Generation of cAMP-Activated Chloride Currents by Expression of CFTR

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Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) cause cystic fibrosis. In order to evaluate its function, CFTR was expressed in HeLa, Chinese hamster ovary (CHO), and NIH 3T3 fibroblast cells, and anion permeability was assessed with a fluorescence microscopic assay and the whole-cell patch-clamp technique. Adenosine 3′,5′-monophosphate (cAMP) increased anion permeability and chloride currents in cells expressing CFTR, but not in cells expressing a mutant CFTR (ΔF508) or in nontransfected cells. The simplest interpretation of these observations is that CFTR is itself a cAMP-activated chloride channel. The alternative interpretation, that CFTR directly or indirectly regulates chloride channels, requires that these cells have endogenous cryptic, chloride channels that are stimulated by cAMP only in the presence of CFTR.

CYSTIC FIBROSIS (CF), THE MOST common lethal genetic disease of Caucasians, is caused by mutations in the gene for CFTR (*1–5*). The predominant clinical manifestations of the disease result from improper function of epithelial tissues (*6*), and CFTR is expressed predominantly in epithelia (*2*). In epithelia affected by CF, Cl^- transport is abnormal. Airway epithelia from patients with CF fail to secrete Cl^- because cAMP-dependent phosphorylation does not activate apical membrane Cl^- channels (*7*). Expression of CFTR, but not CFTR containing the most common mutation found in CF chromosomes (CFTRΔF508), corrects the Cl^- channel defect in epithelial cells from patients with CF (*4, 5*). However, this experiment does not identify the function of CFTR. As a first step in defining the function of CFTR, we expressed CFTR in normal epithelial and nonepithelial cells and assessed cAMP-stimulated Cl^- channel activation.

We used HeLa (a human cervical carcinoma cell line), CHO (a Chinese hamster ovary cell line), NIH 3T3 (a mouse fibroblast cell line), and T84 cells (a human colon carcinoma epithelial cell line). We previously found that T84, but not HeLa, 3T3, or CHO cells, contain CFTR by using antibodies that immunoprecipitate CFTR (*3*). When we used a more sensitive technique, the polymerase chain reaction (PCR), we failed to detect CFTR mRNA in HeLa or 3T3 cells (*8*).

To express CFTR, we used the vaccinia-T7 hybrid expression system developed by Moss and co-workers (*9*). We previously used this system to express functional CFTR in CF airway epithelial cells (*3, 4*); it has also been used to express a functional K^+ channel in several cell types (*10*). We assessed cell membrane halide permeability in single cells by measuring the fluorescence intensity of intracellular 6-methoxy-*N*-(3-sulfopropyl) quinolinium (SPQ) (*11*). Fluorescence intensity was measured first while cells were bathed in NaI, and then after replacement of NaI with $NaNO_3$. Iodide quenches SPQ fluorescence; NO_3^- does not. In this assay, increased anion permeability results in a faster rate of increase of the fluorescence after substitution of NO_3^- for I^- (*4*).

Addition of forskolin to nontransfected HeLa cells or 3T3 fibroblasts did not increase halide permeability (not shown), indicating that in the absence of CFTR there is no measurable cAMP-regulated Cl^- channel activity in these cells (see also Fig. 4) (*12*). When cells were transfected with pTM-CFTR-4 (coding for CFTR) (Fig. 1, A and B), cAMP stimulated the rate of fluorescence increase. In control studies, transfection with pTM-CFTR-3ΔF508 (coding for CFTRΔF508) (Fig. 1, C and D) did not induce a response to cAMP. The transfection procedure resulted in equal expression of CFTR and CFTRΔF508 in HeLa cells (Fig. 1F). Thus expression of

