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Development of an epithelium-specific expression cassette with human DNA regulatory elements for transgene expression in lung airways

(cystic fibrosis transmembrane conductance regulator/transgenic mice/intron enhancer/cell specificity/secreted alkaline phosphatase)

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ABSTRACT **The efficient expression of therapeutic genes in target cells or tissues is an important component of efficient and safe gene therapy. Utilizing regulatory elements from the human cytokeratin 18 (K18) gene, including 5′ genomic sequences and one of its introns, we have developed a novel expression cassette that can efficiently express reporter genes, as well as the human cystic fibrosis transmembrane conductance regulator (CFTR) gene, in cultured lung epithelial cells. CFTR transcripts expressed from the native K18 enhancer/promoter include two alternative splicing products, due to the activation of two cryptic splice sites in the CFTR coding region. Modification of the K18 intron and CFTR cDNA sequences eliminated the cryptic splice sites without changing the CFTR amino acid sequence, and led to enhanced CFTR mRNA and protein expression as well as biological function. Transgenic expression analysis in mice showed that the modified expression cassette can direct efficient and epithelium-specific expression of the *Escherichia coli LacZ* gene in the airways of fetal lungs, with no detectable expression in lung fibroblasts or endothelial cells. This is the first expression cassette which selectively directs lung transgene expression for CFTR gene therapy to airway epithelia.**

Cystic fibrosis (CF) is the most frequent lethal inherited disease in the Caucasian population. This disorder, caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, affects various secretory epithelial tissues, but the major cause of morbidity and mortality is respiratory failure (1, 2). Since the structure and function of the CFTR gene have been well characterized (1), it has been used as a model for designing expression cassettes for lung gene therapy. Both viral and nonviral vectors have been utilized in recent years for CF gene therapy. Although clinical benefits for CF patients have yet to be shown, phase I clinical trials have demonstrated the feasibility of gene transfer to CF patients (3). However, among the major impediments to the development of gene therapy treatments is the lack of suitable expression cassettes for directing selective transgene expression in target cells. We have been interested in employing human DNA regulatory elements for the construction of expression cassettes for CF gene therapy, since the trans-acting factors that naturally interact with these elements are present in the target cells and it is theoretically preferable to avoid viral genomic sequences.

We chose to study promoter elements of the human cytokeratin 18 (K18) gene because it is expressed predominantly in epithelia of internal organs (lung, liver, kidney, and intestine) (4), a distribution pattern that shares several features with that of CFTR (1). The genomic fragment containing the entire K18 gene, including exons, introns and large 5′ and 3′ untranslated regions, has been cloned and shown to confer appropriate tissue specificity, as well as copy number-dependent expression, in transgenic animal studies (5). The minimal promoter and a 2.2-kb upstream region of the K18 gene were reported to be solely responsible for the efficient and copy number-dependent expression of the human K18 gene in lungs of the transgenic mice, suggesting that it contained a lung-specific locus control region. Enhancer activity has also been located within the first intron of the K18 gene (6), which contains binding sites for the transcription factors AP-1 and ETS, occupancy of which correlates with induced K18 expression (7, 8). The regulatory elements in K18 intron 1 have been studied extensively because of the implicated significance in oncogenic signal transduction, yet little is known about the regulation of expression by the 5′ upstream regulatory elements of the K18 gene.

We have analyzed several DNA elements from the K18 gene for gene expression activity and have developed a novel expression cassette based on the results of our analysis. We demonstrate here that this expression cassette can express functional human CFTR protein in cultured cell lines, express reporter genes in primary cultures of lung epithelium, and direct efficient expression of the *LacZ* reporter gene in the lung airways of transgenic mice.

MATERIALS AND METHODS

Expression Constructs. PCRs were performed on genomic DNA of A549 cells (9) to isolate the first intron, minimal promoter, and two 5′ untranslated regions of human K18 gene, based on published sequences (GenBank accession nos. M24842, M19353, and X12799). PCR products were cloned into the polylinker region of pSEAP (Tropix, Bedford, MA) that contains a reporter gene encoding secreted alkaline phosphatase (SEAP).

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Abbreviations: CF, cystic fibrosis, CFTR, CF transmembrane conductance regulator; SEAP, secreted alkaline phosphatase; TE, translational enhancer; CMV, cytomegalovirus; X-gal, 5-bromo-4-chloro-3-indolyl β-D-galactodise.

The sequence of human CFTR cDNA was modified by PCR to improve the translation initiation signal using a CFTRp1 primer (5′-GAGACCATGGAGAGGTCG). A linker containing the alfalfa mosaic virus translational enhancer (TE) sequence (5′-GTTTTTATTTTTAATTTTCTTTCAAATACTTCCA) (10) was inserted immediately upstream of the start codon. The addition of the translational enhancer was to promote the CFTR mRNA translation since the CFTR sequence included in this construct does not contain the 5′ untranslated region required for translation. The SEAP coding region in K18EpilongSEAP was then replaced with the TE-4.6-kb CFTR cDNA fragment, resulting in the K18EpilongTECFTR construct.

