

Correlation Between DNA Transfer and Cystic Fibrosis Airway Epithelial Cell Correction After Recombinant Adeno-Associated Virus Serotype 2 Gene Therapy

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ABSTRACT

Recombinant adeno-associated virus serotype 2 (rAAV2)-based human gene therapy for cystic fibrosis has progressed through a series of preclinical studies and phase I and II clinical trials. This agent has shown an encouraging safety profile, consistent levels of DNA transfer, and positive evidence of short-term clinical improvement in lung function in a prospective, placebo-controlled phase II trial of aerosol administration. Nonetheless, it has been difficult to assess the relationship between its molecular action and the observed clinical improvements, because of the lack of positive results from a highly specific assay for vector mRNA. This issue is further complicated by the fact that the clinical vector utilizes a small cryptic rAAV2 promoter sequence that is less robust for mRNA expression than typical viral promoters. In this paper, we report the results of more sensitive assays performed on primary nasal cells harvested from rAAV2-CFTR gene therapy recipients. These studies demonstrate a correlation between the presence of rAAV2-CFTR vector genomes, CFTR mRNA expression, and cAMP-activated chloride channel function in these cells. The observation of sizeable physiological correction in the face of low mRNA levels may reflect the regulatory role of low levels of CFTR protein as an activator of other chloride channels.

OVERVIEW SUMMARY

Clinical trials of an AAV vector, tgAAVCF, containing the cDNA for the CFTR gene in cystic fibrosis patients have shown reproducible DNA transfer and growing evidence of functional correction as judged by a reduction of the pro-inflammatory cytokine, interleukin 8, in maxillary sinus lavage fluid and expectorated sputum and improvement in FEV₁. However, it has been difficult to demonstrate an intermediate biological effect, such as mRNA expression, that would be consistent with the apparent clinical responses. To bridge that gap, in one clinical trial we harvested epithelial cells from the site of vector delivery in the nasal epithelium and cultured them *in vitro* under conditions favorable for expansion of the epithelial cell population to permit a more extensive characterization of the molecular and functional consequences of tgAAVCF DNA delivery. These studies

show that the presence of tgAAVCF DNA in epithelial cells after *in vivo* delivery in patients can be correlated with detectable CFTR mRNA expression and restoration of cAMP-activated chloride channel activity in these cells.

INTRODUCTION

RECOMBINANT ADENO-ASSOCIATED VIRUS SEROTYPE 2 (rAAV2) vectors utilizing the AAV2 inverted terminal repeat (ITR) promoter elements to drive expression of full-length cystic fibrosis transmembrane conductance regulator (CFTR) were originally developed to address the issue of packaging the large CFTR-coding sequence (4.44 kb) into the constrained packaging limit of rAAV2 (Flotte *et al.*, 1993). Including the two ITRs, which are necessary for replication and packaging of vector genomes, the net capacity of the vector particle for de-

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livery of the coding sequence, promoter, and polyadenylation signal is 4.7 kb. This rAAV2-CFTR vector has been highly characterized with respect to their ability to express CFTR chloride channels and regulate other channels in airway epithelial cells. Immortalized cell lines expressing CFTR from this promoter element (C38 and S9 cells) have been used as positive controls for many assays of CFTR function (Egan *et al.*, 1992; Guay-Broder *et al.*, 1995; Imundo *et al.*, 1995; Rubenstein *et al.*, 1997; Rubenstein and Zeitlin, 2000; Braunstein *et al.*, 2004; Virella-Lowell *et al.*, 2004). Furthermore, the clinical version of this vector, tgAAVCF, has undergone extensive testing, including a number of phase I studies of delivery to the nose, the maxillary sinus, a segmental bronchus via bronchoscopy, or the entire respiratory tract via aerosol inhalation (Wagner *et al.*, 1998, 1999; Aitken *et al.*, 2001; Flotte *et al.*, 2003; Moss *et al.*, 2004). Several of these studies have shown consistent, dose-dependent CFTR cDNA transfer. The most recent phase II study of aerosol administration of tgAAVCF also showed an improvement in lung function, as measured by the forced expiratory volume at 1 sec (FEV₁), and a decrease in the key proinflammatory cytokine, interleukin 8 (IL-8), in induced sputum samples (Moss *et al.*, 2004).

