

CFTR expression from a BAC carrying the complete human gene and associated regulatory elements

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Abstract

The use of genomic DNA rather than cDNA or mini-gene constructs in gene therapy might be advantageous as these contain intronic and long-range control elements vital for accurate expression. For gene therapy of cystic fibrosis though, no bacterial artificial chromosome (BAC), containing the whole *CFTR* gene is available. We have used Red homologous recombination to add a to a previously described vector to construct a new BAC vector with a 250.3-kb insert containing the whole coding region of the *CFTR* gene along with 40.1 kb of DNA 5' to the gene and 25 kb 3' to the gene. This includes all the known control elements of the gene. We evaluated expression by RT-PCR in CMT-93 cells and showed that the gene is expressed both from integrated copies of the BAC and also from episomes carrying the *oriP/EBNA-1* element. Sequencing of the human *CFTR* mRNA from one clone showed that the BAC is functional and can generate correctly spliced mRNA in the mouse background. The BAC described here is the only *CFTR* genomic construct available on a convenient vector that can be readily used for gene expression studies or *in vivo* studies to test its potential application in gene therapy for cystic fibrosis.

Keywords: CFTR • BAC • expression • recombineering • bacterial invasion

Introduction

Cystic fibrosis is a common, fatal genetic disease that affects 1/2500 Caucasians and is caused by mutations in the *CFTR* gene, which encodes a *cAMP*-regulated Cl⁻ channel in epithelial cells of many organs including the lungs, the intestine, the pancreas

and the reproductive tracts (reviewed by [1]). The most severe complications that finally lead to death are those in the airway epithelium [2].

The *CFTR* gene, located on chromosome 7, is 189-kb long [3] and comprises 27 exons. It shows a tightly regulated temporal and spatial pattern of expression [4, 5] although its proximal promoter shares features with promoters of house-keeping type genes as it is CpG rich, contains no TATA box and has multiple transcription start sites and several potential binding sites for the transcription factor Sp1 [6]. There are apparently no tissue-specific regulatory elements in this region, suggesting that other elements outside the proximal promoter are probably involved in 'tissue-specific' regulation of transcription.

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DNase I hypersensitive sites (DHS) that are often, but not always, associated with regulation of transcription, might be indicative of the elements outside the promoter that are involved in controlling the expression of the *CFTR* gene. Several DHS have been identified across 400 kb of DNA flanking the *CFTR* gene. These lie 5' to the gene at -79.5 and -20.9 kb with respect to the translation start site [7], in introns 1 [8], 2, 3, 10, 16, 17a, 18, 20 and 21 [9] and 3' to the gene at +5.4, +6.8, +7, +7.4 and +15.6 kb, respectively, to the end of translation [10]. The presence of some of these DHS has been found to correlate with the 'tissue-specific' expression of the *CFTR* gene. The DHS at -20.9 kb might be involved in transcriptional regulation as a *CFTR* YAC transgene lacking the site was expressed at 60% lower levels than the wild-type YAC in Caco-2 human colon carcinoma cells [11]. The DHS in intron 1 has been shown to contain a tissue-specific regulatory element and to be necessary for normal CFTR expression in the intestine [12]. Similarly, the DHS in introns 20 and 21 [13] and +15.6 kb [10] are associated with tissue-specific enhancer activity. However, the DHS at -79.5 kb is probably not involved in regulation of CFTR. A 310-kb YAC carrying the *CFTR* gene and 58.4 kb of upstream DNA but not the -79.5-kb DHS [14] and 3' flanking DNA at least as far as the +15.6-kb DHS (10), was shown to give full levels of copy-number dependent expression in human epithelial Caco-2 cells [15] and also to give 'tissue-specific' expression adequate to correct the CF phenotype in CF null mice [16].

The relatively easy access to the airway epithelium, the previous cloning and characterization of the *CFTR* gene [3, 17] and the expectation that low levels of its expression could have a clinical benefit [18] make cystic fibrosis an ideal target for gene therapy. Previous experiments with small mini-gene or cDNA constructs that obviously could not cover the whole ~189-kb region of the gene showed some expression of CFTR in transgenic mice [19–24] and low levels of transient correction in patients [25–30]. However, such constructs are not expressed sufficiently in the appropriate tissues to achieve clinical improvement in patients. A large genomic construct spanning ~250 kb including all the known long-range controlling elements of the *CFTR* gene should give full levels of 'tissue-specific' expression and might be advantageous for gene therapy of cystic fibrosis. A 310-kb YAC genomic construct has previously been used to express the *CFTR* gene in human cells [15], in mouse cells [31] and in CF null transgenic mice [16] and also to incorporate and express it from a 5.5 Mb naturally occurring mini-chromosome [32]. However, YACs are very difficult to purify for any gene delivery purposes and therefore it is desirable to have the *CFTR* gene available on a BAC vector. Although various BAC containing parts of the *CFTR* gene have been described, none are available covering the whole genomic CFTR locus. Such a vector needs to be constructed.

We had previously developed a method to link the inserts of two overlapping BACs into a single BAC clone using the Red homologous recombination system [33] and had used this method to recombine together two BACs spanning the transcribed region and 7.6 kb 5' of the *CFTR* gene [34]. Here we have linked

the insert of a third BAC, carrying all the known regulatory elements 5' to the *CFTR* gene, to the previously described BAC by recombinering. We show that human CFTR can be expressed from this BAC after bacterial delivery into mouse CMT-93 cells.

