Generation of a Conditional Null Allele for Cftr in Mice

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Summary: The cystic fibrosis transmembrane conductance regulator (CFTR) gene encodes a cAMP-regulated chloride channel that is important in controlling the exchange of fluid and electrolytes across epithelial cells. Mutation of CFTR can lead to cystic fibrosis (CF), the most common lethal genetic disease in Caucasians. CF is a systemic illness with multiple organ systems affected including pulmonary, gastrointestinal, pancreatic, immune, endocrine, and reproductive systems. To understand the role of CFTR in the various tissues in which it is expressed, we generated a murine conditional null allele of Cftr (CftrloxP) in which loxP sites were inserted around exon 10 of the Cftr gene. The CftrloxP allele was validated by generating constitutive Cftr null (Cftr<sup>−/−</sup>) mice using the protamine-cre system. The Cftr<sup>loxP/loxP</sup> mice displayed almost identical phenotypes to previously published CF mouse models, including poor growth, decreased survival, intestinal obstruction, and loss of Cftr function as assessed by electrophysiology measurements on gut and nasal epithelium. Mice containing the conditional null Cftr allele will be useful in future studies to understand the role of Cftr in specific tissues and developmental time points and lead to a better understanding of CF disease.

Key words: Cftr; cystic fibrosis; mouse model; conditional knockout; ion transport; intestinal obstruction

Mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene can lead to cystic fibrosis (CF), an autosomal recessive disorder which occurs in about 1 in every 3,000 Caucasian live births. The CFTR protein is a chloride channel involved in the exchange of fluid and electrolytes across epithelial cells (Gadsby et al., 2006). CF is a systemic disorder consistently characterized by poor growth, chronic lung infection, exocrine pancreatic insufficiency, intestinal malabsorption, reduced fertility, and shortened lifespan. Many patients with CF experience meconium ileus, distal intestinal obstruction syndrome, delayed puberty, liver disease, and diabetes. Although these phenotypes ultimately arise as a consequence of reduced or absent CFTR function, the wide expression of CFTR throughout the body makes it difficult to establish the mechanism behind the variety of phenotypes. For example, poor growth may be due to loss of CFTR in the intestinal tract, pancreas, neuroendocrine cells, or any combination thereof. CFTR is expressed in many epithelial tissues such as lung (Engelhardt et al., 1992; Trezise and Buchwald, 1991; Trezise et al., 1993a), nose (Engelhardt et al., 1992), salivary glands (Manson et al., 1997; Trezise and Buchwald, 1991), intestine (Manson et al., 1997; Trezise and Buchwald, 1991), stomach (Manson et al., 1997), pancreas (Trezise and Buchwald, 1991; Trezise et al., 1993a), liver (Yang et al., 1993), gall bladder (Yang et al., 1993), sweat gland (Kartner et al., 1992), kidney (Todd-Turla et al., 1996), male and female reproductive tracts (Trezise et al., 1993a,b), thyroid (Devuyst et al., 1997) and the early embryo (Ben-Chetrit et al., 2002) as well as nonepithelial tissues such as heart (Davies et al., 2004), brain (Mulberg et al., 1998), smooth muscle (Robert et al., 2004) and lymphocytes (Yoshimura et al., 1991). The identification of CFTR’s role in each of these tissues will facilitate the understanding of CF disease.

Currently available mouse models of CF were created using conventional gene targeting strategies leading to constitutive null or hypomorphic alleles of Cftr. While the lung pathology so prevalent in human CF patients is mostly absent in these mouse models, Cftr-deficient mice do display multiple CF phenotypes including poor growth, intestinal obstruction, delayed puberty, infertility, pancreatic abnormalities, gall bladder abnormalities, and decreased survival (Colledge et al., 1995; Delaney et al., 1996; Grubb and Boucher, 1999; Hasty et al., 1995; Hodges et al., 2008; Jin et al., 2006; Kent et al., 1996; O’Neal et al., 1993; Ratcliff et al., 1993; Rozmahel et al., 2002) as well as
et al., 1996; Snouwaert et al., 1992; Zeiher et al., 1995). As in the human, it is not clear how the etiology of these phenotypes relate to each other, because in these global knockouts, the physiology of the tissues involved overlap. Thus, fully understanding the effects of losing Cftr in individual tissues or organs of the mouse cannot be accomplished through conventional gene knockout approach.

