Cell, Vol. 64, 681–691, Feb. 22, 1991, Copyright © 1991 by Cell Press

Expression of the Cystic Fibrosis Gene in Non-Epithelial Invertebrate Cells Produces a Regulated Anion Conductance

Norbert Kartner,* John W. Hanrahan,† Tim J. Jensen,* A. Leonard Naismith,* Shizhang Sun,* Cameron A. Ackerley,* Evangelica F. Reyes,* Lap-Chee Tsui,*‡ Johanna M. Rommens,* Christine E. Bear,*§ and John R. Riordan*‖
*Research Institute
The Hospital for Sick Children
Toronto, Ontario M5G 1X8
§Department of Physiology
‡Department of Molecular and Medical Genetics
‖Department of Biochemistry and Clinical Biochemistry
University of Toronto
Toronto, Ontario M5S 1A8
†Department of Physiology
McGill University
Montreal, Quebec H3G 1Y6
Canada

Summary

The nature of involvement of the cystic fibrosis gene product (CFTR) in epithelial anion transport is not yet understood. We have expressed CFTR in Sf9 insect cells using the baculovirus expression vector system. Reactivity with antibodies against 12 different epitopes spanning the entire sequence suggested that the complete polypeptide chain was synthesized. Immunogold labeling showed localization to both cell-surface and intracellular membranes. Concomitant with CFTR expression, these cells exhibited a new cAMP-stimulated anion permeability. This conductance, monitored both by radioiodide efflux and patch clamping, strongly resembled that present in several CFTR-expressing human epithelial cells. These findings demonstrate that CFTR can function in heterologous nonepithelial cells and lend support to the possibility that CFTR may itself be a regulated anion channel.

Introduction

The properties of the product of the cystic fibrosis gene (CFTR) that are predicted by its sequence, and the epithelial tissue specificity of its expression, lead to the suggestion that it may contribute to the abnormal regulation of anion conductance in these tissues (Riordan et al., 1989). The complementation of this regulation of anion conductance in cultured cystic fibrosis (CF) epithelial cells derived from both airways (Rich et al., 1990) and pancreatic ducts (Drumm et al., 1990) after successful expression of exogenous normal CFTR cDNA has been regarded as confirmation of this initial postulate. However, since the CF cells infected or transfected in these experiments possessed at least one type of epithelial Cl^- channel (Schoumacher et al., 1990), little additional insight into the mechanism of restoration of normal activation by CFTR was obtained. With the aim of determining if CFTR is itself capable of constituting a regulated anion conductance pathway in cells with membranes exhibiting few if any ionic currents (Klaiber et al., 1990), we have infected the Sf9 insect cell line derived from ovarian tissue of the Fall armyworm Spodoptera frugiperda (Summers and Smith, 1987) with recombinant baculoviruses containing the CFTR coding sequence. This expression system has been employed most frequently for the generation of large amounts of protein under the control of the powerful, late-acting polyhedrin promoter of Autographa californica nuclear polyhedrosis virus (AcMNPV; Luckow and Summers, 1989). Recently, BEVS (baculorovirus expression vector system) expression has been used to produce a number of different membrane proteins that were shown to be functionally active (Possee, 1986; Domingo and Trowbridge, 1988; Greenfield et al., 1988; George et al., 1989; Johnson et al., 1989; Vialard et al., 1990), including ion channels (Klaiber et al., 1990).

Results

Construction of a CFTR-Expressing Recombinant Baculovirus

A full-length CFTR cDNA (J. M. Rommens and L.-C. Tsui, unpublished data) was cloned into the PstI site of the baculovirus transfer vector, pVL1393 (Luckow and Summers, 1988a). Sf9 insect cells were cotransfected with DNA from this construct and wild-type AcMNPV DNA. Viral stocks were collected from the growth media and used to infect cells for the identification of plaques arising from recombinant CFTR-containing viruses. These were detected both visually, by the absence of polyhedrin-containing occlusion particles, and by probing plaque lifts with a CFTR probe. Six were isolated, but only one of these has been extensively characterized in this study.

Expression of CFTR in Sf9 cells infected with these recombinant viruses was monitored by both RNA blot hybridization (Figure 1A) and immunoblotting (Figure 1B), using an antibody generated against a peptide corresponding to the 16 C-terminal amino acids of CFTR. The fact that very intense bands of the appropriate size were detected in the Northern blots after only 20 min of film exposure indicated a high level of transcription of the CFTR sequence. That these transcripts are successfully translated in the Sf9 cells is evidenced by the strong immunostaining band of approximately 140 kd observed in the Western blot (Figure 1B, lane 3). Since the molecular weight predicted from the primary sequence is about 170 kd, the mobility of the CFTR expressed in Sf9 cells is somewhat greater than anticipated, yielding a relative molecular size estimated at 140 kd. CFTR synthesized endogenously in T84 cells, when detected in a cell membrane fraction with the same antibody (Figure 1B, lane 1), has an apparent size of approximately 170 kd; however, upon N-glycanase treatment to remove N-linked carbohydrate (Figure 1B, lane 2), this size is reduced to a value

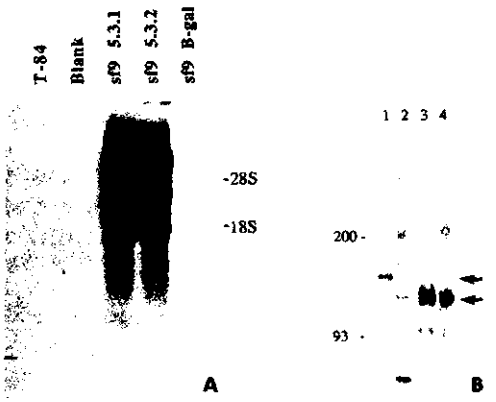


Figure 1. Expression of CFTR mRNA and Protein in Sf9 Cells

(A) Northern blot analysis of CFTR mRNA. Forty-eight hours after infection with recombinant CFTR-baculovirus clones 5.3.1 and 5.3.2 or a β-galactosidase-baculovirus clone, total RNA (15μg) from T84 cells and Sf9 cells was subjected to formaldehyde agarose gel electrophoresis and transferred to nitrocellulose and hybridized with a ^{32}P-labeled CFTR cDNA. The relative positions of the 28S and 18S ribosomal RNAs were as indicated.

(B) Western blot analysis of CFTR protein in T84 cell membranes and Sf9 cell lysates. A crude postmitochondrial membrane was isolated from T84 cell monolayers, and 100 μg of membrane protein was subjected to SDS–PAGE (5% polyacrylamide gel) after incubation without (lane 1) or with (lane 2) N-glycanase to remove N-linked oligosaccharide chains from glycoproteins. A total Sf9 cell lysate was prepared from CFTR-expressing cells, and 15μg of protein was electrophoresed after incubation without (lane 3) or with (lane 4) N-glycanase. Western blots of these gels were probed with an affinity-purified rabbit antibody against residues 1465 to 1480 of CFTR and developed with alkaline phosphatase–conjugated goat anti-rabbit second antibody. Arrows indicate positions of 170 and 140 kd bands.

