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Ostedgaard et al.

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(54) **TRUNCATED CMV PROMOTERS AND VECTORS CONTAINING SAME**

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(65) **Prior Publication Data**

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Related U.S. Application Data

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(51) **Int. Cl.**

C12N 15/70 (2006.01)
C12N 15/74 (2006.01)
C12N 15/85 (2006.01)
C12N 15/86 (2006.01)
C12N 15/864 (2006.01)
A61K 48/00 (2006.01)

(52) **U.S. Cl.** **435/320.1**; 435/325; 435/456; 536/24.1; 424/93.2; 514/44

(58) **Field of Classification Search** .... None
See application file for complete search history.

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(57) **ABSTRACT**

The present invention relates to nucleic acid molecules comprising certain truncated forms of the human cytomegalovirus (CMV) immediate-early enhancer-promoter, either alone or operably linked to transgenes of interest, including those encoding partially-deleted CFTR proteins. This invention further relates to vectors comprising these nucleic acid molecules and host cells transformed by such vectors. The nucleic acid molecules, vectors and transformed host cells of the present invention are useful for treating a variety of genetic, metabolic and acquired diseases, including inter alia cystic fibrosis (CF) airway disease.

16 Claims, 13 Drawing Sheets

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Fig. 1

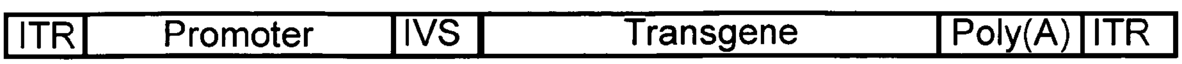

Fig. 2

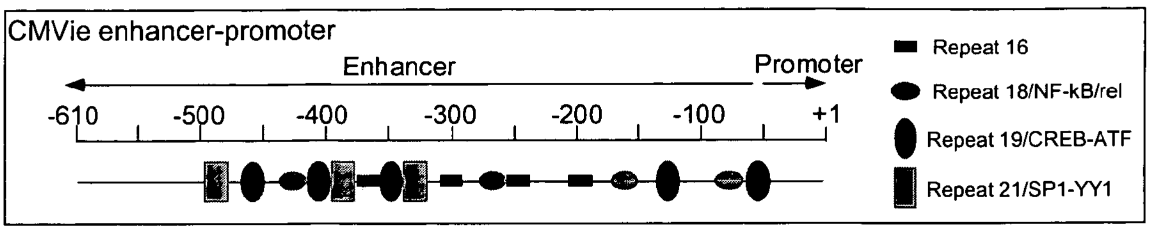
CMVie enhancer-promoter
Enhancer
Promoter
-610
-500
-400
-300
-200
-100
+1
Repeat 16
Repeat 18/NF-kB/rel
Repeat 19/CREB-ATF
Repeat 21/SP1-YY1

Fig. 3

(SEQ ID NO. 4)

CMV173-CFTRΔR

GCGGCCGCACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGT
TTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAATAACCCCGCCCCGTTGACG
CAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAA
CCGT**CAGAATTCTCGAGTGATCGAAAGAGCCTGCTAAAGCAAAAAAGAAGTCACC**ATGC
AGAGGTCGCCTCTGGAAAAGGCCAGCGTTGTCTCCAAACTTTTTTTCAGCTGGACCAGA
CCAATTTTGAGGAAAGGATACAGACAGCGCCTGGAATTGTCAGACATATACCAAATCCC
TTCTGTTGATTCTGCTGACAATCTATCTGAAAAATTGGAAAGAGAATGGGATAGAGAGC
TGGCTTCAAAGAAAAATCCTAAACTCATTAATGCCCTTCGGCGATGTTTTTTCTGGAGA
TTTATGTTCTATGGAATCTTTTTATATTTAGGGGAAGTCACCAAAGCAGTACAGCCTCT
CTTACTGGGAAGAATCATAGCTTCCTATGACCCGGATAACAAGGAGGAACGCTCTATCG
CGATTTATCTAGGCATAGGCTTATGCCTTCTCTTTATTGTGAGGACACTGCTCCTACAC
CCAGCCATTTTTGGCCTTCATCACATTGGAATGCAGATGAGAATAGCTATGTTTAGTTT
GATTTATAAGAAGACTTTAAAGCTGTCAAGCCGTGTTCTAGATAAAATAAGTATTGGAC
AACTTGTTAGTCTCCTTTCCAACAACCTGAACAAATTTGATGAAGGACTTGCATTGGCA
CATTTCGTGTGGATCGCTCCTTTGCAAGTGGCACTCCTCATGGGGCTAATCTGGGAGTT
GTTACAGGCGTCTGCCTTCTGTGGACTTGGTTTCCTGATAGTCCTTGCCCTTTTTCAGG
CTGGGCTAGGGAGAATGATGATGAAGTACAGAGATCAGAGAGCTGGGAAGATCAGTGAA
AGACTTGTGATTACCTCAGAAATGATTGAAAATATCCAATCTGTTAAGGCATACTGCTG
GGAAGAAGCAATGGAAAAAATGATTGAAAACTTAAGACAAACAGAACTGAAACTGACTC
GGAAGGCAGCCTATGTGAGATACTTCAATAGCTCAGCCTTCTTCTTCTCAGGGTTCTTT
GTGGTGTTTTTATCTGTGCTTCCCTATGCACTAATCAAAGGAATCATCCTCCGGAAAAT
ATTCACCACCATCTCATTCTGCATTGTTCTGCGCATGGCGGTCACTCGGCAATTTCCCT
GGGCTGTACAAACATGGTATGACTCTCTTGGAGCAATAAACAAAATACAGGATTTCTTA
CAAAAGCAAGAATATAAGACATTGGAATATAACTTAACGACTACAGAAGTAGTGATGGA
GAATGTAACAGCCTTCTGGGAGGAGGGATTTGGGGAATTATTTGAGAAAGCAAAACAAA
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GGCGGTTGCTGGATCCACTGGAGCAGGCAAGACTTCACTTCTAATGATGATTATGGGAG
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TTTTCCTGGATTATGCCTGGCACCATTAAAGAAAATATCATCTTTGGTGTTTCCTATGA
TGAATATAGATACAGAAGCGTCATCAAAGCATGCCAACTAGAAGAGGACATCTCCAAGT
TTGCAGAGAAAGACAATATAGTTCTTGGAGAAGGTGGAATCACACTGAGTGGAGGTCAA
CGAGCAAGAATTTCTTTAGCAAGAGCAGTATACAAAGATGCTGATTTGTATTTATTAGA
CTCTCCTTTTGGATACCTAGATGTTTTAACAGAAAAAGAAATATTTGAAAGCTGTGTCT
GTAAACTGATGGCTAACAAAACTAGGATTTTGGTCACTTCTAAAATGGAACATTTAAAG
AAAGCTGACAAAATATTAATTTTGCATGAAGGTAGCAGCTATTTTTATGGGACATTTTC
AGAACTCCAAAATCTACAGCCAGACTTTAGCTCAAAACTCATGGGATGTGATTCTTTCG
ACCAATTTAGTGCAGAAAGAAGAAATTCAATCCTAACTGAGACCTTACACCGTTTCTCA
TTAGAAGGAGATGCTCCTGTCTCCTGGACAGAAACAAAAAAACAATCTTTTAAACAGAC

Fig. 3 (continued)

TGGAGAGTTTGGGGAAAAAAGGAAGAATTCTATTCTCAATCCAATCAACTCT***ACGC
TTCAGGCACGAAGGAGGCAGTCTGTCCTGAACCTGATGACACACTCAGTTAACCAAGGT
CAGAACATTCACCGAAAGACAACAGCATCCACACGAAAAGTGTCACTGGCCCCTCAGGC
AAACTTGACTGAACTGGATATATATTCAAGAAGGTTATCTCAAGAAACTGGCTTGGAAA
TAAGTGAAGAAATTAACGAAGAAGACTTAAAGGAGTGCTTTTTTGATGATATGGAGAGC
ATACCAGCAGTGACTACATGGAACACATACCTTCGATATATTACTGTCCACAAGAGCTT
AATTTTTGTGCTAATTTGGTGCTTAGTAATTTTTCTGGCAGAGGTGGCTGCTTCTTTGG
TTGTGCTGTGGCTCCTTGGAAACACTCCTCTTCAAGACAAAGGGAATAGTACTCATAGT
AGAAATAACAGCTATGCAGTGATTATCACCAGCACCAGTTCGTATTATGTGTTTTACAT
TTACGTGGGAGTAGCCGACACTTTGCTTGCTATGGGATTCTTCAGAGGTCTACCACTGG
TGCATACTCTAATCACAGTGTCGAAAATTTTACACCACAAAATGTTACATTCTGTTCTT
CAAGCACCTATGTCAACCCTCAACACGTTGAAAGCAGGTGGGATTCTTAATAGATTCTC
CAAAGATATAGCAATTTTGGATGACCTTCTGCCTCTTACCATATTTGACTTCATCCAGT
TGTTATTAATTGTGATTGGAGCTATAGCAGTTGTCGCAGTTTTACAACCCTACATCTTT
GTTGCAACAGTGCCAGTGATAGTGGCTTTTATTATGTTGAGAGCATATTTCCTCCAAAC
CTCACAGCAACTCAAACAACTGGAATCTGAAGGCAGGAGTCCAATTTTCACTCATCTTG
TTACAAGCTTAAAAGGACTATGGACACTTCGTGCCTTCGGACGGCAGCCTTACTTTGAA
ACTCTGTTCCACAAAGCTCTGAATTTACATACTGCCAACTGGTTCTTGTACCTGTCAAC
ACTGCGCTGGTTCCAAATGAGAATAGAAATGATTTTTGTCATCTTCTTCATTGCTGTTA
CCTTCATTTCCATTTTAACAACAGGAGAAGGAGAAGGAAGAGTTGGTATTATCCTGACT
TTAGCCATGAATATCATGAGTACATTGCAGTGGGCTGTAAACTCCAGCATAGATGTGGA
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AACCTACCAAGTCAACCAAACCATACAAGAATGGCCAACTCTCGAAAGTTATGATTATT
GAGAATTCACACGTGAAGAAAGATGACATCTGGCCCTCAGGGGGCCAAATGACTGTCAA
AGATCTCACAGCAAAATACACAGAAGGTGGAAATGCCATATTAGAGAACATTTCCTTCT
CAATAAGTCCTGGCCAGAGGGTGGGCCTCTTGGGAAGAACTGGATCAGGGAAGAGTACT
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GTCTTGGGATTCAATAACTTTGCAACAGTGGAGGAAAGCCTTTGGAGTGATACCACAGA
AAGTATTTATTTTTTCTGGAACATTTAGAAAAAACTTGGATCCCTATGAACAGTGGAGT
GATCAAGAAATATGGAAAGTTGCAGATGAGGTTGGGCTCAGATCTGTGATAGAACAGTT
TCCTGGGAAGCTTGACTTTGTCCTTGTGGATGGGGGCTGTGTCCTAAGCCATGGCCACA
AGCAGTTGATGTGCTTGGCTAGATCTGTTCTCAGTAAGGCGAAGATCTTGCTGCTTGAT
GAACCCAGTGCTCATTTGGATCCAGTAACATACCAAATAATTAGAAGAACTCTAAAACA
AGCATTTGCTGATTGCACAGTAATTCTCTGTGAACACAGGATAGAAGCAATGCTGGAAT
GCCAACAATTTTTGGTCATAGAAGAGAACAAAGTGCGGCAGTACGATTCCATCCAGAAA
CTGCTGAACGAGAGGAGCCTCTTCCGGCAAGCCATCAGCCCCTCCGACAGGGTGAAGCT
CTTTCCCCACCGGAACTCAAGCAAGTGCAAGTCTAAGCCCCAGATTGCTGCTCTGAAAG
AGGAGACAGAAGAAGAGGTGCAAGATACAAGGCTTTAG**AATAAAACATCTTTATTTTCA**
TTACATCTGTGTGTTGGTTTTTTGTGTG$\boxed{\text{GCGGCCGC}}$

