Functional Cystic Fibrosis Transmembrane Conductance Regulator Expression in Cystic Fibrosis Airway Epithelial Cells by AAV6.2-Mediated Segmental *Trans*-Splicing

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Abstract

Cystic fibrosis is characterized by deficiency of the cystic fibrosis transmembrane conductance regulator (CFTR), a Cl[−] transporter. The packaging constraints of adeno-associated viral (AAV) vectors preclude delivery of both an active promoter and CFTR cDNA to target cells. We hypothesized that segmental *trans*-splicing, in which two AAV vectors deliver the 5' and 3' halves of the CFTR cDNA, could mediate splicing of two pre-mRNAs into a full-length, functional CFTR mRNA. Using a segmental *trans*-splicing 5' donor–3' acceptor pair that split the CFTR cDNA between exons 14a and 14b, cotransfection of donor and acceptor plasmids into CFTR[−] cells resulted in full-length CFTR message and protein. Microinjection of plasmids into CFTR[−] cells produced cAMP-activated Cl[−] conductance. Vectors created with an engineered human serotype, AAV6.2, were used to deliver CFTR donor and acceptor constructs, resulting in full-length CFTR mRNA and protein as well as cAMP-activated Cl[−] conductance in CFTR[−] cells, including human CF airway epithelial IB3-1 cells. Thus, segmental *trans*-splicing can be used with AAV vectors to mediate expression of CFTR, a strategy potentially applicable to individuals with CF.

Introduction

CYSTIC FIBROSIS, a common autosomal recessive disorder in the white population, is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene (Riordan, 1989; Rommens *et al.*, 1989; Welsh and Smith, 1993; Riordan *et al.*, 2008). In the lung, these CFTR mutations result in the absence of or reduction in airway epithelial apical chloride channel activity, leading to salt and water imbalances across the epithelium, with resulting accumulation of mucus and colonization by pathogens (Riordan, 2008). Gene transfer studies have attempted to restore CFTR function to human airway epithelium via transfer of the normal CFTR cDNA (Zabner *et al.*, 1993; Crystal *et al.*, 1994; Sorscher *et al.*, 1994; Wilson *et al.*, 1994; Caplen *et al.*, 1995; Knowles *et al.*, 1995; Flotte *et al.*, 1996, 2007; Harvey *et al.*, 1999; Wagner *et al.*, 1999; Moss *et al.*, 2004, 2007; Griesenbach *et al.*, 2006). Although the data from *in vitro* models are compelling, an efficient method for persistent, efficient expression of CFTR in the airway epithelium remains elusive owing to multiple barriers to gene transfer to the airway *in vivo*, including innate and acquired immunity to vectors, physical barriers, inefficient interaction

of vectors with airway cells, limits on the genetic payload of some vectors, and limits on the persistence of others (Griesenbach *et al.*, 2006; Flotte *et al.*, 2007).

Adeno-associated viral (AAV) gene transfer vectors are capable of mediating persistent gene expression in the airway epithelium in immunocompetent animals, and thus have been the focus of interest for gene therapy for the respiratory manifestations of CF (Flotte *et al.*, 1993b; Halbert *et al.*, 1997, 2001; Zabner *et al.*, 2000; Sirninger *et al.*, 2004; Limberis and Wilson, 2006; Fischer *et al.*, 2007). However, AAV vectors are small, unable to accommodate an expression cassette containing the full 4.5-kb CFTR cDNA with an active promoter and relevant 3' noncoding sequences. Although a number of strategies for overcoming this packaging constraint have been proposed, the challenge of using AAV vectors to deliver a large cDNA remains (Flotte *et al.*, 1993a,b; Zhang *et al.*, 1998; Duan *et al.*, 2000a, 2001; Yan *et al.*, 2000; Liu *et al.*, 2002, 2005; Sirninger *et al.*, 2004; Ostedgaard *et al.*, 2005). Toward this end, the present study uses a strategy known as pre-mRNA “segmental *trans*-splicing” (STS), which relies on two pre-mRNAs sharing an intronic hybridization domain that can lead the spliceosome apparatus to mediate a splicing event that will

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combine the 5' upstream ("donor") sequence of one mRNA with the 3' downstream ("acceptor") sequence of the second mRNA (Pergolizzi *et al.*, 2003; Pergolizzi and Crystal, 2004; Liu *et al.*, 2005; Nakayama *et al.*, 2005). To create an intact, full-length CFTR cDNA, we used two nonfunctional, vector-encoded partial human CFTR cDNAs, one of which encodes the 5' untranslated and upstream coding sequence through exon 14a and one of which encodes the downstream coding sequence starting with exon 14b and ending with 3' untranslated region signals for RNA processing. After demonstration that plasmids encoding the 5' and 3' CFTR cDNA fragments could together direct a functional CFTR mRNA, fragments were used to create separate AAV 5' donor and 3' acceptor vectors. Using AAV serotype 6.2, an AAV6 derivative with a single amino acid change in the capsid (F129L) that results in highly efficient transduction of respiratory epithelium (Rutledge *et al.*, 1998; Limberis *et al.*, 2009), these vectors were used to successfully alter cells lacking CFTR (CFTR⁻ cells) to become functional CFTR-expressing cells (CFTR⁺ cells), including epithelial cells derived from an individual with CF, supporting the concept that segmental *trans*-splicing may be a strategy that can be used to treat the pulmonary manifestations of CF.

Materials and Methods

Cell culture

Fischer rat thyroid (FRT) cells stably expressing a Cl⁻-sensitive yellow fluorescent protein (YFP-H148Q) and FRT cells stably transfected with CFTR cDNA were kindly provided by A.S. Verkman (University of California, San Francisco, San Francisco, CA) (Galiotta *et al.*, 2001a,b). The cells were cultured in Coon's modified F-12 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 5% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT), penicillin (100 U/ml), and streptomycin (100 µg/ml; Invitrogen, Carlsbad, CA). HEK-293 cells and 293orf6 cells (Graham *et al.*, 1977; Brough *et al.*, 1996) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% (v/v) FBS, sodium pyruvate (1%), penicillin (50 U/ml), and streptomycin (50 µg/ml). IB3-1 cells were provided by P. Zeitlin (Johns Hopkins University, Baltimore, MD) and A. Prince (Columbia University College of Physicians and Surgeons, New York, NY) (Zeitlin *et al.*, 1991). IB3-1 cells were maintained in LHC-8 medium (Invitrogen) supplemented with 10% (v/v) FBS, penicillin (50 U/ml), and streptomycin (50 µg/ml). All cells were kept in a humidified incubator at 37°C and 5% CO₂.

