MOUSE MODELS OF CYSTIC FIBROSIS

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MUIS MODELLEN VOOR CYSTIC FIBROSIS

Proefschrift

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van rector magnificus
Prof. Dr P.W.C. Akkermans M.A.
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op
woensdag 13 november om 15.45 uur

door
Joanneke Henriëtte van Doorninck
geboren te Den Helder

Promotiecommissie

Promotoren:	Prof. Dr F.G. Grosveld Prof. Dr H. Galjaard
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The studies described in this thesis were performed in the Medical Genetics Centre South West Netherlands at the department of Cell Biology & Genetics at the Erasmus University Rotterdam. The project was financially supported by grants of HGO/TNO and the Association Française de Lutte contre Mucoviscidose.

Omslagontwerp: Mirko Kuit. Foto's van blz. 103 en 118.

Print: Offsetdrukkerij Ridderprint B.V., Ridderkerk

Whenever man comes up with a better mousetrap, nature immediately comes up with a better mouse.
(James Carswell)

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EXPERIMENTAL WORK

CHAPTER I
CYSTIC FIBROSIS

I. GENERAL INTRODUCTION

Cystic Fibrosis (CF) is the most common, lethal, inherited disease in the Caucasian population with an incidence of about 1 in 2500 newborns in Western-Europe and the United States. CF is a multi-organ disease caused by a single autosomal recessive gene defect. Most prominent disease symptoms are an obstruction of the intestine with intestinal content at birth (meconium ileus), pancreas insufficiency, malabsorption and chronic infections of the lungs. During the past decades improvement of therapeutic procedures has led to a prolongation of the life expectancy and the average age of a CF patient is now about 30 years, with male patients living 2-3 years longer then females (278).

Most of the affected organs in CF patients have an epithelial cell layer lining a lumen. Electrophysiological measurements have shown that chloride transport is abnormal in CF epithelial cells (36, 183, 186). The abnormal ion transport and associated iso-osmotic water transport offers an explanation for the sticky, dehydrated mucus found in most of the affected organs (81). A widely accepted diagnostic test for CF, the sweat test, is based on this aberrant ion transport as sweat glands of CF patients produce sweat with a much higher chloride and sodium content than normal (80, 107). The gene which is mutated in CF, the cystic fibrosis transmembrane conductance regulator (CFTR) gene was cloned in 1989, encodes for a membrane protein and proved to be a cAMP regulated chloride channel (5, 21, 139, 200). The most common mutation is a deletion of one amino acid, a phenylalanine at position 508 in the protein (ΔF508) (139). This mutation results in a block of post-translational processing and the protein can not reach its normal localization at the apical membrane of epithelial cells (52). However, cells can be treated such, that the processing block is alleviated and the ΔF508 CFTR reaches the plasma membrane (76, 210).

I.1 SCOPE OF THE THESIS

As no natural animal model of CF was available, the purpose of this study was to create and analyze a mouse model for CF containing the ΔF508 mutation found in the majority of CF patients. Gene targeting techniques have been utilized to completely disrupt the endogenous mouse *Cftr* gene generating so-called, knock-out mouse models (85, 119, 171, 189, 205, 225). An overview of techniques to mutate endogenous genes in mouse cells is given in chapter II and a comparison of the different mouse models presently available is presented in chapter III. Mice with a complete disruption of the *Cftr* gene have been generated by several groups. In view of the special characteristics of the ΔF508 protein (see Introduction I.10), a mouse with the ΔF508 mutation in the *Cftr* gene seemed a more appropriate animal model

for comparison with CF patients, and for the study of the molecular pathogenesis of the disease and the development of putative therapeutic strategies. This thesis describes the generation and characterization of a mouse model for CF with the ΔF508 mutation (Chapter IV-VI). Chapter VII and VIII describe the experiments done in our group with a mouse strain with a complete disruption of the *Cftr* gene.

I.2 CLINICAL SYMPTOMS IN PATIENTS WITH CYSTIC FIBROSIS

The clinical symptoms have been extensively reviewed by Welsh *et al.* 1995 (278) and will only be briefly described here.

Respiratory tract

CF patients do not show abnormalities in the respiratory tract at birth (231). The first changes involve the submucosal glands located in the upper regions of the lung. These glands consist of serous and mucous acini which produce a seromucous secretion transported through the duct to the lung epithelium. The serous acini and ductal lumina of submucosal glands in CF patients become dilated and filled with mucus (232). Later, mucous obstruction is found in the bronchioli associated with inflammatory symptoms (292). These features arise prior to colonization with pathogenic bacteria, pointing at inadequate clearance of mucus (141, 232). The mucus accumulation is not caused by defective ciliary function as this is normal in CF patients (137, 206). The sticky mucus could be caused either by increased mucous cell secretion or by decreased serous cell secretion in the submucosal glands. Another source of mucus in the lungs are the goblet cells although they secrete an estimated 40 times less mucus than the submucosal glands (194). During progression of the disease, however, goblet cells become more abundant and extend to the lower bronchioli, and may then augment mucus accumulation. Further, abnormalities in the mucus composition itself, as increased sulphation (38, 50), might augment its viscosity and result in sticky mucus and clogging of the small airways. Bacterial infection with pathogens as *Staphylococcus aureus* and especially *Pseudomonas aeruginosa* are typical of CF. The higher NaCl concentration in the surface fluid of CF airway epithelium impaires the killing of bacteria (223). Further, CF airway cells have more receptors for *P.aeruginosa* than normal cells and are less able to phagocytose the bacteria (128, 178, 289). Infection with bacteria provokes a rapid deterioration of the lung histology and results in bronchiolitis, bronchitis and hypertrophy of submucosal glands and of goblet cells. Several proteases released during inflammation are also able to stimulate mucus secretion by glands and goblet cells (143) and may enhance bacterial adherence (169). Ongoing obstruction and recurrent infection cause bronchiectasis and bronchiolectasis and finally stenosis, emphysema and fibrosis which eventually causes death of the patient (278).

Pancreas

The original description of the disease was "cystic fibrosis of the pancreas". Pancreatic ducts secrete a basic fluid that transports the digestive enzymes, produced by the pancreatic acini, to the intestine. In case of CF, already midtrimester fetuses, at a time when the pancreas has not yet started to produce enzymes, the pancreatic ducts may show inspissated secretions (41, 175). When pancreatic acini start to produce pancreatic enzymes, blockage of the secretory ducts prevents normal transport of enzymes that instead autolyse the acini. The destruction leads to cystic spaces in the acini and the pancreatic tissue is gradually replaced by fibrotic and fatty tissue. Later, also the islands of Langerhans can be affected leading to diabetes mellitus. Obstruction of the ducts results in an impaired flow of pancreatic enzyme into the digestive tract and hence to maldigestion and malabsorption of fat, proteins and fat-soluble vitamins. 85% of the CF patients need pancreatic enzyme supplements (278).

Intestinal tract

10-20% of the CF patients are born with an obstruction in the ileum with the fetal intestinal content (meconium ileus). Already prenatally, hyperplasia of goblet cells in the crypts of Lieberkuhn and dilatation of the Brunners glands in the duodenum have been observed (41, 175). CF patients without meconium ileus show mild abnormalities in the intestine consisting of hypertrophic goblet cells in the crypts of the ileum, appendix and colon accompanied by mucus accumulation (242). In the duodenum, the glands of Brunner are hypertrophic and show mucus accumulation in the ducts (174). A distal intestinal obstruction syndrome (DIOS), occurs in more than 20% of adult patients (82). Meconium ileus and DIOS are probably due to a decreased fluidity of the intestinal content although DIOS like symptoms is also related to high pancreatic enzyme replacement intake or digestion defects (224). A malabsorption of essential fatty acids can still be present independent of the pancreatic enzyme deficiency (228). Patients who are treated with pancreas enzyme supplements to restore intestinal digestion may also have fat loss in the stool, together with unabsorbed bile acids (274).

Liver and biliary system

The liver parenchyme in 25% of the CF patients shows focal biliary cirrhosis and periportal fibrosis (79). An excess of mucus in the bile ducts has been observed in infants under three months (172). The bile ducts show hyperproliferation, inflammation and intracanalicular obstruction by secretions (66). Gallbladder disease is present in more than 40% of the CF patients (152) and patients have a decreased bile acid and water output from the gallbladder (276). The gallbladder is occasionally small; thick mucus can be present (17) and stones consisting of calcium and protein can be found (10).

Genital tract

Male CF patients are infertile in 95% of the cases. The vas deferens, the epididymis and the seminal vesicles may be absent, atrophic or fibrotic and the genital tract can be obstructed with inspissated secretions (237). Males without clear CF symptoms but presenting congenital bilateral absence of the vas deferens (CBAVD), frequently carry CF mutations (11). Female CF patients have irregular or no menstruations probably due to malnutrition but many are fertile (150, 218). The cervical mucus is often thick and dehydrated with abnormal biochemical characteristics (149, 173) which might prevent sperm passage.

I.3 THE CFTR GENE

Cloning and structural analysis of the CFTR gene

The CF locus was mapped on chromosome 7, band q31.2 (259), close to the proto-oncogene MET and the D7S8 marker (271, 279). To clone the gene, additional DNA markers were isolated and positioned through somatic cell hybrid mapping, linkage analysis and long range restriction mapping with pulse field gel electrophoresis. Analysis of recombination events in patients and their families allowed accurate positioning of the CF gene in-between the known markers. In the region presumed to contain the gene, CpG islands were identified and the area was searched through chromosome walking and jumping. Genomic clones of this region were tested for cross-hybridization with other species and one DNA fragment proved to contain a 113 bp fragment, coding for exon 1 (202). Additional screening of cDNA libraries and genomic libraries eventually resulted in the isolation of the complete cDNA and a contig spanning the complete genomic area. The cloned gene was immediately recognized as the right candidate as it seemed to code for a transmembrane protein related to the multi drug resistance pump (MDR) and mutations were found in both alleles of CF patients (139, 200). The gene, named cystic fibrosis transmembrane conductance regulator (*CFTR*) consists of 27 exons spanning a region of 230 kb (200, 291). It codes for a mRNA of 6.5 kb, expressed in the epithelial tissues affected in CF, and is translated into a glycosylated protein of 1480 amino acids (200). This was the first time a gene for a genetic disorder was isolated on the basis of map location but without the availability of internal chromosomal rearrangements or deletions which facilitated the cloning of other human disease genes as in Duchenne muscular dystrophy (147), retinoblastoma (96) and chronic granulomatous disease (203).

The protein structure shows homology to the ATP-binding cassette (ABC) family of transporters of which p-glycoprotein or MDR, bovine adenyl cyclase, the yeast mating factor Ste-6 and bacterial amino acid transport proteins are members (125, 200). This family is characteristically involved in pumping substrates into or out of the cell using ATP hydrolysis as a means for active transport. Most members of this family have 12 transmembrane segments (TM) arranged in two membrane spanning domains (MSD) of six TMs each, and two nucleotide (ATP) binding

domains (NBD) (Figure 1). CFTR has an additional highly charged regulatory (R) domain, in between the two MSD and NBD domains, with more than 10 potential phosphorylation sites for protein kinase A and at least two for protein kinase C (PKA and PKC). The R domain in CFTR is not found in the other members of the ABC family nor has it homology to any other known protein. Although the CFTR protein shows a symmetrical structure and there is a high similarity between the two parts, there is no high identity (200) and is therefore not likely to be the result of a duplication of one of the MSD + NBD regions. Also exon duplication of regions coding for the MSDs or NBDs is unlikely, since exon-intron boundary positions are nor shared between the two halves (200).

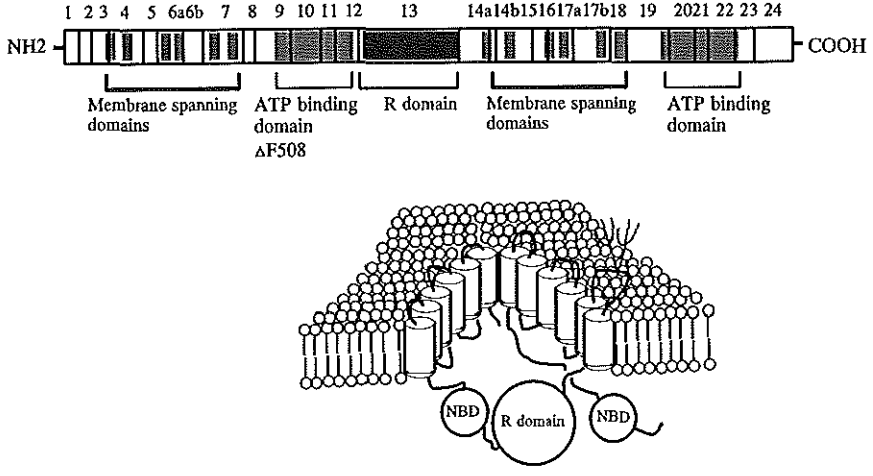

Figure 1. The CFTR gene and the CFTR protein. In the upper figure, the CFTR gene is drawn with its 27 exons. In the lower figure, a model for the insertion of the CFTR protein in the plasmamembrane is shown. The 12 hydrophobic transmembrane segments are shown as cylinders traversing the phospholipid membrane. The two nucleotide binding domains (NBD) and the regulatory domain (R domain) are located at the cytoplasmic site. On the 4th extracellular loop are two glycosylation sites.

Protein sequence conservation

Homologous CFTR genes have been cloned for a variety of species including rodents, cattle and amphibiae (83, 94, 138, 161, 236, 238, 260, 285). In general, the amino acid sequence is well conserved compared to human CFTR, ranging from 72% identity (85% similarity) in dogfish (161) to 91% identity (95% similarity) in sheep (238). In figure 2, the conservation of the different segments of CFTR are shown with

black lines for highly homologous regions as the NBD's and some of the transmembrane segments and grey lines for moderately conserved regions as the R domain and the middle two transmembrane segments of each domain (83, 238). These data suggest that the middle two segments in each domain do not contribute as much to the actual chloride pore of CFTR as the other transmembrane segments. High conservation is also found for the cytoplasmic loops in between the TM segments. Also in the MDR family the inner loops are strongly conserved and a mutation in this region affects substrate specificity (55, 207). The cytoplasmic loops could thus have an important role in CFTR functioning. The glycosylation site, between TM7 and TM8 is conserved in all species although the surrounding extracellular region is highly variable (260).

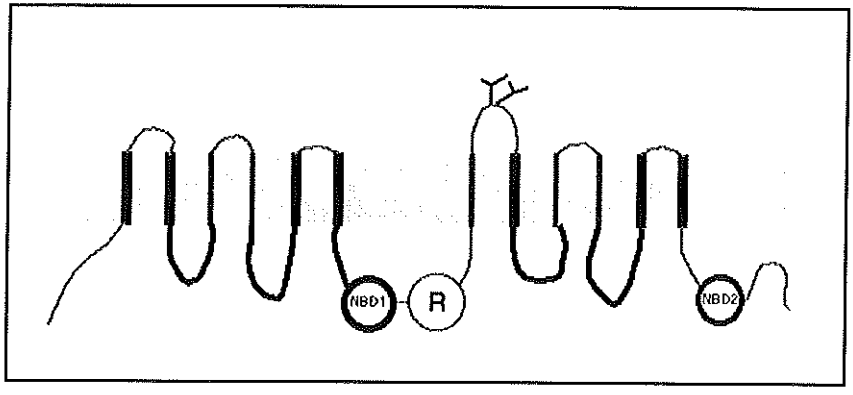

Figure 2. Conservation of CFTR in mouse, rabbit, guinea pig, monkey, sheep, cow, sharck and Xenopus. Different segments of CFTR are shown with black lines for highly homologous regions and grey lines for moderately conserved regions compared to the human CFTR protein (83, 94, 138, 161, 200, 238, 260, 285). Alignment of the sequences shows that the extracellular domains are not well conserved while the cytoplasmic loops are well conserved.

Transcriptional regulation

The specific expression of CFTR in epithelial cells is not distinctively reflected in transcriptional elements in the CFTR promoter. The upstream region does not contain a TATA box and has a high GC content, usually associated with housekeeping genes. Potential binding sites for transcription factors can be recognized as Sp1 binding sites, a cAMP responsive element, glucocorticoid elements, AP1 and AP2 sites and a CCAAT like element (56, 180, 287). The inverted and imperfect CCAAT box in the CFTR promoter was shown to be the mediator of basal and cAMP

induced expression on a heterologous gene. The CCAAT-enhancer binding protein δ (C/EBPδ), present in pancreatic extracts binds the CFTR CCAAT box in a nuclear protein complex together with activating transcription factor/cAMP responsive element binding protein (ATF/CREB) (180). There is a 28 bp stretch with high homology to a regulating element for the human α1(I) collagen gene and some small regions with homology to the promoter of the secretory leukoprotease inhibitor gene, which is epithelial cell specific (287).

Multiple transcriptional start sites are found, probably related to the lack of a TATA box, with variable use in different cell types (148, 287). A correlation was reported between the use of particular transcription initiation sites and the level of expression of the mRNA (148).

Methylation of CpG islands has only some correlation with transcription levels in cell lines but no correlation was found in mouse or human tissues (74, 148). DNAse hypersensitive sites have been located in the upstream region of the gene but they do not seem to be major transcription elements (148, 221, 286).

Cellular differentiation affects the CFTR mRNA levels as seen both temporally and spatially in the developing vas deferens (117, 247, 255) and during cycling of the rodent uterus (201, 256). Rat liver regeneration increases CFTR mRNA in a biphasic way (250), differentiation of intestinal cell line HT29 increases CFTR mRNA (165, 290) and in lymphocytes CFTR expression is cell cycle dependent (48). CFTR mRNA expression is decreased by PMA (252), calcium ionophores/divalent cations (15, 16), IFN-γ (30) and TNF-α (168) and is induced by oestrogen (201), cAMP (45, 180) and sodium-butyrate (51). PMA decreases the CFTR mRNA and protein levels in multiple ways: by transcriptional repression, by decreasing the mRNA stability and by destabilization of the CFTR protein via PKC (44). Divalent cations solely affect the transcription rate (15) while IFN-γ reduces CFTR mRNA half-life (30). cAMP has a dual role in that it not only increases the mRNA levels but also activates the CFTR protein (see section I.4). CFTR mRNA expression has been upregulated by mRNA stabilization without a detectable increase of chloride transport (226), thus expression is not always directly related to an increase in CFTR function on the plasma membrane.

These observations indicate that expression of CFTR involves a complex mechanism with several regulation steps at transcriptional, post-transcriptional and post-translational levels.

Alternative splicing

Alternative splicing of the human CFTR mRNA has been described for exons 4, 9 and 12 (43, 59). In healthy individuals, variations of 0-92% of exon 9^- CFTR are present as analyzed by nested PCR (58), while exon 4^- and exon 12^- CFTR mRNA's are varying from 2-30% (43). Since the exon 9^- variant is so dominant, it was expected to have some function. Transfection studies in HeLa cells indicated however,

that the mutant 9^- protein is processing defective and is unable to reach the plasma membrane (73, 230). This implies that there is no apical function for a 9^- CFTR. Other alternative splice products have also been found as CFTR exon 5^- in mouse and rabbit heart, an extra exon 11b in mouse testis and an extra 10b exon in human lymphocytes (73, 123, 281). The tissue restricted pattern together with the lack of function of the 5^- and 9^- variants (73) were reasons to postulate that the alternative splicing would be a means of gene expression regulation (290). However, also a genetic base has been found for the variable 9^- and 12^- expression. There is a correlation between the length of the polypyrimidine tract near the splice acceptor site and the amount of splice products from the splice acceptor site of exon 9 (57, 142). The shorter the T tract the more 9^- RNA is present. It seems that with a polypyrimidine tract of 5 T's (T5), 50% of the RNA's lack exon 9, with a tract of 7 T's 12.5% and with a tract of 9 T's, 5% of the RNA's lack exon 9. Thus, in a person with two T5 alleles, 90% of the RNA and CFTR protein lack exon 9. Therefore, an individual with one CF mutation and a T5 tract on the normal allele, has only 5% normal 9^+ CFTR protein. Such CF heterozygotes do not show disease symptoms (142). This means that this amount of CFTR is sufficient to keep tissues functional.

I.4. THE CFTR PROTEIN IS A CHLORIDE CHANNEL

Evidence that CFTR is directly involved in CF came from transfection experiments of the cDNA in CF airway and pancreatic cells showing correction of the defect in cAMP regulated chloride channel activity (87, 196). Further, transfection in various heterologous cell types, mammalian as well as non-mammalian, resulted in the presence of cAMP regulated chloride channels on the plasma membrane that were absent in untransfected cells (6, 20, 34, 61, 69, 136). Reconstitution in an artificial lipid bilayer of isolated membrane vesicles of high CFTR expressing cells or purified CFTR protein, showed that the isolated CFTR protein could form a channel with the same characteristics as in endogenously expressing cells (21, 246). This shows that CFTR does not require other proteins but forms the Cl^- channel by itself. CFTR forms a low conductance, 5-10 pS, chloride channel with a linear current voltage relationship. It is selective for anions over cations and has a halide permeability sequence of $Br^->Cl^->I^-$ (7), which is different from most other Cl^- channels which have $Br^->I^->Cl^-$ (97).

The 12 TM segments contain 6 basic residues, putatively involved in the pore formation. Mutation of 2 of those amino-acids into acidic residues, predicted to lie towards the outer half of the pore, altered the ion selectivity of the channel. Two other changes from basic to acidic residues, predicted to lie towards the inner half of the channel, did not change ion permeability (5). These data clearly show that the gene mutated in CF patients, encodes a CFTR protein which functions by itself as a chloride channel. Its absence in CF tissues explains the observed chloride transport.

Regulation of CFTR activity

CFTR mediated chloride current is stimulated by intracellular cAMP, but not by intracellular Ca^{2+} ions alone. In the basal state, CFTR does not pass substantial current as was shown in *in vitro* experiments but the protein can be activated by protein kinase A (PKA) and ATP (53, 109). To open the CFTR channel both phosphorylation of the R domain and binding and hydrolysis of ATP at the NBDs are necessary (4, 18, 26, 234).

Phosphorylation: CFTR activity is regulated by protein kinases and phosphatases. Phosphorylation is mediated by cAMP dependent PKA, and to a lesser extent by protein kinase C (PKC) regulated by Ca^{2+} in combination with diacylglycerol or independent of Ca^{2+} (27). On the basis of patch clamp analysis, PKC has been reported to augment the effect of subsequently added PKA (234), possibly by phoshorylating different sites. In intact cells, activation of PKC by phorbolester activates CFTR in the absence of changes in cAMP or Ca^{2+} concentrations (12, 13, 261, 264). cGMP dependent protein kinases phosphorylate CFTR and activate chloride transport in normal intestinal epithelial cells but not in CF cells (71). In lymphocytes, CFTR could be activated with nitric oxide through a cGMP dependent mechanism (84). Purified cGMP-dependent protein kinase type II, expressed at high levels in intestinal epithelium (160), activates CFTR when added to excised membrane patches. In contrast, the more common type I isoform failed to activate CFTR under similar conditions (95). Membrane bound phosphatases are able to inactivate the channel (23, 27, 234). The tyrosine kinase inhibitor, genistein, is able to activate CFTR (126, 127, 153, 213) and can fully activate CFTR following it's partial activation by PKA (French, personal communication).

Different levels of active kinases and phosphatases may result in different levels of CFTR activity under basal and stimulated conditions in specific tissues. Of the 10 putative phosphorylation sites, 8 serines, located inside the R domain can be phosphorylated by PKA (53, 177) although *in vivo*, 4 or 5 serines are phosphorylated (53, 177, 197). Mutation of some or even all 10 phosphorylation sites still did not completely abolish CFTR stimulation by PKA, although the open time of the channel (open probability, P_o) decreased dramatically (49, 53, 197). This indicates that other, unrecognized phosphorylation sites may be present. The function of the R domain can be understood structurally by imagining the R domain as a plug in the channel which upon phosphorylation bends away from the pore and opens the channel. This is suggested by experiments with a CFTR protein lacking part of the R domain, which is almost constitutively active without the need for phosphorylation (198). The complex phosphorylation pattern of CFTR might serve as a fine tuning mechanism to regulate the P_o and therefore the total chloride transport by CFTR according to the circumstances.

Regulation by ATP: *In vitro* binding studies suggest that both NBDs can bind ATP (118, 245). The two NBD's were identified based on their homology with

Walker motifs previously shown to be the binding sites for ATP in a number of ATP binding proteins. Removal of ATP abolishes channel activity but addition of hydrolysable ATP restores activity both in transfected cells and in primary epithelial cells (4, 8). Increasing amounts of ATP increase the P_o of the channel, with a simple saturation relationship until a maximum of P_o=0.44 is reached (268). Mutations created in NBD1 (exons 9-12) affect the channel activity more than analogous mutations made in NBD2 (exons 19-23) (110). A model has been proposed by Gunderson & Kopito (113), which postulates a NBD1 cycle and a NBD2 cycle of ATP binding and hydrolysis. After phosphorylation, ATP binding and hydrolysis at NBD1 would bring the channel in an active but still closed conformation. Binding of ATP to NBD2 results in the open state of the channel. Dissociation of ATP from NBD2 brings the channel back to the closed state or hydrolysis of the ATP at NBD2 brings the channel in a higher conductance open state, which lasts only for a short period.

Although hydrolysable ATP is necessary for activation and opening of the channel, hydrolysis by the NBDs has not been shown directly. Non-hydrolysable ATP analogs, in the presence of ATP, are able to increase the P_o of CFTR (112, 124) and increase apical Cl^- conductance in sweat duct cells and T84 cells (25, 187). The proposed model explains the prolonged open state that non-hydrolysable ATP analogs cause. Dissociation of the ADP or the P_i hydrolysis products from NBD2 results in closing of the channel while dissociation of ADP from NBD1 converses the channel to an inactive state (113).

From expression studies with glycosylation defective mutants it became apparent that glycosylation of CFTR is not a prerequisite for a proper function in transfected cells (110).

I.5 CFTR EXPRESSION

CFTR mRNA and protein show a very distinct localization in tissues which are affected in CF such as lung, pancreas and intestine (3, 90, 91, 129, 200, 208, 229, 251, 253, 290). A list of expressing organs is presented in Table 1. In all these tissues, expression of CFTR, either on mRNA level or on protein level is found in the epithelial cells. CFTR is also expressed in tissues in which no CF pathology has been found, e.g. in stomach, placenta and brain (choroid plexus) and was demonstrated in non-epithelial cells as cardiac myocytes and T-lymphocytes.

Expression in airway epithelium

The expression of CFTR mRNA in the lung is not detectable by Northern blot analysis (200) but quantitative PCR analysis shows that airway epithelial cells contain an average of 1-2 transcripts of CFTR mRNA per cell (251). CFTR protein can be detected in all regions of the airway (nasal epithelium, pharynges, trachea, bronchus, bronchioli and alveoli) (90, 91, 129, 182, 290). CFTR protein in the lung surface epithelium is found on the apical membrane of non-ciliated cells (90) and in ciliated

cells of nasal epithelium (182).

Table 1. Expression of CFTR mRNA in different tissues

Respiratory tract	(3, 90, 91, 129, 200, 208,
Pancreas	229, 251, 253, 290)
Intestine	
Gallbladder and biliary tract	(63, 229)
Sweat gland	(62)
Vas deferens	(117, 254)
Parotid gland	(200)
Salivary gland	(138, 247)
Endometrium	(225, 249, 256)
Placenta	(43, 93, 200)
Endodermal yolk sac	(102)
Stomach	(138, 225, 229)
Kidney	(43, 138)
Oesophagus	(225)
Brain	(138)
Non-epithelial cells:	
Ventricular myocytes	(154)
T-Lymphocytes	
Neutrophils	(43, 48, 286)
Alveolar macrophages	
Lung fibroblasts	

CFTR mRNA is expressed in epithelial cell layers of the organs named above, except when indicated.

The highest levels of expression is present in submucosal glands which are located in the bronchi (90) and in the trachea (129). In the ciliated and collecting ducts of bronchial submucosal glands, 1-2% of the columnar cells have a high CFTR expression. Virtually all of the cells in the serous tubules express CFTR while the mucous tubules are negative. The localization of CFTR is primarily on the apical side of the cells but also in the cytoplasm (90). Submucosal glands in the trachea show a different expression pattern in that CFTR is present both in serous and mucous cells

(129). Furthermore, the localization in serous cells is not at the apical membrane but rather associated with secretory granule membranes. In the mucous cells, CFTR was observed at the apical side and at the basolateral side (129). Sweat gland duct also shows both apical and basolateral staining (62, 135) in agreement with the Cl^- ion transport across both membranes (191). Whether mucous cells of the tracheal submucosal gland ducts also show Cl^- transport across both apical and basolateral membranes is not known.

In the fetal lung, expression of CFTR mRNA starts at 7 weeks and becomes higher than later in adult lung. At birth, CFTR mRNA is downregulated (102, 117, 163, 164, 247, 248, 255). Cytoplasmic CFTR is seen in the developing epithelium while submucosal glands are not yet present. In the trachea, a weak immunocytochemical staining is found in the cells, while at 26 weeks the signal becomes confined to individual cells in the trachea and is by then also seen on the apical membrane. The submucosal glands are present at this time and express CFTR (102).

CF patients are not known to have any developmental defect in their lungs (231). Therefore, the role of CFTR in lung embryonal development does not seem to be critical and other chloride channels may be more significant (111).

Expression in the gastrointestinal tract

In the fetal pancreas of 12-26 weeks, CFTR is found in immature cell types in ductal termini next to developing acini (247, 255). These duct termini develop eventually in the small intralobular ducts and centro-acinar cells, where CFTR expression on the apical side of the epithelial cells has been found in the adult stage (67, 159).

In bile ducts and in gallbladder, CFTR expression is found throughout the epithelium both in early fetal development when bile formation starts, and in adult life (63, 247).

The intestine shows the highest CFTR expression in jejunum, gradually decreasing to low CFTR expression in ileum and colon. CFTR is found in the bases of the crypts of Lieberkuhn with no distinction in mRNA localization between Paneth cells, goblet cells or undifferentiated cells (229). The villi have CFTR expression as shown in Western blot analysis (de Jonge, pers.comm) but immunocytochemically only about 3% of the cells on the villus are positive. These cells express CFTR in much higher levels of CFTR than is found in the crypts (3, 229, 253). These cells are found in the duodenum and jejunum, but not in distal ileum and in colon (229). High expressing cells are observed in human and rat intestine (3) but are absent in mice (Ameen, pers. comm.). In contrast to the surrounding epithelial cells, the high expressing cells do not contain absorptive enzymes and their function is not known (3). The intestinal expression during fetal development is similar to that in adulthood (247, 255).

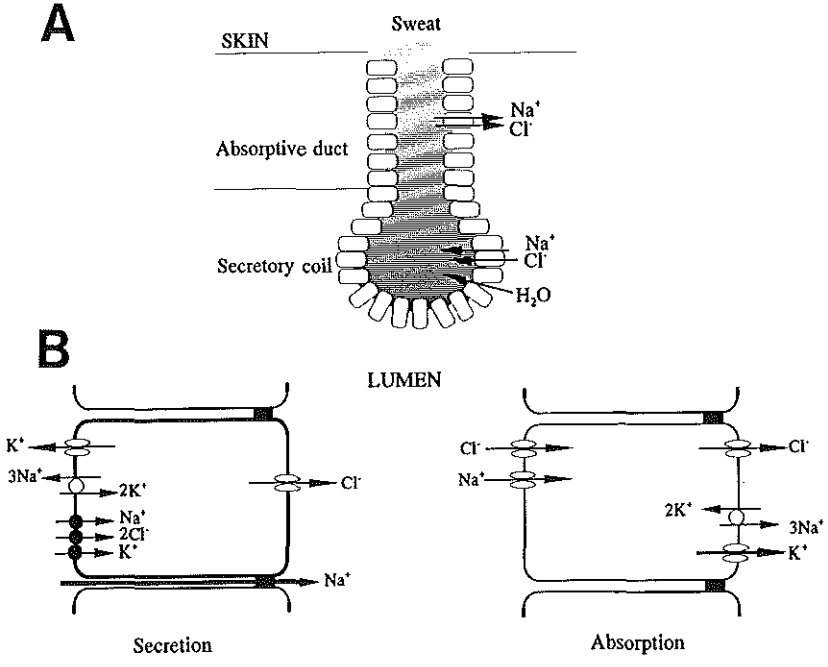

Figure 3. A. Schematic drawing of electrolyte transport in the sweat gland. The secretory coil secretes isotonic fluid and in the waterimpermeable absorptive duct Na^+ and Cl^- ions are absorbed. In CF patients, the cAMP regulated Cl^- channels in the secretory coil and the Cl^- channel in the duct are defective.
B. General mechanisms for salt secretion and absorption. The transporters involve apical Cl^- and Na^+ channels and a basolateral Na^+/K^+ ATPase, a $Na^+/K^+/Cl^-$ cotransporter and a K^+ channel. Na^+ in secreting cells passes paracellularly through the epithelial cells.

I.6 EPITHELIAL ION TRANSPORT AND CFTR

Epithelial cells are polarized, with an apical side and a basolateral side, and actively transport electrolytes and/or water. Epithelial tissues can have a secretory function, an absorptive function or both, depending on the state of activation. Salt secretion can be accomplished by activation of a chloride channel, localized on the apical membrane (Figure 3). Both cAMP activated CFTR and other types of regulated

chloride channels can be present. Positively charged Na^+ will follow paracellularly. At the basolateral side of the cell, a $Na^+/K^+/Cl^-$ cotransporter pumps Cl^- into the cell. Na^+ and K^+ leave the cell either through a basolateral Na^+/K^+ ATPase pump or through basolateral K^+ channels. Resorption of salt occurs through apical Na^+ channels, whereby chloride ions are transported through apical and basolateral chloride channels. The transport of salt is accompanied by an iso-osmotic water flux.

Sweat duct

The first ion transport abnormality found in CF patients was the increased NaCl concentration in the sweat. A sweat gland consists of two parts, a proximal secretory coil and a distal reabsorptive duct. The coil produces isotonic sweat by active Cl^- transport while Na^+ and water follow. The duct, which is water-impermeable, actively absorbs Na^+ while Cl^- ions follow passively. In CF sweat ducts, the transepithelial voltage is increased and has an abnormal response to chloride substitutions. This indicated that the basic defect in CF is an inability to transport chloride ions (36, 183, 186). The lack of Cl^- ion transport in the duct results in higher NaCl levels in sweat which is used as a diagnostic test for CF (107, 265). The sweat duct epithelial cells have CFTR expression both at the apical membrane and at the basolateral membrane (62, 135, 191). The basal state in the sweat duct has a high Cl^- conductance which might indicate a constant stimulation due to high levels of cAMP or to little dephosphorylation (191).

The secretory coil of the sweat gland also has CFTR activity. Normally, the coil increases sweat production in response to cholinergic (Ca^{2+} increase) and to ß-adrenergic (cAMP) stimulation. These pathways are located in two different cell types (190, 192). In CF patients, the coil does not produce sweat in response to ß-adrenergic stimulation and heterozygotes produce 50% of the normal amount (24, 209).

Airway epithelium

In CF airway epithelial cells and nasal epithelium, the increased transepithelial voltage is indicating an ion transport abnormality (144). The Ca^{2+} activated chloride transport is normal in tracheal epithelial cells and nasal polyp cell cultures of CF patients (269, 280). The cAMP dependent chloride transport is absent (8, 65, 269, 280). cAMP induced chloride transport accompanied by water transport, is mediated by CFTR as is supported by the fact that CF fetal lung explants are unable to respond to cAMP with fluid secretion (162). Also in the adult CF lung, a defect of fluid secretion by cultured airway epithelial cells in response to cAMP stimulation has been observed (132, 222). Although this defective water transport would explain insufficient clearance of dehydrated mucus it is not known how defective CFTR activation leads to the ultimate lung pathology.

Pancreas

In normal pancreatic acini, Cl^- is secreted into the lumen and in the ducts the Cl^- is absorbed and exchanged for HCO_3^- ions. Water secretion follows the transported ions. The exchanged HCO_3^- is necessary to increase the pH in the duodenum and the ducts add fluid to the enzymes produced by the acinar cells. Defective CFTR mediated Cl^- transport in the pancreatic ducts (108, 159) leading to defective water transport, explains the obstruction of the ducts by dehydrated mucus. Pancreatic enzymes, produced by the acinar cells are normally released via the ducts, but now, instead, cause endogenous digestion resulting in pancreas fibrosis.

Intestine

The CF intestine shows an increased transepithelial voltage attributable to defective chloride permeability. In addition, stimulation with a number of cAMP, Ca^{2+}, or cGMP increasing agents, as forskolin, theophylline, Ca-ionophore, cholera toxin, prostaglandin E_2 and heat stable *E.coli* toxin STa, do not result in increased chloride permeability in CF intestine (19, 29, 37, 71, 72). In contrast to other tissues, chloride transport in the intestine is generally not regulated by Ca^{2+} regulated Cl^- channels (8, 12, 261). The response to Ca^{2+} ionophores or carbachol may be mediated by PKC phosphorylation of CFTR (12, 13, 27, 234, 262, 264) and by the stimulation of basolateral K^+ channels, resulting in an increased driving force for chloride secretion at the apical membrane (78, 235). Although a decreased chloride and water transport in the distal intestine and colon may explain the occurrence of meconium ileus and DIOS, also other functions are defective in the intestine. CF patients often have steatorrhoea resulting from defective fat absorption. Also, bile acids which are normally absorbed in the duodenum/jejunum are excreted with the faeces in CF patients. Malabsorption of fatty acids can result from the reduction in bile acid concentration as bile acids form micelles with fatty acids and monoglycerides enabling their absorption by the enterocytes. The relationship between malabsorption and CFTR function is not yet well defined. CFTR is found in crypts and in villus enterocytes and in cells with high CFTR expression scattered in the villi of duodenum and jejunum. Those cells do not express intestinal enzymes as sucrase and lactase like the majority of the enterocytes (3). Rat intestinal villi show cAMP dependent chloride transport (146, 227). Moreover, X-ray microanalysis of ion contents in human jejunum cells showed a significant difference in intracellular Na^+, K^+ and Cl^- content between normal and CF villus cells (170). A decreased fluid transport in CF patients dehydrates mucus and could influence the microenvironment in the proximal intestine. A change in the unstirred layer lining the epithelial cells could disturb the proper formation of micelles or the endocytosis of the micelle. The relative contribution of crypt and villus cells and the high CFTR expressing cells to electrolyte and fluid secretion still remains to be elucidated.

