ANALYSIS OF TURKISH CYSTIC FIBROSIS CHROMOSOMES

by

ALPER TUNGA AKARSUBAŞI

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TO THOSE SUFFERING FROM CF

Ben manevi miras olarak hiç bir ayet, hiç bir dogma, ve kalıplaşmış kural bırakmıyorum.

Benim manevi mirasım ilim ve akıldır. Benden sonrakiler, bizim aşmak zorunda kaldığımız çetin ve köklü zorluklar karşısında, belki gayelere tamamen eremediğimizi, fakat asla taviz vermediğimizi, akıl ve ilmi rehber edindiğimizi tasdik edeceklerdir.

Zaman süratle ilerliyor; milletlerin, toplumların, kişilerin mutluluk ve mutsuzluk anlayışları bile değişiyor. Böyle bir dünyada asla değişmeyecek hükümler getirdiğini iddia etmek, aklın ve ilmin gelişimini inkar etmek olur.

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Mustafa Kemal ATATÜRK

(Kaynak: Atatürk'ün Görüş ve Direktifleri, 1. kitap, Genel Kurmay Başkanlığı Yayınları,1984)

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The aim of my work was to better the lives of those suffering from CF. I hope that I have been able to help them in a small way.

ABSTRACT

Cystic fibrosis (CF) results from mutations in the gene encoding the cystic fibrosis transmembrane regulator (CFTR), a protein that regulates chloride ion transport in exocrine glands. Since the cloning of the gene, more than 375 disease-causing mutations have been identified. The major mutation in the gene is ΔF508, a deletion of three bp in exon 10 that removes a phenylalanine residue at position 508 and is found in 68 per cent of CF chromosomes worldwide. In this study, heteroduplex analysis was performed for detection of the mutations ΔF508 and 1677delTA. The former was found in 15.5 per cent of CF chromosomes in Turkey. The latter was found in relatively high frequency (4.5 per cent) with respect to other mutations in our population. Further, DGGE analysis was used to detect mutations in exon 10, a mutation hot spot. A rare mutation, S466X, found in one patient, was the first case reported in the Turkish population. The DGGE migration pattern for the polymorphism 1540A/G was identified by DNA sequencing. It was confirmed with Hph I enzyme digestion and found with high frequency (37 per cent). Also, again by using Hph I digestion, the CF chromosomes were screened for an interesting mutation, 3849+10 kb C->T, which is associated with a mild type of disease. None of the patients were found to carry this mutation.

ÖZET

Kistik fibroz, dış salgı bezlerinde klor taşınmasını düzenleyen kistik fibroz trans membran regülatör proteinini kodlayan gen bölgesindeki mutasyonlardan kaynaklanmaktadır. Genin klonlanmasından bu yana, hastalığa neden olan 375'ten fazla mutasyon belirlenmiştir. Gen üzerinde oluşan en yaygın mutasyon, proteinin 508 inci pozisyonundaki fenilalanin amino asidini ortadan kaldıran, ekson 10 bölgesindeki üç baz çiftlik delesyondur (ΔF508). Bu mutasyon dünya genelinde, bu hastalığa yol açan mutasyonların yüzde 68'ini oluşturur. Bu çalışmada ΔF508 ve 1677delTA mutasyonlarının belirlenmesi için heterodubleks analizi kullanılmıştır. Bunlardan ΔF508'in Türk popülasyonunda yüzde 15.5 oranında olduğu saptanmıştır. 1677delTA ise, toplumumuzda diğer mutasyonlara göre daha yüksek oranda bulunmuştur. Gende mutasyon yoğunluğunun yüksek olduğu onuncu ekson bölgesi, gradiyentli denature edici jel elektroforezi (DGGE) yöntemi kullanılarak taranmıştır. Bir hastada saptanan ve dünya genelinde seyrek olarak görülen S466X mutasyonu ilk kez bu çalışmada rapor edilmiştir. Ayrıca, 1540A/G polymorfizmi yüksek bir oranda bulunmuş (yüzde 37) ve sonuç dizi analizi ve Hph I enzimi kesimi yapılarak kanıtlanmıştır. İlginç bir mutasyon olan ve hastalığın nispeten hafif seyrettiği 3849+10 kb C-T mutasyonu için hasta DNA örnekleri Hph I enzimi kesimi ile taranmıştır. Bu mutasyondan hiçbir hastada gözlenmemiştir.

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ABBREVIATIONS

APS	ammonium persulphate
ARMS	amplification refractory mutation system
ASO	allele specific oligonucleotide
ATP	adenosine triphosphate
bp	base pair
BPB	bromophenol blue
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulatory
ddNTP	2',3'-dideoxynucleoside 5'-triphosphate
DTT	DL-dithiothreitol
DGGE	denaturing gradient gel electrophoresis
dsDNA	double-stranded deoxyribonucleicacid
dNTP	2'-deoxynucleoside 5'-triphosphate
EDTA	ethylenediaminetetraaceticacid
EtBr	ethidium bromide
min	minute
NBF	nucleotide binding fold
PCR	polymerase chain reaction
PI	pancreatic insufficiency
PS	pancreatic sufficiency
R-domain	regulatory domain
SDS	sodium dodecyl sulphate
SSCP	single strand conformational polymorphism
TEMED	N', N', N', N'-tetramethylethylenediamine
Tm	melting temperature
XC	xylene cyanol

I. INTRODUCTION

A. What is CF ?

Cystic fibrosis (CF) is a severe autosomal recessive genetic disorder. It has a high incidence in Caucasian populations, with a frequency of approximately one in 2500 live births. This implies a gene frequency of one in 50 and a carrier frequency of one in 25 (The Cystic Fibrosis Genetic Analysis Consortium, 1993). The disorder is infrequent in Black-Americans (1:17.000) and Asian populations (<1:100.000) (Mcintosh et al., 1992). Both sexes are affected with equal frequency, and parents of affected children show no clinical symptoms. Affected males are almost invariably sterile and have vas deferens absence, while females with the disorder can reproduce.

B. Medical Implications

The clinical severity of patients with CF varies enormously. There are two reasons for this complexity : Firstly, several different organs and systems may be affected, essentially in terms of mucus glands. Secondly, the pattern of organ systems involved varies from patient to patient in terms of severity and development of the disease. Some patients die in childhood, while the majority live well into their third decade. Cystic fibrosis affects children, adolescents and young adults and presents with the following fundamental traits: Insufficiency of the pancreas, chronic bronchopulmonary disease and abnormally high levels of sodium and chloride in the sweat (Dean et al., 1993). The dominating clinical feature of CF is the involvement of the respiratory tract, manifested by the obstruction of the airways by thick, viscous mucus and subsequent infection, especially by Pseudomonas. There is also an involvement of the gastrointestinal tract in most patients, with 85 per cent of them having pancreatic insufficiency as a result of obstruction of the pancreatic ducts and destruction of exocrine function. Five to 10 per cent of newborns with CF are born with a

form of intestinal obstruction called meconium ileus, and two to five per cent develop liver disease during the course of the illness (Santis et al., 1990).

C. Historical Background

Recent improvements in therapy of the disease have increased the median survival age to 29 years. However, CF is not a newly identified disease. Medical literature as early as 1705 describes children with probable CF. In 1938 Andersen published the first comprehensive description of CF as a distinct disease, naming it cystic fibrosis of pancreas (Romano et al., 1970). DiSant Agnese demonstrated that excessive salt loss occurs in the sweat of patients with CF (Aitken et al., 1993), a finding that later led to the acceptance of high levels of sodium and chloride in sweat as the diagnostic criteria for the disease. When performed correctly, the sweat test is highly sensitive and specific for CF. Test results which are greater than 60mEq/L are indicative in diagnosis of CF. In 1955, the CF Foundation, a privately funded organization to promote CF research and patient care, was established. In the 1980's several investigators outlined the specific ion channel abnormalities. In 1989, the CF gene and the ion channel protein it encoded was described (Riordan et al., 1989).

D. Identification of the CFTR Gene

The strategy for isolation and identification of the CF gene required a great collaborative effort and was applied by Kerem et al. in 1989. A candidate gene was cloned using the positional cloning strategies of reverse genetics. The first evidence for the localization of the CF gene on chromosome seven came from linkage analysis in CF families in which DNA polymorphisms on chromosome seven were used as genetic markers, first by Tsui et al. (1985), then by Wainwright et al. (1985) and White et al. (1985). A large number of additional markers were later identified and used in linkage studies to map the

CF locus more precisely to band 7q31. Despite the fact that the CF locus was narrowed down to about 500 kb, it was difficult to identify the gene. At this stage, chromosome jumping was used to construct a jumping library in which each phage clone contained sequences that were widely separated on the chromosome. The next step was chromosome walking, in which overlapping clones were isolated from a conventional phage library. The end result was a 280 kb of DNA cloned into overlapping cosmid and phage clones. The following step was to determine whether any of those clones contained coding sequences. The strategy was to test clones for cross-hybridization to DNA samples of various animal species on Southern zoo blots. The presence of CpG islands in one of the probes from the cosmids suggested that the region might contain the start of a gene. A probe from this sequence was used to screen a cDNA library made from cultured normal sweat gland cells. A homologous clone was identified and the sequence of the gene coding region was determined. Later the gene region was foud to be approximately 250 kb in length, and the sequences of the 27 exons and the associated exon-intron boundaries were determined (Rommens et al., 1989). A messenger RNA of approximately 6.5 kb encodes a polypeptide of 1480 amino acids, termed the cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan et al., 1989). Sequence analysis of the 5' flanking region of the CFTR gene reveals structures similar to those found in several housekeeping genes. It has a high guanine and cytosine content (CpG islands), does not contain a TATA box, and transcription is initiated at more than one position. It is rather surprising that while the housekeeping genes are not expressed in a tissue specific manner, CFTR exhibits organ and tissue specific expression. There are a number of SP1 and AP1 sites instead of TATA or CAAT boxes. Polyadenylation of the transcript occurs at 17 nucleotides downstream of the single AATAAA signal found at the three-prime terminal region.

The sizes of the 27 exons were found to vary greatly, with exon 14b being the smallest (38 bp) and exon 13 being the largest (724 bp). Sequence analysis indicated that the splice junction sequences obeyed the invariant GT-AG rule (Mount, 1982). The length of the introns varied from 1.1 kb for intron 6a to 40 kb for intron 3 (Zielenski et al., 1991). A number of short and long interspersed repetitive elements were detected in the intron regions near the intron/exon boundaries (Jelinek and Schmid, 1982; Bains, 1986; Singer and Skowronski, 1985). For example, five Alu repeats which were found in introns 2, 6a, 8, 14a, 14b, and 23 were identified soon after the gene was isolated. Moreover, a number of simple sequence repeats (microsatellites) were also detected. Two dinucleotide repeats of the (CA)n type (Miesfeld et al., 1981; Hamada et al., 1982) were found in introns 8 and 17b and one of the (TA) in again intron 17b (Morral et al., 1992). These simple repeats serve as polymorphic DNA markers convenient for linkage analysis.

