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A mouse model for the cystic fibrosis ΔF508 mutation

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Most cystic fibrosis (CF) patients produce a mutant form (ΔF508) of the cystic fibrosis transmembrane conductance regulator (CFTR), which is not properly processed in normal cells but is active as a chloride channel in several experimental systems. We used a double homologous recombination ('Hit and Run') procedure to generate a mouse model for the ΔF508 mutation. Targeted embryonic stem (ES) cells (Hit clones) were found; of these either 80 or 20% of the clones had lost the ΔF508 mutation, depending on the distance between the linearization site in the targeting construct and the ΔF508 mutation. Correctly targeted clones underwent a second selection step resulting in ES cell clones (Run clones) heterozygous for the ΔF508 mutation with an efficiency of 2–7%. Chimeric mice were generated and offspring homozygous for the ΔF508 mutation showed electrophysiological abnormalities in nasal epithelium, gallbladder and in the intestine, and histological abnormalities in the intestine, typical of CF. Our data suggest that the ΔF508 mice have residual ΔF508 CFTR activity which would explain the mild pathology of the ΔF508 mice. The ΔF508 mouse may provide a useful model for the study of the processing defect of ΔF508 CFTR and for the development of novel therapeutic approaches based on circumvention of the processing block.

Keywords: animal model/embryonic stem cells/gene targeting/Hit and Run procedure/protein processing

Introduction

Cystic fibrosis (CF) is a lethal autosomal recessive disease affecting ~1 in 2500 Caucasians (Boat *et al.*, 1989). The mutated gene is the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which is expressed in several epithelial tissues (Riordan *et al.*, 1989; Crawford *et al.*, 1991; Denning *et al.*, 1992a). CFTR protein functions as an apical cAMP-regulated Cl^- channel (reviewed in Riordan, 1993; Sferra and Collins, 1993) and is presumably involved in osmotic water transport (Quinton, 1990). Malfunction of this chloride channel in CF patients is associated with intestinal malabsorption, obstruction and inflammation of airways, pancreatic ducts, intestine and bile ducts and absence of the vas deferens. Mortality in CF patients is at present mainly due to recurrent pulmonary infections (Boat *et al.*, 1989).

Over 450 different mutations have been described in CF patients (Cystic Fibrosis Genetic Analysis Consortium). The most common mutation is a deletion of a phenylalanine residue at amino acid position 508 of the protein (ΔF508). It is found in 70% of the CF chromosomes (Kerem *et al.*, 1989; Riordan *et al.*, 1989), and therefore 90% of the CF patients have at least one ΔF508 allele. The mutant protein cannot be processed to its mature glycosylated form (Cheng *et al.*, 1990; Gregory *et al.*, 1991). It behaves like a temperature-sensitive processing mutant (Denning *et al.*, 1992b) which is active as a cAMP-regulated chloride channel under permissive conditions (Dalemans *et al.*, 1991; Drumm *et al.*, 1991; Bear *et al.*, 1992; Li *et al.*, 1993). Consequently, it has been suggested that circumvention of the processing block could lead to a therapeutic strategy for CF (Denning *et al.*, 1992b; Yang *et al.*, 1993; Pind *et al.*, 1994). A mouse model expressing the ΔF508 form of CFTR would be very helpful to develop novel therapeutic approaches. Animal models for CF with a complete disruption of the mouse *cftr* gene are available and these show several pathological and electrophysiological abnormalities characteristic of CF patients (Snouwaert *et al.*, 1992; O'Neal *et al.*, 1993; Ratcliff *et al.*, 1993). The mouse exon 10 amino acid sequence is highly homologous to the human sequence and there is no mismatch around the ΔF508 site (Tata *et al.*, 1991). Therefore, we would expect that mouse CFTR with a deletion of the same phenylalanine at position 508 has properties similar to the human counterpart.

To introduce a mutation into the mouse genome, one can choose several strategies. Since we wanted to obtain a mutant *cftr* gene without possible effects of transcriptional interference, the use of a replacement construct with a neomycin gene in the intron next to the mutated ΔF508 exon (Deng *et al.*, 1993) did not seem ideal. One alternative is a two-step selection procedure, 'Hit and Run' (Hasty *et al.*, 1991), which would result in a mutated exon without selection marker genes or plasmid sequences in the intron structure of the gene. In this report, we describe the successful use of this procedure to generate mouse embryonic stem cells (ES cells) carrying the ΔF508 mutation in the endogenous *cftr* gene. Injection of the ES cells into blastocysts resulted in germline transmission. Our data show that mice homozygous for the ΔF508 mutation have abnormalities characteristic of CF.

Results

Targeted integration of a mutant exon in the ES cell genome

We used the 'Hit and Run' procedure (Hasty *et al.*, 1991) to introduce the ΔF508 mutation into the endogenous