Materials and methods

Construction of plasmids

For the modification of BAC 205G13, the pBISHomABeloHomOSloxZeo vector was constructed (Fig. 1). Initially, HomA was amplified from BAC 68P20 (32) by PCR using primers HomA'L: 5'ATTGTGCGACCAAGAGCCTCTGTCCACCACA and HomA'R: 5'ATTTGGATCCATTGCACATGGGCACTAACCA and cloned into *Bam*H I + *Sal* I cut pBIS1 [34] to give pBIS1HomA. At the same time pBIS2Belo [34] was modified by digesting it with *I-Ppo* I and *Not* I and ligating it to a pair of complementary synthetic oligonucleotides, 2modL: 5'GTGAGCGAATTCATATCTCGAGATCCGCGGGC and 2modR: 5'GGCCGCCCGCGGATATCTCGCGATATGAATTCGCTCACTTAA to give pBIS2BeloMod with the *I-Ppo* I site destroyed, the *Cla* I site replaced by an *Eco*R I site and the order of *Sac* II and *Xho* I sites reversed. The *Zeo*^R gene was then excised from pSV40Zeo (Invitrogen, Paisley, UK) as a 1-kb *Eco*R I-*Xho* I fragment and cloned into *Eco*R I + *Xho* I cut pBIS2BeloMod to give pBISBeloZeo. HomOS was amplified from BAC 205G13 by PCR using primers HomOSL: 5'ATTTCTCGAGAGATAAAGTTCGTATAATGTATGCATACGAAG TTATGCATCCAGTTCCAA (that includes the sequence of a *loxP* site) (6556–6568 of BAC 205G13) and HomOSR: 5'ATTTCCGCGGAGGCTGCAAATCAGCCTAA (7562–7543 of BAC 205G13) and cloned into *Xho* I + *Sac* I cut pBIS2BeloZeo to give pBIS2BeloZeoHomSlox. This plasmid was then digested with *Sal* I + *Sac* I and the 6.6-kb fragment was purified and cloned into *Xho* I + *Sac* I cut pBIS1HomA to give pBISHomABeloHomOSloxZeo.

Homologous recombinations

Preparation of electrocompetent EL350 bacteria [33], electroporation of BAC 205G13 into EL350 bacteria, preparation of electrocompetent EL350 bacteria induced for Red recombination and electroporation of linearized BACLinkZeo were all carried out using the protocols described previously [34]. Selection of bacteria was done using 5 µg/ml gentamycin or 25 µg/ml zeocin as appropriate.

For the introduction of pRetroNeoOE into the CFTR1, 2, 3 BAC and the construction of the CFTR1, 2, 3 OE BAC, 200 ng of pRetroNeoOE were electroporated into EL350 bacteria containing the CFTR1, 2, 3 BAC. After electroporation, bacteria were incubated at 30°C with shaking for 1.5 hrs in the presence of 0.2% arabinose to induce the *Cre* gene and then plated in the presence of 5 µg/ml gentamycin and 50 µg/ml spectinomycin.

PCR assays for checking recombination events

The following primers were used to check that correct homologous recombinations had occurred as described in the results section and Fig. 1. CFTR12165: 5'TAGTCATTGTCACATGCC (12165–12184 of BAC68P20, labelled 3 in Fig. 1).

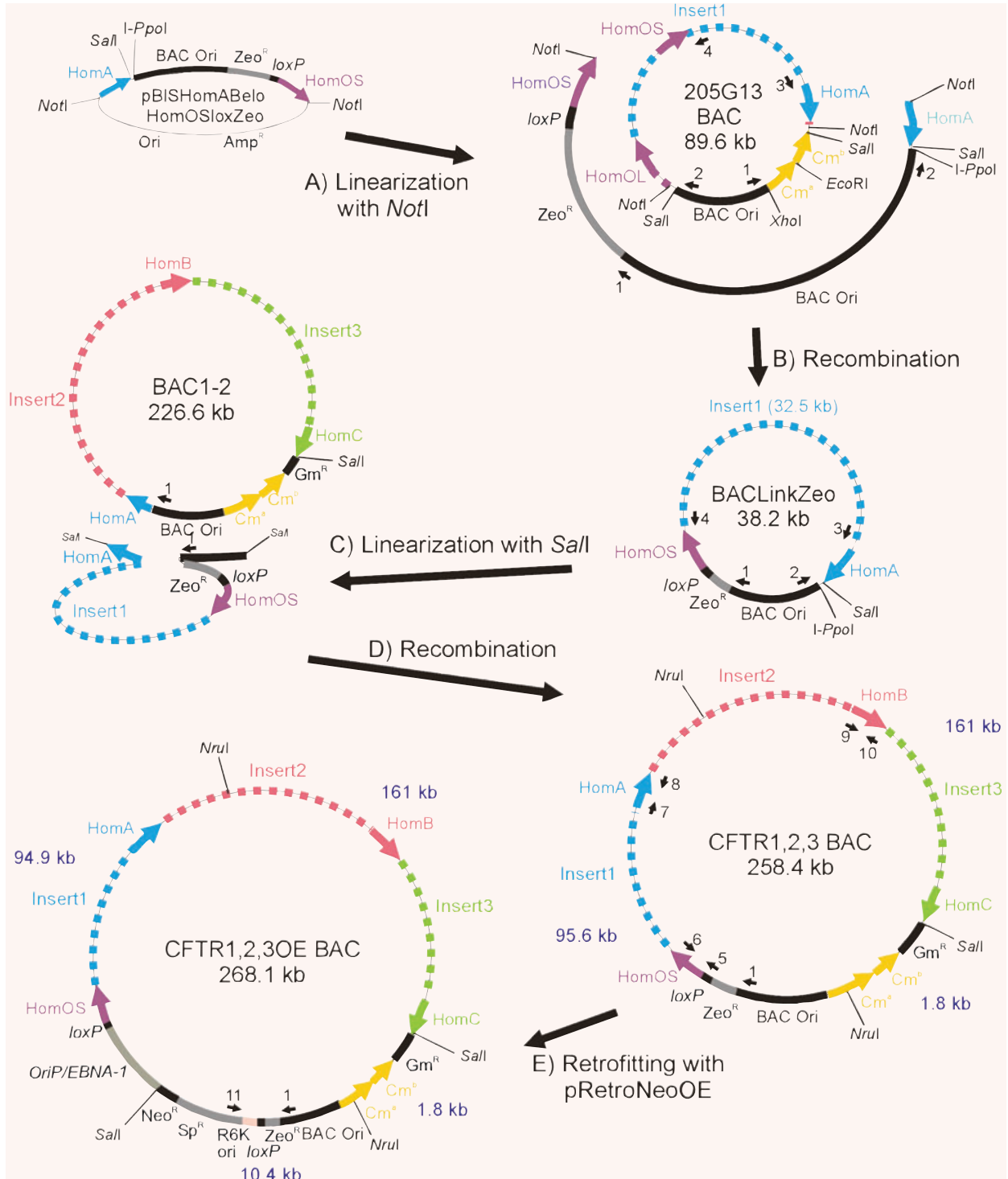


Fig. 1 Recombination strategy for the construction of CFTR1, 2, 3 and CFTR1, 2, 3 OE BACs. The primers used to check each recombination event are indicated as arrows labelled 1 to 11. The sizes of the fragments after digestion with *NruI* + *Sall* are indicated for CFTR1, 2, 3 and CFTR1, 2, 3 OE BACs.

