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Quantitative detection of CFTR mRNA in gene transfer studies in human, murine and simian respiratory tissues *in vitro* and *in vivo*

Research Article

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Abbreviations: CF, Cystic Fibrosis; **CFTR**, Cystic Fibrosis Transmembrane conductance Regulator; **RT-PCR**, reverse transcriptase polymerase chain reaction

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Summary

Pre-clinical and clinical studies aimed to correct the basic defect of cystic fibrosis (CF) by transferring the wild type gene into the airway cells have already shown promising results. One of the main unsolved questions of these studies is the quantitation of the amount of gene required to correct the defect. In this respect, suitable technologies able to measure how much gene is actually transferred into the airway cells are under development. In this article we present a series of application of a single-tube competitive RT-PCR assay to quantitate the vector-encoded CFTR mRNA after gene transfer in human, mouse or monkey respiratory cells. These assays are able to measure very low levels of mRNA, with advantages over traditional expression systems for vector-encoded transcript (Northern analysis) or protein (immunocytochemistry). In some instances they may indicate directly the ratio of vector-encoded versus endogenous transcript, being applicable with both viral-derived and synthetic vectors. The assays presented here are instrumental for future application in gene delivery pre-clinical and clinical trials designed to identify corrective doses of the vector, by providing the basic information on the amount of vector-encoded CF gene transcript expressed in the airway cells.

I. Introduction

Cystic Fibrosis (CF) is a common autosomal recessive disease affecting many organs, pulmonary morbidity being the most life-limiting aspect of the disease. The wild type CF gene encodes for a protein termed Cystic Fibrosis Transmembrane conductance Regulator (CFTR), involved in the transport of Cl^- and probably of other molecules across several epithelia. The most common mutation of the CF gene is the deletion of 3 bases encoding phenylalanine at position 508 in the aminoacidic chain (ΔF508); defective CFTR ultimately leads to chronic lung infection and respiratory insufficiency (Welsh et al, 1995). CFTR is expressed in all serous cells of the submucosal glands of the proximal respiratory tract (Engelhardt et al, 1992) and in ~ 2-5 % of the surface cells of the distal airways (Engelhardt et al, 1994). Pre-clinical studies in which CF gene transfer vectors have been delivered to airways of mice or monkeys are providing important information to compare different vectors in terms of safety, level and duration of transgene expression. Also, clinical trials aiming at transferring the wild type gene into the airway cells of CF patients have already given evidence of the expression of the transgene in the cells collected from nasal or lung mucosa a few days after vector administration (Bellon et al, 1997; Caplen et al, 1995; Crystal et al, 1994; Gill et al, 1997; Hay et al, 1995; Knowles et al, 1995, 1998; Porteous et al, 1997; Wagner

et al, 1998; Zabner et al, 1993, 1996, 1997). Among the different unsolved questions arising from these studies, a key issue is the development of the detection system(s) to quantitate the amount of transgene required to correct the defect *in vivo*. The measurement of the transmucosal potential difference *in vivo* can be an efficient tool to distinguish the CF phenotype as a function of ion transport impairment (Knowles et al, 1981), but the application of this technique to the human bronchial mucosa is still confined to a small number of experienced laboratories (Alton and Geddes, 1997). The relationship between CFTR functional correction and the extent of wild type CF gene transfer has been studied also *in vitro* in CF polarized respiratory cells (Johnson et al, 1992; Zabner et al, 1994) and in CF bronchial xenografts (Goldman et al, 1995). These studies agree that complete correction of Cl^- transport can be achieved with as little as 6-10 % of the CF cells transduced. In this respect, a semi-quantitative evaluation to detect the fraction of cells expressing wild type CFTR protein by classic immunocytochemistry or single cell digital imaging (Renier et al, 1995) can provide useful information. An additional possibility could be to quantitate the wild type CFTR mRNA encoded by the gene transfer vectors. The mRNA approach is particularly interesting in view of the recent understanding that the classic CF phenotype is expressed when the wild type CFTR mRNA decreases below 1-3 % of the normal amount (Estivill, 1996), and that approximately 5 % of normal CFTR mRNA is sufficient to correct the electrical abnormality in CF mouse models (Dorin et al, 1996). In order to provide a suitable technological support to this approach, we developed a series of single-tube competitive mRNA detection assays for both endogenous and vector-encoded CFTR transcripts, both in absolute and relative terms, from human, murine and simian respiratory cell and tissue samples. The evaluation of the expression of the vector-encoded versus the endogenous CFTR transcript can be directly applied to pre-clinical and clinical gene therapy studies utilising different types of viral-derived or synthetic vectors.

II. Results

A. Vector-encoded CFTR transcript expression in human airway cells *in vitro*

We addressed the issue of mRNA quantitation by using a PCR assay, in which a homologous competitor differing only a few bases from the target transcript is amplified in the same reaction tube. The principle of the assay is summarised in **Figure 1**, panel **A**. Competitive PCR was initially applied to quantitate the absolute amount of CFTR transcript encoded by the adenovirus-derived CFTR vector, Ad.CFTR (Rosenfeld et al, 1992), in the human tumour respiratory cell line A549, which is negative for endogenous CFTR expression (Renier et al, 1995). A representative electrophoretogram in which different amounts of the CFTR competitor pTG6525 are amplified together with reverse transcribed exogenous CFTR template is shown in **Figure 1**, panel **B**. The signal ratio of the pTG6525 competitor over the template is plotted against the number of copies of the competitor itself, as shown in **Figure 1**, panel **C**. From the equivalence of the ratio signal of the fitted equation the number of copies of reverse transcribed template can be calculated. Since the DNA competitor is double-stranded while the target cDNA is single-stranded, the value obtained at the equivalence point was multiplied by a factor of 2. In the example reported in **Figure 1**, panel **C**, assuming that reverse-transcription efficiency was equal to 1, the exposure of one plaque-forming unit (pfu) of Ad.CFTR every 10 cells for 24 h produced a steady-state level of 3280 copies of Ad.CFTR-encoded mRNA over 50 ng total RNA.

