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Aminoglycoside suppression of a premature stop mutation in a *Cftr*^{-/-} mouse carrying a human *CFTR*-G542X transgene

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Abstract Cystic fibrosis (CF) is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein. Since ~5% of all mutant *CF* alleles are stop mutations, it can be calculated that ~10% of CF patients carry a premature stop muta-

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tion in at least one copy of the *CFTR* gene. Certain ethnic groups, such as the Ashkenazi Jewish population, carry a much higher percentage of CF stop mutations. Consequently, a therapeutic strategy aimed at suppressing this class of mutation would be highly desirable for the treatment of this common genetic disease. We have shown previously that aminoglycoside antibiotics can suppress premature stop mutations in the *CFTR* gene in a bronchial epithelial cell line [Nat Med (1997) 3:1280]. To address whether aminoglycosides can suppress a *CFTR* premature stop mutation in an animal model, we constructed a transgenic mouse with a null mutation in

the endogenous *CFTR* locus (*Cftr*^{-/-}) that also expressed a human *CFTR*-G542X cDNA under control of the intestinal fatty acid binding protein promoter. We then investigated whether the daily administration of the aminoglycoside antibiotics gentamicin or tobramycin could restore the expression of a detectable level of CFTR protein. Immunofluorescence staining of intestinal tissues from *Cftr*^{-/-} *hCFTR*-G542X mice revealed that gentamicin treatment resulted in the appearance of hCFTR protein at the apical surface of the glands of treated mice. Weaker staining was also observed in the intestinal glands following tobramycin treatment. Short-circuit current measurements made on intestinal tissues from these mice demonstrated that a significant number of positive cAMP-stimulated transepithelial chloride current measurements could be observed following gentamicin treatment ($P=0.008$) and a near significant number following tobramycin treatment ($P=0.052$). When taken together, these results indicate that gentamicin, and to a lesser extent tobramycin, can restore the synthesis of functional hCFTR protein by suppressing the *hCFTR*-G542X premature stop mutation in vivo.

Keywords Aminoglycoside · Cystic fibrosis · *CFTR*-G542X transgene · Premature stop mutation

Introduction

Cystic fibrosis (CF) is the most prevalent autosomal recessive disorder in the Caucasian population, affecting 1 in 2,500 newborns. According to the CF Genetic Consortium [1], approximately 1,000 unique mutations have been identified in (or near) the *CFTR* gene. However, the most common CF mutation, a deletion of the phenylalanine residue at position 508 (*CFTR*- Δ F508), represents ~70% of all CF mutations. The *CFTR*-G542X premature stop mutation, the second most common mutation, represents ~3.4% of all CF mutations. The CFTR protein functions as an apical cAMP-regulated chloride channel in epithelial cells. The loss of CFTR activity in CF patients is associated with mucous obstruction and inflammation of airways, intestinal malabsorption, and blockage of pancreatic and intestinal ducts. Mortality in CF patients is mainly due to recurrent pulmonary infections [2].

Over the last several years, a number of mouse models containing null mutations in the *CFTR* gene have been produced [3, 4, 5]. These mouse models exhibited both the characteristic chloride channel defect and much of the pathology observed in humans with this disease, including a lack of cAMP-stimulated chloride channel activity in nasal and intestinal tissues, goblet cell hyperplasia, and intestinal obstruction and perforation during the first month of life. In general, the absence of CFTR in most *Cftr*^{-/-} mouse models does not result in significant lung disease. Although genetic modifier loci have been shown to influence the severity of CF-associated lung disease in some mice [6], most studies using these mouse models have focused on the intestinal pathology.

Pharmacologic agents such as aminoglycoside antibiotics have been shown to reduce the efficiency of translation termination at premature stop mutations in eukaryotes [7, 8, 9, 10, 11]. It has been shown that this decrease in translational fidelity can restore the translation of full-length CFTR protein in cultured mammalian cells carrying a premature stop codon in the *CFTR* gene [12, 13]. This suggests that a pharmacologic approach aimed at suppressing stop mutations may represent a viable therapeutic treatment for a genotypic subset of patients with diseases such as CF.

In the present study, we asked whether aminoglycoside treatment could restore the expression of the human *CFTR* (*hCFTR*) gene in a CF mouse model system. To do this, a transgenic mouse expressing a *hCFTR* cDNA containing the G542X stop mutation under control of the rat intestinal fatty-acid binding protein (*FABP*) promoter was constructed. We then examined the ability of aminoglycosides to induce hCFTR expression in *Cftr*^{-/-} *hCFTR*-G542X mice. We found that daily administration of the aminoglycosides gentamicin or tobramycin can induce a low level of hCFTR protein expression and function in the intestinal glands of these mice. These results provide evidence that gentamicin (and to a lesser extent, tobramycin) can suppress the *hCFTR*-G542X mutation in vivo.

Materials and methods

Plasmid construction

The plasmid containing *hCFTR*-G542X under control of the rat *FABP* promoter was derived from a *FABP*-*hCFTR*-WT plasmid [14]. The G542X premature stop mutation was introduced by the direct exchange of a 3043 bp *BspE1*/*NcoI* fragment from pDB436, yielding the *FABP*-*CFTR*-G542X plasmid pDB488. In addition to the G542X mutation, this plasmid contains an additional *Sall* restriction site that was introduced at codon 764 of the *CFTR* cDNA. This new restriction site does not alter the amino acid sequence of the CFTR protein.

