Co-ordinate regulation of the cystic fibrosis and multidrug resistance genes in cystic fibrosis knockout mice

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INTRODUCTION

Mutations in the cystic fibrosis transmembrane conductance regulator gene (CFTR) cause cystic fibrosis (1–3). Cystic fibrosis affects a number of organ systems, including the respiratory, digestive and reproductive systems (4), and its underlying cause is the aberrant expression and/or function of a cAMP-regulated chloride channel encoded by CFTR (5–7). As well as functioning as a chloride channel, CFTR also regulates other ion channels (8,9).

The cystic fibrosis (Cftr) and multidrug resistance (Mdr1) genes encode structurally similar proteins which are members of the ABC transporter superfamily. These genes exhibit complementary patterns of expression in vivo, suggesting that the regulation of their expression may be co-ordinated. We have tested this hypothesis in vivo by examining Cftr and Mdr1 expression in cystic fibrosis knockout transgenic mice (Cftrtm1CAM). Cftr mRNA expression in Cftrtm1CAM/Cftrtm1CAM mice was 4-fold reduced in the intestine, as compared with litter-mate wild-type mice. All other Cftrtm1CAM/Cftrtm1CAM mouse tissues examined showed similar reductions in Cftr expression. In contrast, we observed a 4-fold increase in Mdr1 mRNA expression in the intestines of neonatal and 3- to 4-week-old Cftrtm1CAM/Cftrtm1CAM mice, as compared with age-matched +/+ mice, and an intermediate level of Mdr1 mRNA in heterozygous Cftrtm1CAM mice. In 10-week-old, Cftrtm1CAM/Cftrtm1CAM mice and in contrast to the younger mice, Mdr1 mRNA expression was reduced, by 3-fold. The expression of two control genes, Pgk-1 and Mdr2, was similar in all genotypes, suggesting that the changes in Mdr1 mRNA levels observed in the Cftrtm1CAM/Cftrtm1CAM mice are specific to the loss of Cftr expression and/or function. These data provide further evidence supporting the hypothesis that the regulation of Cftr and Mdr1 expression is co-ordinated in vivo, and that this co-ordinate regulation is influenced by temporal factors.

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expression is accompanied by an 80% decrease in CFTR expression. Both effects are reversed following removal of the drug (17).

The creation of cystic fibrosis knockout transgenic mice has allowed us to test the co-ordinate regulation hypothesis in vivo. Cystic fibrosis knockout transgenic mice have been constructed, independently, by several groups (18–23). We have used RNA in situ hybridisation to examine Cfr and Mdr1 expression in the Cfrtm1CAM null mice (19). These mice were created using a replacement targeting strategy and have a severe cystic fibrosis phenotype with most animals succumbing to intestinal blockages early in life. No Cfr-related chloride conductances can be detected in the intestinal, tracheal or pancreatic duct epithelia of Cfrtm1CAM/Cfrtm1CAM mice (18,19,24). It is possible that in the absence of Cfr expression/function, the cellular response may be to activate other chloride channels by increasing the expression of genes encoding chloride channel regulators, such as Mdr1. The hypothesis of co-ordinate regulation of Cfr and Mdr1 expression predicts that an alteration in Cfr expression should lead to changes in Mdr1 expression. Here we show that the level of Cfr mRNA is greatly reduced in all tissues examined in Cfrtm1CAM/Cfrtm1CAM mice, as compared with wild-type mice. This decrease in Cfr mRNA levels is accompanied by altered Mdr1 mRNA expression, providing supporting evidence for the hypothesis that expression of Cfr and Mdr1 is co-ordinately regulated in vivo.

