phenotype, it is demonstrated that the severity and the course of pulmonary disease cannot be predicted by the genotype (The Cystic Fibrosis Genotype-Phenotype Consortium, 1993). There is a wide variability in the extent of pulmonary disease within each genotypic group. Some patients with pancreatic sufficiency have severe pulmonary manifestations, while many patients with pancreatic insufficiency have normal pulmonary function.

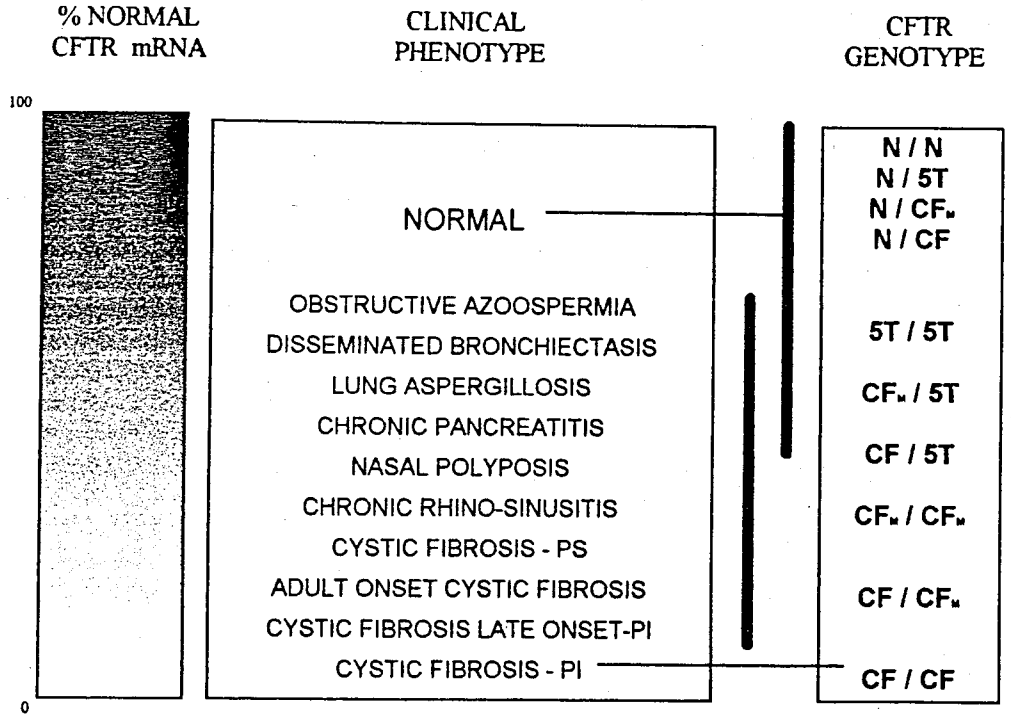

Figure I.3. Clinical phenotypes associated with the CFTR mutations. The level of functional CFTR depends on the nature of the mutations in the two alleles of CFTR gene. Two severe mutations lead to a non-functional or absent CFTR and give rise to the classical CF phenotype with pancreatic insufficiency (CF-PI). Genotypes that preservepartial CFTR function or low levels of normal CFTR produce a wide range of phenotypes. Thenormal phenotype includes carrier status for a CFTR mutation, but also compound heterozygosity for a CF mutation and IVS8-6(5T), and homozygosity for IVS8-6(5T).

Table I.1. Missense mutations in cystic fibrosis patients with pancreatic sufficiency (PS). and in patients with obstructive azoospermia either bilateral (CBAVD) or unilateral (CBUVD). MSD, Membrane-spanning domain; NBF, nucleotide binding fold; R, regulatory domain

CFTR Domain	CBAVD/CUAVD	CF-PS CBAVD/CUAVD	CF-PS
Missense MSD1	MIV,S50Y,S50P, R75Q, R75L, R117C	E92K, R117H, L206W, R258G, R347H	E193K, P205S, 1336K T338I, R347P, R352Q
NBF1 D579G	G576A	S549N, P574H	A455E, G551S,
R	R668C		D614G
MSD2	A1067V, A1067T, R1070W	F1052V R166H D1152H, D1154G	
NBF2	S1235R, D1270N N1303I, D1277H		S1251N
SPLICE		IVS8-6(5T) 2789+5A→G 3272-26A→G 3849+10kbC→T	711+5G→A

G. Mutations in the CFTR gene

More than 600 disease-causing mutations have been identified in the CFTR gene (The Cystic Fibrosis Genetic Analysis Consortium, 1996), 42 per cent of which are missense, 23 per cent frameshift, 16 per cent splice-site, 15 per cent nonsense changes and 4 per cent account for other defects, including amino acid deletions and larger DNA deletions. Exons 9-11, 20, 21 and 23 in which the largest number of mutations has been observed worldwide (Cutting et al., 1992) are the hot spots for mutations. The five main molecular mechanisms of CFTR dysfunction arising from different mutations are described below (Welsh, J.M., 1993) (Table I.2.).

1. Classification of the CF Mutations

a. Class I Mutations: Defective Protein Production

There are mutations located throughout the CFTR gene that produce premature termination signals because of splice site abnormalities, nonsense mutations, and frameshifts due to insertions and deletions. A truncated protein or an aberrant protein having deletions or novel amino acid sequences in the carboxyl terminus may be produced. Such proteins are often unstable, thus would usually be expected to be degraded relatively rapidly, or have little or no function. In other cases, such as the nonsense mutations G542X and R553X, the mutations result in an unstable mRNA and no detectable protein (Hamosh et al., 1991; Hamosh et al., 1992).

Table I.2. Five main classes of CFTR dysfunction arising from different mutations.

CLASS	DEFECT	EXAMPLES	TYPE	CLINICAL
I	Production	G542X	nonsense	CF-PI
		3905insT	frameshift	CF-PI
		621+1G→T	splice	CF-PI
II	Processing	ΔF508	aminoacid deletion	CF-PI
		N1303K	missense	CF-PI
III	Regulation	G551D	missense	CF-PI
		G551S	missense	CF-PS
		R668C	missense	OA
IV	Conduction	R1066C	missense	CF-PI
		R334W	missense	CF-PI/PS
		R117H	missense	CF-PS/OA
		D1152H	missense	OA
V	Synthesis	1811+1.6kbA→G	splice	CF-PI
		3849+10kbC→T	splice	CF-PI/CF-PS/OA
		2789+5G→A	splice	CF-PS/OA
		IVS8-6(5T)	splice	CF-PS/OA

b. Class II Mutations: Defective Protein Processing

Class II mutations prevent the protein from reaching the epithelial membrane. This includes the most common mutation, ΔF508, within the first NBF and another relatively frequent mutation, N1303K, in the second NBF of the CFTR gene (Osborn et al., 1992). The failure of proteins to progress through the biosynthetic pathway can be found in assessing their state of glycosylation. That the phenotypic impacts of these mutations are similar is supported by in vitro studies which showed that neither the ΔF508 nor the N1303K mutant CFTR protein is fully glycosylated, and that both of the mutant CFTR proteins are associated with similar defects in chloride permeability (Gregory et al., 1991). Soon after production, the partially glycosylated mutant protein is degraded. As a result, the mutant proteins ΔF508 or N1303K cannot reach the cell membrane.

c. Class III Mutations: Defective Regulation

Class III mutations in regulatory domains result in an anomalous regulation of the CFTR Cl^- channel. Because intracellular ATP molecules regulate the opening of the CFTR Cl^- channels through direct interaction with the nucleotide-binding domains, mutations such as G551D, G551S and R668C that cause defective regulation result in the decrease of the net Cl^- channel activity. Also, a mutation in the R-domain is likely to alter the phosphorylation-dependent regulation of the R-domain.

d. Class IV Mutations: Defective Ion Conduction

This class is represented by mutations in the transmembrane domain that cause an abnormal conduction of the Cl^- current. A number of CF-associated missense mutations have been identified in the membrane-spanning domains, which contribute to the channel pore. Three mutations in the first membrane-spanning domain, R117H, R334W and R347P, affect arginine residues located in the putative membrane-spanning sequences.

e. Class V Mutations: Reduction in the Synthesis of normal CFTR

Class V contains mutations that cause a reduction in the synthesis of normal CFTR, generally due to splice defects. The mutations IVS8-6(5T) (Chillon et al., 1995) or 3849+10 kb C→T (Highsmith et al., 1994) are two examples. The mutation 3849+10 kb C→T leads to the creation of a partially active splice site in intron 19 and to the insertion into most CFTR transcripts of a new 84-base-pair "exon" containing an in-frame stop codon, between exons 19 and 20. Normally spliced transcripts were also detected at a level of approximately 8 per cent of that found in normal subjects. This mutation is associated with abnormal nasal epithelial and sweat acinar epithelial functions.

H. Mutation Detection in the CFTR

Methods that can detect small changes in DNA sequences, in particular, single base substitutions, deletions, and insertions, have become important tools in the diagnosis of human genetic diseases, such as CF. Nearly 600 different CF mutations have been identified. The presence of such a high number of mutations necessitates the application of various methods. There is yet no single method which can detect all of the known CF mutations. The various methods have complementing strengths. Current methods of mutation detection can be broadly divided into two categories: (1) diagnostic methods, devised to detect known mutations; and (2) scanning methods, devised to screen exons for mutations.

Techniques used for diagnostic purposes are based on direct determination of known disease alleles. The methods included in this category are useful as diagnostic tools to detect changes at defined sites, but cannot be used effectively to screen for randomly distributed mutations or polymorphisms. On the other hand, the techniques used for mutation screening are applied to scan a certain gene region for possible mutations. Hence, scanning methods are devised to screen hundreds or thousands of bases of DNA that might harbor a mutation, without the need for DNA sequencing. All of the techniques in both

categories are based on amplifications of the DNA samples by the polymerase chain reaction (PCR) prior to analysis.

1. The Diagnostic Methods

a. Heteroduplex Analysis

If both mutant and normal template sequences are present in a PCR reaction, heteroduplexes between the two different DNA species can form during the late cycles, or later by a short procedure. Heteroduplex molecules with a single base-pair variance may have different mobilities from homoduplexes in native polyacrylamide gels. This phenomenon is thought to be caused by sequence dependent conformational changes in the dsDNA. Recently, new gel matrices such as Hydrolink MDE (AT Biochem) have become available. They markedly enhance the mutation-induced mobility shifts in heteroduplex molecules. In CF, this technique has been applied successfully for detection of mutations resulting from deletions, such as ΔF508 and 1677delTA (Rommens et al., 1990). Electrophoretic size fractionation of the amplified fragments on a 12 per cent polyacrylamide gel reveals the presence of normal and mutant alleles that are different from each other in length, along with the mobility shift in heteroduplexes.

b. Restriction Enzyme Site Alterations

In this method, base changes which alter any particular restriction enzyme cleavage site are detected. Many point mutations generate or remove a cleavage site, as in the case of the mutations Q220X (Shackleton et al., 1994), L346P (Boteva et al., 1994) and 3849+10 kb C→T (Highsmith et al., 1994). Digestion of the amplification product with the appropriate restriction enzyme and subsequent electrophoresis on either agarose or polyacrylamide gels resolve the DNA fragments for the normal and the mutant alleles that differ in size.

c. Amplification Refractory Mutation Systems (ARMS)

An amplification refractory mutation system (ARMS) is a general technique for the analysis of any point mutation or small deletion (Newton et al., 1989). It is mainly a modification of the polymerase chain reaction, allowing direct detection of mutations after agarose gel electrophoresis. The basis of the system is the observation that an oligonucleotide that is complementary to a given sequence except for a mismatch at its 3' OH residue will not function as a primer in PCR under appropriate conditions. A typical ARMS test consists of two complementary reactions. The first reaction contains an ARMS primer specific for the normal DNA sequence at a given locus and cannot amplify its mutant allele. Similarly, the second reaction contains a mutant-specific primer and does not amplify the normal allele. The genotype of an individual can be determined by analyzing the amplification products. A normal individual's DNA sample generates a PCR product only in the normal reaction, while a heterozygote gives products in both reactions, and a homozygous mutant individual does so only in the mutant reaction. The use of two reactions with internal controls ensures that false-negative results are not obtained. Combination of ARMS tests for the most common CF mutations into a multiplex suitable for use in clinical laboratories rendered this technique simple and rapid (Fortina et al., 1992; Ferrie et al., 1992).

d. Dot Blot Hybridization

In this method, mutations are detected by using allele-specific oligonucleotide probes (ASO). These probes are designed to hybridize selectively to either the normal or the mutant allele and can be used for any locus where the nucleotide sequences of the mutant and normal alleles are known. Two oligonucleotides complementary to sequences flanking the mutation site are used as primers to amplify a fragment that contains the mutation site. Two other oligonucleotides, one complementary to the normal allele and the other complementary to the mutant sequence, are used individually as probes. Samples of the amplification mixture are denatured and transferred in duplicate to membranes with the help of a dot blot apparatus. The membranes are then hybridized with radioactively labeled

normal or mutant ASO separately. However, the utility of this general method is limited, due to the variable hybridization conditions for each mutation and its requirement for radioactive or other kind of labeling. Nevertheless, for the most common mutations specific to a particular gene region, this is the most widely used technique for CF analysis (Shuber et al., 1993).

e. Reverse Dot Blot Hybridization

Reverse dot-blot hybridization is performed especially in screening carriers in order to detect several CF mutations at a time. The method is simple, quick, reliable and nonradioactive. It utilizes the sensitivity of the PCR coupled with colored or chemiluminescent substrates for mutation detection. This technology appears to be the method of choice for the wide scale carrier screening of several CF mutations simultaneously in populations which carry a few predominant mutations (Chehab et al., 1992).

2. The Scanning Methods

a. Denaturing Gradient Gel Electrophoresis (DGGE)

Denaturing gradient gel electrophoresis (DGGE) is an efficient method for detecting nucleotide changes (mutations or polymorphisms) in a chosen DNA region. Double-stranded DNA (dsDNA) amplified by PCR is electrophoresed at 60°C through a polyacrylamide gel containing a linear gradient of increasing concentrations of denaturing agents (urea and formamide). The two DNA strands in the helix progressively dissociate at discrete domains with different melting temperatures as DNA migrates along the gel. The addition of a specifically designed GC-rich region of about 40 nucleotides at the 5' end of one of the primers greatly increases the sensitivity of the technique by ensuring that the amplified sequence has a high melting temperature (Tm). Melting of the dsDNA leads to

an abrupt decrease in its mobility. DNA molecules differing by as little as a single base can be detected with a 95 per cent sensitivity if all is fine in the construction of the experimental set (Myers et al., 1987). DGGE identifies the regions with mutations but does not identify the mutations themselves. DNA samples showing migration patterns altered from that of the normal allele can be further analyzed by direct sequencing of the amplified DNA. DGGE multiplex systems are designed for simultaneous analysis of several hot spot exons of the CFTR gene. With the use of only five DGGE multiplex systems, nearly 89 per cent of the non-ΔF508 CF alleles were detected in the Celtic population (Férec et al., 1992).

b. Single-stranded Conformational Polymorphism (SSCP)

SSCP is also considered to be a powerful method in mutation detection. It is based on the principle that conformation of a single stranded DNA fragment depends on its nucleotide sequence (Orita et al., 1989). A nucleotide change as small as a substitution, deletion, or insertion of a single base in a 100-400 bp DNA fragment results in an altered electrophoretic mobility. However, there is one major point which has to be precise concerning the efficiency of SSCP: 95 per cent rate of detection can be achieved by analyzing the same DNA fragments under at least three gel conditions (varying the percentage of glycerol, the temperature of the electrophoresis, or the strength of the TBE buffer). Shortening the size of PCR fragments by enzymatic restriction also enhances mutation detection rate. The detection of single base sequence differences by SSCP analysis thus demands a considerable amount of technical work compared to the DGGE method.

