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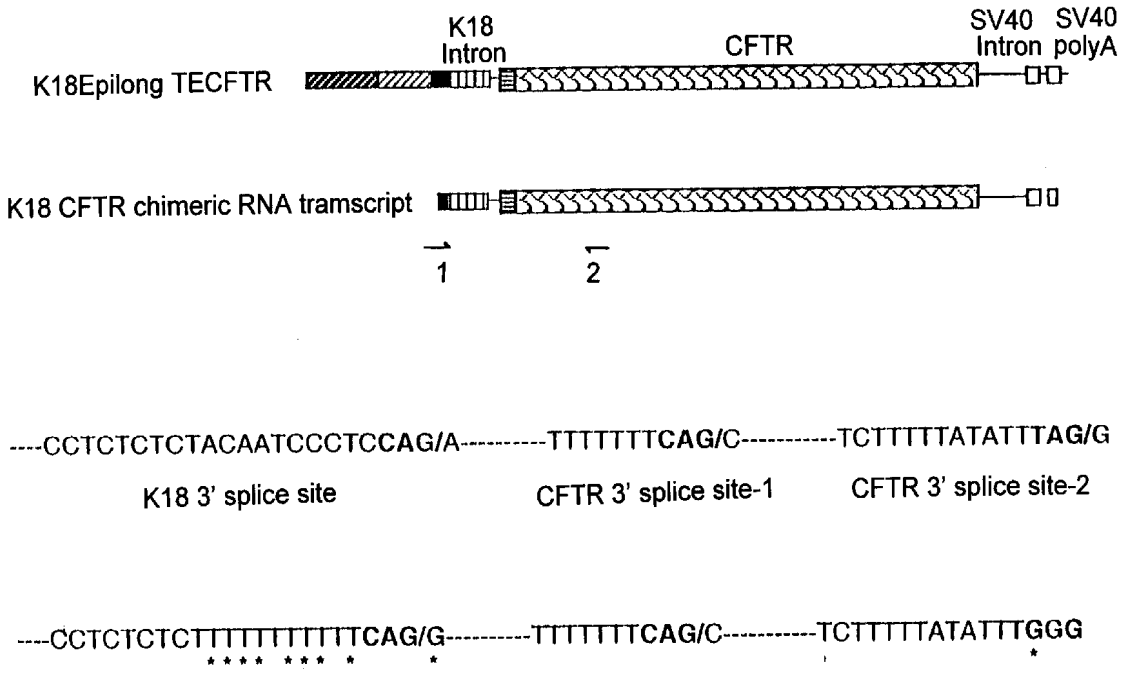
(54) **CASSETTES POUR L'EXPRESSION EPISOMALE EN THERAPIE GENIQUE**

(54) **EPISOMAL EXPRESSION CASSETTES FOR GENE THERAPY**



(57) L'invention consiste en des cassettes pour l'expression épisomale d'un transgène en thérapie génique. Les cassettes comprennent des éléments régulateurs du gène humain de la cytokératine et un transgène. L'invention concerne également des liposomes pour la transfection du tissu épithélial avec les cassettes dans le traitement de la fibrose kystique, de l'emphysème, des cancers d'origine épithéliale affectant les poumons ou d'autres organes.

(57) The invention consists of episomal expression cassettes for expression of a transgene in gene therapy. The expression cassettes consist of regulatory elements of the human cytokeratin gene and a transgene. The invention also includes liposomes for transfection of epithelial tissue with the cassettes in treatment of cystic fibrosis, emphyserma, cancers of epithelial origin arising in the lung or other organs.

Abstract

The invention consists of episomal expression cassettes for expression of a transgene in gene therapy. The expression cassettes consist of regulatory elements of the human cytokeratin gene and a transgene. The invention also includes liposomes for transfection of epithelial tissue with the cassettes in treatment of cystic fibrosis, emphyserma, cancers of epithelial origin arising in the lung or other organs.

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TITLE: **EPISOMAL EXPRESSION CASSETTES FOR GENE THERAPY**

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EPISOMAL EXPRESSION CASSETTES FOR GENE THERAPY

Field of the Invention

The invention relates to gene therapy episomal expression cassettes to express a transgene in epithelial cells.

Background of the Invention

1.1 Gene delivery Demonstration of the feasibility of gene transfer to humans by a number of clinical trials stimulated considerable interest in gene therapy in the scientific community even though no therapeutic benefit has yet been offered to patients (7). Epithelial tissue, particularly lung epithelial tissue, has considerable potential as a target for gene therapy. The lung is a highly suitable organ for *in vivo* gene therapy treatment of patients with potentially lethal lung disorders, such as cystic fibrosis, cancers of epithelial origin and emphysema because of its large accessible epithelial and endothelial surface area (15). Both virus-based and non-virus-based methods can be used to deliver genes to lungs (6, 15). The use of liposomes as gene transfer agents seems to have some significant advantages for *in vivo* lung gene therapy (6, 15). First, liposomes offer a wide margin of safety with low toxicity and have already been used to deliver drugs to humans. They can be administered into the lungs as an aerosol, by direct lavage or following intravenous injection. A clinical trial in nasal epithelia showed no adverse effects; nasal biopsies showed no immuno-histological changes (4). Secondly, liposome-complexed DNA can be used to transfect both resting and dividing cells. In addition, large DNA constructs can be accommodated with liposomes for transfection. Finally and most importantly, liposome-mediated gene expression is episomal, thereby avoiding or reducing the risk of random chromosomal insertions. However, one of the major impediments to liposome-mediated *in vivo* gene therapy is that the currently available expression vectors only offer a very low level of transient transgene expression (15). Therefore, enhancement of the therapeutic gene expression would not only increase the efficacy, but also effectively decrease the already low levels of toxicity by reducing the dose of therapeutic reagent.

1.2 Control of gene expression The inefficient expression of transgenes in lung is, at least in part, due to the lack of proper lung-specific gene expression cassettes (15). An ideal expression cassette for human lung gene therapy should be safe and confer an appropriate level of tissue-specific expression for a reasonable duration. The rational design of expression cassettes for lung gene therapy relies on our knowledge of regulation of gene expression. Regulation of eukaryotic gene expression is a very complicated process. A particular gene may be expressed in only one type of cell or tissue while others are expressed in most cell types or tissues. For example, cytokeratin genes are expressed predominantly in epithelial cells (26). In contrast, genes encoding proteins involved in translation (protein synthesis) are expressed in every cell type. The activity of a eukaryotic gene can be regulated at any stage during the course of its expression, such as transcription, RNA splicing, RNA stability, translation, or post-translational modification. Current knowledge indicates that transcription and RNA splicing are the major steps for regulation of many eukaryotic genes.

1.2a Transcriptional regulation Transcription of eukaryotic genes is catalyzed by an RNA polymerase which is recruited to the promoter by multiple protein factors involved in transcription initiation. Regulation of transcription can be attributed to tissue-specific DNA elements (enhancers or silencers) that stimulate or repress transcription through interaction with tissue-specific transcription factors (25). However, these elements may not function if they reside in an inappropriate location on a chromosome, suggesting that chromosomal position and structure also affect gene expression. This has led to discovering a type of regulatory elements called locus control region (LCR) (13). These LCRs, when integrated into chromosomes, confer copy number-dependent and location-independent gene expression. The first LCR was discovered in 5' region of the human β-globin gene cluster (9, 10, 13). LCRs are now known to be associated with other genes (28, 36) including human cytokeratin 18 and rat LAP (C/EBPb) which direct gene expression in lung cells of transgenic mice (28, 36). Although currently there is no evidence to show that LCRs enhance episomal gene expression, this possibility can not be ruled out since information about the interactions of LCRs with other regulatory elements is still limited. If LCRs increase gene expression, they would be useful in the design of episomal expression cassettes. As lung epithelial cells are not actively dividing, the delivered plasmid DNA may be wrapped by histones or other nuclear factors and kept in a transcriptionally inactive conformation. Although it is generally believed that plasmids when transferred into nucleus do not form chromatin structures, recent

experiments by Jeong and Stein demonstrate that some of the transfected DNAs are in chromatin form (17). The presence of a functional LCR in expression cassettes may allow a plasmid to stay in an open conformation.

1.2b Regulation through RNA processing Regulation of RNA splicing is also very important for tissue-specific and developmentally regulated gene expression (35). This type of regulated RNA splicing or alternative RNA splicing can lead to the production of different proteins from a single gene by inclusion of different exons in different mRNAs. Some introns contain strong enhancers and their exclusion from expression constructs would lead to diminished gene expression. For example, the first intron of the human cytokeratin 18 contains a strong enhancer which is required for expression of the cytokeratin 18 gene (29). Other introns that do not contain enhancers may also affect gene expression. For example, the presence of rpL32 intron 3 leads to a 30-fold increase in mRNA level relative to the intronless rpL32 minigene (21). However, different introns clearly have different effects. For instance, inclusion of intact thymidylate synthase gene intron 4 alone at its normal position in the thymidylate synthase (TS) coding region leads to a decrease in the level of expression relative to that observed with a the intronless TS minigene (21). The details of this splicing regulation of expression are unknown.

1.3 Gene expression in lung epithelial cells Efficient tissue-specific gene expression can be achieved, in theory, by using tissue-specific promoters, promoter elements, RNA processing signals, and tissue-specific RNA-stabilizing elements. Cell-specific gene expression primarily results from either tissue-specific promoters, and/or tissue-specific regulatory elements, such as enhancers, silencers, and locus control regions (LCRs). However, it is very difficult to design a cassette for lung gene therapy because there is not enough information known about regulation of lung gene expression. Currently, no suitable expression vector for lung gene therapy has been reported. There is a pressing need for an effective expression vector because a number of human CF gene therapy trials have been conducted (7). The SV40 promoter was used to direct CFTR expression in the clinical trial by Caplen *et al. (4);* we observed that SV40 promoter is not very active even in cultured lung epithelial cells (see Fig. 5) and its expression in rat lung primary cells is undetectable (Plumb and Hu, unpublished results). That might explain the large amounts of plasmid DNA (10 mg to 300 mg/per nostril) used in the study (4). Recently, several *cis*-acting elements and *trans*-acting factors regulating lung epithelial gene expression have been identified. The promoters

of the SP-A (surfactant protein A), SP-B (surfactant protein B), SP-C (surfactant protein C), SP-D (surfactant protein D) and CC10 (Clara cell 10 kD protein) genes have been extensively analyzed (22, 31, 40, 41). Because these genes are predominately expressed in type II or Clara cells (22), their promoters, unless modified, would not be suitable for expressing genes in epithelial cells of conducting airways, which represent the primary target for CF lung gene therapy.

1.4 Epithelial expression cassette for lung gene therapy Because of the low efficiency in liposome-mediated gene expression, strong viral promoters are often used in gene therapy studies. However, this may not be the ideal approach for liposome-mediated lung gene therapy. For example, the CMV major immediate early gene promoter has been shown to be very strong for transient expression of transgenes in cultured cells, but two studies have shown it to be a poor promoter for lung gene expression in transgenic mice (1, 33). There is no evidence to show the CMV promoter can confer sustained episomal gene expression *in vivo*. Although it is unreasonable to expect a permanent transgene expression from an episomal plasmid, long lasting expression even at a low level may offer considerable clinical benefits to gene therapy patients. In addition, viral promoters may not confer tissue-specificity. Since currently the nuclear uptake of delivered DNA is highly inefficient (44) in addition to the low efficiency of liposome-mediated gene expression, no one would worry about the effect of non-specifically expressing a therapeutic gene *in vivo*. However, when the nuclear uptake and liposome-delivery technology are improved, this has to be seriously considered because there must be an advantage for nature to select genes, such as the cystic fibrosis transmembrane conductance regulatory gene (CFTR), to be epithelium-specific.

If human DNA regulatory elements could direct tissue-specific expression of therapeutic genes at a comparable level to that from strong viral promoters in lung epithelial cells, and sustain gene expression longer than the viral promoters, it would be advantageous to use them for lung gene therapy. These DNA regulatory elements would also be useful to direct tissue-specific expression of therapeutic genes in epithelial cells of other organs. However, one must be aware that successful expression of a reporter gene in the right cell type by an expression vector does not guarantee a positive outcome when a therapeutic gene is inserted in the same cassette if the DNA sequence of the therapeutic gene interferes with transcription or subsequently RNA splicing. One skilled in the art can modify the expression

construct to accommodate a therapeutic gene. The level of expression activity of a modified construct can be measured in an animal model according to methods known in the prior art.

At present, there is no suitable expression vector for epithelial tissue gene therapy. There is a need to develop gene therapy cassettes that use human DNA regulatory elements which naturally express genes in epithelial cells and can be used to direct the expression of therapeutic genes. It would be particularly useful if there was an expression cassette that could direct a high level of reporter gene expression *in vivo* and *in vitro*. The expression cassette should be safe and confer an appropriate level of tissue-specific expression for a reasonable duration. The expression cassette should be capable of use in epithelial cells, such as submucosal cells.

Brief Description of the Drawings

FIG.1 Improvement of GUS Reporter Gene System. A) Modification of the GUS reporter gene. B) Chemiluminescent assays of GUS gene expression. RFLE, rat fetal lung primary epithelial cells. The Bioorbit Luminometer (model number 1253) was used and 1 reading unit from this model equals 10,000 reading units from other models such as Berthold Lumat LB 9501.

FIG. 2 Expression of GLP reporter in A547, IB3 and rat fetal lung primary epithelial cells. A) Expression of the green fluorescent protein in cultured human lung cells. A549 cells were transfected with pGREENLANTERN-1(GIBCO BRL) and visualized under a fluorescent microscope (bottom panel) 2 days post-transfection. The phase-contrast view of the same cells is shown in the top panel. B) Expression of the green fluorescent protein in human cystic fibrosis bronchial epithelial cells. IB3 cells were transfected with pGREENLANTERN-1(GIBCO BRL) and visualized under a fluorescent microscope (bottom panel) 2 days post-transfection. The phase-contrast view of the same cells is shown in the top panel. C) Expression of the green fluorescent protein in rat lung primary cells. Rat fetal lung epithelial cells were transfected with pGREENLANTERN-1 (GIBCO BRL) and visualized under a fluorescent microscope (bottom panel) 2 days post-transfection. The phase-contrast view of the same cells is shown in the top panel.

FIG. 3 Optimization of cell transfection conditions for gene expression. Cells were transfected with pCEP4SEAP complexed with DODAC:DOPE at 2.5 nmol/cm^2. Each sample corresponds to 50µl of culture medium conditioned by the transfected cells.

FIG. 4 Schematic diagrams of SEAP and CFTR expression constructs. A) The genomic structure of the human cytokeratin 18 gene (K18), in which exons 1 through 7 are depicted as solid boxes and DNAse I hypersensitive sites as arrows. Intron-1 fragment covers from the end of exon 1 to the beginning of exon 2. The minimal promoter fragment spans 310 base pairs between a unique *Xho*I (X) site and the K18 translation initiation, excluding the start codon. Enhancer-long and Enhancer fragments cover regions from *Hind* III (H) to *Nsi* I sites and from *Nsi* I to *Xho* I sites, respectively. B) The simplified structures of the promoterless SEAP construct (CloneTech) and a series of its derivatives which contain various segments of K18 untranslated sequence, as well as their relative expression levels. C) The structure of K18EpilongTECFTR, which is identical to K18EpilongSEAP except that the reporter gene SEAP is replaced by CFTR cDNA with a translational enhancer (adapted from Alfalfa Mosaic Virus RNA4) immediately upstream of the CFTR coding sequence.

FIG. 5 Expression pattern of K18 constructs in comparison with SV40- or CMV-promoter directed expression in selected cell lines. A549, WI38, or COS-7 cells are transfected with DNA-lipid complex in parallel. Culture media were collected and assayed for SEAP activity 48 hr post-transfection.

FIG. 6 Long lasting gene expression in cells transfected with K18EpilongSEAP. Shown are expression kinetics of K18EpilongSEAP versus CMVSEAP. A549 and COS-7 cells were transfected with DNA:lipid mix at 1:10 ratio. Culture media were collected at days post-transfection as indicated, prior to media change, and stored at -80°C. SEAP reporter assay was performed according to standard procedure.

FIG. 7 Expression of K18EpilongSEAP in rat fetal lung primary cells. Both epithelial cells and fibroblast cells were transfected with plasmid DNA and DODAC:DOPE at 1:10 ratio. The plasmid, pINXCAT was used as a negative control. pCEP4SEAP contains the CMV promoter.

FIG. 8 I-Efflux of COS7 cells transfected with CFTR expression cassettes. Functional analysis for CFTR by iodide efflux assay. COS-7 cells were transfected with K18EpilongTECFTR, pCMVnot6.2CFTR as a positive control, or a negative control plasmid. 48 hr post-tranfection, cells were loaded with iodide for one hour followed by extensive washes. AMP-dependent channel activity was then assessed as iodide concentration in the wells before and following the addition of the agonist, forskolin, at 0 time point.

FIG. 9 Splicing of the K18-CFTR chimeric RNA transcript. A) Schematic diagram of the K18-CFTR chimeric RNA transcript and positions of the primers used in RT-PCR. B) RT-PCR products from total RNAs isolated from the CFTR transfected IB3 and rat fetal primary epithelial cells. The types of cells and primer sets are indicated on the top. Lane 4 shows the 1kb ladder. The 712-and 640-bp bands are the expected PCR products from these two primer sets. The stars indicate the mis-spliced products. RNAs from untransfected cells do not yield any bands (data not shown). C) The K18 intron 1 sequences critical for splicing.

