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Basal expression of the cystic fibrosis transmembrane conductance regulator gene is dependent on protein kinase A activity

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ABSTRACT **The cystic fibrosis transmembrane conductance regulator (CFTR) functions as a Cl^- channel that becomes activated after phosphorylation by cAMP-dependent protein kinase (PKA). We demonstrate that PKA also plays a crucial role in maintaining basal expression of the CFTR gene in the human colon carcinoma cell line T84. Inhibition of PKA activity by expression of a dominant-negative regulatory subunit or treatment with the PKA-selective inhibitor *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89) caused a complete suppression of CFTR gene expression without affecting other constitutively active genes. Basal expression of a 2.2-kb region of the CFTR promoter linked to a luciferase reporter gene (CFTR-luc) exhibited the same dependence on PKA. The ability of cAMP to induce CFTR over basal levels is cell-type specific. In T84 cells, both the endogenous CFTR gene and CFTR-luc exhibited only a modest inducibility (≈2-fold), whereas in the human choriocarcinoma cell line JEG-3, CFTR-luc could be induced at least 4-fold. A variant cAMP-response element is present at position −48 to −41 in the CFTR promoter, and mutation of this sequence blocks basal expression. We conclude that cAMP, acting through PKA, is an essential regulator of basal CFTR gene expression and may mediate an induction of CFTR in responsive cell types.**

The cystic fibrosis transmembrane conductance regulator (CFTR) gene is expressed in a variety of tissues, including respiratory and nasal epithelium, sweat and pancreatic glands, and intestinal epithelial cells. The gene encodes a cAMP- and ATP-regulated Cl^- channel that plays a vital role in electrolyte and fluid transport across apical epithelial surfaces. Numerous mutations have been identified in the CFTR gene that result in either a complete loss of protein expression or a varying degree of impaired function. Patients with mutations in both alleles of the CFTR gene develop cystic fibrosis (CF), a chronic debilitating disease characterized by progressive respiratory failure and infection, exocrine pancreatic dysfunction, gastrointestinal disorders, and infertility. CF is remarkably prevalent in the Caucasian population, affecting 1 in 2000–2500 live births with a carrier frequency of 1 in 20–25 (1). This prevalence suggests that heterozygotes may have a selective advantage that likely includes decreased susceptibility to cholera toxin (2), an activator of G-protein-coupled adenylate cyclase.

Regulation of Cl^- efflux through the CFTR channel is achieved by the concerted action of ATP at conserved nucleotide-binding folds and phosphorylation of multiple serines by cAMP-dependent protein kinase (PKA) in the putative regulatory (R) domain (3, 4). PKA phosphorylation sites on CFTR have been identified and mutation of four serines within the R domain of the CFTR inhibits cAMP-dependent activation of the channel (3). The mechanism of R domain regulation may involve the obstruction of the channel until phosphorylation of the R domain prevents this blockage, since a deletion of most of the R domain results in high constitutive Cl^- channel activity (5). Protein kinase C (PKC) also phosphorylates CFTR and increases Cl^- channel current, although to a lesser degree than the activation by PKA (6).

In addition to regulating the functional properties of the CFTR channel, protein kinases may also be involved in the regulation of CFTR gene expression. Several groups have shown that treatment of cultured cells with phorbol 12-myristate 13-acetate (PMA) results in down regulation of CFTR mRNA (7–10). Breuer *et al.* (11) demonstrated an increase in CFTR protein after stimulation with agents that increase cAMP. Several studies (12–14) have characterized the CFTR promoter and identified potential regulatory elements that may modulate expression. Multiple start sites of transcription and an alternative splice variant transcript of the CFTR gene have been described in T84 and Caco-2 cells (13, 14). The upstream region does not contain any consensus TATA or CCAAT motifs but does contain a highly G+C-rich promoter region and several consensus Sp1-binding sites (12, 14). There are segments within the CFTR promoter that resemble AP-1 binding sites, a cAMP-response element (CRE), and glucocorticoid response elements. In addition, the CFTR promoter contains sequences similar to AP-2 sites that might confer cAMP or phorbol ester inducibility (14). On the basis of these studies, expression from the CFTR gene is likely to be influenced by physiological stimuli. Here, we show that basal transcription of the CFTR gene is dependent on PKA activity and that CFTR expression may also be increased by elevation of cAMP in certain cell types.