The above mentioned irresponsiveness of the CF intestine to cholera toxin

might be an advantage in case of cholera infection. Heterozygotes are expected to secrete less fluid during a secretory diarrhoea which would increase their chance of survival (35, 184, 185). The knock-out mouse model (described in Chapter III) was used to test this hypothesis and the results show that cholera toxin induced water secretion in heterozygous mice, with one intact *Cftr* allele, was about half the amount of secretion of normal mice (100). A heterozygote advantage in the case of excessive diarrhoea, might explain the incidence of CF carriers which is unusually high for a lethal autosomal recessive disease.

I.7 MUTATIONS

Since the discovery of the CFTR gene, more than 400 different mutations have been found in CF patients (Cystic Fibrosis Genetic Analysis Consortium). The mutations vary from missense mutations creating amino acid substitutions (55%), deletions of one or a few amino acids, to point mutations resulting in nonsense mutations, frame shifts and splice mutations (258). Surprisingly for this large gene, only one big deletion has been observed. The majority of the mutations lead to the production of a mutant form of CFTR protein, which can have partial activity. The most common mutation in the Caucasian population is the ΔF508 mutation with an allele frequency of 70% in the white Northern American population (139). In Europe, the ΔF508 mutation in CF alleles shows a North-South gradient with 87% in the Danish population to 50% in southern Europe (92). The incidence of CF in Caucasians is much higher than in any other population due to the high ΔF508 mutation frequency. For example, while in the Netherlands the CF incidence is 1/3600, in the Hawaiian population it is 1/90.000 (241, 282). The ΔF508 mutation has a single origin and is thought to have arisen at least 52.000 years ago, spreading in the Neolithic age (166). While the high frequency of the ΔF508 mutation might be the result of a selective advantage for heterozygotes in case of secretory diarrhoea, other high mutation frequencies such as the W1282X mutation that occurs in 60% of the Ashkenazic Jewish CF chromosomes (217), could also result from genetic drift or founder effects.

The different mutations found in CF chromosomes have been classified in 4 groups (Figure 4, 277). Mutations that lead to truncated CFTR such as stop codon mutations, frame shifts and splice site abnormalities, have been named type I mutations. These are expected to be null mutants although some truncations can still produce chloride channels in transfection assays (215). One putative mutation in the promoter region of *CFTR* has been described (32). Alternatively spliced variants are excluded from type I mutations. Type II mutations consist of mutant CFTR proteins that are not properly processed nor targeted to their functional cellular location but can be functional. The best example of this type is the ΔF508 mutation. The processing defect of the ΔF508 CFTR will be discussed in more detail below (section I.10). CFTR proteins which are properly processed but can not be activated, are categorized as type III mutants. Most of the these mutations affect the ATP binding regions and

lead to defective binding or hydrolysis of ATP. In exon 13 which encodes at least part of the phosphorylated R domain, some mutations have been found (258). These mutations are not likely to cause defective phosphorylation. The cause of defective CFTR function in exon 13 mutants is not known. CFTR molecules which can be activated by ATP and phosphorylation but do not generate the normal Cl^- current are considered type IV. Three mutations in the MSD1 have been assayed *in vitro* and cause low conductance and/or a reduced open probability (e.g. R117H)(216). The type III and type IV mutation classification is in some cases arbitrarily.

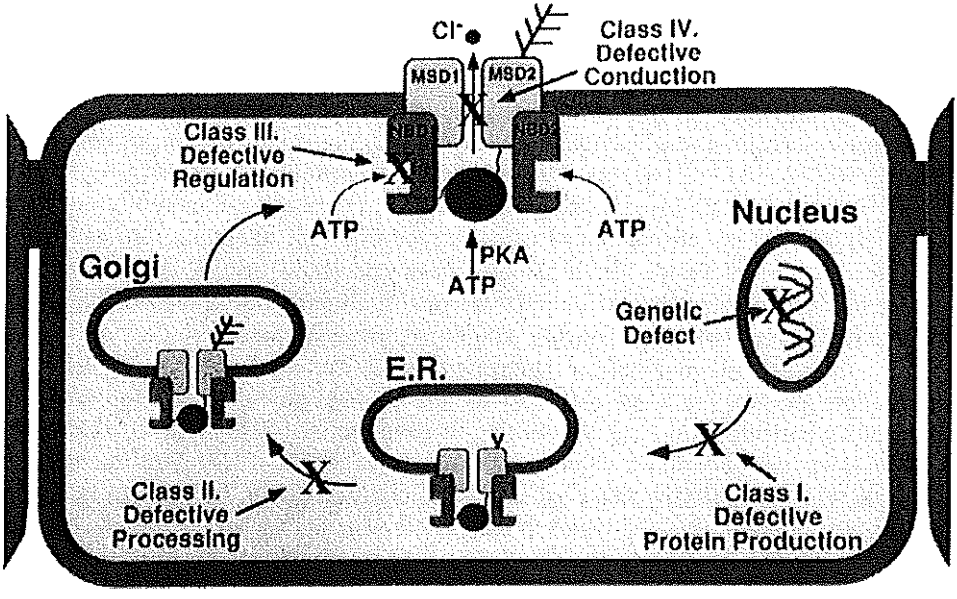

Figure 4. Four classes of CFTR mutations. Representation of the biosynthetic route of CFTR and the effects of CFTR mutations. Class I mutations affect the transcription and translation of CFTR. CFTR with class II mutation type is inserted in the endoplasmic reticulum membrane but fails to fold correctly and is degraded. Class III and IV mutated CFTR molecules are processed through the Golgi apparatus but have defective functioning on the plasmamembrane with none or partial functioning. MSD=membrane spanning domain, NBD=nucleotide binding domain, R=regulatory domain (Figure from 277).

I.8 GENOTYPE-PHENOTYPE CORRELATION

The clinical phenotype of CF patients is highly heterogeneous and not solely linked to the type of the CFTR mutation. Other genetic factors, the nutritional state, the pathogenic environment and the therapeutic regime, like aggressive antibiotic treatment and physiotherapy, are of major importance to delay the morbidity and mortality of CF patients.

A good correlation between specific CF mutations and being pancreatic insufficient (PI) has been found. These mutations are called 'severe' mutations. The severe mutations are stopcodons, splice junctions, frameshift mutations, the two single amino-acid deletions ΔI507 and ΔF508 and missense mutations affecting the NBDs. In a population with a 70% incidence of the ΔF508 mutation, 49% of the patients is homozygous for ΔF508 and 42% of the patients carries one ΔF508 allele together with another mutated allele. The pancreas sufficient (PS) have one or two 'mild' mutations. These are mostly amino acid substitutions affecting the MSD and belonging to category III or IV mutants (151). Also splice junction mutations which affect regulatory elements for the splice junction, result in a milder phenotype as they presumably permit a small amount of normal splicing. Only two mutations in the NBD1, A455E and P574H, give a mild pancreatic phenotype (151, 267).

The lung pathology, which causes the main morbidity and mortality problems in CF patients, is not clearly linked with the type of CFTR mutation. Generally, it is accepted that homozygous ΔF508 patients have pancreatic insufficiency, poor lung function, chronic pseudomonas colonization and high mortality (140) and, overall, a more severe phenotype than patients with other missense mutations.

Patients with homozygous stopcodons generally have a very severe phenotype (68), although there are a few patients with a reasonable physical condition. Some of the G542X patients show residual chloride transport activity (267). The G542X CFTR protein does not show apical chloride transport activity (266). Therefore, the residual chloride channel activity observed in these patients might be derived from an alternative chloride channel which is expressed at a higher level in these patients (266).

Recently, CFTR mutations, predominantly the R117H mutation, have been found in 60-65% of healthy, infertile men (11). The R117H mutation apparently does cause infertility with the same genital tract abnormalities as CF patients, but it does not lead to obvious other CF symptoms. Upon closer examination of these R117H males, some of the CF parameters as abnormal sweat chloride levels, abnormal nasal potential difference, chronic sinusitis and subclinical lung disease (31) were found but in a very mild form. The R117H mutation in combination with another CF mutation can lead to CF disease symptoms when an inefficient splice site is on the same allele. The linkage of clinical phenotype to alternative splicing is not an absolute feature since a ΔF508/R117H-T7 combination may or may not lead to pancreas deficiency indicating other variables in the development of clinical features (142).

Genotype-phenotype correlations can be studied best in a homogeneous

genetic and environmental background. Therefore, sibling and twin studies are being organized and also different CF mouse models may provide valuable information.

There is considerable variability in lung disease development, both in time and severity, among patients with the same mutations which is likely to be influenced by other factors such as genetic background, treatment, nutrition and environment. Alternative chloride transport have been found to alleviate CF symptoms in some patients, (267) and in mice (60, 205). Therefore, not only the introduction of a normal CFTR gene in CF patients by gene therapy is a possible solution to cure patients (39), but therapy could also be directed towards increasing expression or functioning of alternative chloride channels.

I.9 RELATIONSHIP OF CFTR WITH OTHER PROTEINS

From the complex electrophysiological parameters in epithelial tissues it became clear that CFTR is CFTR is not only functioning as a single chloride channel but that it is linked to other proteins in the cell. Epithelial sodium channels, Ca^{2+} regulated chloride channels, the outward rectifying chloride channel (ORCC) and the P-glycoprotein/MDR protein seem to be connected in some way with CFTR generated chloride current. CFTR has also been related to general cell biological processes as endo- and exo-cytosis and in the acidification of cytoplasmic vesicles.

Epithelial Na^+ channels have a prolonged open state in CF epithelial cells (54), accounting for the suggested increased rate of Na^+ absorption observed in human airway epithelium (40, 145). Expression of CFTR in CF cells reduces the Na^+ transport, even in the absence of chloride ions (133, 233), suggesting a direct inhibiting effect of activated CFTR on the sodium channels. However, these results can also be explained by an increase of the Na^+ driving force due to the lack of chloride channels (33).

Ca^{2+} regulated chloride transport is upregulated in CF cell lines, and transfection of CFTR reduces the Ca^{2+} mediated chloride current (133). CF knock-out mice (chapter III), do not show pancreas and lung defects as in human CF patients, which can be explained by the observed higher Ca^{2+} mediated chloride current in the mouse pancreas and lung compared to humans (60). Other chloride channels may therefore serve as alternative pathways for chloride transport and their activation and upregulation may fully or partially revert disease symptoms in case of a CFTR defect.

The ORCC was originally claimed to be the chloride channel defective in CF (156, 211). Only after the cloning of the *CFTR* gene it became clear that the ORCC was not identical to the CFTR gene product (136, 234). However, there is a connection of CFTR with the ORCC. Proof that the ORCC and CFTR are distinct channels but interacting came from transfection experiments and studies in the knock-out mice in which it was shown that ORCC is not regulated by PKA and ATP if CFTR is absent (89, 101). It was also shown that a mutated CFTR protein, A455E,

can regulate the ORCC once they are expressed on the plasma membrane, but others, as G551D, can not (99). However, the G551D mutant, which can be phosphorylated, does activate the ORCC in the presence of extracellular ATP (134). Apparently, the phosphorylated G551D CFTR mutant is able to generate a yet unknown signal which activates the ORCC. It has been suggested that ATP acts via a purinergic receptor which in turn activates the ORCC (212) and CFTR was proposed not only to be a chloride channel but also to be able to transport ATP molecules from the inside to the outside of the cell (195). The latter hypothesis has been overturned by careful experiments in 4 different systems expressing CFTR which show that ATP can not be transported by CFTR (193). The interaction of CFTR with the ORCC would greatly augment the chloride transport as the ORCC has a large conductance of 30-70 pS and CFTR is a 5-10 pS channel.

The P-glycoprotein/ MDR protein, which shares homology with CFTR (125) can regulate chloride conductance, either directly or indirectly (116, 263). Further, it has been reported that high expression of CFTR can confer a multidrug resistant phenotype (275). MDR localization seems to be inversely related to CFTR localization in differentiating intestinal cells and in the cycling uterus (257) but the complementarity of the function functionality of the two channels is not known.

CFTR is involved in pH regulation of cytoplasmic vesicles (14). Vesicles of the trans Golgi network, prelysosomes and endosomes, but not lysosomes were less acidic in CF airway epithelial cells. The enzymes involved in sulphation and sialylation in Golgi vesicles are pH dependent. A more alkaline pH could explain the increased sulphation and decreased sialylation found for CF mucins (1) and the higher expression of the asialo receptor for *P. Aeruginosa* (128). Further, CFTR is involved in endo- and exocytosis (42, 157, 181). The membrane recycling requires an activated and functional CFTR. Cells from CF patients are unable to exocytose in response to cAMP and have increased endocytosis (42, 181).

The loss of Cl^- conductance due to the absence of CFTR does not seem to give sufficient explanation for the complex CF phenotype; however, the possible connection of CFTR with other proteins may clarify some of the varied features in CF patients.

I.10 CFTR PROTEIN PROCESSING

ΔF508 CFTR is a processing mutant

In most Caucasian CF populations, the ΔF508 mutation is the most common mutation with an allele frequency of 70%. This means that 90% of the CF patients carry at least one ΔF508 allele (214). The deletion of the phenylalanine at position 508 of the protein does not affect the mRNA stability (251) and the RNA is translated into a ΔF508 CFTR protein.

Immunolabeling of CFTR in transfected cells showed that ΔF508 CFTR is not found on the apical membrane, in contrast to wild-type CFTR (76), but instead has a

perinuclear localisation and was therefore thought to be present in the ER (52, 283). Confirmation of the aberrant localization of the ΔF508 protein was found in primary airway epithelial cells, airway submucosal glands and sweat duct glands from ΔF508 patients (77, 90, 135).

Normal CFTR processing involves transport from the cytoplasm of the nascent polypeptide to the ER and acquiring a proper conformational structure in the ER. In the ER the protein also gets its core-glycosylation, whereas complete glycosylation occurs after transport from the ER to the Golgi compartment. Glycosylation can thus serve as a marker for the progress of CFTR through the biosynthetic pathway. Western blot analysis of CFTR transfected cells showed different sizes of protein for wild type CFTR and ΔF508 CFTR. Wild type CFTR has a molecular weight of around 170 kD (migrating as band C), and ΔF508 CFTR about 140 kD (band B) (52). Since a CFTR mutant completely lacking glycosylation, runs at a molecular weight of 135 kD (band A), it was concluded that the two forms reflect different stages of glycosylation. The mature CFTR is fully glycosylated whereas the 140 kD mutant protein was thought to be partially glycosylated. Further evidence for the nature of the glycosylation came from experiments where the CFTR protein was treated with glycosidases cleaving specific sugar groups. Normally processed CFTR is sensitive to N-Glycanase treatment, indicating Golgi glycosylation, and is reduced to band A, non-glycosylated CFTR. ΔF508 CFTR migrating as band B, but not mature wild type CFTR, can be reduced by endoglycosidase H, which cleaves N-linked high mannose oligosaccharides present on proteins which have not undergone mannose trimming in the medial cisternae of the Golgi apparatus (52, 179, 272). The observed incomplete glycosylation of ΔF508 CFTR is not the molecular cause of disease. A CFTR mutant that can not be glycosylated, is expressed on the plasma membrane and has normal chloride channel activity (109). These experiments indicate that ΔF508 CFTR is not transported through the Golgi apparatus and it was concluded that the cause of the disease is the inability of ΔF508 CFTR to be processed correctly and reach the plasma membrane (52). For a number of other CF mutations as ΔI507 and G480C, a similar trafficking defect has been observed (52, 110, 220).

Chaperones guide protein processing

Some proteins contain a primary structure which makes them fold spontaneously in their native structure independent of other factors (9). However, most proteins need some guidance in their processing to a mature, correctly folded, protein. During synthesis of a protein the polypeptide emerges from the ribosome as an unfolded amino acid chain, which can not be stabilized until a complete domain has been synthesized. Hydrophobic domains present in those proteins are thus exposed to the aqueous solvent during these processes which can lead to irreversible aggregation of the protein (130). To prevent incorrect inter- or intra-molecular association of unfolded polypeptide chains, proteins which do not fold spontaneously need chaperone

proteins (105, 121). The nascent polypeptide goes through cycles of binding, release and rebinding with chaperones until after being released it completes its folding by forming intramolecular interactions, assembles with other proteins or is transported to another cell compartment. If one of these processes does not happen, the protein will be bound again by chaperones. The assistance in proper folding and assembly of a protein requires ATP hydrolysis. Once the native state of a stable tertiary structure of the protein has been acquired the chaperones are permanently released. Membrane and secreted glyco-proteins follow a transport route from the ER through the Golgi apparatus to reach their proper folding, glycosylation and final destination in the cell.

Mutations in the protein or stress conditions in the cell increase the probability of misfolding of proteins. Hence, the time of interaction with the chaperones is also increased. The expression of chaperones is induced both as a direct response to stress conditions as heatshock and decreased Ca^{+} concentration and by a feedback mechanism to the presence of misfolded proteins (103). Higher concentration of chaperones may delay the formation of misfolded proteins or their aggregation.

The Hsp70 family of chaperone proteins, including the cytosolic hsp72/73 proteins, the resident ER protein BiP and the constitutively expressed hsc70, stabilizes newly synthesized proteins and promote protein translocation across membranes (105). BiP is involved in folding of proteins in the ER and a mammalian homologue of Hsp60 (TriC), is involved in folding of actin and tubulin in the cytosol (98, 121). Calnexin is a new chaperone identified in the mammalian ER membrane where it binds to glycoproteins (120, 176).

Protein processing of normal and ΔF508 CFTR

Synthesis of the complete CFTR chain by the ribosome takes 9.2 min and the nascent chain thus has to be shielded by chaperones during this time to prevent premature folding (272). Nascent chains of CFTR with one synthesized transmembrane region are already associated with two translocation-associated proteins forming part of the translocon of the ER membrane, Sec61P and SSrα (47, 167, 219). Integration into the ER membrane does not occur until four transmembrane segments have been synthesized (219). In yeast, homologs of Hsp70 proteins assist in the translocation of polypeptides into the ER (188) and have functions, separate from, and not replaceable by Bip (46).

Association of immature wild-type CFTR and ΔF508 CFTR has been found with the cytosolic Hsp/Hsc70 proteins (284) and with the ER membrane protein calnexin (179). The folding of CFTR thus seems to be guided by at least those two chaperones. No association was found with the luminal ER chaperones BiP or Grp94 (glucose response protein) (179, 284). Association of Hsp/Hsc70 with a plasma membrane protein has not been reported previously, but calnexin binds specifically to glycoproteins, assisting in folding and retaining of transport-incompetent misfolded proteins (28, 114, 115, 176). Extrapolating from the known functions of Hsp70 and calnexin, it is probable that Hsp/Hsc70 proteins protect newly synthesised CFTR from

premature folding and aggregation and perhaps assist the protein towards a folding machinery (22, 98) while calnexin would assist the proper conformation of CFTR in the ER membrane (120).

Immunoprecipitation experiments showed that complexes of Hsp70 proteins and calnexin with ΔF508 CFTR are more stable than complexes with normal CFTR (179, 284). This prolonged binding of ΔF508 with Hsp/Hsc70 and calnexin chaperones might reflect the repeated cycles of binding, release and rebinding by the chaperones to assist the protein in finding its proper conformation. Both chaperones remain bound to the immature CFTR, until it reaches its complete tertiary structure. The F508 amino acid is positioned in a region which has a ß sheet structure. A synthetic peptide containing the ΔF508 mutation, has a reduced stability of the ß sheet structure (244) and this might prevent folding of the ΔF508 molecule (243). Hsp70 would be predicted to bind the cytosolic domain of CFTR including the mutant ß sheet in the case of ΔF508 CFTR. Calnexin has been shown to associate with proteins through glycosylated residues (120) and therefore binds CFTR from the ER side of the membrane. For CFTR processing, it is not easy to appreciate the role of calnexin: the glycosylation mutant of CFTR is processed correctly as it is able to reach the plasma membrane and functions as a normal CFTR chloride channel (110) but neither the glycosylation mutant nor wild type CFTR treated with deglycosylation agents bind calnexin (199).

The processing kinetics of CFTR and ΔF508 CFTR have been studied in a variety of cell lines. Pulse-chase experiments show that only 25% of the initially synthesized normal CFTR molecules becomes fully mature with a T1/2 of around 40-45 min (158, 179, 272). This is a surprisingly small amount compared to other proteins were 95% of newly synthesized polypeptides fold into the mature form (64, 104). This implies that normal CFTR is inefficiently processed and intrinsically unstable. Newly synthesized CFTR molecules in the ER are protease sensitive, but in the presence of ATP, 25% acquires protease resistance and can be transported to the Golgi. This indicates that the ATP dependent process making CFTR protease resistant is the step towards the final properly folded CFTR molecule, able to be transported to the Golgi apparatus (158). The folding assistance of several chaperones is known to require ATP hydrolysis and thus the necessity of ATP for CFTR to become protease resistant, might reflect the association with Hsp70 as it has an ATPase domain (105, 121). For calnexin it has also been shown that ATP is required *in vivo* for the association with substrates (270).

The remaining 75% of the ER localized CFTR form, core glycosylated band B, is degraded with a similar T1/2 of 30 min for the wild-type CFTR and ΔF508 CFTR. No difference was observed between the degradation patterns of normal and ΔF508 CFTR indicating that the degradation pathways are similar (158, 272).

In order to study the mechanism by which CFTR is degraded, different pharmacological agents were tested which affect lysosomal degradation and

proteasome degradation. Inhibitors of lysosomal proteolytic enzymes did not affect the degradation rate of ΔF508 CFTR. A block on the transport from the ER to other compartments by treatment with Brefeldin A also did not affect the degradation rate of ΔF508 CFTR. This indicates that ΔF508 CFTR is degraded in a non-lysosomal, pre-Golgi compartment (158, 272). Inhibitors of the 20S subunit of the cytosolic 26S proteasome complex did delay degradation resulting in an accumulation of the immature, core-glycosylated, CFTR (131, 273). Some inhibitors still allowed maturation of CFTR while others completely inhibited this process. This indicates that more than one degradation system is involved (131). In no case an increase in the amount of mature ΔF508 CFTR protein was observed. The accumulated material is poly-ubiquitinated and by using a dominant-negative mutant of ubiquitin Ward *et al.* show that ubiquitination is a prerequisite for CFTR degradation (273).

Rescue mutations

The ΔF508 processing defect can be 'rescued' by an other mutation in the CFTR molecule. Experiments with the homologous protein Ste-6, a yeast mating factor, show that when the ΔF508 was introduced at the homologous site in the Ste-6 protein the yeast cells were deficient in mating. Revertants of this phenotype contained new mutations as R553Q, R553M or R555K which restored the mating ability of the ΔF508 Ste-6 protein (239, 240). One of these reversion mutations has also been found in a patient with a ΔF508 and a R553Q mutation on one allele, who had a mild phenotype (86). The 'rescue' probably involves a release of the processing defect. This suggest that with an extra R553Q/M or R555K mutation the aberrant folding of the ΔF508 CFTR is adjusted. Other systems in which CF mutations were tested were in the bacterial LIV-I system which transports amino acids and in the human P-glycoprotein (MDR) (106, 122). CF mutations were made in homologous sites in these proteins and resulted in similar defects as in mutated CFTR such as aberrant processing or aberrant function. These studies did not involve the screening for revertants, as in Ste-6 mutants.

Channel characteristics of ΔF508 CFTR

Mammalian cells transfected with ΔF508 cDNA and kept under normal culture conditions do not express ΔF508 CFTR on the plasma membrane. However, ΔF508 CFTR is present on the plasma membrane in insect Sf9 cells infected with recombinant baculovirus (155), in injected Xenopus oocytes (88), recombinant vacciniavirus mediated expression in Vero cells (69) and cDNA transfected 3T3 cells grown at low temperature (76). In all these systems it was shown that ΔF508 protein had a cAMP mediated chloride channel activity. In the transfected Sf9 cells and in planar lipid bilayers (21, 155), all of the channel characteristics, as conductance, anion selectivity, open time kinetics as well as the open probability were the same for the normal and mutant protein. In the other systems, 3T3 cells, Vero cells and Xenopus Oocytes, the ΔF508 CFTR showed a threefold reduced open probability. The reduced

open probability was due to longer periods of closing of ΔF508 CFTR while the duration of open times were comparable. This difference could be caused by the lower amount of PKA used in the latter systems. The longer closing times might suggest a difficulty in opening of the channel which would implicate the F508 amino acid being involved in ATP binding and hydrolysis or in phosphorylation of ΔF508 CFTR. However, structural analysis of the F508 amino acid does not indicate a function in ATP binding or hydrolysis (125), as this is thought to be directed by the Walker A and B motifs. Also, a synthetic peptide of the Walker A region around the F508 shows the same affinity for binding ATP with and without the ΔF508 mutation (244). The ΔF508 mutation is not located near known phosphorylation sites and although it is not known to what extent the ΔF508 causes steric hindrance in the mature protein, the observed lower open probability of the ΔF508 CFTR in some systems is not explained. Several factors that activate CFTR and most importantly, ΔF508 CFTR are being tested. Combinations of non-hydrolysable ATP, forskolin and phosphatase inhibitors activate ΔF508 CFTR (23, 124). The most promising substance at the moment, is genistein, a protein tyrosine kinase inhibitor. A combination of forskolin and genistein increased the chloride transport to one third of the normal level in a heterologous expression system (70). This treatment does not lead to detectable ΔF508 CFTR on the apical - membrane but is apparently able to excessively stimulate the few ΔF508 CFTR molecules that escaped the quality control mechanism. The observation that ΔF508 CFTR can function as a near normal chloride channel is very important for future therapeutic strategies on therapy for ΔF508 carrying CF patients. All these patients carry a protein which is potentially functional although it is not expressed on the apical membrane of the cell.

Systems expressing active ΔF508 CFTR

Xenopus oocytes and insect Sf9 cells expressing ΔF508 cDNA showed ΔF508 CFTR expression on the plasma membrane (88, 155). Since these cells are cultured at lower temperatures than 37°C the impression arose that it was not the specific cell type which allowed ΔF508 to be processed, but instead, the lower temperature. Consequently, fibroblasts were transfected with ΔF508 cDNA and cultured on a temperature of 23-30°C. This lead to a partial shift of band B CFTR to band C CFTR indicating formation of mature fully glycosylated ΔF508 CFTR (75, 283). At a lower temperature, the ΔF508 CFTR is apparently able to fold somewhat more productive than at 37 °C. Another system in which ΔF508 CFTR was found on the plasma membrane was in overexpression experiments with transfected cells (51, 69) and ΔF508 human airway epithelial and pancreas cell lines treated with the CFTR mRNA inducing agent sodium-butyrate (51). Overloading the cells with ΔF508 CFTR apparently pushes the system, so that part of the mutant protein slips through and emerges on the plasma membrane. However, primary ΔF508 airway epithelial cells and the colonic cell line HT29 did not respond to sodium-butyrate with a cAMP

stimulated chloride efflux (45, 51). Whether this reflects solely the limitations of the primary cultured cells and tissue specificity, and whether sodium-butyrate can still be used *in vivo* to restore CFTR function in ΔF508 patients is not yet known.

Recently, it was found that glycerol can effectively promote processing of ΔF508 CFTR in transiently transfected and stable cell lines into mature and active CFTR Cl^- channels (210). Of normal CFTR, 25% reaches maturity (158, 179, 272) and glycerol treatment of ΔF508 cells resulted in 3-8% mature ΔF508 CFTR. Glycerol retarded the degradation process both of normal CFTR and of ΔF508 CFTR, but from the experiments described above, it is known that preventing degradation does not lead to correction of the processing pathway. Therefore, it is hypothesized that glycerol stabilizes an intermediate folding form of ΔF508 CFTR. Wild type CFTR maturation was not increased by glycerol treatment. The effects of temperature and glycerol are similar and may be additive. Other processing mutants which are temperature sensitive, as K464A and K464R, are also processed when the cells are treated with glycerol treatment while a temperature insensitive K464W mutant was not influenced by glycerol. This observation, including the inability of glycerol to promote maturation of three other mutants, indicates that glycerol does not alter the general processing machinery but functions rather specifically for CFTR (210). The relative specificity for any processing promoting drug is very important for putative therapies with such agents, as suppressing the general quality control mechanism would lead to the expression of other incorrect proteins with possible toxic effects.

DISCUSSION

A number of human genetic diseases are caused by a failure of the mutated protein to traffic from the ER to the Golgi apparatus. Half of the mutations in the low-density lipoprotein receptor found in patients with familial hypercholesterolaemia result in a transport block in the ER, some rare cases of Tay-Sachs and the Z mutation in the α_1-antitrypsin protein also cause a defective intracellular transport (2). For the Z type α_1-antitrypsin it has been shown that the single amino-acid substitution alters the conformation of the protein structure, leading to the processing defect and aggregation (288).

Basic information about the folding of wild-type CFTR and ΔF508 CFTR and the exact nature of their interaction with chaperones is now emerging, but the nature of the temperature sensitivity of ΔF508 is not yet known. It is clear from the degradation studies that simply preventing degradation will not lead to mature ΔF508 CFTR but that the folding pathway itself has to be modified. However, the fact that ΔF508 CFTR can be brought to the plasma membrane in cells, provides the rationale to study putative therapeutic approaches aimed at translocating the ΔF508 protein in patients to the plasma membrane. This could be combined with therapies involving a hyper activation of ΔF508 CFTR channels with cAMP agonists, phosphatase inhibitors and the tyrosine kinase inhibitor genistein (70). Since ΔF508 CFTR is, at

least partially, a functional chloride channel, such a treatment may eventually cure the disease symptoms.

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Abbreviations

ABC	ATP-binding cassette
ATP	adenosine 5' triphosphate
BiP	binding protein, chaperone
cAMP	adenosine cyclic 3', 5'-monophosphate
CBAVD	congenital bilateral absence of the vas deferens
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
cGMP	guanosine cyclic 3', 5'-monophosphate
DIOS	distal intestinal obstruction syndrome
ER	endoplasmic reticulum
ES	embryonic stem cells
FIAU	1-(2-deoxy, 2-fluoro-ß-D-arabinofuranosyl)-5
Grp94	glucose response protein 94 KD
GTP	guanosine 5' triphosphate
HGPRT	hypoxanthyl, guanosine phosphoribosyl transferase (minigene)
hsp/hsc	heat shock protein
LIF	leukemia inhibitory factor
MDR	multi drug resistance pump
ME	meconium ileus
MSD	membrane spanning domain
NBD	nucleotide binding domain
Neo	neomycin
PD	potential difference
PGK	phosphoplycerate kinase (promoter)
PI	pancreas insufficient
PKA	cAMP-dependent protein kinase
PKC	Ca^{2+}/phospholipid-dependent protein kinase
PKG	cGMP-dependent protein kinase
PMA	phorbol myristate acetate
P_o	open probability
PS	pancreas sufficient
R domain	regulatory domain
T5	polypirimidine tract of 5 T's
TK	thymidine kinase promoter
TM	transmembrane segment

CHAPTER II

THE STUDY OF GENE FUNCTION THROUGH THE CREATION OF MOUSE MODELS

Summary

Two different types of manipulated mutant mice exist: transgenic mice, in which a DNA construct is integrated at random in the mouse genome and mice in which an endogenous mouse gene has been modified by homologous recombination. This chapter describes various ways by which endogenous mouse genes can be disrupted or mutated. Disruption or the introduction of a mutation in a gene enables the investigation of it's function in intact, differentiated tissues. Hundreds of mutant mice have been made this way and this has contributed tremendously to the knowledge of the function of the individual proteins and the significance of their expression in different tissues (4).

Mouse models are made through the use of embryonic stem cells (ES cells, Figure 1)(reviewed in 24, 46). These toti-potent cells, originally isolated from mouse blastocysts, are kept as a cell line *in vitro*. The ES cells will remain toti-potent and undifferentiated if cultured in proper conditions. ES cell lines require specific growth factors, provided by conditioned medium or feeder cells, to maintain there toti-potency. LIF (leukaemia inhibiting factor), also called differentiation inhibiting activity (DIA), is the most important growth factor in this respect. The ES cells can either be injected into 3.5 day old mouse blastocysts or aggregated with 8 cell stage embryo's and grown to the blastocyst stage (3, 36). The blastocysts are implanted in a foster mother and as the toti-potent ES cells will contribute to the formation of the embryo, the pups born will be chimeric with tissues partly derived from the host blastocyst and partly from the injected ES cells. Those different origins can be identified by the coat colour of the chimaera: generally, the host blastocysts are derived from a mouse strain with a black coat colour and the ES cells from a mouse strain with chinchilla coat colour. The object is to obtain chimeric mice with an ES cell derived germline.

The gene of interest can be genetically manipulated in ES cells by transfection of homologous DNA construct containing the desired alteration in the DNA. This results in homologous recombination with the endogenous gene in the ES cells. Chimeras generated from these ES cells give offspring heterozygous for the mutated gene. These heterozygotes can be intercrossed to produce homozygote mutant mice (46).

Mouse models have been made for a variety of human diseases: Gaucher disease (58), adenosine deaminase deficiency (ADA) (33), for Fragile X syndrome (1), X-linked chronic granulomatous disease (41), the multigene defect in atherosclerosis (review in (51) and for cystic fibrosis (5, 7, 13, 19, 38, 42, 47, 52, 60, 62). The most common way of studying a defective gene is by disrupting the gene and ablating the function (knock-out strategy). Other strategies are designed to disrupt the function of the gene in tissues or at a specific stage in development (Cre-LoxP strategy), or to

introduce specific mutations into the gene (Hit & Run and other strategies).

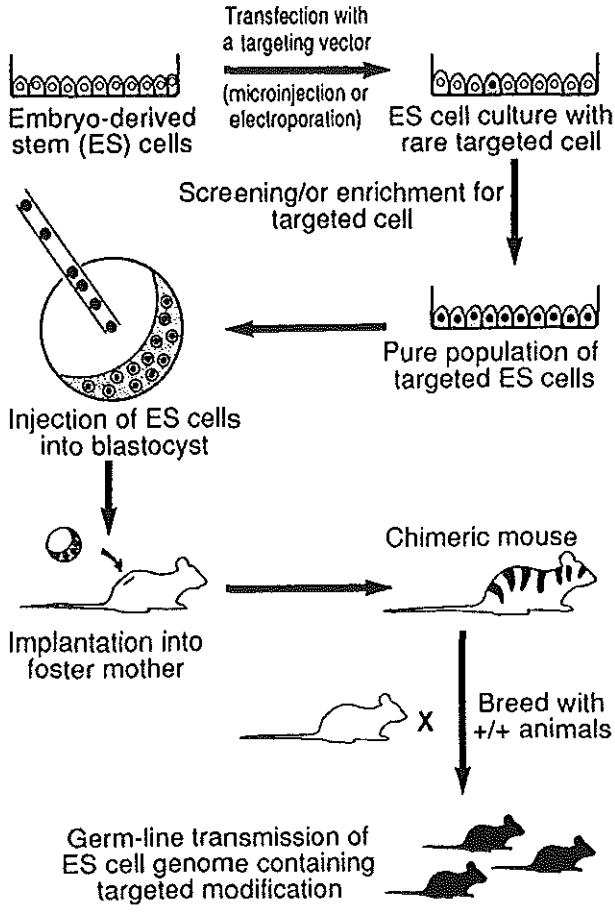

Figure 1. Generation of chimeric mice. Embryonic stem cell lines derived from mouse blastocysts cell cultures, can be genetically manipulated *in vitro*. Correctly targeted clones are then injected into 3.5 day mouse blastocysts from another mouse strain. After implantation of the injected blastocysts into a pseudo-pregnant foster mother, the ES cells will take part in the formation of the embryo. The resultant mouse is chimeric, apparent from the two coat colours derived from the ES cell line mouse strain and from the host blastocyst. If the germline of the mouse is derived from the injected ES cells, half of the offspring of the chimaera will be heterozygote for the introduced genetic alteration. Crossing of the heterozygotes gives homozygote mice. (figure from Capecchi, 1989)

Knock-Out mutations

Knock-out mice will not produce any functional protein of the disrupted gene and are therefore called null mutants. To knock-out a gene, part of the endogenous gene is replaced by a construct containing a disrupting feature (56, Figure 2). In a genomic stretch of DNA, homologous to the gene of interest, an exon is disrupted by a selectable marker, or exons are deleted and replaced by the selectable marker. This construct, upon transfection into ES cells, can align with the endogenous sequence and replace it through a double homologous recombination. Cells which perform a homologous replacement and also cells which integrate the construct randomly in their genome, will contain the selectable marker and be resistant to the selection applied. Screening by Southern analysis or PCR of the selection resistant clones reveals the homologous recombinants. The mutated exon containing the selectable marker, should not be too large, as exon skipping might take place (34, 45). If the resulting mRNA stays in frame, the encoded protein might have a partial function and influences the phenotype of the mouse. Homologous recombination is an inefficient process in mammalian cells, and can be improved by increasing the region of homology (8, 21, 56). Both linear (21, 50) and exponential (8) relationships between the length of the construct and the targeting efficiency have been reported. In addition, a positive-negative selection strategy (31) in which a negative selectable marker is included at the border of the homologous region decreases the number of random integrants found in the selection procedure. Upon homologous recombination, this negative selectable marker will be lost and the cells survive the selection against this gene, while cells which randomly incorporated the construct, still contain the negative selectable marker and die in the selection step. Other ways to improve the targeting efficiency for genes which are expressed in the ES cells, are the use of a 'promoter trap' or an 'enhancer trap' construct whereby the selectable marker has no promoter or only a basic promoter which is dependent on an enhancer. Only homologous recombination, or random integration in the vicinity of a promoter or enhancer, leads to expression of the selectable marker. These strategies have been used both for high and low expressing genes (26, 49). The same principle is used in a polyA trap, where homologous recombination leads to correct polyadenylation of the selectable marker, and thus expression of the protein (12). In addition, a viral internal ribosome entry site (IRES) can be used, which improves the translation efficiency of the marker gene (35).