FIG. 10 Identification of the cryptic 3' splice-sites in the CFTR coding region and improvement of the splicing efficiency of the K18-CFTR chimeric RNA transcript by mutagenesis. A) Schematic diagram of the structures of K18EpilongTECFTR and the RNA transcript. Primers used for RT-PCR in fig.11 are depicted as arrows. B) DNA sequences of K18EpilongTECFTR at 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.

FIG. 11 Splicing patterns of K18-CFTR chimeric RNA transcripts. Shown are 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 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 present along with the 696 bp band.

FIG. 12 Functional analysis of the CFTR channel activity by iodide efflux assay. COS-7 cells were transfected with K18EpilongTECFTR, K18EpilongmCFTR, pCDM8.1CFTR as a positive control, or a negative control plasmid (K18Epilong).

Forty-eight hours post-transfection, cells were loaded with iodide for one hour followed by extensive washes. cAMP-dependent channel activity was then assessed as iodide concentration in the wells before and following the addition of the agonist, forskolin, at 0 time point.

FIG. 13 Targeting expression of the *LacZ* reporter gene in mouse lung epithelia. The lung was dissected out from a 14 day transgenic mouse fetus and stained with X-gal for 3 hr. The K18mLacZ has been demonstrated clearly expressing in airways of the lung.

FIG. 14 A lung of a normal mouse fetus. The lung was excised out from a 14 day mouse fetus and stained with X-gal overnight.

FIG. 15 Enhancer activity of the 1.4 kb DNA fragment from 5' region of the human K18 gene. A549 cells and COS-1 cells were transfected with K18EpiSEAP or K18EpilongSEAP which contains the distal enhancer. SEAP activities in the culture media are normalized to total protein.

FIG. 16 The position effect of K18 intron 1 on reporter gene expression. In K18EpilongSEAPi construct, the intron was moved to the down stream of the SEAP coding region. Relocating this intron abolished the expression of the SEAP reporter gene by the construct.

Summary of the Invention

Understanding how genes are expressed in lung epithelial cells and other epithelial cells allows design of strategies to treat fatal diseases, such as cystic fibrosis. The expression of the cystic fibrosis transmembrane conductance regulator (CFTR) gene in human lungs after birth is localized predominately in the epithelial cells of trachea and large bronchi (37), especially in the submucosal cells (11, 12). Mortality of CF patients is often due to lung failure.

The invention satisfies the need for a suitable expression vector for epithelial tissue gene therapy. The expression cassettes of this invention contain human DNA regulatory elements which naturally express genes in epithelial cells and direct the expression of therapeutic genes. They also direct a high level of reporter gene expression *in vivo* and *in vitro*. The expression cassettes are safe and confer an appropriate level of tissue-specific

expression for a reasonable duration. The expression cassettes may be used in epithelial cells, such as submucosal cells.

The invention utilizes human DNA regulatory elements that naturally express genes in epithelial cells to direct the expression of therapeutic transgenes for gene therapy in lung and other organs. The invention includes the following steps: first generating a series of DNA constructs that were assessed in cell lines for the expression of reporter genes or the human CFTR gene, then examining selected constructs in primary cells and whole tissue sections, and finally testing selected constructs in mice and humans. We developed an expression cassette that directed a high level of reporter gene expression in human epithelial cells *in vivo* and *in* vitro and in rat fetal lung primary epithelial cells. In addition, we extensively modified the first generation of cassette; the modified cassette efficiently directs expression of the human CFTR gene with a change in the CFTR coding sequence. The modified expression cassette directs efficient and cell-specific gene expression in lung epithelia of the transgenic mice and human epithelial cells *in vivo* and *in vitro*.

The invention is an expression cassette for the episomal expression of a transgene in targeted epithelial cells, which consists of regulatory elements of the human cytokeratin gene and a transgene. In one embodiment of the invention, the expression cassette is targeted to a lung epithelial cell. The regulatory elements may comprise a promoter, the 5 ′ region and modified intron 1 of the human cytokeratin 18 gene.

In the cassette, the human cytokeratin gene is the human cytokeratin 18 gene. The regulatory elements of the cassette are from the 5 ′ region of the human cytokeratin 18 gene. The regulatory elements may also consist of a promoter, the 5 ′ region and intron 1 of the human cytokeratin 18 gene. The cassette may also contain an enhancer.

The transgene in the cassette can be the cystic fibrosis transmembrane conductance regulatory gene. In another embodiment, the transgene in the cassette can consist of an enhancer and a modified cystic fibrosis transmembrane conductance regulatory (CFTR) gene.

The cells targeted by the cassette may be epithelial cells, such as submucosal cells.

A liposome may be used to deliver the expression cassette construct.

Cells may be transfected by the expression cassette construct. In one embodiment, the cells are part of tissue in a lung.

The invention also includes a method of treating a patient having a lung disorder, by administering to the patient a liposome containing the cassette so that the cassette transfects a targeted lung cell. The method of administration of the liposome may be selected from a group consisting of aerosol administration, direct lavage and intravenous injection. The expression cassette can be used in treatment of a disorder such as cystic fibrosis, emphysema, and cancers of epithelial origin arising in the lung or other organs.

Detailed Description of the Invention

The invention overcomes the difficulties of designing safe and clinically effective episomal expression cassettes for use in gene therapy. The DNA regulatory elements used in the episomal expression cassettes of the invention are from human genome. Therefore, these elements offer better compatibility when used for human gene therapy because the authentic protein factors interacting with these DNA elements are present in targeted cells. Secondly, these cassettes are epithelium-specific and highly efficient; the cell-specificity increases the efficacy and avoids any adverse effects resulting from expression of the therapeutic gene in non-targeted cells. The high efficiency of gene expression is also critical to minimize the dosage of the therapeutic reagents from gene therapy. Additionally, even in cultured cells, the expression from these constructs last longer than the viral promoter based-expression cassette (see Fig. 6).

The expression cassettes are useful in other epithelial tissue as well because the K18 gene is expressed in the epithelial cells of other internal organs (see Example 3).

Example 1-Development of reporter genes for liposome-mediated plasmid gene transfer

For functional analysis of transcription regulatory elements, more than one reporter gene system is normally required because an extra reporter gene under a different promoter is needed to serve as an internal control to normalize the effects resulted from variation in transfection. In addition a particular reporter gene may not be compatible with a particular expression cassette. Therefore, we developed or adapted the following convenient reporter gene systems for lung gene expression studies:

1.1) GUS reporter system. A bacterial gene (*E. coli* GUS, coding for b-glucuronidase) has worked well as a reporter gene in plants; its expression can be detected by either highly sensitive chemiluminescent assays (2, 3) or cell staining (16). Although b-glucuronidase activity is present in some mammalian cells, the optimal pH value for the

mammalian enzyme is around 4-5 whereas that of the bacterial enzyme is around 7. We have subcloned the GUS gene into pCEP4 (Invitrogen) and transfected different cell lines and primary cells. We demonstrated that GUS can be a sensitive reporter for quantification of gene expression in lung cells. In order to further improve the sensitivity of GUS gene as a reporter, we added a translational enhancer (18) and a DNA sequence encoding a nuclear localization signal (19) to the 5' end of the GUS coding sequence. As shown in Fig. 1, GUS expression was greatly enhanced. We will test and optimize the conditions for cell staining.

1.2) SEAP (secreted alkaline phosphatase) reporter system. We adapted SEAP as a primary reporter for gene expression in cultured cell lines and lung primary cells (Figs. 3, 5 and 6) because the system is more economical and less labor-intensive than CAT or other reporter gene systems. The expression of SEAP can be quantified simply by chemiluminescent assay of the alkaline phosphatase secreted in culture media (2, 3).

1.3) GLP reporter system. We also adapted the Green Lantern Protein (GLP, a modified version of green fluorescent protein) as a reporter to mark the cells transfected with liposome/DNA complex (Fig. 2).

Example 2-Optimization of transfection conditions

We carried out experiments to optimize the transfection conditions because liposome-mediated gene expression in cell lines of lung origin is very inefficient. We used cell lines, such as, A549 (Human Lung Carcinoma cell line), IB3 (Cystic fibrosis bronchial epithelial cell line transformed with adeno-12-SV40;(45)), COS7 (SV40 transformed African Green monkey kidney), and WI38 (Human Lung diploid of fibroblast origin). There are many types of liposomes commercially available. We used DODAC:DOPE (INEX) because it is effective and large quantities will be available for clinical trials. For most of these cell lines, we found that about 2.5 nmol of DODAC:DOPE/cm^2 is optimal. Fig. 3 shows the effect of DNA:lipid ratio on gene expression in A549 and COS7 cells.

Example 3-Construction of K18 expression constructs

Cytokeratins are major components of the epithelial cytoskeleton and different sub-types characterize different epithelia (26). The cytokeratin 18 gene is expressed predominately in internal organs (lung, liver, kidney and intestine) and brain. It is highly epithelium-specific and has been a useful marker of epithelial cell transitions in the remodeling adult lung (42, 43). The 2.5 kb sequence from the 5' region is able to direct lung gene expression in a copy number-dependent and position-independent manner in transgenic

mice (28). Therefore, this region can be considered as a lung LCR (locus control region). A 3.5-kb 3' flanking sequence is required for gene expression in liver and intestine. There is a strong enhancer present in the first intron (29). To construct an expression cassette with the human cytokeratin 18 gene regulatory elements, we isolated the K18 minimal promoter, intron 1 and two 5' fragments by PCR-cloning (Fig. 4). We found that any one of the elements alone could not direct SEAP expression in A549 or COS7 cells. The minimal promoter plus intron 1 has a low level of activity and the two fragments from the 5' region can greatly enhance the level of gene expression (Fig. 4). Since the 5' region and the intron 1 of the K18 gene are critical for gene expression, we decided to keep these elements in their original configuration as much as possible in construction of our first expression cassette, K18EpilongSEAP, to preserve the potential interactions among the transcription factors bound to these elements. In this reporter expression construct, the transcription will start from the K18 promoter, but protein translation will start from the first codon of the reporter gene because most of the K18 exon 1, including all the coding sequence, is deleted.

Example 4-Episomal expression of K18 constructs in cultured cells

To show that the episomal expression directed by K18 regulatory elements has epithelial specificity, we expressed K18EpiSEAP in A549 (human lung epithelial origin) and WI38 (human lung fibroblast origin). As shown in Fig. 5, K18EpiSEAP expressed the reporter gene only in A549, but not WI38, while the viral promoter, CMV, expressed in both cell lines (Fig. 5). The SV40 promoter was not active in these lung cell lines although it was functional in COS7 cells which are monkey kidney cells transformed with SV40 large T antigen. Our results showed that K18EpilongSEAP is about 3 times more active than K18EpiSEAP (Fig. 15) and its expression lasted much longer than the CMV promoter in cell lines (Fig. 6). *In vivo*, the low levels of long lasting expression of the CFTR gene by K18Epilong may offer more clinical benefits to patients in lung gene therapy than the transient expression from viral promoters. K18EpilongSEAP also exhibit clear cell specificity in that its expression can only be detected in A549 cells, but not WI38 or another human lung fibroblast line, HLF (data not shown).

Example 5-Expression of K18Epilong in primary lung epithelial cells

Because promoters active in cell lines are often not active in primary cells, we decided to test the K18EpilongSEAP in rat lung primary cells. Although the K18Epilong expression

in cell lines was much lower than that of CMV promoter, its expression in rat lung primary cells was better or comparable to that of CMV promoter (Fig. 7).

Example 6-K18 CFTR expression in cell lines and in primary cells

Because the K18Epilong can direct a high level of SEAP expression in rat lung primary cells, we built a CFTR expression cassette by replacing the SEAP coding sequence with CFTR cDNA (Fig. 4c). The CFTR gene contains 27 exons and 26 introns, spanning over 250 kb on the long arm of human chromosome 7 (20, 30, 38); but the entire coding sequence is about 4.5 kilobases in length. In order to enhance CFTR protein synthesis, we added a translational enhancer (18) to the 5' end of the CFTR coding sequence and optimized the translation initiation sequence according to the Kozak sequence (23). To show that the CFTR gene was expressed from our expression cassette, we transfected COS7 cells with K18EpilongCFTR. Fig. 8 shows that the transfected cells have cAMP-dependent iodide effluxes, indicating that the episomally expressed CFTR can form functional channels in transfected cells. But, the activity of the CFTR channels was not as high as expected, indicating that the CFTR expression by this construct is not optimized. As shown in Fig. 9, we detected three CFTR mRNA species from transfected rat lung primary cells or IB3 cells using RT-PCR, indicating that two cryptic RNA splice-sites are activated; according to the sizes of the three PCR products, only about 25% of the mature CFTR species (the top band) are properly processed. Therefore, we modified the construct to improve the RNA splicing efficiency.

Example 7-Optimizing RNA splicing

There is not much known about the regulation of RNA splicing in lung cells, despite the important role that splicing can play in tissue-specific gene expression (35); e.g. the presence of rpL32 intron 3, which does not contain an enhancer, led to a 30-fold increase in mRNA relative to the intronless rpL32 minigene (21). Although the mechanism for stimulation of gene expression by regular introns is not clear, it is likely that the RNA splicing machinery may preferentially protect the intron-containing pre-mRNAs from nuclease degradation or facilitate the transport of the spliced mRNAs to cytoplasm. Because intron 1 of the cytokeratin 18 gene contains a strong enhancer that is required for gene expression, we included it in the K18-based CFTR expression cassette. But, incorporation of a heterologous intron into a cDNA sequence could potentially activate the cryptic splice-sites in the intron or in the cDNA and cause mis-splicing or alternative splicing. One potential

solution to this problem is to put the intron after the coding sequence of the cDNA as long as the intron and/or intron-containing enhancer works from downstream. When the K18 intron 1 in K18EpilongSEAP is moved down stream of the reporter gene, expression of the reporter gene is greatly diminished (Fig. 16). Therefore, we modified the K18Epilongcftr to enhance the desired RNA splicing and to eliminate undesired RNA splice-sites.

Typical eukaryotic introns contain relatively conserved, short sequences recognized by the splicing machinery, spliceosome (27). The consensus sequences for the 5' splice site, the branch site and 3' splice site in mammals are AG/GURAGU, YNYURAC, and YAG/G, respectively (R=purine, Y=pyrimidine, N=any nucleotide, and / indicates a splice site; the underlined nucleotides are completely conserved). In addition, a polypyrimidine tract is often present near the 3' splice site. We PCR-cloned the cDNA sequences derived from the alternatively spliced mRNAs and identified the splice-site junctions by DNA sequencing (Fig. 10 B). We then realized that the poly U (uracil) sequence is the preferred polypyrimidine tract for the epithelial cells we used (Fig. 10B). We also noticed that the K18 intron 5' splice-site (AG/GUAAGG), putative branch-site (UUUUCAC), and 3' splice-site (CAG/A) are not highly conserved and can be potentially improved, since introns with more conserved sequences are, in general, spliced more efficiently (21). We modified the DNA sequence of pK18EpilongTECFTR corresponding to the polypyrimidine tract of the K18 intron 1 by changing five Cs (cytosine residues) and three As (adenine residues) into Ts (thymine residues), which will be translated into Us in the pre-mRNA sequence (Fig. 10 C). We also modified the 3' splice site of the K18 intron by changing the first nucleotide, A, of the following exon to G (Fig. 10C). Since these nucleotides are not in the CFTR coding region, these changes would not effect the protein produced from the expression plasmid. In addition, we have made a single nucleotide change (A to G) in the CFTR coding region (see Fig. 10 C) to destroy the second cryptic 3' splice site. We engineered the change in such a way so that the protein sequence remains the same and thus, the CFTR function will not be affected by this modification. This new construct was designated K18 EpilongmCFTR, or pK18mCFTR [SEQ ID NO: 1], and the previous version of plasmid was referred as K18EpilongTECFTR. As shown in Fig. 11, these changes very effectively eliminated the alternative RNA splicing and increased the steady state level of the CFTR mRNA.

To show that the new construct expresses functional CFTR channels, we transfected COS7 cells and performed iodide efflux assays. As shown in Fig. 12, a higher level of CFTR

channel activity was observed in cells transfected with K18EpilongmCFTR than in cells transfected with the previous construct.

Example 8-Expression analysis of the K18 regulatory elements in transgenic mice. To demonstrate that the modified K18 5' regulatory elements and intron 1 can direct cell-specific gene expression in lung epithelia *in vivo*, we carried out a transgenic analysis (28). The transgenic fetuses were identified by PCR and Southern blot analyses of the genomic DNA; the lungs of the 14 day fetuses were dissected out and stained with X-gal solution. These modified K18 DNA regulatory elements direct efficient and cell-specific expression of *E. coli LacZ* gene in lungs of the transgenic fetuses (Fig. 13-14).

Example 9-Expression in Calu-3 cells

Since the human CFTR gene is heavily expressed in submucosal cells (12), we show that our epithelial expression cassettes function in these cells. The current available cell line that resembles the human submucosal cells is Calu-3 which was derived from a lung adenocarcinoma (available from the American Type Culture Collection). These cells express leukocyte protease inhibitor, lysozyme, and all markers of serous gland cells (34). They also express a high level of CFTR and when confluent, show polarization typical of epithelia.

To show that our expression cassettes direct gene expression in Calu-3 cells, we transfect these cells with K18EpilongSEAP and we perform quantitative assays of secreted alkaline phosphatase activity. The SEAP reporter system is the most convenient assay system because only a small amount of culture medium is required for each assay. The *E. coli LacZ* gene is also a useful reporter.

