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Complementation of null CF mice with a human *CFTR* YAC transgene

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We have made transgenic mice carrying a 320 kb YAC with the intact human cystic fibrosis transmembrane regulator (*CFTR*) gene. Mice that only express the human transgene were obtained by breeding with Cambridge null CF mice. One line has approximately two copies of the intact YAC. Mice carrying this transgene and expressing no mouse cftr appear normal and breed well, in marked contrast to the null mice, where 50% die by ~5 days after birth. The chloride secretory responses in these mice are as large or larger than in wild-type tissues. Expression of the transgene is highly cell type specific and matches that of the endogenous mouse gene in the crypt epithelia throughout the gut and in salivary gland tissue. However, there is no transgene expression in some tissues, such as the Brunner's glands, where it would be expected. Where there are differences between the mouse and human pattern of expression, the transgene follows the mouse pattern. We have thus defined a cloned fragment of DNA which directs physiological levels of expression in many of the specific cells where CFTR is normally expressed.

Keywords: CFTR/cystic fibrosis transmembrane conductance regulator/transgenic mouse/YAC/yeast artificial chromosome

Introduction

Cystic fibrosis (CF) is characterized by abnormalities in epithelial cell function in the respiratory, digestive and reproductive tracts and sweat glands. The disease is very common in the Caucasian population, affecting about 1 in 2000 live births, and shows an autosomal recessive pattern of inheritance. The gene responsible was cloned and sequenced in 1989 and named the cystic fibrosis transmembrane conductance regulator (CFTR) (Kerem *et al.*, 1989; Riodan *et al.*, 1989; Rommens *et al.*, 1989). It has since been shown to encode a cAMP-activated chloride channel which is normally located in the apical membrane of many epithelial cells. In addition to being a chloride channel itself, it also has effects on the regulation of other channels in the membrane (Stutts *et al.*, 1995).

Several groups have made mouse models with complete null alleles of the *Cftr* gene by replacement of a section of exon 10 (Colledge *et al.*, 1992; Snouwaert *et al.*, 1992; Ratcliff *et al.*, 1993), by insertion into exon 3 (O'Neal *et al.*, 1993), by introducing a stop site in exon 2 (Hasty *et al.*, 1995) or by disrupting exon 1 (Rozmahel *et al.*, 1996). The Cambridge null mouse (*cftr*^{m1CAM}) (Ratcliff *et al.*, 1993) is typical of these models and has a severe gut pathology characterized by mucus accumulation with distension of the crypts and flattening of the villi, leading to the development of obstruction which leads to death of ~50% of the homozygous null mice 2–5 days after birth and a further period of mortality at weaning. The pancreas is only slightly affected, with some dilation and blockage of ducts in ~50% of the animals. The lacrimal glands are sometimes affected, with the mice prone to eye infections. The lungs show very few histological abnormalities. The cAMP-dependent chloride secretory responses in these null mice are completely abrogated.

Cystic fibrosis is a prime target for gene therapy because the disorder is recessive and the major affected tissues, the lung and the gut, are accessible for DNA delivery by inhalation of nebulized DNA solutions or by instillation. Experiments on delivering DNA to null mouse models using cDNA constructs complexed with liposomes have led to some correction of chloride transport (Alton *et al.*, 1993; Hyde *et al.*, 1993). Clinical trials have also been carried out which have led to low levels of transient correction (Zabner *et al.*, 1993; Caplen *et al.*, 1995). However, to obtain clinical improvement in CF patients the efficiency of correction will need to be increased significantly, particularly as it will be important to correct the defects in chloride and sodium transport. The latter should be automatically corrected if normal levels of CFTR can be achieved in a sufficient number of epithelial cells (Stutts *et al.*, 1995).

One of the main reasons for low levels of correction is certainly the inefficiency of delivery of the therapeutic DNA. However, another reason may be that the promoter and cDNA constructs used are not expressed sufficiently in the target cells. *CFTR* has been shown to have a very precise cellular distribution of and developmental and hormonal regulation of expression (Crawford *et al.*, 1991; Marino *et al.*, 1991; Trezise and Buchwald, 1991; Engelhardt *et al.*, 1992, 1994; McCray *et al.*, 1992; Tizzano *et al.*, 1993; Trezise *et al.*, 1993a,b; Strong *et al.*, 1994). Transgenic mice have been made carrying a human

CFTR cDNA construct driven by the rat intestinal fatty acid binding protein gene promoter (Zhou *et al.*, 1994). However, although there were high levels of expression of the *CFTR* gene in the gut, the gene was expressed in the epithelia of the villi rather than the cells of the crypts and led to only ~25% correction of cAMP-sensitive chloride conductance in the gut. Clearly this would be an inefficient construct for gene therapy. The same may well be true for the CMV, SV40 and adenoviral promoters, which have been used in gene therapy experiments, though transgenic mice expressing *CFTR* from these promoters have not been described.

Transgenic mice have been made with genes driven by the *CFTR* promoter. When 2.2 kb of *CFTR* promoter were used to drive the SV40 T antigen, the only cells to be affected were ependymal cells in the brain, while those cells where *Cftr* is known to be highly expressed were unaffected (Perraud *et al.*, 1992). When 3.8 kb were used to drive a *lacZ* gene, no expression was observed (Griesenbach *et al.*, 1993). The low level of expression may be due to the constructs not containing the necessary controlling elements and, indeed, DNase I hypersensitive sites have been detected at –20.5 kb and –79.5 kb from the start of transcription (Smith *et al.*, 1995).

Yeast artificial chromosome (YAC) clones are large enough to contain the intact *CFTR* gene with all its 27 introns spanning 230 kb. In addition, a YAC could contain all the DNA elements necessary for full levels of expression which may be located tens of kilobases away from the gene itself. Clearly a piece of DNA hundreds of kilobases in size is very large for efficient delivery in gene therapy. However, if the clone is shown to drive full expression of the *CFTR* gene it should be possible to delete segments to derive a construct small enough for use in gene therapy while retaining high levels of expression. Towards this aim we have introduced a 320 kb YAC carrying the intact human *CFTR* gene into transgenic mice. The transgene has then been bred onto the Cambridge null CF background to determine how efficiently it can complement the null phenotype. RNA *in situ* hybridization has been used to assess whether the transgene faithfully mimics cell type-specific expression of the endogenous, wild-type *Cftr* and epithelial transport has been used to assess physiological function of the protein.

Results

Transgenic mice carrying the intact human CFTR gene and no functional mouse gene

The intact human *CFTR* gene has been cloned previously in a YAC clone (37AB12) (Anand *et al.*, 1991). This YAC contains an insert of ~320 kb and appears to contain the intact *CFTR* gene, which consists of 27 exons spanning ~230 kb of DNA (Figure 1). Long range restriction analysis was carried out using the enzymes *Not*I and *Sal*I, which cut in the vector arms but not in the insert, *Bss*HII and *Sac*II, which cut upstream of exon 1, *Nru*I, which cuts in exon 4, and *Sfi*I, which cuts twice in the gene (Figures 1 and 2A). By comparing the restriction fragments of the YAC (Figure 1) with the long range mapping data from the human genome (Rommens *et al.*, 1989), we deduced that the YAC contains ~70 kb of genomic DNA upstream of exon 1 and that the 3′-end of the gene is very close to the right arm of the YAC (the *Eco*RI fragment spanning the last exon is intact). These sizes are only accurate to within ~10 kb as determined by pulsed-field gel electrophoresis and there can be large shifts in relative mobility between marker lanes, yeast DNA and mammalian DNA. Thus, this YAC contains the intact gene and may contain all the DNA necessary for full levels of tissue-specific and regulated expression in transgenic mice.

Seven transgenic mice were made by pronuclear injection of gel-purified, intact YAC DNA. Three of these had only part of the YAC, while the other four carried the left and right arms of the YAC as well as exons 1, 7, 11, 20 and 24 as determined by PCR. Of the four founders carrying most of the YAC, two were bred to give the lines T30 and T57, while the other two did not breed. The transgenic lines are called TgN(yCFTR)T30 and TgN(yCFTR)T57 and are referred to as T30 and T57 respectively.

Long range analysis of the DNA in the transgenic mice was carried out with *Sal*I, *Sac*II and *Nru*I. As can be seen in Figure 2B, the T30 transgene carries an intact copy of the YAC which is cleaved out as a 320 kb fragment by digestion with *Sal*I, which is present in both vector arms. The internal *Sac*II site is present, though digestion is not quite complete. However, internal digestion with *Nru*I was not observed, probably due to methylation of the YAC DNA in the mouse genome. Similar long range analysis of the T57 line did not give the 320 kb *Sal*I fragment, indicating that the YAC could be rearranged in this line or that the *Sal*I sites, which are embedded in prokaryotic DNA in the vector arms, are methylated.

Further investigations were made to determine the intactness of the T57 and T30 transgenes. The 50 kb *Bss*HII fragment upstream of the first exon, the 50 kb *Sfi*I fragment spanning exons 15–19 and a 2 kb *Eco*RI fragment spanning the 3′-terminal exon were all present in both lines. Finally, a 5 kb *Eco*RI band spanning the DNase I hypersensitive site at –20 kb (Smith *et al.*, 1995) and a 4 kb *Hin*dIII fragment spanning the hypersensitive site in the first intron (Smith *et al.*, 1996) were also present.

A *Hin*dIII digest probed with exon 4 showed a copy number of three to four for T30 but a probe for the 3′-end of the gene gave a copy number of two, suggesting that there are one or two incomplete copies of the YAC as well as two intact copies (data not shown). T57 had a copy number of one (data not shown).