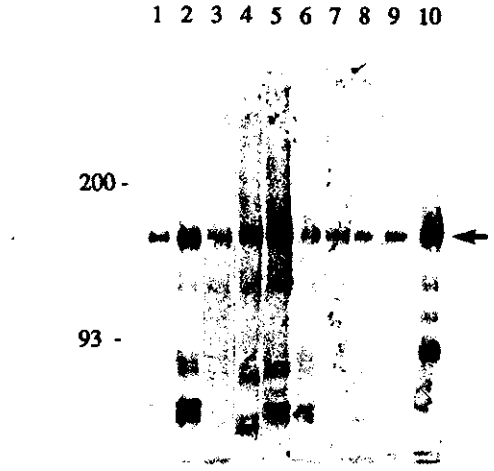


Figure 2. Immunoblotting of CFTR-Infected Sf9 Cell Lysates with Antibodies Recognizing Different Parts of the Protein

Replicate aliquots of the cell lysate containing 15μg of protein were electrophoresed and blotted. The 10 individual blots were probed with affinity-purified rabbit antibodies that had been raised against keyhole limpet hemocyanin-coupled peptides (Marcel et al., 1987) or pGEX (Smith and Johnson, 1988) fusion proteins corresponding to the following segments of the CFTR sequence: lane 1, residues 28–45; lane 2, residues 58–75; lane 3, residues 104–117; lane 4, 279–294; lane 5, 348–698; lane 6, 725–746; lane 7, 712–756; lane 8, 1066–1080; lane 9, 1197–1480; lane 10, 1465–1480. The large polypeptide antigens represented in lanes 5, 7, and 9 were synthesized as bacterial fusion proteins, the others by chemical peptide synthesis.

very close, if not identical, to that of the protein produced by the Sf9 cells. On this basis, it would appear that the complete polypeptide is synthesized in the infected insect cells but little or no oligosaccharide addition occurs. Sf9 cells are known to be incapable of the maturation of complex N-linked oligosaccharide chains (Vialard et al., 1990). Interestingly, when the P-glycoprotein multidrug transporter, which has many structural features in common with CFTR, is expressed with the BEVS in Sf9 cells, it also is not glycosylated (Germann et al., 1990). It is also known that the oligosaccharide moieties of P-glycoprotein play no role in its function (Riordan and Ling, 1985). Further evidence that the complete CFTR polypeptide is synthesized in insect cells is seen in Figure 2, which shows that the same 140 kd band is recognized by 10 different polyclonal antisera raised against different segments of the protein sequence, which span essentially its entire length. The basis of the apparently anomalous mobility of CFTR in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) is presently not understood; however, there is ample precedent for such behavior among membrane proteins. For example, the nucleotide sequence of the photoreceptor cyclic GMP-gated channel predicts a molecular mass of 79,601 daltons, whereas the mobility in SDS–PAGE yields a M_r value of only 63 kd (Kaupp et al., 1989).

Regulated Anion Permeability in CFTR-Infected Cells

CFTR is believed to be involved, directly or indirectly, in the activation of a Cl^- conductance in epithelial cells in which it is normally expressed (Welsh, 1990), and its introduction into CF cells of this type restores defective activation (Rich et al., 1990; Drumm et al., 1990). We have assayed for the presence of a regulated Cl^- conductance pathway in CFTR-infected Sf9 cells by the same techniques that were employed with epithelial cells (Clancy et al., 1990; Venglarik et al., 1990). Such a regulated conductance pathway is not normally present in uninfected Sf9 cells (see Klaiber et al., 1990 and conductance measurements described below).

First, infected cells that had been preloaded with $^{125}I^-$ were stimulated to rapidly increase their intracellular cAMP levels by addition of a cocktail containing forskolin, dibutyryl cAMP, and IBMX, and the time course of isotopic efflux was monitored. Figure 3 shows two important features of $^{125}I^-$efflux in CFTR-expressing cells. First, the basal rate of efflux is greater than in control β-galactosidase–expressing cells, suggesting that CFTR may increase anion permeability to some extent even in the absence of stimulation. It could be argued that this effect is a consequence of perturbation of the plasma membrane of Sf9 cells by the overexpression of CFTR; however, a similar observation was made by Rich et al. (1990) when they introduced CFTR into epithelial cells from CF patients. Second, stimulation causes a substantial increase in the rate of efflux from CFTR-infected cells, but not from

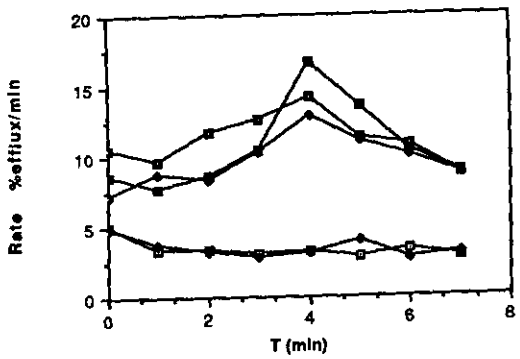


Figure 3. cAMP Stimulation of Radioiodide Efflux from Sf9 Cells

The experiment was performed as described in Experimental Procedures, 36 hr after infection with a CFTR recombinant baculovirus (upper three curves) or a β-galactosidase recombinant baculovirus (lower two curves).

mock-infected cells expressing β-galactosidase. The response of the CFTR-expressing Sf9 cells has a distinctive "signature" that as yet cannot be adequately explained. The stimulated efflux is not instantaneous, but rather builds to a peak over 2–5 minutes. The efflux rate then decays towards the basal unstimulated level within 10 min of the first addition of the cocktail, which is added fresh at each one-minute time point during the time course. Interestingly, this same complex kinetic "signature" is seen in almost precise detail in endogenous CFTR-expressing cells such as the Caco-2 colon carcinoma cell line (C. E. Bear, unpublished data) and in the extensively studied airway epithelial cells of Venglarik et al. (1990). It should be emphasized that this stimulated efflux is similar both quantitatively and qualitatively to that previously characterized by others in CFTR-expressing epithelial cells.

To establish that the appearance of this epithelial-like response correlates with the appearance of CFTR protein, the two parameters were assessed at varying times following infection of the Sf9 cells. Figure 4 represents the results of these experiments. No stimulation of efflux rate from CFTR-infected cells was observed at times earlier than 24 hr after infection (Figure 4A). At this time the presence of the protein is first detected by immunoblotting (Figure 4C). By 36 hr, a quite substantial stimulation of efflux occurs, which is apparently maximal since the curves obtained at 60 hr and at 72 hr are little changed. The amount of CFTR protein continues to increase over this entire period, in agreement with the time course of expression observed for other recombinant proteins driven by the late-expressing polyhedrin promoter. Hence, it would appear that the magnitude of the stimulated efflux is roughly proportional to the amount of CFTR made, at least until a certain level is reached. However, this relationship should not be overemphasized since it does not take into account the localization of the protein, which is certainly not all in the cell surface membrane (see below). The β-galactosidase–infected cells did not respond, regardless of the time post-infection when they were stimulated (Figure 4B).

Many of the epithelial cells in which CFTR is normally expressed have at least two separate Cl^- conductances, the activations of which are mediated by either cAMP or Ca^{2+} ions (Cliff and Frizzell, 1990). The data presented above are consistent with the idea that CFTR contributes to the former. To determine if the CFTR-infected Sf9 cells also exhibit a Ca^{2+} activated anion permeability, similar flux assays were done using the ionophore A23187 as stimulant. Figure 5 shows that there is little, if any, response of the CFTR-infected cells above that of the β-galactosidase–infected cells, consistent with the notion that mutations in the CF gene do not influence the Ca^{2+} activated anion pathway. As in Figure 3, there is greater efflux