BELO6951: 5'GGTTATGTGGACAAAATACCTGG (6874-6951 of pBeloBAC11 [34], labelled 2 in Fig. 1).
 CFTR7969: 5'TCCAGTGGGCTGAGATTAC (7988-7969 of BAC 205G13, labelled 4 in Fig. 1).
 BELO2658: 5'TTTGTACAGGGTTAAGGGC (2658-2638 of pBeloBAC11 [34], labelled 1 in Fig. 1).
 HomOSL: 5'ATTTCTCGAGAGATAAATCTCGTATAATGTATGCTATACGAAGT-TATGCA
 TCCAGTCCAA (that includes the sequence of a *loxP* site) (6556-6568 of BAC205G13, labelled 5 in Fig. 1).
 HomOSR: 5'ATTTCCGCGGAGGCTGCAAATCAGCCTAA (7562-7543 of BAC205G13, labelled 6 in Fig. 1).
 HomAL: 5'CCACCCGCGGCAAGAGCCTCTGTACCACA (12202-12221 of BAC 68P20 [34], labelled 7 in Fig. 1).
 HomAR: 5'CAAATCGAGATTGCACATGGGCTACTAACA (13962-13943 of BAC 68P20 [34], labelled 8 in Fig. 1).
 HomBL: 5'ATTTGGATCCAGTGGTTCAGTCAGGTTGGG (21266-21285 of BAC 133K23 [34], labelled 9 in Fig. 1).
 HomBR: 5'ATTTGTGACAACTCAGACCCCGTTCACAC (22558-22539 of BAC 133K23 [34], labelled 10 in Fig. 1).
 Sp1: 5'TTTGCAACTGCGGGTCAAGG [34], labelled 11 in Fig. 1.
 PCR was carried out with bacteria picked directly from colonies with a toothpick into 50- μ l reactions, using the *Taq* PCR Master Mix Kit (Qiagen, Crawley, UK).

BAC DNA preparation and pulsed-field electrophoresis

Small amounts of BAC DNA were prepared from 1.5 ml of saturated culture using a standard alkali miniprep protocol [35]. Larger amounts of BAC DNA were prepared from 500 ml of saturated culture using the Qiagen Plasmid Maxi Kit with the protocol for low copy plasmids.

BAC DNA was analysed by restriction enzyme digestion followed by separation by pulsed-field gel electrophoresis. 1% agarose gels were cast in $0.5 \times$ TBE. Electrophoresis was conducted in $0.5 \times$ TBE at 14°C, 5.9 V/cm, with a fixed angle of 120°, usually with switching times from 5 to 15 sec. for 18 hrs using a Chef-DRIII and a Mini Chiller model 1000 with a variable speed pump (BioRad, London, UK). Gels were stained for 30 min. with ethidium bromide and subjected to UV light for visualization of the DNA.

Cell culture, transfection using Lipofectamine 2000 and Bacterial invasion

CMT-93 (mouse rectal carcinoma, ATCC CCL-223) cells were grown in DMEM medium (Invitrogen) supplemented with 10% FCS (Invitrogen) at 37°C and 5% CO₂. Transfection using Lipofectamine 2000 (Invitrogen) was carried out as previously described [36]. For the bacterial invasion, 2×10^5 CMT-93 cells/well were plated in six-well plates and incubated overnight. Bacterial strains containing either the CFTR1, 2, 3 BAC or the CFTR1, 2, 3 OE BAC, generated by electroporation of the BAC into BM4573 *Escherichia coli* [37, 38], were grown with shaking at 30°C overnight in brain heart infusion broth (BHI, Merck, Cramlington, UK) supplemented with 0.5 mM diaminopimelic acid (DAP, Sigma, Athens, Greece) and zeocin 25 mg/ml. The next day, bacteria were collected by centrifugation at 5000 rpm for 10 min., resuspended in DMEM supplemented with 0.5 mM DAP to the original volume and diluted with DMEM supplemented with 0.5 mM DAP

to give a multiplicity of infection (MOI) of 100 in 2 ml final volume (an OD₆₀₀ of ~1.2, corresponds to ~10⁹ cfu/ml). The bacteria were overlaid onto the monolayer cultures that had been washed twice with $1 \times$ PBS, and chloroquine was added to 20 μ M. The six-well plates were centrifuged at 1000 rpm in a Sorvall RT-7 centrifuge for 10 min. and incubated at 37°C/5% CO₂ for 2 hrs. The cells were then washed twice with $1 \times$ PBS and were supplied with fresh medium containing 20 μ g/ml kanamycin. After 24 hrs incubation, the cells were transferred to 10-cm culture dishes where selection was added after one more day incubation. Selection of stable CMTCF and CMTCF OE clones was done in the presence of zeocin at 100 μ g/ml and G418 at 300 μ g/ml, respectively.

RT-PCR

Total RNA was prepared from confluent cells grown in a well of a six-well dish using the Qiagen RNeasy Protect Mini kit according to the manufacturer's protocol and collected in 40 μ l final volume. cDNA was synthesized using the Qiagen Omniscript RT-PCR kit according to the manufacturer's protocol from 2- μ l template RNA and with 10- μ M final concentration of Random hexamers. PCR was performed in 50 μ l final volume containing 2 μ l cDNA, 2.5 μ l of 10 μ M primers, 10 μ l of 5 \times buffer (with 3.5mM MgCl₂), 1 μ l of 10 mM dNTPs, 0.5 μ l of Taq polymerase and 21.5 μ l of H₂O, and under the following cycling conditions: initial denaturation at 94°C for 4 min., then 94°C for 30 sec., 60°C for 30 sec., 72°C for 1 min. (35 cycles) and 72°C for 10 min.

