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 (Snooewart 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
mouse cfr gene. The first step involves targeted integration of a mutant construct containing 5.7 kb of isogenic genomic mouse DNA, into the mouse cfr exon 10 region (Figure 1A). The ΔF508 mutation in the construct was created six nucleotides from a new SspI 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 (PGKT) 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 PstI 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 NsiI site 600 bp downstream of the ΔF508 mutation, showed the 11 kb PstI 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 SspI site diagnostic for the ΔF508 mutation. This new SspI site results in a 1.5 kb SspI 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 SspI site diagnostic of the ΔF508 mutation, which resulted in a new 8.7 kb fragment (Figure 2B, lane 3). Since the NsiI 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 SfiI site, 1600 bp downstream from the ΔF508 mutation, only 20% of the Sfi-Hit clones had

Fig. 2. Southern blot analysis of Hit clones. (A) PstI 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) SspI digests hybridized with probe B to check the introduced ΔF508/SspI 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/SspI site.

Fig. 1. Hit and Run procedure. (A) The 5.7 kb targeting construct containing exon 10 with the ΔF508 mutation was linearized at the NsiI or the SfiI 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 cfr allele and is integrated. The result is a duplication of 5.7 kb spaced by the plasmid and the selectable markers [P = PstI, E = EcoRI, N = NsiI, (N) = disrupted NsiI site, Sf = SfiI, S = SspI; only relevant EcoRI and SspI sites are shown]. (B) A correct integration is detected with a PstI digest and probe A outside the integration construct. The 5' duplicate generates a new 11 kb PstI fragment, whereas the normal allele has a 9 kb PstI fragment. A randomly integrated construct does not hybridize with this probe. A SspI digest shows the presence of the ΔF508 mutation with probe B. A correctly integrated construct has a 1.5 kb SspI 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 cfr allele with a normal genomic organization but with a mutant exon 10. Clones are digested with SspI 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.
Table I. Genotype and phenotype of FIAU-resistant clones

<table>
<thead>
<tr>
<th>Hit clone</th>
<th>Hit clone cultured on 2 μM FIAU for 2 days</th>
<th>Hit clone cultured for 3 p. on G418; 2 p. on G418”; 0.4 μM FIAU for 10 days</th>
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<tr>
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<td>No. of TK− clones analyzed     Genotype</td>
<td>No. of TK− clones analyzed     G418-sensitive     Genotype</td>
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<tr>
<td></td>
<td></td>
<td>Wild</td>
</tr>
<tr>
<td>155</td>
<td>3</td>
<td>0</td>
</tr>
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<td>B47</td>
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<td>2</td>
</tr>
<tr>
<td>B84</td>
<td>69</td>
<td>11</td>
</tr>
</tbody>
</table>

p. indicates passages; ND, not determined.

lost the SspI site (2/11 colonies), while the targeting efficiency was in the same range (5%, 13/279 colonies) as for NsiI 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 inter-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 SspI fragment, generated by an SspI site outside of the integrated construct and the SspI 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 SspI 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 SspI 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.

**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
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 medium (Figures 6 and 7c). This gluconate response was quantitatively similar to the response of control littermates (Figure 6). Since cftrm1Cam 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 (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 Ca2+ 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 cftrm1Cam 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

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 runtting 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 5c). 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 cftrm1Cam 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.

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 SspI (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 SspI 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).
A mouse model for the CF ΔF508 mutation

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 cfr<sup>Cam</sup> 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 cfr<sup>Cam</sup> 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 cfr<sup>Cam</sup> 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 cfr 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/SpI site, 84% of the Hit clones had lost the diagnostic SpI 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 SpI 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/SpI 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<sup>6</sup>–10<sup>7</sup> 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
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
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 cftrnullICl 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 cftrnullICl 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 cftrnullICl 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). Ca²⁺-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 Ca²⁺-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 AF508 CFTR in differentiated mouse epithelial cells. The mouse and human processing systems may differ with respect to the kinetics of their interactions with the AF508 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

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**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) Ileum 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 (glu, 133 mM)], w = wash.
Table II. Responses to forskolin or gluconate of ΔF/ΔF and cftr<sup>delCam</sup> – / – mice (ΔPD in mV)

<table>
<thead>
<tr>
<th>ΔF/ΔF</th>
<th>Controls</th>
<th>P*</th>
<th>– / –</th>
<th>Controls</th>
<th>P*</th>
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<td>(n = 13)</td>
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Control mice were heterozygous and homozygous wild-type littersmates. Median values are given, with \(n\) indicating the number of experiments. Mann–Whitney test: \(P^* = P\) value comparing CF mice with littersmates, and \(P^{##} = P\) value comparing ΔF/ΔF mice with – / – cftr<sup>delCam</sup> 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% gelatin-coated dishes in DMEM/10% 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 LIP (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 EcoRI fragment containing exon 10 of the cftr gene was mutated using standard site-directed mutagenesis technology to introduce a ΔF508 deletion and a SapI restriction site (AAAT ATC ATC TTT = amino acids NIF to AT AT ATT AT ... T = amino acids NIL). This fragment of the mouse cftr gene was enlarged to 5.7 kb by insertion of a 4 kb NsiI fragment between the NsiI site of the EcoRI fragment and the PstI 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 (PGKNeoPA, Soriano et al., 1991) was introduced as a PGKTK–PGKNeo Sall fragment into the XhoI site of pESEN7, generating pESTΔF<sup>+</sup> (Figure 1A). This construct contains a unique NsiI linearization site at 600 bp from the ΔF508 and a unique SfiI 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<sup>5</sup>) were electroporated in PBS (Ca<sup>2+</sup>- and Mg<sup>2+</sup>-free) with 5–10 µg linearized plasmid in a Progenitor II, PG200, Hoefer Gene pulser at 250 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 HaelIII fragment upstream of the construct, to probe B, a HindIII–NsiI fragment starting from the second HindIII site in exon 10 or to probe C, a BglII–HindIII fragment ending on the first HindIII 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<sup>6</sup> 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: gacagaaacagtagataga, MCF2: aagcagacgaaat, MCF3: caacactctctctctct, MCF4: ctctctctctctct). Part of the product was digested with SphI, which cuts the introduced SphI 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 Alcian 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<sub>2</sub>, 1.0 MgCl<sub>2</sub>, 20.2 NaHCO<sub>3</sub>, 0.4 Na<sub>2</sub>PO<sub>4</sub>, 0.3 Na<sub>2</sub>HPO<sub>4</sub>, 10 HEPES, saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, 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 recorded for liquid junction potentials. The basic perfusion solution consisted of (mM): 133 NaCl, 5 KCl, 1.5 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 0.4 NaH<sub>2</sub>PO<sub>4</sub>, 0.3 Na<sub>2</sub>HPO<sub>4</sub>, 5.5 glucose and 5 mM HEPES pH 7.4, 37°C.

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A mouse model for the CF ΔF508 mutation


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