Expression and Chromosome Localization of the Murine Cystic Fibrosis Transmembrane Conductance Regulator

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A 13.5-kb genomic fragment of the mouse cystic fibrosis transmembrane conductance regulator (CFTR) gene was isolated from a C57BL/6J liver DNA library, using a human CFTR exon 10 probe. This region of the human gene includes the most common cystic fibrosis mutation (deletion of the Phe608 residue) in the first nucleotide binding domain of CFTR. Sequence analysis demonstrated 87% identity between the predicted mouse and the normal human CFTR exon 10 sequences, including conservation of the Phe608 residue. Northern analysis revealed that the mouse gene is expressed in intestine, lung, stomach, kidney, and salivary gland. In contrast to human CFTR, murine CFTR transcripts were not detectable by Northern analysis in the liver or pancreas. More sensitive PCR analysis, however, revealed that the mouse CFTR gene is weakly expressed in other tissues, including liver and pancreas. During development, mouse CFTR transcripts were observed as early as Embryonic Day 13. Southern analysis of mouse × Chinese hamster somatic cell hybrid DNAs mapped the mouse CFTR locus (Cftr) to Chromosome 6 (Chr 6). Subsequent typing of the progeny of an interspecies backcross revealed that Cftr is closely linked to the proto-oncogene c-met locus (Met) in the centromeric region of mouse Chr 6, consistent with the observation that there is a conserved chromosomal segment on human chromosome 7 and mouse Chr 6.

INTRODUCTION

Cystic fibrosis (CF) is a lethal autosomal recessive disorder in humans, with a carrier frequency estimated at 5% among Caucasians (Boat et al., 1989). Recent attempts to understand the molecular nature of this disease have focused on the isolation of the affected gene. As a result, the human CF gene has been successfully identified using genetic and molecular approaches based on its chromosome localization (Rommens et al., 1989). From the predicted protein structure, the CF gene product is similar to a number of membrane-associated active transport proteins and is likely to be involved in membrane ion transport (Riordan et al., 1989). Thus, the CF gene product has been termed the cystic fibrosis transmembrane conductance regulator (CFTR). CFTR is an N-linked glycoprotein that consists of two repeats containing a nucleotide binding domain (NBD) and six transmembrane segments; the two repeats are separated by a regulatory (R) domain (Riordan et al., 1989). A number of CFTR mutations have been identified, including the deletion of a phenylalanine residue at position 508 (ΔF508) in the first NBD (Riordan et al., 1989; Kerem et al., 1989). In addition to the prevalent ΔF508 mutation, which is present in approximately 70% of CF patients, numerous additional mutations have been identified, many of which are also located within the first NBD (Cutting et al., 1990; Kerem et al., 1990).

The availability of an animal model for CF would provide a suitable system for determining the efficacy of novel treatments, including gene therapy, based on the molecular defects that have been identified in the CF gene, and allow more detailed examinations of the basic molecular defect, as well as the progression of the disease. The production of mouse models for human genetic diseases is possible through manipulation of homologous disease-related genes in pluripotent mouse embryonic stem (ES) cells and has been aided by recent advances in enrichment and analysis of gene targeting events (Frohman and Martin, 1989). The creation of a mouse CF model depends upon the presence of a mouse homologue for the gene that is affected in human CF patients. In particular, only a part of the gene that spans the region to be altered is required for homologous recombination in ES cells. To this end, we have isolated a fragment of the mouse CFTR (mCFTR) gene that is homologous to the region of the human CFTR (hCFTR) gene containing exon 10. For the present study, the isolated mCFTR sequence was used as a molecular probe to examine the expression of the mCFTR homologue and to perform definitive mapping of the mouse gene.

MATERIALS AND METHODS

Polymerase chain reaction (PCR). Genomic DNA templates were amplified as described elsewhere (Saiki et al., 1988). As standard con-
ditions, we used 30 cycles of PCR (denaturation for 30 s at 94°C, annealing for 1 min at 55°C, and extension for 2 min at 72°C) following an initial denaturation at 94°C for 4 min. The accuracy of the thermocycler was controlled using an external device (Stamm et al., 1991). The amplified fragments were analyzed by agarose gel electrophoresis of one-tenth of the reaction mixture. For subcloning purposes, the remainder of the reaction product was extracted with phenol and precipitated. The PCR fragments were then digested with the appropriate restriction enzymes to generate the flanking restriction sites, which were synthesized on the ends of the PCR primers. The resulting fragments were purified by electrophoresis through low-melting-point agarose (FMC, Rockland, ME) and subcloned into a pSP64 vector (Promega, Madison, WI).