Example 10-Expression in lung sections

We show the activity of the expression cassettes *in vivo*. A recently revived technique of lung slice culture (24, 39) is valuable for assessment of expression cassettes. Lungs of mice or rats are excised from anesthetized animals and inflated with 2% liquid agarose at 37°C through trachea. Following cooling to 4°C, the lungs are cut into 0.2 to 1.0 mm thick slices and cultured overnight in cell culture medium. Cells in these lung slices can survive up to seven days (24, 39). Since more cell-cell interactions are maintained in the lung sections, gene expression in these sections should have more relevance to the gene expression *in vivo*. In addition to the preservation of cell-cell interactions, there are other reasons for utilization of this method; the transfection conditions for lung slices can be easily controlled and one mouse lung can be sectioned into many slices for testing many constructs at once while more

animals have to be used for the same experiment *in vivo*. The mouse lung slices are transfected with K18EpilongLacZ construct with DODAC:DOPE in the same way as for cultured cells (see above) in a 6 well dish and cultured at 37°C for two days. We use the LacZ as a reporter in lung slices because its β-galactosidase activity can be easily measured with chemiluminescent assays as well as cell-staining with X-gal. The transfected tissue slices are homogenized for β-galactosidase activity assay or fixed for (i) cell staining, (ii) *in situ* hybridization to detect cell-specificity of RNA expression, and (iii) fluorescent immunostaining of reporter gene products (the anti-β-galactosidase antibody is available from Clontech).

Example 11- Expression in model animals

Gene expression studies in model animals are probably necessary for any expression cassette to be used for gene therapy because regulation of gene expression in model animals resembles that in human better than any other *in vitro* systems. We transfect CD1 mice in triplicates with K18EpilongLacZ using an intra-tracheal instillation technique established by Dr. O'Brodovich's group (at the Hospital for Sick Children, Toronto, Canada) and others. A negative control plasmid, K18Epilong (vector) is included in the study. The β-galactosidase activity in lung cells will be determined initially 2 days after transfection by using the chemiluminescent assays. To carry out a time course study; transfected mice will be sacrificed at day 7, 14, 21, 28 post-transfection and the β-galactosidase activity in lung cells will be assayed.

The best animal models available for cystic fibrosis are the CF knock-out mice, that are available in the Hospital for Sick Children, animal facility (Toronto, Canada). We will test whether K18EpilongCFTR expresses in CF knock-out mice. Dr. O'Brodovich has confirmed the observations (14) that UNC CF mice have a higher basal potential difference (PD) and fail to change their PD in response to lowed lumenal chloride concentration. We transfect the UNC CF knockout mice with our CFTR expression construct through intra-tracheal instillation. A vector plasmid is used as a negative control. The cell-specific expression of the human CFTR mRNA is assessed by fluorescent *in situ* RT-PCR and the human CFTR protein is detected by fluorescent immunostaining. Although there are not many high quality antibodies to CFTR available for *in vivo* detection, Demolombe et al. (8) have recently optimized the conditions for immunofluorescent staining of human CFTR with

a monoclonal antibody, MATG 1031. We also transfect the UNC CF mice with the same CFTR construct by nasal instillation and measure the nasal PD of the transfected mice.

The expression cassettes of this invention may be used in epithelial tissue gene therapy, particularly lung epithelial tissue gene therapy. The pharmaceutical compositions of this invention used to treat patients having degenerative diseases, disorders or abnormal physical states of the epithelial tissue could include an acceptable carrier, auxiliary or excipient. The conditions which may be treated by the expression cassettes include cystic fibrosis, emphysema, and cancers of epithelial origin arising in the lung or other organs.

The pharmaceutical compositions can be administered to humans or animals by methods such as aerosol administration, direct lavage and intravenous injection. Dosages to be administered depend on patient needs, on the desired effect and on the chosen route of administration. The expression cassettes may be introduced into epithelial cells using *in vivo* delivery vehicles such as liposomes. They may also be introduced into these cells using physical techniques such as microinjection and electroporation or chemical methods such as coprecipitation and incorporation of DNA into liposomes. The expression cassette may be introduced into epithelial cells, such as submucosal cells, using these techniques. The expression cassettes may also be used in gene expression studies.

The pharmaceutical compositions can be prepared by known methods for the preparation of pharmaceutically acceptable compositions which can be administered to patients, and such that an effective quantity of the expression cassette is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985).

On this basis, the pharmaceutical compositions could include an active compound or substance, such as an episomal expression cassette and one or more genes to be expressed, in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and isoosmotic with the physiological fluids. The methods of combining the expression cassettes with the vehicles or combining them with diluents is well known to those skilled in the art. The composition could include a targeting agent for the transport of the active compound to specified sites within the epithelial tissue.

Materials and Methods

Construction of reporter gene and CFTR expression cassettes. Polymerase chain reactions (PCR) were performed with *pfu* polymerase (Stratagene) and primer pairs (K18P3-5'GCAACGCGTCAGGTAAGGGGTAGG [SEQ ID NO: 2]/K18P4-5'CGAAGATCTGGAGGGATTGTAGAGAG [SEQ ID NO: 3]), (K18XH5'-5'CATAATAACGTCATTTCCTGCCC [SEQ ID NO: 4]/ K18P2*-5'GCTACGCGTGAGAGAAAGGACAGGACTC [SEQ ID NO: 5]),(K18NsiI-5'CTCACAGTAGGTGCTGAATGC [SEQ ID NO: 6]/K18XH3'-5'GACACGGACAGCAGGTGTTGTTG [SEQ ID NO: 7]) K18P1-5'CGAG GTACCAATAACAGTAAAAGGCAGTAC [SEQ ID NO: 8]/K18NsiIR-5'CACCGGTATATCACCTTTCCTGC [SEQ ID NO: 9]) on genomic DNA of human lung epithelial cells (A549) to isolate the first intron, minimal promoter, and two 5' untranslated regions, respectively, of human K18 gene. PCR products were verified by restriction mapping, according to restriction patterns predicted from published sequence, before cloning into the polylinker region of pSEAP(Tropix), via naturally-occurring restriction sites or sites introduced by PCR primers. The primers were purchased from ACGT Corp., Toronto and the PCR machine (DNA Engine, PTC-200), was purchased from Fisher.

The translation initiation sequence of the human CFTR cDNA was modified to introduce an *Nco* I site as well as to improve the initiation signal, according to Kozak's rule, by PCR using a cftrp1 primer (of sequence 5'GAGACCATGGAGAGGTCG [SEQ ID NO: 10]). A linker containing the alfalfa mosaic virus translational enhancer (TE) sequence (5'GTTTTTATTTTTAATTTTCTTTCAAATACTTCCA [SEQ ID NO: 11]) was inserted immediately upstream of the *Nco* I site. The SEAP coding region in K18EpilongSEAP was then replaced with the TE-4.6 kb CFTR cDNA fragment, resulting in the K18EpilongTECFTR construct.

PCR mutagenesis was performed on K18EpilongTECFTR using a 2-step nested PCR strategy. First-round PCR reactions incorporate primer pairs (TE2-5'GTCCGCAAAGCCTGAGTCCTGTCC [SEQ ID NO: 12]/K183'SS-5'AAATTAAAAATAAAAACAGACCTGAAAAAAAAAAAGAGAGAGGTTGTTCCATG A [SEQ ID NO: 13]) and (TEtop-

5'GATCTGTTTTTATTTTTAATTTTCTTTCAAATACTTCCACCATGGCCCC [SEQ ID NO: 14]/cftr3'SS-5'GGTGACTTCCCCCAAATATAAAAAG [SEQ ID NO: 15]). Products from the first-round reactions were mixed and served as templates for the second-round PCR using TE2 and cftr3'SS primers. K18mCFTR construct was then generated by cloning the second-round PCR product back into K18EpilongTECFTR to replace the corresponding parental fragment.

Tissue culture and transfection. A549, a human lung carcinoma cell line, and COS-7 cells were cultured in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (FBS). Human lung fibroblasts, WI38, were maintained in alpha minimum essential medium (alpha-MEM) with 10% FBS. IB3, a human cystic fibrosis bronchial epithelial cell line, was cultured in LHC-8 with 5% FBS. Day 19 rat fetal lung epithelium and fibroblast cells were isolated according to standard procedure and maintained in alpha-MEM with 10% FBS.

For transfection, cells were seeded at 50-80% confluency in six-well plates and allowed to settle in their regular media for overnight. The cells were then transfected in serum-free media with 1mg DNA premixed with 12 mg of lipofectamine (GibcoBRL) per well according to the recommended procedure. Primary cells were transfected with premixed DNA:lipid complexes consisting of 1.66 mg DNA and 16.6 mg DODAC: DOPE (1:1 dioleyldimethylammonium chloride:dioleoylphosphatidylethanolamine, INEX) in serum-free media for 24 hr.

Reporter assay. Culture media from transfected plates were collected at indicated time points post-transfection, before changes of media, and centrifuged 1 min at 16,000 x g. Supernatant was frozen at -80°C or assayed immediately. Secreted alkaline phosphatase activities in the media were detected with Phospha-Light chemiluminescent assay system (Tropix) as recommended and measured on a luminometer (BioOrbit).

Detection of CFTR mRNA. DNase I treated total RNA from transfected cells, prepared with RNeasy column (Qiagen), was subjected to reverse transcription, followed by PCR (30 cycles) using TE1 (5'CTGTCCTTTCTCTCACGCGTCAG [SEQ ID NO: 16]) or TE2 in

combination with cftrp2 (5'GAGGAGTGCCACTTGC [SEQ ID NO: 17]) or cftrp3 (5'GTTGTTGGAAAGGAGACTAACAAG [SEQ ID NO: 18]) primers.

Functional analysis of CFTR protein. Iodide efflux assays were performed 48 hr post-transfection as previously described(5). Slight modifications were made on compositions of the loading buffer, which is 136 mM NaI, 4 mM KNO_3, 2 mM $Ca(NO_3)_2$, 2 mM $Mg(NO_3)_2$, 11 mM glucose, and 20 mM HEPES, pH 7.4, and the agonists, 20 mM forskolin, 0.5 mM 8-(4-chlorophenylthio)-adenosine 3'; 5'-cyclic monophosphate (CPT-cAMP), and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX).

Production of Transgenic mice. The K18mLacZ construct was constructed by replacing the human *CFTR* coding region in the K18mCFTR plasmid with the *E. coli LacZ* gene. The K18mLacZ expression cassette was released by digestion with *Kpn* I. The DNA fragments were separated by agarose gel electrophoresis and purified through elutip (Schleicher & Schuell) following electroelution. The DNA fragments were microinjected into the pronuclei of fertilized eggs of STL/Bl6 mice. Fertilized eggs that proceeded into 2-cell stage were transferred to pseudo-pregnant CD1 recipients. The lungs of the 14 day fetuses were dissected out and stained with X-gal solution and the transgenic fetuses were identified by PCR and Southern blot analyses of the genomic DNA.

The present invention has been described in terms of particular embodiments found or proposed by the present inventor to comprise preferred modes for the practice of the invention. It will be appreciated by those of skill in the art that, in light of the present disclosure, numerous modifications and changes can be made in the particular embodiments exemplified without departing from the intended scope of the invention. All such modifications are intended to be included within the scope of the appended claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

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SEQUENCE LISTING

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(ii) TITLE OF INVENTION: Episomal Expression Cassettes for Gene Therapy

(iii) NUMBER OF SEQUENCES: 18

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
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(C) OPERATING SYSTEM: PC-DOS/MS-DOS
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PATENT AGENT INFORMATION
NAME: Deeth Williams Wall
REFERENCE NUMBER: 1786/0003

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12143 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "Mixture of genomic DNA, cDNA and other types."

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE:

(vi) ORIGINAL SOURCE:

(vii) IMMEDIATE SOURCE:

(viii) POSITION IN GENOME:
CHROMOSOME/SEGMENT:
MAP POSITION:
UNITS:

(ix) FEATURE:
(A) NAME/KEY: enhancer
(B) LOCATION:8..2570
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:/standard_name= "K18 Enhancer/Promoter"
/note= "DNA fragment was obtained by PCR-cloning and minor modifications were introduced for the purpose of PCR."

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(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:/standard_name= "K18 intron 1"
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(A) NAME/KEY: misc_feature
(B) LOCATION:7949..7984
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:/standard_name= "pBluescript II KS(+) multiple cloning site"

(ix) FEATURE:
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(B) LOCATION:9178..9212
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(A) NAME/KEY: polyA_signal
(B) LOCATION:12021..12055
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:/standard_name= "SV40 polyadenylation signal"

(ix) FEATURE:
(A) NAME/KEY: rep_origin
(B) LOCATION:9562..10205
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:/standard_name= "pUC origin of replication"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION:11283..11353
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:/standard_name= "Ampicillin resistance gene"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION:11345..11800
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:/standard_name= "f1 single strand DNA origin"

(x) PUBLICATION INFORMATION
AUTHORS:
TITLE:
JOURNAL:
VOLUME:
ISSUE:
PAGES:
DATE:
DOCUMENT NUMBER:
FILING DATE:
PUBLICATION DATE:
RELEVANT RESIDUES IN SEQ ID NO.:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GGTACCAATA ACAGTAAAAG GCAGTACATA GCTTGTTGAC TCCACATACT TTATTATAAA 60

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TGCCTCCCAC CTGGCAGGTC CACTGGCCAG GCTTTTCCAG GGTCCCTTCT CTCCCAGGTC 420

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CCACTCCAGA GCAAAGAACA CAGCCAAATG TGTCATACCA GGCCCTGCCA GAAAAACGAG 540

CTGCTGGAGC TGACAAACTT GAAGGCCAAA CACCTAAGGT TCCCCCCAAC ACTTCATTCA 600

GCAGGGATGG TCATTCAGCT TCAGGGGGCA GGCAGCATGA AAGCCTCCCT ACCTCCATCC 660

TTCTCACACA GAGGCTGGGG AGAGCATCTT GGAGGATGCA GTCCCCTGGG GCCAGGCTTC 720

TAATCCAGAC AGCCCTTACA AGGGGGGACA GGGGAAGGAC TGGCTTGGAG AAAAGTCCTA 780

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TATTTATTTT GAGTCAGAGT TTCGCTCTGG TTGCCCAGGC TGGAGCGCGA CGGCACGATC 960

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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(vii) IMMEDIATE SOURCE:

(viii) POSITION IN GENOME:
CHROMOSOME/SEGMENT:
MAP POSITION:
UNITS:

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..24
(D) OTHER INFORMATION:/note= "K18P3 synthetic DNA
oligo-nucleotide - amplification primer for obtaining K18"

(x) PUBLICATION INFORMATION
AUTHORS:
TITLE:
JOURNAL:
VOLUME:
ISSUE:
PAGES:
DATE:
DOCUMENT NUMBER:
FILING DATE:
PUBLICATION DATE:
RELEVANT RESIDUES IN SEQ ID NO.:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

GCAACGCGTC AGGTAAGGGG TAGG 24

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(vii) IMMEDIATE SOURCE:

(viii) POSITION IN GENOME:
CHROMOSOME/SEGMENT:
MAP POSITION:
UNITS:

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..26
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:/note= "K18P4 synthetic DNA
oligo-nucleotide - amplification primer for obtaining K18"

(x) PUBLICATION INFORMATION
AUTHORS:
TITLE:
JOURNAL:
VOLUME:
ISSUE:
PAGES:
DATE:
DOCUMENT NUMBER:
FILING DATE:
PUBLICATION DATE:
RELEVANT RESIDUES IN SEQ ID NO.:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CGAAGATCTG GAGGGATTGT AGAGAG 26

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(vii) IMMEDIATE SOURCE:

(viii) POSITION IN GENOME:
CHROMOSOME/SEGMENT:
MAP POSITION:
UNITS:

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..23
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:/note= "K18XH5' synthetic DNA
oligonucleotide - amplification primer for obtaining K18 "

(x) PUBLICATION INFORMATION
AUTHORS:
TITLE:
JOURNAL:

VOLUME:
ISSUE:
PAGES:
DATE:
DOCUMENT NUMBER:
FILING DATE:
PUBLICATION DATE:
RELEVANT RESIDUES IN SEQ ID NO.:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CATAATAACG TCATTTCCTG CCC 23

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(vii) IMMEDIATE SOURCE:

(viii) POSITION IN GENOME:
CHROMOSOME/SEGMENT:
MAP POSITION:
UNITS:

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..28
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:/note= "K18P2* synthetic DNA
oligo-nucleotide - amplification primer for obtaining K18"

(x) PUBLICATION INFORMATION
AUTHORS:
TITLE:
JOURNAL:
VOLUME:
ISSUE:
PAGES:
DATE:
DOCUMENT NUMBER:

FILING DATE:
PUBLICATION DATE:
RELEVANT RESIDUES IN SEQ ID NO.:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCTACGCGTG AGAGAAAGGA CAGGACTC 28

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(vii) IMMEDIATE SOURCE:

(viii) POSITION IN GENOME:
CHROMOSOME/SEGMENT:
MAP POSITION:
UNITS:

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..21
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:/note= "K18NsiI synthetic DNA
oligo-nucleotide - amplification primer for obtaining K18"

(x) PUBLICATION INFORMATION
AUTHORS:
TITLE:
JOURNAL:
VOLUME:
ISSUE:
PAGES:
DATE:
DOCUMENT NUMBER:
FILING DATE:
PUBLICATION DATE:
RELEVANT RESIDUES IN SEQ ID NO.:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

CTCACAGTAG GTGCTGAATG C 21

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(vii) IMMEDIATE SOURCE:

(viii) POSITION IN GENOME:
CHROMOSOME/SEGMENT:
MAP POSITION:
UNITS:

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..23
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:/note= "K18XH3' synthetic DNA
oligo-nucleotide - amplification primer for obtaining K18"

(x) PUBLICATION INFORMATION
AUTHORS:
TITLE:
JOURNAL:
VOLUME:
ISSUE:
PAGES:
DATE:
DOCUMENT NUMBER:
FILING DATE:
PUBLICATION DATE:
RELEVANT RESIDUES IN SEQ ID NO.:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GACACGGACA GCAGGTGTTG TTG 23

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(vii) IMMEDIATE SOURCE:

(viii) POSITION IN GENOME:
CHROMOSOME/SEGMENT:
MAP POSITION:
UNITS:

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..30
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:/note= "K18P1 synthetic
oligo-nucleotide - amplification primer for obtaining K18"

(x) PUBLICATION INFORMATION
AUTHORS:
TITLE:
JOURNAL:
VOLUME:
ISSUE:
PAGES:
DATE:
DOCUMENT NUMBER:
FILING DATE:
PUBLICATION DATE:
RELEVANT RESIDUES IN SEQ ID NO.:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CGAGGTACCA ATAACAGTAA AAGGCAGTAC 30

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(vii) IMMEDIATE SOURCE:

(viii) POSITION IN GENOME:
CHROMOSOME/SEGMENT:
MAP POSITION:
UNITS:

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..23
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:/note= "K18NsiIR synthetic DNA
oligo-nucleotide - amplification primer for obtaining K18"

(x) PUBLICATION INFORMATION
AUTHORS:
TITLE:
JOURNAL:
VOLUME:
ISSUE:
PAGES:
DATE:
DOCUMENT NUMBER:
FILING DATE:
PUBLICATION DATE:
RELEVANT RESIDUES IN SEQ ID NO.:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CACCGGTATA TCACCTTTCC TGC 23

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(vii) IMMEDIATE SOURCE:

(viii) POSITION IN GENOME:
CHROMOSOME/SEGMENT:
MAP POSITION:
UNITS:

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..18
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:/note= "cftrp1 synthetic DNA
oligonucleotide - amplification primer for PCR mutagenesis"

(x) PUBLICATION INFORMATION
AUTHORS:
TITLE:
JOURNAL:
VOLUME:
ISSUE:
PAGES:
DATE:
DOCUMENT NUMBER:
FILING DATE:
PUBLICATION DATE:
RELEVANT RESIDUES IN SEQ ID NO.:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

GAGACCATGG AGAGGTCG 18

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..34
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:/note= "TE"

(x) PUBLICATION INFORMATION
AUTHORS:
TITLE:
JOURNAL:
VOLUME:
ISSUE:
PAGES:
DATE:
DOCUMENT NUMBER:
FILING DATE:
PUBLICATION DATE:
RELEVANT RESIDUES IN SEQ ID NO.:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GTTTTTATTT TTAATTTTCT TTCAAATACT TCCA 34

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(vii) IMMEDIATE SOURCE:

(viii) POSITION IN GENOME:
CHROMOSOME/SEGMENT:
MAP POSITION:
UNITS:

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..24
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:/note= "TE2 synthetic DNA
oligo-nucleotide - amplification primer for PCR mutagenesis"

(x) PUBLICATION INFORMATION
AUTHORS:
TITLE:
JOURNAL:

VOLUME:
ISSUE:
PAGES:
DATE:
DOCUMENT NUMBER:
FILING DATE:
PUBLICATION DATE:
RELEVANT RESIDUES IN SEQ ID NO.:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GTCCGCAAAG CCTGAGTCCT GTCC 24

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(vii) IMMEDIATE SOURCE:

(viii) POSITION IN GENOME:
CHROMOSOME/SEGMENT:
MAP POSITION:
UNITS:

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..54
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:/note= "K183'SS synthetic DNA
oligo-nucleotide - amplification primer for PCR mutagenesis"

(x) PUBLICATION INFORMATION
AUTHORS:
TITLE:
JOURNAL:
VOLUME:
ISSUE:
PAGES:
DATE:
DOCUMENT NUMBER:

FILING DATE:
PUBLICATION DATE:
RELEVANT RESIDUES IN SEQ ID NO.:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

AAATTAAAAA TAAAAACAGA CCTGAAAAAA AAAAAGAGAG AGGTTGTTCC ATGA 54

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 49 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(vii) IMMEDIATE SOURCE:

(viii) POSITION IN GENOME:
CHROMOSOME/SEGMENT:
MAP POSITION:
UNITS:

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..49
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:/note= "TEtop synthetic DNA
oligo-nucleotide - amplification primer for PCR mutagenesis"

(x) PUBLICATION INFORMATION
AUTHORS:
TITLE:
JOURNAL:
VOLUME:
ISSUE:
PAGES:
DATE:
DOCUMENT NUMBER:
FILING DATE:
PUBLICATION DATE:
RELEVANT RESIDUES IN SEQ ID NO.:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

GATCTGTTTT TATTTTTAAT TTTCTTTCAA ATACTTCCAC CATGGCCCC 49

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(vii) IMMEDIATE SOURCE:

(viii) POSITION IN GENOME:
CHROMOSOME/SEGMENT:
MAP POSITION:
UNITS:

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..25
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:/note= "cftr3'SS synthetic DNA
oligo-nucleotide - amplification primer for PCR mutagenesis"

(x) PUBLICATION INFORMATION
AUTHORS:
TITLE:
JOURNAL:
VOLUME:
ISSUE:
PAGES:
DATE:
DOCUMENT NUMBER:
FILING DATE:
PUBLICATION DATE:
RELEVANT RESIDUES IN SEQ ID NO.:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GGTGACTTCC CCCAAATATA AAAAG 25

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 23 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..23
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:/note= "TE1 synthetic DNA
oligo-nucleotide - amplification primer for PCR analysis of
CFTR
mRNA"

(x) PUBLICATION INFORMATION
AUTHORS:
TITLE:
JOURNAL:
VOLUME:
ISSUE:
PAGES:
DATE:
DOCUMENT NUMBER:
FILING DATE:
PUBLICATION DATE:
RELEVANT RESIDUES IN SEQ ID NO.:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CTGTCCTTTC TCTCACGCGT CAG 23

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(vii) IMMEDIATE SOURCE:

(viii) POSITION IN GENOME:
CHROMOSOME/SEGMENT:
MAP POSITION:
UNITS:

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..16
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:/note= "cftrp2 synthetic DNA oligo-nucleotide - amplification primer for PCR analysis of CFTR mRNA"

(x) PUBLICATION INFORMATION
AUTHORS:
TITLE:
JOURNAL:
VOLUME:
ISSUE:
PAGES:
DATE:
DOCUMENT NUMBER:
FILING DATE:
PUBLICATION DATE:
RELEVANT RESIDUES IN SEQ ID NO.:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

GAGGAGTGCC ACTTGC 16

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(vii) IMMEDIATE SOURCE:

(viii) POSITION IN GENOME:
CHROMOSOME/SEGMENT:
MAP POSITION:
UNITS:

(ix) FEATURE:
(A) NAME/KEY: -
(B) LOCATION:1..24
(C) IDENTIFICATION METHOD:
(D) OTHER INFORMATION:/note= "cftrp3 synthetic DNA
oligo-nucleotide - amplification primer for PCR analysis of
CFTR mRNA"

(x) PUBLICATION INFORMATION
AUTHORS:
TITLE:
JOURNAL:
VOLUME:
ISSUE:
PAGES:
DATE:
DOCUMENT NUMBER:
FILING DATE:
PUBLICATION DATE:
RELEVANT RESIDUES IN SEQ ID NO.:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

GTTGTTGGAA AGGAGACTAA CAAG 24

We claim:

1. An expression cassette for the episomal expression of a transgene in targeted epithelial cells, comprising
   - regulatory elements of the human cytokeratin gene, and
   - a transgene.
2. The expression cassette of claim 1, wherein the epithelial cells are lung epithelial cells.
3. The cassette of claim 1, wherein the human cytokeratin gene is the human cytokeratin 18 gene.
4. The cassette of claim 1, wherein the regulatory elements are from the 5 ′ region of the human cytokeratin 18 gene.
5. The cassette of claim 4, wherein regulatory elements comprise a promoter, the 5 ′ region and intron 1 of the human cytokeratin 18 gene.
6. The cassette of claim 1, wherein regulatory elements comprise a promoter, the 5 ′ region and modified intron 1 of the human cytokeratin 18 gene.
7. The cassette of claim 4, further comprising an enhancer.
8. The cassette of claim 1, wherein the transgene is the cystic fibrosis transmembrane conductance regulatory (CFTR) gene.
9. The cassette of claim 1, wherein the transgene comprises an enhancer and a modified cystic fibrosis transmembrane conductance regulatory (CFTR) gene.
10. The cassette of claim 1, wherein the targeted cells are epithelial cells.
11. The cassette of claim 1, wherein the epithelial cells are submucosal cells.
12. A liposome comprising the construct of claim 1.
13. A transfected cell comprising the construct of claim 1.
14. Lung tissue comprising the cell of claim 13.
15. A lung comprising the lung tissue of claim 14.

16. A method of treating a patient having a lung disorder, by administering to the patient a liposome containing the cassette of claim 1, whereby the cassette transfects targeted lung cells.

17. The method of claim 16, wherein the method of administration is selected from a group consisting of aerosol administration, direct lavage and intravenous injection.

18. The method of claim 16, wherein the disorder is selected from a group consisting of cystic fibrosis, cancers of epithelial origin and emphysema.

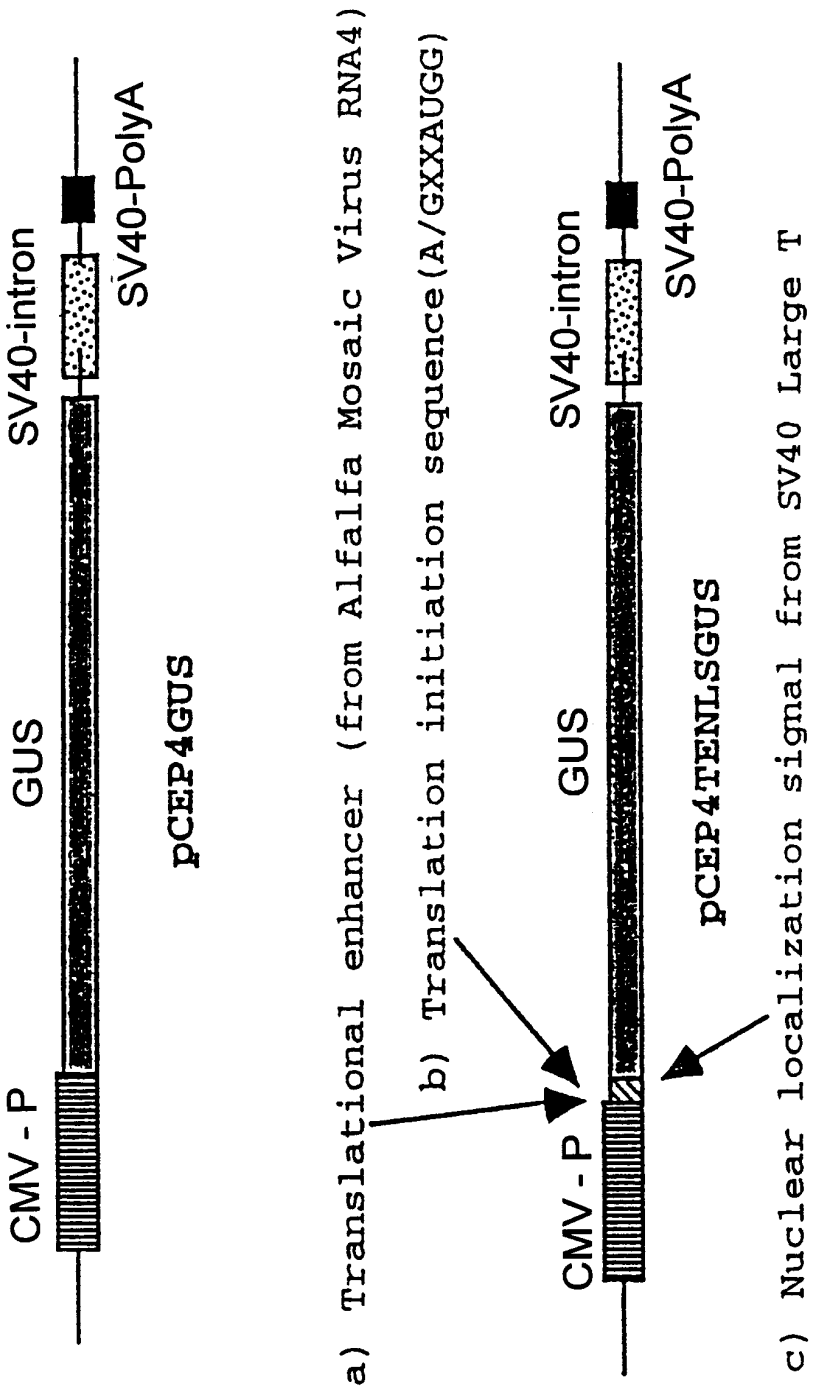

CMV - P
GUS
SV40-intron
SV40-PolyA
pCEP4GUS
a) Translational enhancer (from Alfalfa Mosaic Virus RNA4)
b) Translation initiation sequence(A/GXXAUGG)
CMV - P
GUS
SV40-intron
SV40-PolyA
pCEP4TENLSGUS
c) Nuclear localization signal from SV40 Large T

FIG. 1A

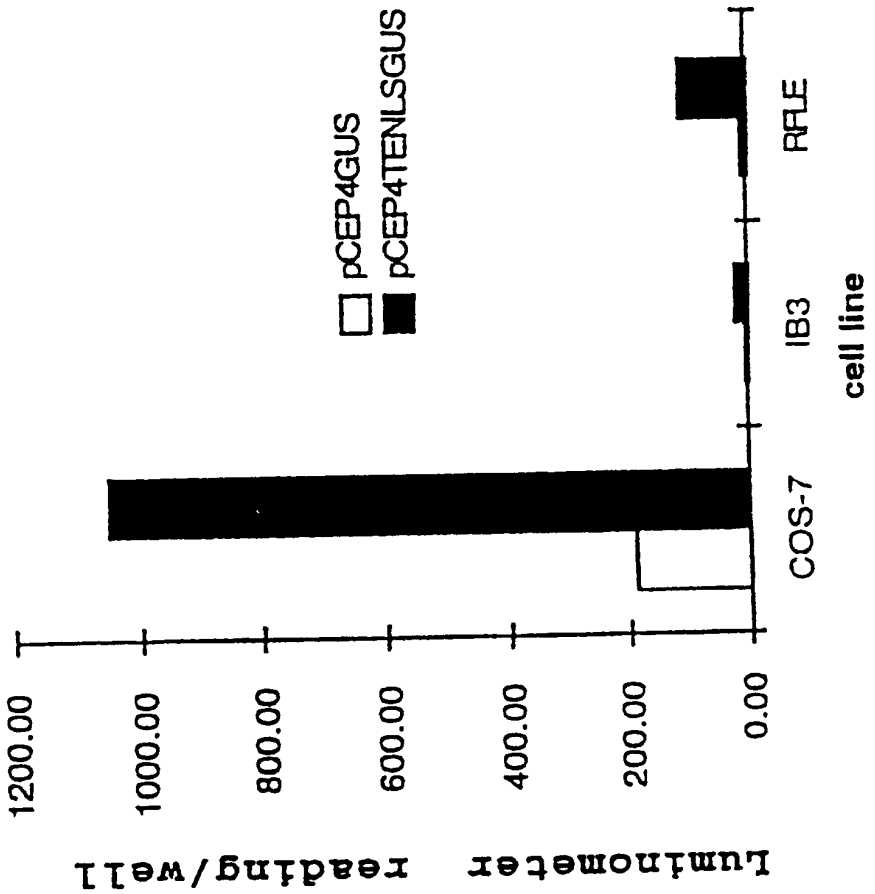

pCEP4GUS
pCEP4TENLSGUS
COS-7
IB3
RALE
cell line
0.00
200.00
400.00
600.00
800.00
1000.00
1200.00
luminometer reading/well