RESULTS
Nature of Cfr mRNAs expressed in Cfrtm1CAM mice

We investigated the nature of the mRNAs transcribed from the Cfrtm1CAM locus to assess the effectiveness of the transcription termination signals inserted in exon 10. Figure 1 shows the structure of part of the Cfrtm1CAM locus surrounding the targeted disruption in exon 10, and a number of possible RNAs that could be transcribed from this gene. The Cfr promoter has not been altered in the Cfrtm1CAM mice and, therefore, transcription initiation is not expected to be affected by the gene disruption. If RNAs initiated at the Cfr promoter are terminated at the targeted disruption, as predicted, then the transcript shown in Figure 1a would be produced. The constitutive Pkg-1 promoter, which was introduced at the targeted disruption in Cfr exon 10, is also expected to initiate transcription. If these transcripts are terminated at the Pkg-1 poly(A)'/transcription termination signals then they would only contain a HPRTK coding sequence (transcripts not shown). However, if the Pkg-1 poly(A)'/transcription termination signals are not 100% efficient, then two other RNAs containing the Cfr coding sequence are predicted. One of these is initiated from the Pkg-1 promoter (Fig. 1b), and the other from the endogenous Cfr promoter (Fig. 1c). It is also possible that a Cfr mRNA spliced directly from exon 9 to exon 11 may be produced (Fig. 1d), although this would result in a frame-shift mutation and the introduction of downstream, in-frame translation stop codons. Cryptic splice sites in and around exon 10 and the targeted mutation might exist, although there is no evidence for such sites being used in the Cfrtm1CAM mice. None of the predicted Cfr mRNAs shown in Figure 1 can encode a functional Cfr protein, and electrophysiological analysis of the Cfrtm1CAM mice demonstrates a null Cfr phenotype (18,19,24).

We used reverse transcription-polymerase chain reaction (RT-PCR) and RNase protection analyses to investigate the nature of Cfr transcripts expressed from the Cfrtm1CAM locus. In tissues that usually express Cfr, all the possible Cfr mRNAs shown in Figure 1 would be anticipated, whereas in tissues that do not usually express Cfr only the Cfr mRNA shown in Figure 1b is predicted. In the RT-PCR, two Cfr fragments were amplified, one from exons 4–6 upstream of the targeted disruption (Fig. 2, lanes ‘u’), and the other from exons 11–13 downstream of the disruption (Fig. 2, lanes ‘d’). Amplification of β-actin mRNA (Fig. 2, lanes ‘a’) was used as a positive control. In Cfrtm1CAM/Cfrtm1CAM mice, the upstream primers generated a 480 bp product from total RNA extracted from lung, kidney, uterus, testis, small intestine, colon, brain and stomach (Fig. 2, lanes ‘u’ and data not shown). However, no upstream product could be amplified from spleen, liver, heart and skeletal muscle RNA (Fig. 2, lanes ‘u’ and data not shown). So, amplification of the upstream PCR product coincides accurately with the known tissue distribution of Cfr mRNA (25,26). In +/- mice, the downstream PCR product (375 bp) could only be amplified from RNA prepared from tissues known to express Cfr (Fig. 2, lanes ‘d’ and data not shown), whereas in the Cfrtm1CAM/Cfrtm1CAM mice, the downstream Cfr primers amplified a 375 bp PCR product from RNA isolated from all tissues examined, including those where no upstream PCR product was observed (Fig. 2, lanes ‘d’ and data not shown). Presumably, this is due to transcription initiation from the ubiquitously expressed Pkg-1 promoter (contained within the targeting construct) and some read-through of the transcription termination signals of the HPRTK mini-gene (see Fig. 1b).

To assess the extent of transcriptional read-through of the HPRTK mini-gene, we used RNase protection analysis with a probe specific for Cfr exon 10 and spanning the targeted disruption. A 191 bp RNA fragment from this probe (plasmid pE16) will be protected by wild-type Cfr mRNA and this was observed with RNA isolated from testis, kidney and intestine of +/- and +/-Cfrtm1CAM animals, but not Cfrtm1CAM/Cfrtm1CAM mice (Fig.3A). A 71 bp fragment was protected by RNA isolated from the testsis of Cfrtm1CAM/Cfrtm1CAM mice (Fig. 3A, lane +/– T and data not shown), the size predicted for RNA containing the 5’ region of Cfr exon 10 upstream of the inserted HPRTK mini-gene. The decreased signal from this 71 bp fragment, compared with the 191 bp fragment in the +/- mice, is due in part to the decreased size of the protected fragment, but also suggests that the amount of truncated Cfr mRNA (Fig. 1a) was greatly reduced. Inability to detect this 71 bp fragment in the kidney and intestine, which express similar Cfr mRNA levels to the testsis, suggests differential stability of the truncated Cfr mRNA (Fig. 1a) in different tissues. A second probe, containing the 3’ region of exon 10 (generated from plasmid p987), would be predicted to generate a 122 bp protected fragment when hybridised either to wild-type Cfr mRNA or to the fused HPRTK-Cfr transcrpts resulting from read-through of the poly(A)’ signal sequence (Fig. 1b and c). A 122 bp fragment was protected by RNA from the testsis, kidney and intestine of +/- and +/-Cfrtm1CAM animals, but not Cfrtm1CAM/Cfrtm1CAM animals, and not Cfrtm1CAM/Cfrtm1CAM animals (Fig. 3B), indicating that the only downstream sequences detected by RNase protection were from wild-type Cfr mRNA. In Cfrtm1CAM/Cfrtm1CAM mice, the detection of Cfr mRNAs containing sequences downstream of the targeted interruption by RT-PCR, but not by RNase protection, suggests that transcriptional read-through of the Pkg-1 termination signal is very low. This analysis establishes that the
**Cftr<sup>tm1CAM</sup> Targeted Locus**