Fig. 4

(SEQ ID NO. 5)

113CMV-CFTRΔR

GCGGCCGCAAAATCAACGGGACTTTCCAAAATGTCGTAATAACCCCGCCCCGTTGACGC
AAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGCTCGTTTAGTGAAC
CGT**CAGAATTCTCGAGTGATCGAAAGAGCCTGCTAAAGCAAAAAAGAAGTCACC**ATGCA
GAGGTCGCCTCTGGAAAAGGCCAGCGTTGTCTCCAAACTTTTTTTCAGCTGGACCAGAC
CAATTTTGAGGAAAGGATACAGACAGCGCCTGGAATTGTCAGACATATACCAAATCCCT
TCTGTTGATTCTGCTGACAATCTATCTGAAAAATTGGAAAGAGAATGGGATAGAGAGCT
GGCTTCAAAGAAAAATCCTAAACTCATTAATGCCCTTCGGCGATGTTTTTTCTGGAGAT
TTATGTTCTATGGAATCTTTTTATATTTAGGGGAAGTCACCAAAGCAGTACAGCCTCTC
TTACTGGGAAGAATCATAGCTTCCTATGACCCGGATAACAAGGAGGAACGCTCTATCGC
GATTTATCTAGGCATAGGCTTATGCCTTCTCTTTATTGTGAGGACACTGCTCCTACACC
CAGCCATTTTTGGCCTTCATCACATTGGAATGCAGATGAGAATAGCTATGTTTAGTTTG
ATTTATAAGAAGACTTTAAAGCTGTCAAGCCGTGTTCTAGATAAAATAAGTATTGGACA
ACTTGTTAGTCTCCTTTCCAACAACCTGAACAAATTTGATGAAGGACTTGCATTGGCAC
ATTTCGTGTGGATCGCTCCTTTGCAAGTGGCACTCCTCATGGGGCTAATCTGGGAGTTG
TTACAGGCGTCTGCCTTCTGTGGACTTGGTTTCCTGATAGTCCTTGCCCTTTTTCAGGC
TGGGCTAGGGAGAATGATGATGAAGTACAGAGATCAGAGAGCTGGGAAGATCAGTGAAA
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GAAGAAGCAATGGAAAAAATGATTGAAAACTTAAGACAAACAGAACTGAAACTGACTCG
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AAAAGCAAGAATATAAGACATTGGAATATAACTTAACGACTACAGAAGTAGTGATGGAG
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GAGCAAGAATTTCTTTAGCAAGAGCAGTATACAAAGATGCTGATTTGTATTTATTAGAC
TCTCCTTTTGGATACCTAGATGTTTTAACAGAAAAAGAAATATTTGAAAGCTGTGTCTG
TAAACTGATGGCTAACAAAACTAGGATTTTGGTCACTTCTAAAATGGAACATTTAAAGA
AAGCTGACAAAATATTAATTTTGCATGAAGGTAGCAGCTATTTTTATGGGACATTTTCA
GAACTCCAAAATCTACAGCCAGACTTTAGCTCAAAACTCATGGGATGTGATTCTTTCGA
CCAATTTAGTGCAGAAAGAAGAAATTCAATCCTAACTGAGACCTTACACCGTTTCTCAT
TAGAAGGAGATGCTCCTGTCTCCTGGACAGAAACAAAAAAACAATCTTTTAAACAGACT
GGAGAGTTTGGGGAAAAAAGGAAGAATTCTATTCTCAATCCAATCAACTCT***ACGCT
TCAGGCACGAAGGAGGCAGTCTGTCCTGAACCTGATGACACACTCAGTTAACCAAGGTC
AGAACATTCACCGAAAGACAACAGCATCCACACGAAAAGTGTCACTGGCCCCTCAGGCA

Fig. 4 (continued)

AACTTGACTGAACTGGATATATATTCAAGAAGGTTATCTCAAGAAACTGGCTTGGAAAT
AAGTGAAGAAATTAACGAAGAAGACTTAAAGGAGTGCTTTTTTGATGATATGGAGAGCA
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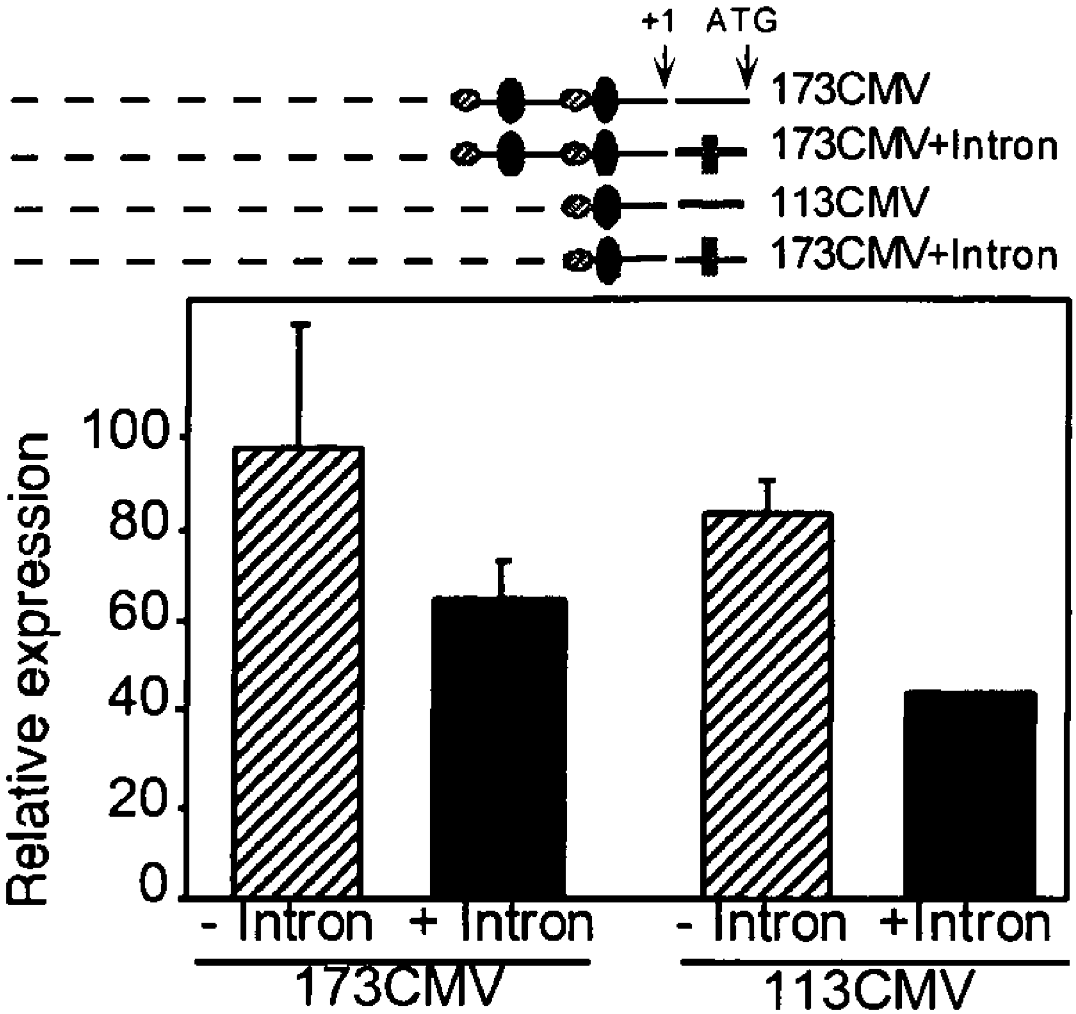
AACCCAGTGCTCATTTGGATCCAGTAACATACCAAATAATTAGAAGAACTCTAAAACAA
GCATTTGCTGATTGCACAGTAATTCTCTGTGAACACAGGATAGAAGCAATGCTGGAATG
CCAACAATTTTTGGTCATAGAAGAGAACAAAGTGCGGCAGTACGATTCCATCCAGAAAC
TGCTGAACGAGAGGAGCCTCTTCCGGCAAGCCATCAGCCCCTCCGACAGGGTGAAGCTC
TTTCCCCACCGGAACTCAAGCAAGTGCAAGTCTAAGCCCCAGATTGCTGCTCTGAAAGA
GGAGACAGAAGAAGAGGTGCAAGATACAAGGCTTTAG**AATAAAACATCTTTATTTTCAT**
TACATCTGTGTGTTGGTTTTTTGTGTG$\boxed{\text{GCGGCCGC}}$

Fig. 5



+1
ATG
173CMV
173CMV+Intron
113CMV
173CMV+Intron
Relative expression
100
80
60
40
20
0
- Intron
+ Intron
- Intron
+Intron
173CMV
113CMV