FIG. 1B

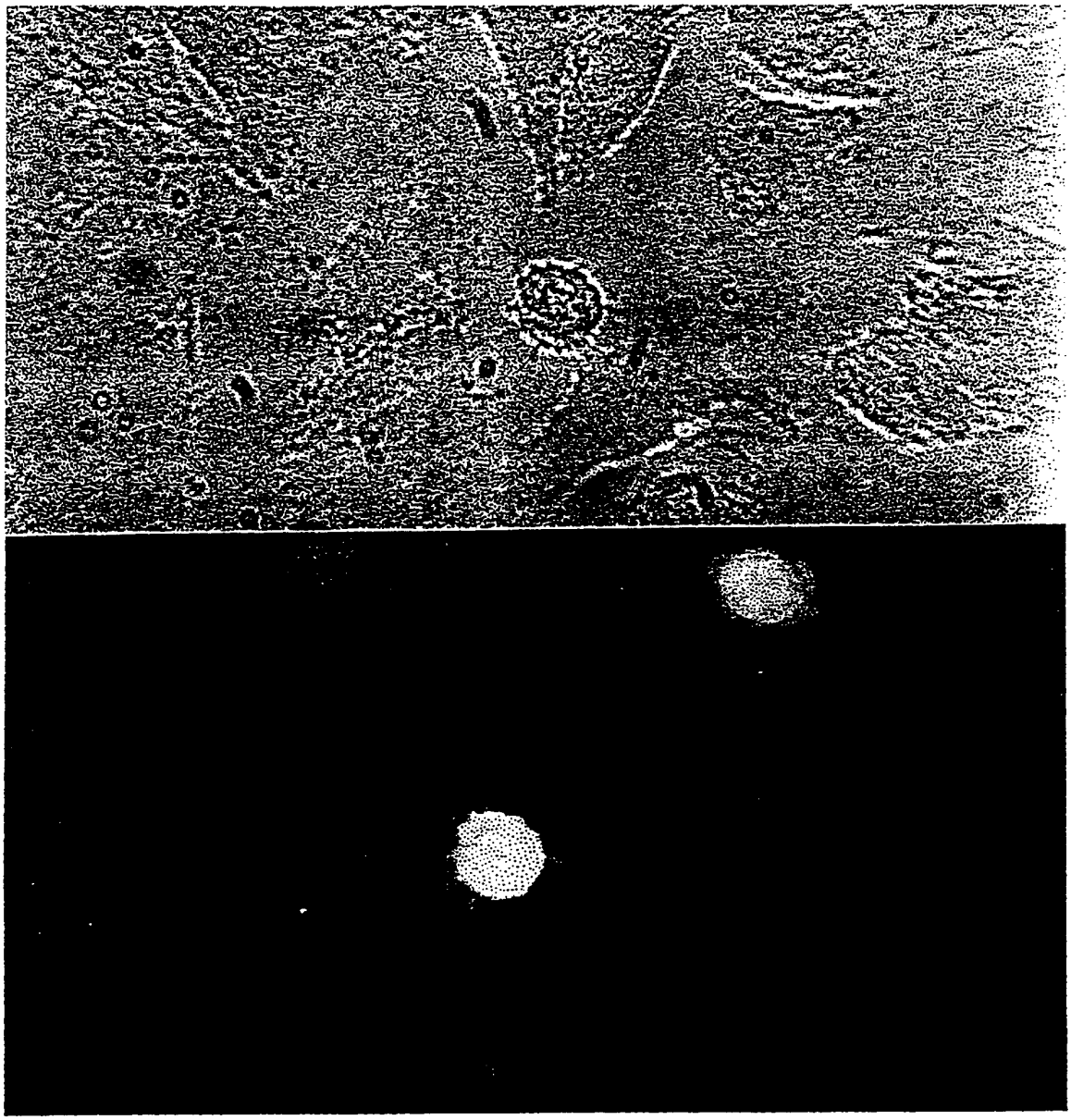


FIG. 2A

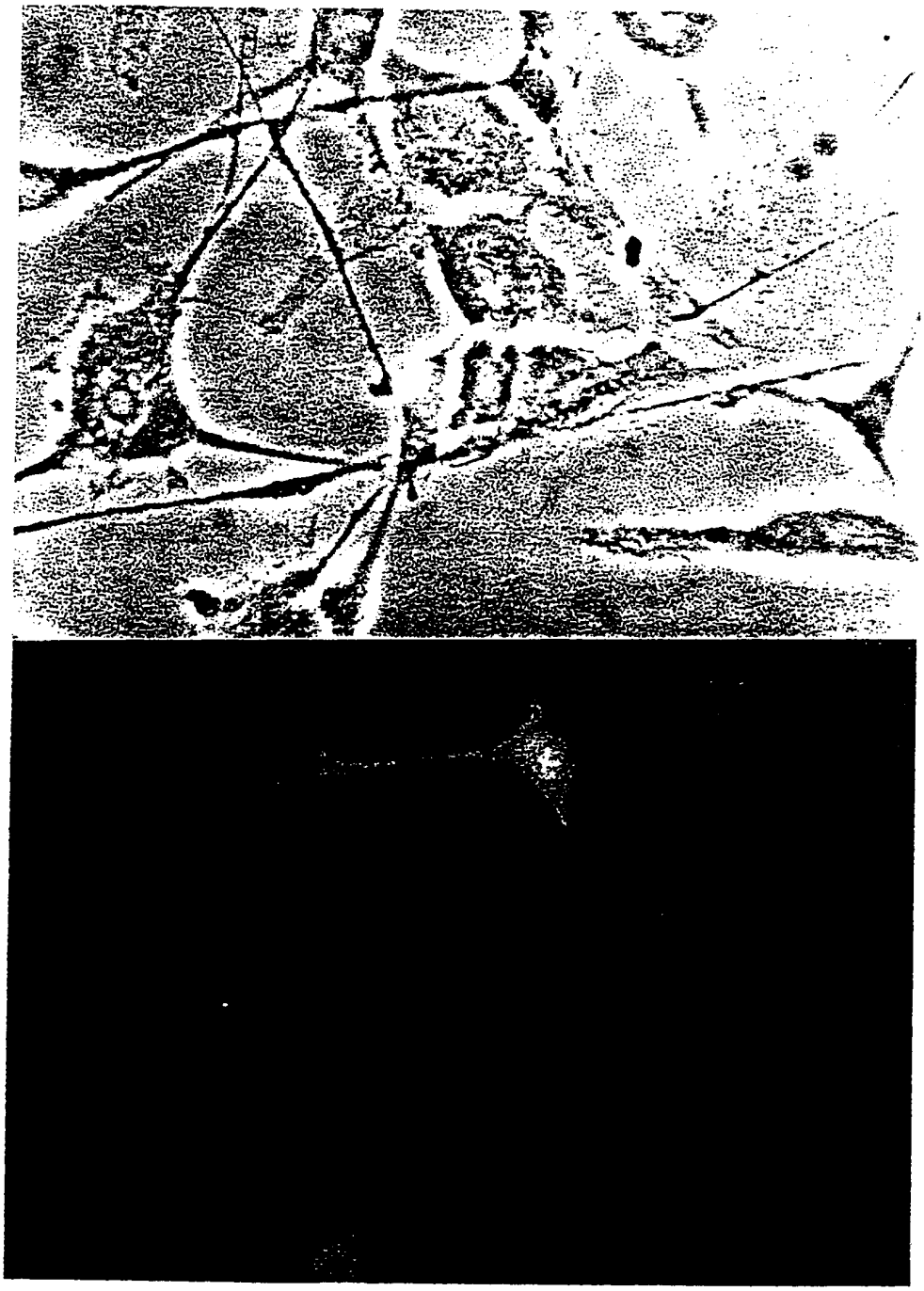


FIG. 2B

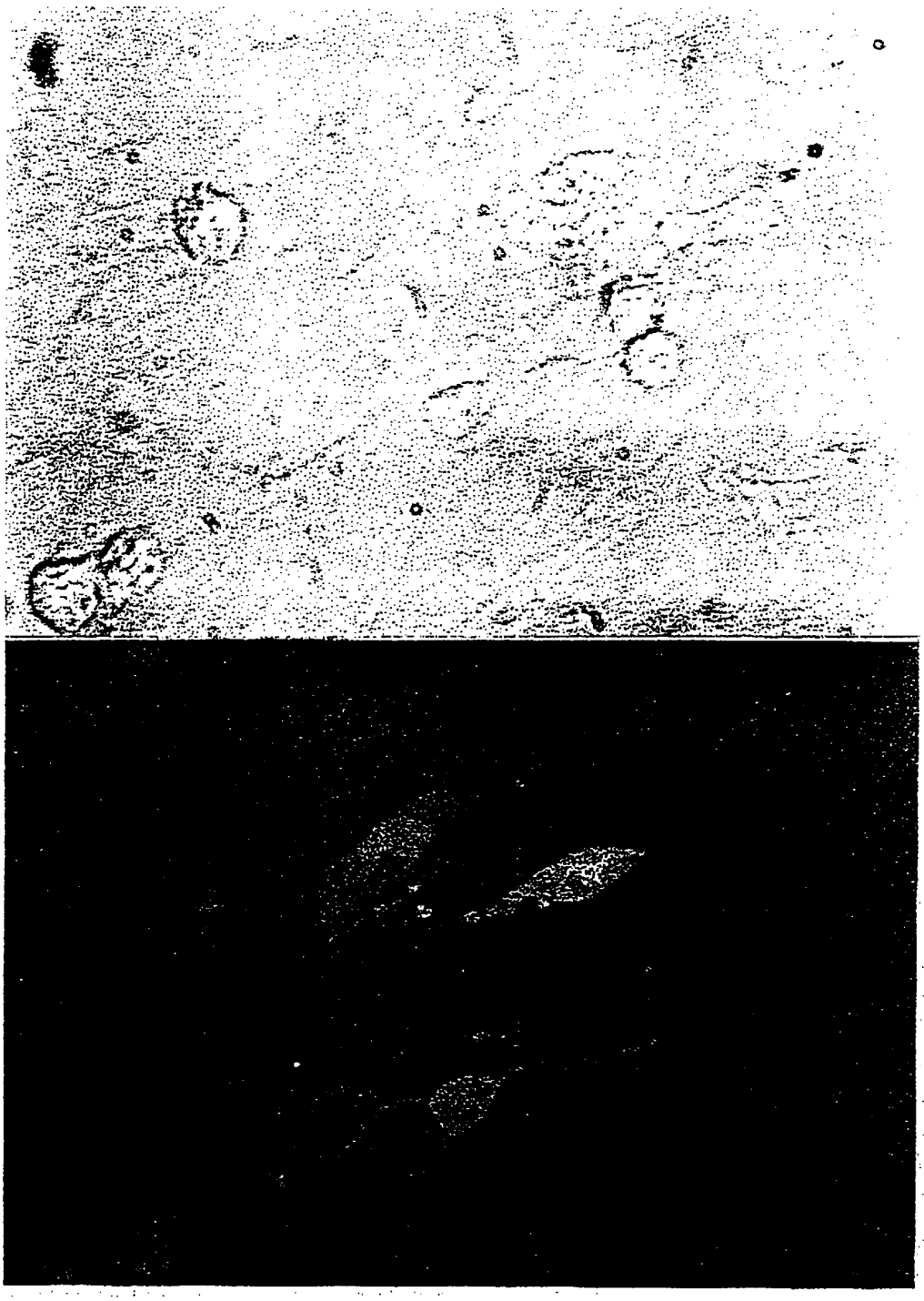


FIG. 2C

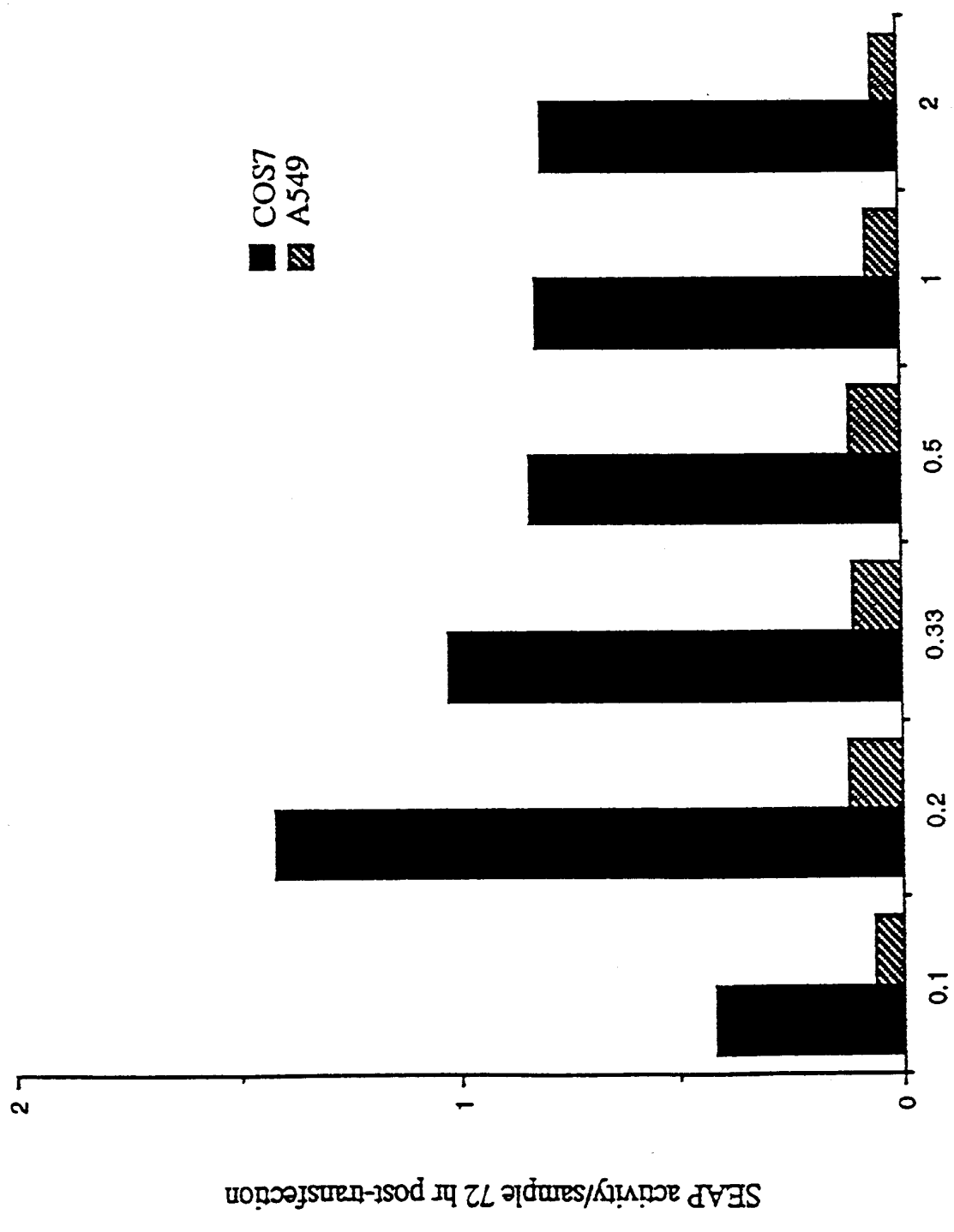

COS7
A549
SEAP activity/sample 72 hr post-transfection
0
1
2
0.1
0.2
0.33
0.5
1
2
DNA/Lipid

FIG. 3

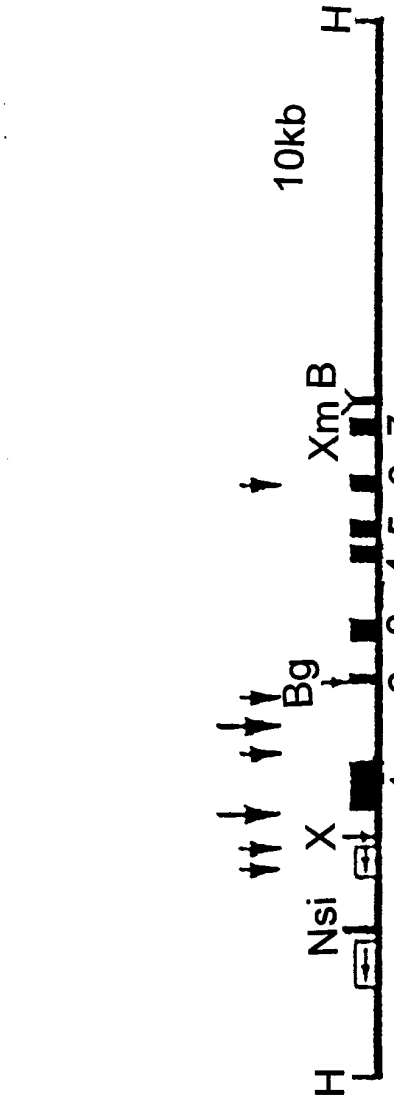

H
Nsi
X
Bg
Xm
B
Y
1
2
3
4
5
6
7
10kb
H
K18
intron-1
promoter
Enhancer
Enhancer-long