To better understand the physiologic relationship of CF-affected tissues and organs, we created a conditional null Cftr allele in the mouse. Here we describe the generation of a murine Cftr allele with loxP sites flanking exon 10 allowing conditional deletion of exon 10 by directed expression of the bacterial Cre recombinase. Exon 10 of Cftr was chosen as a target because of its known importance in protein function. Not only are many exon 10 mutations associated with CF in humans (Tsui, 1992) but also the disruption of exon 10 in mice is known to create severe CF phenotypes (Colledge et al., 1995; Ratcliff et al., 1993; Snouwaert et al., 1992; Zeiher et al., 1995). In addition, future comparison of this model with other null or hypomorphic Cftr alleles of exon 10 should be straightforward because the same region of the gene is altered in other Cftr mouse models.

The mouse Cftr gene consists of 27 exons and spans over 150 kb. To generate a conditional null allele of Cftr, we constructed a targeting vector in which exon 10 of Cftr was flanked by loxP sites (see Fig. 1). Southern blot analysis of three correctly targeted ES cell lines along with the wild-type R1 cell line. Correctly targeted ES cells displayed a unique 7.5-kb band when compared with wild-type cells. (c) PCR strategy for detecting the conditional null allele. P1 and P2 amplify 361- and 408-bp fragments from the wild-type (Cftr10) and conditional null allele (CftrD10) respectively. P1 and P3 amplify a 154-bp fragment from the exon 10 deleted allele (CftrD10). (d) A representative agarose gel image of the PCR results.
The neomycin cassette was removed in subsequent generations of mice (referred to as CfrR10) using Cre recombinase to circumvent any possible complications caused by the presence of the neomycin cassette. Mice homozygous for the conditional null allele (CfrR10R10) were indistinguishable from wild-type mice in growth, survival, and Cfr activity.

To validate the utility of our conditional null Cfr allele, we generated a constitutive deletion of exon 10 by crossing mice with the CfrR10 allele to mice expressing Cre recombinase during the final stages of spermatogenesis through a protamine promoter (O’Gorman et al., 1997). Male mice heterozygous for the CfrR10 allele and positive for protamine Cre recombinase were mated to wild-type females resulting in a portion of offspring heterozygous for a deleted exon 10 allele (CfrA10/A10). The CfrA10/A10 mice were mated to generate CfrA10/A10 mice which were verified by PCR analysis (see Fig. 1). The transmission of the CfrA10 allele followed Mendelian inheritance proportions with 22 (24.4%) wild-type Cfr1/1, 46 (51.1%) heterozygote CfrD10/1, and 22 (24.4%) homozygote mutant mice CfrD10/D10.

The removal of exon 10 in Cfr creates an in-frame deletion in which Cfr is transcribed but exon 10 is absent from the RNA (see Fig. 2). Antibodies raised to Cfr will not distinguish between Cfr containing and lacking exon 10 and so functional assays were employed to determine the effects of the allele. To test for Cfr function, we assayed Cfr’s ability to conduct chloride across the nasal and intestinal epithelium in CfrR10, Cfr1/1, and CfrD10/D10 mice. Nasal potential difference (NPD) across the nasal epithelium was measured before and after the addition of a chloride-free solution containing forskolin (Fig. 3a). Forskolin stimulates adenylate cyclase, leading to activation of Cfr through protein kinase A-mediated phosphorylation. Challenge with a nominally chloride-free solution provides a concentration gradient in favor of chloride secretion. NonCF animals display chloride secretion in response to this challenge, generat-
ing a lumen-negative potential difference, while CF mice do not respond (Fig. 3a). After solution perfusion, \( \text{Cftr}^{+/+} \) animals showed a change in NPD of \(-15.8 \pm 5.2\) mV, \( \text{Cftr}^{-/+} \) animals showed a change in NPD of \(-25.0 \pm 6.8\) mV, while \( \text{Cftr}^{+/+} \) animals showed a change of only \(-0.5 \pm 0.5\) mV \((P < 0.01\) for \( \text{Cftr}^{+/+} \) vs. either control group; three mice per genotype). The lack of change in NPD in \( \text{Cftr}^{+/+} \) mice is consistent with absence of Cftr activity.