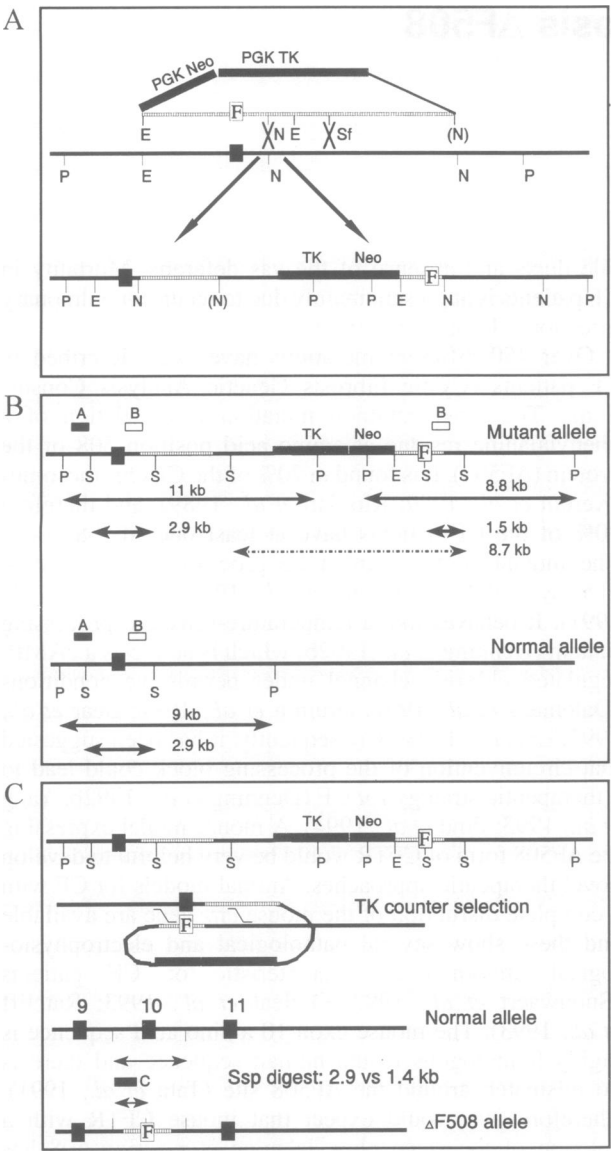

Fig. 1. Hit and Run procedure. (**A**) The 5.7 kb targeting construct containing exon 10 with the ΔF508 mutation was linearized at the *Nsi*I or the *Sfi*I site and transfected into ES cells which were then selected for the expression of the neomycin gene. The DNA of the construct aligns with the endogenous *cftr* allele and is integrated. The result is a duplication of 5.7 kb spaced by the plasmid and the selectable markers [P = *Pst*I, E = *Eco*RI, N = *Nsi*I, (N) = disrupted *Nsi*I site, Sf = *Sfi*I, S = *Ssp*I; only relevant *Eco*RI and *Ssp*I sites are shown]. (**B**) A correct integration is detected with a *Pst*I digest and probe A outside the integration construct. The 5′ duplicate generates a new 11 kb *Pst*I fragment, whereas the normal allele has a 9 kb *Pst*I fragment. A randomly integrated construct does not hybridize with this probe. A *Ssp*I digest shows the presence of the ΔF508 mutation with probe B. A correctly integrated construct has a 1.5 kb *Ssp*I digest, while the normal allele and the 5′ duplicate have a 2.9 kb fragment. A repaired integration shows a 8.7 kb fragment. In a correct Hit clone there is a 2:1 ratio of the 2.9 and 1.5 kb fragment. (**C**) Cells which lost the TK gene will be resistant to selection with FIAU. A homologous cross-over 3′ of the exons generates a normal allele, while a cross-over event 5′ of the exons will result in the desired *cftr* allele with a normal genomic organization but with a mutant exon 10. Clones are digested with *Ssp*I and hybridized with probe C. Correct Run clones have a 1:1 ratio of the 2.9 versus a new 1.4 kb fragment and no neomycin, TK or plasmid sequences.

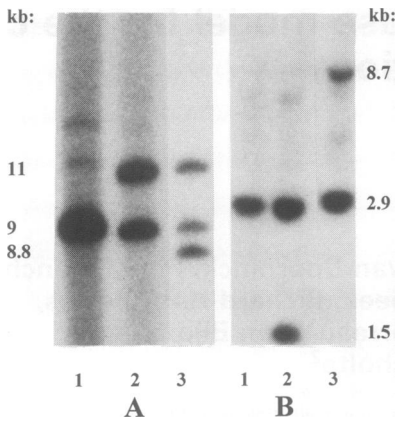

Fig. 2. Southern blot analysis of Hit clones. (**A**) *Pst*I digest of E14 control DNA (lane 1) and Hit DNA (lane 2) hybridized with probe A lying outside of the transfected construct. Lane 3 is as lane 2 but hybridized with the internal probe B. (**B**) *Ssp*I digests hybridized with probe B to check the introduced ΔF508/*Ssp*I site (see Figure 1B). Lane 1 is a E14 control, lane 2 is a correct Hit clone and lane 3 is a Hit clone which lost the ΔF508/*Ssp*I site.

mouse *cftr* gene. The first step involves targeted integration of a mutant construct containing 5.7 kb of isogenic genomic mouse DNA, into the mouse *cftr* exon 10 region (Figure 1A). The ΔF508 mutation in the construct was created six nucleotides from a new *Ssp*I restriction site, which did not change the amino acid sequence and was used in the screening (see Materials and methods). After transfection of mouse ES cells, the linearized construct aligns with its homologous endogenous counterpart and integrates at the site of linearization. This results in a duplication of 5.7 kb of genomic DNA, one part with the normal exon 10 and the other part with the ΔF508 exon, separated by plasmid sequences and a neomycin (PGKNeo) plus thymidine kinase (PGKTK) selection marker cassette (Figure 1). ES cell colonies obtained by selection for G418 resistance were analyzed by Southern hybridization. Probe A, which lies outside of the targeting construct, shows a 9 kb *Pst*I fragment for a normal allele and an 11 kb fragment in the case of successful integration (Figures 1B and 2A). We found that 8% (68/800) of the G418-resistant colonies, obtained with the construct linearized at an *Nsi*I site 600 bp downstream of the ΔF508 mutation, showed the 11 kb *Pst*I band with probe A, indicating a correct targeting event (Figure 2A, lane 2). The internal probe B showed the additional 8.8 kb band from the 3′ duplication (Figure 2A, lane 3). Any random integrations would give an extra band with this probe, but none were found in any of the correctly targeted clones. The *Nsi*-Hit clones were checked for the presence of the *Ssp*I site diagnostic for the ΔF508 mutation. This new *Ssp*I site results in a 1.5 kb *Ssp*I fragment from the 3′ duplicate while the 5′ duplicate and the normal allele both give a 2.9 kb fragment (Figures 1B and 2B, lane 2). Out of 25 *Nsi*-Hit clones, 21 clones (84%) had lost the *Ssp*I site diagnostic of the ΔF508 mutation, which resulted in a new 8.7 kb fragment (Figure 2B, lane 3). Since the *Nsi*I site was rather close to the mutation (600 bp), we chose another linearization site further away from the mutation to test whether this would affect the process. With linearization at the *Sfi*I site, 1600 bp downstream from the ΔF508 mutation, only 20% of the *Sfi*-Hit clones had

Table I. Genotype and phenotype of FIAU-resistant clones

Hit clone	Hit clone cultured on 2 μM FIAU for 2 days				Hit clone cultured for 3 p. on G418; 2 p. on G418⁻; 0.4 μM FIAU for 10 days			
	No. of TK⁻ clones analyzed	Genotype			No. of TK⁻ clones analyzed	G418-sensitive	Genotype	
		Wild	Hit	Scrambled			Wild	ΔF508
155	3	0	0	3	206	61	60	1
B30	157	157	0	0	ND			
B47	39	39	0	0	ND			
B73	33	2	28	3	198	113	99	14
B84	69	11	51	7	170	115	108	7

p. indicates passages; ND, not determined.

lost the *Ssp*I site (2/11 colonies), while the targeting efficiency was in the same range (5%, 13/279 colonies) as for *Nsi*I linearization. These data suggest that a repair mechanism is active around the integration site with a preference for the endogenous chromosomal sequence.