The h/mEx2-5': 5'CTTCTGYTGATTCWGCTGAC and h/mEx6a/b: 5'GATCTCTGTACTTCAATC primers which anneal equally to the mouse and human sequences and amplify from exon 2 to exon 6a/b to give a ~610-bp product [31] and the F1R: 5'ACGTGAAGAAAGATGACATC [39] and 523F: 5'GTTGGCATTCCAGCATTGCTT [40] primers which amplify from exon 19 to exon 24 from the human sequence specifically to give a ~650-bp product, were used to assess expression of the human *CFTR* gene.

Human-specific primers FA: 5'TAGTAGTCTTTGGCATTAG (64-83 on human mRNA), R1: 5'GAAATTACTGAAGAAGAGGC (1431-1412), F3: 5'GGTACTCTCTTGGAGCA (1214-1233), RC: 5'ATCCTCTCGATGCATTCATTTGT (2314-2289), FC: 5'AGGAGATGCTCCTGTCTCCTGGAC (2148-2171), RB: 5'TCTGAAGAGGAGTGTTCCTCAAGGAG (2804-2779), F4: 5'GCCTTTTGTGATGATGGAG (2627-2646), R3: 5'TCGAGATTTGGC-CATTCCTTG (3697-3678), F5: 5'GTAACCTACCAAGTCAACC (3650-3669), R4: TCCCATGTCAACATTTATGC (4601-4581), RA: GAGTCAGTTTCAGTTCT-GTT (1021-1002), FB: 5'TTTCCTGATAGTCTTGCC (816-835), R2: 5'CAAGGAGCCACAGCACAACC (2784-2766), R6: 5'CTGTCTATCACAGATCTGAGC (4122-4101) were used for sequencing of the human mRNA.

Fluorescence *in situ* hybridization (FISH)

FISH analysis was carried out on CMTCF OE clones approximately 5 weeks after their generation by bacterial invasion as described previously [41]. Human CFTR sequences were detected with a probe made of DNA from BAC CFTR1, 2, 3 labelled using the Biotin-Nick Translation Mix (Roche Burgess Hill, UK). The *OriP/EBNA-1* sequences were detected with pRetroNeoOE [36] labelled using the Dig-Nick Translation Mix (Roche Burgess Hill, UK) as a probe. The DNA was incubated with the Nick translation mix at 15°C overnight and the probes were purified using MicroSpin S-300 HR columns (Amersham Biosciences, Little Chalfont, UK). Detection of the biotin-labelled probe was carried out using Texas Red

Avidin DCS and biotinylated anti-Avidin D (Vector Labs, Orton, UK). Detection of the Digoxigenin-labelled probe was carried out using anti-Digoxigenin-Fluorescein, (Roche) and FITC-anti sheep IgG made in rabbit (Vector Labs, Orton, UK). Signals were visualized with an oil immersion $\times 100$ objective on a Leica fluorescence microscope and digitally captured using a Leica image analysis system.

Results

Construction of the human CFTR BAC

The aim of this work was to make a BAC carrying intact the complete human *CFTR* gene using homologous recombination. We have previously constructed BAC1-2 [34] by linking BAC 68P20 (Ac001115) to BAC 133K23 (Ac000061) giving a BAC with a 219.8-kb insert running from 7.6 kb upstream from the start of translation in exon 1 to 25 kb beyond the end of translation in exon 24 of the human *CFTR* gene. A further 32.5 kb of DNA from BAC 205G13 has now been added 5' to the *CFTR* gene giving a BAC with a 250.3-kb insert with 40.1 kb of upstream and 25 kb of downstream DNA.

Figure 1 shows the overall strategy for using homologous recombination to recombine BAC 205G13 and BAC1-2. In the first step, the desired region of the insert of BAC 205G13 was subcloned, by homologous recombination, into a new BAC-linking vector to give BACLinkZeo. In the second step, BACLinkZeo and BAC1-2 were recombined together using one region of homology in the overlapping region of the two BACs (HomA), and the other region in the vector (the BAC vector origin).

Modification of BAC 205G13

The first step of the overall strategy shown in Fig. 1 was to 'subclone' the BAC 205G13 insert, by homologous recombination, into a new BAC vector. For this, the subcloning vector pBISHomABeloHomOSloxZeo was constructed (Fig. 1). This carries two regions of homology, HomA and HomOS, which lie at either end of the region to be recombined onto BAC1-2. It was linearized with *Not*I, and the 11.4-kb fragment with HomA and HomOS at its ends was purified and electroporated into Red-induced EL350 bacteria containing BAC 205G13. This should result in recombination at HomA and HomOS resulting in subcloning of 32.5 kb of BAC 205G13 insert into BACLinkZeo (Fig. 1). Zeocin resistant clones were screened by PCR using primers CFTR12165/BELO6951 (labelled 3 and 2 in Fig. 1) and CFTR7969/ BELO2658 (labelled 4 and 1 in Fig. 1) across homologies HomA and HomOS, respectively. Twelve clones positive by PCR across both homologies were also

analysed by PFGE after *Sal*I and *Sal*I + *Nru*I digestion and were shown to contain the correctly subcloned 32.5-kb insert (not shown).

Combination of the two BACs

The BAC CFTR1, 2, 3 was made by recombining the 38.2-kb BACLinkZeo with BAC1-2 using homologous recombination. Ten micrograms of BACLinkZeo was linearized with *Sal*I, ethanol precipitated and resuspended in 10 μ l final volume. Two micrograms of BACLinkZeo DNA was electroporated into Red-induced EL350 bacteria containing BAC1-2. This should result in recombination within HomA and the BAC origin region and linking of the two BACs. Seventeen clones doubly resistant to zeocin and gentamycin were screened by PCR using primers that amplify within HomO, HomA and HomB (labelled 5-6, 7-8 and 9-10, respectively, in Fig. 1) and 16 were found to contain all three homology regions. DNA was prepared from these 16 clones, digested with *Nru*I, *Sal*I and *Nru*I/*Sal*I and run on a pulsed field gel. All 16 clones showed the expected fragments for the 250.3-kb CFTR insert (one is shown in Fig. 2).

