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Journal of Cystic Fibrosis 3 (2004) 25–28
Journal of Cystic Fibrosis
www.elsevier.com/locate/jcf

Non-PCR methods for the analysis of *CFTR* transcripts

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Available online 17 July 2004

Abstract

Cystic fibrosis transmembrane conductance regulator gene (*CFTR*) shows a complex mechanism of tissue-specific and temporal regulation. *CFTR* mRNA detection and measurement are extremely difficult because of the low to very low levels of its endogenous expression. In this paper, we describe four different non-PCR methods optimized to analyze *CFTR* transcripts in epithelial cell lines, primary cell lines and native tissues that express significant amounts of *CFTR* transcript.

Keywords: Northern blotting; In situ hybridization; RACE

1. Introduction

The expression of the *CFTR* gene is tightly regulated both temporally and spatially. CFTR mRNA is known to be more abundant in the airway epithelium during the second trimester of human development than after birth. This change in abundance might reflect additional or different roles for CFTR in the developing airway epithelium. Multiple start sites and alternative exon usage have also been described for the *CFTR* transcript. These modifications of the *CFTR* transcript could result in alterations in the protein, which could be functionally important. However, so far, no clear functional role has been elucidated for the products of any of these different alternative *CFTR* transcripts. Even though the analysis of *CFTR* transcripts is crucial for a better understanding of the function and role of *CFTR*, these have proven extremely difficult to study because of the low to very low level of endogenous expression. This is particularly relevant for the alternative transcripts that in most cases represent only a small percentage of the transcript population. This is one of the reasons why PCR-based methods are largely used to study *CFTR* transcripts [1,2]. In this paper, we describe three different non-PCR methods optimized to detect and measure *CFTR* transcripts in epithelial cells that express significant amounts of *CFTR* transcript, as well as primary cell lines and native tissues. Although Northern blotting using total RNA is extremely difficult in native tissues and primary cell lines, due to very low levels of endogenous expression, we report a method that allows the detection of endogenous *CFTR* mRNA in epithelial cells that express *CFTR* in significant amounts, the colonic cell lines HT-29 [5] and Caco-2 [3] as well as the respiratory Calu-3 cells [4], a line derived from the submucosal gland and can also be used with polyA$^+$ RNA from primary tissues or to analyze the expression of *CFTR* in heterologous systems expressing high levels of *CFTR* such as CHO or BHK stably expressing *CFTR*. The variant of this technique that we described was successfully used to study the surface expression of the *CFTR* mutant, F508del [6]. The second method is an RNA in situ hybridization optimized for detection of *CFTR* mRNA in vivo [7]. We address a number of critical factors such as tissue fixation, sectioning, type and labeling choice of probe, probe penetration of tissue, background blocking, hybridization, and method of signal detection that combine to determine the

Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; RACE, rapid amplification of cDNA ends; EtBr, ethidium bromide; RB, running buffer; RT, reverse transcriptase; RNA-ISH, RNA in situ hybridization.

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doi:10.1016/j.jcf.2004.05.005

overall sensitivity of the procedure. The last method described is a rapid amplification of cDNA ends (RACE). This method was used successfully to identify alternative 5′ exons of the ovine *CFTR* gene [8]. All protocols discussed here are described in detail at the European Working Group on CFTR Expression website [9].