The number of CFTR mRNA transcripts was measured as a function of different Ad.CFTR infection doses, from 0.01 to 1. The plot in **Figure 1**, panel **D** indicates that the assay makes it possible to detect CFTR transcripts in a dose-dependent fashion, which reflects the consistency of the method. The detection limit of this assay has been calculated by a dilution assay in the minimum level of 20 transcript copies, which can be obtained in our experimental conditions by infecting A549 cells at the infection dose of 0.01 MOI. As expected, the detection limit of this transcript assay is much lower than that observed by detecting gene expression by protein analysis, e.g. by the classic APAAP immunocytochemistry assay applied to CFTR protein detection (Cordell et al, 1984; Renier et al, 1995). In parallel experiments of infection of A549 cells with Ad.CFTR *in vitro*, we found that the lowest infection dose allowing a detectable signal with APAAP assay was higher by two orders of magnitude (1-5 MOI, data not shown).

B. Expression of endogenous CFTR transcript in human airway cells *ex vivo*

Levels of vector-encoded transcript can be compared in parallel to the endogenous transcript for estimation of the extent of gene replacement. Therefore, single-tube competitive RT-PCR was applied to measure the steady-state copies of endogenous CFTR mRNA in respiratory cells. Surface nasal cells from human subjects were brushed and analysed for endogenous transcript expression.

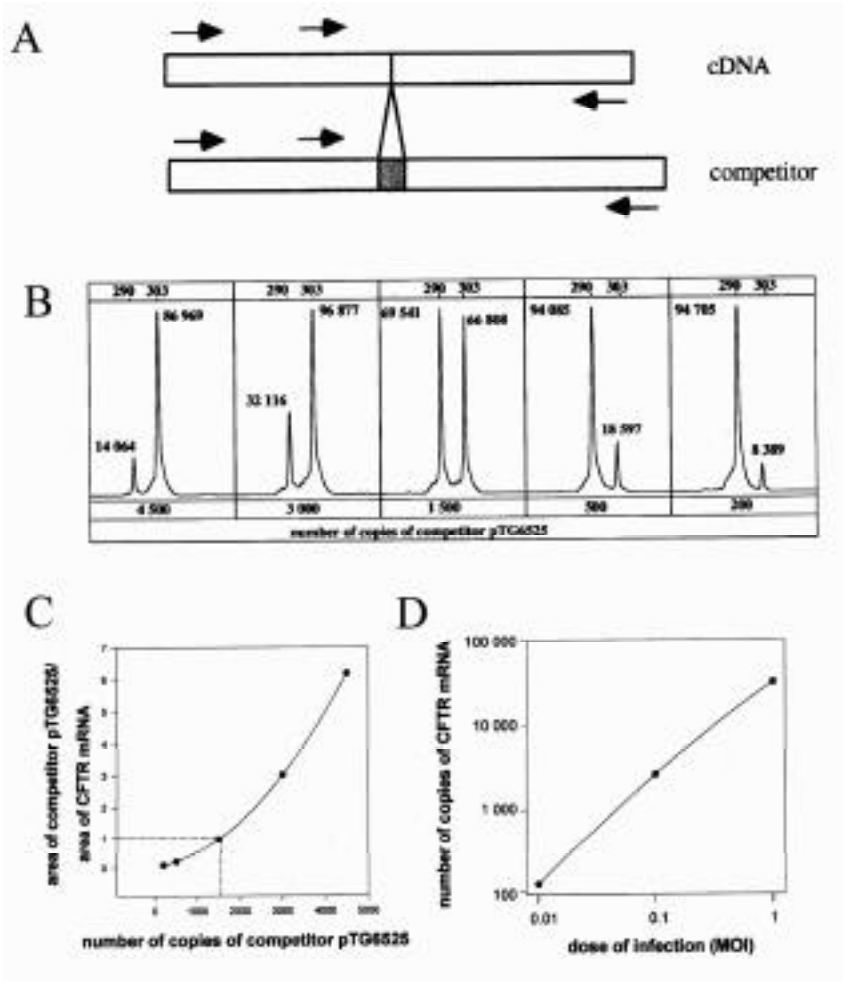

Figure 1. Expression of vector-encoded CFTR mRNA in human respiratory cells *in vitro*.

(**A**) Two consecutive PCR amplifications were performed in the presence of reverse transcribed cDNA (upper fragment), homologous competitor (lower fragment) containing a small insertion (shaded box) and appropriate upstream and downstream primers (solid arrows), as specified in each quantitation. Amplified fragments were labelled by one PCR cycle (run-off) using an internal 5-fluorescently-labeled primer.

(**B**) A549 cells were infected for 24 h with Ad.CFTR at MOI 0.1. The number of copies of competitor template pTG6525 added in the first PCR is indicated on the bottom of each panel. Fragment size related to Ad.CFTR mRNA (290 nt) and pTG6525 (303 nt) is indicated on top. Peak areas are reported close to each peak. (**C**) Competitor over target areas were plotted on the y-axis versus the number of copies of competitor (x-axis), and fitted by least-square regression analysis to quadratic equations. The copies of target mRNA were quantified as twice as much those of the competitor corresponding to the equivalence of competitor-target areas (y-axis value=1). Representative of 5 independent experiments. (**D**) Dose-response of CFTR mRNA transfer to A549 cells. The number of copies of CFTR mRNA corresponding to the infection doses of 0.01, 0.1 and 1 MOI were 123, 2640 and 31874, respectively. Representative of 3 independent experiments.