Site-directed mutagenesis was conducted on K18EpilongTECFTR by 2-step nested PCR. First-round PCR reactions incorporated primer pairs (TE2–5′-GTCCGCAAAGCCTGAGTCCTGTCC/K183′SS-5′-AAATTAAAAATAAAAACAGACCTGAAAAAAAAAAAGAGAGAGGTTGTTCCATGA) and (TEtop-5′-GATCTGTTTTTATTTTTAATTTTCTTTCAAATACTTCCACCATGGCCCC/CFTR3′SS-5′GGTGACTTCCCCCAAATATAAAAAG). Products from the first-round reactions were mixed and served as templates for the second-round PCR using TE2 and CFTR3′SS primers. The K18mCFTR (K18EpilongmCFTR) construct was then generated by cloning the second-round PCR product back into K18EpilongTECFTR to replace the corresponding parental fragment.

Tissue Culture, Transfection, and Reporter Assay. A549, COS-1, WI-38, and CCD-32Lu cells were maintained as recommended by American Type Culture Collection. IB3, a human cystic fibrosis bronchial epithelial cell line (provided by P. Zeitlin, ref. 11), was cultured in Lechner and Laveck medium (Biofluid, Rockville, MD) with 5% fetal bovine serum. Primary cultures of day 19 rat fetal lung epithelium and lung fibroblast cells were isolated and maintained similar to published methods (12). The cells were transfected at 50–80% confluency with 1 μg DNA premixed with 12 μg of lipofectamine (GIBCO/BRL) per 35-mm well according to the recommended procedure. SEAP activities in the culture media were detected using the Phospha-Light chemiluminescent assay system (Tropix) as recommended, and measured on a luminometer (BioOrbit, Turku, Finland).

Functional Analysis of CFTR Protein. Iodide efflux assays were performed as previously described (13, 14), with slight modifications on compositions of the loading buffer [136 mM NaI/4 mM KNO_3/2 mM $Ca(NO_3)_2$/2 mM $Mg(NO_3)_2$/11 mM glucose/20 mM Hepes, pH 7.4] and the agonists, [20 μM forskolin, 0.5 mM 8-(4-chlorophenylthio)-adenosine-3′; 5′-cyclic monophosphate (CPT-cAMP), and 0.5 mM 3-isobutyl-1-methylxanthine]. Net iodide efflux from stimulated and nonstimulated cells was measured potentiometrically using an iodide-specific electrode (Orion, Boston).

Transgenic Embryos. K18mTELacZ was generated by replacing the CFTR cDNA in K18mCFTR with a modified *Escherichia coli* β-galactosidase gene that contains a simian virus 40 large T antigen nuclear localization signal (15) fused in-frame to the 5′ end of the gene. The expression cassette was purified as a 7.5-kb fragment, complete with K18EpilongmTE sequences, nuclear localization signal-*LacZ*, and simian virus 40 polyadenylation signal, without any vector sequence, and injected into pronuclei of mouse SJL/B6 fertilized eggs. Two-cell stage embryos were implanted into pseudopregnant CD1 surrogate mothers by standard procedures.

RESULTS

Development and Analyses of K18-Based Expression Cassettes. A 2.5-kb DNA sequence of the K18 gene was isolated by PCR-cloning as three fragments (see Fig. 1*a*): a 290-bp minimal promoter and two upstream "enhancer" fragments of size 800 bp and 1.4 kb, that can be linked together to reconstruct the genomic sequence. K18 intron 1 was obtained as a 750-bp fragment, complete with 5′ and 3′ splicing consensus sequences. Promoter/enhancer activity in these sequences was evaluated using a SEAP reporter system (Tropix).