Construction of an *oriP/EBNA-1* CFTR BAC vector

For the construction of a BAC vector carrying the human *CFTR* gene and an *oriP/EBNA-1* element, Cre-catalysed homologous recombination targeted at *loxP* sites was used to retrofit plasmid pRetroNeoOE [36] into the CFTR1, 2, 3 BAC to give BAC CFTR1, 2, 3 OE (Fig. 1). Clones doubly resistant to spectinomycin and gentamycin were screened by PCR using primers BELO2658 and Sp1 (labelled 1 and 11 in Fig. 1) and by PFGE. Twelve correctly retrofitted CFTR1, 2, 3 OE BAC clones were obtained and one of them is shown in Fig. 2.

Assessment of CFTR1, 2, 3 and CFTR1, 2, 3 OE BACs in CMT-93 cells

The expression of the human *CFTR* gene from BAC CFTR1, 2, 3 was assessed in CMT-93 mouse cells as these cells have previously been found to express the gene [31]. In addition, they allow assessment of the relative level of expression of the human *CFTR* mRNA by direct comparison to the level of expression of the endogenous mouse *CFTR* mRNA.

Initially, CFTR1, 2, 3 and CFTR1, 2, 3 OE BACs were transfected into CMT-93 cells using lipofectamine 2000. All clones obtained were subjected to RT-PCR with human/mouse degenerate primers that amplify between exons 2 and 6a/b [31] on the mRNA and also with human-specific primers that amplify between exons 19 and 24 [39, 40]. None of the clones were PCR positive for both regions suggesting rearrangements of the BAC DNA (data not shown). A high frequency of rearrangements has also been observed with

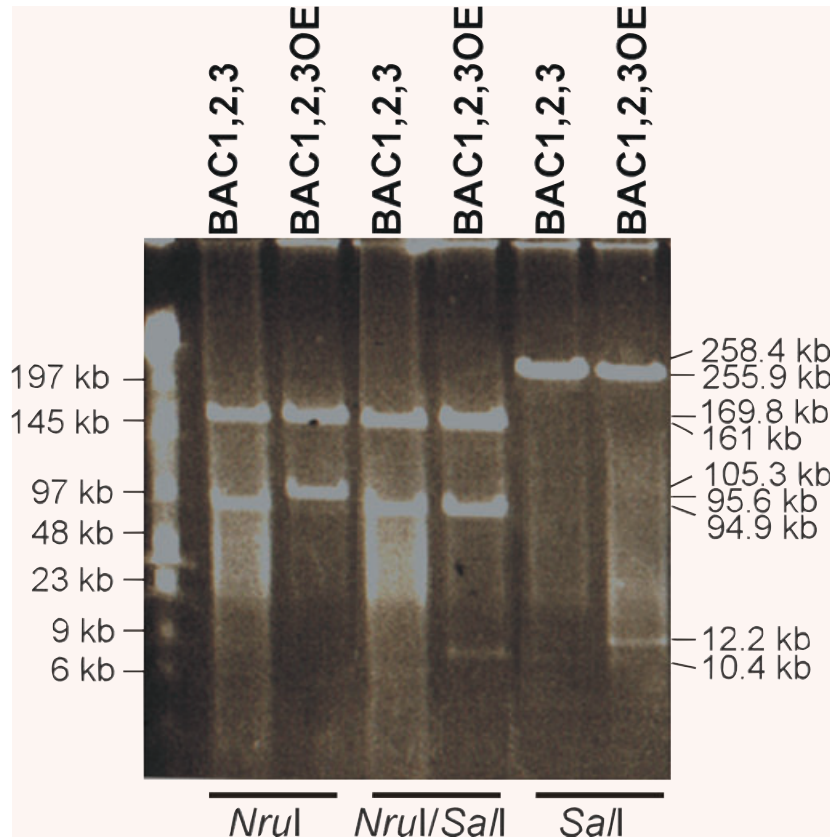


Fig. 2 Pulsed field gel electrophoresis of BAC1, 2, 3 and BAC1, 2, 3 OE clones. BAC1, 2, 3 digested with *Nru* I gives two fragments of 162.8 and 95.6 kb (see Fig. 1). When digested with *Nru* I + *Sal* I it gives three fragments of 161, 95.6 and 1.8 kb (too small to be seen on this gel) and with *Sal* I it gives one fragment of 258.4 kb. Digestion of BAC1, 2, 3 OE with *Nru* I, *Nru* I + *Sal* I and *Sal* I gives two fragments of 162.8 and 105.3 kb, four fragments of 161, 94.9, 10.4 and 1.8 kb (too small to be seen on this gel) and two fragments of 255.9 and 12.2 kb, respectively.

three different BACs, all larger than 130 kb in size, when transfected into B16F10 (mouse melanoma, I.J. Fidler, Texas Medical Center, Houston, TX, USA) cells using lipofectamine 2000. These data together suggest that transfection using lipofectamine 2000 is not a good method for delivering large vectors into mammalian cells intact. In contrast, delivery of large BACs intact into mammalian cells using invasive bacteria has proven very successful.

Delivery of CFTR1, 2, 3 and CFTR1, 2, 3 OE BACs into CMT-93 cells by bacterial invasion

Direct delivery of very large BAC DNA into mammalian cells can be achieved by bacterial invasion [37, 38]. Specifically, the *E. coli* strain BM4573 has been modified to (1) express invasins from *Yersinia pseudotuberculosis* (the *inv* gene), which binds to β 1-integrins on mammalian cells leading to internalization, (2) have impaired cell wall synthesis due to DAP auxotrophy which causes lysis after internalization and (3) express listeriolysin O from *Listeria monocytogenes* (the *hly* locus), which is a pore-forming cytolysin that allows escape from the vacuole after entry. BACs CFTR1, 2, 3 and CFTR1, 2, 3 OE were first electroporated into BM4573 bacteria to generate BMCFTR and BMCFTROE strains, respectively. Subsequently, CMT-93 cells were invaded by

BMCFTR and BMCFTROE. Three zeocin-resistant clones were obtained from the invasion by BMCFTR (CMTCF1-3) and 8 neomycin-resistant clones were obtained from invasion by BMCFTROE (CMTCF1-9).