Cftr function in the intestinal epithelium was assessed by measuring short-circuit current across intestinal sections before and after stimulation by a cocktail of forskolin and IBMX to raise cAMP levels. This maneuver increases short-circuit current in nonCF animals but not in CF animals (Fig. 3b). Intestinal sections from the ileum, jejunum, duodenum, cecum, and colon were tested for change in short-circuit current and all showed similar differences between \( \text{Cftr}^{+/+} \), \( \text{Cftr}^{-/+} \), and \( \text{Cftr}^{+/+} \) animals (Table 1). The lack of increase in short-circuit current in \( \text{Cftr}^{+/+} \) mice is consistent with the absence of Cftr function in the intestinal epithelium.

The \( \text{Cftr}^{+/+} \) mice displayed decreased growth and decreased survival (see Fig. 4) similar to other published reports of CF mouse models (Colledge et al., 1995; Delaney et al., 1996; Hasty et al., 1995; O’Neal et al., 1993; Ratcliff et al., 1993; Rozmahel et al., 1996; Snouwaert et al., 1992; Zeiher et al., 1995). Growth was reduced in \( \text{Cftr}^{+/+} \) mice by an average of 17%–39% depending on the age of the animals (see Fig. 4) similar to the 20%–50% growth reduction observed in \( \text{Cftr}^{+/+} \) null models. The \( \text{Cftr}^{+/+} \) mice also displayed decreased survival compared with littermates with only 32% surviving up to 40 days similar to the 5%–40% survival observed in \( \text{Cftr}^{+/+} \) null models. The majority of deaths in \( \text{Cftr}^{+/+} \) mice occurred shortly after weaning (20–30 days) due to intestinal obstruction (Figs. 4c and 5). Further examination of the intestine of \( \text{Cftr}^{+/+} \) mice revealed typical intestinal histology observed in CF mouse models including increased luminal mucus accumulation and goblet cell hyperplasia (see Fig. 5).

**Table 1**

Effect of Forskolin/IBMX on Short-Circuit Current Across Intestinal Epithelium

<table>
<thead>
<tr>
<th>Intestinal tissue</th>
<th>( \text{Cftr}^{+/+} ) ( \Delta I_{sc} ) (μA/cm²)</th>
<th>( \text{Cftr}^{-/+} ) ( \Delta I_{sc} ) (μA/cm²)</th>
<th>( \text{Cftr}^{+/+} ) ( \Delta I_{sc} ) (μA/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duodenum</td>
<td>87.81 ± 20.6</td>
<td>153.66 ± 24.6</td>
<td>1.10 ± 1.0*</td>
</tr>
<tr>
<td>Jejunum</td>
<td>92.17 ± 27.8</td>
<td>121.61 ± 22.6</td>
<td>3.47 ± 1.5*</td>
</tr>
<tr>
<td>Ileum</td>
<td>98.90 ± 24.8</td>
<td>238.25 ± 24.4</td>
<td>2.15 ± 1.8*</td>
</tr>
<tr>
<td>Cecum</td>
<td>155.16 ± 37.4</td>
<td>291.57 ± 77.6</td>
<td>0.49 ± 0.2*</td>
</tr>
<tr>
<td>Colon</td>
<td>131.00 ± 42.7</td>
<td>129.49 ± 19.1</td>
<td>0.32 ± 0.4*</td>
</tr>
</tbody>
</table>

\( ^* \) \( P < 0.05 \) vs. \( \text{Cftr}^{+/+} \) or \( \text{Cftr}^{-/+} \).