Countering these positive results has been the lack of detectable CFTR mRNA as determined by RNA-specific polymerase chain reaction (RS-PCR) assays on RNA isolated from cells brushed from the bronchial surface. This RS-PCR assay is specific for vector mRNA, utilizing an oligonucleotide linker ligated to the cDNA reverse transcribed from vector CFTR message, so as to exclude the possibility of false signals from input vector DNA sequences that could theoretically self-prime from the ITR (Snyder, 2000; Gerard *et al.*, 2003). However, it is uncertain whether the sensitivity is sufficient to detect vector mRNA at low levels that may still be corrective, as the physiologic level of endogenous CFTR mRNA in normal individuals is only about one copy per cell (Trapnell *et al.*, 1991). Thus, the absence of signal does not necessarily indicate that mRNA is not expressed at corrective levels. In the current paper, we describe a number of studies that utilize a series of sensitive techniques to detect vector DNA sequences, CFTR mRNA, and CFTR function on cells recovered from subjects after vector delivery in one of the phase I studies of tgAAVCF.

MATERIALS AND METHODS

Phase I clinical trial

The details and clinical outcomes of this phase I clinical trial have been published elsewhere (Flotte *et al.*, 2003). The trial was performed in compliance with regulatory and advisory committee oversight from the following bodies: the National Institutes of Health (NIH, Bethesda, MD) Office of Biotechnology Activities/Recombinant DNA Advisory Committee; the Center for Biologics Evaluation Research of the U.S. Food and Drug Administration (Rockville, MD); Institutional Review Boards of Johns Hopkins Hospital (Baltimore, MD) and the University of Florida (Gainesville, FL); Institutional Biosafety Committees of Johns Hopkins University and the University of Florida; Human Use of Radiation committees of both universities; National Heart, Lung, and Blood Institute (Bethesda, MD) program officers; and General Clinical Research Center Advi-

sory Committees of both universities. All subjects gave written informed consent for all research procedures.

Doses of tgAAVCF administered to the nasal and bronchial epithelium of adult CF patients in this study ranged from 3×10^1 to 1×10^9 replication units (RU), approximately equivalent to 6×10^4 to 2×10^{12} DNase-resistant particles (or vector genomes). Twenty-five subjects were enrolled in the original trial, but the additional studies described here were attempted only on the first 18 subjects (identified as Part 1 in the original report) (Flotte *et al.*, 2003), and a sufficient sampling of brushed cells was not available for the additional assays in many patients. The specific doses for individual subjects from whom samples were analyzed here are indicated in Results. The administration of vector to the endobronchial lumen was done through a long polyethylene catheter threaded through the suction port of a fiberoptic bronchoscope. The administration of vector to the nose was accomplished with the subject sitting upright, with the head in a neutral position, using a handheld otoscope and nasal speculum, to deliver vector through a polyethylene catheter along the surface of the inferior turbinate, in a volume of approximately 0.2 ml.

Test of vector identity and bioactivity

Before vector instillation, the biological activity of the vector was confirmed by transducing cultures of the CFTR-deficient IB3-1 bronchial epithelial cell line under the following conditions: cells were plated and grown to approximately 1×10^4 cells per well in 96-well multiplates. Aliquots of vector corresponding to either 0, 0.01, or 0.1 RU per cell (0, 100, or 1000 RU total per well) were inoculated directly into the medium. Medium was changed 3 days after infection, and cells were assayed by a previously published ³⁶Cl⁻ efflux assay 9 days after vector transduction (Schwiebert *et al.*, 1998). This time interval was chosen on the basis of prior studies in IB3-1 cells, demonstrating this to be the peak time for expression from rAAV2 vectors (Afione *et al.*, 1999).

Southern blot analysis of nested PCR products from nasal and bronchial brushings

Nasal samples were harvested with sterile cytology brushes passed through a handheld otoscope with a nasal speculum, whereas bronchial brushes were obtained with bronchoscopy brushes (Olympus, Tokyo, Japan) passed through the suction channel of a 5.2-mm fiberoptic bronchoscope. Separate brushes were obtained for DNA-PCR and RT-PCR. DNA extraction was performed by a standard proteinase K predigestion method (Maniatis *et al.*, 1989), whereas RNA extraction was performed with TRIzol reagent (GIBCO-BRL Life Technologies, Grand Island, NY), according to the manufacturer's instructions.