For isolation of partial cDNA sequences and mRNA analysis, mouse tissue RNAs were annealed with antisense oligonucleotide primers and extended with M-MLV RNase H- reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD), according to the manufacturer's instructions, for 1 h at 37°C. The reaction volume was then adjusted to 50 µl and the cDNA template was stored at −20°C. The first-strand cDNA templates were PCR amplified as described above. For expression analysis, the PCR reactions were modified by the addition of 2.5 µl of 3000 Ci/mmol [α-32P]dCTP (NEN/DuPont, Boston, MA) to each reaction. The PCR-generated reaction products were then analyzed by autoradiography after electrophoresis through 5% polyacrylamide, 7 M urea gels.

**Oligonucleotide primers.** Based on the published hCFTR exon 10 sequence (Riordan et al., 1989), two oligonucleotides, KK009 (5'-GGAATTCGATCAGTTCTCTTCGGATGAG-3') and KK010 (5'-GGATCCCTTCTTCTAGTGTCGATGTT-3'), were synthesized for PCR amplification and cloning of the 335-bp hCFTR exon 10. KK009 and KK010 were designed with EcoRI and BamHI restriction endonuclease sites (underlined) on their 5' ends, respectively, for subcloning into the hCFTR exon 10 fragment.

Oligonucleotides KK005 (5'-GGATTCCAGATCACTCTCTTCGGATGAG-3') and KK036 (5'-GGATACCTTCAATGACATGTT-3') contained EcoRI and BamHI restriction endonuclease sites (underlined) on their 5' ends, respectively, were synthesized to generate a PCR-amplified mCFTR exon 10 fragment from C57BL/6J genomic DNA. These oligonucleotides were based on the sequence-derived from the isolated mCFTR genomic clone containing exon 10 (Fig. 2).

The oligonucleotide KK059 (5'-GGATTTCGGGATACCTCTCTTCGGATGAG-3') was based on the sequence at the 5' end of mCFTR exon 9, as determined by sequence analysis of a PCR-generated partial cDNA clone (pmCF-3). KK059 is identical to KK036, except for incorporation of an additional SaI restriction site on the 5' end. KK009 and KK010 were used to examine mCFTR expression by PCR amplification of KK009-primed, first-strand cDNA from total tissue RNAs.

The oligonucleotides KK055 (5'-GGATACCTTCAATGACATGTT-3') and Act-1 (5'-AACATGATCCTGTTGTTGCT-3') were based on the published sequence of a mouse cytoskeletal β-actin cDNA (Tokunaga et al., 1986). KK055 contains a BamHI restriction endonuclease site (underlined) on its 5' end. These oligonucleotides were used for PCR amplification of Act-1 primed, reverse-transcribed actin cDNA from total tissue RNAs.

**PCR-generated probes.** The standard PCR reaction described above was modified to produce small, double-stranded DNA probes with high specific activity (3–5 × 106 cpm/µg). The templates used to produce these probes were PCR products amplified from either genomic or plasmid DNA. The reaction conditions were the same as those used for the standard PCR reactions described above, except dCTP was replaced with 250 µM (0.53 pmol, 1.7 µCi) of 3000 Ci/mmol [α-32P]dCTP (NEN/DuPont) and the 72°C incubation was increased to 4 min. The hCFTR exon 10 probe was produced using oligonucleotides KK009 and KK010, and the mCFTR exon 10 probe was generated with oligonucleotides KK035 and KK036. The 32P-labeled PCR fragments were purified through push-columns (Stratagene, La Jolla, CA), denatured at 100°C for 5 min, and used as hybridization probes for genomic library screening, as well as Southern and Northern analyses. It is somewhat surprising that we obtained full-length PCR products using a dCTP concentration of 1.7 µM, which is lower than the Kₘ of about 10–100 µM (Williams, 1989). This suboptimal concentration can probably be compensated for by the longer extension time, which was found by other groups to be useful for obtaining full-length PCR products (Schwoller and Sommer, 1989; Jansen and Ledley, 1989).