FIG. 4A

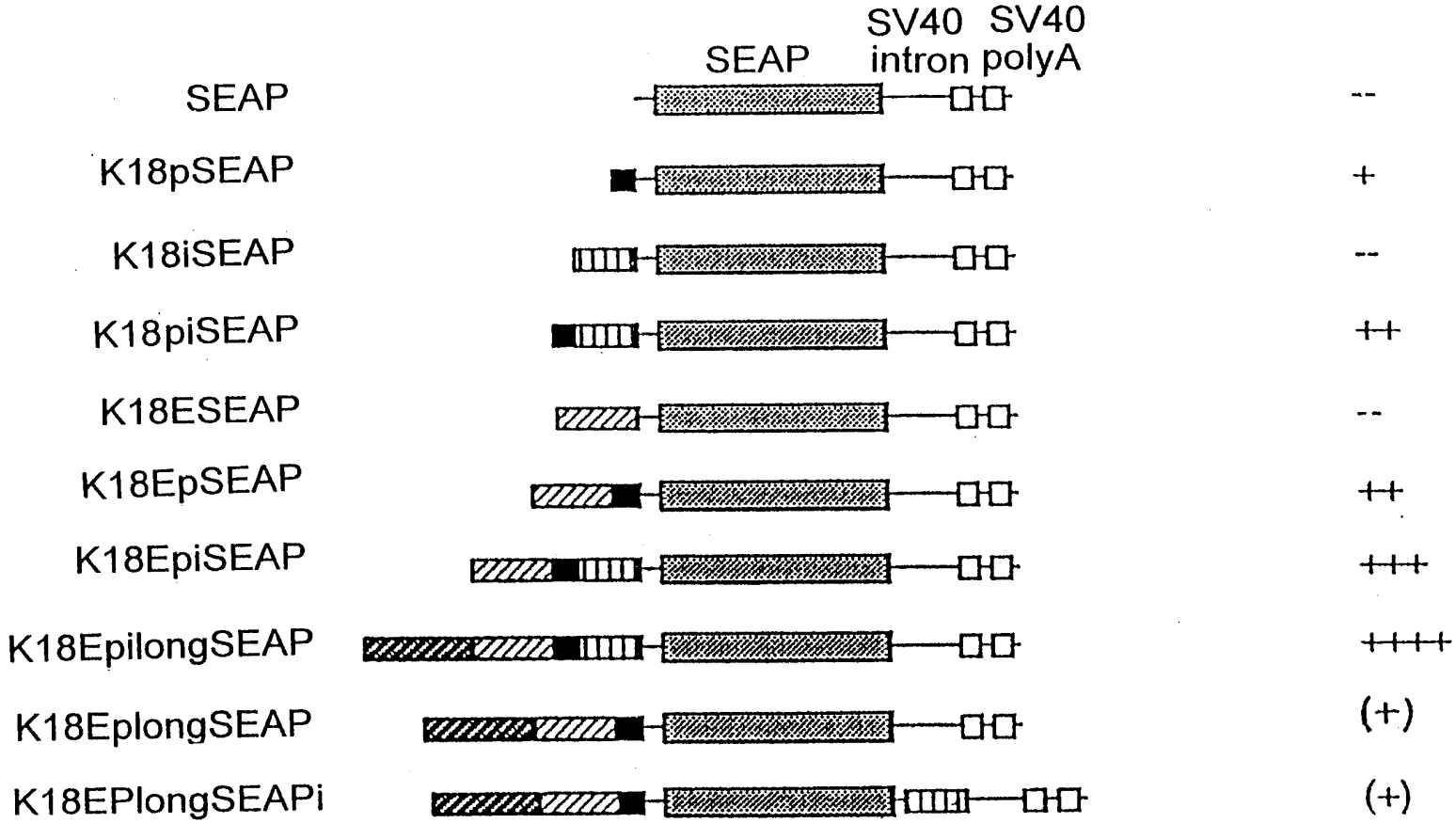


FIG. 4B

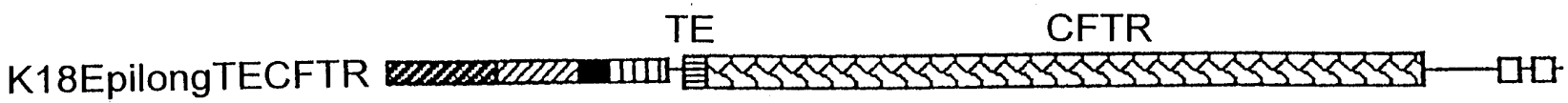


FIG. 4C

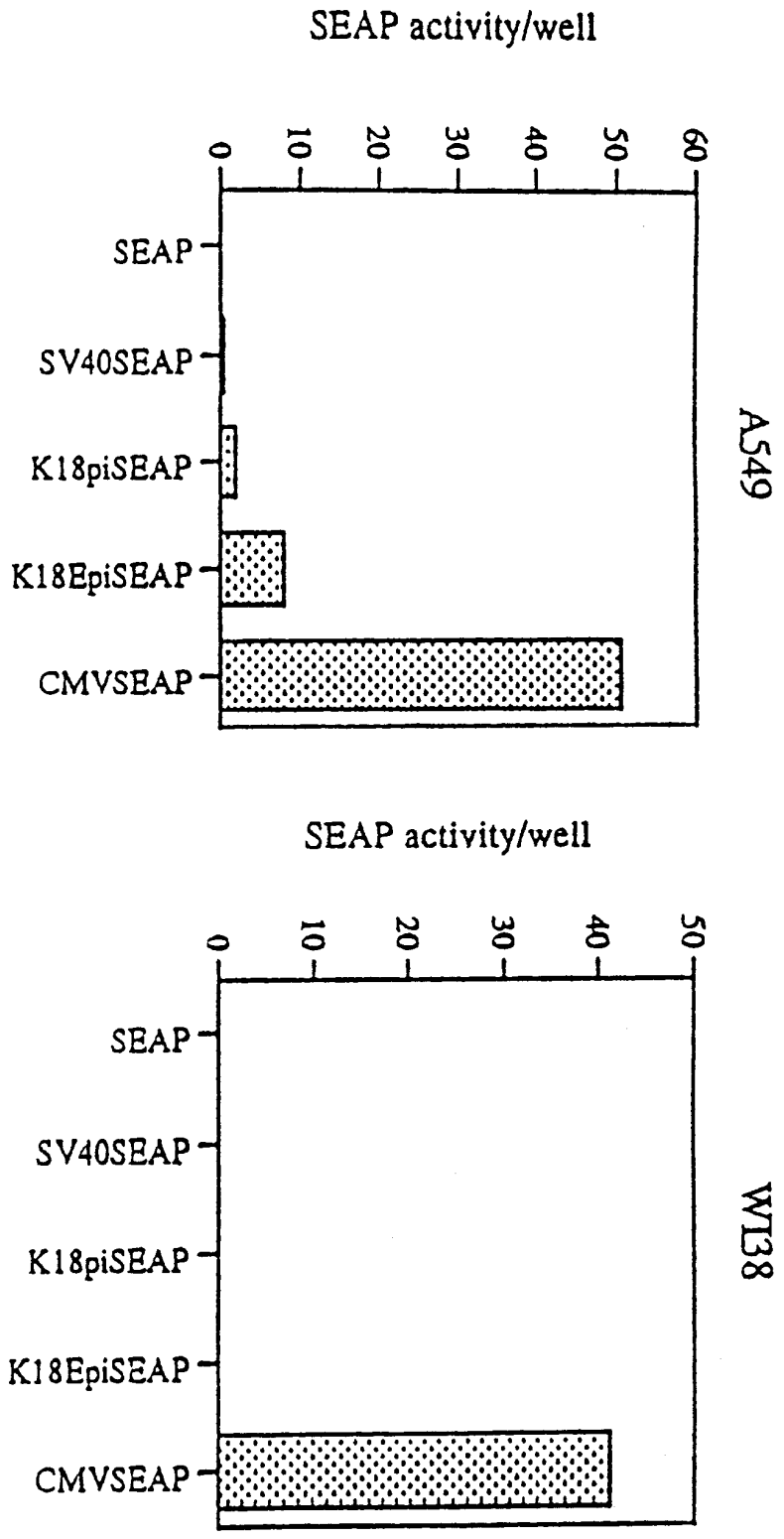


FIG. 5 (continued on next page)

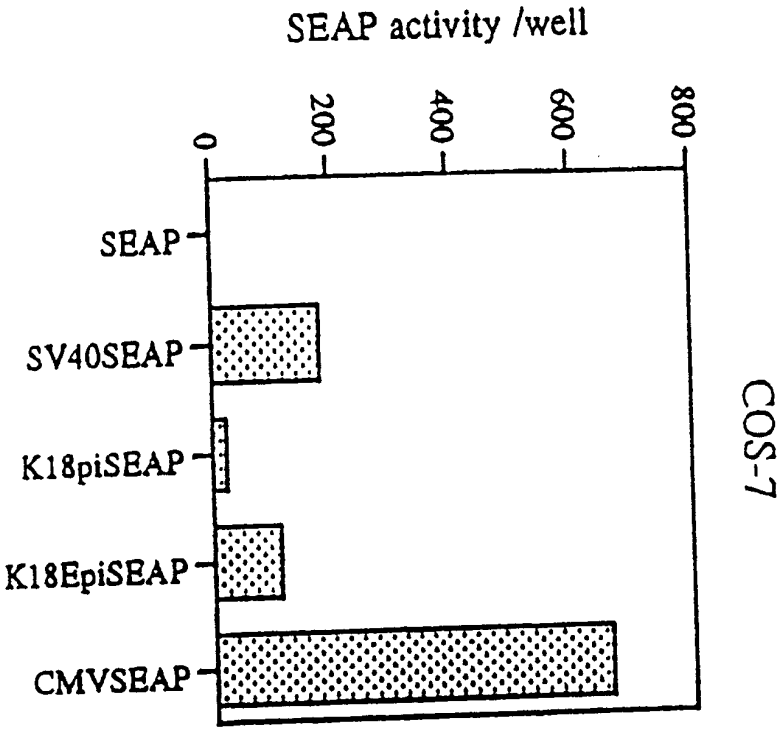

SEAP activity /well
0
200
400
600
800
SEAP
SV40SEAP
K18piSEAP
K18EpiSEAP
CMVSEAP
COS-7

FIG. 5

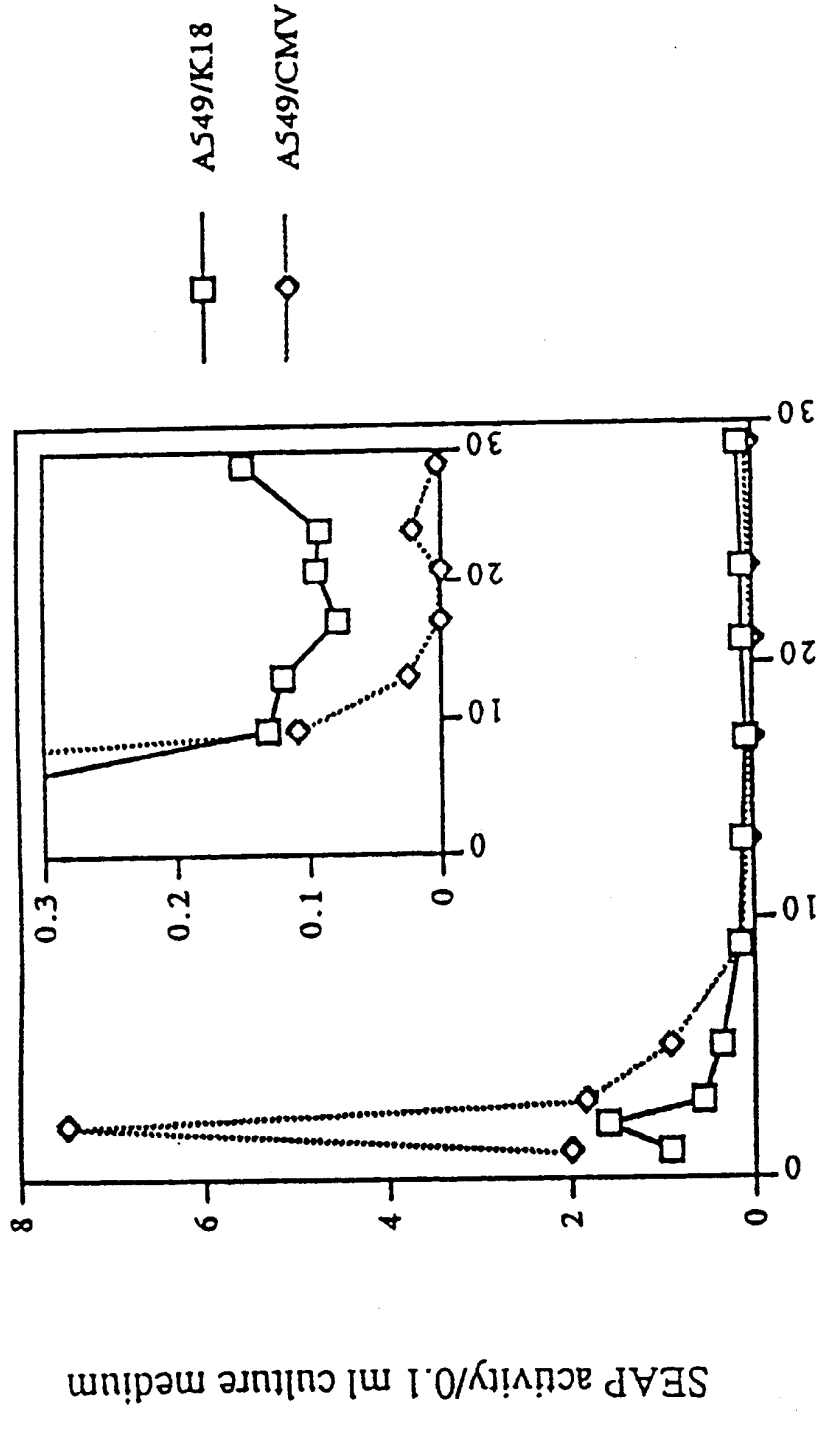


FIG. 6 (continued on next page)

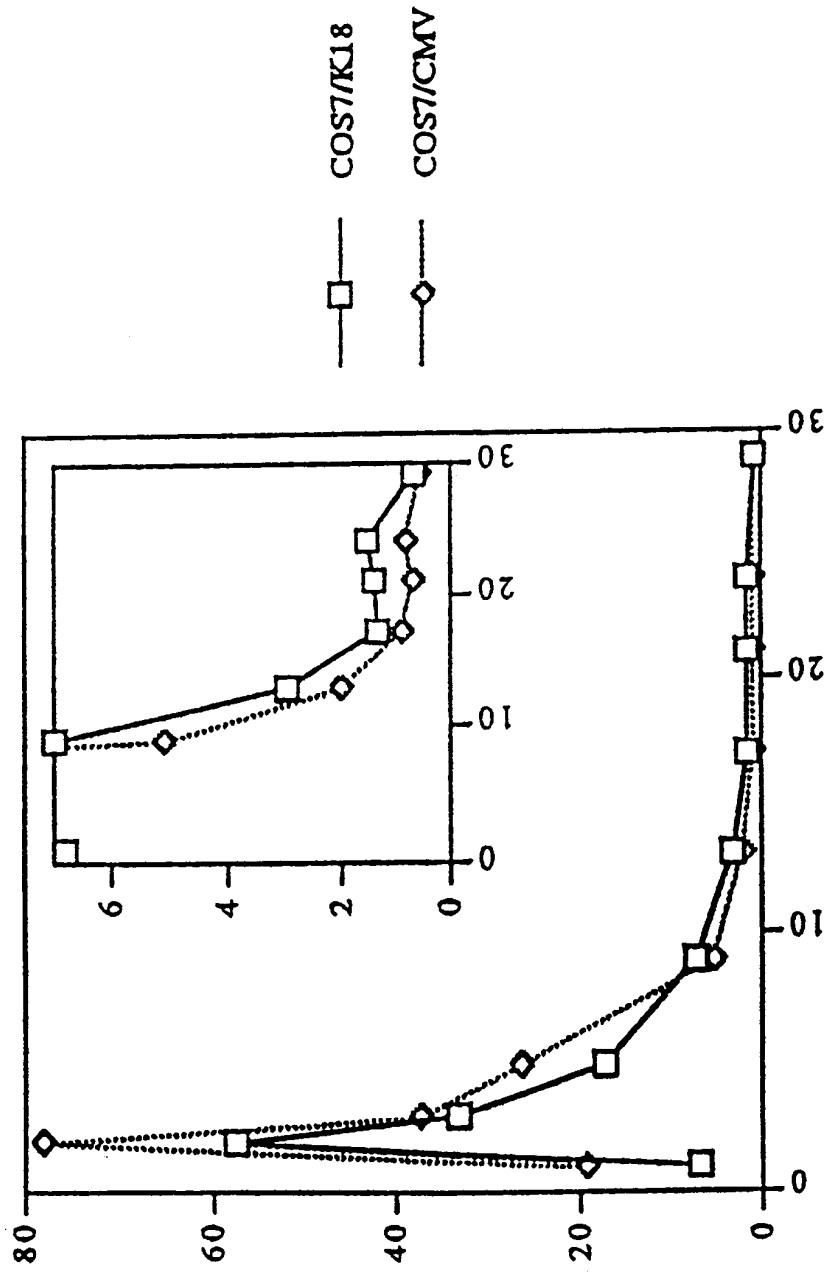

COS7/K18
COS7/CMV
SEAP activity /0.1 ml culture medium
DAY
0
20
40
60
80
0
10
20
30
0
2
4
6
0
10
20
30

