Chloride secretion in the trachea of null cystic fibrosis mice: the effects of transfection with pTrial10–CFTR2

L. J. MacVinish*, D. R. Gill†, S. C. Hyde†, K. A. Mofford†, M. J. Evans‡, C. F. Higgins†, W. H. Colledge‡, L. Huang§, F. Sorgi§, R. Ratcliff‡ and A. W. Cuthbert*‖

*Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, †Imperial Cancer Research Fund, Nuffield Department of Clinical Biochemistry and the Institute of Molecular Medicine, John Radcliffe Hospital, University of Oxford, Oxford OX3 9DU, ‡Wellcome–CRC Institute of Cancer and Development Biology, Tennis Court Road, Cambridge CB2 1QR, UK and §Laboratory of Drug Targeting, University of Pittsburgh, W1316 Biomedical Science Tower, Pittsburgh, PA 15261, USA

1. An improved novel plasmid backbone, pTrial10, has been developed. We have used this vector to deliver the cDNA for the cystic fibrosis transmembrane conductance regulator (CFTR) to cells, both *in vitro* and *in vivo*, complexed with cationic liposomes.

2. Human 293 kidney epithelial cells (HEK 293) showed expression of an immunoprecipitable 165 kDa protein corresponding to CFTR when transfected *in vitro* with pTrial10–CFTR2, but not when the vector pTrial10 was used.

3. HEK 293 cells transfected with pTrial10–CFTR2, but not pTrial10, demonstrated a cAMP-dependent anion conductance, measured by fluorescence microscopy using a halide-sensitive probe, SPQ.

4. The CFTR-dependent, cAMP-sensitive chloride secretory response in murine tracheal epithelium could be measured if the calcium-dependent chloride secretory process was first maximally stimulated with a mixture of the Ca^{2+}-ATPase inhibitor, TBHQ, and the calcium ionophore, A23187. With these conditions wild-type and CF-null (transgenic animals in which the cystic fibrosis (CF) gene has been disrupted so that no CFTR is produced) murine tracheas could be distinguished. The difference between the current elicited by forskolin in wild-type and CF tracheas was highly significantly different ($P < 0{\cdot}001$), giving a CFTR-dependent current of $11{\cdot}2\ \mu A\ cm^{-2}$.

5. Transfection of the airways with pTrial10–CFTR2, but not pTrial10, significantly ($P < 0{\cdot}01$) increased the CFTR-dependent chloride secretory current in CF tracheas. The degree of correction was greater when intra-tracheal installation rather than nasal insufflation was used to deliver the plasmids.

Cystic fibrosis (CF) is a monogenic autosomal recessive disorder. The CF gene encodes an epithelial chloride channel (the cystic fibrosis transmembrane conductance regulator, CFTR) which is located in the apical membranes of many epithelial cell types and is activated by cAMP-dependent protein kinase A and ATP. A loss of activity of this channel in the airways of CF patients is the major cause of morbidity and premature mortality (Riordan *et al.* 1989; Anderson *et al.* 1991). The physiological relevance of this study is to explore how the human disease is reflected in the behaviour of an airway epithelium from transgenic mice and to discover a method to dissect the CFTR-dependent component of the chloride secretory response from CFTR-independent ones.

There is now considerable effort being invested in the development of gene therapy for the pulmonary aspects of cystic fibrosis (Wilson, 1995). Two types of gene delivery vector have received most attention. Adenoviral vectors are tropic for the airway epithelium and can, potentially, deliver the CF gene with high efficiency. However, single and repeated application of adenoviral vectors in animals and

‖ To whom correspondence should be addressed.

L. J. MacVinish and D. R. Gill have contributed equally to this work.

humans generates an unacceptable immunological and inflammatory response (Marshall, 1995). An alternative approach is to use cationic liposomes to deliver the CFTR cDNA to the airway epithelia (Caplen *et al.* 1995).

The generation of CF transgenic mouse models has provided an important experimental tool for preclinical studies in gene transfer prior to clinical trials. Indeed, ethical committees require proof of concept in an experimental model before clinical trials can begin. A mouse model is important in preclinical studies for testing improved vectors, determining duration of action, dosage frequency and establishing safe procedures. In CF-null mice (Ratcliff *et al.* 1993) no CFTR is expressed and epithelial cells fail to show cAMP-dependent increases in chloride permeability. We have previously shown that the delivery of the CFTR cDNA as a plasmid pREP8–CFTR complexed with cationic liposomes to CF-null mice restores chloride secretion in the trachea in response to forskolin, an activator of adenylate cyclase (Hyde *et al.* 1993). However, in that study only young animals (< 10 g) could be used as an alternative chloride conductance develops in older animals, both in CF null and ΔF508 CF mice (Colledge *et al.* 1995). In older animals chloride secretion in the mouse trachea results from both cAMP-mediated and calcium-mediated processes. As forskolin also increases intracellular calcium in the airways, the secretory response is composed of both components, only part of which is CFTR mediated (Grubb, Paradiso & Boucher, 1994). The differences with age only apply to airway epithelia and not, for example, those of the alimentary tract (Cuthbert, MacVinish, Hickman, Ratcliff, Colledge & Evans, 1994). In this study we have developed a method for measuring the cAMP-dependent chloride transporting activity in the tracheas of CF mice of any age, circumventing the complications imposed by the calcium-dependent component. This was achieved by using 2,5-di-*tert*-butyl hydroquinone (TBHQ: Oldershaw & Taylor, 1990; Chao, Kouyama, Heist, Dong & Gardner, 1995) to mobilize calcium from internal stores in the presence of A23187, a calcium ionophore. Following this treatment, the subsequent short circuit current (SCC) responses to forskolin were shown to reflect CFTR-mediated chloride transport allowing wild-type and CF-null tracheas to be differentiated in older animals.

Here we also describe the development of a novel plasmid for use in CFTR gene therapy. In order to circumvent potential adverse immunological responses against proteins expressed during CFTR cDNA delivery, we developed a plasmid backbone which permits efficient replication and production in *Escherichia coli* hosts, but in mammalian hosts should be unable to express any protein. The CFTR cDNA was cloned into this vector (pTrial10) and was under control of the Rous sarcoma virus 3′ long terminal repeat (RSV-3′LTR) promoter. In this study we show that this plasmid is capable of expressing functional CFTR in cultured cells and can correct the CF defect in the trachea of transgenic CF-null mice. No toxic effects of pTrial10–CFTR2 have been recorded; it has received ethical approval and has been used in two human clinical trials on the epithelium of the nose without apparent toxic effects on nasal biopsies.