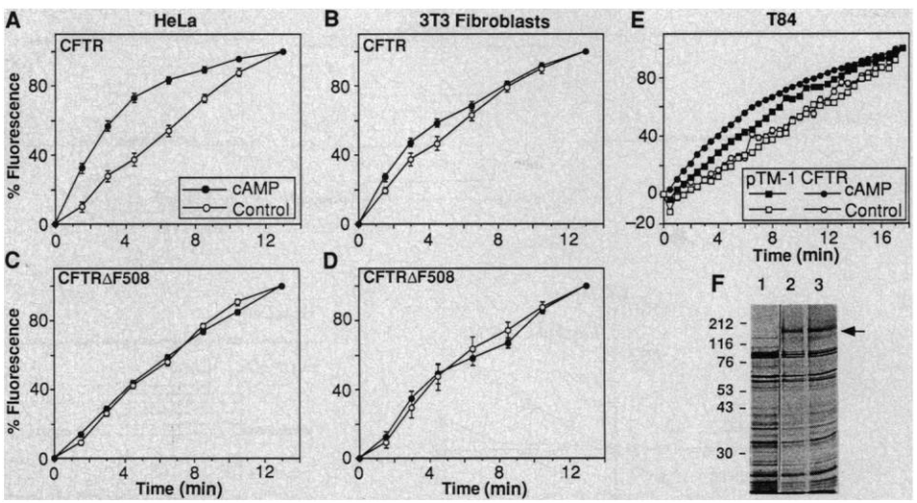

Fig. 1. The change in fluorescence of SPQ-loaded HeLa cells (A and C), 3T3 fibroblasts (B and D), and T84 cells (E) after substitution of NO_3^- for I^- in the bathing medium. Open symbols, basal conditions; closed symbols, stimulated [20 μM forskolin and 100 μM 3-isobutyl-1-methyl-xanthine (IBMX)] conditions. Cells were transfected with either pTM-CFTR-4 (**A and B**), pTM-CFTR-3ΔF508 (**C and D**), or pTM-CFTR-4 (●, ○) and pTM-1 (■, □) (**E**) (*26*). Data are mean ± SEM for 34 control and 75 stimulated cells in (A); 14 control and 77 stimulated cells in (B); 55 control and 83 stimulated cells in (C); 6 control and 36 stimulated cells in (D); 75 control and 429 stimulated CFTR and 68 control and 409 stimulated pTM-1 cells in (E). For T84, we compared cells transfected with pTM-CFTR-4 to cells transfected with pTM-1 to control for effects of vaccinia virus infection and plasmid transfection; infection and transfection with pTM-1 slightly reduced both the basal and the cAMP-induced increase in the rate of fluorescence change (without altering the relation between the two) when compared to noninfected-nontransfected controls. In some cases the standard error bars are smaller than the symbols. Data points were collected every 90 s in (A) through (D) and every 30 s in (E). Stimulated values at 3 min are significantly greater than basal values for cells transfected with pTM-CFTR-4 ($P < 0.05$). In (E), stimulated values for cells transfected with pTM-CFTR-4 and pTM-1 are greater than basal values ($P < 0.001$) and stimulated values for cells transfected with pTM-CFTR-4 are greater than those for cells transfected with pTM-1 ($P < 0.001$). (**F**) Expression of CFTR in transfected HeLa cells. HeLa cells were transfected (*26*) with pTM-1 (lane 1), pTM-CFTR-2 (lane 2), or pTM-CFTR-2ΔF508 (lane 3). Twelve hours after transfection, cells were incubated with [^{35}S]methionine (25 μCi/ml) for 1 hour and collected in (20 μl per dish) 25 mM 4-morpholinepropane sulfonic acid (pH 8.0), 2.5% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate, 185 mM KCl, 1 mM (ethylenedinitrilo)-tetraacetic acid, aprotinin (100 μg/ml), and 17 μM phenylmethylsulfonyl fluoride. Cell lysate (20 μl) was analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography. The gel was exposed to film for 3 days.

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CFTR, but not CFTRΔF508, resulted in the appearance of a cAMP-stimulated halide permeability not previously observed in these cells.

T84 cells express high concentrations of CFTR (*2*) and large cAMP-activated Cl^- currents (*13, 14*). Transfection of these cells with pTM-CFTR-4 increased cAMP-activated halide permeability to values greater than those observed in pTM-1 transfected cells (Fig. 1E). This result indicates that overexpression of CFTR causes a further increase of anion permeability in cells that already express CFTR. This finding is consistent with earlier suggestions of a dosage relation between CFTR and Cl^- permeability (*4, 15*) and suggests that the amount of CFTR determines the magnitude of the cAMP-stimulated anion permeability.