A knock-out gene can also be generated by homologous integration of a linearized construct containing a stopcodon and a selectable marker into the gene (Figure 3). Integration does not lead to exchange of the endogenous DNA for the construct as in homologous recombination but results in a duplication of the region.

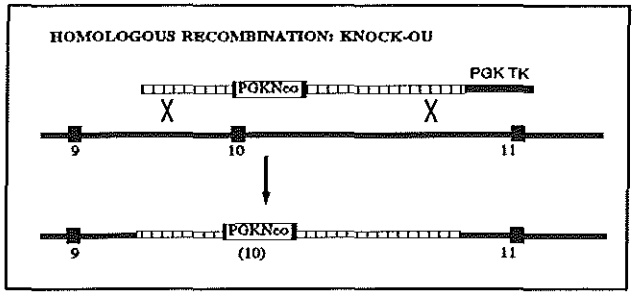

Figure 2. Complete disruption of an endogenous gene. A genomic DNA construct homologous to the target region, with a selectable marker PGK-Neo in an exon, is transfected into ES cells. Upon homologous recombination the endogenous exon in the gene is disrupted by the selectable marker and no normal mRNA can be transcribed.

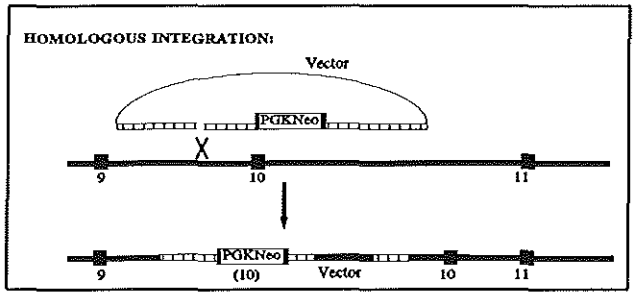

Figure 3. Integration of a targeting construct. A genomic DNA construct with the target region and a disrupting selectable marker gene in an exon is linearized inside the region of homology. Correct integration will lead to a duplication of the exon, one of which is disrupted and this will terminate transcription.

As integration is 5-20 x more efficient than replacement for some loci (18, 22), this approach is often more favourable. The double strand break in the integration vector might stimulate the recombination process (55). There are however loci were integration is equally efficient as replacement (18, 27). In the *Cftr* gene, integration mediated disruption has been used to target exon 3 (38) and exon 10 (11, 14). This method can work very well if no alternative splicing is possible. Alternative splicing can skip the introduced mutant exon and will instead splice to the normal duplicant. Around the area of *Cftr* exon 3, no alternative splicing occurs and transcription is blocked by a stopcodon caused by a frameshift due to the duplication of exon 3 (38). However, around exon 10 it appeared that there was a low level of alternative splicing (15). This resulted in up to 10 % of exon skipping of the mutant exon and thus enabled the transcription of normal mRNA which makes the method not always func-

tional. Alternative splicing can be caused by the artificial increase in length of an exon (34, 45).

Screening of colonies to find homologous recombinants can be done both by Southern analysis or by PCR. PCR has the disadvantage that false-positive clones are often found. When using an integration construct, PCR screening can be more reliable if instead of simply linearizing the construct, a gap is introduced. A homologous integration event will be accompanied by repair of this gap (Gap formation and repair mechanism) so that a primer located in this region will only detect the repaired homologous integrant and not randomly integrated constructs which lack this sequence (25). The gaps can be quite substantial of up to 2.5 and 4.2 kb (23), but loss of the total amount of homologous sequence and loss of adjacent homologous ends might decrease the targeting efficiency.

A variation on the knock-out approach is to introduce a LacZ sequence in frame with the translation startcodon of the gene. This disrupts the gene and at the same time, allows monitoring of the cell specific localization pattern through the LacZ expression (32).

Another possibility is to make knock-outs only in specific cell types with the phage Cre-*LoxP* mechanism (17, 48, 54)(see Figure 4) or the yeast Flp recombinase (37). These recombinases are able to mediate site-specific recombination in mammalian cells, and in mice, transgenic for the system components (30, 40). The Cre enzyme recognizes the short sequences of 2 *loxP* sites and excises the intermediate sequence if the *loxP* sites are in the same orientation or inverts the sequence if they are in opposite orientation. The *loxP* sites can be anywhere in the chromosome, even on two different chromosomes (10). The remaining *loxP* site can be used again to insert a sequence with the proper *loxP* ends. Thus, a mouse generated from ES cells with an exon, or part of the gene, with flanking *loxP* sites (floxed exon), is crossed with a mouse transgenic for the Cre enzyme. The Cre enzyme can be under the control of a promoter with the desired tissue or stage specific expression, and will cause the loss of the floxed gene or exon, resulting in a mouse, with a knocked-out gene in a particular tissue or at a specific stage. As the Cre enzyme remains expressed in the cells, the fragment might pop in and out of the *loxP* site as long as it is not degraded (29, 64).

For all disrupted genes, it should be kept in mind that truncation of a gene might result in proteins with residual function. Therefore, deletion of the 5' end of the gene is preferred. Other genes or regulatory elements should not be affected by the disruption and the effect of the expression of the selectable marker can modify the expression of neighbouring genes (39). Targeting vectors should preferably include Cre or Flp recombinase sites so that transient expression of the recombinase in ES cells gene removes the marker gene (16).

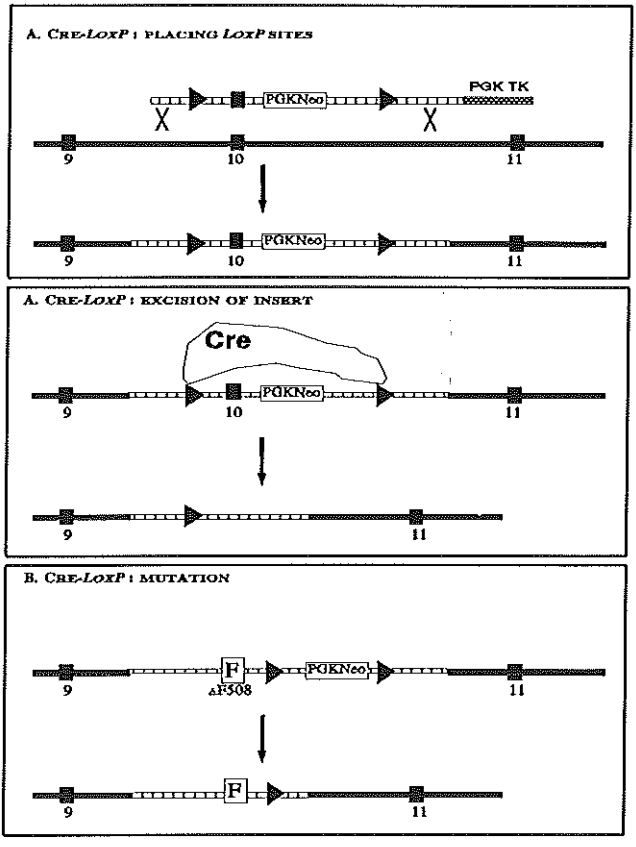

Figure 4. Cre-LoxP excision of (part of) the gene. A. Recognizing lox signal sequences for the Cre recombinase are introduced around the part of the gene which has to be deleted, either in one homologous recombination step or with two separate recombination constructs. Expression of the Cre recombinase under the control of a tissue specific promoter results in excision of the DNA between the two lox sites. B. If in the first recombination steps a mutation in an exon is introduced and the selectable marker is enclosed by lox sites, the Cre recombinase will take out the selectable marker leaving a gene with the mutation in the exon and one lox site in the intron.

Introduction of mutations

The introduction of a specific mutation in the gene rather than complete disruption is sometimes preferred when the mutant protein is known or expected to have relevant properties, as can be the case in point mutations found in human genetic diseases. Also, for genes which are essential for viability in any cell or in the early embryo it can be worthwhile to introduce an impeding mutation rather than a complete

disruption.

The introduction of specific mutations has been accomplished in various ways. Introduction of a mutation and the selectable marker in one step has been published by Deng *et al.* (9). The creation of a ΔF508 CFTR cell line was reported, with the highest frequency for replacement in this area of the *Cftr* gene ever reported (14 %). The construct consisted of a 12 kb genomic stretch of DNA with the desired mutation in the exon and the selectable marker gene in the intron adjacent to it. Transcription of the selectable marker gene is in opposite direction to the gene itself. The same method was used by Colledge *et al.*, (5) to produce a ΔF508 *Cftr* allele with a selectable marker in intron 10. In humans with a ΔF508 allele, the ΔF508 allele and the normal allele produce equal amounts of mRNA (57). On analysis of the mice produced with the two ΔF508-selectable marker constructs, it appeared that the mRNA signals from the ΔF508 allele were greatly reduced in some of the organs (5, 62). This is not due to the ΔF508 mutation itself as in the ΔF508 mouse model that we made, which does not contain a selectable marker, the mRNA levels are normal (60, 61). Therefore, the expression of the mRNA should be carefully monitored in several tissues in order to be confident that an observed phenotype is caused by the desired mutation in the protein and not by lower levels of expression of the mRNA and protein.

Another method which has been used to introduce a mutation into an endogenous gene is cotransfection of a construct with the homologous region and the desired mutation together with a selectable marker (6, 44). The construct replaces the homologous region in the genome resulting in a targeting event, while the selectable marker is integrated somewhere else in the genome. This one-step introduction of a mutation has the advantage that the selectable marker is not inserted into the gene of interest. The efficiency for correct targeting of the HGPRT (hypoxanthine phosphoribosyl transferase) gene was 1/5000 G418 resistant clones (6). However, most of the integrants will integrate at random as concatamers. An additional drawback of this method is that both the selectable marker and targeting construct can disrupt other genes and disturb normal gene function by randomly integrating. This seriously compromises the analysis of the phenotype. If the gene to be mutated is known to be very efficiently targeted this method can be useful, as well as direct micro-injection of the targeting construct into ES cells without the use of a selectable marker (63).

To make a ΔF508 CFTR mouse model, we chose to use a procedure which introduced the mutation without an interfering selectable marker in the intron. One method is to use a Two-Step replacement procedure (Figure 5) (43). In the first step a construct with the bacterial GPT gene (guanine/xanthine phosphoribosyl transferase) or HGPRT, is transfected into a HGPRT negative cell line as a knock-out step, and cells are selected for GPT/HGPRT expression with HAT (hypoxanthine, aminopterine, thymidine). Afterwards, the cells can be transfected with the same construct containing

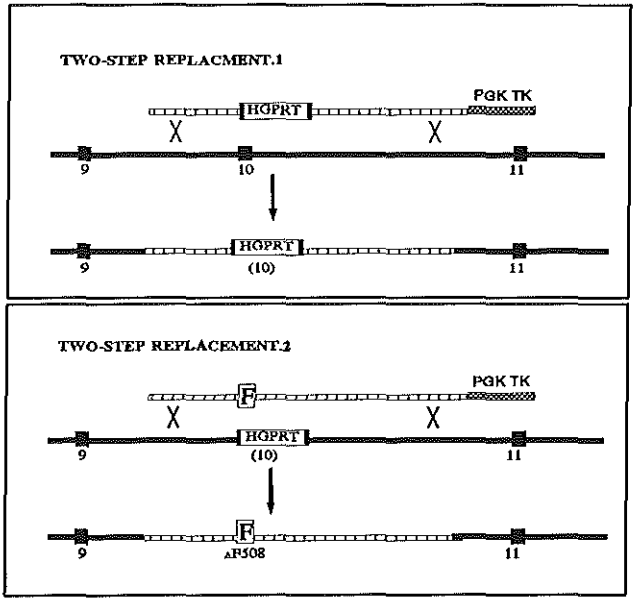

Figure 5. In the two-step-replacement procedure, the first step consists of the introduction of a positive and negative selectable marker in the targeting exon. The second homologous recombination targets with a construct containing the desired mutation. Homologous recombinants contain the mutated exon and have lost the marker gene and will therefore survive selection against it.

the desired mutation in the exon instead of the GPT/HGPRT gene, which upon homologous replacement in the cell will delete again the acquired GPT/HGPRT gene. These cells will then be able to survive the selection against the GPT/HGPRT gene with 6TG (6-thioguanine). The Two-Step replacement strategy has been used to replace the mouse α-Lactalbumin with the human gene (53) but also proved to work with a Neomycin-thymidine kinase cassette so that no HGPRT $^-$ cell line is needed (2).

An alternative is the Hit & Run, or In-Out, procedure (20, 59) (Figure 6) which involves an initial step of integration of a construct with a mutated exon together with a positive and a negative selectable marker in the plasmid in the homologous region of the mouse genome. The positive selection marker is used for screening of this 'Hit' step. The second step involves pairing of the integrated area with the endogenous homologous area and the subsequent looping out and deletion of one of the duplicates. These 'Run' clones have lost the negative selectable marker and will thus survive screening against it. For several reasons the Hit & Run procedure seemed attractive to introduce mutations in the mouse *Cftr* exon 10. Homologous integration can be 5-20 x more efficient than replacement (18, 22) and the efficiency was reported to be high. The most important advantage is that the procedure would result in a gene which does not have any alterations except for the

ΔF508 mutation.

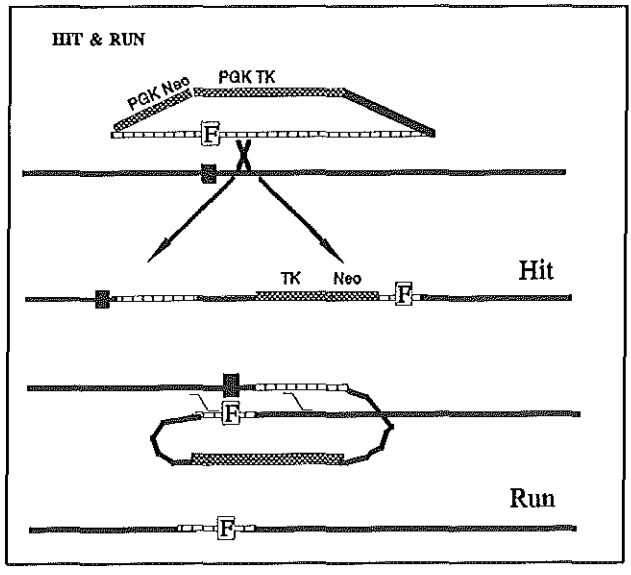

Figure 6. Hit and Run procedure. The targeting construct contains the desired mutation in the exon and a positive and negative selectable marker in the plasmid. The construct is linearized in the region of homology and will integrate in the homologous region of the endogenous gene resulting in a duplication of the region which was introduced, one with the mutated exon, separated by the plasmid and selectable markers. Spontaneous recombination between the two duplicated area's results in a normal gene or in a gene with the mutated exon, in an otherwise unchanged genomic organization.

For a locus which is difficult to target with a replacement vector it can be of great advantage to use the Hit & Run procedure when a single mutation has to be introduced. The data for the *Cftr* gene targeting frequencies vary from 0.04 % to 0.3 % for constructs around 7 kb (28, 52), to 14 % positive clones for a construct of 12 kb, reported after initiation of our study (9). Integration in this area with constructs around 5 kb, was found by Dorin *et al.* (13), Dickinson *et al.* (11) and our group (60), to result in 2-11 % correct integration clones, which shows the effectiveness of integration in the *Cftr* allele.

The lack of a germline competent HGPRT negative cell line and the reported low efficiency of replacements in the *Cftr* gene, made us decide to use the Hit & Run procedure. The results are described in chapter IV and V. Today, it is no longer necessary to use the cumbersome two-step methods to introduce a single mutation in a gene since the Cre and Flp recombinase systems can delete the selectable marker after homologous recombination, leaving the target gene in normal, except for the remaining *LoxP* or FRT site, chromosomal organisation.

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CHAPTER III

Cftr MUTANT MOUSE MODELS: Comparison with the human CF phenotype

Comparison of mouse to human CF phenotype

Endogenous genes in mouse embryonic stem cells can be modified *in vitro* and injected into mouse blastocysts to generate mutant mice. Up to now, there is no other animal species where such modification can be performed. Consequently, there is no choice but to make a mouse model if one wants to create an animal model for a human disease. Following the discovery of the human CFTR gene and the cloning of the mouse homologue, *Cftr* mutant mouse models have been made to be able to study CF *in vivo* and *in vitro* with ample supply of affected tissue (Table I). Although the most common mutation in human patients is the ΔF508 deletion, it is simpler and easier to create a complete disruption of the mouse *Cftr* gene. Therefore, most of the mouse models are knock-out mice, in which the mouse *Cftr* gene has been disrupted by the introduction of a stopcodon followed by a selectable marker into exon 1 (47), exon 2 (30), exon 3 (Bay mouse) (40) and exon 10 (Unc mouse, Cam mouse and HGU mouse) (19, 45, 50). The HGU mouse contains a duplication of the exon 10 area and due to alternative splicing, those mice have up to 10% normal CFTR mRNA.

To better mimic the actual human mutation, we and two other groups made a mouse model with the ΔF508 mutation (10, 59, 64). The ΔF508 mouse model made by our group has no other genomic alteration but the ΔF508 mutation and has normal mRNA levels. The other two ΔF508 models and a G551D mouse model (17) have a selectable marker in intron 10 or intron 11b, resulting in reduced CFTR mRNA levels. In this chapter, the histological and electrophysiological features of the human CF phenotype will be compared with the phenotype of the *Cftr* mouse models. The ΔF508 mouse made by our group is discussed in chapters IV-VI.

All CF mouse models show defects in cAMP regulated chloride transport similar to CF patients. However, differences are observed, both between mouse and human tissues and between the different mouse models. The comparison is complicated as different experimental methods have been used and the mice are bred in different genetic backgrounds. In the following section, major characteristics are described including a short comment on discrepancies.

Gastro-Intestinal tract

10 % of the newborn CF patients present with obstruction of the ileal lumen at the ileocecal valve by a viscid, sticky meconium mass, meconium ileus (ME). Later in life, a distal intestinal obstruction syndrome (DIOS) in the colon is also found. All the knock-out mouse models show similar lethal intestinal obstruction symptoms, although much more frequent than in human CF patients (Table II). The obstructions in mice can be found in the distal intestine, in the colon and also in the junction of the jejunum with the ileum (Unc and Cam mice). The ΔF508 mouse models with low mRNA levels in the intestine also die early although not as frequent as knock-out mice (10, 64). Mice that die of intestinal obstruction show

Procedure	Construct	Reference	Features
Replacement	Neomycin in exon 10	50	complete knock-out
	HGPRT in exon 10	45	complete knock-out
	Neomycin in exon 1	47	complete knock-out
	Stopcodon exon 2	30	complete knock-out
Integration	Neomycin in exon 3	40	complete knock-out
	Neomycin in exon 10	19	10 % normal mRNA
Replacement with missense mutation	ΔF508 mutation, HGPRT in intron 10	10	5-30 % ΔF508 mRNA all organs
	ΔF508 mutation, Neomycin in intron 10	64	10 % ΔF508 mRNA in intestine
	G551D mutation, Neomycin in intron 11b	17	53 % G551D mRNA in all organs
Hit and Run	ΔF508 mutation	59	normal levels ΔF508 mRNA

Table I. Generation of different CF mouse models.

severe destruction of the villi and crypts with inspissated, eosinophilic material. Also prior to death or disease symptoms, dilation of the crypts with accumulated mucus and hyperplastic and hypertrophic goblet cells are found with a proximal to distal increase in severity. Studies in Bay mice without signs of disease symptoms, show that at birth those mice had no abnormalities in the small intestine while at 2-4 days the first signs of goblet cells hyperplasia can be seen. At 3-4 weeks, mucus accumulation, dilation of crypts and focal concretions in the base of the crypts is observed. In the colon of newborn mice, the crypts show mucus accumulation and dilation of the crypts is seen in 3-4 week old mice, progressing to inspissated mucus accumulation in a 6 week old mouse. The HGU mouse with up to 10 % of normal *Cftr* mRNA (20) shows far less ME and DIOS than the complete null mutant mice.

In CF patients, histological abnormalities of the appendix, similar to intestinal crypt abnormalities are observed. DIOS is often associated with inspissated secretions in the appendix. A mouse caecum is normally big and sacklike but the Unc knock-out mice have coiled and wormlike caeca, impacted with faecal pellets.

The Brunner glands are submucosal mucoid secreting glands dispersed in the duodenum and are abnormal in 82 % of the patients with ME. These glands show dilation of the lumen which is filled with eosinophilic material and have a flattened

hyperplastic epithelial cell lining. Mice have only one gland, next to the pylorus, which was completely destroyed in three examined Unc mice with ME but which was normal in the Bay mice.

Electrophysiological measurements of the intestinal tract

Intestinal biopsies of CF patients show a lower potential difference (PD) than normal and have a reduced cAMP activated chloride response in the intestine and also a reduced Ca^{2+} activated chloride response in the colon (5, 6, 16, 53, 60). The mouse models all show similar CF characteristics as they have reduced basal PDs in several intestinal segments and show reduced chloride response to cAMP and Ca^{2+} increasing agents (7, 10, 17, 31, 40, 48, 64).

In the lower colon the reduction of the basal PD in CF mice was less pronounced than in the small intestine. Amiloride was able to decrease the PD which indicates that a substantial part of the basal PD is generated by absorption of Na^+ through, apical, Na^+ channels (7, 12, 48). The stimulation of Cl^- transport by Ca^{2+} via carbachol, is, at least in part, due to an activation of basolateral K^+ channels in the intestinal cells which results in an increased driving force of chloride ions through apical CFTR channels (18, 52).

Direct fluid measurements in isolated intestinal segments of normal mice indicate a basal absorption rate which can be reversed to secretion when stimulated with cAMP. In CF mice, cAMP did not result in secretion of fluid but reduced the absorption rate in the ileum (21). In contrast, jejunal preparations of CF mice did not only fail to respond to cAMP with fluid secretion but are also unable to reduce the absorption rate of Na^+ and Cl^- ions (9). This suggests that CFTR also functions as a regulator of salt and water absorption in at least part of the intestine, possibly through direct or indirect interaction of CFTR, present on villous epithelium, with the Na^+/H^+ exchanger (9).

The hypothesis that the high incidence of CF is caused by a selective advantage of CF carriers in surviving cholera epidemics or other secretory diarrheas (43, 44) was tested in the mouse knock-out model. Cholera enterotoxin and the *E.coli* heat stable toxin STa, induce cAMP levels and thus stimulate chloride and therefore water transport, which can be lethal in case of continuous stimulation during such infections. The fluid transport in mouse intestine in response to cholera toxin was shown to be decreased in CF mice and to be intermediate in heterozygote mice (24), strongly supporting the genetic advantage hypothesis whether it be for cholera or *E.coli* toxins. The difference became evident after 2 hours of stimulation and is not apparent if the chloride current through the epithelium is measured instead of net fluid transport (13). Further, the effect is only observed in an isogenic background inbred CF mouse strains as in a diverse genetic background compensating mechanisms obscure the measurable

	Patients	$cftr^{m1UNC}$
Mutation	Various, 70 % ΔF508	Neomycin exon 10, complete KO
Meconium ileus	10-20% ME at birth, later in life DIOS	40 % first 5 days, 50 % at weaning, ME > DIOS
Growth	reduced growth rate and failure to thrive	10-50 % reduction in weight
Intestinal histology	goblet cell hyperplasia and mucus accumulation in crypts ileum and colon (54)	distended crypts ileum and colon
Brunners glands	hyperplastic glands in 82 % patiens with ME (42)	3/3 mice with ME had destroyed gland
Appendix	hyperplastic mucosa and distended mucosal glands with eosinophilic secretions (38)	coiled, wormlike, impacted with hard fecal pellets
Pancreas	85 % PI, dilation pancreatic acini, eosinophilic material in ductules, distruction pancreas (22)	2/5 mice some enlarged acini, sometimes with eosinophilic material
Lungs	normal at birth, abnormal secretion in first weeks, 95 % patients recurrent infections	patches of goblet cells in proximal airways 5/7 mice; 1 mouse dilation of gland ducts and mucus obstruction airways (49)
Biliary system	25-30 % patients focal biliary cirrhosis in liver; gallbladder hypoplastic, thick mucus in content and calculi (62)	infiltration of polymorphonuclear cells in gallbladder wall
Reproductive system	95 % ♂ infertile, abnormalities reproductive tract; ♀ abnormal mucus in cervix (35)	♂ normal, ♀ reduced fertility and abnormal mucus accumulation cervices (49)
Salivary glands	submandibular, sublingual, submucosal glands have dilated ducts, inspissated secretions, atrophy acini (41)	4/6 mice submaxillary glands show degrees of disruption serous acini

Table II. Comparison of mouse models.

The initial data on patient histology are reviewed by Welsh *et al.* (61), and data of the mice are derived from the initial reports of the *cftr*^{m1Unc} (Unc, 50), *cftr*^{m1Cam} (Cam, 45), *cftr*^{m1HGU} (HGU, 19), *cftr*^{m1Bay} (Bay, 40) mice unless otherwise indicated. nr=not reported.

cftr^{m1Cam}	**cftr^{m1HGU}**	**cftr^{m1Bay}**
HGPRT exon 10, complete KO	Neomycin exon 10, partial KO	Neomycin exon 3, complete KO
80 % first 5 days, 10 % at weaning, ME, later more DIOS, also jejunal blockage	5 % at birth 2 % at weaning	40 % at birth, ME or DIOS, 50 % at weaning,
50 % reduction in weight	no runting in first 30 days	70 % reduced weight
mucus accumulation at 1 day	4/6 mice show mild abnormalities	gradual progression mucus accumulation and crypt abnormalities in small intestine and colon
nr	nr	no abnormalities
nr	nr	nr
50 % mice dilatation and obstruction ducts	no abnormalities	gradual progression atrophy of acinar cells and duct dilatation
no abnormalities	1/6 mice mucin in lung causing focal atelectasis	no abnormalities found, only inflammation secondary to intestinal inflammation
nr	nr	2/3 gross enlargement of gallbladder but no abnormality
nr	1/2 mice gross accumulation mucus in vas deferens	no abnormalities
nr	1/6 mice mild dilatation salivary glands	2/7 mice severe dilatation acini minor sublingual gland, no abnormalities main salivary glands

difference in Cl^- secretion (9). This is compatible with the concept that CF carriers only have a small advantage over non-carriers in surviving secretory diarrheas.

Fluid secretion by intestinal crypts in response to intestinal hormones such as vasoactive intestinal peptide (VIP) and cholinergic agonists is accompanied by a reduction in cell volume. VIP increases intracellular cAMP while cholinergic stimulation leads to Ca^{2+} increase. CF crypt are able to respond to Ca^{2+}-linked carbachol with a volume decrease but not to cAMP-linked VIP (57). Also the regulatory volume decrease (RVD) in response to a hypotonic shock was impaired in knock-out mouse crypts, apparently through a dysfunction of volume-activated K^+ channels. By which mechanism the CF condition leads to a loss of K^+ channels function is presently unclear (58).

Neurogenic stimulation has been observed in normal mouse jejunum in the form of oscillations in the chloride current. These oscillations are absent in CF mouse jejunum, similar to CF patients (4, 27).

It is apparent from the above summarized results that loss of CFTR function does not only affect the cAMP-induced chloride current but also influences other cellular functions through mechanisms which are not yet clear.

Respiratory tract

The lungs of CF patients show abnormalities before infection with pathogens (51). Dilation of submucosal glands and of the acinar structure is later accompanied by mucus obstruction of the small airways and by goblet cell hyperplasia. The extreme detoriation of the lung function is due to the chronic infections. However, the precise mechanisms that are involved in the development of lung pathology are not well understood. The study of CF mouse models might give some insight into certain steps of the pathogenesis of CF lung disease. Generally, not much pathology has been found in young knock-out mice. Some mice show dilated acini with accumulated inspissated material and atrophy of serous glands in the nasal pharyngeal region (Bay, Unc). In the proximal airways patches of goblet cell hyperplasia are seen (Unc). Two older mice showed squamous hyperplasia in trachea and airways filled with mucus (49). The Bay mice only show lung inflammation secondary to intestinal inflammation. To test whether destruction of the airway epithelium would reveal different cellular reaction patterns between CF mice and normal mice, the lungs were treated with 100 % O_2 for 48 hrs but no difference could be observed from control mice (40). A careful comparison of HGU CF mice and controls, shows that even normal mice have quite often small abnormalities, as inflammation and goblet cell hyperplasia, but CF mice seem to be slightly more affected (15).

As patients show severe lung destruction after infection with *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa* and *Burkholderia cepacia*, CF mice were challenged with bacterial strains. In the Unc mice, single or repeated infection with a *S.aureus* strain, isolated from a CF patient, did not lead to

reduced clearance or lung pathology (49). However, 50 % of the HGU mouse show reduced clearance when infected with *S. aureus* and *B. Cepacia* strains. Histological examination of mice infected with *S. aureus* shows that whereas all mice, CF and controls, had lymphoid infiltrate, it is more widespread in CF mice. Obvious differences are found in goblet cell hyperplasia, mucus retention and bronchiolitis in the CF mice. *B. cepacia* infection gives severe pneumonia in CF HGU mice and only minor symptoms in control mice (15). Initial studies with *P. aeruginosa* indicate a diminished phagocytosis capacity by CF mouse epithelial cells similar to human CF airway epithelium (25).

These studies indicate that CF mouse models can be used to study CF lung disease and pathogenic infection studies, despite their short life span and the slight differences in histological structure of the lungs.

Electrophysiology of airway epithelium

The nasal epithelium of CF patients is characterized by an increased basal PD and a larger effect of the Na^+ channel blocker, amiloride. This has been interpreted as a low Cl^- permeability and an increased Na^+ absorption in CF patients (1, 34). *In vivo* measurement of the nasal epithelium of knock-out mice also shows the increased basal PD (19, 23, 29, 48). Amiloride treated cultures of nasal and tracheal CF mouse cells show a difference in basal PD with normal cells. Further, the response to forskolin, which increases intracellular cAMP levels, was absent in CF mouse epithelial cells (7, 40).

To measure electrophysiological characteristics of the mouse trachea, four different approaches have been used: measurement of the trachea *in situ*, mounting of the complete excised trachea in an Ussing chamber, mounting of excised pieces of the trachea in an Ussing chamber and cultures of tracheal cells. Measurement on the trachea *in situ*, on the complete trachea mounted in an Ussing chamber and on cultured fetal tracheal cells, show a small difference between normal mice and CF mice for the basal PD, and no difference in amiloride response and forskolin response (11, 19, 23, 28, 40). Ca^{2+} activated Cl^- channels are present in the apical membrane of tracheal epithelia (8, 23) and a crosstalk between cAMP and Ca^{2+} pathways has been observed in trachea epithelium (28). Thus, stimulation with cAMP can also activate the Ca^{2+} regulated Cl^- channels in CF. A much higher Ca^{2+} Cl^- response masks CFTR regulated Cl^- transport in the mouse trachea (8) so that no difference can be observed between intact normal and CF epithelia.

When instead of the intact trachea, excised pieces of trachea are mounted in an Ussing chamber, or tracheal cells are cultured, both a difference in PD between normal and CF mice and a lower response to forskolin in the presence of amiloride is found in CF mice (7, 31, 45, 48). For the Cam mice, this CF effect could only be observed in young mice, < 20 days (10, 45). While CF patients show an

increased amiloride sensitive sodium absorption in nasal and airway epithelium (2, 33) and CF mice also show a increased sodium absorption in nasal epithelium, in tracheal epithelium, CF mice have a normal or a reduced amiloride sensitive sodium absorption (28, 31, 48). Liposome transfection of CFTR into the lungs of knock-out animals, restores the sodium hypoabsorption to near normal levels (31).

Overall, the data for mouse trachea indicate that intact trachea is not affected in CF mice, while manipulated tracheal tissue, either as excised pieces in an Ussing chamber or as cultured cells, can be treated in such a way that differences become apparent. Whether these differences have any physiological relevance is not known.

Secretion and absorption of water is a continuous process in the lower and upper respiratory tract. The water transport of the intact CF mouse trachea was measured in CF Cam mice in our group. Although the CF mice trachea showed a slightly higher basal PD than normal mice, no altered fluid transport could be observed compared to normal mice. This indicates that CFTR does not play an important role as a mediator of water transport in the mouse trachea (23).

Gallbladder

While CF patients show focal biliary cirrhosis and abnormalities of the gallbladder or bile, similar symptoms were not found in any of the knock-out mice. Some enlargement, inflammation signs and rupture of the gallbladder have been reported (Unc, Bay) but these might be secondary to the intestinal obstruction and associated inflammation.

The Cambridge knock-out mouse was studied in our group to associate the gallbladder abnormalities and bile duct obstructions observed in 25 % of the CF patients, with aberrant mucus or water transport. The results are presented in detail in Chapters VII and VIII. Mucus secretion was not different between cultured gallbladder cells of normal and knock-out mice and stimulation of CFTR with forskolin did not result in an increased mucus secretion. Water transport was measured in intact gallbladders and showed a similar basal absorptive state in normal and knock-out mice. Stimulation with a cAMP mediator resulted in a shift from absorbtion to secretion in the normal gallbladders, while in CF gallbladders the absorption stopped but no secretion was present. This implies an important role for CFTR to regulate the osmotic value of the gallbladder content. If CFTR has a similar role in bile ducts as in the gallbladder, liver disease in CF patients is possibly caused by a reduced fluid secretion resulting in bile duct obstruction. Further, an altered composition of the mucus as hypersulfation might enhance these obstructions.

Pancreas

Pancreas dysfunction is present in 85 % of the CF patients and is due to duct obstruction resulting in autolysis of the pancreas by its own enzymes. No

abnormalities were found in the HGU mouse. Pancreatic abnormalities have been reported in the Unc mice but are attributed to a secondary effect of poor health of the mice and abnormalities have also been found in one third of normal mice (40). Mouse pancreas has less *Cftr* expression and a relatively higher contribution of an alternative, Ca^{2+} activated, chloride channel than human pancreas (7, 50), which might explain the absence of major histological abnormalities in the ducts. Studies on pancreatic enzyme content showed that there is 20-50 % reduction in the amounts of different pancreatic enzymes in CF mice with a normal body weight (32). However, secretion of amylase was not impaired in CF pancreatic tissue (39). Careful analysis of older mice revealed dilation and filling of the acini with aggregated protein (36). An increase in expression of the gp300 protein, a presumed component of digestive enzyme storage vesicles, was found in the acini. This would reflect an inability to secrete the enzymes resulting in the aggregated material in the cells (36).

CF mice show an absence of the cAMP regulated chloride response (8, 26, 63) in pancreatic ducts. In reverse, a higher Ca^{2+} activated chloride current is dominant in mouse pancreas compared to human pancreas (8, 63). Fluid measurements on $_{\Delta}$F508 mouse cultured pancreatic epithelium show a reduced basal absorbing state and a lack of secretion when stimulated with cAMP (64).

Other organs

While 95 % of the male CF patients are infertile due to aspermia, abnormalities of the vas deferens or the epididymis, the male knock-out mice do not show reduced fertility although *Cftr* is expressed in the mouse reproductive system (46, 50, 55, 56). Female mice have reduced litter sizes and analysis of the cervix shows increased mucus accumulation similar to that found in female patients (35, 50).

CF patients show occasional abnormalities of salivary glands; also occasional abnormalities have been observed in knock-out mice as dilation of the acini of the sublingual gland (Unc and Bay) and of the submaxillary glands (Unc). β-adrenergic induced secretion of glycoproteins was reduced in submandibular salivary glands of knock-out mice (39). Although no problems with lacrimal glands or parotid glands have been described for CF patients, acinar dilatation of the lacrimal gland in knock-out mice has been reported (Cam, Bay) including eye infections (Cam) and severe atrophy of the parotid gland (Bay). Whether these aberrations in the mice are due to dysfunction of *Cftr* in these organs or reflects normal variation is not clear.

Transgenic mouse models

Knock-out mice have been complemented with a human CFTR YAC via transgenesis. These mice do no longer show an increased mortality and have a

forskolin response in colon, caecum and jejunum, indicating correction of the mouse *Cftr* disruption by the human CFTR (14). Other transgenic mice in a knock-out background have been made with human *CFTR* cDNA under control of the rat intestinal fatty-acid binding protein (FABPi) (65) promoter or the villin promoter (3, 37). The FABPi-CFTR mice in a knock-out *Cftr* background showed correction of the lethal intestinal defect. This is quite important since the FABhi directs expression in the villi while the highest CFTR expression is found in the crypts. The forskolin response in the intestine was 70 % less than in normal, but is apparently enough to prevent lethal obstruction. The morphology was much improved compared to non-transgenic knock-out mice, but still showed some hyperplasia of goblet cells in intestine and colon (65). The partial correction of the phenotype would imply that fluid transport or possibly the inhibition of absorption, whether in crypts or villi, is sufficient to prevent intestinal blockage.