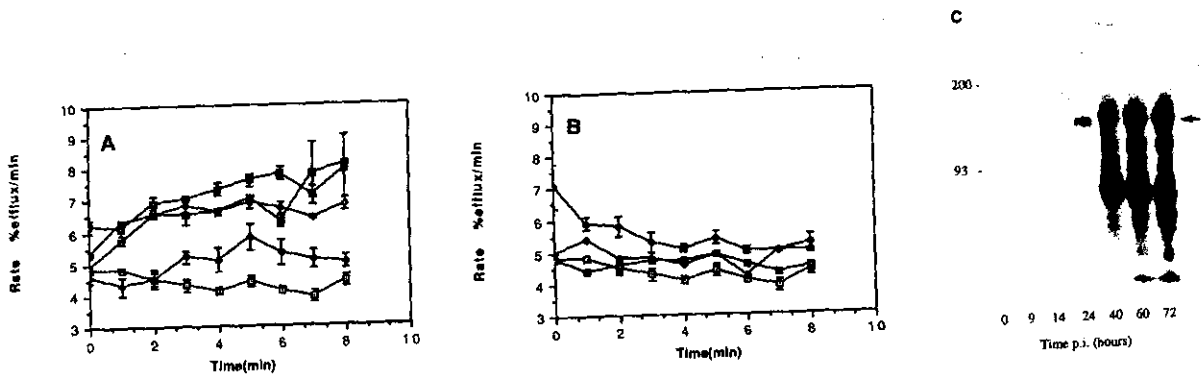


Figure 4. Increase in cAMP-Stimulated ^{125}I Efflux and Amount of CFTR Protein with Time after Infection

(A) cAMP stimulation of radioiodide efflux at varying times after infection with CFTR. Error bars denote standard deviations for triplicate points done in a single representative experiment. Symbols are as follows:
0 time (▣); 24 hr (◆); 36 hr (■); 60 hr (◇); 72 hr (■).
(B) cAMP stimulation of radioiodide efflux at varying times after infection with β-galactosidase. Symbols are as follows:
0 time (▣); 12 hr (◆); 60 hr (■); 72 hr (◇).
(C) Western blot analysis of CFTR protein production at varying times after infection with CFTR. Times post-infection in hours are indicated. The arrow indicates the position of the CFTR polypeptide synthesized in Sf9 cells. The blot was probed with an anti-CFTR mouse monoclonal antibody M3A7 against an epitope between residues 1197 and 1480.

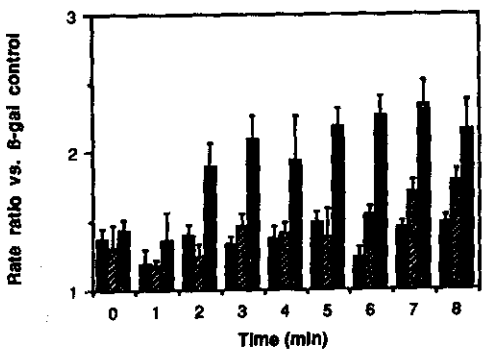
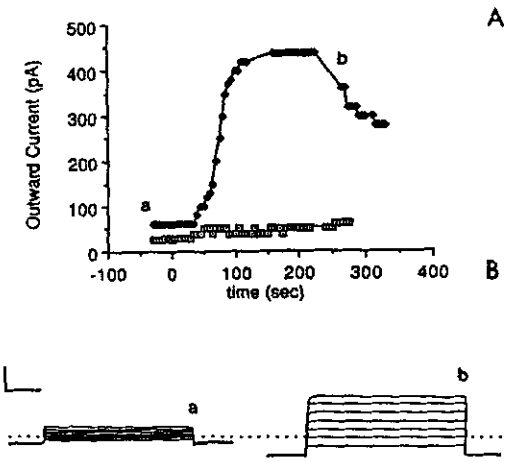


Figure 5. Comparison of the Effects of cAMP and Calcium on $^{125}I^-$ Efflux from CFTR-Infected Sf9 Cells

Relative radioiodide efflux rates from CFTR-infected and β-galactosidase–infected Sf9 cells without stimulation (solid bars) and on stimulation with A23187 (hatched bars) and cAMP (stippled bars).



from CFTR-infected cells than β-galactosidase–infected cells in the absence of any type of stimulation.

Regulated Anion Currents in CFTR-Infected Sf9 Cells

To determine if the permeability changes measured above could also be detected as ionic conductances, the whole-cell patch clamp technique was applied. Stimulation of cells with the same cAMP-elevating cocktail enhanced whole-cell currents in 7 of 14 CFTR-infected Sf9 cells. This frequency is in agreement with previous observations of channel activation in whole-cell recordings of epithelial cells, such as the T84 line (Cliff and Frizzell, 1990). Of the remaining 7 CFTR-infected cells studied, 2 showed spontaneous increases in current without stimulation. The basis of this spontaneous current activation is not clear. None of 9 control cells tested, which had been mock-infected with β-galactosidase–expressing recombinant baculovirus, exhibited a response to the forskolin-containing cocktail.

The results of a typical experiment are shown in Figures 6A and 6B. In agreement with the $^{125}I^-$ efflux experiments, basal current measured at E_m = +30 mV was greater in CFTR-infected cells than in β-galactosidase–infected cells. This difference was significant ($P = 0.001$) for the total number of cells studied. In Figure 6A, it is clear that no change in outward current was detected in the β-galactosidase–infected cells up to 5 min after stimulation. In contrast, outward current was markedly enhanced on stimulation of the CFTR-infected cell. Similar to the iodide efflux response, a lag period of about 1 min preceded the current increase which peaked within 2–4 minutes after treatment. Overall, this time course resembled that observed for cAMP-mediated $^{125}I^-$ efflux in these cells, and it appears to be consistent with the time dependency of cAMP-evoked $^{125}I^-$ efflux from epithelial cells (Venglarik et al., 1990). The currents elicited by the forskolin-containing cocktail did not show any time-dependent voltage effects within the voltage range examined, −40 mV to +30 mV (Figure 6B), another characteristic shared with

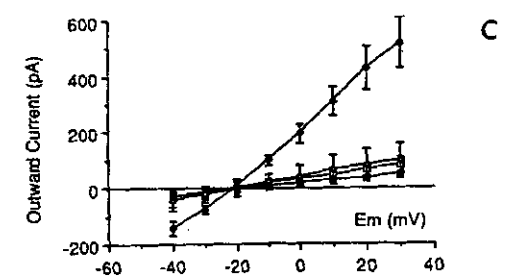


Figure 6. Whole-Cell Cl^- Currents in β-galactosidase and CFTR-infected Sf9 Cells

(A) Time course of whole-cell current measured from control β-galactosidase–infected cell (□) and CFTR-infected cell (◆) following the addition of cocktail containing: forskolin (10 μM), IBMX (1 mM) and dibutyryl cAMP (100 μM). Cocktail was added at time = 0 s. Outward current values measured at E_m = +30 mV.

(B) Whole-cell I–V relationships have been shown at time points (a) and (b), previously indicated on the upper panel for the CFTR-transfected cell. Currents were evoked with depolarizing voltage steps of 10 mV increments from a holding potential of −40 mV to +30 mV. The dotted line indicates the zero current level. The scale for this figure has been shown in the upper left hand corner. The vertical bar indicates 200 pA and the horizontal bar indicates 100ms.

(C) Mean I–V relationships for β-galactosidase–transfected cells (n = 9) before and after cocktail addition (■, □) and responsive CFTR-transfected cells (n = 7) before and after cocktail addition (◆, ◆), respectively. Standard error bars have been shown.

cAMP-evoked currents in epithelial cells (Cliff and Frizzell, 1990).