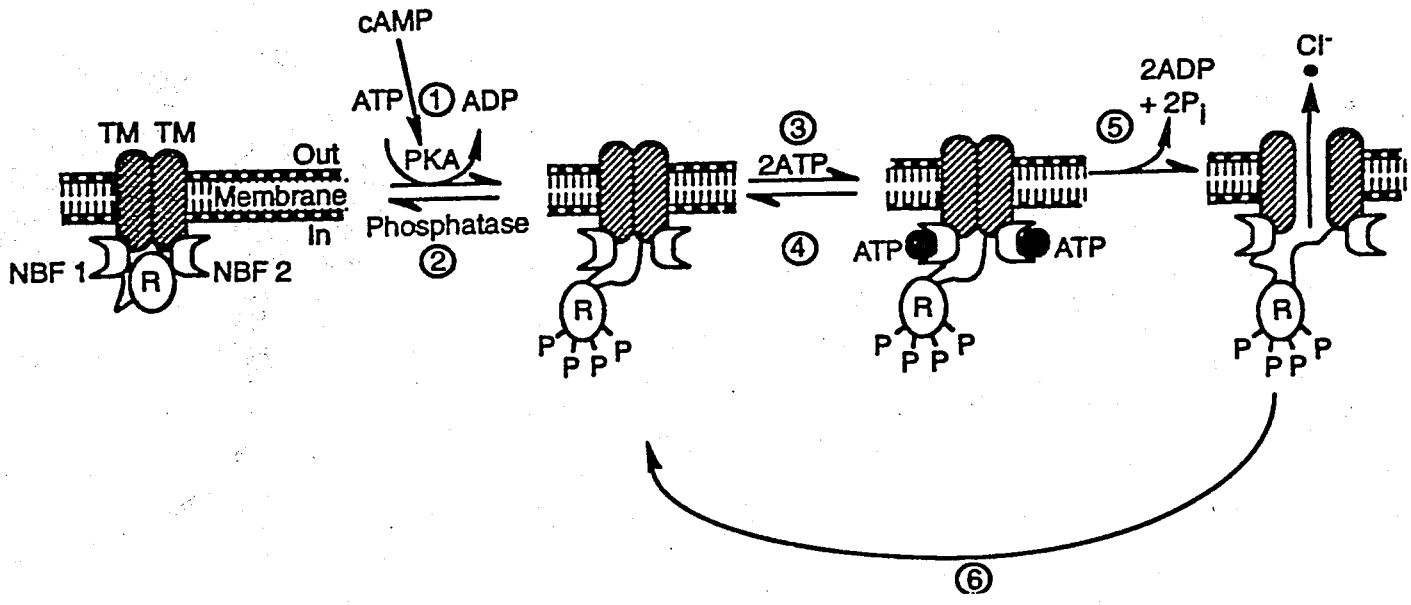


Figure I.1- Hypothesis for the dual control of CFTR by PKA and ATP. In the absence of R-domain phosphorylation, the channel is closed. After cyclic AMP stimulates PKA to phosphorylate one or more serine residues in the R-domain (step 1), the CFTR channel is poised to bind ATP (step 3), which is cleaved to induce a conformational charge (step 5), opening the chloride channel. This is envisioned to decay back (step 6), to a closed state. The seperation of ATP binding, hydrolysis, and channel opening (step 3 and 5), is speculative. Pi, inorganic phosphate, cyclic AMP, and TM transmembrane domain (Collins., 1992).

E. Cystic Fibrosis Transmembrane Conductance Regulator Protein (CFTR)

CFTR is predicted to be an integral membrane glycoprotein consisting of two hydrophobic domains, each composed of six transmembrane segments and two regions assumed to interact with ATP, termed as nucleotide binding folds (NBF). This protein belongs to a superfamily of ATP-dependent transport proteins, which import or export molecules across cell membranes in a process that appears to be coupled with ATP hydrolysis. Members of this group of proteins have the greatest degree of sequence similarity in the putative NBF regions. However, CFTR also has a unique domain. It is the regulatory or R-domain with several charged residues and is located in the middle of the gene, it has multiple sites with a potential for phosphorylation by cAMP dependent kinase (PKA) and protein kinase C (PKC), thus may play a role in the regulation of the CFTR function. It was proposed that the gene product might be either a regulated chloride channel or a regulator of another channel protein (Bear et al., 1992; Collins et al., 1992; Mcintosh et al., 1992).

Two lines of evidence support the proposal that CFTR by itself can function as a chloride channel. First, the anion selectivity of the channel can be altered by mutations in the transmembrane regions of the protein. Second and more convincing is the experiment performed with CFTR purified from over-expressing cell lines (Riordan, J. R., 1993). The protein was reconstituted into lipid bilayers and patch-clamp analysis of these vesicles identified cAMP-regulated currents comparable to those observed in cells expressing CFTR. As no other proteins were present, it is most likely that CFTR is a cAMP-activated chloride channel. A proposed model for the regulation of this protein is illustrated in Figure I.1 According to the model, CFTR is phosphorylated *in vivo* by the cAMP-dependent protein kinase A (PKA). PKA-directed phosphorylation is a necessary priming event for gate openning of the channel. Once phosphorylated, CFTR requires binding of ATP and its hydrolysis for activation. ATP is bound and hydrolyzed either by one or both NBFs. It has been proposed that hydrolysis of ATP causes a conformational change in CFTR that allows passage of chloride ions down the concentration gradient. A CFTR protein mutated at a critical residue in NBF2 can still hydrolyze ATP, suggesting that NBF1 is the primary site for ATP hydrolysis (Fuller et al., 1992; Widdicombe et al., 1991).

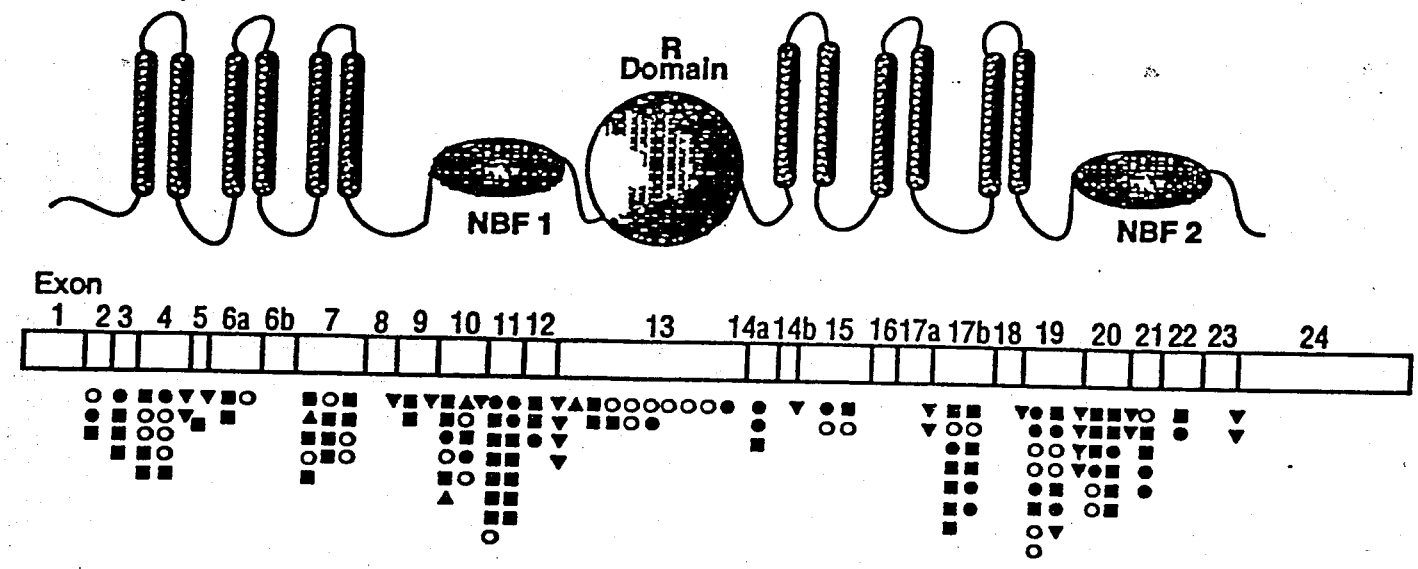


Figure I.2- Spectrum of mutations responsible for CF. The location and nature of CF mutations identified by the Cystic Fibrosis Genetic Analysis Consortium are indicated below a schematic of the CFTR protein. (▲) In-frame deletion; (■) missense mutation; (●) nonsense mutation; (O) frame-shift mutation; and (▼) splicing mutation (Collins., 1992).

F. Mutations in the CFTR gene result in CF

More than 375 disease-causing mutations have been identified in the CF gene (information provided by The Cystic Fibrosis Genetics Analysis Consortium, 1993). Frameshift, missense, nonsense, deletion and RNA splice junction mutations have all been described. Different disease causing mutations have different effects, some influence CFTR localization and stability, while some affects its function (Dean et al., 1994; Melo et al., 1993). One particular mutation, a deletion of three nucleotides resulting in the omission of a phenylalanine residue at codon 508 (ΔF508) (Kerem et al., 1989), is found at a worldwide frequency of 58 per cent, but varies between 30 and 88 per cent in different populations. In Europe, the frequency of this mutation increases along a southeast to northwest gradient, with the highest frequency in Denmark (90 per cent), and the lowest in Israel (20 per cent) and Turkey (15 per cent) (Tsui, 1993). This most common mutation is in exon 10 in the NBF1 region and is shown to prevent maturation of the protein and its delivery to cell surface (Thomas et al., 1992). More recent studies showed that the ΔF508 mutant protein was in fact functional if inserted into the membrane (Barinaga et al., 1992). Furthermore, it is most probably a temperature-sensitive mutant which can be processed and reach the apical membrane at 27°C but not at 37°C (Lukacs et al., 1993).

About half of the CFTR mutations, that have been described, are single base substitutions causing missense mutations. Three of the remaining classes (nonsense, frameshift, splice junction) are more or less equally represented. There are also a few single amino acid and other small in frame deletions. However, large deletions spanning multiple exons are extremely rare. Moreover, no mutations in the promoter region have been described so far. The distribution of the mutations among the functional domains of the protein is not random. The highest concentration is in NBF1 and to a lesser extent, in NBF2 (Tsui, 1993). Whether this characteristics in the distribution of mutations represents certain properties of the nucleotide sequences or functionally important domains is still not known. Several missense mutations may be found at many of the highly conserved amino acid residues within the two predicted NBFs, suggesting that ATP binding is essential for CFTR function. Several of them are located in the transmembrane domains, because any alteration in some of the charged residues in those regions are not tolerated. No disease causing mutations have been found in any of the potential phosphorylation sites (Figure I.2).

CFTR mutant patients may be divided into three general classes according to their properties: Class 1 represents the majority of CF mutations including ΔF508, for which the mutant polypeptide is blocked from being properly inserted into the apical membrane. It is believed that these mutant proteins have a problem in protein folding and maturation. Class

2 mutant proteins are processed correctly, but the channel fails to open upon cAMP stimulation. The third class is believed to have some amino acid substitutions in the transmembrane domains (Tsui, 1993).