Conclusions:

The CF mouse models have proved to mimic some of the CF characteristics and may prove to be of great help in the study of the pathogenesis of CF. The major electrophysiological defect, the absence of a cAMP regulated chloride current, is indeed absent or strongly reduced in the knock-out mice. The intestine of complete knock-out mice shows the typical CF obstructions in small intestine and colon although much more frequent than in CF patients. Small histological abnormalities in the lung and pancreas have been observed and these may become more significant and more reminiscent of CF disease when the mice are challenged with pathogenic bacteria and with increasing age. The gallbladder, pancreas and intestine show defects in absorption and/or secretion of fluid.

Highly important is to characterize the differences between mice and men and to study the effect of genetic heterogeneity. A Ca^{2+} activated Cl^- current an explanation for the difference in intestinal, lung and pancreas disease between mice and men. The exon 1 knock-out mice showed variability in life span which has been attributed to a genetic trait. Mice who survived into adulthood without intestinal obstruction were shown to have an upregulated Ca^{2+} regulated Cl^- conductance which may compensate for the defective CFTR (47). Difficulties originating from the heterogeneous background as in the human situation are therefore present in CF outbred mouse strains but can be controlled and eventually lead to a better understanding of CFTR functioning. It is apparent from the sometimes conflicting results of different mouse models studies, that more factors than currently known are involved in the function of various organs. Factors which are for instance of influence on the survival and bio-electric properties of the CF mice are nourishment, genetic background and residual CFTR activity.

The CF mice can be used as a model for the disease processes occurring in human CF patients as intestinal blockage syndrome, intestinal absorption and presumably for gallbladder disease, some pancreatic abnormalities and lung

infection. Clinically important is that CF mice can be used for testing of therapeutic strategies. ΔF508 mice can be used to study alternative therapies directed at opening the few ΔF508 channels which are able to escape the processing block, or to stimulate the processing pathway of ΔF508 CFTR.

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CHAPTER IV

TARGETING THE MOUSE *Cftr* ALLELE USING THE HIT & RUN PROCEDURE

Introduction

To be able to study the pathogenesis of the various disease symptoms in CF and the effect of specific mutations in the CFTR protein, we wanted to make a mouse model carrying a mutation in the endogenous mouse *Cftr* gene. The most common mutation found in CF patients is a deletion of a phenylalanine at position 508 in the CFTR protein (ΔF508). We used the Hit & Run procedure to introduce the ΔF508 mutation in order not to create any other alterations in the mouse genome. This chapter describes the introduction of the mutation in embryonic stem cells.

The mouse cystic fibrosis transmembrane conductance regulator (*Cftr*) gene is highly homologous to the human *CFTR* gene (12). Of 60 missense mutations found in CF patients, 59 residues are conserved in the mouse *Cftr* gene. Exon 10, which codes for 64 amino acids, including F508 and I507, is highly conserved with only one nonconservative difference (12). Therefore, one would expect a mutated mouse CFTR protein to have similar defects and properties as the mutated human CFTR. In CF patients, the most predominant mutation is ΔF508, 90 % of the patients have at least one *CFTR* allele with the deletion of ΔF508 (10). The features of the ΔF508 CFTR are described in Chapter I.10. This mutation affects the processing of the protein from the endoplasmic reticulum to the golgi apparatus (2, 14). Also a deletion of the isoleucine at position 507 (ΔI507) has been found in CF patients and this leads to defective processing as well (2, 3). Since the ΔF508 CFTR is potentially able to function as a chloride channel, the creation of a ΔF508 mouse model can be used to study therapeutic strategies, designed to target the ΔF508 CFTR to the plasma membrane.

In the experimental work which forms the basis for this thesis we have successfully used of an insertion vector in the first step of the Hit & Run procedure creating the ΔF508 and the ΔI507 mutations in embryonic stem cells. A complete description of the Hit & Run procedure (4) is described in Chapter II.

Results & Discussion

The first step of the Hit & Run procedure consists of the integration of a targeting construct, with the ΔF508 or ΔI507 mutated exon 10, into the homologous region of the endogenous *Cftr* gene in the mouse genome (Figure 1). We isolated a lambda clone with the mouse exon 10 sequence from an EMBL3A lambda Balb/c library screened with a human exon 10 *CFTR* probe. A 3.3 kb *BamHI-XbaI* fragment spanning the *Cftr* exon 10 region was cloned and the deletion of 3 nucleotides coding for an isoleucine at position 507 in the Cftr (ΔI507) was introduced in exon 10 in the construct by site-directed mutagenesis (Figure 1A). A diagnostic *SspI* restriction site was created adjacent to the deletion. A PyTK

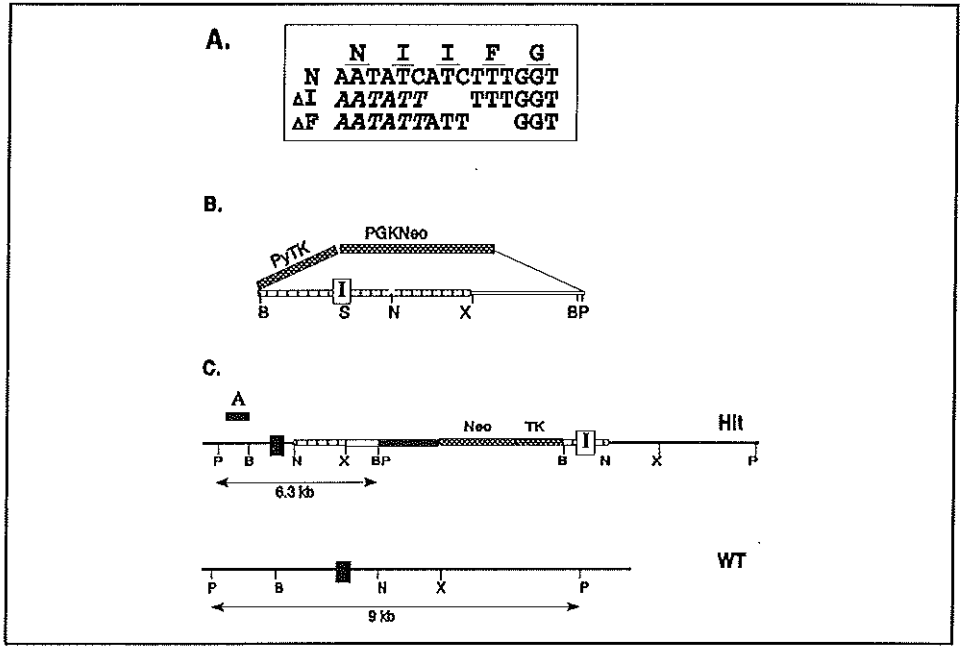

Figure 1. Hit & Run targeting strategy for the *Cftr* gene. A. Mouse exon 10 sequences are mutated to create the ΔI507 or the ΔF508 mutation. An *Ssp1* restriction site (italic) is introduced adjacent to the three basepair deletion, without affecting the amino acid code. B. The construct consists of 3.3 kb genomic sequence containing the ΔI507 exon 10 and selectable markers. The PGKNeo gene is the positive selectable marker used for the Hit step and the PyTK gene serves as a negative selectable marker in the Run step. Linearization was done at the *NsiI* site. C. Correct integration creates a 6.3 kb *PstI* fragment while wild type alleles have a 9 kb *PstI* fragment. B=*BamHI*, S=*SspI*, N=*NsiI*, X=*XbaI*, P=*PstI*.

(Polyoma enhancer-thymidine kinase) gene and a PGKNeo (phosphoglycerate kinase-neomycin) gene were inserted in the plasmid. Neomycin enables selection for integration of the construct in the Hit step while the thymidine kinase expression is used in the Run step. The construct was linearized at the *NsiI* site which results in a 2.3 kb homologous stretch 5' to the *NsiI* site and a 1 kb homologous region 3' of the site. E14 embryonic stem cells were transfected and G418 selection was applied. After 10 days, 500 clones were picked of which 450 were screened by Southern analysis. We used a *PstI* digest which gives a 9 kb fragment for the wild type allele and a 6.3 kb fragment for a *Cftr* allele with a homologous integration of the construct (Figure 1C). The probe is located 5' of the homologous area outside the construct. None of the 450 clones showed any other band than the 9 kb fragment from the wild type allele (results not shown). Hybridization with a probe from the Neomycin gene showed bands of various sizes in all of the clones.

Thus, we did not find a homologous integration of the targeting construct into the mouse *Cftr* exon 10 area with a targeting construct derived from Balb/c DNA.

Homologous replacements experiments have been performed with homologous areas as small as 243 bp (9) but generally should be 2 kb or longer (5), thus our construct was theoretically not too short.

Our inability to find a targeting event with the Balb/c construct was explained when it was found that targeting efficiency is dependent on the source of the DNA used for the construct (8). We had used DNA originating from a Balb/c mouse lambda library while the embryonic stem cells are derived from a 129/Ola mouse strain. Sequence variations between those mouse strains are on average about 0.5 %. With a mouse exon 10 probe, we isolated lambda clones from a 129/Ola library and a 9 kb *PstI* fragment was cloned and partially sequenced. In total, 1678 nucleotides of both mouse strains could be compared and revealed striking sequence divergences. The overall sequence divergence was 2.4 % and consisted both of mismatches as well as unmatched nucleotides. The highest percentage of divergence was 5' of exon 10, primarily due to the different length of a CT stretch. In Balb/c DNA there was a stretch of 30 CT's, including 2 CA's, while 129/Ola DNA had 9 CT's less (Figure 2).

```
Balb/c  GCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCCCA
CTCTGTGTGTATAC
        ||  |||  ||||||||||||||||||  ||||||||||  |  ||
||||||||||||||
129/Ola GCACTCACTCTCTCTCTCTCTCTTTCTCTCTCTC  CACT
```

Figure 2. Sequence divergence. 5' of exon 10 is a highly divergent sequence between the Balb/c DNA of the targeting construct and the endogenous 129/Ola DNA of the mouse *Cftr* gene.

As the sequence divergence was so extensive between the two mouse strains we decided to generate 129/Ola derived constructs. These targeting vectors were basically the same as the Balb/c targeting vector. The constructs consisted of a 5.7 kb *BamHI-NsiI* fragment homologous to the exon 10 area which was mutated with either the introduction of the ΔI507 or the ΔF508 mutation (Figure 3A) both tagged with a diagnostic *SspI* restriction site (Figure 1B). In the plasmid, a cassette of PGKTK and PGKNeo was placed. For the first transfection series, the ΔF508 construct was linearized with *NsiI*; 5' of the linearization site is an area of 2.3 kb homology and 3' is an area of 3.4 kb homology. The transfection and isolation was performed in the same way as for the Balb/c construct. We screened the clones with a *PstI* digest distinguishing between a wild type allele of 9 kb and a targeted

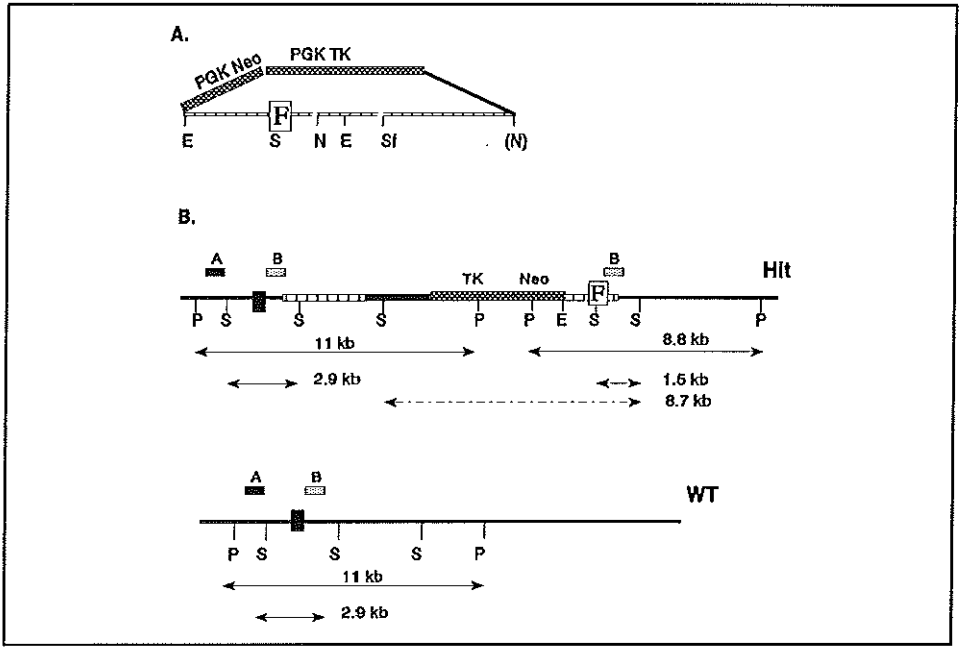

Figure 3. Targeting with 129/Ola construct. A genomic construct of 5.7 kb containing a ΔF508 or a ΔI507 exon was linearized at the *NsiI* site or at the *SfiI* site. B. Correct integration leads to a 11 kb *PstI* fragment while a normal allele generates a 9 kb fragment, detected with a 5' probe A. Hybridization with the internal probe B gives an additional 8.8 kb fragment from the 3' duplication. Detection of the diagnostic *SspI* fragment with internal probe B gives a 1.5 kb fragment in case of correct integration, 2.9 kb fragments from the 5' side and from the normal allele and a 8.7 kb fragment if the diagnostic *SspI* site is lost. E=*EcoRI*, S=*SspI*, N=*NsiI*, Sf=*SfiI*, (N)=disrupted *NsiI*, P=*PstI*.

allele of 11 kb, using the H10 probe, 5' and outside of the targeting construct (Figure 3B). Out of a total of 800 clones analyzed, 68 clones showed both a wild type allele and a targeted allele of 11 kb in a 1:1 ratio (Figure 4A). This targeting frequency of 8 % is very high compared to the efficiencies reported for replacement of the exon 10 area with constructs of similar size, which are in the 0.3-0.04 % range (6, 7, 11). When the Hit clones were hybridized with a neomycin probe, only the 11 kb fragment hybridized, indicating that no other, random, integrations of the targeting construct were present (results not shown). The positive clones were checked for correct integration of the 3' site by using the internal probe B on a *PstI* digest, which hybridizes with both the 3' and 5' duplication in the targeted

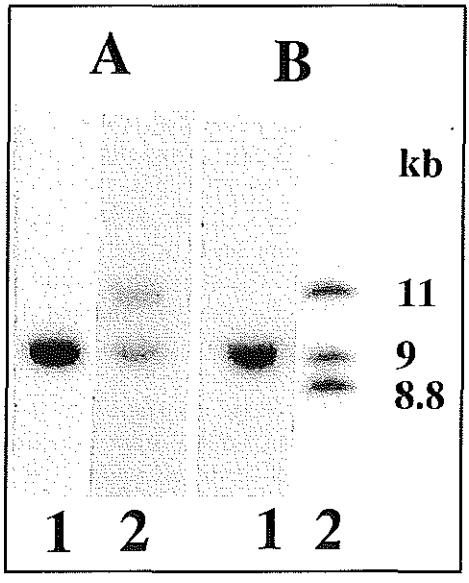

Figure 4. Genomic analysis of Hit clones. Hybridization of a *PstI* digest with the 5' probe A shows a 11 kb fragment from a targeted allele and a 9 kb fragment from the normal allele in Hit clones (lane 2). A wild type clone only has a 9 kb fragment (lane 1). Hybridization with the internal probe B additionally shows the 8.8 kb *PstI* fragment from the 3' duplication.

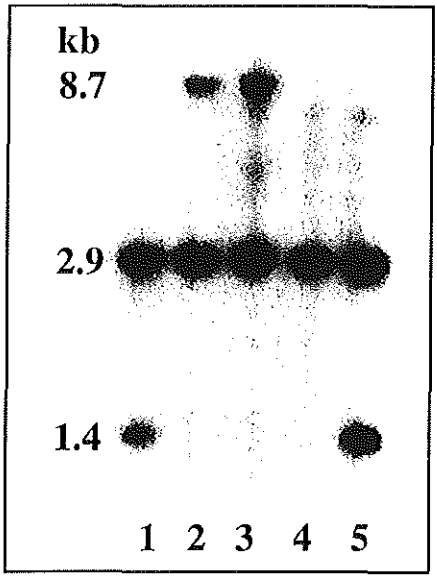

Figure 5. Detection of the diagnostic *SspI* site. Hybridization of an *SspI* digest with the internal probe B shows the 1.4 kb fragment from clones with a correct integration of the construct and a 2.9 kb fragment of the normal allele (lanes 1 and 5). Lanes 2 and 3 show an aberrant *SspI* fragment of 8.7 kb caused by the loss of the diagnostic *SspI* fragment. Lane 4 is a wild type ES cell clone with the normal 2.9 kb *SspI* fragment.

allele. Also an *SspI* digest was done to check whether the ΔF508 mutation was accurately integrated (Figure 3B). The clones showed the integration of the complete construct since in addition to the 9 kb wild type *PstI* fragment and the 5' duplicate of 11 kb, also a 8.8 kb fragment from the 3' duplicate was detected (Figure 4B). However, the diagnostic *SspI* site next to the introduced mutation, was absent in 84 % of the clones analyzed and the detected fragment of 8.7 kb was formed through the use of an *SspI* site in the plasmid sequences further upstream (Figure 3B and 5). This means that during the integration process the diagnostic *SspI* site was lost. During integration, a heteroduplex DNA intermediate is formed, a Holliday junction, containing paired DNA strands from the construct and the chromosomal DNA. Branch migration of the Holliday junction can happen over several kilobases. The *NsiI* linearization site where the Holliday junction starts is

600 bp from the mutation and well within this range of branch migration. Normally, one would expect a mismatch from aligning DNA strands to be repaired at random. Since we found 84 % of the clones to have lost the *SspI* site, this implicates preferential repair towards the endogenous strand. In mammalian cells repair is mostly done through gap formation and repair (1, 13). To ensure the correct repair of the mismatch, the system checks which is the lagging strand since the mismatch is most likely to be caused by aberrant synthesis of the DNA during replication. The lagging strand has a break in the replication fork. In our case, the break is at the linearization site in the targeting construct. Therefore, this strand is deleted from the mismatched gap until the break, deleting also the *SspI* site and the ΔF508 mutation, and a new strand is synthesized with the endogenous leading strand as the mould.

To reduce the effect of this repair mechanism we chose a linearization site further downstream from the exon. A *SfiI* site 1600 bp downstream from the exon and was used to linearize both a construct with the ΔF508 mutation and a ΔI507 targeting construct. Transfection and analysis of the clones was performed as described, showed a targeting efficiency of 5 % for both constructs. Out of 13 targeted clones analyzed, only two had lost the *SspI* site and the remainder were correct Hit clones. Thus, the increased distance resulted in less repair of the *SspI* site without a clear difference in integration efficiency (Table I).

Both for the ΔF508 and for the ΔI507 mutation we have Hit clones with a correct integration of the construct and no other random integrations of the targeting vector. Karyotyping of the clones showed that few had aberrant chromosome numbers, those were excluded from further experiments, which left 5 Hit clones for the ΔF508 and 5 Hit clones for the ΔI507 mutation. The results of the subsequent Run step to resolve the duplication into a ΔF508 allele are described in Chapter V.

Source DNA:cut at	Targeting efficiency	Loss of mutation
Balb/c DNA:		
NsiI site	0 % (0/500)	-
129/Ola DNA:		
NsiI site	8 % (68/800)	84 % (21/25)
129/Ola DNA:		
SfiI site	5 % (13/279)	20 % (2/11)

Table I. Comparison of number of correct Hit clones obtained with two sources of genomic DNA and the frequencies of loss of the *SspI*/ΔF508 mutation with two different linearization sites in the targeting construct.

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CHAPTER V

A MOUSE MODEL FOR THE CYSTIC FIBROSIS ΔF508 MUTATION

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(EMBO J. 14: 4403-4411, 1995)

The EMBO Journal vol.14 no.18 pp.4403–4411, 1995

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

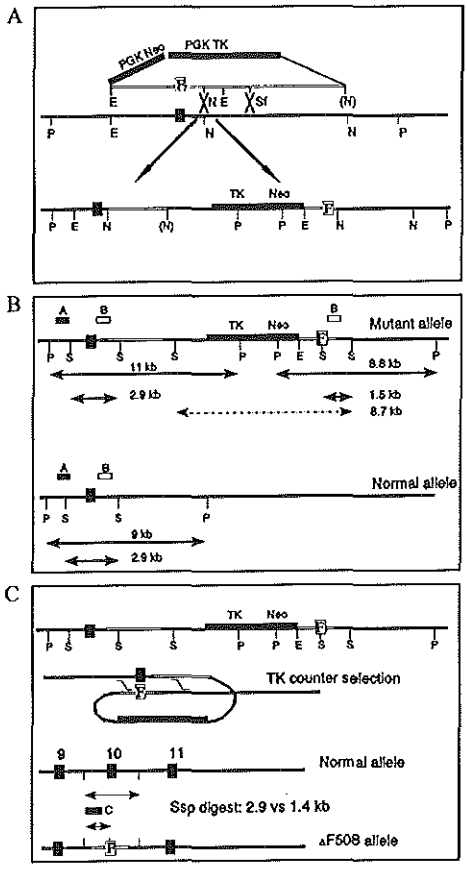

Fig. 1. Hit and Run procedure. **(A)** The 5.7 kb targeting construct containing exon 10 with the ΔF508 mutation was linearized at the *Nsi*I or the *Sfi*I site and transfected into ES cells which were then selected for the expression of the neomycin gene. The DNA of the construct aligns with the endogenous *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.

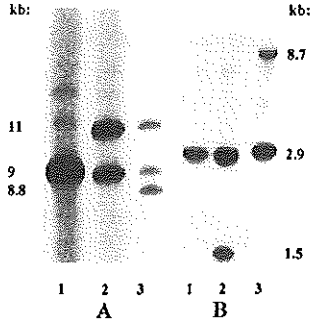

Fig. 2. Southern blot analysis of Hit clones. **(A)** *Pst*I digest of E14 control DNA (lane 1) and Hit DNA (lane 2) hybridized with probe A lying outside of the transfected construct. Lane 3 is as lane 2 but hybridized with the internal probe B. **(B)** *Ssp*I digests hybridized with probe B to check 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 *Sspl* 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 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 *Sspl* fragment, generated by an *Sspl* site outside of the integrated construct and the *Sspl* 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 *Sspl* 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 *Sspl* 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

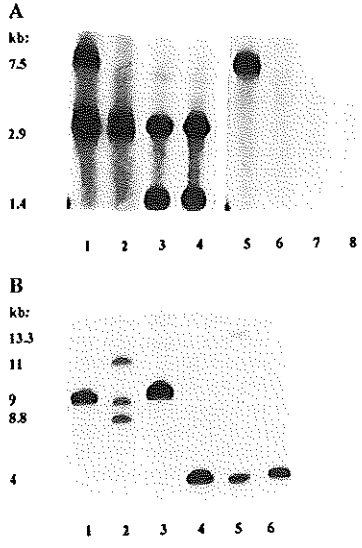

Fig. 3. Southern blot analysis of Run clones hybridized with probe C, 5′ of exon 10. (A) *Sspl* digests of Hit clone DNA (lane 1), E14 control DNA (lane 2) and two Run clones (lanes 3 and 4). Lanes 5–8: the same blot hybridized with a neomycin probe demonstrating the absence of PGKNeo in the Run clones. (B) *PstI* digests (lanes 1–3) and *NsiI* 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.

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

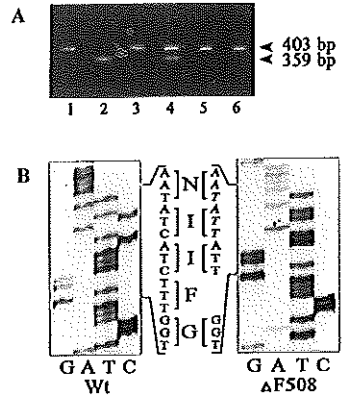

Fig. 4. (A) A RT-PCR was performed with intestinal RNA of a ΔF/ΔF mouse (lanes 1 and 2), a N/ΔF mouse (lanes 3 and 4) and a N/N mouse (lanes 5 and 6) with primers located in exon 8 and 10. The 403 bp PCR products (odd lanes) were digested with *Sspl* (even lanes) which cuts the products of a ΔF508 allele 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 *Sspl* 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 *Sspl* 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 *Sspl* 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

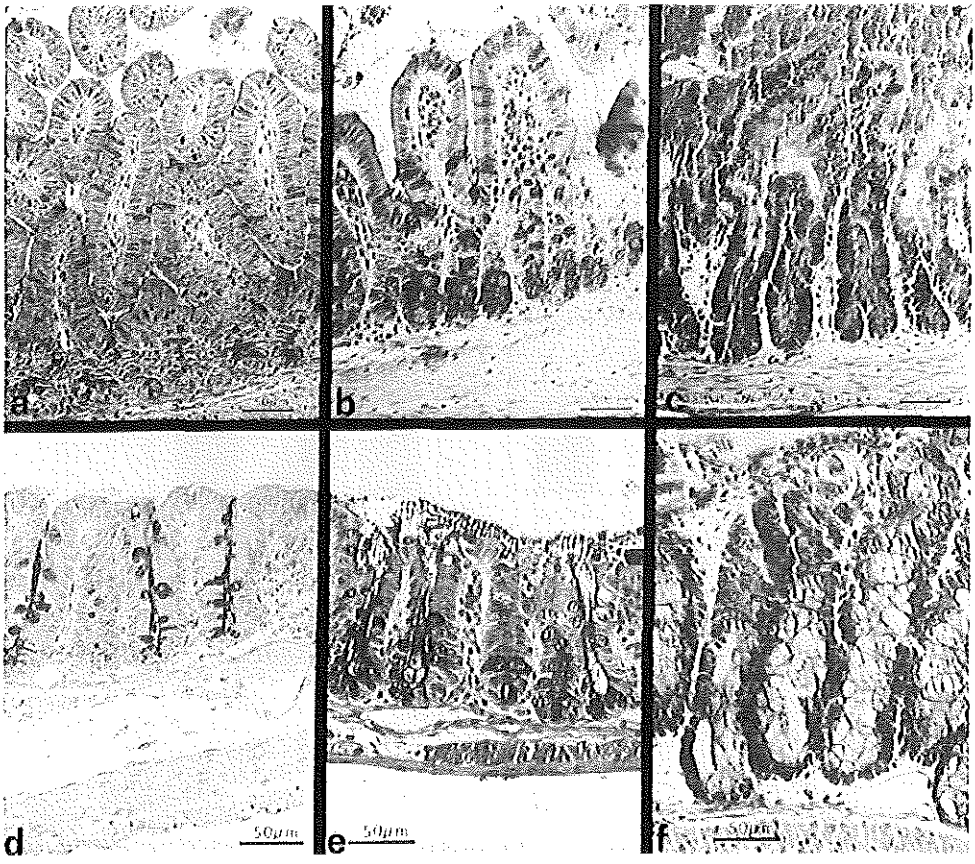

Fig. 5. Histopathology of ΔF508 mice. Sections of the small intestine (**a–c**) and colon (**d–f**) of normal (littermates of ΔF/ΔF) (a and d), ΔF/ΔF (b and e) and *cftr*^{Cam} knock-out mouse (c and f). In the ΔF/ΔF mice, focal hypertrophy of goblet cells was found in crypts of the small intestine (b), 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/*Sspl* site, 84% of the Hit clones had lost the diagnostic *Sspl* 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 *Sspl* 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/*Sspl* 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

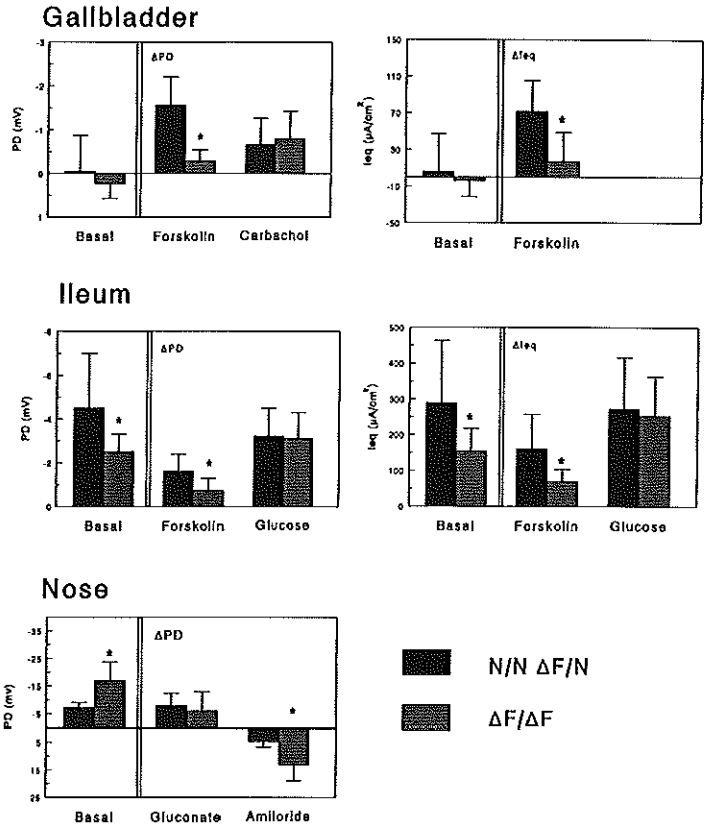

Fig. 6. Summary of the electrophysiological data obtained from six ΔF/ΔF, five ΔF/N and three N/N mice. Left panels: PD, right panels: Ieq. Top: gallbladder. The basal PD and Ieq as well as the peak response to carbachol of ΔF/ΔF mice and normal (N/N or ΔF/N) littermates are comparable. Forskolin (10^{-5} 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

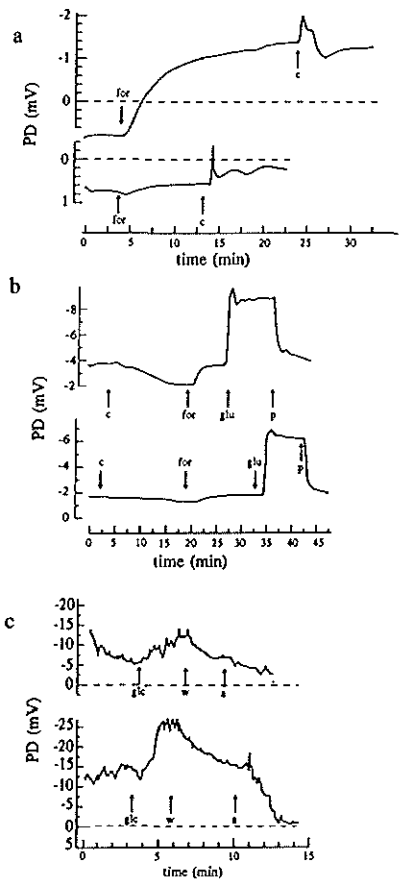

Fig. 7. Representative Ussing chamber tracings from normal mice (upper tracings) and ΔF/ΔF mice (lower tracings) tissue. (a) Gallbladders of ΔF/ΔF mice had a reduced response to forskolin (for) compared with normal littermates, whereas peak responses to carbachol (c) were similar. (b) Ilea of ΔF/ΔF mice 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 $cftr^{mHGU}$ 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 $cftr^{mICam}$ 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 $cftr^{mICam}$ 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.

Acknowledgements

We thank Frank Grosveld for valuable advice and critically reading the manuscript and Professor H.Galjaard for supporting the project. We also thank Rosemary Ratcliff, William Colledge and Martin Evans for providing us with the Cambridge knock-out mice, Dr Martine Jaegle for an introduction to the blastocyst injection techniques, Ellen van Drunen for performing karyotype analyses, Stephan van de Eijnde and Mirko Kuit for photomicroscopical assistance and Dr P.G.M.Mulder for help with the statistical analysis. The project was funded in part by HGO\ TNO, SLW and the Clinical Genetics Foundation.

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Received on May 12, 1995; revised on June 29, 1995

CHAPTER VI

A ΔF508 MUTATION IN MOUSE CFTR RESULTS IN A TEMPERATURE SENSITIVE PROCESSING DEFECT *IN VIVO*.

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(Journal of Clinincal Investigation, in press)

Abstract
The most prevalent mutation (ΔF508) in cystic fibrosis patients inhibits maturation and transfer to the plasma membrane of the mutant cystic fibrosis transmembrane conductance regulator (CFTR). We have analysed the properties of a ΔF508 CFTR mouse model, which we described recently. We show that the mRNA levels of mutant CFTR are normal in all tissues examined. Therefore the reduced mRNA levels reported in two similar models may be related to their intronic transcription units. Maturation of mutant CFTR was greatly reduced in freshly excised oviduct, compared to normal. Accumulation of mutant CFTR antigen in the apical region of jejunum crypt entrerocytes was not observed, in contrast to normal mice. In cultured gallbladder epithelial cells from ΔF508 mice, CFTR chloride channel activity could be detected at only two percent of the normal frequency. However, in mutant cells that were grown at reduced temperature the channel frequency increased to over sixteen percent of the normal level at that temperature. The biophysical characteristics of the mutant channel were not significantly different from normal. In homozygous ΔF508 mice we did not observe a significant effect of genetic background on the level of residual chloride channel activity, as determined by the size of the forskolin response in Ussing chamber experiments. Our data show that, like its human homologue, mouse ΔF508-CFTR is a temperature sensitive processing mutant. The ΔF508 mouse is therefore a valid *in vivo* model of human ΔF508-CFTR. It may help us to elucidate the processing pathways of complex membrane proteins. Moreover, it may facilitate the discovery of new approaches towards therapy of CF.

Introduction.

Cystic Fibrosis is the most common lethal genetic disease in the caucasian population [1, 2]. It is caused by mutations in the gene encoding the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) [3]. CFTR is a cAMP regulated chloride channel, which is expressed in the apical membrane of many epithelia. The most common mutation in CF patients is a deletion of a phenylalanine at position 508 (ΔF508) [4]. This mutation affects correct processing of the protein to its mature, fully glycosylated form [5, 6]. The ΔF508 form of CFTR is retained in the endoplasmic reticulum and degraded, and does not reach the apical plasma membrane [7, 8]. However, ΔF508-CFTR can function as a cAMP regulated chloride channel, both in the endoplasmic reticulum [9] and when expressed on the apical plasma membrane. The latter occurs when ΔF508-CFTR expressing cells are cultured at low temperatures [10] and when Vaccinia expression vectors are used [11]. Cells cultured at lower temperatures, Sf9 insect cells [12] and *Xenopus* oocytes [13], also express functional ΔF508-CFTR on the plasma membrane when transduced with a ΔF508-CFTR expression vector. The clinical significance of this observation is that it may lead to a new approach towards the therapy of CF based on relieving the processing defect. In an attempt to facilitate research in this field we have

recently reported the generation of a mouse model with the ΔF508-CFTR mutation using the 'hit and run' mutagenesis procedure [14]. In this model the intron structure is not disturbed in contrast to two similar models presented [15, 16]. In our previous studies we demonstrated severely reduced chloride permeability in several epithelial tissues, confirming a CF phenotype. However, in Ussing chamber experiments with intact gall bladder and intestinal tissue from mutant mice we observed a small residual cAMP regulated chloride secretion, possibly due to the presence of some functional ΔF508-CFTR [14]. Our present study shows that the mouse ΔF508-CFTR is not processed efficiently to the fully glycosylated form *in vivo*. However, the mutant protein is expressed as functional chloride channels in the plasma membrane of cells cultured at reduced temperature. Furthermore, we could show that the electrophysiological characteristics of the mouse ΔF508-CFTR channels were indistinguishable from normal.

Methods.

Animals. All animal experiments were performed according to the guidelines issued by the Dutch government concerning animal care. Mice with the ΔF508 mutation were described by Van Doorninck et al. [14]. Mice with a targeted disruption in the CFTR gene ($cftr^{tm1cam}$ knockout mice , CF-/-) resulting in complete loss of function were obtained from Dr Ratcliff et al. [17]. All animals were bred under pathogen-free conditions in our transgenic unit. The genotype of each individual animal was tested by Southern blotting of tail DNA. The CF-/- animals in our facility display less severe runting and lower mortality due to intestinal obstruction as reported for the Cambridge colony, which may be due to breeding conditions. All physiological parameters measured, nasal PD's, intestinal and gall bladder PD measurements [14] (Peters, submitted) confirmed the CF-/- phenotype of these animals. The animals used for the experiments reported here were 5-6 weeks old, without obvious signs of disease or discomfort, with an average weight of 23± 3 g. All experiments involving ΔF508 mice were performed with the strain in 129/FVB genetic background [14] using littermates as control in parallel experiments. The experiments presented in figure 7 were performed with three different ΔF508 CFTR mouse colonies as described in text.