Preliminary results showed a great sample to sample variability in the steady-state levels of endogenous CFTR mRNA, suggesting that RNA degradation from these samples obtained *ex vivo* from nasal mucosa is a major problem. This limitation was overcome by quantitating the Human Cytokeratin - 15 (HCK-15) transcript (Leube et al, 1988), which is constitutively expressed in respiratory epithelial cells. Both CFTR and HCK-15 were co-amplified in the presence of their respective homologous competitor templates. **Table 1** reports the CFTR/HCK-15 transcript ratio, in 10 non-CF individuals (mean 1.45, SD = 0.74). Therefore, the method in question can provide reliable evaluation of endogenous CFTR transcripts, after normalisation with a reporter gene.

C. Relative expression of wild type vs. ΔF508 CFTR transcript in human airway cells

Since the most common mutation of the CF gene is the deletion of the three bases encoding for Phe 508 (ΔF508), the assay can be made much simpler and more rapid by direct comparison between the amounts of wt and mutated transcript. This is possible for two reasons. First, it should be recalled that the DNA automated sequencer utilised in these assays can discriminate fragments differing only a few bases in length. Second, in the simultaneous presence of the wt and mutated alleles, each transcript competes with and normalizes the other, without the need for a homologous competitor and analysis of a

reporter gene. This approach was first applied to endogenous transcripts of healthy carriers of the ΔF508 mutation in nasal respiratory cells, obtained *ex vivo* by brushing. **Figure 2**, panel **A**, shows a representative experiment in which very similar levels of transcription were observed for normal and ΔF508 CFTR alleles. The almost equivalent areas of the peaks at 100 and 103 nucleotides correspond to mutated and normal CFTR fragments, respectively. Extension of the analysis to other healthy carriers of ΔF508 mutation gave a wt/ΔF508 CFTR mRNA ratio of 0.90 ± 0.15 (mean ± SD, 4 subjects tested in triplicate; individual values = 0.75 ± 0.17, 0.92 ± 0.05, 1.05 ± 0.07, 0.86 ± 0.10), which is consistent with results obtained by other investigators utilising a different technique (Trapnell et al, 1991). The second application was to test the direct wt/mutated approach in respiratory cells obtained from nasal polyps, excised from CF patients homozygous for the ΔF508 mutation, cultured and infected *in vitro* with Ad.CFTR (72 h at MOI 10). In our experimental conditions, the steady state level of the wt transcript encoded by Ad.CFTR was 3 to 4 times higher than in the case of the endogenous ΔF508 mutation, as judged by the peak areas shown in **Figure 2**, panel **B**. We found a wt/mutated ratio of 3.64 ± 1.27 (mean ± SD, 4 separate cultures from 2 individuals tested in duplicate). Though we consider the direct wt/mutated transcript comparison a simplified application of the competitive method, it seems potentially appropriate for gene transfer experiments as a source of rapid information on relative transcript expression in human clinical trials enrolling CF patients homozygous for ΔF508 mutation.

D. Relative expression of vector-encoded vs. endogenous CFTR transcript in mouse lung

The direct comparison of vector-encoded and endogenous CFTR mRNA can be usefully applied to pre-clinical studies in which interspecies homologies can be utilised to amplify with the same set of primers the human vector-encoded and the endogenous mouse CFTR sequences. Similarly to wt and ΔF508 transcripts, exogenous and endogenous CFTR transcripts can compete each other, and the endogenous transcript directly normalizes for RNA degradation. By choosing a portion of the genes in which the two species differ for at least one base in length, it is possible to distinguish the specific signal by running the samples labelled with a fluorescent primer in a 4-6 % denaturing polyacrylamide gel electrophoresis with the automated sequencer.

Alternatively, by choosing a portion of the genes in which the restriction pattern generated by an endonuclease enzyme is different in the two species, it is possible to perform a selective digestion and distinguish the different transcripts by running the ethidium bromide stained fragments in 2 % agarose gel electrophoresis. In this case, the signal can be collected with a videocamera and quantified by image analysis. For instance, mouse CFTR exon 19 differs from the human homolog at an additional GAA codon.

We took advantage of this difference by reverse transcribing vector-encoded and endogenous mouse transcripts expressed with a sequence specific homologous primer annealing to exon 20. The cDNA was amplified with a first PCR cycle (PCR-1) from exon 20 to 17b and a second PCR cycle (PCR-2) from exon 18 to 19. As summarised in **Figure 3**, panel **A**, the amplified fragments from human and mouse transcripts differ of 3 bases in

Table 1. Quantitation of endogenous CFTR mRNA expression in respiratory cells.

Subject	CFTR/HCK-15 ratio of number of copies
A	1.02
B	1.80
C	1.72
D	0.72
E	1.65
F	0.43
G	2.12
H	1.11
I	1.00
J	2.94
mean	1.45
SD	0.74

After extraction from 4 x 10^5 cells recovered from non-CF individuals (from A to J) by nasal brushing, total RNA (50 ng) was reverse-transcribed into cDNA in the presence of CFTR11-R1 and hCK15-6. CFTR9-D1 plus hCK15-5 primers were added in PCR-1 which was performed in the presence of the competitors pCFTR-ΔC and phCK15-C. PCR-2 was performed in the presence of CFTR9-D1, CFTR10-11R2, hCK15-D2 and hCK15-R2. Run-off reaction was done with CFTR10-D2 FAM and hCK15-D2 FAM. HCK-15 mRNA was utilised as reporter transcript in order to normalise the absolute amount of CFTR mRNA. Normalized data were provided by the ratio of the number of copies of CFTR/HCK-15 mRNA.

length (total lengths were 284 and 287 bp, respectively). The amplified fragments contain restriction sites for the endonuclease *Rsa I* , allowing the distinction of the human and mouse origin. As shown in **Figure 3**, panel **B**, by ethidium bromide stained agarose gel a single band is visible from the human amplification (lane 1), two bands from mouse amplification (lane 2), and the fragments can be easily distinguished in lung samples of animals exposed to the CFTR vector (lane 3). The assay was directly applied to the relative quantitation of the CFTR transcript encoded by two adenovirus-derived vectors instilled in the trachea of SCID mice, in which the quantitation of the CFTR mRNA by Northern analysis was unsuccessful because of low expression level (data not shown). In the example reported in **Figure 3**, panel **C**, it has been possible to observe that intratracheal instillation of the Ad.TG5643 vector resulted in higher level of vector-encoded transcript in comparison to Ad.CFTR vector, particularly in the lungs of mice sacrificed at day 3 and 14. In our hands, the assay is rapid and reproducible. Moreover, since the reverse transcription and amplification is performed on a sequence within the CFTR gene, the assay can be applied to the relative quantitation of the exogenous transcripts encoded by any CFTR vector, either viral-derived or synthetic.