Plasmid constructs

The CFTR donor and acceptor plasmids were based on the segmental *trans*-splicing plasmid derived from pcDNA3.1(+) (Invitrogen) (Pergolizzi *et al.*, 2003) (Fig. 1). Briefly, polymerase chain reaction (PCR)-amplified CFTR coding sequences were cloned into the pcDNA plasmid. The engineered segmental *trans*-splicing junction of CFTR was located between exon 14a and exon 14b. The donor CFTR segmental *trans*-splicing construct (pCFTR Donor) included the cytomegalovirus immediate-early promoter/enhancer, CFTR exons 1–14a, the 5' end of intron 6 cloned from the human vascular endothelial growth factor gene, a unique 160-bp hybridization domain that is not found in the human genome, and a polyadenylation signal (Pergolizzi *et al.*, 2003). The acceptor CFTR

segmental *trans*-splicing construct (pCFTR Acceptor) included the cytomegalovirus immediate-early promoter/enhancer, the complementary sequence of the unique 160-bp hybridization domain in the donor construct, an optimized branch point, a polypyrimidine tract, a splice acceptor, CFTR exons 14b–24, and a polyadenylation signal (Pergolizzi *et al.*, 2003). A positive control plasmid was synthesized to contain the full-length CFTR cDNA (pCFTR). All the plasmids were prepared with kits (Qiagen, Valencia, CA) according to the manufacturer's instructions.

Evaluation of segmental *trans*-splicing-generated CFTR mRNA in transfected cells

The constructs (8 µg/plate) were transfected into HEK-293 cells (2.5 × 10⁶ cells) plated in 60-mm plates, using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Forty-eight hours after transfection, the cells were lysed. Total RNA was isolated with RNeasy mini kits (Qiagen) and reverse transcribed (SuperScript III reverse transcriptase; Invitrogen) using an oligo(dT) primer (Invitrogen). To demonstrate that the exon 14a–exon 14b junction was made by the segmental *trans*-splicing process, PCR was performed with SuperMix High Fidelity (Invitrogen) and the following primers: forward probe (TCA CCG AAA GAC AAC AGC ATC C); reverse probe (TGG TAA GAG GCA GAA GGT CAT CC). PCR was carried out in a 35-cycle reaction, using a 5-min initial 95°C denaturing step. Each repeating cycle consisted of three steps: 30 sec at 94°C, 30 sec at 60°C, and 45 sec at 72°C. PCR products were analyzed on a 1.5% agarose Tris–acetate gel stained with ethidium bromide.

Evaluation of segmental *trans*-splicing-generated CFTR protein in transfected cells

HEK-293 cells (6.5 × 10⁶ cells) plated in 100-mm plates were transfected with pcDNA3.1, pCFTR Donor, pCFTR Acceptor, pCFTR Donor plus pCFTR Acceptor, or pCFTR (24 µg/dish). Forty-eight hours later, the transfected cells were lysed with radioimmunoprecipitation (RIPA) buffer (Sigma-Aldrich) according to the manufacturer's instructions. The protein lysate was immunoprecipitated with 1.6 µg of anti-CFTR monoclonal antibody (clone MAB1660; R&D Systems, Minneapolis, MN), followed by immunoblotting with anti-CFTR antibody (kindly provided by J. Riordan, University of North Carolina School of Medicine, Chapel Hill, NC) (Mall *et al.*, 2004). The blots were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were visualized with an enhanced chemiluminescence (ECL) detection kit (Pierce Biotechnology, Rockford, IL).

Assessment of CFTR protein function after segmental *trans*-splicing in microinjected cells

FRT-YFP cells were harvested and seeded onto a 35-mm plate containing a sterile, 15-mm diameter, round glass coverslip. The plasmids were suspended at 300 µg/ml in injection buffer (140 mM KCl, 10 mM HEPES; pH 7.4). The injection solution contained Alexa Fluor 647 (0.04 mM; Invitrogen) to permit identification of the microinjected cells. Microinjection (~2 pl, corresponding to 0.6 pg of DNA) was performed on an Olympus IX71 inverted microscope (Olympus America,

FIG. 1. Schematic illustration of the strategy for segmental *trans*-splicing of the human cystic fibrosis transmembrane conductance regulator (CFTR) gene. In the segmental *trans*-splicing constructs, the 5' fragment is termed the donor and the 3' fragment is the acceptor. The CFTR donor construct contains (5' to 3') the cytomegalovirus (CMV) immediate-early promoter/enhancer; the 5' portion of the CFTR cDNA including exons 1 through 14a; the 5' portion of vascular endothelial cell growth factor (VEGF) intron 6; and a unique hybridization domain. The CFTR acceptor construct contains (5' to 3') the CMV promoter/enhancer; a unique hybridization domain complementary to the hybridization domain of the donor construct; an optimized branch point; a polypyrimidine tract; a splice acceptor; the 3' portion of the CFTR cDNA, including exons 14b through 24; and a polyadenylation signal. The asterisk (*) marks the location of the *trans*-splicing junction. Below each construct is a schematic showing the predicted pre-mRNA structure, with the splice donor, branch point, and splice acceptor sites, as well as the complementary hybridization domains. The final spliced version of the mRNA is indicated at the bottom. The locations of primer pairs, both forward (PF) and reverse (PR), used for reverse transcription-PCR (RT-PCR) across the splice junction are indicated.

Center Valley, PA) equipped with an injector (Pico Injector model PLI100; Harvard Apparatus, Holliston, MA), micro-manipulator (Narashige, East Meadow, NY), and glass pipettes pulled with a Flaming/Brown micropipette puller (model P-87; Sutter Instrument, Novato, CA). Twenty-four hours after injection, CFTR activity of injected cells was measured by evaluation of YFP fluorescence intensity (Jayaraman *et al.*, 2000; Galletta *et al.*, 2001a,b). Cells plated on coverslips were mounted in a low-volume, heated chamber (model RC-20 with a TC-324B thermostatic controller to regulate stage temperature; Warner Instruments, Hamden, CT), using a multireservoir flow system (model VC-6; Warner Instruments), with an in-line glass heating coil to prewarm the buffers before entering the flow chamber (model 158830; Radnoti Glass Technology, Monrovia, CA) connected to a thermostatically controlled water-circulating pump (model 1104; VWR, West Chester, PA). All buffers also included a final concentration of 0.5% dimethyl sulfoxide (DMSO) to

eliminate the potential for changes in membrane permeability as conditions were changed. The cells were initially perfused with Cl⁻-phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 0.7 mM CaCl₂, 1.1 mM MgCl₂, 1.5 mM KH₂PO₄, 8.1 mM Na₂HPO₄; pH 7.4) at 1.8 ml/min at 37°C. In the first cycle of buffer exchanges, no cAMP activators were included. After equilibration in Cl⁻-PBS, cells were switched to I⁻-PBS in which NaCl was replaced with NaI (137 mM). After equilibration in I⁻-PBS, cells were returned to Cl⁻-PBS and reequilibrated. In a second cycle of buffer exchanges, cells were equilibrated in I⁻-PBS and then switched to I⁻-PBS in the presence of cAMP activators (5 μM forskolin, 50 μM isobutylmethylxanthine). Cell fluorescence was measured every 5 sec on an inverted Olympus IX70 microscope equipped with a cooled charge-coupled device (CCD) camera (Quantix camera operating at 1 MHz; Photometrix, Tucson, AZ) and the fluorescence intensity of individual cells was assessed with MetaMorph image analysis software (Molecular

Devices/Universal Imaging, Downingtown, PA). To assess the relative amount of CFTR activity, the rate of iodide influx (decreasing YFPH148Q fluorescence intensity) was assessed in the presence of cAMP activation and the maximal point-to-point slope was calculated. Background measured in the absence of cells was subtracted.

