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Quantitative methods for the analysis of CFTR transcripts/splicing variants

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Abstract

In cystic fibrosis (CF), transcript analysis and quantification are important for diagnosis, prognosis and also as surrogate markers for some therapies including gene therapy. Classical RNA-based methods require significant expression levels in target samples for appropriate analysis, thus PCR-based methods are evolving towards reliable quantification. Various protocols for the quantitative analysis of CFTR transcripts (including those resulting from splicing variants) are described and discussed here.

Keywords: Transcripts; RT-PCR; Quantitative PCR; Real-time PCR; Splicing; Alternative splicing

1. Introduction

In cystic fibrosis (CF), there are three aspects of clinical utility of transcript analysis and quantification as tools for disease diagnosis and monitoring, namely: (1) the absolute quantification of *CFTR* transcripts in different tissues; (2) assessment of the qualitative and quantitative impact of certain gene variants on splicing, by measurement of the relative levels of aberrantly spliced isoforms; (3) definition of novel molecular parameters as prognostic markers for disease evolution (RNA expression profiling that might provide novel diagnostic or prognostic tools for assessment of CF patients is dealt with in a separate article of the present supplement [1]).

The first of these applications is important for: (i) identification of relevant target tissues/cells for therapy; (ii) determination of the residual levels of normal, full-length *CFTR* transcripts that may have prognostic significance; (iii) as surrogate markers to evaluate response to gene therapy, or to pharmacological agents aimed at increasing the levels of *CFTR* gene expression (e.g., phenyl butyrate).

The classic methods of Northern or Southern blot allow reliable quantification of unamplified DNA or RNA, but require the target molecule to be present at a high-copy number in samples. In contrast, polymerase chain reaction (PCR)-based techniques allow quantification by specific amplification of nucleic acid sequences starting with a very low copy number and/or limited amounts of sample. Reverse transcriptase (RT)-PCR therefore represents a sensi-

Abbreviations: CERES, Composite Exonic Regulatory Element of Splicing; ddNTPs, dideoxynucleotides; NBD, nucleotide-binding domain; OAT, ornithine amino transferase; PCR, polymerase chain reaction; RT, reverse transcriptase; SAB, sum above background.

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tive and powerful tool for analyzing RNA, and quantitative RT-PCR has tremendous potential, although a comprehensive knowledge of its technical aspects is required. Successful quantitative RT-PCR involves correction for experimental variations in individual RT reactions and often correction for different PCR efficiencies using external standards. These parameters are described below in detail.

Recent technological advances allow detection of the increment per cycle of a specific PCR product in ''real-time mode''. This technology has set the stage for the reliable quantification of very small amounts of nucleic acids in extremely small samples, possibly even single cells. This technique has already been successfully applied to the quantification of both CFTR DNA and RNA (including splice variants) from several sources, and is also discussed here.

Besides practical considerations of experimental design, we also address here absolute quantification by RT-PCR, choice of RNA standards (internal vs. external) and a number of quantification strategies, all in a CF context. All protocols referred to here are described elsewhere in further detail [2].

In a scenario of application of some of these techniques to the diagnosis (and possibly prognosis and/or therapy assessment) of CF, we envisage that new guidelines, as well as extensive quality control and standardization programs, must be introduced.

2. Choice of primers

Successful and optimal analysis of transcripts by RT-PCR requires a careful design of oligonucleotide primers. This can be achieved simply by inspection of the sequence to be amplified, or by using software packages either freely available online [3–5] or included with certain real-time thermocycler machines (e.g., PrimerExpress™, Applied Biosystems, Foster City, CA, USA). To quantitatively assess splicing variants, primers can be selected to produce reaction products, which serve as their own internal competitors (by spanning the alternative splice variants). All methods discussed here imply the previous extraction of RNA from samples, as described elsewhere [6].

3. Methods for absolute or relative quantification of CFTR transcripts

3.1. RT-PCR quantification of CFTR mRNA in human samples with one F508del allele

This RT-PCR protocol allows the relative quantification of CFTR transcripts from CF carriers or patients possessing the F508del mutation in one allele. The strategy also allows transcripts with and without skipping of exon 9 [2,7] to be distinguished, and thus quantified. Additionally, by assuming that levels of transcripts from the F508del-CFTR allele are constant among different individuals, this method also allows estimation of the absolute level of non-F508del transcripts present.