**Genomic library construction and screening.** A mouse genomic library was constructed by cloning BamHI-digested C57BL/6J liver DNA into the BamHI restriction site of a pEMBL3 vector (Stratagene, La Jolla, CA). Recombinant plasmids were screened by the method of Benton and Davis (1977), using a PCR-generated hCFTR exon 10 probe. Recombinant phage DNA was prepared and analyzed as described by Kelley and Pitha (1985).

**DNA sequencing.** Double-stranded plasmid DNA was sequenced by a modification of the dideoxynucleotide chain-termination method (Wallace et al., 1981). The chemical sequencing method of Maxam and Gilbert (1980) was used to sequence through one region of the plasmid pmCF-1, which could not be sequenced by the enzymatic chain-termination method.

**Genomic DNA preparation and Southern analysis.** Genomic DNAs were prepared from mutant cell hybrids and mouse liver as described previously (Heisterkamp et al., 1982). The high-molecular-weight DNAs (5–10 µg) were digested with restriction endonucleases, electrophoresed through 1% agarose gels, and transferred to nitrocellulose membranes. The filters were prehybridized for 4–6 h at 37°C and then hybridized with PCR-generated probes for 48 h at 37°C in 5X SSC, 50% formamide. The filters were washed with a final stringency of 0.5× SSC at 50°C for 30 min.

**Tissue RNA preparation and Northern analysis.** Total RNA was isolated by extraction of various mouse tissues in guanidine thiocyanate and centrifugation through a 5.7 M CsCl cushion as described elsewhere (Chirgwin et al., 1979; Glisín et al., 1974). For the developmental study, whole embryo RNA from Days 13, 15, and 17 of gestation was isolated; the embryos were not contaminated with either extraembryonic membranes or maternal tissue. Isolated RNA was denatured and electrophoresed through 1.1% agarose, 2 M formaldehyde gels as described by Lehrach et al. (1977), transferred to nitrocellulose, and hybridized to a PCR-generated mCFTR exon 10 probe for 48 h at 37°C in 5X SSC, 50% formamide. The filters were washed with a final stringency of 0.5× SSC at 50°C for 30 min.

**Isolation of partial cDNA clones.** To verify the intron/exon boundaries of mCFTR exon 10, two partial cDNA clones, pmCF-3 and pmCF-4, spanning mCFTR exons 9–10 and 10–12, respectively, were isolated after PCR amplification of reverse-transcribed C57BL/6J intestinal RNA. The nucleotide sequence for mCFTR exons 9–12 was determined and in complete agreement with the published BALB/c cDNA sequence of Yorifugi et al. (1991), while there are four positions within mCFTR exon 9 that differ from the C57BL/6J sequence published by Tata et al. (1991).

**Chinese hamster × mouse somatic cell hybrids.** The production and characterization of Chinese hamster × mouse somatic cell hybrids have been described previously (Hoggen et al., 1988). Thirteen hybrids were selected from a larger panel of 76 hybrids for this study. Seven of these hybrids were typed by karyology for their mouse chromosome content; the remainder were typed for markers on specific mouse chromosome arms.

**Interspecies backcross.** NFS/N and C57BL/6J mice (Mus musculus) were obtained from the Division of Natural Resources, NIH (Bethesda, MD) and The Jackson Laboratory (Bar Harbor, ME), respectively. Mus tridentatus were provided by Dr. M. Potter (NCI, NIH, Bethesda, MD) from his colony at Hazleton Laboratory (Rockville, MD). NFS/N females were mated with M. spretus males, and the F1 females were mated with C57BL/6J or M. spretus males. DNA was extracted from the livers and spleens of individual progeny, digested with various restriction endonucleases, electrophoresed through 0.4% agarose gels, and transferred to nylon membranes (Hybond N+, Amer- sham). Membranes were hybridized with probes labeled by the ra-
dom primer method. The DNAs were typed for a BamHI restriction fragment length polymorphism of the mouse Cftr locus, using a plasmid clone of mCFTR exon 10 (pmCF-2), and a BamHI restriction enzyme variant of the Met locus, using a 1000-bp fragment of the MET human proto-oncogene obtained from Oncor (Gaithersberg, MD).