G. Genotype-Phenotype Correlation

The degree to which the nature of a mutation (genotype) influences the clinical course of the disease (phenotype) is under investigation. This type of analysis can provide prognostic information for patients and enlightens the functional role of a protein at the cellular, tissue and organ levels. However, the heterogenous expression of the CFTR gene makes the correlation difficult. Among the various clinical findings, the most remarkable one is that affected members within the same family have a similar degree of pancreatic function. This indicates that both the severity of pancreatic disease and its rate of progression are determined by the patient's genotype (Sferra et al., 1993). It has been observed that about 90 per cent of the mutant CFTR alleles are associated with very low levels (<1 per cent of normal) of pancreatic secretion (pancreatic insufficiency, PI), while only 10 per cent of the mutant alleles confer close to normal levels of secretion (pancreatic sufficiency, PS). The molecular basis behind this observation was revealed by mutation analysis. Individuals with one or two copies of the missense alleles (such as R117H) are found to be PS, whereas those with two copies of nonsense, frameshift, splice junction or a subset of missense mutations are invariably PI (Aitken et al., 1993; Tsui, 1993). Other phenotypic differences, such as pulmonary function, sweat chloride level and age of diagnosis were also observed among CF patients, but there is no good correlation between these factors and pancreatic function or other clinical characteristics (Sferra et al., 1993). The mildest CF mutation thus far identified results in male infertility as the only manifestation of cystic fibrosis (Angurano et al., 1992). It is now obvious that genetic and environmental factors apart from CF genotype also play an important role in the overall severity of the disease.

One of the most interesting mutations with a mild phenotype of CF is the 3849+10 kb C→T in intron 19 (Highsmith et al., 1991). The genetic lesion is creation of a new exon due to a resulting intron-exon boundary. Also, the patients have normal sweat chloride levels (≥60 mmol/L). Moreover, the mutation was detected in high frequency in Israel, including one Turkish patient (Abeliovic et al., 1992 Augarten et al., 1993).

The characteristics of the CF phenotypes can be categorized into three levels, probably reflecting the role of CFTR in the tissues involved: The first is the common symptom for all CF patients, the high sweat chloride level. The second type of clinical result is the pancreatic function status. At the third level, the phenotype is believed to be strongly influenced by other factors, since the symptoms may be variable even in patients of the same family who carry the same mutation (Strong, 1991).

H. Mutation Detection in CF

Identification of mutations in the CF gene has many implications for clinical medicine. DNA analysis is used as an aid in diagnosis and carrier testing in affected families. However, not all of the CF mutations have been identified yet, and no single method can detect all of the known CF mutations. In other words, a variety of strategies used to detect different CF mutations.

Procedures for mutation detection can be separated into two distinct groups: The first consists of techniques which efficiently identify known disease alleles, thus are used in population screening for carriers of the common CF mutations. The second group consists of methods to scan gene regions for unknown mutations. While several useful technologies for the detection of sequence heterogeneity exist, no single method is applicable for all situations. The ability to amplify DNA enzymatically via the polymerase chain reaction represents the most important advance in mutation detection technology. All the procedures described below rely on PCR amplification of the sample DNA prior to analysis.

1. Identification of Known Mutations

If mutant and normal template sequences are present together in a PCR reaction, heteroduplexes between the two different DNA species can form during the late cycles. Heteroduplex molecules with a single base variance may show a different mobility from homoduplexes in polyacrylamide gels. This phenomenon is thought to be caused by sequence dependent conformational changes in the dsDNA. This technique is attractive

because of its simplicity and has been applied successfully for the detection of the most common mutation in CF, namely ΔF508, which is a three base deletion.

Some point mutations generate or remove a restriction endonuclease recognition site as in the mutation G551D. Thus, in those cases, digestion of the amplified product with the specific restriction enzyme and subsequent electrophoresis identifies normal DNA fragments and mutant alleles depending on fragment size.

Another method does not require an alteration in a restriction enzyme site, thus is more general and utilizes allele-specific oligomeric probes (ASO) in dot blot hybridization (Diaz-Chico et al., 1988). The PCR product is applied in duplicate to two hybridization membranes. One of the membranes is probed with an oligomer homologous to the normal DNA sequence and the other with an oligomer homologous to the mutation.

There are several other techniques which are used to screen known CF mutations, such as amplification refractory mutation system (ARMS) (Newton et al., 1989) and reverse dot-blot hybridization (Chehab et al., 1991). Those methods are simple, quick and reliable, but they require that 80-90 per cent of the mutations be known for the particular population, to be effective.

2. Identification of Unknown Mutations

Single base alteration is the most common type of mutation in CF. Denaturing gradient gel electrophoresis (DGGE), first described by Fischer and Lerman, 1979, is a highly reliable and rapid method specifically in screening genes for unknown mutations. Once the appropriate GC-clamped PCR primers are obtained and denaturant conditions determined, single base differences can be detected with 95 per cent accuracy in fragments up to 500 bp in length. In the method, dsDNA fragments are electrophoresed through a polyacrylamide gel that contains a linear gradient of increasing denaturant concentration. As the DNA migrates, the two DNA strands progressively in the fragment dissociate in discrete sequence dependent domains at increasingly greater melting temperatures (Tm). Therefore, the molecule forms a branched structure that has a retarded mobility in the gel. Although the presence of a sequence difference can thus be detected in the fragment, its precise location and nature needs to be determined by DNA sequencing.

Single strand conformational polymorphism (SSCP) is another widely used technique to determine unknown sequence variations (Orita et al., 1989). Wild type and mutant target DNA are amplified by PCR, denatured and electrophoresed side by side through a neutral polyacrylamide gel. The two single strands of each denatured PCR product assume a three-dimensional conformation which is dependent on primary sequence.

If a difference exists between the wild type and the mutant DNA sequences, this may result in a different mobility of one or both of the strands of the mutant with respect to those of the normal fragment. PCR products with altered migration patterns are thus indicative of sequences different than normal, and are later analyzed by DNA sequencing for a further detailed result. SSCP analysis detects 70-95 per cent of mutations in PCR products of 200 bp or less. The sensitivity of the method decreases with increasing size of the PCR product.

Both methods described above are capable with varying efficiencies of detecting mutations, but none of them defines precisely the nature of the change. It is the DNA sequencing that defines the location and nature of the change, therefore, sequencing is a necessary final step in any mutation detection method. Sanger's dideoxy chain termination method is the most commonly used sequencing technique. In order to sequence PCR products successfully by this method, it is essential to convert the double stranded PCR product into a single-stranded sequencing template. Several methods can be used to achieve this. In the asymmetric or single-strand PCR (ssPCR), product is amplified in a second reaction in which one of the primers is in 100:1 excess of the other. This creates an abundance of single stranded PCR product for sequencing. Then, ssPCR product is purified from the left-over primers and nucleotide triphosphates by using either agarose gel extraction or spin dialysis membranes, such as Centricon 100. The latter is easier and more efficient, but expensive. The purified sample is freeze-dried and dissolved in water. The molar amount of sample used in the sequencing reaction depends on the method employed.

II. PURPOSE

Cystic fibrosis is the most common recessive disorder in Caucasian populations of Northern European ancestry. Carrier detection based on the protein product of the CF gene is not yet available. The only way to directly diagnose heterozygotes is by mutation analysis, made possible by the cloning of the CFTR gene (Riordan et al., 1989; Rommens et al., 1989) and identification of mutations in CF patients (Kerem et al., 1989; Cutting et al., 1990). But, there is an important restriction in several populations since the efficiency of carrier detection by mutation analysis depends on the fraction of known mutations in the given population (Beaudet et al., 1990). There is no treatment available yet for CF. The prevention of the disease is possible by carrier identification and subsequent prenatal diagnosis in families at risk. In order to design a heterozygote screening program in a given population, two sets of data are of crucial importance: First, the type of mutations and their proportions in the CF patients, and second, the heterozygote frequency in the general population. The aim of this study was to initiate an approach to these two prerequisites with regard to the Turkish population. Thus, exon 10 which is one of the mutation hot spots was screened.

Any *de novo* mutations determined in the Turkish cystic fibrosis patients may help elucidate the problems on the molecular basis of the genetic disorder through phenotype genotype correlations. Identification of unknown mutations is important in this aspect.

III. MATERIALS

A. Equipment

Autoclaves : Medexport, C.W.I.S.
Eyela, Japan

Balances : Electronic Balance Type 1574, Sartorius, Germany
Electronic Balance Libror EB-3200H, Shimadzu, Japan
Precision Balance H72, Mettler, Germany

Cameras : RB67, Mamiya, Japan
Direct Screen Instant Camera, Polaroid, USA

Centrifuges : Sorvall RC-5B Refrigerated Superspeed Centrifuge,
Dupont, USA
Biofuge A, Heraus Christ, Germany
Hettich EBA 3S, Germany

Deepfreezers : -70°C, GFL, Germany
-70°C, Sanyo, Japan
-20°C, Bosch, Germany

DGGE Apparatus : Home made, A. T. Akarsubasi and T. Onay

Electrophoretic Equipment : Vertical apparatus, PS500 XT, Hoeffer Scientific Instruments, USA
Horizon 58, Model 200,Horizontal Gel Electrophoresis Apparatus
Sequencing Apparatus, Bio-Rad, USA
Miniprotean II, Bio-Rad, USA

Freeze-Drier : ChemLab Instruments Ltd, Model SB6, England

Heat-block	: Multi-block Lab-Line, USA
Incubators	: Incubator, Plus Series, Gallenkamp, Germany Oven 300, Plus Series, Gallenkamp, Germany
Power Supplies	: ECPS 3000/150 Constant Power Supply, Pharmacia, Sweden Model 100, BRL, USA
Spectrophotometer	: Lambda 3 UV/VIS, Perkin-Elmer Cetus, USA
Thermo-cyclers	: Model 480, Perkin-Elmer Cetus, USA Thermal Reactor TR1, Hybaid, UK
Transilluminators	: Reprostar II, Camag, Switzerland Fluorescent Table, Consort, Belgium
Gel Dryer	: Vacuum Dryer, Biometra, Germany
Water baths	: Thermomix, BU, Braun, Germany Thermomix 1441, Braun, Germany

B. Chemicals

All chemicals used in this study were purchased from Merck (Germany) or Sigma (USA) unless stated otherwise in the text. Absolute alchohol was from Tekel (Turkey). The enzyme Taq DNA polymerase was from Perkin Elmer Cetus (USA), Promega (USA) or Boehringer Manheim (Germany).