Quantitative PCR analysis. Two mouse specific oligonucleotides MCF2 (5'-AATGACCACAGGCATAATC-3'), and MCF3 (5'-CAACACTCTTATATCTGTAC-3') were synthesized. The sequences are localized in exons 8 and 10 respectively and generate a mouse CFTR mRNA specific 403 bp fragment in RT-PCR analysis. Total RNA was isolated by extraction of various mouse tissues or cultured cells in guanidine isothiocyanate and centrifugation through a 5.2 mol/l CsCl step-gradient. The RNA was reversed transcribed to cDNA by adding 10 units Avian myoblastoma virus reverse transcriptase (AMV-RT) in 20 µl cDNA buffer (7 µg heat denatured RNA, 0.8 µmol/l antisense

oligonucleotide primer MCF3, 10^{-3} mol/l dNTP's, 0.05 mol/l KCl, 0.02 mol/l Tris-HCl pH 8.4, 0.0025 mol/l $MgCl_2$, 0.1 g/l bovine serum albumin and 20 Units of RNasin) for one hour at 37°C. The 50 µl PCR assay contained $0.3.10^{-6}$ mol/l sense and antisense oligonucleotide primer (MCF2 and MCF3, $0.2.10^{-3}$ mol/l dNTP's, 4 µl cDNA mix, 0.05 mol/l KCl, 0.02 mol/l Tris-HCl ph 8.4, $2.5.10^{-3}$ mol/l $MgCl_2$, and 0.1 g/l bovine serum albumin. After denaturing at 94°C, 5 units Taq polymerase was added at 72°C. Standard cycling program: 30 cycles of denaturation for 2 minutes at 93°C, primer annealing for 2 minutes at 47°C, and elongation for 4 minutes at 72°C. Aldolase A oligonucleotide primers, which amplify a 442 bp cDNA fragment, were as in Bremer et al. [18]. To check the quality of the RNA and the PCR procedure, the amplification of aldolase A sequences was done in parallel, under the same conditions as described above for CFTR. PCR products were separated on a 1.5% agarose gel . For quantitative analysis of the PCR reaction, equal amounts of total RNA isolated from different tissues were subjected to an RT-PCR protocol in parallel incubations. Aliquots were withdrawn at regular intervals from the PCR and subjected to Southern blotting. Radioactivity was measured with a Phosphorimager (Molecular Dynamics).

Western blot analysis of oviduct. Female mice of different phenotypes were anaesthetized with ether. The lower abdomen was opened and the oviducts were dissected. Following rapid excision the oviducts from one mouse were pooled and immediately solubilized by vortexing followed by brief sonication in 30 µl of modified Laemmli sample buffer (Tris-HCl 0.06 mol/l, 2% (w/v) SDS, 15% (w/v) Glycerol, 2% (v/v) ß-mercaptoethanol, 0.05 g/l leupeptin, 0.05 g/l soybean trypsin inhibitor, 0.03 g/l phosphoramidon, 10^{-4} mol/l Pefabloc (Boehringer, Mannheim), 10^{-3} mol/l benzamidine, 0.1% (w/v) bromophenol-blue, pH 6.8). The samples were incubated for ten minutes at 37°C and centrifuged (2 min, 8000 g). Samples of the supernatants (10 µl, containing about 20 µg protein) were separated on 6 % poly-acrylamide slabgels using a Bio-Rad Miniprotean apparatus. Proteins were subsequently electroblotted onto nitrocellulose paper (0.1 µm pore size, Schleicher and Schuell) in 0.025 mol/l Tris, 0.192 mol/l Glycine, 20 % (v/v) methanol. The blots were then incubated overnight at 4°C with 0.02 mol/l Tris-HCl, 0.5 mol/l NaCl, 0.05% (w/v) Tween20 (TTBS), followed by an overnight incubation at 4°C with a 1:3000 dilution of affinity purified antibody R3195 in TTBS. The blots were washed three times in TTBS, incubated with peroxidase conjugated anti-rabbit IgG (Tago, Burlingame; 1:3000 in TTBS for 2 hrs), and washed four times with TTBS. Peroxidase activity was detected with bioluminescence reagents (Amersham, Braunschweig) on X-ray film. The rabbit polyclonal antibody R3195 was developed against the 13 aminoacid C-terminal peptide sequence of rodent CFTR, conjugated to bovine thyroglobulin. The antibody was affinity-purified on a peptide-epoxide activated Sepharose column, eluted with 4.9 mol/l $MgCl_2$, dialysed and concentrated.

Immunocytochemistry. Non-fasted mice were killed after inhalation anaesthesia. Intestinal tissues were quickly removed and frozen in OCT embedding medium (Miles Lab., Elkhardt, In.) using liquid nitrogen cooled 2-methylbutane. Cryosections (5 μm) were fixed with 3 % (w/v) paraformaldehyde (10 min.) and methanol (20 min), washed with phosphate buffered saline supplemented with 1% bovine serum albumin and protease inhibitors before incubation with the anti CFTR antibody R3195 (1/500). Antibody labelling was detected with FITC conjugated anti-rabbit F(ab)2 fragments (Boehringer, Mannheim, Germany).

Gall bladder epithelial cell culture. Gall bladders were removed from the animal. After the bile was washed out with RPMI-1640 medium supplemented with 100 U/ml penicillin, 0.1 g/l streptomycin, and 0.02 mol/l Hepes pH 7.2, the gallbladders were cut into small pieces and embedded in 1 mm collagen gels (Sigma, Calf skin type I). The explant cultures were incubated at 37 °C in a humidified 10% CO_2/air mixture in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100 U/ml penicillin, 0.1 g/l streptomycin, 0.002 mol/l glutamine, and 10% fetal calf serum. Mouse gall bladder epithelial cells could be cultured for up to 3 weeks, with medium changes every 2-3 days.

Patch clamp analysis. Patch clamp experiments, data sampling and analysis were performed as previously described [19]. Patch pipettes, borosilicate glass (Clark GC150-TF) were pulled to a resistance of 3-8 MOhm. Excised patches were transferred to a solution exchange compartment [19]. This allowed us to add various substrates (ATP, protein kinase A) and to change the buffer composition to a I^-, F^-, $NO3^-$, Br^-, or gluconate buffer. Pipette (external) and bath (internal) solutions contained (mol/l) 0.14 NaCl, 5.10^{-3}KCl, $1.2.10^{-3}$ $MgCl_2$, 0.15. 10^{-3} $CaCl_2$, 10^{-3} EGTA and 5.10^{-3} Hepes. Final Ca^{2+} concentration was 10^{-8} M, pH 7.4. Low chloride buffer was identical except that it contained 0.14 mol/l Na-gluconate instead of NaCl, except for the low chloride pipet buffer used to analyse normal mouse CFTR. This buffer contained (mol/l) 0.140 N-methyl-d-glucamine (NMDG), 5.10^{-3} $CaCl_2$, 2.10^{-3} $MgCl_2$, 10^{-2} Hepes and 0.1 D-Aspartic acid adjusted to pH 7.2, final chloride concentration 49.10^{-3}. High (0.427 mol/l) chloride buffers contained 0.420 mol/l NaCl. In other buffers 0.140 mol/l NaCl was replaced with either NaI, NaBr, $NaNO_3$, NaF, or Na-Gluconate, all 0.140 mol/l. All experiments were performed at 37°C. A List LM-EPC 7 (Darmstadt, Germany) amplifier was used for current amplification and voltage clamping. Data were digitalized (Sony PCM-F1) and stored on videotape. For analysis, data were filtered at 50 Hz, sample frequency 100 Hz, and transferred to a personal computer (Tulip 386sx). Pipette potential refers to the voltage applied to the pipette interior with respect to the (grounded) bath. Positive (upward) currents represent negative charge flowing out of the pipette. $V_{I=0}$ was corrected for the liquid junction potential. Experiments presented in figure 6 were performed blinded with respect to the genotype of the cells under study.

Ussing chamber experiments. Mouse gallbladder and caecum were mounted in an Ussing chamber, basal electric potential and responses to forskolin (10^{-5} mol/l) were determined as described by van Doorninck et al.[14]
Statistical analysis. Significance analysis was tested with linear regression analysis for the current-voltage data presented in figure 5, and with a Student's t-test (unequal variance) for the channel frequency determination (figure 6)

Results.
ΔF508 mice have normal CFTR mRNA levels. The ΔF508 mouse model from our laboratory was generated by the 'hit and run' procedure. With this method the mutation is introduced in an otherwise normal mouse *cftr* gene. Therefore, CFTR mRNA transcription and processing rates are expected to be normal. This is particularly important as low ΔF508-CFTR mRNA levels were reported in tissues of two other ΔF508-CFTR mouse models which were made while introducing a selectable marker in the intron structure [15,16]. In order to investigate this we have performed quantitative RT-PCR analysis with RNA isolated from heterozygous and homozygous ΔF508 mice. As controls we have used normal littermates and homozygous *cftr* knockout mice [17]. The PCR product from the mutant allele contains an *SspI* restriction site which is not present in the wild type allele [14]. This allowed us to accurately determine the ratio of normal and mutant CFTR mRNA in a single PCR experiment with RNA from a heterozygous animal. With this approach we can rule out variations introduced by differences in reaction conditions and quality of RNA preparations. Our data show that the steady state levels of normal and ΔF508-CFTR mRNA are virtually identical in different regions of the intestine and in salivary gland (Fig 1A-C). Therefore, the mutation that we have introduced does not significantly affect mRNA synthesis processing or stability in epithelial tissues. In experiments with RNA isolated from homozygous animals ΔF508-CFTR, mRNA levels did not differ significantly from normal values (Fig 1D-F). This confirms that also in homozygous deficient animals expression of the gene in these tissues is not reduced.
Abnormal processing of ΔF508-CFTR in mouse oviduct. Western blot analysis (N=3) of mouse CFTR from normal oviduct (Fig 2, +/+) showed the characteristic pattern as observed for human CFTR, a core-glycosylated form (band B) and forms containing complex N-linked oligosaccharides (band C) [3]. In oviduct from a mouse with a complete loss of function mutation (Fig 2, -/-) [17] these bands were not observed. Oviduct from a homozygous ΔF508 mouse (Fig 2, ΔF/ΔF) produced a prominent band B and some band C antigen. The ratio of B over C is increased over fifty fold compared to the normal ratio as estimated by scanning the original radiograph. This strongly suggests that processing and subsequent glycosylation of

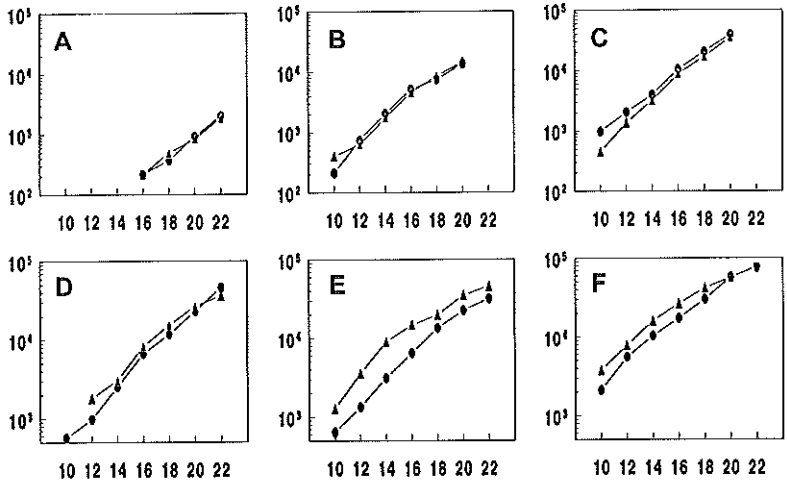

Figure 1. Quantitative analysis of CFTR mRNA expression. Total RNA from normal and mutant mice was isolated from different tissues and subjected to RT-PCR analysis in parallel experiments as described. Specific primers in mouse *Cftr* exons 8 and 10 yielded a 403 bp fragment, which contained an *SspI* site only when the product was initiated on a ΔF508-CFTR mRNA [13]. Samples collected at different cycle intervals were subjected to Southern blotting using the labelled 403 bp fragment as a probe. Data shown here represent the amount of radioactivity per band in arbitrary units. Data from different panels are not corrected for differences in blotting efficiency. PCR products from heterozygous mice (ΔF/+) were digested with *SspI* before Southern analysis (A-C). Data indicate the amount of PCR product from the normal allele (403 bp, ●-●) and from the ΔF508 allele (359 bp, ▲-▲) when RNA was isolated from salivary gland (A), caecum (B) and jejunum (C) of heterozygous mice. PCR products from homozygous normal (●-●) and homozygous ΔF508 mice (▲-▲) were compared in parallel, using RNA isolated from salivary gland (D), caecum (E) and jejunum (F). The data show that there is no significant difference in the levels of mutant and normal CFTR mRNA. RNA from CFTR knockout mice [17] did not produce a PCR product in a parallel experiment whereas the aldolase primers produced comparable amounts of PCR product in all samples tested (not shown).

mouse ΔF508 CFTR is severely affected in this tissue, as described previously for the human form of ΔF508 CFTR [5, 6]. Gallbladder, pancreas and intestine did not give reproducible data with this technique.

Mouse ΔF508-CFTR is not efficiently transported to the apical membrane. Immunocytochemical detection of CFTR antigen in normal jejunal crypt shows intense staining of the apical region of all crypts (N=7, Fig 3 A,B and E, F). This is in accordance with immunocytochemical localization studies published previously for

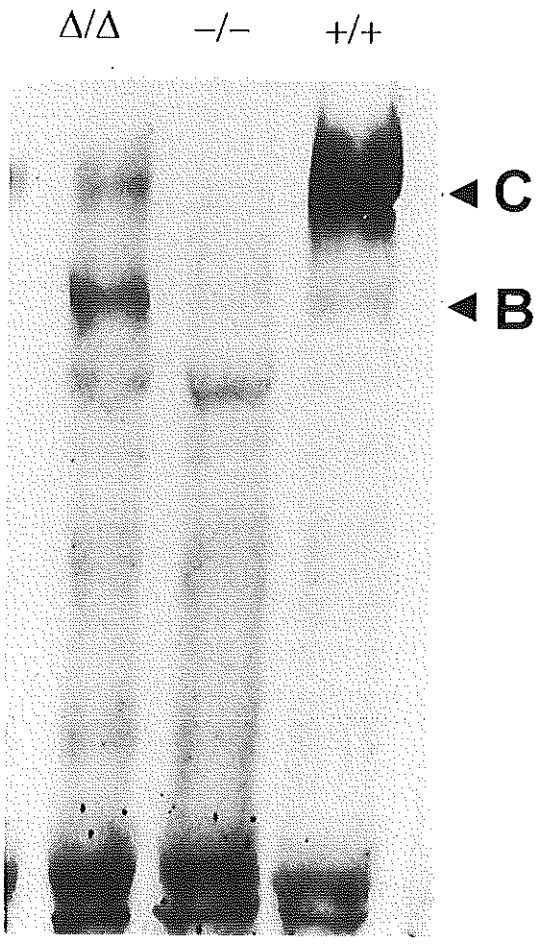

Figure 2. CFTR in mouse oviduct. Oviduct from normal (+/+), homozygous ΔF508 (Δ/Δ), and CFTR deficient (-/-) mice were subjected to Western blot analysis as described in the methods section. C indicates the fully glycosylated form of mouse CFTR antigen, B indicates the core-glycosylated precursor. The data show that ΔF508-CFTR is not glycosylated to the same extent as the normal form.

human intestine [20] . In jejunal crypts from mice with a loss of function mutation [17], no CFTR-specific apical staining is observed (N=6, Fig 3 C,D). This confirms the specificity of the staining. Jejunum crypts of homozygous ΔF508 mice generally showed no clear apical CFTR antigen (N=6, Fig 3 G,H). In some preparations (three out of six) a diffuse cytoplasmic stain was observed in the epithelial crypt cells. The immunocytochemical method used here is characterised by an inherently nonlinear relationship between signal intensity and local antigen density. Moreover the absolute level of staining intensity varies per experiment. Therefore we can neither quantitate the level of correct processing nor the level of antigen expression in homozygous ΔF508 mice with any accuracy. The data presented show that despite the normal CFTR mRNA levels in ΔF/ΔF mice (Fig 1) the amount of apical CFTR antigen in the jejunum crypt is greatly reduced. This is in agreement with our functional studies in intestinal tissue of mouse [14] and human [21] ΔF508-CFTR mutants. Moreover, it is in agreement with immunocytochemical studies in airway cells [22] and in cell culture. [23] Other tissues that we have tested for CFTR specific staining with this method (gallbladder, cystic duct, pancreas) did not show consistent specific staining above background signals observed in tissues from knockout mice.

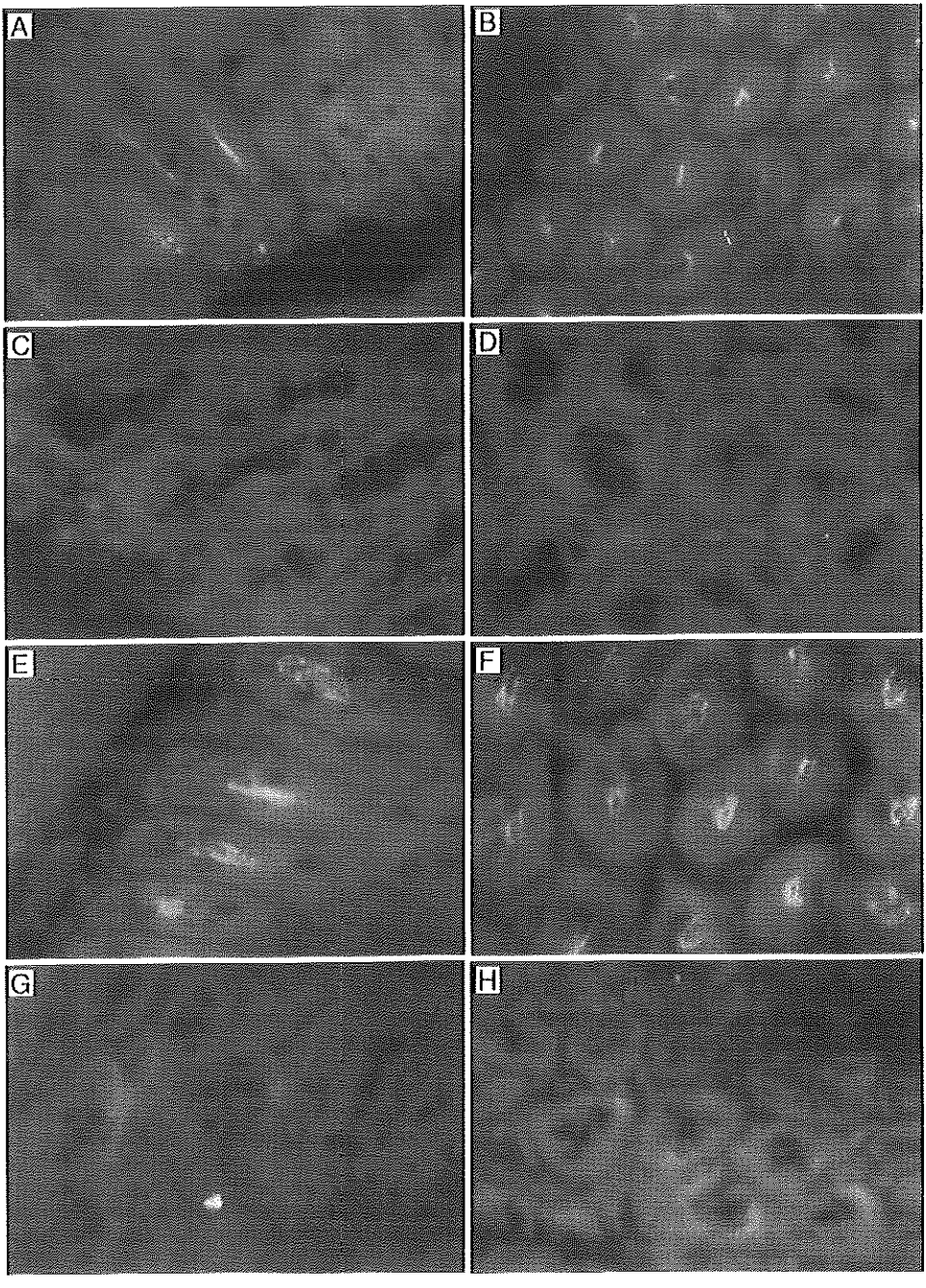
A
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Figure 3. CFTR in mouse jejunum. Immunocytochemical detection of CFTR antigen in jejunal crypts from normal (A-B, +/+ $cftr^{tm1cam}$ colony; E-F, +/+ ΔF508cftr colony), CFTR deficient (-/- $cftr^{tm1cam}$) mice (C-D), and homozygous ΔF508 mice (G-H), and was performed as described in the methods section, using a polyclonal antibody against the murine CFTR carboxyterminal. Normal crypts show intense staining of the apical region of the epithelial cells. This is not observed with crypts from CFTR -/- mice or in ΔF/ΔF mice.

Patch clamp analysis of mouse ΔF508-CFTR. CFTR mRNA is expressed in mouse gallbladder epithelial cells, both *in situ* and in primary culture (Peters, Americ. J. Phys., in press, chapter VIII). We have determined the number and properties of normal and ΔF508-CFTR chloride channels by patch clamp analysis of mouse gallbladder epithelial cells in primary culture. In excised membrane patches from unstimulated normal (+/+ and +/ΔF) cells, a linear 5.1 ± 0.1 pS (SE) could be activated by the addition of protein kinase A in the presence of ATP (Fig 4A). In cell attached membrane patches of forskolin-stimulated normal cells channels were observed at an average of 27.6 ± 16.5 (SEM) per patch (Fig 6A). Excision of the membrane inactivated the channel but it could be reopened by addition of ATP (Fig 4B). Considering the resemblance with human CFTR [3] we conclude that these linear chloride channels represent normal mouse CFTR. The only difference observed between mouse and human CFTR is the lower conductance of the channel (human CFTR 8.0 ± 0.6 pS). [3] In homozygous mutant cells cultured at 37°C the number of functional CFTR channels per patch is about 1% of normal (+/+ and +/ΔF, $P<0.02$) (Fig 6), but not zero. These data confirm our conclusion that the expression of CFTR activity on the plasma membrane of homozygous mutant cells is greatly reduced but not completely absent. [14]

In cells expressing human ΔF508-CFTR an increase of apical channel activity has been observed when cells were grown at reduced temperature, which is associated with a conversion of core- to complex chain glycosylation and increased apical localisation of ΔF508-CFTR. [10] In normal (+/+) and heterozygote (ΔF/+) mouse gall bladder cells grown at 27°C for 3-7 days we observed a twofold decrease in CFTR channel frequency compared to 37°C (Fig 6). In contrast, ΔF/ΔF cells cultured at 27°C show a considerable increase in ΔF508-CFTR channel frequency compared to cells cultured at 37°C ($P<0.05$). The average number of channels per patch was 16 % of the average observed in normal cells cultured at 27°C (Fig 6). The high frequency at which the ΔF508-CFTR channel was observed in cells cultured at lower temperatures enabled us to study its properties in detail. ΔF508-CFTR could be observed in cell attached patches after addition of forskolin. The channel became silent upon excision but could be reactivated by the addition of ATP (Fig 4 D). Excised membrane patches from unstimulated ΔF/ΔF cells were silent, but CFTR-like channel activity was observed by the addition of protein kinase A plus

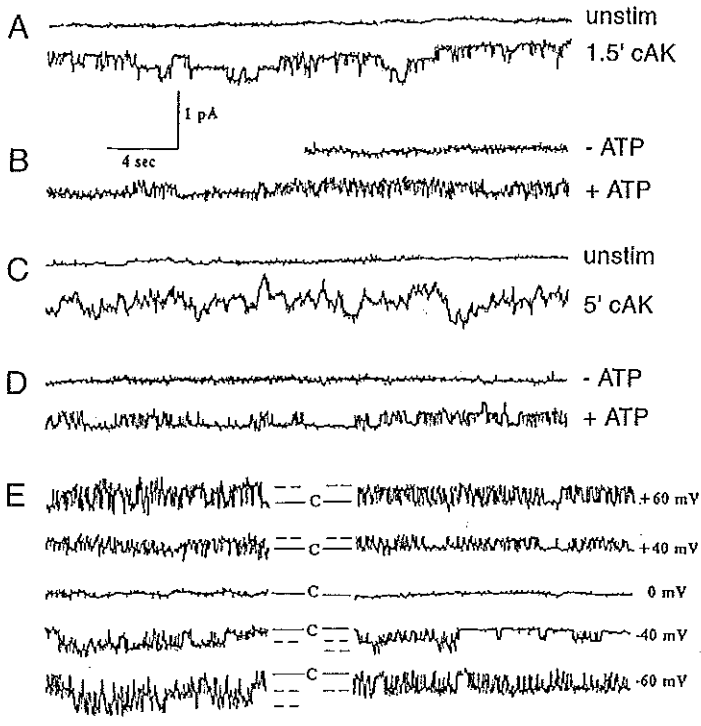

Figure 4. Characterisation of normal and ΔF508 CFTR chloride channel activity. Current tracings of CFTR activity in excised patches from normal (A,B) and ΔF508 (C,D) mouse gallbladder epithelial cells cultured at 27' C. Unstimulated excised, inside-out patches from cultured gallbladder epithelial cells generally showed no channel activity. Upon a short incubation with the catalytic subunit of protein kinase A (cAK, 2 mU/ml) in the presence of 2.10^{-3} mol/l ATP, a low conductance channel was observed in both normal and ΔF508 cells (A resp. C). Active patches of forskolin stimulated cells became silent upon excision (B and D, -ATP), but could be reactivated by the addition of 2.10^{-3} mol/l ATP alone both in normal (B) and ΔF508 CFTR (D, +ATP). E: current tracings at different holding potentials from normal (left tracings) and ΔF508 CFTR (right tracings). Holding potential was +40 mV scale bar as indicated, except for C where the holding potential was + 60 mV, scale bar 4 sec, 1.5 pA.

ATP (Fig 4 C). These data show that mouse ΔF508-CFTR has a normal protein kinase and ATP regulation. Mouse ΔF508-CFTR is a linear 4.9 ± 0.1 pS chloride channel, similar to normal mouse CFTR, 5.1 ± 0.1 (SE) (Fig 5, A-B). Lowering or increasing the chloride concentration in the bath results in a shift in the reversal potential ($V_{I=0}$), indicative for anion selectivity (Fig 5, A-B). The relative cation to anion permeability, P_{cat}/P_{Cl}=0.14 and 0.16 for normal and ΔF508-CFTR respectively (calculated from the least permeant anion, i.e. F^-). When chloride was substitut-

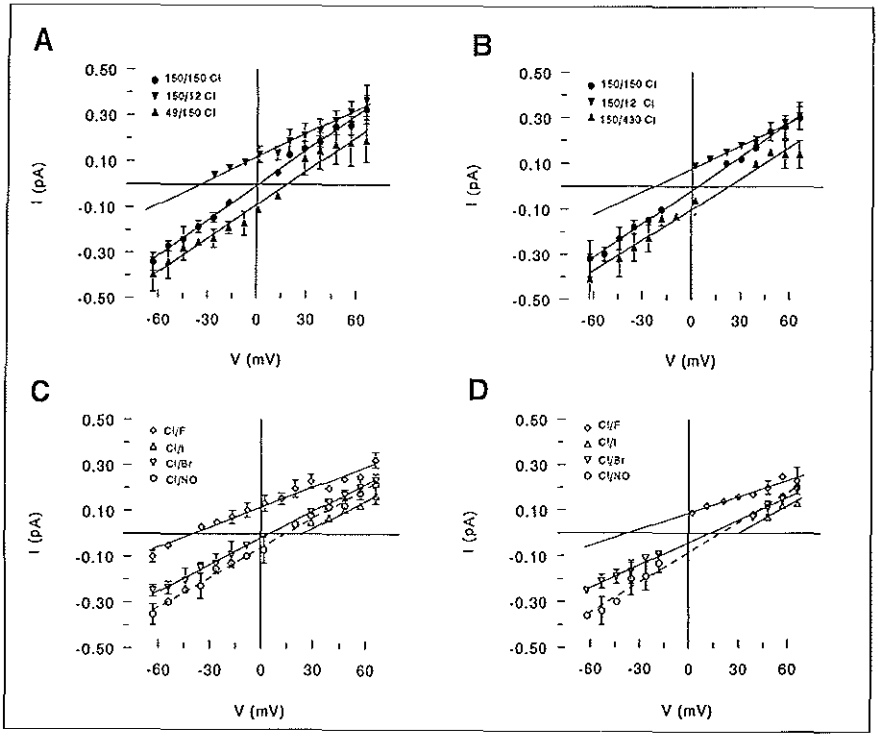

Figure 5. Current-Voltage characteristics and relative anion permeability of normal and ΔF508 mouse CFTR. Current-Voltage relationships of normal (A and C) and ΔF508-CFTR (B and D). At equal pipet and bath chloride concentrations (•-•, 0.150 mol/l) both normal and ΔF508-CFTR show a linear conductance of 5.1 ± 0.1 resp. 4.9 ± 0.1 pS. A shift in the reversal potential ($V_{I=0}$) expected for a chloride channel was observed when bath chloride concentration was higher (▲-▲) or lower (▼-▼) than the pipet concentration (A, B). By replacing chloride in the bath with other anions as indicated (C, D), the relative ion selectivity was determined as NO_3^->Br^->Cl^->>gluconate>F^- for both normal (C) and ΔF508-CFTR (D). Iodine has a high affinity but low permeability in both normal and ΔF508-CFTR, as in human CFTR.

ed for other monovalent anions the relative permeability was determined as NO_3^- >Br^->Cl^->>gluconate>F^- for both normal (Fig 5C) and ΔF508-CFTR (Fig 5D). Iodine had a high affinity but low permeability for both normal and ΔF508-CFTR (Fig 5C and D) which is a characteristic for CFTR. [3] The open probability (Po) of ΔF508-CFTR (0.33 ± 0.04) is comparable to normal mouse CFTR (0.35 ± 0.04) (n=5, values ± SE, excised patches from forskolin activated cells in the presence of ATP). The Po of both normal and mutant CFTR was voltage independent. Channel 'flickering', i.e. the number of closing events during a period of activa-

tion, also did not differ significantly between ΔF/ΔF (3.99 ± 0.70 s^{-1}, n=12) and normal (+/+) cells (3.23 ± 0.55 s^{-1}, n=7 patches, ±SE). We conclude that, similar to human ΔF508-CFTR, trafficking of the mouse ΔF508-CFTR to the plasma membrane is greatly increased at reduced temperature. Moreover, the biophysical properties of the mouse ΔF508-CFTR channel do not differ significantly from the wild type form.

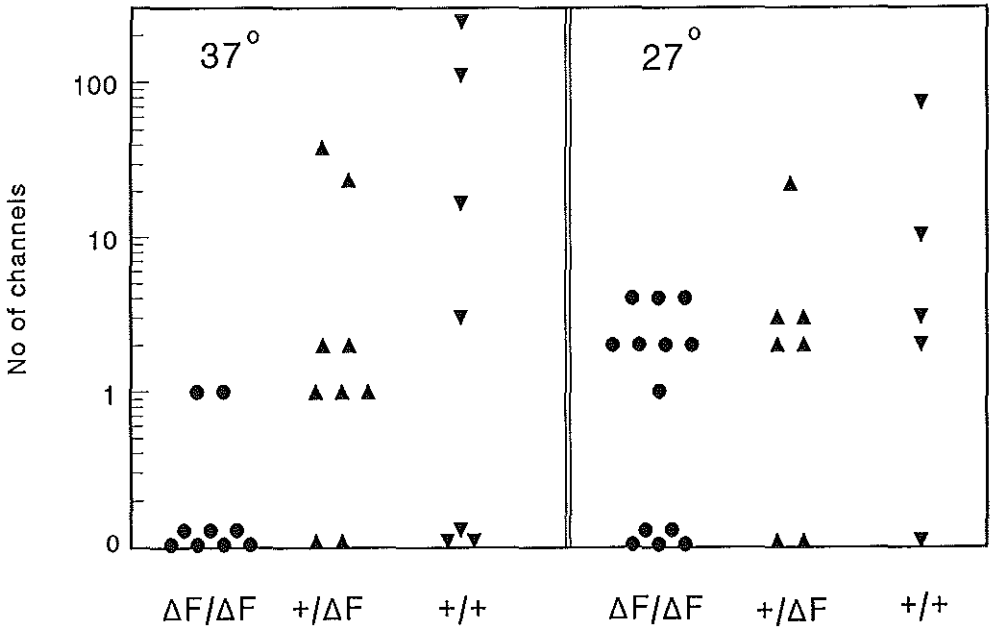

Figure 6. CFTR chloride channels in gall bladder plasma membrane.
CFTR channel activity on plasma membrane of cultured gallbladder epithelial cells was recorded with patch clamp analysis as described. Cells were cultured either at 37° (left panel) or at 27° (right panel) for 3-7 days prior to analysis. Forskolin stimulated cells were recorded for two minutes in the on cell patch clamp configuration, followed by a one to ten minute recording in the excised patch configuration in the presence of 2 mM ATP. CFTR channel frequency was calculated from the equitation $n=I_a/(I_1*Po)$ where n is the calculated number of levels, I_a is the average current in the patch, I_1 is the current of one channel at holding potential, and Po is the open probability. When 4 or less levels of activity were observed in the patch the equitation was not used, in these cases the number of channels correspond directly to the number of current levels observed. Patches which showed no CFTR activity were scored as zero. Cells were obtained from parallel cultures of littermates. ●: homozygous mutant, ΔF/ΔF (cells from 3 mice at 37° and 8 mice at 27°); ▲: heterozygous mutant +/ΔF (3 and 4 mice); ▼: homozygous normal +/+ mice (2 and 1 mouse).

Inefficient processing of mouse ΔF508 CFTR is observed in different genetic backgrounds. The most sensitive available measure of apical CFTR activity is probably the steady state response to forskolin in Ussing chamber experiments. Using this method, we have previously shown low but significant residual ΔF508-CFTR activity in different tissues of homozygous ΔF508 mice when compared to cftr^{tm1cam} knockout mice that showed no activity.[14] Next, we studied whether genetic background influences the level of ΔF508-CFTR processing. The 129*FVB and 129*C57Bl/6 ΔF strains were obtained by introduction of the original mutant clone (129/Ola) in FVB and C57BL/6 blastocysts respectively, ΔF/+ (129*FVB) mice were crossed with cftr^{tm1cam} knockout mice (KO) that are 129*C57BL/6. The forskolin response of gallbladder and caecum of these different ΔF mouse strains was monitored in Ussing chamber experiments. For all strains the forskolin response was equally reduced in homozygous ΔF/ΔF and in ΔF/- mice (Fig 7). All individual measurements from ΔF mutant mice are in the same range with only one exception observed sofar (Fig 7). The data show no evidence for dominant alleles in either the 129/Ola, FVB or C57BL/6 inbred genetic backgrounds that

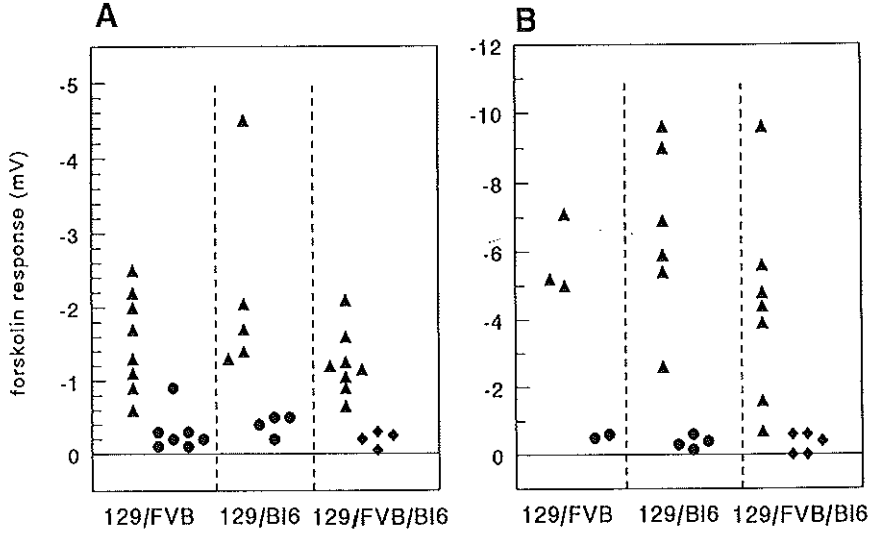

Figure 7. Forskolin responses in homozygous ΔF/ΔF mice gallbladder and caecum in mice strains with different genetic backgrounds. Gallbladder (A) and caecum (B) of mice from ΔF508 CFTR strains with different genetic backgrounds were mounted in the Ussing chamber and monitored for their electrical response to forskolin (ΔPD, mV). 129/FVB and !29/Bl6 were obtained by blastocyst injection and crossing into FVB and C57BL/6 type animals. 129/FVB/Bl6 was obtained by crossing ΔF/+ (129/FVB) with +/- cftrm^{1cam} (129/C57BL6). ▲: +/+, +/Δ and +/- mice; •: ΔF/ΔF mice; ♦ ΔF/- mice.

significantly increase or reduce the level of ΔF508-CFTR processing. However, one homozygous ΔF/ΔF mouse in a 129*FVB background did show a response to forskolin in the normal range using gallbladder (Fig 7A,) and ileum (not shown). Therefore, more complex relationships involving several genetic loci can not yet be excluded and require further study.

Discussion.

CFTR mRNA expression is normal in mutant mice.

Using the 'hit and run' procedure, we have generated a ΔF508-CFTR mouse model without modifying the intron structure of the CFTR gene [14]. Our quantitative PCR data show that in heterozygotes the expression levels of normal and mutant alleles are comparable in intestine and salivary gland. Also in homozygote normal and mutant mice the expression levels are not significantly different. In two other ΔF508-CFTR mouse models created by homologous recombination, low CFTR mRNA levels were reported in salivary glands and intestine [15, 16]. In these models an expression cassette encoding a selectable marker was introduced into the flanking intron, in addition to the ΔF508 mutation in exon 10. Comparison with our data suggests that the presence of this transcription unit affects the CFTR mRNA levels by interfering with RNA polymerase activity or RNA processing.