Cells were defined as being CFTR⁺ if they met three criteria, including (1) slope of YFP fluorescence that exceeded the mean slope of negative control cells (FRT-YFP) by >2 standard deviations (i.e., a change in slope $>0.03 \times 10^{-3}/\text{sec}$), (2) a total decrease in fluorescence intensity of >25% compared with the initial fluorescence intensity before I⁻, and (3) a recovery of fluorescence intensity >15% compared with the initial fluorescence intensity.

Generation of adeno-associated viral vectors expressing CFTR Donor and Acceptor vectors

The AAV6.2 serotype was chosen for expression of the CFTR segmental *trans*-splicing donor and acceptor constructs on the basis of data showing that this capsid conferred high-efficiency gene transfer to airway epithelial cells in rodents, humans, and nonhuman primates (Limberis *et al.*, 2009). The CFTR segmental *trans*-splicing donor or acceptor constructs were cloned into an AAV packaging plasmid containing a cytomegalovirus (CMV) promoter and a simian virus 40 (SV40) polyadenylation site. AAV6.2 vectors packaging CFTR donor or acceptor constructs were produced by a three-plasmid transfection strategy, with the following three plasmids: (1) pAAVCFTR Donor or Acceptor genome plasmid; (2) pAAV6.2 plasmid, which provides Rep proteins and Cap proteins; and (3) pAdΔF6, an adenoviral helper plasmid that provides adenoviral helper functions of E2, E4, and V_A RNAs. To initiate a round of vector production, pAAVCFTR genome plasmid (donor or acceptor, 600 μg each), pAAV6.2 (800 μg), and pAdΔF6 (1.2 mg) were cotransfected into HEK-293 cells (which contain an integrated copy of the Ad E1 region) with PolyFect (Qiagen) according to the manufacturer's instructions. At 72 hr posttransfection, cells were harvested, and AAV particles were purified as previously described (De *et al.*, 2008). Briefly, a crude viral lysate was prepared by three cycles of freeze-thaw, and clarified by centrifugation. AAV6.2CFTR Donor and AAV6.2CFTR Acceptor were purified by iodixanol gradient and HiTrap Q HP Sepharose anion-exchange chromatography (Amersham/GE Healthcare Life Sciences, Piscataway, NJ) as previously described (De *et al.*, 2008). The purified AAV6.2 vectors were concentrated with a Biomax 100 membrane concentrator (Millipore, Billerica, MA) and stored in PBS (pH 7.4) at -80°C until use. Vector genome titers were determined by TaqMan real-time PCR, using a CMV enhancer-specific primer-probe set (Applied Biosystems, Foster City, CA) (De *et al.*, 2008).

Evaluation of segmental trans-splicing-generated CFTR mRNA and protein in AAV6.2-infected cells

293orf6 cells seeded in 60- or 100-mm dishes were infected with AAV6.2CFTR Donor, AAV6.2CFTR Acceptor, or a combination of AAV6.2CFTR Donor and AAV6.2CFTR Acceptor for 90 min at a dose of 2×10^4 genome copies/cell for each vector. Virus infection was performed with serum-free DMEM. After infection, cells were cultured in DMEM containing 100 μM ZnCl₂ and 10% FBS. After 72 hr, infected cells

were lysed and total RNA and protein were isolated to evaluate the expression of CFTR mRNA and protein, using the assays described previously. As a positive control, HEK-293 cells were transfected with a plasmid expressing full-length CFTR cDNA (pCFTR), described previously, according to the manufacturer's instructions. RNA levels were assessed by TaqMan real-time PCR with relative quantitation, using rRNA as reference and reagents from Applied Biosystems. The primer and probes were also from Applied Biosystems, and consisted of a CFTR Donor-specific set (Hs00357004_m1), spanning the junction of exons 9 and 10; a CFTR Acceptor specific set (Hs00357011_m1), spanning the junction of exons 21 and 22; and a segmental *trans*-splicing specific set (Hs01565534_m1), spanning the junction of exons 14a and 14b. Linearity of response was determined by ensuring that the relative amounts were unaffected by dilution of RNA by a factor of 10.

Assessment of segmental trans-splicing-generated CFTR protein function in AAV-infected FRT-YFP cells

FRT-YFP cells (1.5×10^5) seeded on 15-mm round, glass coverslips were infected with AAV6.2CFTR Donor, AAV6.2CFTR Acceptor, or a combination of AAV6.2CFTR Donor and AAV6.2CFTR Acceptor in a volume of 30 μl of OptiMEM medium (Invitrogen) at a concentration of 5×10^4 genome copies/cell. Virus infection was performed for 90 min followed by addition of 2 ml of fresh Coon's modified F-12 medium containing 5% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). After 72 hr, the CFTR functional assay was performed as described previously. FRT-YFP cells stably transfected with CFTR served as the positive control (Galiotta *et al.*, 2001a).

Generation of AdpK7.YFP-H148Q vector

To create an adenoviral gene transfer vector to modify cells to express YFP, the pH-sensitive YFP-H148Q gene was amplified from FRT-YFP cells. It was sequenced and subcloned into pShuttleCMV. This plasmid was recombined with Ad-LacZ pK7 adenovirus (Wickham *et al.*, 1996), and the resulting plasmid was transformed in HEK-293 cells, expanded, and purified as described previously; the addition of the pK7 motif (which includes a stretch of seven consecutive lysine residues in the knob of the fiber protein) is intended to enhance entry into cells not expressing high levels of the adenovirus receptor (Wickham *et al.*, 1996, 1997; Hidaka *et al.*, 1999). To confirm the presence of both YFP and the pK7 fiber construct, the infectivity of the virus was compared with that of Ad5YFP (prepared as described previously, using a conventional E1⁻, E3⁻ adenovirus serotype 5 backbone) for infection of human dermal fibroblasts, known to be deficient in the high-affinity coxsackievirus-adenovirus receptor (Hidaka *et al.*, 1999). AdpK7YFP-H148Q infectivity was >2 logs higher than that of Ad5YFP-H148Q (the same vector without the seven lysine residues) in these cells (data not shown).