Nested DNA-PCR was performed with an outer primer pair consisting of a sequence within the synthetic poly(A) signal (5'-CTGTGAGCCGAGTCTTTAAG-3') coupled with a sequence within exon 19 (5'-CACTCTGATCGATGCATCTG-3'), whereas the inner pair consisted of a primer further downstream within exon 19 (5'-CTTCTCAATAAGTCTTGCC-3') coupled with one within exon 24 (5'-CAATCTGGGTCTTAGACTTG-3'), with 40 cycles for each amplification. These conditions were optimized in preclinical studies in rabbit and nonhuman primate samples (Conrad *et al.*, 1996). Amplified products were separated by 0.7% agarose gel electrophoresis,

blotted onto nylon membranes, and probed with a ^{32}P -labeled internal CFTR-specific probe. For RT-PCR, samples were pretreated with RNase-free DNase (Roche, Indianapolis, IN), and first-strand cDNA was synthesized with reverse transcriptase and random hexamers before utilizing the above-described nested PCR conditions. This assay had a lower limit of detection of 0.018 pg of DNA per sample under these conditions.

Primary culture of nasal epithelial cells harvested after tgAAVCF instillation

Additional cells from the medial surface of the inferior turbinate, taken 10 days after vector instillation, were seeded in primary culture. To separate epithelial cells from fibroblast contaminants, the samples were treated with standard tissue culture-grade trypsin-EDTA solution for 1 hr at 37°C, and then plated on 35-mm-diameter wells (six-well dishes; Nalge Nunc International, Rochester, NY) that had been precoated with a collagen-fibronectin-bovine serum albumin (BSA) solution as previously described (Flotte *et al.*, 1993). Cells were grown in LHC-8E medium (BioFluids, Rockville, MD), in 5% CO₂ incubators at 37°C until confluent. The time to confluence varied from 3 to 10 days.

Vector rescue of tgAAVCF sequences

To rescue vector sequences flanked by AAV2-ITR sequences, primary cultures were seeded into new 35-mm-diameter wells, also collagen-fibronectin-BSA coated, and grown to near confluence (approximately 5×10^6 cells). Cells were then infected overnight in LHC-8E with Ad5 (multiplicity of infection [MOI] of 20 plaque-forming units [PFU]/cell) and AAV2 (10 infectious units [IU]/cell) before changing the medium the next day. Cells were grown for a total of 5 days after infection. On day 5 after infection, cells were harvested

by the Hirt extraction procedure. All samples were tested 5 days after infection. Hirt supernatant and pellet DNA were then separated by Southern blot hybridization and probed with CFTR-specific probes to detect tgAAVCF sequences as described (Afione *et al.*, 1996).

Whole cell current measurement of primary nasal epithelial cells

Whole cell recordings of viable epithelial cells, usually found as small islands of epithelial cells that were darker in appearance, had three-dimensional shape, and had a visible nucleus at $\times 400$ magnification, were performed as described previously in symmetrical 140 mM Tris-HCl solutions (Schwiebert *et al.*, 1994, 1995). Two modifications were made regarding these specialized recordings. Sucrose (60 mM) was added to the extracellular bath solutions to prevent swelling of the cell after the whole cell configuration was initiated. The cells derived from both sides of the nose were each pretreated with the permeable cyclic AMP analog 8-(4-chlorophenylthio)adenosine 3',5'-cyclic monophosphate (CPT-cAMP) so that only those cells with expressed CFTR would have stimulated any measurable currents.