These two transgenic lines, T30 and T57, were crossed with the Cambridge null CF line in which the *Cftr* gene is interrupted with a *HPRT* cassette resulting in a complete null allele ($cftr^{m1CAM}$) (Colledge *et al.*, 1992; Ratcliff *et al.*, 1993). Heterozygotes carrying one copy of the knocked out gene and one copy of the transgene (designated T30 cf/+ or T57 cf/+) were crossed to give offspring expressing no endogenous mouse *Cftr* but carrying the human transgene (designated T30 cf/cf or T57 cf/cf). The homozygosity of the null allele was determined both by PCR and Southern blotting. However, it was not determined whether the YAC transgene was heterozygous or homozygous.

Over the course of various breeding experiments, six out of 11 homozygous null mice not carrying any transgene (cf/cf) died before the age of 40 days. During this same period, one out of 73 mice carrying the T30 transgene on

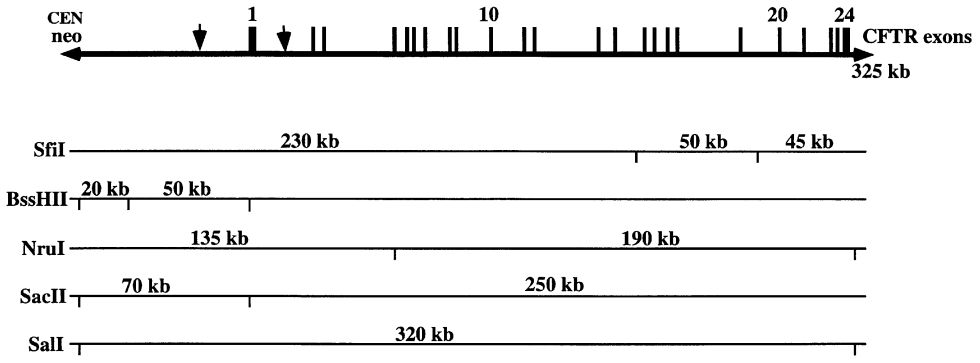


Fig. 1. Map of YAC yCFTR showing the positions of the *Bss*HII, *Nru*I, *Sal*I, *Sac*II and *Sfi*I sites, the *CFTR* exons (vertical lines) and the DNase I hypersensitive sites (arrows). The positions of the internal sites were determined using the cDNA probe, the –20 kb probe and the left and right vector arm probes. The positioning of the exons was extrapolated from the map of the genomic DNA in this region (Rommens *et al*., 1989).

a null background (T30 cf/cf) died (age 12 days), six out of 56 mice carrying the T57 transgene on a null background (T57 cf/cf) died and only three out of 255 mice carrying a wild-type mouse gene died before the age of 40 days. Thus the T30 transgene has effectively rescued the mortality due to CF, whereas there may be some residual phenotype with the T57 transgene. The T30 cf/cf mice are fertile and showed no ill health. However, T57 cf/cf mice had a problem with the lacrimal glands (in some the eyes became swollen) and some females were infertile.

The CFTR protein is functional in the transgenic mice

Epithelial transport is one of the most sensitive ways of detecting functional CFTR. Three epithelial tissues lining the intestine were examined using a standard protocol in which a variety of pharmacological agents were employed to display the transporting activities present in the tissues. Colons from T30 cf/cf animals showed short circuit current (SCC) responses to forskolin, acting via cAMP (Figure 3), of a magnitude comparable with those of wild-type tissues (Figure 4). Responses to forskolin in T57 cf/cf colons were significantly smaller than those of either T30 cf/cf or wild-type colons ($P < 0.01$). However, the responses in T30 cf/cf, T57 cf/cf and wild-type colons were all significantly greater than those in CF null colons (Figure 4). The reduction in SCC in CF null colon epithelia following forskolin was due to stimulation of potassium ion secretion (Cuthbert *et al.*, 1994).

In the jejunum responses to forskolin were not significantly different in T30 cf/cf, T57 cf/cf and wild-type tissues and all produced responses significantly greater than those in CF null jejuna ($P < 0.02$) (Figure 4). The small responses to forskolin in CF null jejuna are due to stimulation of sodium-dependent glucose transport (Grubb, 1995).

No significant differences were recorded between the responses of T30 cf/cf, T57 cf/cf and wild-type caecal epithelium (Figure 4), although in this tissue the responses are rather variable depending on the part of the caecal sac from which the epithelium is taken. Caecal epithelium from CF null animals showed little or no response to forskolin or, indeed, other chloride secretagogues, such as lysylbradykinin (Hyde *et al.*, 1993; Ratcliff *et al.*, 1993).

The mean degree of inhibition of the chloride secretory response by frusemide in T30 cf/cf colons was 58% and in T57 cf/cf colons was 67%, comparable with the inhibition seen in wild-type murine colons of 62% (Cuthbert *et al.*, 1995). This is a further indication that the responses of the transgenic colons are essentially normal.

Although the majority of the observations upon epithelial transport in gut epithelia were made with forskolin, the actions of another agonist were evaluated on a few occasions after washing and allowing the tissues to restabilize. An example with carbachol, which acts through the Ca^{2+}/IP3 cascade, is shown in Figure 3. The response of the T30 cf/cf colon is similar to that observed from wild-type colons (Cuthbert *et al.*, 1994), whereas the response in the T57 cf/cf colon is reminiscent of an effect on potassium ion secretion, reported earlier for CF null epithelia (Cuthbert *et al.*, 1994). One possibility was that although T57 cf/cf gut epithelia did show wild-type responses, the ability to do so was not well maintained, so that on further application of chloride secretagogues the responses reverted to the CF type. When the T57 cf/cf colon of Figure 3 was exposed to carbachol it had already been short circuited for >2 h and had previously been exposed to forskolin. Consequently, experiments were designed to examine if the responses in transgenic animals to forskolin were as well maintained as those of wild-type tissues.

To do this, tissues were treated with 10 μM forskolin or with forskolin and 1 mM isobutyl methyl xanthine (IBMX) continuously for a period of 6 h and continually short circuited. Forskolin will increase the concentration of cAMP within the cells, while IBMX, acting as a phosphodiesterase inhibitor, will prevent hydrolysis of the nucleotide. However, since IBMX also has phosphatase inhibitory activity, it has been shown that it prevents the rundown of activated CFTR chloride channels by preventing dephosphorylation (Becq *et al.*, 1994).

To compare the responses under the two conditions, the value of the SCC increase at 1 h was designated 100% and responses before and after this time were compared with this standard value. The patterns of responses in cf/+ (Figure 5A) colons were very similar to wild-type (Figure 5B), with little fall-off in the response over 6 h in the presence of forskolin and IBMX and a somewhat more rapid fall-off in the presence of forskolin alone. The T30 cf/cf colon (Figure 5C) showed well-maintained responses comparable with those of wild-type tissues. However, T57 cf/cf (Figure 5D) colons maintained SCCs

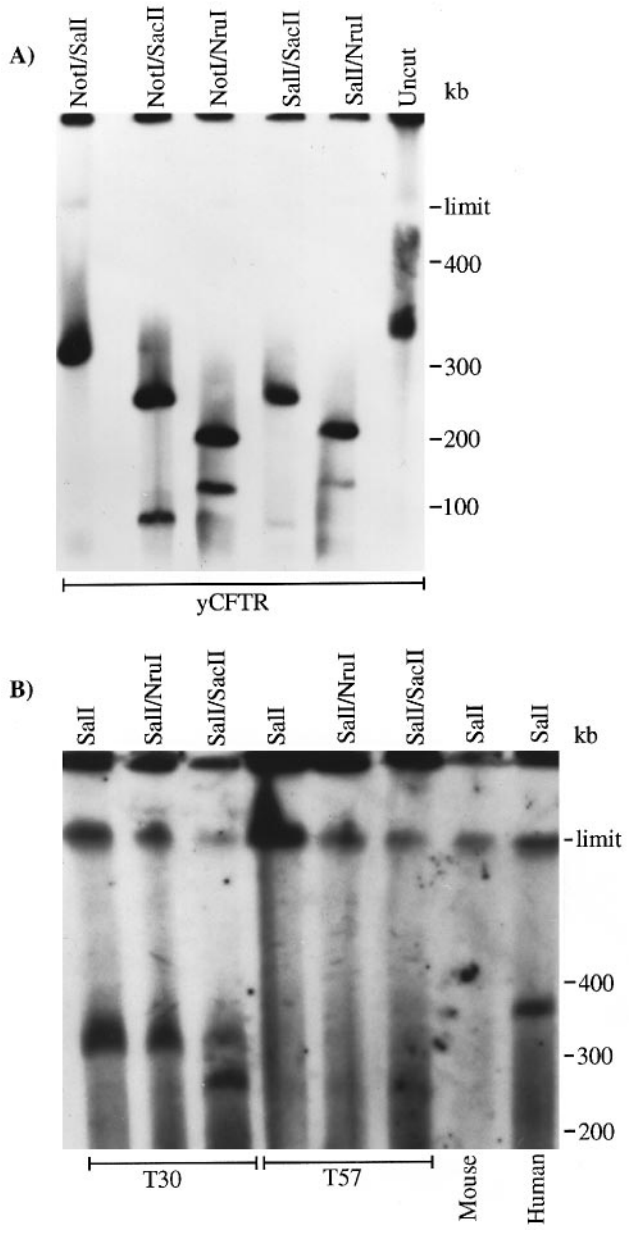


Fig. 2. Long range analysis of the YAC and the transgenic mice. (**A**) High molecular weight DNA from the yeast containing the YAC was digested with *Not*I, *Nru*I, *Sal*I and *Sac*II in the combinations shown above the lanes. (**B**) High molecular weight DNA from a transgenic mouse carrying the T30 transgene, a transgenic mouse carrying the T57 transgene, a wild-type mouse and human DNA were digested with the enzymes shown above the lanes. The DNA was separated by pulsed-field gel electrophoresis, blotted and probed with the human *CFTR* cDNA probe. The positions of λ size markers are shown on the right of the gels. The yeast DNA has run somewhat faster than the mammalian DNA relative to the markers due to differences in complexity of the DNA, which are very difficult to eliminate.

for 1 h or so, after which the responses faded to near zero by 4 h. Thus T57 cf/cf colons not only produce smaller responses to forskolin, but the responses are less well maintained compared with T30 cf/cf and wild-type epithelia.