![Diagram of exons 9–11 of the disrupted Cftr locus and mRNAs predicted to be expressed in Cftr<sup>tm1CAM</sup> mice.](image)

Possible RNA transcripts:

- **a)** initiated at the Cftr promoter and terminated at the introduced mini-gene transcription termination signal.
- **b)** initiated at the introduced Pgk-1 promoter.
- **c)** initiated at the Cftr promoter.

Functional CFTR - Protein

- **No**

<table>
<thead>
<tr>
<th>a</th>
<th>b</th>
<th>c</th>
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**Figure 1.** Diagram of exons 9–11 of the disrupted Cftr locus and mRNAs predicted to be expressed in Cftr<sup>tm1CAM</sup> mice. The RNA shown in (a) would be predicted if RNA transcription initiated normally at the Cftr promoter and was terminated at the introduced mini-gene transcription termination signal. The RNAs shown in (b) and (c) would be predicted if there is read-through of the introduced mini-gene transcription termination signal. The RNA shown in (b) is initiated at the introduced Pgk-1 promoter and the RNA shown in (c) is initiated at the Cftr promoter.

**Figure 2.** Reverse transcription-PCR analysis of Cftr expression in stomach and skeletal muscle of Cftr<sup>tm1CAM</sup> mice. Lane a shows the products of a control amplification of β-actin mRNA sequences. Lane u shows the products of an amplification of exons 4–6 of Cftr mRNA sequences, upstream of the targeted disruption in exon 10. Lane d shows the products of an amplification of exons 11–13 of Cftr mRNA sequences downstream of the targeted disruption in exon 10. Lane M shows size standards. The sizes (bp) of the amplified fragments are shown on the left.

**Cftr expression in Cftr<sup>tm1CAM</sup> mice**

RNA *in situ* hybridisation was used to study the localisation and level of Cftr mRNA expression in Cftr<sup>tm1CAM</sup> mice at three different stages of development: neonatal (1 day), suckling–weaning transition (3–4 weeks) and adult (10 weeks). We used two probes to examine Cftr expression, one upstream of the targeted disruption of the Cftr locus and the other downstream. The upstream Cftr probe hybridised to exons 3–5 (bp 309–695), and the downstream probe recognised exons 11–13 (bp 1771–2112 according to ref. 27). The RT-PCR and RNase protection analyses have shown that the majority of the Cftr mRNA expressed in the Cftr<sup>tm1CAM</sup>/Cftr<sup>tm1CAM</sup> mice was initiated at the Cftr promoter and terminated at the targeted disruption in Cftr exon 10 (see Fig. 1a). So, the hybridisation signal detected with the upstream Cftr probe (see Fig. 4) indicates the localisation and level of mRNAs initiated at the Cftr promoter (Fig. 1a, c and d).

In 1-day-old, +/- mice, the upstream Cftr probe readily detected Cftr mRNA in the intestinal inter villous epithelia from which the intestinal crypts develop during postnatal weeks 1 and 2 (see Fig. 4A and B). In contrast, very little Cftr mRNA could be detected in the intestines of Cftr,<sup>tm1CAM</sup>/Cftr<sup>tm1CAM</sup> mice (Fig. 4G and H); in +/-Cftr<sup>tm1CAM</sup> mice an intermediate amount of Cftr mRNA was observed (Fig. 4D and E). At all times, the experimenters performing the RNA *in situ* hybridisations were blinded with respect to the genotypes of the animals examined. Using RNA *in situ* hybridisation to examine Cftr mRNA levels,
the genotypes of CFtrtm1CAM mice were identified correctly in 28/30 cases and all the CFtrtm1CAM/Cftrtm1CAM mice (nine) were identified correctly.