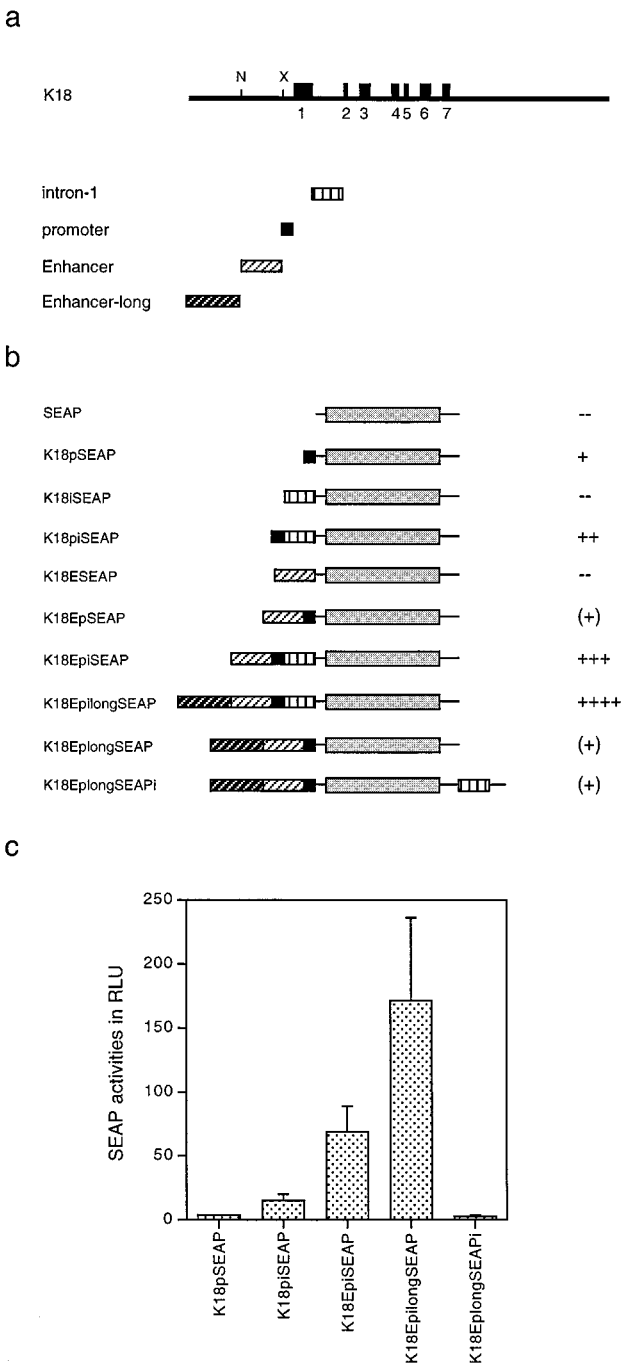

FIG. 1. Construction and analysis of K18-based expression cassettes. Shown in panel *a* are the genomic structure of the human K18 gene, in which exons are depicted as solid boxes, and the spatial relationships of the various untranslated regions isolated. (*b*) The schematic diagrams of the promoterless SEAP construct and a series of its derivatives that contain segments of K18 untranslated sequences, singularly or in combination. Summarized on the right are their relative expression levels, shown as − (negative) and + (positive). Each added + indicates a 3–5 fold increase of expression activity and (+) means marginally positive. (*c*) Expression levels from a subset of K18SEAP constructs. A549 cells were transfected with the indicated plasmids. After normalized to total protein, the expression levels of the K18SEAP constructs are shown as relative light units measured with the luminometer. The results are presented as mean ± SEM (n = 5).

When tested in a human lung epithelial cell line, A549 (9), the K18 minimal promoter directs a low, but detectable, level of expression. While the intron 1 and two enhancer fragments by themselves could not drive SEAP expression, in combination with the minimal promoter they each could enhance the promoter activity 3- to 7-fold (Fig. 1 *b* and *c*). These enhancer activities are additive, as the strongest expression construct (K18EpilongSEAP) contains the entire 2.5-kb 5′ sequence in the genomic configuration plus intron 1 upstream of the SEAP coding region. This K18Epilong construct was shown to contain sufficient information to direct epithelium-specific expression because it drives SEAP expression in A549 cells, but not in the human lung fibroblast cell lines WI-38 or CCD-32Lu (Fig. 2*a*), while the cytomegalovirus (CMV) enhancer/promoter works well in either cell type. These results are consistent with our observations that the K18 enhancer/promoter showed a comparable expression activity in primary rat fetal lung epithelial cells, while its level of expression in rat fetal lung fibroblast cells is only about 10–15% of that conferred by the CMV enhancer/promoter (data not shown).

To test the duration of K18EpilongSEAP expression in A549 cells, the conditioned culture media of the transfected cells were harvested and then replaced with fresh media every two days, and the SEAP activity in the conditioned media was measured. K18EpilongSEAP showed peak expression levels at 2 days posttransfection, which then decreased steadily until about day 10 and then maintained at a level significantly above background (Fig. 2*b*). Although the level of expression of SEAP driven by the CMV enhancer/promoter had a 2–5 fold higher peak level at day 2, it then dropped sharply and after two weeks became undetectable in A549 cells.