Wild-type caecal epithelia do not maintain the forskolin-sensitive current as well over time as colonic epithelia. However, T30 cf/cf caecae are indistinguishable from wild-type in this test (Figure 5E). Only one T57 cf/cf caecum was examined and, like T57 cf/cf colon, it failed to maintain SCC as well as the other genotypes (Figure 5E).

Overall, the responses in T30 cf/cf gut epithelial tissues were equal to those of wild-type responses and these were well maintained over time. T57 cf/cf colons gave somewhat smaller responses than wild-type, though the other tissues gave good responses. However, the responses in T57 cf/cf tissues decreased rapidly after ~1 h.

Relatively few observations were made on airway epithelia from the mice, particularly as only one tracheal preparation of 2.3 mm^2 could be obtained from each mouse. Using the protocol described in Materials and methods to eliminate the calcium stores and raise intracellular levels, the SCC increase in response to forskolin was 6.6 ± 2.0 $\mu A/cm^2$ ($n = 3$) in T30 cf/cf mice. This value is significantly greater ($P < 0.013$, single tailed *t*-test) than the value in CF null mice of 2.5 ± 0.6 $\mu A/cm^2$ ($n = 7$) but not different ($P < 0.063$, single tailed *t*-test) from the value in wild-type mice of 13.7 ± 2.3 $\mu A/cm^2$ ($n = 9$). In three other tracheae from T57 cf/cf mice the forskolin response was 4.3 ± 1.1 $\mu A/cm^2$, which was not different ($P < 0.09$) from the response of CF null tracheae.

Expression of the human CFTR mRNA

We used RNA *in situ* hybridization to assess whether the human *CFTR* transgenes faithfully mimic the tissue- and cell type-specific expression of the endogenous mouse *Cftr* gene. The species specificity of the mouse and human *Cftr* probes was demonstrated by hybridizing both sense and antisense probes to consecutive sections of wild-type mouse ileum. The sense probes gave no signal, as expected. The mouse antisense probe detected the mouse mRNA while the human antisense probe did not give any signal, indicating that under the hybridization conditions used here the mouse and human *Cftr* probes are species specific.

Expression of the human transgene was investigated in the ileum of transgenic mice (Figure 6). *Cftr* is predominantly expressed in the crypt epithelia throughout the intestine, with a decreasing gradient of expression from the duodenum to the colon in both mouse and human tissues (Trezise and Buchwald, 1991; Trezise *et al.*, 1992, 1993b; Strong *et al.*, 1994). Figure 6A–F shows the ileum of a T30 cf/+ mouse; Figure 6B shows expression of the mouse gene (one copy), Figure 6E shows expression of the human T30 transgene (two or four copies because the mouse could be homozygous or heterozygous). The T30 transgene is expressed in the crypts, as is the endogenous mouse *Cftr* gene. In addition, it is possible to say that the level of expression of the T30 transgene is roughly the same as that of the mouse gene, as the signal is of roughly the same intensity after hybridization with similarly labelled probes and exposure for the same length of time. Figure 6J–O shows the ileum of a T57 +/+ mouse. The human T57 transgene (one or two copies) (Figure 6N) is expressed in very similar cells, though at a somewhat lower level than the mouse gene (two copies) (Figure 6K). Clearly, both human *CFTR* transgenes are being expressed appropriately in the crypts of the ileum and the level of expression is similar to that of the mouse gene.

We also examined expression of the transgenes in different regions of the intestines. In addition to being

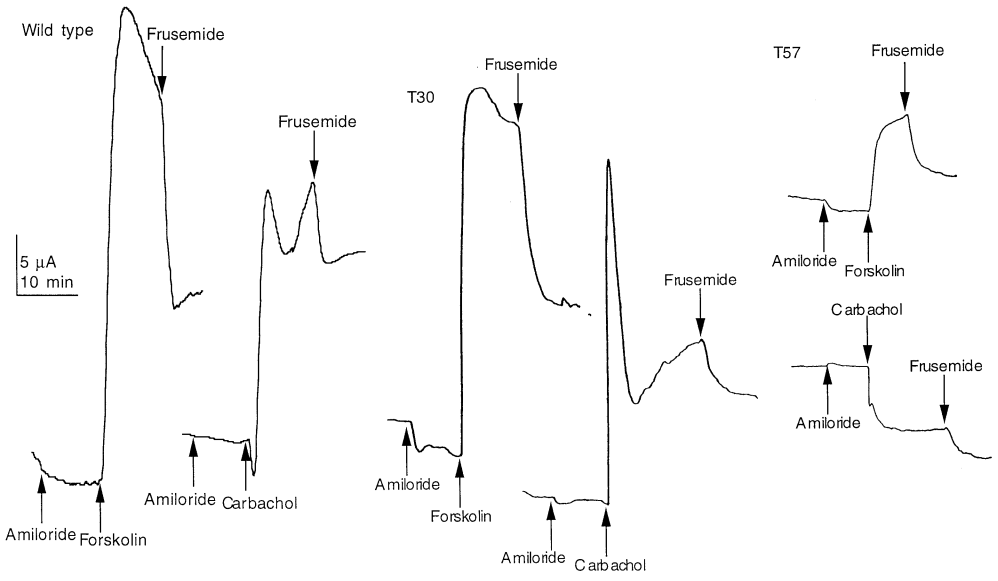


Fig. 3. Typical SCC responses from colonic epithelia (20 mm^2) from wild-type, T30 cf/cf and T57 cf/cf animals. After exposure to amiloride, tissues were exposed to forskolin and then frusemide. After repeated washing, and when the SCC had returned to a low and stable baseline, the protocol was repeated with carbachol as the secretagogue. Drug concentrations were: amiloride, 10 μM given apically; forskolin, 10 μM applied both sides; frusemide, 1 mM applied basolaterally; carbachol, 10 μM applied basolaterally.

expressed in the cells in the crypts, *CFTR* has also been shown to be expressed in ~1% of villous cells in the duodenum of rats and humans, but not mice (Trezise and Buchwald, 1991; Trezise *et al.*, 1993b; Strong *et al.*, 1994), though the precise nature of these rare villous cells remains to be identified. Figure 6G–I shows the duodenum of a T30 cf/cf mouse. Figure 6H shows human *CFTR* expression (there is no endogenous mouse *Cftr* expression), which is present in the crypts as expected. An interesting feature is the lack of transgene expression in any of the villous cells of the duodenum. In this respect the transgene appears to be following the mouse pattern of *Cftr* expression, rather than the human pattern. Expression of the human T30 transgene is seen to be very similar in both the cf/+ mouse (Figure 6E) and the cf/cf null mouse (Figure 6H), indicating that expression of the mouse gene does not greatly affect the human transgene.

CFTR has also been shown to be expressed in the salivary glands of both rodents and humans (Trezise and Buchwald, 1991; Kartner *et al.*, 1992). In the transgenic mice, the T57 *CFTR* transgene faithfully mimicked expression of the endogenous mouse *Cftr* gene. Figure 7C1–C4 shows sections of the submaxillary gland and three major structures are identified in these sections; serous acini (S), mucous acini (M) and salivary gland ducts (D). Endogenous mouse *Cftr* is expressed in the ductal epithelium and in the mucous acini, but not the serous acini. Figure 7C4 clearly shows expression of the T57 transgene in the ductal epithelium and the mucous acini but not the serous acini, matching the endogenous gene (T30 was not analysed).

The pancreas is a further site of *CFTR* expression in both rodents and humans. However, in human pancreatic ducts *CFTR* is expressed at quite high levels (Riodan *et al.*, 1989; Crawford *et al.*, 1991; Marino *et al.*, 1991; Trezise *et al.*, 1993b; Strong *et al.*, 1994), while in rats and mice the levels of *Cftr* expression in this tissue are very low (Trezise and Buchwald, 1991). We were not able to detect expression of the human *CFTR* transgene in the pancreatic ducts of either line of transgenic mice regardless of the *Cftr* genetic background (data not shown). It is not possible to say whether the transgene is not being expressed at all or whether it is being expressed at a very low level, similar to the endogenous expression of mouse *Cftr*. In any case, it is clear that the transgene is not being expressed in a typical human pattern, which would direct high level *CFTR* expression in the pancreatic ducts.

The lung is a particularly important organ in CF pathogenesis in humans. In the adult human bronchus there is low level expression of *CFTR* in the epithelium with much higher expression in the submucosal glands (Engelhardt *et al.*, 1992, 1994). However, the level of expression in human adult lung is very low and much lower than in fetal tissues (Crawford *et al.*, 1991; Trezise *et al.*, 1993b). The glands and ducts are effectively absent in the rodent lung (Trezise and Buchwald, 1991) and, indeed, neither endogenous mouse nor human transgene expression was detected in a T57 +/+ mouse (data not shown).