METHODS

Construction of pTrial10 and pTrial10–CFTR2

Plasmid pTrial4 consists of base pairs (bp) 1–2446 of pAT153 (Twigg & Sherratt, 1980) recircularized via a 26 bp synthetic polylinker sequence: 5′CTGCAGAGGCCTCTCGAGCCCG GGAA3′, creating unique restriction endonuclease sites for *Pst*I, *Stu*I, *Xho*I, *Sma*I and *Eco*RI. The pAT153 portion of pTrial4 incorporated two minor sequence changes, A to T at nucleotide 29 and A to G at nucleotide 86 (pAT153 numbering GenBank accession number LO8853).

The 1081 bp *Sal*I fragment from pREP8 (Invitrogen, NV-Leek, The Netherlands) containing the RSV-3′LTR promoter and SV40 terminator expression cassette, was inserted into the *Xho*I site in pTrial4 to create plasmid pTrial10.

A 4522 bp *Eco*RV–*Xho*I fragment containing the entire CFTR coding sequence (Riordan *et al.* 1989) was introduced into the unique *Pvu*II–*Xho*I sites of pTrial10 to create pTrial10–CFTR2. The cDNA incorporated two minor changes from the published sequence, T to C at nucleotide 936 (Cheng *et al.* 1990) and A to C at nucleotide 1990 (Gregory *et al.* 1990) and included a Kozak translation initiation sequence (Kozak, 1986) immediately 5′ to the translation initiation codon. The entire nucleotide sequence of pTrial10–CFTR2 was confirmed prior to use and its structure is given in diagrammatic form in Fig. 1.

Plasmid pTrial4 and its derivatives were propagated in *E. coli* NSURE, a tetracycline-sensitive derivative of *E. coli* SURE (Stratagene, Cambridge, UK), in growth media supplemented with 10 μg ml^{-1} of tetracycline and purified using Qiagen-tip 2500 MEGA columns (Qiagen, Hilden, Germany) for the cellular studies. For the mouse work the plasmid was purified by the standard alkaline lysis method followed by CsCl density gradient purification.

Liposomes

Cationic liposomes were formulated from 3β(*N*-(*N*′*N*′-dimethylaminoethane)-carbamoyl) cholesterol (DC-Chol) and dioleoyl phosphatidylethanolamine (DOPE). DC-Chol–DOPE in a 6:4 molar ratio was prepared by microfluidization at a final concentration of 2 μmol ml^{-1} in water, equivalent to 1·2 mg ml^{-1} of total lipid (Gao & Huang, 1991).

Transfection of cultured epithelial cells

Each batch of liposomes was tested for transfection efficiency by lipofecting HeLa Ohio cells and human epithelial kidney (HEK 293) cells (Graham, Smiley, Russell & Nairn, 1977), assaying chloramphenicol acetyl transferase (CAT) reporter gene activity expressed from plasmid pREP4CAT (Invitrogen). Plasmid pREP4 was included as a negative control.

HeLa Ohio cells were seeded at 3×10^6 cells per 90 mm-diameter dish and grown for 18 h after which they were transfected with 30 μg of plasmid DNA and 300 nmol DC-Chol–DOPE. The DNA and liposomes were each diluted into 9 ml of Opti-MEM I cell culture medium (Gibco BRL), mixed, incubated for 5 min and then added to the cells. The medium was replaced after 24 h and CAT

activity assayed at 3 days using a CAT assay enzyme system (Promega). Assays with HEK 293 cells were carried out essentially as above except the cells were seeded at 5×10^5 cells per 35 mm-diameter dish. A 5 μg aliquot of plasmid DNA and 50 nmol of DC-Chol–DOPE, each diluted in 1·5 ml Opti-MEM I, were mixed, incubated and added to the cells. The medium was replaced after 2 h and the cells assayed after 2–4 days. Each assay was carried out in triplicate. Liposome transfection efficiencies were greater than 0·2 CAT units (mg total protein)$^{-1}$.

Immunoprecipitation of CFTR protein

Immunoprecipitated proteins from lysates of HEK 293 cells that had been transfected with pTrial10–CFTR2 or pTrial10 were phosphorylated using the catalytic subunit of protein kinase A and γ-^{32}P-ATP essentially as described by Cheng *et al.* (1990). The anti-CFTR antibody used was MATG1104 (a kind gift from Transgene, France) raised against the R-domain of CFTR. γ-^{32}P-labelled immune precipitates were electrophoresed on a 7·5% polyacrylamide gel and CFTR visualized by autoradiography.

SPQ fluorescence measurements

HEK 293 cells were transfected with plasmids pTrial10 and pTrial10–CFTR2 as above, except that the cells were cultured on glass coverslips. The cells were then loaded with the halide-sensitive fluorophore 6-methoxy-*N*-(3-sulphopropyl)-quinolinium (SPQ) by hypotonic shock in NaI buffer for 20 min, and allowed to recover in isotonic NaI buffer (Chao, Dix, Sellars & Verkman, 1989). The cells were assayed for the presence of cAMP-activated halide efflux as described by Rich, Gregory, Anderson, Manavalan, Smith & Welsh (1991). Fluorescence was detected every 30 s using a Leica DMIRBE fluorescence microscope (Leica, UK) in conjunction with an Improvision intracellular ion-imaging system (Improvision, UK). Excitation was at 350 nm and emission was collected at 420 nm and above. Fields of cells (typically 25–100 cells) were visualized at ×200 magnification. To quench SPQ fluorescence, cells were initially incubated in a buffer containing NaI. After 120 s of control recording, the bathing medium was changed to a buffer containing $NaNO_3$. After a further 300 s the bathing medium was changed to $NaNO_3$ buffer containing 100 μM 3-isobutyl-1-methyl-xanthine (IBMX) plus 25 μM forskolin. The brightest regions of interest (ROIs) were selected for quantification of fluorescence from the last image without knowledge of the rate of fluorescence change. The data are presented as the relative fluorescence, which is $100 \times (F_t/F_0)$, where F_t is fluorescence intensity at time t and F_0 is the fluorescence intensity at time zero.

