ELSEVIER

Journal of Cystic Fibrosis 3 (2004) 7–10

Journal of Cystic Fibrosis

www.elsevier.com/locate/jcf

Analysis of genomic *CFTR* DNA

Claude Férec[a], Cédric Le Maréchal[a], Marie-Pierre Audrézet[a], Carlos M. Farinha[b,c], Margarida D. Amaral[b,c], Sabina Gallati[d], Javier Sanz[d], Bernhard Steiner[d], Nathalie Mouchel[e], Ann Harris[e], Martin J. Schwarz[f,*]

[a] *Inserm U613, University Hospital, 29275 Brest Cédex, France*
[b] *Center of Human Genetics, National Institute of Health, Lisboa, Portugal*
[c] *Department of Chemistry and Biochemistry, University of Lisboa, Portugal*
[d] *Department of Clinical Research, Human Molecular Genetics, Children's Hospital, Lab. G2/818, Bern 3010, Switzerland*
[e] *Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DS, UK*
[f] *Regional Molecular Genetics Laboratory, Royal Manchester Children's Hospital, Pendlebury, Manchester M27 4HA, UK*

Available online 2 July 2004

Abstract

There are numerous methodologies available for the analysis of genomic *CFTR* DNA. We present here the basic tools to allow a thorough investigation of the *CFTR* gene, beginning with the identification of potential regulatory regions using DNase I hypersensitive sites, and continuing with methods for the detection of mutations: denaturing High Performance Liquid Chromatography (dHPLC), Single Strand Conformation Polymorphism (SSCP), and allele-specific oligonucleotide (ASO) hybridisation. Also provided is a comprehensive set of PCR primers for the amplification of most regions of the *CFTR* gene. Full details of the methodologies given are to be found at the European Working Group on CFTR Expression website http://central.igc.gulbenkian.pt/cftr/vr/transcripts.html.

Keywords: CFTR; Mutation detection; DNase I; dHPLC; SSCP; ASO

1. Introduction

There are numerous methodologies at the disposal of the molecular geneticist for the analysis of genomic *CFTR* DNA. We present here the basic tools to allow a thorough investigation of the *CFTR* gene and the development of a strategy for the identification of all sequence changes in the *CFTR* gene. Beginning with the identification of potential regulatory regions using DNase I hypersensitive sites, we then describe three different protocols for the detection of mutations: denaturing High Performance Liquid Chromatography (dHPLC), Single Strand Conformation Polymorphism (SSCP), and allele-specific oligonucleotide (ASO) hybridisation. Also provided is a comprehensive set of PCR primers that can be used for the amplification of most regions of the *CFTR* gene. Full details of the methodologies given are to be found in the found at the European Working Group on CFTR Expression website [1].

2. Identification of potential regulatory elements

2.1. Assay for DNase I hypersensitive sites in chromatin

This protocol describes the extraction of chromatin from tissues and cells to subsequently map and evaluate DNase I hypersensitive sites that are often associated with regulatory elements in a gene. The method of extraction of chromatin from tissues is adapted from Becker et al. [2]. The extraction of chromatin from cells was adapted from Higgs et al. [3]. The whole procedure has to be performed at 4 °C. Tissue is roughly mashed and added to buffer A

Abbreviations: ASO, allele-specific oligonucleotide; DGGE, denaturing gradient gel electrophoresis; dHPLC, denaturing High Performance Liquid Chromatography; HD heteroduplex (NP-40), Nonidet P-40; PBS, phosphate buffered saline; PMSF, phenyl methyl sulfonyl fluoride; SSCP, Single Strand Conformation Polymorphism.

* Corresponding author. Tel.: +44-161-727-2567; fax: +44-161-727-2328.
E-mail address: Martin.Schwarz@CMMC.nhs.uk (M.J. Schwarz).

1569-1993/$ - see front matter © 2004 European Cystic Fibrosis Society. Published by Elsevier B.V. All rights reserved.
doi:10.1016/j.jcf.2004.05.003