Maturation of mouse ΔF508-CFTR is abnormal. Immunochemical analysis of CFTR expression in intact mouse tissues is difficult with the available antisera. This can be attributed in part to relatively low expression levels as in gallbladder tissue (Peters et al submitted) and to proteolytic activity, especially in intestinal tissue (De Jonge, unpublished data). This limits the scope of our investigations to tissues that give consistent and reproducible data. We were able to make reproducible western blots of total oviducts from normal and mutant mice with a purified antibody raised against the carboxyterminus of rodent CFTR (Fig 2). The data show that CFTR antigen is produced in both normal (+/+) and mutant (ΔF/ΔF) mice, but not in CFTR deficient (-/-) mice. However, the proportion of mature, fully glycosylated CFTR is severely reduced in homozygous mutant. We conclude that the ΔF508 mutation in the mouse CFTR protein results in a processing defect similar to that observed with human ΔF508-CFTR. The available evidence suggests that transfer to the Golgi system and subsequent full glycosylation of the human mutant protein does not occur, because proper chaperonin-dependent folding is arrested in the endoplasmic reticulum [7, 8, 10]. With the same antibody as used for western blot analysis, we could show specific staining of the apical region of normal mouse jejunum crypt epithelial cells (Fig 3A-B, E,F). Specificity of the staining was verified with serum preabsorbed with the antigenic peptide (not shown) and jejunum of CFTR-deficient mice (Fig 3, C-D). This is in agreement with localisation studies in human intestine [20] and rat. [24] In jejunum crypts of mice homozygous for the ΔF508 mutation we did not observe a preferential staining of

the apical region. Most crypts seemed devoid of specific staining. However, with this method we cannot rule out that cytoplasmic CFTR is present (note that also in control cells no specific cytoplasmic staining above background can be seen Fig 3). In several preparations some crypts did stain more intensely than background but the signal was not concentrated to the apical region as in normal crypts. These observations are in agreement with a processing defect of mouse ΔF508-CFTR that prevents translocation of the protein to the apical region.

Temperature dependent apical targeting of ΔF508-CFTR. Airway cells of ΔF508 mutant mice in primary culture have increased cAMP-induced chloride conductance when cultured at reduced temperature [16]. We were unable to detect CFTR chloride channel activity in primary airway cells using patch clamp single channel analysis (French, unpublished data). In contrast, cultured mouse gall bladder cells provide a convenient system for the study of CFTR with this method. This allowed us to show that the actual frequency of active mutant CFTR molecules at the plasma membrane is increased by incubation of the gallbladder cells at low temperature. In homozygous mutant cells cultured at 37°C few CFTR channels were observed, i.e. 1 % of the normal level at this temperature (Fig 6). The expression of ΔF508-CFTR channels increased to 16 % of normal levels when cells from homozygous mutants were grown at 27°C (Fig 6). A similar behaviour was observed with human ΔF508-CFTR. [10]

Channel activity of ΔF508-CFTR. The channel conductance and anion selectivity of mouse ΔF508-CFTR is in our analysis indistinguishable from normal mouse CFTR (Fig 4). In addition, the open probability (Po) and number of closing events during a burst of activity of ΔF508-CFTR are normal. Studies of human ΔF508-CFTR are inconsistent at this point as two groups reported normal conductance but reduced Po for human ΔF508-CFTR [10, 11] whereas others claimed a normal conductance and Po. [25] Since the activity of the CFTR chloride channel depends on a number of parameters in particular the state of phosphorylation of the protein, the reported difference in relative Po may result from differences in experimental conditions.

Residual activity of ΔF508-CFTR. Both Ussing chamber experiments [14] (Fig 7), and patch clamp analysis (Fig 6) in our model indicated a low but significant level of apical activity of mouse ΔF508-CFTR at physiological temperature. This is confirmed by the western blot analysis (Fig 2), which shows a low level of fully glycosylated CFTR. Interestingly, a minority of human ΔF508 CFTR homozygotes possess a small residual intestinal chloride permeability, which correlates with a relatively mild clinical status [21]. Unpublished data showed that in some, but not all, cases this activity is insensitive to the channel blocker DIDS (H. Veeze *pers. commun.*). This would suggest that in these cases the residual activity is due to ΔF508-CFTR. It is possible therefore, that a significant level of correct processing is also present in a subgroup of human ΔF508 CFTR homozygotes. Immunocytochemical analysis of airway cells from ΔF508 CFTR patients seem to confirm this

[26]. It will be of great interest to analyze the genetic and environmental factors which contribute to this phenomenon. Our current data indicate that the level of mutant CFTR activity, as determined by Ussing chamber experiments, is quite constant in the different genetic backgrounds tested (Fig 7). The one exception to this rule we found sofar is intriguing, but extensive further studies are required to establish a genetic basis for this effect. The level of ΔF508-CFTR processing could differ in various tissues and small variations in apical activity levels could have profound effects on pathology. Our electrophysiological data, supported by immunochemical data, show that in all epithelial tissues studied sofar the ΔF508 mice have severely reduced apical activity. However, a careful analysis of ΔF508-CFTR processing kinetics may reveal subtle differences between cell types.

Applications of the ΔF508 mouse model. Our data show that the tissues of the ΔF508 mouse model provide a valid model for the processing defect of the human ΔF508-CFTR mutation. It gives us the opportunity to study several aspects of CFTR function in more detail in intact tissues. CFTR is active in intracellular compartments [9], possibly affecting the pH of vesicular compartments. This could explain why CFTR deficient cells produce mucins and surface glycoproteins with abnormal carbohydrate structures [27-31]. These abnormalities may contribute to the formation of abnormal secretions and to the reduced clearance of airway pathogens characteristic of CF. CFTR is involved in regulated endocytosis and exocytosis in some cell types [32, 33]. In addition, recent evidence suggests that CFTR either directly or indirectly interacts with other ion transport systems [34, 35]. ΔF508-CFTR expressed in epithelial cells may be partially active in these processes. Therefore, we may expect to observe differences between ΔF508 mice and mice with complete loss of function mutations (knockout). It will be interesting to compare ΔF508 mice with knockout mice in an experiment in which the mice are challenged with lung pathogens [36]. Furthermore, the ΔF508 mouse model will allow us to study conditions that may enhance the activity of ΔF508-CFTR at the apical epithelial membrane *in vitro* and *in vivo*. Processing of normal CFTR involves chaperonin mediated folding and core glycosylation at the endoplasmic reticulum. This is followed by full glycosylation in the Golgi system and transport to the apical membrane. It is a rather inefficient process since approximately 75 % of normal CFTR is degraded in immature form by cellular proteases [7, 8]. Processing of ΔF508-CFTR to a fully glycosylated apical form is severely reduced. The striking stimulating effect of reduced temperature on ΔF508-CFTR expression, both with human and mouse mutants, suggests that the processing block can be relieved within the range of physiological conditions. We can consider several approaches towards finding therapeutic substances. Compounds which interfere with ΔF508-CFTR-chaperonin interaction may improve processing by relaxing the quality control mechanism. An example of this may be glycerol, which has an effect comparable to low temperature.[37] Inhibition of CFTR degradation may improve

processing of mutant CFTR, although this approach has not been successful to date [7]. CFTR at the plasma membrane is activated by ATP binding and multi-site phosphorylation of the large cytoplasmic regulatory domain [3, 38]. Therefore, substances that stabilize the open state of CFTR [39, 40] or inhibit protein phosphatases [41, 42, 43] are expected to improve the activity of both normal and ΔF508-CFTR. These studies may lead to new developments, not only in our understanding of membrane protein processing, but also towards effective therapies for CF.

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Acknowledgements. We thank Wendy Boomaars for expert technical assistance and Mirko Kuit for excellent photography. We thank Dr W. Colledge, Dr R. Ratcliff and Dr M. Evans (Cambridge, UK) for providing us with the $cftr^{tm1cam}$ knockout mice. This work was funded in part by HGO/TNO, SLW and the Clinical Genetics foundation.

CHAPTER VII

CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR MEDIATES THE cAMP INDUCED FLUID SECRETION BUT NOT THE INHIBITION OF RESORPTION IN MOUSE GALLBLADDER EPITHELIUM.

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(Hepatology, in press)

Abstract.
We have studied the physiological role of the cystic fibrosis (CF) gene product (CFTR) in gallbladder epithelium using a knockout mouse model for CF. We found that normal mouse gallbladder epithelium expresses functional CFTR as shown by RT-PCR analysis and Ussing chamber experiments. Gallbladders from *Cftr* -/- mice were structurally intact as shown by microscopic and physiological parameters but lacked the cAMP induced chloride current observed in normal gallbladders. In fluid transport measurements, normal and *Cftr* -/- gallbladders were equally active in basal resorption. The addition of forskolin, which activates CFTR anion channel activity through the cAMP system, resulted in net fluid secretion in normal gallbladders. In contrast, *Cftr* -/- gallbladders were unable to secrete fluid while a complete inhibition of resorption by forskolin was observed. We conclude that in normal mouse gallbladder epithelium, cAMP provoked fluid secretion involves simultaneous inhibition of apical sodium chloride resorption and activation of CFTR. Our data support the hypothesis that gallbladder disease in CF is at least in part due to a deficient secretory response to the endogenous cAMP linked hormones VIP and secretin.

Introduction

Hepatobiliary abnormalities are quite common and severe in cystic fibrosis (CF) patients, although lung disease is still the main cause of mortality and morbidity in CF (5). These abnormalities include obstruction of intrahepatic biliary ducts and subsequent hepatic fibrosis (4, 28), but also gallbladder dysmorphia and frequent gallstones (36, 39, 49). One may presume that as the treatment of lung disease in CF improves, hepatic disease will become even more manifest. The primary defect in CF is related to mutations in a gene encoding the Cystic Fibrosis Transmembrane conductance Regulator (CFTR). CFTR is an anion channel, localised in active form at the apical plasma membrane of polarised epithelial cells. The channel is activated by phosphorylation of the intracellular domain by protein kinases (13, 45). CFTR is thought to play a role in net fluid transport across epithelia (45) but direct evidence for such a role is limited to mouse intestine (21) and cultured airway epithelium (25, 50). The involvement of CFTR in plasma membrane recycling (6, 42) and acidification of intracellular vesicles (2) has also been suggested. Recent evidence shows that CFTR can influence the activity of a parallel chloride conductance possibly through a regulated ATP efflux function, however the physiological significance of this mechanism remains to be established (47).

In the liver, CFTR mRNA and protein are expressed in bile duct epithelial cells, as shown for rat (20) and man (12, 51). In isolated rat bile duct epithelial cells a cAMP dependent chloride conductance, with properties typical of CFTR, was

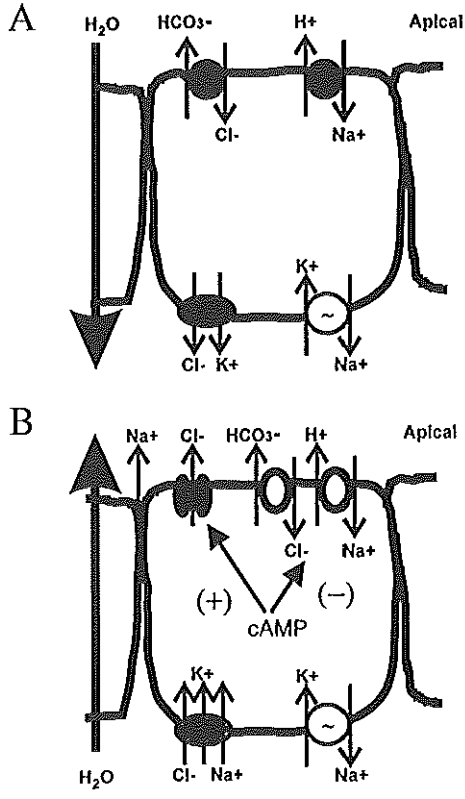


Fig. 1. Iontransport systems in gallbladder epithelium

A. A model for isosmotic fluid resorption in the gallbladder, as suggested by Reuss *et al.* for various species (44). Net transport of NaCl to the serosal side is mediated by the combined action of a Cl^-/HCO_3^- and a Na^+/H^+ exchanger at the apical membrane, activated by ion gradients that are generated by the Na^+/K^+ ATPase at the basolateral membrane. Part of the luminal bicarbonate dissociates as CO_2 plus H_2O.

B. Data presented in this paper suggest that fluid secretion that is induced by an increase of the intracellular cAMP concentration, involves inhibition of the apical exchange carrier systems and activation of CFTR, the apical chloride conductance (see text for discussion). The basolateral chloride conductance has not been described in detail in this tissue. By analogy with other chloride secreting epithelial cells, a Na-K-Cl cotransport system could be involved in chloride secretion (23).

observed (20, 32). In mouse (41, 53) and human (18, 51) gallbladder epithelium CFTR expression and chloride transport activity has been reported. Similarly, in Necturus gallbladder a cAMP dependent homologue of CFTR was described (15, 24, 26). However, the relationship between the complex and variable hepatic abnormalities in CF patients and the primary function of CFTR, i.e. chloride transport at the apical epithelial membrane, is not resolved now. By analogy with its role in other tissues, CFTR in biliary and gallbladder tissue is likely to be involved in regulated isosmotic fluid transport, which determines bile output and composition. However, this has not been shown directly. The mechanism of fluid resorption in the gallbladder has been described in literature (Fig 1A) (44, 46). Inhibition and even reversal of fluid resorption has been observed upon beta-adrenergic stimulation in several species (52). Using a CF mouse model, we show here that CFTR is required for cAMP induced isosmotic fluid secretion in the intact gallbladder, and that cAMP mediated inhibition of resorption is independent of CFTR in this tissue.

Methods.

Animals.

All animal experiments were performed according to the guidelines issued by the Dutch government concerning animal care. A colony of mice with a lesion in the CFTR gene resulting in complete loss of function (43) was bred under pathogen-free conditions in our transgenic unit. The genotype of each individual animal was tested by Southern blotting of tail DNA. The *Cftr* -/- animals in our facility display less severe runting and mortality due to intestinal obstruction than initially reported for the Cambridge colony. This is probably due to breeding conditions. All physiological parameters, nasal PD, intestinal and gallbladder PD responses (53), (this paper) confirmed the *Cftr* -/- phenotype of these animals. The animals selected for the experiments reported here were 5-6 weeks old, without obvious signs of disease or discomfort, average weight 23.5 +/- 2.7 g for *Cftr* -/- and 23.0 +/- 4.4 g for their normal (*Cftr* +/+ and +/-) littermates.

RT-PCR analysis.

Two mouse-specific oligonucleotides MCF1 (5'-GCAGAAACAAGAGTATAAAG-3'), and MCF4 (5'-CTGCTGTAGTTGGCAAG-3') were synthesized. The sequences are localised in exons 8 and 10 respectively (58) and generate a mouse CFTR mRNA specific 459 bp fragment in RT-PCR analysis. Total RNA was isolated by extraction of various mouse tissues in guanidin isothiocyanate and centrifugation through a 5.2 mol/l CsCl stepgradient. The RNA was reverse transcribed to cDNA by adding 10 units Avian myoblastoma virus reverse transcriptase (AMV-RT) in 20 µl cDNA buffer (7 µg heat denatured RNA, 0.8 µmol/l antisense oligonucleotide primer MCF4, 10^{-3} mol/l dNTP's, 0.05 mol/l KCl, 0.02 mol/l Tris-HCl pH 8.4, $2.5x10^{-3}$ mol/l $MgCl_2$, 0.1 mg/ml bovine serum albumin and 20 U RNAsin) for one hour at 37°C. The 50 µl PCR assay contained $3x10^{-7}$ mol/l sense and antisense oligonucleotide primer (MCF1 and MCF4), $0.2x10^{-3}$ mol/l dNTPs, 4 µl cDNA mix, 0.05 mol/l KCl, 0.02 mol/l Tris-HCl pH8.4, $2.5x10^{-3}$ mol/l $MgCl_2$, and 0.1 g/l bovine serum albumin. After denaturing at 94°C, 5 units Taq polymerase was added at 72°C ('hot start'). Standard cycling program: 30 cycles of denaturation for two minutes at 93°C, primer annealing for two minutes at 47°C, and elongation for four minutes at 72°C. Aldolase A oligonucleotide primers, which amplify a 442 bp cDNA fragment, were as in Bremer et al. (8). Amplification of aldolase A fragment was done as a control, in parallel with CFTR amplification, under the same conditions. PCR products were separated on a 1.5% agarose gel. For quantitative analysis of the PCR reaction, equal amounts of total RNA isolated from different tissues were subjected to an RT-PCR protocol in parallel incubations. Samples were withdrawn at regular intervals from the PCR and subjected to Southern blotting. Radioactivity was measured with a Phosphorimager (Molecular Dynamics).

Electronmicroscopy.
Gallbladders from normal and *Cftr* -/- mice were obtained and incubated as described for the fluid transport experiments. After a preincubation of 10 minutes the tissues were incubated for 15 minutes either with forskolin or with an equivalent amount of solvent. Subsequently, the gallbladders were fixed in 0.15 mol/l cacodylate buffer pH 7.3 containing 2.5% glutaraldehyde. The tissues were post fixed with 1% osmium tetroxide and embedded in Epon 812. Ultrathin sections stained with uranyl acetate and lead citrate were examined and photographed in a Philips CM100 electron microscope at 80 kV.

Ussing chamber experiments.
Freshly excised mouse gallbladders were used for measurements of transepithelial potential differences and short circiut currents. Adult *Cftr* -/- and normal (*Cftr* +/- and +/+) littermates were used in this study. The mice were anaesthetized by intraperitoneal injection of 80-100 µl Hypnodyl (50 g/l metomidate, Janssen Pharmaceuticals). Gallbladders were removed from the animal and cut open lengthwise. Bile salts were washed out with ice-cold Meyler's solution ($x10^{-3}$ mol/l) (105 NaCl, 4.7 KCl, 1.3 $CaCl_2$, 1 $MgCl_2$, 20.2 $NaHCO_3$, 0.4 NaH_2PO_4, 0.3 Na_2HPO_4 and 10 Hepes, pH7.4). In low chloride ($7x10^{-3}$ mol/l) buffer NaCl is replaced with isosmolar Na-gluconate. The transepithelial potential difference across the gallbladder was measured in an Ussing chamber, essentially as described by Verbeek et al. (54). Briefly, the fresh gallbladder was mounted between two compartments (aperture size 0.8 mm^2) so that the potential difference and electrical resistance could be measured with two sets of calomel and platinum electrodes. Electrical resistance of the gallbladder was calculated from the potential shift induced by a standard current pulse corrected for the resistance of the buffer. The two compartments were filled with Meyler's solution, saturated with a 95% O_2 plus 5% CO_2 gas mixture (pH 7.4). Forskolin (10^{-5} mol/l), diphenylamine-2-carboxylic acid (DPC, Fluka, $2x10^{-3}$ mol/l) and carbachol ($5x10^{-5}$ mol/l) were added to both the serosal and mucosal compartment.

Fluid transport in mouse gallbladder.
Adult mice were anaesthetized with Hypnodyl as described above. The gallbladder with the cystic duct attached was removed, and placed in ice-cold Meyler's solution. The gallbladder was cut open where it joined the cystic duct, and a small plastic cannula was inserted into the gallbladder. The bile was washed out with cold Meyler's solution. After filling the lumen of the gallbladder, the cannula was removed, and a ligature around the neck of the gallbladder was secured to prevent leakage. In some experiments ethyl-isopropyl amiloride (EIPA, $5x10^{-5}$ mol/l, Merck) in DMSO was added to the lumen. The gallbladder was placed in a bath of carbogenated Meyler's solution at 37°C. Net transfer of fluid across the gallbladder was determined by periodic measuments changes of the weight of the gallbladder. At intervals, the gallbladder was lifted from the bathing solution and weighed to the nearest 0.05 mg. In some cases, ethanol or forskolin was added to the bath

after 45 minutes. Because of the marked differences in the sizes of individual gallbladders, each was cut open to measure the surface area using a calibrated micro-grid. The net fluid transport was expressed as $\mu l/cm^2.h$.

Results

***Cftr* -/- mouse gallbladders have normal morphology.**

The *Cftr* -/- animals bred in the Rotterdam pathogen free facility do not display the high mortality due to intestinal obstruction previously reported for this strain (43). Mortality is less than 40% in the first four weeks. This may be due to differences in breeding conditions. The animals selected for study were between five and six weeks old, without obvious signs of severe disease or discomfort. We have observed no histological evidence of obstructive liver disease or severe gallbladder dysmorphia in our colony. Freshly excised *Cftr* -/- gallbladders did not show obvious morphological abnormalities or signs of inflammation as shown by light (not shown) and electronmicroscopy (Fig. 2). We could observe intact tight junctions, typical extensive lateral protrusions, apical microvilli and many subapical vesicles in both normal and *Cftr* -/- gallbladder epithelial cells (Fig. 2 A, C). Treatment of the tissues with forskolin under conditions used for fluid transport measurements (Fig. 5) did not cause any obvious morphological changes in the tissue (Fig 2 B, D). The large opaque amorphous inclusions seen in Fig 2C and D are not typical for *Cftr* -/- tissues but were also observed in normal cells. We conclude that the *Cftr* -/- gallbladder tissue used in this study is intact and has a normal morphology.

Expression of CFTR in mouse gallbladder.

CFTR mRNA is expressed in mouse gallbladder and cystic duct as shown by RT-PCR analysis (Fig 3A). Although the aldolase RT-PCR control of liver RNA was positive (Fig 3B) we did not detect a CFTR specific product at 24 PCR cycles with ethidium bromide staining (Fig 3A). To obtain an estimate of relative CFTR mRNA levels in different tissues we did a quantitative RT-PCR experiment (Fig 3C). The relative amount of CFTR transcripts in mouse gallbladder was approximately thirty-fold lower than in intestine and tenfold higher than in liver as determined with a quantitative RT-PCR assay (Fig. 3C). This is in agreement with the notion that also in mice CFTR is expressed exclusively in bile duct and gallbladder epithelium, as bile duct cells represent only a few percent of the total liver volume. In RT-PCR experiments with tissues from *Cftr* -/- mice, CFTR mRNA was not detectable (Peters, unpublished data).

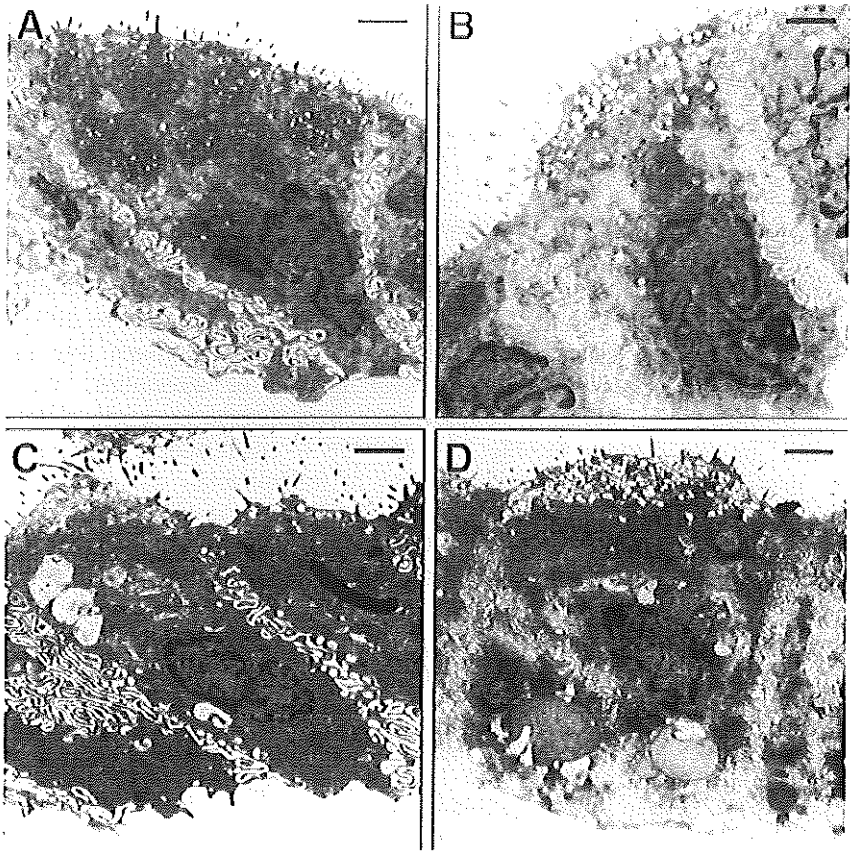


Fig. 2. Electronmicroscopic analysis of normal and CF tissues.
The effect of forskolin on normal and *Cftr* -/- mouse gallbladders, under the conditions described for the fluid transport measurements (Fig. 5). The upper panels show electron-micrographs of *Cftr* +/+ gallbladders incubated with solvent (A) or with forskolin (B, 10^{-5} mol/l). The lower panels show *Cftr* -/- gallbladders in the presence of solvent (C) or forskolin (D) . The bars in panels A, B, C and D are 1.3, 1.0, 1.2 and 1.5 μm respectively.

Electrophysiological analysis of normal and mutant gallbladder.
To demonstrate the presence of active CFTR, the bio-electrical properties of intact mouse gallbladders were measured in an Ussing Chamber. The baseline electrical potential difference (PD, mV) and the resistance (R, Ω/cm^2) were low as expected for a leaky epithelium (Fig. 4B, C), and did not differ significantly between normal (*Cftr* +/+ or +/-) and mutant (*Cftr* -/-) mice. All gallbladders from normal mice displayed a forskolin-induced PD change (ΔPD, Fig. 4A, B). Also short circuit current (Isc) measurements showed a forskolin induced response (Fig 4C). This electrogenic forskolin response can be attributed to activation of an apical chloride

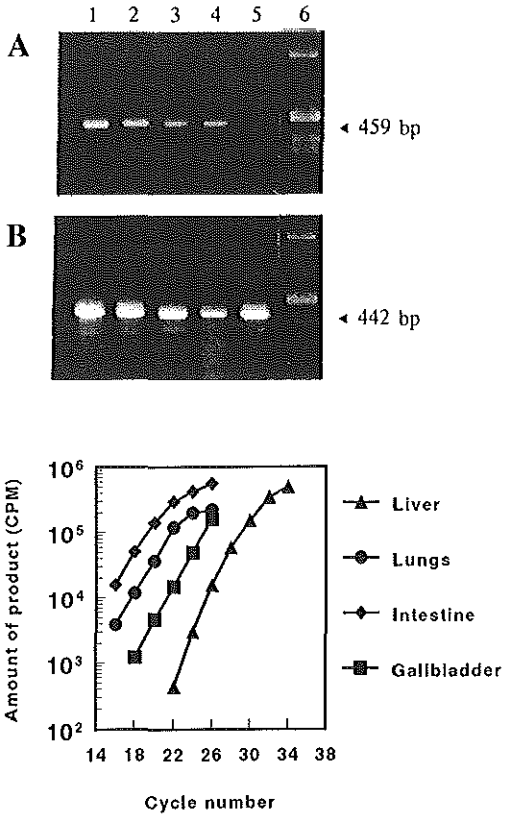


Fig. 3. Expression of CFTR mRNA in mouse gallbladders.
Agarose gel electrophoresis (ethidium bromide stain) of RT-PCR products obtained with CFTR primers (A) or aldolase primers as a control (B). Total RNA was isolated from mouse cystic duct (*lane 1*), gallbladder (*lane 2*), lungs (*lane 3*), kidney (*lane 4*), liver (*lane 5*). Size marker (1018, 517/506, 396, 344, 298 bp) (*lane 6*). Although the Aldolase signal in the liver sample is clearly seen with ethidium bromide staining, the CFTR signal is undetectable under these conditions (24 cycles). (C): The amount of CFTR RT-PCR product as a function of the number of PCR cycles was determined as described in the methods section. The data indicate that in mouse gallbladder the CFTR mRNA level is higher than in total liver and lower than in intestine. In tissues from *Cftr* -/- mice no signal was observed in this assay (data not shown).

channel based on the following arguments. First, in low chloride buffer both the PD and short circuit current responses to forskolin were greatly reduced in normal gallbladders (Fig 4B,C). Furthermore, the response was inhibited to a large extent by the chloride channel blocker DPC, which has been shown to inhibit CFTR activity specifically (30, 48). Finally, none of the gallbladders from mice homozygous for a lesion in the CFTR gene (*Cftr* -/-) responded to forskolin (Fig. 4). This defect was not due to nonspecific damage to the epithelium as shown by morphological analysis (Fig. 2) and the electrical resistance of the *Cftr* -/- tissue, which was in the same range as normal littermates (Fig. 4D). We conclude that in the normal, but not in the *Cftr* -/-, mouse gallbladder, the apical chloride conductance, presumably CFTR, is increased by forskolin-induced protein Kinase A activity (45). Low chloride buffer causes a more negative PD and an associated increase in basal Isc (Fig. 4B,C). This is probably due to hyperpolarisation of the apical membrane revealing apical sodium conductance (3). The calcium agonist carbachol added after forskolin causedelicited a complex response in both normal and *Cftr* -/- gallbladders. The initial transient phase (Fig 4A, B, C, peak) was also observed in low chloride buffer in normal gallbladders, suggesting that it is associated with

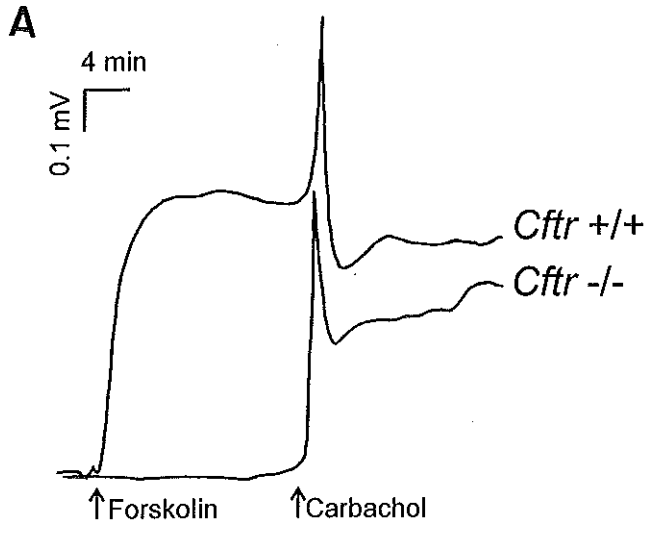
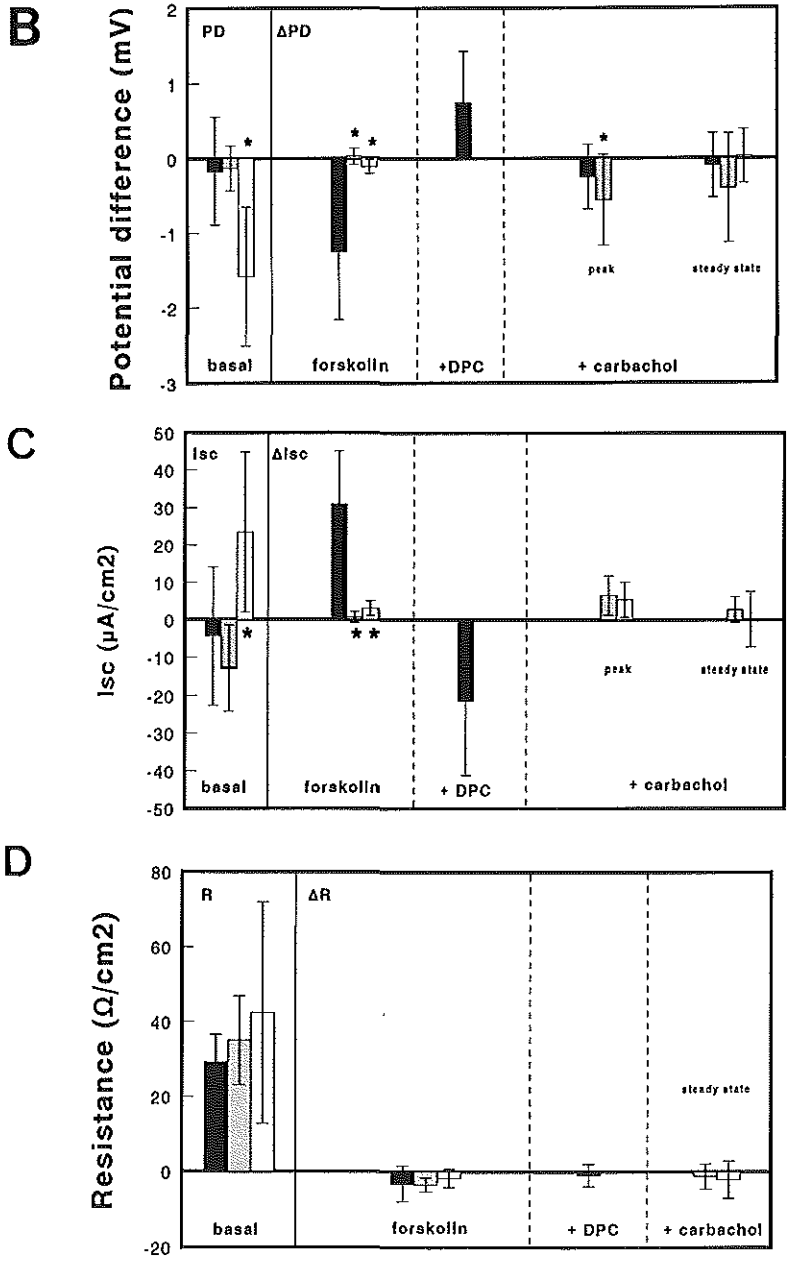


Fig. 4. Effect of forskolin on transepithelial electrical potential in mouse gallbladder. Electrical potential differences (A, B), short circuit currents (C) and electrical resistance (D) were measured using an Ussing chamber as described. A: a representative PD trace shows the kinetics of the responses observed. B-D: normal (*Cftr* +/- and +/+, solid bars) and *Cftr* -/- (hatched bars) gallbladders in standard Meyler's solution, and normal gallbladders in low chloride (7 mM) Meyler' solution (open bars). Bars represent mean ± S.D. PD: steady state basal potential difference in mV; ΔPD is the change from steady state PD in mV upon addition of an agonist. Net anion transport to the mucosa gives a negative value. Isc: short circuit current, ΔIsc change in short circuit current ($\mu A/cm^2$; normal N=6, *Cftr* -/- N=4, low Cl N=6) R: electrical resistance calculated from the PD response to a series of 1 μA current pulses (Ω/cm^2; normal N=15, *Cftr* -/- N=11, low Cl N=6), ΔR change in resistance. Addition of 10^{-5} mol/l forskolin was followed by addition of either $2x10^{-3}$ mol/l DPC or $5x10^{-5}$ mol/l carbachol. Means that are significantly different (Mann-Whitney, $P<0.05$) from normal gallbladder in standard Meyler's solution are indicated (*).

activation of basolateral potassium channels rather than with activation of the apical chloride conductance. Compared to the effect of forskolin in normal tissue, the effect of carbachol on the steady state PD and Isc was small in *Cftr* -/- mice (Fig 4B,C). This response can be attributed to chloride channels other than CFTR (31, 33, 34). It should be noted that these channels are not activated by cAMP in intact gallbladder, as shown by the absence of a forskolin response in *Cftr* -/- mice. The effect of agonists on electrical resistance is characteristically small in this leaky epithelium (Fig 4C), as it is determined mainly by paracellular ion conductance. Our data do not show a significant effect of forskolin on tissue resistance.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Resistance (Ω/cm2)
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basal
forskolin
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+ carbachol
steady state

Fluid transport in intact mouse gallbladders.

To study the role of CFTR in fluid transport in mouse gallbladder epithelium we have applied a classical protocol (17). Normal (*Cftr*+/+ and +/-) mouse gallbladders are active in fluid resorption, as shown by a steady decrease in weight during incubation in a physiological buffer solution (Fig. 5). This process has been studied extensively in gallbladders from several species. The combined action of a Na^+/H^+ exchanger plus a Cl^-/HCO_3^- exchanger at the apical epithelial membrane, and the Na^+/K^+ ATPase plus a potassium chloride transporter at the basolateral surface, result in active NaCl resorption from the lumen of the gallbladder (44, 46). This in turn drives isosmotic fluid transport (Fig. 1A). We did not observe a significant difference between homozygous normal (*Cftr* +/+) and heterozygous (*Cftr* +/-) animals in this assay. We therefore consider them both as phenotypically normal. The Na^+/H^+ exchanger is required for the basal resorption in the mouse gallbladder as is shown by the effect of EIPA (Fig. 5B), which is a specific inhibitor of this transportsystem (9, 59). Gallbladders from *Cftr* -/- mice show normal fluid resorption rates (Fig. 5). This confirms that the *Cftr* -/- tissues are intact and functional. Furthermore, this indicates that CFTR is not required for NaCl resorption, which is in agreement with the model (Fig. 1A). The addition of forskolin to normal gallbladders results in reversal of the fluid flow from net resorption to net average secretion (Fig. 5A). This was not observed when solvent (ethanol) was added (Fig. 5A). Since EIPA had no significant effect on forskolin induced secretion (Fig. 5B) we can conclude that the Na^+/H^+ exchanger is not involved in this process. Flame-photometric measurements in luminal fluid of normal gallbladders that were treated with forskolin, showed no increase in potassium or sodium concentration compared to the bath (N=5, data not shown). If secretion was caused by apical KCl extrusion, we would have expected a considerable increase in the luminal potassium concentration (from 5 to 15 mM). Therefore, isosmotic fluid secretion is most likely associated with a paracellular sodium flux (Fig. 1B). In contrast to normal, addition of forskolin to *Cftr* -/- gallbladders resulted in a complete inhibition of resorption to zero flow in all experiments, but never in fluid secretion (Fig. 5B). Addition of forskolin plus solvent does not affect the morphology (Fig. 2D), the electrical resistance (Fig. 4C) or the ion transport capacity of *Cftr* -/- gallbladders, as shown by the carbachol response (Fig. 4A,B). These observations allow us to draw two conclusions. First, the resorption mechanism in mouse gallbladder is completely inhibited by forskolin and independent of CFTR activity. Second, the secretion component of the net fluid transport is completely dependent upon CFTR activity (Fig. 1B). In addition, the low variance in the data obtained with *Cftr* -/- gallbladders in the presence of forskolin (Fig. 5B, table 2) clearly show that the considerable variance observed under all other conditions in not due to instrumental artefacts but reflect biological variability of the tissues.