Endogenous *CFTR* is also expressed in the submucosal glands of the intestine, the Brunner's glands, in both mouse and human tissue (Strong *et al.*, 1994; A.Trezise, unpublished data). Neither of the human transgenes was expressed in this structure of the intestine, though the endogenous gene clearly is (Figure 7B3 and B4 for T57; data for T30 not shown). Other tissues where the human transgenes were not expressed but the mouse gene is expressed include the pyloric glands of the stomach (Figure 7A1–A4; T57 and T30 analysed with data for T30 not shown) and the initial segment of the epididymis (Figure 7D1–D4; T57 analysed, T30 not analysed). As both the T30 and T57 transgenes were not expressed in the Brunner's glands and the pyloric glands it is very unlikely to be due to effects of the position of integration.

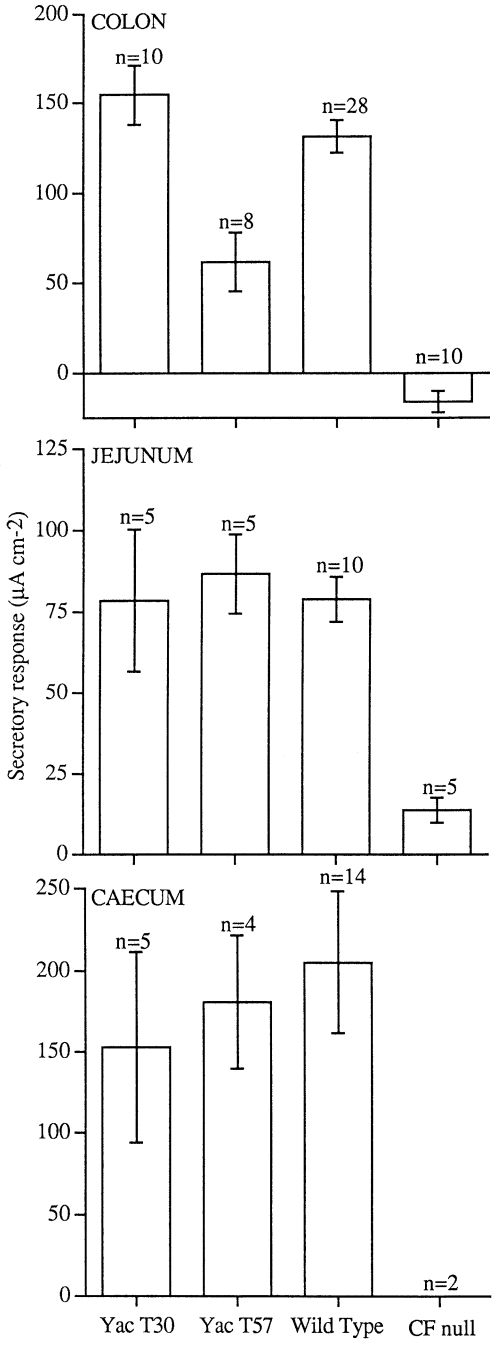


Fig. 4. Composite data from gut epithelia from four T30 cf/cf and three T57 cf/cf mice. One to three pieces of colonic, jejunal and caecal epithelia were taken for SCC recording. Each bar shows the mean responses ± SEM for the SCC increase in response to forskolin (10 μM). *n* values refer to the number of tissues. Responses in colonic epithelia are shown at the top, jejunal epithelia in the middle and caecal epithelia at the bottom. In the first set of columns responses of T30 cf/cf tissues are given, in the second T57 cf/cf responses, wild-type responses are given in the third and in the fourth column responses from CF null tissues. The data for the wild-type and CF null epithelia are taken from other of our publications (Ratcliff *et al*., 1993; Cuthbert *et al*., 1994, 1995), except those for CF null jejuna, which are new to this study. Responses in T30 cf/cf, T57 cf/cf and wild-type colons are all significantly greater ($P < 0.01$) than in CF nulls, while those in T57 cf/cf colons are significantly smaller ($P < 0.01$) than those of T30 cf/cf and wild-type colons. There were no significant differences between the responses in T30 cf/cf, T57 cf/cf and wild-type jejunal epithelia, which were all significantly larger than those of CF null jejunal epithelia ($P < 0.02$). Similarly, the responses of the three types of caecal epithelia were not significantly different from one another.

Discussion

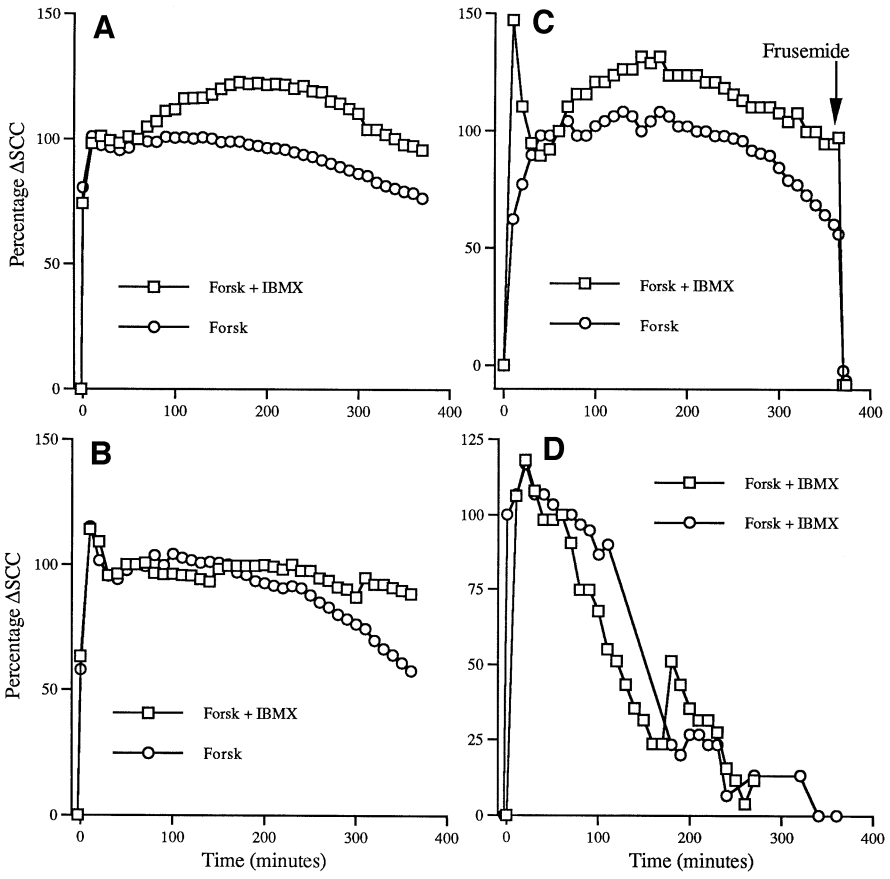
YAC 37AB12 contains the intact human *CFTR* gene consisting of 27 exons spread over ~230 kb and ~70 kb of upstream DNA. This YAC DNA was introduced into transgenic mice giving two lines which express the *CFTR* gene. In T30 there are two copies of the intact YAC and one or two partial copies. In T57 there is a single copy of the YAC DNA which is largely unrearranged, though it could not be cleaved out as an intact fragment and hence may be rearranged or may be methylated at the ends. Both of these transgenes were crossed onto the Cambridge null CF background giving mice which express no mouse *Cftr* but which do express the human gene. The T30 cf/cf mice are fully healthy and breed well. The T57 cf/cf mice have a mild CF phenotype, with the females being sub-fertile and the lacrimal glands not functioning normally. In both lines the histology of the gut is essentially normal.

Electrogenic chloride transport is a sensitive way of measuring function of the CFTR protein. In T30 cf/cf mice the amplitude of response to cAMP was found to be normal in colon, jejunum and caecum and probably airway epithelium. Colonic tissues responded to amiloride, forskolin, carbachol and frusemide in an essentially normal fashion. Finally, the duration of the response to forskolin or forskolin and IBMX in the colon and caecum was effectively normal. This full correction in T30 cf/cf tissues is consistent with physiological levels of cell type-specific expression from the intact YAC DNA.

There were, however, significant differences between the responses of tissues taken from T57 cf/cf and wild-type animals. Colons from T57 cf/cf animals gave significantly smaller ($P < 0.001$) responses than wild-type, though significantly greater ($P < 0.01$) than in cf/cf null colons (~40% correction). Furthermore, the responses in T57 cf/cf tissues fell after 1 h or so, even though IBMX was present. A plausible hypothesis for the small non-maintained currents in T57 cf/cf tissues is that the density of chloride channels in the apical membrane is less than in wild-type membranes and the rate of synthesis is unable to keep pace with the rate at which inactivated channels are removed from the membrane. Acute responses in jejunal and caecal epithelia from T57 cf/cf animals gave responses in the range of those shown by wild-type tissues. Even so, a single T57 cf/cf caecal epithelium was unable to maintain the Cl^- secretory current as well as wild-type or T30 cf/cf tissues.

RNA *in situ* hybridization was used to determine the cell type specificity of expression of the YAC transgenes. The T30 and T57 transgenes were found to have the same cell type specificity of expression in all tissues examined. The level of expression of the T30 and T57 transgenes was comparable with that of the endogenous mouse genes as judged from the intensity of the signal from hybridization with equally labelled probes. This implies that expression from the YAC is largely independent of the position of integration and each copy is expressing approximately physiological levels of mRNA. In addition, the level and cell type specificity of expression was not affected by whether the mouse was expressing endogenous mouse *Cftr* or not.