Mean current–voltage relations were generated in control and CFTR-infected cells (Figure 6C). In the experiments depicted, the current reversal potentials were in the range of approximately −20 mV (near the equilibrium potential for chloride under these conditions), indicating that Cl^- is the predominant current-carrying ion. With additional experiments, a more rigorous comparison between the current–voltage relationships for unstimulated CFTR-infected (n = 8) and β-galactosidase–infected (n = 13) Sf9 cells was possible and revealed that the reversal

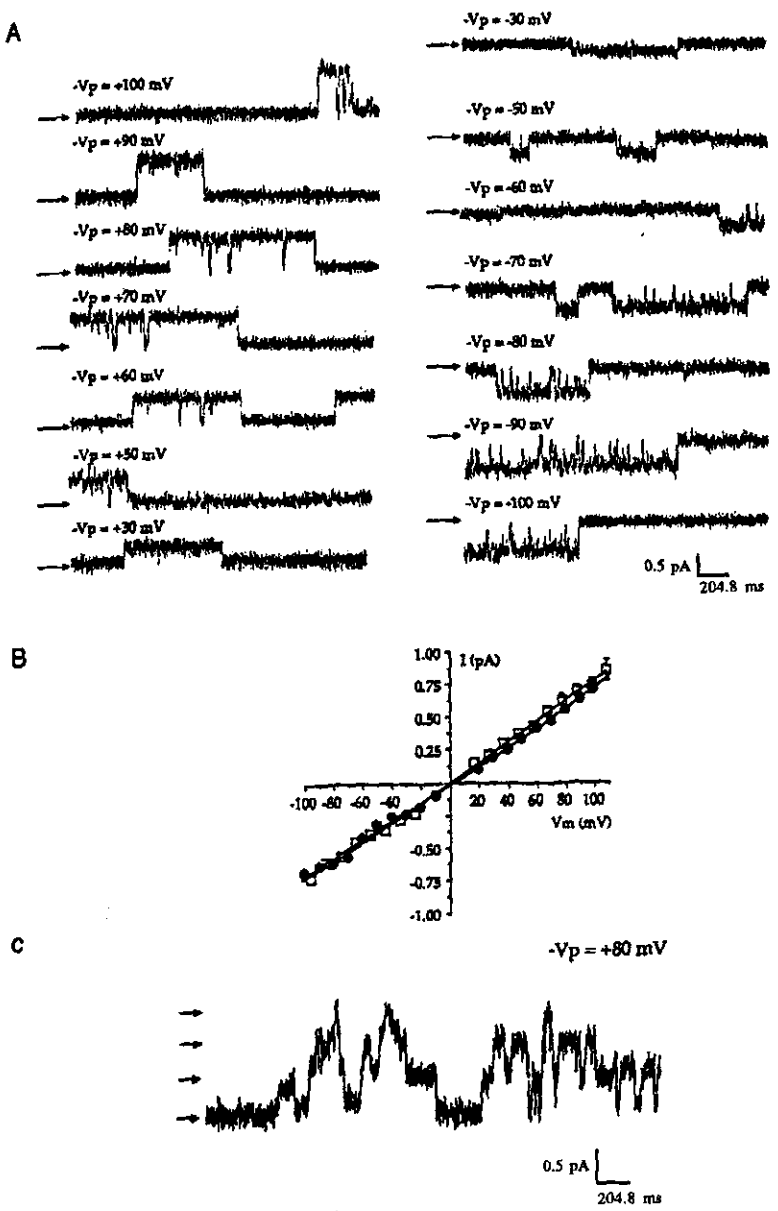


Figure 7. Cell-Attached Recordings on CFTR-Infected Sf9 Cells

(A) Single channel recorded at different voltages on an unstimulated CFTR-infected Sf9 cell.

(B) Current voltage relationships obtained on cAMP-stimulated cells in the cell-attached configuration with (●) 150 mM NaCl or (□) 150 mM N-methyl-D-glucamine chloride in the pipette.

(C) Recording obtained from a cAMP stimulated cell showing wave-like pattern of channel openings.

potentials were −22 mV and −14 mV, respectively, confirming that the predominant conductance path is more anion selective in the former than in the latter.

The anion selectivity of the conductance route in CFTR-infected cells was further substantiated by partial substitution of bath NaCl (135 mM) with sodium gluconate (121 mM). This experimental maneuver resulted in the shift of reversal potential from −22 mV to +10 mV, corresponding to a 1:0.1 permeability ratio for chloride to gluconate (n = 2). Complete replacement of extracellular NaCl with NaI caused a shift in the zero current level to −34 mV (n = 3), corresponding to a selectivity ratio for iodide to chloride of 1.1:1 (data not shown). Because this conductance path is permeable to iodide, it probably accounts for the $^{125}I^-$ fluxes described above. The I–V relationship of cAMP-activated currents was nonrectifying, comparable to that observed for whole-cell currents activated in CFTR-expressing epithelial cells (Cliff and Frizzell, 1990).

Single Channel Studies

Cell-attached patch recording was used to identify the channels that underlie the whole-cell chloride conductance described above. CFTR-infected cells were found to have a high density of low-conductance channels, which were not noticeably voltage-dependent and which usually became inactive when patches were excised. Single channels were observed in 2 of 6 patches on unstimulated CFTR-infected Sf9 cells. Data from one of these is shown in Figure 7A. Hence, in all three assays some spontaneous activity was detected in the CFTR-infected cells. How-

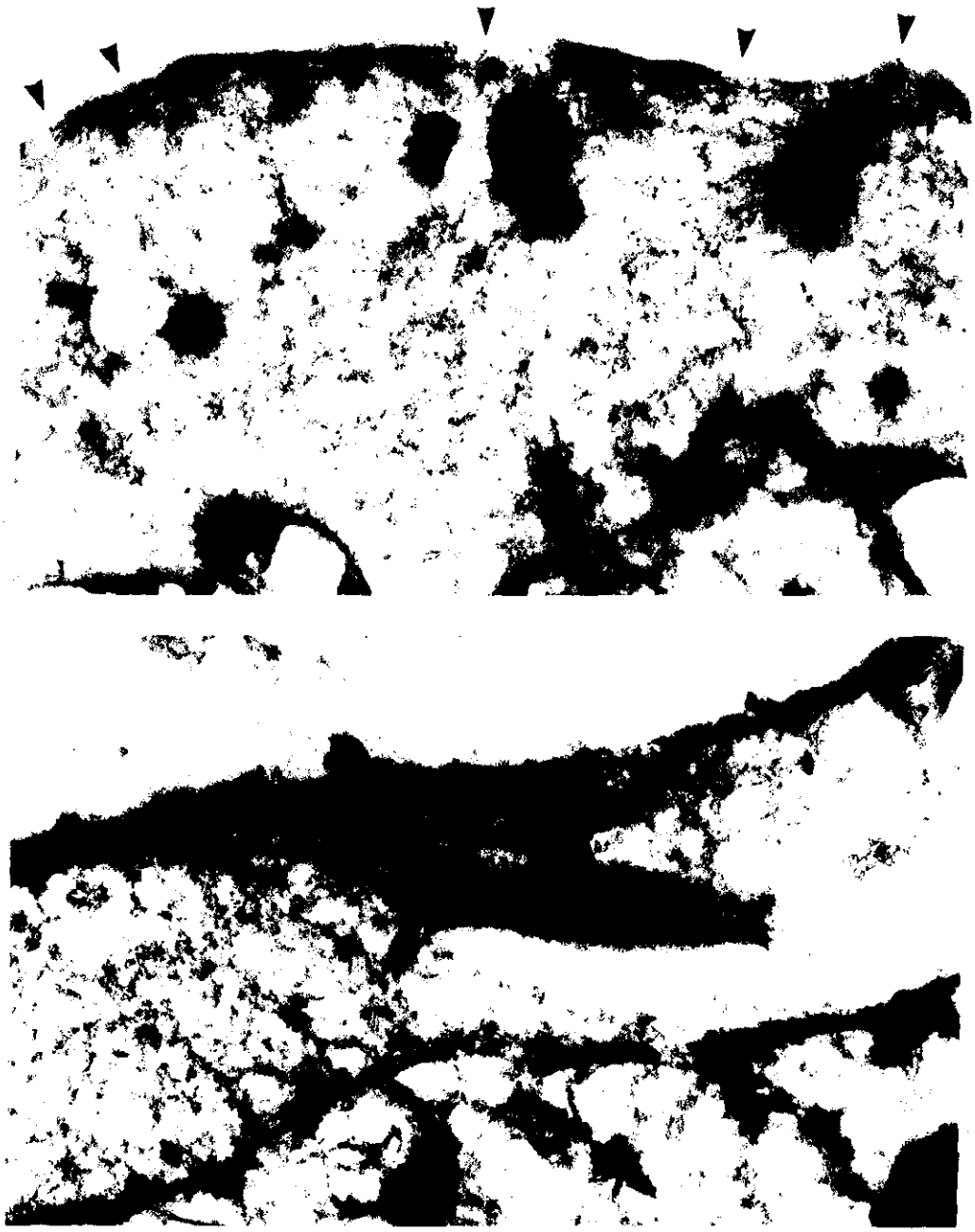


Figure 8. Electron micrographs of Immunogold-Labeled CFTR-infected and Control Sf9 Cells