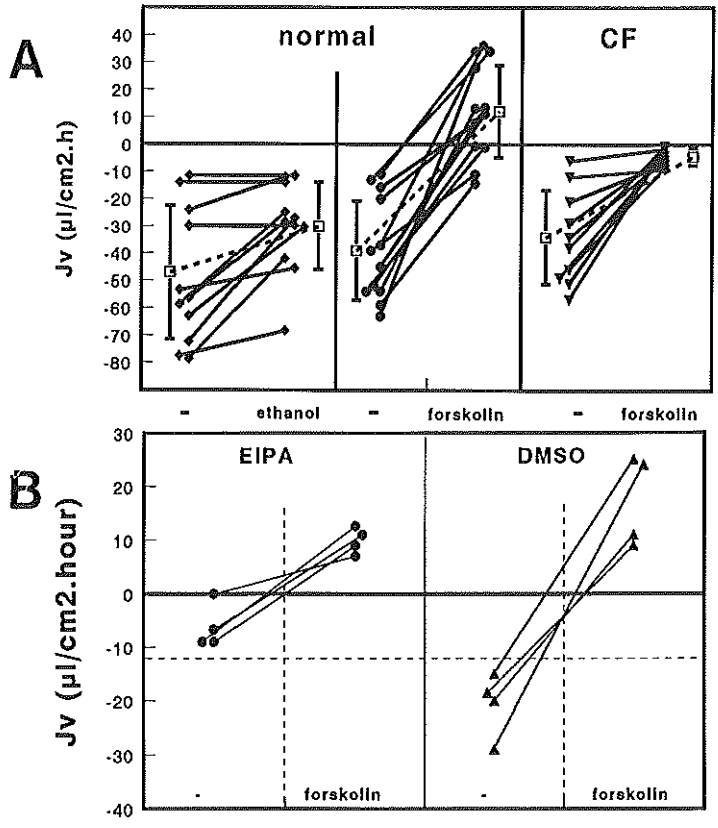

Fig.5: Fluid transport by normal and CF mouse gallbladder.
A. The net water transport (Jv, µl/cm^2.h) in normal (*Cftr* +/+ and +/-) and mutant (*Cftr* -/-) gallbladders was determined as described before and after addition of 10^{-5} mol/l forskolin or an equivalent amount of solvent to the bath. A decrease of weight in time (negative Jv) shows net resorption of fluid. In normal gallbladders a steady rate of resorption is observed, which is reversed to net secretion by forskolin at 45 minutes (N=12). Solvent (ethanol) alone had no significant effect on basal resorption (N=11). In *Cftr* -/- gallbladders forskolin completely inhibits basal resorption, but no net secretion is observed (N=10). Average values are shown as open symbols ± SD (Bars). Significance analysis was done with the Mann-Whitney/Wilcoxon rank test (2-tailed, corrected for ties). There is no significant difference in basal resorption between the *Cftr* -/- and the control groups. *Cftr* -/- plus forskolin differs significantly from normal plus solvent ($P<0.001$) and from normal plus forskolin ($P<0.01$).
B: Net water flux (Jv, µl/cm^2.h) in normal gallbladders before and after addition of forskolin. Either 5.10^{-5} mol/l EIPA, an inhibitor of the Na^+/H^+ exchanger, or an equivalent amount of solvent (DMSO) was added to the lumen at t=0. The basal resorption rate is significantly reduced by EIPA compared to solvent alone (Mann-Whitney, $p<0.05$), whereas secretion after addition of forskolin is not different from control in the presence of EIPA.

Discussion.

Our RT-PCR (Fig 3) show that, like in rat (20, 32) and man (12, 18, 51), CFTR is expressed in mouse biliary and gallbladder epithelium. Normal mouse gallbladders respond to the cAMP agonist forskolin with a change in PD and short circuit current. This response is dependent on chloride concentration and is inhibited by the CFTR specific channel blocker DPC. Gallbladders from mice with a targeted disruption of the CFTR gene (43) do not display a response to forskolin (Fig 4). The *Cftr* -/- gallbladders used in this study were intact and functional, as judged by morphological criteria (Fig. 2), by the electrical resistance and response to carbachol (Fig. 4), and by the normal rate of basal fluid resorption (Fig. 5). We conclude that CFTR is involved in the cAMP induced increase of apical chloride permeability in gallbladder epithelium of the mouse. In the airway epithelium of the mouse a parallel calcium dependent chloride channel is activated by forskolin treatment (10, 22). A similar chloride anion channel has also been described in rat bile duct epithelial cells (31, 33, 34). The complex response to the calcium agonist carbachol (Fig. 4) could be attributed in part to such a pathway. However, we do not observe activity of this channel in gallbladder epithelium of *Cftr* -/- mouse treated with forskolin. Possibly, the "crosstalk" between the cAMP and calcium second messenger systems is less pronounced in gallbladder epithelium than in trachea epithelium. This would make the gallbladder a more convenient tissue to test CFTR function than trachea in the mouse.

Normal and *Cftr* -/- gallbladders absorb fluid at a comparable rate (Fig. 5A). Resorption is sensitive to a specific inhibitor of the sodium-proton exchanger (Fig. 5B). This is in agreement with the current model of isosmotic fluid transport, which allows resorption of NaCl through a system of electroneutral exchangers, independent of chloride channels (Fig. 1A). In *Cftr* -/- mice, forskolin inhibits fluid resorption in the gallbladder completely (Fig. 5A). This can be explained by a cAMP mediated inhibition of the apical ion exchangers, as has been observed previously in Necturus and Guinea pig gallbladder (44). Recent studies showed that the Na+/H+ exchanger (NHE-3), which is expressed on the apical membrane of epithelial cells (37), is inhibited by protein kinase A mediated phosphorylation (35, 56). Therefore, the CFTR independent inhibition of resorption by forskolin that we observe is most likely due to this mechanism. A markedly different result was found in a recent study of the mouse jejunum. Here, cAMP mediated inhibition of Na resorption was observed in normal but not in *Cftr* -/- mice, suggesting that CFTR was involved in this phenomenon (11). This indicates that tissue specific differences in the regulation of fluid resorption exist.

The active fluid secretion seen in normal gallbladders in the presence of forskolin is not observed in *Cftr* -/- gallbladders (Fig. 5A). Fluid secretion in the mouse gallbladder apparently requires activation of CFTR. Our data indicate that the normal fluid secretion response in gallbladder epithelium involves simultaneous inhibition of resorption and CFTR dependent increase of apical chloride conduc-

tance (Fig. 1B). Though the most simple explanation for our observations is that the forskolin induced chloride current is carried by CFTR, we cannot rigorously exclude the possibility that CFTR activates parallel chloride channels in an indirect way (47). Our morphological data (Fig. 2) and electrical resistance measurements (Fig. 4C) do not show a change in the paracellular conductance pathway by forskolin.

The involvement of CFTR in isosmotic fluid transport has been shown previously in cultured human airway cells (25, 50) and in the intact intestine in a CF mouse model (21). The physiological significance of fluid secretion in gallbladder epithelium remains to be determined. *In vivo*, the hormones VIP and secretin, which are released in response to feeding (55), regulate cAMP levels in bile duct and gallbladder (44). On the basis of our data it seems reasonable to suggest that these hormones affect fluid transport in gallbladder *in vivo*, not only by inhibiting resorption, but also by inducing secretion through activation of CFTR. In CF patients, as in Cftr -/- mice, the secretion response will be absent. This may contribute to the frequent gallstones formation and gallbladder malformations observed in CF patients.

The intrahepatic duct epithelium has many morphological and functional features in common with the gallbladder epithelium, including CFTR expression. However, expression of the apical Na^+/H^+ exchanger NHE-3 is undetectable in rat and human liver (7, 40). Therefore, fluid resorption is probably not a prominent feature of this tissue, in contrast to gallbladder. Bile fluid volume is determined by the amount of bile salts secreted by parenchymal cells and by a component contributed by the ductal system (19). Secretin (27) and VIP (38) increase ductular bile flow through the cAMP mediated second messenger pathway. In addition, cAMP stimulated bicarbonate secretion in isolated rat bile duct cells involves activation of a chloride conductance (1). These data, and the fact that CFTR is expressed in intrahepatic bile duct epithelium (12, 20, 51) suggest that CFTR is involved in biliary bicarbonate secretion and fluid volume regulation. Consequently, CFTR deficiency is expected to result in insufficient hormone induced fluid secretion from the biliary tree. This could explain the progressive bile duct obstruction observed in CF patients (4, 28, 36, 39, 49). Future studies of hormone regulated bile secretion in the CF mouse model could supply further evidence for this. In the CF mouse model that we have studied no liver pathology reminiscent of CF in humans was observed. Steatosis was seen in all *Cftr* -/- animals (data not shown), but this may be related to malnutrition caused by intestinal malabsorption in these mice (43). The apparent absence of hepatic fibrosis or plugging of biliary ducts in *Cftr* -/- mice may have several reasons. No mice that were older than five weeks were studied in our experiments. This may be insufficient for detectable liver disease to develop. Furthermore, the relative contribution in mice of canalicular versus ductular bile flow or the bile composition may be different from human. Finally,

the genetic background of the mice may play a role. In a recently published report on a G551D CFTR mouse mutant, abnormalities similar to human liver disease were observed in a minority of the animals investigated (16).
Current treatment of hepatic disease in CF is aimed at increasing canalicular bile flow with ursodeoxycholate (14, 19). Our data would support the validity of this approach. Gene therapy with adenoviral vectors that express CFTR in the ductal epithelium has been put forward as a possible alternative (29, 57). Further study of chloride conductances other than CFTR in biliary epithelium (31, 33, 34) may lead to new ways to improve ductular flow in CF patients.

Acknowledgements
We thank Dr Henk Veeze MD, and Dr Hugo de Jonge for critically reading the manuscript, Dr Hans Morreau, MD, for evaluating histological sections and Dr P. Mulder for advice on statistical analysis. This study was supported by a grant from HGO/TNO and the SLW .

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This study was supported by HGO/TNO, the Dutch Medical Genetics Centre and the Association Francaise de Lutte contre Mucoviscidose.

CHAPTER VIII

CFTR EXPRESSION AND MUCIN SECRETION IN CULTURED MOUSE GALLBLADDER EPITHELIAL CELLS.

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(American Journal of Physiology, in press)

Abstract.
Dysfunction of the Cystic Fibrosis Transmembrane conductance Regulator (CFTR) in humans is frequently associated with progressive liver disease which appears to result from obstruction of biliary ducts with mucous material. CFTR in the liver is expressed in the biliary epithelium. Using a mouse model for cystic fibrosis (CF) we have studied the relationship between CFTR expression and glycoprotein secretion in primary culture of mouse gallbladder epithelial cells (MGBC). MGBC in culture maintain a well differentiated phenotype as shown by microscopy. The cells produce CFTR mRNA to levels comparable to the intact tissue. With patch clamp analysis we could frequently observe a linear protein kinase A regulated chloride channel which shows all the major characteristics of human CFTR, although its conductance is lower (5 pS compared to 8 pS). MGBC in culture produce and secrete high molecular weight glycoproteins (HMG) in a time dependent and temperature sensitive manner. Secretion of HMG was not stimulated significantly by either cAMP, Ca^{2+} or protein kinase C agonists in this system. High concentrations (3 mM) of extracellular ATP stimulated secretion threefold but low concentrations (0.3 mM) had no effect. Approximately one third of the HMG produced and secreted consisted of mucin. Cultured MGBC from CFTR deficient mice produced and secreted mucin to a similar extent as normal cells. We conclude that cultured mouse gallbladder cells are a convenient model to study both CFTR function and mucin secretion. In this system, we found no evidence for a direct link between mucin secretion and CFTR activity, as has been suggested for other cell types.

Introduction.
The intrahepatic bile ducts as well as the cystic duct and gallbladder are lined with epithelial cells with distinct morphology. This epithelial cell layer plays an important role in the regulation of bile volume and bile pH using a combination of ion-transport systems situated in both apical and baso-lateral membranes. In addition to performing regulated ion-transport, bile duct cells have a secretory function. Many subapical vesicles can be observed in electron micrographs of biliary epithelium. Mucins are prominent among the products secreted from this intracellular pool (37). The physiological function of mucin secretion is supposedly the formation of an extracellular gel protecting the tissue against the aggressive biliary environment. The regulation of mucin secretion in biliary epithelium has not been studied in detail.

Cystic fibrosis (CF) is a recessive inherited disease with a high incidence in the caucasian population (6, 60). It is caused by mutations in a cAMP regulated chloride channel (CFTR) situated in the apical membranes of a variety of epithelial cells (49). CFTR is involved in the regulation of isosmotic fluid transport. CFTR dysfunction results in a complex phenotype characterized by recurrent lung infections, pancreatic insufficiency and malabsorption. Morbidity and mortality in CF is mainly associated

with lung disease. However, hepatobiliary abnormalities are quite common in CF patients. Obstruction of biliary ducts, focal or multilobular biliary cirrhosis was reported in over 50% of adult CF patients at autopsy (5, 33, 59). Micro-gallbladder and high incidence of gallstones is also observed (41, 42, 52). CFTR in the liver is mainly expressed in bile duct cells, as shown for rat (21) and man (13, 55). The pathogenesis of liver disease in CF remains to be elucidated. Defective isosmotic fluid transport may result in decreased solubility and subsequent precipitation of bile components. In support of this, studies with a mouse model for CF in our laboratory showed that CFTR is required for cAMP induced fluid secretion across the biliary epithelium (Peters, in press, chapter VII). On the other hand, there may be additional reasons for the abnormal properties of mucous secretions in CF epithelia. First, CFTR dysfunction affects membrane recycling (7, 45) and possibly secretion (20, 39, 50) in several cell types. Second, CFTR mutations can result in abnormal glycosylation and sulphation of glycoproteins (11, 16, 35, 64). Biliary mucins are implicated as promoting factors in the formation of bile stones (1, 9). Therefore, both increased secretion of mucins and abnormal mucin structure could contribute to hepatic disease in CF.

We have studied the relationship between CFTR and mucin secretion in cultured mouse biliary epithelial cells using a mouse model of CF. We show here that primary cultures of mouse gallbladder epithelium maintain many characteristics of the original tissue, including CFTR expression and mucin synthesis.

Methods

Materials. Sepharose CL-4B was from Pharmacia (Uppsala, Sweden) and Guanidinium chloride from Merck (Darmstadt, Germany). Heparitinase I (Heparinase III) from *Flavobacterium heparinum*, Keratanase (*Pseudomonas* species) and hyaluronidase from *Streptomyces hyalurolyticus* were purchased from Sigma Chimie (Saint Canteen Fallavier, France), chondroitinase ABC from *Proteus vulgaris* was from Seikagaku Corporation (Tokyo, Japan). The ECL™ glycoprotein system, the enhanced chemiluminescence Western blotting detection system and the nitrocellulose membrane Hybond™-C extra were from Amersham (Little Chalfont, UK). Scintillation cocktail was Aqualite™ from Baker (Deventer, Holland).

Animals. All animal experiments were performed in compliance with the guidelines issued by the Dutch government concerning animal care. All experiments not involving CF-/- mice shown in this paper were performed with C57/bl6 animals. Pilot studies showed that good explant cultures could also be obtained with BCBA or Balbc mice. A colony of mice with a lesion in the CFTR gene resulting in complete loss of function in C57/Bl6 genetic background (47) was bred under pathogen-free conditions in our transgenic unit. The genotype of each individual animal was tested by Southern blotting of tail DNA.

Mouse gallbladder epithelial cell culture. Gallbladders were removed from the animal and cut open lengthwise. After the bile was washed out with RPMI-1640 medium supplemented with 100 U/ml penicillin, 0.1 g/l streptomycin, and 20 mM

Hepes pH 7.2, the gallbladders were cut into small pieces and embedded in 1 mm collagen gels (Sigma, Calf skin type I). The explant cultures were incubated at 37°C in a humidified 10% CO2/air mixture in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 100 U/ml penicillin, 0.1 g/l streptomycin, 2 mM glutamin, and 10% fetal calf serum. Mouse gallbladder epithelial cells could be cultured for up to 3 weeks, with medium changes every 2-3 days.

Light- and electron microscopy. Cells cultured on collagen were either fixed in phosphate-buffered saline containing 4% paraformaldehyde for light microscopy, or in 0.15 mol/l cacodylate buffer pH 7.4 containing 2.5% glutaraldehyde for electron microscopy. The glutaraldehyde fixed cells were post fixed with 1% osmium tetroxide and embedded in Epon 812. Ultrathin sections stained with uranyl citrate were examined and photographed in a Philips CM100 electron microscope at 80 kV. The paraformaldehyde fixed cells were dehydrated and embedded in paraffin. Sections were made at 5-10 µm and stained with hematoxylin/eosin.

Quantitative PCR analysis. Two mouse specific oligonucleotides MCF1 (5'-GCAGAAACAAGAGTATAAAG-3'), and MCF4 (5'-CTGCTGTAGTTGGCAAG-3') were synthesized. The sequences are localized in exons 8 and 10 respectively and generate a Mouse CFTR mRNA specific 459 bp fragment in RT-PCR analysis. Total RNA was isolated by extraction of various mouse tissues or cultured cells in guanidine isothiocyanate and centrifugation through a 5.2 mol/l CsCl step-gradient. The RNA was reversed transcribed to cDNA by adding 10 units Avian myoblastoma virus reverse transcriptase (AMV-RT) in 20 µl cDNA buffer (7 µg heat denatured RNA, 0.8 µmol/l antisense oligonucleotide primer MCF4, 10^{-3} mol/l dNTPs, 0.05 mol/l KCl, 0.02 mol/l Tris-HCl pH 8.4, 0.0025 mol/l $MgCl_2$, 0.1 g/l bovine serum albumin and 20 U RNasin) for one hour at 37°C. The 50 µl PCR assay contained $0.3.10^{-6}$ mol/l sense and antisense oligonucleotide primer (MCF1 and MCF4), $0.2.10^{-3}$ mol/l dNTPs, 4 µl cDNA mix, 0.05 mol/l KCl, 0.02 mol/l Tris-HCl pH 8.4, $2.5.10^{-3}$ mol/l $MgCl_2$, and 0.1 g/l bovine serum albumin. After denaturing at 94°C, 5 units Taq polymerase was added at 72°C ('hot start'). Standard cycling program: 30 cycles of denaturation for 2 minutes at 93°C, primer annealing for 2 minutes at 47°C, and elongation for 4 minutes at 72°C. Aldolase A oligonucleotide primers, which amplify a 442 bp cDNA fragment, were as in Bremer et al.(8). The amplification of aldolase A sequences was done in parallel, under the same conditions as described above for CFTR. PCR products were separated on a 1.5% agarose gel. For quantitative analysis of the PCR reaction, equal amounts of total RNA isolated from different tissues were subjected to an RT-PCR protocol in parallel incubations. Aliquots were withdrawn at regular intervals from the PCR and subjected to Southern blotting. Radioactivity was measured with a Phosphorimager (Molecular Dynamics).

Patch clamp analysis. Patch clamp experiments, data sampling and analysis were performed as previously described (27). Patch pipets, borosilicate glass (Clark GC150-TF) were pulled to a resistance of 3-8 MOhm. Excised patches were trans-

ferred to a solution exchange compartment, (27), this allowed us to add various substrates (ATP, protein kinase A, glybenclamide) as well as to change the buffer composition to a I^-, F^-, $NO3^-$, Br^-, or gluconate buffer. Pipet (external) and bath (internal) solutions contained ($.10^{-3}$ mol.l) 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 0.15 $CaCl_2$, 1 EGTA and 5 Hepes. Final Ca^{2+} concentration was 10^{-8} mol/l, pH 7.4. Low (0.05 mol/l) chloride buffer was identical except for NaCl (0.043 mol/l); 0.155 mol/l mannitol was added to adjust the osmolarity of the buffer. High (0.427 mol/l) chloride buffers contained 0.42 mol/l NaCl. Other buffers replaced 0.14 mol/l NaCl for either NaI, NaBr, $NaNO_3$, NaF, or Na-Gluconate, all 140 mM. All experiments were performed at 37°C.

Mucin secretion. Normal mouse gallbladder epithelial cells were incubated with 6 μCi (6-^{3}H)-glucosamine hydrochloride (25.10^3 Ci/mol) in 2 ml culture medium for 20 hours. After the radio-labeling, the cells were rinsed three times in serum free medium (RPMI-1640 supplemented with 100.000 U/l penicillin, 0.1 g/l streptomycin, and 0.02 mol/l Hepes). The secretion experiment was started by adding 0.75 ml medium to the cells. The incubation was performed at 37°C or at 4°C in 95% air plus 5% CO_2 in a humidified atmosphere. After 1 hour a potential secretagogue was added to the cells. At different time points, the incubation medium was replaced, and the cells were rinsed to remove the adherent mucin. At the end of the incubation period the cells were lysed with 0.1% Triton-X100. The labelled glycoproteins in the medium samples and the cell lysates were washed with deionized water by centrifugation through a 10.000 MW Millipore filter, until no unincorporated ^{3}H-glucosamine was detectable in the eluate, and lyophilized. Part of the samples were used for scintillation counting, part was subjected to SDS-PAGE on 5.5% gels. The gels were fixed in 10% methanol-10% acetic acid, incubated in Amplify (Amersham) and dried. The radio-labeled glycoproteins in the gel were made visible by exposing the gels at -70°C to X-ray films (Kodak).

Analysis of mucins from CF-/- and normal cells. Gallbladder explant cultures (8 days) from 8 CF(-/-) and 7 control littermates (+/- and +/+) were incubated for 16 hours with 5 μCi/ml ^{3}H-glucosamine (20-30 Ci/mmol) in culture medium. The medium was collected, the cells were washed and incubated for 20 hours at 37°C in serum free medium. After the incubation, the cells were lysed with 0.1% Triton-X100. The medium samples (overnight labeling medium and chase) and cell lysates were lyophilized in three aliquots, after extensive washing to remove the unincorporated label and used for further investigation. Enzymatic digestion:
an aliquot of each sample was solubilized in 0,3 ml of 0.2 mol/l Tris/acetate buffer, pH 7.4, containing 2.10^{-3} mol/l $CaCl_2$ and 0.02 % sodium azide and digested with a glycosaminoglycan degrading enzyme cocktail (GAG cocktail) composed of hyaluronidase 1.5 U, chondroitinase ABC 0.75 U and heparitinase 1.5 U for 18 h at 37 °C. Each fraction was then dialysed, freeze dried and subjected to gel filtration on Sepharose CL-4B (46). Non reductive alkaline degradation: samples obtained from cell lysates were subjected to β-elimination in 1 ml of 0.05 mol/l NaOH at 45 °C for 16 h.

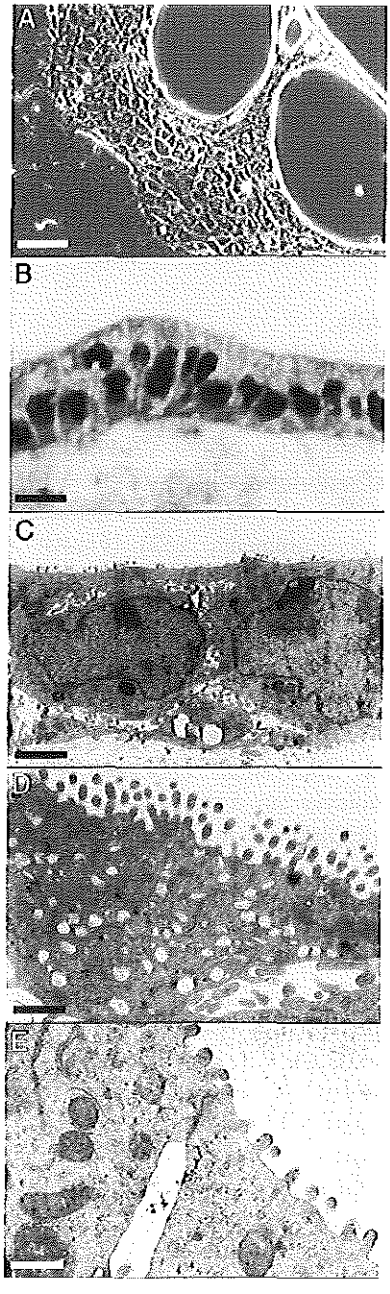
After neutralization with acetic acid, the treated material was analyzed on Sepharose CL-4B columns. Fractionation on Sepharose CL-4B: an aliquot of each sample was fractionated on a Sepharose CL-4B (32 x 1 cm diameter) in 6 mol/l guanidinium chloride. The flow rate was 24 ml/h and 1 ml fractions were collected. Radioactivity was assayed in a Beckman S 3801 liquid scintillation counter after mixing 0.1 ml of each eluted fraction with 2 ml of Aqualite.

Results.

In this study we used a knockout mouse model for CF (47). These mice suffer from a complete loss of CFTR function due to a disruption of exon 10. The CF-/- animals in our facility display less severe mortality due to intestinal obstruction as reported for the Cambridge colony (20% in the first two weeks in Rotterdam, compared to 80% in the Cambridge colony (47)). This may be due to differences in breeding conditions. However, all physiological parameters measured, nasal PD's, intestinal and gallbladder PD measurements (57) (French submitted) confirmed the CF phenotype of our animals. In particular, no cAMP induced chloride transport (57) or fluid secretion (Peters, in press, chapter VII) was observed in intact gallbladders of CF-/- animals, in contrast to control. The animals used for the experiments reported here were 5-6 weeks old, without obvious signs of disease or discomfort, average weight 23.5 ± 2.7 g for CF-/- and 23.0 ± 4.4 for their normal (CF+/+ and CF+/-) littermates.

Cultured mouse gallbladder cells. Mouse gallbladder epithelial cells (MGBC) grow from small (1-4 mm^2) pieces of embedded mouse gallbladder tissue as a monolayer on a collagen gel (Fig 1A, see methods section). The outgrowth finally results in holes in the collagen gel (Fig 1A top right) as cells cover both the upper and lower surface of the gel . Mouse gallbladder epithelial cells proliferate for up to 20 days under these conditions to approximately ten times the original surface area of the epithelium. Microscopic analysis of the cells growing on collagen for seven to fourteen days showed a morphology highly reminiscent of the intact tissue (58, 61): a polarized high cuboid epithelium (Fig 1B,C) with tight junctions (Fig 1E), apical microvilli (Fig 1C,D,E), extensive lateral villi (Fig 1C,D), and numerous mitochondria (Fig 1C,E). Clusters of vesicles similar to those found in native tissue were routinely observed as seen with electromicroscopic analysis (Fig 1D). The morphology of cultured gallbladder epithelial cells from CF -/- mice is normal (light microscopy, not shown). On morphological criteria (light and electron microscopic analysis) no significant outgrowth of fibroblasts was observed under these conditions. After prolonged incubation (> 20 days), clusters of fibroblasts were sometimes observed in the open spaces in the collagen gel. All experiments described below were performed with explant cultures of less than three weeks old with typical epithelial morphology (Fig 1).

Fig 1: Light- and electron micrographs of cultured mouse gallbladder epithelial cells.
a) Primary culture of mouse gallbladder epithelial cells (MGBC) (12 days), unstained cells, light microscopy. Epithelial cells grown from fragments of mouse gallbladder are cultured on collagen gel as described in the methods sections (Bar= 40 µm)
b) Paraffin section of MGBC in culture (eosin, hematoxylin), showing high cuboid polarized epithelium on collagen matrix (lower part). (Bar= 7 µm).
c) Electronmicrograph of cultured mouse gallbladder cells with apical and lateral microvilli (Bar = 1.6 µm). d) Electronmicrograph showing apical microvilli and vesicles (Bar= 0.5 µm). e) Electronmicrograph showing tight junctions, apical villi and mitochondria (Bar= 0.5 µm).



CFTR expression in cultured mouse gallbladder cells. The expression of the cAMP dependant chloride channel CFTR in cultured mouse gallbladder cells was examined with two different methods. RT/PCR analysis demonstrated that cultured mouse gallbladder cells produce CFTR mRNA at levels comparable to the intact tissue (Fig 2A-C). Intestinal tissues of normal mice (Ileum, Caecum) give a more than tenfold higher level of expression than gallbladder in this assay (Peters, in press, chapter VII). However these tissues were negative in CF-/- mice, which confirms the absence of full length CFTR mRNA in these animals (data not shown).

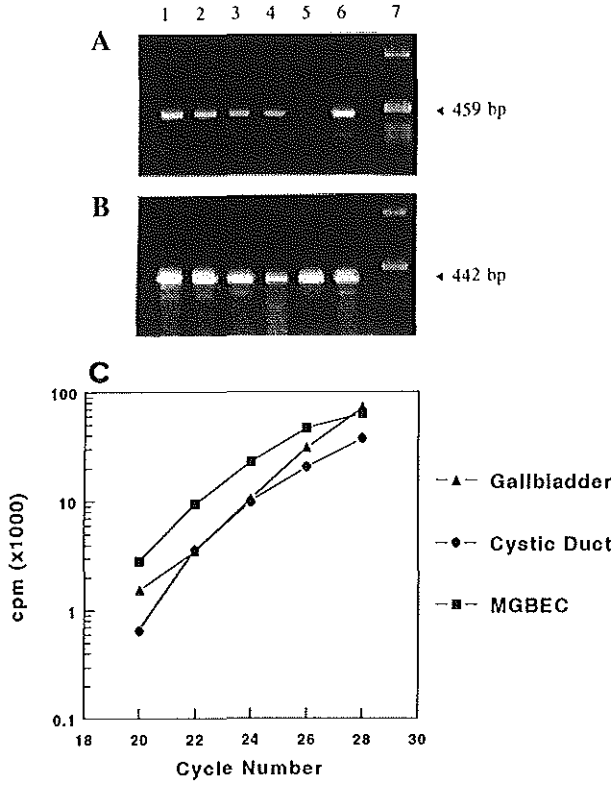


Fig.2: Expression of CFTR mRNA in cultured mouse gallbladder epithelial cells. Agarose gelelectrophoresis (ethidium bromide stain) of RT-PCR products obtained with CFTR primers (A) and aldolase primers as a control (B). Total RNA was isolated from murine cystic duct (lane 1), gallbladder (lane 2), lungs (lane 3), kidney (lane 4), total liver (lane 5), and cultured mouse gallbladder epithelial cells (lane 6). Size marker (1018, 517/506, 396, 344, 289 bp) (lane 7). (C): The amount of CFTR RT-PCR product as a function of number of PCR cycles was determined as described in the methods section. The data indicate that the cultured cells produce CFTR mRNA at levels comparable to the epithelial cells of cystic duct or intact gallbladder.

Patch-clamp analysis allows us to detect single channel activity in membrane patches sealed to a microelectrode. During on-cell recording the cAMP agonist forskolin induced the activity of small linear channels. These channels were inactive after the membrane patches were excised but could be activated with the cAMP dependant protein kinase A (PKA) plus ATP (Fig 3A). The voltage-current characteristic of this channel is linear with a conductance of 5 pS in 150 mM NaCl (Fig 3B). The ionselectivity of the channels was determined by exchanging the bath solution with low chloride buffer. This resulted in a shift of the reversal potential (I=0) expected of an anion selective channel (Fig 3b). In this way the relative ionselectivity was determined as NO_3^->Br^-=Cl^->F^->>I^-=gluconate. This channel is very similar to the human CFTR although we and other authors find a higher conductance for human CFTR (8 pS) (4, 17, 48). On average 6 (± 5, n=10) CFTR channels per patch were observed in this way. In five successful patch clamp protocols with CF-/- cells not one chloride channel of this type was observed. These observations are consistent with the conclusion that cultured normal mouse gallbladder cells express the mouse homologue of human CFTR. A different chloride channel frequently observed was a 25 pS outward rectifying channel which often appeared upon sustained hyperpolarization of the membrane. This channel was not

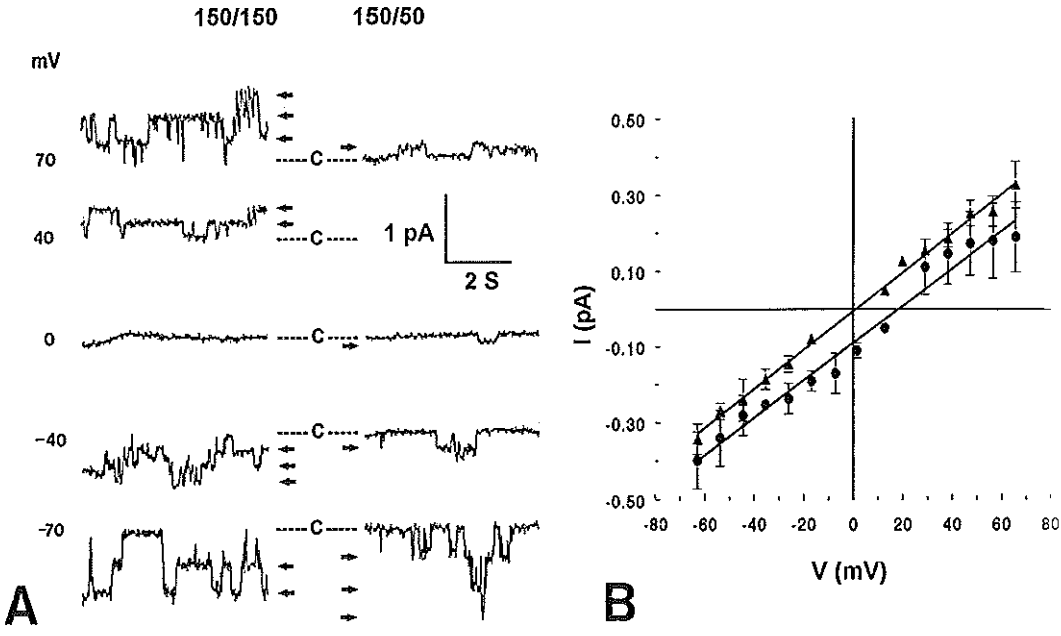


Fig.3: Patch clamp analysis Detection of mouse CFTR channel activity in cultured mouse gallbladder cells with patch clamp analysis was performed as described in the method section. A: a typical pipet current recording (pA) of mouse CFTR activity at different clamp voltages (mV). -C- indicates closed state of the channel, arrows indicate single channel open state levels. Left: 150 mM chloride in pipette and bath. Right: 0.05 mol/l chloride in bath. B: Current voltage relationship of the 5 pS channel after activation with protein kinase A and ATP at 0.15 mol/l chloride in pipette and bath (▲-▲) and at 0.05 mol/l chloride in bath, 0.15 mol/l in pipette (●-●). The shift of the reversal potential (at which current is zero) indicates the ionselectivity of the channel.

activated by PKA plus ATP. Glybenclamide (10^{-4} mol/l) reduced the conductivity and induced channel flickering. The ionselectivity of this channel was determined as NO_3^- >I^-=Br>Cl>>F>gluconate (n=3), and was also observed in cells from the CFTR deficient (CF-/-) mice (n=2). This channel is very similar to the outward rectifying chloride channel (ORCC) described previously in human cells (23, 51).