Fig. 6

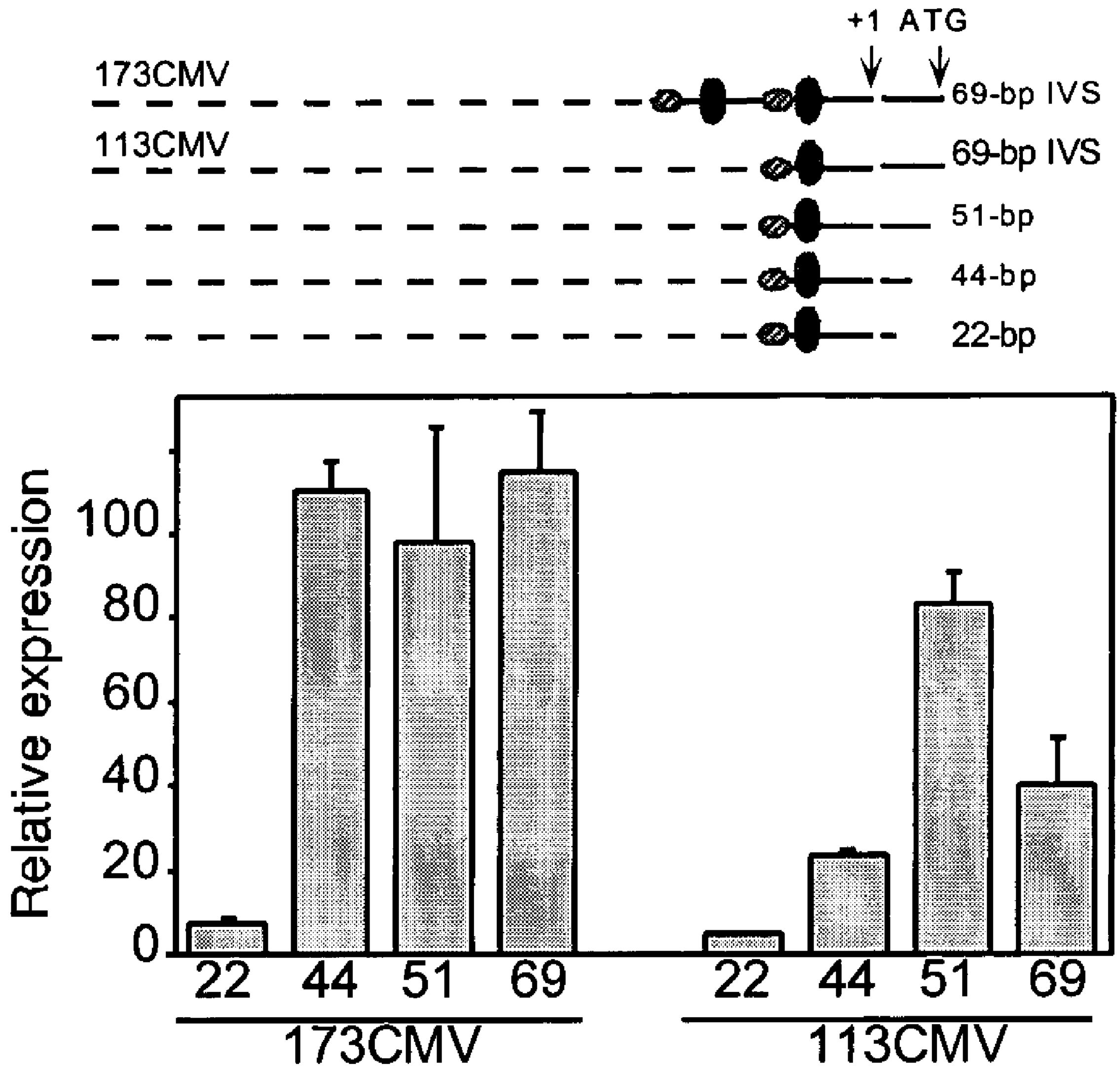

+1 ATG
173CMV
69-bp IVS
113CMV
69-bp IVS
51-bp
44-bp
22-bp
Relative expression
100
80
60
40
20
0
22
44
51
69
173CMV
22
44
51
69
113CMV

Fig. 7

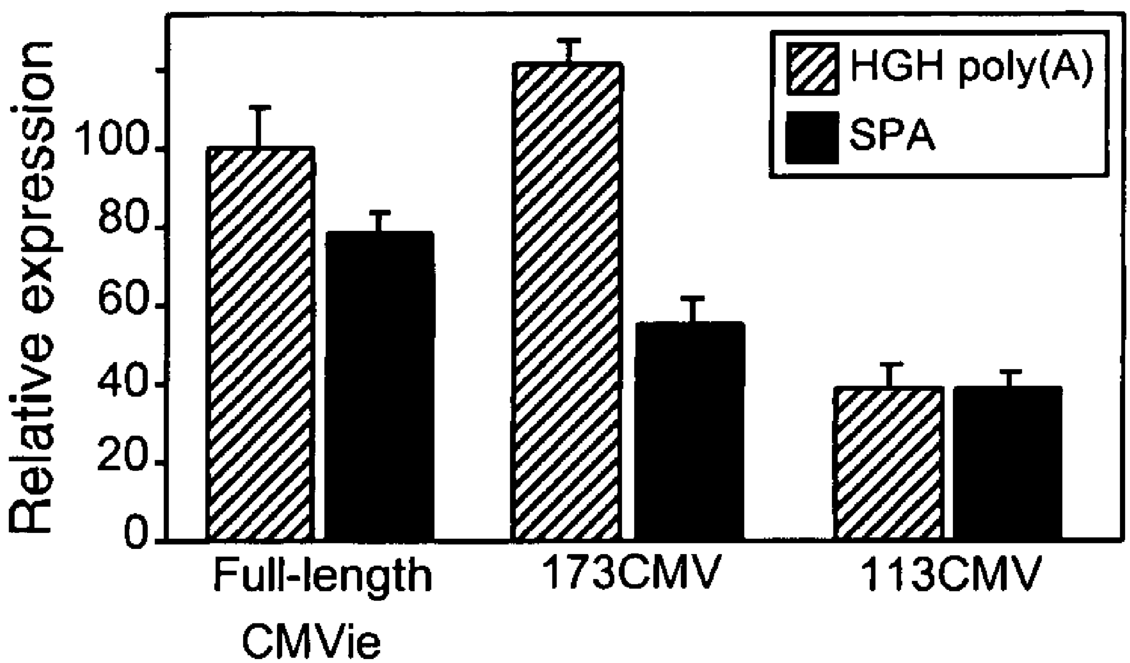

HGH poly(A)
SPA
Relative expression
0
20
40
60
80
100
Full-length CMVie
173CMV
113CMV

Fig. 8

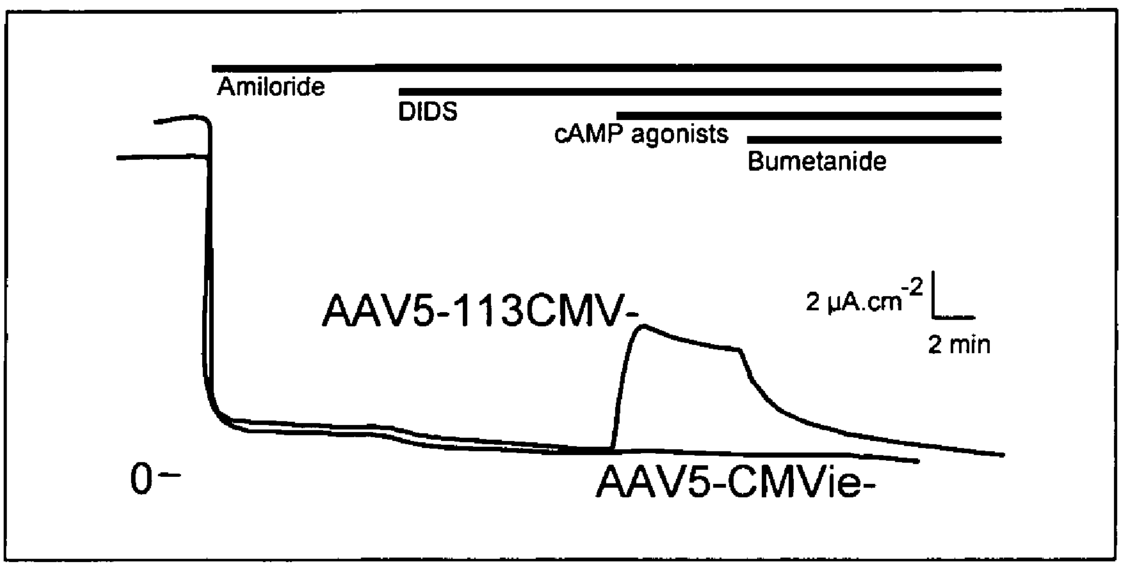
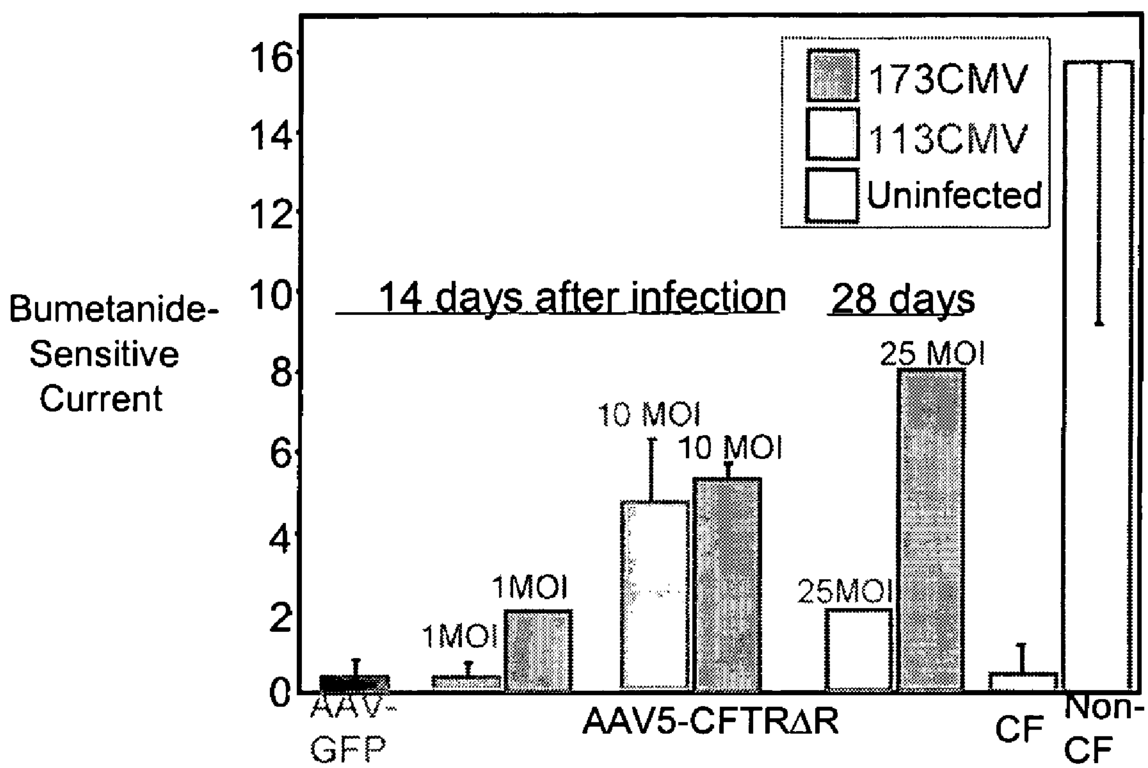

Amiloride
DIDS
cAMP agonists
Bumetanide
AAV5-113CMV-
2 μA.cm-2
2 min
0-
AAV5-CMVie-

Fig. 9



173CMV
113CMV
Uninfected
Bumetanide-Sensitive Current
14 days after infection
28 days
1MOI
1MOI
10 MOI
10 MOI
25MOI
25 MOI
0
2
4
6
8
10
12
14
16
AAV-GFP
AAV5-CFTRΔR
CF
Non-CF