2. Methods

2.1. Northern blotting

After RNA extraction, RNA concentration is determined by measurement of absorbance at 260 nm. To be able to compare, the same amount of total RNA must be loaded in each lane. To each RNA sample (about 20 μg resuspended in a maximum of 4.5 μl), add formamide, formaldehyde and 5 × Running Buffer (RB) (40 mM MOPS, 10 mM sodium acetate, 1 mM EDTA pH 7.0) to the final concentrations of 50% (v/v), 2.3 M and 1 ×, respectively. Denature the samples for 15 min at 65 °C, immediately cool on ice and add the loading buffer (50% (v/v) glycerol, 1 mM EDTA, 0.04% (w/v) bromophenol blue and 0.04% (w/v) xylene cyanol blue) to a final volume of 22 μl. Load an appropriate molecular weight standard (e.g., RNA ladder 1.24–9.5 kb) and the RNA samples on a 1% or 2% (w/v) agarose gel prepared in 1 × RB and formaldehyde 2.3 M. Run electrophoresis either at 100 V for 2–3 h or overnight (16–18 h) at 50 V in 1 × RB. After electrophoresis, cut off the lane corresponding to the RNA ladder, stain it with ethidium bromide (EtBr) 0.5 μg/ml for 15 min, remove the excess of EtBr in two distilled water washes (5 min each) and photograph under UV. Rinse the gel two to three times with distilled water for 5 min to remove the excess of formaldehyde and soak it into 20 × SSC (3 M NaCl and 0.3 M sodium citrate pH 7.0) for 30 min to neutralize it. Set the blot to transfer overnight using 20 × SSC as described elsewhere [10]. To speed up and increase efficiency of transfer, change the wet paper towels for a new set of dry ones at least once during the transfer. After transfer, wash the nitrocellulose/nylon filter twice rapidly with 2 × SSC, air dry it at room temperature and incubate it for 2 h at 80 °C. Pre-hybridize filters carrying RNA samples in hybridization buffer for at least 4 h at 42 °C. During this time, 200 ng of the *Eco*RI *CFTR* cDNA fragment corresponding to the region 2226–3714 are radioactivity labeled according to the manufacturer's instructions using the Multiprime DNA Labeling System (Amersham). The non-incorporated nucleotides are removed by gel filtration using Sephadex G-50 microcolumn (Boehringer Mannheim), according to the manufacturer's instructions. The labeled probe can be stored at − 20 °C at this step. Denature the probe by boiling it for 3–5 min and add it to the minimum possible volume of hybridization solution (5 × SSC, 0.02% (w/v) Ficoll, 0.02% (w/v) PVP, 0.02% (w/v) BSA, 0.1% (w/v) SDS, 50% (v/v) formamide and 0.5 mg/ml sonicated salmon sperm DNA) needed to cover entirely the whole surface of the filter. Hybridize overnight at 42 °C. Wash the filter twice for 15 min at room temperature with wash solution I (2 × SSC, 0.1% (w/v) SDS) and once for 15 min at 50 °C with wash solution II (0.1 × SSC, 0.1% (w/v) SDS). Scan the filter surface with Geiger counter and if necessary wash once more for 15 min with wash solution II at 50 °C. Air dry filter, expose it to Biomax film with intensifying screens for 48 h at − 70 °C or to phosphorimager. For re-probing, wash filter for 2 h at 80 °C with wash solution II and proceed directly to the pre-hybridization step.

Other *CFTR* cDNA fragments are commonly used as probe. Primer sets needed to prepare *CFTR* cDNA fragment to be used as a probe and a fragment of the mouse house keeping gene β-actin (used as a loading control) by RT-PCR are listed in Table 1.

2.2. RNA in situ hybridization[1]

The sensitivity of the procedure is determined by how thoroughly tissue is fixed since the fixation step halts the process of RNA degradation and to preserve the cellular and tissue architecture during sectioning (for additional fixation notes, see Ref. [11]). Post-fixation of tissue after sectioning does not preserve the RNA sufficiently well to allow detection of *CFTR* mRNA.

Fixative (Fix) A is 0.1 M NaOH, 40 g/l paraformaldehyde and 13.6 g/l sodium acetate. Heat and stir to dissolve, do not exceed 65 °C. Cool to 4 °C. Adjust the pH to 6.5 with glacial acetic acid. Fix B is 0.1 M NaOH, 40 g/l paraformaldehyde and 0.05 M sodium tetraborate. Heat and stir to dissolve, do not exceed 65 °C. Cool to 4 °C. Adjust the pH to 9.5 with HCl. For tissue storage and post-fixation, make a 10% (w/v) sucrose Fix B. If ice crystal damage is still a problem, the percentage sucrose can be increased anything up to 30% (w/v). The correct preparation of microscope slides (including coating with 3-aminopropyltriethoxysilane is crucial for success [11]. Collect the required tissues into Fix B with 10% sucrose and keep tissue at 4 °C for at least 48 h before sectioning. Tissue can be kept in this way for many months. If perfusion fixation is not possible, then collect tissue directly into Fix B + 10% (w/v) sucrose as soon as possible after surgical removal. Place on a gently rocking platform for 24 h at 4 °C. Keep the tissue samples as small as possible to aid with fixative penetration.