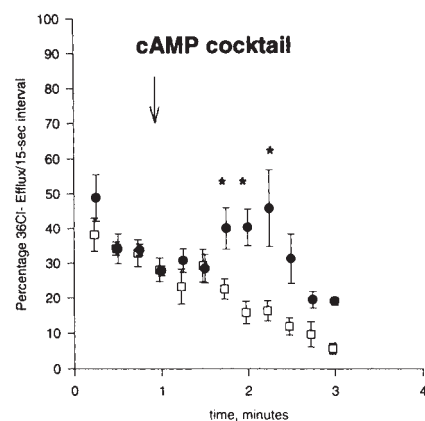
RESULTS

Confirmation of *in vitro* biological activity of rAAV-CFTR channel function

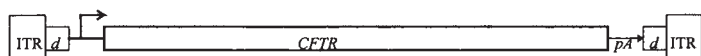
The clinical lot of AAV-CFTR vector carrying the AAV2-ITR-flanked CFTR-coding sequence packaged into rAAV2 capsid virions was tested for potency of CFTR correction, using a cAMP-activated $^{36}\text{Cl}^-$ efflux assay (Fig. 1A). The details of the sequence of the tgAAVCF vector used in these studies

FIG. 1. Confirmation of biological activity of tgAAVCF vector for CFTR correction. Before instillation into subjects in a phase I clinical trial, an aliquot of the tgAAVCF vector was tested for identity and biological activity as indicated by restoration of cAMP-activated $^{36}\text{Cl}^-$ efflux. (A) Percentage of isotope effluxed per 15-sec interval in the presence (solid circles) or absence (open squares) of the cAMP cocktail (forskolin, IBMX, and cAMP analog). (B) Schematic depiction of the vector genome. ITR, AAV2 inverted terminal repeat; *d*, AAV2-ITR *d* sequence, that is, AAV2 nucleotides 125–145; *CFTR*, cystic fibrosis transmembrane conductance regulator-coding sequence; *pA*, synthetic polyadenylation signal.

A



B



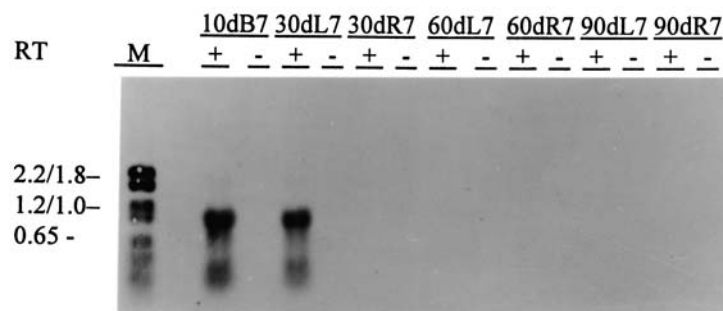
have been presented elsewhere (Flotte *et al.*, 2003), but the key features are identified in Fig. 1B. The promoter sequence driving CFTR expression is a cryptic promoter element near the *a/d* junction of the AAV2-ITR (Flotte *et al.*, 1993; Baudard *et al.*, 1996; Haberman *et al.*, 2000). The version of CFTR cDNA used contains the entire amino acid-coding sequence, with most of the 5'- and 3'-untranslated regions deleted. Immortalized CFTR-deficient CF bronchial epithelial cells (IB3-1 cells) when mock transduced show no increase in $^{36}\text{Cl}^-$ efflux after treatment with a cocktail of agents that increase intracellular cAMP (forskolin, 3-isobutyl-1-methylxanthine [IBMX], and cAMP analog), whereas tgAAVCF-transduced IB3-1 cells show a significant increase in $^{36}\text{Cl}^-$ efflux between 1.5 and 3 min after addition of the cAMP cocktail. This response is characteristic of CFTR function (Schwiebert *et al.*, 1998), and indicates that the vector genome sequences are, in fact, capable of expression and of functional CFTR correction under optimal tissue culture conditions.