C. Buffers and Solutions

1. DNA Isolation Buffers

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

Buffer A (1X) : 67 mM Tris-HCl (pH 8.8)
16.6 mM $(NH_4)_2SO_4$
6.7 mM $MgCl_2$
0.17 mg/ml BSA
10 mM β-Mercaptoethanol

Buffer B (1X) : 10 mM Tris (pH 8.3)
50 mM KCl
1.5 mM $MgCl_2$

3. Restriction Enzyme and Its Digestion Buffer

Hph I	: 200 units (4000 units/ml)
NE buffer	: 50 mM Potassium acetate 20 mM Tris acetate 10 mM Magnesium acetate 1 mM DTT, (pH 7.9)

4. Electrophoresis Buffers and Gel Systems:

a. Electrophoresis Buffers:

5X Tris-Borate (TBE) Buffer	: 445 mM Tris-base 445 mM Boric acid 10 mM Na_2EDTA
20X Tris-Acetate (TAE) Buffer	: 0.8 M Tris-base 0.4 M Sodium acetate 0.02 mM Na_2EDTA
10X Loading Buffer A	: 2.5 mg/ml BPB 1% SDS in glycerol
10X Loading Buffer B	: 20% Sucrose, 10 mM Tris (pH 7.8), 1 mM Na_2EDTA 0.25% BPB 0.25% XC
Ethidium Bromide	: 10 mg/ml

b. Gel Systems

Agarose gel	: Agarose in 0.5X TBE
NuSieve	: 2% NuSieve 1% Agarose in 0.5X TBE
30% Acrylamide (19: 1)	: 28.5% Acrylamide 1.5% Bis-acrylamide (N, N-Methylene-bis-acrylamide)
40% Acrylamide (19: 1)	: 38% Acrylamide 2% Bis-acrylamide
Instagel Solution (19: 1)	: 8% Acrylamide (prepared from 37.5:1, 40% stock)) 7 M Urea 1X TBE Buffer (pH 8.3)
40% Acrylamide (37.5: 1)	: 37.5% Acrylamide 1% Bis-acrylamide
0% Stock Denaturing Gel	: 6.5% Acrylamide/Bis-acrylamide (37.5:1) 1X TAE Buffer (pH 7.4)
80% Stock Denaturing Gel	: 6.5% Acrylamide/Bis-acrylamide (37.5:1) 5.6 M Urea 32% Deionized Formamide 1X TAE Buffer (pH 7.4)
10% Ammonium persulfate	: 1 g APS in 9 ml H_2O peroxidisulphate (APS)

D. Others

The radionucleotide [^{35}S]-dATP α S (1000 Ci/mmol) was bought from Amersham (USA).
Centricon spin dialysis membranes were from Amicon (USA).
Instant films (Polaroid 667) were from Polaroid (USA).
X-ray films (X-Omat AR) and the developing solutions were from Kodak (USA).

E. Oligonucleotide Primers:

All primers used in DGGE analysis were purchased from MAM-TUBITAK (Turkey). The specific primers for heteroduplex analysis were bought from GenSet (France) and from TUBITAK.
The sequences for all the oligonucleotide primers used in this study are given in Table IV.1.

F. Blood and Fetal Samples

Blood, amniotic fluid or chorionic villi 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 Pediatrics, Istanbul University Cerrahpasa Medical School (Istanbul).

Table IV.1- Oligonucleotide primer sequences used in PCR amplification reactions.

Region	Oligonucleotide Primer Sequence
ΔF	5' GTTTTCCTGGATTATGCCTGGCAC 3' 5' GTTGGCATGCTTTGATGACGCTTC 3'
Exon 10	5' *CCGCCGCGCCCCGCGCCCGCGGGCCTCCCGCCGCCCGCGCC CAGTGTGAAGGGTTCATATGCA 3' 5' CACTTCTGCTTAGGATGATAAT 3'
Intron 19	5' AGGCTTCTCAGTGATCTGTTC 3' 5' GAATCATTCAGTGGGTATAAGCAG 3'

* 40 bp GC clamp.

IV. METHODS

A. DNA Extraction

1. DNA Isolation from Whole Blood

Two to ten ml of peripheral blood was drawn into K_2EDTA containing tubes to prevent coagulation. Tubes were stored either at four degrees centigrade for a maximum of two days or at -20°C for longer periods. Of the existing DNA isolation procedures, the most common ones are still based on the use of toxic organic solvents such as phenol and chloroform. Furthermore, organic DNA extraction techniques are very slow, inconvenient and relatively expensive. Miller et al. (1988) have applied the well known protein salting out technique for isolating high molecular weight DNA. In our adapted procedure (Middleton L., personal communication) NH_4OAc was used instead of the NaCl in Miller's procedure, thus avoiding the presence of NaCl in the DNA sample after the salting out procedure.

To lyse the cells, the blood sample was transferred to a 50 ml Sorvall centrifuge tube and ice cold lysis buffer was added; (30 ml of buffer per 10 ml of blood sample), mixed well and left in the cold room (+4°C) for 15 min or until the sample became clear. It was later centrifuged at 5 K at +4°C for 10 min to collect leukocyte (white blood cell: WBC) nuclei. All centrifugations were carried out in SS34 rotor in Sorvall RC-5B Refrigerated Superspeed Centrifuge from Dupont (USA). This step was repeated using 10 ml lysis buffer to wash the pellet. The supernatant was discarded and the pellet (WBC nuclei) was ready to be treated with any of the extracting methods, such as salting out or phenol-chloroform extraction. The nuclear pellet was suspended in three ml of nuclei lysis buffer and vortexed till clumps disappeared completely. The sample was incubated with proteinase K (150 µg/ml) and SDS (0.14 per cent) at 37°C for overnight or at 56°C for two to four hours to degrade the proteins. Afterwards, the viscous sample was transferred to a smaller tube and 0.37 ml of 9.5 M ammonium acetate per ml of lysate was added. The tube was shaken vigorously untill it had a milky appearance. The sample was centrifuged at 10 K at room temperature for 20 min.

The DNA in the supernatant was precipitated by addition of two volumes of absolute ethanol. The tube was gently inverted several times until DNA thread became visible, which were later washed in 70 per cent ethanol. Finally, DNA was taken into 0.5-1.0 ml of TE buffer and left overnight at room temperature to dissolve.

2. DNA Extraction from Chorionic Villus Samples (CVS)

DNA for prenatal diagnosis was extracted from CVS samples received in 0.15 M NaCl solution. The samples were treated immediately by the method described by L. Middleton (personal communication). Ten to 15 µl of CVS was washed in nuclei lysis buffer. Fourhundred-fifty µl of nuclei lysis buffer was added and mixed well. Twenty-five µl of proteinase K (20 mg/ml) and 25 µl of 10 per cent SDS were added and the sample was incubated at 56°C for two hours. Two hundred µl of the sample was transferred into an Eppendorf tube. The rest of the sample was saved in case anything went wrong. After mixing with 80 µl of 9.5 M NH_4OAc, centrifugation was performed in the microcentrifuge at 10 K at room temperature for five minutes. The supernatant was transferred into a clean tube and two volumes of absolute ethanol was added. DNA was precipitated by inverting the tube several times, taken out using a micropipette tip and suspended in 0.1 ml of TE buffer. The amount of DNA was determined on a minigel.

B. Qualitative and Quantitative Analysis of Isolated DNA Samples

There are two widely used approaches for the quantitative and qualitative analysis of DNA samples: Spectrophotometric assay and minigel method. The former was used for precisely determining the concentration and purity of the DNA, while the latter gave a fast estimate of its quantity.

1. Spectrophotometric Analysis

The concentration of DNA was calculated as a function of the optical density at 260 nm by applying the formula :

50 μg/ml × OD_{260} × dilution factor = concentration in μg/ml

(50 μg of double stranded DNA has an absorbance of 1.0 at 260 nm)

The purity of DNA was determined by calculating the ratio between the optical densities at 260 nm and 280 nm. OD_{260}/OD_{280} as 1.8 is the reference value for a sample of high purity.

2. Minigel Method

The minigel was prepared by using 0.8 or 1.0 per cent (w/v) agarose in 0.5XTBE buffer containing ethidium bromide (0.5 μg/ml). One or two ml of the DNA sample was mixed with loading buffer and applied to the agarose gel. After electrophoresis at 10V/cm for 15-20 min, the band was visualized under UV light. The amount of DNA was estimated by comparing its intensity to a standard quantity of DNA, typically a 200 ng sample.

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 here are based on PCR amplification of the sample DNA prior to analysis.

1. PCR for Detection of the Mutations ΔF508 and 1677delTA via Heteroduplex Formation Analysis

A double stranded target sequence of 98 bp was amplified in a 25 µl reaction volume. The sequences of the synthetic oligonucleotide primers namely ΔF forward (ΔF F) and ΔF reverse (ΔF R), (Kerem et al., 1989) are shown in Table IV.1. The reaction samples contained 1XGuy's buffer, 0.75 pmole of each primer, 0.4 mM dNTP, 200 ng genomic DNA and 0.5 units of Taq DNA polymerase in 25 µl. The sample was overlayered with a drop of mineral oil to prevent evaporation. The reaction tubes were placed in the thermocycler set to program A: Initial denaturation at 94°C for three minutes, followed by 94°C for 30 seconds for denaturation and 60°C for two minutes for primer annealing and chain elongation for a total of 30 cycles. A final three-minute elongation step allowed annealing of any single stranded fragments. (Table IV.2).

After the thermocycler program was completed, three to five microliters of the PCR reaction was assayed on a 1.2 or 1.5 per cent agarose gel to estimate the quantity of the product.

2. Heteroduplex Analysis

The PCR product was transferred onto a piece of parafilm to get rid of the oil and the aqueous phase was taken into a clean tube. The sample was heated to 95°C for five minutes then reannealing was allowed at 65°C for five minutes, which should facilitate heteroduplex formation in heterozygotes. Heteroduplexes formed between wild type and mutant PCR products can be detected on polyacrylamide gels, because they migrate more slowly than the corresponding homoduplexes. Thus a heteroduplex is easily identified.

If a patient is homozygous for either ΔF508 or 1677delTA, the presence of the mutation is revealed by the slightly higher mobility of the homoduplexes upon polyacrylamide gel electrophoresis. However, the efficiency of detection may be improved by heteroduplex analysis. Therefore, the PCR product of a patient was heteroduplexed with that of a normal person's after mixing them in equal amounts (15 µl each). The samples were assayed on a 12 per cent polyacrylamide gel using as marker the heteroduplex samples from heterozygote individuals for the mutations ΔF508 and 1677delTA as markers. Two heteroduplex bands with lower mobility than either homoduplex bands indicated the presence of the respective mutation.

Table IV.2- Conditions for PCR amplifications of exon 10 and intron 19.

	Length of PCR product	Buffer*	Denaturation	Annealing	Extension
Detection of dF & del TA	98 bp	A	94°C/1 min	60°C/2 min	60°C/3 min
DGGE	370 bp	B	94°C/30 sec	55°C/30 sec	72°C/2 min
ss PCR for sequencing	370 bp	B	94°C/30 sec	55°C/30 sec	72°C/2 min
Intron 19 3849+10 kb C→T	435 bp	B	94°C/30 sec	55°C/30 sec	72°C/2 min

* Buffer A: 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris (pH 8.8)
Buffer B: 67 mM Tris, 16.6 mM $(NH_4)_2SO_4$, 6.7 mM $MgCl_2$, 0.17 mg/ml BSA.

The gel was prepared in a 15 ml volume and contained six ml of acrylamide:bis-acrylamide from a 30 per cent stock (19:1), three ml of 5X TEB buffer, six ml of dH_2O, 100 µl of 10 per cent APS and nine ml of TEMED. It was run in 1X TEB buffer for approximately two hours at 10V/cm.