Generation of transgenic mice

A 1.2 kb DNA fragment containing the rat intestinal fatty-acid binding protein (*FABP*) promoter (kindly provided by Jeffrey Gordon, Washington University, St. Louis) was used to direct expression of the human *CFTR* cDNA containing the G542X mutation to the intestinal epithelial cells of mice using methods previously described [14]. The chimeric *FABP*-*hCFTR*-G542X gene construct was microinjected into fertilized oocytes. These oocytes were then implanted into pseudopregnant foster mothers to produce transgenic mice in an FVB/N background. The *FABP*-*hCFTR*-G542X transgene was detected by Southern analysis in founder mice using a 2.54 kb *Bam*HI fragment from the *hCFTR* gene as a probe. Mice that carried the transgene were bred with wild-type C57BL/6 J mice, and progeny with the transgene were identified by PCR. Animals that expressed the *FABP*-*hCFTR*-G542X transgene were then bred with heterozygous *Cftr*^{+/+}/*Cftr*^{tm1Cam} mice (hereafter referred to as *Cftr*^{+/-} mice). The *Cftr*^{+/-} mice carried the *HPRT* gene inserted into exon 10 of the mouse *Cftr* gene [15]. The original ES cell line used to generate the *Cftr*^{tm1Cam} allele, TG4, contained multiple copies of a provirus containing a *Neo*^R cassette that was not linked to the *Cftr* locus (R. Ratcliff, personal communication). Animals that contained single copies of both the

Cftr^{tm1Cam} allele and the *FABP-hCFTR-G542X* transgene were interbred until the *Neo^R* marker was eliminated and the progeny were homozygous for the *FABP-hCFTR-G542X* transgene. *Cftr*^{+/-} mice that were homozygous for the *FABP-hCFTR-G542X* transgene were then intercrossed to generate the desired *Cftr*^{-/-} progeny carrying the transgene. Animals with this genotype were obtained at the expected 25% frequency. In some experiments, *Cftr*^{-/-} mice expressing a wild type human *CFTR* transgene under *FABP* promoter control (*FABP-hCFTR-WT*) were used [14].

DNA was extracted from tail snips using a Puregene DNA isolation kit (Gentra Systems) to determine genotypes. Amplification of the *FABP-hCFTR-G542X* transgene DNA was carried out using the primers DB985 (5'-CAAGATAGAA AGAGGACAGT TGTT-3') and DB986 (5'-TTGAGGGTTG ACATAGGTGC TTGAA-3'). This primer pair generated a 1557 bp fragment containing both the G542X mutation and the new *SalI* site. The primers used to amplify the wild type murine *Cftr* gene were DB987 (5'-TCCTGAT-GTT GATTTGGGA-3') and DB988 (5'-TGGCTGTCTG CTTCTGACT ATG-3'), which amplified a 350 bp fragment. Primers used to detect the *Cftr^{tm1cam}* mutant allele were DB989 (5'-TGCCGACCCG CAGTCCCAGC GTCG-3') and DB990 (5'-CGTGGGGTC CTTTACCA GC -3'), which amplified a 450 bp fragment.

mRNA expression in intestinal tissue

To determine whether the *FABP-CFTR-G542X* transgene was expressed, mRNA was isolated from intestinal tissues of *FABP-hCFTR-G542X* or *FABP-hCFTR-WT* transgenic mice. Avian reverse transcriptase was used to synthesize *hCFTR* cDNA, which was then PCR-amplified using the primers DB985 and DB986. The *hCFTR-G542X* PCR product was digested with the restriction enzyme *SalI* to demonstrate that the predicted 954 and 603 bp fragments were produced.

Pathology

Mice were humanely euthanized by administration of pentobarbital sodium (Abbott Labs) to achieve deep surgical anesthesia, followed by exsanguination. All major organs were examined for gross lesions and fixed by submersion in alcoholic formalin (10% formalin in 70% ethanol). Sections (5 μ m) of paraffin-embedded tissues were stained by standard methods with hematoxylin and eosin or periodic-acid Schiff method, and subsequently evaluated histopathologically in a blinded manner.

Aminoglycoside treatment

Aminoglycoside treatment of homozygous *Cftr*^{-/-} mice carrying the *FABP-hCFTR-G542X* transgene was initiated 16 days after birth (1 week before weaning). Whenever possible, littermates were divided into aminoglycoside treatment and control groups. The treatment group was given a single subcutaneous injection of 34 μ g/g body weight of either gentamicin or tobramycin each day. To determine the distribution of this dose, the serum gentamicin concentration of treated mice was determined by fluorescence polarization immunoassay (FPIA). Two control groups of mice were also examined. One group was maintained without any treatment, while a second group was treated with 0.4 mg/ml sulfamethoxazole and 0.08 mg/ml trimethoprim (smx/tmp) in the drinking water (Elkins-Sinn). This latter control group was included to ensure that any increase in survival observed in aminoglycoside treated animals was not due solely to an antibiotic effect. All mouse pups were weaned 23 days after birth.

Short-circuit current measurement

Intestinal tissues were harvested for short-circuit current (I_{sc}) measurements from ~100 day old mice. Tissue segments approximate-

ly 5 mm in length from jejunum or ileum were mounted as a flat sheet in a modified Ussing chamber (area of ~0.16 cm²) and I_{sc} recordings were made as previously described [16]. The mucosal bathing solution (37°C; pH 7.4) contained 150 mM Na⁺, 5 mM K⁺, 129.8 Cl⁻, 1.2 mM Ca²⁺, 1.2 mM Mg²⁺, 25 mM HCO₃⁻ and 4.2 mM PO₄. The serosal surface of the tissue was bathed in a Ringer's solution (37°C; pH 7.4) containing 145 mM Na⁺, 5 mM K⁺, 124.8 mM Cl⁻, 1.2 mM Ca²⁺, 1.2 mM Mg²⁺, 25 mM HCO₃⁻, 4.2 mM PO₄, and 10 mM glucose. Tetrodotoxin (TTX) 1.54 \times 10⁻³ μ M) was added to the serosal side of the tissue to block Na⁺ channels, which are activated by action potentials. Forskolin (5 μ M) was added to both the mucosal and serosal solutions while the I_{sc} was continuously monitored. Apical glucose was omitted to exclude electrogenic Na⁺ transport pathways [17]. In control experiments, glibenclamide was found to block the forskolin-activated I_{sc} in *Cftr*^{+/-} mice carrying the *hCFTR-G542X* transgene.