FIG. 6

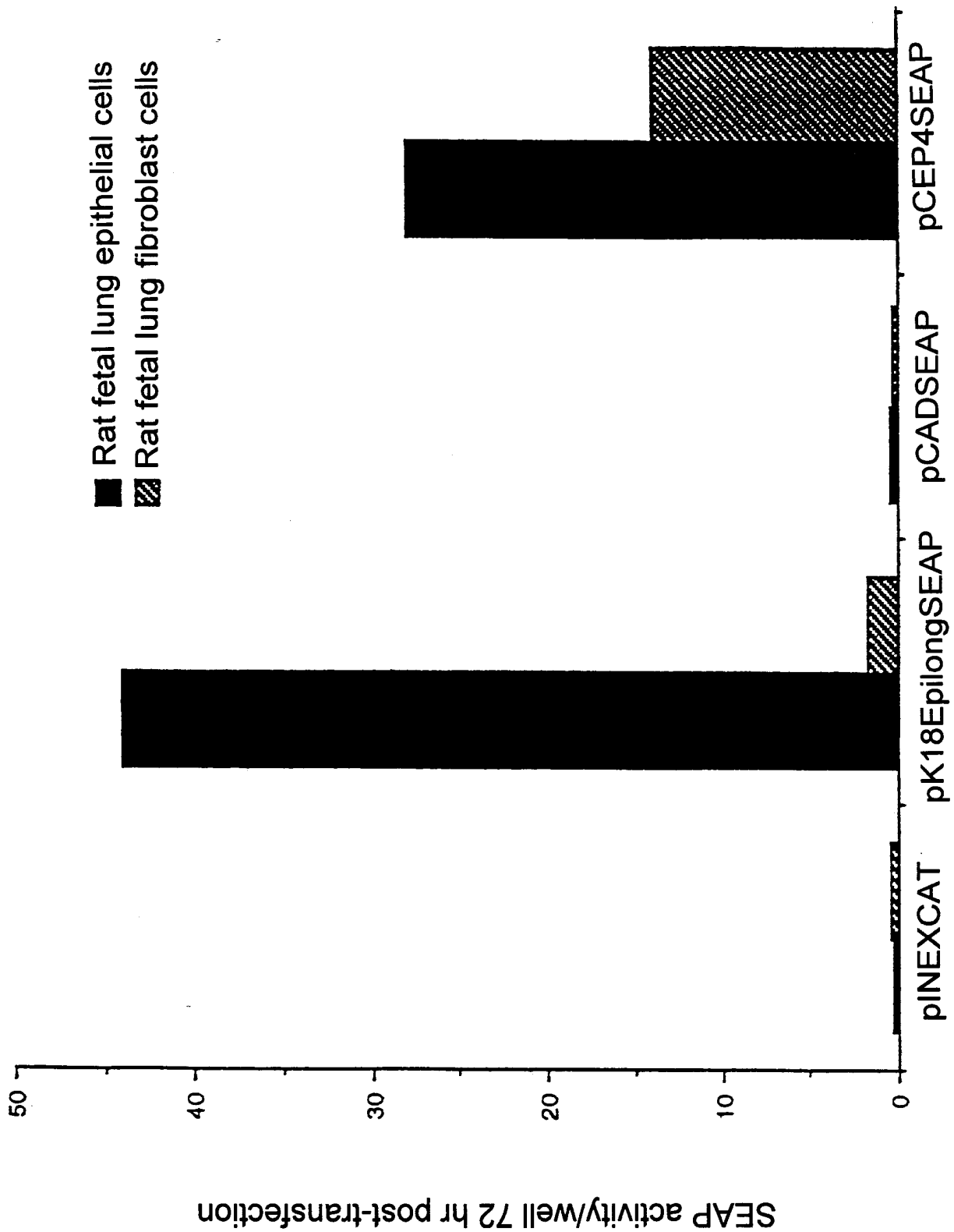
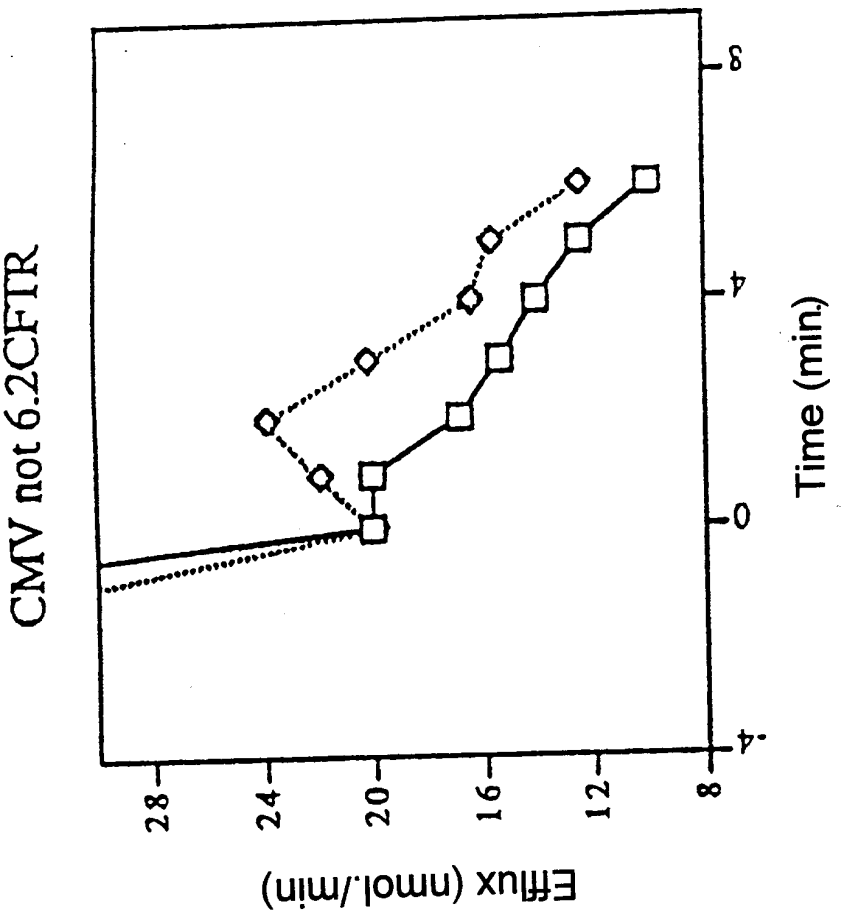

Rat fetal lung epithelial cells
Rat fetal lung fibroblast cells
SEAP activity/well 72 hr post-transfection
0
10
20
30
40
50
pINEXCAT
pK18EpilongSEAP
pCADSEAP
pCEP4SEAP

FIG. 7



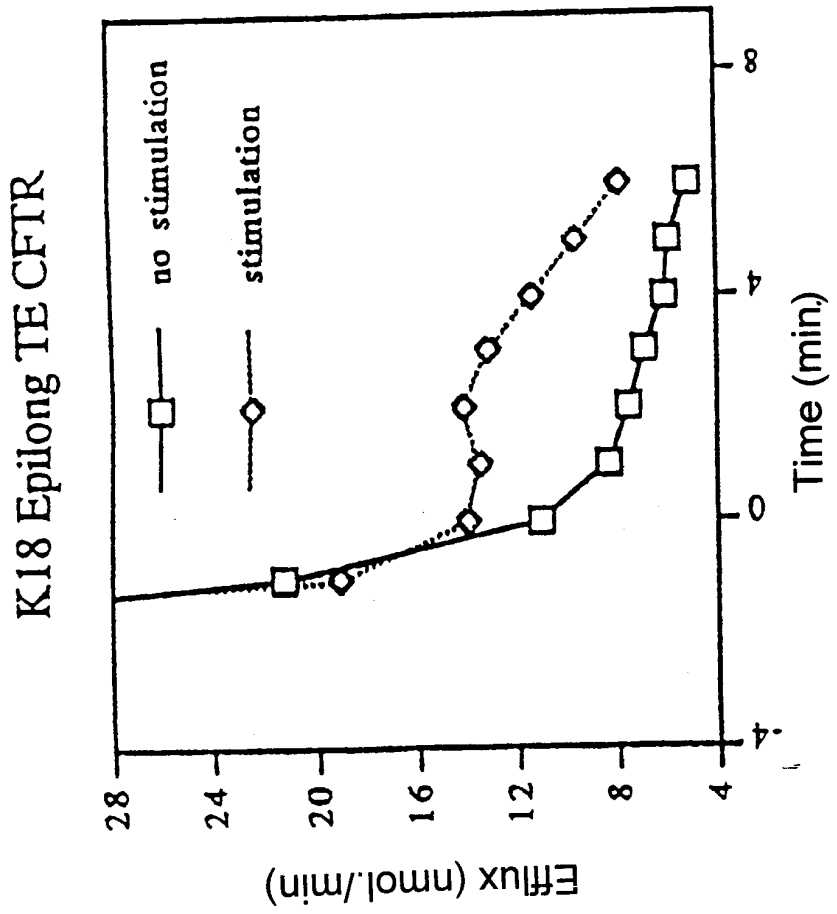


FIG. 8 (continued on next page)

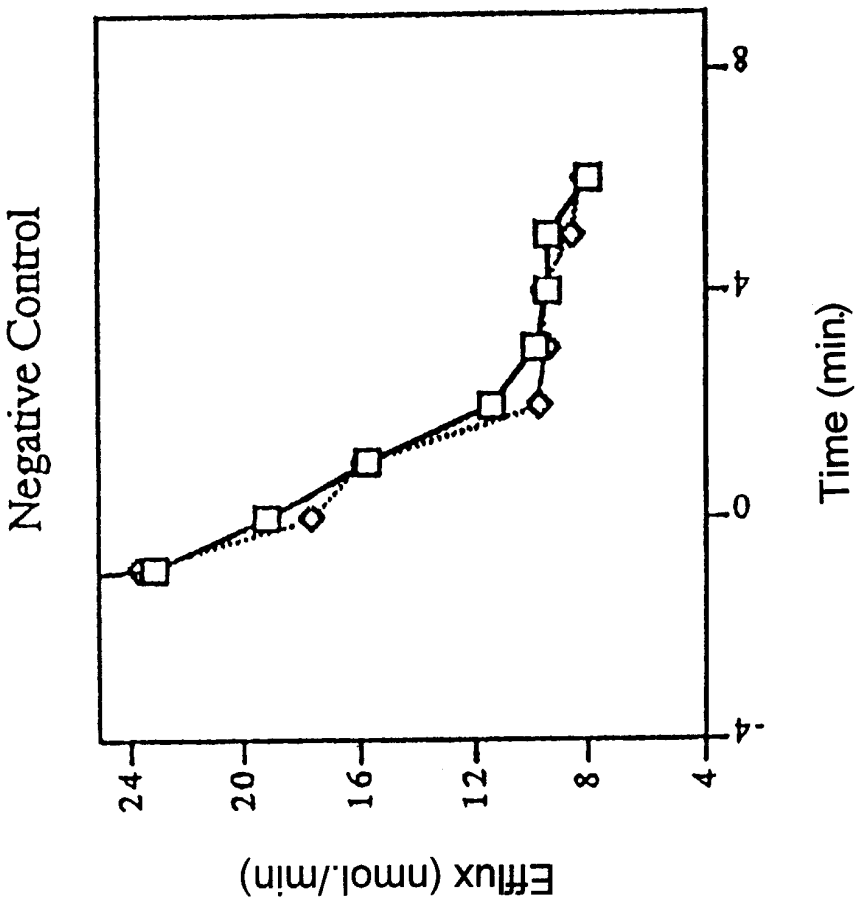


FIG. 8

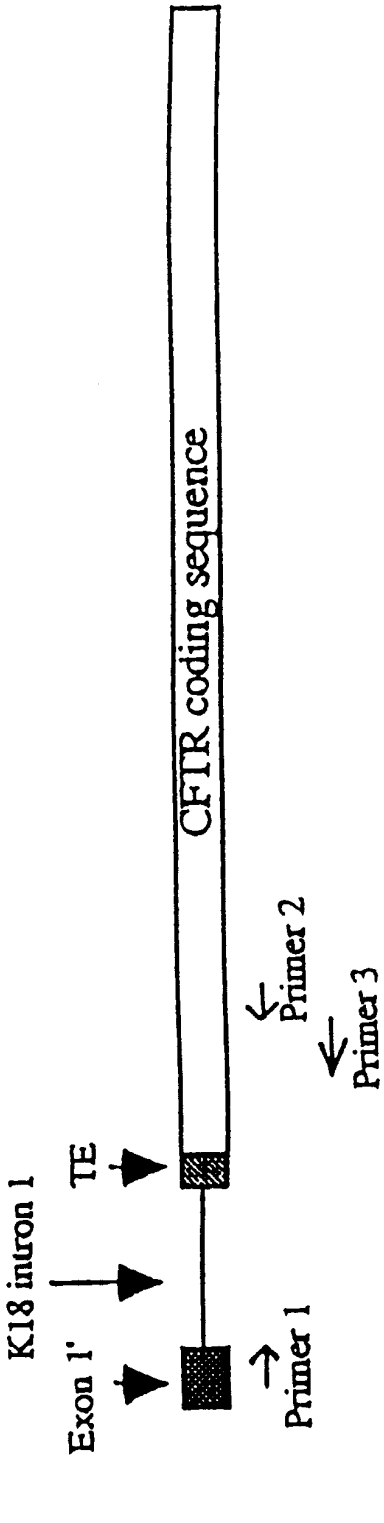

CFTR coding sequence
Primer 2
Primer 3
TE
K18 intron 1
Exon 1'
Primer 1

FIG. 9A

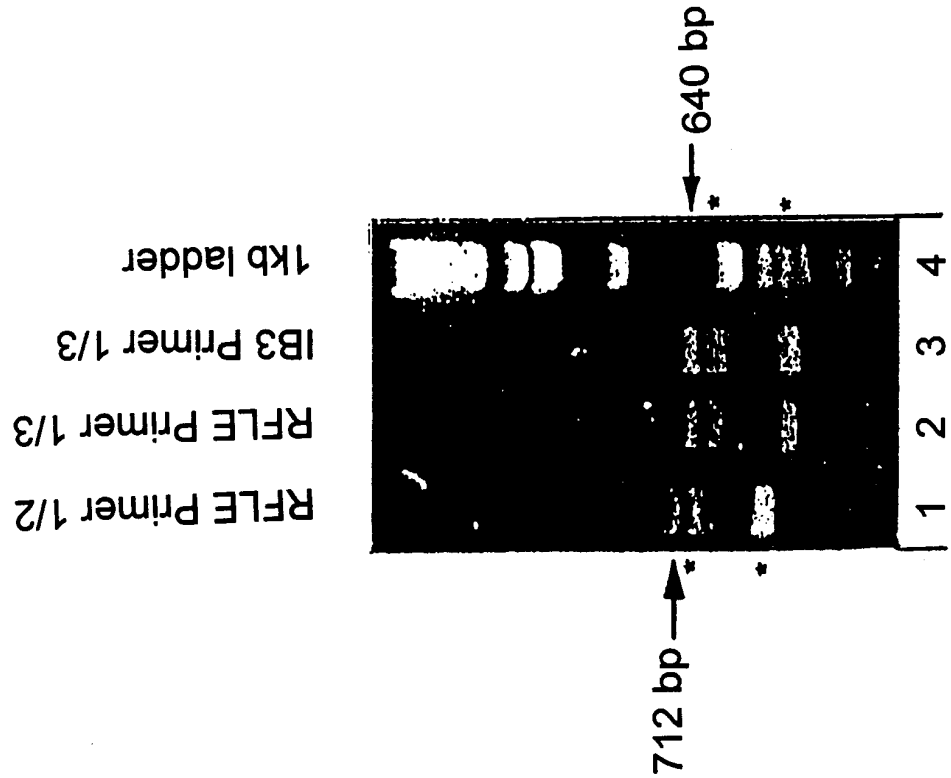

640 bp
712 bp
1
2
3
4
RFLE Primer 1/2
RFLE Primer 1/3
IB3 Primer 1/3
1kb ladder

FIG. 9B

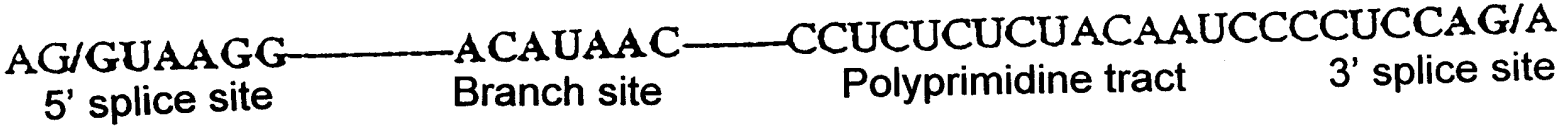
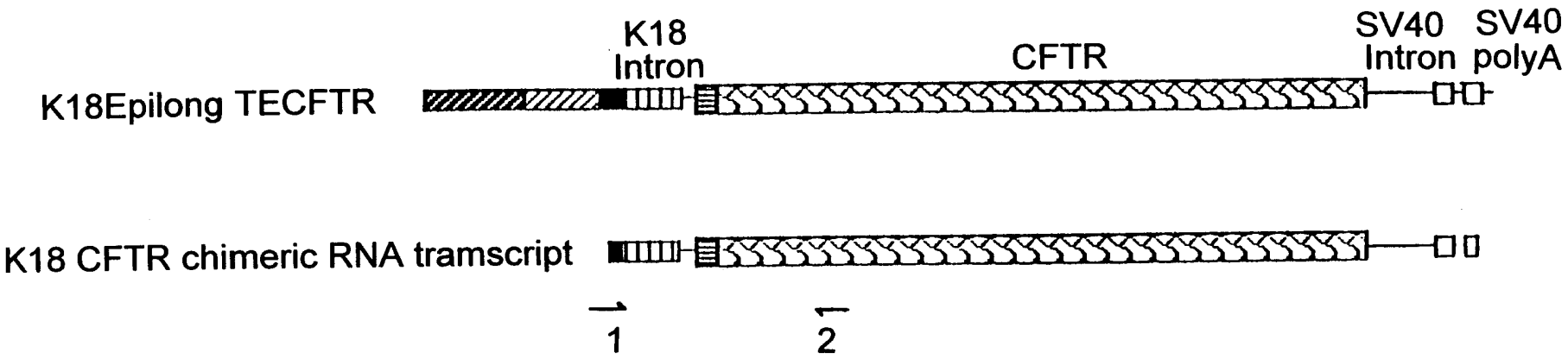