Detection of tgAAVCF vector genomes and CFTR mRNA in brushed nasal and bronchial samples

Volunteer subjects participating in this trial had nasal and bronchial epithelial brushings performed at intervals out to 90 days after the initial administration of vector to localized areas of the nose and subsegmental bronchus (superior segment of the right lower lobe). These samples were subjected to vector DNA-PCR and RT-PCR assays, using a nonquantitative nested PCR assay in which the outer primer pair included one vector-specific primer and the inner primer pair was entirely within the CFTR cDNA. One of the subjects, subject 7, who received a nasal dose of 1×10^5 RU (approximately equivalent to $2 \times$

10^6 vector genomes) and a bronchial dose of 3×10^4 RU (approximately equivalent to 6×10^7 vector genomes), showed evidence of CFTR mRNA in the bronchial brush sample 10 days after vector delivery and in the left nasal sample 30 days after vector delivery (Fig. 2A, lanes 10dB7 and 30dL7). In each case, control mRNA samples assayed without reverse transcription (Fig. 2A, -RT lanes) were negative, indicating that these signals were not from contaminating vector DNA sequences. These DNA sequences were removed from all mRNA samples by treatment with RNase-free DNase. Sufficient vector mRNA was not available to perform this assay at all of the time points (data not shown), but separate brushings were taken from this same subject 7 for vector DNA-PCR (Fig. 2B). These results indicated that left nasal samples were positive for vector DNA 3, 10, and 30 days after vector delivery, thus bracketing the time frame in which the positive CFTR mRNA signal was observed in the left nasal sample. It is important to note that the validated clinical assay for vector mRNA established as a clinical end point for the primary study and reported in the original paper (Flotte *et al.*, 2003) was not done in the samples from these subjects, whereas these "research only" assays were done on subjects from the first nine cohorts, from whom sufficient additional brushing material was available. It is also important to note that vector DNA was administered to only one nostril of the patients and that the observed positive signals in the samples did not always correlate with the sidedness of vector administration. This phenomenon could be explained by cross-contamination of vector through the posterior nasopharynx, as has been observed with nonviral vectors (Konstan *et al.*, 2004). Nonetheless, the presence of vector DNA did correlate with the presence of detectable mRNA, as demonstrated here.

A



B

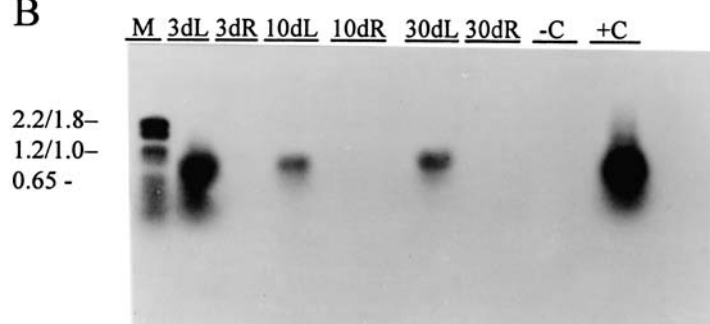


FIG. 2. Detection of vector CFTR mRNA and DNA in brush samples from the sites of vector delivery. Brushings were taken, at various times after vector delivery (3d, 3 days; 10d, 10 days; etc.), from the bronchial surface [lanes in (A) with B in the label designation], left nasal epithelium (lanes with L in the label designation), or right nasal epithelium (lanes with R in the label designation) from subject 7, and were analyzed by nested PCR-Southern blot assays for vector-expressed CFTR mRNA (A) or tgAAVCF vector DNA (B). Controls included parallel samples analyzed without reverse transcriptase [-RT lanes in (A)], vector plasmid as a positive control for the DNA PCR [+C lane in (B)], and water substituting for input DNA as a negative control [-C lane in (B)]. Lanes M, ^{32}P -labeled linear double-stranded DNA molecular weight markers.

Primary culture of nasal epithelial cells from the site of vector injection and subsequent rescue of vector DNA sequences

In an attempt to overcome some of the limitations of small sample volumes from the epithelial brushings, we chose to expand some of these cell populations taken from the site of vector delivery by growing them in primary cell culture. A vector rescue method was used as an extremely sensitive assay for detection of vector DNA sequence. In this technique, primary cells from the site of vector delivery were superinfected with wild-type Ad5 and AAV2, at MOIs of 20 PFU/cell and 10 IU/cell, respectively. Our group had used a similar technique to identify vector DNA sequences in bronchial epithelial samples taken from the site of vector delivery in rhesus macaques treated with this same vector through a fiberoptic bronchoscope (Afione *et al.*, 1996). In the cultures taken from CF patients in this phase I study, we first examined Hirt supernatant and pellet fractions by Southern blot hybridization and observed signals from the left nasal sample from subject 5, who like subject 7 received a nasal dose of 1×10^3 RU (approximately equivalent to 2×10^6 vector genomes) (Fig. 3A). Note that genomic DNA fractions were not consistently recovered from all samples. In further studies focusing on the Hirt supernatants, similar signals of res-