Relative levels of mRNA expression were determined objectively by densitometric analysis of X-ray film contact sheets made from slides prior to autoradiography (see Materials and Methods). This analysis showed that the hybridisation signal in 1-day-old, +/+ mice was clearly detectable, while the signal in CFtrtm1CAM/Cftrtm1CAM mice was not above background level.

We also examined CFtr expression in the intestines of 3- to 4-week-old (suckling–weaning transition) and 10-week-old (adult) mice. The results of these experiments are summarised in Table 1. Compared with +/+ mice, the upstream CFtr probe detected reduced, but still clearly detectable levels of CFtr mRNA in the submucosal (Brunner’s) glands of the intestine of 3- to 4-week-old CFtrtm1CAM/Cftrtm1CAM mice. However, the downstream probe detected very little CFtr mRNA in the Brunner’s glands of CFtrtm1CAM/Cftrtm1CAM mice, much less than the upstream probe. In wild-type mice, both probes detected approximately equivalent amounts of CFtr mRNA. Identical results were obtained from the 10-week-old CFtrtm1CAM mice. These data suggest the presence of significant levels of the truncated CFtr mRNA (Fig. 1a) in the Brunner’s glands of neonatal, +/+ mice (data summarised in Table 1). In CFtrtm1CAM/Cftrtm1CAM mice, the upstream probe detected CFtr mRNA in the intra- and interlobular salivary gland ducts, while the downstream CFtr probe did not detect CFtr mRNA expression. This pattern of expression is similar to that seen in the intestinal Brunner’s glands of the CFtrtm1CAM mice, and again suggests the presence of the truncated CFtr mRNA (Fig. 1a) in the salivary gland ducts of CFtrtm1CAM/Cftrtm1CAM mice.

**Table 1.** CFtr mRNA expression in the intestines and salivary glands of wild-type (+/+) and CFtrtm1CAM/Cftrtm1CAM mice

<table>
<thead>
<tr>
<th>Tissue and age</th>
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<th>Upstream CFtr probe</th>
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<tr>
<td>Intestinal Brunner’s glands</td>
<td>+/+</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>(weaning and adult mice)</td>
<td>CFtrtm1CAM/Cftrtm1CAM</td>
<td>+++</td>
<td>+</td>
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<tr>
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<td>+/+</td>
<td>+++</td>
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<tr>
<td>(weaning and adult mice)</td>
<td>CFtrtm1CAM/Cftrtm1CAM</td>
<td>+ (4-fold reduced)</td>
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<td>+/+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>(neonates)</td>
<td>CFtrtm1CAM/Cftrtm1CAM</td>
<td>+++</td>
<td>–</td>
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Relative signal intensities: ++++ = high; +++ = moderate; ++ = low; + = just detectable; – = not detectable.

Age groups: neonatal = 1–3 days; weaning = 3–4 weeks; adult = 10 weeks.

The salivary glands are also a major site of CFtr expression (25,28). Similar levels of CFtr mRNA were detected with both the CFtr probes in the intra- and interlobular ducts of the salivary glands of neonatal, +/+ mice (data summarised in Table 1). In CFtrtm1CAM/Cftrtm1CAM mice, the upstream probe detected CFtr mRNA in the intra- and interlobular salivary gland ducts, while the downstream CFtr probe did not detect CFtr mRNA expression. This pattern of expression is similar to that seen in the intestinal Brunner’s glands of the CFtrtm1CAM mice, and again suggests the presence of the truncated CFtr mRNA (Fig. 1a) in the salivary gland ducts of CFtrtm1CAM/Cftrtm1CAM mice.

**Mdr1 expression in CFtrtm1CAM mice**

Co-ordinate regulation of CFtr and Mdr1 would predict that alterations in the expression of one gene would lead to subsequent changes in the expression of the other. In rodents, the homologue of the human MDR1 gene has been duplicated and the two mouse genes are designated Mdr1a and Mdr1b (also known as Mdr3 and Mdr1, respectively, ref. 11). Since we are interested in combined Mdr1a and Mdr1b expression in the CFtrtm1CAM mice, we used a probe which hybridised to both Mdr1a and Mdr1b sequences. We will refer to mouse Mdr1 expression throughout to denote combined Mdr1a and/or Mdr1b expression. The sequences of exon 6 of mouse Mdr1a and Mdr1b contain only five differences out of 192 bp, so exon 6 of mouse Mdr1b was used to examine Mdr1 expression. Southern blotting analysis demonstrated that this probe recognised both Mdr1a and Mdr1b, but not Mdr2 (data not shown).