II. AIM OF THE STUDY

Cystic Fibrosis (CF) is one of the most common and severe autosomal recessive genetic disorders worldwide. The gene defects causing the disorder are extremely heterogeneous, and point mutations account for a very high percentage. There is no treatment yet for CF. Carrier detection based on the protein product of the CFTR gene is not available. However, the introduction of DNA-based methods has radically improved its accurate diagnosis either by linkage analysis or direct detection of mutations. The identification of point mutations in the CFTR gene presents a challenge because of the large size of the gene and the high number of mutations. A number of rapid screening methods has been developed to screen such large genes for unknown mutations. We have chosen the denaturing gradient gel electrophoresis (DGGE) method to screen for point mutations in the CFTR gene. The variants in the gene will give an idea about the nature and localization of both the mutations and the polymorphisms. The study should reveal the molecular genetics of CF in the Turkish population.

III. MATERIALS

A. Blood Samples

Blood samples from CF patients and their family members were obtained from the Institute of Child Health in Ege University Medical School (Izmir), the Department of Pediatrics, Marmara University Medical School (Istanbul) and the Department of Child Health and Disease, Istanbul University (Istanbul).

B. Equipment

Autoclave	: Eyela Autoclave, MAC-601, Japan
Balances	: Electonic Balance Type 1574, Sartorious, Germany Electronic Balance Libror EB-3200H, Shimadzu, Japan Precision Balance H72, Mewttler, Germany
Camera	: BioDoc Video Documentation System, Biometra, Germany
Centrifuges	: Sorvall RC-5B Refrigerated Superspeed Centrifuge, Dupont, USA Biofuge A, Herauschrist, Germany Hettich EBA 35, Germany Eppendorf Centrifuge 5415C, Germany

Deepfreezers	: -70°C, GFL, Germany -70°C, Sanyo, Japan -20°C, Bosch, Germany
Electrophoresis Equipments	: DGGE, PS500 XT, Hoeffer Scientific Instruments, USA Horizon 58, Model 200, Horizontal Gel Electrophoresis Apparatus, Bio-Rad, USA Miniprotean II, Bio-Rad, USA
Heat-block	: Multi-block Lab-Line, USA
Incubator	: Incubator, Plus Series, Gallenkamp, Germany
Oven	: Oven 300, Plus Series, Gallenkamp, Germany
Microwave Oven	: Microwave Oven, Vestel, Turkey
Shaker	: VIB, InterMed, UK
Thermo-cyclers	: Model 480, Perkin-Elmer Cetus, USA Thermal Reactor TR1, Hybaid, UK Biometra, Germany
Spectrophotometers	: Lambda 3UV/VIS, Perkin Elmer, USA UV/Visible Spectrophotometer, Bio-Project, GmgH, Germany
Waterbath	: Clifton, UK
Magnetic Stirrers	: Mini mag, Iled DK-4000 Roskilde, Intermed, Denmark Chiltern Scientific Hotplate Magnetic Stirrer HS31, UK

C. Chemicals

All chemicals used in this study were purchased from Merck (Germany) or Sigma (USA) unless stated otherwise in the text. Absolute alcohol was purchased from Tekel (Turkey). The enzyme Taq DNA polymerase was purchased from Boehringer Mannheim (Germany).

D. Buffers and Solutions

1. DNA Isolation Buffers

Cell Lysis Buffer	: 155 mM NH_4Cl
	10 mM $KHCO_3$
	0.1 mM EDTA
Nucleus Lysis Buffer	: 10 mM Tris-HCl (pH 8.0)
	400 mM NaCl
	2 mM Na_2 EDTA
Sodium dodecyl sulfate	: 10% (SDS) stock solution
Proteinase K	: 20 mg/ml in H_2O
Ammonium acetate	: 9.5 M stock solution
TE Buffer	: 20 mM Tris-HCl (pH 8.0)
	0.1 mM Na_2EDTA

2. Polymerase Chain Reaction Buffers

Guy's Buffer (1X)	: 16.6 mM $(NH_4)_2SO_4$
	6.7 mM $MgCl_2$
	0.17 mg/ml BSA
	10 mM β-Mercaptoethanol
	67 mM Tris -HCl (pH 8.8)
Boehringer Mannheim	: 1.5 mM $MgCl_2$
Buffer (1X)	50 mM KCl
	10 mM Tris-HCl (pH 8.8)

3. Restriction Enzymes and Digestion Buffers

*Bst*U-I	: 1000 units (10000 units/ml)
NE Buffer 2 (1X)	: 50 mM NaCl
	1 mM DTT
	10 mM MgCl
	10 mM Tris-HCl (pH 7.9)
Mae III	: 50 units (2 units/μl)
Mae III Incubation buffer	: 275 mM NaCl
	6 mM $MgCl_2$
	7 mM 2-mercaptoethanol
	20 mM Tris-HCl (pH 8.2)

4. Electrophoresis Buffers and Gel Systems

a. Electrophoresis Buffers :

5X Tris-Borate (TBE) Buffer	: 445 mM Boric acid
	10 mM Na_2EDTA
	445 mM Tris-base (pH 8.3)
20X Tris-acetate (TAE) Buffer	: 0.4 M Sodium acetate
	0.02 mM Na_2EDTA
	0.8 M Tris-base (pH 7.4)
10X Loading Buffer I	: 2.5 mg/ml BPB
	1% SDS in Glycerol
10X Loading Buffer II	: 20% Sucrose
	1 mM Na_2EDTA
	0.25% BPB
	0.25% XC
	10 Mm Tris (pH 7.8)
Ethidium Bromide	: 10 mg/ml

b. Gel Systems

Agarose gel	: Agarose in 0.5X TBE
NuSieve-agarose gel (2:1)	: 2% NuSieve
	1% Agarose in 0.5X TBE
40% Acrylamide (37.5:1)	: 37.5% Acrylamide
	1% Bis-acrylamide
80% Stock Denaturing Gel	: 6.5% Acrylamide/bis-acrylamide (37.5:1)
	5.6 M Urea

32% Deionized Formamide

1X TAE Buffer

10% Ammonium peroxidisulphate (APS) : 1 g APS in 10 ml H_2O

5. Buffers for Silver Staining

Pre-Stain Fixation Buffer : 10% Ethanol

0.5% Acetic acid

Staining Buffer : 0.1% $AgNO_3$

Developing Buffer : 1.5% NaOH

0.01% $NaBH_2$

0.15% Formaldehyde

Fixing Buffer : 0.75% .Na_2CO_3

E. Oligonucleotide Primers

All primers used in DGGE analysis were kindly supplied by the European Community Concerted Action for Cystic Fibrosis (ECCACF). ARMS amplification system was kindly provided by the Department of Genetics in the Hospital for Sick Children in Perth, Australia. All other primers used in restriction enzyme analysis were purchased from MAM-TUBITAK (Turkey). The sequences for all the oligonucleotide primers used in this study are given in Tables III.1, III.2 and III.3.

Table III.1. Sequences of the PCR primers used in DGGE Analysis

Exon	Oligonucleotide Primer Sequence (5'-3')
2	F-CCAAATCAAGTGAATATCTG R-[40 GC]TAATAATATGAATTTCTCTCTT
3	F-CCAAATCAAGTGAATATCTG R-[40 GC]TTCGTAGTCTTTTCATAATC
4	F-TGTGTTGAAATTCTCAGGGT R-[40 GC]CAGAATATATGTGCCATGGG
5	F-[35 GC] TATTTGTATTTTGTTTGTTGA R-CTTTCCAGTTGTATAATTTA
6a	F-[40 GC] TGGAAGATACAATGACACCTG R-GCATAGAGCAGTCCTGGTTT
6b	F-TATGACTTAAAACCTTGAG R-[40 GC] AAGGACAGAATTACTAACAA
7	F-CATCCTGAATTTTATTGTTA R-[50 GC] ATCATAGTATATAATGCAGC
8	F-[50 GC] TAAAGTAGATGTAATAATGC R-ATTTTATTCGCCATTAGGAT
9	F-TGAAAATATCTGACAAACTC R-[40 GC] CCTTCCAGCACTACAAACTA
10	F-TCCTGAGCGTGATTTGATAA R-[35 GC] ATTTGGGTAGTGTGAAGGG
11	F-[35 GC] CAGATTGAGCATACTAAAGTG R-CATTTACAGCAAATGCTTGCTAG
12	F-ATGACCAGGAAATAGAGAGG R-[30 GC] GCTACATTCTGCCATACCAA
13	F-[35 GC] TATATCTTAAAGCTGTGTCTGT R-TCCCTGCTCAGAATCTGGTA
14a	F-[35 GC] GGTGGCATGAAACTGTACTG R-TGTATACATCCCCAAACTATCT
14b	F-AATAGGTGAAGATGTTAGAA R-[40 GC] ATAAAACACAATCTACACAA
15	F-TCAGTAAGTAACTTTGGCTGC R-[40 GC] CCTATTGATGGTGGATCAGC
16	F-[25 GC] TCTGAATGCGTCTACTGTGA R-GCAATAGACAGGACTTCAAC
17a	F-[35 GC] TGCAATGTGAAAATGTTTAC R-CTCTTATAGCTTTTTTACAA
17b	F-[40 GC] TTTGTGTTTATGTTATTTGC R-TGCAGCCATTTTATTCATTGA

Exon	Oligonucleotide Primer Sequence (5'-3')
18	F-TAGGAGAAGTGTGAATAAAG R-[40 GC] ATACTTTGTTACTTGTCTGA
19	F-GTGAAATTGTCTGCCATTCT R-[45 GC] AGGCTACTGGGATTCACTTA
20	F-[35 GC] TATGTCACAGAAGTGATCCC R-TGAGTACAAGTATCAAATAGC
21	F-TGAAATATTTTACAATACAATAAGGG R-[40 GC] GCCATTTGTGTTGGTATGAG
22	F-TTTTAGAATGTCAACTGCTT R-[50 GC] ATGATTCTGTTCCCACTGTG
23	F-[40 GC] CTGTTCTGTGATATTATGTG R-TTTCTGTCCCTGCTCTGGTC

F: forward primer, R: reverse primer

Table III.2. The Sequences of Allele Specific Primers Used for the ARMS Test

Mutation	Code	Oligonucleotide Primer Sequence (5'-3')
N1303K	NK-C NK-N NK-M	F-TTCTTTATTCTAAAGACATTGG R-CCACTGTTCATAGGGATCCAAG R-CCACTGTTCATAGGGATCCAAC
W1282X	WX-C WX-N WX-M	F-TTTTACCTTATAGGTGGGCCTC R-TATCACTCCAAAGGCTTTCCAC R-TATCACTCCAAAGGCTTTCCAT
Internal Control	11i3 11i5	F-CAACTGTGGTTAAAGCAATAGTGT R-GCACAGATTCTGAGTAACCATAAT

Table III.3. The Sequences of the Primers Used in Restriction Enzyme Analysis

Exon	Oligonucleotide Primer Sequence (5'-3')
Exon 6a	F-TTAGTGTGCTCAGAACCACG R-CTATGCATAGAGCAGTCCTG
Exon 7	F-AGACCATGCTCAGATCTTCCAT R-GCAAAGTTCATTAGAACTGATC

F. Dna Markers for DGGE Assay

The markers for most of the exons were provided by Centre Hospitalier Henri Mondor Laboratoire De Biochimie in France, as part of the ECCACF.

IV. METHODS

A. DNA Extraction

Five to ten ml of blood samples from patients and family members were collected into K_2EDTA containing tubes to prevent coagulation. For best results, it is advised to use fresh blood samples or samples stored for less than three days. The most commonly used methods for DNA extraction require use of phenol-chloroform. Recently salting out of proteins is preferred because it is safer (no hazardous organics), easy to use, economical, and results in high recovery of DNA. When ammonium acetate was used instead of NaCl, contamination of DNA samples with Na^+ is avoided. The effectiveness of ammonium acetate for removing proteins was reported immediately following the publication of Miller's salting out procedure which required NaCl (Miller et al., 1988; Crouse et al., 1988). Hence, in our adapted procedure, DNA extraction in the presence of ammonium acetate rather than NaCl improved the recovery of DNA.

For extraction of its DNA, the blood sample was transferred into a Sorvall centrifuge tube, and ice cold lysis buffer was added (30 ml of buffer per 10 ml of blood sample), then mixed. The sample was kept at +4°C for 15 min to allow lysis of cell membranes. It was later centrifuged at 5000 rpm for 10 min at +4°C to collect leucocyte nuclei. The fixed angle rotor was used in all centrifugations. The supernatant was discarded, and after resuspending the nuclear pelletin the lysis buffer, centrifugation was repeated. The pellet was washed with one to two ml of lysis buffer in order to remove contaminants remaining in the tube. After the second centrifugation step, the pellet (leucocyte nuclei) was ready to be treated with Proteinase K. The nuclei were suspended in three milliliters of nucleus lysis buffer and vortexed till clumps disappeared completely. The sample was incubated with proteinase K (150 µg/ml) and SDS (0.14 %) at 37°C overnight or at 56°C for three hours to degrade cellular proteins. Afterwards 0.6 ml of 9.5 M ammonium acetate per ml of the lysate was added. The tube was shaken vigorously to enhance the separation of DNA from the proteins and other cell debris. The sample was

centrifuged at 10000 rpm at room temperature for 20 min. The supernatant containing DNA was taken into a clean Falcon tube and the DNA was precipitated with the addition of two volumes of absolute ethanol. The tube was gently inverted several times until DNA thread became visible. The DNA precipitate was fished out, put into 0.5 to 1 ml of TE buffer in an Eppendorf tube and left overnight at room temperature to dissolve completely.

B. Qualitative and Quantitative Analysis of the Isolated DNA Samples

There are two different approaches for quantitative and qualitative analyses of the extracted DNA samples: Spectrophotometric assay and the minigel method. The former was used for precise determination of the concentration and purity, while the latter was preferred for its quick and easy application.

1. The Spectrophotometric Method

The concentration of DNA was calculated after reading the optical density at 260nm and applying the formula :

$$50\ \mu g/ml \times OD_{260} \times \text{dilution factor} = \text{concentration in } \mu g/ml$$

Since 50 μg of double stranded DNA has an absorbance of 1.0 at OD_{260}. Also, the purity of the DNA sample was determined by taking the ratio between the optical densities at 260 nm and 280 nm. $OD_{260/280}$ as 1.8 is the reference value for a sample of high purity.

2. Agarose Gel Electrophoresis

One per cent agarose gel was prepared by boiling 1 g agarose in 100 ml of 0.5XTBE buffer and then adding 5 µl ethidium bromide (10mg/ml). The gel was poured onto an electrophoresis plate and left at room temperature to polymerize. One or two µl of extracted DNA sample was mixed with 5 µl 1X loading buffer and applied to the agarose gel. The gel was electrophoresed at 150V and the band was visualized under UV light. The amount of DNA was estimated by comparing the intensity of its fluorescence with that of a known sample, typically a 200 ng DNA.

C. Polymerase Chain Reaction (PCR)

Amplification of a DNA segment via polymerase chain reaction using a thermostable DNA polymerase represents the most important advance in mutation detection technology (Saiki et al., 1985). All procedures described in this study are based on PCR amplification of the sample DNA prior to analysis.

1. PCR for the Amplification Refractory Mutation System (ARMS) Test

In screening for the mutations W1282X and N1303K in exon 20 and exon 21, respectively, a modified version of the PCR technique (ARMS) was applied. ARMS is based on the requirement of a primer to be perfectly matched to the template DNA at its 3' end for annealing and primer extension during the amplification reaction. An amplification system provided kindly by the Department of Genetics in the Hospital for Sick Children in Western Australia included ARMS primers specific for the two normal alleles (normal

primers NK-N and WX-N), two primers identical in sequence to the normal ones except for the terminal 3' nucleotides, thus, specific for the mutations W1282X and N1303K (mutant primers NK-M and WX-M), two common primers (NK-C, WX-C), and the primers flanking exon 11 (11i3 and 11i5). The lattest primers served as an internal control in the amplification reactions (Ferrie et al., 1992).