Top panel shows CFTR-infected Sf9 cell stained with anti-CFTR mouse monoclonal antibody L12B4 (which localizes between amino acids 348 and 698) and 15 nm colloidal gold-labeled second antibody. Arrows indicate plasma membrane labeling, but intracellular membranes, mainly endoplasmic reticulum, are also stained. No staining is seen over the nucleus (not shown). Bottom panel shows mock-infected Sf9 cell treated as above. No staining is apparent in these control cells. Similar results were obtained with the mouse monoclonal antibody, M3A7, which is directed towards a different epitope (between amino acids 1197 and 1480) on the CFTR molecule. Magnification is 25,000×.

ever, on stimulation with cAMP, similar channels were recorded in 45 of 51 patches where their activity was much higher (typically 4–8 active channels per patch), but were not observed in uninfected cells ($n = 5$) or in cells infected with the β-galactosidase gene ($n = 25$). The single channel current–voltage relationship was almost linear when the pipette solution contained 150 mM NaCl (Figure 7B). Ignoring the slight outward rectification, linear regression of the entire I–V curve between −100 mV and +100 mV yields a single channel conductance of 8.4 ± 0.2 pS ($n = 8$). Currents reversed within 2 mV of the membrane potential. Identical I–V relationships were obtained when

the pipette solution containing sodium was replaced with one containing the impermeant cation N-methyl-D-glucamine (150 mM NMDG). The conductance under these conditions was 7.5 ± 1.2 pS (n = 3), and currents reversed at +1 mV after correction for the liquid junction at the agar bridge electrode. In two experiments, it was possible to estimate iodide permeability under bi-ionic conditions using excised patches. Negative currents increased slightly when bath chloride was replaced with iodide, and the reversal potential shifted by 6 mV, which suggests an iodide-to-chloride permeability ratio of approximately 1.2. The channel was relatively insensitive to DIDS (see Experimental Procedures) and remained active for several minutes at moderate potentials (± 60 mV) when 100 μM DIDS was included in the pipette solution (n = 13). However, DIDS did appear to cause some decrease in open probability at large positive potentials. Kinetics have not yet been studied quantitatively; however, one property was obvious immediately: channels frequently opened in distinctive waves of activity, as described previously for low conductance chloride channels in rat pancreatic duct (Gray et al., 1988) and the human colonic cell line T84 (Tabcharani et al., 1990).

We tested for the presence of outwardly rectifying anion channels in 14 excised patches by clamping the membrane potential at +120 mV, a maneuver that normally activates outward rectifiers in other cell types. However, outwardly rectifying anion channels were not observed in these or in any of the approximately 60 cell-attached patches studied under various conditions. Two other channels were observed occasionally: one had a conductance of approximately 40 pS and became active at large hyperpolarizing potentials (>−60 mV). It may be cation selective, because it was observed when the pipette solution contained sodium but not NMDG. The other unidentified channel type had very low conductance (<4 pS), and it was observed only rarely. However, its density was difficult to assess, because it could only be resolved when the seal resistance was extremely high. Both these channel types appear to be endogenous to Sf9 cells because, unlike low conductance chloride channels, they were observed in both CFTR-infected and control β-galactosidase–infected cells.

Localization of CFTR Expressed in Sf9 Cells

Several different BEVS-expressed proteins at least partially localize to the same cellular compartment(s) in Sf9 cells as in the tissue where they are endogenously expressed (Luckow and Summers, 1988b). However, since very high-level expression can occur under the control of the polyhedrin promoter, there may be spillover into compartments other than the native one (Luckow and Summers, 1989). We have used antibodies to CFTR to detect localization of the protein by immunogold staining (Figure 8). At 36 hr after infection, when substantial amounts of protein were already detected in immunoblots (Figure 4) and there was a strong response to stimulation of ion transport, immunogold labeling could be seen in several cellular compartments but not at all in mock-infected cells. As expected from the regulated anion permeability imparted on the cells, gold particles are seen in the cell surface membrane; they are also present in association with intracellular membranes.

Discussion

We have shown that introduction of an expressible human CFTR cDNA into nonepithelial cells of invertebrate origin, which normally do not exhibit voltage-activated ionic currents (Klaiber et al., 1990), produces a regulated anion conductance. This conductance has properties in common with that expressed endogenously in several epithelial cell types containing CFTR and which is believed to account for cAMP-stimulated Cl^- secretion. Whole-cell currents in epithelial cells have linear I–V relationships which may be accounted for by the 5–10 pS chloride channel that has been described in several epithelial cell types (Gray et al., 1988; Christensen et al., 1989; Tabcharani et al., 1990). Single channel measurements made on cell-attached patches confirmed that virtually all CFTR-expressing Sf9 cells displayed a 8.4 pS linear chloride channel on activation by cAMP. The low conductance chloride channel was by far the predominant channel in CFTR-infected Sf9 cells, but it was not observed in any of the control cells. The linear current–voltage relationship of the low conductance channel, its permeability to iodide, and its increased activity during stimulation by cAMP suggest that it may mediate the macroscopic current observed in whole-cell recordings. Its single channel conductance, relative insensitivity to DIDS, and distinctive wave-like pattern of channel openings are closely similar to low conductance chloride channels reported previously in pancreatic duct (Gray et al., 1988) and T84 cells (Tabcharani et al., 1990). Estimates of the iodide-to-chloride permeability ratio (1.2) are higher than reported for whole-cell currents in T84 cells (0.4; Cliff and Frizzel, 1990) but are in reasonable agreement with those for low conductance channels in pancreatic duct (approximately 1.0; Gray et al., 1990). Insensitivity to DIDS matches the lack of response of transepithelial chloride secretion and of low conductance chloride channels (Tabcharani et al., 1990) and cAMP-stimulated whole-cell currents (Cliff and Frizzell, 1990) in T84 cells.

Furthermore, there is a correlation between the amount of CFTR protein synthesized in infected Sf9 cells and the magnitude of the stimulation of anion permeability mediated by cAMP. These findings could be explained by invoking a scenario where latent preexisting Cl^- channels are activated in Sf9 cells as a consequence of CFTR expression. However, even if cryptic channels are present prior to infection, it seems unlikely that their properties would be so similar to those of mammalian epithelial cells. Hence, a more likely scenario is that CFTR itself constitutes the regulated conductance pathway. Using the terminology that we initially employed to suggest possible functions of CFTR (Riordan et al., 1989), one could be tempted to speculate that the molecule might not be "either channel or regulator" but actually "channel and regu-

lator." Our data, however, cannot provide definitive proof of such a model, and our present speculation will only be rigorously tested when we have purified and reconstituted CFTR into artificial lipid membranes. We are hopeful that the relatively large amounts of protein that can accumulate in recombinant baculovirus-infected Sf9 cells will facilitate these kinds of experiments.

It is important to stress that the possibility that CFTR may contribute directly to the regulated anion permeability is not at odds with any existing data. The "correction" of the CF ion transport defect on introduction of CFTR into epithelial cells derived from patients (Rich et al., 1990; Drumm et al., 1990) could have resulted from either the restoration of the regulation of existing channels or from the presentation of new regulatable channels. In fact, evidence that channels activated by Ca^{2+}- or cAMP-mediated events are distinct entities (Cliff and Frizzell, 1990) is more consistent with the latter possibility than the former. The finding of a chloride permeability activated by cAMP but apparently not by Ca^{2+} would support the interpretation that the two major epithelial chloride channel types are entirely separate.