ES cell clones heterozygous for the ΔF508 mutation

The next step of the procedure (Run) involves the removal of the duplication plus the intervening plasmid sequences via an intra-chromosomal homologous recombination (Figure 1C). This leads to the loss of either the mutant or the wild-type exon (Figure 1C). The latter case gives us the desired clone with a mutant ΔF508 exon 10 in an otherwise normal genomic organization of the *cftr* gene. In both cases, recombinants will be resistant to the toxic thymidine kinase substrate 1-(2-deoxy, 2-fluoro-β-D-arabinofuranosyl)-5 (FIAU) and sensitive to G418. The correct Run clone is identified by a new 1.4 kb *Ssp*I fragment, generated by an *Ssp*I site outside of the integrated construct and the *Ssp*I site in the mutated exon 10 (Figure 1C). Different Hit clones with a correct karyotype were subjected to selection with 2 μM FIAU for 2 days. Resistant clones were picked after 7–10 days and screened by Southern analysis. In initial experiments, we found either wild-type cells with two copies of the 2.9 kb *Ssp*I fragment, or cells which had no genomic rearrangement compared with Hit clones (Table I). The latter presumably represented TK mutants. A few clones showed a rearranged and scrambled genotype. To reduce the possibility of contaminating wild-type cells in the population, we cultured the Hit cells for three passages in the presence of G418 before the FIAU selection. This decreased the number of FIAU-resistant clones 10- to 100-fold. In addition, we screened the resultant clones for G418 sensitivity to avoid Southern analysis of clones with a mutation in the TK gene. When we started the FIAU selection the day after ending the G418 selection, we did not find any G418-sensitive cells (0/42 colonies). However, if we cultured the cells for two passages in non-selective medium prior to FIAU selection, 30–70% of the clones were G418 sensitive (Table I). Of these, 2–7% had the 1.4 kb *Ssp*I fragment of the desired ΔF508 Run clone. (Table I, Figure 3A). No rearrangements of the *cftr* gene were observed, as other digests show a wild-type restriction pattern (Figure 3B). Further, hybridization with the neomycin probe does not show a signal with any of the Run clones (Figure 3A, lanes 6–8). These results confirm the loss of the duplication and the normal genomic organization of the ΔF508 allele in the Run clones.

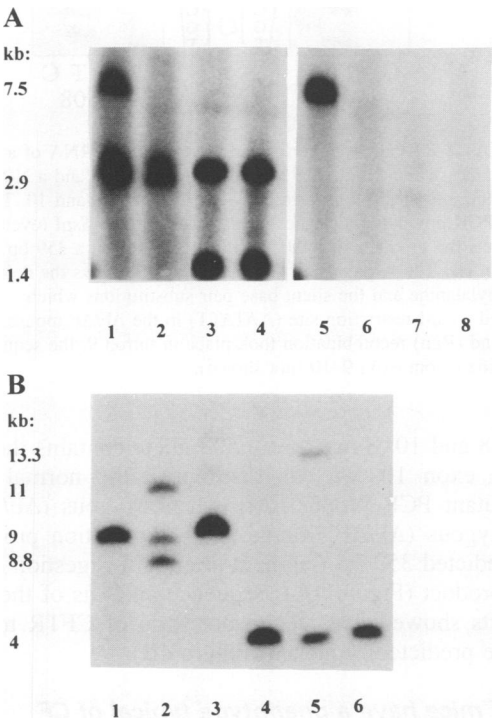

Fig. 3. Southern blot analysis of Run clones hybridized with probe C, 5′ of exon 10. (**A**) *Ssp*I digests of Hit clone DNA (lane 1), E14 control DNA (lane 2) and two Run clones (lanes 3 and 4). Lanes 5–8: the same blot hybridized with a neomycin probe demonstrating the absence of PGKNeo in the Run clones. (**B**) *Pst*I digests (lanes 1–3) and *Nsi*I digests (lanes 4–6) of respectively E14 DNA, Hit clone DNA and Run clone DNA hybridized with probe C were used to assay the correct chromosomal structure of the Run clones.

Generation of a ΔF508 CFTR mouse strain

Cells of 11 Run clones derived from three different Hit clones, with a correct karyotype and heterozygous for the ΔF508 mutation, were injected into blastocysts and gave rise to sex conversion and coat color chimerism ranging from 20 to 100%. Two male chimeras showed full germline transmission in a cross with FVB mice, as indicated by coat color and Southern analysis (using the same procedure as for the Run clone analysis, results not shown). To check the correct transcription of the ΔF508 allele, we isolated intestinal RNA from normal and mutant mice and performed a nested RT-PCR analysis with primers in

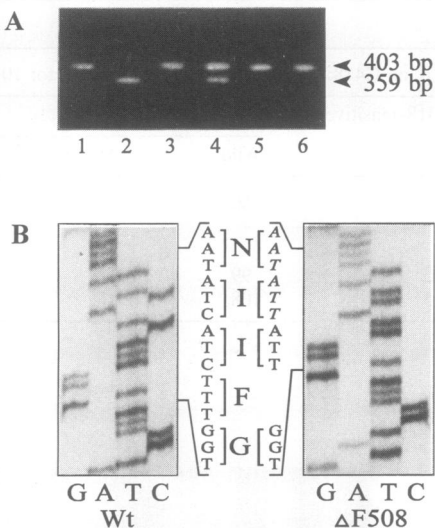

Fig. 4. (**A**) A RT-PCR was performed with intestinal RNA of a ΔF/ΔF mouse (lanes 1 and 2), a N/ΔF mouse (lanes 3 and 4) and a N/N mouse (lanes 5 and 6) with primers located in exon 8 and 10. The 403 bp PCR products (odd lanes) were digested with *Ssp*I (even lanes) which cuts the products of a ΔF508 allele resulting in a 359 bp fragment. (**B**) The sequence of the PCR products shows the deletion of the phenylalanine and the silent base pair substitutions which generated a *Ssp*I restriction site (AATATT) in the ΔF/ΔF mouse. Since the second (Run) recombination took place in intron 9, the sequence was verified from exon 9–10 (not shown).

exons 8 and 10. Since the ΔF508 allele contains the *Ssp*I site in exon 10, we can distinguish the normal from the mutant PCR product. Mice heterozygous (ΔF/N) or homozygous (ΔF/ΔF) for the ΔF508 mutation produced the predicted 359 bp fragment after *Ssp*I digestion of the PCR product (Figure 4A). Sequence analysis of the PCR products showed that the mutant form of CFTR mRNA had the predicted sequence (Figure 4B).