In order to determine the presence or absence of W1282X and N1303K, PCR amplification was carried out in two different PCR reactions. One reaction tube (Tube N) contained 2.5 µl Taq polymerase buffer, 40 µM dNTP, 1.5 mM $MgCl_2$, 50 pmole of reverse primers (NK-C, WX-C) together with the same amount of normal primers (NK-N, WX-N) and the primers for exon 11, 200-300 ng of genomic DNA and 1U of Taq polymerase enzyme in a total volume of 25 µl. The content in the second reaction tube (Tube M) was similar except that here, primers complementary to the 3' residues of each mutation (NK-M and WX-M) were used instead of the normal primers. The samples were covered with a few drops of mineral oil to prevent evaporation. They were then placed into the thermocycler, namely Perkin Elmer Cetus DNA thermal cycler, and the following program with a ramp time was performed: 5 minutes denaturation at 94°C, 28 cycles consisting of 30 seconds at 94°C, 30 seconds primer annealing at 55°C and 2 minutes extension at 72°C. Final extension was at 72°C for 8 minutes.

5 µl of the PCR product was mixed with 5 µl of 1X loading buffer and run on a two per cent agarose gel in order to detect the allele specific products. The gel was electrophoresed at 150V and the band was visualized under UV light.

2. Screening for the Mutations L346P and Q220X by Restriction Enzyme Analysis

The principle of restriction enzyme analysis method is based on digestion of an amplified exon with the suitable restriction enzyme. The cutting sites are either generated or abolished by a point mutation. In the case of the mutation L346P, a change of the C to a T at nucleotide 1169 results in the substitution of Leucine by Proline at codon position 346 (Boteva et al., 1994). This change creates a recognition site (CG↓CG) for the restriction

enzyme *Bst*U-I in addition to three already-existing sites located within the GC clamp, which is at the 5' end of one of the primers in the 308 bp fragment. Thus, fragments of 201 bp, 87 bp and smaller fragments (9, 7 and 4 bp) are obtained as a result of digestion of the clamp. The GC clamp of the primer is not important for this technique. In the absence of the mutation, the resulting fragments are a 288 bp piece and smaller ones originating from the GC clamp. On the other hand, the mutation Q220X which is a T to C substitution at nucleotide 790 (Shackleton et al., 1994), abolishes the only *Mae*III cutting site (↓GTNAC) in the 270 bp fragment. In normal alleles 121 bp and 149 bp digestion fragments are visualized.

a. PCR Amplification of Exon 6a and Exon 7

In screening for the mutations L346P and Q220X in exon 7 and exon 6a, respectively, primers which had been synthesized by TUBITAK according to the sequences published by Zielenski et al., (1989) were used (Table III.1). Amplification of the exons were carried out in similar reaction conditions except that the use of exon specific primers differed. A volume of 25 µl contained 2.5 µl of 10X Guy's Buffer, 25 pmole of each corresponding primer (7i3 and 7i5 for exon 7, and 6ai3 and 6ai5 for exon 6a), 0.3 mM dNTP, 200-300 ng of genomic DNA and 1-2 units of Taq polymerase enzyme. The following PCR program was performed: 5 minutes initial denaturation at 94°C, then 30 seconds at 94°C, 30 seconds primer annealing at 55°C and 2 minutes extension at 72°C for a total of 35 cycles. Final extension was at 72°C for 5 minutes.

b. Purification of the PCR product

Before restriction enzyme analysis, amplified DNA was purified by ethanol precipitation according to the following procedure: 20 µl of the amplified product was taken into a 0.5 ml tube. First 2 µl of ammonium acetate (3 M), then twice the volume absolute ethanol were added. The mixture was centrifuged at 10000 rpm for 15 minutes in a minifuge. In washing the pellet with 70 per cent cold ethanol, the centrifugation step was

repeated, but this time at 5000 rpm for five minutes. The supernatant was discarded and the pellet was left to dry at 56°C for about ten minutes, then dissolved in 10 µl of distilled water.

c. Digestion with Restriction Enzymes

Digestion for L346P detection was carried out at 60°C overnight according to the assay conditions recommended by the supplier. 10 µl of the PCR product of exon 7 was digested with 1U of *Bst*U-I enzyme in a total volume of 20 µl: A volume of 2 µl 10X resriction enzyme buffer (NE Buffer II) and 8 µl water were also added. Incubation for Q220X detection was carried out in a total of 15 µl which contained 1U of *Mae*III restriction enzyme, 1.5 µl 10X incubation buffer mixed with 3 µl DNA and 10.5 µl water. Total mix was then left for digestion at 55°C overnight as was recommended by the supplier.

d. Analysis of the enzyme-treated fragments

5 µl.of each enzyme-treated fragment was mixed with 5 µl 1X loading buffer and run on a 2:1 NuSieve-agarose gel in order to detect the digestion products. The gel was electrophoresed at 150V and the bands were visualized under UV light.

3. Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE is a gel system which detects variations among amplified DNA fragments by taking advantage of differences in their melting properties. Electrophoresis of a dsDNA fragment in a gradient of increasing denaturants results in its denaturation when it reaches the concentration of denaturants equivalent to its melting temperature (T_m). When the

molecule is at its T_m the branching caused by partial melting sharply decreases its mobility in the gel. The corresponding domains of different DNA fragments differing by as little as a single base substitution melt at slightly different denaturant concentrations. One of the primers in the pair bears an additional GC rich region, called a GC clamp, which creates a high temperature melting domain, to facilitate the detection of sequence variations that are in higher temperature domains.

a. PCR for DGGE Analysis

All of the gene except for exons 1 and 24 was studied by denaturing gradient gel electrophoresis. Fifteen of the exons were analyzed in five triplex systems, each of which amplified three exons in the same reaction. The exon fragments in each triplex system have different melting temperatures (Tm), but a common annealing temperature for their PCR primers (Table V.1). All of the triplex premixes were provided by the European Community Conserted Action for the Coordination of Cystic Fibrosis Research and Therapy (ECCACF) and contained all the reagents needed in PCR except for the DNA template and the Taq polymerase. PCR amplifications were carried out in 50 µl. Each reaction was prepared by mixing 40 µl of the premix, 2 µl of diluted DNA (100ng/µl), 1 unit of Taq polymerase and 8 µl of water. Each primer was used at 0.2 µM. After an initial denaturation at 94°C for 5 minutes, 40 PCR cycles of 1 minute at 94°C, 1 minute at the suitable annealing temperature and 2 minutes at 72°C were performed for each triplex. There was a final incubation at 72°C for 7 minutes. As a thermocycler, Cetus was preferred over Hybaid and Biometra because of the necessity for a proper ramp time which was obviously needed for the optimization of the PCR conditions.

A total of 11 exons (not included in the triplex reactions) from each patient were amplified individually, in a volume of 25 µl which contained 1X Guy's buffer, 0.2 µM of each primer, 0.35 mM of each dNTP, 0.5 units of Taq polymerase and 200 ng of genomic DNA. The amplification was carried out in Cetus with the same program that was used for triplex amplification, but a suitable annealing temperature was chosen for each pair of primers (Table V.2).

After PCR was completed, a 3.5 µl aliquot the product was mixed with 5 µl 1X loading buffer I and electrophoresed on a 2:1 NuSieve-agarose gel, and the quality and quantity of the amplification was determined under UV light on a transilluminator.

b. Heteroduplex Formation

Four µl of each PCR product was mixed with the same amount of a known normal sample. The mixture was heated to 95°C for 5 minutes to allow denaturation. The samples were then renaturated at 65°C for 15 minutes in order to enhance heteroduplex formation between the wild-type and the mutant alleles.

It became obvious that during the application of the DGGE technique, one particular homozygous mutant genotype remained indistinguishable from the homozygous wild-type, having similar DGGE patterns. It was thus justified that the search for mutations by DGGE analysis was needed to be further improved by heteroduplex analysis.

c. Preparation of the Gel System and Electrophoresis

Just prior to the assembly of the DGGE apparatus, the glass plates were cleaned with ethanol to remove any dust and oily fingerprints. The side spacers which were 0.75 mm thick were placed along the length of the gel. Clean plates are essential to pour gels without bubbles which disrupt the continuous formation of the gradient and electrophoresis. After sealing across the full width of the spacers using 1 per cent agarose, the plates were inserted between two side frames containing several screws for tightening, and placed on a base. The screws were tightened but not very firmly, pressing the glass plates against the spacers. The use of the clamps ensured that the bottom edge of the plates were firmly sealed above the base. In addition, pouring one per cent agarose all along the base prevented any possible leakage arising especially when the two plates do not match well.

One of the most important factors in the DGGE method is the formation of a continuous linear concentration gradient. For this purpose, a gradient maker which had two chambers connected to each other by a stopcock was used. The right chamber was connected to the peristaltic pump by another stopcock. The pump reached the gap between the glass plates through a thin plastic tube with a needle at its end. Peristaltic pump ensured a constant flow of acrylamide. Schematic model of the DGGE apparatus is shown in Figure IV.1. Two gel solutions were prepared by diluting the two 6.5 per cent acrylamide stock solutions (one with 80 per cent denaturant concentration, the other being nondenaturing) so that when 8 ml of each gel solution were mixed, the desired denaturant concentration range was obtained. Seven molar urea and 40 per cent formamide together correspond to 100 per cent denaturant concentration. A 3 ml solution without denaturant was also prepared. 80 µl APS and three µl TEMED were added to the gel solutions just before pouring them into each the chamber. The higher denaturant solution was poured into the right chamber while the lower denaturant solution was poured into the left chamber of the gradient maker. The peristaltic pump was turned on. The higher denaturant solution was the first to exit the gradient maker, and when it entered the gel chamber of the electrophoresis apparatus, the stopcock between the chambers of the gradient maker was opened. The solutions were allowed to be totally transferred to the gel chamber. A 1-3 cm space remained below the 15-tooth comb. Within 15 minutes, the appearance of a thin straight line on top of the gel signalled the start of polymerization. Three ml acrylamide solution without denaturant was poured to fill this space. The gel was allowed to polymerize for at least one and a half hours. Prior to loading, 3 µl of the PCR product was mixed with 5 µl of loading buffer II and applied to the gel. Products were run at constant voltage (160V) and temperature (60°C) in 1XTAE buffer, but the run time varied from one exon to another. The electrophoresis conditions, i.e. the range of denaturant concentrations in the gel and the duration of electrophoresis for both the triplex systems and the individual exons, are shown in Table V.1 and Table V.2. After electrophoresis was completed, the DGGE apparatus was disassembled by separating the glass plates.

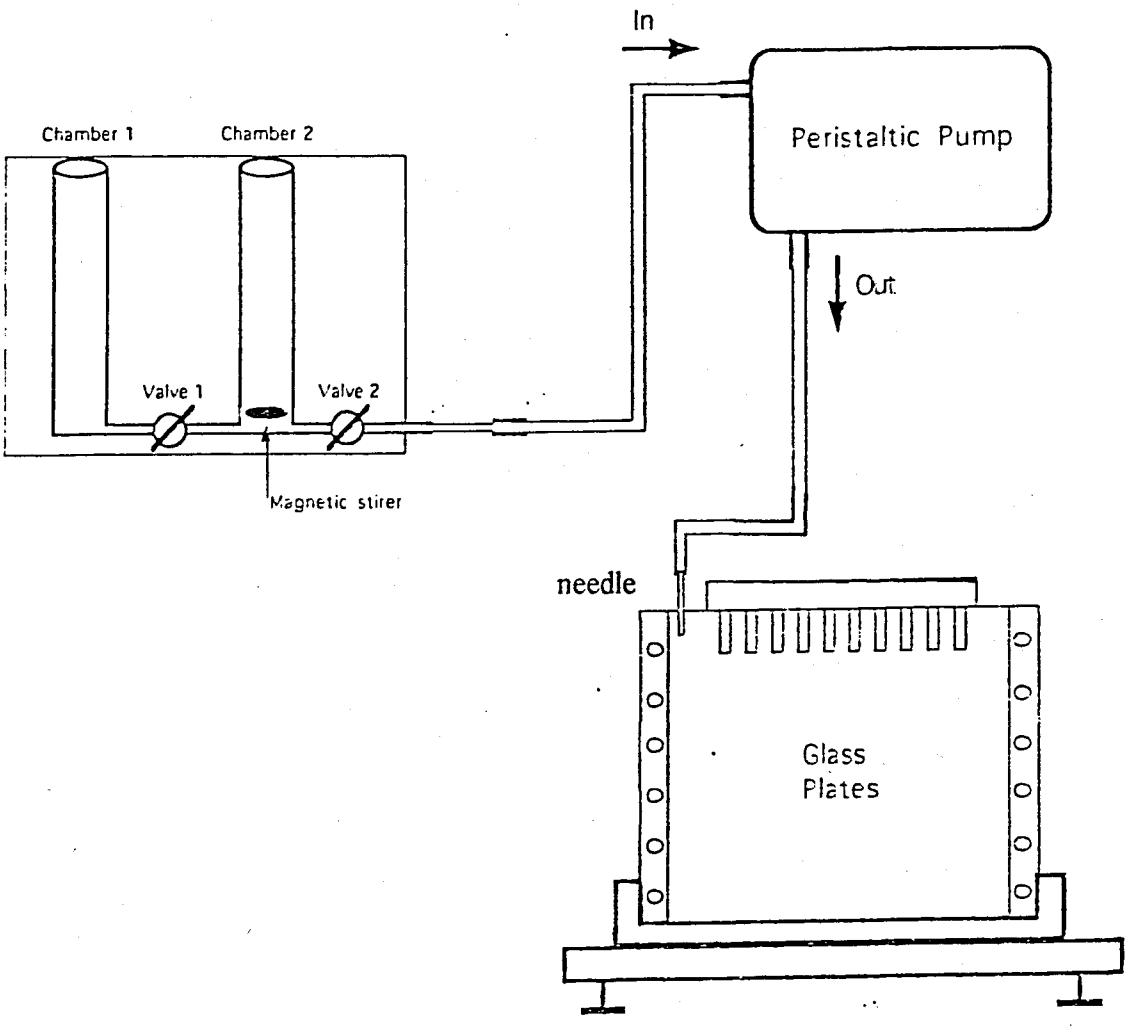

Figure IV.1. Schematic model of the DGGE apparatus. A gradient maker with two chambers connected to each other by valve 1 was used. The right chamber was connected to the peristaltic pump by valve 2. The pump reached the gap between the glass plates through a thin plastic tube with a needle at its end.

d. Staining

In the early part of the study, the DGGE gel was stained with ethidium bromide (EtBr). Later silver staining was preferred because a highly sensitive detection of nucleic acids, i.e. in the nanogram range, can only be achieved by this method. A photochemical reaction in which silver binds to nucleic acid bases provided a level of sensitivity considerably higher (at least five fold) than that of staining with ethidium bromide. In addition, separation on polyacrylamide lacks the resolution power with ethidium bromide especially for bands which are very close to each other. Gels were stained with silver using the method of Sanguinetti et al., 1994. A similar protocol was previously reported for various purposes such as protein assay or SSCP anaysis (Lenk et al., 1993). The protocol consists of four steps:

1. Pre-Stain Fixation Step: The gel was incubated twice with 300 ml of 10 per cent ethanol and 0.5 per cent acetic acid solution for three minutes to prevent diffusion of the DNA.
2. Staining Step: The gel was stained with 150 ml of 0.1 per cent $AgNO_3$ for 15 minutes and washed twice with Milli-Q water.
3. Developing Step: The gel was washed twice with Milli-Q water to remove excess silver ions and then incubated with a freshly prepared mixture of 1 per cent NaOH, 0.01 per cent $NaBH_4$ and 0.15 per cent formaldehyde until the bands became well visible.
4. Fixation Step: The gel was washed again with Milli-Q water and soaked for up to 5 minutes with a 0.75 per cent Na_2CO_3 in order to fix the stain.