3. PCR for Denaturing Gradient Gel Electrophoresis (DGGE)

Amplification of exon 10 of the CFTR gene yielded a 370 bp fragment. One of the two oligonucleotide primers had a clamp of 40 base long GC rich segment in its five prime end to create a high melting temperature domain (Table IV.1). PCR reactions for DGGE analysis were performed in a total volume of 50 µl. Reaction mixtures contained 1XGuy's buffer, 0.8 mM of dNTP, 0.75 pmole from each primer, approximately 200 ng genomic DNA and 0.5 units of Taq DNA polymerase. Thermocycler program B for PCR was as follows: Initial denaturation was at 94°C for five minutes, then at 94°C for 30 seconds for denaturation, 55°C for 30 seconds for annealing of the primers, 72°C for two minutes for elongation for a total of 32-35 cycles, and lastly, two minute extension of the last annealing step.

4. PCR for Sequencing

Two subsequent PCR reactions were carried out for sequencing reactions: A double-stranded (dsPCR) and an asymmetric or single-stranded (ssPCR) PCR (Gyllensten et al., 1988).

a. Double-Stranded PCR (dsPCR)

Amplification conditions were the same as described in the previous section for DGGE, except that the total volume was 25 µl.

b. Single-stranded PCR (ssPCR)

One µl of template from dsPCR product was used for a 100 µl single-stranded amplification reaction. This was preferred over using a purified or agarose extracted dsDNA sample as template, as was suggested in protocols, since nothing in the sample was expected to interfere with the synthesis of ssPCR. A reaction mix for ssPCR contained 1X Guy's buffer, 0.4 mM dNTP, one pmole of primer exon 10 GC-R, 100 pmole of exon 10 F primer, one µl template DNA and 0.5 units of Taq DNA polymerase and was subjected to thermocycler program B (35 cycles). Since one of the primers had a very low concentration, after the first few cycles, amplification continued linearly only on one strand. PCR product was assayed on a 1.5 per cent agarose gel in which typically two different bands were observed, one ds and the other ss.

5. PCR for 3849+10 kb C → T in Intron 19

The genomic region containing the site of the mutation 3849 + 10 kb C to T in intron 19 was amplified by PCR using two oligonucleotide primers given in Table IV.1. PCR reaction was performed in 25 ml and prepared as follows: 1X Guy's buffer, 0.75 mole of each primers, 0.4 mM dNTP, 200 ng genomic DNA and 0.5 units of Taq DNA polymerase. The reaction tubes were placed in thermocycler, and program B was performed as indicated in part 3 of methods.

D. Denaturing Gradient Gel Electrophoresis (DGGE)

In this method, dsPCR products are electrophoresed on a polyacrylamide gel that contains a linearly increasing gradient of denaturants, namely urea and formamide (a combination of 7 M urea and 40 per cent formamide corresponds to 100 per cent denaturant concentration). DGGE facilitates mutation detection because mutant DNA migrates differently from its wild type counterpart. Each fragment (homoduplex or

heteroduplex) melts at a unique denaturant concentration and once denatured, migrates extremely slowly.

1. Preparation of a Denaturing Gradient Gel

One of the most important factors in the DGGE method is the formation of an undisrupted linear concentration gradient during gel casting. For that purpose, a gradient maker was used which had two chambers separated with a stopcock via the u-tube principle and a peristaltic pump to ensure constant acrylamide flow into the vertically placed glass plates with 0.75 mm thick spacers. A gradient of desired denaturant concentration was prepared by dilution from two 6.5 per cent acrylamide stock solutions, one of which had 80 per cent denaturants while the other had none. The chambers contained eight ml of 6.5 per cent acrylamide:bisacrylamide (37.5:1), one with denaturants with uppermost and the other with lowermost concentration. Eighty µl APS and 3 µl TEMED were added to the acrylamide solutions just before pouring them into the chambers. Lastly, 5 milliliters of acrylamide solution without any denaturant was poured to the uppermost part where slots were to be formed. After polymerization for an hour, the gel was stored at four degrees centigrade untill application time, which was usually the next day.

2. Electrophoresis

Amplified DNA samples were freeze-dried down to about half volume (15-20 µl) and mixed with 2-3 µl of loading buffer before they were applied to the gel. The gel was mounted on a TAE buffer containing tank which had been preheated to 60°C. The temperature was kept constant throughout electrophoresis. A constant temperature and a uniformly heated gel was essential for obtaining sharp bands. After seven hours at five V/cm, the gel was stained with ethidium bromide (EtBr) for five minutes and visualized under ultraviolet light.

E. Sequencing with Sequenase Version 2.0 Kit

Once a gene region has been identified as different from normal, DNA sequence analysis is performed to determine the sequence difference. DNA sequencing defines the location and nature of the change, thus is the necessary final step for mutation detection. For this purpose, the Sequenase Version 2.0 kit (USB) is used, which is based on Sanger's dideoxy chain termination method. All necessary components for the reaction were supplied with the kit.

1. Purification of the PCR Product

Both the quantity and the purity of the DNA template are very important for sequencing to function efficiently. For this purpose, after checking the amount of the asymmetric PCR product on a gel as described in section IV.B. 2, the remaining product (~100 µl) was purified by spin dialysis using a Centricon-100 microconcentrator. Centricon-100 was chosen because the size of one of the primers was long. Two ml of dH_2O was added to the amplified product in the Centricon-100, which was then centrifuged at 3.5 K for 25 min. This step was repeated with 1.5 ml dH_2O. Later, the filter was inverted and spinned at 5 K for two minutes to collect the purified product, which was then freeze-dried and dissolved in 10-12 µl of dH_2O. The sample was checked once more on an agarose gel for quality and quantity. There should be no primers.

2. Sequencing Reactions

Dideoxy termination sequencing method using the Sequenase Version 2.0 kit (USB) consists of three steps: Annealing, extension and termination. A modified procedure was used instead of the annealing step recommended by the manufacturer. In the modified procedure, the sample was heated to 37°C for 15 minutes rather than to 65°C for 2 min and the sample was cooled to 35°C for annealing. At most 3 µl of template DNA was used and

sterile dH_2O was added to 7 µl. One µl of primer exon 10 F and 2 µl of reaction buffer was added, mixed and left in a 37°C water bath for 15 min for annealing. In the extension step, 1 µl DTT, 2 µl diluted labelling mix (1:10), 0.5µl [^{35}S] dATP and 2 µl of diluted (1:8) sequenase polymerase were added, mixed and incubated at room temperature for approximately 4 min. In the termination step, 3.5 µl of the labeling reaction was transferred into 4 tubes, each containing one type of dideoxy-NTP and the incubation was continued at 37°C for 5 min. The reactions were stopped by addition of 4 µl of stop solution. Samples could be stored up to one week at -20°C.

3. Electrophoresis and Autoradiography

The sequencing reactions were run on an 8 per cent polyacrylamide gel containing 7 M urea. Samples were denatured just before application. Three µl of reaction mixtures were loaded in the order of A, C, G and T onto a gel preheated to 55°C. Typical running conditions were 50-55 W, 27-29 mA and 40 V/cm. Since BPB and XC that were included in the stop buffer were used as a measure for the extent of migration, the extent of electrophoresis could be monitored to resolve DNA of desired length.

After drying the gel in the vacuum drier for at least two hours at 90°C, an X-ray film was placed over the gel and left at room temperature for one to several days depending on the activity of the [^{35}S]dATP.

F. Restriction Enzyme Digestion

The Hph I restriction endonuclease was used to detect the mutation 3849+10 kb C→ T and also for the confirmation of the polymorphism 1540A/G.

1. Screening for the Mutation 3849+10 kb C → T by Enzyme Digestion

Intron 19 was amplified for the purpose of screening for the mutation 3849+10 kb C →T as described in methods part C. 3. To purify the sample, the amplified 435 bp product of 50 µl was precipitated by addition of 5 µl NaAc (3M) and 51 µl isopropanol. The mixture was centrifuged at 13 K for 10 minutes. The supernatant was discarded and the pellet was washed with 40 µl of 70 per cent, EtOH to wash the pellet. Finally, the pellet was dissolved in 10-15 µl water and made ready for digestion. The digestion mixture was prepared as follows:

2 µl Buffer (1X)
0.2 µl Hph I (1 unit)
7.3 µl H_2O
10 µl purified PCR product

Samples were incubated at 37°C for overnight, applied to a 2:1 NuSieve gel and electrophoresed for half an hour at 15 V/cm.

2. Confirmation of the Polymorphism 1540A/G by Restriction Endonuclease Digestion

Samples were amplified as in the Methods (part C.3). Five to 10 µl of amplified product was used directly (without purification) for digestion. All the other digestion and electrophoresis conditions were as given above.

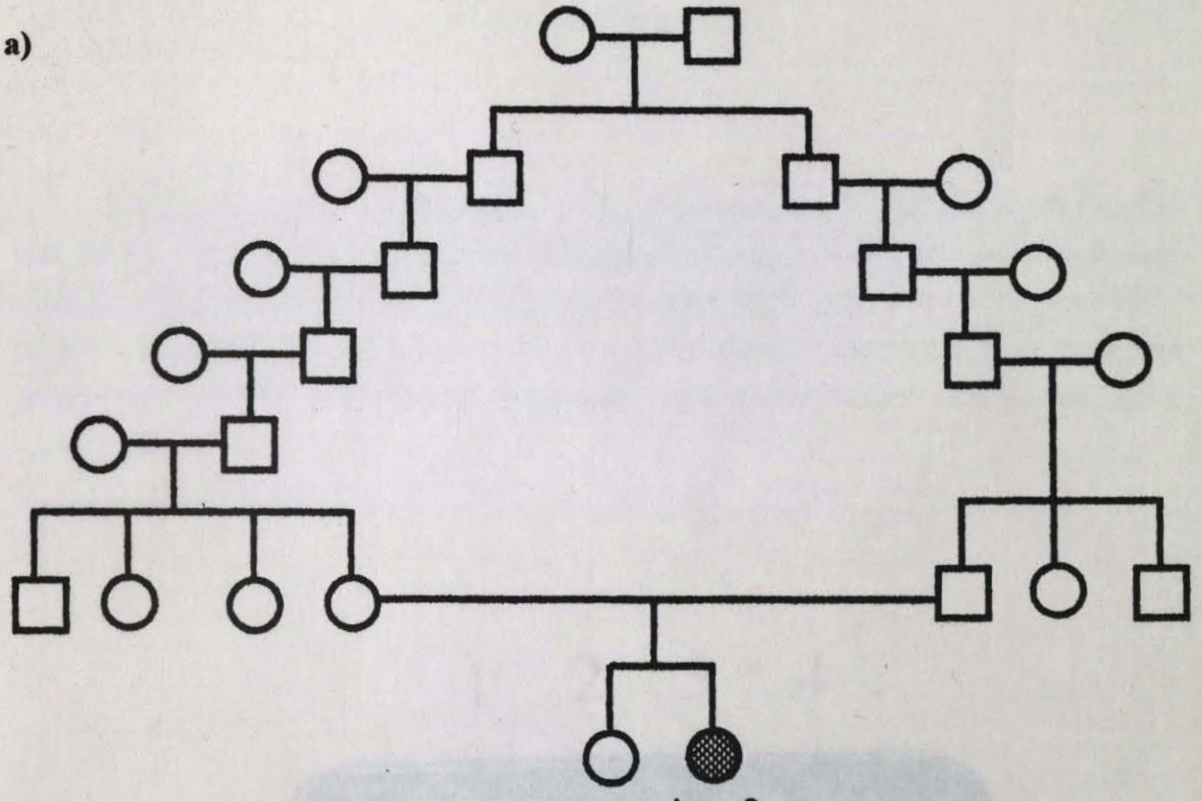
V. RESULTS

A. Heteroduplex Analysis for Detecting the Mutations ΔF508 and 1677delTA

Fifty unrelated CF patients and ten parents corresponding to 110 CF chromosomes were screened for the mutations ΔF508 and 1677delTA by heteroduplex analysis. Parents were related in two families. ΔF508 was detected in 17 out of 110 alleles. The frequency of the mutation ΔF508 is thus 15.5 per cent in our study. The mutation results in a 95 bp instead of the normal 98 bp PCR product. The patterns for individuals who are normal, heterozygous and homozygous with respect to ΔF508 are shown in Figure V.1. In the first lane is a marker DNA from a ΔF508 heterozygote, supplied kindly by Dr. Cahill at the University of California San Diego. DNA in the second lane is a normal (non-CF) control DNA. The DNA of patient 48 in lane 3 was observed to have migrated a little faster than a normal sample (lane 2), suggesting a ΔF508 homozygote genotype. To confirm the homozygotes, the normal DNA sample was mixed with that of the patient to facilitate heteroduplex formation as mentioned in Methods, part C.2. The heteroduplex pattern in lane 4 is typical of a ΔF508 heterozygote. In the pedigree of the family, the parents are indicated to be distantly related.