RESULTS

Human and Mouse CFTR Homology

A 193-bp hCFTR exon 10 fragment was PCR-amplified from human placental DNA using sense and antisense oligonucleotides from the 5' and 3' ends of this exon, respectively (Fig. 1A). The resulting PCR product was used as a template to prepare a PCR-generated hCFTR exon 10 probe. A majority of the PCR-generated radiolabeled fragments span the entire length of the amplified region (Fig. 1B). To assess the suitability of the hCFTR exon 10 PCR probe for the detection of homologous mouse CFTR sequences, a Southern analysis of human and mouse DNAs was performed. The human exon 10 PCR probe hybridizes to a 23-kb BamHI fragment and a 4.4-kb EcoRI fragment in human DNA (Fig. 1C), as expected from the previously published hCFTR restriction map (Rommens et al., 1989). This probe also cross-hybridizes to a 13.5-kb BamHI fragment and a 2.2-kb EcoRI fragment in mouse DNA (Fig. 1C), indicating that there is sufficient homology to allow identification and isolation of a mouse genomic fragment containing mCFTR exon 10 sequences.

Isolation of a Genomic Fragment Containing mCFTR Exon 10

A λEMBL3 library was prepared from BamHI-digested C57BL/6J liver DNA. Recombinant plaques (2.5 × 10⁵) were screened with the hCFTR exon 10 PCR probe. A single positive clone was plaque purified and further characterized. This mouse genomic clone (AmCFTR10) contained a 13.5-kb BamHI insert, as expected from the Southern analysis of mCFTR. A 2.1-kb BamHI/EcoRI fragment from λmCFTR10, which hybridizes to the hCFTR exon 10 probe, was subcloned into pSP64 to generate the plasmid pmCF-1. This subcloned fragment was used as a hybridization probe for Southern analysis of human and mouse DNAs to confirm that the genomic clone contained mCFTR exon 10 sequences in comparison to hCFTR exon 10-probed mouse DNA. Unfortunately, the presence of repetitive sequence elements in the pmCF-1 subclone probe made it impossible to verify that mCFTR exon 10 sequences had been isolated (data not shown).

The entire sequence of the 2078-bp pmCF-1 insert was determined (Fig. 2). Comparison with the hCFTR exon 10 sequence revealed an analogous 193-bp region in pmCF-1. This region shares 84% homology to hCFTR exon 10 at the nucleotide level and 87% in the predicted amino acid sequence. The phenylalanine 508 (Phe508)
FIG. 2. Nucleotide sequence and deduced amino acid sequence of the mCFTR gene spanning the region with homology to hCFTR exon 10. The sequence includes the entire 2078-bp BamHI/EcoRI insert from pmCF-1. The underlined AG and GT at the 5' and 3' intron/exon boundaries represent the consensus splice donor and acceptor sites, and their locations were inferred from homology to hCFTR (Riordan et al., 1989). The nucleotides beneath the mouse sequence are derived from hCFTR and represent the positions at which the human and mouse sequences are different within the protein-coding region. The CTT triplet indicated by asterisks is the sequence which is deleted in the AF508 mutation in human CF patients. These sequence data have been deposited in the GenBank database under Accession No. M84614.

Genetic Mapping

Southern blot analysis identified 2.2- and 23-kb EcoRI fragments in mouse and hamster DNAs, respectively, which are cross-reactive with a mCFTR exon 10 probe (Fig. 3). Thirteen Chinese hamster × mouse somatic cell hybrids were typed for this restriction fragment length polymorphism. The mouse 2.2-kb EcoRI fragment was present in eight of the 13 hybrids (Fig. 3). Examination of the chromosome content of these lines showed a perfect correlation with Chromosome 6 (Chr 6), indicating that the mouse gene for CFTR, designated Cfr, is on Chr 6 (Table 1). To provide a more specific map location, progeny of the cross (NFS/N X M. spretus) × C57BL/6J or M. spretus were typed. Southern blot analysis of the parental DNA identified CFTR-reactive BamHI fragments of 12.2 kb in M. spretus and of 13.5 kb in NFS/N and C57BL/6J (Fig. 4). Analysis of the progeny identified the M. spretus 12.2-kb CFTR segment in 63 of 97 mice, and the pattern of segregation was compared with that of 90 marker loci on all linkage groups. Close linkage was observed between Cfr and Met on Chr 6 (recombination frequency was 1/97 = 1.0 ± 1.0 cM), indicating that Cfr is closely linked to Met.
TABLE 1