Evaluation of segmental trans-splicing-generated functional CFTR expression in IB3 cells

IB3-1 cells grown on 15-mm round, glass coverslips were infected with 30 μl of rAAV-containing DMEM. IB3 cells were treated with 40 μM *N*-acetyl-L-leucyl-L-leucyl-L-norleucine

(LLnL; Boston Biochemical, Boston, MA), a proteasome inhibitor that enhances AAV-mediated gene expression (Duan *et al.*, 2000b). In separate experiments, the ability of LLnL to enhance transgene expression by a factor of >10-fold after AAV infection of IB3-1 cells with AAV6.2LacZ vector was confirmed (data not shown). IB3 cells were transferred to fresh medium containing LLnL (40 μ M) and AAV6.2CFTR Donor, AAV6.2CFTR Acceptor, or a combination of AAV6.2CFTR Donor and AAV6.2CFTR Acceptor under the infection conditions described previously for FRT cells. After 90 min, cells received fresh medium containing 40 μ M LLnL for a period of 24 hr. Cells were then transferred to medium without LLnL for 24 hr followed by infection with AdpK7.YFPH148Q (3000 particles/cell) for 60 min. The cells were then trypsinized, replated on 18-mm round coverslips, and incubated overnight. Coverslips were used to evaluate CFTR function assay as described previously. As a positive control, naive IB3 cells were infected with both AdCFTR (5000 particles/cell) (Harvey *et al.*, 1999) and AdpK7YFP-H148Q (3000 particles/cell) for 60 min and incubated for 24 hr before assessment of CFTR activity.

Statistical assessment

Data are presented as means $\pm$ standard error (SE), where mean values for a condition were calculated on the basis of mean values from each coverslip tested for that condition. Tests for significance of difference between groups employed the Student *t* test, where $p < 0.05$ was taken as an indication of a significant difference between groups.

Results

Detection of segmental *trans*-splicing-generated CFTR mRNA and protein in transfected cells

To test the feasibility of CFTR segmental *trans*-splicing *in vitro*, 293 cells were transfected with the plasmids containing either 5' sequences (CFTR exons 1–14a followed by an intron with a splice donor, "CFTR Donor"), 3' sequences (an intron with a splice acceptor sequence followed by CFTR exons 14b–24, "CFTR Acceptor"), or both constructs. CFTR gene expression was evaluated at the mRNA and protein levels. Intact CFTR message produced by segmental *trans*-splicing was assessed by collecting total RNA from transfected cells and conducting RT-PCR with primers that bridged the splice junction. The presence of a single 623-bp product was diagnostic for the presence of the upstream forward primer-binding site in the donor sequence (CFTR nucleotides 2349–2370) and the downstream reverse primer-binding site in the acceptor sequence (CFTR nucleotides 2949–2971) on a contiguous stretch of nucleic acids that could only result from a splicing event (Fig. 2A). The presence of the 623-bp band required reverse transcriptase activity as well as the presence of both the donor and acceptor plasmids, indicating that the splicing event was a result of a rearrangement at the RNA level rather than at the DNA level. Although the amplified product size matched a PCR product generated from intact CFTR message (pCFTR plasmid), further confirmation of correct splicing was provided by sequencing the PCR product, which showed that the mRNA contained the correct CFTR mRNA sequence through the splice junction (Fig. 2B). To determine whether cells transfected with pCFTR Donor

plus pCFTR Acceptor produced CFTR protein, CFTR protein expression was evaluated by Western analysis after immunoprecipitation. In agreement with the RT-PCR data, the 170-kDa CFTR protein was detected in 293 cells transfected with a plasmid containing full-length CFTR cDNA (pCFTR) or with a pair of plasmids containing the segmental *trans*-splicing donor and acceptor pair (pCFTR Donor plus pCFTR Acceptor; Fig. 2C). The 170-kDa band was absent from naive cells (data not shown) and from cells transfected with either the donor or acceptor construct alone. Two additional bands at 55 and 25 kDa resulted from immunoreactivity of the immunoglobulin used during immunoprecipitation; these bands were less prominent in the pCFTR sample, where less total protein was loaded. The banding pattern and size of immunoreactive protein products were similar to those of the product produced from 293 cells transfected with full-length CFTR cDNA. As expected, CFTR protein were not found in 293 cells transfected with the control plasmids pcDNA3, pCFTR Donor plus pcDNA3, or pCFTR Acceptor plus pcDNA3.

CFTR protein function after segmental *trans*-splicing in microinjected cells

A fluorescence-based assay was used to assess functional expression of CFTR (Galiotta *et al.*, 2001a,b). To evaluate CFTR function after segmental *trans*-splicing, microinjection (~ 0.6 pg/cell) was used to deliver CFTR segmental *trans*-splicing plasmids into the nuclei of Fischer rat thyroid cells (FRT; known to be CFTR negative) that stably express a Cl^- -sensitive yellow fluorescent protein (YFP-H148Q). Microinjected cells were identified by codelivery of a soluble red fluorophore (Alexa Fluor 647). After microinjection, CFTR function was assessed in naive and microinjected cells by measuring YFP fluorescence as extracellular Cl^- was replaced with I^- . Channel activity was stimulated by addition of the adenylate cyclase activator, forskolin, and the phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX). Although all cells contained yellow fluorescent protein due to stable expression of YFP-H148Q (displayed in the green channel), only microinjected cells contained red fluorescence (displayed in the red channel). Viable injected cells were identified by the presence of both YFP and Alexa Fluor 647 (colocalization of digital green and red signals appears yellow/orange; Fig. 3A, white arrowheads). Some microinjected cells became nonviable; these cells appear red in digital images because of a lack of detectable YFP-H148Q (Fig. 3A, black arrowheads).

One day after injection, CFTR function was evaluated through the change in YFP-H148Q fluorescence during exchange of chloride and iodide followed by addition of cAMP activators (Galiotta *et al.*, 2001a,b). In the absence of CFTR, the change in YFP fluorescence was negligible in FRT-YFP cells after the addition of cAMP activators (Fig. 3B, black triangles). These data confirmed earlier reports that FRT cells have low basal halide permeability (Galiotta *et al.*, 2001a). A significant decrease in YFP fluorescence was observed in FRT-YFP cells microinjected with a plasmid containing CFTR cDNA (Fig. 3B, green triangles) after addition of cAMP activators (IBMX/forskolin), whereas YFP fluorescence increased rapidly when the cell medium was returned to a buffer containing Cl^- (Fig. 3B); some of the microinjected cells were negative for CFTR activity. In FRT-YFP cells injected with pCFTR