Briefly, a PCR product in the region of exons 8–10 is amplified. Aliquots are collected at different cycle numbers (e.g., n, $n+1$, $n+2$, $n+3$) to verify that the reaction is still in the exponential phase, and the products separated and detected in an automatic sequencer. Unequal amplification efficiencies of transcripts with and without exon 9 are corrected for by use of control plasmids. Percentage of each CFTR mRNA species is estimated relative to total CFTR present, by integration of the corresponding PCR product peak areas using GeneScan™ software (Applied Biosystems). The absolute amount of transcripts from the non-F508del CFTR allele is then calculated, using raw data obtained from integration of peak areas from separate analysis of samples from each individual (CF carrier or patient). Here, F508del transcripts are considered as internal standards, i.e., assumed as non-variant among individuals.[1]

3.2. Relative quantification of CFTR mRNA by RT-PCR using β-actin as an internal standard

This protocol allows the relative quantification of CFTR transcripts (target) in mRNA samples, by comparison with the mRNA abundance of β-actin (control), a housekeeping gene which is assumed to be expressed at roughly equal levels in different cell types and under different conditions. The protocol [2] differs from the one above in that it does not require presence of the F508del allele, and is thus a more general method for detection of differences in CFTR mRNA expression levels among different samples. Briefly, a duplex RT-PCR is performed using FAM-labelled primers to amplify CFTR and β-actin cDNAs from the same RNA samples, originating products of similar (and relatively small) size to minimize differences in amplification efficiencies. As above, aliquots are taken after increasing numbers of PCR cycles, the products are run on an automatic sequencer, and PCR product abundance estimated by integrating the corresponding peak areas using GeneScan™ software. Provided both products are still in the logarithmic phase of amplification (see above and also Ref. [6]), which can be shown by plotting peak areas against number of cycles for both products, CFTR mRNA abundance is then calculated as a percentage of β-actin expression. For very low abundance mRNAs (e.g., CFTR, in most cell types) it is also possible to mix normal and competitive non-extendable β-actin primers, synthesized with dideoxynucleotides (ddNTPs) at their 3′ ends, to artificially ''tune'' the amplification reactions of both transcripts (target and control) to the logarithmic phase and thereby allow comparison.

[1] Almost all F508del chromosomes detected in CF patients and carriers all over the world are associated the same extended haplotype and TG10T9 [28].

Values obtained should be validated by experimental replication ($n = 3$ or more independent experiments) followed by statistical analysis. It is important to remember that this is a relative method of quantification, and resulting values can only be compared with others obtained in the same reaction or under identical experimental conditions, particularly if competitive actin primers are used.

3.3. Quantitative sheep, human and mouse CFTR transcript analysis by real-time PCR (TaqMan™)

The protocol that we optimized for the quantitative analysis of sheep *CFTR* transcripts [2] used a probe and primer set designed by ABI PrimerExpress™ 1.0 software and is specific for RT-PCR as the probe spans the boundary of exons 6a and 6b of ovine CFTR. This area of the transcript has been shown by our laboratory not to exhibit any significant alternative splicing. The probe can be labelled with FAM and TAMRA. More recently probe and primer sets have been optimized for the detection of human CFTR with a probe spanning the boundaries of exons 5 and 6a and mouse CFTR with a probe spanning the boundaries of exons 16 and 17a.

TaqMan™ equipment provides instructions on how to determine ideal probe and primer reaction concentrations. This may have to be determined each time a new probe is ordered, even if the sequence is the same. A small amount of detectable target RNA should be used in the optimization procedure.

To obtain meaningful values, the same experimental sample should be assayed with an internal control. Due to the lack of availability of a suitable, well-characterized, sheep-specific internal control, we used a ribosomal RNA assay.[2] This assay works for species as diverse as *Saccharomyces cerevisiae* and human. However, it has the major disadvantage that 18S rRNA is expressed at an extremely high level compared to *CFTR*, and, as a result, extensive dilution of the sample is required to generate reproducible results. In general, it is best to use an internal control with equivalent expression levels to the gene under investigation.

It is sometimes possible to assay control and target gene expression in the same sample within the same tube if different labels are used as on each probe and the probe/primer sets function efficiently in the same reaction conditions.

3.4. RT-PCR based quantification of CFTR YAC copy number

This RT-PCR based quantitative method is designed for detection of YAC transgene expression in both human cells and transgenic mice, normalized per YAC copy number. RNA samples prepared from cell lines or transgenic mouse tissues is first reverse transcribed with oligo-dT primer, and an aliquot of the cDNA thus produced is used in the specific PCR reaction with a radio-labelled primer [2].