E. Relative expression of vector-encoded vs. endogenous CFTR transcript in monkey lung

Even though the human-mouse CFTR sequence homologies are quite different from those of the human-monkey (Tata et al, 1991; Wine et al, 1998), the set of primers and the PCR conditions utilised for relative quantitation of vector-encoded versus mouse endogenous CFTR mRNA can be directly applied also to CFTR transcript from Rhesus monkey (*Macaca mulatta*). The single-tube competitive RT-PCR assay again generates two fragments of 284 and 287 bp, from human and monkey CFTR transcripts, respectively. The *Rsa I* digestion of exons 18/19 gives the same restriction pattern, since monkey and human CFTR sequences are highly homologous. On the other side, the fragment amplified from exon 18 to 19 of monkey CFTR contains a unique *ScrF I* restriction site, generating two fragments of 163 and 124 bp. The relative quantitation assay has been applied to evaluate the level of CFTR gene expression in relation to ICAM-1 transcript induction. We have previously found that steady-state levels of ICAM-1 transcript are increased in the lung portions of Rhesus monkeys instilled by bronchoscope with high doses of the E1-E3-deleted vector Ad.CFTR (Nicolis et al, 1998).

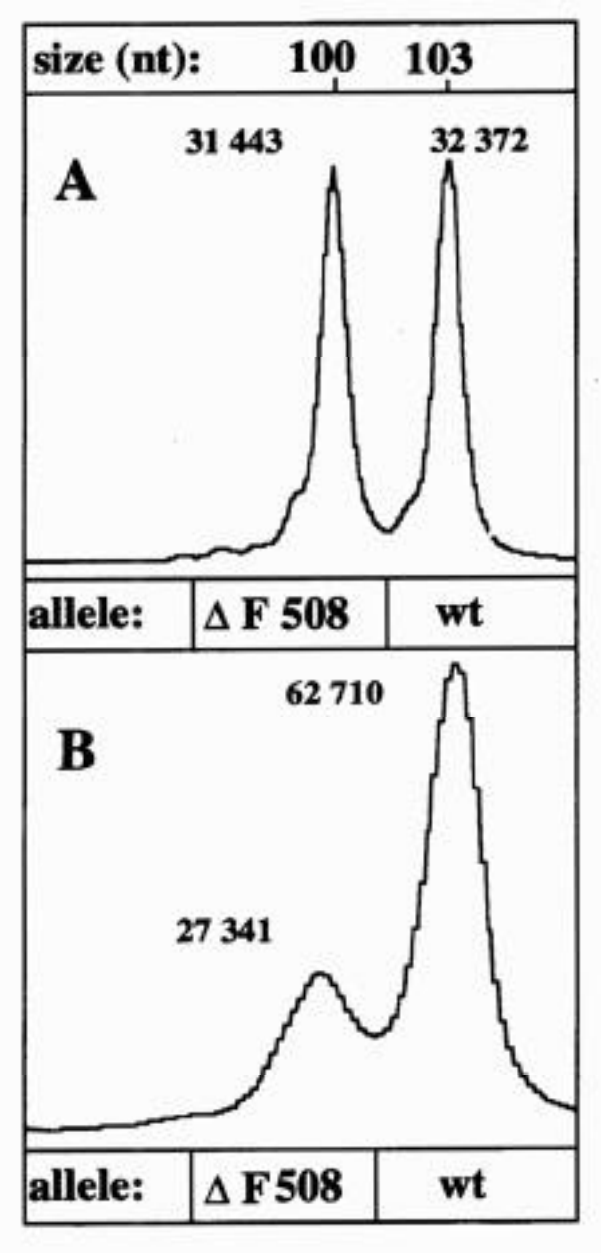

Figure 2. Relative expression of human wt and ΔF508 CFTR mRNA.

(**A**) Electrophoretogram of endogenous wt and ΔF508 mutated CFTR transcripts from human nasal respiratory cells obtained *ex vivo* from a ΔF508 healthy carrier.

(**B**) Electrophoretogram of the relative expression of vector-encoded wt and endogenous ΔF508 CFTR mRNA from primary cultures of nasal polyp cells from a CF subject homozygous for ΔF508 mutation. Cells were infected with Ad.CFTR at MOI 10 for 72 h, as previously described (Renier et al, 1995). Peak areas at 103 and 100 nt in the electrophoretogram correspond to the amounts of amplification products of wt and ΔF508 CFTR mRNA, respectively.