ΔF/ΔF mice have a phenotype typical of CF

Mice homozygous for the ΔF508 mutation (ΔF/ΔF) are viable and do not show severe disease symptoms, such as runting or meconium ileus. However, the ΔF/ΔF mice are growth retarded, as is apparent from the significant difference in weight ($P<0.05$) between the ΔF/ΔF mice (average age 42 days: 17.9 g ± 4.4, $n = 6$) and their normal, N/ΔF and N/N, littermates (average age 39 days: 23.4 g ± 2.6, $n = 7$). Mice were sacrificed at 5–7 weeks and used for histological and electrophysiological studies. In the small intestine of ΔF/ΔF mice, focal hypertrophy of goblet cells was observed in the crypts of Lieberkuhn (Figure 5b). The hypertrophy of goblet cells was predominantly seen in deep crypts, though a few hypertrophic cells were found at the crypt–villus border. The crypts containing hypertrophic goblet cells appeared to be located in clusters. The majority of the intestine appeared normal. Crypts in the colon showed some mucus retention and slight dilatation (Figure 5e). A layer of thick mucus on top of the crypts could also be noticed. We did not observe dramatic distention of the crypts in the small intestine or complete intestinal obstruction either in the ileum or colon, as reported in *cftr* knock-out mice (Figure 5c and f, data from a cftr^{m1Cam} mouse, Ratcliff *et al.*, 1993). So far, we have not seen abnormalities or stasis of inspissated mucus in lungs, pancreas, liver bile ducts, vas deferens and salivary glands.

Electrophysiological abnormalities in ΔF/ΔF mice

The reduced chloride permeability of the epithelium due to CFTR dysfunction causes typical abnormalities in the ion transport of different epithelia (Quinton, 1990). An increased nasal potential difference (PD) is characteristic of CF patients (Knowles *et al.*, 1983; Alton *et al.*, 1990) and is also observed in *cftr* knock-out mice (Grubb *et al.*, 1994a; P.J.French, unpublished data). The basal *in vivo* nasal PD of ΔF/ΔF mice was significantly higher than in normal littermates (Figures 6 and 7c). Consequently, the net effect of the sodium channel inhibitor amiloride, which would block net ion movement in a CF mouse, was higher than normal (Figure 6). The nasal PD in ΔF/ΔF mice increased in response to a large chloride gradient created by substitution of chloride by gluconate in the superfusion solution (Figures 6 and 7c). This gluconate response was quantitatively similar to the response of control littermates (Figure 6). Since cftr^{m1Cam} knock-out mice do not show a significant response to gluconate under our conditions (Table II), we conclude that the response we observe in the ΔF/ΔF mouse is indicative of a residual chloride conductance in nasal epithelium.

Human CF epithelial tissues do not respond to forskolin, which activates CFTR through an increase of $[cAMP]_i$ (Riordan, 1993). The adult excised ΔF/ΔF mouse trachea shows a forskolin response comparable with normal mice (P.J.French, unpublished data). This is also observed in the *cftr* knock-out mouse trachea (Grubb *et al.*, 1994b). In this tissue, forskolin can apparently activate calcium-dependent chloride channels (Clarke *et al.*, 1994) through a cross-talk mechanism which is currently not understood. In the gallbladder, the ΔF/ΔF mice showed a markedly reduced PD response to forskolin compared with normal littermates in Ussing chamber experiments (Figures 6 and 7a). This reduced forskolin response was not due to aspecific tissue damage, as both the electrical resistance and the peak response to the Ca^{2+} agonist carbachol were normal (Figures 6 and 7a). In the ileum, the initial PDs and equivalent short circuit currents (Ieq) were significantly lower in ΔF/ΔF mice compared with normal littermates (Figure 6). Luminal hyperpolarization induced by forskolin was significantly reduced in ΔF/ΔF mice, whereas the response to glucose addition, which activates the Na^+/glucose co-transporter (Wright, 1993), was normal (Figures 6 and 7b). In Table II, the responses to forskolin of ΔF/ΔF mice are compared with those responses in cftr^{m1Cam} knock-out mice. Knock-out mice lack forskolin responses in ileum and gallbladder, whereas the homozygous ΔF/ΔF mice exhibit residual activity. This is in agreement with the observed residual chloride permeability in ΔF/ΔF nasal epithelia (Table II).

Discussion

We have generated mice with a mutation analogous to the frequently occurring temperature-sensitive processing mutation in CF, ΔF508. We intend to use this mouse to study the interaction between ΔF508 CFTR and processing proteins such as calnexin (Pind *et al.*, 1994) and hsp70 (Yang *et al.*, 1993) in intact tissues. This might lead to