A. Identification of microinjected YFP cells

B. Plasmid microinjection, YFP cells

C. Quantitative assessment, YFP cells

FIG. 3. Functional analysis of CFTR produced via plasmid-mediated CFTR segmental *trans*-splicing in FRT-YFP cells. FRT cells constitutively expressing the chloride-sensitive YFP mutant, H148Q, were microinjected with solution containing a red tracer dye (Alexa Fluor 647) and plasmids (pCFTR, pCFTR Donor, pCFTR Acceptor, or pCFTR Donor plus pCFTR Acceptor). (A) Identification of microinjected FRT-YFP cells. Cells were evaluated by fluorescence microscopy for endogenous YFP expression (green), Alexa Fluor 647 dye (red), or colocalization of the

two (yellow/orange), indicating the presence of red dye in healthy, intact FRT-YFP cells (white arrowheads). Cells that were positive for YFP (green) but negative for Alexa Fluor 647 were not microinjected. Cells that were positive for Alexa Fluor 647 but negative for YFP (black arrowheads) were nonviable microinjected cells and were excluded from the analysis. Data on CFTR activity were collected from both microinjected cells and noninjected cells (negative control) in each culture. Scale bar: 50 μ m. (B) Representative data showing changes in YFP fluorescence intensity. YFP fluorescence intensity was evaluated every 5 sec during the course of the experiment and expressed as a percentage of the initial fluorescence. To determine CFTR-mediated chloride flux, cells were equilibrated in Cl^- , transferred to I^- , transferred to I^- supplemented with forskolin and isobutylmethylxanthine (FSK/IBMX) to elevate intracellular cAMP, and then returned to Cl^- at the times indicated by the arrows above the fluorescence trace. A decrease in YFP fluorescence intensity in the presence of I^- and FSK/IBMX was taken as an indication of the presence of CFTR-mediated chloride conductance. FRT-YFP cells with constitutive expression of CFTR were used as a positive control. FRT-YFP cells microinjected with a plasmid that did not contain an expression cassette (pcDNA3) were used as a negative control. (C) Summary of CFTR activity data. Chloride conductance was quantified by determining the maximal slope per 5-sec interval during iodide influx in the presence of FSK/IBMX. In cultures that received microinjected plasmid, all viable microinjected cells in the field (identified by the presence of both YFP and Alexa Fluor 647 fluorescence) were evaluated for CFTR activity. Cells were considered positive for CFTR if they demonstrated the following three criteria: a slope of YFP fluorescence >2 standard deviations above that of naive cells; a total decrease in YFP fluorescence of $>25\%$ of the initial observed intensity on addition of I^- with cAMP activators; and a total increase in YFP fluorescence of $>15\%$ of the initial observed intensity on replacement of extracellular Cl^- . No CFTR⁺ cells were observed for negative control groups. Data for both CFTR⁺ and CFTR⁻ cells are shown for cells injected with pCFTR and with pCFTR Donor plus pCFTR Acceptor. The maximal slope was plotted as the maximal change in fluorescence intensity per second (mean $\pm$ SE) from naive FRT cells ($n = 3$ experiments, total of 18 cells measured), pCFTR-injected FRT cells ($n = 6$ experiments, total of 12 CFTR⁺ cells measured, 9 CFTR⁻ cells measured), and cells injected with pCFTR Donor plus pCFTR Acceptor ($n = 4$ experiments, 14 CFTR⁺ cells measured, 22 CFTR⁻ cells measured).

the chloride/iodide exchange ($\sim 60\%$ of pCFTR-injected cells and $\sim 40\%$ of pCFTR Donor/pCFTR Acceptor-coinjected cells). To assess CFTR activity quantitatively, the maximal slope per 5-sec interval during iodide influx in the presence of forskolin/IBMX was calculated; this parameter has previously been established as the best index of CFTR activity

(Galiotta *et al.*, 2001b). Multiple cells from at least three independent experiments were examined for each condition (see the caption to Fig. 3 concerning panel C). Whereas microinjection of either the pCFTR Donor or pCFTR Acceptor plasmid alone failed to change the slope of the change in YFP fluorescence, microinjection of either pCFTR or the combination of

pCFTR Donor and pCFTR Acceptor led to significant increases in the slope of the change in YFP fluorescence ($p < 0.001$ for both conditions compared with uninjected cells; Fig. 3C). The mean slopes for the pCFTR and pCFTR Donor plus pCFTR Acceptor groups were the same ($p > 0.1$).

Functional expression of CFTR by segmental trans-splicing using AAV vectors

Among the AAV serotypes that have been used for gene transfer to the airway epithelium, evidence suggests that a novel capsid designated AAV6.2 is highly efficient (Limberis *et al.*, 2009). After cloning the CFTR Donor and CFTR Acceptor expression cassettes into an AAV packaging plasmid, each plasmid was cotransfected with a plasmid containing AAV6.2 *cap* genes, resulting in production of AAV6.2CFTR Donor and AAV6.2CFTR Acceptor.

To test the ability of AAV6.2CFTR Donor and AAV6.2CFTR Acceptor to produce CFTR through segmental *trans*-splicing, the two vectors were used to infect 293orf6 cells alone or in combination. Infection of 293 cells by this serotype was confirmed with AAV6.2LacZ under the same conditions, with $>80\%$ of the cells staining positive with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal). As a positive control, 293orf6 cells were transfected with pCFTR as described previously. The presence of an intact 14a/b junction at the mRNA level was confirmed by RT-PCR (Fig. 4A). In the absence of reverse transcriptase, no PCR fragments were generated. In the presence of reverse transcriptase, a 623-bp fragment was generated in the positive control and in the culture that was infected with both AAV6.2CFTR Donor and AAV6.2CFTR Acceptor. Cells infected with either AAV6.2CFTR Donor or AAV6.2CFTR Acceptor alone did not exhibit an intact 14a/b junction. Sequencing of the PCR fragment from cells infected with AAV6.2CFTR Donor and AAV6.2CFTR Acceptor confirmed that the junction formed correctly (Fig. 4B). Western analysis for CFTR demonstrated the presence of a 170-kDa band in cells infected with AAV6.2CFTR Donor and AAV6.2CFTR Acceptor together, but not in cells infected with AAV6.2CFTR Donor or AAV6.2CFTR Acceptor alone (Fig. 4C). These data confirmed that RNA transcripts derived from AAV vectors were able to participate in segmental *trans*-splicing leading to production of CFTR protein.

To estimate the efficiency of segmental *trans*-splicing, TaqMan real-time PCR with relative quantitation was used. RNA from 293orf6 cells infected with AAV6.2CFTR Donor, AAV6.2CFTR Acceptor, or both was analyzed, using rRNA as a relative reference and human tracheal epithelium RNA as a calibrator. Three primers and probe combinations were compared: the first within the CFTR Donor; the second within the CFTR Acceptor, and the third spanning the STS junction (Fig. 4D). As expected, the TaqMan primer and probe combination specific to the CFTR Donor detected mRNA only in the samples in which AAV6.2CFTR Donor was used for infection. Similarly, the TaqMan primer and probe combination specific to the CFTR Acceptor detected mRNA only in the samples in which AAV6.2CFTR Acceptor was used for infection. In the doubly infected cells, the apparent levels for the AAV6.2CFTR Donor-specific mRNA and AAV6.2CFTR Acceptor-specific mRNA were comparable, showing both vectors had comparable transduction efficiencies. The TaqMan primer and probe combination spanning the segmental *trans*-

splicing junction showed amplification only in the RNA from cells that received both vectors. The amount was 4.7% of the estimated amount of RNA from the AAV6.2CFTR Donor and 12.1% of the amount from the AAV6.2CFTR Acceptor. Moreover, the overall amount of CFTR mRNA was greater than was seen in human tracheal epithelium, suggesting that physiological levels of CFTR may be obtained after *in vivo* gene transfer.