[Figure 3. RNase protection analysis of CFtr expression in CFtrtm1CAM mice. CFtr expression was analysed in testis (T), kidney (K), intestine (I), heart (H) and skeletal muscle (M). (A) RNase protection using a probe specific for CFtr exon 10 and spanning the targeted interruption. Arrows indicate specifically protected fragments at 191 and 71 bp. (B) RNase protection using a probe to the 3′ region of CFtr exon 10. The arrow indicates a specifically protected fragment at 122 bp.]

**Table 1.** CFtr mRNA expression in the intestines and salivary glands of wild-type (+/+) and CFtrtm1CAM/Cftrtm1CAM mice

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Relative signal intensities: ++++ = high; +++ = moderate; ++ = low; + = just detectable; – = not detectable.

Age groups: neonatal = 1–3 days; weaning = 3–4 weeks; adult = 10 weeks.
Figure 4. Cftr mRNA expression in 1-day-old, Cftr<sup>tm1CAM</sup> mice. (A, D and G) Brightfield images of cryostat sections of the small intestine that have been hybridised to the antisense, upstream Cftr probe. Arrows indicate intestinal inter villous epithelia, from which the intestinal crypts will develop during post-natal weeks 1 and 2. (B, E and H) Darkfield images of the same sections allowing visualisation of the hybridisation signal (white dots). The intensity of the hybridisation signal gives an indication of the amount of mRNA present. (C, F and I) Darkfield images of consecutive sections hybridised with the corresponding sense strand probe, which serves as a negative control. The sections in (A), (B) and (C) are from a +/+ mouse, those in (D), (E) and (F) from a +/Cftr<sup>tm1CAM</sup> mouse and those in (G), (H) and (I) from a Cftr<sup>tm1CAM</sup>/Cftr<sup>tm1CAM</sup> mouse. The size bar equals 200 µm.
Reduced levels of Cftr mRNA expression were observed in the intestines of 1-day-old and 3- to 4-week-old Cftr<sup>tm1CAM</sup>/Cftr<sup>tm1CAM</sup> mice (Fig. 4). Therefore, Mdr1 expression was studied in a series of consecutive sections. The cellular patterns of Cftr and Mdr1 expression observed in 3- to 4-week-old mice were similar to those found in the 1-day-old mice. In +/+ mice of both age groups, low levels of Mdr1 mRNA were observed in the intestinal intervillous and villous epithelia (Fig. 5A and B). The expression of Mdr1 in both intervillous and villous epithelial cells differed from the pattern of expression in adult +/+ mice where expression was restricted to the villous epithelia (ref. 15, and see Fig. 6). In 1-day-old and 3- to 4-week-old Cftr<sup>tm1CAM</sup>/Cftr<sup>tm1CAM</sup> mice, Mdr1 mRNA expression was also observed in intervillous and villous epithelia, but the level of Mdr1 expression was increased 4-fold compared with +/+ mice (Fig. 5G and H). We also examined intestinal Mdr1 expression in 3- to 4-week-old heterozygous mice. The cellular distribution of Mdr1 mRNA was identical in all three mouse genotypes, and an intermediate level
of Mdr1 expression was observed in the +/+Cftrtm1CAM mice as compared with +/+ and Cftrtm1CAM/Cftrtm1CAM mice (Fig. 5, compare E with B and H, respectively).