To determine if the increase in halide permeability was due to Cl^- channel activation, we used the whole-cell patch-clamp technique (*16*) to directly measure Cl^- currents in HeLa cells (Fig. 2A). Elevation of cAMP did not alter current in any of the nontransfected cells (current ± SEM was 15 ± 3 pA before and 12 ± 3 pA after stimulation; n = 8) or cells transfected with CFTRΔF508 cDNA (current was 22 ± 5 pA before and 29 ± 8 pA after stimulation; n = 7), a result consistent with data obtained with the SPQ assay (Fig. 1). In contrast, cAMP activated currents in cells transfected with CFTR cDNA (Figs. 2A and 4) (current was 62 ± 101 pA before and 887 ± 1608 pA after forskolin; n = 16; $P <$ 0.025; Wilcoxon paired sample test). Although we cannot completely exclude the possibility that CFTRΔF508 has some effect on Cl^- curent, our data suggest that such an effect is at most 3% of that observed in cells transfected with CFTR cDNA.

Of the 16 HeLa cells transfected with CFTR cDNA and studied by patch clamp, 8 showed an acute increase in current with cAMP stimulation; in those cells that responded, current increased from a baseline of 93 ± 48 to 1739 ± 697 pA. The fact that not every cell responded is consistent with our previous studies with this expression system in airway epithelial cells from patients with CF (*4*) and with the heterogeneous response in HeLa and 3T3 cells with the SPQ assay.

Currents were measured with the relatively impermeant cation *N*-methyl-D-glucamine (NMDG), and changes in Cl^- concentration shifted the current-voltage (*I-V*) relation as expected for a Cl^- current (Fig. 2, B and C). When forskolin was removed from the bath, current returned to basal values (Fig. 2D). Current could also be stimulated by addition of 8-(4-chlorophenylthio) adenosine 3′,5′-monophosphate (CPT-cAMP) and inhibited by the Cl^- channel blocker diphenylamine-2-carboxylic acid (DPC) (3 mM DPC inhibited the current by 63% and 36% in two cells studied). Depolarizing voltages had minimal time-dependent effects on the cAMP-activated Cl^- current (Fig. 2, A and C). The cAMP-activated Cl^- current had a linear *I-V* relation (Fig. 2B). All of these properties are similar to cAMP-activated whole-cell Cl^- currents in epithelial cells that normally express CFTR (*14*).

Transfection of CHO cells with pTM-CFTR-4 (*17*) caused the production of a similar current in response to cAMP stimulation (Fig. 3, A through C, and Fig. 4). The cAMP-induced currents in CHO cells were similar to those in HeLa and CF airway cells (*4*) (Fig. 3A), although we sometimes observed a small time-dependent increase in current at depolarizing voltages. The *I-V* relation reversed as expected for a Cl^- current (Fig. 3B). Figure 3C shows an example of the time course of cAMP-activated Cl^- currents in a cell with a very large current response.

Speculation about the function of CFTR has been based on several observations: CFTR is a membrane-associated phosphoprotein (*3*); cAMP-dependent (phosphorylation-dependent) Cl^- channel activation is defective in CF epithelial cells (*7*); expression of CFTR but not CFTRΔF508 corrects the Cl^- channel defect in CF epithelial cells (*4, 5*); and portions of the deduced amino acid sequence of CFTR show similarity to that of some nucleotide binding proteins (*2, 18*). These observations have led to the development of three main hypotheses about the relation between CFTR and Cl^- channels: (i) CFTR is itself a cAMP-activated Cl^- channel. (ii) CFTR directly regulates Cl^- channels. CFTR might associate with Cl^- channels and in so doing regulate their function (that is,