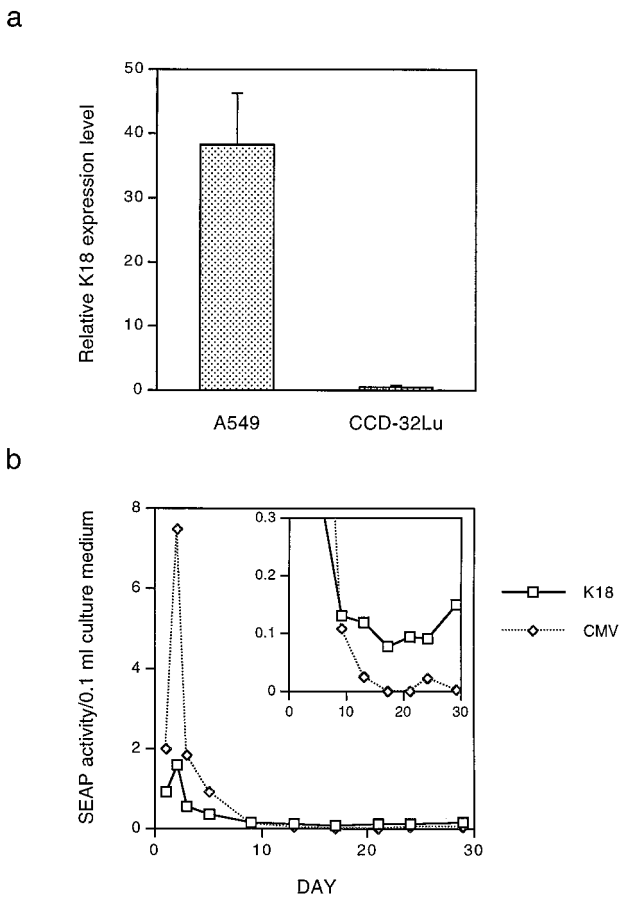

FIG. 2. Expression specificity and kinetics of K18EpilongSEAP. (*a*) Preferential expression of K18EpilongSEAP in epithelial cells. A549 (human lung epithelial) and CCD-32Lu (human lung fibroblast) cells were transfected with K18EpilongSEAP and CMVSEAP in parallel. The expression levels from K18EpilongSEAP in either cell lines are shown as percentage of CMVSEAP expression levels. Results are from three independent experiments, each as average of triplicates, and presented as mean ± SEM. (*b*) Expression kinetics of K18EpilongSEAP vs. CMVSEAP. A549 cells were transfected with DNA:lipid mix at 1:10 ratio. Culture media was collected, prior to media change, at days posttransfection as indicated and stored at −80°C until being assayed.

Expression and Differential Splicing of K18CFTR Chimeric Transcripts. To test if CFTR protein can be expressed by the K18 enhancer/promoter, the SEAP coding sequence in K18EpilongSEAP was replaced with the human CFTR cDNA (Fig. 3*a*). A TE (10) was added in front of the CFTR coding sequence and the translation initiation signal was modified according to the Kozak's consensus (16) to optimize translation efficiency.

PCR amplification of reverse-transcribed total RNA from transfected cells was performed to detect K18CFTR chimeric transcripts. The PCR product derived from the mature K18CFTR transcript, with the K18 intron spliced, should be 696 bp. In K18EpilongTECFTR transfected A549 (Fig. 3*d*, lane 3), IB3 and rat fetal lung fibroblast cells (not shown), two faster migrating species of PCR products are present along with the 696-bp band. These three fragments were isolated and subcloned for sequence analysis, which confirmed that the 696-bp reverse transcription–PCR product is derived from the K18-CFTR transcript accurately joined at the 5′ and 3′ splice sites of K18 intron 1, and that the two faster migrating bands result from the usage of two cryptic 3′ splice sites within the CFTR coding region (Fig. 3*b*). Because these alternatively spliced transcripts lack the authentic CFTR start codon, translation initiation could only take place at start codons further downstream, causing N terminus truncation or frame shift. As a result, <25% of the total transcript could give rise to full-length CFTR protein.

Evaluation of the splicing signals revealed that the K18 3′ splice site contains only a suboptimal consensus (17), which might be responsible for utilization of other cryptic 3′ splice sites. Two strategies could be employed to avoid the alternative splicing problem: to move the K18 intron 1 downstream of the CFTR coding sequence and to improve the K18 3′ splicing signal with point mutations. We did not pursue the former option after we observed that when K18 intron 1 was placed downstream of the SEAP cDNA, the resulting reporter construct, K18EplongSEAPi, produced only a minimal amount of SEAP (Fig. 1*c*).

In previous genetic studies, varied lengths of a thymidine track have been noted in front of the splice-acceptor site of intron 8 of the CFTR gene. The length appears to correlate with the efficiency of splicing, with the shortest variant (5T) producing ≈95% exon 9-minus mRNA (18, 19). These observations suggest that a long polythymidine track might be favored by the splicing machinery in epithelial cells. By PCR site-directed mutagenesis, the 3′ splice junction sequence of K18 intron 1 was changed to CAG/G, and the polypyrimidine tract was altered to include a stretch of 11 T's (Fig. 3*c*). The second cryptic 3′ splice site in the CFTR coding region, which contains a good consensus sequence, was destroyed by introducing a silent mutation. Together, these mutations, which did not alter the amino acid sequence of CFTR, effectively eliminated the alternative RNA splicing and resulted in a 10-fold enrichment of accurately spliced mRNA (Fig. 3*d*, lane 1).