Fig. 10A

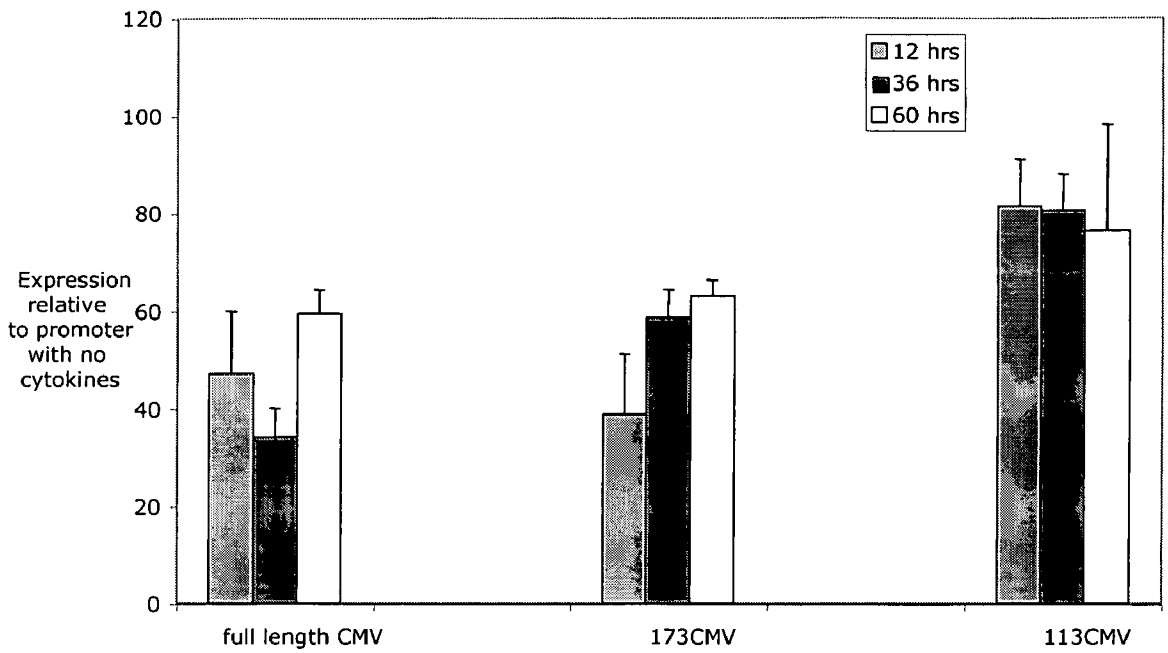
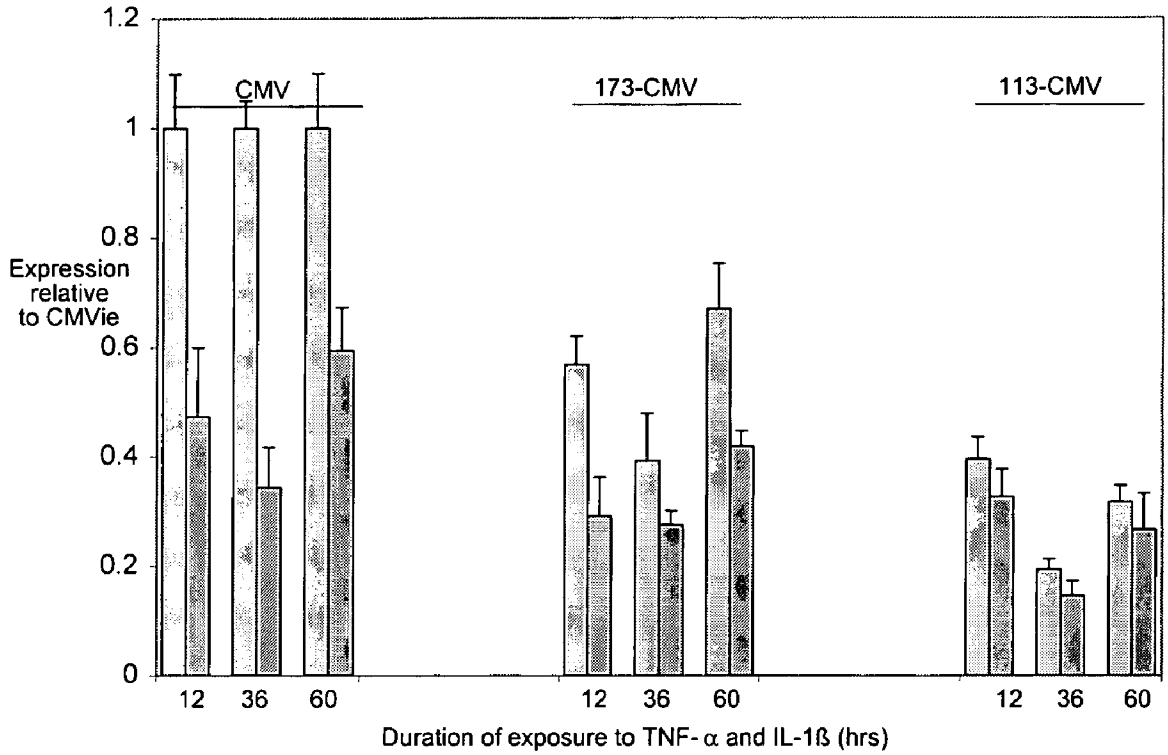

120
100
80
60
40
20
0
Expression relative to promoter with no cytokines
12 hrs
36 hrs
60 hrs
full length CMV
173CMV
113CMV

Fig. 10B


1.2
1
0.8
0.6
0.4
0.2
0
Expression relative to CMVie
CMV
173-CMV
113-CMV
12
36
60
12
36
60
12
36
60
Duration of exposure to TNF-α and IL-1ß (hrs)

TRUNCATED CMV PROMOTERS AND VECTORS CONTAINING SAME

RELATED APPLICATIONS

The present application claims the benefit of U.S. Application No. 60/527,146 filed Dec. 5, 2003 which is incorporated herein by reference in its entirety. This disclosure contains information related to research performed with government support under grant HL61234 awarded by the National Institutes of Health. The government may have certain rights herein.

FIELD OF THE INVENTION

This invention relates to nucleic acid molecules comprising certain truncated forms of the human cytomegalovirus (CMV) immediate-early promoter, either alone or operably linked to transgenes of interest, including those encoding partially-deleted CFTR proteins. This invention further relates to vectors comprising these nucleic acid molecules and host cells transformed by these vectors. The nucleic acid molecules, vectors and transformed host cells of the present invention are useful for the treatment of a variety of genetic, metabolic and acquired diseases, including inter alia cystic fibrosis (CF) airway disease.

BACKGROUND OF THE INVENTION

Airway disease is the major cause of morbidity and mortality in cystic fibrosis (CF), an autosomal recessive disease caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel. Welsh et al., *The Metabolic and Molecular Basis of Inherited Disease,* eds. Scriver CR, Beaudet AL, Sly WS, Valle D, Childs B, Vogelstein B, McGraw-Hill, New York, 1995. Gene transfer offers the potential for a new and effective treatment for CF airway disease. For reviews see Davies et al., 2001, *J. Gene Med.* 3:409-417; Flotte, 1999, *Curr. Opin Mol. Ther.* 1:510-516; and Welsh, 1999, *J. Clin. Invest.* 104:1165-1166. Previous studies have shown the feasibility of transferring the CFTR cDNA to CF airway epithelial cells in vitro and in vivo. However, with most vectors, two main problems limit gene transfer: gene transfer across the apical surface of differentiated airway epithelia is inefficient, and expression of the transferred gene is transient. See Davies et al., 2001, *J. Gene Med.* 3:409-417; Flotte, 1999, *Curr. Opin Mol. Ther.* 1:510-516; and Welsh, 1999, *J. Clin. Invest.* 104:1165-1166.

Adeno-associated virus (AAV) vectors offer several potential advantages as vectors for the transfer of the CFTR gene to CF airway epithelial cells. First, they have an excellent safety record in the lab and in humans. Second, they target both dividing and non-dividing cells like those in airway epithelia. Third, they do not induce a cell-mediated immune response. Fourth, they have been reported to generate long-term transgene expression. Fifth, unlike adenovirus and other human AAV serotypes, serotype 5 of human AAV (AAV5) targets the apical surface of differentiated airway epithelia. See Zabner et al., 2000, *J. Virol.* 74:3852-3858.

The utility of AAV vectors for CF gene transfer, however, is limited by their packaging capacity. The single-stranded genome of AAV5 is 4642 bp in length, which is similar to that of other AAV serotypes (Chiorini et al., 1999, *J. Virol.* 73:1309-1319), making it likely that AAV vectors will package only relatively small genomes. The only cis components required for replication and packaging of the recombinant genome into AAV5 virions are the two AAV5 ITRs, each 167 bp in length. Rabinowitz et al., 2000, *Virology* 278:301-308. The full-length CFTR cDNA is 4443 bp in length from the ATG through the stop codon. Thus, the length of the ITRs and a full-length CFTR cDNA (4777 bp) exceeds the length of the wild-type AAV5 genome. Moreover, an AAV expression cassette must also include a promoter, an intervening sequence (IVS) between the transcription and translation start sites, and a poly(A) addition sequence. See FIG. **1** for a schematic representation showing a typical arrangement of these elements in an AAV expression cassette.

Dong et al. studied DNA of various sizes and concluded that the optimal packaging limit for the AAV2 serotype was 4.9 kb; above this limit, packaging efficiency dropped precipitously. Dong et al., 1996, *Human Gene Therapy* 7:2102-2112. This observation is consistent with the previous findings that AAV vectors containing transgene inserts substantially longer than 4.9 kb have been difficult to produce.

In general, vectors that contain cDNA encoding truncated CFTR with short promoters or encoding full-length CFTR with no promoter other than the ITR were packaged with varying efficiency. See Flotte et al., 1993, *J. Biol. Chem.* 268:3791-3790; Zhang et al., 1998, *Proc. Natl. Acad. Sci. USA* 95:10158-10163; Wang et al., 1999, *Gene Therapy* 6:667-675. Recombinant vectors longer than 4900 bp were packaged less efficiently or not at all.

For example, Flotte et al. found that a 5010 bp DNA encoding CFTR was not packaged. However, they were able to package a 4647 bp vector that contained cDNA encoding CFTR with residues 1-118 deleted. In this vector, the ITRs were used as a promoter. Flotte et al., 1993, *J. Biol. Chem.* 268:3791-3790. Similarly, Zhang et al. reported that they could package a 4837 bp sequence containing a full-length CFTR cDNA with no promoter other than the ITRs, but the vector generated no CFTR Cl-current. They also reported that a 4727 bp cassette with a p5 promoter and a CFTR containing deletions in both the C-terminus and the R domain generated Cl^- current, as detected by the very sensitive patch-clamp technique in isolated cells. Zhang et al., 1998, *Proc. Natl. Acad. Sci. USA* 95:10158-10163. Wang et al. also produced a 4983 bp AAV vector with CFTR under control of the p5 promoter and reported detectable CFTR expression by whole-cell patch clamp in JME CF cells, but that genome was packaged much less efficiently than the 4837 bp genome of Zhang et al. Wang et al., 1999, *Gene Therapy* 6:667-675.

From these studies, it is clear that the relatively small packaging size limit of AAV vectors places severe constraints on the generation of AAV-based vectors for transfer of the CFTR cDNA. There are no reports of AAV-based vectors containing CFTR-encoding constructs longer than 5 kb. There are some reports of limited packaging into AAV virions for CFTR constructs of 4.9 and 5 kb in length. However, evidence that CFTR protein was expressed in cells transduced by these vectors relied on very sensitive patch-clamp detection techniques in single cells, and there was no evidence that expression was sufficient to generate trans-epithelial Cl^- current in an epithelium.