MATERIALS AND METHODS

Cell Culture. Human colon adenocarcinoma T84 cells, the neomycin-resistant control clone P3, and the mutant R subunit (R_{AB})-expressing R5 and R6 clones were maintained as described (15).

Northern Blot Analysis. Total RNA was extracted from T84 cell cultures at 60–70% confluence by a modification of the guanidine hydrochloride method (16). The CFTR probe was created by using T7 RNA polymerase (New England Biolabs)

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Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; PKA, cAMP-dependent protein kinase; R, regulatory; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; CRE, cAMP-response element; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; MT, metallothionein; H-89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide; 8-cpt-cAMP, 8-(4-chlorophenylthio)-cAMP; CREB, CRE-binding protein.

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to synthesize antisense ^{32}P-labeled CFTR RNA with 340 bp of CFTR cDNA from clone P 10-1 (17) in pBluescript SK(−) as template. The blot was also probed with antisense ^{32}P-labeled RNA for human glyceraldehyde-3-phosphate dehydrogenase (G3PDH; Clontech).

Solution Hybridization. Total nucleic acid was harvested from T84 cells after treatment with 30 μM H-89 {*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide} (Seikagaku America, Rockville, MD), 100 μM 8-(4-chlorophenylthio)-cAMP (8-cpt-cAMP), or 100 nM PMA, hybridized to ≈5000 cpm of ^{32}P-labeled CFTR probe and analyzed and quantified as described (18). Solution hybridization with the ^{32}P-labeled antisense G3PDH RNA probe was also performed for each sample.

Construction of CFTR-Promoter Reporter Vector. PCR was used to amplify the region from the *Xba* I restriction site at −2247 to position +52 of the CFTR promoter by using a fragment of the promoter kindly provided by Douglas Jones (University of Iowa). Promoter locations are relative to the major transcription start site in T84 cells (14). The PCR fragment, containing a *Bam*HI restriction site at the 3′ end, was ligated with an oligonucleotide *Sac* I/*Xba* I adapter into the inh-luc construct (19) digested with *Sac* I and *Bgl* II to construct CFTR-luc. The CFTR(mut)-luc construct was generated by using the Altered Sites protocol (Promega) to change the CRE-like sequence from TGACaTCA to TACTaGTA. The ΔP-luc is a promoterless control construct.

Transient Transfections. T84 cells were transfected at ≈50% confluence by using a modification of the Lipofectin protocol (GIBCO/BRL). Reporter plasmid (10 μg per 100-mm dish) and 100 μl of Lipofectin were added to 5 ml of Opti-MEM on cells and incubated for 24 h at 37°C. Transfected cells were then replated onto 6-well dishes, allowed to recover for 3 h, and treated with H-89 or forskolin as indicated in the figure legends. Cells were then lysed and assayed for luciferase activity as described (20). Luciferase activity was divided by the amount of protein present (Bio-Rad), and the basal expression level (in the absence of H-89 or forskolin) was set at 100%.

Human JEG-3 choriocarcinoma cells were grown on 24-well plates (21) and transfected as described (22) except that 100 ng of CFTR-luc or 2.5 ng of α168-luc was used as the luciferase reporter. For those experiments examining R_{AB}, the Zn^{2+}-inducible expression vector metallothionein (MT)-R_{AB} (21) was also added. As a control for possible promoter competition, the amount of MT-containing vector was kept constant by the inclusion of parental MT vector that lacks an R_{AB} insert. After forskolin or H-89 treatment, cells were harvested and assayed for luciferase and β-galactosidase activity as described (23).