cued vector were observed from the left nasal sample taken from subject 7 (Fig. 3B). This was taken at the 10-day time point, thus correlating directly with the direct vector DNA signal seen in Fig. 2B, and the direct vector mRNA signal seen in Fig. 2A. As mentioned above, the sidedness of these findings did not correspond with the side of vector administration, that is, the right nostril was the site of dosing for patient 7.

Functional correction of CFTR function in primary cells harvested after vector delivery correlates with the presence of tgAAVCF sequences

In some cases, cell samples obtained from both the left and right nostrils were of sufficient size to be grown in primary culture, and the presence of vector DNA within these samples was correlated with cAMP-activated whole cell chloride currents. In the case of subject 9 (who received a nasal dose of 1×10^5 RU or 2×10^8 vector genomes of tgAAVCF), a unique opportunity was present because there were primary cultures both from the primary site of vector instillation on day 10 after vector delivery and from nasal polyp tissue taken from both sides on day 21. Nested PCR analysis for tgAAVCF DNA sequences showed a positive signal, only from the primary culture of the 10-day left nasal brush sample and not from the right nasal

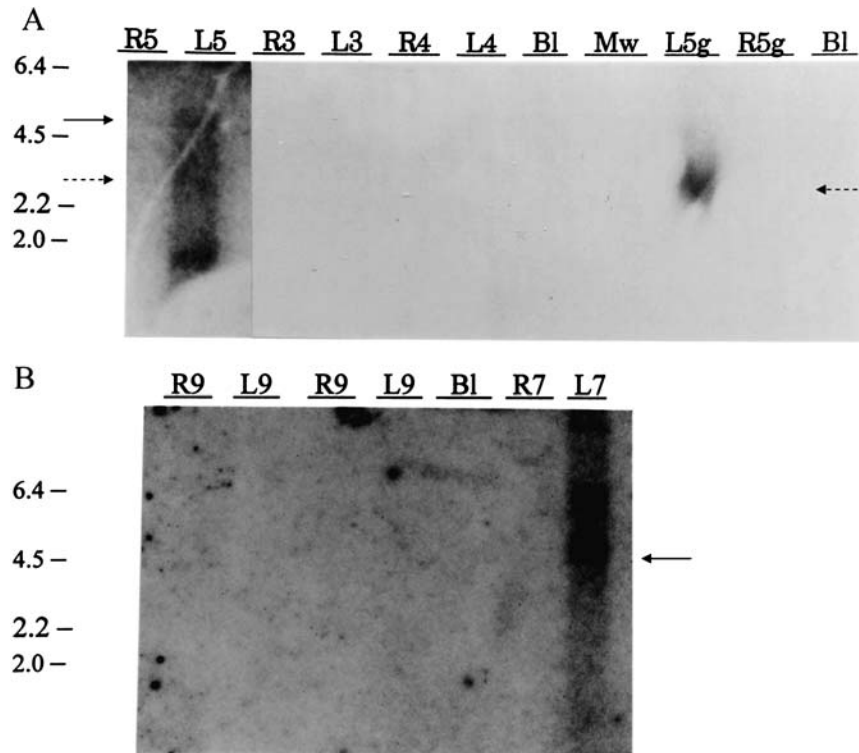


FIG. 3. Viral rescue of tgAAVCF vector DNA sequences. Epithelial cells grown in primary culture after harvesting from nasal epithelium of the indicated side (L, left; R, right; and subject number 3, 4, 5, 7, or 9) 10 days after vector delivery were superinfected with wild-type Ad5 and AAV2 at multiplicities of infection of 20 PFU/cell and 10 IU/cell, respectively. Five days later, cells were harvested for Hirt extraction and fractionation into low molecular weight and genomic fractions [lanes designated *Lw* and *g*, respectively, in (A)]. Unless otherwise indicated, the low molecular weight Hirt supernatants were analyzed by Southern blot with a CFTR-specific probe. Bl, blank lanes. Solid arrows indicate bands of the predicted size (4.8 kb) for double-stranded monomeric linear replicating forms of the vector genome; dashed arrows indicate bands of the predicted size (2.4 kb) for single-stranded linear monomers. Larger bands may represent dimeric or larger multimeric episomal forms.