Immunofluorescence

The mice used for immunolocalization experiments were 100–150 days old. Immediately following sacrifice, intestinal tissue was placed in a cryomold (Miles Laboratories, Naperville, Ill.) containing OCT embedding medium. Samples were flash-frozen by immersion into a metal cup filled with 2-methyl butane that had been pre-chilled with liquid nitrogen. Frozen blocks were then sectioned using a cryostat. Five μ m sections were collected and fixed in 3% formaldehyde in PBS for 45 min at room temperature. After blocking with 25–50% normal goat serum for 20 min at room temperature to reduce non-specific antibody binding, samples were incubated with a 1/200 dilution of CFTR antibody no. 4562. The antigen used to raise this polyclonal rabbit antibody included CFTR NBD1 and a portion of the R domain (amino acids 521–828) fused to the *Escherichia coli* TrpE protein [18]. After incubation with this antibody for 1 h at 37°C, the samples were washed with four changes of PBS and again blocked with 25–50% normal goat serum for 20 min at room temperature. The samples were then incubated for 1 h at 37°C with 25 μ g/ml goat anti-rabbit IgG conjugated to AlexaFluor-488 (Molecular Probes no. A-11001). Finally, samples were washed twice with PBS and incubated with 20 μ g/ml Hoechst 33258 for 4 min at room temperature. To confirm the specificity of the signal obtained, samples were incubated with pre-immune rabbit serum in place of the CFTR-specific serum using the same protocol.

In vitro transcription/translation

A modified form of a readthrough reporter system previously used to monitor the suppression of stop mutations by aminoglycosides [10] was used to determine whether the aminoglycosides gentamicin and tobramycin could suppress the *CFTR-G542X* mutation in an in vitro rabbit reticulocyte lysate translation system (Promega TNT system). RNA synthesized from these constructs was driven from the *SP6* promoter. The transcribed reporter gene consists of three major components: an initial open reading frame encoding a 25 kDa protein; a readthrough cassette that contains the stop mutation and surrounding sequence context to be tested for suppression; and an open reading frame downstream of the readthrough cassette that encodes another 10 kDa of protein. In this study, the readthrough cassette contained the *CFTR-G542X* premature stop mutation and six upstream and downstream codons from the *hCFTR* gene. A DNA fragment containing these 13 codons was introduced into the *Bam*HI and *Hind*III sites of the readthrough cassette region of pDB654 by ligation of the annealed oligonucleotides DB809 and DB810. The resulting plasmid, pDB664 was then expressed in a mammalian translation system in the presence or absence of aminoglycosides. Translation products were monitored by the incorporation of ³⁵S-methionine incorporation into protein followed by SDS-PAGE. Efficient translation termination at the G542X stop mutation produced a 25 kDa protein, while suppression of the stop codon resulted in the synthesis of a 35 kDa protein. PhosphorImager (Molecular Dynamics) analysis was used

to determine the relative abundance of each translation product. Suppression of the G542X mutation was expressed as percent readthrough, which was calculated as the [35 kDa protein / (25 kDa + 35 kDa proteins)] × 100.

Results

Genetic characterization of a *Cftr*^{-/-} mouse expressing the *hCFTR*-G542X transgene

The objective of this study was to determine whether the aminoglycosides gentamicin or tobramycin can suppress a *hCFTR* premature stop mutation in an animal model. Since the *Neo^r* cassette frequently used as a selectable marker in knockout mice inactivates many aminoglycosides, this selectable marker had the potential to interfere with our study. As a result, we bred *FABP-hCFTR*-G542X mice with *Cftr*^{+/-} mice that carried the *HPRT* gene inserted in exon 10 of the mouse *Cftr* gene [4, 15]. Male and female *Cftr*^{+/-} mice that were homozygous for the *FABP-hCFTR*-G542X transgene were produced and used as breeders for all subsequent experiments. From these breeder pairs, offspring homozygous for both the *Cftr*^{tm1Cam} allele and the *FABP-hCFTR*-G542X transgene were obtained at a frequency of 25%.

To confirm the presence of the *FABP-hCFTR*-G542X transgene in these animals, a PCR reaction with *hCFTR*-specific primers was carried out using genomic DNA isolated from both the *FABP-hCFTR*-G542X and *FABP-hCFTR*-WT transgenic mice. A PCR product of 1,557 base pairs (bp) was obtained, indicating that the transgene containing the *hCFTR* cDNA was present. As a unique identifier, the *hCFTR*-G542X transgene also contained a new *SalI* restriction site polymorphism that does not alter the amino acid sequence of the *hCFTR* protein. Digestion of the PCR products with *SalI* produced the expected restriction fragments of 954 and 603 bp from the PCR product from the *hCFTR*-G542X mouse, but not from the *hCFTR*-WT mouse. This result confirmed the presence of the *FABP-hCFTR*-G542X transgene.

We next analyzed the expression of the *FABP-hCFTR*-G542X transgene. Since the *FABP* promoter is expressed predominantly in intestine [14], mRNA was isolated from intestinal tissues from either *hCFTR*-G542X or *hCFTR*-WT transgenic mice. RT-PCR was then carried out using the same primers as above. As can be seen in Fig. 1, a 1,557 bp fragment could be detected in both the *hCFTR*-G542X and *hCFTR*-WT transgenic mice. In addition, digestion of the cDNA fragment generated by RT-PCR with *SalI* site again confirmed that a *SalI* site was present exclusively in the mouse line carrying the *hCFTR*-G542X transgene. These results confirmed that the *FABP-hCFTR*-G542X construct is expressed in intestinal tissue from the *FABP-hCFTR*-G542X mice.