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**FIG. 4.** Phenotype of \( \text{Cftr}^{-/-} \) mice created from the conditional null allele. (a) Growth curve for \( \text{Cftr}^{+/+} \), \( \text{Cftr}^{-/+} \), \( \text{Cftr}^{+/+} \), and \( \text{Cftr}^{+/+} \) mice. (b) Representative picture of \( \text{Cftr}^{+/+} \) (left) and \( \text{Cftr}^{-/+} \) (right) littermates (20 days old). (c) Survival curve for \( \text{Cftr}^{+/+} \), \( \text{Cftr}^{-/+} \), \( \text{Cftr}^{+/+} \), and \( \text{Cftr}^{+/+} \) mice. (There was no difference in growth or survival among \( \text{Cftr}^{+/+} \), \( \text{Cftr}^{-/+} \), and \( \text{Cftr}^{+/+} \), so they were combined).
These results indicate that loss of exon 10 results in a functionally null phenotype in mice, displaying decreased growth, high incidence of intestinal obstruction, goblet cell hyperplasia, and loss of normal transepithelial ion transport. As this allele can be generated in a conditional fashion using the \textit{Cftr}/10 mouse and regulated Cre expression, it provides a tool to study tissue-specific and temporal effects of losing Cftr. For example, decreased growth and intestinal obstruction in CF mice have been hypothesized to be due to loss of Cftr activity in the intestinal epithelium. This hypothesis can be tested by crossing the \textit{Cftr}/10 mouse with a mouse in which Cre recombinase expression is driven by the villin promoter so that Cre is expressed specifically in the intestinal epithelium (Madison \textit{et al.}, 2002). Delayed growth in CF mice may also have a neuroendocrine origin given the multiple reports of Cftr expression in the brain (Mulberg \textit{et al.}, 1998; Weyler \textit{et al.}, 1999). Possible Cftr function in the nervous system could be examined by crossing the \textit{Cftr}/10 mouse with a mouse in which Cre expression is driven by the nestin promoter so that Cre is expressed specifically in the central and peripheral nervous system (Tronche \textit{et al.}, 1999). Finally, one can test the importance of Cftr activity on the development of the mouse (e.g., in utero, postnatal, pubertal, and adult) by deleting Cftr function in the whole mouse at different time points by crossing the \textit{Cftr}/10 mouse with a mouse in which Cre expression is driven by an inducible promoter (e.g., Badea \textit{et al.}, 2003). The ability to delete Cftr in a tissue or time-dependent manner in the mouse will not only help understand how Cftr function contributes to the function and development of each tissue but how the absence of CFTR in these same tissues leads to CF disease in humans.

METHODS AND MATERIALS

Generation of the \textit{Cftr} Floxed Allele

Genomic DNA was isolated from R1 cells. The targeting vector consisted of amplified fragments of \textit{Cftr} that included 4.4 kb of intron 9 (5' arm), 4.5 kb of intron 10 (3' arm), and 0.5 kb consisting of exon 10 and ~150 bp of intron 9 and 10 on each side. These fragments were subcloned into pBluescript. Two \textit{loxP} sites surrounding the phosphoglycerol kinase promoter attached to a neomycin-resistance gene (PGK-neocassette) were inserted downstream of exon 10 while the third \textit{loxP} site was inserted upstream of exon 10. A HTK cassette was inserted downstream of intron 10. A schematic of the targeting construct is shown in Figure 1. The linearized targeting vector was transfected into R1 ES cells and neomycin resistant colonies were screened by Southern blot and PCR genotyping to confirm germline transmission of the conditional \textit{Cftr} null allele.

PCR

Genotyping was completed by PCR analysis using DNA extracts from ear biopsies. To detect the \textit{Cftr}/10 (or \textit{Cftr}/10) allele (408 bp), the \textit{Cftr} allele (361 bp), and the \textit{Cftr}/10 allele (154 bp), primers P1 (5'-GTA-GGGGCTCGGTCTTCTTCTT-3') and P2 (5'-GTACCAGCA-
TAATCCAAGA-3') and P3 (5'-AGCCCGTCGGAGGACC- 
TAAT-3') were used (Fig. 1c,d). PCR reactions were 
completed for 40 cycles of 95°C for 30 seconds, 58°C for 
1 min and 72°C for 1 min.

Lungs and intestines were collected from Cftr +/-, 
+-/-, and --/- animals and total RNA was isolated using 
Trizol (Invitrogen) per the manufacturer's instructions. 
One μg of RNA was reverse transcribed into cDNA with 
MMLV. cDNA was used in PCR analysis to amplify Cftr 
exons 8–11 to detect wild-type allele (563 bp) and 
excised exon 10 allele (371 bp) expression. PCs were 
completed for 40 cycles of 95°C for 30 sec, 55°C for 30 
sec, and 72°C for 1 min with primers (5'-CCACAGGCA- 
TAATCATGGAA-3') and (5'-TGTGACTCCACCTTCTC- 
CAA-3').

Histology

Mouse intestines were isolated and fixed in 10% for-
malin, embedded in paraffin and sectioned every 5 μm. 
Periodic acid Schiff (PAS) staining of these sections were 
completed to detect mucus accumulation.