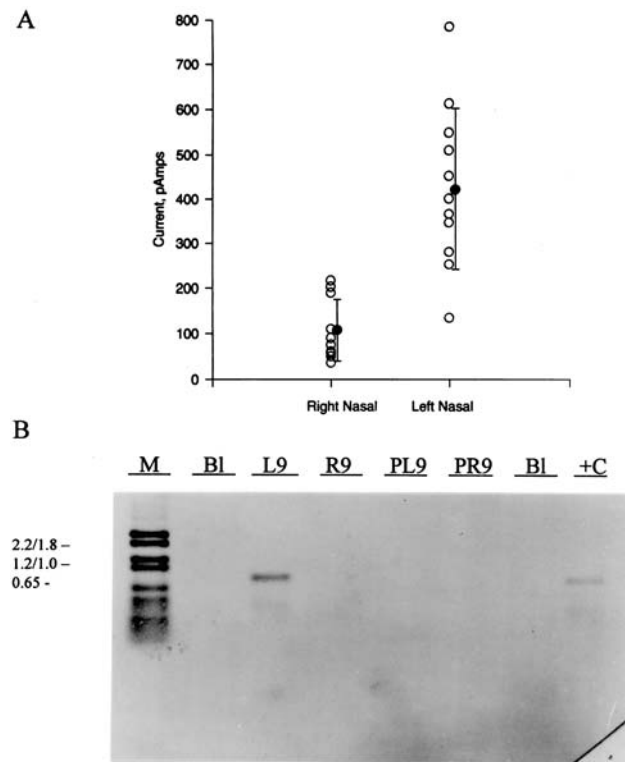


FIG. 4. Correlation of functional CFTR correction as indicated by whole cell current analysis and presence of vector DNA sequences. Primary cell cultures from the nose of subject 9, including 10-day brushings from the inferior turbinate surface on each side (L9 and R9) and 21-day polyp samples from each side (PL9 and PR9), were analyzed. **(A)** Comparison between the maximal whole cell current measured from individual cells after activation with a cAMP cocktail. Individual cells from the 10-day right inferior turbinate sample are indicated as Right Nasal, whereas corresponding currents from cells taken from the left side are indicated as Left Nasal. The solid circles and error bars indicate mean values and standard deviations, respectively. **(B)** Southern blot hybridization of nested PCR products from each of the four cultures are shown.

brush sample, nor from either culture of nasal polyp tissue (Fig. 4B). The presence of vector DNA in this left nasal primary cell population corresponded with a significant increase in cAMP-activated chloride currents (Fig. 4A) as compared with the levels observed from the PCR-negative culture taken from the contralateral side. No such increase was noted in the primary culture of cells from the polyp tissue (data not shown), which was taken at a later time point and from an anatomical location superior and posterior to the site of vector administration.

Unfortunately, sufficient numbers of primary cells could be sustained in culture to complete functional chloride channel assays on only 5 of 19 patients in the first part of this study. The results of the CFTR-PCR and nasal chloride studies on these five patients are summarized in Table 1. These data indicate that the three patients with at least one positive PCR were at

the higher dose (2×10^6 DNase-resistant vector genome particles), and that two of these three had significant positive results on the chloride current assay (Table 1). Patient 7 would have been an ideal candidate for functional assays, but the primary cells split off for this assay did not survive.