RT-PCR was performed on all 11 cell lines with the human-specific primers F1R and 523F that amplify from exon 19 to exon 24 (Fig. 3A). All clones showed expression of the human CFTR mRNA but the levels cannot be determined as the PCR reactions were carried out under saturating conditions.

All 11 clones were also analysed by RT-PCR with the h/m2-6 primers [31] that amplify a 610-bp region from exon 2 to exon 6 from both mouse and human mRNA. The origin of the resulting PCR product was determined after restriction enzyme digestion with *Nru* I (Fig. 3B). *Nru* I will cut the human *CFTR* gene product but not mouse-derived product. To exclude the possibility of partial digestion, the reaction was performed twice with excess enzyme and for longer incubation periods than normal. In addition, 5 μ g of plasmid DNA were digested with *Nru* I under the same conditions to confirm complete digestion (not shown). This suggested that the 610-bp DNA fragment remaining after digestion was the PCR product derived from the mouse CFTR and not uncut human product. Again all the cell lines were found to express some human mRNA. As both the human and mouse mRNA were amplified competitively with the same

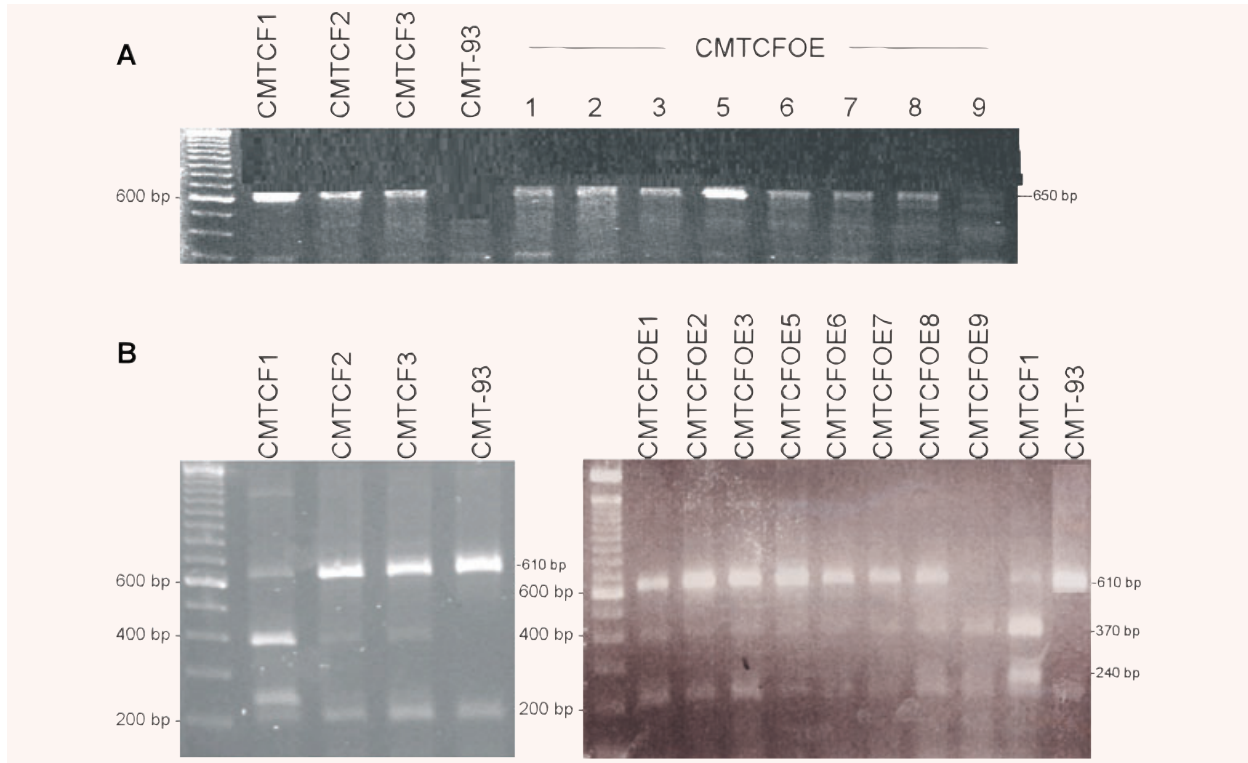


Fig. 3 RT-PCR analysis of CMTCF and CMTCF OE clones. **(A)** RT-PCR with human-specific primers which amplify between exons 19 and 24. A 650-bp product is amplified only from the human mRNA. **(B)** RT-PCR with primers which amplify between exons 2 and 6. A 610-bp product is amplified from both mouse and human mRNA but only the human product cuts with *Nru I* to give two fragments of 370 and 240 bp.

primers in the same reaction, the relative levels of the human and mouse products reflected the starting levels of the mRNAs. Thus, CMTCF1 expressed human *CFTR* mRNA more highly than mouse *CFTR* as the human-specific 370-bp product was considerably stronger than the mouse-specific 610-bp product. The levels of human mRNA were considerably lower relative to the mouse product in lines CMTCF2 and 3. Low levels of human mRNA expression were also detected in CMTCF OE1, 2, 3, 5, 6, 7 and 8. CMTCF OE9 appears to have more human than mouse mRNA in this region but the product for exons 19–24 was not good in this cell line.

CMTCF OE clones were further subjected to FISH analysis to determine their episomal or integrated status and their copy number. Three clones, CMTCF OE5, 6 and 7 (Table 1), were found to contain episomes with no other detectable integrated BAC-derived DNA and with both the *CFTR* (BAC CFTR1, 2, 3) and the OE (pRetroNeoOE) signals present on all episomes (Fig. 4). Clones 6 and 7 contained episomes in more than 10 copies per metaphase while clone 5 contained less than 10 episomes per metaphase. Three clones, CMTCF OE1, 3 and 8 (Table 1), contained only integrations with both the *CFTR* and the OE signals detected. The *CFTR* signal from these integrations was stronger