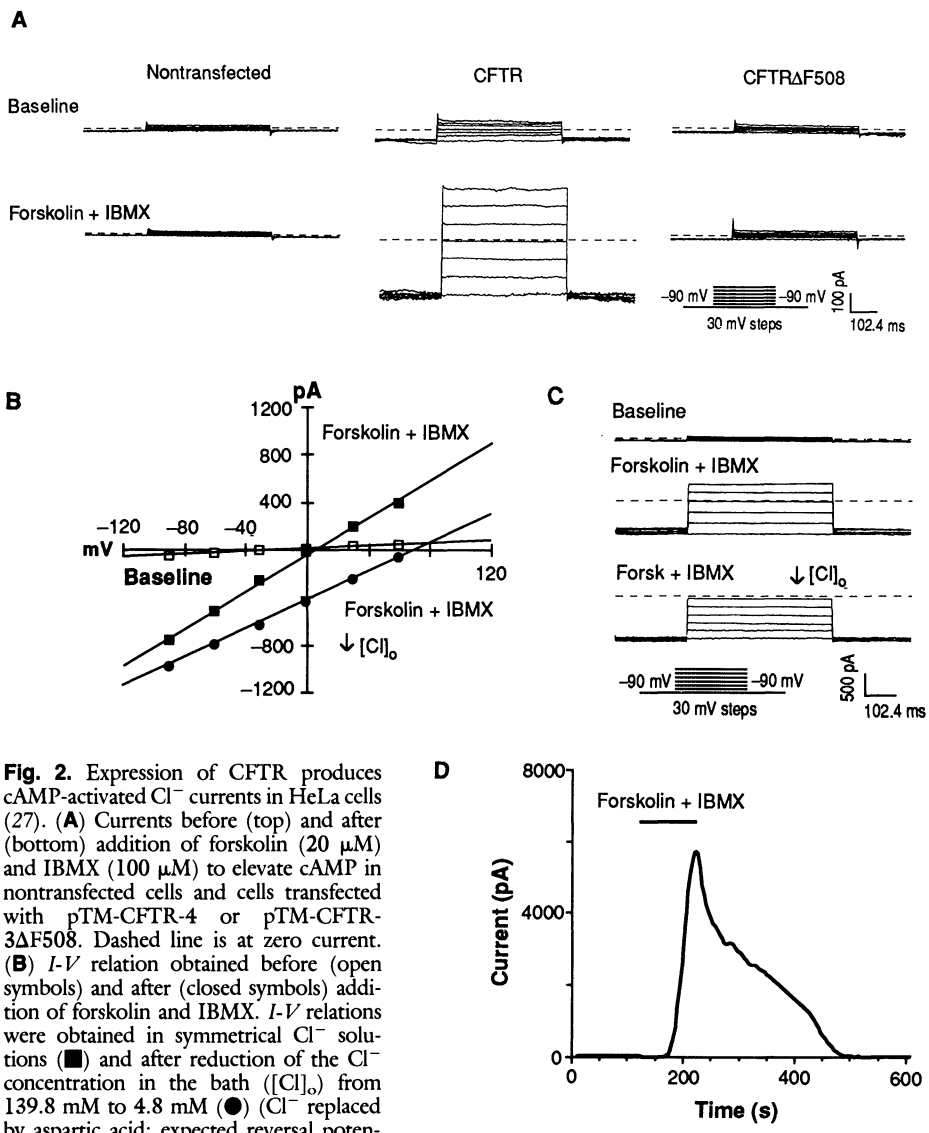

Fig. 2. Expression of CFTR produces cAMP-activated Cl^- currents in HeLa cells (*27*). (**A**) Currents before (top) and after (bottom) addition of forskolin (20 μM) and IBMX (100 μM) to elevate cAMP in nontransfected cells and cells transfected with pTM-CFTR-4 or pTM-CFTR-3ΔF508. Dashed line is at zero current. (**B**) *I-V* relation obtained before (open symbols) and after (closed symbols) addition of forskolin and IBMX. *I-V* relations were obtained in symmetrical Cl^- solutions (■) and after reduction of the Cl^- concentration in the bath ($[Cl]_o$) from 139.8 mM to 4.8 mM (●) (Cl^- replaced by aspartic acid; expected reversal potential, +84.8 mV). (**C**) Current tracings used to generate the data in (B). (**D**) Time course for activation and reversal of currents; bar, time during which forskolin and IBMX were present.