A different heteroduplex pattern was previously observed with another patient. The PCR product was sequenced by M. Telatar and shown to be the mutation 1677delTA, first described by Ivaschenko et al., 1991. The sample was used later as a control DNA for this rare mutation in analyzing other patients. In figure V.2, the heteroduplex pattern of patient 33 containing the 96 bp and the 98 bp fragments (lane 4) could easily be distinguished from the markers ΔF508 heterozygote (98 and 95 bp) in lane 3 and normal samples in lanes 1 and 2. Since the information on this mutation was limited and we had only a few patients carrying this particular mutation, we cooperated with some colleagues in the Black Sea region and Cyprus, where the 1677delTA had also been detected in several cases (Angelicheva et al., 1994). The mutation has a relatively high frequency in our patients (4.5 per cent) and accounts for 5.4 per cent of non-ΔF 508 chromosomes (Table V.1). The collaborative study was extended to cover the clinical phenotypes and haplotypes (Angelicheva et al., 1994).

a)



b)

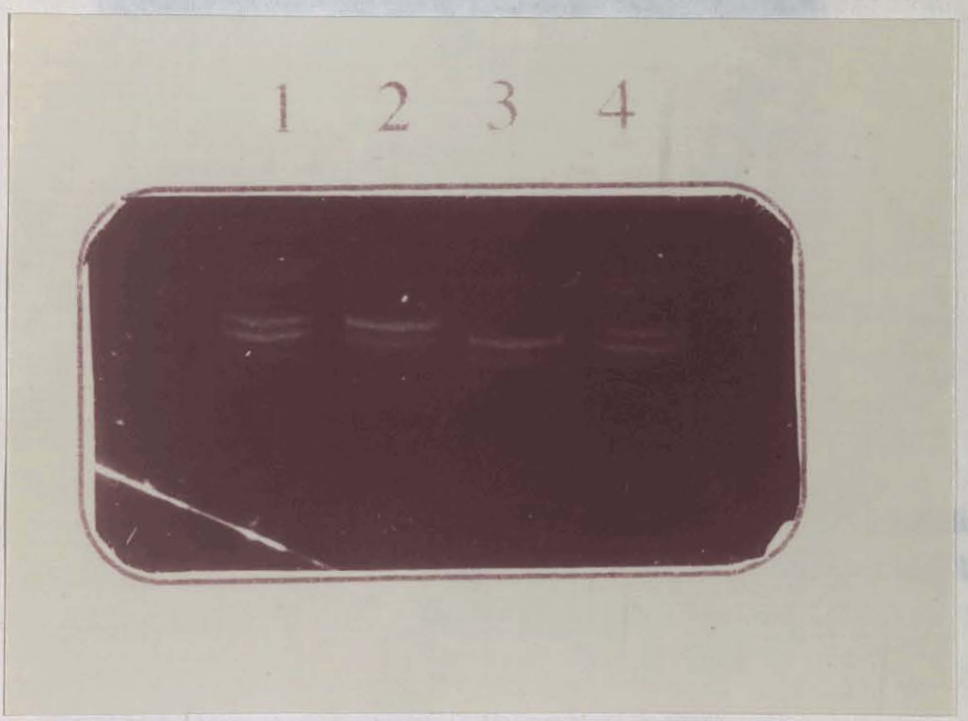


Figure V.1- a) The pedigree of the family 48, b) Heteroduplex analysis for detecting the mutation ΔF508, lane 1: ΔF508/N control DNA, lane 2: N/N control DNA, lane 3: patient 48 (ΔF508/ΔF508), lane 4: heteroduplex formation 48/N

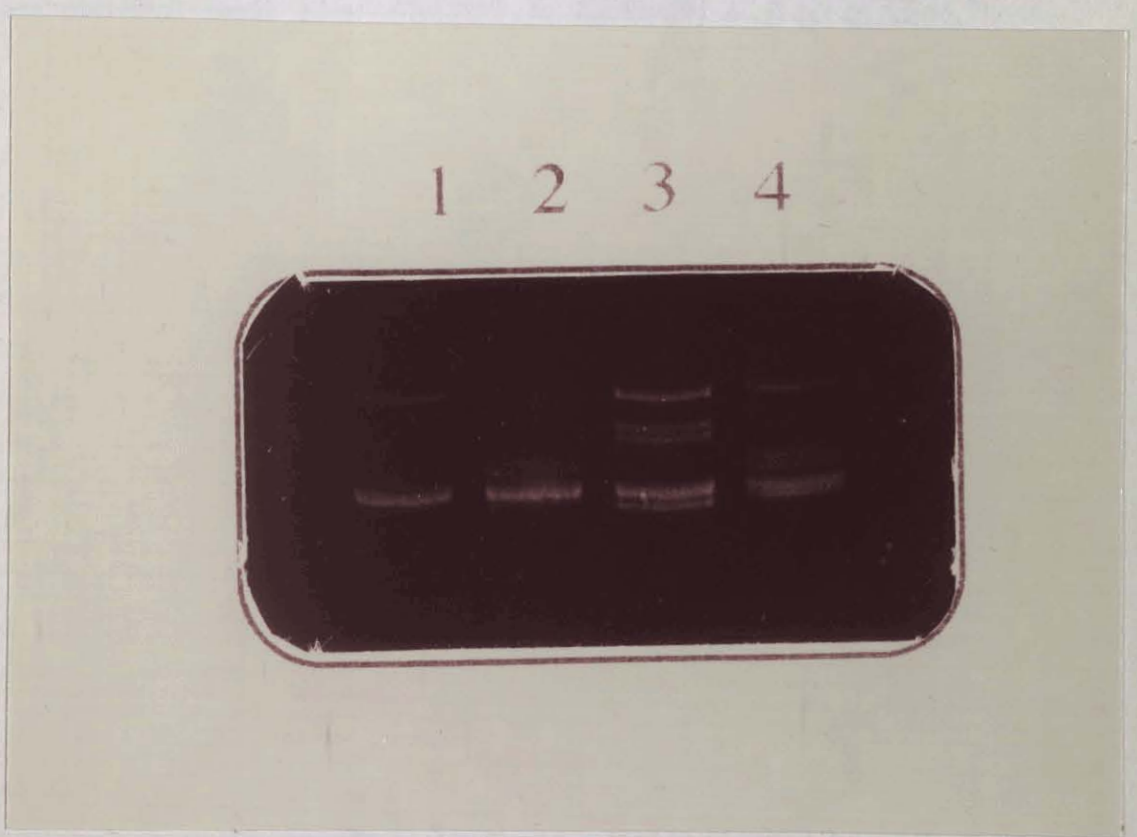


Figure V.2 12% Polyacrylamide gel stained with EtBr. The bands were visualized under UV light. Lane 1: Patient 32 (N/N), lane 2: N/N control DNA, lane 3: ΔF508/N control DNA, lane 4: Patient 33 (1677delTA/N).

B. Prenatal Diagnosis

During this study, prenatal diagnosis was requested by a family (CF26) who was at risk for CF since there was an affected child (lane 3 in Figure V. 3). A blood sample was obtained from the affected child and DNA analysis identified him to be a homozygote for ΔF508. The fetal CV sample was analyzed by heteroduplex formation and the fetus was found to be a ΔF508 carrier (lane 4). As a result, the pregnancy was allowed to continue.

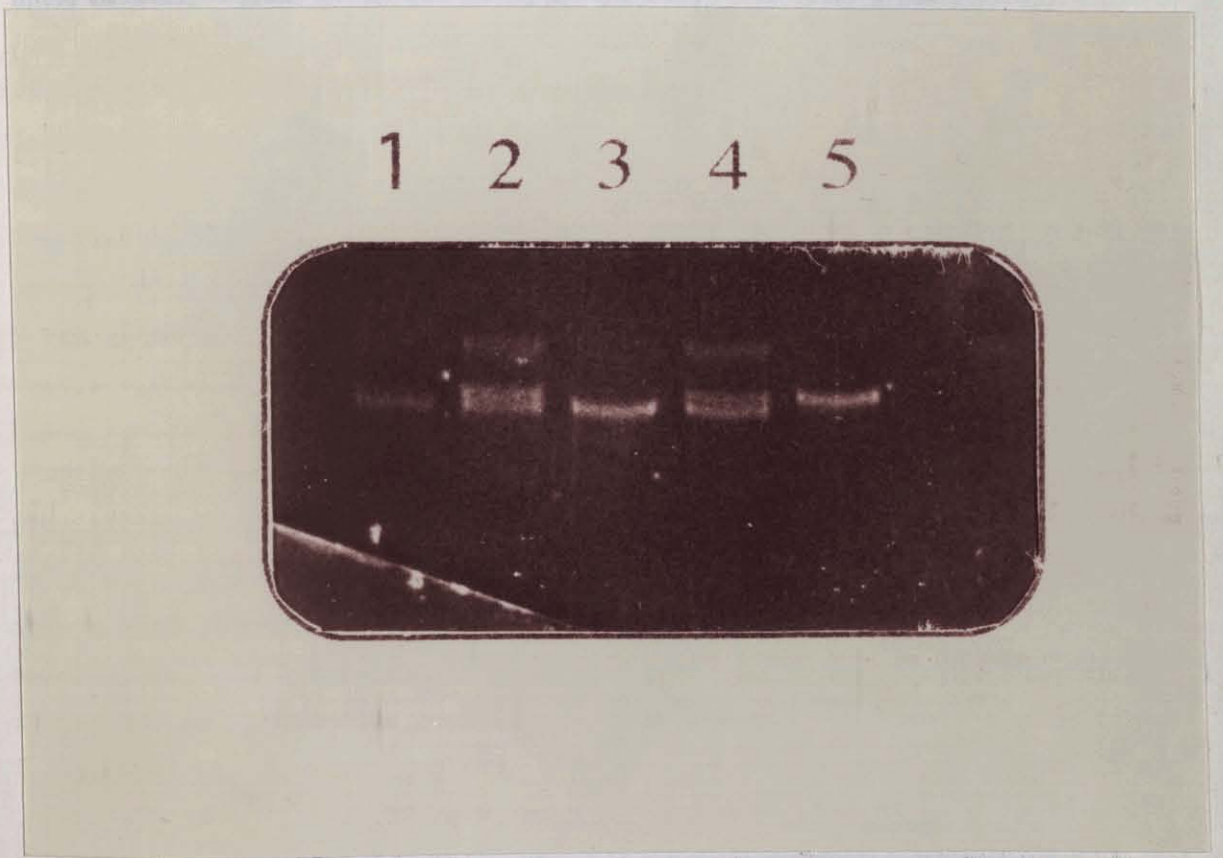


Figure V.3 Prenatal diagnosis in family 26 Lane 1: ΔF508 homozygote control DNA, lane 2: ΔF508 heterozygote control DNA, lane 3: affected child (ΔF508 homozygote), lane 4: fetal sample (ΔF508 heterozygote), lane 5: normal control DNA

C. DGGE Analysis of Exon 10

In this study, DGGE was used to detect any mutations other than ΔF508 and 1677delTA in exon 10 of the CFTR gene. A total of 74 CF chromosomes were screened by DGGE in order to detect any samples with unique migration patterns.