The longest components contained within the AAV expression cassette (FIG. **1**) are usually the promoter and the transgene. Thus, the two most likely ways in which the length of the expression cassette may be reduced would be to shorten the promoter or to shorten the transgene. The coding sequence of full length CFTR is 4450 bp. Riordan et al., 1989, *Science* 245:1066-1073. Addition of the two inverted terminal repeats of AAV (300 bp), and minimal 3' and 5' untranslated regions (~100 bp) yields an insert (4850 bp), which leaves little room for enhancer-promoter elements, most of which are >600 bp. However, several groups have shown that selective deletion of portions of the coding region of a gene can decrease the overall size of the gene while still allowing expression of an active protein molecule. This approach has been successfully employed to create a mini-dystrophin gene for use in gene therapy for Duchenne muscular dystrophy (DMD; Phelps et al., 1995, *Hum. Mol. Genet.* 4:1251-1258) and also for CFTR (Zhang et al., 1998, *Proc. Natl. Acad. Sci.* 95:10158-10163; and Flotte et al., 1993, *J. Biol. Chem.* 268: 3781-3790).

The R (regulatory) domain of the CFTR protein extends approximately from residues 634-708 at the N-terminus to approximately 835 at the C-terminus. See Ostedgaard et al., 2001, *J. Biol. Chem.* 276:7689-7692; Ostedgaard, et al., 2000, *Proc. Natl. Acad. Sci. U.S.A.* 97:5657-5662; and Csandy et al., 2000, *J. Gen. Physiol.* 116:477-500. Previous work has shown that a peptide encompassing residues 708-831 regulates activity, but in solution forms a predominantly random coil. Ostedgaard, et al., 2000, *Proc. Natl. Acad. Sci. U.S.A.* 97:5657-5662.

Several earlier studies showed that CFTR molecules in which portions of the R domain had been deleted still retained some CFTR function as a chloride ion channel. See Rich et al., 1991, *Science* 253:205-207; Rich et al., 1993, *Receptors Channels* 1:221-232; Ma et al., 1997, *J. Biol. Chem.* 272: 28133-28141; Zhang et al., 1998, *Proc. Natl. Acad. Sci. U.S.A.* 95:10158-10163; Vankeerberghen et al., 1999, *Biochemistry* 38:14988-14998; and Xie et al., 2000, *Biophys. J.* 78:1293-1305. However, at least some of these deletions induced channel activity in the absence of phosphorylation, reduced the response to PKA-dependent phosphorylation, and/or reduced net channel activity. See Rich et al., 1991, *Science* 253:205-207; Rich et al., 1993, *Receptors Channels* 1:221-232; Ma et al., 1997, *J. Biol. Chem.* 272:28133-28141; Zhang et al., 1998, *Proc. Natl. Acad. Sci. U.S.A.* 95:10158-10163; Vankeerberghen et al., 1999, *Biochemistry* 38:14988-14998; Xie et al., 2000, *Biophys. J.* 78:1293-1305; and Ostedgaard et al. 2001, *J. Biol. Chem.* 276:7689-7692. Moreover, previous studies have only examined CFTR expressed in heterologous cell lines and studied activity using the patch-clamp technique, planar lipid bilayers, or anion efflux. There was little information about their function in airway or other epithelia, which is critical in assessing the value of these proteins in gene transfer applications because deletions could alter protein-protein interactions, targeting to the apical membrane, constitutive and stimulated activity, phosphorylation-dependent regulation, and perhaps toxicity.

In contrast, Ostedgaard et al. have developed a shortened CFTR transgene (CFTR-ΔR) in which biosynthesis, localization, and Cl^- channel function of this CFTR-ΔR protein were demonstrated to be the same as wild-type CFTR in airway epithelia. Ostedgaard et al., 2002, *Proc. Natl. Acad. Sci. USA* 99:3093-3098. In these studies, however, an adenoviral vector, which has a much greater packaging capacity than AAV-based vectors, was employed to transfer the DNA sequence encoding the CFTR-ΔR into the airway epithelial cells. Incorporation of the same CFTR-ΔR expression cassette employed by Ostedgaard et al. into an AAV vector would still impose some packaging limitations. Thus, some truncation of the promoter sequence would still be necessary to achieve efficient rescue of this expression cassette in AAV vectors.

The cytomegalovirus immediate early (CMVie) enhancer-promoter is one of the most widely-employed promoters in gene transfer vectors. See Stinski, 1999, In *Gene Expression Systems: Using Nature for the Art of Expression.* Academic Press, New York. 1999. pp. 211-233. The CMVie enhancer-promoter directs expression in many different cell types, generates higher levels of expression than most other enhancer-promoters, and functions in many viral and non-viral vectors. For example, in the airway epithelial lines ELM and CFT1, the CMVie enhancer-promoter generated much greater expression of a reporter gene than promoters of a housekeeping gene (ubiquitin B), a cytokine gene (interleukin 8), a signaling ligand gene (nitric oxide synthase; NOS), the tissue-specific genes MUC1, CC10 and SPC, or another viral promoter (adenovirus E1a). Yew et al., 1997, *Human Gene Therapy* 8:575-584. Importantly, this relative expression pattern also was observed in vivo in mouse lung, and CMVie enhancer-promoter drives CFTR expression and corrects the CF Cl^- transport defect in cultured airway cell lines (JME/CF15) and primary cultures of differentiated airway epithelia. See Ostedgaard et al, 2002, *Proc. Natl. Acad. Sci. USA* 99:3093-3098; Jiang et al., 1996, *Am. J. Physiol.* 271:L527-L537. Forms of the CMVie enhancer truncated at nt −348 or nt −222 were observed to retain some activity. Stinski and Roehr, 1985, *J. Virol.* 55:431-441.

SUMMARY OF THE INVENTION

The present invention provides novel nucleic acid molecules for the regulation of gene transcription. These molecules are based on various functional truncated forms of the human cytomegalovirus immediate-early (CMVie) enhancer-promoter that promote expression of an operably-linked transgene.

In particular, the present invention provides functional truncated forms of the cytomegalovirus (CMV) immediate-early promoter that are useful in promoting the transcription of transgenes. Being relatively small in size, such promoters are especially useful in applications where the overall size of the expression cassette is a limiting factor, such as when AAV-based vectors are used to transfer genes with relatively long coding regions (i.e. those longer than approximately 3 to 4.5 kb). These truncated CMV promoters are therefore useful in the development of CFTR-expressing AAV vectors for use in gene therapy for CF.

In particular nonlimiting embodiments, the nucleic acid molecules of the present invention comprise functional variants of the human cytomegalovirus immediate-early enhancer-promoter region that are lacking the 16 and 21 base pair (bp) repeat units otherwise found in the wild-type human CMVie enhancer-promoter. The truncated CMVie enhancer-promoter elements of the present invention may contain one or more copies of the 18 and 19 bp repeat units present in the wild-type human CMVie enhancer-promoter. In particular nonlimiting embodiments, the nucleic acid molecules of the present invention comprise the nucleic acid sequence of SEQ ID NO:1 or SEQ ID NO:2. In specific nonlimiting embodiments, the nucleic acid molecules of the present invention are the nucleic acid sequence of SEQ ID NO:1 or SEQ ID NO:2.

In other nonlimiting embodiments, these truncated CMVie enhancer-promoter elements may be operably linked to a heterologous gene (i.e. a "transgene"), so that transcription of the transgene is regulated by the truncated CMVie enhancer-promoter elements of the present invention.

In particular nonlimiting embodiments, these truncated CMVie enhancer-promoter elements are operably linked to a transgene encoding a CFTR protein. In certain nonlimiting embodiments, the transgene encode CFTR proteins from which all or part of the R domain has been deleted. The CFTR-encoding transgenes operably linked to the truncated CMVie enhancer-promoter element may comprise, in addition to deletion of all or part of the R region, deletions in other regions provided that the proteins encoded by these transgenes exhibit functional chloride ion channel activity when expressed in a CF airway epithelia cell. In a particular non-limiting embodiment, the transgene encoding a CFTR protein from which all or part of the R domain has been deleted comprises the nucleic acid sequence of SEQ ID NO:3. In another specific nonlimiting embodiment, the transgene encoding a CFTR protein from which all or part of the R domain has been deleted consists essentially of the nucleic acid sequence of SEQ ID NO:3. In another specific nonlimiting embodiment, the transgene encoding a CFTR protein from which all or part of the R domain has been deleted is the nucleic acid sequence of SEQ ID NO:3.

Also provided are vectors comprising the nucleic acid molecules of the present invention. In particular nonlimiting embodiments, the vectors comprise nucleic acid molecules encoding functional variants of the human cytomegalovirus immediate-early enhancer-promoter region that lack the 16 and 21 bp repeat units, which are found in the wild-type human CMVie enhancer-promoter. In vectors of the present invention, the truncated CMVie enhancer-promoter elements may be operably linked to a transgene, including but not limited to a transgene encoding a CFTR protein from which all or part of the R domain has been deleted. In particular nonlimiting embodiments, the vectors of the present invention may comprise without limitation a cosmid, a phagemid, a bacteriophage, a bacterial artificial chromosome, a yeast artificial chromosome, a human artificial chromosome, a retrovirus vector, an adenovirus vector, an adeno-associated virus vector, a herpes virus vector, an Epstein-Barr virus vector, a vaccinia virus vector, or combinations or chimerics thereof. Those of ordinary skill in the art will recognize that the truncated CMVie enhancer-promoter elements of the present invention also will be useful in other vectors employed in the fields of gene expression and gene transfer, especially those in which the overall size of the transgene expression cassette is a limiting factor. In particular nonlimiting embodiments, the truncated CMVie enhancer-promoter elements of the present invention are incorporated into vectors derived from serotype 5 of human adeno-associated virus (AAV5).

The vectors of the present invention may further comprise various transgenes, intervening sequences, polyadenylation signals, and/or other elements known to those of ordinary skill in the art to express transgenes from certain vectors. In nonlimiting embodiments, the vectors of the present invention are derived from AAV5 that express a human CFTR protein or functional variants thereof. In other nonlimiting embodiments, the present invention provides vectors comprising a truncated CMVie enhancer-promoter elements, in accordance with the present invention, operably linked to other transgenes encoded by nucleic acid sequences greater than approximately 3 kb in length. In specific nonlimiting embodiments, the transgene is encoded by a nucleic acid sequence of approximately 3.1, 3.2, 3.3, 3.4, 3.5, 3.6, 3.7, 3.8, 3.9, 4.0, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, or 5.0 kb in length.

Also provided by the present invention are cells transformed by the vectors described herein. In particular nonlimiting embodiments, transformed host cells include, but are not limited to, cultured airway epithelial cells, such as the A549 or H441 cell lines, primary airway epithelial cell cultures derived from CF or wild-type donors, or airway epithelial cells from CF or non-CF individuals in vivo et situ.

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1**. Schematic representation of a typical arrangement of an AAV expression cassette. ITR represents the AAV inverted terminal repeat. IVS represents an intervening sequence located between the transcription and translation initiation sites. Poly(A) represents a polyadenylation signal sequence.

FIG. **2**. Schematic representation of the cytomegalovirus immediate-early (CMVie) enhancer-promoter. Relative locations of identified transcription factor binding sites are indicated by the various symbols. The transcription start site is indicated as "+1."