V. RESULTS

A. Screening for the Mutations N1303K and W1282X using the ARMS Test

A total of 124 chromosomes from 62 patients were investigated in the framework of this study. DNA samples were subjected to the ARMS test according to the method described in Section IV.C.1. Two patients were found to carry the mutation W1282X, whereas only one patient carried the mutation N1303K. The results of the ARMS analysis are presented in Figure V.1. In one of the patients with the mutation W1282X, the other CF allele was ΔF508, the most common mutation. In the other patient, the second mutation is not yet identified. In the patient with the mutation N1303K, who died of CF at the age of seven, the other allele was already known to carry the insertion mutation 2184Ains in exon 13. The pedigree of this family, whose three other affected children died soon after birth while two other children are healthy, is shown in Figure V.2. Mutation analysis indicated that the maternal CF allele was 2184Ains, and the paternal CF allele was W1282X and linked to a variant DGGE pattern in exon 6a. When the 24-year old sister of the patient requested a DNA analysis for herself and her husband, who is also her cousin, she was already eleven-week pregnant. The methods, ARMS and DGGE were applied to the sister's and her husband's DNA samples. The results of the analyses showed that the sister had inherited W1282X together with the yet uncharacterized variation in exon 6a, whereas her husband was found not to carry any of the two mutations in the family, nor any variation in the remaining exons screened by DGGE.

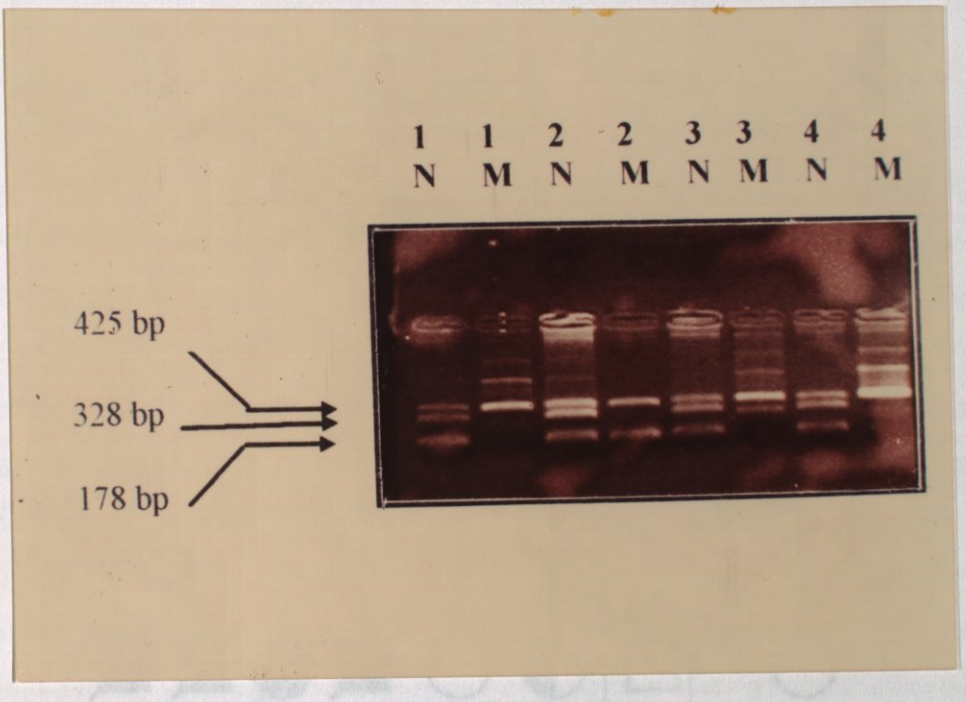

Figure V.I. ARMS analysis for the two mutations. Amplified fragments were analyzed on 2:1 NuSieve-agarose gel. The numbers correspond to patients. Lanes indicated N contain samples amplified with the normal primers, those with M with the mutant primers. The uppermost band is the internal control, the second and third bands are ARMS bands for the mutations N1303K and W1282X, respectively. The first and the last patients are normal with respect to the two mutations. The second patient is heterozygous for the mutation W1282X, and the third patient for N1303K.

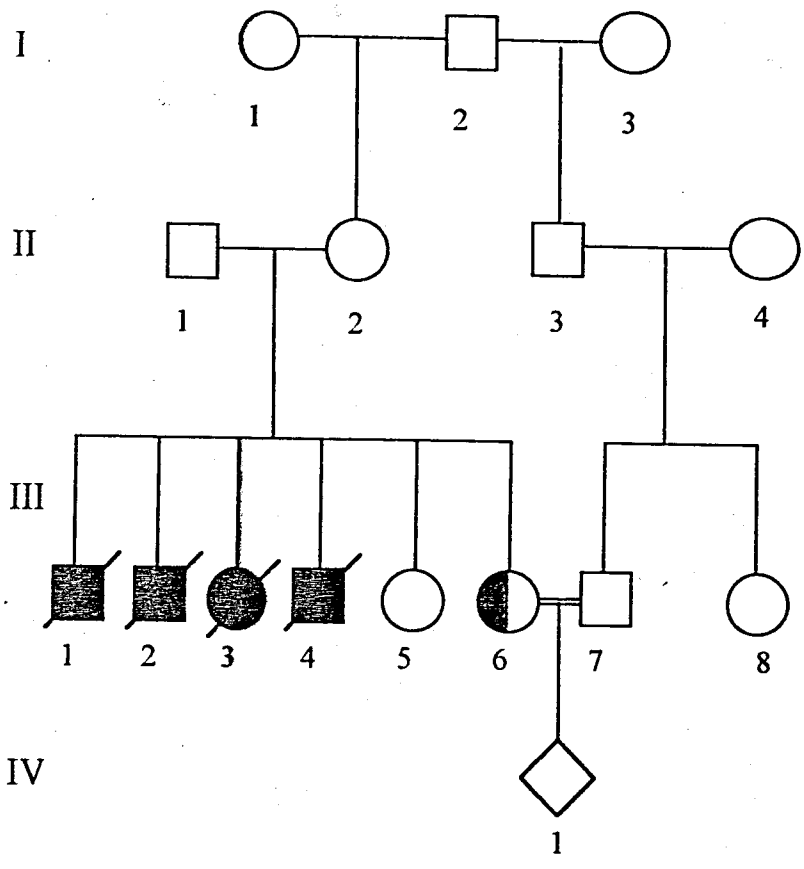

Figure V.2. The pedigree of Family 1. The methods DGGE and ARMS when applied to patient III.4 revealed the presence of two mutations (W1282X in exon 20 and 2184Ains in exon 13), heterozygosity for the polymorphism 1540A/G in exon 10 and another yet uncharacterized variation in exon 6a. The pregnant sister (III.6) was found to carry both the mutation W1282X and the uncharacterized variation in exon 6a. Her husband, who is also her cousin, does not carry any of these mutations, nor any other mutation in the exons screened. As a result, the fetus was not at risk.

B. Restriction Enzyme Analysis

Restriction enzyme digestion was used to screen for the mutations L346P in exon 7 and the mutation Q220X in exon 6. For this purpose, the region containing these mutation sites were PCR-amplified using the amplification protocol described in Section IV.C.2.a, and then digested with the corresponding restriction enzymes: *Mae*III for Q220X and *Bst*U-I for L346P. The digested products were resolved on a 2:1 NuSieve-agarose gel. Figure V.3 demonstrates the *Mae*III digestion of the 270 bp fragment of exon 6a PCR product. The presence of a 121 bp and a 149 bp fragments indicated the absence of the mutation Q220X in the patients screened.

The digestion results of exon 7 by *Bst*U-I is shown in Figure V.4. The presence of a 288 bp fragment resulting from digestion of only the GC clamp indicates the absence of the mutation L346P in all patients. The digested products were run on a 2:1 NuSieve-agarose gel. The control sample in lane 3 is a 308 bp fragment not treated with the enzyme.

The two screening studies included all of the CF chromosomes and indicated that neither of the mutations was present in any of the chromosomes analyzed.

C. Screening of twenty-five exons by DGGE Analysis

The low frequencies in our CF patients of the most common CF mutation, ΔF508, as well as the other worldwide frequent mutations such as N1303K and W1282X, and the absence of Q220X and L346P which are frequent in the neighboring geographical areas led us to extend our investigation in the direction of screening the whole gene exon by exon using an efficient and reliable method. Hence, in our large-scale screening program, we included the application of the denaturing gradient gel electrophoresis (DGGE) which is well suited to the rapid characterization of any sequence variation in the coding and adjacent regions.

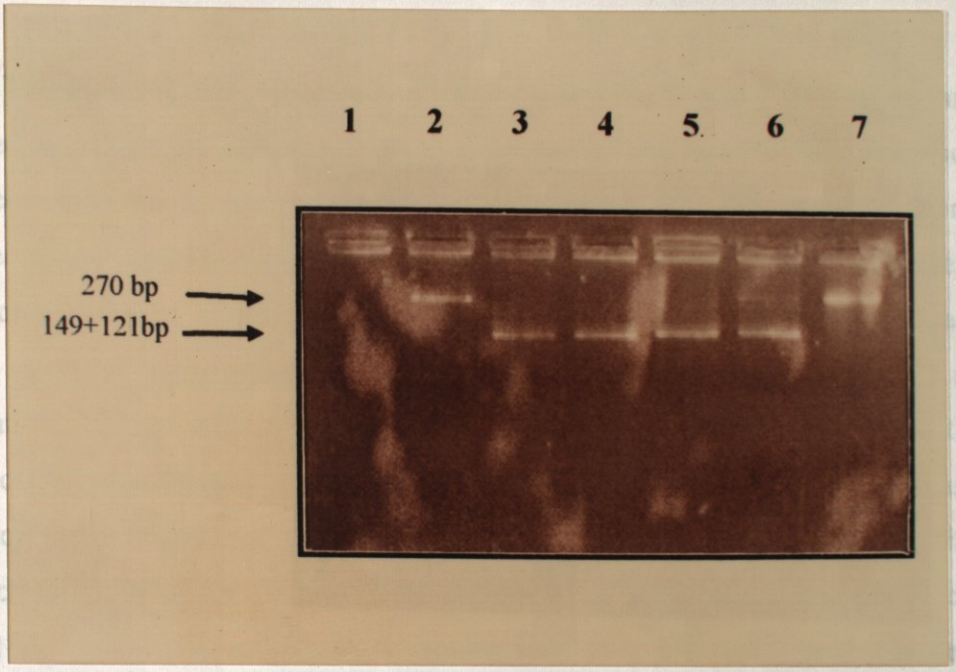

Figure V.3. Restriction enzyme analysis for the mutation Q220X using *Mae*III. Lanes 2, 3, 4 and 5 are all digested samples, thus are normal with respect to Q220X. The control samples in lanes 2 and 7 are fragments not treated with the enzyme. Lane 1 is blank.

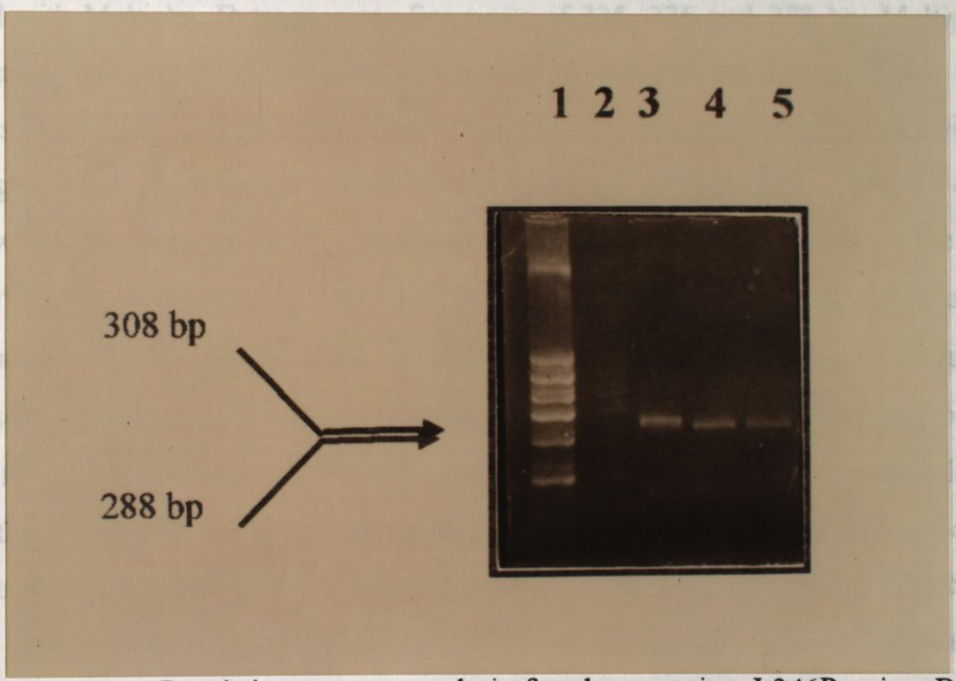

Figure V.4. Restriction enzyme analysis for the mutation L346P using BstU-I. Lanes 4 and 5 are samples digested only at the GC-clamp which carries several cutting sites, thus giving rise to fragments of less than 10 bp which cannot be detected on the gel. The control sample in lane 3 is the 308 bp fragment not treated with the enzyme. The sample in lane 1 is a size marker.

Twenty five CFTR exons out of 27 (excluding the first and the last exons) were amplified by using GC-clamped primers as described in Section IV.C.3.a. The nucleotide sequences of the primers are given in Materials (Section III.E). Fifteen of the exons were amplified in five triplex systems, each of which facilitate the amplification of three exons. The choice of three exons in each set were according to three experimental parameters: Similar annealing temperatures of the primers in the PCR reaction, different melting temperature (T_m) and the same electrophoresis time. The second condition was that, T_m values of the fragments in the same set must differ sufficiently to allow reasonable separation in electrophoresis (Costes et al., 1993). Optimized conditions for amplification and electrophoresis of the triplex systems are shown in Table V.I. Multiplex A contains exons 17b, 11 and 14b to give PCR fragments of 266, 223 and 168 bp, respectively, whereas exons 14a, 15 and 20 were amplified with Multiplex B to give rise to 276, 390, and 302 bp fragments. Multiplex C analysis involved simultaneous amplification of exons 3, 12, and 23 which are 323, 296, and 242 bp, respectively. While exon 6a, 9 and 21 were amplified with Multiplex D to generate fragments of 335, 375 and 272 bp, Multiplex E amplification was performed to analyze exons 5, 8 and 18 simultaneously to obtain fragments of 235, 302 and 277 bp. All other remaining exons were amplified individually according to their annealing temperatures (T_a) indicated in Table V.2. The T_a of the primers which were selected to generate fragments suitable for DGGE analysis were either 45°C, 50°C or 55°C (Fanen et al., 1992). The proper selection of the range of denaturant concentrations and the electrophoresis run time ensured maximum resolution. 25 µl PCR volume was sufficient for single amplifications. However, 50 µl reaction volume was essential for proper annealing and extension of the primers in triplex systems. In addition, because the presence of heteroduplexes is crucial to this type of analysis, PCR conditions which are described in Section IV.C.3.a were used to optimize the annealing of allelic DNA strands. Amplification products were checked on a 2:1 per cent NuSieve-agarose gel. Figure V.5 shows the PCR products of the exons which were amplified either one by one or with Multiplex systems.