RESULTS

CFTR mRNA Expression Is Decreased in Stable T84 Transformants Expressing the Dominant-Negative R Subunit of PKA. We have shown that R5 and R6, T84 clones independently transfected with the dominant-negative PKA expression vector MT-R_{AB}, demonstrate inhibited PKA activity and fail to show an increased anion conductance in response to agents that elevate cAMP (15). CFTR mRNA levels were measured by Northern blotting in wild-type T84 cells, the control T84 clone P3 (transfected with a vector containing only the gene for neomycin resistance), and mutant clones R5 and R6. As shown in Fig. 1, the expected 6.5-kb CFTR band was present in the T84 wild type and control P3 cells but was absent in the R5 and R6 clones. The blot was reprobed with ^{32}P-labeled G3PDH RNA probe, and all samples revealed equal intensity of the predicted 1.0-kb band (Fig. 1). Ribonuclease protection analysis also demonstrated a lack of CFTR RNA in the PKA-deficient cell lines (data not shown).

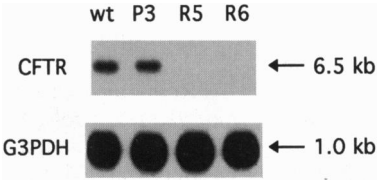

FIG. 1. Absence of CFTR mRNA in human epithelial T84 subclones deficient in PKA. Northern blot of total cellular RNA (10 μg per lane). Lane 1, T84 wild-type (wt); lane 2, T84 control clone (P3); and lanes 3 and 4, T84 PKA-deficient clones R5 and R6.

CFTR mRNA Is Regulated by PKA in Wild-Type T84 Cells. To determine whether pharmacological kinase inhibition would also result in decrease CFTR expression, we treated wild-type T84 cells with the specific PKA inhibitor H-89 (24) and examined CFTR mRNA expression by solution hybridization. Treatment with 30 μM H-89 resulted in a marked downregulation of CFTR expression by 24 h (Fig. 2*A*) in comparison with control T84 cells. G3PDH mRNA levels were unchanged by H-89 treatment (data not shown).

To determine whether increases in PKA activity would increase CFTR mRNA in T84 cells, we performed solution hybridizations after treating cells with the cAMP analog 8-cpt-cAMP. Activation of PKA by 8-cpt-cAMP produced a 2-fold increase in the level of CFTR mRNA after 8 h of stimulation (Fig. 2*B*); there was no change in G3PDH mRNA (data not shown). We also treated wild-type T84 cells with 100 nM PMA over the same time course. PMA treatment resulted in a decrease in the number of CFTR mRNA molecules per cell, with maximal suppression 16 h after treatment (Fig. 2*B*), similar to previous results showing downregulation of CFTR expression after phorbol ester treatment (7–10). The data presented in Fig. 2 suggest that PKA might regulate either the transcription of CFTR or the stability of CFTR RNA.

The CFTR Promoter Is Regulated by PKA in Wild-Type T84 Cells and JEG-3 Cells. To examine potential regulation of the CFTR promoter by PKA, we ligated a 2.2-kb fragment of the human CFTR promoter to a luciferase reporter gene. We also created a mutant (mut) CFTR construct in which the CRE-like

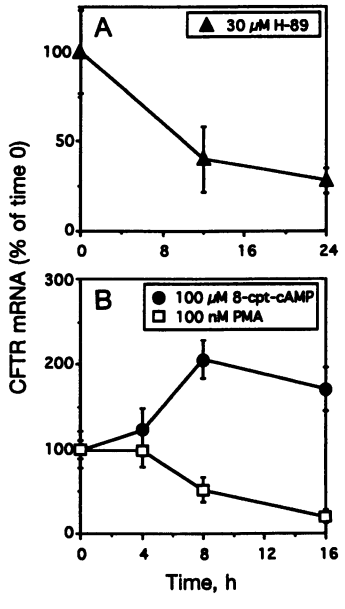

FIG. 2. Regulation of CFTR mRNA by PKA and PKC. (*A*) T84 wild-type cells treated with 30 μM H-89. (*B*) T84 wild-type cells treated with 100 μM 8-cpt-cAMP (●) or 100 nM PMA (□). CFTR mRNA was quantified by comparison with a standard curve by CFTR riboprobe hybridization. Error bars represent the range of values from duplicate dishes.