Remove tissue from Fix B, rinse twice in 0.9% saline and blot dry with filter paper. Using a disposable plastic mold, embed tissue in O.C.T. embedding medium (Tissue Tek) and freeze in liquid nitrogen. Store cut sections and remaining frozen blocks at − 80 °C in an airtight container. Before use, place slides under vacuum at room temperature overnight to dry sections onto slides or incubate the slides at 55 °C for 4 h.

[1] More details are available on request from the author A. Trezise: ann.trezise@uq.edu.au.

Table 1
Sequences, location and GenBank accession number of oligonucleotides used to prepare cDNA probe specific to the human *CFTR*, human actin γ or mouse actin β gene by RT-PCR (h—human, m—mouse)

Primer sequence	Orientation	Gene and Location	GenBank accession number
5′ACTGGAGCAGGCAAGACTTCA	sense	h*CFTR*, 1510–1530	NM_000492
5′CAGTGTGATTCCACCTTCTC	antisense	h*CFTR*, 1757–1775	NM_000492
5′AAGAACGTGCTTGTGGAAGAC	sense	hγActin, 2402–2422	M19283
5′GGTTACGGCAGCACTTTTATT	antisense	hγActin 2832–2812	M19283
5′TGTGATGGTGGGAATGGGTCA	sense	mβActin, 206–227	NM_007393
5′TTTGATGTCACGCACGATTTCC	antisense	mβActin, 698–719	NM_007393

The optimal length for a cRNA probe is 300–500 bp. The template for the probe synthesis reaction is a linear plasmid. Add 10 μl 5 × Transcription Buffer (200 mM Tris, pH 8.0, 40 mM $MgCl_2$, 10 mM spermidine, 250 mM NaCl), 1 μl 10 mM ATP, 1 μl 10 mM CTP, 1 μl 10 mM GTP, 1 μl 750 mM DTT, 1 μl RNase inhibitor (RNasin), 12.5 μl ^{35}S-UTP (Amersham, 10 mCi/ml, >1000 Ci/mmol), 18 μl sterile water, 1 μl DNA template (1 μg), 1 μl T3 or T7 RNA polymerase. Incubate at 37 °C for 3–4 h. Add 1 μl RNase free DNase (RQ1 DNase, Promega) and incubate a further 10 min. Add 1 μl 0.5 M EDTA and 50 μl SET + DTT (1% sodium dodecyl sulfate (SDS) in 10 mM Tris, pH 7.5, 1 mM EDTA, 10 mM DTT) and vortex briefly. Prepare RNase Free spin columns as described in Ref. [5] and apply probe, spin, wash and collect eluate into a fresh tube. Count incorporation of label which should be about 90–100% incorporation with a final concentration of $1–3 \times 10^6$ cpm/μl in the probe eluate from the column. Labeled probes should be stored at −70 °C and used a soon as possible after labeling.

To pre-hybridize the slide-mounted frozen sections use de-ionized, distilled water to make up wash solutions and stock reagents at room temperature. Pre-heat the solution A (25 ml of 1 M Tris–HCl (pH8.0), 250 μl of 10 mg/ml proteinase K (Boehringer), 25 ml 0.5 M EDTA (pH 8.0) in 200 ml of distilled water) to 37 °C. Add slides and place container in 37 °C water bath for 2–20 min. The exact timing of the incubation will depend on the tissue being examined and the abundance of the mRNA of interest [11]. Rinse in 0.2% glycine to stop the Proteinase K digestion. Post-fix in Fix A for 5 min. Rinse in 0.1 M triethanolamine (TEA), pH 8.0, for 2 or 3 min. Acetylate for 10 min at room temperature (625 μl of acetic anhydride (99% minimum purity) in 250 ml 0.1 M TEA pH8.0) to block positive charges on tissue induced by proteinase K digestion. Rinse briefly and gently in 2 × SSC. Dehydrate quickly in ascending concentrations (50–100%) of ethanol. Drain slides (5 min) and dry with desiccant under vacuum at room temperature for at least 1 h until hybridization (can be left overnight).