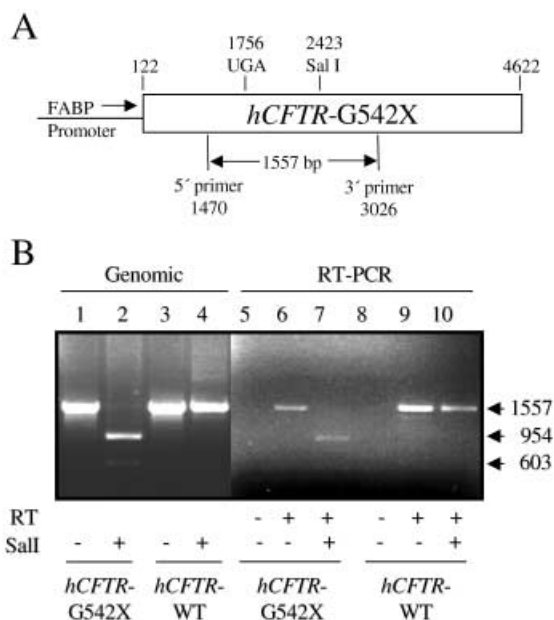
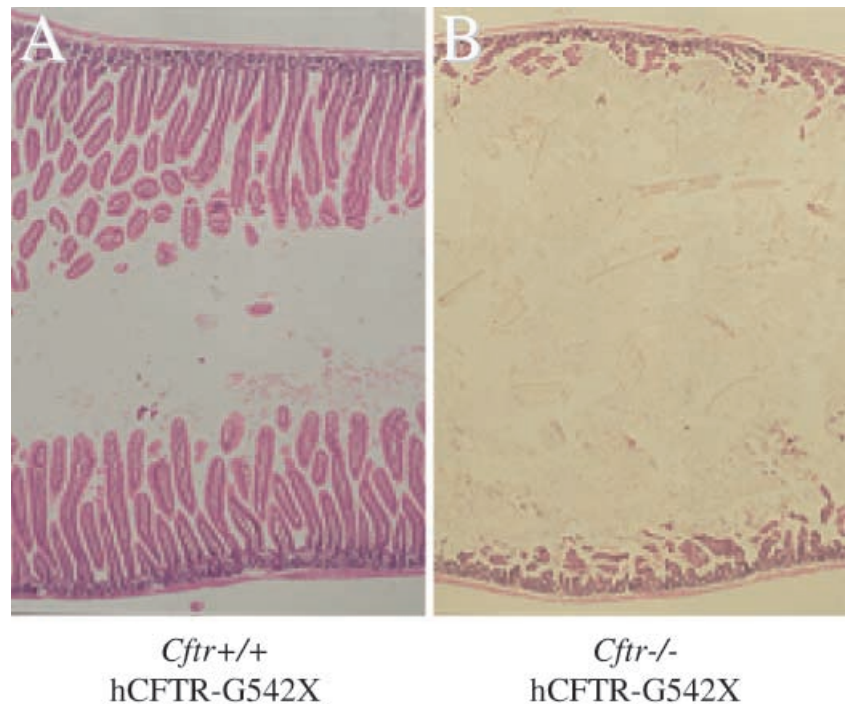


Fig. 1A, B Generation of *Cftr*^{-/-} *FABP-hCFTR*-G542X mice. **A** Schematic diagram of the *FABP-hCFTR*-G542X construct. **B** PCR from genomic DNA showing the presence of the *hCFTR*-G542X transgene (left) and an RT-PCR showing that the *hCFTR*-G542X transgene is expressed in intestinal tissues (right). See text for further details

Pathologic characterization of *Cftr*^{-/-} mice expressing the *hCFTR*-G542X transgene

Mice homozygous for both the *Cftr*^{-/-} allele and the *FABP-hCFTR*-G542X transgene were generally smaller than their heterozygous littermates, as observed in various *Cftr*^{-/-} mouse models [19]. It was previously shown that *Cftr*^{-/-} mice exhibit little or no lung pathology, possibly due to the presence of other chloride channels that can functionally substitute for CFTR in the lung epithelium or the paucity of submucosal glands in the mouse airways. While the survival of the *Cftr*^{-/-} *hCFTR*-G542X mice was generally high during the first few weeks after birth, we found that most of these animals died in the days following weaning. This finding was consistent with prior studies that also found that *Cftr*^{-/-} mice frequently experience intestinal blockage shortly after weaning [3, 4, 5]. To determine the primary cause of death of the *Cftr*^{-/-} *hCFTR*-G542X mice, a comprehensive histopathologic examination was carried out on several animals. In particular, our attention focused on comparing healthy and sick *Cftr*^{-/-} *hCFTR*-G542X mice in the days following weaning. In sick mice, we found that most tissues were normal. However, examination of the intestinal tract frequently revealed severe distension of much of the small intestine by ingesta, severe thinning of the intestinal wall, distension of the crypts by PAS positive mucus in the distal half of the small intestine, compression and loss of the villi in the lower jejunum and upper ileum, and impaction of feces in the ileum and proximal large intestine. Some of these findings are il-

Fig. 2 Histopathology observed in the jejunum of: **A** a *Cftr*^{+/+} mouse carrying the *hCFTR*-G542X transgene, and **B** an ill *Cftr*^{-/-} mouse carrying the *hCFTR*-G542X transgene. $\times 30$ magnification



illustrated in Fig. 2. A similar analysis of *Cftr*^{+/+} *FABP*-*hCFTR*-G542X littermates found that all tissues were normal. These results confirmed that the *cftr*^{-/-} *FABP*-*hCFTR*-G542X mice, like other *Cftr*^{-/-} mouse models, frequently experience intestinal blockage. However, little or no intestinal pathology was observed in *Cftr*^{-/-} *hCFTR*-G542X mice that were not ill, other than a minimal retention of mucus in the crypts of the small and large intestine (data not shown).

Determination of the serum gentamicin levels following subcutaneous gentamicin injection

In a previous study, the subcutaneous injection of gentamicin at a concentration of 34 $\mu\text{g/g}$ body weight was used to show that aminoglycoside treatment could suppress a premature stop mutation in the *Dystrophin* gene [20]. In preliminary experiments, we found that the subcutaneous administration of gentamicin at a similar dose was well tolerated in the *FABP*-*hCFTR*-G542X mice. To determine the serum level of gentamicin produced by this treatment, we carried out subcutaneous injections of gentamicin and collected serum samples at various times post injection (Fig. 3). Fluorescence polarization immunoassay measurements indicated that the serum concentration of gentamicin reached a level of 64 $\mu\text{g/ml}$ within 27 min of injection, and then dropped rapidly to ~ 20 $\mu\text{g/ml}$ by 40 min after injection. Since this dose and route of administration produced a robust serum level of gentamicin without causing any detectable side effects, it was used throughout the remainder of our study.