Transfection of CF-null mice

CF-null mice (Ratcliff *et al.* 1993) anaesthetized with Avertin (Bromethol, Bayer Products Ltd) given intraperitoneally (0·02 ml (g body wt)$^{-1}$) were transfected using cationic liposomes complexed with plasmids pTrial10–CFTR2 or pTrial10 either intra-tracheally or intra-nasally. The transfecting agents were prepared by mixing 10 μg of plasmid DNA with 100 nmol of DC-Chol–DOPE and diluted to a volume of 100 μl in Krebs–Hepes buffer (mM): NaCl, 140; KCl, 6; $MgCl_2$, 1; $CaCl_2$, 2; glucose, 10; and Hepes, 10; pH 9. A 100 μl aliquot of the formulation was delivered into the trachea through a blunt-ended steel tube attached to a syringe in 20 μl aliquots over a 30 min period. Animals were allowed to recover from the anaesthetic, returned to their cages and used 2 days later. The procedure for nasal transfection was similar except that the liposomes were diluted in water. Small volumes, 2 μl, were placed in turn on each nostril and were drawn into the airways as the animal breathed, until 100 μl had been administered. The animals were used 2 days after the transfection had taken place.

Measurement of short circuit current (SCC) in murine tracheal epithelium

Wild-type and CF-null mice were killed by exposure to a rising concentration of CO_2 and the trachea and colon quickly removed and placed into fresh, cold Krebs–Henseleit Solution (KHS). The trachea was opened below the larynx by cutting through the centre of the cartilaginous rings. Only one preparation was obtained from each mouse and a piece about 2 mm square was fashioned from the opened tube. This was mounted in an Ussing chamber with a window area of 2·3 mm^2. The tissue was continually short circuited using a WPI dual voltage clamp with series resistance voltage compensation. Each side of the tissue was bathed in KHS, kept at 37 °C, and bubbled with 95% O_2–5% CO_2. SCC was recorded either on a pen recorder or the data were collected and stored using a MacLab in conjunction with an Apple Macintosh Computer. Drugs could be added to either the apical or basolateral side of the tissue by adding them to the reservoirs from which the warmed KHS was circulated. As the tissue window was at the cone-shaped end of each half-chamber the diffusion of drugs into this less well-stirred space was sometimes slow. To overcome this problem, the bath solution was bled from the voltage-sensing electrodes. These consisted of fine polythene tubes filled with KHS which led via a 3 M KCl–agar plug to a 3 M KCl solution containing a calomel cell. As the ends of the voltage-sensing electrodes were closely apposed to the tissue, bleeding for a few seconds drew the bulk bath solution into the tissue region.

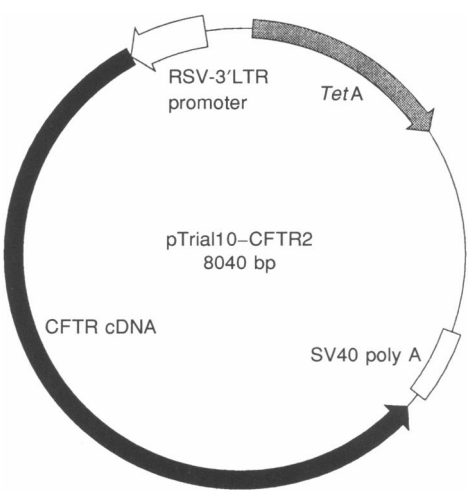

Figure 1. A plasmid map of pTrial10–CFTR2

The Rous sarcoma virus 3′ long terminal repeat promoter (RSV-3′LTR) at position 7858–7407 bp, and the Simian virus 40 polyadenylation signal (SV40 poly(A)) at position 2859–2463 bp control expression of the human CFTR coding sequence (CFTR cDNA) at position 7394–2955 bp. The *Tet*A gene from *E. coli* (89–1276 bp) encodes resistance to the antibiotic tetracycline and contains a mutation of the ATG initiation codon to GTG at position 89 as described in Methods.

Colonic epithelia (20 mm^2) were mounted in Ussing chambers as described previously (Cuthbert *et al.* 1994). The KHS had the following composition (mM): NaCl, 118; KCl, 4·7; $CaCl_2$, 2·5; $MgSO_4$, 1·2; KH_2PO_4, 1·2; $NaHCO_3$, 25·0; and glucose, 11·1. The solution was bubbled with 95% O_2–5% CO_2 and had a pH of 7·4 at 37 °C.

Experimental criteria for the inclusion of data from tracheas

While the tracheal preparations were stabilizing, amiloride (100 μM) and forskolin (10 μM) were applied to the colons from the same mouse followed by frusemide (furosemide; 1 mM). In CF colons, forskolin causes a decrease in SCC due to potassium secretion which is reversed by frusemide (Cuthbert *et al.* 1994). This procedure was used as an internal control for the genotype determined with the polymerase chain reaction and Southern blotting, but no further use was made of the colonic tissues.

Each tracheal epithelium was exposed in turn to a mixture of TBHQ (25 μM) and A23187 (1 μM) on both sides, then forskolin (10 μM) on both sides, and finally frusemide (1 mM) on the basolateral side. Amiloride (100 μM) was added apically either at the beginning of the experiment or after the response to TBHQ–A23187 had been established. If the SCC response to TBHQ–A23187 failed to increase by more than 15 $\mu A\ cm^{-2}$ (approximately 1 in 5 of all mice) the tissue was abandoned. Peak responses to TBHQ–A23187 could reach 70 $\mu A\ cm^{-2}$ and when they were less than 15 $\mu A\ cm^{-2}$ it was unsafe to assume that the calcium stores had been discharged. For tracheas meeting the inclusion criterion the response to TBHQ–A23187 was recorded both at the peak SCC and again after 30 min when forskolin was added followed by frusemide (see Fig. 6). In this study forty-three mice (9 wild-type, 34 CF) met the above inclusion criteria but a further four were eliminated from the analysis. All four were from the groups receiving transfection by intra-tracheal instillation of pTrial10 or pTrial10–CFTR2 and showed unusual behaviour, basal SCCs were 3–4 times greater than normal, responses to TBHQ–A23187 did not decay with time and the responses to forskolin were greater than the group mean by 8–20 times the S.D., clearly representing another population. We suspect that tracheal damage, either during transfection or mounting, was responsible.