3.4.1. Analysis of transgenic mouse tissues

For the analysis of transgenic mouse tissues, RT-PCR products are digested with *Nru*I (digests the human YAC product) and *Hin*dIII (digests the mouse product) and then separated by agarose gel electrophoresis. The latter is dried prior to exposure to a phosphorimager screen for radioactivity counting. After recording the sum above background (SAB) counts from the 566 bp mouse digestion product fragment (mouse-SAB) and the 390 bp human digestion product (human-SAB), the mouse-SAB is divided by 326 and the human-SAB by 221 for normalization, accounting for the AT content of each fragment. Each normalized SAB is then corrected to a copy number of 2 (e.g., if the YAC copy number has been estimated at 3, divide the normalized SAB by 2/3) for comparison.

3.4.2. Analysis of human cell lines

For the analysis of human cell lines, a very similar protocol is used, except that digestion of RT-PCR products is carried out with restriction enzymes *Cla*I (specific for the human YAC product) and *Bcl*I (specific for the endogenous gene). These yield radioactive products of 590 bp (endogenous gene digestion product) and 590 bp (YAC digestion product). Following exposure to a phosphorimager screen and recording of SAB from these products, SAB values normalized for their respective AT content are also corrected to a copy number of 2, as above, for comparison.

3.5. Methods for differentiation/quantification of normal and mutant CFTR mRNAs. Example: E822X

Nasal epithelial cells are collected as described [6] from individuals with mutation E822X (G>T at 2596), non-CF controls and heterozygotes for the polymorphism R668C, and mRNA extracted. First strand cDNA is synthesized using random hexanucleotide primers. RT-PCR is performed using Hot Star polymerase (Qiagen, Hilden, Germany) as described [2,9]. An aliquot of the final products is loaded on an automatic DNA sequencer. Each analysis is repeated three times to ensure accurate quantification. Relative amounts of mutant and normal cDNA products are sized and quantified using the Fragmentor analysis software, and are expressed as percentage of total cDNA.

3.5.1. E822X

RNA samples with E822X are reverse-transcribed with an initial primer set spanning exons 13 to 14a. The forward primer was designed to contain a 3′ mismatch (G>A at 2594[3]) which in combination with the normal sequence at

[2] Closer inspection of the sequences of primers included in the 18S rRNA kit (Applied Biosystems) revealed that the 3′ primer actually overlapped with the probe by a single thymidine residue. All 18SrRNA reactions performed by our laboratory (Institution 3) used these sequences despite this error and results were reproducible.

[3] Numbering system according to Zielenski et al. [29].

E822 (G at 2596, E822X: G>T at 2596) creates a restriction site for *Hph*1, whereas the mutant transcript remains intact. The reverse primer is end labeled with the fluorescent moiety Texas-Red.

3.5.2. Quantification of mutant/normal cDNA

A modification of the RT-PCR protocol is used to quantify the levels of mutant/normal (822X/E822) mRNA by one-step extension of the fluorescent primer, using an aliquot of previously amplified CFTR cDNA as template. The product is *Hph*1 digested and analyzed on an automatic sequencer as above. The one-step primer extension avoids formation of normal/mutant heteroduplexes that remain uncleaved by the restriction enzyme, leading to false ratios. The quantification is validated by including homozygous mutant and normal controls.

4. Detection and quantification of CFTR splicing variants

Several CFTR splicing mutations have been shown to generate both correctly and aberrantly spliced transcripts. Some quantification strategies for splice variants are discussed here.

4.1. One-step RT-PCR method to determine low levels of aberrantly spliced CFTR mRNAs

4.1.1. General strategy of the assay

This protocol describes a sensitive, accurate and rapid real-time one-step RT-PCR method for measuring low abundance *CFTR* splice variants. The use of well-validated boundary-spanning primers has been shown to be the most accurate method of real-time PCR for quantification of splice variants, which differ greatly in abundance [10,11].