The transgenes are expressed in a highly cell type- and



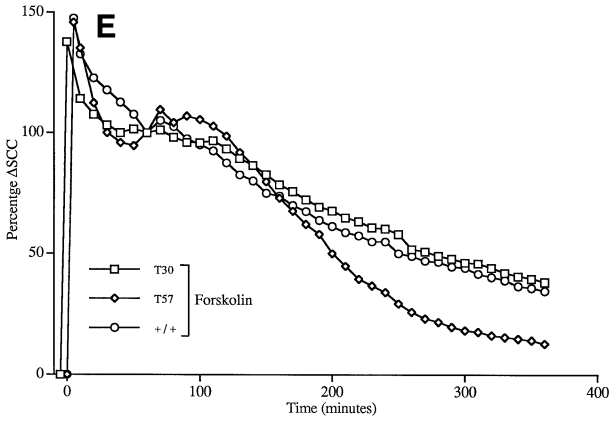


Fig. 5. Duration of the response to forskolin in mouse colon epithelium. Tissues were exposed to forskolin (10 μM, both sides) or forskolin plus IBMX (10 μM and 1 mM respectively) and short circuited for up to 6 h. The SCC values obtained 60 min after the agent(s) was added was designated 100% and the SCCs at other times were calculated with respect to this value. (**A**) Mean values for five pairs of colons from separate heterozygous cf/+ animals are shown. The basal SCC was 12.0 ± 4.2 μA/cm^2 (mean ± SE, n = 10) and the mean increase in SCC caused by forskolin or forskolin and IBMX was 197.0 ± 14.5 μA/cm^2 (n = 10). (**B**) Mean values from two pairs of colons from wild-type animals are given. The values of basal SCC and the increase caused by forskolin, with or without IBMX, were respectively 37.5 ± 10.1 and 243.8 ± 20.5 μA/cm^2 (n = 4 for both). (**C**) Results for the colon of a single T30 cf/cf animal. The basal SCC was 37.5 μA/cm^2 (n = 2) and the increase caused by forskolin or forskolin and IBMX was 207.5 μA/cm^2 (n = 2). (**D**) Data from colons of two T57 cf/cf mice are shown, the basal SCC was 4.4 μA/cm^2 (n = 2) and the increase caused by forskolin plus IBMX was 38.8 μA/cm^2 (n = 2). (**E**) Duration of the response to forskolin in mouse caecal epithelia. Responses are shown for T30 cf/cf caecae (basal SCC 1.5 μA/cm^2, ΔSCC forskolin 258.7 μA/cm^2, both n = 2), a T57 cf/cf caecum (basal current 2.0 μA/cm^2 and ΔSCC forskolin 270 μA/cm^2) and a wild-type (+/+) caecum (basal current 2.0 μA/cm^2 and ΔSCC forskolin 295 μA/cm^2).

tissue-specific manner and no expression was observed in any cell types which did not express the endogenous mouse *Cftr* gene. Expression was observed in the crypts of the gut, as has previously been observed for mouse and human tissues (Trezise and Buchwald, 1991; Trezise *et al.*, 1992). The transgenes were also expressed in the submaxillary glands, as expected (Trezise and Buchwald, 1991; Kartner *et al.*, 1992). In tissues where the expression is known to differ between mouse and human, the pattern of expression seemed to follow the mouse pattern rather than the human; thus transgene expression levels were very low in pancreas and adult lung and were not observed in any villous cells in the duodenum. As the levels of expression of both the endogenous mouse gene and the human transgene were too low to be detected in the adult lung and the pancreas, we cannot predict whether there would be good expression in the equivalent human tissues. This will be tested in human cell lines in tissue culture.

However, there were a number of tissues, including the pyloric glands, Brunner's glands, epididymis and sublingual gland, where expression was not found, even though expression of the endogenous mouse gene was

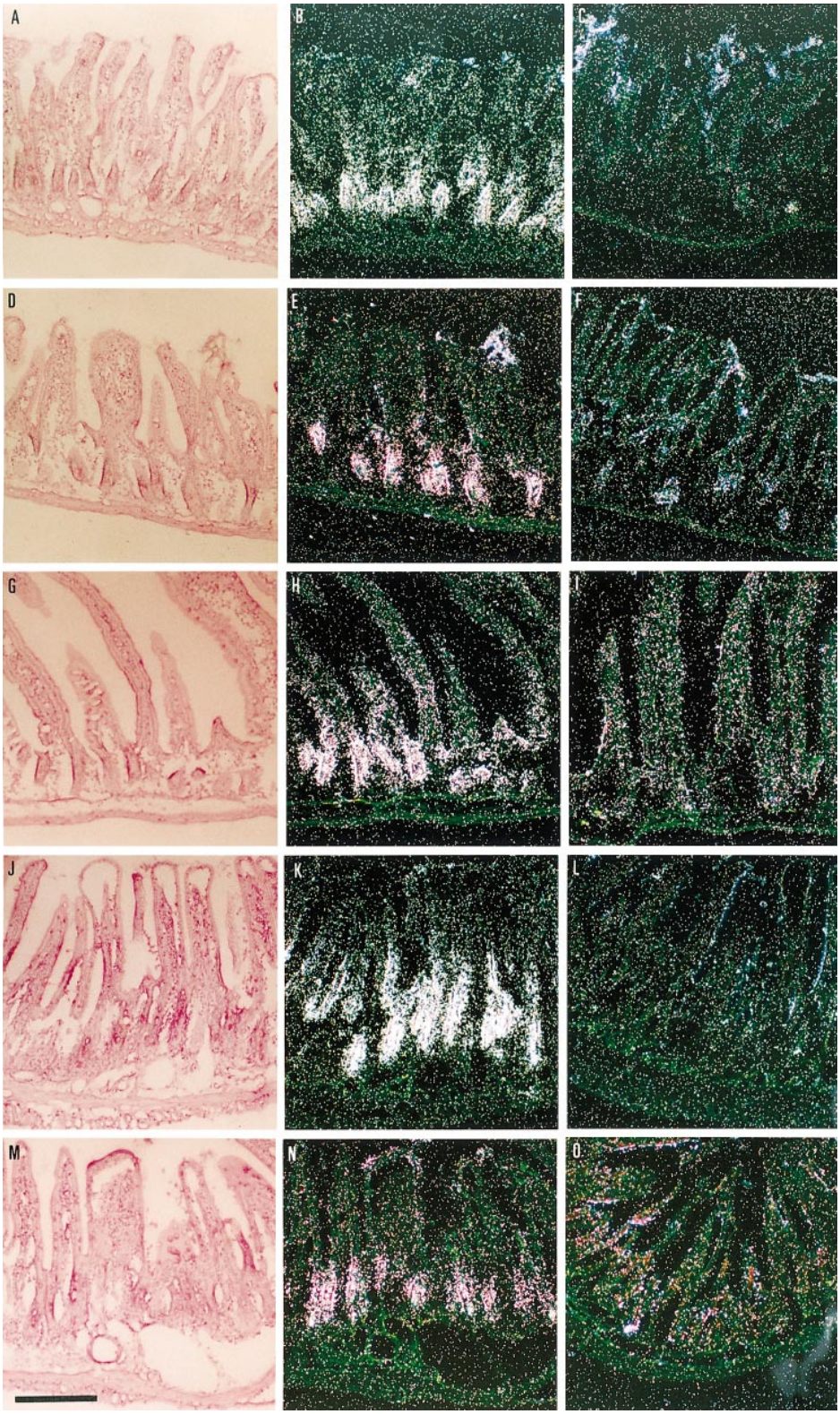


Fig. 6. Cell type-specific expression of the human gene in the gut of transgenic mice. Sections of the ileum from a T30 cf/+ mouse: (**A**) brightfield and (**B**) darkfield with mouse *Cftr* antisense probe; (**C**) darkfield with mouse sense probe; (**D**) brightfield and (**E**) darkfield with human antisense probe; (**F**) darkfield with human sense probe. Sections of the duodenum from a T30 cf/cf mouse: (**G**) brightfield and (**H**) darkfield with human antisense probe; (**I**) darkfield with human sense probe. Sections from a T57 +/+ mouse; (**J**) brightfield and (**K**) darkfield with mouse antisense probe; (**L**) darkfield with mouse sense probe; (**M**) brightfield and (**N**) darkfield with human antisense probe; (**O**) darkfield with human sense probe. In each case the brightfield and the darkfield with the antisense probe are images of the same section, while the sense probe is hybridized to a consecutive section. Scale bar 200 μm.

