
Short communication

Lymphocyte mRNA as a resource for detection of mutations and polymorphisms in the CF gene

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The most common mutation in the cystic fibrosis gene, deletion of a phenylalanine residue at amino acid 508, occurs in about 70% of CF patients in North America and northern Europe.^{1,2} A collection of alternative mutations in the CF gene has already been defined by members of the CF Genetic Analysis Consortium. Most of these mutations have been detected by amplification of individual CF gene exons from genomic DNA followed by DNA sequence analysis. Clearly this approach is laborious since each of the 27 exons has to be analysed independently. An alternative means of searching for mutations is through the examination of mRNA. This would enable much larger segments of the gene to be examined at each analysis. However, this approach is not readily applied to CF since the CF gene is apparently only expressed at significant levels in tissues that are primarily involved in the pathology of the disease, such as the epithelium lining the lungs and respiratory system, pancreatic ducts, gut, and sweat gland ducts.^{1,3} Apart from nasal epithelium, such tissues are not readily available for mRNA analysis. To circumvent this problem we have developed a method to amplify the CF mRNA from peripheral blood lymphocytes. This method relies on cDNA synthesis, followed by two rounds of polymerase chain reaction using nested primers.⁴ 'Ectopic' transcription of other genes, including the dystrophin and factor VIII genes, has recently been detected in lymphocytes.⁵⁻⁷

Lymphocytes were isolated from whole blood by Histopaque (Sigma) centrifugation. Cells were washed by phosphate buffered saline and then RNA was extracted by single step guanidinium thiocyanate phenol extraction.⁸ Alternatively, RNA was isolated from lymphocytes or lymphoblastoid cell lines by guanidinium thiocyanate lysis followed by caesium chloride centrifugation.⁹ The CF cDNA sequence was divided into eight segments (A to H) as is shown in fig 1. Segments A to F broadly correspond to predicted functional domains of the protein, and segments G and H to the 3' non-coding region of the mRNA. In order to amplify the CF cDNA from lymphocyte RNA, in which the CF gene message is represented at an extremely low level, it was necessary to have two 30 to 35 cycle rounds of polymerase chain reaction (PCR) for each segment, using nested sets of primers. The primer sequences are shown in the table. The first round of PCR was carried out using primer set 1 following cDNA synthesis primed by the 3'20-mer (IL) of each PCR reaction. The second round of PCR amplification was done using primers located inside those used in the first round reaction (primer set 2). Fifty nanograms of the 3' primer for each CF gene segment was annealed to 0.5 to 1 µg of total RNA at 65°C. cDNA was synthesised from this primer with reverse transcriptase (Gibco BRL) in 50 mmol/l Tris-HCl, pH 8.5, 75 mmol/l KCl, 5 mmol/l DTT, 3 mmol/l MgCl₂, 1.25 mmol/l dNTPs, and 50 units of placental ribonuclease inhibitor (Boehringer, Mannheim) in a total volume of 20 µl at 42°C for 60 minutes. To this was added 30 µl of a premix of PCR buffer containing 67 mmol/l Tris-HCl, pH 8.8, 167 mmol/l (NH₄)₂SO₄, 6.7 mmol/l MgCl₂, 0.17 mg ml⁻¹ BSA, 0.07% β-mercaptoethanol, 500 ng of the 5' primer, 450 ng of the 3' primer, and five units of Taq polymerase (Cetus). The following conditions were used for the PCR reaction: after five minutes denaturation at 94°C, 30 cycles of 60 seconds denaturation at 94°C, 60 seconds annealing at 60°C, five to seven minutes extension at 72°C

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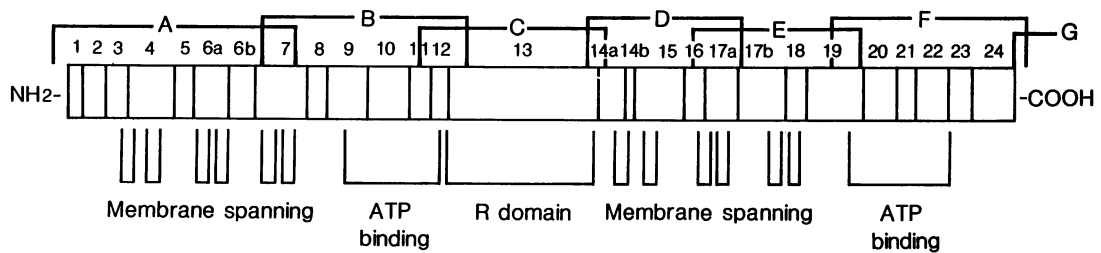