4.1.2. Quantitative one-step RT-PCR

Quantification of *CFTR* mRNA is difficult because of its low abundance with only 1–2 copies per cell in the respiratory tract [12] and cell yields of nasal brushings from children are low. Consequently, to reach a good degree of sensitivity and specificity, three methods were combined. First, CFTR transcripts with and without exon 9 ($ex9^+$; $ex9^-$) are reverse transcribed with the same reverse primer in exon 10, as the selection of gene specific primers for low abundance transcripts is recommended [13]. The first five bases of the forward primer for both transcripts are complementary to exon 10. The residual 16 bases of the forward primer are identical to the 3′ end of exon 9 for CFTR 9 transcripts and to the 3′ end of exon 8 for CFTR $ex9^-$ transcripts. These primers not only discriminate between $ex9^+$ and $ex9^-$ transcripts, but also prevent amplification of contaminating genomic DNA. Secondly, for the real-time PCR a hot-start approach was used for reduction of non-specific products. Thirdly, the rapid cycle DNA amplification is a LightCycler specific PCR technique with improved product specificity and fast cycle times [14]. With this technique, an allele-specific assay can even discriminate between a 3-bp or a 1-bp mismatch at the 3′ end of the corresponding primers [15].

4.1.3. Diagnostic accuracy and reproducibility

Identical amplification efficiency of the two splice variants is essential for reliable and reproducible relative and absolute quantitative real-time RT-PCR. All calibration curves for CFTR $ex9^+$ and $ex9^-$ standards (each diluted from 10^7 to 10^3 copies of single-stranded DNA per reaction mixture) showed linearity over the entire range with correlation coefficients >0.99, indicating a precise log-linear relationship. The intrarun variability, calculated from duplicates of the two targets, showed a maximal average S.D. of 3.8%. For interrun variability the threshold cycle was determined in duplicates for both splice variants for each sample in four independent analytical runs, and mean threshold cycle was used to determine a maximal interassay S.D. of 6.0%.

4.2. Semi-quantitative RT-PCR for alternatively spliced exons: 3849+10 Kb C->T and IVS8-polyT

Quantification of CFTR splice variants is performed by fluorescent RT-PCR analysis. The PCR primers flank the alternatively spliced exon, and, therefore, generate two products in each PCR reaction, one including and one lacking the alternatively spliced exon. One of the primers is fluorescently labeled, thus all PCR products are fluorescent and can be quantified in an automated sequencer. The analysis is performed using GeneScan™ software (see above). The level of any given transcript (aberrantly or correctly spliced) is determined as the signal peak area of the corresponding PCR product divided by the sum of the signal peak areas of both aberrantly and correctly spliced PCR products. The PCR is performed under semi-quantitative conditions as determined by serial tertiary dilutions of the cDNA, and is defined for each set of primers. The quantitative range lies within the linear range, so assays should be designed accordingly.

4.2.1. The 3849+10 Kb C->T mutation

The primers used are as described elsewhere [2] and bind to sites in exons 18 and 20. The reverse primer is fluorescently labelled with 6-FAM. The PCR products of the correctly and aberrantly spliced transcripts are 304 and 388 bp, respectively. The aberrantly spliced transcript includes a cryptic ‘exon’ of 84 bp (between exons 19 and 20), which bears a stop codon [16]. Therefore, the total amount of the transcripts has to be calculated [17].

4.2.2. The IVS8-polyT alleles

The primers used bind to exons 8 and 10, respectively. The reverse primer is fluorescently labelled with 6-FAM.

The PCR products of the correctly and aberrantly (ex9^{-}) spliced transcripts are 513 and 330 bp, respectively. The primer sequences and PCR protocol are available at the European Working Group on CFTR Expression website [2].

5. Minigene functional analysis of CFTR splicing transcripts

5.1. General strategy of the hybrid minigene transient transfection assay for the identification and analysis of splicing defects

Intronic and exonic sequences contain several splicing regulatory elements important for correct processing of mRNA. Human mutations interfering with these regulatory elements may cause splicing defects. The most common splicing mutations affect canonical elements such as the conserved 3′ (AG) or 5′ (GT) splice sites, or the branch site. However, pre-mRNA splicing alterations that modify non-canonical regulatory elements are more difficult to detect by sequence inspection alone. As RNA samples from patients are not always available, hybrid minigenes represent an important tool for the study and characterization of the effect of human genetic variations/mutations on the pattern or efficiency of pre-mRNA splicing. Any region of interest suspected to cause a splicing defect (e.g., a specific exon along with flanking intronic sequences) is introduced into a minigene and transfected into cell lines. The minigene described here is transcribed by polymerase II into pre-mRNA and accurately spliced in the cell. This approach, initially described for the study of basic mechanisms of alternative splicing [18], has been recently validated for the study of the effect of mutations and/or polymorphic variants on the processing of the nascent RNA in several genes, including CFTR [8,19–22]. The mRNA is analyzed by RT-PCR with primers that amplify processed transcripts derived from the minigene. Two different examples are described here, and detailed protocols can be found at the European Working Group on CFTR Expression website [2].