Hyde et al. (1990) have presented deductive arguments that CFTR should not be a chloride channel. However, these are simplistic and not based on any experimental data. Their point that CFTR is in no way similar to neuronal chloride channels was already evident to CF researchers, since attempts had been made in several groups, including our own, to clone homologs of these channels as CF gene candidates. These attempts failed. It was also suggested that ion movement via a molecule with transporter-like properties should not occur at rates comparable to those mediated by a channel. However, the classical distinctions between carriers, transporters, channels, and other types of membrane transport molecules are becoming far less clear as we begin to gain some knowledge of their molecular structures and realize that in some cases, interconversions between them can even occur (Dierks et al., 1990a, 1990b). It should be appreciated that only a very small proportion of transport molecules known functionally have been cloned and sequenced, and structure–function relationships have been elucidated for even fewer. It is, however, reasonable to expect that CFTR, which appears to belong to the TM6-NBF superfamily of membrane transporters, should in fact transport something (Hyde et al., 1990; Riordan et al., 1990).

Although it is still possible that the primary function of CFTR is unrelated to epithelial anion transport, both the previous, so-called complementation experiments and the heterologous expression reported here strongly suggest an involvement in regulated Cl^- conductance. At least three different types of involvement can be considered. First, CFTR may interact with other polypeptides that constitute the channel and thereby regulate it. However, if the primary CF defect is in a permeability pathway that necessitates the involvement of several components, only one of which is CFTR, mutations in these other components would also be expected. As yet, no CF patient has been found who does not have a mutation in the CFTR gene.

Second, as has been suggested by others, on the basis of the structural similarity between CFTR and P-glycoproteins or the yeast STE6 gene product, all of which transport small hydrophobic molecules (Riordan et al., 1990), CFTR might transport lipophilic channel modulators. Arachidonic acid or its metabolites have been suggested (Ringe and Petsko, 1990), since high concentrations of this fatty acid can inhibit chloride channels (Hwang et al., 1990), but there is no evidence that these can serve as substrates of CFTR.

Both of the two types of involvement alluded to above imply that CFTR acts to control the activity of a separate channel molecule. A third possibility, which we think is favored by the results described in this report, is that CFTR is itself an actively regulated channel. There are cation channels of known primary structure that are directly gated by nucleotides (Kaupp et al., 1989), though these do not involve an active transport process as part of their regulation. There are also ATP-dependent K^+ channels in pancreatic β cells and in brain, though their structures have yet to be determined (Ashcroft, 1988). Hence, it is does not seem unreasonable to suppose that there are metabolically regulated channels in epithelial cells, in which transcellular ion movements are controlled by a variety of stimuli (Welsh, 1990). CFTR may be one such channel. One of us has recently speculated that CFTR may function as a very tightly regulated channel, by virtue of its ability to actively maintain a configuration in which the R-domain is held in apposition to the inner charged mouth of the pore formed by the membrane-spanning segments, such that the passage of anions can then be acutely controlled by the changes in the phosphorylation state of the R-domain (Riordan, 1991).

While such models will require extensive further experimental testing before they are taken very seriously, they at least illustrate that one can readily reconcile the concepts of CFTR having both an active transport function, consistent with it being a member of the large TM6-NBF transporter superfamily, and a channel function. These two aspects of the function would be distinguished by their substrates: the R-domain being the substrate of an abortive active export step, chloride being the channel substrate. Alternative models incorporating these concepts and others could equally well be put forward at this time.

We have confirmed the demonstration of Klaiber et al. (1990) that the BEVS in Sf9 cells is well suited to the study of heterologous ion channels. The fact that expression of the CF gene in these cells causes the appearance of a cAMP-activated Cl^- conductance suggests that one is not necessarily restricted to the use of epithelial cells derived from CF patients in attempts to evaluate the basic structure–function relationships or the pharmacology of CFTR.

Experimental Procedures

Construction of a CFTR-Containing Baculovirus Transfer Vector

A 4.7 kb fragment containing the complete CFTR coding sequence and extending from nucleotides 72 to 4721 of the complete cDNA sequence as originally published (Riordan et al., 1990) had been prepared by J. M. Rommens and L.-C. Tsui (unpublished data) by the in-

troduction of PstI sites at these locations. This fragment was excised from a Bluescript (Stratagene) plasmid with this enzyme and cloned into the PstI site of the pVL1393 transfer plasmid (Luckow and Summers, 1989).

Northern Blot Analysis of CFTR Expression

Total RNA was isolated according to Chirgwin et al. (1979), electrophoresed on 1% agarose gels in the presence of 7% formaldehyde, and transferred to nitrocellulose filters. Filters were prehybridized for at least 3 hr at 42°C in 5 × SSC containing 25 μg/ml denatured salmon testis DNA, 0.2% bovine serum albumin (BSA), 0.2% Ficoll 400, 0.2% polyvinyl pyrrolidine, 0.1% SDS, and 50% deionized formamide. Hybridization was in the same solution containing 10% dextran-sulfate and 10^6 cpm/ml of ^{32}P-labeled full-length CFTR cDNA. The blots were rinsed several times in 2 × SSC containing 0.1% SDS at room temperature followed by extensive washing in 0.2 × SSC, 0.1% SDS at 65°C, and exposed to Kodak X-Omat XAR-5 films at −80°C with intensifying screens.

Membrane and Cell Lysate Preparations

A crude membrane fraction was prepared from T84 cells by homogenizing them in a hypotonic buffer containing 10 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris–HCl (pH 7.4). Nuclei and mitochondria were removed by microcentrifugation at 4000 × g for 5 min, and membranes were collected at 9000 × g for 15 min. Membrane pellets were dissolved directly in SDS–PAGE sample buffer at 37°C for 10 min for electrophoresis and immunoblotting. Sf9 cell lysates were prepared by scraping cells into a minimal volume of buffer containing 50 mM Tris–HCl (pH 6.8) and 50 μg/ml DNAase I. Cells were sonicated 3 × 10 s on ice at low power, incubated 10 min on ice, and then solubilized by addition of 5× SDS–PAGE sample solubilizing buffer and incubating at 37°C for 10 min. CFTR-enriched lysates were prepared by swelling Sf9 cells in a hypotonic buffer consisting of 10 mM NaCl, 1 mM $MgCl_2$, 10 mM Tris–HCl (pH 7.4) for 1 hr on ice at a density of approximately 4 × 10^7 cells per ml, then adding a detergent mix that brings the final concentrations to 1% Tween 40 and 0.5% sodium deoxycholate. The cell suspension was then cycled 5 times through a 30 gauge needle, microcentrifuged at 2200 rpm for 3 min at 4°C, and pellets were collected and solubilized as above.

Glycosidase Digestion

T84 crude membranes (100 μg) or Sf9 cell lysate (15 μg) was dissolved in 2% SDS at room temperature for 15 min. Ten volumes of buffer was then added, containing 50 mM Tris–HCl (pH 8.0), 25 mM EDTA, 1% Triton X-100, 1% 2-mercaptoethanol, and 0.7 U of N-glycanase (N-glycosidase F; Boehringer-Mannheim). Samples were incubated for 15 hr at 37°C. Proteins were precipitated by the addition of 4 vol of cold ethanol, collected by microcentrifugation, and dried under vacuum. The dry pellets were taken up in SDS–PAGE sample buffer and electrophoresed as usual.