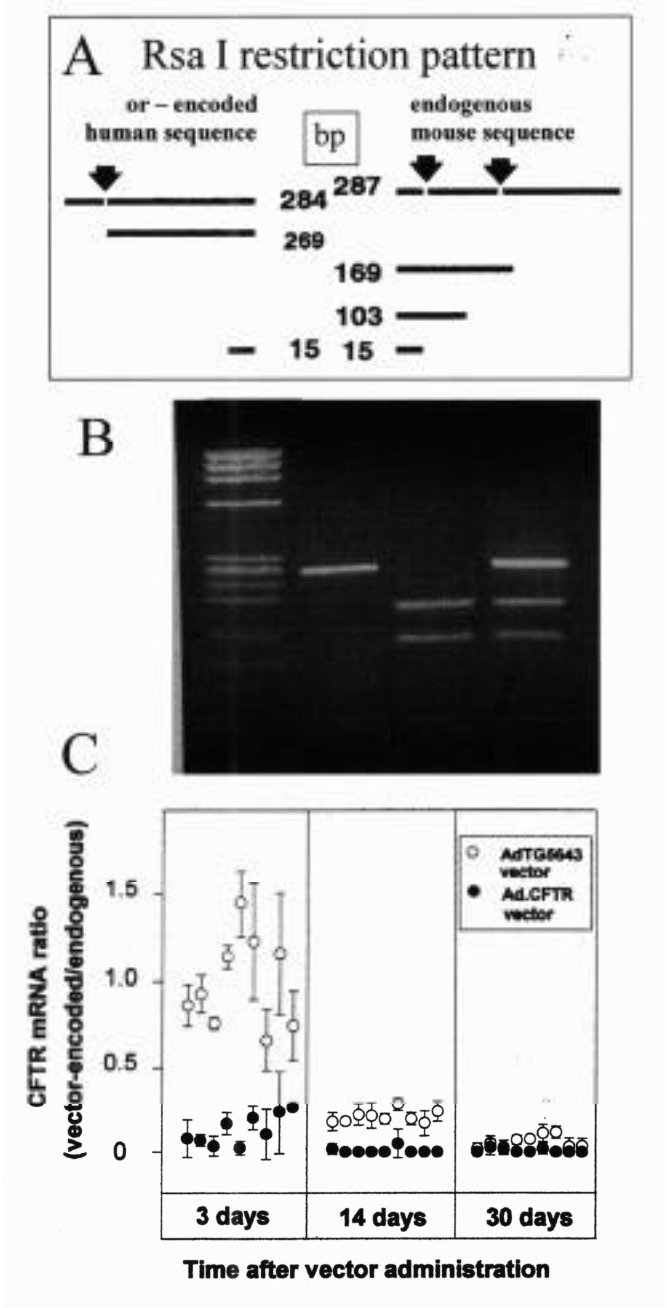

Figure 3. Relative expression of vector-encoded versus endogenous CFTR mRNA in mouse.

(**A**) Schematic drawing of the expected restriction pattern with *Rsa I* endonuclease enzyme of human vector-encoded and mouse endogenous sequences after RT-PCR. The length of the fragments (bp) is indicated.

(**B**) Ethidium bromide-stained agarose gel electrophoresis of the amplified fragments digested with *Rsa I*. Lane 1: human CFTR cDNA from Ad.CFTR vector. The restriction fragment of 15 bp is usually not visible; lane 2: mouse CFTR mRNA reverse transcribed and amplified from total RNA extracted from the lungs of a mouse not infected with CFTR vectors; lane 3: total RNA reverse transcribed and amplified from lungs of a mouse infected with AdTG5643 *in vivo*, containing both endogenous and vector-encoded amplified fragments. The relative migration of the molecular weight marker (M) φX174 cleaved with *Hae III* is shown (apparent sizes are 1353, 1078, 872, 603, 310, 281/271, 234, 194, 118 and 72 bp).

(**C**) Relative expression of CFTR mRNA from lungs of SCID mice infected with CFTR vectors Ad.CFTR (closed circles) and Ad.TG5643 (open circles) and sacrificed 3, 14 or 30 days after intratracheal instillation. Data are mean ± SD of determinations performed in 3 different days.

To test whether the upregulation was directly related to the efficiency of infection of the vector, we studied ICAM-1 mRNA expression as a function of vector-encoded CFTR mRNA by the relative quantitation assay. **Figure 4** shows that ICAM-1 transcript increases in direct relation to the levels of Ad.CFTR-encoded CFTR mRNA. In particular, ICAM-1 transcript levels increase above the basal levels (crosshatched area) in the lung portions in which vector-encoded CFTR mRNA is expressed more than 10 % the levels of Rhesus monkey endogenous CFTR mRNA (vector-encoded/endogenous ratio > 0.1). Therefore, the present results confirm the dependence of ICAM-1 mRNA induction as a function of the infection efficiency of the E1-E3-deleted adenovirus-derived vector Ad.CFTR.

III. Discussion

Northern blot, slot-blot, RNase protection assay, in situ hybridisation and run-on assay can be utilised for detection of RNA expression (Trapnell, 1993). Unfortunately, these techniques suffer different drawbacks

in effective quantitation of mRNA transcripts expressed at low level. Most of them require relatively large amounts of RNA, involve cumbersome steps or simply are indicated for qualitative but not quantitative detection. To overcome at least some of these limitations, we designed a further development of a single-tube competitive RT-PCR method (Pannetier et al, 1993), with specific application to wt and mutated CFTR gene transcripts, either endogenous or encoded by a gene-transfer vector in three different species. The main advantages of the method here described are based on the possibility of detecting and linearly quantitating few copies of cDNA derived from reverse transcription of more than one target, without the need of radioactive molecules. In fact, the assay is able to detect as few as 10 initial copies of cDNA and the linearity of the signal ranged over 2 orders of magnitude (data not shown), which is a wider range compared to that obtained by densitometric analysis of autoradiograms (Pannetier et al, 1993). On the other side, the calculation of the number of copies of transcript is conditioned by RNA degradation, efficiency of reverse transcription and specific set-up of PCR.