Enhancement of Functional CFTR Expression. Expression of CFTR was examined by Western blot analysis. In COS-1 cells transfected with K18mCFTR, the unmodified, immature form (≈170 kDa) and the fully glycosylated, mature form (≈200 kDa) of CFTR protein were detected by an anti-CFTR antibody (Fig. 4*a*). To test if adequate CFTR protein was expressed to confer cAMP-dependent anion channel function, we employed a whole cell population-based iodide (I^-) efflux assay (13, 14). A characteristic cAMP-stimulated I^- efflux was

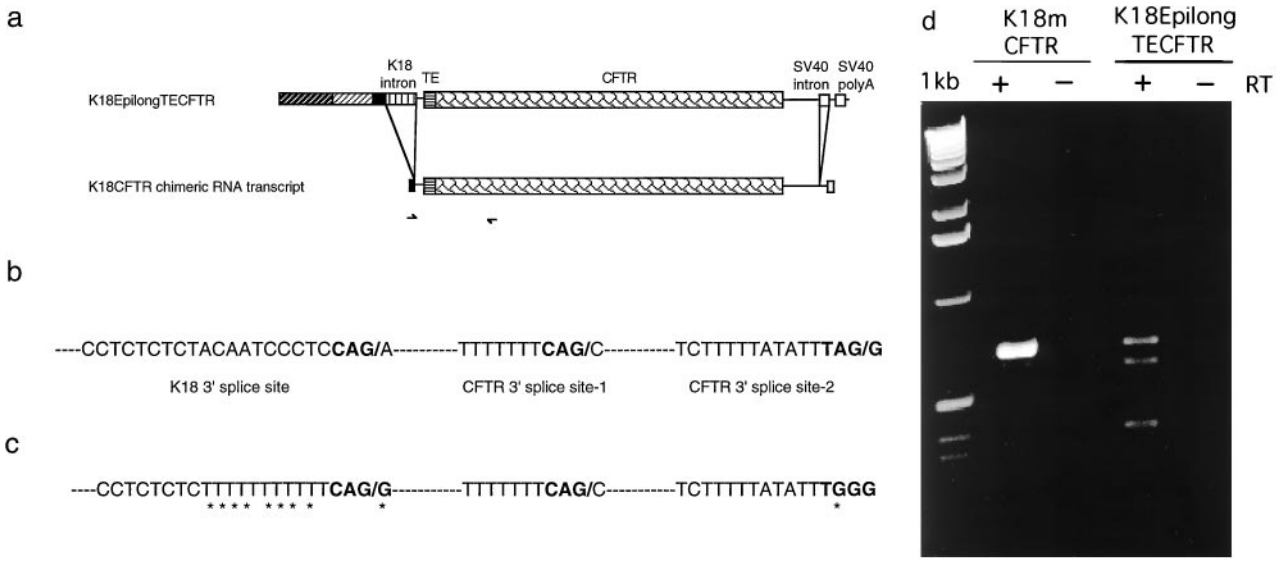

FIG. 3. Splicing of the K18-CFTR chimeric RNA transcript. (*a*) Schematic diagram of the structures of K18EpilongTECFTR and the mature RNA transcript. Primers used for reverse transcription–PCR in *d* are depicted as arrows. (*b*) DNA sequences of K18EpilongTECFTR at the K18 3′ splice site and two cryptic splice sites in the CFTR coding region. (*c*) DNA sequences of K18mCFTR at respective sites. Mutations introduced are indicated by asterisks. (*d*) PCR products from reverse-transcribed (RT+) total RNAs isolated from A549 cells transfected with the indicated plasmids. The correctly spliced transcript yields a 696-bp band, which is the only species found in K18mCFTR-transfected cells. In K18EpilongTECFTR transfected cells, two faster-migrating species, corresponding to splicing products utilizing the cryptic splice sites in the CFTR coding region, are also present.

observed from cells transfected with K18EpilongCFTR and K18mCFTR, but not from the negative control, K18Epilong (Fig. 4*b*). Consistent with the reverse transcription–PCR results, the mutated splicing signals in the K18mCFTR construct lead to a much higher level of CFTR channel activity.

Targeting Expression of the LacZ Reporter in the Airways of Lung. To examine the tissue distribution of expression of the K18Epilong construct *in vivo*, we generated transgenic mice using an *E. coli LacZ* reporter gene. Five out of sixteen embryos at gestational age 15.5 days were shown to carry the K18mTELacZ transgene by PCR and Southern analysis on yolk sac DNA. The day 15.5 lungs of these transgenic embryos as well as their nontransgenic littermates were also harvested and stained with 5-bromo-4-chloro-3-indolyl β-D-galactodise (X-gal) to detect β-galactosidase activity. As shown in Fig. 5 *a* and *b*, the airway epithelium in four lungs from the transgenic embryos stained blue with X-gal, although to various degrees. The different expression levels may be due to variation in developmental maturity and mosaic distribution of the transgene. Higher magnification of sections of these lungs revealed that the reporter gene expression was largely restricted to the epithelium lining the major airways (Fig. 5*c*). These results indicate that the modified K18 expression cassette is capable of conferring lung epithelium-specific expression *in vivo*.