Statistical treatment of data

Student's t test was used to compare different sets of data and $P < 0{\cdot}05$ was taken to indicate a significant difference.

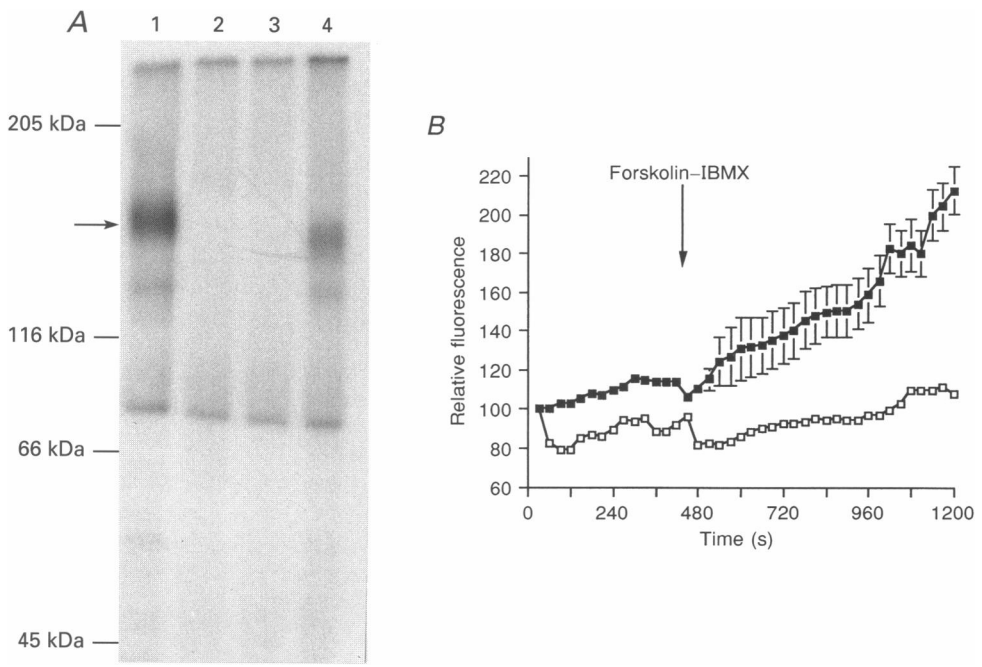

Figure 2. Expression of CFTR from plasmid pTrial10–CFTR2

A, immune precipitation of CFTR using anti-CFTR antibody MATG1104. Lane 1, C127–CFTR cells (Marshall *et al.* 1994) expressing CFTR. Lane 2, untransfected HEK 293 cells. Lane 3, HEK 293 cells transfected with pTrial10. Lane 4, HEK 293 cells transfected with plasmid pTrial10–CFTR2. The arrow indicates the diffuse 165 kDa CFTR band in lane 1. The slight difference in migration observed for human CFTR expressed in the murine C127–CFTR cells and the HEK 293 cells, may reflect differences in glycosylation. The positions of the molecular mass markers are shown. *B*, functional analysis of CFTR using the SPQ fluorescence assay. The change in fluorescence of SPQ is shown for HEK 293 cells transfected with plasmid pTrial10 (□) and plasmid pTrial10–CFTR2 (■). At 120 s the perfusing buffer was changed from one containing NaI to one containing $NaNO_3$ and at the arrow forskolin (25 μM) and IBMX (100 μM) were added to the NO_3^- buffer. Relative fluorescence values are shown as means ± S.E.M. for 56 regions of interest (ROIs) transfected with pTrial10–CFTR2, and 63 ROIs for cells transfected with pTrial10. For the latter the S.E.M. values fall within the symbols. Note each ROI corresponds normally to a single cell or occasionally to a pair of cells. These were always chosen without prior knowledge of the change in fluorescence which had occurred.

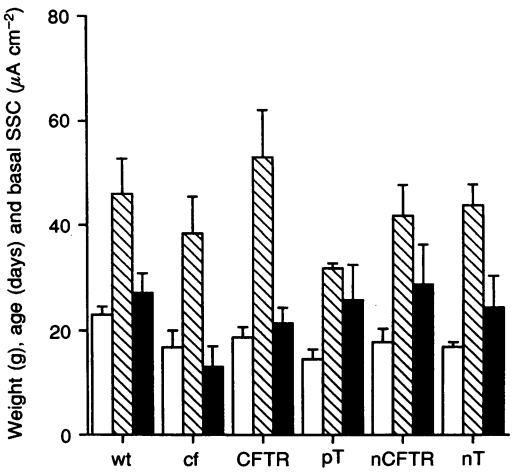

Figure 3. Basal characteristics of tracheas
Weights (g; □), age (days; ▨) and basal SCC (μA cm^{-2}; ■) for tracheas from the 6 groups of mice used in the study. Means ± S.E.M. are given. The number of animals in each group (n) were as follows: wt (wild-type untreated), $n = 9$; cf (CF-null untreated), $n = 7$; CFTR (CF-null tracheally lipofected with pTrial10–CFTR2), $n = 5$; pT (CF-null tracheally lipofected with pTrial10), $n = 6$; nCFTR (CF-null nasally lipofected with pTrial10–CFTR2), $n = 6$; and nT (CF-null nasally lipofected with pTrial10), $n = 6$. Basal SCC was significantly less in the cf group compared with the wt group ($P < 0{\cdot}05$). Basal SCCs in the treated groups were not sigificantly different from either the wt or cf values.

RESULTS

Expression of functional CFTR from pTrial10–CFTR2 *in vitro*

HEK 293 kidney epithelial cells were transfected with plasmids pTrial10 and pTrial10–CFTR2 *in vitro* as described in Methods. CFTR protein expression was detected by immunoprecipitation (Fig. 2*A*). Cells transfected with pTrial10–CFTR2, but not those transfected with pTrial10, produced the highly glycosylated 165 kDa CFTR protein.