5.2. Nucleotide substitutions in exonic regulatory elements: missense and silent mutations in Composite Exonic Regulatory Elements of splicing in CFTR exon 12

Exonic sequence variations overlap with coding sequences and thus are frequently considered only for their effect on the protein, e.g., when missense mutations modify amino acid composition. Synonymous or silent variations are largely ignored, but may have unexpected and unpredictable effects on splicing, with variable amounts of exon skipping. Exonic substitutions may affect novel regulatory elements termed Composite Exonic Regulatory Elements of Splicing (CERES) [22]. These are short exonic RNA sequences (5–

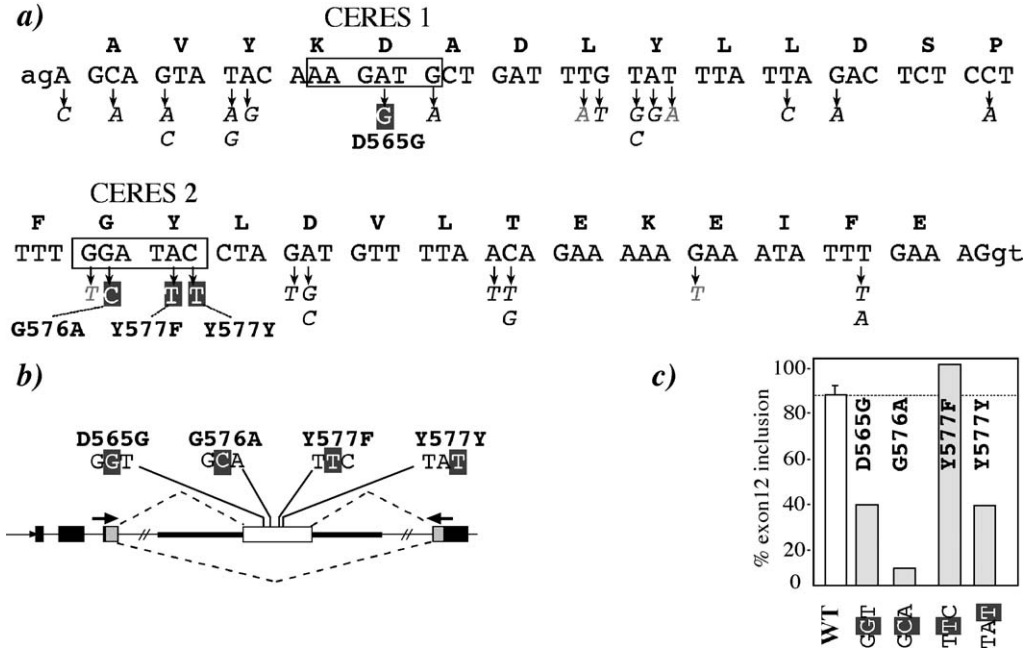

Fig. 1. Mutations in Composite Regulatory Elements of Splicing (CERES) in CFTR exon 12. (a) Nucleotide sequence of the CFTR exon 12 showing the list of available point substitutions in the CF database [23]. Some missense and silent variations (highlighted) are located in the CERES elements. Nucleotide substitutions never analyzed for splicing efficiency are shown in *italics*. (b) Schematic representation of the three hybrid minigenes used for analysis of alternative splicing of CFTR exon 12. These minigenes contains normal exon 12 (WTex12) or the four natural mutations occurring in CERES elements. The α-globin, fibronectin, and CFTR exons are shown as black, shaded and white boxes, respectively. Arrows indicate RT-PCR primers. Transcription is driven by the SV40 enhancer (small arrow at the 3′ end). For details see Ref. [2]. (c) The effect of natural CFTR exon 12 mutants. The graph shows the percentage of exon 12 inclusion. The wt exon 12 minigene shows about 80% of exon inclusion. D565G, G576A and Y577Y induce exon skipping, while Y577F increases the percentage of exon 12 inclusion. Data are from Ref [22].