CFTR protein function after segmental trans-splicing in FRT-YFP cells infected with AAV6.2CFTR Donor and Acceptor vectors

To determine whether CFTR protein produced by AAV-mediated segmental *trans*-splicing was functional, we evaluated CFTR activity in FRT-YFP cells infected with AAV6.2CFTR Donor and AAV6.2CFTR Acceptor individually or in combination. Infection of FRT-YFP cells with this serotype was confirmed with AAV6.2LacZ under the same conditions, with $18 \pm 1\%$ of the cells staining positive with X-Gal. CFTR function was assessed by analysis of the fluorescence intensity of the YFP-H148Q mutant as described previously. Fluorescence intensity profiles of individual cells were evaluated, and the slope of the decrease in fluorescence intensity was calculated after the replacement of extracellular chloride with iodide in the presence of cAMP activators (Fig. 5A). Whereas naive FRT-YFP cells showed little variation in fluorescence intensity after addition of iodide and cAMP activators, approximately 50% of the FRT-YFP cells expressing CFTR exhibited a decrease in fluorescence on addition of the cAMP activators. In cultures that received either AAV6.2CFTR Donor or AAV6.2CFTR Acceptor, cells never exhibited a decrease in YFP fluorescence intensity, but cells that received both AAV6.2CFTR Donor and AAV6.2CFTR Acceptor had clear decreases in YFP fluorescence intensity on addition of cAMP activators to iodide-containing medium. Cells were designated as CFTR⁺ when they met the three criteria described in Materials and Methods and the slope of fluorescent change was assessed from multiple cells per group from at least three independent experiments (see Fig. 5B caption). Consistent with the fraction of cells infected with AAV6.2LacZ under these conditions, 19% of the cells infected with AAV6.2CFTR Donor plus AAV6.2CFTR Acceptor vectors were functionally CFTR positive. The CFTR-negative cells were assumed to be uninfected or to have been infected with only AAV6.2CFTR Donor or AAV6.2CFTR Acceptor, but not both. The rate of change of fluorescence intensity in cells infected with AAV vectors carrying the CFTR donor/acceptor pair was significantly less than in cells with endogenous expression of CFTR ($p < 0.01$; Fig. 5B), but based on the knowledge that 5–10% of CFTR activity is necessary to correct CFTR mutations (Trapnell *et al.*, 1991; Johnson *et al.*, 1992; Farnen *et al.*, 2005), the function mediated by the donor/acceptor vector is likely sufficient.

Correction of CFTR Cl[−] conductance in IB3-1 cells

Expression of functional CFTR in FRT cells after infection with AAV6.2CFTR Donor and AAV6.2CFTR Acceptor demonstrated the principle that segmental *trans*-splicing was successful in a cell that was CFTR negative. We sought to determine whether the process would work in cells containing mRNAs encoding mutant forms of CFTR. To answer this

FIG. 4. CFTR mRNA production via AAV6.2-mediated CFTR segmental *trans*-splicing (STS) in 293orf6 cells. **(A)** Evaluation of CFTR segmental *trans*-splicing *in vitro* by RT-PCR. 293orf6 cells were infected with AAV6.2CFTR Donor and AAV6.2CFTR Acceptor vectors. RT-PCR analysis was carried out on total RNA from infected cells, using primers PF and PR (see Fig. 1), 72 hr after infection. To show that RNA, and not DNA, was amplified, a set of control reactions (lanes 1–4) was done without the addition of reverse transcriptase. Lane 1, mock transfected; lane 2, AAV6.2CFTR Donor only; lane 3, AAV6.2CFTR Acceptor only; lane 4, AAV6.2CFTR Donor plus AAV6.2CFTR Acceptor. The same conditions were tested in the presence of reverse transcriptase (lanes 5–8). Lane 9, positive control, 293orf6 cells transfected with a plasmid containing the full-length CFTR cDNA. An RT-PCR product of 623bp was observed in lanes 8 and 9. **(B)** Sequence of the RT-PCR product of CFTR segmental *trans*-splicing isolated from 293orf6 cells infected with AAV6.2CFTR Donor plus AAV6.2CFTR Acceptor vectors (**A**, lane 8). Data show the sequence around the splice junction (asterisk in Fig. 1) in the forward orientation. The observed sequence is identical to the expected sequence. **(C)** Western analysis of CFTR protein expression. CFTR protein expression was evaluated in 293orf6 cells by immunoprecipitation with mouse anti-CFTR followed by SDS-PAGE and transfer to nitrocellulose. Blots were probed with mouse anti-CFTR. Conditions included the following: lane 10, 293orf6 cells transfected with pCFTR as a positive control; lane 11, 293orf6 cells infected with AAV6.2CFTR Donor; lane 12, 293orf6 cells infected with AAV6.2CFTR Acceptor; lane 13, 293orf6 cells infected with AAV6.2CFTR Donor plus AAV6.2CFTR Acceptor vectors. The anticipated molecular mass of CFTR (170 kDa) is marked. Bands visible in all lanes of the Western analysis represent IgG heavy and light chains from antibodies included in the immunoprecipitation (55 and 25 kDa). **(D)** Assessment of Donor, Acceptor, and segmental *trans*-spliced RNA levels after *in vitro* gene transfer. For replicate cultures, RNA was extracted and converted to cDNA and the relative amount of CFTR mRNA was determined in comparison with rRNA by TaqMan real-time PCR. Human tracheal RNA was used as a calibrator. Three different primer and probe combinations were used to detect Donor, Acceptor, or segmental *trans*-spliced product. Data represent the mean of $n = 3$ determinations at two different RNA concentrations.

question, IB3-1 cells, SV40-transformed airway epithelial cells derived from an individual with CF (Zeitlin *et al.*, 1991), were used as the model cell line for the study. Unlike FRT-YFP cells, IB3-1 cells do not constitutively express the chloride-sensitive YFP-H148Q. For the purpose of detecting CFTR expression, an adenoviral vector expressing the YFP-H148Q gene was

created. Although the vector was based on adenovirus serotype 5, the construct employed a modified form of the adenoviral fiber protein with a stretch of seven lysine residues in the globular head domain (AdpK7). The pK7 fiber was designed to confer efficient gene transfer with broad tropism conveyed by the polylysine motif (Wickham *et al.*, 1996). The