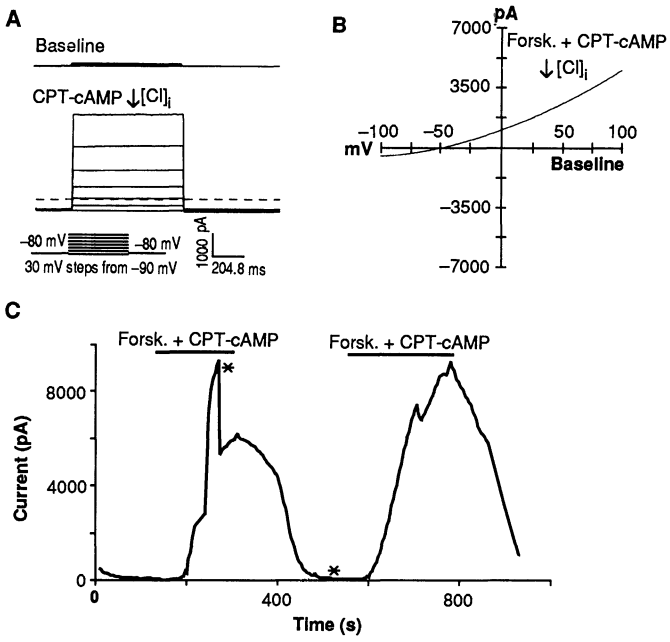

Fig. 3. Expression of CFTR induces cAMP-activated Cl^- current in CHO cells (*27*). (**A**) Currents before and after addition of CPT-cAMP (500 μM) in a cell transfected with pTM-CFTR-4. The Cl^- concentration in the pipette ($[Cl]_i$) was reduced to 11.8 mM. (**B**) *I-V* relation before and after addition of forskolin (20 μM) and CPT-cAMP (500 μM) in a cell transfected with pTM-CFTR-4. (The *I-V* relation before stimulation is hidden under the line forming the *x* axis.) The *I-V* relation was obtained with a voltage ramp from −100 to +100 mV. The Cl^- concentration in the pipette was reduced to 11.8 mM(Cl^- replaced with aspartic acid; expected reversal potential, −65.6 mV). The *I-V* relation likely rectifies because of asymmetric Cl^- concentrations. (**C**) Time course for activation and reversal of currents; bars, time during which forskolin and CPT-cAMP were present. Because the current was so large, series resistance compensation (*16*) was temporarily turned off between the asterisks.

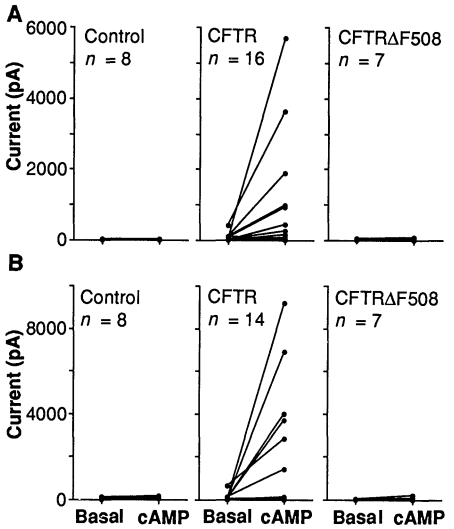

Fig. 4. Comparison of currents measured at +80 mV before (Basal) and after (cAMP) addition of agents that increase cAMP in HeLa (**A**) and CHO (**B**) cells. Forskolin (20 μM) alone, forskolin with IBMX (100 μM), and CPT-cAMP (500 μM) alone all gave similar results. cAMP significantly increased the current in pTM-CFTR-4–transfected cells ($P < 0.004$ for HeLa; $P < 0.01$ for CHO). cAMP did not significantly increase currents in either of the other two groups. There were no statistically significant differences in baseline current between the groups; *n*, number of cells studied in each group. The peak current response to cAMP in pTM-CFTR-4–transfected cells was observed 177 ± 29 s (HeLa) and 155 ± 11 s (CHO) after addition of agents that increase cAMP. Membrane capacitance was similar for all three groups, indicating that differences in current cannot be attributed to differences in cell size. In HeLa cells, membrane capacitance was 24 ± 4 pF for nontransfected, 18 ± 1 for CFTR, and 17 ± 4 for CFTRΔF508. In CHO cells, membrane capacitance was 29 ± 7 pF for nontransfected, 22 ± 2 for CFTR, and 25 ± 3 for CFTRΔF508. Statistical significance was tested with the Wilcoxon paired-sample test.

cause them to become sensitive to cAMP). (iii) CFTR indirectly regulates Cl^- channels. Proponents of this alternative argue that CFTR is a pump and not a channel (*18*) on the basis of the observation that the deduced amino acid sequence of CFTR contains putative nucleotide binding folds and membrane spanning sequences; this overall sequence organization of CFTR resembles that of several other eukaryotic and prokaryotic proteins (*19*) (some, but not all, of which are involved in active transport). How might a pump regulate Cl^- channels? CFTR might pump a Cl^- channel inhibitory factor out of the cell or pump a factor into the cell that is required for cAMP to activate Cl^- channels (*20*).