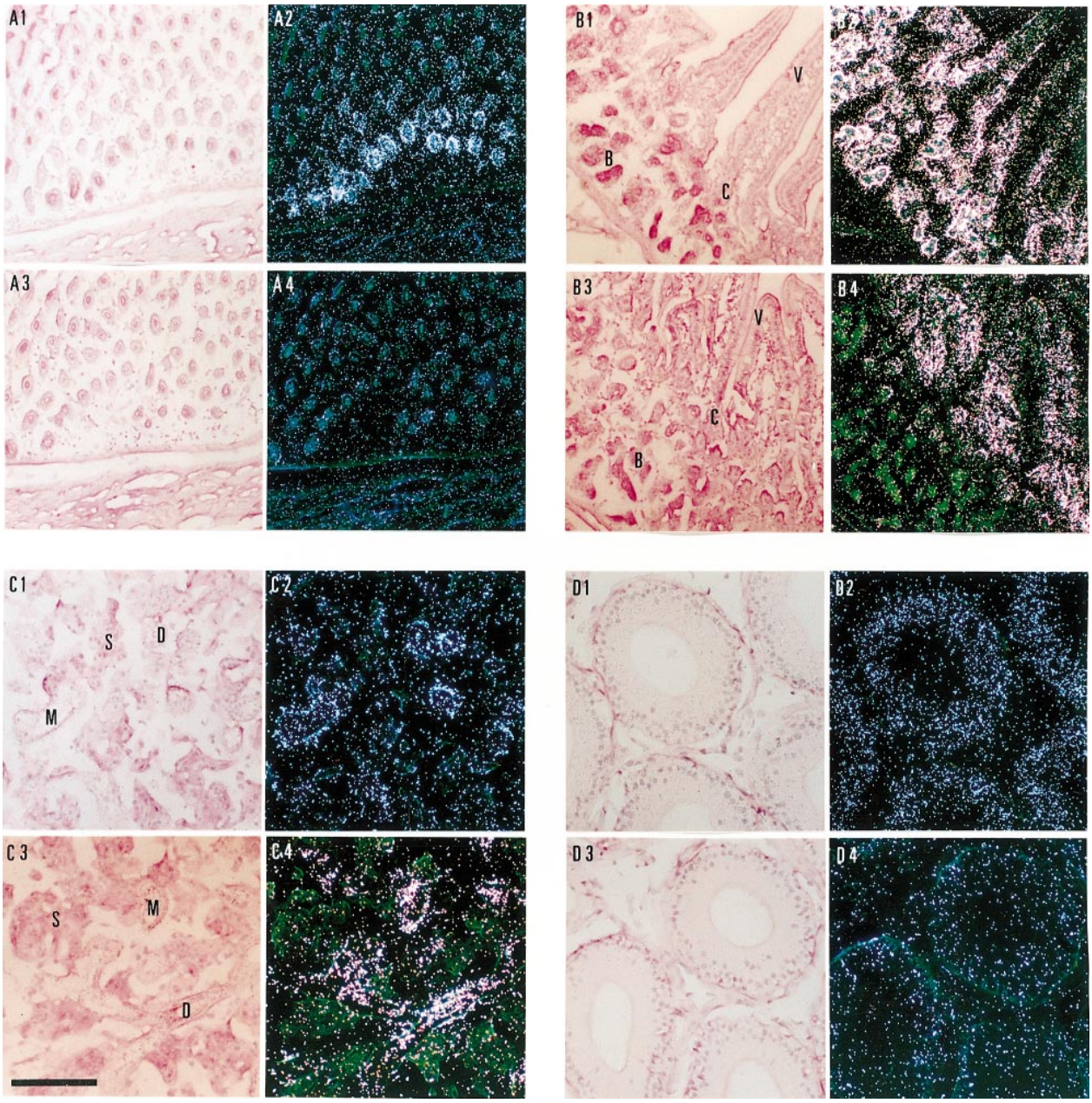


Fig. 7. Expression of the human *CFTR* gene in various tissues. (**A**) Expression in the pyloric glands of the stomach: (**A1**) brightfield and (**A2**) darkfield with mouse antisense probe; (**A3**) brightfield and (**A4**) darkfield with human antisense probe. From a T57 +/+ mouse. (**B**) Expression in the duodenum and Brunner's glands: (**B1**) brightfield and (**B2**) darkfield with mouse antisense probe; (**B3**) brightfield and (**B4**) darkfield with human antisense probe. B, Brunner's glands; C, crypts of Lieberkuhn; V, villus. From a T57 +/+ mouse. (**C**) Expression in the submaxillary gland: (**C1**) brightfield and (**C2**) darkfield with mouse antisense probe; (**C3**) brightfield and (**C4**) darkfield with human antisense probe. M, mucous acinus; S, serous acinus; D, duct. From a T57 +/+ mouse. (**D**) Expression in the initial segment of the epididymis: (**D1**) brightfield and (**D2**) darkfield with mouse antisense probe; (**D3**) brightfield and (**D4**) darkfield with human antisense probe. From a T30 cf/+ mouse. In each case the brightfield image is the same section as the neighbouring darkfield. For panels (A1)–(A4) and (B1)–(B4), scale bar 200 μm; for panels (C1)–(C4) and (D1)–(D4), scale bar 100 μm.

observed. In the case of the Brunner's glands it is known that the endogenous *CFTR* gene is expressed at high levels in the human tissue as well as the mouse (Strong *et al.*, 1994). This lack of expression of the transgene in tissues where the mouse and human genes are known to be expressed normally could be due to the YAC not containing elements needed for tissue-specific expression, such as the DNase I hypersensitive site at –79 kb (Smith *et al.*, 1995), which is not present in the YAC. This could be tested by using a larger YAC including this region. It could also be due to a lack of the correct interaction between the mouse transcription machinery and the human DNA due to evolutionary divergence. This could be tested by introducing the YAC into an organism where there is greater conservation of the *CFTR* promoter sequence with man.

Other mouse models indicate that between 10 and 50% of normal levels of expression of the mouse gene are needed for physiological complementation. Thus, heterozygous null mice are indistinguishable from normal mice

(Ratcliff *et al.*, 1993). Whereas 'knock-out' mice expressing ~10% of the normal *Cftr* levels (*cftr*^{m1HGU}) (Dorin *et al.*, 1992, 1994) are easily distinguished from the wild-type by electrophysiological measurements and most have an abnormal gut histology, though only ~10% die before adulthood (Dorin *et al.*, 1992, 1994). It is possible that the genetic background of the mice is giving a mild phenotype. It has been shown that the C57BL/6J background contains at least one genetic modifier which reduces the severity of the gut pathology of *Cftr* null mice due to up-regulation of a calcium-regulated Cl^- channel (Rozmahel *et al.*, 1996). However, in our crosses we observe a high death rate of the cf/cf mice, so the mild phenotype is almost certainly due to the human *CFTR* gene.

Previous *CFTR* transgenes have not fully complemented the electrophysiological defects in null mice or have not matched the tissue specificity of the endogenous gene. Mice carrying a human *CFTR* cDNA construct driven by the rat intestinal fatty acid binding protein gene promoter on a null background had abundant expression of the human *CFTR* mRNA in the ileum, jejunum and duodenum (Zhou *et al.*, 1994). However, it was expressed in the epithelial cells of the intestinal villi, not in the crypts of Lieberkuhn. This expression corrected the gross goblet hyperplasia in the ileum but the chloride secretory responses were only ~25% of those in wild-type tissues and were insensitive to inhibition by loop diuretics. The complete correction of chloride secretory responses, including a normal degree of inhibition by frusemide, seen here, together with the tissue specificity of expression in the crypts as observed by *in situ* hybridization argues that expression in the crypts is more efficient in giving electrogenic correction than expression in the villi. A transgenic mouse with human *CFTR* expressed from the human surfactant protein promoter has also been described (Whitsett *et al.*, 1992). However, although abundant mRNA and protein was detected in the respiratory epithelial cells, no electrophysiological measurements were made.

We conclude that the human *CFTR* gene carried on the YAC is able to drive physiological levels of expression in many of the cells where *Cftr* is normally expressed and the human protein effectively restores chloride secretory responses in otherwise null mice. The high degree of correction suggests that the YAC DNA will be valuable for use in gene therapy applications, at least in the gut. It is probably not possible to deliver 320 kb fragments efficiently with presently available methods. However, it should be possible to reduce the size of the construct by removing unimportant DNA while retaining the high levels of tissue-specific expression.

Materials and methods

Preparing the YAC DNA for microinjection

YAC 37AB12 has been isolated previously and shown to contain the intact human *CFTR* gene in the insert (Anand *et al.*, 1991). A version of this YAC, which had been retrofitted with the vector pLNA-1 as described previously (Riley *et al.*, 1992), was used to make the transgenic mice. High concentration plugs of the YAC were made and the DNA separated on preparative pulsed-field gels made of SeaPlaque agarose, as described previously (Gnirke *et al.*, 1993). The YAC DNA was excised from the gel, without irradiating it with UV light, and the agarose slice was equilibrated with agarase buffer (10 mM bis Tris–HCl, pH 6.5, 0.2 mM EDTA, 100 mM NaCl). The agarose was melted (68°C, 10 min), cooled (40°C, 5 min) and treated with agarase (1 U/100 mg agarose, 40°C for 2 h). The resulting solution was spun for 25 min in a microcentrifuge (13 000 *g*) at room temperature to clear the DNA of particles which otherwise block the microinjection needles. A Millipore Ultrafree-MC 30 000 NMWL Filter Unit (Millipore catalog no. UFC3 TTK 00) was cleaned by loading with 400 µl oocyte grade water (Sigma W1503) and spinning at 6000 r.p.m. in a microcentrifuge until all the water had passed through the filter. An aliquot of 400 µl DNA solution was loaded onto the filter and spun at 6000 r.p.m. in a microcentrifuge until 200 µl had passed through the filter. A Millipore dialysis filter (Millipore catalog no. VMW 02500) was cleaned by placing it on the surface of 30 ml microinjection buffer (10 mM Tris, pH 7.4, 0.2 mM EDTA, 100 mM NaCl, in oocyte grade water) in a Petri dish for several hours. The filter was then transferred to clean microinjection buffer and 200 µl DNA solution were placed on the filter. The DNA solution was dialysed for several hours. The DNA was transferred to a 1.5 ml tube and stored at 4°C for up to 1 month prior to injection. The YAC DNA was always handled with pipette tips which had been cut off to give a wide bore so as to prevent shearing of the DNA.

Mouse strains

F1 mice (C57BL/6J×CBA/Ca) were intercrossed to produce eggs for microinjection. Subsequent generations of mice were crossed to F1 mice for maintenance or transgenic mice were crossed to each other giving a heterogeneous background of CBA/Ca and C57BL/6J. The Cambridge null mice are on a 129/MF1 mixed background and were bred directly to the transgenic animals. Doubly heterozygous mice were crossed to establish the transgene on a null CF background with the genetic background being a mixture of CBA/Ca, C57BL/6J, 129 and MF1.