Table V.1. Optimized conditions for amplification and electrophoresis of multiplex systems

Multiplex	Exon	Amplified product (bp)	Annealing temperature (°C)	Denaturant range (%)	Running time (h)
A	11	224	50	10-60	3
	14b	168			
	17b	266			
B	14a	276	55	10-60	5
	15	390			
	20	302			
C	3	323	50	10-60	3
	12	296			
	23	242			
D	6a	345	55	10-60	5
	9	375			
	21	272			
E	5	235	45	10-60	3
	8	302			
	18	277			

Table V.2. Optimized conditions for individual amplification and electrophoresis for the exons 2, 4, 6b, 7, 10, 13, 16, 17a, 19 and 22

Exon	Amplified product (bp)	Annealing temperature (°C)	Denaturant range (%)	Running time (h)
2	240	50	10-60	3
4	369	55	20-70	5
6b	301	50	10-60	4
7	365	50	10-70	6
10	336	55	10-60	4
13	516	55	10-60	4
16	323	55	10-60	3
17a	283	45	10-60	4
19	407	55	20-70	5
22	340	55	30-80	5

Three fragments amplified in a single reaction were analyzed in a single lane of the DGGE gel. The PCR products of all five Multiplex systems were electrophoresed on a 6.5 per cent polyacrylamide gel containing a linearly increasing denaturant gradient from 10 to 60 per cent at 160V with a run time of 5 hours for Multiplex B and D, and 3 hours for A, B and E. Optimized conditions for individual amplification and electrophoresis for the exons 2, 4, 6b, 7, 10, 13, 16, 17a, 19 and 22 are shown in Table V.2. The DGGE parameters for primer pairs specific to each exon were described in Fanen et al., 1992. However, modifications in the denaturant range for some of the exons were necessary to obtain optimum resolution of the bands, which was essential for observation of possible heteroduplexes and homoduplexes properly. Hence, the optimization of the technique under our laboratory conditions was essential.

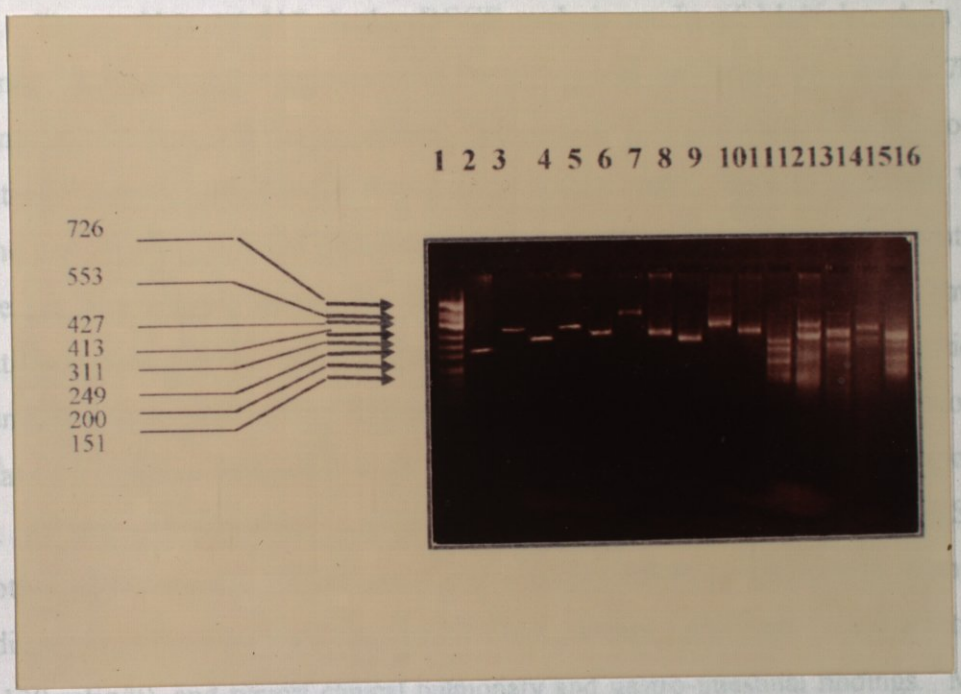

Figure V.5. PCR amplification products of the exons which were amplified either individually or in a triplex system. The fragments were resolved on a 2:1 per cent NuSieve-agarose gel. Lane 1 (M) is the ϕX174 Hinf I DNA size marker. Lanes 2-11 represent the amplification products of the exons 2, 4, 6b, 7, 10, 13, 16, 17a, 19 and 22, respectively. Lanes 12-16 show the PCR amplification products of Multiplex systems A, B, C, D and E, respectively.

1. Analysis of the Spot Patterns

Samples were subjected to staining either with silver or ethidium bromide after electrophoresis, and the bands were analyzed for the presence of altered migration patterns. Silver staining method was preferred after comparison of the efficiencies of the two staining methods. When the analysis of 25 exons was completed, a complete spot pattern was generated, consisting of 47 spots for the 25 exons.

a. Analysis with Multiplex A

Figures V.6 and V.7 display DGGE analysis results of Multiplex A in a set of patients. Altered patterns were detected for exon 11 in one patient, for exon 17b in four patients and for exon 14b in two patients. Because exon 11 and exon 17b are known to be mutation hot spots, their simultaneous analysis is very useful. As seen in Figure V.6 (lane 4), the patient who displayed variations in both exon 17b and exon 14b was identified in a single gel lane using multiplex A. Upon comparision with mutant marker samples, the variation in exon 17b was identified as the mutation R1069Q, a missense mutation in the transmembrane domain of the CFTR protein. R1069Q accounts for three variations out of a total of four cases detected in exon 17b, constituting a total allelic frequency of 2.4 per cent. All cases were heterozygous for the mutation. One of the patients carried S466X on the other allele, whereas another patient carried a yet unknown variation in exon 10. The CF diagnosis in those patients was based on two positive sweat tests (varying between 120 and 130 mEq/lt), and typical clinical pulmonary and gastro-intestinal findings. Meconium ileus (MI) was not present, while pancreatic insufficiency was common. The clinical data suggested that the severity of the disease varied possibly with the mutation on the other allele. The fourth variation in exon 17b displayed a different migration pattern than the mutation R1069Q (Lane 5 in Figure V.8).

Only one variation was detected in exon 11 and it was in a homozygous case. This variation could not be detected without the formation of heteroduplexes with a normal control, and was deduced to be G542X after comparision with a mutant marker. The banding pattern totally agreed with that of the marker DNA kindly supplied by the Laboratory of Molecular Pathology in the University Obstetrics and Gynaecology Hospital in Sofia. The clinical expression was very similar to that patients reported for the the same mutation (Klaydjieva et al., 1991; Osborn et al., 1992). In this patient, CF was diagnosed very early (soon after birth) by a positive sweat chloride test (130mEq/lt). The patient was born to related healthy but parents, and developed diarrhea, excreted fatty stools and had recurrent pulmonary infections.

The homozygous variant in exon 14b migrated a little slower than a normal sample and is shown in lane 13 in Figure V.6 and lanes 8 and 9 in Figure V.8 This variation could be easily distinguished from a normal sample, unlike the case of G542X. As shown in Figure (V.8), Multiplex A and C were coelectrophoresed in order to accelerate the DGGE analysis. T_m values corresponding to each exon differ significantly, thus the migration of all six exons slowed down at different points in the DGGE gel.

b. Analysis with Multiplex B

Multiplex B analysis involved simultaneous amplification of exons 14a, 15 and 20. Analysis with this triplex system characterized the second most common polymorphism 2694T/G (exon 14a) after 1540A/G (exon 10) (Zielenski et al., 1991). Their frequencies are 12 per cent and 37 per cent, respectively. The mutation W1282X in exon 20 was found in two alleles, confirming the results of the ARMS test. The heteroduplex migration pattern which is specific to this mutation can be observed in lane 4 in Figure V.9. Another variant pattern in exon 20 was detected in one heterozygous individual. No variants in exon 15 was observed. Only one variation in exon 14a was observed, which was in a homozygous individual. The heteroduplex analysis identified a different migration pattern than that of the polymorphism 2694T/G.

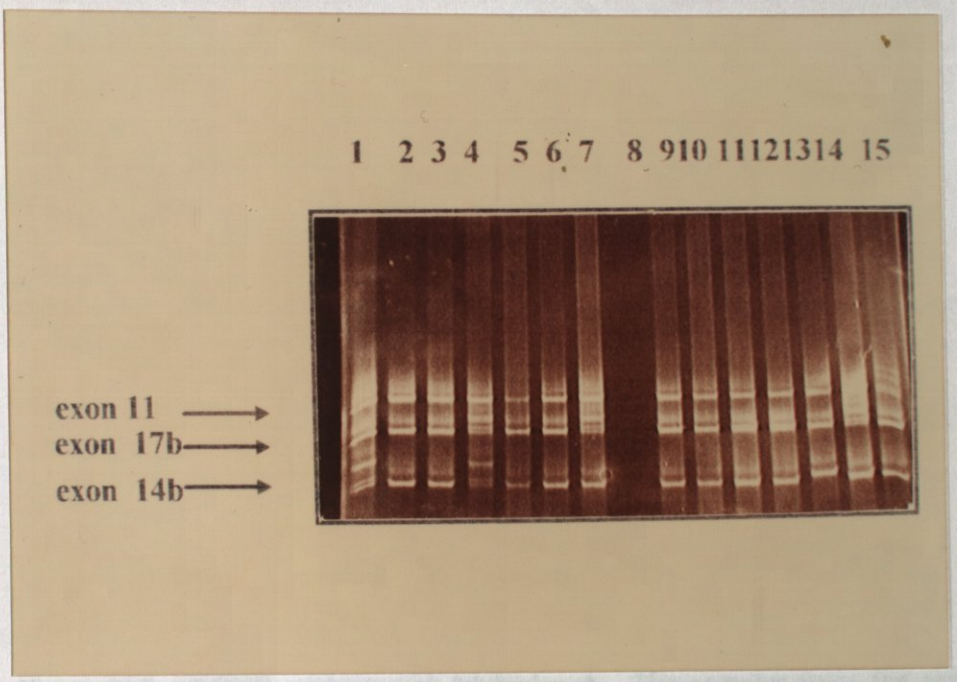

Figure V.6. DGGE analysis with Multiplex A. Samples were stained with ethidium bromide after electrophoresis, and the bands were analyzed for the presence of altered migration patterns. The patient who displayed variations in both exon 17b and exon 14b can be seen in lane 4. The variant pattern in exon 17b can also be observed in lanes 7 and 14, and was identified as the mutation R1069Q. Lane 15 contains the marker DNA which is heterozygous for G542X. Lane 13 shows the homozygous case in exon 14b which migrated a little slower than the normal samples. Lane 1 shows the migration pattern of the sample in lane 13 after being heteroduplexed with a normal sample. Lane 8 does not contain any sample.

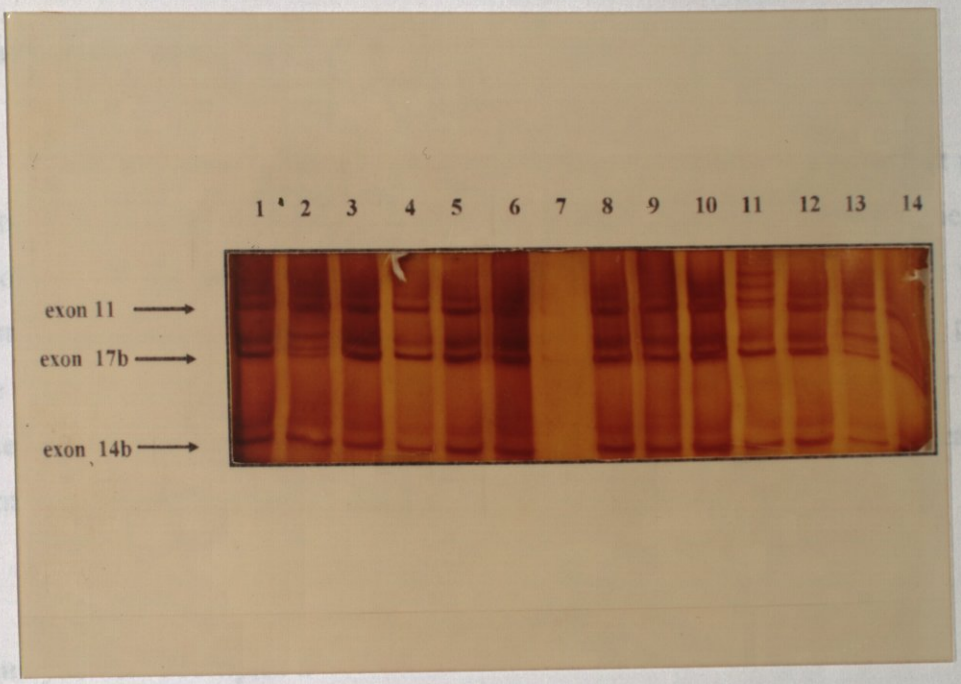

Figure V.7. DGGE analysis with Multiplex A. The samples were the same as in Figure V.6, but were subjected to staining with silver after electrophoresis.

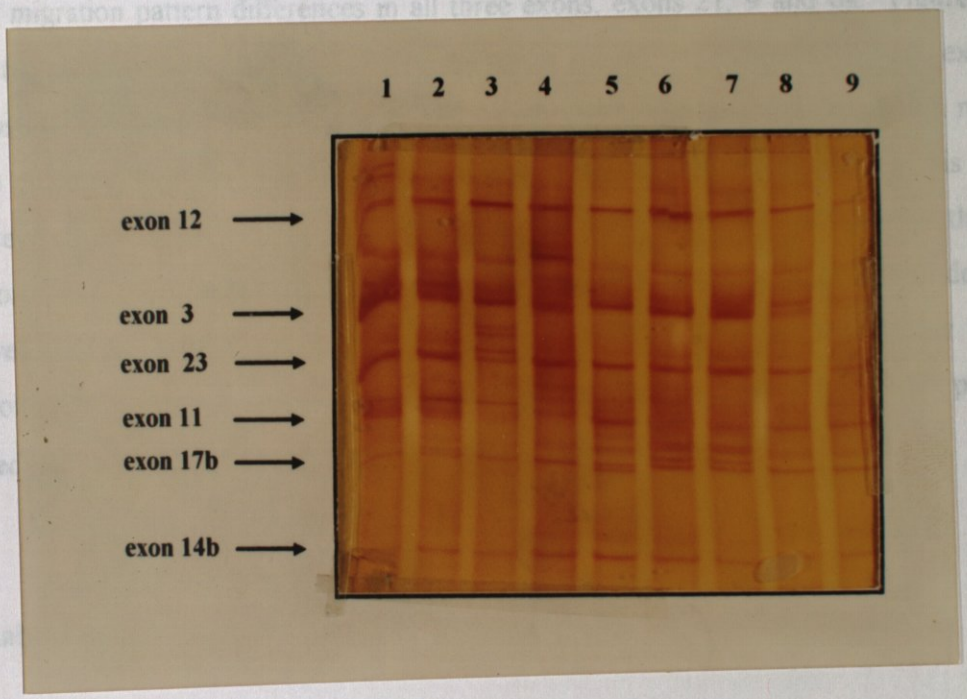

Figure V.8. Multiplex A and C were coelectrophoresed in order to accelerate the DGGE analysis. T_m values corresponding to each exon differ significantly, thus the migration of all six exons slowed down at different points in the DGGE gel.

c. Analysis with Multiplex C

DGGE analysis with Multiplex C revealed the presence of two variant spot patterns in exon 3, both in heterozygous cases (Figure V.10). In one of the two patients, the diagnosis of CF was made at 2 years of age because of repeated bronchitis, but was not confirmed by the sweat chloride test, which was normal (60mEq/lt). However, the pedigree of the family drawn according to the information given by the parent revealed other possibly affected family members. They all had the same phenotype with severe lung disease. No variant was observed in exons 12 and 23.

d. Analysis with Multiplex D

Multiplex D is the second useful triplex system because all three exons that can be analyzed with this system are hot spot regions for mutations. It was possible to detect several migration pattern differences in all three exons, exons 21, 9 and 6a. Figure V.11 shows the results of the DGGE analysis for a set of patients. The spot pattern in exon 21 was observed and confirmed to be N1303K, by by ARMS test and comparing to a marker known to be a N1303K heterozygote. No other variant was observed in this exon. Interpretation for exon 9 is difficult because of the various alleles generated by the two polymorphisms, $(TG)_n$ and T_n repeats in intron 8. The sole variant in exon 6a was detected in eleven patients, leading us to believe that this might be a polymorphism rather than a mutation. Comparison of the migration pattern of this variant to the marker spot patterns revealed that it was the same as the polymorphism 875+40A→G.

e. Analysis with Multiplex E

No variant DGGE patterns was observed in any of the exons amplified with Multiplex E, i.e. exons 8, 5 and 18.