Exon 10 was amplified to give a 370 bp product with a 40 bp GC-clamp. Primer sequences and the conditions for amplification are described in Methods (IV. 3). The amplified product was electrophoresed on a 6.5 per cent polyacrylamide gel containing a linear denaturant gradient from 30 to 60 per cent at six V/cm for seven hours.

ΔF508, delTA1677 and normal (non-CF) DNA samples were used as markers to distinguish between different migration patterns (Figure V.4). Specifically, the two heteroduplex bands which are formed by annealing of the two strands of a mutated allele to those of the normal alleles strongly enhanced the resolution of the DGGE. In lane 12, Δ F508 heteroduplex bands could be observed. The same mutation in homoduplex form was seen in lane 11. Instead of a single homoduplex band, three discrete bands were observed in a normal individual (lanes 9 and 10), while a single band was seen at a defined position in another normal individual (lane 3) as expected. A normal DNA sample displaying three bands was sequenced (part V.E) and was found to be heterozygous for the polymorphism 1540A/G. The sample in lane 5 exhibited a different homoduplex pattern which was later detected to carry G/G allele at 1540 by using Hph I digestion. The heteroduplex in patient 44 (first lane) displayed a totally different migration pattern than the others. Later, the sequence analysis revealed that this individual carried the mutation S466X. Four other samples with either heteroduplexes or homoduplexes with unique migration patterns were sequenced later to determine the nature of the mutations.

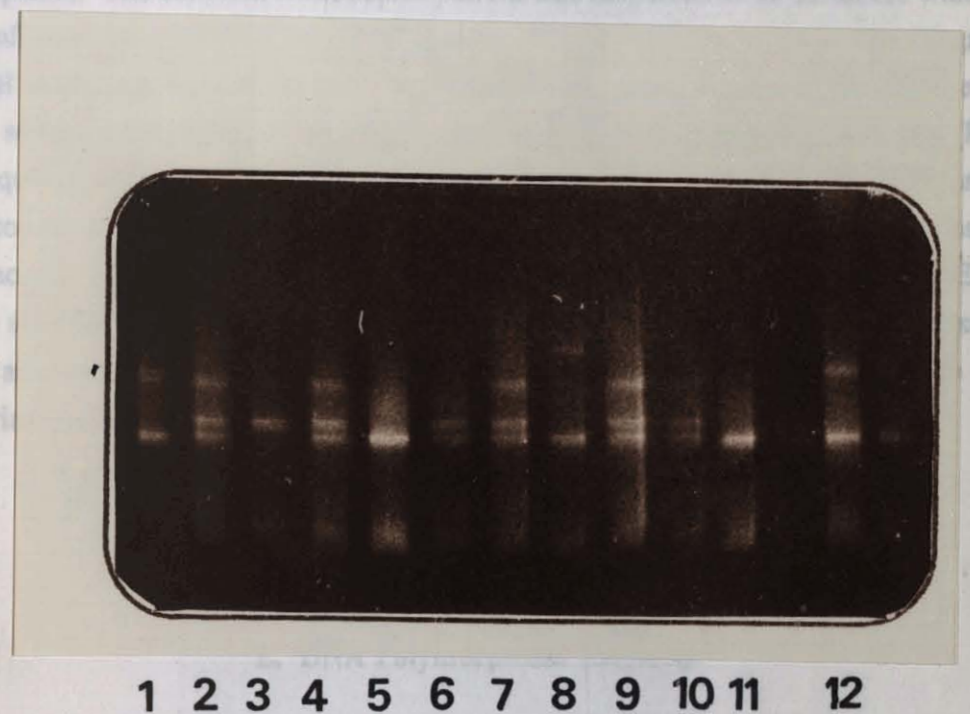


Figure V.4 DGGE analysis of exon 10. Lane 1: Patient 44 (S466X), lane 2: N/N (1540A/G), lane 3: N/N (1540A/A), lane 4: N/N, lane 5: N/N (1540G/G), lane 6: N/N, lane 7: N/N, lane 8: yet undetermined, lane 9: N/N, lane 10: N/N, lane 11: ΔF508 homozygote, lane 12: ΔF508/N.

D. Sequencing

Six samples which displayed different migration patterns in DGGE analysis were subjected to sequence analysis. In four of them the whole coding region and a few bases from each intron were sequenced. One of the samples was determined to have the G/G alleles of the polymorphism 1540A/G (Figure V.5) and another to be heterozygous for the polymorphism. The common homoduplex pattern was suspected to be 1540A/A which was later confirmed by restriction enzyme digestion. The DGGE analysis of exon 10 gave an abnormal pattern in one of the patients. This pattern was not seen in any of the other patients screened by DGGE. The sample was found to be a S466X heterozygote (Patient 44). Sequence analysis revealed a C to G base change at nucleotide position 1529, creating a stop codon at amino acid position 466 (Figure V.6). The fourth patient was found to have a normal sequence in exon 10 inspite of a different migration pattern in DGGE. The last two samples were sequenced up to approximately 50 bases in the 5'-region of exon 10. Further analysis can be carried out by means of longer electrophoresis run time to detect base variations for the remaining part of the exon, if there is any.

E. DNA Polymorphism 1540A/G

The unusual heterozygote band formation in normal (non-CF) individuals as well as in several patients lead us to sequence the fragment. As a result, the substitution of G for A at nucleotide 1540 was found to be responsible for this specific band pattern. The nucleotide substitution results in the substitution of valine by methionine at amino acid position 470. The restriction site for Hph I is created endonuclease at the polymorphic site. Therefore, the polymorphism could be confirmed by restriction enzyme digestion. The patients were screened using Hph I and the polymorphism was found to occur at a high frequency (36/96). The digestion patterns of Hph I are shown in Figure V.7.

Figure V.5- DNA sequence analysis of the sample in lane 2 of Figure V.4 carrying alleles A/A at position 1540.

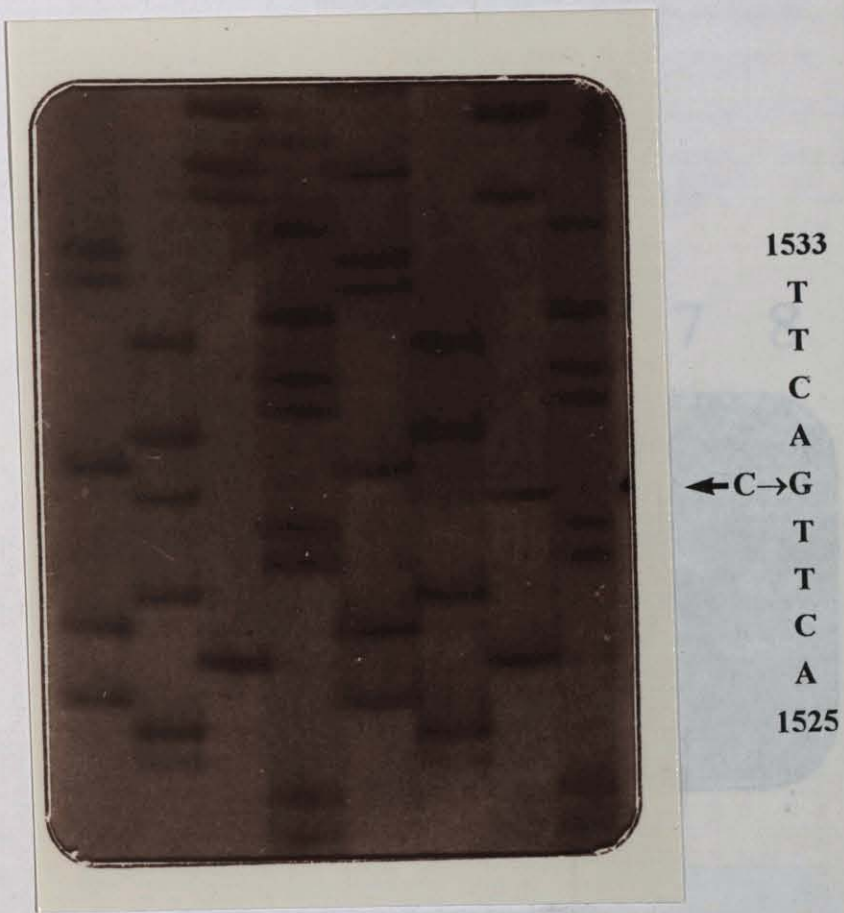


Figure V.6- DNA sequence analysis of exon 10 of patient 44 heterozygous for the mutation S466X.

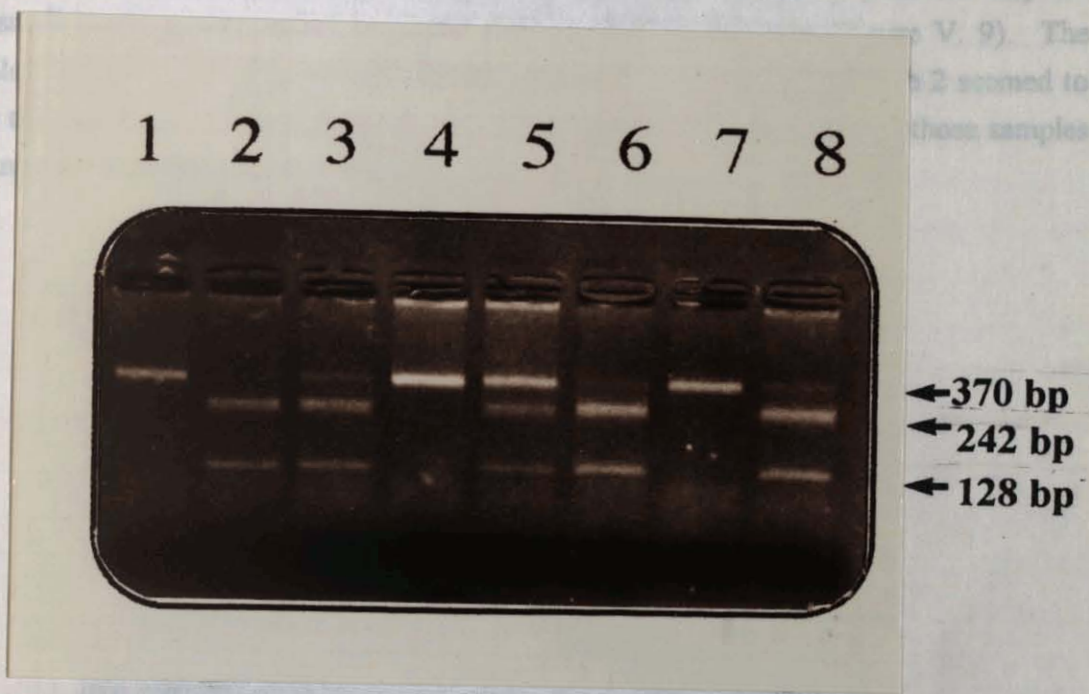


Figure V.7 Confirmation of the polymorphism 1540A/G by Hph I digestion. The 370 bp PCR product is digested into two fragments of 242 bp and 128 bp. There was no digestion in lanes 1, 4 and 7 (1540A/A). All three bands exist in lane 5, indicating that the sample is a heterozygote and 1540G/G homozygotes are seen in lanes 2, 3, 5 and 8.