12 bases) that may contain enhancer or silencer sequences for splicing regulation where different substitutions at nearby (or the same) positions can have opposing effects on splicing efficiency [22]. CFTR exon 12 contains several sequence variations (see CF mutations database [23]) (Fig. 1a) associated with different CF phenotypes, including classical and non-classical CF. Some are considered benign polymorphic variants and for several, a clear disease-causative role is missing. In particular, two missense mutations, D565G and G576A (previously considered a neutral polymorphism), showed no clear association with loss of protein function or disease phenotype. The effect of these variations on the splicing process was evaluated using a suitable hybrid minigene (Fig. 1b). Skipping of exon 12 removes a highly conserved region encoding part of the first nucleotide-binding domain (NBD1) of CFTR, rendering the protein non-functional. Using the hybrid minigene, these natural mutations were found located in CERES elements. D565G and G576A induce variable levels of exon 12 skipping, thus reducing levels of normal transcripts. Another missense mutation, Y577F, reported in a patient with severe CF [23], increased the amount of transcript with this exon in comparison to non-CF controls (Fig. 1c), suggesting that this amino acid substitution was directly responsible for the severe phenotype. Interestingly, the synonymous Y577Y substitution caused exon skipping indicating that "benign" variants have to be assessed for splicing efficiency. This study was reinforced by characterization of the splicing pattern in patient samples where the patterns with the minigene and in cells harbouring the natural mutation were in concordance [22]. CERES-like elements have also been observed in CFTR exon 9 [21] and have been suggested to occur in exons 10 and 11 [24].

5.3. Minigene assay for the evaluation of CFTR exon 9 splicing efficiency

CFTR exon 9 is frequently and extensively skipped, and is caused primarily by an abbreviated version of the polypyrimidine tract in the splice acceptor of exon 9 called the 5T allele. High proportions of transcripts lacking exon 9 combined *in trans* with a severe CFTR mutation can lead to atypical CF, most commonly congenital bilateral absence of the vas deferens (CBAVD). Minigenes provide a controlled system with which to isolate *cis* and *trans* factors that affect the splicing of this and other exons [16,19,20,25,26]. To study factors affecting *CFTR* exon 9 splicing, we designed a transfectable minigene that incorporated exon 9 and its flanking intronic sequence. To retain the appropriate *cis* elements and approximate the in vivo splicing context for exon 9, the 3′ and 5′ ends of exons 8 and 10, respectively, were also included. We have used this minigene to assess the contribution of splice sites, the polypyrimidine tract (Tn), and the adjacent TG dinucleotide repeat (TG)m to exon 9 skipping [26,27]. CFTR exon 9 minigenes have been also used to identify specific RNA *trans*-acting factors

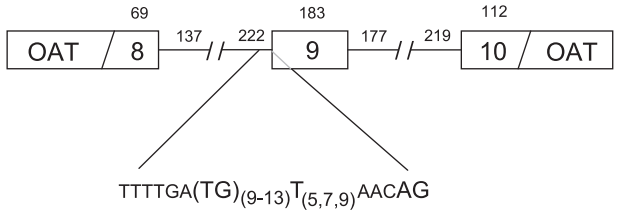

Fig. 2. CFTR–OAT hybrid minigene.

binding to the (TG)m repeat [30,31]. An example of a CFTR exon 9 minigene is shown in Fig. 2.

5.3.1. Minigene construction and assay

The minigene was created by amplifying genomic DNA from a control sample. Exon 9, portions of exons 8 and 10, and flanking intronic sequences are included (Fig. 2). Genomic DNA was amplified by PCR, introducing unique restriction sites to enable subsequent ligation of the minigene components. These were introduced in-frame to an ornithine amino transferase (OAT) cDNA and inserted into the expression plasmid pBK-RSV (Clontech, Palo Alto, CA, USA). The minigene construct can be introduced into any transfectable cell line, e.g., HEK293, and allowed to express for 24 h, after which total RNA is harvested, cDNA is synthesized, and RT-PCR performed. The 5′ primer is specific for the 5′ OAT sequence, and is 6-FAM-labelled. The unlabelled 3′ primer is specific for *CFTR* exon 10. PCR products are analysed in an automatic sequencer. ABI's GeneScan™ software automatically calculates peak area measurements, which are used to estimate the total amount of product derived from each splice variant. A detailed description of the protocol used in the analysis of hybrid minigenes can be found online [2].

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