FIG. **3**. Nucleotide sequence of the 173CMV-CFTRΔR expression cassette. The NotI restriction endonuclease recognition sites are shown in the boxes. The CMV promoter region is shown in underlined type. The intervening sequence region is shown in bold, underlined type. The ATG translation initiation codon is shown in dashed underlining. The asterisks indicate the site of the R domain deletion. The TAG stop codon is shown in double underlining. The SPA sequence is shown in bold, double underlined type.

FIG. **4**. Nucleotide sequence of the 113CMV-CFTRΔR expression cassette. The NotI restriction endonuclease recognition sites are shown in the boxes. The CMV promoter region is shown in underlined type. The intervening sequence region is shown in bold, underlined type. The ATG translation initiation codon is shown in dashed underlining. The asterisks indicate the site of the R domain deletion. The TAG stop codon is shown in double underlining. The SPA sequence is shown in bold, double underlined type.

FIG. **5**. Effect of the presence or absence of an intron on expression in A549 cells of a β-galactosidase gene driven by a CMVie promoter truncated as indicated. Expression is presented as β-galactosidase activity of the indicated construct relative to that obtained from the full-length CMVie enhancer-promoter.

FIG. **6**. Effect of length of intervening sequence on expression in A549 cells of a β-galactosidase gene driven by a CMV promoter truncated as indicated followed by an intervening sequence (IVS) of 22, 44, 51, or 69 bp. Expression is presented as β-galactosidase activity of the indicated construct relative to that obtained from the full-length CMVie enhancer-promoter followed by a 51 bp IVS.

FIG. **7**. Effect of replacement of the human growth hormone poly(A) tail by a synthetic polyadenylation signal sequence on expression in A549 cells of a β-galactosidase gene driven by full-length and truncated CMVie enhancer-promoters. Expression is presented as β-galactosidase activity of the indicated construct relative to that obtained from the full-length CMVie enhancer-promoter with a full-length hGH poly(A) tail.

FIG. **8**. Short-circuit current (I_{SC}) tracings from differentiated CF epithelia treated two weeks after treatment by an AAV5 vector, at a multiplicity of infection (MOI) of 10, containing either a full-length CMVie promoter driving GFP or a shortened 113-bp CMV promoter driving CFTR-ΔR. During the times indicated by the bars, the following agents were present: 100 μM mucosal amiloride, 100 μM mucosal DIDS, 10 μM forskolin and 100 μM IBMX (cAMP agonists), and 100 μM submucosal bumetanide.

FIG. **9**. Short circuit current (I_{SC}) following addition of bumetanide to inhibit trans-epithelial Cl^- transport. AAV5 vectors were applied to apical surface of differentiated CF epithelia for 30 minutes and trans-epithelial current was measured 2 weeks later. Data on the right in open bars are means±SD of cAMP-stimulated bumetadine-sensitive current from untreated CF (n–=64) and non-CF (n=458) epithelia. The data show that both short CMV promoters direct expression of cAMP-regulated CFTR channels in airway epithelia 14 days and 28 days following infection.

FIG. **10**. (A) Expression relative to CMVie. FIG. **10**A shows beta-gal expression relative to that seen with a full length CMV promoter in the airway cell line A549 in the presence and in the absence of two cytokines, TNF-alpha (10 ng/ml) and IL-1beta (50 ng/ml) at 12, 36 and 60 hours after addition of cytokines. (B) Expression relative to promoter with no cytokines. FIG. **10**B shows beta-galactosidase expression in the presence of cytokines relative to each individual promoter without cytokine. The data indicate that cytokine downregulation of CMV-promoter-driven beta-galactosidase expression from either of the short CMV promoters is not greater than that observed using the full length CMV promoter.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based on the surprising finding that the human cytomegalovirus immediate-early (CMVie) enhancer-promoter element, which in the wild-type form contains a constellation of 16, 18, 19 and 21 bp repeat units as depicted schematically in FIG. **2**, may be truncated to remove completely the 16 and 21 bp repeat units without eliminating its activity as a transcriptional regulator. The relatively small sizes of the promoters created by the various truncations described herein make them well-suited for incorporation into transgene expression cassettes in which length is otherwise a limiting factor, for example when the transgene to be expressed is encoded by a gene or cDNA whose size is relatively large, i.e. greater than approximately 3 kb in length, and the vector to be used for delivery of the transgene has a relatively small packaging limit, e.g. adeno-associated viruses (AAV), which have a packaging limit of approximately 5 kb.

In particular nonlimiting embodiments, the truncated CMVie enhancer-promoter elements of the present invention have the following nucleic acid sequences:

```
                                       (SEQ ID NO: 1)
5'-ACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGT
TTGTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAATAACCC
CGCCCCGTTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATA
TAAGCAGAGCTCGTTTAGTGAACCGT-3',
or

                                       (SEQ ID NO: 2)
5'-AAAATCAACGGGACTTTCCAAAATGTCGTAATAACCCCGCCCCGTTG
ACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAGAGC
TCGTTTAGTGAACCGT-3',
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corresponding to the truncated CMVie enhancer-promoters CMV173 and CMV113, respectively. However, those of ordinary skill in the art will recognize that the CMVie enhancer-promoters of the present invention are not limited merely to those of SEQ ID NO:1 and SEQ ID NO:2. Indeed, one of ordinary skill in the art will recognize that the present invention encompasses any and all promoters derived from the wild-type human CMVie enhancer-promoter that lack the 16 and 21 bp repeat units while retaining one or more copies of the 18 and 19 bp repeat units present in the wild-type human CMVie enhancer-promoter. Thus, the CMVie enhancer-promoters of the present invention include nucleic acid sequences in which nucleotide substitutions, deletions or insertions have been made, provided that these substitutions, deletions or insertions do not alter the sequences of the one or more copies of the 18 and 19 bp repeat units retained in the truncated CMVie enhancer-promoter, and further provided that these substitutions, deletions or insertions do not diminish the activity of the resulting promoter elements to less than approximately 10% of the activity of the CMVie enhancer-promoters of SEQ ID NOS:1 or 2. A person of ordinary skill in the art would appreciate that the strength of a given promoter could be readily determined using any one of a number of standard reporter gene assays including, but not limited to, a β-galactosidase reporter gene assay such as that employed in Example 1 below.

In further embodiments, the truncated CMVie enhancer-promoter elements of the present invention may be operably linked to a heterologous gene (i.e. a "transgene"), so that transcription of the transgene is regulated by a truncated CMVie enhancer-promoter element. In particular nonlimiting embodiments, the truncated CMVie enhancer-promoter elements of the present invention are be operably linked to a transgene encoding a CFTR protein. In other nonlimiting embodiments, the truncated CMVie enhancer-promoter elements of the present invention are operably linked to a transgene encoding a CFTR protein from which all or part of the R domain has been deleted. The CFTR-encoding transgene operably linked to the truncated CMVie enhancer-promoter element may comprise, in addition to deletion of all or part of the R region, deletions in other regions provided that the protein encoded by the transgene produces functional chloride ion channel activity when expressed in CF airway epithelia cells. In a specific nonlimiting embodiment, the transgene encoding a CFTR protein from which all or part of the R domain has been deleted comprises one of the following nucleic acid sequences:

```
                                       (SEQ ID NO: 3)
5'-ATGCAGAGGTCGCCTCTGGAAAAGGCCAGCGTTGTCTCCAAACTTTT

TTTCAGCTGGACCAGACCAATTTTGAGGAAAGGATACAGACAGCGCCTGG

AATTGTCAGACATATACCAAATCCCTTCTGTTGATTCTGCTGACAATCTA

TCTGAAAAATTGGAAAGAGAATGGGATAGAGAGCTGGCTTCAAAGAAAAA

TCCTAAACTCATTAATGCCCTTCGGCGATGTTTTTTCTGGAGATTTATGT

TCTATGGAATCTTTTTATATTTAGGGGAAGTCACCAAAGCAGTACAGCCT

CTCTTACTGGGAAGAATCATAGCTTCCTATGACCCGGATAACAAGGAGGA

ACGCTCTATCGCGATTTATCTAGGCATAGGCTTATGCCTTCTCTTTATTG

TGAGGACACTGCTCCTACACCCAGCCATTTTTGGCCTTCATCACATTGGA

ATGCAGATGAGAATAGCTATGTTTAGTTTGATTTATAAGAAGACTTTAAA

GCTGTCAAGCCGTGTTCTAGATAAAATAAGTATTGGACAACTTGTTAGTC

TCCTTTCCAACAACCTGAACAAATTTGATGAAGGACTTGCATTGGCACAT

TTCGTGTGGATCGCTCCTTTGCAAGTGGCACTCCTCATGGGGCTAATCTG

GGAGTTGTTACAGGCGTCTGCCTTCTGTGGACTTGGTTTCCTGATAGTCC

TTGCCCTTTTTCAGGCTGGGCTAGGGAGAATGATGATGAAGTACAGAGAT

CAGAGAGCTGGGAAGATCAGTGAAAGACTTGTGATTACCTCAGAAATGAT

TGAAAATATCCAATCTGTTAAGGCATACTGCTGGGAAGAAGCAATGGAAA

AAATGATTGAAAACTTAAGACAAACAGAACTGAAACTGACTCGGAAGGCA

GCCTATGTGAGATACTTCAATAGCTCAGCCTTCTTCTTCTCAGGGTTCTT

TGTGGTGTTTTTATCTGTGCTTCCCTATGCACTAATCAAAGGAATCATCC
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TCCGGAAAATATTCACCACCATCTCATTCTGCATTGTTCTGCGCATGGCG
GTCACTCGGCAATTTCCCTGGGCTGTACAAACATGGTATGACTCTCTTGG
AGCAATAAACAAAATACAGGATTTCTTACAAAAGCAAGAATATAAGACAT
TGGAATATAACTTAACGACTACAGAAGTAGTGATGGAGAATGTAACAGCC
TTCTGGGAGGAGGGATTTGGGGAATTATTTGAGAAAGCAAAACAAAACAA
TAACAATAGAAAAACTTCTAATGGTGATGACAGCCTCTTCTTCAGTAATT
TCTCACTTCTTGGTACTCCTGTCCTGAAAGATATTAATTTCAAGATAGAA
AGAGGACAGTTGTTGGCGGTTGCTGGATCCACTGGAGCAGGCAAGACTTC
ACTTCTAATGATGATTATGGGAGAACTGGAGCCTTCAGAGGGTAAAATTA
AGCACAGTGGAAGAATTTCATTCTGTTCTCAGTTTTCCTGGATTATGCCT
GGCACCATTAAAGAAAATATCATCTTTGGTGTTTCCTATGATGAATATAG
ATACAGAAGCGTCATCAAAGCATGCCAACTAGAAGAGGACATCTCCAAGT
TTGCAGAGAAAGACAATATAGTTCTTGGAGAAGGTGGAATCACACTGAGT
GGAGGTCAACGAGCAAGAATTTCTTTAGCAAGAGCAGTATACAAAGATGC
TGATTTGTATTTATTAGACTCTCCTTTTGGATACCTAGATGTTTTAACAG
AAAAAGAAATATTTGAAAGCTGTGTCTGTAAACTGATGGCTAACAAAACT
AGGATTTTGGTCACTTCTAAAATGGAACATTTAAAGAAAGCTGACAAAAT
ATTAATTTTGCATGAAGGTAGCAGCTATTTTTATGGGACATTTTCAGAAC
TCCAAAATCTACAGCCAGACTTTAGCTCAAAACTCATGGGATGTGATTCT
TTCGACCAATTTAGTGCAGAAAGAAGAAATTCAATCCTAACTGAGACCTT
ACACCGTTTCTCATTAGAAGGAGATGCTCCTGTCTCCTGGACAGAAACAA
AAAAACAATCTTTTAAACAGACTGGAGAGTTTGGGGAAAAAAGGAAGAAT
TCTATTCTCAATCCAATCAACTCTACGCTTCAGGCACGAAGGAGGCAGTC
TGTCCTGAACCTGATGACACACTCAGTTAACCAAGGTCAGAACATTCACC
GAAAGACAACAGCATCCACACGAAAAGTGTCACTGGCCCCTCAGGCAAAC
TTGACTGAACTGGATATATATTCAAGAAGGTTATCTCAAGAAACTGGCTT
GGAAATAAGTGAAGAAATTAACGAAGAAGACTTAAAGGAGTGCTTTTTTG
ATGATATGGAGAGCATACCAGCAGTGACTACATGGAACACATACCTTCGA
TATATTACTGTCCACAAGAGCTTAATTTTTGTGCTAATTTGGTGCTTAGT
AATTTTTCTGGCAGAGGTGGCTGCTTCTTTGGTTGTGCTGTGGCTCCTTG
GAAACACTCCTCTTCAAGACAAAGGGAATAGTACTCATAGTAGAAATAAC
AGCTATGCAGTGATTATCACCAGCACCAGTTCGTATTATGTGTTTTACAT
TTACGTGGGAGTAGCCGACACTTTGCTTGCTATGGGATTCTTCAGAGGTC
TACCACTGGTGCATACTCTAATCACAGTGTCGAAAATTTTACACCACAAA
ATGTTACATTCTGTTCTTCAAGCACCTATGTCAACCCTCAACACGTTGAA
AGCAGGTGGGATTCTTAATAGATTCTCCAAAGATATAGCAATTTTGGATG
ACCTTCTGCCTCTTACCATATTTGACTTCATCCAGTTGTTATTAATTGTG
ATTGGAGCTATAGCAGTTGTCGCAGTTTTACAACCCTACATCTTTGTTGC
AACAGTGCCAGTGATAGTGGCTTTTATTATGTTGAGAGCATATTTCCTCC
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In other specific, nonlimiting embodiments, the transgene encoding a CFTR protein comprises a CFTR protein in which amino acids 708-835, amino acids 708-759, amino acids 708-723, 749-783 and 832-835, amino acids 708-723, 749-783 and 819-835, amino acids 708-759 and 819-835, amino acids 760-835, amino acids 708-783, or amino acids 708-783 and 823-835 have been deleted. In other specific, nonlimiting embodiments, the CFTR protein consists essentially of one of these CFTR variants. In a specific, nonlimiting embodiment, the CFTR protein consists essentially of SEQ ID NO:3.

Non-CFTR transgenes whose transcription also may be regulated by the CMVie enhancer-promoter elements of the present invention may include any transgene of interest in gene transfer or gene therapy applications, such as those for the treatment of metabolic disorders, infectious diseases, acquired diseases, or oncogenic diseases. See e.g. U.S. Pat. No. 5,872,005. The enhancer-promoter elements of the present invention are particularly useful in regulating transcription of transgenes containing relatively long coding regions (i.e. regions longer than approximately 3 to 4.5 kb). One example of a gene with a relatively long coding region that is of importance in gene therapy applications is the dystrophin gene, the cDNA of which is approximately 14 kb in length. Those of ordinary skill in the art will recognize other gene expression or gene transfer applications that would potentially benefit from the application of the truncated CMVie enhancer-promoters of the present invention.

The present invention also provides for vectors comprising nucleic acid molecules of the invention including, without limitation, nucleic acid molecules comprising the truncated CMVie enhancer-promoter elements lacking the 16 and 21 bp repeat units otherwise found in the wild-type human CMVie enhancer-promoter. In these vectors, the truncated CMVie enhancer-promoter elements may be operably linked to a transgene, such as those discussed immediately above. In particular nonlimiting embodiments, these vectors comprise the truncated CMVie enhancer-promoter elements operably linked to a transgene encoding a CFTR protein from which all or part of the R domain has been deleted. In particular nonlimiting embodiments, these vectors comprise truncated CMVie enhancer-promoter elements operably linked to transgenes having the nucleic acid sequence of SEQ ID NO:3, or encoding CFTR proteins from which amino acids 708-835, amino acids 708-759, amino acids 708-723, 749-783 and 832-835, amino acids 708-723, 749-783 and 819-835, amino acids 708-759 and 819-835, amino acids 760-835, amino acids 708-783, or amino acids 708-783 and 823-835 have been deleted.

In further embodiments, vectors comprising the truncated CMVie enhancer-promoter elements of the present invention may include, but are not limited to, a plasmid, a cosmid, a phagemid, a bacteriophage, a bacterial artificial chromosome, a yeast artificial chromosome, a human artificial chromosome, a retrovirus vector, a lentivirus vector, an adenovirus vector, an adeno-associated virus vector, a herpes virus vector, an Epstein-Barr virus vector, an alphavirus, or a vaccinia virus vector. Those of ordinary skill in the art will recognize that the truncated CMVie enhancer-promoter elements of the present invention also will be useful in other vectors employed in the fields of gene expression and gene transfer, especially those in which the overall size of the transgene expression cassette is a limiting factor. In particular nonlimiting embodiments, the truncated CMVie enhancer-promoter elements of the present invention are incorporated into vectors derived from human adeno-associated virus (AAV), especially vectors derived from serotype 5 of human AAV (AAV5).

The vectors of the present invention may further comprise various transgenes, intervening sequences, polyadenylation signals, or other elements known to those of ordinary skill in the art to be beneficial to the expression of transgenes from the particular vector being examined. Particularly preferred are vectors derived from AAV5 that express human CFTR protein or functional variants thereof. Alternative vector embodiments may include those in which the truncated CMVie enhancer-promoter elements of the present invention are operably linked to other transgenes encoded by nucleic acid sequences greater than approximately 3 kb in length.

Also provided by the present invention are cells transformed by the vectors described herein. In this context, transformation refers to any process by which heterologous nucleic acid material is introduced into and expressed within a cell. Thus, transformation as used herein includes "transient" transfection procedures, including but not limited to those mediated by electroporation, cationic lipid/DNA complexes, protein/DNA complexes, calcium phosphate-mediated pinocytosis, virus vectors, etc., where a nucleic acid introduced into the host cell exists extrachromosomally. Moreover, transformation as used herein may refer to so-called "stable" transfection methods, wherein a particular nucleic acid is introduced into a host cell in combination with a second nucleic acid encoding a selectable marker (e.g. resistance to an antibiotic), which enables the positive selection of cells in which the transfected nucleic acids have been integrated into the genome of the host cell. In particular nonlimiting embodiments, such vector-transformed cells include, but are not limited to, cultured airway epithelial cells, such as the A549 or H441 cell lines, primary airway epithelial cell cultures derived from CF or wild-type donors, or airway epithelial cells from CF or non-CF individuals in vivo et situ.

The present invention provides methods for treating genetic, metabolic or acquired diseases. In a nonlimiting embodiment, the present invention provides a method for expressing a nucleic acid molecule of the invention in a cell, the method comprising contacting the cell with a sufficient amount of a nucleic acid molecule and/or vector of the present invention. Preferably, the method is performed under conditions in which the transgene of interest is expressed in the cell. The transgene-expressing cell may be transduced with the transgene in vitro, ex vivo, or in vivo.

In another nonlimiting embodiment, the present invention provides a method for treating a subject having, or at risk of having, cystic fibrosis (CF) airway disease, the method comprising contacting the subject in need of such treatment with a sufficient amount of a nucleic acid molecule, vector, and/or cell of the present invention. Preferably, the treatment is performed under conditions in which the transgene of interest is expressed in the subject. The nucleic acid molecule, vector, and/or cell of the present invention may be contacted with the cells or tissues of the subject by any suitable mode of administration. For example, a vector of the invention may be administered topically on differentiated airway epithelia. In another example, a vector of the invention may be administered topically on the apical membrane of an epithelial cell. In yet another example, a nucleic acid molecule and/or vector of the present invention may be contacted with the cells or tissues of the subject by intranasal administration. The subject may be mammalian, preferably human. The effectiveness of the treatment may be assayed using any suitable method available to one of ordinary skill in the art.

In particular nonlimiting embodiments, the present invention provides methods for treating a subject with cystic fibrosis (CF) airway disease. The method may comprise contacting the subject in need of such treatment with a sufficient amount of a vector, wherein the vector comprises a nucleic acid having a functional truncated human cytomegalovirus immediate-early enhancer-promoter region, wherein the full-length human cytomegalovirus immediate-early enhancer-promoter region comprises nucleotide repeat units of 16, 18, 19 and 21 base pairs, wherein said truncated human cytomegalovirus immediate-early enhancer-promoter lacks the nucleotide repeat units of 16 and 21 base pairs, wherein the nucleic acid is operably linked to a transgene, and wherein the transgene expresses in the subject a nucleic acid molecule encoding a CFTR protein having a deletion in the R domain.

The present invention is further illustrated by the following examples which in no way should be construed as further limiting the invention.

EXAMPLES

Example 1

Construction and Analysis of Truncated CMVie Enhancer-promoters

FIG. **2** shows a diagram of the 610-bp human CMVie enhancer-promoter, which contains clusters of nucleotide repeats that bind specific transcription factors. Stinski, 1999, In *Gene Expression Systems: Using Nature for the Art of Expression*. Academic Press, New York. 1999. pp. 211-233. Coordination between these transcription factors determines promoter activity. To maintain promoter activity while reducing length, sequences that contained different combinations of these nucleotide clusters were systematically removed and inserted into a β-galactosidase reporter plasmid. The resulting plasmids then were transfected into two airway epithelial cell lines (A549 and H441) and into non-polarized, primary cultures of human airway epithelial cells by lipofectamine-mediated transfection, where promoters that were functional in airway epithelia could be identified.

The initial constructs examined were the CMVie enhancer-promoter truncated at nt −348 or −222 previously studied by Stinski. Stinski and Roehr, 1985, *J. Virol.* 55:431-441. These constructs produced β-galactosidase activity in all airway cells tested. Shorter promoter constructs that retained either two pairs of the 18-bp and 19-bp repeats (173CMV) or a single set of the 18-bp and 19-bp repeats (113CMV) then were examined. The nucleotide sequences of 173CMV and 113CMV are shown within the context of a complete expression cassette in FIGS. **3** and **4**, respectively. As shown in FIG. **5**, when expressed in the airway cell lines and in primary cultures of airway epithelia, these two shortened constructs maintained much of the high-level expression of the full-length CMVie.