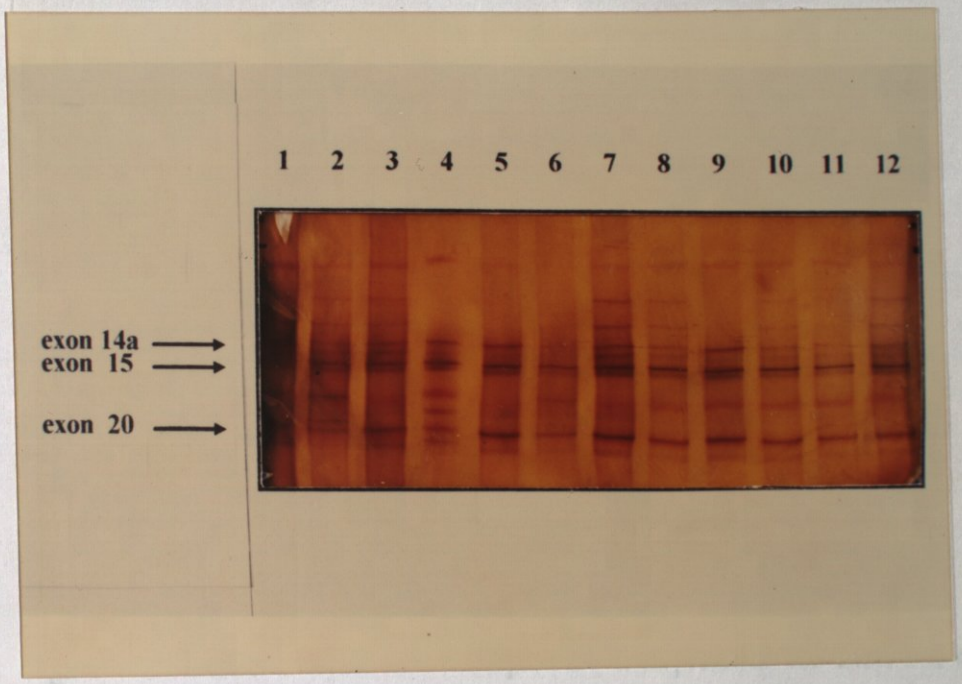

Figure V.9. DGGE analysis with Multiplex B. The polymorphism 2694T/G in exon 14a is observed in lanes 1, 2, 3, 7, 8, 10, and 12. Lanes 1 and 2 contain marker DNA which are heterozygous for Q890X and G1244E, respectively. Lane 4 shows the heteroduplex migration pattern for W1282X.

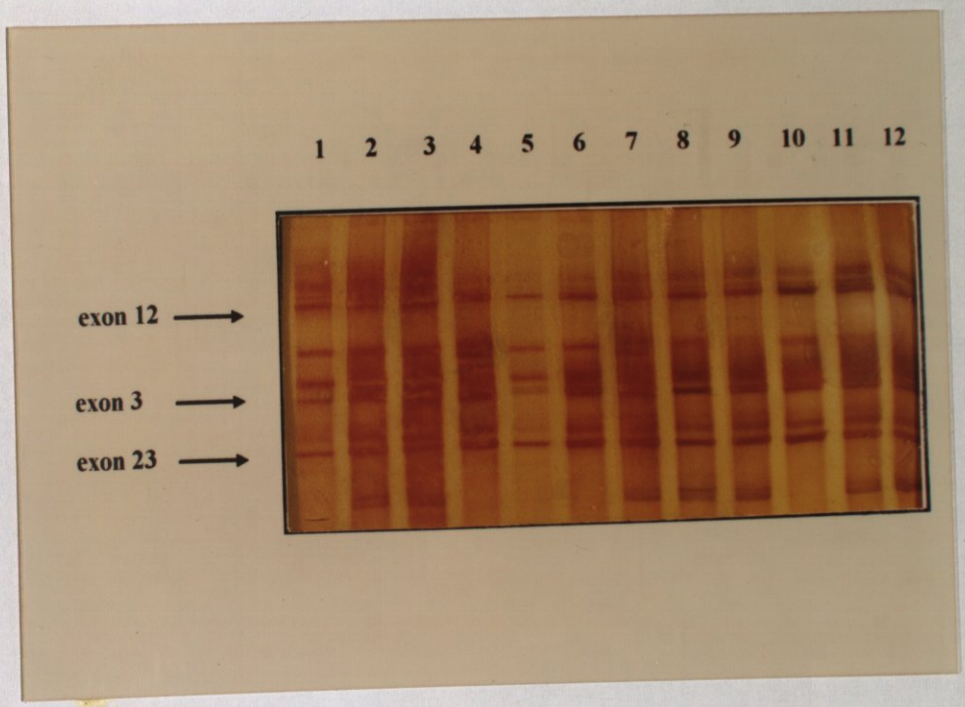

Figure V.10. DGGE analysis with Multiplex C. In lanes 4 and 11 two different spot patterns in exon 3, both in heterozygous cases, can be observed. The PCR product in lane 4 is similar to that of the lane 7. No variant was observed in exons 12 or 23.

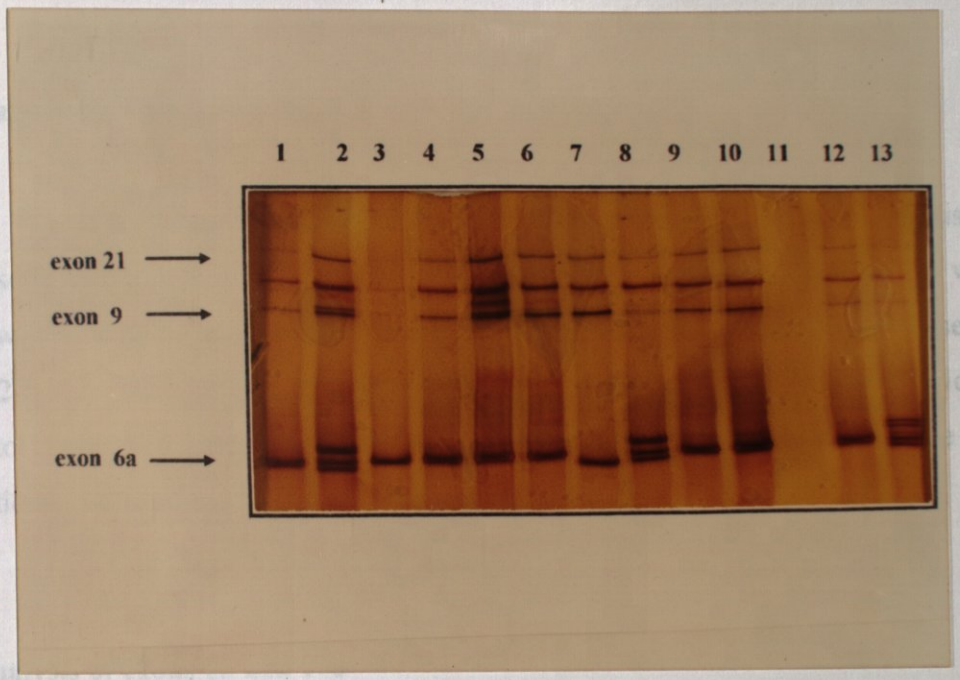

Figure V.11. DGGE analysis with Multiplex D. Interpretation for exon 9 is difficult because of the various alleles generated by the two polymorphisms, $(TG)_n$ and T_n repeats in intron 8, as seen in lanes 2, 5, 6, 8 and 12. A variant in exon 6a is observed in lanes 2, 7 and 8. Lane 7 shows the homozygous case which migrated a little faster than the normal samples. Lane 13 contains the marker DNA which is heterozygous for Q220X.

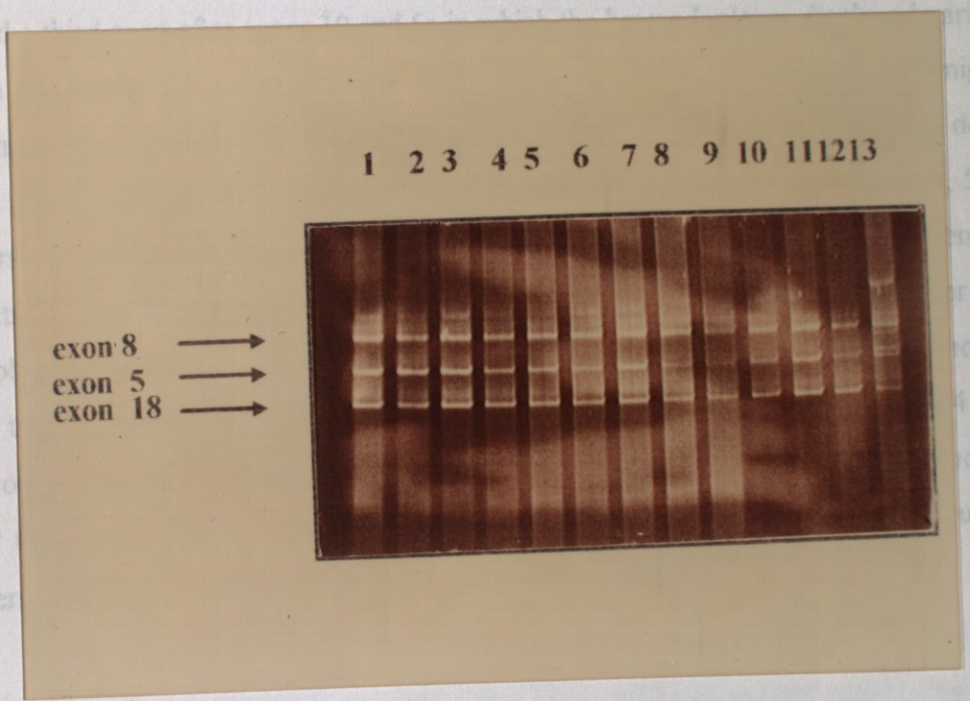

Figure V.12. DGGE analysis with Multiplex E. Samples were stained with ethidium bromide after electrophoresis. No variant DGGE pattern was observed in any of the exons 8, 5 and 18. Lane 13 contains the marker DNA which is heterozygous for 711+1 G→T.

f. Analysis of exons 2 and 16

Figure V.13 shows the DGGE results of exon 2 and exon 16. The analysis of the two exons was possible on a single gel. The upper band corresponds to exon 16, whereas the lower band to exon 2. A different pattern of migration in exon 16 can be observed in lanes 2 and 3. The samples in the first lane are heterozygous DNA markers which were used to check that denaturation was taking place during the optimization of the DGGE conditions. The analysis displayed no variants in exon 2.

g. Analysis of exons 6b and 17a

Another example for the electrophoresis of two exons together is the analysis of exons 6b and 17a. The upper band corresponds to exon 6b and the lower band to exon 17a in Figure in V.14. Variant patterns could be observed in two patients in exon 17a, both with different migration patterns and in heterozygous cases (lanes 2, 4). Exon 6b, however, is the the third exon after exons 10 and 6a in which the heteroduplexes displayed variants at a high frequency: 18 per cent of the uncharacterized alleles. Three different migration patterns in this exon could be observed in lanes 2, 4 and 5. The variant in lane 2 is detected in five alleles, whereas the variant in lane 4 in one allele, and the variant in lanes 4, 5 and 6 appears to be the most common one, in total 11 allelic cases. In two of the patients with variants in exon 6b, already two other allelic variants had been detected in other exons. This observation led us to believe that this variant in exon 6b might be a polymorphism rather than a mutation. But since the two patients carried the same variant in exon 4 seen in three other patients, we tended to believe this variant in exon 4 might be a polymorphism rather than the variant in exon 6b. The samples in the first lane are heterozygous DNA markers, 1001+11C→T in exon 6b and I1027T in exon 17a.

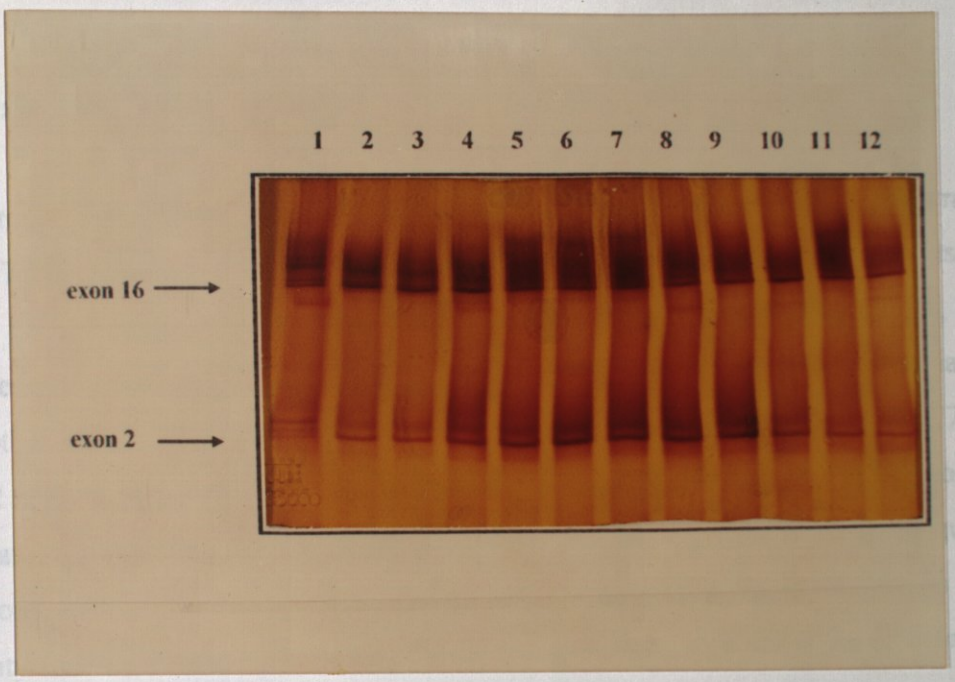

Figure V.13. DGGE analysis of exons 2 and 16. The upper band corresponds to exon 2 and the lower band to exon 16. Different variants in exon 16 can be observed in lanes 2 and 3. The samples in the first lane are heterozygous DNA markers which are R31C in exon 2 and 3120+ 1G→T in intron 16. No variant was observed in exon 2.