A. AAV6.2-mediated delivery of CFTR, YFP cells

B. Quantitative assessment, YFP cells

FIG. 5. Functional analysis of CFTR produced via AAV6.2-mediated CFTR segmental *trans*-splicing in FRT-YFP cells. FRT-YFP cells (see Fig. 3) were infected with AAV6.2CFTR Donor, AAV6.2CFTR Acceptor, or AAV6.2CFTR Donor plus AAV6.2CFTR Acceptor. (A) Representative data showing changes in YFP fluorescence intensity. YFP fluorescence intensity was evaluated as described in Fig. 3. FRT-YFP cells with constitutive expression of CFTR were used as a positive control. Naive FRT-YFP cells were used as a negative control. (B) Summary of CFTR activity data. Chloride conductance was quantified by determining the maximal slope per 5-sec interval during iodide influx in the presence of FSK/IBMX. For data from FRT-YFP cells infected with AAV6.2CFTR Donor plus AAV6.2CFTR Acceptor, <5% of the cells were deemed CFTR⁺ by the three criteria described in Fig. 3. All FRT-YFP-CFTR cells were positive for CFTR activity and data for both CFTR⁺ and CFTR⁻ cells are provided. The maximal slope was plotted as the maximal change in fluorescence intensity per second (mean $\pm$ SE) for naive FRT cells ($n=3$ experiments, total of 30 cells measured), cells infected with AAV6.2CFTR Donor plus AAV6.2CFTR Acceptor ($n=4$ experiments, 9 CFTR⁺ cells measured, 40 CFTR⁻ cells measured), AAV6.2CFTR Donor-infected FRT cells ($n=4$ experiments, total of 40 cells measured), and AAV6.2CFTR Acceptor-infected FRT cells ($n=3$ experiments, total of 30 cells measured).

resulting adenoviral vector (AdpK7-YFPH148Q) efficiently expressed chloride-sensitive YFP in a manner independent of the expression of the coxsackievirus-adenovirus receptor, indicating that the pK7 fiber was functioning appropriately (data not shown). To enhance the efficiency of AAV-mediated gene expression in IB3-1 cells, the cells were cultured with LLnL, a proteasome inhibitor, previously shown to enhance expression of transgenes from AAV serotype 2 vectors *in vitro* in human polarized epithelia (Duan *et al.*, 2000b). We tested the ability of LLnL to enhance AAV6.2-mediated gene expression in IB3-1 cells, using both LacZ and GFP as marker genes. In all cases, cells treated with LLnL showed superior expression compared with expression without LLnL treatment (data not shown).

To evaluate gene expression, IB3-1 cells were infected with AAV6.2CFTR Donor and AAV6.2CFTR Acceptor individually or together, treated with LLnL-containing medium, infected with AdpK7-YFPH148Q, and assessed for YFP fluorescence intensity after exchange of iodide for chloride in

extracellular medium (Fig. 6A). Infection of IB3 cells with this serotype was confirmed, using AAV6.2LacZ under identical conditions, with $39 \pm 5\%$ of the cells staining positive with X-Gal. The results in IB3-1 cells mirrored those in FRT-YFP cells. Naive IB3-1 cells showed no chloride conductance. A large proportion of IB3-1 cells infected with AdCFTR demonstrated clear chloride conductance. Cells infected with either AAV6.2CFTR Donor or AAV6.2CFTR Acceptor had no chloride conductance, whereas a small percentage of cells infected with both AAV6.2CFTR Donor and AAV6.2CFTR Acceptor were positive for chloride conductance. The percentage of doubly infected cells that were functionally CFTR positive was lower (11%) than expected on the basis of the efficiency of infection with AAV6.2LacZ. When evaluating only cells that were considered positive for CFTR conductance by the criteria described in Materials and Methods, positive cells were found only among cells that received AAV6.2CFTR Donor and AAV6.2CFTR Acceptor, whereas cells that received only one vector were never positive. Among positive

A. AAV6.2-mediated delivery of CFTR, IB3-1 cells

B. Quantitative assessment, IB3-1 cells

FIG. 6. Functional analysis of CFTR produced via AAV6.2-mediated CFTR segmental *trans*-splicing in IB3-1 cells derived from airway epithelial cells of an individual with cystic fibrosis. IB3-1 cells were infected with AAV6.2CFTR Donor, AAV6.2CFTR Acceptor, or AAV6.2CFTR Donor plus AAV6.2CFTR Acceptor in the presence of the proteasome inhibitor, N-acetyl-L-leucyl-L-leucyl-L-norleucine (LLnL), and then maintained in LLnL-containing medium for 24 hr. Forty-eight hours after infection, the YFP-H148Q protein was expressed in IB3-1 cells via infection with an adenoviral vector (AdpK7.YFP-H148Q). After an additional 24 hr, CFTR activity was assessed. **(A)** Representative data showing changes in YFP fluorescence intensity. YFP fluorescence intensity was evaluated as described in Fig. 3. IB3-1 cells infected with AdCFTR were used as a positive control. Naive IB3-1 cells were used as a negative control. **(B)** Summary of CFTR activity data. Chloride conductance was quantified by determining the maximal slope per 5-sec interval during iodide influx in the presence of FSK/IBMX. In the culture infected with both AAV6.2CFTR Donor and AAV6.2CFTR Acceptor, cells were included in the analysis if they were “positive” for CFTR activity by the three criteria cited in Fig. 3. The slope was plotted as the maximal change in fluorescence intensity per second (mean $\pm$ SE) for naive IB3-1 cells ($n = 4$ experiments, total of 40 cells measured), AdCFTR-infected IB3-1 cells ($n = 4$ experiments; total of 40 cells measured), cells infected with AAV6.2CFTR Donor plus AAV6.2CFTR Acceptor ($n = 4$ experiments; 5 CFTR⁺ cells measured; 40 CFTR⁻ cells measured), AAV6.2CFTR Donor-infected IB3-1 cells ($n = 3$ experiments; total of 30 cells measured), and AAV6.2CFTR Acceptor-infected IB3-1 cells ($n = 4$ experiments; total of 40 cells measured).

cells, the slope of the conductance in CFTR⁺ cells was significantly greater than the slope observed in control cells (Fig. 6B). The rate of change of fluorescence intensity in cells infected with AAV vectors carrying the CFTR donor/acceptor pair was less than for cells infected with AdCFTR ($p < 0.01$), but the level was likely sufficient to correct the Cl⁻ conductance defect.