DISCUSSION

The rationale for using human cell-specific DNA regulatory elements to develop expression cassettes for gene therapy is that the human *trans*-acting factors that normally interact with these elements are present in the target human cells. Viral promoters, which are often chosen for their strength, are generally nonspecific in regard to cell expression (20). Our results with the CMV enhancer/promoter not only confirmed the lack of tissue specificity but also revealed its transient expression. Moreover, although the CMV promoter directs higher expression levels in cultured cells, its activity in rat primary epithelial cells is comparable to that of the human K18 promoter. Previous transgenic studies have shown that the CMV enhancer/promoter was not functional in the mouse

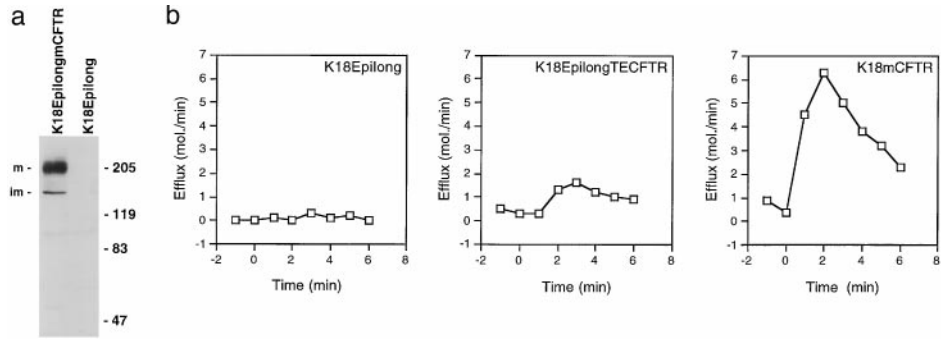

FIG. 4. Expression of the human CFTR protein and functional analysis of its chloride channel activity. (*a*) Whole cell lysates of transfected COS-1 cells were prepared with RIPA lysis buffer at 48 hr posttransfection, fractionated by SDS/PAGE, and probed with a CFTR C terminus-specific mAb (Genzyme). Bands correspond to mature (m) and immature (im) forms of CFTR protein are indicated. (*b*) Functional analysis for CFTR by iodide efflux assay. COS-1 cells were transfected with K18EpilongTECFTR, K18 mCFTR, or a negative control plasmid (K18Epilong). Forty-eight hours posttransfection, cells were loaded with iodide for 1 hr followed by extensive washes. Differences of iodide concentration in the wells between cAMP stimulated and nonstimulated cells, before and following the addition of the agonists at 0 time point, represent the cAMP-dependent channel activity.

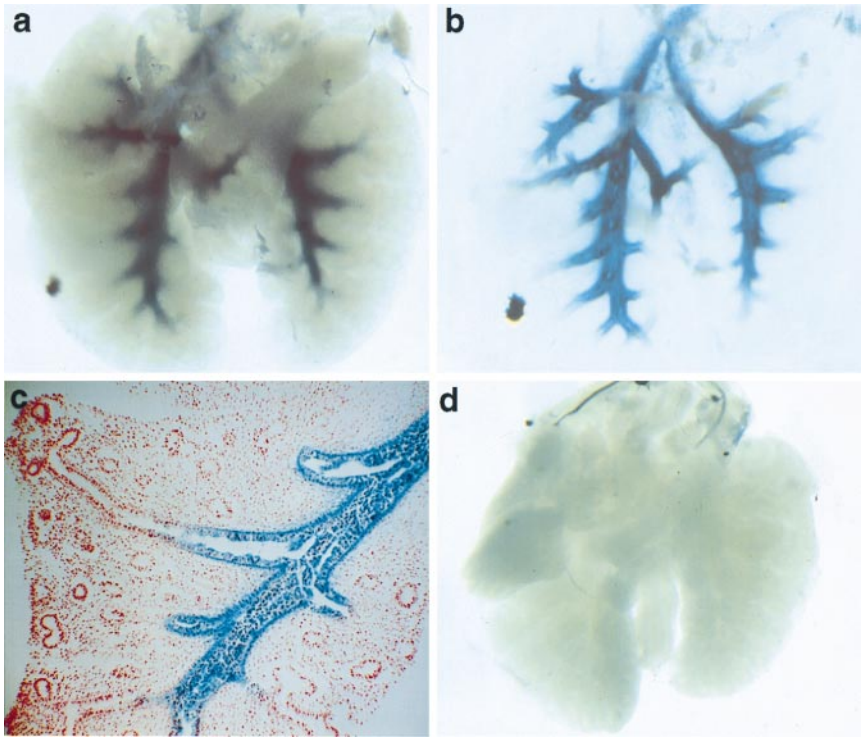

FIG. 5. Transgenic expression of the K18 mlacZ construct in mouse lungs. (*a*) F_0 transgenic embryos were harvested at 15.5 days postconception and the lungs were dissected, fixed, washed, and stained with X-gal for 3 hr. (*b*) The same lung shown in *a* was cleared by methyl salicylate after dehydration in methanol (2 changes, 30 min each). The LacZ expression pattern is restricted in the major airways. (*c*) The lung was subsequently sectioned in paraffin and counterstained with neutral red. Microscopic examination confirmed that the X-gal staining is only present in the epithelium lining the airways. (*d*) A control lung from a 15.5 day nontransgenic embryo was stained with X-gal for 16 hr.