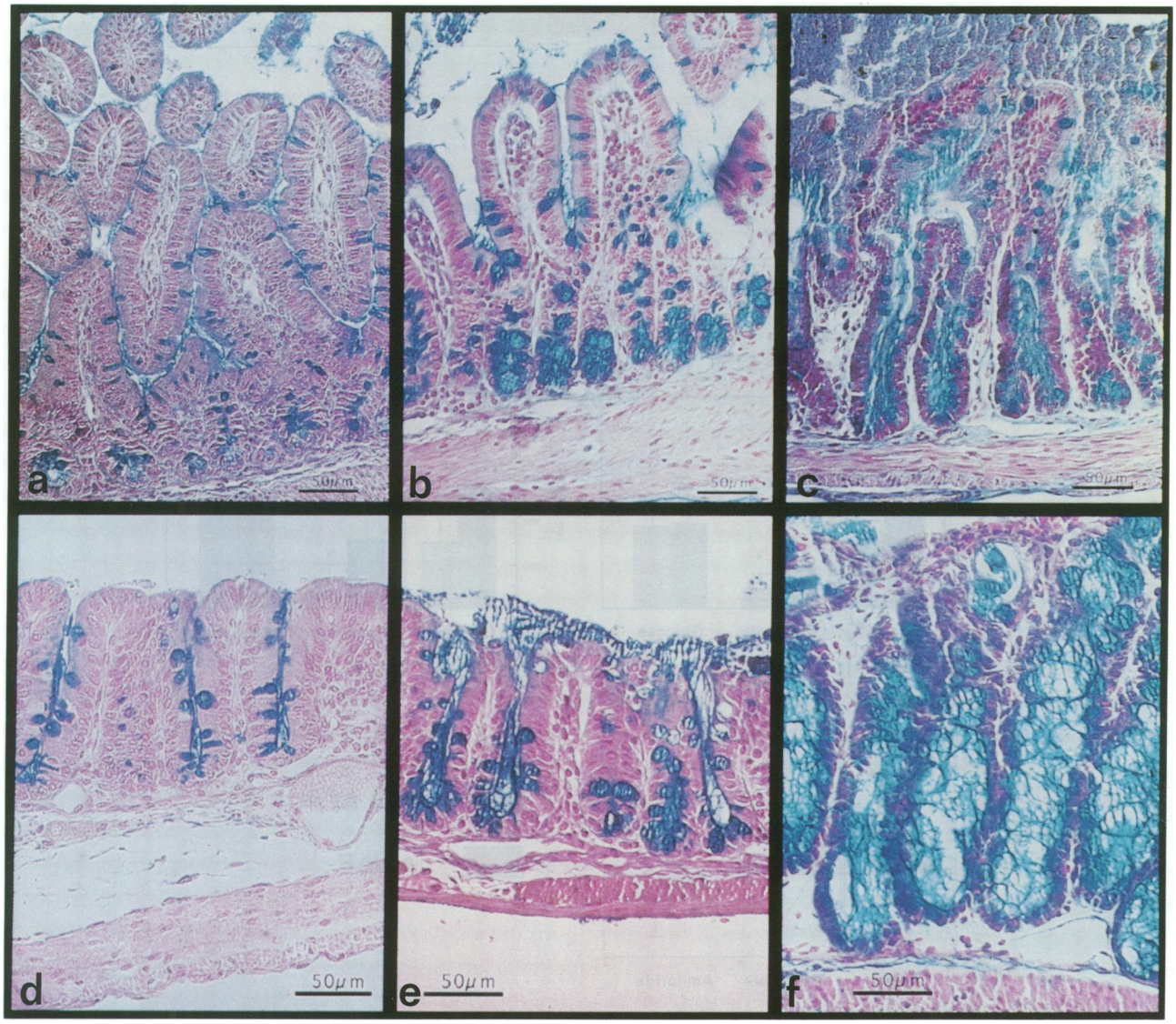

Fig. 5. Histopathology of ΔF508 mice. Sections of the small intestine (**a–c**) and colon (**d–f**) of normal (littermates of ΔF/ΔF) (a and d), ΔF/ΔF (b and e) and *cftr*^{Cam} knock-out mouse (c and f). In the ΔF/ΔF mice, focal hypertrophy of goblet cells was found in crypts of the small intestine (b), though not as severe as the ileum of a *cftr*^{Cam} knock-out mouse showing extensive goblet cell hyperproliferation, increased mucus accumulation and luminal obstruction (c). Colon of a ΔF/ΔF mouse with moderate mucus accumulation and distension of the crypts (e) compared with the colon of a *cftr*^{Cam} knock-out with massive goblet cell hyperplasia and hypertrophy (f).

novel therapeutic approaches for the 90% of CF patients carrying a ΔF508 mutation.

Hit and Run procedure

We found integration of the targeting construct into the mouse *cftr* locus in 5–8% of the G418-resistant ES cell colonies. This is comparable with the frequencies found by Dickinson *et al.* (1993) for integration of a targeting construct near exon 10. When we used a linearization site relatively close (600 bp) to the ΔF508/*Ssp*I site, 84% of the Hit clones had lost the diagnostic *Ssp*I restriction site. Since we observed no rearrangement in the exon 10 area, we concluded that the plasmid DNA is preferentially repaired using the endogenous DNA as a template. The mechanism of this repair might be through gap formation and repair (Valancius and Smithies, 1991). We never found the appearance of the *Ssp*I site in both exons nor did we find it only in the 5′ duplication. This implies that, during our Hit procedure, mismatch heteroduplex repair and migration of the Holliday junctions across the mutations are rare (Hasty and Bradley, 1993). The usage of a restriction site 1600 bp away from the ΔF508/*Ssp*I site resulted in only 20% loss of the mutation, confirming previous observations that the frequency of loss depends on the distance between the break and the mutation (Valancius and Smithies, 1991; Deng *et al.*, 1993)

The overall frequency of a correct Run clone (ΔF/N) is ~$1/10^6$–10^7 cells in the original population used for selection, and 2–7% of the clones screened by Southern analysis. In order to find these Run clones, we had to adapt our screening protocol by selecting against wild-type contaminants and by allowing time for the cells to perform an intra-chromosomal recombination. Screening for the loss of G418 resistance further reduced the number of clones analyzed by Southern analysis (Table I). On the basis of the length of the homologous regions involved