Discussion

Cystic fibrosis, a common lethal hereditary disorder, is caused by mutations in the cystic fibrosis transmembrane conductance regulator gene (Riordan *et al.*, 1989; Rommens *et al.*, 1989; Welsh and Smith, 1993; Riordan, 2008). Although multiple organs are affected by the CFTR mutation, the lung disease is responsible for most of the morbidity and mortality in individuals with CF. Hence, the major focus of gene therapy for CF is to correct CFTR function in the airway epithelium. Among the candidate gene therapy vectors for CF, AAV appears to be a good choice because of its ability to confer

persistent, high-level gene expression in immunocompetent animal models of genetic diseases (Warrington and Herzog, 2006; Wu *et al.*, 2006). However, for application to CF, the AAV vector is faced with the challenge that the packing capacity of AAV cannot accommodate the full-length CFTR cDNA in combination with an active promoter and relevant 3' signals (Flotte *et al.*, 1993a,b; Zhang *et al.*, 1998; Sirninger *et al.*, 2004; Ostedgaard *et al.*, 2005). In this study, segmental *trans*-splicing was used to solve this challenge. This strategy relies on delivery of two halves of the CFTR cDNA in different vectors (Pergolizzi *et al.*, 2003; Pergolizzi and Crystal, 2004; Liu *et al.*, 2005; Nakayama *et al.*, 2005). When the two vectors infect the same cells, the two pre-mRNAs come together by virtue of complementary sequences inserted into the 3' and 5' noncoding regions of the upstream and downstream vectors, respectively. By including relevant splicing signals near the complementary hybridization sequences, the nuclear splicing machinery joins the two half-transcripts into an intact mRNA. In this study, the CFTR cDNA was divided between exon 14a and exon 14b. RT-PCR and Western analysis results

after plasmid transfection in 293 cells demonstrated expression of correctly *trans*-spliced CFTR mRNA and full-length protein. A CFTR functional assay after microinjection confirmed the presence of functional CFTR after segmental splicing.

After demonstrating the feasibility of the strategy, the constructs were packaged into AAV capsids to determine whether AAV-mediated expression of the constructs was consistent with segmental *trans*-splicing. A novel AAV capsid, AAV6.2, was chosen for the vector because of its highly efficient *in vitro* infection of human polarized airway epithelial cultures and highly efficient *in vivo* infection of mouse airway (Limberis *et al.*, 2009). Coinfection of 293orf6 cells with AAV6.2CFTR Donor and AAV6.2CFTR Acceptor vectors led to the production of correctly spliced CFTR RNA and full-length protein. After coinfection with the same pair of vectors, chloride conductance was observed in FRT-YFP cells as well as in IB3-1 cells, an airway epithelial cell line derived from an individual with CF (Zeitlin *et al.*, 1991).

The efficiency of segmental *trans*-splicing in this study, with no optimization of splicing signals, was measured as 4.7–12.1%. However, several previous studies involving dual-AAV vector gene delivery strategies that rely on genome concatenation have been published (Halbert *et al.*, 2002; Reich *et al.*, 2003; Lai *et al.*, 2006; Ghosh *et al.*, 2007; Yan *et al.*, 2007). Although these studies demonstrate *in vivo* efficacy, there are several limitations that prevent efficiency comparisons with the RNA-based *trans*-splicing approach presented here. These include the use of different promoters controlling gene expression, the use of reporter gene expression as readout as opposed to the percentage of donor and acceptor incorporated into mature mRNA, and the use of coding sequences with differing gene-splitting sites, which affects the efficiency of the process. Several studies compare the efficiency of the dual-gene delivery strategy with small reporter genes with that of a single vector containing the full-size coding sequence. In the case of CFTR, these types of comparisons are not possible because the CFTR coding sequence cannot be packaged into a single AAV vector (Halbert *et al.*, 2002; Reich *et al.*, 2003; Ghosh *et al.*, 2007). The most analogous measurement of splicing efficiency to that presented in our current study involved quantitation of RNA accumulation by RNase protection assay after dual-vector delivery of a mini-dystrophin gene (Lai *et al.*, 2005, 2006). These studies measured the level of donor cleaved at the splice donor site as a measure of successful *trans*-splicing; however, it is possible that all donor cleaved at the splice site may not be correctly spliced into the intended acceptor and, therefore, efficiency comparisons with our study, which measures the amount of product formed from successful splicing of donor to acceptor, cannot be made.

Although the efficiency of the segmental *trans*-splicing process needs to be improved, together, the data reveal that segmental *trans*-splicing may be a feasible strategy to use in conjunction with AAV6.2 to accomplish sustained expression of CFTR in the airway of individuals with CF.

Overcoming AAV packaging limitations

A variety of other strategies have been used in attempts to overcome the packaging limit of AAV vectors as it relates to CFTR gene therapy. In some studies, investigators dispensed

with the promoter region, relying instead on latent promoter activity in the inverted terminal repeat (ITR) of the AAV genome (Flotte *et al.*, 1993a,b). The low efficiency of the ITR as a promoter may have led to reduced levels of AAV-mediated gene expression, using this strategy. In other studies, a truncated form of an active promoter was used with a full-length CFTR cDNA (Zhang *et al.*, 1998; Sinniger *et al.*, 2004). Yet another strategy employed a truncated, functional form of the CFTR gene with an active promoter (Ostedgaard *et al.*, 2005). Finally, a novel strategy used the ability of AAV genomes to undergo intermolecular concatamerization between two independent AAV vectors, resulting in end-to-end continuous DNA segments (Duan *et al.*, 2000b, 2001). If this strategy was applied to two vectors expressing two halves of the CFTR gene, then this mechanism would have the potential to create a complete gene encoding full-length CFTR.

Finally, conventional (as opposed to segmental) *trans*-splicing has been used to correct endogenous CFTR mRNA (Mansfield *et al.*, 2000; Liu *et al.*, 2002, 2005). In this strategy, a single downstream construct was delivered to cells and *trans*-splicing was accomplished by using a hybridization sequence that was complementary to an intron sequence upstream of the exon containing the common $\Delta F508$ mutation. *Cis*-splicing (joining of exons in the original, endogenous CFTR pre-mRNA) occurred preferentially over *trans*-splicing (joining of the upstream endogenous CFTR pre-mRNA exons to the downstream exogenous, corrected CFTR pre-mRNA exons). Rare, but productive, *trans*-splicing resulting in CFTR activity was observed in some treated cells. The potential advantages of classical *trans*-splicing are that only one gene transfer vector is needed and that the production of protein is limited by the amount of endogenous CFTR message and, thus, inherently relies on endogenous regulatory mechanisms that act on the CFTR promoter. However, CFTR exhibits low endogenous transcription rates, and thus classical *trans*-splicing efficiency is limited by the concentration of one of the two partners in the bimolecular hybridization reaction.

The present study uses segmental *trans*-splicing, a method developed, in part, to increase the efficiency of *trans*-splicing by expressing high levels of both of the hybridization partners in the bimolecular hybridization reaction (Pergolizzi *et al.*, 2003; Pergolizzi and Crystal, 2004; Nakayama *et al.*, 2005). In prior studies using segmental *trans*-splicing, sufficient segmental *trans*-splicing product was used to mediate phenotypes and correct a genetic defect. Any increase in efficiency of CFTR production through the high expression of both upstream and downstream splicing partners, using the segmental *trans*-splicing strategy, will have to be balanced against any reduction in efficiency that may occur given the requirement to have a single cell infected by two different AAV vectors. This question will be answered only by further studies using complex *in vitro* models (e.g., polarized human epithelium) or *in vivo* models (e.g., CF knockout mice).

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Author Disclosure Statement

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