Alternatively, CFTR might be both a channel and an adenosine triphosphatase (ATPase) (or at least bind nucleotides). The presence of nucleotide binding folds [which conserve the "Walker motif" for ATP binding (*21*)] does not exclude the possibility that some of the membrane spanning regions of CFTR form a transmembrane pore or channel. For example, the chloroplast ATP synthase (*22*) has both catalytic and channel activity.

Our data show that expression of CFTR produced cAMP-activated Cl^- currents in cells that do not normally express CFTR. In addition, overexpression of CFTR in cells that already express large amounts of CFTR caused a cAMP-stimulated anion permeability that was greater than that in cells transfected with control plasmid. In contrast, expression of CFTRΔF508 did not induce cAMP-activated Cl^- channels. The magnitude of the currents induced by expression of CFTR was sometimes very large (5,000 to 10,000 pA) (Figs. 2D, 3C, and 4); thus, if CFTR affects silent Cl^- channels, CHO and HeLa cells must have a large number of such silent channels.

The cAMP-activated Cl^- currents we report here are similar to those seen with the whole-cell patch-clamp technique in both normal cells expressing endogenous CFTR (*14*) and CF cells expressing recombinant CFTR (*4, 5*). Those Cl^- currents have a relatively linear *I-V* relation and lack time-dependent voltage effects. Recent single-channel patch-clamp studies have also reported cAMP-activated Cl^- currents with a linear *I-V* relation in normal cells (*23*), although comparable studies have not been done in cells from CF patients. At present, it is not clear how these observations can be reconciled with previous single-channel studies, which showed defective regulation of an outwardly rectifying Cl^- channel in CF airway epithelia (*24*) and a cAMP-activated outwardly rectifying Cl^- channel in other epithelia (*25*). The resolution of this apparent difference will be important in the future.

If CFTR is not a Cl^- channel, our data require that HeLa, CHO, and 3T3 cells have Cl^- channels that become cAMP-sensitive only in the presence of CFTR. Because the vaccinia virus system inhibits synthesis of cellular proteins, it is unlikely that CFTR stimulates the synthesis of new Cl^- channels. If CFTR pumps a regulatory factor into or out of the cell, or if CFTR associates with and thereby regulates Cl^- channels, these cells must contain Cl^- channels that can respond to such a factor or such an association. Although our data do not rigorously disprove such hypotheses, the simplest interpretation is that CFTR is itself a cAMP-activated Cl^- channel.

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thiocyanate, and samples were treated with deoxyribonuclease to remove potentially contaminating cDNA from plasmid or, in the case of 3T3 cells, genomic DNA. RNA was reverse transcribed with primer 5′-CAGTTTACAGACACAGCT-3′ (anti 1914). PCR was performed on the cDNA [H. A. Erlich, *PCR Technology: Principles and Applications for DNA Amplification* (Stockton Press, New York, NY, 1989)] with primers from exons 9 (1399, 5′-AATGGTGATGACAGCCTCTTC-3′) and 13 (anti 1914, 5′-CAGTTTACAGACACAGCT-3′). CFTR cDNA was found in untransfected T84, untransfected airway epithelial cells [D. M. Jefferson *et al., Am. J. Physiol.* **259**, L496 (1990)], transfected HeLa, and transfected 3T3 cells but not in untransfected HeLa cells. cDNA from 3T3 cells was also negative when we used primers from exon 13 (1900, 5′-CTGTGTCTGTAAACTGATGCC-3′ and anti 2601, 5′-TTTAAGTCTTCTTCGTTAAT-3′) that share only two mismatches each (near the 3′ end) with the sequence of mouse CFTR cDNA. R. C. Crystal and co-workers [*Ped. Pulmonol. Suppl.* **5**, 59 (1990)] have also reported that CFTR mRNA is not found in HeLa cells or in human fibroblasts.