Example 2

Effect of an Intervening Sequence on the Function of the Truncated CMVie Enhancer-promoters

In previous studies utilizing the full-length CMVie enhancer-promoters, it was observed that the inclusion of an intron increased expression levels. Yew et al., 1997, *Human Gene Therapy* 8:575-584. Therefore, the effect of including the 19S/16S intron from SV40 in the intervening sequence between the transcription and translation start sites of the 173CMV and 113CMV constructs were examined. As shown in FIG. **5**, this intron did not enhance β-galactosidase activity in A549 cells, but instead slightly reduced promoter activity.

To determine how the length of the intervening sequence (IVS) between the transcriptional and translational start sites affected promoter activity, a series of IVSs of differing lengths that maintain a minimum of predicted secondary structure were examined. The IVS in our original vector was 69-bp and was a composite of a multiple cloning site and the 5' untranslated region upstream of the β-galactosidase coding sequence (from the Clontech plasmid pCMVβ). For both the 173CMV and 113CMV promoters, the effect of varying the length of the IVS on the relative β-galactosidase expression was tested. As shown in FIG. **6**, a 22-bp IVS with no Kozak sequence markedly reduced expression. When the IVS included the Kozak translation consensus sequence, length had little effect on expression from the 173CMV promoter. In contrast, in the 113CMV promoter, a 51-bp IVS generated substantially more activity than either a longer 69-bp or a shorter 44-bp IVS.

Example 3

Effect of Polyadenylation Signals on the Function of the Truncated CMVie Enhancer-promoters

The 49-bp consensus synthetic poly(A) addition (SPA) sequence (AATAAA(22nt)GTx/Tx) has been reported to be equivalent to or better than a typical full-length poly(A) addition sequence in expression constructs. Levitt et al., 1989, *Genes Dev.* 3:1019-1025. To determine whether this SPA could replace the full-length 630-bp human growth hormone (hGH) poly(A) sequence present in pCMV5-β-gal, constructs containing these two alternative poly(A) signals were prepared and tested in airway epithelial cell cultures. As shown in FIG. **7**, these two poly(A) addition signals yielded nearly equivalent expression levels when tested with the full-length CMVie and the 113CMV promoters. However, expression from the 173CMV promoter was reduced when the SPA was used.

Example 4

Construction and Testing of AAV5-based Vectors Incorporating the Truncated CMVie Enhancer-promoters

Based on these results, two AAV expression cassettes were prepared, one with the 173-bp CMVie truncated enhancer-promoter (SEQ ID NO:1) and a second one with the 113-bp CMVie truncated enhancer-promoter (SEQ ID NO:2). The nucleotide sequences of these expression cassettes are depicted in FIGS. **3** and **4**, respectively. The enhancer-promoter sequences were ligated directly to the AAV5 ITR sequence (167 bp), resulting in the retention of only minimal plasmid sequences (26 bp of plasmid sequence and 8 bp corresponding to the NotI restriction endonuclease recognition site; see Table 1). The expression cassette included the 51-bp IVS followed by the CFTRΔR transgene (SEQ ID NO:3) and the 49-bp SPA (Table 1 and FIGS. **3** and **4**). The 173CMV expression cassette was 4940 bp in length and the 113CMV expression cassette was 4880 bp in length (Table 1).

These two expression cassettes were used to test the hypothesis that an AAV5 vector containing an cassette in which transcription of the CFTR-ΔR gene was regulated by a truncated CMVie enhancer-promoter could correct the Cl^- transport defect when applied to the apical surface of well differentiated CF airway epithelia. AAV5 viral vectors containing these expression cassettes were rescued and applied to the apical surface of CF airway epithelia, where they partially corrected the CF Cl-transport defect. FIG. **8** shows an example of a short-circuit current trace taken 2 weeks after applying 10 MOI, as determined by an infectious center assay (Hernandez et al., 1999, *J. Virol.* 73:8549-8558), of an AAV5 vector to the apical surface of an airway epithelium. The ratio of particles to infectious particles was ~1000:1. Transepithelial current in the vector-transduced cells increased with cAMP agonists and then fell when transepithelial current was blocked by bumetanide (FIG. **8**).

As shown in FIG. **9**, the amount of current produced in the transduced epithelia was dose-dependent, with transduction by 10 MOI of vector producing significantly greater amounts of conductance that transduction by 1 MOI. Average currents from a large number of CF and non-CF airway epithelia are shown at the right of FIG. **9** for purposes of comparison. As can be seen, transduction by 10 MOI of the AAV vectors in which either the 173CMV or 113CMV truncated enhancer-promoter elements were used to drive the transcription of the CFTR-ΔR gene restored approximately 30% of the amount of conductance observed in non-CF epithelia. These findings indicate that an AAV5 vector with a shortened promoter and transgene can partially correct the Cl^- transport defect when applied to the apical surface of well-differentiated CF airway epithelia (for example, by restoring approximately 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the amount of conductance observed in non-CF epithelia). These studies provide the first evidence that an AAV vector can express functional CFTR in differentiated CF airway epithelia.

Example 5

Expression in Human Tissue Cultures of Adenoviral-based Vectors Incorporating Truncated CMVie Enhancer-promoters

Cultures of human airway epithelia are obtained from CF bronchus and/or trachea (e.g., Delta F508/Delta F508 or Delta F508/other genotypes) and cultured at the air-liquid interface. Epithelia are used at least 14 days after seeding when they are well differentiated, e.g., epithelia comprising ciliated cells, goblet cells, and other nonciliated cells. Preferably, the epithelia retain certain functional properties of airway epithelia such as, for example, trans-epithelial electrolyte transport and resistance.

Epithelia are infected with multiplicities (e.g., 50, 100, 200, 500) of infection of adenoviral vectors of the invention using 5 mM EGTA applied to the apical surface to transiently disrupt the tight junctions as described.

Example 6

Expression in CF Mice of Adenoviral-based Vectors Incorporating Truncated CMVie Enhancer-promoters

For in vivo analysis in animals, 6- to 8-wk-old Delta F508 homozygote CF mice are used. Mice are lightly anesthetized in a halothane chamber. Adenoviral vectors of the invention (e.g., 10^8 particles, 10^9 particles, 5×10^9 particles, 10^{10} particles) are administered intranasally as Ad:CaPi coprecipitates in two 5-μl instillations delivered 5 min apart. The adenoviral vector may encode a partially-deleted CFTR protein, for example. Four days later, animals are anesthetized with ketamine and xylazine, and the trans-epithelial electric potential difference across the nasal epithelium (Vt) is measured. During measurement of Vt, the nasal mucosa is perfused at a rate of 50 μl/min with a Ringer's solution containing: 135 mM NaCl, 2.4 mM KH2PO4, 0.6 mM K2HPO4, 1.2 mM CaCl2, 1.2 mM MgCl2, and 10 mM Hepes (pH 7.4 with NaOH). Three solutions are used: (i) Ringer's solution containing 100 μM amiloride; (ii) Ringer's solution containing 135 mM Na-gluconate substituted for NaCl plus amiloride; and (iii) Na-gluconate Ringer's solution containing 10 μM isoproterenol and amiloride. Measurements are made after perfusion for 5 min.

The infected nasal epithelia are treated epithelia with amiloride to inhibit Na+ channels and then Vt is measured in response to perfusion with solutions containing a low Cl- concentration and isoproterenol to elevate cellular cAMP levels. Expression of the adenoviral vectors of the invention expressing a partially-deleted CFTR protein correct the nasal voltage defect to a similar extent as wild-type CFTR and to levels similar to those of non-CF mice. This method demonstrates the successful introduction of the adenoviral vectors of the invention, and an appropriate biosynthesis, localization, and functional activity of the transgene.

Example 7

Expression in Humans of Adenoviral-based Vectors Incorporating Truncated CMVie Enhancer-promoters

Adenoviral vectors of the invention (e.g., 10^8 particles, 10^9 particles, 5×10^9 particles, 10^{10} particles) are administered to a human intranasally as Ad:CaPi coprecipitates in multiple instillations delivered several minutes apart. The adenoviral-infected nasal epithelium expresses the transgene at relatively high levels. The encoded protein appropriately is trafficked through the cell and demonstrates functional and/or therapeutic activity. In a human with CF airway disease, the expression of the transgene in the adenoviral-infected nasal epithelium ameliorates certain symptoms of the disease.

The foregoing merely illustrates the principles of the present invention. Various modifications and alterations to the described embodiments will be apparent to those of ordinary skill in the art in view of the teachings herein. It will thus be appreciated that those of ordinary skill in the art will be able to make and use the present invention in ways that, although not explicitly shown or described herein, embody the principles of the invention and are thus within the spirit and scope of the invention.

The contents of all publications and references cited herein are hereby incorporated herein by reference in their entireties

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We claim:

1. An isolated nucleic acid comprising:

a functional truncated human cytomegalovirus immediate-early enhancer-promoter region that comprises the nucleic acid sequence of SEQ ID NO:1 and that lacks the nucleotide repeat units of 16 and 21 base pairs of a full length human cytomegalovirus immediate early enhancer-promoter region.

2. The isolated nucleic acid of claim **1** operably linked to a transgene.

3. The isolated nucleic acid of claim **2**, wherein the transgene comprises a nucleic acid encoding a CFTR protein having a deletion in the R domain.

4. The isolated nucleic acid of claim **3**, wherein the nucleic acid encoding a CFTR protein having a deletion in the R domain has the sequence of SEQ ID NO:3.

5. A vector comprising the nucleic acid of claim **2**.

6. A vector comprising the nucleic acid of claim **3**.

7. A vector comprising the nucleic acid of claim **4**.

8. The vector of claim **5**, wherein the vector is selected from the group consisting of a plasmid, a cosmid, a phagemid, a bacteriophage, a bacterial artificial chromosome, a yeast artificial chromosome, a human artificial chromosome, a retrovirus vector, an adenovirus vector, an adeno-associated virus vector, a herpes virus vector, an Epstein-Barr virus vector, and a vaccinia virus vector.

9. The vector of claim **6**, wherein the vector is selected from the group consisting of a plasmid, a cosmid, a phagemid, a bacteriophage, a bacterial artificial chromosome, a yeast artificial chromosome, a human artificial chromosome, a retrovirus vector, an adenovirus vector, an adeno-associated virus vector, a herpes virus vector, an Epstein-Barr virus vector, and a vaccinia virus vector.

10. The vector of claim **7**, wherein the vector is selected from the group consisting of a plasmid, a cosmid, a phagemid, a bacteriophage, a bacterial artificial chromosome, a yeast artificial chromosome, a human artificial chromosome, a retrovirus vector, an adenovirus vector, an adeno-associated virus vector, a herpes virus vector, an Epstein-Barr virus vector, and a vaccinia virus vector.

11. The vector of claim **8**, wherein the adeno-associated virus vector is an AAV5-based vector.

12. The vector of claim **9**, wherein the adeno-associated virus vector is an AAV5-based vector.

13. The vector of claim **10**, wherein the adeno-associated virus vector is an AAV5-based vector.

14. A cultured cell comprising the vector of claim **5**.

15. A cultured cell comprising the vector of claim **6**.

16. A cultured cell comprising the vector of claim **7**.

* * * * *