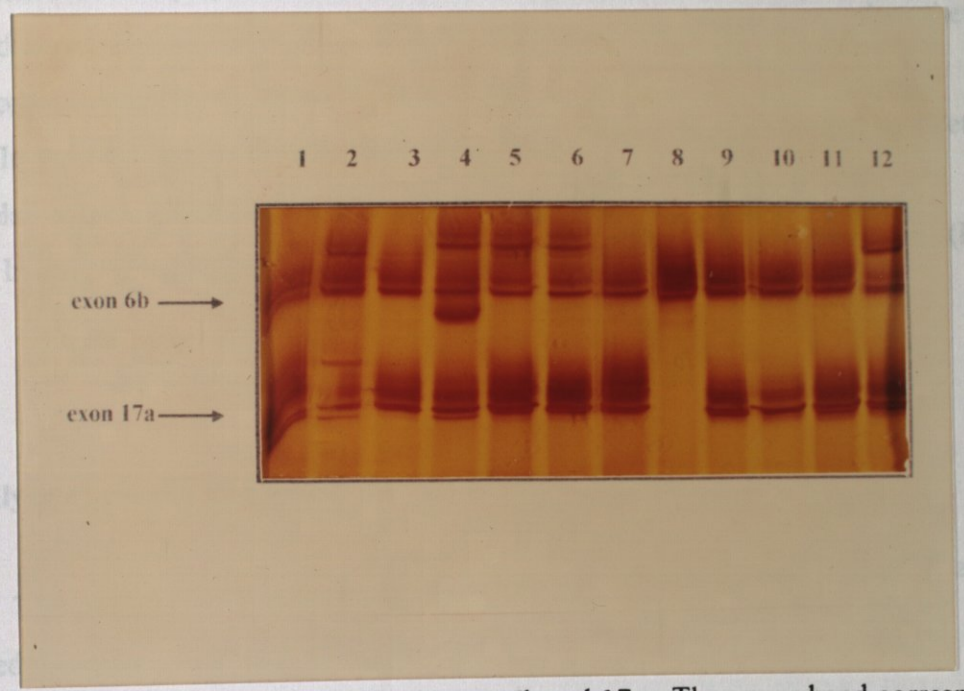

Figure V. 14. DGGE analysis of exons 6b and 17a. The upper band corresponds to exon 6b whereas the lower band to exon 17a. Three different variant patterns specific to exon 6b could be observed in lanes 2, 4 and 5. The heterozygotes in exon 17a displayed variants in lanes 2, 4. The samples in the first lane are heterozygous DNA markers, 1001+11C→T in exon 6b and I1027T in exon 17a.

h. Analysis of exons 4, 10, 13 and 19

Studies on exon 4 by using DGGE method identified variant migration patterns on the gel. Three patients were observed to have the same pattern. One of the patients was found to be homozygous whereas the other two were heterozygous. Surprisingly, only those three patients among 62 patients were found to carry already two additional variations on the other exons. The additional variations were: In Patient 1 in exon 3 and 17a, in Patient 2 in exon 6a and exon 6b, and in Patient 3 in exon 6b and 1677delTA in exon 10. The variations concerning exon 4 are shown in Table V.3, but excluded from the frequency calculations, since those patients were already two mutation candidates. There is a small probability that a CF chromosome might bear two mutations, this variation in exon 4 is believed to be a polymorphism (Figure V.15).

The previously detected mutations ΔF508 and 1677delTA in exon 10 displayed different migration patterns. Various types of heteroduplex migration patterns arose due to the presence of the polymorphism 1540A/G. Apart from the migration spot patterns observed with these mutations, three different variants (one in homozygous, the other two in heterozygous cases) were detected(Figure V.16).

In exon 13, besides the previously identified 2184Ains, another variant was detected in 5 alleles, four of which were in homozygous cases (Figure V.17).

In exon 19 only one variant was detected and it was in a homozygous case, (Figure V.18).

i. Analysis of exons 7 and 22

The analysis with individually amplified and electrophoresed exons 7 and 22 revealed no variants by DGGE analysis.

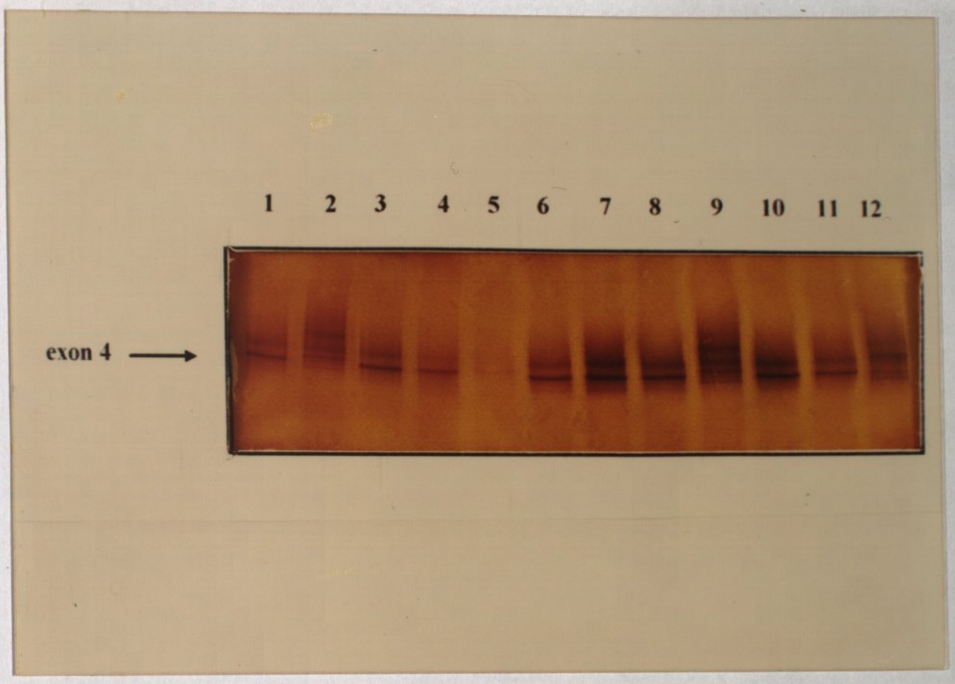

Figure V.15. DGGE analysis of exon 4. A variants pattern was observed in lanes 2, 9 and 12.

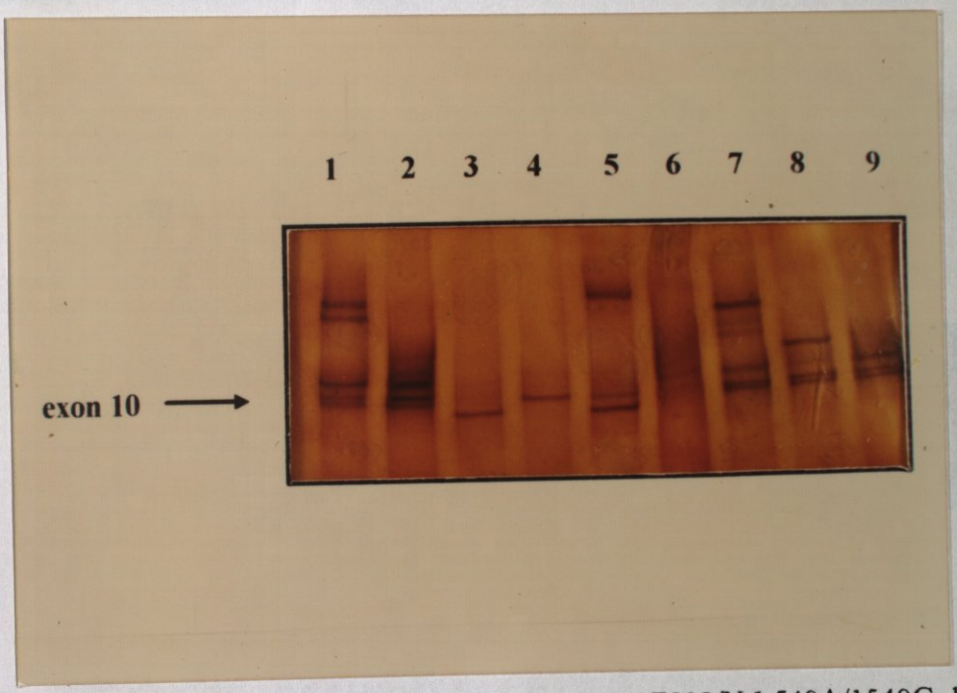

Figure V.16. DGGE analysis of exon 10. Lane 1: ΔF508/N,1 540A/1540G; lane 2: N/N, 1540A/1540G; lane 3: DelTA/DelTA, 1540G/1540G lane 4: N/N, 1540A/1540A; lane 5: ΔTA/N, 1540A/1540G; lane 6: N/N, 1540A/1540G; lane 7: DelTA/N, 1540A/1540G; lane 9: N/N, 1540A/1540G; lane 8 contains a different migration pattern, indicating a yet uncharacterized variant.

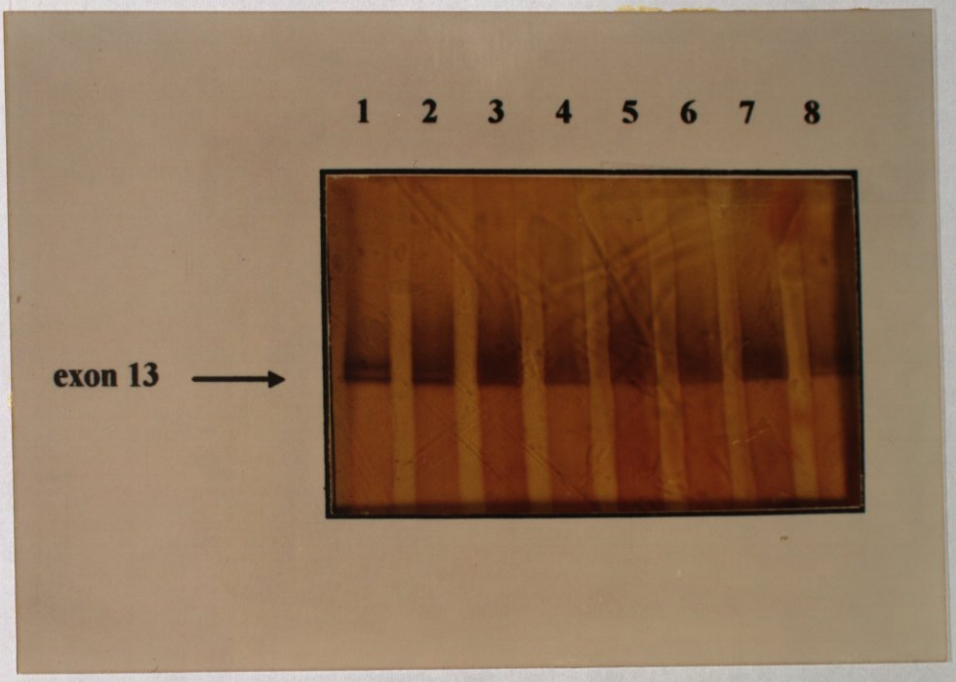

Figure V.17. DGGE analysis of exon 13. The first lane contains the mutation 2184Ains, the others have no variants.

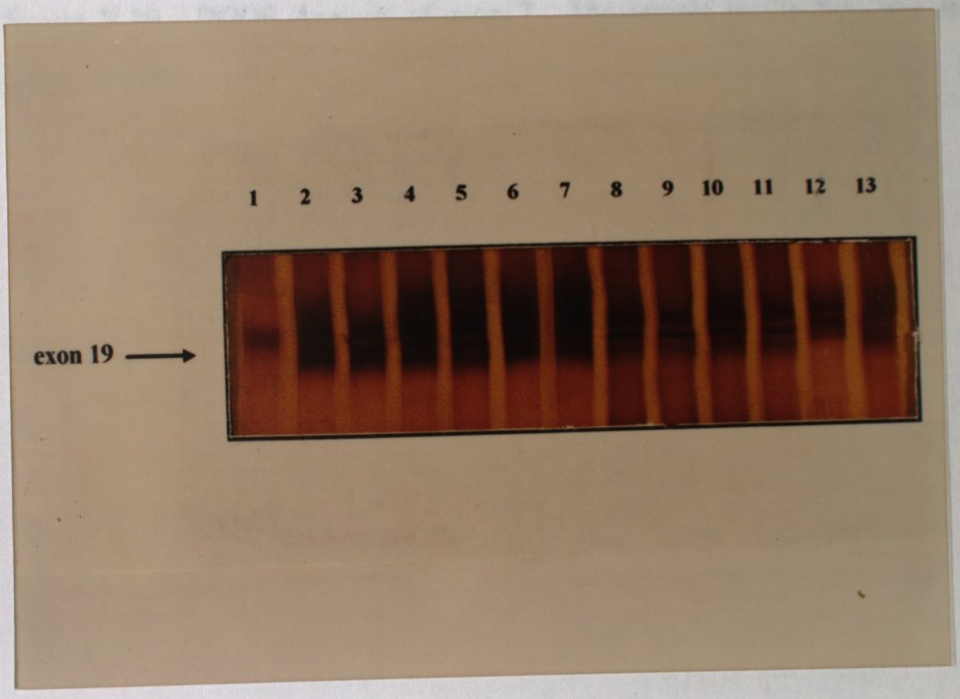

Figure V.18. DGGE analysis of exon 19. Lane 12 shows a variant in a homozygous case. Lane 1 contains the PCR product of exon 22.

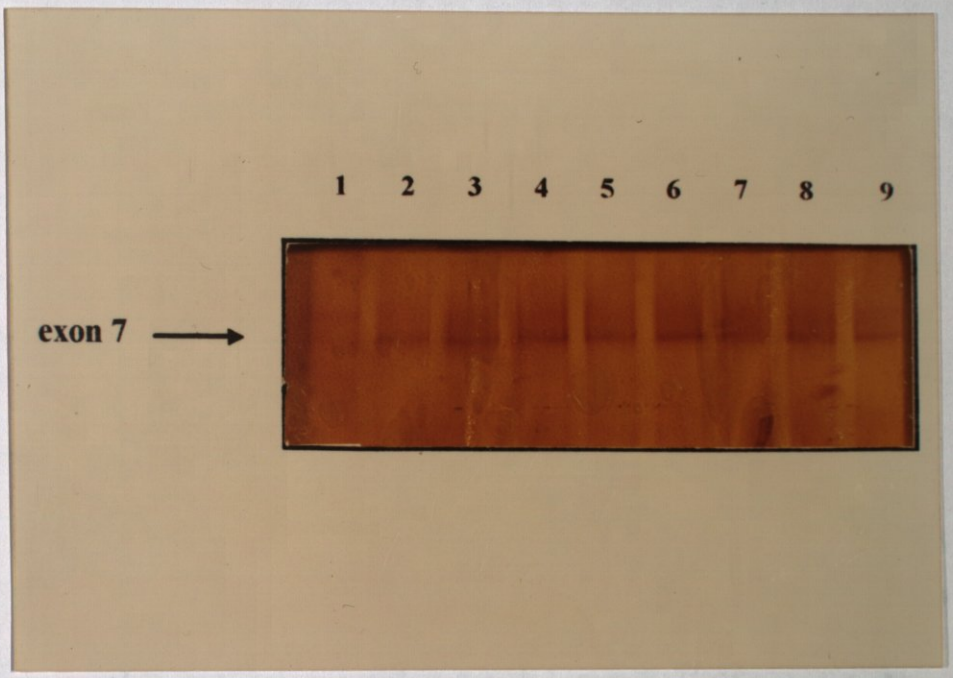

Figure V.19. DGGE Analysis of exon 7. The sample in the first lane is DNA marker which is heterozygous for I351S. Other samples have no variants.

Table V.3. Distribution of variants among the CFTR exons. Data includes 95 uncharacterized alleles excluding the known alleles (29 out of 124) and the two polymorphisms (2694T/G and 1540A/G). Exon 9 which is hard to interpret due to the various alleles generated by the two polymorphisms, $(TG)_n$ and T_n repeats in intron 8, is also excluded.