Bioelectric Measurements

The potential difference (PD) across the nasal epithe-
lium of the mice was measured according to procedures 
previously described elsewhere (Brady et al., 2001; Kel-
ley et al., 1997), with some modifications. Mice were 
anesthetized with 8.5 mg/ml ketamine, 1.7 mg/ml xyla-
zine, and 0.3 mg/ml acepromazine in sterile saline. Ani-
mals were dosed intraperitoneally with 0.012 ml/g 
mouse weight. A PE-10 tube stretched to one-half the 
original diameter was inserted 2 mm into the mouse nos-
tril and placed against the septum. With the use of a sy-
ringe pump (model A-99; Razel Scientific Instruments) 
and 3-ml syringes, N-2-hydroxyethylpipеразине-Н-2эти-
ленаэусульфоновую кислоту (HEPES)-buffered Ringer's solution perfused into the nostril at a rate of 5 ml/min. Valves 
controlled the solution which entered the nostril, with a 15-
sec delay until the new solution reached the nasal epi-
thelium. Readings were taken until a steady-state value 
was reached before perfusing the nasal epithelium with 
a new solution. Paper wicks were used to absorb excess 
liquid from the mouth and opposite nostril of the mouse 
being tested. A trachea tube consisting of an Angiocath 
IV Catheter was inserted into the mouse trachea to facil-
itate breathing. Bridges (4% agar) connected the tubing 
to calomel electrodes, and each bridge was made in the 
same solution as the perfusate it measured (HEPES-buff-
ered Ringer’s or chloride-free HEPES-buffered Ringer’s).

A needle containing 4% agar in HEPES-buffered Ringer’s 
was placed subcutaneously in the mouse’s back and con-
ected to another calomel electrode to serve as a refer-
ence. The measuring and reference calomel electrodes 
were situated in a KCl bath in which one end of the agar 
bridge was also immersed. The other end of the meas-
uring electrode’s bridge was in contact with the perfus-
ate 20 cm from the mouse nose. The transepithelial dif-
fERENCE was measured with a voltmeter (model ISO-
DAM-D; World Precision Instruments), and the signal 
was recorded on a chart recorder (model BD112; Kipp 
and Zonen). The PD measurements were corrected for 
junction potentials, and changes in PD values were cal-
culated by subtracting the value after 4 min of perfusion 
with chloride-free Ringer's solution containing forskolin 
from the value when the perfusion began. Before mea-
surements, the system was zeroed by adjusting the 
voltmeter after connecting the reference needle to 
the tube normally placed in the mouse nose to complete 
the circuit.

Electrolyte transport was investigated in the intestine 
of 8- to 12-week-old animals that were fasted overnight. 
Mice were rendered unconscious by CO2 asphyxiation 
and killed by exsanguination. The large and small intes-
tines were removed and immediately placed in ice-cold 
HEPES-buffered Ringer's solution (in mM: 138 NaCl, 5 
KCl, 2.5 NaHPO4, 1.8 CaCl2, 1.0 MgSO4, and 10 HEPES-
NaOH, pH 7.4) for dissection. The intestine was cut lon-
gitudinally, and the contents were removed with a stream 
of Ringer’s solution. The tissue was stretched and 
injected into a Ussing chamber with an aperture of 0.125 
em2. Tissues were bathed on both sides by 6–8 ml of 
mammalian Krebs-Ringer bicarbonate solution (in mM: 
115 NaCl, 25 NaHCO3, 5 KCl, 2.5 NaHPO4, 1.8 CaCl2, 
1.0 MgSO4, and 10 glucose, pH 7.4 (mannitol replaced 
glucose in the apical bathing solution)], which was 
warmed to 37°C and circulated with 95% O2–5% CO2 
through gas lifts. Transepithelial electrical voltage differ-
ence (Vms) was measured between two Ringer-agar 
bridges. Calomel cells connected the bridges to a high-
impedance voltmeter (DVC 1,000 Voltage/Current Clamp, World Precision Instruments). Current from an 
external DC source was passed by silver-silver chloride 
electrodes and Ringer-agar bridges to clamp the sponta-
neous Vms to zero. The current required (short-circuit 
current, Isc) was corrected for solution resistance 
between the tips of the voltage-sensing electrodes and 
recorded. Tissues were maintained with Vms clamped to 
zero (short-circuit conditions). At 60-sec intervals, Vms 
was clamped to +2 mV for 3 sec to calculate transep-
ithelial resistance (Rms). Tissues were mounted and 
observed until the Isc stabilized (generally 10–15 min), at 
which time experimental maneuvers were begun.

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LITERATURE CITED