To show that the CFTR protein formed in HEK 293 cells functioned as a chloride conductance, SPQ fluorescence microscopy was used as described in Methods. Cells transfected with pTrial10–CFTR2, but not those treated with pTrial10, showed a marked increase in fluorescence when stimulated with forskolin and IBMX (Fig. 2*B*).

Observations on murine tracheal epithelium

Baseline characteristics of wild-type and CF-null mice. Data were obtained for tissues from thirty-nine mice; thirty CF-null mice and nine with wild-type characteristics. Those with wild-type characteristics comprised four homozygotes and five heterozygotes and data for these two groups have been pooled since their electrophysiological properties could not be distinguished (Ratcliff *et al.* 1993; Colledge *et al.* 1995; Cuthbert, Halstead, Ratcliff, Colledge & Evans, 1995).

The CF mice were allotted to one of five groups while wild-type mice formed a sixth group. The groups, of size n, were designated as follows: (a) wild-type untreated (wt), $n = 9$; (b) CF-null untreated (cf), $n = 7$; (c) CF-null treated by tracheal instillation of pTrial10–CFTR2 (CFTR), $n = 5$; (d) CF-null treated by tracheal instillation of pTrial10 (pT), $n = 6$; (e) CF-null treated by nasal instillation of pTrial10–CFTR2 (nCFTR), $n = 6$; and (f) CF-null treated by nasal instillation of pTrial10 (nT), $n = 6$. Figure 3 shows the weights, ages and baseline SCC values of the six groups. All animals were in the age range of 25–89 days with a mean age of 43·3 ± 2·3 days. All groups had a mean age close to this value except pT, which were younger with the ages closely clustered. All the CF groups (b–f) had mean weights less than those of the wild-type group. This is expected since CF mice fail to thrive as well as wild-type and achieve lower weights at a comparable age. Basal SCC was significantly lower ($P < 0{\cdot}05$) in the untreated null animals than in the wild-type group, while the other CF groups all had basal SCC values similar to those of wild-

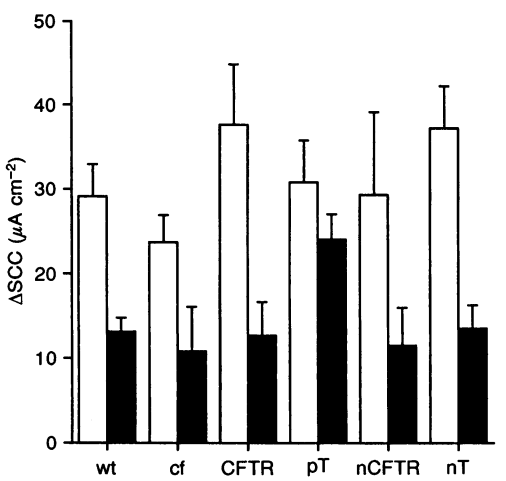

Figure 4. Effects of TBHQ–A23187
The increase in SCC caused by TBHQ–A23187 applied at time zero (□) and the SCC increase remaining after a further 30 min (■). The numbers and codings in each of the 6 groups are as in Fig. 1.

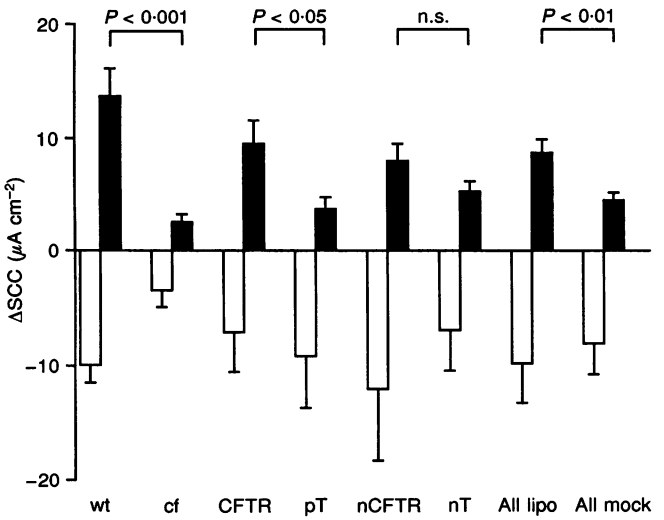

Figure 5. Effects of amiloride and forskolin on tracheas

Changes in SCC (ΔSCC) caused by amiloride (100 μM; □) and forskolin (10 μM; ■). Means + S.E.M. are given. The numbers of observations in each of the 6 groups are given as in Fig. 1. The P values at the top of the diagram refer only to the values of the current increases caused by forskolin. To the right is shown the result of combining data for all the transfected animals (11 with pTrial10–CFTR2 (all lipo) and 12 with pTrial10 (all mock)). The value of the response to amiloride in the wt group was significantly greater than in the cf group ($P < 0{\cdot}05$).

type. All the groups (c–f) had been treated in some way, whereas groups (a) and (b) were untreated.

Figure 4 shows the combined effect of TBHQ and A23187 on SCC in the six groups of tracheas. All groups achieved a mean initial peak value close to 30 μA cm^{-2}, which was twice the exclusion value (15 μA cm^{-2}), the level at which tissues were abandoned. Over a period of 30 min in the presence of the calcium mobilizing agents, the SCC fell to about 12 μA cm^{-2} above baseline, except the pT group where the current was more maintained. There was no obvious reason for this difference.

Amiloride and cAMP mediated responses in wild-type and CF tracheal epithelia. Figure 5 gives the changes in SCC caused by amiloride and forskolin in the six groups of murine tracheas. The response in the cf group to amiloride was significantly less than in the wt group (3·5 ± 1·5 *versus* 9·9 ± 1·5 μA cm^{-2}, means ± S.E.M., $P < 0{\cdot}05$). However, basal SCC was also less in the cf group compared with wt (13·0 ± 3·6 *versus* 27·1 ± 3·5 μA cm^{-2}, means ± S.E.M., $P < 0{\cdot}05$; Fig. 3) so that the percentage inhibition of the basal SCC by amiloride was similar in the two groups (26·7% in cf and 36·6% in wt). Nevertheless it appears that