Correlation between Specific Mouse Chromosomes and Cftr in 13 Chinese Hamster × Mouse Somatic Cell Hybrids

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* Symbols represent the presence (+/) or absence (-/) of the mouse CFTR 2.2-kb EcoRI restriction fragment as related to the presence (+/) or absence (-/) of a particular mouse chromosome. The number of discordant observations is the sum of the +/- and -/+ observations. Seven of the hybrids were karyotyped; the remainder were typed for the presence or absence of markers on specific mouse chromosomes.

which has been positioned at the centromeric end of this chromosome (Lyon and Kirby, 1991; Hillyard et al., 1991).

mCFTR Expression

Northern analysis of mouse tissue RNAs using a mCFTR exon 10 PCR probe revealed that mCFTR is expressed in intestine, lung, stomach, salivary gland, and kidney in adult C57BL/6J mice (Fig. 5) and during embryogenesis (data not shown). The size of the transcript detected by Northern analysis (6-7 kb) is in the range of what has been observed for human CFTR (Rommens et al., 1989; Riordan et al., 1989). The apparent lack of mCFTR transcripts in pancreas and liver was surprising since hCFTR mRNA levels are relatively high in these tissues (Riordan et al., 1989).

To characterize mCFTR expression further, the pattern revealed by Northern analysis was reexamined by PCR amplification of reverse-transcribed tissue RNAs. An antisense oligonucleotide (KK042) from the 3' end of mCFTR exon 10 was used as a primer for reverse transcription of total tissue RNAs. The newly synthesized first-strand cDNA was then PCR-amplified using KK042 and a sense oligonucleotide from the 5' end of mCFTR exon 9 (KK059). This combination of oligonucleotides results in a PCR fragment amplified from reverse-transcribed cDNA that can be distinguished from fragments produced by amplification of any contaminating genomic DNA in the RNA sample. PCR amplification of reverse-transcribed mCFTR transcripts revealed an expression pattern similar to that detected by the Northern analysis (Fig. 6A). Use of actin primers for analysis of reverse-transcribed RNA revealed actin-specific transcripts in pancreas and liver comparable to those in other tissues, suggesting that the absence of CFTR transcripts was not due to RNA degradation (Fig. 6B). A more sensitive analysis of the PCR fragments generated from reverse-transcribed mCFTR transcripts was performed by the addition of 2.5 μCi of [α-32P]dCTP to the PCR reactions. The resulting PCR fragments were analyzed by autoradiography after electrophoresis through 5% polyacrylamide gels. This analysis revealed low levels of expression in tissues that appear to be negative by Northern analysis, including the pancreas and

FIG. 4. Southern analysis of the progeny of an interspecies backcross. DNAs from progeny of the cross (NFS/N × M. spretus) × C57BL/6J were typed for the presence of a BamHI restriction fragment length polymorphism that was detected between M. spretus and M. musculus (C57BL/6J and NFS/N). M. spretus CFTR is characterized by the presence of a 12.2-kb BamHI fragment, while the C57BL/6J or NFS/N CFTR gene is identified by a 13.5-kb BamHI fragment. Lanes 1–4 are four representative progeny from the (NFS/N × M. spretus) × C57BL/6J cross, which contain either the M. musculus (lanes 1 and 3) or the M. musculus and M. spretus (lanes 2 and 4) CFTR BamHI fragments. The positions of λ SmaI/HindIII marker fragments are indicated.
FIG. 5. Northern analysis of mCFTR expression. Total tissue RNAs isolated from an adult male C57BL/6J mouse were electrophoresed through 1.1% agarose, 2.2 M formaldehyde gels as described (Lehrach et al., 1977). After transfer of the RNA samples to nitrocellulose, the filter was hybridized with a mCFTR exon 10 PCR probe. The positions of the 18 S and 28 S ribosomal RNAs are indicated.

liver (Fig. 6C). Mouse CFTR transcripts were also detected as early as Embryonic Day 13 (E13), which is the earliest developmental stage examined to date (Fig. 6C).