DISCUSSION

Studies of clinical trials with tgAAVCF have shown reproducible DNA transfer and growing evidence of functional correction as judged by a reduction of the proinflammatory cytokine, IL-8, in maxillary sinus lavage fluid and expectorated sputum and improvement in FEV₁. However, these studies lacked any demonstration of intermediate biological effects that

TABLE 1. PCR AND NASAL PRIMARY CELL CHLORIDE CURRENT RESULTS FOR PATIENTS FOR WHOM BOTH WERE AVAILABLE^a

Patient number	Nasal dose (DNase-resistant vector genomes)	PCR result	Nasal primary cell chloride current result
3	2×10^5	Negative	Negative
4	2×10^5	Negative	Negative
8	2×10^6	Positive	Negative
9	2×10^6	Positive	Positive
12	2×10^6	Positive	Positive ($p = 0.000054$) ($p = 0.011$)

^aPCR results are reported as positive if there were any positive results from the first 30 days after vector delivery, and as negative if there were none.

might support the plausibility of the apparent clinical events. The current set of observations attempts to bridge that gap by means of an approach based on harvesting epithelial cells from the site of vector delivery and culturing them under conditions favorable for expansion of the epithelial cell population (and the exclusion of nonadherent inflammatory cells) so that more extensive characterization of the molecular and functional consequences of tgAAVCF DNA delivery might be performed. These studies clearly show that the presence of tgAAVCF DNA in epithelial cells after *in vivo* delivery in patients can be correlated with detectable CFTR mRNA expression and restoration of cAMP-activated chloride channel activity in these cells.

There are a number of key controls present in these samples. Importantly, all RT-PCR samples had undergone prior DNase treatment and data were used only if control samples amplified without RT remained negative. The nature of the vector rescue assay itself indicates that only ITR-flanked CFTR sequences will be detected. This assay has been found to be sensitive in previous nonhuman primate studies of vector administration (Afione *et al.*, 1996). Although the sidedness of intended vector administration in the nose did not always correlate with the site at which positive samples were found, this phenomenon of cross-contamination has been seen with other nasal gene therapy vector studies (Konstan *et al.*, 2004). There was consistent correlation between the presence of vector DNA and the observation of downstream consequences, such as positive mRNA signals and correction of cAMP-activated chloride currents.

There are some caveats regarding the primary cell culture technique. First, the cells undergo several rounds of additional replication and some other phenotypic changes after harvesting could affect the fate and activity of vector DNA. It has been reported that vector DNA administered at the apical surface of airway epithelial cells may be trafficked to an extranuclear compartment, but enter the nucleus later if appropriate stimuli are provided (Duan *et al.*, 2000; Yan *et al.*, 2004). Thus harvesting of cells followed by primary culture could affect the cells in such a way that vector DNA trafficking to the nucleus and downstream biological effects of the vector might be artifactually enhanced. It is also possible that other events downstream from vector attachment could have been enhanced, such as leading-strand DNA synthesis or transcriptional activation. On the other hand, expansion of cells in primary culture could also have generated a false negative result by decreasing the biological effect of episomal vector DNA sequences, which are lost when cells become mitotically active in other systems (Flotte *et al.*, 1994; Afione *et al.*, 1996, 1999; Kearns *et al.*, 1996; Nakai *et al.*, 2001; Song *et al.*, 2004).

Despite the limitations, these observations provide some evidence that functional correction of the CF defect can occur in airway cells that are exposed to rAAV2 vectors *in vivo* in a living patient. The functional data were available only from a limited subset of patients in this study. Thus, it is not possible to draw firm conclusions about the dose-response relationship. However, significant functional correction of chloride transport was seen both in these studies and in the other clinical trials, despite the fact that CFTR mRNA could be detected only by the most sensitive of techniques. This does not necessarily imply regulation of sodium transport, which was not studied here. Unless the several different independent positive results occurred by pure chance and artifact, these results suggest that the vector DNA is capable of providing a sizeable correction

of the CF defect with low levels of mRNA expression. If this latter explanation is entertained, it implies that the primary role of CFTR is as a regulator of other chloride channels rather than as a mass-action chloride channel. This conclusion is certainly in agreement with a number of observations of CFTR function in normal cells, including the low endogenous CFTR mRNA levels in the bronchial epithelium and the numerous documented interactions of CFTR with other channels in the epithelium (Trapnell *et al.*, 1991; Schwiebert *et al.*, 1994, 1995, 1998). If this conclusion is correct, then CFTR mRNA levels may not be the ideal pharmacokinetic variable on which to evaluate candidate vectors for CF gene therapy.

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