lung (20, 21). The simian virus 40 early enhancer/promoter was also tested and found to be inactive in cultured lung cells in our system (data not shown). Taken together, our results suggest that these widely used viral promoters might not be suitable for lung gene therapy. On the other hand, the K18 expression cassette is epithelium-specific and functions well both *in vitro* and *in vivo*. More importantly, expression from the K18 enhancer/promoter could be sustained for up to 4 weeks, although at a low level. This latter point may not be critical because 5% of the normal level of CFTR expression can confer 50% correction of the chloride ion transport defect in the mouse model (22). Thus, the long-lasting expression from the K18 cassette even at a low level might offer considerable clinical benefits in gene therapy.

Incorporation of cDNAs into expression cassettes requires careful design. The data presented here illustrate several important points in the design, in particular the effect of introns on the expression and the correct and efficient RNA splicing. During the development of the SEAP reporter expression constructs, we noted that the presence and the position of K18 intron 1 contributes significantly to efficient expression from the K18 promoter. Removing or misplacing the intron caused substantial reduction of SEAP expression, probably because crucial protein interactions between the intron and the 5′ upstream region were disrupted. Placing the K18 intron 1 immediately behind the promoter and upstream of the coding region, however, revealed a potential problem of aberrant RNA processing, due to the suboptimal splicing signal at the splice acceptor site. In the case of CFTR, at least two cryptic splice sites were activated, resulting in alternative splicing and reduced protein production. By improving the 3′ splicing signals of K18 intron 1 and silencing a strong 3′ splice site in the CFTR coding sequence without altering the amino acid sequence, we were able to correct the splicing anomaly and greatly enhance CFTR protein production. Such incompatibility between the intron sequence and downstream coding region is certainly not unique to CFTR gene, and should be taken into consideration when introns are used in transgene expression cassettes. Indeed, we observed improved expression of various reporter genes, including chloramphenicol acetyltransferase, *LacZ*, GUS, but not SEAP, with the mutated K18Epilong cassette but not with the original version (data not shown). Although demonstrating the efficient expression of reporter genes is interesting, one has to be aware that expression of a transgene with heterologous DNA regulatory elements in gene therapy is not without potential limitations. For example, the level of the transgene expression may not be desirable in target cells, or hormonal and environmental regulation of transgene may be quite different from that under its native promoter. However, these potential limitations can be evaluated in transgenic animals.

Cis-acting and trans-acting factors regulating lung epithelial gene expression have been recently identified. The promoters of surfactant protein A, B, C, D, and CC-10 (Clara cell 10-kDa protein) genes have been analyzed extensively (23–27). Since these genes are predominantly expressed in alveolar type II cells or Clara cells, their promoters, unless modified, might not be suitable for expressing genes in the epithelial cells of the human upper airways that must be targeted for CF gene therapy. In contrast, the human K18 gene is expressed in the epithelia lining the upper airway of the lung. Similar K18-*LacZ* reporter constructs have been used by another group to study the expression pattern in transgenic animal systems (28). In this previous work, the expression of the *LacZ* reporter gene did not accurately reflect the developmental pattern of K18, nor did it show tissue specificity. Although the reason for the lack of correct *LacZ* expression is still unclear, it could be due to the aberrant splicing we described above or compromised stability of the K18LacZ fusion protein resulting from the constructs used in the previous study. By avoiding the N-terminal coding region in the first exon of K18 and by modifying the splicing signals of the intron 1 in our constructs,

we were able to detect efficient and cell-specific expression of the reporter β-galactosidase in epithelial cells along major airway branches in lungs of the transgenic embryos up to day 17.5. No detectable expression could be seen in lung interstitial, smooth muscle, or endothelial cells. Our results show that human DNA regulatory elements can be effectively used to create expression cassette for transgene expression in lung. We are currently analyzing temporal and spatial expression pattern of the K18mlacZ construct in transgenic mice. Our preliminary results showed that the LacZ reporter is expressed in brain, kidney, intestine, testis, and epididymis, in addition to lung (data not shown), consistent with the distribution profile of human cytokeratin 18 (4). To our knowledge, this is the first expression cassette that can direct transgene expression in the proximal airway of the lung.