(mM): 60 KCl; 15 NaCl; 15; Tris pH 7.5, 500 sucrose; 0.5 EGTA; 2 EDTA; 0.5 spermidine; 0.15 spermine; 0.5 β-mercaptoethanol; 5 DTT; 1 phenyl methyl sulfonyl fluoride, PMSF (10 ml/g of tissue). Spermidine, spermine, β-mercaptoethanol, DTT and PMSF are added just before use. The tissue is then homogenized and filtered 2× through sterile gauzes. The volume is then adjusted to 50 ml with buffer A and the suspension is centrifuged at 3000 × *g* for 10 min at 4 °C. The pellet is resuspended in 5 ml of buffer A and the cells lysed by adding 5 ml of buffer A containing 0.8% (v/v) triton X100. The resulting suspension is then mixed and incubated on ice for 10 min. This is then centrifuged at 3000 × *g* for 15 min at 4 °C and the resultant pellet resuspended in 10 ml of buffer A. This wash is repeated three times, before filtering through a 70 μm cell strainer. The product is then centrifuged at 1000 × *g* for 10 min at 4 °C, and the pellet resuspended in 50 ml of RBS (mM): 10 Tris HCl pH 7.5; 10 NaCl; 3 $MgCl_2$. This is then centrifuged at 1000 × *g* for 10 min at 4 °C and the pellet resuspended in 0.8 ml of RSB, before proceeding to DNase I digestion of samples.

To extract chromatin from cells, 10^8 cells are trypsinised or harvested for suspension cultures; the cells are washed twice with phosphate-buffered saline, PBS (0.02% (w/v) KCl; 0.02% (w/v) KH_2PO_4; 0.8% (w/v) NaCl; 0.115% (w/v) Na_2HPO_4 pH7.4), centrifuged at 200 × *g* for 5 min at 4 °C and resuspended in 50 ml of PBS. After three washes the cell pellet is resuspended in 20 ml PBS. Cells are lysed and nuclei released by adding 20 ml of RSB containing 0.2% (v/v) Nonidet P-40 (NP-40). After 10 min on ice, the cells are centrifuged at 200 × *g* for 5 min at 4 °C and the nuclei resuspended in 50 ml of RSB. The nuclei are then centrifuged at 200 × *g* for 5 min at 4 °C and resuspended in 1 ml of RSB. 200 μl of nuclei suspension is aliquoted into six tubes containing 800 μl of RSB.

DNase I (Amersham Pharmacia, Little Chalfont, UK, cat no.: 27-0514-02) is used for digestion. One tube with no DNase I remains at 4 °C, and a second tube with no DNase I is incubated at 37 °C as a control for any endogenous DNase I activity. The remaining four tubes that contain increasing amount of DNase I are incubated at 37 °C (about 20 min) until the penultimate tube is clear (not viscous) when mixed with 50 μl of lysis buffer (10 mM Tris HCl pH 8.0; 10 mM NaCl; 10 mM EDTA pH 8; 0.5% (w/v) SDS). 1 ml of lysis buffer is added to the remaining samples to stop the reaction. DNase I digestion may also be carried out with the same amount of enzyme in each tube but increasing incubation times. 10 μl of proteinase K (50 mg/ml) is added to each sample and incubated at 37 °C overnight. If the DNA solution is still viscous, more proteinase K is added and incubated until the solution is no longer viscous. After one phenol/chloroform extraction and two chloroform extractions, the samples are precipitated in two volumes of ethanol (100%) plus 1/10 volume of 5 M NaCl. This is incubated at −20 °C for 2 h, after which it is centrifuged at 2000 × *g* for 30 min at 4 °C. The pellet is then air-dried and resuspended in 200 μl of TE (10 mM Tris; 0.1 mM EDTA; pH 8.0), before storage at −20 °C.

3. Detection of mutations in the *CFTR* gene

3.1. Amplification of CFTR exons and coding regions

Numerous PCR primers have been described for amplification of the *CFTR* gene. A comprehensive list of tried and tested primers currently in use can be found in a 'Table of Primers for *CFTR* gene Amplification' at the European Working Group on CFTR Expression website [1]. Whilst it is relatively easy to design primers to amplify a particular exon, some thought must be given to the application the PCR products are intended for. Some of the primers can be used for more than one application (e.g. sequencing, single-stranded conformational polymorphism, SSCP, etc.) but others are restricted to a specific purpose.

3.1.1. Notes to 'Table of Primers for CFTR gene Amplification' [1][1]

1. Primers named (exon)i5 and (exon)i3 are those suggested by Zielenski et al. [4]. These generally give a PCR product of 400–500 bases and may be used for general screening of the relevant exon.
2. Primers with the prefix 'DCF' are derived from Fanen et al. [5] and are the primers for denaturing gradient gel electrophoresis (DGGE) from that paper, less the GC-clamp. These primers generally give a PCR product of a size more useful for SSCP and sequencing than the Zielenski primers [4].
3. Primers containing the letters 'sp' have been designed especially for sequencing and are not usually used in a pair.