To determine whether the changes in Mdr1 expression observed in 1-day-old and 3- to 4-week-old Cftrtm1CAM/Cftrtm1CAM mice were affected by intestinal development, we also examined Mdr1 expression in 10-week-old (adult) Cftrtm1CAM mice. In adult +/+ mice, Mdr1 expression was restricted to the duodenal and ileal villous epithelia with no evidence of expression in the crypt epithelia or Brunner’s glands (Fig. 6A and B and data not shown), typical of the mature pattern of intestinal Mdr1 expression (15). Comparison of Mdr1 mRNA expression in all three age groups of +/+ mice revealed a gradual contraction in expression from the intervillous/crypt and villous epithelia seen in mice up to the age of weaning, to the villous epithelia of adult mice. There was also an increase in the level of Mdr1 expression in the villous epithelia after weaning was complete. In contrast to our observations in the younger Cftrtm1CAM/Cftrtm1CAM mice, we observed a marked decrease in Mdr1 mRNA expression in the duodenal and ileal villous epithelia of 10-week-old Cftrtm1CAM/Cftrtm1CAM mice (Fig. 6C and D), as compared with +/+ mice (Fig. 6A and B). The levels of Mdr1 mRNA were 3-fold less in the small intestines of 10-week-old Cftrtm1CAM/Cftrtm1CAM mice, compared with +/+ mice. Our observations suggest that the transition through weaning results in Mdr1 mRNA expression changing from overexpression in immature Cftrtm1CAM/Cftrtm1CAM intestine, to underexpression in adult Cftrtm1CAM/Cftrtm1CAM intestine. This is in contrast to the developmental changes in Mdr1 expression in +/+ intestine, where an overall increase in Mdr1 mRNA levels was observed after weaning.

To establish that the changes in Mdr1 expression observed in the Cftrtm1CAM/Cftrtm1CAM mice are specific to the loss of Cftr expression and/or function, and not a result of a general alteration in expression of all genes in the Cftrtm1CAM/Cftrtm1CAM mice, we examined Mdr2 and Pgk-1 expression in 3- to 4-week-old and 10-week-old Cftrtm1CAM mice (Fig. 7). The mouse Mdr2 probe recognised Mdr2 exon 21, and Southern blotting showed that this probe hybridised specifically to mouse Mdr2, but not to Mdr1a or Mdr1b (data not shown). A human PGK-1 probe, which cross-hybridises with mouse Pgk-1 sequences (29), was used to assess mouse Pgk-1 expression. Mdr2 is not normally expressed in the intestine (30) and no transcripts were detected in the intestines of either +/+ or Cftrtm1CAM/Cftrtm1CAM mice (data not shown). Pgk-1 was found to be expressed in the duodenal and ileal crypt and villous epithelia (Fig. 7). The highest level of Pgk-1 mRNA was observed in the crypt epithelia, with a decreasing gradient of

![Figure 6](image_url)
expression along the crypt–villous axis. Pgk-1 mRNA was not detected in the Brunner’s glands of the intestine (Fig. 7A–H). In both age groups of \( \text{Cftr}^{\text{tm}1\text{CAM}} \) mice, the level and cellular distribution of expression of Pgk-1 mRNA in the intestine was similar in ++ and \( \text{Cftr}^{\text{tm}1\text{CAM}}/\text{Cftr}^{\text{tm}1\text{CAM}} \) mice (Fig. 7). Therefore, we conclude that the changes in \( \text{Mdr}1 \) expression observed in the \( \text{Cftr}^{\text{tm}1\text{CAM}}/\text{Cftr}^{\text{tm}1\text{CAM}} \) mice are specific to the loss of \( \text{Cftr} \) expression and/or function.

DISCUSSION

\textbf{Cftr expression in \( \text{Cftr}^{\text{tm}1\text{CAM}} \) mice}

The \( \text{Cftr}^{\text{tm}1\text{CAM}} \) mice were produced by a replacement targeting event in which an \( \text{HPRT}^\text{R} \) mini-gene was inserted into exon 10 of the \( \text{Cftr} \) locus. RT-PCR, RNase protection and RNA \textit{in situ} hybridisation data suggest the level of read-through of the \( \text{HPRT}^\text{R} \)
Mini-gene is low and that the majority of the Cftr mRNA detected in the Cftr tm1CAM/Cftr tm1CAM mice is the truncated Cftr mRNA shown in Figure 1a. We were able to detect the truncated Cftr mRNA (Fig. 1a) in the Brunner’s glands and salivary gland ducts, but not in the intestinal crypt epithelia of the Cftr tm1CAM/Cftr tm1CAM mice. Cftr is expressed at similar levels in the salivary gland ducts and intestinal crypt epithelia of wild-type mice. This suggests tissue-specific differences in the stability of the truncated Cftr mRNA, and that the truncated mRNA is less stable than wild-type Cftr mRNA in crypt epithelia. This is not surprising since exons 11–24 and the 3′ untranslated region (3′ UTR) have been deleted, and the 3′ UTR often contains sequence motifs important in determining mRNA stability (31). This finding suggests that the 3′ UTR may also be important for Cftr mRNA stability in some cell types. It is also possible that the alternations in Cftr mRNA levels may be due to changes in the rate of transcription initiation from the Cftr tm1CAM promoter, and that these changes in transcription are further complicated by the altered stability of the mutant Cftr mRNAs. As the disruption of the Cftr tm1CAM locus is contained entirely within exon 10 it is unlikely that this event alters the rate of transcription initiation from the Cftr tm1CAM promoter, although this has not been demonstrated formally. Overall, these studies show that Cftr mRNA levels are greatly reduced in the Cftr tm1CAM/Cftr tm1CAM mice and any Cftr mRNA expressed is largely terminated at the targeted interruption.