sequence at nucleotide −48 was mutated from TGACaTCA to TACTaGTA. The CFTR(wt)-luc and CFTR(mut)-luc constructs are depicted in Fig. 3*A*. In transient transfections of T84 cells, basal expression of CFTR(wt)-luc was 4- to 5-fold above the expression of the promoterless control ΔP-luc construct, which was similar to the untransfected control (data not shown). A 6-h forskolin treatment (30 μM) produced an 80% increase in CFTR-luc expression (Fig. 3*B*), similar to the effect of 8-cpt-cAMP on endogenous CFTR mRNA (Fig. 2). A 24-h H-89 treatment (30 μM) inhibited basal CFTR-luc expression by 60% (Fig. 3*B*). The forskolin-mediated stimulation of CFTR-luc was also inhibited over the same range of H-89 concentrations (data not shown). In the experiment depicted in Fig. 3*B*, comparison of CFTR(wt) and CFTR(mut) activities revealed that the CFTR(mut) promoter had lost 90% of its basal activity, although a weak activation by cAMP was still observed. As a control for cAMP responsiveness, we used the cAMP-inducible promoter from the glycoprotein hormone α subunit (α168-luc) (25). α168-luc activity was stimulated 4-fold by 30 μM forskolin, while treatment with H-89 inhibited basal α168-luc expression by 75%, as shown in Fig. 3*C*. The experiments depicted in Fig. 3 demonstrate that the basal expression of CFTR-luc is dependent on PKA activity, similar to the expression of endogenous CFTR mRNA in T84 cells. Furthermore, the cAMP-mediated stimulation of CFTR-luc closely matches the stimulation of endogenous CFTR mRNA in T84 cells. The α168 promoter is also regulated by PKA activity in T84 cells, but the 4-fold stimulation observed here is much weaker than the induction seen in other cell types (25).

The modest induction of both CFTR-luc and α168-luc by PKA in T84 cells prompted us to examine these promoters by using JEG-3 cells, a cell line that we have used previously to study cAMP-mediated gene expression (20, 22, 25). Although JEG-3 cells do not express the endogenous CFTR gene, as assayed by ribonuclease protection or solution hybridization (data not shown), we observed a substantial activity of CFTR-luc in transient transfection assays. Basal expression of CFTR-luc was 4-fold higher than the expression of the promoterless control ΔP-luc, and forskolin induced CFTR-luc a further 3- to 4-fold (Figs. 4 and 5). Fig. 4*A* depicts an experiment in which JEG-3 cells were transfected with CFTR-luc and then treated with forskolin for 6 h, resulting in a 3-fold induction. H-89, added 30 min prior to addition of forskolin, inhibited the induction with an IC_{50} of 3 μM. Only a slight inhibition of the basal expression of CFTR-luc by H-89 was observed over the 6-h time course in Fig. 4*A*, whereas increasing the time of H-89 exposure to 24 h completely suppressed basal expression (Fig. 4*C*). The cAMP-induced expression of the α168-luc construct (Fig. 4*B*) showed the same sensitivity to H-89 inhibition. The relative luciferase activity of cells transfected with ΔP-luc did not change significantly with different drug treatments (data not shown).

We also examined the ability of the dominant-negative PKA R subunit expression vector (MT-R_{AB}) to inhibit basal and forskolin-stimulated CFTR-luc activity in JEG-3 cells. When JEG-3 cells were cotransfected with MT-R_{AB} and CFTR-luc, both the basal and forskolin-stimulated activities of CFTR-luc were inhibited (Fig. 5*A*). In a parallel experiment, forskolin-stimulated α168-luc activity was also inhibited by MT-R_{AB} (Fig. 5*B*). These results demonstrate that the transiently expressed CFTR promoter responds to a genetic inhibition of PKA. Stimulation of α168-luc by forskolin in JEG-3 cells was more dramatic than that seen in T84 cells (Figs. 3 and 4, and data not shown), suggesting that PKA elicits a more dynamic effect on gene expression in JEG-3 cells. Our results from the JEG-3 cells support the findings from our transient transfection assays in T84 cells demonstrating that the CFTR promoter is regulated by changes in PKA activity.