Some of our RNA samples were intrinsically prone to degradation because of high RNAase content of the mucosa of the airways. Moreover, RNA can be further degraded during the step of digestion of vector-encoded cDNA. These artefacts induce large sample to sample variability, reduce the sensitivity of the assay and may result in underestimating the absolute number of copies of transcript. Even though the risk was reduced by performing RT reaction immediately after RNA extraction, in some cases we have been obliged to normalise the data of the target mRNA with those of a reporter mRNA, which is extracted and processed in the same tube. Assuming that under the same experimental conditions both target and reporter transcripts are stable to the same extent, normalisation can cope with the presence of RNA degradation, with the only disadvantage of giving the result in terms of relative and not absolute number of copies. It should be recalled that we utilised HCK-15 as reporter gene (Trapnell et al, 1991), which is a marker expressed in respiratory epithelial cells (Hamosh et al, 1991) on levels similar to those of endogenous CFTR mRNA, rendering the co-amplification convenient for further appropriate and simultaneous computerised analysis.

An uncompleted efficiency of reverse transcription (RT) of mRNA into cDNA may also result in underestimation of the transcript copies. Efficiency of RT can be calculated by adding known amounts of RNA transcript, that is an external standard RNA, before reverse transcription. Similarly, for homologous competitors it is possible to utilise alternatively cRNA or cDNA sequences (Becker-Andrè and Hahlbrock, 1989; Santagati et al, 1993; Wang et al, 1989). However, if the use of RNA standards or cRNA competitors might overcome the variability of efficiency of cDNA synthesis, they do not correct at the same time for losses of RNA. In this respect, we preferred to assume arbitrarily the maximal efficiency of reverse transcription while controlling RNA degradation, both with normalisation and by choosing cDNA competitors, which are also easily synthesised and accurately measured without need of radiolabeled compounds (Santagati et al, 1993).

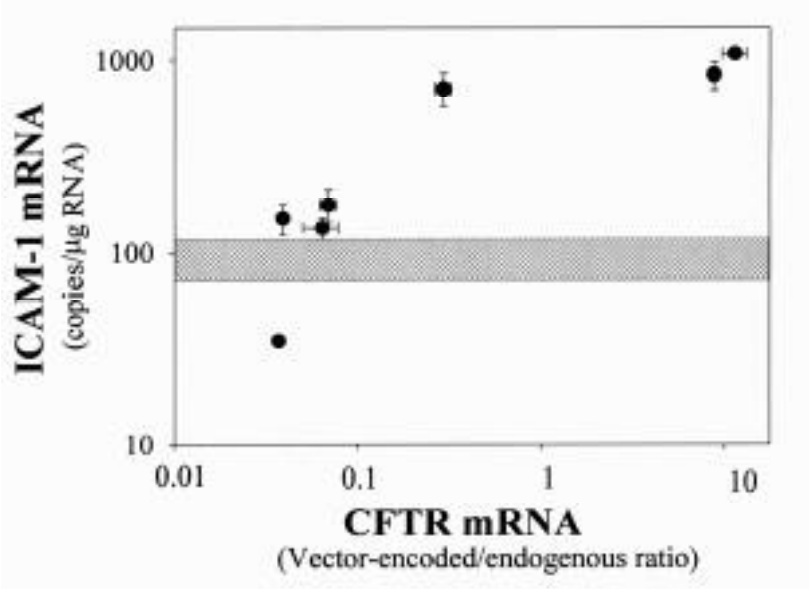

Figure 4. ICAM-1 induction as a function of vector-encoded CFTR mRNA in Rhesus monkeys.

Copies of ICAM-1 mRNA are plotted against the relative expression of vector-encoded over endogenous transcript from total RNA extracted from the lungs of Rhesus monkeys instilled with Ad.CFTR (1.5×10^{10} pfu) as previously reported (Bout et al, 1994; Nicolis et al, 1998). Data are mean ± SD of both transcript quantitation. Crosshatched area is the 99 % confidence interval of the mean of ICAM-1 expression in lungs of Rhesus monkeys exposed to saline solution instead of Ad.CFTR vectors, as previously reported (Nicolis et al, 1998).

In the present assay, the between-run variation in efficiency of PCR of the target (or reporter) is rendered practically irrelevant since quantitation is based on direct comparison with the respective homologous competitor templates processed in the same reaction tube. Generation of competitor sequences diverging only few bases from the targets is relatively easy either by consecutive digestion, filling and ligation, as described in Material and Methods, or by mutagenesis through PCR (Higuchi, 1989). In most quantitative PCR reaction, amplification must be maintained in exponential phase, which requires difficult and long steps for setting conditions. In case of homologous competitors, amplification is not required to run in the exponential phase. It was previously demonstrated that running to saturation, in the plateau phase, did not affect accuracy of quantitation (Becker-Andrè and Hahlbrock, 1989 ; Pannetier et al, 1993 ; Porcher et al, 1992).

Assuming arbitrarily that in our experimental conditions the stability of target and reporter mRNAs are similar and that the efficiency of RT is maximal, this assay seems a convenient method to quantitate steady-state levels of transcripts expressed in low copies. On the other side, we presented data in which the wild type vector-encoded transcript was competing with the endogenous CFTR transcript itself, as for the human ΔF508 allele, the mouse and monkey CFTR mRNA. In these cases, the limitation of different RNA degradation and efficiency of reverse transcription between target and competitor should be considered solved, since the endogenous transcript is a normalizer itself. Also, since the primers anneal inside the CFTR gene, the relative exogenous/endogenous assay can be utilised irrespectively from the type of vector utilised, either viral or synthetic, in pre-clinical and clinical studies.

IV. Materials and Methods

CF gene transfer *in vitro*

A549 cells (American Type Culture Collection, CCL 185, Rockville Pike, MD, USA) and primary cultures of nasal polyp cells were grown as previously described (Renier et al, 1995). The replication-defective adenoviral-derived vectors encoding for wt CFTR gene, Ad.CFTR, which is deleted in the E1 and E3 regions (Major Late promoter), and Ad.TG5643, which is deleted in the E1, E3 and E4 regions (CMV promoter), were prepared and titrated by plaque-forming units (iu) assay as described (Lusky et al, 1998). Infection dose was expressed as Multiplicity Of Infection (MOI), where 1 MOI corresponds to 1 pfu per cell. Primary cultures of nasal polyp cells from a CF subject homozygous for ΔF508 mutation were infected *in vitro* with Ad.CFTR (MOI 10, 72 h), as previously described (Renier et al, 1995).