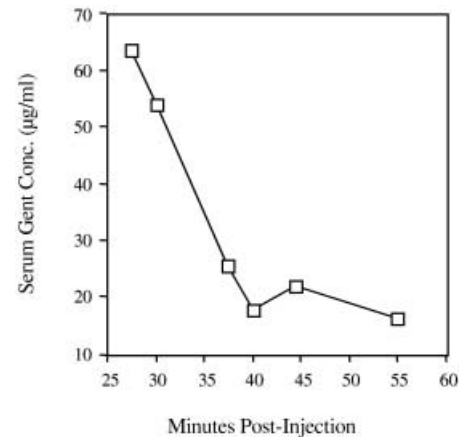


Fig. 3 Serum gentamicin levels following subcutaneous injection. A single blood sample was taken from different mice at the indicated times following the injection of 34 $\mu\text{g/g}$ gentamicin. The gentamicin concentration in each serum sample was determined by fluorescence polarization immunoassay (FPIA)

Effect of aminoglycoside treatment on the survival of *Cftr*^{-/-} *hCFTR*-G542X mice

We first asked whether the subcutaneous administration of the aminoglycosides increased the survival of *Cftr*^{-/-} mice carrying the *hCFTR*-G542X transgene. To do this, the survival of untreated animals was compared to animals that received a daily subcutaneous injection of 34 $\mu\text{g/g}$ gentamicin or tobramycin. As a control for any change in survival of these animals caused by antibacterial effects rather than suppression of the *hCFTR*-G542X stop mutation, we also treated a control group of mice

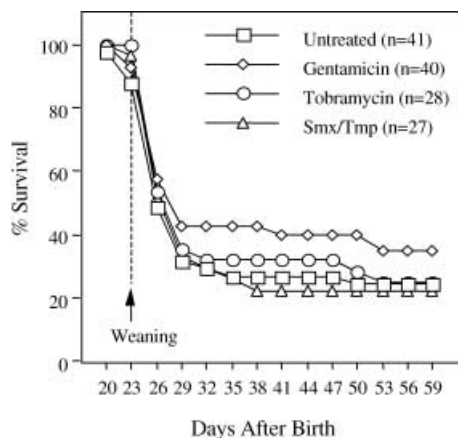


Fig. 4 Survival of homozygous *Cftr*^{-/-} *hCFTR*-G542X mice. Survival (indicated in days after birth) of mice that were untreated, gentamicin-treated, tobramycin-treated, or smx/tmp-treated is shown. Weaning was carried out on day 23

with the non-aminoglycoside antibiotics sulfamethoxazole and trimethoprim (smx/tmp) in the drinking water.

We found that the survival of untreated *Cftr*^{+/-} mice was similar to their wild type siblings, with >95% surviving well beyond weaning (data not shown). Groups of both treated and untreated *Cftr*^{-/-} *hCFTR*-G542X mice had a high rate of survival up to 20 days after birth (Fig. 4). However, following weaning on day 23, the survival of each group decreased precipitously. As described above, morphologic examination revealed that the decrease in survival following weaning resulted from intestinal obstruction. By day 26 (3 days after weaning), 50–60% survival was observed in each group. By day 38 (15 days after weaning), only 27% of the untreated mice survived, while the groups treated with smx/tmp and tobramycin showed 22% and 32% survival, respectively. Only the gentamicin treated group exhibited what appeared to be a trend toward increased survival (43%). This trend was maintained in the gentamicin treated group through day 60, but never became large enough to attain statistical significance. The survival of all groups stabilized by 2 weeks after weaning, and several animals from treated and untreated groups survived a year or longer.

hCFTR activity is detected in *Cftr*^{-/-} *hCFTR*-G542X mice following aminoglycoside treatment

The CFTR protein is a cAMP-activated chloride channel that facilitates transepithelial chloride conductance following its activation by cAMP agonists. In isolated intestinal tissue from the ileum or jejunum, this activity can be measured as I_{sc} following the addition of forskolin to raise the intracellular cAMP level. In intestinal tissue from untreated *Cftr*^{+/-} *hCFTR*-G542X mice, we observed a strong increase in transepithelial chloride current that ranged from 6.2 to 26.6 $\mu\text{A}/\text{cm}^2$ following forskolin addition, demonstrating the presence of endogenous CFTR activity in these heterozygous animals. When in-

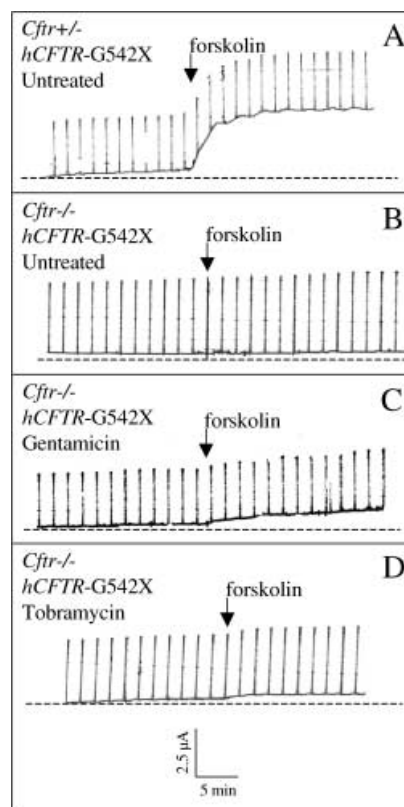


Fig. 5A–D Representative forskolin-stimulated I_{sc} tracings from mouse intestinal tissues. **A** Tracing from the ileum of a heterozygous *Cftr*^{+/-} mouse carrying the *hCFTR*-G542X (without aminoglycoside treatment). **B** Tracing from the ileum of a homozygous *Cftr*^{-/-} *hCFTR*-G542X mouse (without aminoglycoside treatment). **C** Tracing from the ileum of a homozygous *Cftr*^{-/-} *hCFTR*-G542X (following gentamicin treatment). **D** Tracing from the ileum of a homozygous *Cftr*^{-/-} *hCFTR*-G542X (following tobramycin treatment). The area of the Ussing chamber was 0.16 cm^2 . Scale depicts the current/time relationship