3.2. Mutation detection of CFTR gene by HPLC

Denaturing High Performance Liquid Chromatography (dHPLC) has been described in the past years as a method of scanning DNA samples for point mutations or variations in the genome. Initially, HPLC was a tool to separate DNA fragments as small oligonucleotides, PCR fragments, etc. The development of columns containing alkylated non-porous particles has opened a new approach to research development in the field of mutation/polymorphism detection. This technique involves subjecting PCR products to

[1] The author (M. Schwarz) will be pleased to respond to requests for further information.

ion-pair reverse-phase liquid chromatography in a column containing alkylated non-porous particles.

Within a linear acetonitrile gradient and under partial heat denaturation, heteroduplexes (HDs) that may be present in a PCR product display a reduced column retention time compared with their counterpart wild-type (wt) homoduplexes. The pattern of profile of these HDs is in the majority of cases easily detected and additional techniques are used (such as sequencing) to confirm the presence of a mutation or sequence variation.

The list of genes studied by dHPLC as at October 2003 is available elsewhere [6]. More than 250 genes responsible for human diseases have now been totally or partially analysed by dHPLC and this technique is becoming one of the most popular for mutation scanning. The dHPLC technique has been compared to other methods with regard to sensitivity, cost and time taken to obtain the results. The conclusion of most of these studies was that dHPLC is effective, fast and sensitive for the detection of point mutations and polymorphisms. This is also our conclusion after having designed primers to enable complete scanning of the 27 exons of the *CFTR* gene by dHPLC. For this reason, we believe dHPLC is now the 'Gold Standard' for analysing the *CFTR* gene, which is one of the most highly mutated genes in the human genome.

3.3. CFTR gene analysis by SSCP

3.3.1. General strategy of the assay

The large size of many disease genes and the multiplicity of mutations complicate the design of an adequate assay for the identification of disease causing sequence variants. In the past 10 years, SSCP and HD analysis have rapidly gained popularity as two of the most straightforward and versatile methods to screen for DNA alterations [7]. The principle reasons for the popularity of these two methods are their technical simplicity and their high sensitivity for the detection of mutations. Traditionally, SSCP and HD are prepared separately involving time-consuming (12–14 h), labour-intensive, and cumbersome electrophoresis of radioactively labelled DNA samples under non-denaturing conditions in a high resolving cross-linked polyacrylamide gel and affording relatively low sensitivity of mutation detection (60–90%) [7]. To increase sensitivity and specificity of SSCP and HD analysis, several factors have to be optimised. For SSCP analysis, these factors include: choice of gel matrix, electrophoretic conditions, presence of neutral additives, fragment size, and G+C content. For HD analysis, the principle factors influencing sensitivity are the gel matrix and the identity of the base mismatch. Thus varying these parameters, we optimised the sensitivity of our combined SSCP/HD protocol, simultaneously performing SSCP and HD analysis on the same gel and therefore increasing the sensitivity of both methods [8]. We defined uniform SSCP/HD conditions, such as 12% (w/v) non-denaturing polyacrylamide gel, 7% (v/v) glycerol, two-phase buffer system, 90 min electrophoresis at 12–15 °C, and silver staining. These conditions can be used for mutation screening in all 27 *CFTR* exons, or in any other genes. Additionally, restriction enzyme digest of the amplification product of each *CFTR* exon further increases mutation detection.

3.3.2. Screening strategy and mutation detection rate

We present a three-step screening strategy allowing analysis of the sequences with first priority within 5 h and analysis of the entire coding and adjacent noncoding regions within 1 week, including sequence analysis of the variants. The screening strategy is based on the abundance of mutations in the *CFTR* gene [8]. The first priority includes: exon 3, 4, 7, 10, 11, 13, 17b, 19, 20, 21 and intron 19. The second priority: exon 2, 5, 6a, 12, 14b, 15, 16, 17 and intron 11. The third priority: exon 1, 6b, 8, 9, 14a, 18, 22, 23, 24 and the promoter sequence. Many of the fragments are longer than 300 bp, but this does not seem to be a disadvantage in identifying point mutations. The reason why particular mutations can only be identified with, or without, digestion of the PCR product may be based on the fact that digestion alters the position of the sequence variant within the amplification product and in consequence also the conformation of the single-strand and/or the HD formation allowing or preventing the detection of a band shift. In our initial study, a total of 80 known CF mutations (28 missense, 22 frameshift, 17 nonsense, 13 splicesite) and 20 polymorphisms was analysed resulting in a detection rate of 97.5% including the 24 most common mutations worldwide [8]. More recently, the protocol proved to be able to detect mutations independent of their nature, frequency, and population specificity, which is also confirmed by the identification of novel mutations (i.e. 420del9, 1199delG, R560S, A613T, T1299I) in Swiss CF patients. 93 different mutations have been detected in 1200 patients analysed so far. This protocol represents a general model for point mutation analysis in genetic disorders and is successfully used for the analysis of a number of disease-causing genes. In conclusion, this protocol provides firm evidence that this SSCP/HD technique is very fast and has the ability to detect at least 97% of all sequence variants in the coding region of the *CFTR* gene. Thus, we consider it to be a high-yield scanning method that can be recommended to laboratories involved in the mutation screening of genetically heterogeneous populations.