CF gene transfer in mouse and Rhesus monkey *in vivo*

Immunodeficient mice C.B17-scid/scid (Iffa Credo, Lyon, France) were instilled intratracheally with $1.5x10^9$ pfu/animal of Ad.CFTR or Ad.TG5643 vectors diluted in a solution of 0.9 % NaCl in a volume of 25 µl. Animals were sacrificed 4, 14 or 30 days post-infection. Lungs portions were snap-frozen before total RNA extraction. Lung portions from Rhesus monkeys subjected to Ad.CFTR instillation by bronchoscope (1.5×10^{10} pfu) (Bout et al, 1994), in which ICAM-1 induction was previously observed (Nicolis et al, 1998), were analysed for relative vector-encoded versus simian endogenous CFTR mRNA expression.

Primers for reverse transcription and PCR

Oligonucleotides were synthesised using the 392 Applied Biosystems DNA/RNA synthesiser, following the Supplier's instructions. FAM (5-carboxyfluorescein) and TAMRA (N,N,N',N'-tetramethyl-6-carboxyrhodamine) (Applied Biosystems-Perkin Elmer) are fluorescent dye-N-hydroxyl succinimide esters (Dye-NHS ester) that have been conjugated to different oligonucleotides. Oligonucleotides utilised in competitive RT-PCR are reported in **Table 1.** Primers for endogenous CFTR transcript expression and for relative vector-encoded vs. endogenous expression for human ΔF508 alleles, murine and simian transcripts are designed inside CFTR gene sequence.

Homologous competitors for human CFTR mRNA and Cytokeratin 15

The competitor phCK15-C was obtained as follows. Total RNA from A549 cells was reverse-transcribed using the primer hCK15-6, as described by Others (Trapnell et al, 1991). A 589-bp PCR product was generated with primers hCK15-5 and hCK15-6 (Trapnell et al, 1991), then cloned into the pCRII vector using the TA cloning kit (Invitrogen) as specified by the Supplier. Eight bases were then inserted in the cloned hCK-15 sequence by digestion with *Nco I*, after which the protruding termini thus created were filled with DNA polymerase I Klenow fragment and ligated. The construct obtained was named phCK15-C. The competitor phCFTR-ΔC was prepared as follows: a fragment of ΔF508 CFTR cDNA sequence was reverse-transcribed from total RNA, extracted from epithelial respiratory cells of ΔF508 homozygous individuals, using the primer hCFTR11-R1. PCR reaction was then performed in the presence of upstream and downstream primers specific for human CFTR sequence (hCFTR7-D1 and hCFTR11-R1, respectively). The PCR product was further amplified with the internal upstream primer hCFTR7-D2 and hCFTR11-R1. A 706-bp fragment was generated and cloned in the pCRII vector as described above. The construct obtained was named pCFTR-ΔC and utilised as a homologous competitor for quantitation of human endogenous wt CFTR mRNA.

Table 2 Sequence of primer oligonucleotides

Primer name	Oligonucleotide sequence	Localization	Position
***Human* CFTR gene** (Riordan et al, 1989)			
hCFTR7-D1	5' –GAAGGCAGCCTATGTGAGATAC- 3'	exon 7	1023-1044
hCFTR7-D2	5' –TGTGCTTCCCTATGCACTAATC- 3'	exon 7	1095-2016
hCFTR9-D1	5' –GGACAGTTGTTGGCGGTTGC- 3'	exon 9	1483-1502
hCFTR10-D2- FAM	FAM- 5' –GCCTGGCACCATTAAAGAA- 3'	exon 10	1626-1644
hCFTR11-R1	5' –AGAAATTCTTGCTCGTTGACCTCC- 3'	exon 11	1803-1780
hCFTR10/11-R2	5 '-CTTGGAGATGTCCTCTTCTAGTTG- 3'	exon 10-11	1728-1705
***Murine* CFTR gene (*Mus musculus*)** (Tata et al, 1991)			
mCFTR17b-D1	5'-TCCATTTTAACAACAG-3'	exon 17b	3473-3488
mCFTR20-R1	5'-CCTGATCCAGTTCTTCC-3'	exon 20	3870-3855
mCFTR18-D2	5'-ATGAATATCATGAGTA-3'	exon 18	3530-3545
mCFTR19-R2	5'-GAAATGTTCTCTAATA-3'	exon 19	3816-3801
mCFTR18/19-D3-TAMRA	TAMRA-5'-TGATGCGATCTGTGAG-3'	exon 18-19	3588-3603
***Simian* CFTR gene (*Macaca mulatta*)** (Wine et al, 1998)			
mCFTR17b-D1	5'-TCCATTTTAACAACAG-3'	exon 17b	3424-3439
mCFTR20-R1	5'-CCTGATCCAGTTCTTCC-3'	exon 20	3821-3805
mCFTR18-D2	5'-ATGAATATCATGAGTA-3'	exon 18	3481-3496
mCFTR19-R2	5'-GAAATGTTCTCTAATA-3'	exon 19	3767-3752
mCFTR18/19-D3-TAMRA	TAMRA-5'-TGATGCGATCTGTGAG-3'	exon 18-19	3539-3554
Ad.CFTR vector gene (Senè et al, 1995)			
OTG3042	5' –GCAGTTGATGTGCTTGGCTAGAT- 3'	exon 22	4185-4207
OTG5349	5' –TTGTGAAATTTGTGATGCTATTGC- 3'	SV40 polyA	4696-4673
hCFTR24-D2	5' –CATAGAAGAGAACAAAGTGCGGC- 3'	exon 24	4377-4399
OTG4741 FAM	FAM 5' –GTAACCATTATAAGCTGCAATAAAC- 3'	SV40 polyA	4665-4641
Human cytokeratin 15 (HCK-15) gene (Trapnell et al, 1991)			
hCK15-5	5' –TGAAGGAGTTCAGCAGCCAGCTGG- 3'		804-827
hCK15-6	5' –ACTGACTCTTCTACATTGATGTGG- 3'		1392-1369
hCK15-D2-FAM	FAM- 5'-GCAGAGATGAGGGAGCAGTAC- 3'		888-909
hCK15-R2	5' –GCTGGTCTGGATCATTTCTGTTG- 3'		1016-995