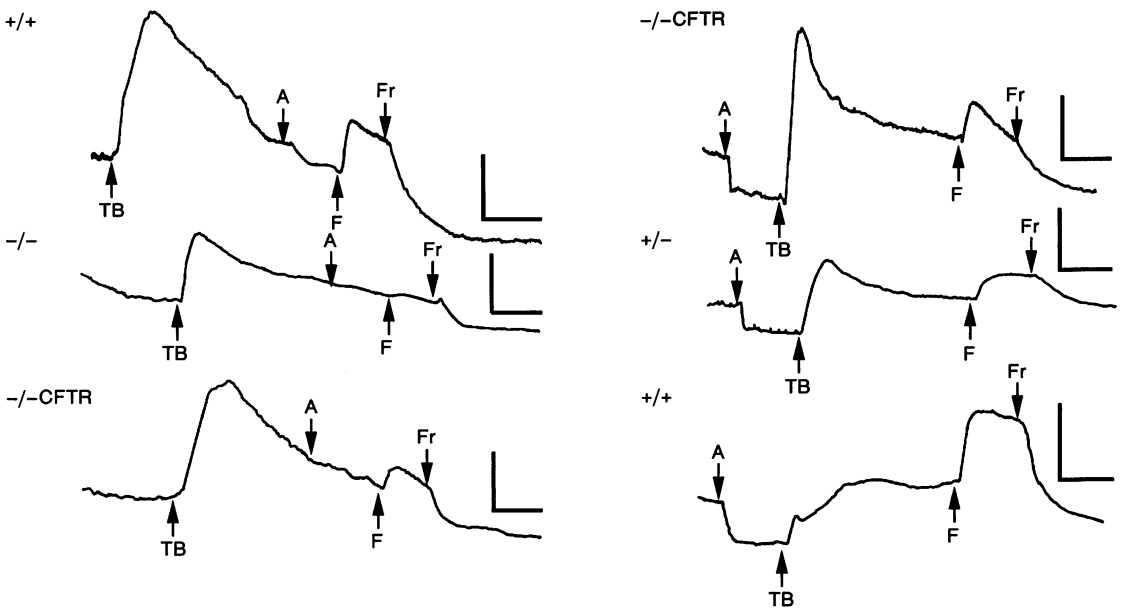

Figure 6. Examples of records from wt and CF-null tracheas

Two of the null tracheas had been transfected with pTrial10–CFTR2 2 days previously. Genotypes are indicated on each trace. In 3 experiments amiloride was added before TBHQ–ionomycin (right traces), while in the others it was added afterwards (left traces). All calibrations are 0·5 μA and 10 min. Abbreviations are: A, amiloride; TB, TBHQ–A23187; F, forskolin; Fr, frusemide; +/+, wild-type homozygote; +/−, wild-type heterozygote; −/−, CF null; −/−CFTR, CF-null transfected with pTrial10–CFTR2.

CF tracheas are hypo-absorbing sodium compared with wt as shown previously (Hyde *et al.* 1993). The amiloride-sensitive SCCs in groups c–f were similar to those of wt (i.e. group a) tracheas. All of these CF groups had been treated, but two groups (pT and nT) had not received transfection with a CFTR-expressing plasmid so that the increase in the amiloride response cannot be related to CFTR. The amiloride-sensitive current in groups CFTR and nCFTR were not different from wt tracheas.

Responses to forskolin after amiloride and TBHQ–A23187 pretreatment allow wt and cf tracheas to be differentiated. Forskolin increased SCC in cf by $2{\cdot}5 \pm 0{\cdot}6$ μA cm^{-2} compared with $13{\cdot}7 \pm 2{\cdot}3$ μA cm^{-2} in wt, these values being significantly different ($P < 0{\cdot}001$).

Two days following tracheal transfection of CF-null mice with pTrial10–CFTR2 the animals were killed and the tracheas removed for assessment. The response to forskolin following the standard protocol to mobilize calcium was $9{\cdot}5 \pm 2{\cdot}0$ μA cm^{-2} in the CFTR group, compared with $2{\cdot}5 \pm 0{\cdot}6$ μA cm^{-2} in the cf group, these values being significantly different ($P < 0{\cdot}01$). However, it is more appropriate to compare the group transfected by instillation of pTrial10–CFTR2 into the trachea with a group treated with the plasmid vector alone (pTrial10). Compared in this way the group receiving pTrial10–CFTR2 had a significantly greater response to forskolin than the pTrial10 group ($9{\cdot}5 \pm 2{\cdot}0$ *versus* $3{\cdot}7 \pm 1{\cdot}1$ μA cm^{-2}, $P < 0{\cdot}05$). Instillation of the CFTR-containing plasmid via the nose also significantly increased the forskolin response in the trachea (group nCFTR) compared with untreated CF tracheas ($8{\cdot}0 \pm 1{\cdot}4$ *versus* $2{\cdot}5 \pm 0{\cdot}6$ μA cm^{-2}, $P < 0{\cdot}01$), although the response was not significantly larger when compared with the more appropriate control group in which pTrial10 was instilled via the nose ($8{\cdot}0 \pm 1{\cdot}4$ *versus* $5{\cdot}3 \pm 0{\cdot}8$ μA cm^{-2}). It is known that plasmid DNA reaches the trachea by the nasal route, as an equal volume of Methylene Blue solution instilled in the same way stains the trachea and the lungs, although exposure is less than by direct instillation in the trachea because of losses in the nasal passages. Combining the data for all mice transfected with pTrial10–CFTR2 by either route (all lipo, $n = 11$) with those transfected with pTrial10 (all mock, $n = 12$), there was a significant difference with a higher probability than with the tracheally transfected group alone ($8{\cdot}7 \pm 1{\cdot}1$ μA cm^{-2} compared with $4{\cdot}5 \pm 0{\cdot}7$ μA cm^{-2}, $P < 0{\cdot}01$). All the data from the transfection studies are given in Fig. 5.

At the end of each experiment frusemide (1 mM) was added to inhibit chloride secretion by inhibiting the Na^+–K^+–$2Cl^-$ cotransporter, but complete inhibition of SCC was not seen in any group, either because chloride ions can enter the cells by other than on the cotransporter or other unidentified currents were present. However, there was no significant difference between the degree of frusemide inhibition of SCC in any of the groups, which were as follows: wt, $57{\cdot}0 \pm 5{\cdot}7$%; cf, $63{\cdot}3 \pm 15{\cdot}9$%; CFTR, $67{\cdot}2 \pm 6{\cdot}4$%; pT, $45{\cdot}3 \pm 11{\cdot}9$%; nCFTR, $76{\cdot}0 \pm 9{\cdot}8$; and nT, $44{\cdot}2 \pm 13{\cdot}2$%. Traces typical of the SCC records from individual experiments are given in Fig. 6 where the effects of TBHQ–A23187, amiloride, forskolin and frusemide are shown.