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17. Patch-clamp studies were performed on CHO but not 3T3 cells, and SPQ studies were performed on 3T3 but not CHO cells, because 3T3 cells were difficult to patch and SPQ loading of CHO cells was not adequate for reliable measurements.
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19. This family of proteins (*2, 18*) consists of two repeated elements, each consisting of six membrane spanning regions and a nucleotide binding fold (in some of the prokaryotic members of the family different domains are supplied by separate proteins). CFTR also has some unique features not shared by other members of this family. For example, CFTR has a large, polar R-domain that contains multiple phosphorylation consensus sequences, and 10 of 12 transmembrane regions contain amino acids with charged side chains (*2*), a property similar to transmembrane regions of ion channels.
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26. Cell culture: All cells were plated at ~5×10^4 cells per square centimeter on collagen-coated glass cover slips 24 hours prior to infection. Infection and transfection: Recombinant vaccinia virus vTF7-3 was added to the cells for 1 hour in serum-free media at a high multiplicity of infection (10 to 20). Cells were then transfected with recombinant plasmids (5 μg of plasmid per 10^6 cells) with lipofectin (BRL) (20 μg of lipid per10^6 cells) and incubated at 37°C (*3, 4*). pTM-CFTR-4 contains a mutation at nucleotide 936 (T to C), which destroys a cryptic bacterial promoter (*3*) and thereby allows expression of a high copy number of the CFTR plasmid without changing the amino acid sequence (R. J. Gregory and A. E. Smith, manuscript in preparation). pTM-1 is the parent control plasmid. SPQ fluorescence: Fluorescence was assayed 8 to 14 hours after transfection (*4*). Cells were loaded with SPQ by including 10 mM SPQ in the media for 9 to 12 hours. SPQ fluorescence was initially quenched by incubating cells for 25 to 45 min in a buffer containing 135 mM NaI, 2.4 mM K_2HPO_4, 0.6 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaSO_4$, 10 mM Hepes (pH 7.4), and 10 mM dextrose. Forskolin (20 μM) and IBMX (100 μM) were added 5 min before the anion substitution. After measuring fluorescence for at least 2 min, the 135 mM NaI solution was replaced by one containing 135 mM $NaNO_3$ (time zero) and fluorescence was measured for another 13 to 17.5 min. Fluorescence of SPQ in single cells was measured with a Nikon inverted microscope, a SPEX digital imaging system, and a DAGE SIT66 camera. Excitation was at 350 nm and emission was at >410 nm. Studies were done at room temperature. Cells were chosen for quantitation of fluorescence without knowledge of the rate of change; the area of measurement was chosen from the last fluorescence image. Depending on the area studied and cell density, 3 to 85 cells were studied per field. One field was examined per experiment and at least three different transfected cultures (experiments) were studied for each condition. Only cells that had greater than 7 units of fluorescence increase were included. To correct for cell-to-cell differences in absolute fluorescence intensity (due to differences in cellular concentration of SPQ and differences in cell size), we present the data as % fluorescence, which is $100 \times (F_t - F_0)/(F_{13} - F_0)$, where F_0, F_{13}, and F_t are fluorescence intensity at time zero, 13 min, and at time t, respectively. The T84 studies (Fig. 1E) were longer, and $F_{17.5}$ was used for calculation. At 13 and 17.5 min, there was sufficient discrimination between basal and stimulated conditions, yet leakage of SPQ and phototoxicity were minimized. (A longer duration would magnify, rather than reduce, the difference between basal and cAMP conditions.) Statistical significance between groups of averaged data was assessed by unpaired t test at the 3-min time point; 3 min was selected on the basis of our previous work (*4*) and because, at this time, basal and cAMP conditions were easily distinguished.
27. Currents were measured with the whole-cell patch-clamp technique (*16*). For HeLa cells the pipette contained 120 mM NMDG, 5 mM Hepes, 3 mM $MgCl_2$, 1 mM CsEGTA, 1 mM MgATP, pH 7.2 (117.5 mM HCl). The external bath solution contained 140 mM NMDG, 10 mM Hepes, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM dextrose, pH 7.2 (135 mM HCl). Membrane voltage was maintained at −90 mV and depolarized to +80 mV for 500 ms every 3 s. For CHO cells the pipette contained 120 mM NMDG, 115 mM aspartic acid, 5 mM Hepes, 3 mM $MgCl_2$, 1 mM CsEGTA, pH 7.2 (5.8 mM HCl), 1 mM MgATP. Membrane voltage was maintained at −80 mV and depolarized to +80 mV for 500 ms every 4 s. HeLa cells were studied 13 ± 1 hours after transfection; CHO cells were studied 21 ± 2 hours after transfection; the time for CFTR and CFTRΔF508 expressing cells was the same. Series resistance and cell capacitance were compensated (*16*). Cells were studied at 30° to 35°C.
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