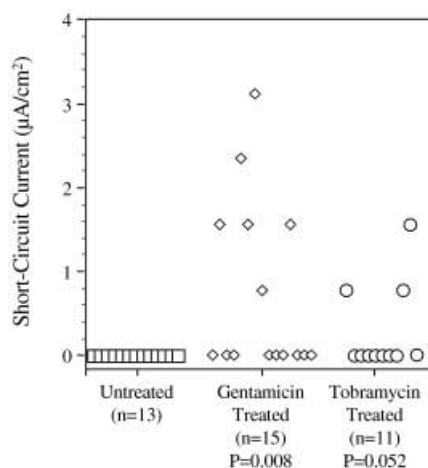
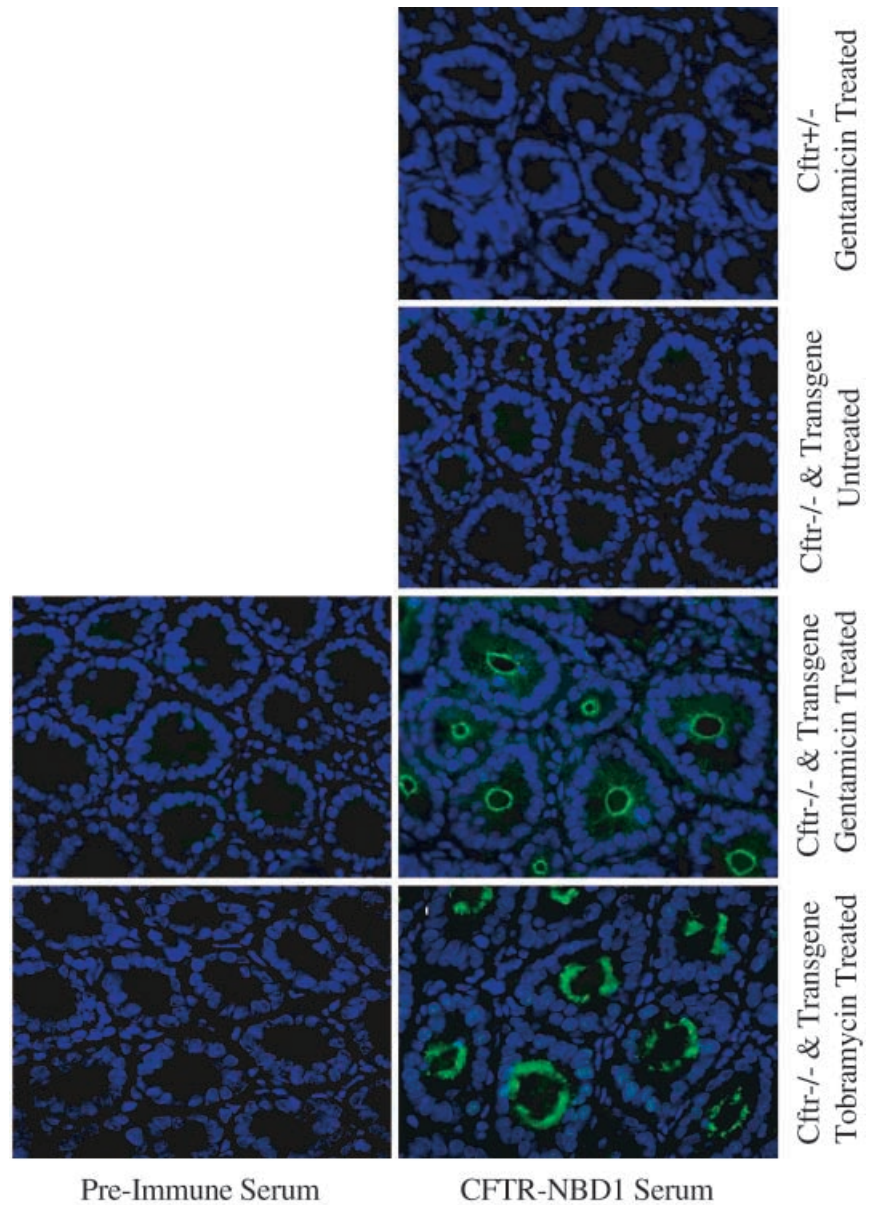


Fig. 6 Summary of I_{sc} data obtained in untreated, gentamicin-treated, and tobramycin-treated mice. I_{sc} values were calculated based on the 0.16 cm^2 surface area of the Ussing chamber. The “n” value represents the total number of samples assayed under each condition

Fig. 7 CFTR immunofluorescence in intestinal tissues. Samples from the duodenum of homozygous *Cftr*^{-/-} *hCFTR*-G542X mice (harvested from untreated, gentamicin-treated, or tobramycin-treated animals) were incubated with either pre-immune serum or CFTR-NBD1 serum. Similar samples from heterozygous *Cftr*^{+/-} animals that lacked the *hCFTR*-G542X transgene were also examined following gentamicin treatment. After incubation of the sample with a fluorescent secondary antibody, the samples were visualized by fluorescence microscopy. Other details are described in the Materials and methods



intestinal tissue from untreated *Cftr*^{-/-} *hCFTR*-G542X mice was examined following forskolin treatment, no change in the transepithelial chloride current was detected in 13 independent samples (Figs. 5, 6). These results demonstrated the specificity of the I_{sc} measurement as a measure of CFTR activity in this animal model.