Other approaches used to dissect components of the chloride secretory response. Initial attempts to deplete the calcium stores were made with thapsigargin. Like TBHQ, thapsigargin increased SCC after which responses to forskolin could be obtained in wild-type tracheas. However, thapsigargin proved to be extremely difficult to remove from the walls of the apparatus so there were apparent effects on current in subsequent experiments before agents were added. Consequently TBHQ was used in preference to thapsigargin. An alternative strategy was to use BAPTA to chelate calcium ions intracellularly. However it was not possible to differentiate between calcium-independent responses, and responses in which the intracellular buffer had become saturated so revealing a calcium-dependent current.

A further alternative was to use a blocking agent of calcium-sensitive chloride channels, and 4,4′diisothiocyanate-stilbene-2,2′-disulphonic acid (DIDS) has been reported to have this effect (Clarke, Grubb, Yankaskas, Cotton, McKenzie & Boucher, 1994). Figure 7 shows results from experiments carried out in the presence of DIDS (100 μM). In large CF

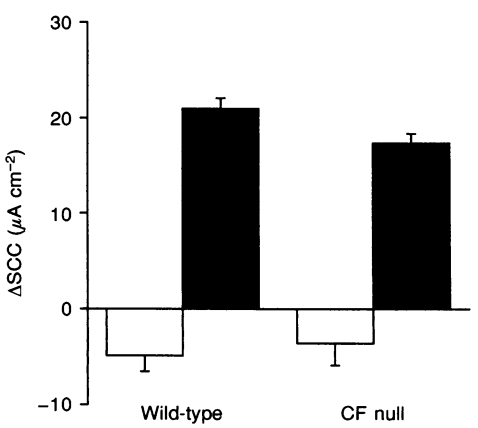

Figure 7. Effects of DIDS

Responses to amiloride (100 μM; □) and forskolin (10 μM; ■) in the presence of DIDS (100 μM) for tracheas from 3 wild-type and 3 CF-null mice.

mice only the calcium component of the forskolin response should be present which will, theoretically, be blocked by DIDS. It is seen in Fig. 7 that the responses in the presence of DIDS were considerably larger than after treatment with TBHQ–A23187, especially for CF-null tracheas ($17{\cdot}4 \pm 1{\cdot}0$ *versus* $2{\cdot}5 \pm 0{\cdot}6\ \mu A\ cm^{-2}$, $P < 0{\cdot}001$). Thus it was not possible to use this method to differentiate wild-type and CF tracheas.

DISCUSSION

The ion-transporting activity of airway epithelia has many components, including CFTR-dependent and -independent chloride secretory activity together with electrogenic sodium absorption. Methods have been developed to dissect these components in the tracheal epithelium of the mouse, both wild-type and a transgenic animal with a CF-null mutation. CF not only eliminates the possibility of one component of chloride secretion but other transporting activities show adaptations to the absence of CFTR. We have investigated these and explored the possibility of restoring normal physiological function by transfection using a new plasmid complexed with cationic lipids. The basal transporting activity (SCC) of the tracheal epithelium in the six groups were not different except for the cf group, where the amiloride-sensitive current was significantly ($P < 0{\cdot}05$) less than in the wt group, indicating that in CF animals the tracheas are hypo-absorbing sodium, a feature we have previously reported for CF-null and ΔF508 CF animals (Hyde *et al.* 1993; Colledge *et al.* 1995), while nasal epithelia show an enhanced amiloride-sensitive sodium current (Grubb, Vick & Boucher, 1994). We have not shown here that transfection with CFTR restores the wild-type level of sodium absorption, even though responses in the treated animals were not different from the wild-type, as this occurs even when treatment was with pTrial10.

In this study one aim was to establish a reliable test to determine phenotype using the mouse trachea. Cultured airway epithelia derived from the nasal passages or the trachea of CF mice fail to respond to forskolin, yet do respond to the calcium ionophore, ionomycin, the latter effect being blocked by DIDS. It is concluded from these earlier studies that CF cells have a Ca^{2+}-regulated Cl^- conductance (Clarke, Grubb, Gabriel, Smithies, Koller & Boucher, 1992; Clarke *et al.* 1994). However, the situation in fresh, intact epithelium from the trachea is different from the properties of cultured tracheal epithelia. First, only in tracheas from young mice is there a detectable difference in the chloride secretory responses to forskolin between wild-type and CF animals (Hyde *et al.* 1993; Colledge *et al.* 1995). Further, forskolin increases intracellular calcium (Ca_i^{2+}) in freshly isolated cells from both CF and wild-type tracheas, the response disappearing if the cells are cultured for a few days (McCann, Bhalla & Welsh, 1989; Grubb *et al.* 1994). Therefore, a major problem is to distinguish the CFTR component of the forskolin response from the calcium component. Two approaches were used: either to eliminate the calcium component or to maximally stimulate it before measuring the cAMP contribution. The latter approach allowed wild-type and CF tracheas from mice of any age to be distinguished according to genotype. Although it is not known by what mechanism forskolin increases Ca_i^{2+} it seems probable that cAMP influences Ca^{2+} release, as has been shown in other systems (Burgess, Bird, Obie & Putney, 1991). For this reason it was considered important to use a Ca^{2+}-ATPase inhibitor to empty intracellular stores. Originally, thapsigargin was chosen because of proven effects on epithelia (Brayden, Hanley, Thastrup & Cuthbert, 1989) but because of difficulties with adhesion to the chambers an alternative, TBHQ, was used. This agent has been shown to be as equally effective as thapsigargin (Oldershaw & Taylor, 1990) and to act on epithelia (Chao *et*