FIG. 9C



----CCTCTCTCTACAATCCCTC**CAG/A**----------TTTTTTT**CAG/C**----------TCTTTTTATATTT**AG/G**

K18 3' splice site CFTR 3' splice site-1 CFTR 3' splice site-2

----CCTCTCTCTTTTTTTTTTT**CAG/G**----------TTTTTTT**CAG/C**----------TCTTTTTATATTT**GGG**

* * * * * * * * *

FIG. 10

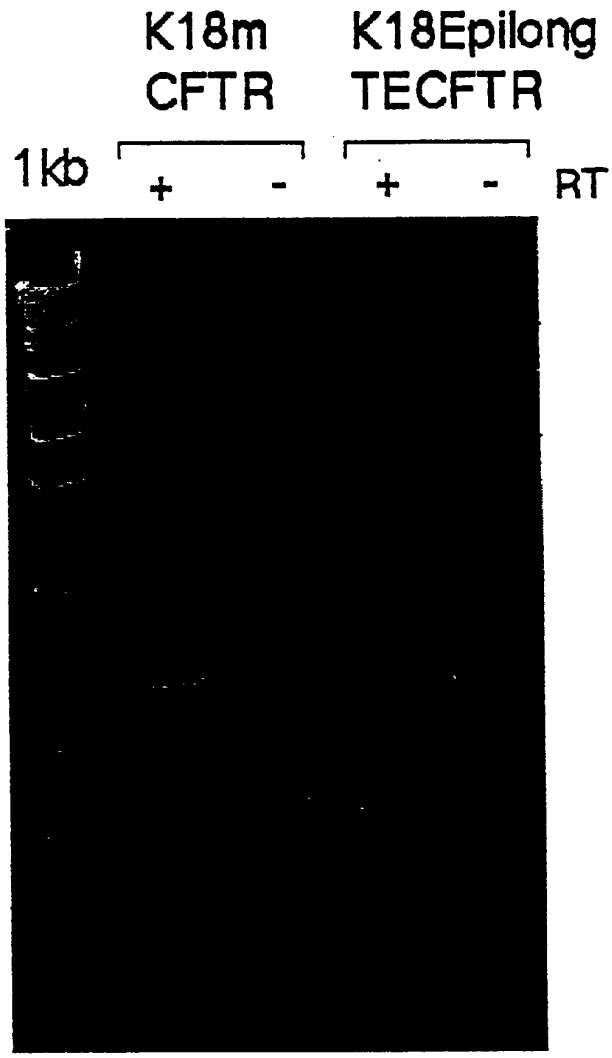
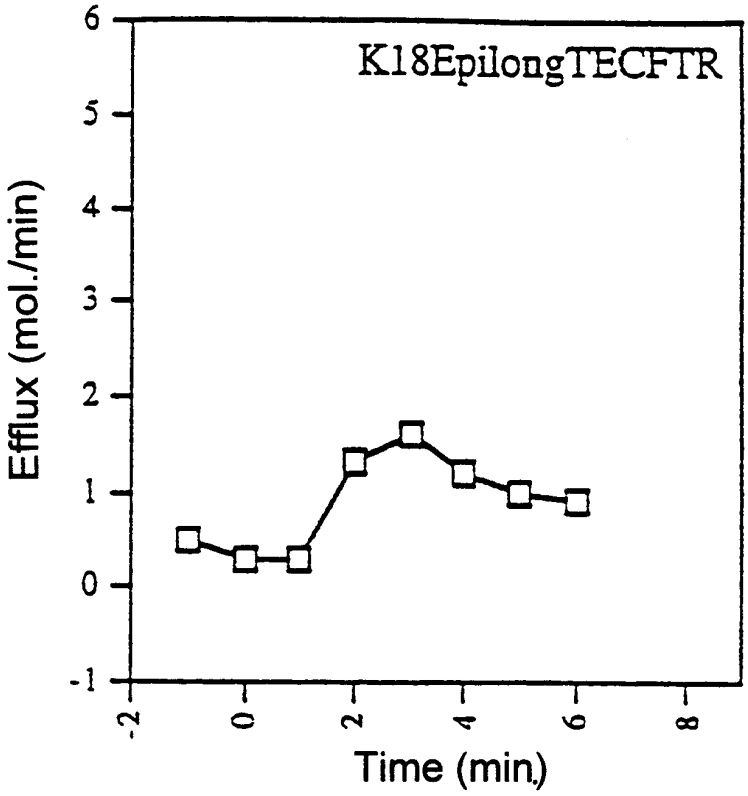


FIG. 11



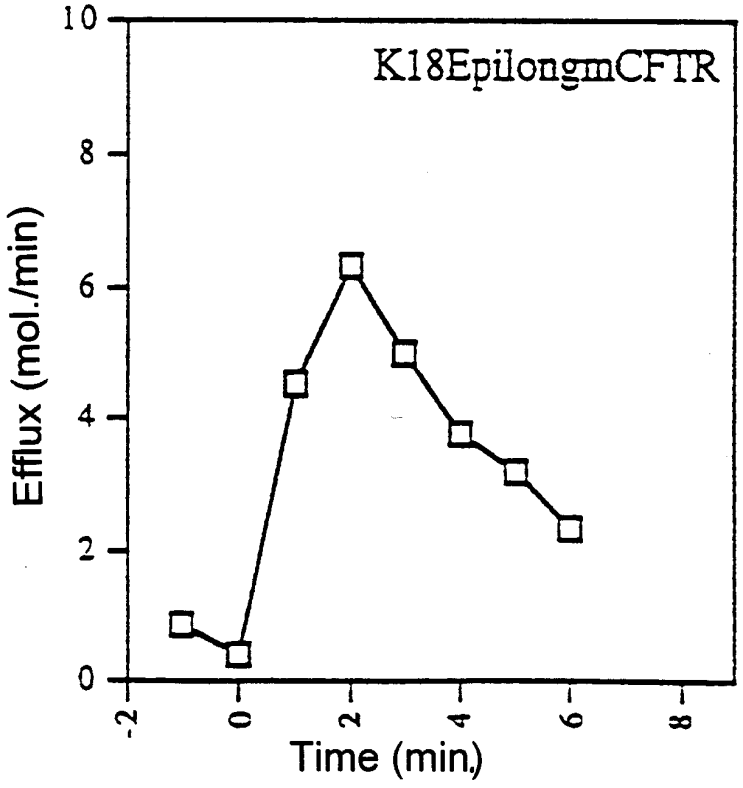
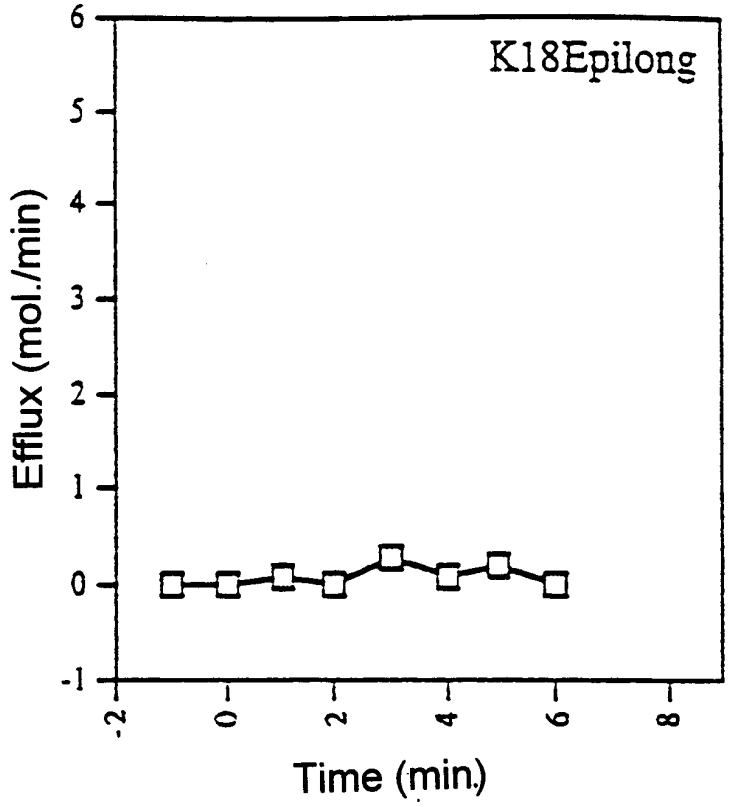


FIG. 12 (continued on next page)


K18Epilong
Efflux (mol./min)
6
5
4
3
2
1
0
-1
-2
0
2
4
6
8
Time (min.)

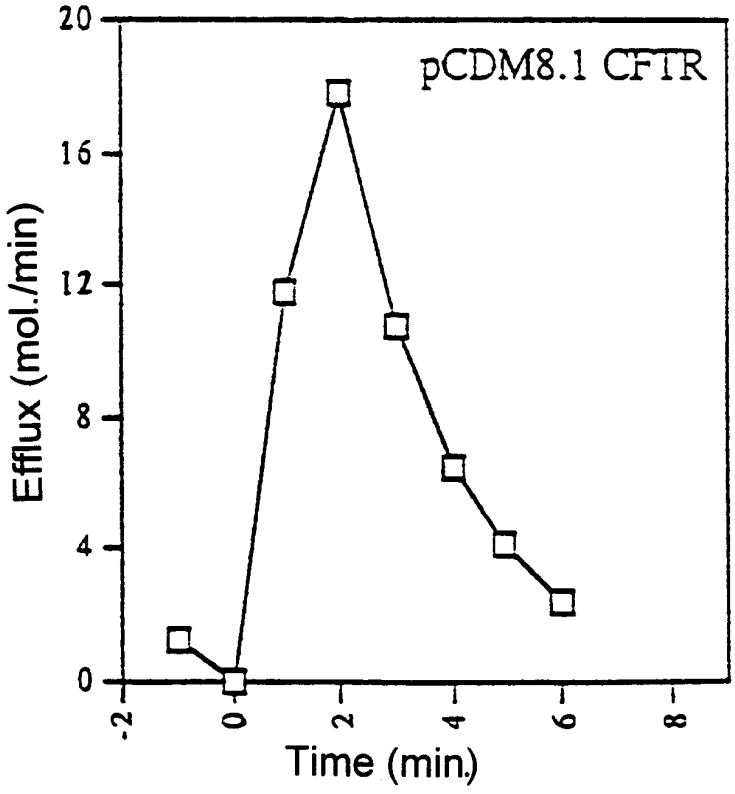

pCDM8.1 CFTR
Efflux (mol./min)
20
16
12
8
4
0
-2
0
2
4
6
8
Time (min.)

FIG. 12

2,205,076

UNSCANNABLE ITEM

RECEIVED WITH THIS APPLICATION

(ITEM ON THE 10TH FLOOR ZONE 5 IN THE FILE PREPARATION SECTION)

DOCUMENT REÇU AVEC CETTE DEMANDE

NE POUVANT ÊTRE BALAYÉ

(DOCUMENT AU 10 IÈME ÉTAGE AIRE 5 DANS LA SECTION DE LA

PRÉPARATION DES DOSSIERS)

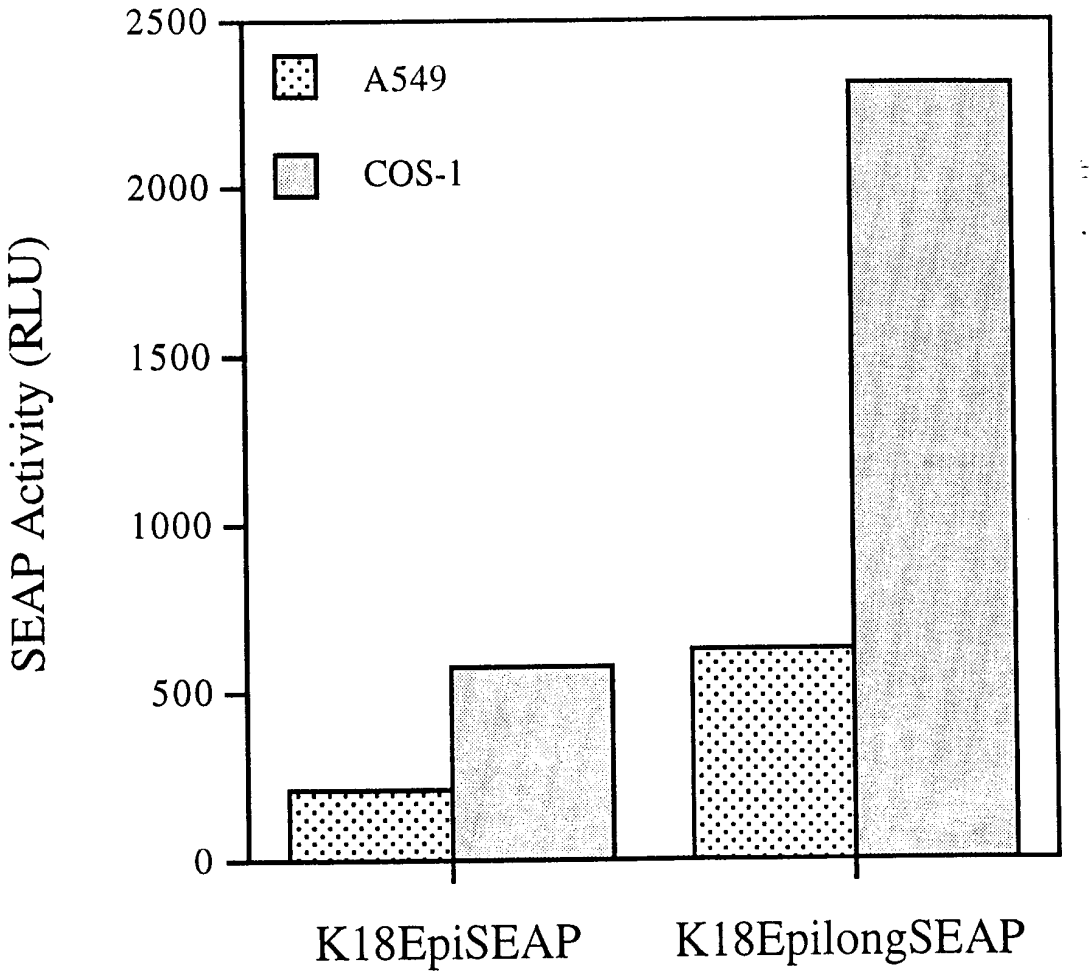

2500
2000
1500
1000
500
0
SEAP Activity (RLU)
A549
COS-1
K18EpiSEAP
K18EpilongSEAP

FIG. 15

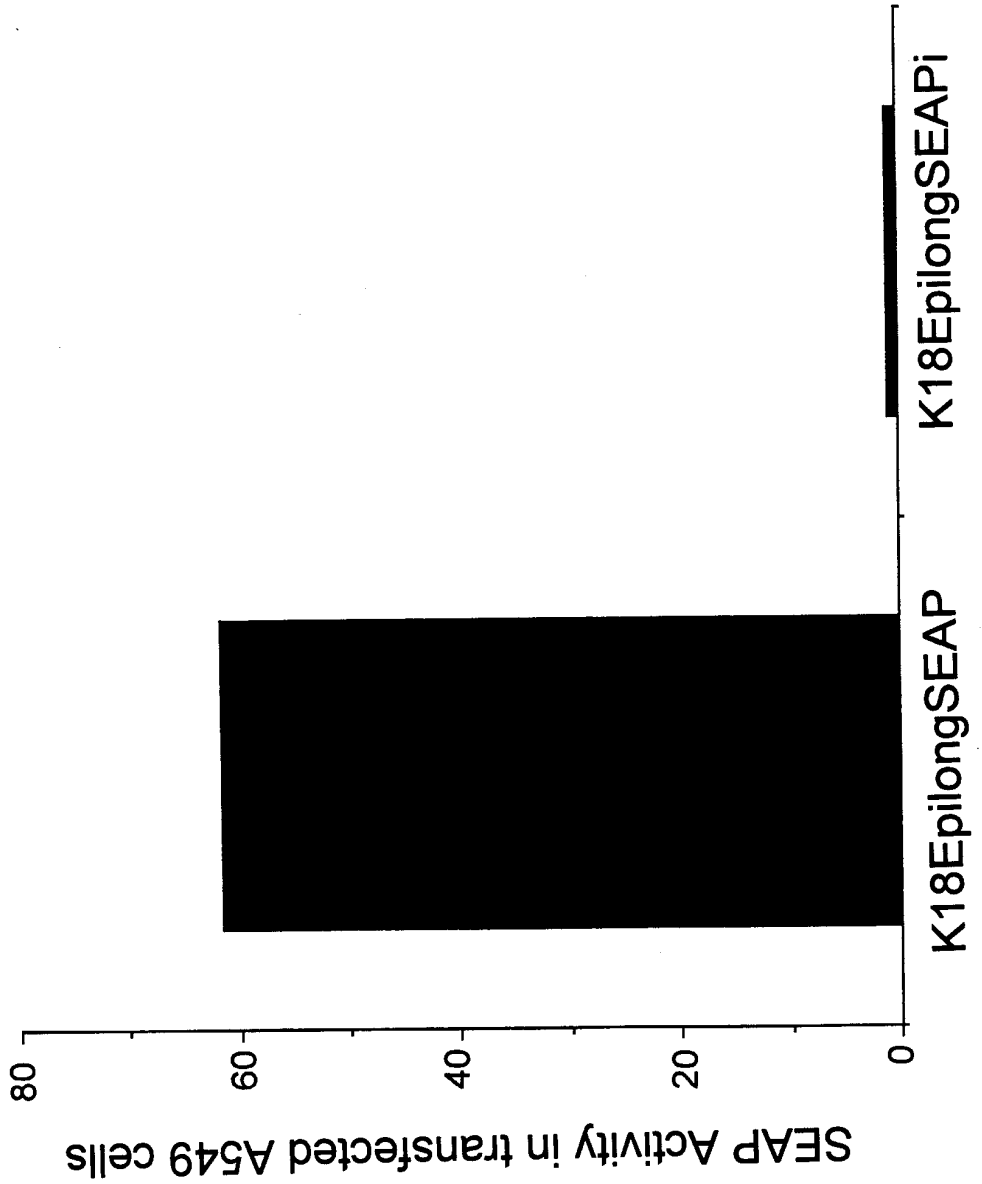

SEAP Activity in transfected A549 cells
0
20
40
60
80
K18EpilongSEAP
K18EpilongSEAPi

FIG. 16