Genotyping

At 3 weeks of age, mice were weaned and 0.5 cm sections of the tail taken for DNA preparation. PCR was carried out to detect the left arm of the YAC, the right arm of the YAC and various exons (Zielenski *et al.*, 1991). In addition, the knock-out CF allele was detected with an assay specific for the human *HPRT* gene, which is part of the construct used to disrupt the mouse *Cftr* gene. The normal mouse allele was detected with a PCR assay which crosses exon 10 and which cannot amplify from the disrupted allele. Thus mice heterozygous for the knock-out are positive for both the *HPRT* and mouse exon 10 assays, whereas mice homozygous for the knock-out are positive only for the *HPRT* gene. The genotype of the mouse alleles was confirmed using Southern blotting. The tail DNA was cut with *Kpn*I and probed with the cloned PCR product of mouse exon 10 (Ratcliff *et al.*, 1993).

There is no easy way of determining whether the mice are homozygous or heterozygous for the transgene except by densitometry, which we did not find very reliable, or by laborious breeding inheritance studies. For the mice analysed in this paper we do not know whether the transgene is heterozygous or homozygous, but two thirds are expected to be heterozygous.

Pulsed-field gels and Southern blotting

High molecular weight DNA was prepared from spleens. The spleens were homogenized and the cells washed twice in phosphate-buffered saline (PBS). The cells were then embedded at 2×10^7 cells/ml in 1% agarose (SeaPlaque; FMC) in PBS and the agarose blocks treated with LDS solution (1% lithium dodecyl sulfate, 100 mM EDTA, 10 mM Tris, pH 8.0) for 1 h and then over night at 37°C. The plugs were washed in NDS solution (0.2% lauryl sarcosine, 100 mM EDTA, 2 mM Tris, pH 9.0) twice for 2 h at room temperature, then twice for 30 min in TE and then twice for 30 min in 1× restriction buffer before treatment with restriction enzymes. The digested DNA was separated by pulsed-field gel electrophoresis using a DRII Chef apparatus (BioRad). Gels were made and run in 0.5× TBE at 14°C with 27 s switching for 21 h. DNA was also prepared in solution and separated by standard agarose gel electrophoresis. The DNA was transferred by Southern blotting onto Hybond N membrane (Amersham) using the protocol recommended by the manufacturer. Probes were labelled using the Megaprime kit (Amersham) and hybridized and washed using standard conditions.

The following probes were used: the *Pst*I *CFTR* cDNA fragment from pSV-CFTR (cDNA probe) (Alton *et al.*, 1993); pBE2.5, a 2.5 kb *Bam*HI–*Eco*RI fragment located just upstream of the –20 kb DNase I hypersensitive site (–20 kb probe) (Smith *et al.*, 1995); a subcloned PCR product between primers IA1R and TSR4 (Smith *et al.*, 1996) located just upstream of the DNase I hypersensitive site in intron 1 (Smith *et al.*, 1996); a subcloned 438 bp PCR product spanning exon 4 using previously

described primers (exon 4 probe) (Zielenski *et al.*, 1991); a subcloned PCR product spanning bp 4579–5508 (Riodan *et al.*, 1989) from exon 24 (3′ exon probe) of the *CFTR* gene; the large and small *Bam*HI–*Pvu*II fragments of pBR322 as probes for the left and right arms of the YAC respectively.

For quantification, transgenic mouse DNA and varying amounts of human genomic DNA were run out in adjacent lanes by standard agarose gel electrophoresis. The gel was blotted and probed with either the exon 4 probe or the 3′ exon probe. The amount of genomic DNA in the lanes was determined by the OD_{260} of the DNA solution and also by the intensity of ethidium bromide staining on the gel. The intensity of the radioactive hybridization was determined using a Phosphorimager and Imagequant software.

Electrophysiology

SCCs were measured from epithelial tissues using standard procedures described elsewhere (Hyde *et al.*, 1993; Cuthbert *et al.*, 1994). For the three gut tissues used, i.e. colon, jejunum and caecum, the overlying muscle layers were dissected away and the epithelia were mounted in Ussing chambers (window area 20 mm^2) sandwiched between Parafilm washers. The transepithelial potential was controlled at zero potential using a WPI dual voltage clamp and the SCC recorded continuously. Fluid resistance compensation was applied to allow for the potential drop between the ends of the potential monitoring electrodes and the tissue surface. Forskolin (10 μM) was used throughout to increase the cAMP content of the epithelium and was applied to both sides of the epithelium. Other agents were used as follows: amiloride, 10 μM, applied apically; carbachol, 10 μM, applied basolaterally; frusemide, 1 mM, applied basolaterally.

With the tracheal epithelium only one preparation of 2.3 mm^2 could be obtained from each trachea. To overcome the problem of Ca^{2+} release by forskolin (Grubb *et al.*, 1994), the stores were first discharged with 25 μM THBQ in the presence of 1 μM A23187 for 30 min before 10 μM amiloride and then 10 μM forskolin were added (L.J.MacVinish *et al.*, in preparation).

For statistical analysis Student's *t*-test was used, $P < 0.05$ being considered significant. In this study four T30 cf/cf mice (22.8 ± 1.0 g) and three T57 mice (20.7 ± 0.9 g) were used. Wild-type and heterozygous CF mice were used as necessary to provide the controls. In the YAC-containing mice up to three pieces of colon, two of jejunum and caecum and a single tracheal preparation were taken for recording.

RNA in situ hybridization

Animals were killed by a lethal injection of anaesthetic and tissues fixed by whole body perfusion with 4% paraformaldehyde. *In situ* hybridization was carried out essentially as described previously (Trezise *et al.*, 1993a). Cryostat sections (10 μm) were hybridized overnight to ^{35}S-labelled, single-stranded RNA probes. Following hybridization, sections were treated with RNase A, which digests any non-hybridized probe and other single-stranded RNA, and were washed at a stringency of 0.1× SSC at 60°C. Slides were dipped in Kodak NTB-2 emulsion, left to expose for 2 weeks at 4°C, developed and counterstained with haematoxylin and eosin. Sections were photographed under brightfield and darkfield illumination using a Nikon Optiphot-2 microscope equipped with a Nikon UFX-DX automatic camera.

In all cases, consecutive sections were hybridized with either an antisense RNA probe or the corresponding sense RNA probe. The ^{35}S-labelled antisense probe will hybridize to the mRNA and label cells expressing the gene of interest. The sense probe is identical to the mRNA and serves as a negative control, allowing identification of non-specific signals. The radioactive probe exposes the emulsion which, after development, appears black under brightfield illumination and white under darkfield illumination. The brightfield images of the sections also show the morphology of the tissue. Comparison of the darkfield images of sections hybridized with the antisense and sense RNA probes identifies any specific hybridization signals.

Hybridizations were performed with species-specific mouse and human CFTR probes (Trezise *et al.*, 1993a,b). The mouse *Cftr* cDNA probe encompassed the last 141 bp of exon 10, all of exons 11 and 12 and the first 210 bp of exon 13 of the mouse *Cftr* gene. The human *CFTR* cDNA probe corresponded to nucleotides 1977–2461 and is entirely contained within exon 13 (numbering according to Riodan *et al.*, 1989). Sequence comparison reveals that these probes are 88% identical. Control hybridizations (data not shown) were carried out with a human *PGK1* probe, encompassing 68 bp of intron 2 and 121 bp of exon 3. The nucleotide sequence of this region of human *PGK1* was sufficiently similar to the equivalent region of mouse *Pgk1* to allow cross-hybridization of the human probe to the mouse *Pgk1* mRNA. The plasmid containing the human *PGK1* insert was kindly given to us by Dr J.Firth (Firth *et al.*, 1994). All cDNA inserts were cloned into Bluescript vectors (Stratagene) to allow *in vitro* transcription of the cDNA insert using T3 and T7 RNA polymerases.

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References

Alton,E.W.F.W. *et al.* (1993) Non-invasive liposome-mediated gene delivery can correct the ion transport defect in cystic fibrosis mutant mice. *Nature Genet.*, **5**, 135–142.

Anand,R., Ogilvie,D.J., Butler,R., Riley,J.H., Finniear,R.S., Powell,S.J., Smith,J.C. and Markham,A.F. (1991) A yeast artificial chromosome contig encompassing the cystic fibrosis locus. *Genomics*, **9**, 124–130.

Becq,F., Jensen,T.J., Chang,X.-B., Savoia,A., Rommens,J.M., Tsui,L.-C., Buchwald,M., Riordan,J.R. and Hanrahan,J.W. (1994) Phosphatase inhibitors activate normal and defective CFTR chloride channels. *Proc. Natl Acad. Sci. USA*, **91**, 9160–9164.

Caplen,N.J. *et al.* (1995) Liposome-mediated CFTR gene transfer to the nasal epithelium of patients with cystic fibrosis. *Nature Med.*, **1**, 39–46.

Colledge,W.H., Ratcliff,R., Foster,D., Williamson,R. and Evans,M.J. (1992) Cystic fibrosis mouse with intestinal obstruction. *Lancet*, **340**, 680.

Crawford,I., Maloney,P.C., Zeitlin,P.L., Guggino,W.B., Hyde,S.C., Turley,H., Gatter,K.C., Harris,A. and Higgins,C.F. (1991) Immunocytochemical localization of the cystic fibrosis gene product CFTR. *Proc. Natl Acad. Sci. USA*, **88**, 9262–9266.