4. Detection of F508del by ASO hybridization

The complete protocol can be used to screen for known mutations in *CFTR* DNA, either in genomic DNA (from patients, carriers) or in plasmids (e.g. to screen for mutants

after a mutagenesis protocol). It can be found online at the European Working Group on CFTR Expression website [1].

Two oligonucleotide probes (one complementary to the mutant sequence and another to the wt sequence in the same DNA region) should be used. Samples (PCR amplicons or plasmids) that fail to hybridize with one probe should hybridize positively with the other probe. Exceptions are amplicons from individuals who are heterozygous for the mutation under analysis. For these, positive signals (though weaker) should be seen with both probes. When a mixture of plasmids (wt and mutant) is present a similar result is obtained.

Positive controls for each sequence (acting as negative controls for the other) should always be included on the same filter as samples.

4.1. Protocol outline

After soaking the membrane first in distilled water and then into 2 × SSC, it is assembled into the appropriate dot-blot apparatus [1]. Following denaturing in appropriate solution, DNA samples under test are then applied on the membrane with the help of the three-valve system that regulates vacuum flow. Samples on apparatus wells are washed with 2 × SSC (with vacuum off). The vacuum is turned on again and after solution absorption, the apparatus is disassembled, the membrane soaked into 2 × SSC and air-dried, before incubation at 80 °C for 2 h for DNA fixation.

Stringent hybridization with oligonucleotidic probes[2] (radiolabelled with [γ-^{32}P]dATP by T4 polynucleotide kinase) is carried out at 50 °C for 2 h, after overnight pre-hybridization at the same temperature. The hybridized membrane is then washed under stringent conditions (including two 30-min washes at a higher temperature) to remove unspecifically bound probe and air-dried. Before exposure to an X-ray film, with intensifying screens for 2 h at −80 °C.

Acknowledgements

The preparation of this review was supported by the EU-European Thematic Network on Cystic Fibrosis and Related Diseases (EU-QLK3-CT-1999-00241).

References

[1] The Online Virtual Repository of Reagents and Methods for CFTR Research (Section A); 2004. http://central.igc.gulbenkian.pt/transcripts.html.
[2] Becker P, Renkawitz R, Schutz G. Tissue-specific DNaseI hypersensitive sites in the 5′-flanking sequences of the tryptophan oxygenase and the tyrosine aminotransferase genes. EMBO J 1984;3(9):2015–20.
[3] Higgs DR, Wood WG, Jarman AP, Sharpe J, Lida J, Pretorius IM, et al. A major positive regulatory region located far upstream of the human alpha-globin gene locus. Genes Dev 1990;4(9):1588–601.
[4] Zielenski J, Rozmahel R, Bozon D, Kerem B, Grzelczak Z, Riordan JR, et al. Genomic DNA sequence of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Genomics 1991;10(1):214–28.
[5] Fanen P, Ghanem N, Vidaud M, Besmond C, Martin J, Costes B, et al. Molecular Characterization of Cystic Fibrosis: 16 Novel Mutations Identified by Analysis of the Whole Cystic Fibrosis Conductance Transmembrane Regulator (CFTR) Coding Regions and Splice Site Junctions. Genomics 1992;13(3):770–6.
[6] The Stanford Genome Technology Center [http://www-sequence.stanford.edu/], maintained by Peter Oefner (oefner@genome.stanford.edu).
[7] Liechti-Gallati S, Schneider V, Neeser D, Kraemer R. Two buffer PAGE system-based SSCP/HD analysis: a general protocol for rapid and sensitive mutation screening in cystic fibrosis and any other human genetic disease. Eur J Hum Genet 1999;7(5):590–8.
[8] Nataraj AJ, Olivos-Glander I, Kusukawa N, Highsmith Jr WE. Single-strand conformation polymorphism and heteroduplex analysis for gel-based mutation detection. Electrophoresis 1999;20(6):1177–85.

[2] Sequence of probes for F508del detection is available elsewhere [1].