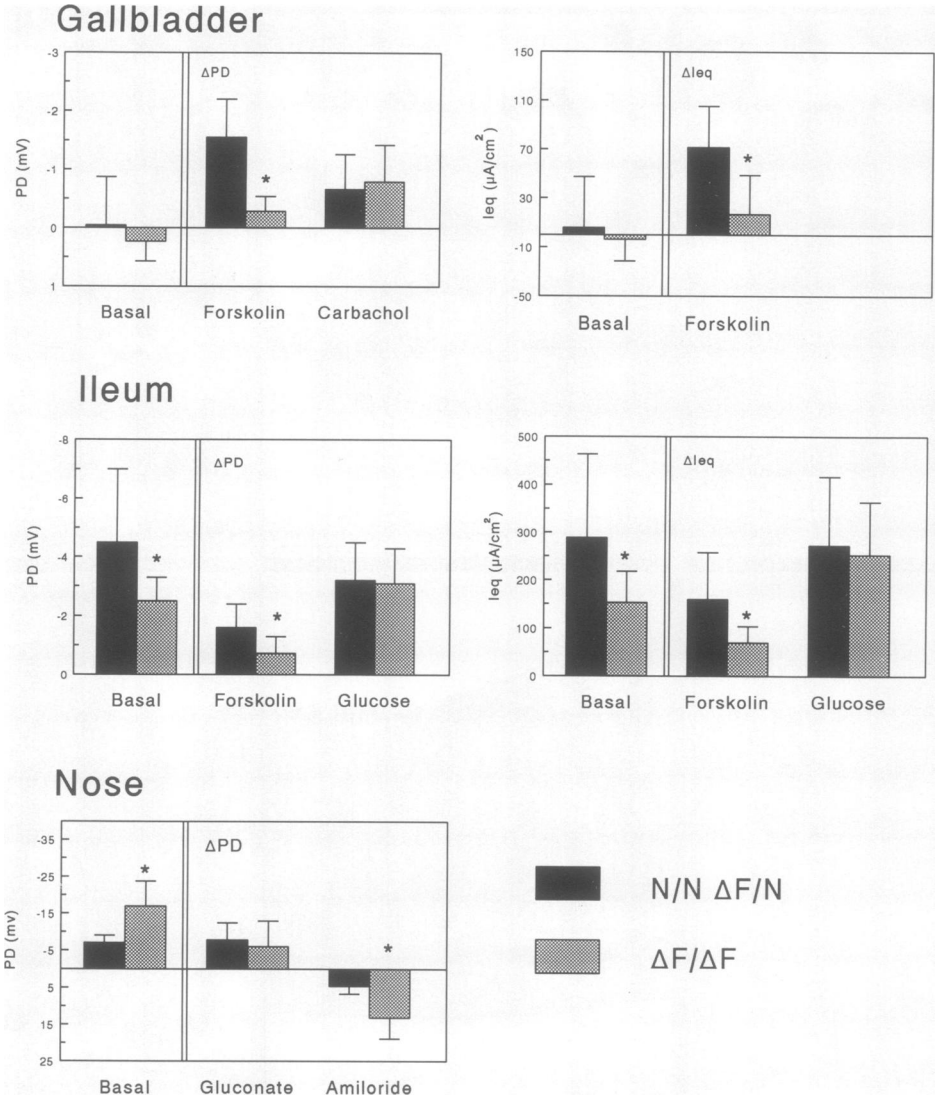

Fig. 6. Summary of the electrophysiological data obtained from six ΔF/ΔF, five ΔF/N and three N/N mice. Left panels: PD, right panels: Ieq. Top: gallbladder. The basal PD and Ieq as well as the peak response to carbachol of ΔF/ΔF mice and normal (N/N or ΔF/N) littermates are comparable. Forskolin (10^{-5} M) responses, however, were markedly reduced in the ΔF/ΔF gallbladder ($P<0.001$, nine tissue samples from six mice) compared with normal (eight tissue samples from eight mice). Middle: ileum. ΔF/ΔF ilea (12 tissue samples from six mice) had a significantly reduced basal PD and Ieq ($P<0.02$) compared with their normal littermates (14 tissue samples from eight mice). Responses to forskolin were reduced in the ΔF/ΔF ileum ($P<0.01$). Lower: nasal PD. In ΔF/ΔF mice the *in vivo* basal PDs were significantly higher than their control littermates ($P<0.01$). Both normal ($n = 6$) and ΔF/ΔF ($n = 6$) nasal epithelia responded to a chloride gradient [153 mM chloride replaced gluconate (133 mM), final Cl^- concentration 10 mM]. Responses to the sodium channel inhibitor amiloride were significantly higher in ΔF/ΔF nasal epithelia ($P<0.02$), resulting in a similar residual PD. Values are averages ± SD. Statistical analyses were performed with the student's *t*-test.

in the two possible Run recombinations, one would expect to find ~25% ΔF508 Run clones (Figure 1C). However, the actual ratio of ΔF508 to wild-type recombinants (± 1/20) does not seem to follow this simple rule. This may be due to cryptic sequence preferences in the recombination process.

The Hit and Run procedure has previously only been used to produce the mutant Hoxb-4 mouse (Ramírez-Solis *et al.*, 1993). The possible drawback of the 'Hit and Run' procedure compared with a one-step replacement protocol is the number of passages under selective pressure that is involved. The starting passage of our E14 cells was 24; selections with both G418 and FIAU plus several expansion steps lead to ΔF508 clones of around passage 57 (assuming about three divisions per passage). The blastocyst injections showed that germline chimeras can still be made with these cells. However, in Run clones derived from the B84 Hit clone, loss of totipotency was observed (20% chimerism, sex-reversal rate 1.4 and no germline transmission).

ΔF/ΔF mice have a CF phenotype

The Hit and Run procedure resulted in mice which produce ΔF508 CFTR mRNA with the predicted sequence (Figure 4B). The heterozygous ΔF/N mice do not show significant abnormalities in any of our assays. We therefore consider them phenotypically normal. ΔF/ΔF mice show a phenotype typical of CFTR dysfunction, including failure to grow to normal weight, histological abnormalities in the intestine (Figure 5) and electrophysiological abnormalities in nasal epithelium, gallbladder and intestine (Figures 6 and 7). We did not observe lethal obstruction of the