The hybridization mix A consists of 25 ml freshly deionised formamide (Fluka), 5 g dextran sulfate (Pharmacia), 3 ml 5 M NaCl, 1 ml 50 × Denhardt's solution (1 g of Ficoll (Sigma), 1 g of polyvinylpyrrolidone (Gibco) and 1 g of Bovine Serum Albumin (BSA) Fraction V (Sigma) in 100 ml of sterile water), 0.5 ml 1 M Tris, pH 8.0 and sterile water up to 40 ml final volume. Vortex thoroughly and heat at 50–60 °C to dissolve dextran sulphate. Dilute your probe in this solution before incubating with the tissue section. For 10 ml final volume of hybridization solution mix 500 μl tRNA (Sigma) 10 mg/ml in distilled water (kept frozen), 100 μl 1 M DTT (kept frozen) × μl of probe to obtain a final probe concentration of 5×10^6 to 2×10^7 cpm/ml and sterile water to bring the volume to 2 ml. Add 8 ml of hybridization mix A and vortex thoroughly. The probe mix can be stored at −70 °C. The hybridization solution stored at −70 °C should be heated for 10 min at 65 °C for denaturation and centrifuged before use. Once diluted probes are stable at −70 °C for 2 weeks. Apply hybridization solution to cover slip: use about 80 μl for a 50 × 22 mm cover glass, proportionally less for smaller ones, then place inverted slide carrying tissue section onto coverslip, and place upright. Seal edges of cover glass with a line of liquid D.P.X. mountant (BDH). Incubate overnight at 50 °C on a slide warming tray in a fume hood.

For the post-hybridization RNase treatment and washes use a rocking or rotating table for gentle agitation. Allow slides to cool gradually to room temperature (30 min). Peel off DPX. Place slide in slide rack immersed in 4 × SSC. Soak off cover slips in 4 × SSC rinse (with gentle agitation for20 min.). Lift slides out of rack, and let the cover glass slide off. Place slides in another slide rack immersed in 4 × SSC (RNase Staining Rack) and rinse twice in 4 × SSC. Pre-heat RNase solution (500 μl of 10 mg/ml RNase A (Boehringer) (stored at −20 °C), 25 ml of 5 M NaCl, 2.5 ml of 1 M Tris (pH 8.0), 500 μl of 0.5 M EDTA (pH 8.0) and 221.5 ml of distilled water) to 37 °C. Add slides and place container in a 37 °C water bath for 30 min. RNase digestion removes most of the non-specifically bound probe. Rinse and wash at progressively increasing stringency of SSC (starting from 2 × up to 0.1 ×) with 1 mM dithiothreitol (DTT) added to all solutions by gently lifting slides in and out of each solution five times and placing them on rocking platform for 5 min for each wash at room temperature. For the last wash preheat the washing solution (0.1 × SSC) to 60 °C and place the slides in the 60 °C water bath for 30 min. Briefly rinse the slides at room temperature in 0.1 × SSC with DTT to cool slides and dehydrate quickly in ethanol with salt and DTT (add 1 ml 20 × SSC and 250 μl 1 M DTT per 250 ml) as before. Drain well (5 min.) and vacuum dry at

room temperature for 60 min. Alternatively, dry under a stream of air for 60 min or in oven at 50 °C for 60 min. Slides are now stable, and can be stored at room temperature.

Slides can be exposed to X-ray film and tissue sections will produce a contact print image on the X-ray film with the signal intensity proportional to the amount of bound probe and therefore underlying gene expression. Relative levels of mRNA expression can be obtained by densitometric analysis of contact sheet images [12].