Table 1 Summary of FISH analysis of CMTCF OE clones

Clone	Episomes	Integrations	CFTR signal on episomes
CMTCF OE1	No	Yes	NA
CMTCF OE2	Yes (few)	Yes	Yes
CMTCF OE3	No	Yes	NA
CMTCF OE5	Yes (<10)	No	Yes
CMTCF OE6	Yes (>10)	No	Yes
CMTCF OE7	Yes (>10)	No	Yes
CMTCF OE8	No	Yes	NA
CMTCF OE9	Yes	Yes	

than that from the episomes of the first three clones. The remaining two clones, CMTCF OE2 and 9 (Table 1), contained integrations of both the *CFTR* and the OE DNA and also episomes. Episomes in clone CMTCF OE2 seemed to contain both *CFTR* and OE DNA. On the other hand, clone CMTCF OE9 contained episomes with only the OE DNA and no detectable *CFTR* signal. In all

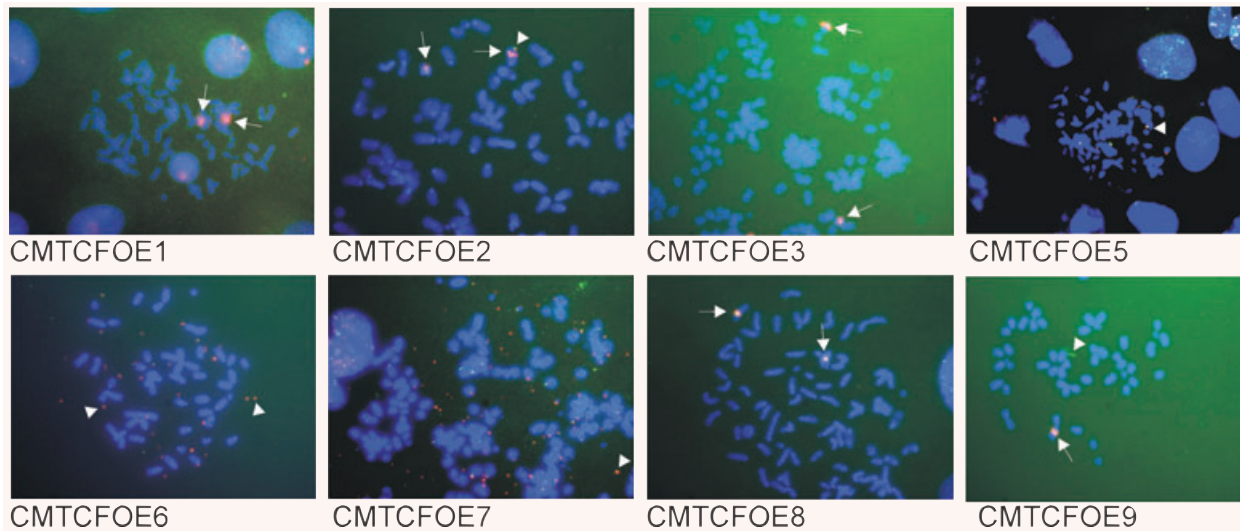


Fig. 4 FISH analysis of CMTCFOE clones. All clones were hybridized with a CFTR1, 2, 3 BAC probe (red) and vector pRetroNeoOE probe (green). Chromosomes were counterstained with DAPI (blue). Red, green and blue signals overlaid are presented. Episomes are indicated with arrowheads and integrations with arrows.

clones, no CFTR signal was detected alone without the OE signal, suggesting that the probe was hybridizing with the human *CFTR* either on an episome or integrated and not with the endogenous mouse *CFTR*.

Clone CMTCF1 was further analysed by sequencing of the human *CFTR* mRNA to show that no mutations had occurred to the CFTR1, 2, 3 BAC during the recombineering processes. This was done by amplification of the whole human mRNA using human-specific primers in five overlapping parts and sequencing of each of the five RT-PCR products separately as shown in Fig. 5. For some of the RT-PCR products the same primers used for their amplification were also used for their sequencing. For other RT-PCR products new sequencing primers were designed. Sequences were aligned individually with the human and mouse *CFTR* sequence. The sequences shown in Fig. 5 were 100% identical to the human *CFTR* and 80% on average identical to the mouse *CFTR* confirming that they correspond to un-mutated human *CFTR* mRNA.

Each amplification with each pair of primers resulted in a single RT-PCR product except with primers F3 and RC, where three bands of approximately 1.1, 0.9 and 0.8 kb were obtained (not shown). After sequencing of these three F3RC RT-PCR products, it was found that one of them (product A, ~1.1 kb) represented the correctly spliced part of the human mRNA shown in Fig. 5 and contained all the exons in the region between F3 and RC (exons 8, 9, 10, 11, 12 and part of exon 13), product B (~0.9 kb) was missing exon 9 (about 200 bp shorter than A) and product C (~0.8 kb and about 100 bp shorter than B) was missing exon 9 and probably another exonic DNA

sequence that was not defined by sequencing. Transcript variants lacking both exons 9 and 12 have been reported previously [42] and product C is a candidate for this exon skipping but this was not confirmed.

DISCUSSION

The construction of a 250.3-kb insert BAC vector carrying the complete human *CFTR* gene with its 27 exons, 40.1 kb of DNA 5' to the gene (with respect to the start of translation) and 25 kb of DNA 3' to the gene (with respect to the end of translation) is shown. This area covers all the known regulatory elements except the -79.5-kb DHS which is not thought to be important in *CFTR* expression.

We applied homologous recombination in *E. coli* as previously described [34] and developed plasmids that will be of general use for linking contiguous overlapping BACs. Indeed, our group succeeded in linking two BACs covering the entire factor VIII gene into a single vector using the same system [38]. Another system based on Red recombination has also been devised for linking overlapping BACs [43], but it cannot be used for linking more than two BACs and is limited as it depends on the presence of only one common restriction site in the overlapping region of the two BACs. For linking more than two BACs, the pBACLinkSp and pBACLinkGm vectors [34] or the vector described here can be used. We chose to develop a new vector and not make use of the