Cuthbert,A.W., MacVinish,L.J., Hickman,M.E., Ratcliff,R., Colledge,W.H. and Evans,M.J. (1994) Ion-transporting activity in the murine colonic epithelium of normal animals and animals with cystic fibrosis. *Pflügers Arch.*, **428**, 508–515.

Cuthbert,A.W., Halstead,J., Ratcliff,R., Colledge,W.H. and Evans,M.J. (1995) The genetic advantage hypothesis in cystic fibrosis heterozygotes: a murine study. *J. Physiol.*, **482**, 449–454.

Dorin,J.R. *et al.* (1992) Cystic fibrosis in the mouse by targeted insertional mutagenesis. *Nature*, **359**, 211–215.

Dorin,J.R., Stevenson,B.J., Fleming,S., Alton,E.W.F.W., Dickinson,P. and Porteous,D.J. (1994) Long-term survival of the exon 10 insertional cystic fibrosis mutant mouse is a consequence of low level residual wild-type *Cftr* gene expression. *Mamm. Genome*, **5**, 465–472.

Engelhardt,J.F., Yankaskas,J.R., Ernst,S.A., Yang,Y., Marino,C.R., Boucher,R.C., Cohn,J.A. and Wilson,J.M. (1992) Submucosal glands are the predominant site of CFTR expression in the human bronchus. *Nature Genet.*, **2**, 240–248.

Engelhardt,J.F., Zepeda,M., Cohn,J.A., Yankaskas,J.R. and Wilson,J.M. (1994) Expression of the cystic fibrosis gene in adult human lung. *J. Clin. Invest.*, **93**, 737–749.

Firth,J.D., Ebert,B.L., Pugh,C.W. and Ratcliffe,P.J. (1994) Oxygen-regulated control elements in the phosphoglycerate kinase 1 and lactate dehydrogenase A genes: similarities with the erythropoietin 3′ enhancer. *Proc. Natl Acad. Sci. USA*, **91**, 6496–6500.

Gnirke,A., Huxley,C., Peterson,K. and Olson,M.V. (1993) Microinjection of intact 200- to 500-kb fragments of YAC DNA into mammalian cells. *Genomics*, **15**, 659–667.

Griesenbach,U., Suen,T.C., Frumkin,A., Olek,K. and Tsui,L.-C. (1993) Study of the promoter of the human cystic fibrosis transmembrane conductance regulator in transgenic mice. *Am. J. Hum. Genet.*, **53** (suppl.), 691 (Abstract).

Grubb,B.R. (1995) Ion transport across the jejunum in normal and cystic fibrosis mice. *Am. J. Physiol.*, **268**, G505–G513.

Grubb,B.R., Vick,R.N. and Boucher,R.C. (1994) Hyperabsorption of Na^+ and raised Ca^{2+}-mediated Cl^- secretion in nasal epithelia of CF mice. *Am. J. Physiol.*, **266**, C1478–C1483.

Hasty,P. *et al.* (1995) Severe phenotype in mice with termination mutation in exon 2 of cystic fibrosis gene. *Somat. Cell Mol. Genet.*, **21**, 177–187.

Hyde,S.C., Gill,D.R., Higgins,C.F., Trezise,A.E.O., MacVinish,L.J., Cuthbert,A.W., Ratcliff,R., Evans,M.J. and Colledge,W.H. (1993) Correction of the ion transport defect in cystic fibrosis transgenic mice by gene therapy. *Nature*, **362**, 250–255.

Kartner,N., Augustinas,O., Jensen,T.J., Naismith,A.L. and Riordan,J.R. (1992) Mislocalization of ΔF508 CFTR in cystic fibrosis sweat gland. *Nature Genet*., **1**, 321–327.

Kerem,B.-S., Rommens,J.M., Buchanan,J.A., Markiewicz,D., Cox,T.K., Chakravarti,A., Buchwald,M. and Tsui,L.-C. (1989) Identification of the cystic fibrosis gene: genetic analysis. *Science*, **245**, 1073–1080.

Marino,C.R., Matovcik,L.M., Gorelick,F.S. and Cohn,J.A. (1991) Localization of the cystic fibrosis transmembrane conductance regulator in pancreas. *J. Clin. Invest*., **99**, 812–816.

McCray,P.B.,Jr, Wohlford-Lenane,C.L. and Snyder,J.M. (1992) Localization of cystic fibrosis transmembrane conductance regulator mRNA in human fetal lung tissue by *in situ* hybridization. *J. Clin. Invest*., **90**, 619–625.

O'Neal,W.K., Hasty,P., McCray,P.B., Casey,B., Rivera-Perez,J., Welsh,M.J., Beaudet,A. and Bradley,A. (1993) A severe phenotype in mice with a duplication of exon 3 in the cystic fibrosis locus. *Hum. Mol. Genet*., **2**, 1561–1569.

Perraud,F. *et al.* (1992) The promoter of the human cystic fibrosis transmembrane conductance regulator gene directing SV40 T antigen expression induces malignant proliferation of ependymal cells in transgenic mice. *Oncogene*, **7**, 993–997.

Ratcliff,R., Evans,M.J., Cuthbert,A.W., MacVinish,L.J., Foster,D., Anderson,J.R. and Colledge,W.H. (1993) Production of a severe cystic fibrosis mutation in mice by gene targeting. *Nature Genet*., **4**, 35–41.

Riley,J.H., Morten,J.E.N. and Anand,R. (1992) Targeted integration of neomycin into yeast artificial chromosomes (YACs) for transfection into mammalian cells. *Nucleic Acids Res*., **20**, 2971–2976.

Riodan,J.R. *et al.* (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science*, **245**, 1066–1073.

Rommens,J.M. *et al.* (1989) Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science*, **245**, 1059–1065.

Rozmahel,R. *et al.* (1996) Modulation of disease severity in cystic fibrosis transmembrane conductance regulator deficient mice by a secondary genetic factor. *Nature Genet*., **12**, 280–287.

Smith,A.N., Wardle,C.J.C. and Harris,A. (1995) Characterization of DNase I hypersensitive sites in the 120kb 5′ to the CFTR gene. *Biochem. Biophys. Res. Commun*., **211**, 274–281.

Smith,A.N., Barth,M.L., McDowell,T.L., Moulin,D.S., Nuthall,H.N., Hollingsworth,M.A. and Harris,A. (1996) A regulatory element in intron 1 of the cystic fibrosis transmembrane conductance regulator gene. *J. Biol. Chem*., **271**, 9947–9954.

Snouwaert,J.N., Brigman,K.K., Latour,A.M., Malouf,N.N., Boucher,R.C., Smithies,O. and Koller,B.H. (1992) An animal model for cystic fibrosis made by gene targeting. *Science*, **257**, 1083–1088.

Strong,R.V., Boehm,K. and Collins,F.S. (1994) Localization of cystic fibrosis transmembrane conductance regulator mRNA in the human gastrointestinal tract by *in situ* hybridization. *J. Clin. Invest*., **93**, 347–354.

Stutts,M.J., Canessa,C.M., Olsen,J.C., Hamrick,M., Cohn,J.A., Rossier,B.C. and Boucher,R.C. (1995) CFTR as a cAMP-dependent regulator of sodium channels. *Science*, **269**, 847–850.

Tizzano,E.F., Chitayat,D. and Buchwald,M. (1993) Cell-specific localization of CFTR mRNA shows developmentally regulated expression in human fetal tissues. *Hum. Mol. Genet*., **2**, 219–224.

Trezise,A.E. and Buchwald,M. (1991) *In vivo* cell-specific expression of the cystic fibrosis transmembrane conductance regulator. *Nature*, **353**, 434–437.

Trezise,A.E., Romano,P.R., Gill,D.R., Hyde,S.C., Sepulveda,F.V., Buchwald,M. and Higgins,C.F. (1992) The multidrug resistance and cystic fibrosis genes have complementary patterns of epithelial expression. *EMBO J*., **11**, 4291–4303.

Trezise,A.E., Linder,C.C., Grieger,D., Thompson,E.W., Meunier,H., Griswold,M.D. and Buchwald,M. (1993a) CFTR expression is regulated during both the cycle of the seminiferous epithelium and the oestrous cycle of rodents. *Nature Genet*., **3**, 157–164.

Trezise,A.E.O., Chambers,J.A., Wardle,C.J., Gould,S. and Harris,A. (1993b) Expression of the cystic fibrosis gene in human fetal tissues. *Hum. Mol. Genet*., **2**, 213–218.

Whitsett,J.A. *et al.* (1992) Human cystic fibrosis transmembrane conductance regulator directed to respiratory epithelial cells of transgenic mice. *Nature Genet*., **2**, 13–20.

Zabner,J., Couture,L.A., Gregory,R.J., Graham,S.M., Smith,A.E. and Welsh,M.J. (1993) Adenovirus-mediated gene transfer transiently corrects the chloride transport defect in nasal epithelia of patients with cystic fibrosis. *Cell*, **75**, 207–216.

Zhou,L., Dey,C.R., Wert,S.E., DuVall,M.D., Frizzell,R.A. and Whitsett,J.A. (1994) Correction of lethal intestinal defect in a mouse model of cystic fibrosis by human *CFTR*. *Science*, **266**, 1705–1708.

Zielenski,J., Rozmahel,R., Bozon,D., Kerem,B.-S., Grzelczak,Z., Riordan,J.R., Rommens,J. and Tsui,L.-C. (1991) Genomic DNA sequence of the cystic fibrosis transmembrane conductance regulator (CFTR) gene. *Genomics*, **10**, 214–228.

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