Glycoprotein secretion from cultured Mouse gallbladders. Cultured mouse gallbladder cells produced high molecular weight glycoproteins (HMG) as shown by SDS PAGE analysis of ^{3}H-glucosamine labelled proteins (Fig 4B). Labelled HMG is also observed in the culture medium (Fig 4A). Transport of HMG to the medium is

time and temperature dependant, as expected for active secretion (Fig 5A). Cells passaged once on collagen gels had similar morphology (light microscopy) but secreted HMG at a lower rate than primary cells (compare Fig 5A and 5B). Upon testing the effect of possible activators of secretion (Fig 5B) we found a

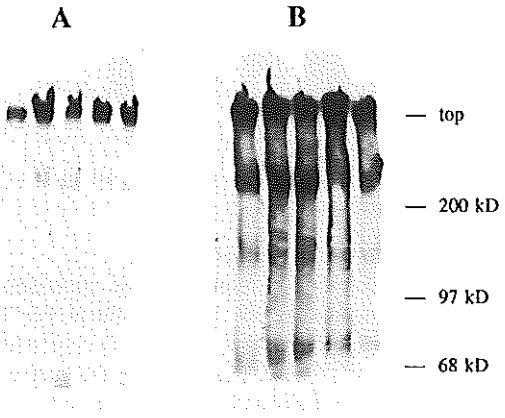
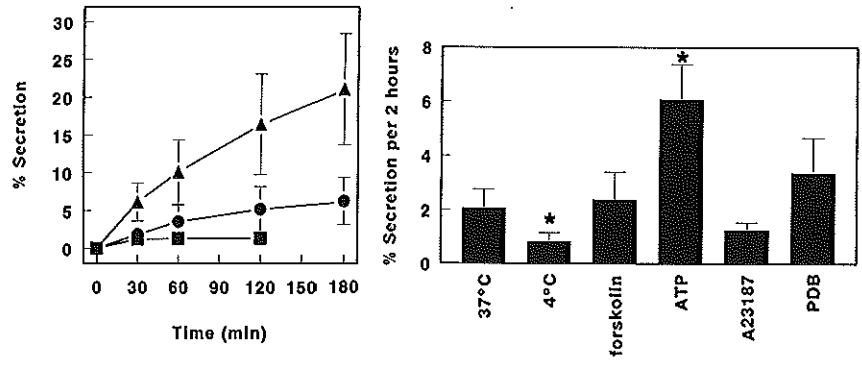


Fig.4: SDS-PAGE of ^{3}H-labeled glycoproteins secreted from cultured mouse gallbladder epithelial cells.
Cultured mouse gallbladder epithelial cells pre-incubated for 16 hours with ^{3}H-glucosamine were washed and incubated in fresh medium for 30 minutes at 37°C. After the incubation medium was collected and the cells were lysed with triton-X100. The incubation media from five separate dishes (A) and the associated cell lysates (B) were subjected to SDS-PAGE on a 5.5% acrylamide gel (90 % of the total samples). The ^{3}H-labeled glycoproteins were visualized by autoradiography. Both the medium samples and the cell lysates contain a prominent high molecular weight component on the top of the gel, indicative of mucins.

twofold stimulation by 3 mM ATP. However, lower concentrations of ATP (0.3 mM) had little effect on basal secretion rates (not shown). Forskolin which increases the intracellular cAMP concentration had no significant effect. This suggests that in these cells there is no direct link between CFTR activation and glycoprotein secretion and also that the glycoprotein secretion mechanism is not directly regulated by intracellular cAMP. Calcium ionophore or phorbol esther (PMA) alone or in combination (not shown) did not induce mucin secretion. In conclusion, the cells show active secretion of HMG, probably from the pool of subapical vesicles. The secretion apparently occurs mainly via a constitutive (non regulated) pathway. The HMG were further analyzed by column chromatography. Part of the labelled HMG found in the cell lysates was excluded from a Sepharose CL4B column (Fig 6A). This material is insensitive to enzymes known to break down glycosaminoglycans but is sensitive to



A B

Fig.5: Glycoprotein secretion from cultured mouse gallbladder epithelial cells.
A) Time and temperature dependence of glycoprotein secretion. Cultured mouse gallbladder epithelial cells and NIH-3T3 cells as a negative control (approximately 30 % confluent per 3.5 cm dish) were preincubated for 16 hours with ^{3}H-glucosamine to label cellular glycoproteins. Dishes were washed and incubated for 3 hours at 4°C or 37°C as described in the methods section. Samples were collected and analyzed at the intervals indicated. Finally, the cells were lysed and the secretion from the cells, as a percentage of total labelled glycoprotein was calculated (values are averages of 7 dishes ± SD). Data show a steady secretion of high molecular weight glycoproteins from Mouse gallbladder cells at 37° C (▲-▲) which is significantly reduced at 4°C (●-●). Less than 2% of total labelled glycoproteins were secreted by NIH-3T3 cells at 37°C (■-■). B) The effect of different agonists on glycoprotein secretion. In a parallel experiment, MGBC passaged once on collagen gels were labelled with ^{3}H-glucosamine (N indicates number of dishes), washed and incubated for 60 minutes in label free medium as indicated. The medium was then replaced by medium without additions at 37°C (N=5) or at 4°C (N=5), with 10^{-6} mol/l forskolin (N=4), 3.10^{-3} mol/l ATP (N=3), 5.10^{-7} mol/l calcium ionophore (A23187) (N=4) or 5.10^{-7} mol/l phorbol-dibutyrate (PBD) (N=5) followed by a 2 hour incubation. Medium was collected, cells were subsequently lysed and the release of high molecular weight glycoprotein to the medium was expressed as the percentage of the. *) indicates a significant difference with control at 37°C (Student's T-test).

alkaline degradation, suggesting an O-glycosyl attachment of carbohydrate chains. These data show that the labelled HMG excluded from the column consist mainly of mucins. The partially included peak possibly corresponds to the 300 kD band on the PAGE gel (Fig 4) and has similar properties with respect to enzyme and alkali sensitivity (Fig 6A). This material may be precursors or breakdown products of mucins. The labelled HMG secreted into the medium consists in part of mucins as shown by column chromatography, sensitivity to enzymes (Fig 6B) and carbohydrate staining on westernblots (data not shown). The 300 kd mucin component is less

prominent than in cell lysates (compare Figs 4 and 6) suggesting that this material is not actively secreted. The peak in the column volume (23 ml) presents a smear of labelled material on a 5-15% PAGE gel in which no specific products could be identified (not shown). As this component was not prominent in short secretion experiments (Fig. 4) it may represent a mixture of low molecular weight glycoproteins and breakdown products of High molecular weight components.

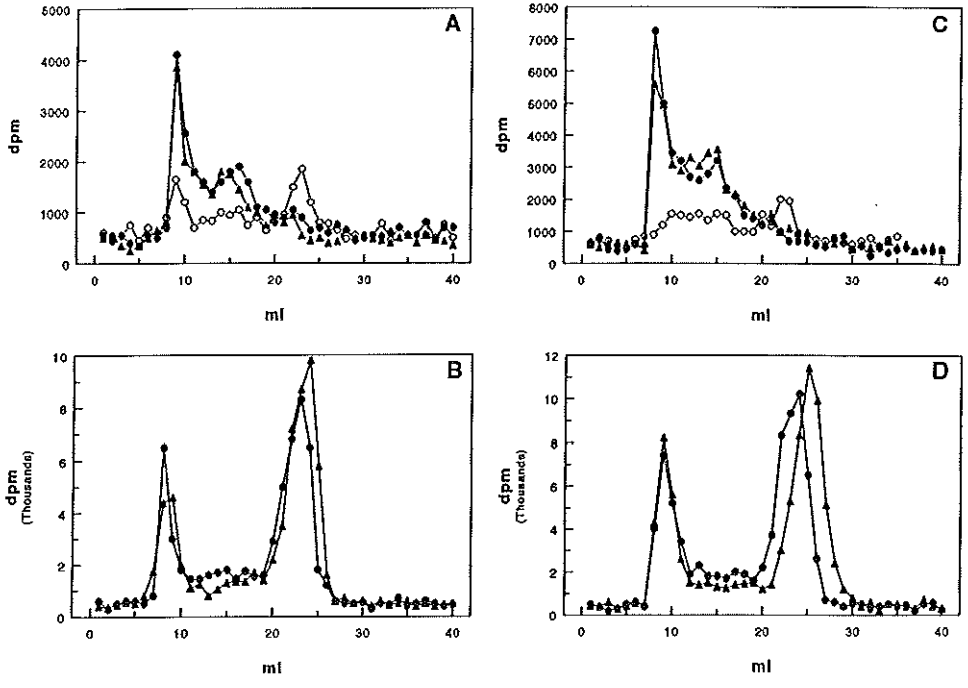


Fig.6. Column chromatography of labelled glycoproteins of Normal and CF-/- mouse cells. Labelled glycoproteins were collected from cell lysates (A,C) and culture media (B,D) of normal (A,B) and CF-/- (C,D) mouse gallbladder epithelial cells in culture as described in the methods section. Samples were analyzed on Sepharose CL4B columns either without further treatment (●-●), after incubation with a mixture of heparitinase, hyaluronidase and chondroitinase (■-■) or alkaline degradation (○-○).

Mucin secretion by CF-/- cells. Cells of CF-/- mice also produce and secrete mucins as shown by column chromatography, enzyme and alkali sensitivity (Fig 6C,D). Quantitative analysis of total tritium labelled HMG and the material excluded from the Sepharose column (Table 1) shows that there is no significant difference

between normal and CF-/- cells with respect to the rate of basal secretion of ^{3}H-Glucosamine incorporated in HMG and in mucins., either expressed as percentage of total amount produced or by comparing the amount secreted per dish of culture cells. We conclude that CFTR is not required for either accumulation or basal secretion of glycoprotein including mucins in cultured mouse gallbladder cells.

Table 1.
Secretion of high molecular weight glycoproteins by cultures of normal and CF-/- mouse gallbladder epithelial cells.

	Control (DPM)	CF-/- (DPM)
^{3}H-HMG Medium (Chase)	92.000	96.000
^{3}H-HMG Medium (overnight)	100.000	96.000
^{3}H-HMG Cell Lysate	92.000	175.000
Total ^{3}H-HMG	282.000	367.000
% ^{3}H-HMG secreted	68 %	52 %
^{3}H-mucin Medium (Total)	42.000	60.000
^{3}H-mucin Cell lysate	36.000	69.000
Total ^{3}H-mucin	78.000	129.000
% ^{3}H-mucin secreted	53 %	48 %

Primary explant cultures of normal (N=7) and CF-/- (N=8) gallbladders were labelled with ^{3}H-glucosamine overnight. The medium was collected and replaced by unlabelled medium (chase). High molecular weight glycoproteins (HMG) of medium samples and cell lysates (expressed as dpm ^{3}H) were determined by ultrafiltration as described. The combined medium samples and the cell lysates were analyzed by gelfiltration (Fig 6) to determine the mucin component of the HMG. % mucin and HMG secreted is the percentage of material in the combined media of the total.

Discussion

We have established and characterized a primary culture system for mouse gallbladder epithelial cells. The cells we obtained have many of the morphological characteristics of fully differentiated biliary epithelium and can therefore be considered a valid model for this tissue (Fig 1). Similar results have been obtained with gallbladder cells from other species including man (62) and dog (28, 43). In contrast to these systems, however, we found that the mouse cells could not be passaged regularly without senescence of the cells and fibroblast overgrowth. Therefore, the experiments described in this paper were performed with cells grown from primary explants or with cells passaged once to collagen gels.

Our data show that CFTR mRNA is produced in cultured mouse gallbladder cells with levels comparable to intact tissue (Fig 2). Patch clamp analysis revealed the presence of a small chloride channel with characteristics very similar to human CFTR (Fig 3). We conclude that these cells express the mouse counterpart of human CFTR, providing the first description of this chloride channel. The number of chloride channels per patch in these cells compares favourably to other primary epithelial cells in culture (24) or even colon carcinoma cell lines (HT29, T84) which are often used as a model for CFTR expression (2, 56). Cultured MGBC therefore are a convenient model for the study of CFTR activity in a well differentiated epithelial cell in primary culture. This is particularly important in view of the availability of several mouse models of CF. These include loss of function mutants (44, 47, 54) and more recently the prevalent ΔF508 processing mutant (14, 57, 63) and a G551D mutant (15). Other CFTR mutations are likely to follow.

An additional interesting feature of the cultured MGBC is that they produce and secrete glycoproteins (Fig 4,5), including mucins (Fig 6). Mucin secretion has been shown for cultured human (62) and dog (29, 43) gallbladder epithelial cells. Mucin secretion in cultured dog epithelial cells was reported to be stimulated by prostaglandins (PGE2), possibly by increasing the intracellular cAMP concentration (29). In contrast, our results suggest that in cultured MGBC glycoprotein secretion occurs via a constitutive pathway. Forskolin, Ca-ionophore and phorbol-esther had no significant effect on glycoprotein release in these cells. This suggests that an increase in intracellular cAMP, Ca or protein kinase C activity is not sufficient to induce secretion (Fig 5). The effect of extracellular ATP is difficult to interpret. Since 0.3 mM ATP had no effect (not shown) the activity of a P2 purinergic receptor seems excluded. Possibly, aspecific effects of ATP on membrane permeability (18) are involved.

As pointed out in the introduction, several observations in the literature suggest a relationship between CFTR activity and mucin secretion. This could contribute to the development of liver disease in CF. In bronchial segments the beta-adrenergic secretion stimulus observed in normal tissue is reduced in CF patients (50). In this case the coupling could be indirect as CFTR is mainly expressed in serous cells of

submucosal glands (19), reduced output from serous acini could impair mucin transport. Mucus secretion in airway goblet cells is under purinergic rather than cAMP control (32). In the intestinal crypt, mucin secreting Goblet cells are stimulated by Ca^{2+} agonists but not by cAMP (25). In isolated submandibular salivary gland a significant reduction of cAMP induced secretion was observed in human CF patients (39) and in a CF mouse model (40). In these cells, mucin release is triggered by an increase of intracellular Ca^{2+} (34). Therefore, the exact relationship between CFTR and secretion is not yet clearly established. Our current data show that MGBC from mice with a loss of function mutation of the CFTR gene produce and secrete labelled glycoproteins including mucin to a similar extent as normal cells, as determined by 3H-Glucosamine labelling (Fig 6, table 1). Therefore, neither activation of CFTR by forskolin nor absence of CFTR has a marked effect on glycoprotein secretion. This does not support the notion that in gallbladder cells CFTR function and secretion are closely coupled, as suggested by experiments with Neomycin selected dog gallbladder cell clones expressing CFTR from an expression vector (28). However, since the amount of mucins we can recover are too small to analyze their molecular structure, we can not exclude that the mucins from CF deficient cells differ from normal with respect to carbohydrate structure and protein-carbohydrate ratio. CFTR deficiency in humans results in increased sialation and sulphation of glycoproteins, and abnormal carbohydrate structure were observed (10, 11, 35, 36). The exact cause of this phenomenon is not known but it has been suggested that it relates to an effect of CFTR of acidification of intracellular vesicles (3). Therefore, It is possible that the mucins produced by CFTR deficient gallbladder and biliary cells are abnormal due to increased sulphation or aberrant branching as in human salivary and respiratory mucins (10, 35). Abnormal biliary mucin structure could contribute to the development of liver disease in CF patients. The amount of material that can be recovered from the present mouse gallbladder cell cultures does not allow a careful analysis of mucin carbohydrate molecular structure. We are presently attempting to increase the yield of cultured cells by crossing the ΔF508 CFTR mouse model that we recently produced (57), with a mouse producing a temperature sensitive version of Large T antigen. In this model we can study the effect of CFTR dysfunction on mucin structure and membrane recycling.

Cells expressing CFTR show a marked cAMP dependent inhibition of endocytosis and CFTR internalization, which is not observed in CFTR deficient cells (7, 45). CFTR apparently can affect regulated membrane trafficking. An interesting observation in this context is that the cAMP agonist secretin induced release of a fluid phase marker from an endocytotic compartment in isolated rat biliary duct cells. Similarly, the columnar intestinal crypt cells which express CFTR release vacuolar content in response to cAMP agonists (25). It would be interesting to observe the effect of cAMP on uptake and release of fluid phase markers in normal and CF MGBC. It is possible that the cells contain vesicular compartments with different regulation of secretion.

CF mice do not develop histological liver abnormalities, at least not at the age

we usually study them (less than three months). The same is probably true for a majority of young human CF patients. The development of liver disease in CF patients does not necessarily correlate with a severe clinical phenotype (lung disease, pancreas dysfunction) (59). CFTR dysfunction apparently is not the only determinant. One other contributing factor may be the presence of other chloride channels in the biliary cells (38) which may partially compensate for CFTR dysfunction in some tissues (12). Where expression of this compensating activity becomes the limiting factor, i.e. in CFTR mutants, species and individual differences in pathology can be expected

It is possible that cultured gallbladder cells behave differently from cells in intact biliary tissue. There is evidence that prostaglandins (30) and cholecystokinin (CCK) (58) stimulate mucin release from intra hepatic duct cells. CCK activates the phospho-inositol pathway rather than intracellular cAMP. There is no compelling evidence that cAMP directly affects mucin release in the intact biliary tract. cAMP agonists do activate biliary fluid and bicarbonate secretion in vivo (31). Fluid secretion is dependent on CFTR activity as was shown for the mouse gallbladder (Peters, in press, chapter VII), intestine (22) and cultured airway epithelium (26, 53). Future *in vivo* studies in the CF mouse model may allow us to determine the effect of agonists on mucin and fluid output from the intrahepatic biliary system. Available data point at reduced fluid output rather than increased mucin secretion as the major factor in the development of CF liver disease.

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DISCUSSION

Since the discovery of the *CFTR* gene in 1989, research in the CF field has made enormous progress. Genotyping of patients and prenatal diagnostics made diagnosis far more accurate than before. Pregnancies in families at high risk for CF are being screened for the most common CF mutations. The protein defective in CF patients is a chloride channel on the plasma membrane of epithelial cells. This confirmed electrophysiological data that indicated a defect in chloride transport in CF patients (see chapter I). However, the exact involvement of the chloride channel in characteristic features of CF, such as mucus accumulation, susceptibility for lung infections and intestinal malabsorption is still not understood. Tissues from CF patients are not easily obtained, primary cultures from the affected epithelial tissues are difficult to maintain and those cultured cells may not have the same properties as intact tissues. Therefore, an animal model, even with some different characteristics, is vital for basic and applied research of CF. It can be used to study the primary and secondary effects of CFTR dysfunction and to develop new therapy strategies.

The most predominant mutation in the Caucasian population is a deletion of an amino acid at position 508 (ΔF508). This mutation inhibits the processing of the protein, resulting in absence of CFTR on the plasma membrane. It does not, or slightly, affect its function as a chloride channel. This implies that 90 % of CF patients carry at least one ΔF508 mutation and thus have a CFTR protein that could be functional, if only it passed through the processing machinery. Some experimental cell systems have shown that it is possible to direct ΔF508 CFTR to the plasma membrane (see chapter I.10). If necessary, the ΔF508 CFTR can subsequently be hyperactivated with genistein, a tyrosine kinase inhibitor (French, in preparation). These results give hope for an alternative therapy, aimed at bringing ΔF508 CFTR to its functional site and stimulating it to its maximum capacity.

Mouse models have been made for CF (see chapter III), with a complete disruption of the gene and with specific mutations as a G551D mutated mouse and the ΔF508 mouse model. The ΔF508 mouse model that was made by our group (ΔF508-EUR) has normal ΔF508 CFTR mRNA levels. In contrast, ΔF508 mouse models made by two other groups (4, 24) and the G551D mouse model (6), show decreased mRNA levels from the mutated allele. These mice were made by homologous recombination of a construct containing the ΔF508 or G551D mutation and the selectable marker in intron 10 or intron 11b. The levels of mRNA expression from the mutated allele varied in different organs. The lowest levels were found in the intestine in all these three mouse models, ranging from 15 % to 50 %. We have found no reduction of mRNA levels in the ΔF508-EUR mouse, which does not contain any other genomic alteration but the introduced mutation. Therefore, the selectable marker in the intron must have some adverse affect on

mRNA transcription regulation although no regulating elements are yet defined in either intron 10 or 11b.

The ΔF508-EUR mice have lower body weights than normal mice, reflecting the malabsorption syndrome also seen in CF patients. In electrophysiological assays, the ΔF508-EUR mouse has clear defects characteristic for CF but it has some residual chloride activity. The ΔF508-EUR mice do not show intestinal obstructions, which are fatal in the CFTR knock-out mice and to a lesser extent in the other mutated mouse models. These observations indicate that mouse ΔF508 CFTR has sufficient residual function to relieve severe intestinal syndromes. Similar to human ΔF508 CFTR, mouse ΔF508 CFTR is clearly abnormally glycosylated, is not observed at the apical membrane and has the temperature sensitive processing defect. Tissues of ΔF508 patients generally do not show a residual chloride activity. The mouse ΔF508 CFTR may therefore be less severely impeded in its processing than human ΔF508 CFTR (see chapter V and VI). It is not yet known whether this property is related to the mouse ΔF508 CFTR or to the mouse processing system. On the one hand, we know that human ΔF508 CFTR expressed in mouse 3T3 cells is not processed to the mature form (2). On the other hand, some ΔF508 patients show residual chloride activity, not attributable to a Ca^{2+} regulated chloride channel (Veeze, pers.comm.). This would suggest that the level of residual processing is determined by genetic background. One way to address this question is to make a transgenic human ΔF508 CFTR mouse to see whether the human ΔF508 CFTR is processed similarly as mouse ΔF508 CFTR.

The molecular aspects of CFTR processing begin to emerge but they do not yet provide an understanding of the folding pathway. Basic research of the processing must involve the characterization of proteins that show an interaction with ΔF508 CFTR and immature normal CFTR. Two chaperones associate with CFTR, i.e. cytosolic hsp70 proteins and the ER resident calnexin. ΔF508 CFTR has a prolonged binding time with both of these chaperones (16, 22). Calnexin is not likely to be involved in the processing defect, as a glycosylation mutant of CFTR does not bind calnexin but is still processed correctly (17). The prolonged chaperone binding of ΔF508 CFTR reflects the inability of the cellular machinery to assist in proper folding of the mutant protein. Inhibition of the degradation pathway does not result in increased mature ΔF508 CFTR (13, 18). Other known chaperons, as Bip and Grp94 were not associated with CFTR forms. To understand more of the processing pathway and to open ways of intervening with the folding process, interactions of chaperons with CFTR should be further studied. A way to bypass the quality control mechanism in the ER might come from the observed alleviation of the processing defect under low temperature (30°C) or when cells are treated with glycerol (7, 18). No studies have yet been published concerning the mechanism of this partial relieve of the processing block. An alternative approach would be to study revertants of the ΔF508 phenotype. In an experiment in which the ΔF508 mutation was introduced in the yeast Ste-6 mating protein, a member of

the ABC family, revertants could be found by screening for regained mating ability. These revertants contained new mutations in Ste-6 itself (19, 20). In a mammalian system, one would expect to find revertants with mutations in proteins like chaperones apart from revertant mutations in ΔF508 CFTR itself. Such a revertant as been found in a CF patient with a mild phenotype (8). Since CFTR is not an essential protein, this can not be done directly. However, a ΔF508 based processing defective mutant could be made in proteins with homology to CFTR but with a selectable property. The multidrug resistance pump MDR or the multi drug related protein MRP could be used for this purpose (3, 10). A chimeric MDR-like_ΔF508 domain protein could be made, that possesses the ΔF508 processing defect and can be used as a selectable marker. This would be invaluable in an efficient and rapid screening for drugs which alleviate the processing defect. Alternatively, an immuno tagged CFTR that can be demonstrated on the plasma membrane could be used for such strategies (11) or fluorescent dyes which are specifically taken up in CFTR positive cells (21).

It is not likely that a CFTR specific chaperone will be found and therefore the treatment of patients with agents that interfere with the processing machinery will also result in the processing of other mutant proteins which should have been degraded. Only experiments in the ΔF508 mouse model will be able to show the effects and side effects of such treatment.

Several clinical aspects of human CF may be studied in CF mouse models. The histology and electrophysiology of mouse and human organs are comparable but not identical. For example, in the lung humans have abundant submucosal glands in the trachea, while mice have only few but have more predominant Clara cells (14). As expression of CFTR is highest in the human submucosal glands, this might be an explanation for the virtual absence of spontaneous lung pathology in the CF mouse models. However, CF mice developed more serious inflammation than normal mice when challenged repeatedly with specific bacteria (5). Increased adherence and decreased phagocytosis of *Pseudomonas Aeruginosa* by CF cells and specifically by ΔF508 CFTR cells has been found (12, 15, 23). To study *Pseudomonas Aeruginosa* clearance, infection, and pathogenesis, an infection model for the ΔF508 mouse is being developed in our group.

Intestinal malabsorption is prevalent in patients and is probably only partially caused by pancreatic enzyme insufficiency. All CF mouse models show intestinal dysfunction. The complete knock-outs often die of intestinal blockage in the first three weeks. However, the death rate is less when mice are fed a special, liquid diet (9), or when kept in clean mouse facilities (6). It seems therefore, that mice lacking CFTR are highly susceptible to intestinal blockage and this is modified by the diet and stable conditions, possibly related to pathogen exposure. All mice that survive, either knock-outs or ΔF508 mice, still have lower body weights than their normal littermates. This indicates a malabsorption syndrome similar to

CF patients. Whether the few pancreatic abnormalities present in knock-out mice have any part in the intestinal malabsorption is not yet known. The absorption of different compounds can be measured in the mouse CF models, by perfusion of an isolated intestinal segment *in vivo* and by modifying the environment. Factors which might influence the absorption are the amount and type of bile acids, the pH of the intestinal content, properties of the mucus and the unstirred water layer.

CF patients have quite often abnormalities of the biliary tract and gallbladder, as liver cirrhosis and bile stones. These aberrations might come from obstruction of biliary ducts with thick and sticky mucus, either caused by an increased mucus secretion or by a decreased fluid secretion in CF patients. The knock-out mouse model was used to study the direct relationship between the pathogenesis of biliary tract disease and the absence of CFTR. While no increased mucus secretion was found in cultured mouse gallbladder cells, a clear dysfunction in fluid secretion by the gallbladder was observed (chapter VII and VIII). Gallbladders from normal and CF mice absorb fluid in the basal state. However, while normal gallbladders respond to cAMP increase with fluid secretion, CF gallbladders show inhibition of absorption but no secretion. This indicates that CF gallbladder is not able to secrete fluid in response to the endogenous cAMP increasing hormones VIP and secretin, which are released in response to feeding. This lack of fluid secretion might contribute to the formation of bile stones and a similar defect in the biliary tree to obstruction of bile ducts in CF patients.

The pH of Golgi vesicles and endosomes is more alkaline in CF epithelial cells. This change of pH can alter the effectiveness of enzymes responsible for sialylation and sulfation of proteins. This could explain the aberrant sulfation and sialylation found for CF glycoproteins and glycolipids (1). As mentioned above, human ΔF508 epithelial cells have better adherence but are less able to phagocytose *P.aeruginosa* than normal cells, and also than other CF mutants. This is related to the number of asialylated receptors on the membrane of the CF cells (12, 15, 23). The ΔF508 mouse can be used to analyze the protein production and specific glycosylation compared to knock-out mice and in relation to the pH of intracellular vesicles.

In conclusion, the specific properties of the ΔF508 mouse make it a suitable model system to study clinical symptoms in comparison to knock-out mice i.e. infection with *P.aeruginosa*, to study the role of ΔF508 CFTR in vesicles and in glycosylation. Most importantly, possible therapeutic agents which promote processing of CFTR to the plasma membrane and agents which can activate the ΔF508 CFTR can be studied and tested in the ΔF508 mouse.

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SUMMARY/SAMENVATTING

Summary

Cystic fibrosis (CF) is the most common, lethal, inherited disease in the Caucasian population. It affects organs with epithelial cell layers, as lung, pancreas and intestine. Disease symptoms are mucus obstructions in the lung accompanied with severe and chronic bacterial infections, pancreas deficiency and malabsorption in the intestine. Patients die from respiratory complications or heart failure due to the destruction of the lungs by bacterial infections. The average age is presently around 30 years. The genetic defect lies in the CFTR gene, which codes for a chloride channel. The dysfunction of the mutant chloride channel disturbs the transepithelial salt transport and therefore the accompanying water transport. Lack of water transport is presumably the cause of blockage of the airways and of the pancreatic ducts with viscous mucus. The treatment involves pancreatic enzymes to supplement the pancreas deficiency, antibiotic treatment against the recurrent lung infections and intensive physiotherapy to clean the airways. For some patients a lung transplantation is possible. Trials to test the safety and efficacy of gene therapy and pharmacological drugs which stimulate water transport in the lungs are being done.

More than 400 different mutations in the CFTR gene have been reported, but by far the most common CF mutation is a deletion of one amino acid, a phenylalanine, at position 508 in the CFTR protein (ΔF508). Because of this mutation, the protein can not be transported towards the plasma membrane were it has it's function. Electrophysiology of single chloride channels shows that ΔF508 CFTR channels behave in almost the same way as normal CFTR. This means that the 90% of CF patients that carry a ΔF508 allele have a ΔF508 CFTR channel that is not localized at the correct site, but is in principle functional.

In this thesis, the creation and analysis of mouse models for CF is described. A CF mouse model opens possibilities to study pathogenesis and therapy at a molecular level in intact tissues, which is not possible with human material. We have made a ΔF508 mouse model where the nature of the processing defect can be studied in intact tissues and possible therapeutic agents can be tested. Those agents should be able to target ΔF508 CFTR to the plasma membrane or to increase the activity of the small amount of ΔF508 CFTR that reaches the plasma membrane.

The introduction, chapter I, gives an overview of the disease symptoms in patients with CF, the properties of the CFTR chloride channel and the electrophysiology of CF tissues. Also the nature of the ΔF508 mutation and its effect on CFTR protein processing is described. Chapter II describes the generation of different types of mouse models and various ways in which endogenous mouse genes can be mutated. In chapter III, pathology and electrophysiology of different CF mouse models and CF patients are compared. The CF mouse models show important characteristics of CF, as intestinal obstruction and electrophysiological

defects in airways, intestine, pancreas and gallbladder but also differences are observed between mice and men.

Chapters IV and V describe the experimental work of the generation of a ΔF508 mouse model, using the Hit & Run procedure. This is a two step procedure in which a construct containing the ΔF508 mutation together with selectable markers, is first integrated into the homologous site in the mouse CFTR gene (chapter IV). The second step involves the deletion of the selectable markers, together with the normal exon, leaving the mutated exon in the gene. This results in a mouse CFTR gene which has no other genomic alteration but the ΔF508 mutation (chapter V). The ΔF508 mouse has histological abnormalities in the intestine and shows electrophysiological defects in nasal epithelium, intestine and gallbladder characteristic of CF. The electrophysiological responses are not the same as in knock-out mice, which has a complete disruption of the CFTR gene, since the ΔF508 mouse has some residual CFTR activity. In chapter VI, the nature of this residual activity is studied in more detail. Protein staining shows it to be aberrantly localized in the intestinal crypt cells. Similar to the human ΔF508 CFTR, the mouse ΔF508 CFTR can be brought to the plasma membrane by reducing the culture temperature.

In chapters VII and VIII, experiments with gallbladders from knock-out mice are described. Abnormalities of gallbladder and of obstruction of the biliary tract, are found in 40 % of the CF patients. We studied the transport of water by the mouse gallbladder (chapter VII) and the production of mucus in cultured gallbladder epithelial cells (chapter VIII). In contrast to normal gallbladders, knock-out mouse gallbladders are unable to secrete water when stimulated with cAMP increasing agents. There was no difference in mucus secretion between cultured gallbladder cells from normal and knock-out mice. Therefore, viscous mucus is not caused by increased production of mucus but rather by decreased fluid secretion by the gallbladder cells. The lack of water secretion might lead to the obstruction of biliary ducts and the formation of bile stones in the gallbladder that are found in CF patients.

Samenvatting

Cystic Fibrosis (taaislijmziekte) is de meest voorkomende, dodelijke, erfelijke ziekte in de Caucasische populatie. Organen met een epitheliale cellaag zijn het meest aangedaan, zoals long, alvleesklier en de darm. Eén van de ziekte symptomen is een opeenhoping van taai slijm wat in de long samengaat met zware en chronische longinfecties en in de alvleesklier enzym deficiëntie veroorzaakt. Ook hebben patiënten malabsorptie in de dunne darm. Patiënten overlijden door complicaties van de luchtwegen of door een hartfalen veroorzaakt door de slechte longfunctie. De gemiddelde leeftijd van een patiënt is nu ongeveer 30 jaar. Het genetisch defect van cystic fibrosis (CF) ligt in het CFTR gen wat codeert voor een chloride kanaal. De dysfunctie van het gemuteerde chloride kanaal verstoort het trans-epitheliale zout transport en het hiermee samenhangende water transport. Het tekort aan watertransport is waarschijnlijk de oorzaak van de obstructie van de luchtwegen en de alvleesklierducten met taai slijm. De behandeling bestaat uit enzym suppletie om het tekort aan alvleesklier enzym aan te vullen, antibiotica tegen de recidiverende luchtweg infecties en intensieve fysiotherapie om de luchtwegen schoon te houden. Voor sommige patiënten is een longtransplantatie mogelijk. Momenteel worden er studies verricht naar de mogelijkheid van gentherapie en farmacologische therapie.

Meer dan 400 verschillende mutaties zijn nu gevonden in het CFTR gen en de meest voorkomende mutatie is een deletie van 1 aminozuur, een fenylalanine, op positie 508 van het CFTR eiwit (ΔF508). Door deze mutatie kan het ΔF508 CFTR eiwit niet getransporteerd worden naar de celmembraan waar het zijn functie heeft. Electrophysiologie van chloride kanalen heeft aangetoond dat ΔF508 CFTR chloride kanalen zich bijna hetzelfde gedragen als normale CFTR kanalen. Dit betekent dat de 90 % van de CF patiënten die een ΔF508 mutatie hebben, alle een chloride kanaal bezitten wat in principe functioneel is maar alleen niet juist gelocaliseerd is.

In dit proefschrift wordt het maken en analyseren van muis modellen voor CF beschreven. Een muis model voor CF opent de mogelijkheden om pathologie en therapie op een moleculair niveau te bestuderen in intacte weefsels, wat niet mogelijk is met humaan materiaal. Wij hebben een ΔF508 muis model gemaakt waarin de oorzaak van het transport defect bestudeerd kan worden in intacte weefsels en waarin mogelijk therapeutische drugs getest kunnen worden. Deze drugs zouden in staat moeten zijn om ΔF508 CFTR naar de celmembraan te brengen of om het kleine gedeelte ΔF508 CFTR wat op de celmembraan terecht is gekomen, extra te stimuleren.

De introductie, hoofdstuk I, geeft een overzicht van de ziekte symptomen van CF patiënten, eigenschappen van het CFTR chloride kanaal en de electrofysiologie van CF patiënten materiaal. Ook kenmerken van de ΔF508 mutatie en haar

invloed op het CFTR eiwit transport worden hier besproken. Hoofdstuk II beschrijft het maken van verschillende types van muis modellen en de manieren waarop genen gemuteerd kunnen worden. In hoofdstuk III worden de pathologie en electrofysiologie van verschillende CF muis modellen en humane CF patiënten met elkaar vergeleken. De CF muis modellen vertonen belangrijke CF karakteristieken zoals darm obstructie en electrofysiologische afwijkingen in luchtwegen, darm, alvleesklier en galblaas, maar er zijn ook verschillen tussen mens en muis CF.

De hoofdstukken IV en V beschrijven het experimentele werk wat door gebruik te maken van de Hit & Run procedure geleidt heeft tot de generatie van een ΔF508 muis model. Deze procedure bestaat uit twee stappen waarin eerst een construct met het gewenste gemuteerde exon en selectie markers geïntegreerd wordt in het homologe gebied in het muis CFTR gen. De tweede stap wordt bepaald door de deletie van de selectie markers tegelijkertijd met het normale exon, waarbij het gemuteerde exon achterblijft. Dit resulteert in een muis CFTR gen waarin niets dan het exon is gemuteerd en de genomische organisatie volledig intact is (hoofdstuk V). De muis die met deze procedure is gemaakt heeft histologische abnormaliteiten in de darm en vertoont electrofysiologische afwijkingen in neus epitheel, darm en galblaas die karakteristiek zijn voor CF. De electrofysiologische responsen zijn niet dezelfde als die in een knock-out muis, waarbij het gehele CFTR gen is gedeleteerd, daar residuele CFTR activiteit in de ΔF508 muis gevonden is. In hoofdstuk VI wordt deze residuele activiteit nader bestudeerd. Eiwit kleuring toont de afwijkende lokalisatie in intestinale crypt cellen. Net als de humane ΔF508 CFTR vorm, kan ook het muizen ΔF508 CFTR naar de cel membraan gebracht worden door de temperatuur te verlagen.

In de hoofdstukken VII en VIII worden experimenten met galblazen van knock-out muizen beschreven. Afwijkingen van de galblaas en de galducten wordt gevonden in 40 % van de CF patiënten mogelijk veroorzaakt door mucus obstructies. Hier beschrijven we de studies van water transport in muis galblaas (hoofdstuk VII) en de mucine secretie door gekweekte galblaascellen (hoofdstuk VIII). In tegenstelling tot normale galblazen, kunnen knock-out galblazen geen water secreteren als respons op cAMP stimulatie. Er was geen verschil in mucus secretie tussen gekweekte galblaascellen van normale en knock-out muizen. Dit betekent dat taai en dik mucus niet veroorzaakt worden door een toename in de productie van mucus maar aannemelijker is dat dit door een tekort aan water secretie veroorzaakt wordt. Het gedehydreerde mucus zou galstenen in de galblaas en biliaire obstructies in de galwegen kunnen veroorzaken.

Curriculum Vitae

De schrijfster van dit proefschrift werd geboren op 10 Mei 1965 te Den Helder. Het voorbereidend wetenschappelijke onderwijs werd genoten op het Huygens Lyceum te Voorburg en afgesloten in juni 1983. De studie van voorkeur was in dat jaar nog niet begonnen en zo kreeg Biologie te Leiden er een tijdelijke passant bij zonder dit evenwel met een propadeuse af te sluiten. In september 1994 ging uiteindelijk de gewenste studie Gezondheidswetenschappen, in Leiden, van start. Korte stages werden gevolgd bij Medische Biochemie, RUL olv Marc Timmers en Prof. van der Eb en bij Medische Gerontologie, RUL olv Inge de Greef en Prof. Hijmans. De hoofdvakstage werd ook bij Medische Biochemie olv Marc Timmers gedaan en werd gevolgd door nog een verlengde "korte" stage Immuno Haematologie, AZL olv Rudi Hendriks, Ruud Schuurman en Prof. van Rood. Uiteindelijk werd het doctoraal examen afgelegd in september 1990. De verkregen bul was voor doctorandus in de Biomedische Wetenschappen Leiden, aangezien de studie onderwijl van naam veranderd was. In october 1990 begon het onderzoek beschreven in dit proefschrift op de afdeling Celbiologie & Genetica, onder leiding van Dr. Bob Scholte en Prof. Galjaard. In september 1995 mocht een nieuw onderzoek begonnen worden als wetenschappelijk medewerker bij hetzelfde instituut ditmaal olv Prof. Frank Grosveld.

Nawoord

Liever praat en lach en ouwehoer ik met jullie dan ieder hier apart op te noemen en te zeggen dat dank zij jullie het lab in stand gehouden wordt, werkdiscussies gehouden worden, mijn artikelen gecontroleerd worden, essentiële onderdelen voor mijn experimenten gegeven worden, ik allerlei nuttige informatie krijg en ook dat jullie met me samenwerkten al was dat soms helemaal geen lolletje. Ik zou niet weten wat ik liever doe met m'n leven dan op een lab werken en dat wordt ook bepaald door de uiterst aangename, bijzonder prettige werkomgeving.
Veel, heel veel dank hiervoor !