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1. Welsh, M. J., Tsui, L.-C., Boat, T. F. & Beaudet, A. L. (1995) in *The Metabolic and Molecular Basis of Inherited Disease*, eds. Scriver, C. R., Beaudet, A. L., Sly, W. S. & Valle, D. (McGraw-Hill, New York), pp. 3799–3876.
2. Tizzano, E. F., O'Brodovich, H., Chitayat, D., Benichou, J. C. & Buchwald, M. (1994) *Am. J. Respir. Cell Mol. Biol.* **10,** 355–362.
3. Crystal, R. G. (1995) *Science* **270,** 404–410.
4. Moll, R., Franke, W. W., Schiller, D. L., Geiger, B. & Krepler, R. (1982) *Cell* **31,** 11–24.
5. Neznanov, N., Thorey, I. S., Cecena, G. & Oshima, R. G. (1993) *Mol. Cell. Biol.* **13,** 2214–2223.
6. Oshima, R. G., Abrams, L. & Kulesh, D. (1990) *Genes Dev.* **4,** 835–848.
7. Pankov, R., Neznanov, N., Umezawa, A. & Oshima, R. G. (1994) *Mol. Cell. Biol.* **14,** 7744–7757.
8. Pankov, R., Umezawa, A., Maki, R., Der, C. J., Hauser, C. A. & Oshima, R. G. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 873–877.
9. Lieber, M., Smith, B., Szakal, A., Nelson-Rees, W. & Todaro, G. (1976) *Int. J. Cancer* **17,** 62–70.
10. Jobling, S. A. & Gehrke, L. (1987) *Nature (London)* **325,** 622–625.
11. Zeitlin, P. L., Lu, L., Rhim, J., Cutting, G., Stetten, G., Kieffer, K. A., Craig, R. & Guggino, W. B. (1991) *Am. J. Respir. Cell. Mol. Biol.* **4,** 313–319.
12. O'Brodovich, H., Rafii, B. & Post, M. (1990) *Am. J. Physiol.* **258,** L201–L206.
13. Mohamed, A., Ferguson, D., Seibert, F. S., Cai, H. M., Kartner, N., Grinstein, S., Riordan, J. R. & Lukacs, G. L. (1997) *Biochem. J.* **322,** 259–265.
14. Chang, X. B., Tabcharani, J. A., Hou, Y. X., Jensen, T. J., Kartner, N., Alon, N., Hanrahan, J. W. & Riordan, J. R. (1993) *J. Biol. Chem.* **268,** 11304–11311.
15. LaCasse, E. C. & Lefebvre, Y. A. (1995) *Nucleic Acids Res.* **23,** 1647–1656.
16. Kozak, M. (1986) *Cell* **44,** 283–292.
17. Moore, M. J., Query, C. C. & Sharp, P. A. (1993) in *The RNA World*, eds. Gesteland R. F. & Atkins J. F. (Cold Spring Harbor Lab. Press, Plainview, NJ), pp. 303–357.
18. Zielenski, J., Patrizio, P., Corey, M., Handelin, B., Markiewicz, D., Asch, R. & Tsui, L. C. (1995) *Am. J. Hum. Genet.* **57,** 958–960.
19. Chillon, M., Casals, T., Mercier, B., Bassas, L., Lissens, W., Silber, S., Romey, M. C., Ruiz, R. J., Verlingue, C., Claustres, M., Nunes, J., Ferec, C. & Estivill, X. (1995) *N. Engl. J. Med.* **332,** 1475–1480.
20. Schmidt, E. V., Christoph, G., Zeller, R. & Leder, P. (1990) *Mol. Cell. Biol.* **10,** 4406–4411.
21. Baskar, J. F., Smith, P. P., Nilaver, G., Jupp, R. A., Hoffmann, S., Peffer, N. J., Tenney, D. J., Colberg, P. A., Ghazal, P. & Nelson, J. A. (1996) *J. Virol.* **70,** 3207–3214.
22. Dorin, J. R., Farley, R., Webb, S., Smith, S. N., Farini, E., Delaney, S. J., Wainwright, B. J., Alton, E. W. & Porteous, D. J. (1996) *Gene Ther.* **3,** 797–801.
23. Venkatesh, V. C., Planer, B. C., Schwartz, M., Vanderbilt, J. N., White, R. T. & Ballard, P. L. (1995) *Am. J. Physiol.* **268,** L674–L682.
24. Wert, S. E., Glasser, S. W., Korfhagen, T. R. & Whitsett, J. A. (1993) *Dev. Biol.* **156,** 426–443.
25. Sawaya, P. L., Stripp, B. R., Whitsett, J. A. & Luse, D. S. (1993) *Mol. Cell. Biol.* **13,** 3860–3871.
26. Rooney, S. A., Young, S. L. & Mendelson, C. R. (1994) *FASEB J.* **8,** 957–967.
27. Korfhagen, T. R., Glasser, S. W. & Stripp, B. R. (1994) *Curr. Opin. Pediatr.* **6,** 255–261.
28. Thorey, I. S., Meneses, J. J., Neznanov, N., Kulesh, D. A., Pedersen, R. A. & Oshima, R. G. (1993) *Dev. Biol.* **160,** 519–534.