Figure 1 Diagram of the CF gene cDNA to show overlapping segments amplified by each set of clustered PCR primers (A-F) and their relation to the predicted functional domains of the protein.

Primer sequences used for PCR amplification of CF gene cDNA segments.

Primer	Sequence	Fragment size	
A1R	124	CGAGAGACCATGCAGAGGTC	1033
A2R	156	GGCCAGCGTTGTCTCCAAC	
A2L		gccgagtgaccgccatgcgc	1189
A1L		gctccaagagagtcatacca	1232
B1R	998	GACAAACAGAACTGAAACTG	898
B2R	1022	GGAAGGCAGCCTATGTGAGA	
B2L		agccatcagtttacagacac	1920
B1L		cagtttcttaaatgttcc	1972
C1R	1777	GTGGAGGTCAACGAGCAAGA	769
C2R	1812	AGCAGTATACAAAGATGCTG	
C2L		tcttcacttattccaagcc	2581
C1L		actccttaagcttcttcg	2626
D1R	2490	GACAAACAGCATCCACACGAA	729
D2R	2530	GCAAACCTTGACTGAACTGGA	
D2L		gttgtttgagttgctgtgag	3259
D1L		aattggactcctgccttcag	3283
E1R	3055	AGATTCTCAAAGATATAGC	699
E2R	3082	GATGACCTTCTGCCTCTTAC	
E2L		ctgtgagatctttgacagtc	3781
E1L		gaaatgtgtctaataatgac	3824
F1R	3722	ACGTGAAGAAAGATGACATC	877
F2R	3754	GGCCAAATGACTGTCAAAGA	
F2L		catgtcaacatttatgctgc	4599
F1L		cgagctccaattccatgagc	4627
G1R	4524	GCTGCTCTGAAAGAGGAGAC	760
G1L		agattataacagccctttcc	
H1R	5127	GAATACCACAGGAACCACAA	945
H1L		gcagcttcttaagagtcag	

depending on the length of the cDNA segment; a final polymerisation step was carried out at 72°C for five minutes. A total of 1 µl of the first PCR reaction was then transferred to 49 µl of a PCR buffer premix as above but containing 500 ng of each primer in the inside pair of the clustered set and 0.5 mmol/l dNTPs. A further 30 to 35 cycles of amplification

were done using the same parameters as for the first reaction.

The products of each PCR reaction are shown in fig 2, where one-fifth of a second round PCR reaction on 1 µg of total RNA from lymphoblastoid cell lines (1) and from peripheral blood lymphocytes (2) have been analysed on a 5% polyacrylamide

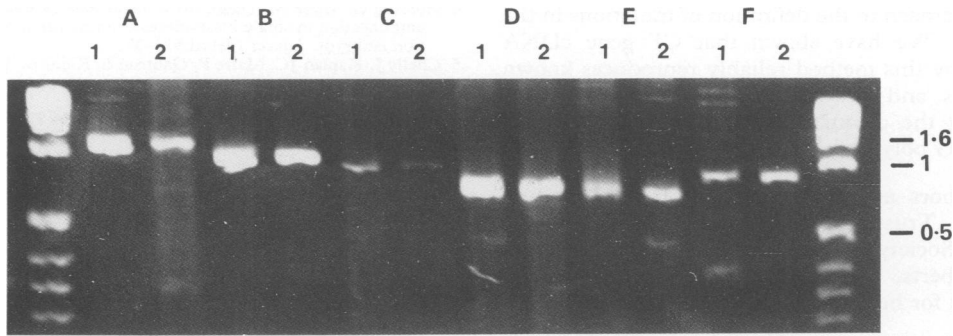


Figure 2 Fragments A to F of the CF gene, PCR amplified after cDNA synthesis from 1 μ g of total RNA from (1) a lymphoblastoid cell line and (2) lymphocytes; 10 μ l of a 50 μ l PCR reaction separated on a 5% polyacrylamide gel.

minigel. All sets of primers functioned efficiently, though the yield with set C was always lower than with the other primer sets in the same RNA sample. Lowering of the annealing temperature of the PCR reaction to 58°C increased the yield. Each CF cDNA fragment was digested with at least one restriction enzyme to confirm the identity of the PCR product, based on the published cDNA sequence (data not shown).