We next examined the effect of gentamicin or tobramycin treatment on cAMP-stimulated transepithelial chloride current. In *Cftr*^{-/-} *hCFTR*-G542X mice treated with gentamicin, we observed a modest increase in I_{sc} upon forskolin addition in 6 of 15 samples. The magnitude of these currents ranged from 0.8 to 3.1 $\mu\text{A}/\text{cm}^2$. This number of positive responses observed with tissues from gentamicin treated mice was statistically significant ($P=0.008$). In *Cftr*^{-/-} *hCFTR*-G542X mice treated with tobramycin, we observed an I_{sc} increase in 3 of 11 samples following forskolin addition, which approached sta-

tistical significance ($P=0.052$). However, the magnitude of these responses was somewhat weaker, with I_{sc} ranging from 0.8 to 1.5 $\mu\text{A}/\text{cm}^2$. These results suggest that both gentamicin and tobramycin can restore a low level of hCFTR activity in *Cftr*^{-/-} *hCFTR*-G542X mice.

hCFTR protein can be detected in intestinal glands following aminoglycoside treatment

Since our functional analysis detected the presence of cAMP-activated I_{sc} currents in intestinal tissues of *Cftr*^{-/-} *hCFTR*-G542X mice following aminoglycoside treatment, we next asked whether the hCFTR protein could be detected. To do this, an immunofluorescence assay using an hCFTR-specific polyclonal antiserum was carried out (Fig. 7). In gentamicin-treated *Cftr*^{-/-} *hCFTR*-G542X

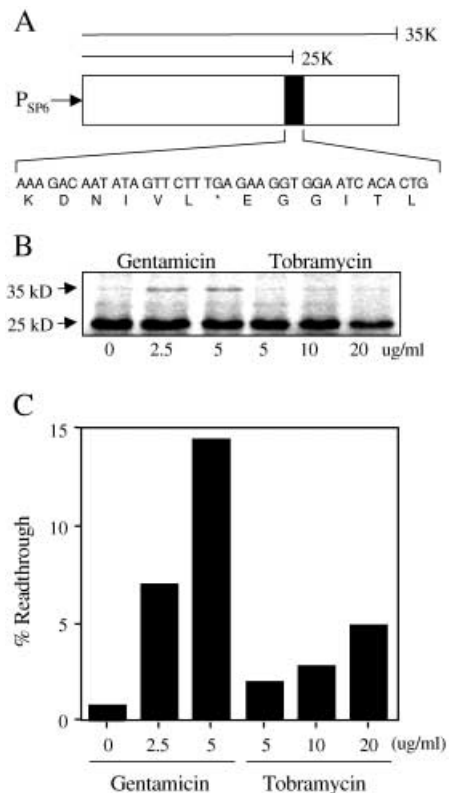


Fig. 8A–C In vitro translation reactions with gentamicin and tobramycin. **A** Schematic of readthrough reporter construct and the *hCFTR* sequence surrounding the premature stop mutation. **B** Example of gentamicin- and tobramycin-mediated suppression of the *hCFTR*-G542X stop mutation. **C** Quantitation of data shown in B

mice, we found that the hCFTR antiserum could detect hCFTR protein in the apical epithelium of the submucosal glands of the duodenum, along with a weaker signal in the mucosal glands. Similar results were obtained with tobramycin-treated mice, although the signal observed in the apical epithelium of the submucosal and mucosal glands was weaker and more irregular than was observed in tissue from gentamicin-treated mice. No signal was observed in the epithelium of either the crypts of Lieberkuhn or the intestinal villi. Furthermore, a positive signal was not observed in tissues from gentamicin-treated *Cftr*^{-/-} *hCFTR*-G542X mice when a pre-immune serum was used, or when the hCFTR-specific antiserum was used on samples from mice that had not been treated with gentamicin. We also confirmed that a specific signal was not observed in *Cftr*^{+/-} mice that did not carry the *hCFTR*-G542X transgene following gentamicin treatment. This demonstrated that the hCFTR-specific signal was not an artifact of gentamicin treatment, and also confirmed that the antibody could detect the human, but not mouse, CFTR protein. When taken together with the functional data presented above, these results indicate that treatment of *Cftr*^{-/-} *hCFTR*-G542X mice with either gentamicin or tobramycin can promote the synthesis of full-length, functional CFTR that localizes to the apical epithelium of the submucosal and mucosal glands of this transgenic mouse.

Previous studies have shown that both the termination codon and the following nucleotide (referred to as the tetranucleotide termination signal) determine the susceptibility of a translation termination signal to aminoglycoside-mediated suppression [10, 11]. We previously reported that tobramycin is unable to induce readthrough of a UGAC tetranucleotide termination signal in an in vitro system [10]. More recently, we found that while tobramycin cannot induce readthrough at the UGAC and UGAA tetranucleotide signals, it can induce weak readthrough at other tetranucleotide termination signals in an in vitro translation system [21]. To more directly determine the ability of gentamicin and tobramycin to suppress the *hCFTR*-G542X mutation, we constructed a readthrough reporter construct for use in an in vitro translation system that contains the *hCFTR*-G542X stop mutation and six flanking codons upstream and downstream of the premature stop mutation (Fig. 8). When in vitro translation reactions were carried out with this construct, we found that increasing amounts of gentamicin induced as much as 14% readthrough, while tobramycin induced ~5% readthrough. These results confirm that the UGAG tetranucleotide signal is susceptible to suppression by both gentamicin and tobramycin, although tobramycin is ~3-fold less effective than gentamicin.

Discussion

Several recent studies have suggested that the suppression of premature stop mutations may provide a viable therapeutic option for CF patients carrying premature stop mutations. For example, in vitro studies have shown that the aminoglycosides G418 and gentamicin can suppress premature stop mutations in the *CFTR* gene, as indicated by the restoration of both CFTR protein expression and cAMP-activated chloride channel activity in human cell lines [12, 13]. More recently, two clinical studies also provided evidence that the aminoglycoside gentamicin can suppress these *CFTR* premature stop mutations in affected patients. In the first study, a nasal potential difference (PD) assay was used to demonstrate that the topical administration of gentamicin to the nasal epithelium of CF patients with premature stop mutations in the *CFTR* gene produced a partial correction of transepithelial chloride transport [22]. A second clinical study confirmed these results using systemic gentamicin administration [23]. This latter study also demonstrated that gentamicin did not improve airway chloride transport in CF patients that lacked a *CFTR* premature stop mutation, showing that the aminoglycoside-induced appearance of CFTR activity only occurs when a premature stop mutation is present. This genotype-specific recovery of CFTR activity suggested that the partial restoration of CFTR activity is attributable to suppression of premature stop mutations located in the *CFTR* gene.