Mdr1 expression in the Cftr tm1CAM mice

The data presented here show that Mdr1 expression is developmentally regulated in the intestines of wild-type mice. In neonatal and suckling–weaning mice, Mdr1 was expressed at low levels throughout the villous/crypt and villous epithelia, while in adult mice the level of Mdr1 expression was increased and restricted to the villous epithelia. The transition through weaning is a crucial stage of intestinal development and is known to produce changes in the expression of a number of genes that play important roles in intestinal physiology (32,33). The developmental regulation of Mdr1 expression in the intestine is consistent with such a role for Mdr1 in normal intestinal function. We have shown previously that Cftr and Mdr1 exhibit complementary patterns of expression in vivo, suggesting that the regulation of expression of the two genes may be co-ordinated (15). The results of this study provide further evidence for the co-ordinated regulation of Cftr and Mdr1 expression in vivo.

In the intestinal epithelia of neonatal (1-day-old) and suckling–weaning (3- to 4-week-old) Cftr tm1CAM/Cftr tm1CAM mice, Mdr1 mRNA expression was increased compared with wild-type mice, while in adult mice Cftr tm1CAM/Cftr tm1CAM mice Mdr1 was underexpressed. The expression of two control genes was not altered in the Cftr tm1CAM/Cftr tm1CAM mice, indicating that the observed changes in Mdr1 expression were specific to the loss of Cftr tm1CAM expression and/or function. Furthermore, as the +/Cftr tm1CAM mice are phenotypically wild-type (19), this suggests that the changes in Mdr1 expression are not due to a general stress response associated with the severe intestinal phenotype of the Cftr tm1CAM/Cftr tm1CAM mice. The changes in Mdr1 expression observed in the Cftr tm1CAM/Cftr tm1CAM mice are also influenced by the developmental stage of the intestine. The transition through weaning results in Mdr1 expression in Cftr tm1CAM/Cftr tm1CAM mice survive to 10 weeks, it is possible that these mice may represent a sub-population, and that down-regulation of Mdr1 expression in 10-week-old Cftr tm1CAM/Cftr tm1CAM mice does not represent the true pattern of Mdr1 expression in the general population of adult Cftr tm1CAM mice. If this were the case then we would expect to see some immature Cftr tm1CAM/Cftr tm1CAM mice showing underexpression of Mdr1 in their intestines. We analysed Mdr1 expression in nine neonatal and suckling–weaning Cftr tm1CAM/Cftr tm1CAM mice and always observed increased intestinal Mdr1 expression. For similar reasons, we suggest that the modifier locus, which modulates the severity of the cystic fibrosis phenotype in the Cftr tm1Rm5 mice (22), is unlikely to have an impact on Mdr1 expression in the Cftr tm1CAM mice.

This work, together with our previous studies of the expression of Cftr and Mdr1, suggests that the expression of both these genes is subject to spatial, hormonal, temporal and co-ordinate regulation (15,26,34). A number of possible mechanisms underlying the co-ordinated regulation of Cftr and Mdr1 can be envisaged. It is possible that a reduction or loss of Cftr function influences Mdr1 mRNA expression. This model is consistent with the observation of increased Mdr1 expression in the Cftr tm1CAM/Cftr tm1CAM mice, which express no functional Cftr protein, and the intermediate increase in Mdr1 mRNA levels in +/Cftr tm1CAM mice. However, it is equally possible that some other aspect of Cftr expression influences Mdr1 expression, for example mRNA levels. Any proposed model will also have to take into account the interaction between mechanisms directing the co-ordinate regulation of Cftr and Mdr1 and the temporal regulation of Mdr1.