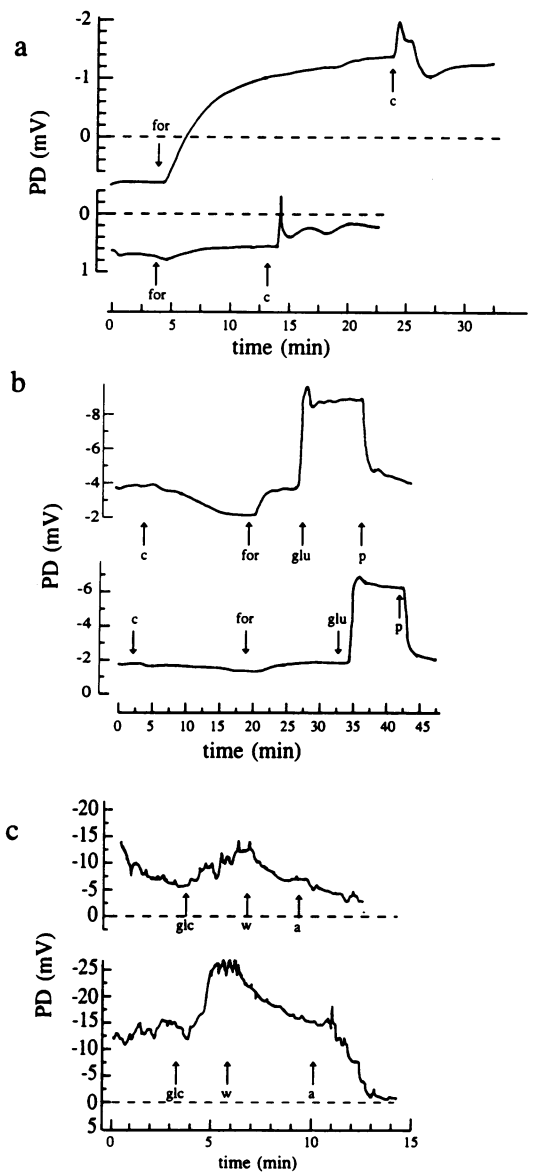

Fig. 7. Representative Ussing chamber tracings from normal mice (upper tracings) and ΔF/ΔF mice (lower tracings) tissue. (**a**) Gallbladders of ΔF/ΔF mice had a reduced response to forskolin (for) compared with normal littermates, whereas peak responses to carbachol (c) were similar. (**b**) Ilea of ΔF/ΔF mice had a reduced response to forskolin (for) compared with normal littermates. Responses to 30 mM glucose (glu) and subsequent 0.2 mM phloridzin (p), activating and inhibiting the Na^+/glucose transporter (Wright, 1993) respectively, were similar. Responses to carbachol (c) on average caused luminal depolarization and an increase in tissue resistance. (**c**) Nasal epithelia of ΔF/ΔF mice had a higher basal PD and an increased response to amiloride (a) but did respond to a chloride gradient [from 153 to 10 mM Cl^-, chloride replaced gluconate (glc, 133 mM)], w = wash.

intestine in ΔF/ΔF mice. Meconium ileus, i.e. obstruction of the intestine, is diagnosed in only 10% of neonatal CF patients, and those patients show mucus obstruction in the crypts. Other CF patients do not show severe mucus accumulation but have distended glands and goblet cell hypertrophy similar to our ΔF/ΔF mice (Thomaidis and Arey, 1963). Another typical feature of CF, progressive lung inflammation accompanied by accumulation of viscous mucus (Boat *et al.*, 1989), is not observed in either ΔF/ΔF or knock-out mice under normal conditions. However, it has been shown recently that CF mice, when challenged with lung pathogens, do show increased pathology compared with normal littermates (Davidson *et al.*, 1995). It would therefore be interesting to subject the ΔF/ΔF mice to a similar protocol. As many abnormalities in CF patients develop with age, further histological analysis will be done with mice older than those of 5–7 weeks which we used for this study.

The virtual absence of lethal intestinal obstruction in ΔF/ΔF mice differs from the *cftr* knock-out models described earlier. The latter were reported to have a 50–90% mortality rate in the first 4 weeks, mainly due to intestinal obstructions (Snouwaert *et al.*, 1992; O'Neal *et al.*, 1993; Ratcliff *et al.*, 1993). The $cftr^{m1HGU}$ insertion mutant (Dorin *et al.*, 1992) expresses low levels of normal CFTR (Dorin *et al.*, 1994) and has a low mortality rate. This suggests that the mild phenotype of the ΔF/ΔF mice is related to the residual chloride permeability observed. However, mortality rates may prove a rather misleading parameter. The colony of $cftr^{m1Cam}$ mice in the Rotterdam animal facility has a considerably lower mortality rate than reported (<40% in the first 4 weeks versus 80% in the first 5 days, Ratcliff *et al.*, 1993). Apparently, the breeding conditions have a major impact on mortality rates. The phenotypic effect of residual chloride permeability may be revealed by challenging knock-out and ΔF/ΔF mice with pathogens in parallel experiments.

Residual CFTR activity in ΔF/ΔF mice

The small but significant forskolin response observed in ileum and gallbladder, and the response to a chloride gradient in the nasal epithelium (Figures 5 and 6) of the ΔF/ΔF mouse might be due to residual ΔF508 CFTR activity. An alternative chloride channel (Clarke *et al.*, 1994) seems a less plausible explanation since $cftr^{m1Cam}$ knock-out mice completely lack a forskolin response in the gallbladder (Table II) and intestine (Table II, Clarke *et al.*, 1992; Cuthbert *et al.*, 1994) and show no significant gluconate response in the nasal PD measurements (Table II). Ca^{2+}-activated chloride channels appear absent in the ileum, as the ileum does not respond to carbachol with a luminal hyperpolarization (Figure 7). Thus, the cAMP-induced activation of Ca^{2+}-dependent chloride channels as described in mouse trachea (Grubb *et al.*, 1994b) seems not to occur in the ileum or the gallbladder. It is conceivable that the mouse ΔF508 CFTR is partially processed into mature ΔF508 CFTR protein reaching the plasma membrane. At present, it is not known whether the same phenomenon occurs in CF patients. Residual intestinal chloride transport activity, observed in the minority of ΔF/ΔF patients, is correlated with mild disease (Veeze *et al.*, 1994). Since this has not yet been analyzed at the level of single chloride channels, this activity cannot be attributed unequivocally to CFTR. Further experiments should determine the channel characteristics of the mouse ΔF508 CFTR and the processing kinetics of ΔF508 CFTR in differentiated mouse epithelial cells. The mouse and human processing systems may differ with respect to the kinetics of their interactions with the ΔF508 CFTR protein, and the mouse ΔF508 CFTR may have different properties from the human ΔF508 form. The ΔF508 mouse model gives us an opportunity to study the CFTR processing

Table II. Responses to forskolin or gluconate of ΔF/ΔF and cftr^{m1Cam} –/– mice (ΔPD in mV)

	ΔF/ΔF	Controls	*P**	–/–	Controls	*P**	*P#*
Gluconate:							
Nose	–5.3 *n* = 6	–8.3 *n* = 6	ns	–2.5 *n* = 11	–7.0 *n* = 11	<0.001	ns
Forskolin:							
Gallbladder	–0.2 *n* = 9	–1.5 *n* = 6	<0.001	0.0 *n* = 6	–1.2 *n* = 10	<0.001	<0.01
Intestine	–0.5 *n* = 13	–1.5 *n* = 13	<0.001	0.05 *n* = 6			<0.001

Control mice were heterozygous and homozygous wild-type littermates. Median values are given, with *n* indicating the number of experiments. Mann–Whitney test: *P** = *P* value comparing CF mice with littermates, and *P#* = *P* value comparing ΔF/ΔF mice with –/– cftr^{m1Cam} mice. ns, not significant.

defect observed in a majority of CF patients in more detail and allows us to test novel approaches for therapy of CF *in vivo*.