The objective of the current study was to construct a mouse model that would allow us to test more rigorously whether aminoglycosides can induce the expression of

full-length CFTR from a transgene containing the *hCFTR*-G542X premature stop mutation in vivo. The results obtained provide both biochemical and functional evidence that CFTR expression can be restored in this new mouse model, as indicated by the detection of both hCFTR protein and cAMP-activated chloride channel activity in intestinal tissues. To optimize our chance of detecting the appearance of hCFTR protein produced by suppression of the *hCFTR*-G542X mutation, we administered 34 $\mu\text{g/g}$ gentamicin by subcutaneous injection once daily. This dosing regimen produced a peak serum level of ~ 64 $\mu\text{g/ml}$, which is roughly 3-fold higher than the 16.4 to 21.0 $\mu\text{g/ml}$ range of peak serum levels obtained in a study of 2,184 patients administered a once daily intravenous dose of 7 mg/kg gentamicin [24]. Thus, while our study provides a good “proof of concept” that stop mutations can be suppressed by aminoglycosides in vivo, further studies are needed to systematically compare the efficacy of lower doses, as well as different administration schedules. However, it should be noted that a recent clinical study examined the ability of gentamicin administered at a dose of 2.5 mg/kg three times daily to suppress CFTR stop mutations [23], which resulted in peak serum gentamicin levels of 8–10 $\mu\text{g/ml}$. The results of that study demonstrated that a statistically significant trend toward correction of cAMP-stimulated chloride currents could be obtained in gentamicin-treated CF patients carrying stop mutations [23]. Thus, a limited amount of functional data already exists showing that clinically acceptable levels of gentamicin can suppress premature stop mutations in the *CFTR* gene in CF patients.

Our results also demonstrate for the first time that tobramycin is capable of weakly suppressing the *hCFTR*-G542X stop mutation in vivo. The *hCFTR*-G542X stop mutation is a UGAG tetranucleotide termination signal. Previous studies have shown that gentamicin can suppress the UGAG tetranucleotide termination signal in cell culture or in vitro translation systems [10, 11]. More recently, it was reported that tobramycin can also suppress a UGAG termination signal in an in vitro translation system, although much less efficiently than gentamicin [21]. These findings are consistent with the results of the present in vivo study. Both gentamicin and tobramycin were found to suppress the *hCFTR*-G542X stop mutation, although gentamicin appeared to mediate this effect much more efficiently than tobramycin.

Previous studies have shown that a significant fraction of *cfr*^{-/-} mice die due to intestinal blockage during or shortly after weaning [3, 4, 5, 19]. However, the threshold of CFTR activity required to eliminate this intestinal pathology is quite low, since as little as 5% of murine *Cfr* expression can restore normal survival in mice [25]. Murine CFTR is normally present in the colon, ileum, and jejunum, where it is located primarily in the crypts of Lieberkuhn and the mucosal and submucosal glands. However, the level of hCFTR required to reverse the intestinal pathology is still poorly defined, since previous studies that examined the ability of

hCFTR to complement mouse *Cfr* knockouts produced conflicting results. In one study, a *hCFTR* cDNA was targeted to the mouse *Cfr* locus using a “knock-in” replacement strategy [26]. This construct was found to produce *hCFTR* mRNA in the epithelial cells of the crypts of Lieberkuhn and the intestinal villi at $\sim 28\%$ of normal murine *Cfr* expression. However, *cfr*^{-/-} animals that expressed this *hCFTR* gene replacement did not show any improvement in either intestinal pathology or survival, suggesting that hCFTR is unable to efficiently substitute for the murine protein. Since the human and murine forms of CFTR retain only 78% identity at the amino acid sequence level, it was suggested that hCFTR may be compromised in its ability to respond efficiently to endogenous murine signaling pathways, or to activate other ion channels present in murine intestinal tissues. A second study used a yeast artificial chromosome (YAC) to express the *hCFTR* gene in a *Cfr*^{-/-} mouse [27]. RNA in situ hybridization demonstrated that *hCFTR* expression was highly cell-type specific and similar to endogenous mouse *Cfr* gene expression. Furthermore, the chloride secretory response in intestinal tissues was robust and the survival of these mice was completely normal. These results clearly demonstrated that *hCFTR* expression can effectively complement the loss of endogenous murine *Cfr* expression. When taken together, the results of these two studies suggest that hCFTR can complement the electrophysiological defects associated with the loss of murine CFTR. However, relatively modest differences in the level of *hCFTR* expression, its expression pattern, or genetic modifier loci [6] may play critical roles in determining the ability of hCFTR to fully complement the intestinal pathology and reduced survival associated with the loss of the endogenous murine CFTR.

A third study used a *FABP* promoter construct to make a *Cfr*^{-/-} mouse that expressed a *hCFTR*-WT transgene [14]. In that study, *hCFTR*-WT expression restored normal survival to *Cfr*^{-/-} mice, even though the correction of both the histologic abnormalities and chloride transport defects was incomplete. *hCFTR* expression was localized primarily to the villi of the ileum, jejunum, and duodenum, but was not found in the crypts of Lieberkuhn or the glands. This difference in localization from endogenous murine *CFTR* could be responsible for the partial complementation observed in that study [14]. In the present study, we used a similar *FABP*-*hCFTR* construct to show that aminoglycosides can suppress the *hCFTR*-G542X mutation. Significantly, we found that gentamicin treatment of the *FABP*-*hCFTR*-G542X mouse resulted in the appearance of CFTR in the mucosal and submucosal glands, but not in the crypts of Lieberkuhn or the intestinal villi. The different patterns of expression observed with these two similar *FABP*-*hCFTR* constructs are probably attributable to position effects caused by the integration of these two transgenes at different chromosomal locations. Since hCFTR expression in the crypts of Lieberkuhn or the intestinal villi are probably necessary for effective complementation, it

is unclear whether a mouse expressing much higher levels of hCFTR with the tissue distribution of our mouse model would exhibit normal survival. Further studies will be necessary using a model that shows a more natural tissue distribution to determine whether a sufficient level of hCFTR can be obtained by aminoglycoside-mediated suppression of stop mutations to provide a therapeutic benefit.

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