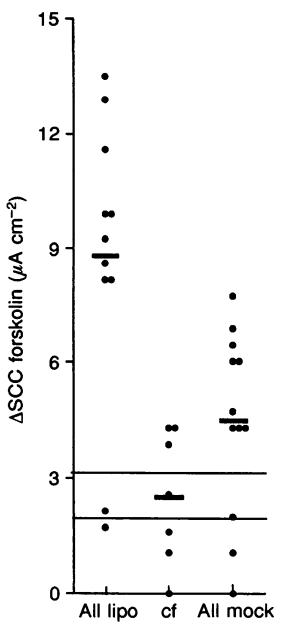

Figure 8. Scatter diagram showing the changes in SCC in response to forskolin in the tracheas of CF mice

Tracheas were transfected with either pTrial10–CFTR2 (all lipo) or pTrial10 (all mock), or were untransfected. Mean values are shown by bars and the horizontal lines indicate ± S.E.M. for the untreated group of CF-null mice (cf).

al. 1995) The combination of TBHQ to release Ca_i^{2+} stores, together with A23187 to promote calcium influx, was considered to provide conditions in which cAMP could not cause a further increase in Ca_i^{2+}. TBHQ and A23187 together produced a large increase in current which declined slowly over 30 min, at which time forskolin was added. Under these conditions and in the presence of amiloride, the responses of wild-type and CF tracheas to forskolin were easily differentiated. CF tracheas still maintained a small response (*ca* 2·5 μA cm^{-2}), but the origins of this current remain unknown. Two other attempts to eliminate the effects of forskolin-released calcium were not successful. When using DIDS to block Ca^{2+}-sensitive chloride channels in CF tracheas the responses to forskolin were significantly ($P < 0{\cdot}001$) greater than those obtained using the TBHQ–A23187 protocol. Thus DIDS is not a sufficiently specific or active antagonist to be useful. Similarly we rejected the use of the Ca_i^{2+} chelator BAPTA, because while it reduced the responses to forskolin there was no way to differentiate whether the residual responses were CFTR mediated or the capacity of the chelator to bind Ca^{2+} was saturated.

By instilling liposomes in the trachea it was possible to increase significantly the response to forskolin in CF tracheas with pTrial10–CFTR2 into the wild-type range. When pTrial10–CFTR2 liposomes were instilled nasally, the forskolin-sensitive current of the trachea was significantly greater than that of CF-null tracheas ($P < 0{\cdot}05$), although not significantly greater compared with those of CF mice receiving pTrial10. However, comparing tracheas from all lipofected animals with those from animals receiving mock transfection showed there was a significant ($P < 0{\cdot}01$) increase in the response to forskolin. When the data are presented as a scatter diagram (Fig. 8) it can be seen that nine of the eleven CF mice receiving pTrial10–CFTR2 had cAMP-sensitive chloride currents in the normal range.

The plasmid used in this study was specifically designed for use in a clinical trial to be delivered complexed with cationic lipsomes to the nasal epithelium of CF patients. In an earlier study (Hyde *et al.* 1993) plasmid pREP8–CFTR, containing the ampicillin-resistant gene, was used. It is a formal possibility that such a plasmid could be transmitted to resident airway bacteria and could lead to low-level resistance to β-lactam antibiotics. Plasmid pTrial10–CFTR2 encodes a gene for resistance to tetracycline, an antibiotic unlikely to be used as a treatment for lung infections,

It is known that expression of bacterial genes can raise an immune response in animal models (Bronte *et al.* 1995). In the present study, several steps were taken to limit the possible expression of the tetracycline-resistant gene in human airway cells during a clinical trial. These included the subcloning of the bacterial gene in the opposite direction to the CFTR gene and the screening of the sequence to avoid inclusion of potential eukaryotic expression signals. As a further precaution, the ATG initiation codon of the tetracycline-resistant gene was changed to GTG. Whereas bacterial ribosomes are able to initiate at both AUG and GUG, eukaryotic ribosomes initiate exclusively at AUG codons (Stewart, Sherman, Shipman & Jackson, 1971; Sherman, McKnight & Stewart, 1980). For example, a mutation in the CFTR gene in which the initiation codon is changed from ATG to GTG has been identified in three CF patients (Cheadle, Belloni, Ferrari, Millar-Jones & Meredith, 1994).

The DC-Chol–DOPE liposomes used in this study are approved for human use and have been used in several gene-therapy clinical studies (Nabel *et al.* 1993; Caplen *et al.* 1995). These lipsomes successfully deliver plasmid pTrial10–CFTR2 to epithelial cells grown in culture as shown by immune precipitation of CFTR and the presence of a cAMP-sensitive chloride conductance, measured by SPQ fluorescence. While this constitutes proof of principle for the effectiveness of this new plasmid, a whole-animal study is required before a clinical trial.

As already mentioned, adenoviral vectors are limited in use because of immunological problems and the risk of generating a replication-competent infectious virus by combination with ubiquitous wild-types (Alton & Geddes, 1994). The greatest drawback with the liposome approach is the low efficiency of delivery. Apparently it is only necessary to transfect 5% of cells to restore the CFTR-dependent chloride conductance in xenografts (Goldman, Yang & Wilson, 1995), yet in human CF airways there is not only a failure of cAMP-dependent chloride conductance but also an increase in electrogenic sodium absorption. The latter contributes substantially to the dehydration of mucus, perhaps more so than the failure of chloride secretion. When CFTR chloride channels are co-expressed with amiloride-sensitive sodium channels the sodium conductance is limited compared with that found when expressed alone (Stutts *et al.* 1995). Thus CFTR channels regulate sodium channels by an unknown mechanism which apparently influences mean open time (Disser & Fromter, 1989). Thus while transfection of 5% of airway cells restores chloride conductance this level of transfection has no effect on sodium transport (Goldman *et al.* 1995). Therefore CFTR expression will need to be greater than 5% of normal to affect clinical improvement, although restoration of cAMP-sensitive chloride conductance is achieved at low expression levels.

In conclusion the method we have evolved here does allow the CFTR-dependent component of the tracheal response to be determined without interference from the calcium-dependent component, making the CF-null trachea a useful tissue in which to investigate gene therapy *in vivo*. Finally, if the fraction of responding animals could be reproduced in man, and if repeated doses produce no untoward effects, a small step towards clinical treatment will have been made. The report of a successful clinical trial with pTrial10–CFTR2 will be published elsewhere.

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Author's email address

A. W. Cuthbert: awc1000@cam.ac.uk

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