Non-PCR methods for the analysis of CFTR transcripts

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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
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, 0.1% (w/v) SDS, 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

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.
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 MgCl₂, 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 35S-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 DNease (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 × 10⁶ 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 20–20 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. Acetate 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 × 10⁶ to 2 × 10⁷ 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 for 20 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

Table 1

<table>
<thead>
<tr>
<th>Primer sequence</th>
<th>Orientation</th>
<th>Gene and Location</th>
<th>GenBank accession number</th>
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</table>
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 dithiothreitol, 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, 1 μl of 10× reaction buffer, 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

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References