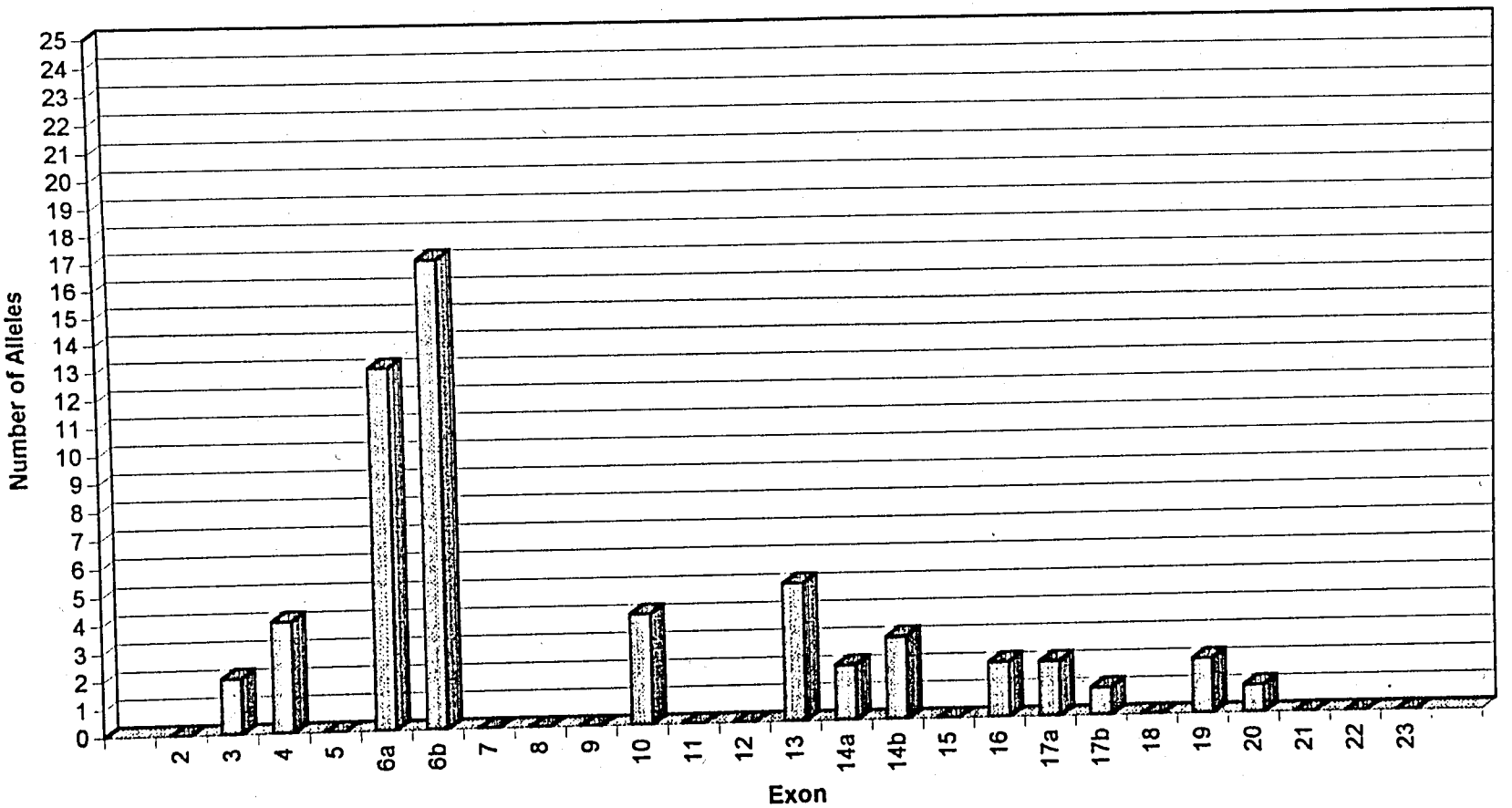

Table V.4. Distribution of characterized mutations and polymorphisms among the CFTR exons in patients. Mutations in 25 per cent of the CF alleles have been characterized with a frequency of 13 per cent for ΔF508, 4 per cent for 1677delTA, 2.5 per cent for R1069Q, 1.6 per cent each for W1282X and G542X, 0.8 per cent each for S466X, 2184Ains and N1303K. The two polymorphisms 2694T/G and 1540A/G have frequencies of 12 per cent and 37 per cent, respectively.

Exon / Patient	10	11	13	14a	17b	20	21
5.3	DelTA/DelTA, 1540G/G						
6.3	ΔF508/ΔF508, 1540A/A						
7.3	ΔF508/ΔF508, 1540A/A						
8.3	ΔF508/N, 1540A/G						
12.3	ΔF508/ΔF508, 1540A/A						
20.3	1540A/G				R1069Q		
24.3	1540A/A				R1069Q		
26.3	ΔF508/ΔF508, 1540A/A						
27.3	1540A/G		2184Ains /N	2694T/G		W1282X/N	
30.3	1540A/G			2694T/G			
31.3	ΔF508/ΔF508, 1540A/A						
33.3	DelTA/N, 1540A/A						
35.3	1540A/A			2694T/G			
38.3	1540A/G			2694T/G			
39.3	DelTA/DelTA, 1540G/G						
40.3	ΔF508/N, 1540A/A						N1303K/N
43.3	1540A/G			2694T/G			
44.3	S466X/N, 1540A/A			2694T/G	R1069Q		
46.3	1540A/A			2694T/G			
48.3	ΔF508/ΔF508, 1540A/A						

Exon / Patient	10	11	13	14a	17b	20	21
50.3	ΔF508/N, 1540A/A						
54.3	1540A/G			2694T/G			
55.3	1540A/G			2694T/G			
56.3	1540A/G			2694T/G			
58.3	1540A/G			2694T/G			
60.3	1540A/G			2694T/G			
62.3	1540A/G			2694T/G			
63.3	1540A/G			2694T/G		W1282X/N	
64.3	ΔF508/N, 1540A/G						
67.3	1540A/A	G542X/ G542X					

Table V.5. Distribution of variants among the *CFTR* exons in patients.

Exon / Patient	3	4	6a	6b	10	13	14a	14b	16	17a	17b	19	20
9.3			V1/N	V1/N									
14.3												V1/V1	
19.3		V1/N	V1/N	V1/N									
20.3					V1/N								
22.3						V1/V1							
24.3								V1/N					
27.3			V1/N										
28.3				V1/N									
29.3					V2/N		V1/V1						
30.3			V1/N										
32.3				V1/N		V2/N							
33.3		V1/V1		V1/N									
35.3				V2/N									
36.3				V1/N									
37.3													
38.3			V1/N										
42.3						V2/V2							
43.3	V1/N		V1/N							V1/N			
45.3				V1/N				V1/V1					
46.3				V1/N									
50.3											V1/N		
52.3			V1/V1		V3/V3								
54.3			V1/N										

Exon / Patient	3	4	6a	6b	10	13	14a	14b		17a	17b	19	20
55.3				V2/N									
56.3			V1/V1										
57.3				V1/N									V1/N
58.3				V2/N									
60.3			V1/N						V1/N				
62.3				V2/N									
64.3				V1/N									
65.3	V2/N	V1/N								V2/N			
68.3				V3/N									
71.3			V1/N	V2/N									
72.3				V1/N					V1/N				

V1: Variant 1; V2: Variant 2; V3: Variant 3

VI. DISCUSSION

The predominant mutation in Caucasions, ΔF508, was also found in our patient population, but at a significantly lower frequency (13%) than in the European populations. Several studies on the Turkish CF patients carried out in European laboratories further supported the low frequency of this particular mutation in our population. The comparably low incidence demonstrates that, in the Turkish population, CF is predominantly caused by mutations other than the deletion ΔF508. In order to characterize the non-ΔF508 and non-1677delTA mutations that account for 83 per cent of cystic fibrosis chromosomes in our patient population,, we started a systematic study: First we screened for some known mutations (Q220X, L346P, N1303K and W1282X) which are frequent in the neighboring geographical areas. For example, Q220X was detected in several of the patients of Turkish origin in Bulgaria (Kalaydjieva, personal communication). L346P was first detected in Cyprus (Boteva et al., 1994) and the mutations N1303K and W1282X were seen in the several of the Jews of Turkish origin in Israel. Two methods were applied to screen for the presence of these mutations: Restriction enzyme analysis and ARMS test. Restriction enzyme analysis included all of the CF chromosomes and indicated that neither L346P nor Q220X was present in the Turkish CF chromosomes analyzed. Because of the large size of the CFTR gene and the high number of mutations reported worldwide (over 600), it was not possible to screen for all of the known mutations with the help of the diagnostic methods described in Section I.H.1. Alternatively, restricting the analysis to the mutations commonly seen in this geographic area and to the mutations detected in patients of Turkish origin in various laboratories abroad could not be justified. The two mutations N1303K and W1282X with their frequencies 0.8 and 1.6 per cent, respectively, lower down the amount of uncharacterized CF chromosomes insignificantly. As a result, an efficient, quick and reliable screening method which would cover all of the coding regions was needed. For this purpose, we used the DGGE technique. To date, the efficiencies of the techniques that facilitate the identification of mutations in cloned genes are still a matter of discussion. However, the DGGE technique is believed to have the highest sensitivity of detection (99%), higher than the other available techniques, such as SSCP.

In this large study, we analyzed all of the gene except for the first and the last exons of 124 CF chromosomes. Sixty-two patients diagnosed on the basis of standard clinical criteria were from all over Turkey. Twelve patients whose clinical data suggested diseases other than CF were excluded from this study. To speed up the screening process by DGGE, fifteen of the exons were analyzed in five triplex systems, each of which amplified and allowed resolution of three exons at one time. In addition, some exons with different T_m values were amplified individually and coelectrophoresed in pairs on a single DGGE gel. For optimization of the amplification and electrophoresis conditions, most of the effort went into finding proper ramp times and proper selections of the range of denaturant concentrations needed for various exons to detect homoduplexes and heteroduplexes. Correct ramp time, which is the time needed for a thermal transition from the denaturation to the annealing temperature during the amplification reaction, has two implications: First, the binding of the primer to its complementary region is ensured. Second, the amplification of non-specific bands were avoided. Non-specific bands occurred from time to time during the amplification process of some multiplexes and single exons. Sometimes they complicated the interpretation of the gels as was in the case of Multiplex C, but most of the time these extra bands did not interfere with the expected bands, thus had no consequence as was in the case of Multiplex D. Hence, the use of markers was absolutely needed and it had two important contributions: First, the interpretation of the correct band in the DGGE gel was ensured, and second, the denaturant range could be optimized. Another problem encountered in this method was the restriction on the use of PCR-amplified samples: A sample amplified in a 50 µl PCR reaction could not be used for more than in two electrophoresis runs, because in order to detect any variation by staining with ethidium bromide, at least 20 µl of each sample had to be loaded onto the gel. When a third analysis was needed for some DNA samples, a second round of amplification became necessary. As a result, in order to save time and materials, especially primers supplied in amounts just sufficient to carry out this study, we adopted the silver staining method in our large scale screening program. Staining had very high efficiency, besides its simplicity in use and low cost. The current price of a 100 ml of staining buffer with which it is possible to stain 30 gels is only 10 U.S dollars. Silver staining was as sensitive as autoradiographic techniques. It is possible to detect PCR bands in as little as 2 µl PCR samples very effectively. Even some bands which did not appear on the same gel stained previously with EtBr, became

visible. As a result, sensitivity of silver staining was at least 10 times more than the currently used EtBr staining.

Our previous hyphothesis that the profile of CF mutations in the Turkish population has great heterogeneity is consistent with the data obtained in the framework of this study. We expected to find many mutations distributed randomly in coding regions of the CFTR gene, each of which would account for a relatively small fraction of the mutations. Hence the identification of a large number of variants in the gene suggested that the heterogeneity might be broader than previously thought, covering 14 exons and with some of them having relatively high frequencies (Exon 6a with a frequency of 14 per cent and exon 6b with a frequency of 20 per cent of a total uncharacterized alleles). No variant DGGE patterns was observed in any of the exons, exons 2, 5, 7, 8, 12, 15, 18. These exons are not functionally important exons, such as those encoding part of the NBFs (exon 9-11, 20, 21 and 23), in which the largest number of mutations has been observed worldwide (Cutting et al., 1992). Table V.3 shows the distribution of uncharacterized variations among exons. The two polymorphism 2694T/G and 1540A/G had frequencies of 12 per cent and 37 per cent, respectively. Not included in this table are the characterized mutations which make up 25 per cent of the cases, revealing a frequency of 13 per cent ΔF508, 4 per cent 1677delTA, 2.5 per cent R1069Q, 1.6 per cent each of W1282X and G542X, 0.8 per cent each of S466X, 2184Ains, and N1303K. Sixty four per cent of the uncharacterized alleles (a total of 95 uncharacterized alleles) could be identified as having a variation. We have not analyzed exons 1 and 24, nor the promoter or intragenic sequences of the CFTR gene. Hence 36 per cent of these uncharacterized chromosomes displayed no variation at all in the coding sequences studied.

In the most common approach for mutation identification, mutations are first localized to small regions of the gene by DGGE and subsequently identified by DNA sequence analysis. It is, however, often desirable to circumvent sequencing by finding out whether a gene alteration is one of previously reported mutations. Alternatively, a variety of PCR-based diagnostic methods for detection of known mutations have been established, but each of these mutations required a set of oligonucleotides for hybridization or amplification as described in the Introduction (I.H).

The mutation resolving capacity of DGGE is based on the fact that similar DNA fragments differing only by a minute sequence alteration, such as a single base substitution, migrate differently due to differences in melting parameters on a denaturing gradient gel. Increased electrophoretic resolution can be achieved by analysis of heteroduplexes which can form in samples with two different alleles. Heteroduplex molecules carry a single base mismatch and will, due to their lower thermostability, be retarded in the gel at a lower concentration of denaturants than the corresponding homoduplexes. Since each base substitution has a particular influence on the melting properties of a DNA fragment, each mutation is characterized by a unique spot pattern on the gel in the form of a composite pattern of homoduplex and heteroduplex bands, providing indication of the identity of the mutation. In our experience, the pattern of heteroduplex bands are specific for each base-pair substitution in a given sequence. Therefore, when a proper set of control mutations is available, it is possible to identify mutations by DGGE by comparing to control markers. In order to verify this suggestion, we obtained the French marker spot patterns. Two different mutations can, however, result in similar band images, even though rarely, and further identification is therefore necessary. But, such identification can be accomplished simply by mixing the test sample with a control sample containing a known mutation, followed by heteroduplex formation and DGGE. If the two mutant fragments are not identical, additional mismatched heteroduplexes will be formed by random reassortment of the different single-strand constituents and reveal novel bands on the gel. This practical approach was performed with the mutations G542X in exon 11 and the polymorphism 2694T/G in exon 14a. We mixed our samples with the control samples known to have the same mutations, and subjected to heteroduplex analysis. It was then observed that, the banding patterns specific to those mutations remained unchanged.

The data support the previous findings that the Turkish population is genetically very heterogeneous. However, the observation that most of the patients were compound heterozygous was unexpected, considering the high frequency of consanguineous marriages in the population. The incidence of CF remains unknown in the population, and the number of families diagnosed are very few. If the incidence were low, then a higher percentage of the patients would have been homozygous. The low number of known CF families is probably be due to low diagnosis because of ignorance of the disease, or to low chloride levels in some patients. Several of the patients in this study have low chloride

levels. An accurate population figure can be detected quickly when the mutations are identified. We have started DNA sequence analysis in the samples with unique DGGE patterns to identify mutations/polymorphisms. This information is not only important for carrier testing and understanding the molecular basis of the disease, but also for understanding the function of the CF gene product, and for correlating clinical findings to different types of mutations.

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