The competitor pTG6525 utilised for Ad.CFTR-encoded transcript, which was obtained by adding 13 bases to the Ad.CFTR cDNA between the 3' end of hCFTR and the SV40 polyA sequence, was a generous gift of Prof. Jean-Luc Imler.

Reverse transcription

Total RNA was extracted by RNAzol^{TM} B (Biotecx, Houston, TX) following the Supplier's instructions. When CFTR gene was transferred with vectors, total RNA was treated for 1 h at 37°C with RNAase-free RQ1 DNAase (10 units/μg of total RNA) (Promega, Madison, WI), in the presence of 800 U/ml RNAase inhibitor (Perkin Elmer, Norwalk, CT), in 40 mM Tris-HCl pH 7.9, 10 mM NaCl , 6 mM $MgCl_2$ and 10 mM $CaCl_2$ (final volume 50 μl), after which the total RNA was ethanol-precipitated. 50 ng of total RNA were reverse transcribed by GeneAmp RNA PCR kit (Perkin Elmer) in 50 mM KCl, 10 mM Tris-HCl, 5 mM $MgCl_2$, 4 mM dNTP, 15 pmol appropriate downstream oligonucleotides (as reported in **Tables 2** and **3**), 20 units RNAase inhibitor and 50 U Moloney murine leukaemia virus RT, final volume 20 μl, for 90 min at 42°C.

Amplification conditions

The total volume of RT reaction was utilised for PCR-1 in 50 mM KCl, 10 mM Tris-HCl, 2 mM $MgCl_2$, 0.8 mM dNTP, 15 pmol appropriate upstream primer, 2.5 units AmpliTaq DNA Polymerase

(Perkin Elmer), and homologous competitor was added to quantitate and normalise the endogenous human and the Ad.CFTR-encoded transcripts (for primers and competitors refer for details to **Table 3**). Relative expression of the transcripts (from human ΔF508 allele, mouse and monkey) did not require addition of exogenous competitors. 3 μl of the PCR-1 were subjected to further amplification (PCR-2) in the same conditions as for PCR-1, except that each sample included 30 pmol of upstream and downstream primers, as detailed in **Table 3**. The amplification conditions for PCR-1 and PCR-2 were: 80°C for 5 min, then 30 cycles of denaturation (94°C for 30 sec), annealing (30 sec at the temperature indicated in **Table 3**), and extension (72°C for 30 sec). A run-off reaction (Pannetier et al, 1993) of 4 μl of the final amplification reaction was performed in 10 μl containing 0.1 μM fluorescently-labeled primer, 50 mM KCl, 10 mM Tris-HCl, 200 μM dNTP, 3 mM $MgCl_2$ and 20 U/ml AmpliTaq DNA Polymerase (Perkin Elmer). Run-off reaction started with a denaturation step at 95°C for 2 min, followed by 30 sec at the appropriate annealing temperature (see **Table 3**) and finally to 15 min at 72°C.

Electrophoresis and gel analysis

2 μl of run-off reaction products were mixed with 0.5 μl of molecular weight standard GS 500 ROX (Applied Biosystems-Perkin Elmer) and 2.5 μl of a 8.3 mM EDTA/formamide solution. After denaturation at 90°C for 2 min, samples were loaded on a 4-6 % polyacrylamide, 8.3 M urea gel, run for 5 h at 1500 volts / 40 watts in an Applied Biosystems 373A DNA Sequencer. Peak area and length of PCR fragments were determined using Genescan 672 Software (Applied Biosystems-Perkin Elmer). For endogenous hCFTR and Ad.CFTR-encoded transcripts, competitor over target areas were plotted on the y-axis versus the number of copies of competitor (x-axis), and fitted by least-square regression analysis to quadratic equations by Sigma Plot software (Jandel Scientific, Erkrath, Germany). The copies of target mRNA were taken to be those of the competitor corresponding to the equivalence of competitor-target areas (y-axis value = 1).

Table 3. RT-PCR assays, The size of the amplified fragments reported refers in the order to the exogenous and endogenous ones, respectively.

RT primer	PCR-1primers *annealing T*	PCR-2 primers *annealing T*	Run-off primer *annealing T*	Run-off fragment size	Competitor
Endogenous human CFTR mRNA normalized with human Cytokeratin 15 mRNA					
hCFTR11-R1	hCFTR9-D1 *55°C*	hCFTR9-D1 hCFTR10-11-R2 *55°C*	hCFTR10-D2-FAM ***55°C***	100–103 nt	phCFTR-ΔC
hCK15-6	hCK15-5 *55°C*	hCK15-5 hCK15-R2 *55°C*	hCK15-D2-FAM *55°C*	136-128 nt	phCK15-C
Ad.CFTR-encoded transcript					
OTG5349	OTG3042 *55°C*	hCFTR24-D2 OTG5349 *55°C*	OTG4741-FAM *55°C*	303-290 nt	pTG6525
Human vector-encoded versus Murine (*mus musculus*) and monkey (*macaca mulatta*) CFTR transcript					
mCFTR20-R1	mCFTR17b-D1 *47°C*	mCFTR18-D2 mCFTR19-R2 *44°C*	mCFTR18/19-D3-TAMRA *43°C*	229-232 nt	none

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