F. Screening Intron 19 for The Mutation 3849+10 kb C→T Using Hph I Digestion

The presence of the mutation abolishes the restriction site for Hph I, thus the enzyme cannot cleave mutant alleles. Patients who are homozygous for the mutation should give rise to a single large band and heterozygotes result in three bands, two being smaller. Restriction enzyme digestion was applied to 48 families as described in the methods IV.F.1. None of the patient samples exhibited any of those patterns; instead, they all had only the two small bands corresponding to normal the state for this mutation (Figure V. 9). The samples in lanes 1, 2 and 6 were partially digested, and the sample in the lane 2 seemed to carry the mutation. However, repeating the digestion showed that neither of those samples nor any others carried this mutation.

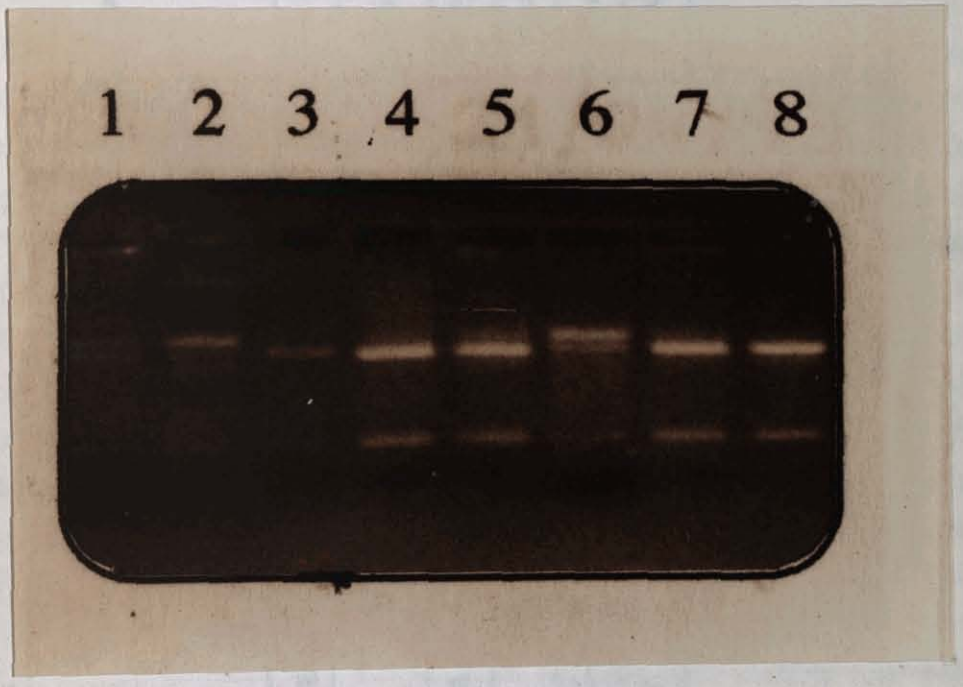


Figure V.8 Hph I digestion patterns for the mutation 3849+19 kb C→T.

Table V.1- Mutations detected in 110 Turkish CF chromosomes.

Base Change	Description	Results	Frequency	Reference
ΔF508	Deletion of 3 bp between bases 1654 and 1656	Deletion of Phe 508	15.5%	Kerem et al., 1989
1677delTA	Deletion of TA at 1677-78	Frameshift	4.5%	Ivaschenko et al., 1991
S466X	C→G at base1529	Ser 466 →Stop	1%	Meitenger et al., 1994
3849+10 kb C→T	84 bp insertion	Creation of a new exon	0%	Highsmith et al., 1991
1540 A/G	Polymorphism (Val by Met)	No change	37%	Kerem et al., 1989

VI. DISCUSSION

Cystic fibrosis results from mutations in the gene coding for the CFTR, a protein that regulates chloride ion transport in exocrine glands. It is of great importance to have information about mutation frequency covering the whole population for the prevention of the disease for which there is yet no efficient treatment. Neither the prevalent mutations nor the frequency of the genetic disorder in the Turkish population is known. Only a few hundred afflicted families have been reported country-wide so far. Therefore, we have established several techniques (including heteroduplex analysis, DGGE and direct sequencing) to scan one of the mutation hot spot regions in CFTR, namely exon 10. Because of ethnic heterogeneity, we expected to find a large number of mutations, each of which will account for a small fraction of the CF mutations.

Heteroduplex analysis which is a quick and simple method was used for identification of mutations ΔF508 and 1677delTA. This method is limited in the sense that only deletion mutations in the particular region can be screened for.

Deletion of codon 508 (CTT) in exon 10 leads to the deletion of phenylalanine 508 (ΔF508) in the protein NBF1 region (Kerem et al., 1989). In other populations, this deletion of three base pairs (ΔF508) is the most common mutation in CF (70 per cent of all affected chromosomes). However, this particular mutation was found in Turkey at a significantly lower frequency (15.5 per cent) than in the European and North American populations. Another study on the Turkish population has reported the frequency of ΔF508 as being approximately 30 per cent (Ozguc M, 1994). This may be due to either a regional difference or to the small number of patients in both of the studies. In any case, both of the frequencies reported so far indicate that the population in Turkey is genetically different, even though the ΔF508 mutation may have a common ancestral origin in all populations.

1677delTA, a rare mutation (8 alleles out of 2127 screened, CF Consortium, 1992) of two nucleotide deletion at position 1677 was observed to be associated with a clinically severe phenotype (Ivaschenko et al., 1991). As a result of the deletion, the protein reading frame is shifted, introducing a termination codon (TAG) at amino acid position 515. In this study, 1677delTA was detected in 4.5 per cent of the CF chromosomes and accounts for 5.4 per cent of non-ΔF508 alleles. The carriers of the mutation have originated from Anatolia and exhibit a severe course of the disease from the first days of life. Mostly they die at infancy, due to meconium ileus. Surviving patients usually have pancreatic insufficiency. A collaborative study with Bulgarians and Cypriots demonstrates that 1677delTA is relatively common among non-ΔF508 CF alleles along the Black Sea and

should be included in the diagnostic panel for the molecular analysis of CF patients in this region (Angelicheva et al., 1994). The geographical distribution of the mutation and its association with a single chromosomal haplotype suggest that the mutation is young in evolutionary terms. The frequency of the mutation is highest among Georgians and gradually declines to the east and south. The Turkish population has the second highest frequency, indicating that this frameshift mutation, originated probably in Georgia, was then spread out rest of the world. The mutation has a frequency gradually declining from Georgia across Turkey to Bulgaria and Cyprus.

Mutations responsible for the majority of the CF cases in the Turkish population are yet unknown. It is of great importance to identify the prevalent mutations in offering prenatal diagnosis for families at risk. New mutations will also facilitate understanding the molecular mechanism underlying the disease. If the proband is alive, mutation analysis can be performed directly on the individual. For cases in which the proband is deceased, the parents can be tested for mutations, but this is difficult when a significant percentage of mutations in the population are currently unidentified. In families where the mutations remain unidentified, linkage analysis with polymorphic DNA markers is the only method available for prenatal diagnosis. The most efficient of the markers are the three intragenic microsatellites, one in intron 8 and two in intron 17b. However, this indirect method has some disadvantages. It requires an extensive family study to establish the linkage pattern. The family of interest must have an affected individual to provide a DNA sample and family members should be informative for the polymorphic markers. Moreover, crossing-over events may lead to misdiagnosis.

A general and a very effective method for screening for both known and unkown mutations is DGGE. This method can be used simply as a nonradioactive diagnostic approach. Once the conditions for the electrophoresis are optimized, the technique is simple and requires small amounts of genomic DNA. A large number of samples can be applied on a gel, thus it is very useful in routine diagnostic tests for analyzing CF mutations. Moreover, DGGE has the additional advantage of detecting any genetic variation that is present within long DNA fragments. This information is especially applicable to cases where the location of the mutation is not known.

DGGE was employed in this study to uncover the remaining unknown mutations in exon 10 after heteroduplex analysis. As a result, the mutation S466X was determined as the first case in a Turkish CF patient. Also, the polymorphism 1540A/G was detected and further confirmed with Hph I restriction endonuclease digestion. This particular polymorphism was found at a high frequency in the patients studied (37 per cent). The heterogeneity of the patients for the polymorphism indicated that CF is old in evolutionary terms in our population. Furthermore, the polymorphism can be used as a genetic marker for linkage analysis for carrier identification and prenatal diagnosis.

We also screened a different region (intron 19) for a particular mutation which is called 3849+10 kb C→T. Patients who carry this mutation have a milder course of the disease as compared to those with mutations ΔF508 and 1677delTA. They live longer and are in better nutritional state. Generally, patients carrying the mutation 3849+10 kb C→T are pancreatic sufficient and have normal or border line sweat chloride levels (Abeliovich et al., 1992). Highsmith et al., 1991, proposed a hypothesis to explain the milder course of the disease: The C to T base substitution in intron 19 creates an alternative splicing site which results in the insertion of 84 base pairs into the CFTR coding region. The change might result in a protein with normal function, together with a non-functioning protein. Alternatively, this mutation might lead to production of a protein that is only partly functional and causes milder disease. There are two lines of data which urged us to screen for this mutation: One of them is that CF cases are believed to escape detection in our population and this might be due to normal sweat chloride levels in the patients. The other evidence was that the mutation was found in Israel, a neighbouring country where also a ethnic Turkish patient with this particular mutation was identified. The present study determined that none of our patients carried the mutation. This mutation may be encountered when more CF petients are investigated.

Further diagnostic tests are required for patients whose clinical course suggest CF, but the sweat chloride test results are normal. In such cases, DNA studies are very useful, especially in populations in which 95 per cent of mutations are detectable. However, the situation is complicated by the fact that more than 375 mutations have been detected so far and many of them in only one or a few patients.

The study is intended to be extended to cover other hot spots in the CFTR gene, such as exons 6, 7, 11, 20 and 21.

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