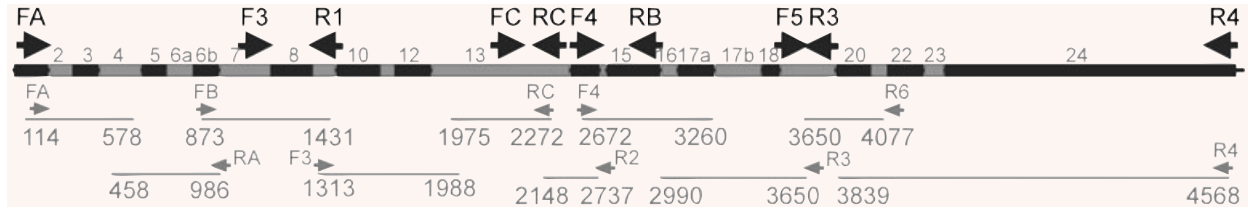


Fig. 5 Sequencing of the human *CFTR* mRNA from clone CMTCF1. Five pairs of primers, FA-R1, F3-RC, FC-RB, F4-R3 and F5-R4 were used to amplify the whole human mRNA. RT-PCR product 1 was then sequenced with primers FA, FB and RA, product 2 with F3 and RC, product 3 with R2, product 4 with F4 and R3, product 5 with R4 and R6. The numbers indicate the start and the end of each sequence obtained with respect to the base number on the mRNA. These sequences put together covered the whole mRNA. The exons of the gene relative to the primers used in amplification and sequencing are also indicated.

pBACLink system for adding the third BAC onto BAC1-2 [34], as our strategy was based on introducing the small fragment of DNA into bacteria already containing the large fragment and not the opposite. Our recombination system makes alternating use of plasmids with a high and/or a single-copy origin. This is advantageous compared to other systems previously described [44] as it allows easy plasmid DNA manipulation and preparation in the presence of the high copy origin and stability of a large insert in the presence of the single copy origin.

Transfection of the CFTR1, 2, 3 or the CFTR1, 2, 3 OE BACs into CMT-93 cells using Lipofectamine 2000 was found to be problematic as no clones containing the entire BAC were obtained. This has also been observed when other large BACs were transfected into CMT-93 and B16F10 cells. On the other hand, delivery of the large CFTR constructs by bacterial invasion eliminated the need of DNA preparation and resulted in all clones obtained containing the input DNA un-rearranged as judged by RT-PCR at both ends of the human *CFTR* mRNA. However, bacterial delivery was found to be less efficient than transfection using Lipofectamine 2000 and this explains why only a small number of CMTCF and CMTCF OE were obtained. Additionally, bacterial delivery of *oriP/EBNA-1* constructs into CMT-93, Hepa1-6 (mouse hepatoma, ATCC CCL-1830), 293A (human embryonic kidney, P. Farrel, Imperial College London, UK) and B16F10 cell lines has previously been found to be inefficient and a similar observation was made with the CFTR constructs.

Although our RT-PCR results from the CMTCF and CMTCF OE clones were not strictly quantitative, the levels of expression of the human *CFTR* could be compared directly with the levels of the endogenous *CFTR* expression in each clone allowing indirect comparison of the expression levels between the clones. Clone CMTCF1 had higher expression of the human *CFTR* than clones CMTCF2 and CMTCF3 either due to difference in the copy number or due to the site of integration. YAC and BAC transgenes (including a YAC with the *CFTR* gene) usually give position independent expression [16, 45–49] so this was expected with the CFTR1, 2, 3 BAC. However, position effects cannot be determined unless more clones with various integration sites and copy numbers are studied.

Clones CMTCF OE5, 6 and 7 were found to contain only episomal copies of the BAC and no integrations. All three clones showed low levels of human *CFTR* mRNA relative to the mouse mRNA. This was similar to the levels obtained previously with a YAC based clone carrying the intact *CFTR* gene and *oriP/EBNA1* elements [31] where level of expression did not seem to correlate with copy number of the episomes.

This study demonstrates the production of full length, correctly spliced and un-mutated human *CFTR* mRNA from the CFTR1, 2, 3 BAC in CMT-93 cells. Some human splicing variants lacking exon 9 and probably exons 9 + 12 have also been detected. Skipping of these exons occurs in a variable proportion of transcripts differing between normal individuals [42, 50, 51]. The extent of exon 9 skipping, in particular, depends on the number of Ts (5, 7 or 9) and of TGs (9–13 repeats) in the exon 9 splice acceptor [52, 53]. The proportion of misspliced variants increases with smaller T alleles (5T) in conjunction with longer TG alleles but was found to differ in cultured cells compared to *in vivo* observations [54]. In our study in CMT-93 cells, skipping of the first 248 or the first 195 nucleotides of exon 13, which has been reported previously [42, 55], was not detected in any of the three F3RC RT-PCR products. Product B was found to contain exon 12, the omission of which has been demonstrated in a significant proportion of transcripts from normal individuals [50]. No other human splicing variants lacking exons flanked at least by one forward and one reverse primer used for sequencing were detected. Detection of variants lacking exons 1, 13, 19 and 24 would have not been possible (even if they existed) because primers FA, FC, RC, F5, R3 and R4 were located within exons 1, 13, 13, 19, 19 and 24, respectively. Finally, bands A, B and C representing the three F3RC RT-PCR products seemed to be of equal intensity but further analysis is required to determine the proportion of missplicing from the CFTR1, 2, 3 BAC which has the (TG)₁₁T₇ genotype and to compare it with the overall proportion of missplicing occurring to living individuals.

The choice of mouse CMT-93 cells for the evaluation of the CFTR BAC vectors was based on their ability to express the human transgene and the potential to compare its levels of expression with those of the endogenous *CFTR* gene. Unfortunately, the presence of the mouse CFTR renders this cell line inconvenient for

studies at the protein level. For instance, the production and correct location of the human CFTR protein at the cell surface could be demonstrated more easily in other cells that can express the human *CFTR* gene driven by its own promoter but which do not express any functional endogenous *CFTR*, preferably a cell line from the airway epithelium of a cystic fibrosis patient. For this however, the low transfection efficiency by bacterial invasion is a major concern. Similarly, a protein functionality study by an ion efflux assay is not applicable in the CMT-93 clones because the human transgene is driven by its own promoter and is not expected to show significantly higher expression than that of the endogenous mouse gene. Future studies will determine whether the BAC-derived CFTR transcript can correct the phenotype of epithelial cells derived from a CF patient.

The CFTR1, 2, 3 BAC described in this study is the only BAC available covering the whole genomic area of the human *CFTR* gene with the flanking regulatory DNA. It should confer full levels of 'tissue specific' and controlled expression and therefore has useful features as a basis for a gene therapy vector.

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