Materials and methods

Embryonic stem cells

E14 ES cells (a gift of M.Hooper, Edinburgh) were cultured on 0.1% gelatine-coated dishes in DMEM/60% Buffalo rat liver-conditioned medium/10% fetal calf serum (FCS) (Smith and Hooper, 1987) supplemented with 1% non-essential amino acids (Gibco BRL, Life Technologies), 0.1 mM 2-mercaptoethanol, antibiotics and 1000 U/ml LIF (ESGRO, Gibco BRL, Life Technologies) and passaged every 2–3 days.

Targeting constructs

CFTR genomic DNA was cloned from a 129/Ola λ library (provided by G.Grosveld). A 2 kb *Eco*RI fragment containing exon 10 of the *cftr* gene was mutated using standard site-directed mutagenesis technology to introduce a ΔF508 deletion and a *Ssp*I restriction site (AAT ATC ATC TTT = amino acids NIIF to *AAT ATT* AT. ..T = amino acids NII.). This fragment of the mouse *cftr* gene was enlarged to 5.7 kb by insertion of a 4 kb *Nsi*I fragment between the *Nsi*I site of the *Eco*RI fragment and the *Pst*I site of the pBluescript vector (pESEN7). A cassette with a thymidine kinase (TK) gene driven by a phosphoglycerate kinase promoter (PGKTK, a gift of N.van der Lugt, Amsterdam) and a neomycin gene driven by a PGK promoter (PGKNeobpA, Soriano *et al.*, 1991) was introduced as a PGKTK–PGKNeo *Sal*I fragment into the *Xho*I site of pESEN7, generating pESTNΔF+ (Figure 1A). This construct contains a unique *Nsi*I linearization site at 600 bp from the ΔF508 and a unique *Sfi*I site at 1600 bp from the ΔF508. The mutant exon and surrounding sequences were checked by sequence analysis.

Selection of integration mutants (Hit clones)

E14 ES cells (10^7) were electroporated in PBS (Ca^{2+}- and Mg^{2+}-free) with 5–10 μg linearized plasmid in a Progenetor II, PG200, Hoefer Gene pulser at 350 V/cm, 1200 μF, 10 ms. Selection was started at 300 μg/ml G418 (Gibco BRL, Life Technologies) the next day. Genomic DNA of G418-resistant clones was digested as indicated, Southern blotted and hybridized to probe A, a *Hae*III fragment upstream of the construct, to probe B, a *Hin*dIII–*Nsi*I fragment starting from the second *Hin*dIII site in exon 10 or to probe C, a *Bgl*II–*Hin*dIII fragment ending on the first *Hin*dIII site in exon 10 (Figure 1B). Targeted clones (Hit clones) with a normal karyotype were used for the next step.

Selection of TK revertants (Run clones)

To obtain independent ΔF508 clones, a Hit clone was grown on G418 for three passages, followed by two passages on medium without G418, on 10 different plates which were kept separate during the whole procedure. To select for TK-negative clones, the cells were plated at 3×10^6 cells per 10 cm dish and FIAU (Bristol Myers, Squibb) was added at a concentration of 0.4 μM for 10 days. Colonies were picked at days 7–10 and transferred to 24-well plates. FIAU-resistant clones derived from the same plate were not considered to be independent. When confluent, a clone was divided into three parts, for testing loss of G418 resistance, for freezing and for DNA analysis. G418-resistant clones were discarded.

Generation and screening of ΔF508 mice

Blastocyst injection and re-implantation were performed as described by Bradley (1987). Male chimeras were crossed with FVB mice. DNA was isolated from tail segments (Laird *et al.*, 1991) and analyzed on Southern blots as described for the Run clones.

Analysis of the ΔF508 allele

To check the correct transcription of the ΔF508 allele, intestinal RNA was isolated from ΔF/ΔF mice and control mice with the LiCl/urea method (Auffray and Rougeon, 1980). A PCR (30 cycles: 2 min 94°C, 2 min 47°C, 4 min 72°C) was carried out with primers MCF1 and MCF4 located in exon 8 and at the 3′ end of exon 10. A nested PCR was done with the same cycle parameters and with primers MCF2 and MCF4 located inside and adjacent to the first primer pair. (5′→3′ sequence MCF1:gcagaaacaagagtataaag, MCF2: aatgaccacaggcataatc, MCF3: caacactcttatatctgt, MCF4: ctgctgtagttggcaag). Part of the product was digested with *Ssp*I, which cuts the introduced *Ssp*I site adjacent to the ΔF508 mutation. The PCR products derived from ΔF/ΔF mice and N/N mice were cloned and sequenced.

Histological analysis of the ΔF/ΔF mice

The ΔF508 mouse strain has a 129/Ola×FVB/N background and was kept in a pathogen-free environment. Tissues were fixed in 40% ethanol, 5% HAC, 3.7% formaldehyde, 45% saline pH 7.3, and paraffin sections were stained with Alcium blue, haematoxylin and eosin.

Electrophysiological analysis

Gallbladder and ileum were mounted in an Ussing chamber, under open circuit conditions. Experiments were performed at 37°C. The electrical resistance of the epithelium was measured at intervals by the voltage deflection following a calibrated current pulse. The basic perfusion solutions for gallbladder and ileum consisted of (mM): 105 NaCl, 4.7 KCl, 1.3 $CaCl_2$, 1.0 $MgCl_2$, 20.2 $NaHCO_3$, 0.4 NaH_2PO_4, 0.3 Na_2HPO_4, 10 HEPES, saturated with 95% O_2 and 5% CO_2, pH 7.4. To the serosal side of the ileum, 5 mM glucose was added. Nasal PD measurements were performed *in vivo* essentially as described by Grubb *et al.* (1994b). Data were corrected for liquid junction potentials. The basic perfusion solution consisted of (mM): 133 NaCl, 5 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 0.4 NaH_2PO_4, 0.3 Na_2HPO_4, 5.5 glucose and 5 mM HEPES pH 7.4, 37°C.

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