In order to establish the fidelity of the 'ectopic' CF gene transcript in lymphocytes, we first attempted to detect the Δ F508 mutation in exon 10 in known carrier subjects. RNA from the B region of the gene sequence which includes this mutation (fig 1) was reverse transcribed, amplified by PCR, and subjected to chemical mismatch analysis.¹⁰ Where the PCR reaction yielded small amounts of non-specific product, the fragment of interest was purified on agarose gels before GeneClean (Bio 101) separation. Fig 3 shows a mismatch reaction with different ratios of control to patient (Δ F508 carrier) DNA in the hybrid annealing step. Though with ratios of 1:10 and 1:20 control:patient DNA we were unable to detect the three base pair deletion at base 1654, when the ratio was increased to 1:30 or 1:40, mismatch bands of about 635 and 265, corresponding to the two cleavage products caused by the Δ F508 deletion, were clearly visible after both osmium and hydroxylamine modification. The three T residues in the mismatch are detected by the osmium tetroxide modification and a C residue adjacent to the deletion, and so destabilised, is modified by hydroxylamine.

A polymorphism has been shown to occur at base 1540 of the CF cDNA sequence¹¹ where an A or G may occur. A 380 bp mismatch band seen on fig 3 corresponds to a mutation 380 bp from the 3' end of fragment B at 1920 bp. Sequence analysis of cDNA and genomic DNA from this subject showed the A1540→G base change (data not shown).

The establishment of a reliable method for detecting the CF gene transcript in peripheral blood lymphocytes and lymphoblastoid cell lines opens up

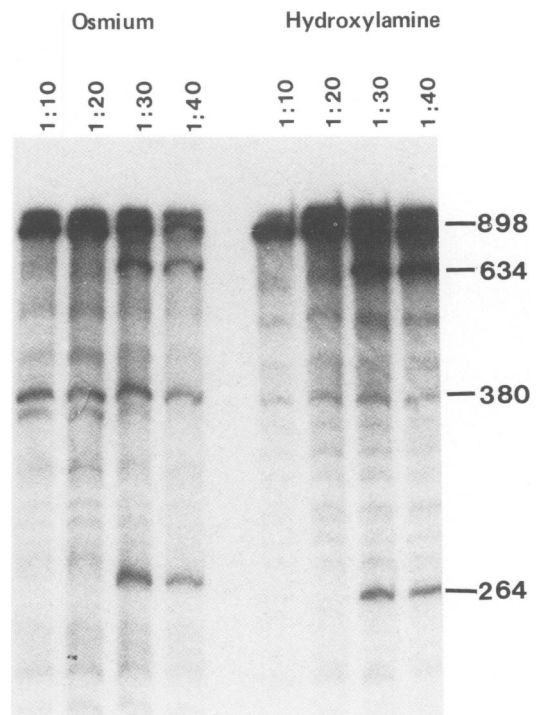


Figure 3 Autoradiograph of a 6% denaturing polyacrylamide gel showing a chemical mismatch reaction between control normal DNA segment B and the same segment from a CF patient, heterozygous for the Δ F508 mutation, after purification of both with GeneClean (Bio 101). Different ratios of probe:patient DNA have been used 1:10 to 1:40. The first four lanes show mismatches after osmium modification and the right four lanes after hydroxylamine modification. Fragment B is 898 bp.

a new approach to the definition of mutations in the CF gene. We have shown that CF gene cDNA isolated by this method reliably reproduces known mutations and polymorphisms in the CF gene including the common $\Delta F508$ mutation and the A1540 \rightarrow G polymorphism.

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