SDS–PAGE and Immunoblotting

Electrophoresis was done essentially according to Laemmli (1970) using 5% or 6% acrylamide gels. Western blots were prepared according to the method of Towbin et al. (1979) and were probed with antibody according to standard methods. Radioiodinated rabbit anti-mouse immunoglobulins (Jackson) were used to develop immunoblots probed with mouse monoclonal antibodies, and alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega) was used for those probed with polyclonal rabbit sera. Rabbit sera were prepared against synthetic peptides by standard methods and were purified by affinity chromatography on peptide columns (S. Sun and J. R. Riordan, unpublished data). Hybridomas were prepared from mice immunized with fusion proteins, as described in the figure legends (N. Kartner and J. R. Riordan, unpublished data). Methods of preparation were similar to those described previously (Kartner et al., 1985).

Quantitation of Radioiodide Efflux

Approximately 2 × 10^6 Sf9 cells were seeded per well in 6-well cluster tissue culture dishes in Ex-Cell 400 medium (JRH Biosciences, Lenexa, KS). After allowing cell attachment for 1 hr, medium was aspirated, and cells were overlaid with 0.3 ml of viral stock per well in medium (approximately 10^8 pfu/ml) and incubated for 1 hr at 27°C. The viral stock was then aspirated, and cells were incubated for up to 72 hr in 1.5 ml of medium per well at 27°C. Except for studies of the time course of expression, $^{125}I^-$ efflux assays were done 36 hr after infection. Wells were rinsed with Grace's medium (Gibco), buffered with 10 mM MES (pH 6.2) (efflux buffer), and then loaded by incubation at 24°C for 1 hr after addition of 15 μCi of $Na^{125}I$ (carrier-free; Amersham) in 1 ml of efflux buffer. Wells were washed 6 times, rapidly, with 1 ml aliquots of efflux buffer and were then equilibrated for 1 min in a final 0.5 ml aliquot. All washes were discarded. At each one-minute time point thereafter, the medium was removed to a gamma counting tube and replaced quickly with a fresh 0.5 ml aliquot. The first four aliquots were used to establish a stable baseline in efflux medium alone. All subsequent time points (the first being denoted time-zero) were done in efflux buffer with the inclusion of agonist cocktail or other experimental additives as noted in the figure legends. Counting errors were generally less than 2%.

Whole-Cell Current Recording

Membrane currents were measured using conventional whole-cell patch clamp techniques (Hamill et al., 1981). The patch pipette was filled with a solution containing (mM): Na gluconate (110), NaCl (25), $MgCl_2$ (8), HEPES (10), disodium ATP (4), sodium EGTA (5) and was adjusted to pH 7.3 with NaOH. The bath contained (mM): NaCl (135), K_2HPO_4 (2.4), KH_2PO_4 (0.8), $MgCl_2$ (3), $CaCl_2$ (1), MES (10), glucose (10), amiloride (0.01), indomethacin (0.003), with the pH adjusted to 6.2. Amiloride was added to inhibit Na^+ conductance and indomethacin to inhibit cyclooxygenase. Membrane potentials were alternatively clamped at −40 mV and +30 mV for 600 ms and 400 ms respectively. I–V relationships were determined at least twice during the experimental period by stepping from −40 mV to +30 mV by 10 mV increments. Cell capacitance was compensated using the cancellation circuitry of the EPC7 patch clamp amplifier. Studies were performed at room temperature. Both β-galactosidase and CFTR-expressing Sf9 cells were studied between 36 hr and 48 hr after infection.

Single Channel Recordings

Most single channel recordings were made in the cell-attached configuration using conventional techniques. The pipette solution contained (mM): NaCl or N-methyl-D-glucamine chloride (150), TES (10), and $CaCl_2$ (2). The bath was perfused throughout experiments with oxygenated insec medium (Ex-Cell 401; JRH Biosciences). To elevate intracellular cAMP levels, N^6,2′-O-dibutyryl cAMP (0.5mM), 3-isobutyl-1-methylxanthine (IBMX; 10 μM) and forskolin (10 μM) were added to the perfusate. In some experiments, 4,4′-diisothiocyanostilbene-2,2′disulfonic acid (DIDS; 100 μM) was added to the pipette solution. DIDS solutions were stored in the dark prior to use. All chemicals were from Sigma (St. Louis, MO). Single channel currents were amplified (Axopatch 1b, Axon Instruments, Inc., Foster City, CA), recorded (DR384, Neurodata Instr. Co., NY), and analyzed (Indec Systems, Sunnyvale, CA.) as described previously (Tabcharani et al., 1990). Measurements were carried out at 27°C ± 1°C.

Electron Microscopy

Cells were fixed in 2% paraformaldehyde and 0.001% glutaraldehyde in 0.1 M HEPES buffer (pH 7.2) for 2 hr. They were then rinsed several times with buffer and infiltrated with 2% agarose (Stierhof et al., 1986). The specimens were then infused with 2.3 M sucrose for several hours and frozen by rapid immersion in liquid nitrogen-cooled Freon 22. Cryosections were obtained using a Reichert Ultracut/FC4 cryoultramicrotome.

After collection on grids on iced agarose-gelatin, the sections were quenched with 80 mM ammonium chloride for 10 min. They were washed in PBS containing 0.5% BSA and 0.15% glycine, followed by normal goat serum diluted 1:20 in PBS/BSA. After further washing, the sections were incubated for 75 min with purified primary mouse monoclonal antibody, L12B4 or M3A7, in PBS containing 0.5% BSA. The sections were washed again, and then incubated for 60 min with 15 nm gold-conjugated goat anti-mouse IgG. After an additional six washes in PBS/BSA and four washes with distilled water, the sections were stabilized in 2% methylcellulose containing 0.2% uranyl acetate and air dried. The sections were then examined in a JEOL 1200 EX II transmission electron microscope.

As controls, sections from the same blocks were processed as de-

scribed above, except that irrelevant purified mouse monoclonal antibodies of the same isotype (Pharmingen) and at the same concentration were substituted for specific antibody in the primary antibody incubation step.

Acknowledgments

We thank Rob Dunn of the Montreal General Hospital for his advice regarding the use of the BEVS and Jim Phillips, Chief of the Department of Pathology at The Hospital for Sick Children, for making the immunopathology facilities available. The work was supported by grants from the Canadian and U. S. Cystic Fibrosis Foundations and the NIH-NIDDK. N. K. is a Kinsman Fellow of the CCFF and J. W. H. is an MRC Research Scholar.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 USC Section 1734 solely to indicate this fact.

Received December 10, 1990; revised January 15, 1991.

References

Ashcroft, F. M. (1988). Adenosine 5'-triphosphate-sensitive potassium channels. Annu. Rev. Neurosci. *11*, 97–118.

Chirgwin, J. M., Przbyla, A. E., MacDonald, R. J., and Rutter, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry *18*, 5294–5299.

Christensen, O., Simon, M., and Randlev, T. (1989). Anion channels in a leaky epithelium. Pflügers Arch. *415*, 37–46.

Cliff, W. H., and Frizzell, R. A. (1990). Separate Cl^- conductances activated by cAMP and Ca^{2+} in Cl^--secreting epithelial cells. Proc. Natl. Acad. Sci. USA *87*, 4956–4960.

Dierks, T., Salentin, A., Heberger, C., and Krämer, R. (1990a). The mitochondrial aspartate/glutamate and ADP/ATP carrier switch from obligate counterexchange to unidirectional transport after modification by SH-reagents. Biochim. Biophys. Acta *1028*, 268–280.

Dierks, T., Salentin, A., Krämer, K. (1990b). Pore-like and carrier-like properties of the mitochondrial aspartate/glutamate carrier after modification by SH-reagents: evidence for a preformed channel as a structural requirement of carrier-mediated transport. Biochim. Biophys. Acta *1028*, 281–288.

Domingo, D. L., and Trowbridge, I. S. (1988). Characterization of the human transferrin receptor produced in a baculovirus expression system. J. Biol. Chem. *263*, 13386–13392.