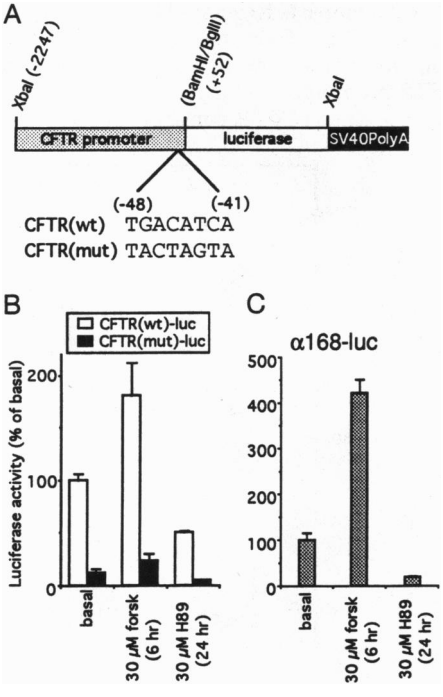

FIG. 3. α168-luc and CFTR-luc expression are regulated by PKA in T84 cells. (*A*) Wild-type (wt) and mutant (mut) CFTR-luc reporter constructs. The sequence of the variant CRE at position −48 and −41 in the wt and mut constructs is shown. (*B*) T84 cells were transfected en masse with CFTR-luc and after replating were treated with 30 μM H-89 (24 h) or 30 μM forskolin (forsk) (6 h). Basal expression of CFTR(wt) was set at 100%. (*C*) T84 cells were transfected with α168-luc and treated with 30 μM forskolin (6 h) or 30 μM H-89 (24 h). Error bars represent the SD of triplicates.

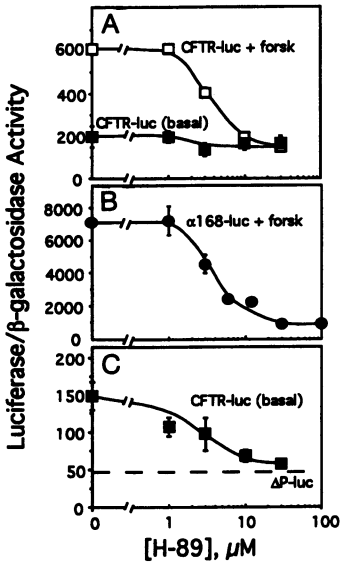

FIG. 4. H-89 inhibits forskolin-stimulated and basal CFTR-luc expression. (*A*) JEG-3 cells were transfected with CFTR-luc and were treated with H-89 at the concentrations indicated. After 30 min, 30 μM forskolin (forsk) (□) or vehicle (■) was added directly to the medium; cells were lysed and assayed 5 h later. (*B*) JEG-3 cells were transfected with α168-luc and subsequently treated with H-89 in the indicated concentrations and 30 μM forskolin as in *A*. (*C*) In a parallel experiment to *A*, cells were transfected with CFTR-luc and treated with H-89 24 h before harvesting. The dashed line represents the relative activity of cells transfected with 100 ng of ΔP-luc per well. The transfections were performed in triplicate and the mean ± SD is shown for each data point. Data are presented as a ratio of luciferase activity to β-galactosidase activity resulting from coexpression of the internal control plasmid, pRSV-lacZ.