Slides are then dipped in X-ray emulsion to be able to detect a signal. Either Kodak Nuclear Tracking (NTB-2) liquid emulsion or Ilford K5 liquid emulsion is used. Dipping and developing is done in the darkroom with a red safelight (Kodak Safelight Filter #2 with a 15-W bulb). NTB-2/K5 is diluted with an equal volume of water plus a couple of drops of glycerol, before dipping. Pre-warm an appropriate amount of water/glycerol to 42 °C then add an equal volume of NTB-2 and place at 42 °C to melt. Mix thoroughly then pour into a Dip Miser Cup (Electron Microscopy Sciences, USA, Cat #70510) and place back at 42 °C. Hand dip each slide individually for approximately 4 s to achieve thin coverage of emulsion and then air dry vertically for approximately 1 h. Place slides in slide boxes and wrap the boxes in four layers of aluminium foil. Put the slides at 4 °C to expose for the appropriate amount of time, 2 weeks in most cases. Kodak D19 developer is used to develop in situ slides. When developing in situ slides, dilute the amount of D19 stock needed 1:1 with water. Make sure the temperature of the developer is 20 ± 2 °C. Develop slides in Diluted Kodak D19 developer for precisely 4 min. Rinse in 0.2% acetic acid to stop development and fix in Kodak non-hardening fixer for 5 min. Rinse slides in running tap water for at least 20 min before counter-staining. After the emulsion has been developed, tissue can be counter-stained with the stain of choice. (More details are available at the Online Virtual Repository [11]).

2.3. Rapid amplification of cDNA ends (RACE)

This technique may be carried out efficiently with the 5′RACE kit from Roche (catalogue number: 1 734 792).

The synthesis of the first cDNA strand is performed using 4 μl of 5 × AMV synthesis buffer (250 mM Tris–HCl, 40 mM MgCl2, 150 mM KCl, 5 mM dithiotreitol, pH 8.5) mix with 2 μl of dNTP 10 mM (mixture of dATP, dTTP, dGTP, dCTP 10 mM each), 1 μl of specific primer 12.5 μM, a total of 0.2–2 μg of RNA and 1 μl (20 U) of AMV reverse transcriptase in a final volume of 20 μl. Incubate the reaction at 55 °C for 1 h and then at 65 °C for 10 min. After briefly spinning down, the reaction the cDNA is purified using the High Pure PCR Product Purification Kit (Roche) as per the manufacturer's instructions. The polyA tailing reaction of cDNA is performed as follows. Mix 19 μl of purified cDNA with 2.5 μl of 10 × reaction buffer (100 mM Tris–HCl, 15 mM MgCl2, 500 mM KCl, pH8.3) and 2.5 μl of 2 mM dATP. Incubate for 3 min at 94 °C. Chill the reaction on ice, briefly spin down the mixture and add 1 μl (10 U) of terminal transferase. Mix and incubate at 37 °C for 20 min. To heat inactivate the enzyme, incubate at 70 °C for 10 min. Briefly spin down the reaction and place the tube on ice. The dA-tailed cDNA is then amplified in 50 μl total volume using 5 μl of dA-tailed cDNA, 1 μl of oligo dT-anchor primer 37.5 μM (5′ GACCACGCGTATC-GATGTCGACTTTTTTTTTTTT TTTV 3′ V = A, C or G), 1 μl of specific primer 12.5 μM, 1 μl dNTP 10 mM, 0.5 μl of Taq DNA Polymerase and 5 μl of 10 × reaction buffer. The PCR conditions, 1 × 2 min at 94 °C followed by 10 × 15 s at 94 °C, 30 s at the specific primer annealing temperature, 40 s at 72 °C and 25 × 15 s at 94 °C, 30 s at the specific primer annealing temperature, 40 s at 72 °C plus a cycle elongation of 20 s for each cycle and 1 × 7 min at 72 °C, will depend on the specific primers used. A second round, nested PCR, is sometimes necessary using 1 μl of the first round PCR reaction and the same PCR condition. The least robust part of this technique is the T-tailing reaction as terminal transferase is a very unstable enzyme.

Acknowledgements

The CF-Chip project (EU-QLK3-CT-2001-01982) supported the preparation of this review.

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