Drumm, M. L., Pope, H. A., Cliff, W. H., Rommens, J. M., Marvin, S. A., Tsui, L.-C., Collins, F. S., Frizzell, R. A., and Wilson, J. M. (1990). Correction of the cystic fibrosis defect in vitro by retrovirus-mediated gene transfer. Cell *62*, 1227–1233.

George, S. T., Arbabian, M. A., Ruoho, A. E., Kiely, J., and Marbon, C. C. (1989). High-efficiency expression of mammalian β-adrenergic receptors in baculovirus-infected insect cells. Biochem. Biophys. Res. Commun. *163*, 1265–1269.

Germann, U. A., Willingham, M. C., Pastan, I., and Gottesman, M. M. (1990). Expression of the human multidrug transporter in insect cells by a recombinant baculovirus. Biochemistry *29*, 2295–2303.

Gray, M. A., Greenwell, J. R., and Argent, B. E. (1988). Secretin-regulated chloride channel on the apical plasma membrane of pancreatic duct cells. J. Membr. Biol. *105*, 131–142.

Gray, M. A., Pollard, C. E., Harris, A., Coleman, L., Greenwell, J. R., and Argent, B. E. (1990). Anion selectivity and block of the small conductance chloride channel on pancreatic duct cells. Am. J. Physiol. *259*, C752-C761.

Greenfield, C., Patel, G., Clark, S., Jones, N., and Waterfield, M. D. (1988). Expression of the human EGF receptor with ligand-stimulatable kinase activity in insect cells using a baculovirus vector. EMBO J. *7*, 139–146.

Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981). Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. Pflügers Arch. *391*, 85–100.

Hwang, T. C., Guggino, S. E., and Guggino, W. B. (1990). Arachidonic acid block of epithelial Cl^- channels in tracheal airway cells. Biophys. J. *57*, 88a.

Hyde, S. C., Emsley, P., Hartshorn, M. J., Mimmack, M. M., Gileadi, U., Pearce, S. R., Gallagher, M. P., Gill, D. R., Hubbard, R. E., and Higgins, C. F. (1990). Structural model of ATP-binding proteins associated with cystic fibrosis, multidrug resistance and bacterial transport. Nature *346*, 362–365.

Johnson, P. W., Attia, J., Richardson, C. D., Roder, J. C., and Dunn, R. J. (1989). Synthesis of soluble myelin-associated glycoprotein in insect and mammalian cells. Gene *77*, 287–296.

Kartner, N., Evernden-Porelle, D., Bradley, G., and Ling, V. (1985). Detection of P-glycoprotein in multidrug-resistant cell lines by monoclonal antibodies. Nature *316*, 820–823.

Kaupp, U. B., Nildome, T., Tanabe, T., Terada, S., Bönigk, W., Stühmer, W., Cook, N. J., Kangawa, K., Matsuo, H., Hirose, T., Miyata, T., and Numa, S. (1989). Primary structure and functional expression from complementary DNA of the rod photoreceptor cyclic GMP-gated channel. Nature *342*, 762–766.

Klaiber, K., Williams, N., Roberts, T. M., Papazian, D. M., Jan, L. Y., and Miller, C. (1990). Functional expression of shaker K^+ channels in a baculovirus-infected insect cell line. Neuron *5*, 221–226.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature *227*, 680–685.

Luckow, V. A., and Summers, M. D. (1988a). Signals important for high-level expression of foreign genes in *Autographa californica* nuclear polyhedrosis virus expression vectors. Virology *167*, 56–71.

Luckow, V. A., and Summers, M. D. (1988b). Trends in the development of baculovirus expression vectors. Bio/Technol. *6*, 47–55.

Luckow, V. A., and Summers, M. D. (1989) High level expression of non-fused foreign genes in *Autographa californica* nuclear polyhedrosis virus expression vectors. Virology *170*, 31–39.

Marcel, Y. L., Innerarity, T. L., Spilman, C., Mahley, R. W., Protter, A. A., and Milne, R. W. (1987). Mapping of human apolipoprotein B antigenic determinants. Arteriosclerosis *7*, 166–175.

Possee, R. D. (1986). Cell-surface expression of influenza virus haemagglutinin in insect cells using a baculovirus vector. Virus Res. *5*, 43–59.

Rich, D. P., Anderson, M. P., Gregory, R. J., Cheng, S. H., Paul, S., Jefferson, D. M., McCann, J. D., Klinger, K. W., Smith, A. E., and Welsh, M. J. (1990). Expression of cystic fibrosis transmembrane conductance regulator corrects defective chloride channel regulation in cystic fibrosis airway epithelial cells. Nature *347*, 358–363.

Ringe, D., and Petsko, G. A. (1990). Cystic fibrosis: a transport problem? Nature *346*, 312–313.

Riordan, J. R. (1991). The cystic fibrosis gene product (CFTR) is a member of the TM6-NBF transporter super family. Bioassays, in press.

Riordan, J. R., and Ling, V. (1985). Genetic and biochemical characterization of multi-drug resistance. J. Pharmacol. Therapeut. *28*, 51–75.

Riordan, J. R., Rommens, J. M., Kerem, B.-S., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.-L., Drumm, M. L., Iannuzzi, M. C., Collins, F. S. and Tsui, L.-C. (1989). Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science *245*, 1066–1073.

Riordan, J. R., Alon, N., Grzelczak, Z., Dubel, S., and Sun, S.-Z. (1990). The CF gene product as a member of a membrane transporter (TM6-NBF) super family. In The Identification of the CF (Cystic Fibrosis) Gene: Recent Progress and New Research Strategies, L.-C. Tsui, G. Romeo, R. Greger, S. Gorini, eds. (New York: Plenum Publishing Corporation).

Schoumacher, R. A., Ram, J., Iannuzzi, M. C., Bradbury, N. A., Wallace, R. W., Tom Hon, C., Kelly, D. R., Schmid, S. M., Gelder, F. B., Rado, R. A., and Frizzell, R. A. (1990). A cystic fibrosis pancreatic adrenocarcinoma cell line. Proc. Natl. Acad. Sci. USA *87*, 4012–4016.

Smith, D. B., and Johnson, K. S. (1988). Single-step purification of

polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene *67*, 31–40.

Stierhof, Y.-D., Schwarz, H., and Frank, H. (1986). Transverse sectioning of plastic-embedded immunolabelled cryosections: morphology and permeability to protein A-colloidal gold complexes. J. Ultrastruct. Res. *97*, 187–196.

Summers, M. D., and Smith, G. E. (1987). A manual of methods for baculovirus vectors and insect cell culture procedures. In Bulletin No. 1555 (College Station, Texas: Texas Agricultural Experiment Station and Texas A&M University), pp. 10–39.

Tabcharani, J. A., Low, W., Elie, D., and Hanrahan, J. W. (1990). Low-conductance chloride channel activated by cAMP in the epithelial cell line T84. FEBS Lett. *270*, 157–164.

Towbin, H., Staehlin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. Proc. Natl. Acad. Sci. USA *76*, 4350–4354.

Venglarik, C. J., Bridges, R. J., and Frizzell, R. A. (1990). A simple assay for agonist-regulated Cl and K conductances in salt-secreting epithelial cells. Am. J. Physiol. *259*, C358-C364.

Vialard, J., Lalumiere, M., Venet, T., Briedis, D., Alkhatib, G., Henning, D., Levin, D., and Richardson, C. (1990). Synthesis of the membrane fusion and hemagglutinin proteins of measles virus using a novel baculovirus vector containing the B-galactosidase gene. J. Virol. *64*, 37–50.

Welsh, M. J. (1990). Abnormal regulation of ion channels in cystic fibrosis epithelia. FASEB J. *4*, 2718–2725.