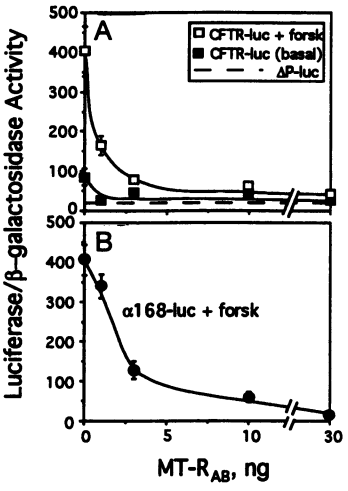

FIG. 5. Forskolin stimulation of CFTR promoter activity is inhibited by R_{AB} coexpression. (*A*) JEG-3 cells were cotransfected with CFTR-luc and R_{AB} expression vector (MT-R_{AB}) in the amounts indicated. After 24 h of DNA exposure and 14 h of Zn^{2+} treatment, cells were exposed to 30 μM forskolin (forsk) (□) or vehicle (■) for 5 h before being assayed. The relative activity of 100 ng of ΔP-luc per well is indicated by the dashed line. (*B*) In a parallel experiment, JEG-3 cells were cotransfected with 2.5 ng of α168-luciferase per well (●) and MT-R_{AB} in the amounts indicated, treated with Zn^{2+} and 30 μM forskolin as above, and assayed for luciferase and β-galactosidase activity. The transfections were performed in triplicate and the mean ± SD is shown.

DISCUSSION

The T84 human colon carcinoma cell line has been used extensively as a cell culture model to study the regulation of the CFTR anion-selective channel that is defective in patients with CF. We have previously characterized mutations in the R subunit of PKA that interfere with cAMP binding and lead to a dominant inhibition of kinase activity (21). When this mutant R subunit (R_{AB}) is expressed in cultured T84 cells, stable transformants can be isolated that lack normal levels of PKA activity, and these subclones lose the ability to regulate ^{125}I efflux in response to vasoactive intestinal peptide, prostaglandin E_2, or forskolin (15). However, ^{125}I efflux is stimulated normally in response to intracellular Ca^{2+} in PKA-deficient subclones. Since at the time of our original report it was thought that the stimulation of anion efflux by elevated intracellular Ca^{2+} was also dependent on CFTR, we concluded that the CFTR protein continued to be expressed and that only the channel phosphorylation by PKA was inhibited (15). More recent evidence has demonstrated that cAMP- and Ca^{2+}-stimulated Cl^- effluxes occur via electrophysiologically distinct channels that differ in anion selectivity and channel-blocker sensitivity (26, 27) and are products of distinct genes (28). Since the PKA-deficient T84 subclones have lost their cAMP-inducible Cl^- efflux, we examined whether these clones continued to express the CFTR gene. Surprisingly, the subclones produced no detectable CFTR mRNA, as determined by Northern blotting, ribonuclease protection, or solution hybridization, indicating that either transcription or mRNA stability might depend on PKA activity. Pharmacological inhibition of PKA also leads to a decrease in CFTR mRNA, whereas agents that stimulate PKA such as 8-cpt-cAMP elicit only a modest induction. These results demonstrate that in T84 cells the expression of CFTR mRNA is completely dependent on constitutive activation of the PKA pathway.

To examine the ability of PKA to directly affect transcription of the CFTR gene, we constructed CFTR-luc, which contains the 2.2-kb CFTR promoter upstream of the luciferase gene. The transient expression of CFTR-luc was examined in both T84 and JEG-3 cells, a human choriocarcinoma cell line that we have used extensively to study PKA-mediated effects on transcription (20, 22, 25). In parallel with the CFTR-luc experiments, we transfected cells with the α168-luc construct, which contains 168 bp of the glycoprotein hormone α subunit promoter upstream of the luciferase coding region (25). This promoter, which contains two tandem consensus CREs, has been shown to be highly responsive to PKA (25). PKA regulates CFTR-luc and α168-luc in transient transfections of T84 cells, though this cell line proved to be much less responsive to cAMP than the JEG-3 cells. Although the induction of CFTR-luc by cAMP was modest, the basal activity of the CFTR-luc construct was completely dependent on PKA activity, as was seen for the endogenous CFTR gene. In the more cAMP-responsive JEG-3 cells, we found that CFTR-luc was stimulated 3- to 4-fold by administration of forskolin and that, as in T84 cells, both basal and forskolin-stimulated CFTR-luc expression were inhibited by H-89. In addition, transient cotransfection of the dominant-negative PKA regulatory subunit (R_{AB}) used to create the mutant T84 cell lines also inhibited basal and forskolin-stimulated CFTR-luc expression in JEG-3 cells. In the JEG-3 cell experiments presented here, α168-luc gave a higher level of basal expression than CFTR-luc and was stimulated by forskolin to a much greater degree (up to 30-fold).