DISCUSSION

The CF gene product has two NBDs that share a high degree of homology with the NBDs of various vertebrate, invertebrate, and even prokaryotic membrane proteins (Riordan et al., 1989), indicating the functional significance of this region. On this basis, we hypothesized that the first NBD of mCFTR should share significant homology with hCFTR, especially within the region flanking the Phe\textsuperscript{508} residue, which is deleted in a majority of CF patients. Our approach to generate a probe for detection of mCFTR was to amplify CFTR exon 10, which encodes the Phe\textsuperscript{508} residue and surrounding NBD sequences, from human DNA using primers predicted from the human cDNA sequence. Using a PCR-generated hCFTR probe, it was determined that the human and mouse CF genes share significant homology within exon 10, and this species cross-hybridization was used to isolate a mouse genomic fragment containing mCFTR exon 10 and flanking intron sequences. The mCFTR exon 10 homologue shares a high degree of sequence homology with hCFTR exon 10, while the absence of other mCFTR exon sequences in the isolated genomic clone is consistent with the organization of hCFTR (Rommens et al., 1989).

In man, the CF locus (CFTR) has been mapped to the long arm of chromosome 7, band q31 (Tsui, 1989). Other closely linked loci, including the c-met proto-oncogene locus (MET), have been identified (Cutting et al., 1989) and were instrumental in the identification of the cystic fibrosis gene (Rommens et al., 1989). Homologues of various genes in this region of human chromosome 7 are known to map to mouse Chr 6, including Met (Lyon and Kirby, 1991, Hillyard et al., 1991). Our somatic cell hybrid mapping data revealed that the mouse genomic clone, which was isolated on the basis of its homology to the human CFTR gene, is part of a genetic locus on mouse Chr 6. In addition to the sequence homology between hCFTR and the isolated mouse clone, this result indicated that the isolated genomic fragment was derived from the mouse CFTR homologue. This conclusion is further strengthened by the interspecies back-
cross that revealed that the murine *Met* locus is closely linked to the locus on mouse Chr 6 from which the isolated mouse genomic fragment was obtained. Thus, the genetic mapping provides strong evidence that the isolated genomic fragment was derived from the murine CFTR homologue and has extended the information on the comparative mapping of these regions in man and mouse.

Northern (RNA) analysis of adult mouse tissues revealed a 6- to 7-kb mRNA that is expressed in some of the same sites as hCFTR, including lung. The size and location of this transcript are similar to those observed for hCFTR and are consistent with the conclusion that the isolated mouse genomic fragment is derived from the mCFTR homologue. This same analysis, however, indicated that mCFTR and hCFTR do not share an identical tissue distribution; although hCFTR and mCFTR are both expressed in lung, intestine, and stomach, there are notable differences. While hCFTR mRNA is readily detected in pancreas and liver (Riordan et al., 1989), mCFTR transcripts could not be detected in these tissues by Northern analysis. It was also previously reported by Tata et al. (1991) that mCFTR is not expressed in mouse liver. Upon further examination, however, we found that mCFTR transcripts are present in mouse liver and pancreas, but at levels that can be detected only by sensitive PCR analysis. This weak expression observed in some tissues, such as liver or pancreas, may reflect expression in a subpopulation of cells. The availability of mCFTR probes will allow this possibility to be examined by in situ hybridization studies. In addition, the initial analyses conducted by Riordan et al. (1989) suggested that hCFTR is not expressed in the kidney, although their subsequent experiments revealed hCFTR kidney transcripts with variability between individual samples. Kidney mCFTR transcripts were observed in the present study, as well as by Tata et al. (1991). The observed variability in kidney hCFTR mRNA levels may reflect normal differences due to the age of the individuals from whom the samples were obtained or allelic variations in tissue-specific cis- or trans-acting regulatory elements or factors. With the availability of isolated mCFTR sequences, it will be possible to determine the developmental expression of mCFTR to address the effect of age on the variability of expression that has been observed in human kidney.

The results of this study, as well as those of Tata et al. (1991) and Yorifugi et al. (1991), have demonstrated the existence of a mouse CFTR homologue that is very similar to hCFTR, which is a prerequisite for the production of various mouse CF mutants through gene targeting. The availability of a mouse genomic fragment containing the coding region that is analogous to hCFTR exon 10 creates the potential for targeted mutagenesis of this region of the mouse CFTR gene.

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