MATERIALS AND METHODS

Mice and reagents

The population of cystic fibrosis knockout mice was created and bred at the Wellcome/CRC Institute in Cambridge (Cftr tm1CAM mice). The genotypes of the mice were established by PCR and Southern blot analysis as described previously (18,19).

In situ hybridisation

Animals were sacrificed by a lethal injection of anaesthetic and tissues fixed by whole body perfusion with 4% paraformaldehyde. In situ hybridisation was carried out essentially as described previously (26). Cryostat sections (10 µm) were hybridised to 35S-labelled, single-stranded RNA probes. The antisense strand probe will hybridise to the mRNA and identify cells expressing the gene of interest. The sense strand probe serves as a negative control.

The upstream and downstream mouse Cftr probes were generated by PCR and have been described previously (26). The mouse Mdr1b exon 6 probe was produced by PCR amplification from the mouse Mdr1b cDNA (a generous gift from Dr P. Gros). The sequence of the PCR DNA fragment exactly matched the published mouse Mdr1b sequence, and corresponded to nucleotides 446–635 (35). The mouse Mdr2 exon 21 probe was
generated by PCR from mouse genomic DNA. The sequence of the mouse Mdr2 exon 21 probe exactly matched the published mouse Mdr2 sequence and corresponded to positions 2663–2864 (36). The human PGK-1 probe, which cross-hybridises with mouse Pkg-1 sequences, corresponded to 68 bp of intron 2 and 121 bp of exon 3 of human PGK-1. The plasmid containing the human PGK-1 insert was kindly provided by Dr J. Firth (29). All DNA fragments used as in situ hybridisation probes were subcloned into pSK* (Bluescript: Stratagene) to allow the in vitro transcription of the insert using T3 and T7 RNA polymerases.

In situ hybridisation signal intensities were estimated by densitometric analysis of contact sheets of in situ hybridisation slides made prior to dipping the slides in photographic emulsion. Contact sheets were obtained using Amersham β-max film and hybridisation signal intensities quantitated using an Seescan densitometer (Cambridge, UK). Hybridisation signals from a given probe were quantitated by averaging readings from a minimum of three sections per probe per animal. In the case of +/+, 1-day-old animals, measurements from two animals were averaged to give a final measure of the level of signal from a given probe. In all other cases, measurements were averaged from three animals.

**Total RNA preparation, reverse transcription-PCR and RNase protection analysis**

Total RNA was prepared using either the acid guanidinium thiocyanate phenol method (37), or by lithium chloride urea extraction (38).

RT-PCR was carried out according to (39). Two pairs of primers were used. One pair amplified a fragment of 480 bp, containing exons 4–6 of murine Cfr upstream of the targeted interruption in exon 10 of the Cfrtm1CAM mice. The other pair of primers amplified a fragment of 375 bp, corresponding to exons 11–13 downstream of the targeted disruption. A pair of primers which amplified a 500 bp fragment of β-actin cDNA (40) were used as a positive control.

The primers for Cfr exons 4–6 were: SM3, 5′-tccag cctgt ctgca tagga aagat-3′ and SM4, 5′-cattg attct gctag cttgac-3′. The primers for Cfr exons 11–13 were: X11F, 5′-GACAT CACCA AGTTT GCAGA A-3′ and SM4, 5′-AACTG GCCGA AAAAT ATGCA-3′. The primers for β-actin were: Actin 1, 5′-ATGGA TGACG ATATG GCTG-3′ and Actin 2, 5′-ACCTG ACAGA CTACCC TCAT-3′.

RNase protection assays were performed using standard techniques (41) except that tRNA was omitted from the reaction. After digestion with RNase A and T1, the digestion products were purified using standard techniques (41) except that tRNA was omitted from the reaction. Contact sheets were obtained using Amersham β-max film and hybridisation signal intensities quantitated using an Seescan densitometer (Cambridge, UK). Hybridisation signals from a given probe were quantitated by averaging readings from a minimum of three sections per probe per animal. In the case of +/+, 1-day-old animals, measurements from two animals were averaged to give a final measure of the level of signal from a given probe. In all other cases, measurements were averaged from three animals.

**REFERENCES**