The use of specific genetic approaches to inhibit PKA has made it possible to examine the role of basal PKA activity in the regulation of specific genes. For example, mouse Y1 adrenal cells expressing R_{AB} exhibit a nearly complete loss of expression of ornithine decarboxylase, P450 side-chain cleavage enzyme, and urokinase mRNAs; these genes are constitutively expressed in control Y1 cells and are induced by elevated levels of cAMP (18). A similar genetic approach involves transient overexpression of the PKA inhibitor PKI, together with promoter–reporter constructs. By using this latter technique, the basal expression of cotransfected enkephalin (29) and prolactin (30) promoters has been shown to be PKA-dependent. In some of these cases, CREs have been identified in the promoter and are thought to mediate PKA effects by binding phosphorylated CRE-binding protein (CREB) or related transcription factors. The contribution of CREBs to both constitutive and inducible expression has been examined by site-directed mutagenesis of CREs within the promoter for ornithine decarboxylase (31), glycoprotein hormone α subunit (25, 32), tyrosine hydroxylase (33), and dopamine β-hydroxylase (34) genes. In all cases, both PKA-inducible and constitutive expression were lost when the CREs were mutated. The CFTR promoter contains one variant CRE sequence (TGACaTCA) that differs from the consensus sequence (TGACGTCA) at the central G residue. An identical variant CRE occurs in the bovine and rat glycoprotein α subunit gene promoter, but disparate results have been reported on its ability to support a cAMP response (32, 35). The human tissue-type plasminogen activator (t-PA) gene promoter also contains a copy of this variant CRE, and in this context it mediates a cooperative interaction with other sites to confer basal and weak cAMP-inducible expression (36). When the variant CRE is mutated in the CFTR promoter, basal expression declines dramatically, although forskolin is still able to elicit a weak stimulation. Clearly the nature of this variant CRE requires further study, since although the sequence will bind CREB *in vitro* (R.P.M. and G.S.M., unpublished observations), we have not determined whether CREB mediates the basal activity *in vivo* or whether the site will support a cAMP response when placed upstream of a heterologous promoter. The CFTR promoter also contains putative binding sites for AP-2, AP-1, glucocorticoid receptor, and CCAAT enhancer-binding protein β (C/EBPβ), and it is possible that the PKA-dependent regulation is mediated by

one or more of these other sites in concert with the CRE, a scenario similar to that observed for the t-PA promoter (36).

The role of cAMP and PKA in CFTR regulation appears to involve at least two levels of control. The rapid opening of CFTR channels and efflux of Cl^- is a direct result of PKA-dependent phosphorylation of the R domain of CFTR. Here, we have shown that what has previously been thought of as constitutive activity of the CFTR gene is actually PKA-dependent transcription. These results suggest that a prolonged decrease in PKA activity will modulate the number of CFTR channels by a direct effect on transcription of the CFTR gene. We also demonstrate the potential for cAMP-mediated induction of CFTR expression, although the magnitude of this effect is cell-type specific. The dual mechanism involving channel phosphorylation and regulation of transcription may have important physiological consequences. Mutations within the promoter, such as those we have described in the CRE-like sequence, could lead to symptoms of CF, although this has yet to be clinically observed. In addition, pharmacological intervention, including the established treatments for airway inflammation such as β_2-adrenergic agonists—e.g., albuterol and terbutaline—and adenosine antagonists—e.g, theophylline—might lead to a stimulation of CFTR gene expression via the PKA pathway. The resulting change in expression of the channel could have therapeutic benefits in CF patients depending on the nature of the CFTR mutations.

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